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Functional Analysis of the Shiga Toxin and Shiga-Like Toxin Type II Variant Binding Subunits by Using Site-Directed Mutagenesis

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The B subunit of Shiga toxin and the Shiga-like toxins (SLTs) mediates receptor binding, cytotoxic specificity, and extracellular localization of the holotoxin. While the functional receptor for Shiga toxin, SLT type I (SLT-I), and SLT-II is the glycolipid designated Gb_3, SLT-II variant (SLT-IIv) may use a different glycolipid receptor. To identify the domains responsible for receptor binding, localization in *Escherichia coli*, and recognition by neutralizing monoclonal antibodies, oligonucleotide-directed site-specific mutagenesis was used to alter amino acid residues in the B subunits of Shiga toxin and SLT-IIv. Mutagenesis of a well-conserved hydrophilic region near the amino terminus of the Shiga toxin B subunit rendered the molecule nontoxic but did not affect immunoreactivity or holotoxin assembly. In addition, elimination of one cysteine residue, as well as truncation of the B polypeptide by 5 amino acids, caused a total loss of activity. Changing a glutamate to a glutamine at the carboxyl terminus of the Shiga toxin B subunit resulted in the loss of receptor binding and immunoreactivity. However, the corresponding mutation in the SLT-IIv B subunit (glutamine to glutamate) did not reduce the levels of cytotoxicity but did affect extracellular localization of the holotoxin in *E. coli*.

Shiga toxin, which is produced by *Shigella dysenteriae* type 1, and the Shiga-like toxin (SLTs), which are produced by enterohemorrhagic strains of *Escherichia coli*, possess similar biological and biochemical properties (18). Nucleotide sequence analysis revealed that Shiga toxin and SLT type I (SLT-I) are essentially identical (22) while SLT-II is 55% homologous to both (8). SLT-II variant (SLT-IIv), which is produced by edema disease-causing strains of *E. coli*, is antigenically related to SLT-II but differs from the other members of the Shiga toxin family with respect to cytotoxic specificity (15). While Shiga toxin, SLT-I, and SLT-II are predominantly cell associated in *E. coli* and are equally cytotoxic for HeLa and Vero cells, SLT-IIv is predominantly extracellular and is markedly more cytotoxic for Vero cells. All members of the Shiga toxin family are bipartite molecules composed of an enzymatic A subunit, which is a 28S rRNA *N*-glycosidase (3, 20), and multiple copies of a binding (B) subunit. The functional receptor for the B subunits of Shiga toxin, SLT-I, and SLT-II is the glycolipid receptor designated Gb_3 (13, 25). The observation that Gb_3 is not the functional receptor for the SLT-IIv B subunit probably explains why the variant toxin differs from the other members of the Shiga toxin family with respect to cytotoxic specificity (2; J. E. Samuel, D. L. Weinstein, V. Ginsburg, and H. C. Krivan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B-74, p. 42; J. E. Samuel, L. P. Perera, S. Ward, A. D. O'Brien, V. Ginsburg, and H. C. Krivan, Infect. Immun., in press).

Studies with hybrid toxins composed of the A and B subunits of Shiga toxin, SLT-I, SLT-II, and SLT-IIv demonstrated that the B moiety dictates cytotoxic specificity and extracellular localization in *E. coli* (26). Comparison of the deduced sequences of Shiga toxin/SLT-I (22), and SLT-II (8) with that of SLT-IIv (27) revealed significant amino acid differences in the B subunits. In an attempt to identify the domains of the Shiga toxin and SLT-IIv B polypeptides involved in receptor binding, localization in *E. coli*, and recognition by monoclonal antibodies, site-directed mutagenesis was used to change the selected amino acid residues that differed among these toxins. In addition, two well-conserved B subunit domains of Shiga toxin, a hydrophilic region near the amino terminus and the single disulfide bond, were targeted for mutagenesis.

MATERIALS AND METHODS

Bacteria, bacteriophage, plasmids, and reagents. The bacteriophage M13mp18 and mp19 vectors were used for nucleotide sequence analysis (19). *E. coli* MC4100 has been described by Casadaban (1). *E. coli* HB101 and LE392 contain *supE* and *supE supF* mutations (14), respectively, which translate an amber termination codon with a glutamine (*supE*) or a tyrosine (*supF*). Plasmid pEW3.0 contains the A and B subunit genes of Shiga toxin (*stx* A and *stx* B) on a 2.5-kilobase (kb) *Bgl*II-*Eco*RI fragment in vector pBR329. Plasmid pDLW5.3 carries the A and B subunit genes of SLT-IIv (*slt*-IIv A and *slt*-IIv B) on a 4.0-kb *Eco*RI-*Cla*I fragment in vector pBR329, and pDLW5.5 carries the *slt*-IIv operon on a 2.3-kb *Aat*II-*Eco*RI fragment in pBR329 (26). All bacterial strains, bacteriophage, and plasmids were propagated in Luria broth or Luria broth agar, supplemented with antibiotics when necessary. Restriction endonucleases, the large (Klenow) fragment of DNA polymerase I, and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). DNA sequence analysis was performed with a Sequenase kit from United States Biochemicals (Cleveland, Ohio).

DNA preparation. Plasmid DNA was purified by methods described by Maniatis et al. (14). DNA fragments used for subcloning were purified by electrophoresis with 0.8% preparative agarose gels and eluted with a model UEA electroeluter (International Biotechnologies Inc., New Haven, Conn.).

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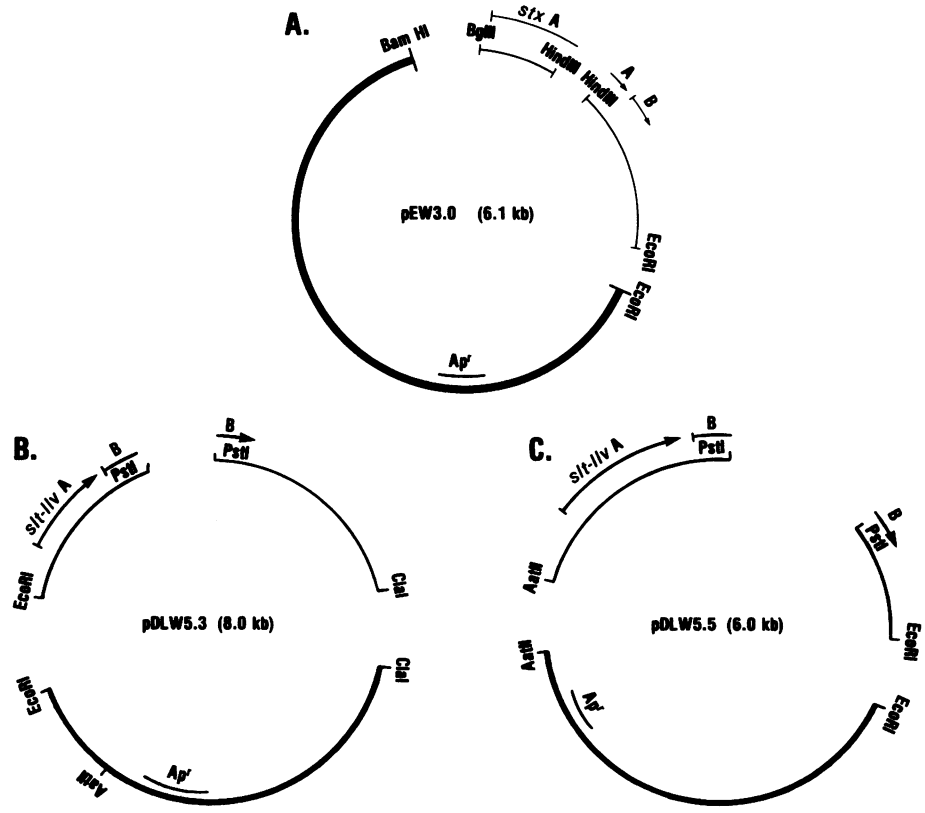

FIG. 1. Construction of the mutagenized *stx* and *slt*-IIv operons by simultaneous ligation of three restriction fragments. (A) Plasmid pEW3.0 encoded the *stx* operon on a 2.55-kb *Bgl*II-*Eco*RI fragment. All mutations in *stx* B were induced in the 1.8-kb *Hin*dIII-*Eco*RI fragment encoding downstream *stx* A sequences and the entire *stx* B gene. (B) Plasmid pDLW5.3 carried the *slt*-IIv operon on a 4.0-kb *Eco*RI-*Cla*I fragment. Mutations N17D and I52K were induced in the 1.5-kb *Eco*RI-*Pst*I fragment encoding the upstream *slt*-IIv B sequences. (C) Plasmid pDLW5.5 encoded the *slt*-IIv operon on a 2.3-kb *Aat*II-*Eco*RI fragment. Mutation Q64E was induced in the 0.5-kb *Pst*I-*Eco*RI fragment encoding the downstream *slt*-IIv B sequences.

Oligonucleotide-directed site-specific mutagenesis. The double-oligonucleotide primer method of Zoller and Smith (28) and was used to introduce point mutations in *stx* B and *slt*-IIv B. Oligonucleotides designed to introduce point mutations were synthesized on the basis of the sequences of *stx* (22) and *slt*-IIv (27) with a model 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). The single-stranded templates used for mutagenesis were M13mp18 containing a 1.8-kb *Hin*dIII-*Eco*RI fragment carrying *stx* B (Fig. 1A), M13mp19 containing a 1.5-kb *Eco*RI-*Pst*I fragment carrying upstream *slt*-IIvB sequences (Fig. 1B), or M13mp18 containing a 0.5-kb *Pst*I-*Eco*RI fragment carrying downstream *slt*-IIv B sequences (Fig. 1C). All mutations were confirmed by DNA sequence analysis. The mutagenic oligonucleotides used in this study are described in Table 1.

Construction of mutagenized operons. Mutagenized *stx* B carried by a 1.8-kb *Hin*dIII-*Eco*RI fragment was isolated from the replicative form of M13 following site-directed mutagenesis. This fragment was simultaneously ligated with a 0.75-kb *Bgl*II-*Hin*dIII fragment carrying upstream *stx* A sequences into *Bam*HI- and *Eco*RI-digested pBR329 to construct the intact *stx* operon (Fig. 1A). To construct double mutations at distant residues, the *Hin*dIII-*Eco*RI fragment cloned in M13mp18 was subjected to two rounds of site-directed mutagenesis before isolation and construction of the *stx* operon.

The mutagenized *slt*-IIv operon was constructed with either a 1.5-kb *Eco*RI-*Pst*I fragment carrying the upstream sequences of *slt*-IIv B (mutations N17D and I52K; Fig. 1B) or a 0.5-kb *Pst*I-*Eco*RI fragment carrying the downstream sequences of *slt*-IIv B (mutation Q64E; Fig. 1C). These restriction fragments isolated from the replicative form of the M13 vector after site-directed mutagenesis were ligated with fragments containing the remainder of the *slt*-IIv operon into pBR329 (Fig. 1B and C). To construct the *slt*-IIv double mutants N17D,Q64E and I52K,Q64E, the *Eco*RI-*Pst*I fragment carrying mutagenized upstream *slt*-IIv B sequences was ligated to the *Pst*I-*Eco*RI fragment carrying mutagenized downstream *slt*-IIv B sequences into *Eco*RI-digested pBR329. All manipulations of high-level toxin-producing clones were conducted under BL3+EK1 containment (4).

Colony blot assay. A colony blot enzyme-linked immunosorbent assay (ELISA) was used to assess the immunoreactivity of mutagenized Shiga toxin B subunits expressed by *E. coli* transformed with the recombinant plasmids described above. Toxin released from bacterial colonies by treatment with polymyxin B (2 mg/ml for 1 h at 37°C) was bound to a nitrocellulose membrane, and an ELISA was performed as previously described (23). Three Shiga toxin B subunit-specific monoclonal antibodies designated 13C4, 19G8, and 16E6 (23) were used in the colony blot assays.

Microcytotoxicity assay. The levels of Vero and HeLa cell toxicity for wild-type and mutant Shiga toxin and SLT-IIv were assessed by using the microcytotoxicity assay of Gentry and Dalrymple (5). Both extracellular (supernatant) and cell-associated (cytoplasmic and periplasmic) fractions were collected for assay as previously described (16). The amount

TABLE 1. Nucleotide sequence of mutagenic oligonucleotides

Toxin and oligonucleotide[a]	Amino acid substitution[b]
Shiga toxin	
G G TATAATCATCACGATACCTTTACAG	D16H, D17H
G AAATATAATAATGACGATACC	D16N
G TATAATGATAACGATACCTTTACA	D17N
G TATAATGATGACAATACCTTTACAG	D18N
G A AAAGTGAGTGGTAAAGAATTA	G25S, D26G
G ACCAACAGATAGAATCTT	W34Amber
T AATCTTCAGCCTCTTCTTCTC	S38P
A TGTAACCATTATAACTAATGCCTG	K53I
GT ACTAATGCCTAGCATAATGGA	C57Amber
A T GCCTGTCATTAGGGAGGGGGA	N59Amber
G A GGATTCAGCTAGGTTATTTTTC	E65Amber
SLT-IIv	
A TAATGAGGATGATACCTTTACTG	N17D
T TGTAACAATCAAATCTAATACCTGC	I52K
C GGCTTTGCCGAGGTGAAGTTTAAC	Q64E

[a] Wild-type sequences are shown above the mutated nucleotides.

[b] Nomenclature of Knowles (11). One-letter amino acid code: A, alanine; R, arginine; N, asparagine; D, aspartate; C, cysteine; E, glutamate; Q, glutamine; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine.

of toxin contained in the last 10-fold dilution of the sample in which ≥50% of the HeLa or Vero cells detached from the plastic, as assessed by A_{620}, was considered the 50% cytotoxic dose (CD_{50}) per milliliter of culture.

Receptor-analog assay. The ability of mutated Shiga toxin to bind the glycolipid receptor Gb_3 was assessed in an ELISA with the receptor analog Gal-Gal-bovine serum albumin as previously described (27). A 1:3,500 dilution of ascites fluid containing the Shiga toxin A subunit-specific monoclonal antibody 4F7.3 (kindly provided by J. E. Brown) was used to detect bound toxin (6).

Minicell analysis. To determine if B subunit with the D16H,D17H double mutation could associate with A subunit to form Shiga holotoxin, ^{35}S-labeled polypeptides were immunoprecipitated from the periplasm of a minicell-producing strain of *E. coli* as described by Meagher et al. (17). Total ^{35}S-labeled polypeptides were extracted with polymyxin B, and Shiga toxin was immunoprecipitated (10) with the B subunit-specific monoclonal antibody 13C4 followed by goat antimouse immunoglobulin G as described by Strockbine et al. (23). The immunoprecipitated extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography to identify labeled A and B subunit polypeptides (12).

RESULTS AND DISCUSSION

It has been proposed that a hydrophilic sequence between residues 10 to 20 in the Shiga toxin B subunit is an antigenic domain of the polypeptide (21). Evidence that this sequence does contribute to antigenicity was obtained by Harari et al. (7), who prepared neutralizing antibodies against synthetic peptides corresponding to this region of the Shiga toxin B subunit. This region is well conserved in the B subunits of Shiga toxin, SLT-I, SLT-II, and SLT-IIv (8, 22, 27) (Fig. 2). Two double and three single amino acid substitutions designed to reduce the hydrophilicity of this region were induced at aspartate residues 16, 17, and 18 of the Shiga toxin B subunit. Only the double mutation of aspartates 16 and 17 to histidines (D16H,D17H [Table 2]) had a significant effect on activity. This double mutation caused the total loss of cytotoxic activity on HeLa and Vero cells and eliminated receptor analog binding. However, the mutated polypeptide was fully immunoreactive in the colony blot ELISA with the three monoclonal antibodies (13C4, 16E6, and 19G8) that were specific for the native Shiga toxin B subunit (Table 2). The aspartate residues at positions 16 and 17 could be part of the receptor binding site. Alternatively, they could be required to maintain the functional conformation of a receptor-

```
 1              5                  10                  15                  20
Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr Lys Tyr Asn Asp Asp Asp Thr Phe
--- Ala         Ala Lys         Ile     Phe Ser             Glu     Asn
    1             5                   10                  15

                25                  30                  35                  40
Thr Val Lys Val Gly Asp Lys Glu Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu
                Ser Gly Arg     Tyr Trp                             Pro
20              25                  30                  35

                45                  50                  55                  60
Leu Ser Ala Gln Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
Gln             Leu                         Ile Ser     Thr     Ser Ser
40              45                  50                  55

                65
Gly Gly Phe Ser Glu Val Ile Phe Arg
Ser         Ala Gln     Lys     Asn
60              65
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FIG. 2. Amino acid sequences of the mature Shiga toxin (21, 22) and SLT-IIv (27) B subunits. The complete sequence of the Shiga toxin B subunit is shown on the upper line beginning with the threonine at position 1. The amino acid sequence of the SLT-IIv B subunit is shown below the Shiga toxin sequence only where the two differ. The SLT-IIv B subunit begins with an alanine at position 1. Amino acids altered by site-directed mutagenesis are underlined.

TABLE 2. Missense mutations in the Shiga toxin B subunit

Mutation[a]	Cytotoxicity (CD_{50}/ml)	Result[b] of colony blot ELISA with:			Receptor analog binding (%)
		13C4	16E6	19G8	
None (pEW3.0)	10^7	+++	+++	+++	100
D16H,D17H	0	+++	+++	+++	0
D16N,D17N	10^7	+++	+++	+++	94
D16N	10^7	+++	+++	+++	100
D17N	10^7	+++	+++	+++	84
D18N	10^7	+++	+++	+++	100
K53I	10^6	++	++	−	18
D18N,K53I	10^6	+++	+++	−	24
G25S,D26G	10^7	++	++	++	100
S38P	10^7	++	++	++	100

[a] Nomenclature is given in footnote *b* of Table 1. Plasmid pEW3.0 expressed wild-type Shiga toxin.

[b] +++, Fully immunoreactive; ++, somewhat immunoreactive; −, not immunoreactive.

TABLE 3. Amber termination mutations in the Shiga toxin B subunit

Host[a] and residue	Cytotoxicity (CD_{50}/ml)	Result[b] of colony blot ELISA with:			Receptor analog binding (%)
		13C4	16E6	19G8	
HB101					
pEW3.0	10^7	+++	+++	+++	100
W34	10^3	+	+	+	0
C57	0	−	−	−	0
N59	10^5	+	+	−	0
E65	0	−	−	−	0
LE392					
W34	10^6	++	+	+	52
C57	0	−	−	−	0
N59	10^7	++	+	−	100
E65	0	−	−	−	0

[a] HB101 is *supE* (= *glnV*); LE392 is *supE supF* (= *glnV tyrT*) (14). Nomenclature is given in footnote *b* of Table 1.

[b] +++, Fully immunoreactive; ++, somewhat immunoreactive; +, weakly immunoreactive; −, not immunoreactive.

binding site located elsewhere in the B subunit. Both the A and B subunits were immunoprecipitated by using monoclonal antibody 13C4 from periplasmic extracts of *E. coli* minicells encoding the mutated *stx* operon (data not shown). This observation established that the D16H,D17H double mutation did not prevent holotoxin assembly.

The remaining single and double mutations induced in the Shiga toxin B subunit (K53I; D18N,K53I; G25S,D26G; and S38P) were based on a comparison with the SLT-IIv B subunit sequence (Fig. 2). Toxin mutated at lysine 53 (K53I and K53I,D18N) was reduced 10-fold in cytotoxicity and expressed approximately 20% of wild-type receptor analog binding (Table 2). In addition, B polypeptide mutated at lysine 53 was not immunoreactive with the monoclonal antibody 19G8 but was recognized by 13C4 and 16E6. The remaining mutations, G25S,D26G and S38P, did not have a significant effect on total cytotoxic levels, immunoreactivity, or receptor analog binding. Our results suggest that lysine 53 is part of the epitope recognized by monoclonal antibody 19G8. No data concerning the structure of the epitopes recognized by monoclonal antibodies 13C4 or 16E6 are available.

Amber termination mutations were introduced at codons corresponding to the following four residues of the mature Shiga toxin B subunit: tryptophan 34, cysteine 57, asparagine 59, and glutamate 65 (Table 3). When the *stx* operon with any of these four amber mutations was expressed in *E. coli* MC4100, which lacks suppressor activity, no toxin was detected in tests for HeLa and Vero cell toxicity, receptor analog binding assays, or colony blot assays with any of the three B subunit-specific monoclonal antibodies (data not shown). Because the carboxyl-terminal-most amber mutation was at codon 65, these results indicate that truncating the B subunit by 5 amino acids at the carboxyl terminus inactivates Shiga toxin.

When the *stx* operons containing amber mutations at codon 34 or 59 were expressed in *E. coli* HB101 or LE392, toxicity was partially to fully restored (Table 3). The level of activity expressed was influenced by the efficiency of chain propagation from the suppressor tRNA encoded by the host (9), with the double suppressor host LE392 expressing 2 to 3 orders of magnitude more cytotoxicity than the single suppressor host HB101 (Table 3). Because the receptor analog assay required the expression of at least 10^6 CD_{50}/ml, no binding activity was exhibited for the W34 and N59 mutations expressed by *E. coli* HB101. Wild-type levels of cytotoxicity and receptor analog binding were restored in LE392 by suppression of the amber mutation at codon 59, with substitution of glutamine or tyrosine for asparagine 59, but the mutated polypeptides were not recognized by the monoclonal antibody 19G8 in the colony blot ELISA (Table 3). Therefore, both lysine 53 and asparagine 59 contribute to the epitope defined by monoclonal antibody 19G8. In a separate study, Surewicz et al. (24) used fluorescence spectroscopy to demonstrate that tryptophan 34 is at or very near the Gb_3 receptor-binding domain. However, replacement of this tryptophan with a glutamine or a tyrosine did not prevent binding to the receptor analog (Table 3), indicating that this tryptophan residue was not directly involved in receptor recognition.

The nonsense mutation at codon 57 was not phenotypically suppressed in *E. coli* HB101 or LE392 (Table 3). Because there are only two cysteine residues in the Shiga toxin B subunit (Fig. 2), replacement of cysteine 57 with a glutamine or tyrosine eliminated the single intrachain disulfide bond. Also, the nonsense mutation at codon 65 was not suppressed. Expression of this mutation in HB101 replaced glutamate 65 with a glutamine, the residue at the corresponding position in B subunit of SLT-IIv (Fig. 2). The glutamate 65-to-glutamine change in the Shiga toxin B subunit rendered the toxin inactive in all assays (Table 3), while the reciprocal change in the SLT-IIv B subunit (Q64E) altered extracellular localization of the toxin in *E. coli* without drastically affecting Vero cell toxicity (Table 4). These results indicate that while the carboxyl terminus of SLT-IIv B was important for release of holotoxin from the periplasm, the carboxyl terminus of the Shiga toxin B subunit was a domain important for the structural integrity and perhaps assembly of the holotoxin. Alternatively, the Shiga toxin B subunit with the glutamate 65 mutation may have been an unstable polypeptide that was degraded by the cell.

Three amino acid residues in the SLT-IIv B subunit were mutated to the corresponding residue in the Shiga toxin B subunit (Fig. 2). Because neither a monoclonal antibody nor a receptor analog ELISA was available for SLT-IIv, the only assay used to analyze mutant toxin was that measuring the

TABLE 4. Missense mutations in SLT-IIv B subunit

Mutation[a]	Cytotoxicity (CD_{50}/ml)	
	Cell associated	Extracellular (%)
None (pDLW5.3)	10^4	10^5 (91)
N17D	10^3	10^4 (91)
I52K	10^2	10^3 (91)
Q64E	10^4	10^2 (1)
N17D,Q64E	10^3	0 (0)
I52K,Q64E	10^3	0 (0)

[a] Nomenclature is given in footnote *b* of Table 1. Plasmid pDLW5.3 expressed wild-type SLT-IIv.

ability to kill Vero and HeLa cells. As with native toxin, the mutated forms of SLT-IIv were cytotoxic for Vero cells only, with no detectable activity for HeLa cells. Compared with native SLT-IIv expressed by pDLW5.3, the glutamine-to-glutamate mutation at residue 64 had no effect on the cell-associated levels of cytotoxicity (10^4 CD_{50}/ml) but reduced the extracellular levels of activity 1,000-fold (from 10^5 to 10^2 CD_{50}/ml [Table 4]. SLT-IIv with the double mutations N17D,Q64E and I52K,Q64E, which included the glutamine 64-to-glutamate change, was present in cell-associated fractions but was not detectable in culture supernatants (Table 4). The SLT-IIv B subunit is a relatively basic molecule with an isoelectric point of 10.2 (23). The pI was reduced by replacing the glutamine with a glutamate, and this change may have affected the localization of SLT-IIv by disrupting ionic interactions with the *E. coli* cell envelope. The remaining mutations induced in SLT-IIv B, N17D and I52K, caused 10-fold and 100-fold reductions in the levels of cytotoxicity, respectively, with no effect on toxin localization.

While no single amino acid could be identified as the B subunit receptor-binding moiety, mutagenesis of the hydrophilic region near the amino terminus of the Shiga toxin B subunit eliminated receptor binding without affecting holotoxin assembly. Furthermore, the carboxyl terminus of Shiga toxin may be important for biological activity and holotoxin assembly, while the carboxyl terminus of SLT-IIv is involved in determining the extracellular localization of that toxin in *E. coli*. Multiple residues distantly separated in the primary structure of the Shiga toxin and SLT-IIv B subunits may be required for receptor recognition. It is not known whether the receptor-binding site lies within a single B polypeptide or is formed by the interaction of adjacent B polypeptides.

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LITERATURE CITED

1. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104**:541–555.
2. **DeGrandis, S., H. Law, J. Brunton, C. Gyles, and C. A. Lingwood.** 1989. Globotetraosyl ceramide is recognized by the pig edema disease toxin. J. Biol. Chem. **264**:12520–12525.
3. **Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and K. Igarashi.** 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eucaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur. J. Biochem. **171**:45–50.
4. **Federal Register.** 1986. Recombinant DNA research guidelines appendix F-IV-H. Fed. Regist. **51**:16972.
5. **Gentry, M. K., and J. M. Dalrymple.** 1980. Quantitative microtiter cytotoxicity assay for *Shigella* toxin. J. Clin. Microbiol. **12**:361–366.
6. **Griffin, D. E., M. K. Gentry, and J. E. Brown.** 1983. Isolation and characterization of monoclonal antibodies to Shiga toxin. Infect. Immun. **41**:430–433.
7. **Harari, I., A. Donohue-Rolfe, G. Keusch, and R. Arnon.** 1988. Synthetic peptides of Shiga toxin B subunit induce antibodies which neutralize its biological activity. Infect. Immun. **56**: 1618–1624.
8. **Jackson, M. P., R. J. Neill, A. D. O'Brien, R. K. Holmes, and J. W. Newland.** 1987. Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933. FEMS Microbiol. Lett. **44**:109–114.
9. **Kaplan, S., A. O. W. Stretton, and S. Brenner.** 1965. *Amber* suppressors: efficiency of chain propagation and suppressor specific amino acids. J. Mol. Biol. **14**:523–533.
10. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**:1617–1624.
11. **Knowles, J. R.** 1987. Tinkering with enzymes: what are we learning? Science **236**:1252–1258.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Lindberg, A. A., J. E. Brown, N. Stromberg, M. Westling-Ryd, J. E. Schultz, and K.-A. Karlsson.** 1987. Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* type 1. J. Biol. Chem. **262**:1779–1785.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Marques, L. R. M., J. S. M. Cryz, and A. D. O'Brien.** 1987. *Escherichia coli* strains isolated from pigs with edema disease produce a variant of Shiga-like toxin II. FEMS Microbiol. Lett. **44**:33–38.
16. **Marques, L. R. M., M. A. Moore, J. G. Wells, I. K. Wachsmuth, and A. D. O'Brien.** 1986. Production of Shiga-like toxin by *Escherichia coli*. J. Infect. Dis. **154**:338–341.
17. **Meagher, R. B., R. C. Tait, M. Betlach, and H. W. Boyer.** 1977. Protein expression in *E. coli* minicells by recombinant plasmids. Cell **10**:521–536.
18. **Montfort, W., J. E. Villafranca, A. F. Monzingo, S. R. Ernst, B. Katzin, E. Rutenber, N. H. Xuong, R. Hamlin, and J. D. Robertus.** 1987. The three-dimensional structure of ricin at 2.8 A. J. Biol. Chem. **262**:5398–5403.
19. **O'Brien, A. D., and R. K. Holmes.** 1987. Shiga and Shiga-like toxins. Microbiol. Rev. **51**:206–220.
20. **Sanger, F., A. R. Coulson, B. G. Barvell, A. J. H. Smith, and B. Roe.** 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**:161–178.
21. **Saxena, S. K., A. D. O'Brien, and E. J. Ackerman.** 1989. Shiga toxin, Shiga-like toxin II variant, and ricin are all single-site RNA N-glycosidases of 28S RNA when microinjected into *Xenopus* oocytes. J. Biol. Chem. **264**:596–601.
22. **Seidah, N. G., A. Donohue-Rolfe, C. Lazure, F. Auclair, G. T. Keusch, and M. Chretien.** 1986. Complete amino acid sequence of *Shigella* toxin B-chain. A novel polypeptide containing 69 amino acids and one disulfide bridge. J. Biol. Chem. **261**: 13928–13931.
23. **Strockbine, N. A., M. P. Jackson, L. M. Sung, R. K. Holmes, and A. D. O'Brien.** 1988. Cloning and sequencing of the genes for Shiga toxin from *Shigella dysenteriae* type 1. J. Bacteriol. **170**:1116–1122.
24. **Strockbine, N. A., L. R. M. Marques, R. K. Holmes, and A. D. O'Brien.** 1985. Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*. Infect. Immun. **50**:695–700.
25. **Surewicz, W. K., K. Surewicz, H. H. Mantsch, and F. Auclair.** 1989. Interaction of *Shigella* toxin with globotriaosyl ceramide

receptor-containing membranes: a fluorescence study. Biochem. Biophys. Res. Commun. **160**:126–132.

26. **Waddell, T., S. Head, M. Petric, A. Cohen, and C. Lingwood.** 1988. Globotriosyl ceramide is specifically recognized by the *Escherichia coli* Verocytotoxin 2. Biochem. Biophys. Res. Commun. **152**:674–679.
27. **Weinstein, D. L., M. P. Jackson, L. P. Perera, R. K. Holmes, and A. D. O'Brien.** 1989. In vivo formation of hybrid toxins comprising Shiga toxin and the Shiga-like toxins and role of the B subunit in localization and cytotoxic activity. Infect. Immun. **57**:3743–3750.
28. **Weinstein, D. L., M. P. Jackson, J. E. Samuel, R. K. Holmes, and A. D. O'Brien.** 1988. Cloning and sequencing of a Shiga-like toxin type II variant from an *Escherichia coli* strain responsible for edema disease of swine. J. Bacteriol. **170**:4223–4230.
29. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA **3**:479–488.