Investigation of Ribosome Binding by the Shiga Toxin A1 Subunit, Using Competition and Site-Directed Mutagenesis

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Shiga toxin (STX) is a multimeric molecule consisting of a single 32-kDa enzymatic A subunit (StxA) covalently associated with a ring of five 7.7-kDa B subunits (StxB) (17). The StxB subunits mediate the binding of toxin to its receptor on eukaryotic membranes, and the entire STX molecule enters cells by endocytosis from coated pits (28). StxA is processed in the trans-Golgi network by the cellular protease furin (11) to release StxA1, which possesses the enzymatic activity, and StxA2, which acts as a bridge between StxA1 and the B pentamer. StxA1 is an N-glycosidase which catalytically removes a specific adenine from a well-conserved tetra-loop of 28S rRNA and thus inhibits protein synthesis by preventing the binding of aminoacyl tRNAs to the ribosome and halting protein elongation (7). StxA1 is a member of the ribosome-inactivating protein (RIP) family of enzymes, which are a large group of protein synthesis inhibitors primarily found in the leaves, seeds, and rootsof dicotyledonous plants (5, 7, 30). The only protein synthesis inhibitors primarily found in the leaves, seeds, and rootsof dicotyledonous plants (5, 7, 30). The only RIPs identified so far which are not produced by plants are the members of the STX family of toxins.

The catalytic sites of StxA1 have been identified by sequence comparison of RIP family members and site-specific mutagenesis. Amino acid substitutions in three regions of StxA1 which are highly conserved among the RIPs identified tyrosine 77, glutamic acid 167, arginine 170, and tryptophan 203 as important. These residues include the ribic A chain as well as other examples of plant toxins. StxA1 catalytically depurinates a well-conserved GAGA tetra-loop of 28S rRNA which lies in the acceptor site of eukaryotic ribosomes. The specific activities of native StxA1, as well as mutated forms of the enzyme with substitutions in catalytic site residues, were measured by a n vitro translation assay. Electroporation was developed as an alternative method for the delivery of purified A1 polypeptides into Vero cells. Site-directed mutagenesis coupled with N-bromosuccinimide modification indicated that the sole tryptophan residue of StxA1 is required for binding it to the 28S rRNA backbone. Northern analysis established that the catalytic site substitutions reduced enzymatic activity by specifically interfering with the capacity of StxA1 to depurinate 28S rRNA. Ribosomes were protected from StxA1 by molar excesses of tRNA and free adenine, indicating that RIPs have the capacity to enter the acceptor site groove prior to binding and depurinating the GAGA tetra-loop.

The enzymatic subunit of Shiga toxin (StxA1) is a member of the ribosome-inactivating protein (RIP) family, which includes the ricin A chain as well as other examples of plant toxins. StxA1 catalytically depurinates a well-conserved GAGA tetra-loop of 28S rRNA which lies in the acceptor site of eukaryotic ribosomes. The specific activities of native StxA1, as well as mutated forms of the enzyme with substitutions in catalytic site residues, were measured by an in vitro translation assay. Electroporation was developed as an alternative method for the delivery of purified A1 polypeptides into Vero cells. Site-directed mutagenesis coupled with N-bromosuccinimide modification indicated that the sole tryptophan residue of StxA1 is required for binding it to the 28S rRNA backbone. Northern analysis established that the catalytic site substitutions reduced enzymatic activity by specifically interfering with the capacity of StxA1 to depurinate 28S rRNA. Ribosomes were protected from StxA1 by molar excesses of tRNA and free adenine, indicating that RIPs have the capacity to enter the acceptor site groove prior to binding and depurinating the GAGA tetra-loop.

Materials and Methods

Bacterial strains and recombinant plasmids. The following strains of Escherichia coli were used in this study: HB101 (supE44 thiS20 rifA15 ara-14 proA2 lacY1 galK2 spdL20 yfi-S mal-1) (1), BL21(DE3) [F lacD1 lacB galompT r m l avi ip3 k A r s u l b c r s c e x ym carb-hsdS28-eryA171 bsc reB ura3-59 cysThr5 (Kan')] (2), and SURE [lacI5 recJ umuC::Tn (Kan')] (31). M15Tn2 (31), and SURE{e14- (Tetr)} M15Tn2 (31), and SURE{e14- (Tetr)}

Preparation of crude toxin extracts. Crude toxin extracts were prepared by sonication of bacterial cultures. Thirty-milliliter overnight cultures were concentrated 10-fold in culture supernatant by centrifugation and lyzed by sonication with a Model 300 Sonic Disembrator (Fisher). Cells were sonicated at 50% power for a total of 3 min in 15-s bursts. Lysates were clarified by centrifugation and filter sterilized. Total protein content was measured, and samples were normalized to contain equivalent concentrations.

Column purification of native and mutated StxA1. The recombinant pET22N (+) vectors carrying native and mutated StxA1 genes were transformed into the E. coli host BL21(DE3)pLysS by the calcium chloride-heat shock method (27). BL21(D3pLysS permits the IPTG (isopropyl-β-D-thiogalactoside) induction of genes carried by the pET vectors (13). BL21(DE3)pLysS growth and protein induction were conducted according to the manufacturer’s (Novagen, Madison, Wis.) recommendations.

Nickel (Ni²⁺-nitrilotriacetic acid) affinity columns (Qiagen, Chatsworth, Calif.) were used to purify wild-type and mutated StxA1. The pET22 vector produces a fusion protein with six His residues at the C terminus which binds with high affinity to the Ni²⁺ resin. Weakly bound proteins were removed by washing with 15 ml of a buffer containing a low concentration of imidazole (30 mM imidazole, 0.5 M sodium chloride, 20 mM Tris [pH 7.9]), and the desired proteins were eluted with 15 ml of 1 M imidazole in the same buffer. One-milliliter fractions were collected, and 20-μl samples were analyzed by sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis. Fractions containing a unique 29-kDa protein were dialyzed overnight against 4 liters of phosphate-buffered saline (0.13 M sodium chloride, 5.5 mM sodium phosphate dibasic, 3.2 mM potassium phosphate monobasic [pH 7.4]) at 4°C. Protein content was determined with the Bio-Rad Protein Assay Kit with bovine serum albumin standard, and samples were stored in aliquots at −70°C. The STX A1 polypeptide purified by this system contained six His residues at the C terminus.

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TABLE 1. Recombinant plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description*</th>
<th>Reference</th>
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<tr>
<td>pEW3</td>
<td>stxAB</td>
<td>19</td>
</tr>
<tr>
<td>pMJ230</td>
<td>stxAB (ΔL201-Y213 in StxA)</td>
<td>18</td>
</tr>
<tr>
<td>pLS10</td>
<td>stxA in pET22b(+)</td>
<td>This study</td>
</tr>
</tbody>
</table>

* All recombinant plasmids, with the exception of pLS10, were constructed with pBR329 (27).

RIBOSOME BINDING BY SHIGA TOXIN A1 SUBUNIT

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoFOR</td>
<td>GACCGAGAATTCATTAAGGTGACACTCTTCTT</td>
</tr>
<tr>
<td>NorREV</td>
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<td>BamHI</td>
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<td>NotI</td>
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<td>XhoI</td>
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<td>XbaI</td>
<td>TCTCCCATCTGCGGACATAGAAGGAACTC</td>
</tr>
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<td>RNA probe I</td>
<td>TGAAGCCTGGTTTCCTCGTGTCATG</td>
</tr>
<tr>
<td>RNA probe II</td>
<td>CAAAATGTCTGAACTCCGGGTTCTC</td>
</tr>
</tbody>
</table>

* Mutated residues are indicated in boldface; restriction sites introduced by PCR are underlined.

a Lowercase “c” corresponds to adenine derived from 28S rRNA by RPs.
solution C (H$_2$O-saturated phenol containing 0.04% octylhydroxyquinoline) by mixing vigorously for 10 s and incubating on ice for 15 min. After centrifugation for 10 min at 4°C, the aqueous phase was removed and reextracted with 500 μl of solution D (5 parts phenol phase of solution C plus 1 part chloroform-isooamyl alcohol [24:1]). The final aqueous phase (500 μl) was precipitated with 1 ml of ice-cold ethanol.

Following overnight precipitation at -20°C, RNA was collected by microcentrifugation at full speed for 10 min at 4°C and resuspended in 10 μl of H$_2$O. RNA concentration was determined spectrophotometrically, with 1 A$_{260}$ unit = 40 μg/ml.

**Northern blot analysis.** Anilin was used to cleave the tRNA backbone at the site depurinated by StxA1 or RTA according to the method of Sallustio and Stanley (26). Briefly, 6.6 μg of RNA was mixed with 13.2 μl of freshly prepared 1.0 M aniline buffer (50 μl of glacial acetic acid, 700 μl of H$_2$O, 100 μl of aniline, and 250 μl of 1.0 M acetic acid), and samples were incubated on ice in the dark for 10 min. The reaction was terminated by the addition of 200 μl of H$_2$O, and the aniline was removed by evaporation of the sample to dryness in vacuo.

Aniline-treated whole-cell RNA or equivalent concentrations of untreated RNA were mixed with 15 μg of glyoxal-dimethylsulfoxide denaturing solution (1.8 ml of 6 M glyoxal, 5.3 ml of dimethyl sulfoxide, 1.0 ml of 100 mM sodium phosphate [pH 7.0], and 1.8 ml of H$_2$O) and incubated at 50°C for 1 h prior to electrophoresis in 1.4% agarose.

Following electrophoresis, gels were stained briefly with ethidium bromide and the RNA was visualized by the IS-1000 Imaging System (Alpha Innotech Corp., San Leandro, Calif.). Gels were soaked in 0.05 M sodium hydroxide for 20 min, rinsed with H$_2$O, and soaked for 45 min in 20× SSC (0.3 M sodium chloride, 0.3 M sodium citrate [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). RNA was transferred overnight to ZetaProbe GT nylon membranes (Bio-Rad) which were baked for 1.5 h at 80°C in vacuo to immobilize the nucleic acids. Membranes were incubated at 65°C for 3 h in 10 ml of a hybridization solution consisting of 30% (vol/vol) 20× SSC, 0.2% (wt/vol) SDS, 0.2% (wt/vol) Ficoll type 400, 0.2% (wt/vol) bovine serum albumin fraction V, 0.2% polyvinylpyrrolidone, and 200 μg of sheared DNA per ml RNA probes I and II (Table 2) were prepared by 5’ end labeling 20 μl of the oligonucleotides with 50 μCi of γ-$^32$P-ATP (3,000 Ci/mmol) in a 20-μl reaction mixture consisting of 2 μl of 10× kinase buffer (1 M Tris [pH 8.3], 0.1 M magnesium chloride, 0.1 M DTT) and 5 U of T4 kinase (Bethesda Research Laboratories, Bethesda, Md.). RNA probe I is complementary to positions 4557 to 4582 in the human 28S rRNA site (23) and spans the site depurinated by RIPs. RNA probe II is complementary to positions 4566 to 4591 in human 28S rRNA and is immediately 5’ to the depurination site. RNA blots were incubated overnight in hybridization solution containing 10 μl of the labeled RNA probe I or II. Blots were washed with 6× SSC at 50°C and exposed to X-ray film for 5 to 6 h with a Coronex Lightning Plus intensifying screen (Dupont, Wilmington, Del.). The IS-1000 Imaging System (Alpha Innotech Corp.) was used to quantify the concentration of RNA in each lane of the Northern blots.

**RESULTS**

**Vero cell cytotoxicity of wild-type and mutated STX.** Vero cells were treated with a 10$^5$-fold dilution of crude preparations containing native STX and STX with the E167D and W203A/G204A/R205A subunit substitutions. Figure 1 shows the average results of six separate experiments. STX reduced cell viability to nearly 0, while STX-E167D was approximately fivefold less cytotoxic. STX-W203A/G204A/R205A was approximately eightfold less cytotoxic than native STX. These cytotoxicity assays provided a qualitative assessment of the effects of the amino acid substitutions on activity since the concentrations of native and mutated STX in the crude lysates may vary.

**Enzymatic activity of native and mutated StxA1.** An in vitro translation assay programmed with luciferase mRNA was used to assess the enzymatic activities of wild-type and mutated StxA1 which was purified by the pET expression system. The concentration of A1 polypeptide which reduced translation (monitored by using luciferase levels) by 50% in comparison to untreated controls was expressed as the 50% ribosome inhibitory concentration (RIC$_{50}$). Because the pET system was used for protein purification, the STX A1 polypeptide used in this study carried six His residues at the C terminus which may have affected activity.

RIC$_{50}$(s) (in nanomolars) of protein synthesis inhibitors were as follows: StxA1, 0.009; StxA1-E167D, 18.0; StxA1-W203A/G204A/R205A, 2.0 × 10$^3$; ricin A, 0.048; puromycin, 160.0. Native StxA1 and the RTA were the most potent inhibitors of protein synthesis, with RIC$_{50}$ of 9 and 48 pM, respectively. StxA1 with the catalytic site mutation E167D was approximately 10$^3$-fold less active than the native enzyme with an RIC$_{50}$ of 18 nM. The W203A/G204A/R205A substitutions, which involved residues in the putative RNA-binding domain of StxA1, reduced the RIC$_{50}$ of the polypeptide to 2.0 μM. Puromycin, which was used as a control because it is a structural analog of aminoacyl-tRNA, had a RIC$_{50}$ of 160 nM.

**Competition between StxA1 and adenine or tRNA.** Since adenine is the product of the StxA1-catalyzed depurination of tRNA (8), it presumably interacts directly with the active site of the enzyme. Attempts to demonstrate binding of $^3$H]adenine to the active site of StxA1 by equilibrium dialysis and immunoprecipitation were unsuccessful. However, preincubation of StxA1 with adenine prior to treatment of the rabbit reticulocyte lysate in an in vitro translation assay did abrogate enzymatic activity. Adenine at a final concentration of 48 μM, which was the maximum concentration that did not affect pro-
protein synthesis (Fig. 2 and 3), restored ribosome activity in a lysate exposed to the RIC50 of StxA1 to near-untreated levels (Fig. 3).

The nucleoside monophosphate AMP and triphosphates ATP and GTP were also tested for the capacity to block the enzymatic activity of StxA1 based on their structural relatedness to adenine. Each of these compounds was tested at 48 μM since this concentration had no inhibitory effect on the in vitro translation assay. In contrast to adenine, which inhibited StxA1, neither ATP, GTP, nor AMP protected the ribosomes in vitro (Fig. 3).

The adenine removed from tRNA by RIP is located in the acceptor site groove of the ribosome. Since this is also the location where tRNA binds during translation, we next examined the ability of an excess concentration of tRNA to compete with StxA1 for binding to the ribosome. As with adenine, it was necessary to first determine the highest concentration of tRNA which would not inhibit the in vitro translation assay. As shown in Fig. 4, 120 nM rabbit liver tRNA did not significantly inhibit the ability of ribosomes to translate luciferase mRNA. This concentration of tRNA was added to the reticulocyte lysate simultaneously with 9 pM StxA1 (t = 0) or 5 min after exposure to the enzyme (t = 5). Simultaneous addition of 120 nM tRNA protected ribosomes from complete inactivation by StxA1 and maintained ribosome activity at 70% of the untreated control values (Fig. 3). In contrast, the addition of excess tRNA 5 min after exposure of the ribosomes to StxA1 had no protective effect; the translational capacity of the lysate remained at 50% as expected for the 9 pM RIC50 of StxA1 (Fig. 3).

Oxidation of Trp203 and its effect on StxA1 activity. The increased RIC50 of StxA1-W203A/G204A/R205A (2.0 μM) compared to wild-type StxA1 (9 pM) indicated that the fidelity of these residues is required for activity. In an attempt to assess the contribution of the sole tryptophan at position 203 of StxA1, this residue was chemically modified with NBS.

Unmodified StxA1 at a final concentration of 41 nM completely inhibited protein synthesis (Fig. 5). In contrast, the same concentration of StxA1 which had been modified with NBS reduced translational activity of the rabbit reticulocyte lysate to 28% of that of the untreated control (Fig. 5). Therefore, the selective oxidation of tryptophan 203 by NBS reduced the activity of StxA1 approximately 4,000-fold: the concentration of NBS-modified enzyme required to reduce ribosome activity was 41 nM in comparison to an RIC50 of 9 pM for the untreated A1 polypeptide (Fig. 5).

Northern blot analysis of tRNA isolated from intoxicated Vero cells. In addition to assays which measured the capacity of native and mutated STX to inhibit protein synthesis and affect cell viability, we used Northern blot analysis to directly assess the capacity of the A1 polypeptide to specifically depurinate 28S rRNA in toxin-treated Vero cells. Depurination was visualized as a 400-nucleotide fragment released from the 3′ end of 28S rRNA from cells which had been exposed to STX or ricin and treated with aniline to cleave the phosphodiester backbone. Because RNA probe II (Table 2) was complementary to the region of 28S rRNA immediately 3′ to the site depurinated by RIPs (A-4324 in mammalian 28S rRNA) (8), it will hybridize with intact 28S rRNA or the 400-nucleotide fragment released from the 3′ end of 28S rRNA by exposure to StxA1 or RTA and aniline.

Northern blot analysis showed that exposure of Vero cells to ricin converted 100% of the 28S rRNA to the 400-nucleotide fragment upon treatment with aniline (Fig. 6, lane 6). Interestingly, crude extracts from E. coli HB101(pBR329), which was a negative control for the strain expressing recombinant STX, expressed some depurinating activity in the Northern blot assay (Fig. 6, lane 2). Lanes 3 and 4 of Fig. 6 show the unmodified 28S rRNA from untreated Vero cells.

Northern analysis of rRNA isolated from Vero cells exposed to crude preparations of STX revealed that 87% of full-length 28S rRNA was converted to the 400-nucleotide fragment (Fig. 7, lane 2). Substitution of the active site glutamic acid residue at position 167 with an aspartic acid reduced the depurinating capacity to 56% of wild-type levels (Fig. 7, lane 4). In contrast to the in vitro translation assays which showed that alteration of tryptophan 203 and adjacent residues profoundly reduced activity, STX with the W203A/G204A/R205A substitutions retained 77% of wild-type depurination activity (Fig. 7, lane 6).

A second oligonucleotide designated RNA probe I (Table 2) was used in Northern blot analysis of rRNA isolated from Vero cells exposed to STX-W203A/G204A/R205A. Because RNA...
probe I is homologous to a sequence of 28S rRNA which spans the RIP depurination site, it simultaneously hybridized with uncleaved 28S RNA and with the 400-nucleotide fragment. Northern analysis with RNA probe I confirmed our previous findings that STX with the W203A/G204A/R205A substitutions in the A subunit retains the capacity to specifically depurinate 28S rRNA (Fig. 8).

**Electroporation of Vero cells.** Since purified forms of native and mutated StxA1 lacked B subunits and were unable to enter Vero cells via receptor-mediated endocytosis, we developed electroporation as an alternate means of delivering and assessing the depurination capacity of these enzymes.

Northern blot analysis was performed on rRNA isolated from Vero cells electroporated with native StxA1 and two mutated forms of the polypeptide. Native StxA1 delivered by electroporation depurinated the 28S rRNA and permitted release of the 400-nucleotide fragment by aniline (Fig. 9, lane 2). A weak band was also detected in lane 3 of Fig. 9, suggesting that StxA1 with the E167D substitution retains some capacity to depurinate 28S rRNA.

**DISCUSSION**

The effects of various amino acid substitutions in the putative active sites of StxA1 have been previously reported (2, 16, 18, 33). Results of the current study provided the specific activities of purified StxA1 and two mutated forms of the polypeptide. The substitution E167D caused a 2,000-fold increase in the RIC50 of StxA1, corroborating earlier mutagenesis studies (16, 18, 33) which showed that this conservative amino acid substitution resulted in a 100- to 1,000-fold reduction in activity by the cell-free translation assay. This glutamic acid has been established as an active site residue among other members of the RIP family of enzymes (24, 29). StxA1 with the W203A-R205A substitution had an RIC50 of 2 μM, confirming a previous mutagenesis study by Yamasaki and coworkers (33) which showed that tryptophan 203 and arginine 205 are important for enzymatic activity.

The target site adenine for RIPs is on a stem-loop structure which protrudes into the acceptor site groove of the large ribosomal subunit (3, 4). This is the RNA domain involved in EF-1-dependent binding of aminoacyl tRNAs to the ribosome and in EF-2-catalyzed GTP hydrolysis and translocation of peptidyl tRNA to the P site. Normally, binding of EF-2 protects 28S rRNA against depurination by ricin (9). However, when Trp-221 of EF-2 was chemically modified with NBS ribosomes, it became sensitive to ricin, indicating that Trp-221 must be part of an amino acid sequence which is involved in the interaction of EF-2 with nucleotides in the ricin-sensitive loop (14). Chemical modification of tryptophan 203 in the STX A1 polypeptide with NBS reduced enzymatic activity 4,000-fold, establishing the role of this residue in activity. Because tryptophan 203 lies in the active site cleft of StxA1 and modification of this residue affects activity, we propose that it interacts with the phosphodiester backbone to properly orient the 28S rRNA prior to depurination.

RIP-mediated cleavage of an N-glycosidic bond in 28S rRNA renders the phosphodiester bonds on either side of the

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**FIG. 7.** Northern blot analysis of rRNA isolated from Vero cells exposed to native and mutated STX. RNA was isolated from Vero cells treated with crude preparations of STX (lanes 1 and 2), STX/E167D (lanes 3 and 4), or STX-W203A/G204A/R205A (lanes 5 and 6) and was hybridized to RNA probe II. The absence (−) or presence (+) of aniline treatment is indicated above each lane. The arrow indicates the 400-nucleotide fragment.

**FIG. 8.** Northern blot analysis of rRNA isolated from Vero cells treated with STX-W203A/G204A/R205A. RNA was isolated from Vero cells treated with a crude preparation of STX-W203A/G204A/R205A (lane 1) or with ricin (lane 2), aniline treated, and hybridized with RNA probe I. The arrow indicates the 400-nucleotide fragment.

**FIG. 9.** Northern blot analysis of rRNA isolated from electroporated Vero cells. RNA was isolated from Vero cells electroporated with a control column fraction (lane 1) or 1.0 × 10^7 molecules of StxA1 (lane 2), StxA1-E167D (lane 3), and StxA1-W203A/G204A/R205A (lane 4) per cell. All samples were treated with aniline and hybridized with RNA probe II. The arrow indicates the 400-nucleotide fragment.
ribose susceptible to a β-elimination reaction which can be catalyzed at an acidic pH by primary amines such as aniline (6). Chain scission releases a small fragment from the 3’ end of rRNA which we quantified by Northern analysis with a specific oligonucleotide probe. Therefore, the effects of amino acid substitutions in the A1 polypeptide were assessed as the conversion of 28S rRNA to the 400-nucleotide fragment. Blobs of rRNA isolated from cells treated with a crude preparation of wild-type STX revealed that 87% of the 28S rRNA was converted to the 400-nucleotide fragment. The E167D substitution in STX reduced this conversion to 56% of the total 28S rRNA in Northern blot assays. Results of the in vitro translation assay using StxA1-E167D corroborated this finding: the enzymatic activity was reduced 1,000-fold in comparison to native STX but was not completely abolished. We hypothesize that the shortened carboxylate side chain of aspartic acid in comparison to glutamic acid was suboptimal for stabilization of the ribose oxycarbonium transition state intermediate which precedes the depurination reaction.

The effects of the W203A/G204A/R205A substitutions in the A1 polypeptide were more puzzling. While the specific activity of this mutated form of StxA1 was reduced 105-fold in comparison to the native A1 polypeptide, Northern blot analysis revealed that 77% of the 28S rRNA isolated from Vero cells intoxicated with STX-W203A/G204A/R205A was converted to the cleaved form. STX carrying these substitutions retains the catalytic site glutamic acid 167 which is required for protonation of the target adenine and stabilization of the oxycarbonium intermediate but is disrupted in the downstream region believed to be involved with stabilizing the interaction of the enzyme with the ribose-phosphate backbone. Therefore, these mutations may have reduced the capacity of StxA1 binding in the relatively short time frame of the translation assay (approximately 2 h) while the longer incubation period required to intoxicate the Vero cells for Northern analysis (16 h) may have permitted additional interactions between the rRNA backbone and amino acids outside the active site cleft of StxA1. These interactions may have been adequate for low-level binding and depurination at a reduced efficiency compared to the wild-type enzyme. The extended incubation time for Vero cell intoxication was required for B subunit-mediated entry and translocation of STX to its target site.

It was necessary to develop electroporation as a means for delivering the purified A1 polypeptide into Vero cells. Due to the inefficiency of electroporation as a delivery method, as well as the high rate of cell death induced by this technique, it was necessary to use a ratio of 1.0 × 107 molecules of StxA1 per Vero cell in these assays. Northern analysis of rRNA isolated from the electroporated cells was consistent with the results with STX. In future studies, the delivery and translocation route of electroporated A1 polypeptide will be compared to the native route mediated by the B subunits.

Competition studies were conducted with tRNA because the adenine removed from 28S rRNA by RIPs is located in the acceptor site groove of ribosomes and the crystal structures of StxA1 and RTA resemble a tRNA molecule. Our results demonstrated that the simultaneous addition of excess tRNA protected ribosomes from inactivation by StxA1 while the addition of tRNA 5 min after StxA1 was not effective. This indicates that StxA1 and tRNA compete for binding to the acceptor site groove of ribosomes.

Since adenine is the product of the N-glycosidase activity of RIPs, we reasoned that it would bind directly to the active site of StxA1 (32). However, attempts to demonstrate direct binding of adenine to the A1 polypeptide by equilibrium dialysis were unsuccessful. This same result was previously reported by Zamboni et al. (34) with RTA. In comparison to the tetra-loop target site in 28S rRNA, free adenine may be too small to bind the RIP active site cleft. Although we could not demonstrate direct binding, a molar excess of adenine was used in the competition experiments to overcome the transient binding of the free base to the active site cleft of StxA1 and effectively inhibit enzymatic activity. These competition studies indicate that it may be possible to develop chemical intervention strategies which could be used therapeutically to protect mammalian cells from intoxication by members of the STX family.

ACKNOWLEDGMENT

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