Identification of the Shiga Toxin A-Subunit Residues Required for Holotoxin Assembly

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Recent X-ray crystallographic analyses have demonstrated that the receptor-binding (B) subunits of Shiga toxin (STX) are arranged as a doughnut-shaped pentamer. The C terminus of the enzymatic (A) subunit presumably penetrates the nonpolar pore of the STX B pentamer, and the holotoxin is stabilized by noncovalent interactions between the polypeptides. We identified a stretch of nine nonpolar amino acids near the C terminus of StxA which were required for subunit association by using site-directed mutagenesis to introduce progressive C-terminal deletions in the polypeptide and assessing holotoxin formation by a receptor analog enzyme-linked immunosorbent assay, immunoprecipitation, and a cytotoxicity assay. Tryptophan and aspartic acid residues which form the N-terminal boundary, as well as two arginine residues which form the C-terminal boundary of the nine-amino-acid sequence, were implicated as the stabilizers of subunit association. Our model proposes that residues 279 to 287 of the 293-amino-acid STX A subunit penetrate the pore while the tryptophan, aspartic acid, and 2 arginine residues interact with other charged or aromatic amino acids outside the pore on the planar surfaces of the STX B pentamer.

Members of the Shiga toxin (STX) family, which includes the Shiga-like toxins (SLT), and of the cholera toxin family, which includes the heat-labile enterotoxins (LT), have significant structural similarities. These toxins are composed of a single enzymatic (A) subunit noncovalently associated with five receptor-binding (B) subunits (7, 20). The A subunits are nicked by a bacterial protease to give two disulfide-linked fragments, designated A_1, which possesses the enzymatic activity, and A_2, which is the bridge between the A_1 polypeptide and the B pentamer. Although the receptor-binding specificities of the STX and cholera toxin B subunits are similar (4, 10), these toxins differ with respect to entry and mode of action (7, 11). Despite the absence of detectable sequence homology between these two toxin families, X-ray crystallographic analysis of the B subunits has revealed a striking degree of structural similarity (27–29).

X-ray crystallographic structures for the B subunits of SLT-I (2, 29) and LT (27, 28) have established that both form doughnut-shaped pentamers presumably penetrated by the A_2 polypeptide. Individual B subunits are composed of antiparallel β-sheets and an α-helix. The cleft formed by the interaction of β sheets in adjacent monomers results in five potential carbohydrate-binding sites per pentamer. Ten β-sheets form the outer surfaces of the SLT-I B pentamer, while the five helices form an 11-Å (ca. 1-nm) neutral pore, in contrast to the highly charged pore of LT (2, 27–29).

While the specific sites of noncovalent association between the A and B subunits of the STX family have not yet been established, biochemical studies (7) as well as X-ray crystallographic analysis (27) have revealed that the A_2 fragment of cholera toxin and LT forms a hairpin structure with 50% of its surface buried in the highly charged central pore of the B pentamer. Stein et al. (29) have extrapolated the findings with LT to the STX family of toxins and proposed that if the

interaction between the A and B subunits of STX and SLT-I is the same as that with LT in which the A subunit lies below the pentamer, then the B pentamer of STX and SLT-I would bind to the receptor and the A subunit would be oriented away from the membrane. In contrast to LT, which enters the cell through a B subunit-induced pore, SLT-I enters the cell by receptor-mediated endocytosis (23, 32) and StxA is translocated to the Golgi apparatus (24).

The purpose of the present study was to establish a structural model for the association between StxA and the STX B pentamer. $StxA_2$ residues which penetrate the pore of the B pentamer and stabilize subunit interaction were identified by introducing C-terminal deletions and mutations. Holotoxin assembly was assessed by using a receptor analog enzyme-linked immunosorbent assay (ELISA), a cytotoxicity assay, and immunoprecipitation with an StxB-specific monoclonal antibody.

MATERIALS AND METHODS

Bacterial strains and recombinant plasmids. The following strains of *Escherichia coli* were used in this study: HB101 [F⁻ *supE44 hsdS20*(r$_B$⁻ m$_B$⁻) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1* λ⁻] (3), ED8767 (*recA56 supE supF hsdS⁻R⁺M⁺ met*) (18), and MC4100 [F⁻ *araD139* Δ(*argF-lac*) U169 *rpsL150 rel ptsF25 rbsR*] (26) were used as hosts for recombinant plasmids. JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* λ⁻ Δ(*lac-proAB*) F′ (*traD36 proAB⁺ lacI^q lacZ* M15)] (35) was host for the recombinant M13 phage used in mutagenesis experiments and sequencing. X1411 (F⁻ T$_6$^{s} *minA minB* λ⁻ *str^s*) (5) was used in minicell experiments.

The recombinant plasmids used in this study are listed in Table 1.

Preparation of plasmid DNA. Plasmid DNA was prepared by the Birnboim and Doly (1) minilysate procedure. Restriction endonuclease fragments were isolated from preparative agarose gels by electroelution with a model UEA electroeluter according to the instructions of the manufacturer (International Biotechnologies Inc., New Haven, Conn.).

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TABLE 1. Recombinant plasmids

Designation	Restriction fragment (gene)	Source
pNAS10	4.4-kb NcoI (stxAB)	30
pEW3.0	2.5-kb BglII-EcoRI (stxAB)	13
pJH356	1.2-kb BglII-EcoRV (stxA)	9
pJH4.1	0.87-kb BglII-NcoI (stxA')	9
pJH7.0	0.97-kb BglII-NruI (stxA')	9
pJH230	1.1-kb BglII-SspI (stxA')	9
pJN26	1.5-kb HindIII-NcoI (slt-IB)	19

Oligonucleotide-directed site-specific mutagenesis. Synthetic oligonucleotides were provided by the Wayne State University Biochemistry Department core facility with an Applied Biosystems model 380A DNA synthesizer with phosphoramidite chemistry. The synthetic oligonucleotides used in this study are listed in Table 2.

Restriction endonuclease sites and codon changes were introduced in the *stxA* gene by site-directed mutagenesis by using the double-primer method described by Zoller and Smith (36). The template used for mutagenesis was the single-stranded form of M13mp18 carrying a 1.8-kb HindIII-EcoRI fragment which contains the downstream sequences of the *stxA* gene and the entire *stxB* gene on the noncoding strand (13). The fidelity of the *stxA* gene sequence carrying the amber mutations was confirmed by nucleotide sequence analysis (25).

Preparation of crude toxin. Bacterial cultures were grown overnight at 37°C with agitation and were lysed directly or following a 10-fold concentration in the culture supernatant by sonication (21) with a model 300 sonic dismembrator (Fisher, Itasca, Ill.). Sonic lysates were clarified by centrifugation at 8,000 × g for 10 min. The protein concentrations of the clarified sonic lysates were measured spectrophotometrically by using a protein assay kit (Bio-Rad).

Receptor analog ELISA. The receptor analog ELISA used to assess the association of A and B subunits was performed as described previously (34). The STX receptor analog, galactose-α-1,4-galactose conjugated to bovine serum albumin, was purchased from Carbohydrate International (Chicago, Ill.). Monoclonal antibody 4F7.3 to the STX A subunit (8) was used at a 1:3,500 dilution. Monoclonal antibody 4F7.3 has been shown to react with an StxA$_1$ epitope in a previous study (9). ELISA values were expressed relative to activities in sonic lysates prepared from strains encoding wild-type STX.

Cytotoxicity assays. Microcytotoxicity assays with Vero cells were according to the procedure described by Gentry and Dalrymple (6).

Minicell analysis. The minicell-producing strain, *E. coli* X1141 (5), was used for the expression of the wild-type toxins and the mutated forms of STX encoded by the plasmids

constructed in this study. Minicell experiments were performed by the method described by Meagher et al. (17). The ^{35}S-labeled proteins in the minicell periplasmic extracts were immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Whole-cell labeling. Periplasmic proteins from 3-ml overnight cultures grown in the presence of 1 mCi of an [^{35}S]methionine and [^{35}S]cysteine mixture (DuPont/NEN, Wilmington, Del.) were extracted with polymyxin B sulfate. ^{35}S-labeled polypeptides were immunoprecipitated and analyzed by SDS-PAGE.

Immunoprecipitation. STX subunit association was assessed by using immunoprecipitation (15) of periplasmic extracts from the ^{35}S-labeled minicells and whole bacterial cells. Monoclonal antibody 13C4, which is specific for the STX B subunit (31), was concentrated 30-fold for immunoprecipitation.

RESULTS

Proteolytic processing of StxA. Separation of StxA into the A$_1$ and A$_2$ polypeptides is the result of proteolytic nicking and reduction of a single disulfide bond. A previous study demonstrated that the StxA nicking site is located between Ala-253 and Ser-254 (33). Site-directed mutagenesis was used to introduce conservative amino acid substitutions and to investigate the importance of Ala-253 and Ser-254 in the proteolytic processing of the A subunit. The mutagenic oligonucleotide designated A253G,S254T (Table 2) was used to introduce the double conservative amino acid substitutions Ala-253 to Gly and Ser-254 to Thr. Periplasmic extracts from *E. coli* HB101 carrying the *stx* operon with the A253G,S254T mutation expressed 100% binding in the receptor analog ELISA and wild-type levels of cytotoxicity (Table 3). In support of these findings, immunoprecipitation of minicell extracts containing StxA with the A253G,S254T substitutions demonstrated that alteration of the amino acids which constitute the nicking site had no effect on subunit association. Full-length StxA and mature StxB were immunoprecipitated by a monoclonal antibody directed against the B subunit (Fig. 1, lane F).

The nicking site was also targeted by site-directed mutagenesis to substitute Ser-254 with an amber termination codon (S254Am [Table 2]). Introduction of the *stxA* gene encoding the S254Am mutation in the nonsuppressor host MC4100 resulted in the expression of a truncated StxA which terminated at Ser-254 and was not active in the receptor analog ELISA or cytotoxicity assay (Table 3). Introducing the *stxA* gene encoding the S254Am mutation into the double suppressor host ED8767 (*supE supF*) resulted in the expression of a full-length StxA with Ser-254 replaced by a Gln or a Tyr at an efficiency of 81% (14). This StxA with the S254Q and S254Y substitutions expressed 10^4 50% cytotoxic doses (CD$_{50}$) per ml

TABLE 2. Synthetic oligonucleotides used for site-directed mutagenesis

Designation	Sequence^a	Position^b
S254Am	GCCAGAATGGCATAGGATGAG	974–994
C261Am	CCTTCTATGTAGCCGGCAGATGG	1000–1020
S279Am	TTGTGGGATTAGTCCACTCTGGGG	1053–1063
M287Am	GGGGCAATTCTGTAGCGCAGAACT	1073–1096
T290Am	CTGATGCGCAGATAGATTAGC	1082–1102
A253G,S254T	GCCAGAATGGGAACTGATGAG	974–994
R288G,R289G	ATTCTGATGGGCGGAACTATTAGC	1079–1102
W277Am,D278Am	AAAATATTGTAGTAGTCATCCACTCTG	1046–1072

^{a} Underlined nucleotides were mutated from the wild-type *stxA* sequence.
^{b} *stxA* gene positions from Strockbine et al. (30).

TABLE 3. Subunit association with mutated STX A polypeptides

Host[a]	Mutation	ELISA (%)[b]	Cytotoxicity (CD$_{50}$/ml)
MC4100	Wild type	100	10^7
	S254Am	0	0
	C261Am	10	0
	W277Am,D278Am	2	0
	S279Am	47	10^3
	M287Am	43	10^3
	T290Am	97	10^5
HB101	Wild type	100	10^7
	S254Am	3	0
	C261Am	8	0
	W277Am,D278Am	1	0
	S279Am	60	10^3
	M287Am	30	10^2
	T290Am	86	10^7
	R288G,R289G	100	10^6
	A253G,S254T	100	10^7
ED8767	Wild type	100	10^7
	S254Am	58	10^4
	C261Am	71	10^2
	W277Am,D278Am	2	0
	S279Am	60	10^5
	M287Am	47	10^4
	T290Am	98	10^7

[a] MC4100, sup^0; HB101, supE = glnV; ED8767, supE supF = glnV tyrT.
[b] Expressed as a percentage of the wild-type optical density value.

in the cytotoxicity assay and 58% receptor analog binding (Table 3). Immunoprecipitation of the STX A polypeptide with the S254Am mutation by using an StxB monoclonal antiserum corroborated these findings. The mutated STX A polypeptide was immunoprecipitated along with StxB when the *stxA* gene containing the S254Am mutation was expressed in the minicell strain X1411 which has the capacity to suppress amber termination mutations (Fig. 1, lane B), while expression in the nonsuppressor host MC4100 resulted in no holotoxin assembly (Fig. 2A, lane A).

Association of StxB with StxA truncated by using restriction endonuclease sites. The *stxA* gene was progressively shortened at the 3′ terminus by using naturally existing restriction sites and the introduction of termination codons. The truncated
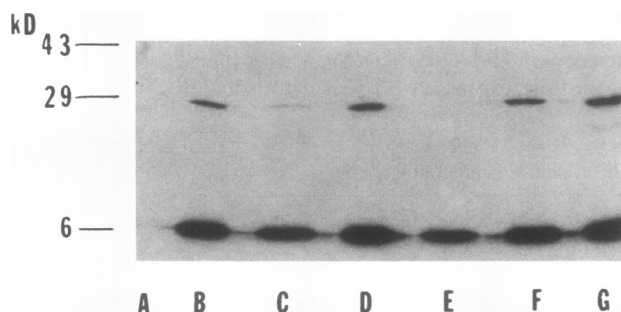

FIG. 1. Immunoprecipitation of ^{35}S-labeled polypeptides synthesized by the *E. coli* minicell strain X1411 with a monoclonal antibody to StxB. Polypeptides were encoded by pBR329 (lane A), pEW3.0 with S254Am (lane B), C261Am (lane C), T290Am (lane D), pNAS10 with the *Hpa*I-*Nru*I deletion (lane E), pEW3.0 with A253G,S254T (lane F), or pNAS10 (lane G). The numbers on the left represent molecular masses in kilodaltons.
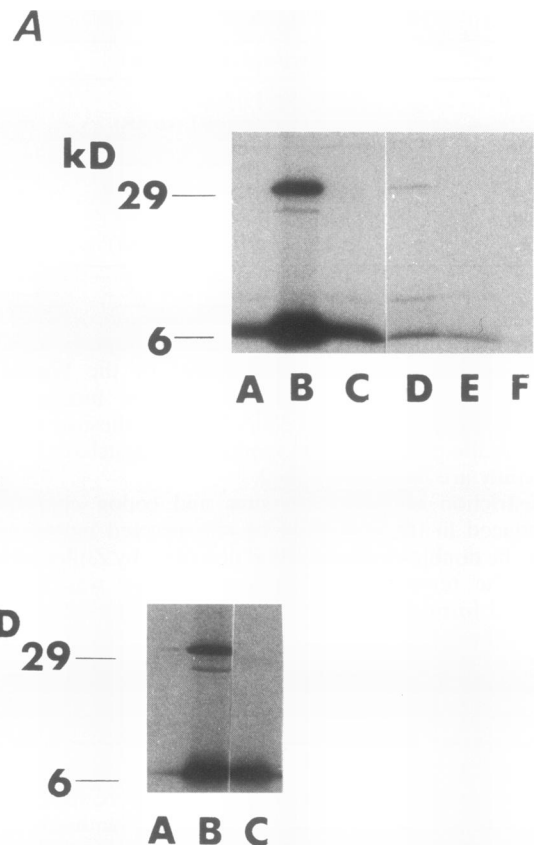

FIG. 2. Immunoprecipitation of ^{35}S-labeled polypeptides produced by the nonsuppressor host MC4100 by using a monoclonal antibody to StxB. (A) pEW3.0 with S254Am (lane A); pNAS10 (lane B); pEW3.0 with C261Am (lane C), T290Am (lane D), or M287Am (lane E); pBR329 (lane F). (B) Lanes: A, pBR329; B, pNAS10; C, pEW3.0 with S278Am. The numbers on the left for both panels are molecular masses in kilodaltons.

STX A polypeptides encoded by pJH4.1, pJH7.0, and pJH230 (Fig. 3) were complemented with STX B subunits encoded by pJN26 (19) (Table 1). Sonic lysates of the *E. coli* HB101 cotransformants were assessed for subunit association by the cytotoxicity assay and receptor analog ELISA with an StxA monoclonal antibody.

An *Nco*I site introduced in a previous study (12) was used to eliminate 80 C-terminal residues from the STX A polypeptide encoded by pJH4.1, and an *Nru*I site that exists upstream of the codons for the proposed nicking site residues (Ala-253 and Ser-254) (33) was used to eliminate 46 amino acids from the StxA C terminus encoded by pJH7.0 (Fig. 3) (Table 1). Because the recombinant strategies used for the construction of pJH4.1 and pJH7.0 resulted in the addition of up to 30 vector-encoded residues to the C termini of these truncated STX A polypeptides, subunit association was disrupted either by truncation or by the addition of these vector-encoded amino acids. Neither subunit association nor cytotoxic activity was detected following complementation with STX B subunits (Table 4). StxA was shortened by 18 C-terminal amino acids by ligating a 1.1-kb *Bgl*II-*Ssp*I fragment of pNAS10 to the *Bam*HI-*Hinc*II-restricted pACYC184 vector (Fig. 3). The resultant plasmid, designated pJH230 (Table 1), encoded a 275-amino-acid STX A polypeptide and two additional vector-encoded amino acids. Subunit complementation with this 277-amino-
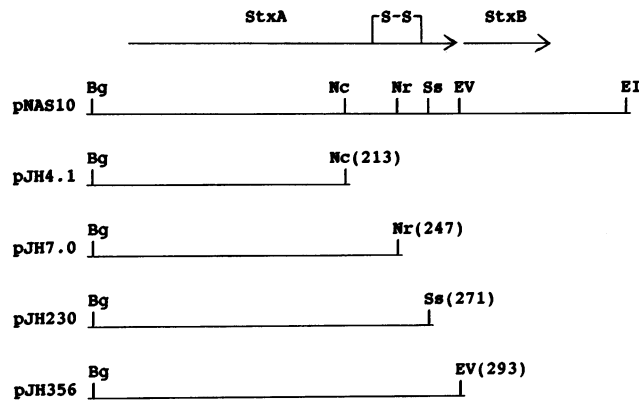
FIG. 3. Restriction maps of the recombinant *stxA* gene. Restriction endonuclease sites were used to introduce 3' deletions in the *stxA* gene. Amino acid positions of the STX A polypeptide are given in parentheses next to the restriction sites. Abbreviations: Bg, *Bgl*II; EI, *Eco*RI; EV, *Eco*RV; Nc, *Nco*I; Nr, *Nru*I; and Ss, *Ssp*I.

acid StxA polypeptide failed to result in holotoxin formation, as determined by the receptor analog ELISA and cytotoxicity assay (Table 4). Only the full-length StxA (293 amino acids) encoded by pJH356 (Fig. 3) (Table 1) associated with the B subunits encoded by pJN26 to give wild-type levels of receptor analog binding and cytotoxicity (Table 4).

The failure of the 277-amino-acid StxA encoded by pJH230 to associate with homologous B subunits indicated that the deletion of 18 amino acids from the C terminus of StxA abrogated its ability to associate with B subunits and form the holotoxin. Therefore, site-directed mutagenesis was used to introduce amber termination codons in the *stxA* gene 3' to the *Ssp*I site which was used to construct pJH230.

Association of StxB with StxA truncated by the introduction of termination codons. Site-directed mutagenesis was used to introduce amber termination codons in the *stxA* gene and progressively shorten the STX A polypeptide. The mutated *stxA* gene was introduced into three different *E. coli* hosts which carried either a single suppressor mutation (HB101), a double suppressor mutation (ED8767), or no suppressor mutation (MC4100). This technique was used to investigate the role of $StxA_2$ amino acid residues 276 to 293, which were deleted from the pJH230-encoded polypeptide, in the association with the STX B pentamer. By using the mutagenic oligonucleotides designated T290Am, M287Am, S279Am, C261Am, S254Am, and W277Am,D278Am, respectively (Table 2), amber termination mutations were introduced at codons corresponding to the following residues of the mature StxA: Thr-290, Met-287, Ser-279, Cys-261, Ser-254, and a double mutation at Trp-277 and Asp-278. In addition, the Arg residues at positions 288 and 289 of the StxA C terminus were

TABLE 4. Subunit association with truncated StxA[a]

Plasmid	StxA length (amino acids)	ELISA (%)[b]	Cytotoxicity (CD_{50}/ml)
pJH356	293	100	10^6
pJH230	275	1	0
pJH7.0	247	2	0
pJH4.1	213	7	0

[a] SLT-I B polypeptides provided in *trans* by pJN26 (19).
[b] Expressed as a percentage of the wild-type optical density value.

replaced with the amino acid Gly by using a single mutagenic oligonucleotide, designated R288G,R289G (Table 2).

The importance of StxA conformation in holotoxin assembly was investigated by introducing an amber termination mutation at Cys 261 by using the oligonucleotide designated C261Am in Table 2. Because StxA has a single disulfide bond (30), alteration of either of the two Cys residues would disrupt the tertiary structure of the polypeptide. Sonic lysates of *E. coli* MC4100, HB101, and ED8767 harboring the *stxA* gene with the C261Am mutation were subjected to the receptor analog ELISA, cytotoxicity assay, and immunoprecipitation. Neither receptor analog binding nor cytotoxic activity was detected in lysates of MC4100 carrying the recombinant *stx* operon with the C261Am mutation (Table 3), although this 261-amino-acid STX A polypeptide expressed full enzymatic activity with an in vitro translation system (9). Loss of receptor analog binding and cytotoxicity as a result of the C261Am mutation was supported by the failure to immunoprecipitate the 260-amino-acid StxA with a monoclonal antibody to StxB (Fig. 2A, lane C). Immunoprecipitation of StxA with the C261Am mutation encoded by the minicell strain *E. coli* X1411, which ostensibly carries a single *sup* mutation, revealed barely detectable levels of A and B subunit association (Fig. 1, lane C). Only StxA with the C261Am mutation expressed by the double suppressor host ED8767, which would replace Cys-261 with a Gln or a Tyr residue, possessed the capacity to associate with the B subunits in the receptor analog ELISA, although the cytotoxicity levels of this mutated STX molecule remained relatively insignificant (Table 3). This indicates that a full-length but unfolded STX A polypeptide expressed by the suppressor hosts ED8767, HB101, and X1411 was enzymatically active and retained the capacity to associate with the STX B pentamer but was incapable of entering and intoxicating Vero cells. While both assays require A and B subunit association, the cytotoxicity assay is more sensitive than the receptor analog ELISA because holotoxin must bind to the native receptor prior to endocytosis. In contrast, the ELISA requires binding to an analog of the receptor association and recognition by the appropriate monoclonal antibody.

Expression of the *stxA* gene containing an amber termination mutation at the codon for Thr-290 (designated T290Am) in *E. coli* MC4100 resulted in the production of an STX A polypeptide lacking four amino acids at the C terminus. This 289-amino-acid StxA retained the capacity to associate with B subunits, as assessed by the receptor analog ELISA (97% of wild-type levels), the cytotoxicity assay (10^5 CD_{50}/ml) (Table 3), and SDS-PAGE of the immunoprecipitated toxin (Fig. 2A, lane D). While the receptor analog values were relatively unaffected, the cytotoxic activity of StxA with the T290Am mutation was increased by 2 orders of magnitude when the mutated *stxA* gene was expressed in *E. coli* HB101 or ED8767, which suppressed the amber mutation and produced full-length StxA with substitution of Gln or Tyr for Thr at position 290 (Table 3). These results suggested that the C-terminal four amino acids of the STX A subunit were not required for subunit association but were required for the expression of wild-type cytotoxicity levels.

Expression of the *stxA* gene with the M287Am and S279Am mutations in the nonsupressor host MC4100 resulted in the production of an STX A polypeptide lacking 7 and 15 amino acids from the C terminus, respectively. These polypeptides were less than 50% reactive in the receptor analog ELISA and expressed 10^3 CD_{50}/ml in the cytotoxicity assay (Table 3), although both of these truncated A subunits retained wild-type levels of enzymatic activity (9). Immunoprecipitation analyses supported the observation that the deletion of residues 279 to

287 from the StxA C terminus dramatically reduced subunit association (Fig. 2). StxA with the S279Am mutation expressed 47% activity in the receptor analog ELISA (Table 3) and was barely detectable in the immunoprecipitation assay (Fig. 2B, lane C), while the M287Am mutation resulted in 43% receptor analog binding and no detectable subunit association in the immunoprecipitation assay (Fig. 2A, lane E).

Expression of the *stxA* gene with the S279Am and M287Am mutations in the suppressor host HB101 or ED8767 increased receptor analog binding and cytotoxicity values, although the levels were significantly less than those of the wild type (Table 3). These results indicate that, unlike the Thr-290 residue which could be replaced with a Gln or Tyr with essentially no effect on subunit association, the residues Ser-279 to Met-287 may play a more significant role in holotoxin assembly.

Because receptor analog binding decreased by over 50% and cytotoxicity was reduced 100-fold by truncating StxA from Thr-290 to Met-287, we predicted that the intervening two residues at positions 288 and 289 may have a critical role in subunit association. Interestingly, positions 288 and 289 are both occupied by Arg, which are the only charged residues in a stretch of 15 amino acids at the C terminus of StxA. Therefore, Arg-288 and Arg-289 were both changed to Gly (R288G,R289G in Table 2) to investigate the role of these residues in holotoxin assembly. While the deletion of Arg-288 and Arg-289 had a significant effect on holotoxin formation (M287Am in Table 3), substituting these two residues with Gly caused only a 10-fold reduction in cytotoxicity and had no effect on receptor analog binding (Table 3).

Because the 277-amino-acid StxA encoded by pJH230 was incapable of associating with the STX B subunits (Table 4) and StxA which was terminated at Ser-279 showed 47% association with the B subunits in the receptor analog ELISA (Table 3), the intervening residues, Trp-277 and Asp-278, were targeted for mutational analysis. The synthetic oligonucleotide designated W277Am,D278Am (Table 2) was used to introduce amber termination codons for these two residues. StxA carrying these mutations in the nonsuppressor host MC4100 was incapable of associating with B subunits, as assessed by the receptor analog ELISA and cytotoxicity assay (Table 3). Unlike the previous amber mutations, suppression of the W277Am,D278Am mutations in HB101 or ED8767 failed to restore receptor analog binding or cytotoxicity (Table 3). In addition, immunoprecipitation analysis revealed that the STX A polypeptide with the W277Am,D278Am mutations expressed in ED8767 did not associate with the STX B subunits (data not shown). These findings implicate these two residues as critical subunit association sites, because the two suppressor hosts HB101 and ED8767 would produce full-length StxA with Trp-277 and Asp-278 replaced by Gln or by Gln and Tyr.

DISCUSSION

As part of a continuing analysis of the structure and function of STX, the present study was conducted to define amino acids in the C terminus of StxA which were required for holotoxin assembly. The capacity of the STX A polypeptide with C-terminal deletions and mutations to associate with the B subunits was assessed. Key A subunit residues required for association with the B pentamer have been identified, and a model for holotoxin assembly has been proposed.

The roles of proteolytic processing and a single disulfide bond in subunit association were investigated by altering the putative nicking site residues Ala-253 and Ser-254 (33) and introducing amino acid substitutions at one of the two Cys residues in StxA. The conservative amino acid substitutions of

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...Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr
   254                 260                                     270

His Asn Lys Ile Leu{Trp Asp}Ser Ser Thr Leu Gly Ala Ile Leu Met{Arg Arg}Thr
                             280                                 290

Ile Ser Ser
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FIG. 4. Sequence of the StxA$_2$ residues which interact with the StxB pentamer to form the holotoxin. The nine StxA$_2$ residues which are underlined penetrate the neutral pore of the StxB pentamer. Amino acids shown in brackets (Trp-278, Asp-279, Arg-288, and Arg-289) stabilize the StxA$_2$-StxB association.

Ala-253 to Gly and Ser-254 to Thr had no effect on cytotoxicity or enzymatic activity (9), indicating that the specific nicking site residues were not important for activation of StxA. Perhaps proteolytic processing of the STX A subunit into A$_1$ and A$_2$ polypeptides is not dictated by a sequence-dependent protease but is dependent on folding which exposes the nicking site.

StxA possesses a single intrachain disulfide bond linking the A$_1$ and A$_2$ polypeptides. Reduction of this disulfide bond in an endocytotic vesicle of the target cell presumably causes release of the enzymatic A$_1$ polypeptide (16, 22). While substitution of Cys-261 with a Gln or Tyr by suppression of the C261Am mutation in ED8767 did not prevent A and B subunit association as assessed by the receptor analog ELISA and immunoprecipitation, it caused a significant reduction in cytotoxic activity. This indicated that StxA which lacked its single disulfide bond retained some capacity to associate with the B subunits, although recognition of the native cell surface receptor by this toxin or release and translocation of the A$_1$ polypeptide into the cytoplasm of the target cell were significantly altered.

Removal of the last four amino acids from the StxA C terminus (T290Am) had little effect, while extension of the C-terminal deletion to Met-287 caused a substantial reduction in holotoxin formation. This finding implicated Arg-288 and Arg-289, the only charged amino acids present in a stretch of 15 nonpolar amino acids at the C terminus of StxA, as key residues which may interact with other charged amino acids outside the nonpolar pore of the StxB pentamer. However, substitution of these two Arg residues with Gly (R288G,R289G) caused only a 10-fold reduction in cytotoxicity, with no effect on receptor analog binding. Therefore, while the M287Am and T290Am mutations established the importance of the intervening amino acids in subunit assembly, the R288G,R289G mutations demonstrated that these residues may be either basic or uncharged. The influence of substituting one or both of these Arg residues with an acidic amino acid could be used to investigate the role of ionic interactions between the STX A$_2$ polypeptide and the B pentamer in holotoxin assembly. In contrast to the Arg residues at positions 288 and 289 of StxA which could be replaced by Gly, the deletion or substitution of Trp at position 277 and Asp at position 278 totally abolished subunit association.

Collectively, the results obtained from the set of truncated and mutated STX A polypeptides facilitated the establishment of a model for the interaction between the STX A polypeptide and the B subunit pentamer. A sequence of nine nonpolar amino acids between residues 279 and 287 of StxA$_2$ may penetrate the B pentamer pore (Fig. 4). Trp-277, Asp-278, Arg-288, and Arg-289 form the boundaries of this nine-residue sequence. Perhaps these four residues stabilize the noncovalent association between subunits through interactions with

other aromatic or charged amino acids present outside the neutral pore on the planar surfaces of the B subunit pentamer. Disruption of the A subunit conformation by elimination of the single disulfide bond did not prevent A and B subunit association, indicating that the StxA$_1$ polypeptide had no significant role in holotoxin assembly. These findings support the model for holotoxin assembly derived from the crystallographic analysis of LT (27) and may be used to corroborate future structural analyses of the STX family members.

ACKNOWLEDGMENT

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