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Identification of amino acids critical for the cytotoxicity of Shiga toxins 1 and 2 in *Saccharomyces cerevisiae*

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Abstract

Shiga toxins (Stx1 and Stx2) are produced by *E. coli* O157:H7, which is a leading cause of food-borne illness. The A subunits of Stx1 (Stx1A) and Stx2 (Stx2A) are ribosome inactivating proteins (RIPs) that inhibit translation by removing an adenine from the highly conserved α -sarcin ricin loop (SRL) of the large rRNA. Here, we used mutagenesis in *S. cerevisiae* to identify residues critical for cytotoxicity of Stx1A and Stx2A. The A subunits depurinated the SRL, inhibited translation and caused apoptotic-like cell death in yeast. Single mutations in Asn75, Tyr77, Glu167 and Arg176 reduced the cytotoxicity of both toxins around 10-fold. However, Asn75 and Tyr77 were more critical for the depurination activity of Stx2A, while Arg176 was more critical for the depurination activity of Stx1A. The crystal structures of the two proteins lack electron density for some surface loops, including one which is adjacent to the active site in both molecules. Modeling these loops changed neither the secondary nor the tertiary structures of the rest of the protein. Analysis of solvent accessible surface areas indicated that Asn75 and Tyr77 are more exposed in Stx2A, while Arg176 is more exposed in Stx1A, indicating that residues with higher surface exposure were more critical for enzymatic activity. Double mutations at Glu167 and Arg176 eliminated the depurination activity and cytotoxicity of both toxins. C-terminal deletions of A chains eliminated cytotoxicity of both toxins, but showed functional differences. Unlike Stx1A, cytotoxicity of Stx2A was lost before its ability to depurinate ribosomes. These results identify residues that affect enzymatic activity and cytotoxicity of Stx1A and Stx2A differently and demonstrate that the function of these residues can be differentiated in yeast. The extent of ribosome depurination and translation inhibition did not correlate with the extent of cell death, indicating that depurination of the SRL and inhibition of translation are not entirely responsible for cell death.

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Conflict of interest

None.

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Keywords

Shiga toxin; *E. coli* O157:H7; ricin; ribosome inactivating protein; ribosome depurination; apoptosis

1. Introduction

Shiga toxin (Stx) producing *E. coli* (STEC) are foodborne pathogens that can cause severe morbidity and mortality, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Paton and Paton, 1998; Pickering et al., 1994). The recent epidemics attributed to STEC contamination of various food products, drinking water, and recreational water clearly illustrate the public health impact of these pathogens (Maki, 2006). There are no antidotes or therapeutics effective against Stx-mediated HUS, which is the most common cause of renal failure in infants and young children in the US (Siegler and Oakes, 2005). Shiga toxins are a family of AB₅ toxins or type II ribosome-inactivating proteins (RIPs), consisting of an enzymatically active A subunit that associates with a pentamer of identical B subunits (Johannes and Romer, 2010). There are two main types of Shiga toxins, Stx1 and Stx2, with variants of each type. Shiga toxins can bind to eukaryotic cells through interaction between the B pentamer and host receptors on the cell surface that are concentrated in lipid rafts. Most known Stx variants have high binding affinity for the neutral glycolipid globotriaosylceramide (Gb3), although other receptors exist (Jacewicz et al., 1986; Lingwood et al., 1987; Waddell et al., 1988). After binding, the holotoxin is internalized by clathrin-mediated endocytosis (Sandvig et al., 2002) and traffics retrograde through the Golgi apparatus. The A subunit of the Shiga toxins can be proteolytically cleaved into an enzymatically active A₁ chain and an A₂ chain, which remains associated with the B pentamer (Garred et al., 1995). In the endoplasmic reticulum (ER) the A₁ chain is released from the A₂-B₅ complex by reduction of the disulfide bond and undergoes retrotranslocation from the ER into the cytosol (Sandvig and van Deurs, 2005). The A₁ domains of Stx1 and Stx2 are *N*-glycosidases that specifically remove a universally conserved adenine from the α -sarcin/ricin loop (SRL) of the large rRNA, resulting in inhibition of protein synthesis in the elongation phase (Endo et al., 1988).

The Stx1 and Stx2 holotoxins have 55 and 57% sequence identity in the A and B subunits respectively. While Stx1 is highly conserved and differs by only one amino acid from Shiga toxin (Stx) produced by *Shigella dysenteriae*, greater sequence variation exists within Stx2 family members (O'Brien et al., 1992). Very few mutagenesis studies have been conducted to identify residues critical for Stx/Stx1 and Stx2 function. Early studies, which were carried out using the holotoxins identified Tyr77, Glu167, Arg170, Tyr114 and Trp203 of the A subunits as important for catalytic activity (Cao et al., 1994; Deresiewicz et al., 1993; Deresiewicz et al., 1992; Hovde et al., 1988; Ohmura et al., 1993; Suhan and Hovde, 1998). The corresponding residues were also important in the enzymatic activity of ricin (Ready et al., 1991; Schlossman et al., 1989).

Stx1 and Stx2 display indistinguishable enzymatic activity in cell free systems (Brigotti et al., 1997; Head et al., 1991; Tesh et al., 1993). However, epidemiological data indicate that Stx2 is more important than Stx1 in the development of HUS (Nataro and Kaper, 1998; Siegler et al., 2003). The cytotoxicity of Stx2 in animal models is much stronger than Stx1 and even among variants with identical B subunits, sequence differences within the A subunit of Stx2 can lead to differences in lethality for mice (Tesh et al., 1993). The X-ray structures of *Shigella* Stx and Stx2 are highly similar (Fraser et al., 1994; Fraser et al., 2004). However, regions of dissimilarity (Smith et al., 2009) and structural differences that might result in differences in the mechanisms of action, have been identified (Fraser et al.,

2006; Fraser et al., 2004). The active site of Stx is blocked by the A2 chain (Fraser et al., 2006). In contrast, the active site of Stx2 is accessible to the adenine substrate and Stx2 cleaves the adenine when it is crystallized in the presence of adenosine (Fraser et al., 2006). Other studies suggest that the B subunit mediates potency (Head et al., 1991). Differences have been observed in the affinity of Stx1 and Stx2 holotoxins for the glycolipid receptor, globotriaosylceramide (Gb3). The binding affinity of Stx1 is higher than Stx2 for Gb3-mimicking receptors, suggesting that the higher potency of Stx2 might not be due to its higher receptor binding ability (Head et al., 1991; Nakajima et al., 2001; Tesh et al., 1993). However, the molecular basis for the differences between the toxicity of Stx1 and Stx2 is not understood.

Here we describe development of the yeast, *S. cerevisiae*, as a model to study the cytotoxicity of the A subunits of Stx1 and Stx2. Studies in yeast cells demonstrated that Stx1A₁ is retrotranslocated out of the ER when it is synthesized *de novo* in the cell (LaPointe et al., 2005). Similar results were obtained with ricin A chain (RTA) expressed in yeast (Li et al., 2007; Simpson et al., 1999). Using *de novo* expression in yeast, we can bypass endocytosis and retrograde transport, and directly compare retrotranslocation of the A₁ subunits and their interaction with the host ribosome. Since only a minor fraction of Stx is thought to reach the ER after endocytosis and such small quantities are difficult to detect, targeting Stx1 and Stx2 to the ER overcomes this technical issue. Here, we carried out a comprehensive mutagenesis of the A subunits of Stx1 and Stx2 (Stx1A and Stx2A) in yeast to identify residues critical for cytotoxicity. We show that Stx1A and Stx2A depurinate the SRL, inhibit translation and cause apoptotic-like cell death in the absence of the B subunits in yeast, indicating that yeast is an attractive model to study the function of the A subunits. Our results demonstrate that enzymatic activity of the A subunits of Stx1 and Stx2 can be differentiated in yeast and provide evidence that depurination of ribosomes and translation inhibition are not entirely responsible for apoptotic-like cell death.

2. Materials and methods

2.1. Yeast expression constructs

Total DNA was isolated from *E. coli* O157:H7 strain ATCC43895 producing both Stx1 and Stx2 using a modified protocol as described (Pollard et al., 1990). The full-length DNAs encoding the A-subunits of Stx1 (Stx1A) and Stx2 (Stx2A) were amplified by polymerase chain reaction (PCR) using bacterial DNA as the template and gene-specific primers. The PCR-amplified DNAs were cloned into pGEMT-easy cloning vector (Promega, Madison, WI) and subsequently cloned into pYES2.1/V5-His-Topo yeast expression vector (Invitrogen, Calsbad, CA) downstream of the *GAL1* promoter, resulting in plasmids NT890 (Stx1A) and NT901 (Stx2A) with 3' V5- and His- tags at their C-termini. The Stx1A DNA includes the N-terminal 22-residue signal peptide and A1/A2 coding sequence, while the Stx2A DNA contains the N-terminal 22-residue signal peptide and the A1/A2 coding sequence. NT890 and NT901 were transformed into *Saccharomyces cerevisiae* strain W303 (*MATa ade2-1 trp1-1 ura3-1 leu2-3.112 his3-11.15 can1-100*) and transformants were selected on synthetic drop-out medium minus uracil (SD-Ura) containing 2% glucose.

2.2. Mutagenesis of Stx1A and Stx2A

Random mutagenesis was carried out by treating NT890 and NT901 plasmids with hydroxylamine as previously described (Hur et al., 1995; Li et al., 2007). Mutated NT890 and NT901 were isolated from the colonies that were able to grow on galactose, transformed into *E. coli* and characterized by nucleotide sequence analysis. In addition, conserved residues at the active site and C-termini were mutagenized using site-directed mutagenesis, which was carried out by the QuikChange site-directed mutagenesis kit (Stratagene, Santa

Clara, CA). All mutants were confirmed by sequence analysis and subsequently transformed into yeast cells.

2.3. Yeast cell viability assay

Yeast cells expressing wild type (wt) or mutant forms of Stx1A and Stx2A were first grown in SD-Ura medium containing glucose to A_{600} of 0.3 and then transferred to SD-Ura medium supplemented with 2% galactose. At 10-hr post induction, serial dilutions of cells (15 μ l) were spotted onto SD-Ura plates containing glucose. Plates were incubated at 30 °C for 48 hr.

2.4. Immunoblot analysis of Stx expression

Yeast cells containing Stx1A/2A plasmids were grown in SD-Ura medium containing 2% galactose for 6 h to induce expression. ER membrane fractions were isolated as previously described (Parikh et al., 2005), separated on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane which was then probed with anti-V5 monoclonal antibody to detect the 14 amino acid V5 epitope at the C-termini of all constructs (Invitrogen, Carlsbad, CA) or with polyclonal antibodies against Stx1A or Stx2A (BeResources, Manassas, VA). The membranes were stripped with 8M guanidine hydrochloride and re-probed with monoclonal antibody against dolichol-phosphate mannose synthase (Dpm1p, Invitrogen, Carlsbad, CA) for confirmation of equal loading.

2.5. rRNA depurination assay by dual primer extension

Ribosomal RNA depurination was analyzed by the dual primer extension assay as described previously (Parikh et al., 2002). The following two [γ - 32 P]-ATP-end labeled primers were used in the same primer extension reaction with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA): the depurination primer Dep, 5'-AGCGGATGGTGCTTCGCGCAATG-3'), which anneals 73 nucleotides 3' of the depurination site (A3137) on the yeast 25S rRNA; and the 25S primer (25S, 5'-TTCCTCGCCGTTACTAAGG-3'), which anneals 100 nucleotides 3' of the 5' end of the 25S rRNA. The primer extension reaction products were resolved on a 7M urea, 5% polyacrylamide gel and quantified with a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA) as previously described (Parikh et al., 2002).

2.6. *In vivo* [35 S]-methionine incorporation

Translation inhibition was measured by *in vivo* [35 S]-methionine incorporation at 6 h post induction when depurination is at maximal levels. Yeast cells harboring Stx1A/2A or the mutants were induced for 6 hr on 2% galactose as above. At time zero, [35 S]-methionine was added to cells. At 15, 30, 45 and 60 min intervals following Met addition, 400 μ l of cells were taken for growth measurement at A_{600} and another 400 μ l of cells were assayed for the incorporation of [35 S]-methionine as previously described (Parikh et al., 2002). Translation rates, expressed as cpm/ A_{600} /min, were calculated from the slope of [35 S]-methionine incorporation curves, which were linear up to 45 min. The translation inhibition by Stx1A, Stx2A and mutants was calculated from three independent experiments as the percentage of total translation by the vector control at 30 min after the addition of [35 S]-methionine.

2.7. Computational methods

The Protein Data Bank (PDB) files used as starting points for the modeling were 1DM0 (2.5 Å resolution) for the toxin from *S. dysenteriae* (Fraser et al., 1994) and 1RP4 (1.77 Å resolution) for Shiga toxin type 2 (Stx2) from *E. coli* (Fraser et al., 2004). The *S. dysenteriae* toxin differs in only one amino acid from Stx1 of *E. coli*, the difference being T45S.

Modeling was performed with the Insight II package from Accelrys (San Diego, California). Solvent accessible surface area calculations were performed by the method of Lee and Richards (Lee and Richards, 1971) with the program ACCESS (Richards, 1985). We analyzed the atom by atom area output of ACCESS with the program BINS to aggregate the area of each amino acid. Kajander *et al.* (Kajander et al., 2000) provide a full description of the program.

In order to examine the effects of mutations on the structures, we minimized the conformational energy after modeling the missing residues in the PDB files. The *Shigella* toxin Protein Data Bank (PDB) file (1DM0), whose length is 287 amino acids, lacks residues 43–46, 184–188 and 243–256. Stx2 (PDB file 1R4P), 297 residues in length, is missing amino acids 243–258. All the missing residues in both structures are surface loops or turns. Conformational flexibility within the crystals could easily account for the lack of electron density. In modeling the loops, a few minimization iterations were run “*in vacuo*,” following which a shell of simulated water molecules 5 Å thick was built around the protein and the minimization continued to convergence. Modeling of mutations began with the water shell present. The models were examined for non-standard geometry with PROCHECK (Laskowski et al., 1993). The *Shigella* toxin was converted to Stx1 prior to modeling the loops by replacing threonine 45 with serine. The changes that resulted from building the loops did not significantly impact the crystal structure; neither the secondary nor the tertiary structures of the rest of the proteins were significantly altered (data not shown). The modeling of loops entailed several steps. Loop candidates were found by searching the Protein Data Bank for loops which met three criteria: (1) correct length (2) the direct end-to-end distance of the loop closely matched the distance between the points on the polypeptide structure to which they would be attached and (3) the direction of the amino and carboxy terminal residues had to be such that joining the loop to the main body of the protein did not result in an abrupt chemically unacceptable change in the chain direction. The Insight II software was used to perform all three of these functions.

For the two short loops in Stx1 (residues 43–46 and 184–188) the backbone conformations agreed very well with the corresponding parts of Stx2 (data not shown). To manage the third requirement for the long loops (residues 243–256 in Stx1 and 243–258 in Stx2), we followed the conventions of the software and designated five residues of the known polypeptide structures at the amino end of the loop attachment site and five at the carboxyl end as “pre-flex” and “post-flex” residues to establish the chain direction (Supplemental Fig. 1). The direction of these pre- and post-flex residues determined whether the loop, which included part of the A₂ region of the A subunit, covered much of the active site as in Stx1 (See also Fraser et al., 2004, 2006). The software presented up to ten loop candidates at a time. The loop backbone conformations varied from one candidate to another, but all met the three criteria described above. Energy minimization was done after the side chains were added and was used simply to relax any steric overlaps and to ensure that electrostatic interactions did not violate well-established conformational requirements.

2.8. Analysis of cell death, nuclear fragmentation and ROS production in yeast cells

Yeast cells expressing wild type and mutant forms of Stx1A and Stx2A were sampled at 0, 2, 4, 6, 10, and 24 hr post induction, stained with 0.05% Evans blue for 30 min, and destained with water for 10 min as previously described (Li et al., 2007). The apoptotic hallmarks such as cell death, ROS production and nuclear fragmentation were analyzed as previously reported (Li et al., 2007). Cells were counted using a Zeiss Axiovert 200 inverted microscope. All experiments were done in triplicate. To detect nuclear fragmentation, cells were resuspended in phosphate-buffered saline buffer (20 mM sodium phosphate, 140 mM NaCl, pH 7.4) and stained with 1 µg/ml DAPI (4,4-diaminido-2-phenylindole) for 5 min at room temperature (Li et al., 2007). The digital images were acquired with a Zeiss Axiocam

digital camera and software for image archival and management (Axiovision 3.0; Carl Zeiss Vision GmbH). ROS staining was carried out with 1 mg/ml diaminobenzidine (DAB) for 10 min followed by washing with water three times and the stained cells were observed under a Zeiss Axiovert 200 inverted microscope as described (Li et al., 2007). Intracellular production of H₂O₂ was detected using the oxidant-sensitive probe 2,7-dichlorodihydrofluorescein diacetate (DCDHF-DA) (Invitrogen, Carlsbad, CA) as previously described (Li et al., 2007). The fluorescence of the supernatant was measured using an HTS700 Perkin Elmer bioassay reader (Wellesley, MA) with excitation at 490 nm and emission at 518 nm.

3. Results

3.1. Mutations at the active sites of Stx1A and Stx2A

The A subunits of Stx1 (Stx1A) and Stx2 (Stx2A), which contain a 22 amino acid signal sequence, were expressed from the *GAL1* promoter in *S. cerevisiae*. Growth inhibition was examined by plating cells on glucose containing medium after galactose induction for different times in liquid media (Li et al., 2007; Parikh et al., 2005). As shown in Fig. 1A and B, Stx1A and Stx2A expression reduced the viability of cells by almost two logs compared to the vector control at 10 h after induction. The doubling time of cells expressing the A subunits of Stx1 and Stx2 was 13.3 h and 14.7h, respectively, while doubling time of cells harboring the empty vector ranged between 4.5–6 h (Table 1). As previously observed with RTA (Li et al., 2007; Parikh et al., 2008), the majority of Stx1A and Stx2A expressed in yeast was associated with the membrane fraction by immunoblot analysis (Fig. 2A). A very low level of protein was detected in the cytosolic fraction (data not shown). The faster migrating band in Fig. 2A is consistent with the signal sequence cleaved mature form, since the V5 tag located at the C-termini of each toxin detects both the precursor (35 kDa) and the mature (32.5 kDa) forms. The blot was reprobed with antibody against Dpm1p as a loading control.

To determine if Stx1A and Stx2A were able to depurinate the rRNA in yeast, total RNA was isolated after 6 hours of induction and depurination was examined by dual primer extension analysis (Parikh et al., 2002). The level of depurination was quantified by comparing the level of depurination (Dep) to the level of the total rRNA (25S) (Fig. 2B; Table 1). Ribosomes were depurinated in yeast expressing the A subunits of each toxin at a similar level (Fig. 2B). To determine if reduction in growth correlated with inhibition of translation, we examined total translation in cells expressing the A subunits of Stx1, Stx2 or harboring the empty vector by [³⁵S]-methionine incorporation (Parikh et al., 2002). Translation rates calculated from three independent experiments at 6 h post induction were expressed as a percentage of the vector control in Fig. 2C. Total translation was reduced to 43% and 45% of the vector control in yeast expressing Stx1A or Stx2A, respectively (Table 1; Fig. 2C).

Asn75 and Tyr77 are highly conserved among the RIPs (Fig. 3A). As shown in Fig. 1A and B, substitution of Asn75 and Tyr77 with Ala (N75A and Y77A) by site-directed mutagenesis reduced cytotoxicity of both Stx1A and Stx2A (Fig. 3B). Immunoblot analysis showed that Y77A was expressed at a higher level than N75A in both proteins (Fig. 2A). Ribosomes were depurinated at a similar level in yeast expressing Stx1A-N75A and Stx1A-Y77A, although Stx1A-N75A was expressed at a lower level (Fig. 2B). Total translation was inhibited by Stx1A with these mutations (Fig. 2C; Table 1). Stx2A-N75A and Stx2A-Y77A had lower depurination activity compared to the same mutations in Stx1A (Table 1; Fig. 2B), although they were expressed at high levels (Fig. 2A), indicating that the lack of depurination was not due to lower level of protein expression. The lack of correlation between protein level and depurination was observed in previous studies (Hudak et al., 2004; Li et al., 2007). In general, nontoxic forms of RIPs are expressed at higher levels than

the toxic forms in yeast, and even very low levels of toxic proteins can depurinate ribosomes, suggesting that a threshold level of expression is needed for depurination (Hudak et al., 2004; Li et al., 2007). Asn75 and Tyr77 mutations reduced the extent of translation inhibition by Stx2A and Stx1A (Fig. 2C; Table 1). We conclude from these results that Asn75 and Tyr77 are more important for the depurination activity of Stx2A than Stx1A.

A comparison of the three dimensional structures of Stx1A and Stx2A is shown in Fig. 4A and C, and solvent accessible surfaces are shown in Fig. 4B and D. Many of the residues, including Asn75 are fully buried in the protein interior in Stx1A (Fig. 4B). In contrast, Asn75 and Tyr77 are considerably more accessible in Stx2A (Fig. 4D). Solvent accessible surface areas for Stx1A and Stx2A were analyzed for the residues mutated in this study and are presented in Table 2. The values in each row represent the changes in solvent accessible surface area for the particular residue shown in Stx1A/Stx2A. The first two columns show the surface accessibility of the mutated residues in the wild type Stx1A and Stx2A, respectively and the third column shows the differences in surface accessibility between the two A subunits (Stx2A-Stx1A). The differences are particularly noticeable for Asn75, Tyr77, Arg176, and Asn241 (Asn240 in Stx2A) as indicated in the third column. Asn75 and Tyr77 are more accessible to the solvent and therefore to substrates and other cellular components in Stx2A than in Stx1A.

Columns 4–9 in Table 2 show the changes in surface area as a result of the particular mutation indicated in each column (e.g. Stx1A N75A). In the wild type Stx1A and Stx2A, Y77 has an accessible area of 48 Å² and 79.8 Å², respectively. N75A mutation reduces the surface accessibility of Y77 by 17.8 Å² (37%) in Stx1A, and by 36.2 Å² (45%) in Stx2A (Table 2). Approximately similar effects are seen at R176, N241/240 and C242/241 with the exception of a small increase at R176 and N240 in Stx2A. This result indicates that N75A mutation causes a general closing down of the active site area plus other changes throughout the protein (Table 2). Replacement of Tyr77 with alanine in both Stx1A and Stx2A results in considerable loss of accessible area, as a much smaller amino acid replaces a larger one which is heavily exposed in the wild type. Y77A mutation reduces the surface accessibility of Tyr77 by 33.3 Å² in Stx1A and by 59.7 Å² in Stx2A (Table 2). The side chain of alanine is equivalent to the beta carbon of tyrosine. Removal of the ring and hydroxyl of tyrosine leaves a “stump” which is deeper in the active site, and this also contributes to the loss in accessible surface area. Asn75, in contrast, is fully buried in Stx1A and heavily so in Stx2A. Replacement by the smaller alanine thus has little effect on the access of this site to the surroundings (Table 2).

To identify other residues critical for cytotoxicity of Stx1A and Stx2A, the yeast expression plasmid containing each toxin was randomly mutagenized by hydroxylamine and 14 screened for toxicity as previously described (Hur et al., 1995; Li et al., 2007). One of the plasmids isolated from this screen that reduced cytotoxicity of Stx2A by almost two logs contained a double point mutation, E167K/R176K (Fig. 1A; B). Alignment of these amino acids in Stx2A with Stx1A, pokeweed antiviral protein (PAP) and RTA showed that Glu167 is conserved among the different RIPs, while Arg176 is conserved only between Stx1 and Stx2 (Fig. 3B). Site-directed mutagenesis was used to introduce the double point mutation into Stx1A to determine if the function of these residues is conserved. The E167K/R176K mutation reduced the cytotoxicity of Stx1A over 10-fold (Fig. 1A; B). To characterize the relative contribution of each mutation to toxicity, we introduced the single mutations into Stx1A and Stx2A. The single mutations, E167K and R176K, reduced toxicity of Stx2A by approximately 10-fold, while the double mutation further reduced toxicity to near vector control levels (Fig. 1B). The doubling time of yeast expressing the double mutants of Stx1A and Stx2A more closely resembled the vector control than yeast expressing the single mutants (Table 1).

Stx1A and Stx2A containing the single (E167K and R176K) or the double mutations (E167K/R176K) expressed proteins as the wild type Stx1A and Stx2A (Fig. 2A). Depurination of the SRL was reduced in yeast expressing Stx1A-E167K and R176K to 19% and 10% of the wild type, respectively, while Stx1A-E167K/R176K did not show any depurination (Fig. 2B; Table 1). Stx2A-E167K/R176K also did not show any detectable depurination, while Stx2A-E167K depurinated the SRL at 22% and Stx2A-R176K depurinated at 75% of the wild type levels (Table 1). A similar level of protein was expressed in these mutants (Fig. 2A). These results demonstrated that the R176K mutation had a greater effect on depurination activity of Stx1A than Stx2A, suggesting that Arg176 was more important for the enzymatic activity of Stx1A. Arg176 is not directly in the active site. Analysis of the solvent accessible surface areas showed that it is actually somewhat more exposed in Stx1 than in Stx2 (Supplemental Fig. 2; Table 2), which may account for the greater effect on Stx1A depurination. Stx1A-E167K or Stx1A-E167K/R176K did not inhibit total translation (Fig. 2C; Table 1). In contrast, Stx1A-R176K inhibited translation even though it depurinated the SRL at only 10% of the wild type Stx1A (Table 1; Fig. 2C). Stx2A with the corresponding single and double mutations showed a lower level of translation inhibitory activity than the wild type Stx2A (Fig. 2C; Table 1).

Arg170 is conserved in Stx1A, Stx2A and other RIPs (Fig. 3B) and has been shown to be critical for the enzymatic activity of Stx1A (Ohmura et al., 1993). The R170A mutation reduced the cytotoxicity of Stx1A and Stx2A in yeast around 10-fold (Fig. 1A; B). Ribosomes were depurinated at 53% and 65% of the wild type in yeast expressing Stx1A-R170A and Stx2A-R170A, respectively (Table 1; Fig. 2B). These results suggest that even moderate reductions in depurination can decrease toxicity. Total translation was inhibited in yeast expressing Stx1A-R170A and Stx2A-R170A (Table 1; Fig. 2C) and the doubling time was similar to yeast expressing the wild type toxins (Table 1). These results provided evidence that ribosome depurination and translation inhibition can be uncoupled from the toxicity of Stx1A and Stx2A.

In Stx1A and Stx2A, Glu167 forms a buried salt bridge with Arg170 with the glutamic acid being somewhat more deeply buried behind the arginine. Mutation of Glu167 to lysine switches its charge from negative to positive. However, this change does not force Arg170, which is also positively charged, away. The region around these side chains is sufficiently constricted, such that the guanidino group of Arg170 can't move very far. Interestingly, the E167K mutation pushes the positively charged Arg176 further into the solvent (an area increase of $25.7 \text{ \AA}^2$), especially in Stx2A, whose arginine at this site was less exposed than that of Stx1A ($93.5 \text{ \AA}^2$ vs $129.0 \text{ \AA}^2$) (Table 2). Arg176 does not have a direct interaction with Glu167 in either protein, so the effect must be mediated through the protein interior. As the interior's dielectric constant is lower than that of water, electrostatic effects will be stronger. It is possible that the presence of two positive charges instead of net zero charge of a salt bridge is sufficient to move Arg176, which is relatively unconstrained by the rest of the protein. The reciprocal change, R170A, uncovers some of Glu167, allowing it slightly greater solvent access in Stx1A (an increase of $14.7 \text{ \AA}^2$ from $0.2 \text{ \AA}^2$) (Table 2). Substitution of an Ala instead of a Lys at Glu167 resulted in a similar reduction in cytotoxicity, depurination and translation inhibition as the E167K mutation in both Stx1A and Stx2A (data not shown). These results indicate that mutations at the active site affect surface exposure not only at the mutation sites, but also at distant sites and the effects on Stx1A and Stx2A are not the same (Table 2).

3.2. Mutations at the C-termini of Stx1A and Stx2A

The C-terminal sequences of ricin and other RIPs contain a C-terminal hydrophobic region that may be critical for mediating toxin translocation across the ER membrane into the cytosol (Baykal and Tumer, 2007; LaPointe et al., 2005; Suhan and Hovde, 1998). To

determine if C-terminal residues are critical for toxicity of Stx1A and Stx2A, sequential deletions were made from the C-terminus of each protein by introducing stop codons (*). The resulting deletion mutants were then assayed for cytotoxicity (Fig. 5A; B), protein expression (Fig. 6A), depurination (Fig. 6B), and translation inhibition (Fig. 6C). The results of these assays are summarized in Table 1. As shown in Fig. 5A, deletion starting at Cys242 (C242*) did not affect the cytotoxicity of Stx1A or depurination of the SRL (Fig. 6B; Table 1). However, deletion starting at Leu240 (L240*) reduced the cytotoxicity of Stx1A relative to the wild type (Fig. 5A; B), as well as its depurination activity (Fig. 6B; Table 1). Protein expression was detected in the C-terminal deletion mutants of Stx1A (Fig. 6A). Our results are consistent with a previous study, which showed that Leu240* mutant of Stx1 is unable to retrotranslocate to the cytosol (LaPointe et al., 2005), which could be why so little protein is detected in this mutant by immunoblot analysis (Fig. 6A). Ile239* mutation caused faster migrating bands to appear (Fig. 6A), suggesting that it allowed proteolysis to occur. The reduction in cytotoxicity of Stx1A correlated with the depurination level which decreased to 53% for Leu240* and to 26% for I239* (Fig. 6B; Table 1). Translation inhibition was comparable to wild type for Stx1A-C242*, N241* and L240* (Fig. 6C; Table 1), but was lost upon introduction of a stop codon at Ile239 (Fig. 6C; Table 1). Depurination activity and cytotoxicity of Stx1A gradually decreased upon sequential deletion of C-terminal amino acids, indicating that activity of the C-terminal deletion mutants of Stx1A correlated with their cytotoxicity.

A similar C-terminal deletion analysis of Stx2A was carried out to compare the function of this region to Stx1A. Introduction of a stop codon at Leu239 (L239*), which corresponded to Leu240 in Stx1A did not eliminate the cytotoxicity of Stx2A (Fig. 5) nor did it cause any loss in its depurination activity (Fig. 6B). Cytotoxicity of Stx2A was lost after introduction of a stop codon at Ile238 (I238*) (Fig. 5). Although I238* was not cytotoxic, it was able to depurinate the SRL at levels comparable to wild type (Fig. 6B; Table 1). However, I238* caused a lower level of translation inhibition than the wild type Stx2A (Fig. 6C; Table 1). These results showed that unlike Stx1A, cytotoxicity of Stx2A was lost before its ability to depurinate the SRL, indicating that the activity of the C-terminal deletion mutants of Stx2A did not correlate with their cytotoxicity.

3.3. Ribosome depurination can be separated from apoptotic-like cell death in yeast

To gain insight into the mechanism of Shiga toxin induced cell death, the hallmarks of apoptosis, including cell death, nuclear fragmentation and reactive oxygen species (ROS) production were examined in yeast expressing wild type and mutant forms of Stx1A and Stx2A from the *GAL1* promoter. To examine cell death, yeast cells grown in liquid culture were stained with Evans blue at different time points after induction with galactose. The extent of staining at 24 h post induction is shown in Fig. 7 and quantified after 0, 6 and 24 h after induction in Fig. 8. In cells expressing the wild type Stx1A or Stx2A, cell death was observed at 6 h after induction and gradually increased up to 24 h (Fig. 8A; B). We observed a decrease in cell death relative to wild type in the active site and the C-terminal deletion mutants that were shown to reduce or eliminate cytotoxicity (Fig. 7A; D). The decrease in cell death correlated very well with the decrease in cytotoxicity. The C-terminal deletions of Stx1A (N241* and C242*) and Stx2A (L239*, N240* and C241*) that were toxic exhibited cell death at 24 h, while nontoxic mutants of Stx1A (I239* and L240*) and Stx2A (I238*) exhibited less than 10% cell death (Fig. 8A; B). The active site mutations that decreased cytotoxicity exhibited a greater percentage of cell death at 24 h in Stx2A, than in Stx1A (Fig. 8A; B). The percentage of cell death was also higher in the wild type and cytotoxic Stx2A mutants than in Stx1A mutants at 6 and 24 hours post induction (Fig. 8A; B). From these results, we conclude that Stx2A caused a higher level of cell death in yeast than Stx1A

and mutations that significantly reduced cell death by Stx1A did not have the same effect in Stx2A.

To determine whether Stx1A- and Stx2A-mediated cell death required ROS, cells were stained with DAB and observed at 24 h post induction (Fig. 7B; E). DAB stained cells were quantified in Fig. 8C and D. DAB staining was visible at 6 h post induction in cells expressing wild type and toxic mutants of Stx1A and Stx2A and increased up to 24 h (Fig. 7B; E). However, cells expressing the nontoxic mutants showed reduced DAB staining. As observed with cell death, a slightly higher level of ROS production was observed in cells expressing the wild type or cytotoxic forms of Stx2A than Stx1A (Fig. 8C; D). In contrast, ROS production did not increase in cells expressing the nontoxic mutants of Stx1A and Stx2A (Fig. 8C and D). L240* mutation reduced ROS production in Stx1A, but the corresponding mutation (L239*) did not have the same effect in Stx2A. The typical hallmarks of apoptosis including chromatin condensation and nuclear fragmentation were observed when yeast cells expressing the wild type Stx1A and Stx2A were stained with DAPI (Fig. 7C; F). The nuclei of these cells were abnormal and fragmented at 24 hr post induction. In contrast, DAPI staining of cells expressing the nontoxic mutants showed normal and single round shaped nuclei (Fig. 7C; F). These results demonstrated that Stx1A and Stx2A induced apoptotic-like cell death in yeast. Mutations at identical residues in Stx1A (L240*) and Stx2A (L239*) differed in their ability to induce cell death and several mutants that depurinated ribosomes did not show morphological changes consistent with apoptosis, indicating that ribosome depurination can be separated from apoptotic-like cell death in yeast.

4. Discussion

4.1. Residues at the active site of Stx1A and Stx2A that differ in their role in enzymatic activity differ in their surface accessibility

Here we used random and site directed mutagenesis to identify residues critical for the cytotoxicity of Stx1A and Stx2A in *S. cerevisiae* and examined the effect of these mutations on depurination of the SRL and inhibition of total translation. Unlike cell-free systems, which failed to detect any differences in enzymatic activity (Brigotti et al., 1997; Head et al., 1991; Tesh et al., 1993), we identified mutations at the active site and C-terminal residues that affect the enzymatic activity of Stx1A and Stx2A differently. The A subunits of Stx1 and Stx2 depurinated the SRL, inhibited translation and caused apoptotic like cell death in the absence of the B subunits in yeast, suggesting that the presence of the A₂ chain did not affect the enzymatic activity of the A subunits or their transport to the cytosol. Consistent with these results, the A₁ domain of Stx1 or Stx2 had similar depurination activity as the A subunit when its expression was induced in yeast (data not shown). Moreover, the A subunits had similar activity with or without the V5 tag, indicating that the V5 tag did not affect enzymatic activity (data not shown).

In the structures of Stx and Stx2, part of the A₂ domain is an alpha helix, which is inserted into the hole in the center of the B subunit pentamer (Fraser et al., 1994; Fraser et al., 2004). The rest of the A₂ chain, which precedes the helix, forms part of the interface between A and the pentamer. These helices are easily seen at the bottom of Figs. 4A and C. It could be argued that removal of the pentamer would cause conformational changes which would compromise the modeling. The C-terminal helices of A₂ would certainly be conformationally destabilized. However, this does not mean that the entire A chain would be destabilized. Except for the part inserted into the pentamer's hole, the A chain is a well formed globular protein (Fraser et al., 2006). Our results indicate that A chains alone are catalytically active. If the conformation were seriously disordered by the absence of the pentamer, the isolated A chain would be highly unlikely to perform catalysis. Examination

of the crystal structures of the holotoxins indicates that one end of the binding cleft is blocked by the pentamer, but the active site around E167 and R170 remains open to the surroundings, consistent with the observation that Stx2 can cleave an adenosine (Fraser et al., 2006). We suggest that in addition to serving as a cell surface docking module, the pentamer ensures that the holotoxins remain inactive within the cells where they are synthesized.

A single point mutation at Asn75 (N75A) or Tyr77 (Y77A) reduced the cytotoxicity of Stx1A and Stx2A and affected ribosome depurination more profoundly in Stx2A than in Stx1A. Tyr77 binds the substrate by stacking with the adenine ring (Fraser et al., 2006; Fraser et al., 2004), and Asn70 in PAP, which corresponds to Asn75 in Stx1A and Stx2A, is thought to interact with the phosphate backbone of the SRL (Rajamohan et al., 2000). Analysis of the solvent accessible surface areas showed that the differences for N75 and Y77 were the largest among the other residues at the active site, except for the surface residue, R176, and both residues were more surface exposed in Stx2. The N75A and Y77A mutations caused a reduction in the surface area of Y77, R176, N241/240 and C242/241, indicating that they led to a general closing down of the active site area, and the decrease was greater for Stx2A.

A double mutation at Glu167 and Arg176 (E167K/R176K) abolished the cytotoxicity of Stx2A and reduced the cytotoxicity of Stx1A over 10-fold. The single mutation at Arg176 affected ribosome depurination and translation inhibition differently in each toxin. Stx1A-R176K depurinated the SRL at only 10% of wild type, but inhibited translation at a similar level as the wild type, while Stx2A-R176K depurinated at 75% of the wild type, but caused less translation inhibition than the wild type. As discussed above, these results indicated that Arg176 is more critical for the depurination activity of Stx1A. Arg176 is somewhat more exposed to solvent in Stx1 than in Stx2 and is not in direct contact with the active site (Supplemental Fig. 2; Table 2). The presence of this residue on the protein surface may explain the greater effect of its mutation on depurination activity of Stx1A when compared with the other active site residues. Its role in the toxins' activity is unclear, though important. It may be involved in ribosome binding rather than catalysis, but further work is needed to elucidate the exact role of this residue.

Recent studies indicate that ribotoxins and RIPs bind to the ribosome through electrostatic interactions that lie outside the catalytic site (Korennykh et al., 2007; Korennykh et al., 2006; Li et al., 2009). RTA interacts with ribosomes in two different ways: nonspecific interactions that do not require an intact ribosomal stalk and stronger, more specific electrostatic interactions with the ribosomal stalk that lead to depurination of the SRL (Chiou et al., 2008; Li et al., 2009). Stx1A₁ also interacts with the ribosomal stalk (McCluskey et al., 2008). Therefore it is possible that the Arg to Lys substitution at position 176 reduces the depurination activity of Stx1A, but does not interfere with its interactions with ribosomes, since the positive charge is conserved. Interaction of the mutant protein with the ribosomal stalk may affect binding of the elongation factors to the stalk and cause translation inhibition independent of depurination of the SRL.

In order to examine the effects of mutations of Stx1A and Stx2A on the structures, we have modeled the substitutions at the active sites and examined their effect on surface accessibility of each residue. *In silico* mutation of the residues at the active site affected the surface accessibility not just near the mutation sites but throughout the proteins, and the effects on Stx1 and Stx2 were not the same (Table 2). The observation that a point mutation at each of the residues described in Table 2 affects the accessible surface area and thus the conformation at several other residues, suggests that changes at a single site can have effects at distant sites. Interestingly, point mutations at residues (Asn75, Tyr77 and Arg176) that

affected enzymatic activity of each toxin differently had the greatest effect on surface accessibility (Table 2). Mutation at these residues led to a general closing of the active site, which would probably cause a decrease in activity.

4.2. C-terminal amino acids have differential roles in Stx1A and Stx2A

The hydrophobic region spanning residues Ile224 through Asn241 in Stx1A was hypothesized to participate in toxin translocation across ER membranes (LaPointe et al., 2005; Suhan and Hovde, 1998). Mutations in this hydrophobic region that disrupted transmembrane propensity reduced cytotoxicity to Vero cells (Suhan and Hovde, 1998). Our results demonstrated that removal of A₂ did not reduce the cytotoxicity or depurination activity of either toxin in yeast. In contrast, C-terminal deletions of the A₁ chains beginning at Leu240 in Stx1A and Ile238 in Stx2A reduced cytotoxicity (Fig. 5). These results are consistent with a previous study, which showed that deletion of residues 240–251 reduced cytotoxicity of the Stx1 A₁ chain in yeast and affected its retrotranslocation from the ER to the cytosol (LaPointe et al., 2005). The corresponding C-terminal motif (residues 249–259) in PAP also mediates its transport to the cytosol (Baykal and Tumer, 2007). Analysis of the solvent accessible surface areas indicated that Stx1A-Leu240 and Stx2A-Ile238 are not surface exposed (Table 2). However, upon cleavage of A₂ chain, surface exposure of the C-terminal residues, Leu240-Arg251 in Stx1A₁, and Leu239-Arg250 in Stx2A₁ increase dramatically (Supplemental Fig. 3), suggesting that this region may trigger transport of the A₁ subunits to the cytosol (LaPointe et al., 2005). Smith et al. (2009) found three regions to be part of the epitope at which a monoclonal antibody binds to Stx2A, thereby inhibiting its activity. It is of interest that two of these involve substantial changes in accessible surface when A₂ is removed. These are region B, residues 42–49, and especially region C, residues 246–250, as shown in Supplemental Fig. S3. Although the extent of depurination correlated with the extent of translation inhibition in the C-terminal deletion mutants of Stx1A, toxicity of Stx2A was lost before its ability to depurinate the SRL, indicating that Stx2A can depurinate ribosomes more efficiently than Stx1A.

4.3. Ribosome depurination and translation inhibition can be separated from apoptotic-like cell death caused by Stx1A and Stx2A in yeast

Apoptosis or necrosis was induced in HeLa cells by internal expression of either Stx B or A subunit (Nakagawa et al., 1999). Shiga toxins trigger monocytic cell apoptosis through the ER stress response, and activation of caspase-8 via a calpain-dependent mechanism (Lee et al., 2008). However, the detailed mechanism of Stx1A- and Stx2A-induced apoptosis is not known. We demonstrated the morphological changes of yeast cells consistent with apoptosis by staining cells with Evans blue, DAB and DAPI. Our results provide the first evidence that Stx1A and Stx2A cause apoptotic-like cell death in yeast and indicate that cell death is accompanied by nuclear fragmentation and ROS production. The involvement of ROS upstream of caspase-3 activation has been demonstrated for signal transduction pathways induced by ricin and Stx in various cell types (Rao et al., 2005). We previously showed that expression of RTA in yeast induces ROS production (Li et al., 2009). The induction of ROS correlated well with cell death mediated by Stx1A and Stx2A, suggesting that ROS may act as an effector of Stx1A- and Stx2A-induced apoptosis and trigger cell death in yeast. We further show that Stx2A has higher potency than Stx1A in yeast and mutations in Stx1A that reduce its cytotoxicity do not have the same effect on Stx2A. The extent of ribosome depurination and translation inhibition did not correlate with the extent of or apoptotic-like cell death induced by Stx1A or Stx2A in yeast, indicating that they are not entirely responsible for cell death.

5. Conclusion

We used mutagenesis in yeast to identify the role of individual amino acids in the biological activity of the A subunits of Stx1 and Stx2 and identified the amino acids at the active site (Asn75, Tyr77, Glu167 and Arg176) and at the C-termini (Stx1A-Leu240 and Stx2A-Leu239) that exhibit functional differences. Mutations at the active site affected surface accessibility not just near the mutation sites, but throughout the proteins and the effects on Stx1A and Stx2A were not the same. Residues with higher surface exposure were more critical for enzymatic activity. We further show that the apoptotic-like cell death induced by Stx1A and Stx2A in yeast is not entirely due to ribosome depurination and translation inhibition. The nontoxic forms of Stx1A and Stx2A may be useful as potential vaccine candidates. They may also be valuable in the development of immunotoxins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Viability of yeast cells expressing the active site mutants of Stx1A and Stx2A. A. Yeast cells were induced for 10 hours in SD -Ura medium containing galactose and serial dilutions were plated on SD-Ura plates containing glucose. The analysis was repeated at least three times with different transformants. B. Colony forming units (CFU) per mL were plotted for yeast cells shown in panel A.

Fig. 2.

Protein expression and rRNA depurination by wild type and mutant forms of Stx1A and Stx2A. **A.** Immunoblot analysis of yeast cells expressing active site mutants of Stx1A and Stx2A. Membrane fractions (15 μ g) were separated on a 12% SDS-acrylamide gel and probed with monoclonal antibody against the V5 epitope. The blot was then stripped with guanidine hydrochloride and re-probed with anti-Dpm1 monoclonal antibody as a loading control. Prestained Benchmark protein standard (BioRad, Hercules, CA) was used as the molecular weight markers. **B.** *In vivo* depurination of rRNA in yeast cells expressing active site mutants of Stx1A and Stx2A. Total RNA (2 μ g) was isolated and subjected to the dual primer extension analysis using the depurination (Dep) primer to measure the extent of depurination and the 25S rRNA primer (25S) to measure the total amount of 25S rRNA. The primer extension products were separated on a 5% polyacrylamide gel containing 7M urea. The experiments were repeated at least twice with multiple colonies for each mutant. **C.** Analysis of total translation in yeast expressing active site mutants of Stx1A and Stx2A. Yeast expressing the wild type or the mutant forms of Stx1A and Stx2A or harboring the vector (VC) were grown in SD-Ura and -Met and 2% galactose for 6 hr to induce protein expression. At time zero, [35 S]-methionine was added and the rate of incorporation into protein was determined as described in the Materials and Methods. The translation rates were calculated from three independent experiments as percentage of total translation in the vector control at 30 min after the addition of [35 S]-methionine.


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A 60 - - - V E L S H B A E L S V T L A L D V T W A T V V G Y R 85 RTA
52 - - - V E L Q S R K E T T I L W R R M L T V M G S 77 PAP
53 D V R G I D P E S G R F N L R L I V E R R M L T V T G V Y 82 Stx1A
53 D I R G L D V Y Q A R F D H L R L I T E Q H M L T V A G F V 82 Stx2A

B 166 S P T I C I Q H I S K A A R F Q Y I E G E M R T R I R - - 193 RTA
165 E F L L V A I Q H V S K A A R F E Y I E R Q V E T - - N - - 190 PAP
156 S A H L R F V T T A K A L R F R Q I Q M E F R Q A L S E - 185 Stx1A
156 S A V L R F V T T A K A L R F R Q I Q M E F R Q A L S E - 184 Stx2A

C 236 R G R F S V Y D V I L I P I I A L H W Y R C A P P F S S Q F 267 RTA
233 R G R K E V S R V D E I K P D V A L L M F - - Y G S S Q T T 262 PAP
220 R V G R I S F S S I N A I L G S V A L L H C R - - H H A R V A R 251 Stx1A
219 R V G R I S F H N I S A I L O T V A V L L C R - - R Q A R S V R 250 Stx2A

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Fig. 3.

Sequence comparison of conserved residues among RIPs. A. Comparison of the amino acid sequence of the substrate binding domain, B. active sites and C. C-termini of PAP, RTA, Stx1A and Stx2A. The residues mutated in this study are shown in bold. Sequence alignment was generated using DNASTAR. The numbering of the amino acids starts at position 1 of the mature protein for each RIP.

Fig. 4. Three-dimensional structure of *Shigella* Stx and Stx2A and solvent accessible surfaces. A. Structure of the main chain and selected side chains of *Shigella* Stx. B. Solvent accessible surfaces of *Shigella* Stx. C. Structure of the main chain and selected side chains of Stx2A. D. Solvent accessible surfaces of Stx2 A. Color scheme: blue = subunit A1; red = subunit A2 except for long loop residues in cyan (Stx residues 243–256 and Stx2 243–258). Amino terminal nitrogens and one of the carboxy terminal oxygens (Stx residue 287, and Stx2 residue 297) and side chains are shown as spheres: magenta = Y77, green = N75, orange = E167, R170 and R176. R176 is not visible in the surface area images (B and D).

Fig. 5.

Viability of yeast cells expressing C-terminal mutants of Stx1A and Stx2A. A. Yeast cells were induced for 10 h in SD-Ura medium containing galactose. At 10 hr post induction, serial dilutions of cells were spotted on SD-Ura plates containing glucose. The analysis was repeated at least three times with different transformants. B. Colony forming units (CFU) per mL were plotted for yeast cells shown in panel A.

Fig. 6. Protein expression and rRNA depurination of Stx1A and Stx2A and their C-terminal deletion mutants in yeast cells. A. Immunoblot analysis of yeast cells expressing C-terminal deletion mutants of Stx1A and Stx2A as described in Fig. 2. B. *In vivo* depurination of rRNA in yeast cells expressing C-terminal deletion mutants of Stx1A and Stx2A as described in Fig. 2. Experiments were repeated at least twice with multiple colonies for each mutant. C. Analysis of total translation in yeast expressing C-terminal deletion mutants of Stx1A and Stx2A as described in Fig. 2.

Fig. 7.

Analysis of cell death, nuclear fragmentation and ROS production in yeast expressing wild type and mutant forms of Stx1A and Stx2A. Cells were stained with Evans blue (A, D), DAB (B, E) or DAPI (C, F) at 24 hr after induction and visualized using a Zeiss Axiovert 200 inverted microscope (magnification, 40X).

Fig. 8. Quantitative analysis of cell death (A, B) and ROS production (C, D) induced by Stx1A, Stx2A and the mutant forms. The percentage of cell death at different hours after induction was quantified and represented as the means $\pm$ standard deviations ($n = 3$). The H_2O_2 production was quantified using DCDHF-DA. The results are represented as the means $\pm$ standard deviations ($n = 3$).

Table 1

Characterization of mutant forms of Stx1A and Stx2A

Yeast Plasmids	Mutation	Cytotoxicity ^d	Depurination (% of wt) ^b	Doubling Time (hr)	Translation (% of VC at 30 min) ^c
Stx1A					
NT890	wild type (wt)	++	100	13.3	43.4
VC		–	0	4.5	100
NT2034 (s.d.) ^f	N75A	+	100	6.3	51.6
NT2014 (s.d.)	Y77A	+	100	6.0	58.7
NT2028	E167K	+	19	7.8	100
NT2041 (s.d.)	R170A	+	53	10.3	44.9
NT2052 (s.d.)	R176K	+	10	6.2	50.1
NT2048 (s.d.)	E167K/R176K	–	0	4.7	100
NT2051 (s.d.)	I239* ^e	–	26	9.4	100
NT2040 (s.d.)	L240*	–	53	8.8	47.3
NT2042 (s.d.)	N241*	++	100	12.3	47.3
NT2033 (s.d.)	C242*	++	100	14.7	47.3
Stx2A					
NT901	wild type (wt)	++	100	14.7	45.3
VC		–	0	6.0	100.0
NT2106 (s.d.)	N75A	+	10	5.3	62.4
NT2109 (s.d.)	Y77A	+	14	4.8	69.0
NT2142 (s.d.)	E167K	+	22	8.6	70.8
NT2108 (s.d.)	R170A	+	65	17.9	24.3
NT2143 (s.d.)	R176K	+	75	11.7	60.2
NT2114	E167K/R176K	–	0	7.2	70.7
NT2130 (s.d.)	I238*	–	94	7.6	72.4
NT2131 (s.d.)	L239*	++	100	8.1	53.7
NT2124 (s.d.)	N240*	++	100	11.0	50.7
NT2113 (s.d.)	C241*	++	100	12.4	48.7

b Depurination levels were determined by the ratio of the depurination band to the 25S rRNA band in Figs. 2B and 6B and expressed as a percentage of wild type Stx1A or Stx2A.

c Total translation was determined by 35S Met incorporation and expressed as a percentage of the vector control.

d s.d., site-directed mutation.

e * null mutation.

Table 2

Solvent accessible surface areas ($\text{\AA}^2$) and changes in area upon mutation in Stx1A and Stx2A

Residue	Stx1A/Stx2A	Stx1A wt ^b	Stx2A wt ^b	Stx2A- Stx1A ^b	Stx1A N75A ^c	Stx2A N75A ^c	Stx1A Y77A ^c	Stx2A Y77A ^c	Stx1A E167K ^c	Stx2A E167K ^c
N75		0.0	20.3	20.3	0.0	-1.4	2.0	12.1	2.4	0.7
Y77		48.0	79.8	31.8	-17.8	-36.2	-33.3	-59.7	-11.8	-9.3
E167		0.2	3.4	3.2	0.1	-3.4	0.3	-3.3	2.8	-0.9
R170		8.5	5.3	-3.2	-4.1	-5.1	5.9	6.5	-4.5	0.5
R176		129.0	93.5	-35.5	-16.9	6.6	4.8	0.8	9.0	25.7
I239/238 ^a		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L240/239		0.4	8.7	8.3	-0.4	3.4	1.6	-5.9	1.2	5.2
N241/240		41.0	23.9	-17.1	-22.1	6.5	-13.5	-6.8	9.7	8.2
C242/241		30.1	35.4	5.3	-12.6	-13.1	12.7	-17.3	4.6	-5.8

Residue	Stx1A/Stx2A	Stx1A wt ^b	Stx2A wt ^b	Stx2A- Stx1A ^b	Stx1A R170A ^c	Stx2A R170A ^c	Stx1A R176K ^c	Stx2A R176K ^c	Stx1A E167K/R176K ^c	Stx2A
N75		0.0	20.3	20.3	3.6	10.2	0.0	4.9	1.9	4.0
Y77		48.0	79.8	31.8	6.9	-12.2	1.9	-28.3	-6.6	-9.5
E167		0.2	3.4	3.2	14.7	9.7	0.7	-3.4	6.1	-1.6
R170		8.5	5.3	-3.2	-7.8	-4.0	-0.3	-4.5	-8.3	-0.2
R176		129.0	93.5	-35.5	5.8	10.3	-5.7	-3.5	-10.6	-0.2
I239/238		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L240/239		0.4	8.7	8.3	1.5	0.6	1.8	-1.3	-0.1	4.1
N241/240		41.0	23.9	-17.1	-3.4	-2.7	-7.5	5.6	-2.5	9.8
C242/241		30.1	35.4	5.3	2.8	-14.3	8.5	-9.2	1.3	-11.8

^aThere is a one-residue gap in the Stx2A sequence so that I239 in Stx1A corresponds to I238 in Stx2A and similarly for subsequent residues.

^bData columns 1-3 correspond to the wild type proteins. The areas (in $\text{\AA}^2$) of the indicated residues are shown in columns 1 and 2. Column 3 shows the difference between the wild type proteins as (Stx2A area) - (Stx1A area). These three columns are the same in the upper and lower halves of the table for ease of reference.

^cData columns 4-9 correspond to the mutants. The data are the *changes* in solvent accessible surface areas upon mutating the wild type residue to the indicated residue. Note that mutation at a given locus, e.g. N75A, produced accessible area changes at other loci, e.g. Y77, which was *not* mutated in the N75A protein.