Characterization of the A subunit mutants of Stx1 and Stx2 in

Saccharomyces cerevisiae

by

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A Thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Microbiology and Molecular Genetics

written under the direction of

Dr. Rong Di

and approved by

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New Brunswick, New Jersey

October 2009

ABSTRACT OF THE THESIS

Characterization of the A subunit mutants of Stx1 and Stx2 in *Saccharomyces cerevisiae*

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Shiga toxins are a family of related toxins with two major groups, Stx1 and Stx2. The most common sources for Shiga toxin are the bacteria *Shigella dysenteriae* and the Shigatoxigenic group of *Escherichia coli* (STEC), which includes serotype O157:H7. Shiga-like toxin 1 and 2 (Stx1 and Stx2) are produced by some *E. coli* strains. Stx produced by *S. dysenteriae* differs from Stx1 by one amino acid. Stx1 and Stx2 have only 56% sequence homology.

E. coli O157:H7 has been the major cause of food-borne illnesses. Symptoms include abdominal cramping, bloody diarrhea, vomiting and a low-grade fever. Serious complications can lead to Hemolytic Uremic Syndrome (HUS).

Stx1 and Stx2 are categorized as and AB_5 toxin. The A complex is responsible for toxicity and has enzymatic activity inside the host cell. The B subunits bind to a receptor on the surface of the host cell. Shiga toxin acts as an N-glycosidase. It cleaves a specific adenine residue in the conserved α- sarcin ricin loop in the 28S subunit of the

eukaryotic rRNA. This interferes with the translocation step in protein synthesis and leads to inhibition of translation and cell death.

When Stx1A and Stx2A cDNA was cloned downstream of the GAL1 promoter and transformed into *Saccharomyces cerevisiae*, cells growth was greatly reduced when expression of the toxin was induced. To identify residues critical for cytotoxicity or Stx1A and Stx2A random and site-directed mutations were made. The ability of the mutant proteins to depurinate ribosomes *in vivo* in yeast was determined by dual primer extension analysis. The amount of translation was measured by incorporation of [^{35}S]-methionine in the cell.

The results of these assays show mutations in N75, Y77, E167, R170, and R176 are critical for toxicity in both toxins. Also C-terminal deletions after L240 in Stx1A and I238 in Stx2A abolished toxicity. Several of these mutants, even though they are non-toxic still have the ability to depurinate ribosomes. This indicates cytotoxicity and depurination may not be directly related. This provides evidence that cytotoxicity is not necessarily a direct result of depurination, and other potential mechanisms may contribute in Stx1A and Stx2A cytotoxicity in yeast.

Acknowledgements

I would like to express my deepest thanks to my thesis advisor Dr. Rong Di. She has shown me guidance and given her assistance ever since my undergraduate years. I want to thanks Dr. Nilgun Tumer for giving me the chance to work in her lab and on this project. I have developed my mind as a scientist and critically think and analyze information. My gratitude also goes out to Dr. Charles Martin for his suggestions and advice on my thesis and serving on my committee. I would like to thank all my past and present lab members, Dr. Ulku Baykal, Dr. Xiao-Ping Li, Dr. John McLaughlin, Qing Yen, Anwar Bin Umer, Jia-Chi Chou, Natasha Mendez, and Varsha Shete. I am indebted to my family and friends for their continuous support in my endeavors throughout my life.

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Background and Significance

Shiga toxins, also known as verotoxins or Shiga-like toxins are produced mainly by enteric bacteria *Shigella dysenteriae* and enterohaemorrhagic *Escherichia coli* (14,24). This paper will focus on *E. coli* O157:H7, which is a Shiga toxin-producing *E. coli* (STEC). *E. coli* O157:H7 is the causative agent in recent food borne outbreaks of gastroenteritis and similar forms of enteric infections. The non-O157 STEC are not as well understood, because outbreaks due to them are rarely identified. In general, the non-O157 serogroup is less likely to cause severe illness compared to *E. coli* O157, but there are some non-O157 STEC serogroups that can cause the most severe cases of STEC illness (5).

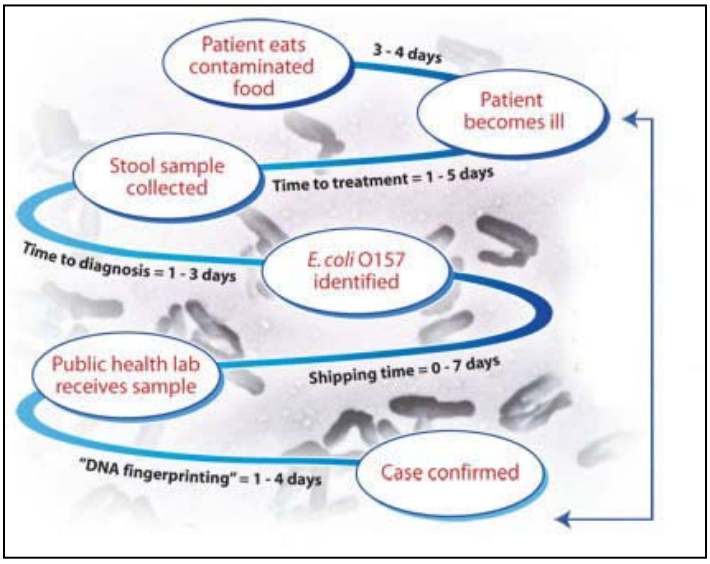

Fig. 1. Timeline for identifying and reporting of *E. coli* cases (4)

When a person consumes food contaminated with *E. coli* O157:H7, it can take up to 3-4 days to develop symptoms like cramping, vomiting, bloody diarrhea. It may also

be accompanied with a low fever. Depending on the severity of the symptoms individuals may take 1-5 days to seek medical attention. Samples need to be collected from infected individuals and confirmed by public health laboratories; this may take up to an addition week (Fig. 1). During this lag period of identifying, confirming and reporting cases of *E. coli* infection, many others may get sick or develop life threatening symptoms.

In recent years the United States had several cases of outbreaks of *E. coli* O157:H7. Beginning in August through October 2006, STEC infection was reported in 199 people in 26 states in association with consumption of fresh spinach. Packages of spinach obtained from infected patients from different states revealed that they came from a single manufacturing facility on the same day (17). Months later in December 2006, STEC was found in the lettuce used by Taco Bell. In this incident 71 cases were reported where 53 people were hospitalized and 8 developed hemolytic-uremic syndrome (HUS). Between July 20, 2007 and October 10, 2007 at least 8 people were hospitalized and 4 people developed HUS resulting in kidney failure due to infection of *E. coli* O157:H7 in Illinois, Kentucky, Missouri, New York, Ohio, Pennsylvania, South Dakota, Tennessee, Virginia and Wisconsin. This multistate outbreak was believed to be caused by consumption of frozen pepperoni pizza which was contaminated with STEC (5). In another incident occurring around the same time frame the USDA issued a recall on 21.7 million pounds of frozen ground beef patties produced by Topp's, located in New Jersey, on September 29, 2007. 21 people across different states were hospitalized and 2 developed HUS (5). More recently in the summer of 2008 STEC was found in ground beef distributed by the Kroger supermarket. This led to a recall for ground beef sold at Kroger. Twenty-seven people were hospitalized and one patient developed HUS (5). STEC can affect anyone of

any age. Also these cases can be wide spread because of how food commodities are produced, handled, and distributed.

STEC infection is acquired by ingestion of contaminated food and water, or by human contact. It can be found to live in the intestines of healthy cattle and other ruminants (26). Cattle, swine, and other domestic animals do not have receptors for Stx so they can harbor the bacteria with no ill effect. Stx requires specific glycolipids to bind to the cell surface (27). The bacteria can be shed in their feces which can lead to cross contamination of other products such as produce. A major source of infection is consumption of undercooked ground beef. Other sources include consumption of unpasteurized milk and juice, and produce like lettuce and spinach (26,27).

Structure

Stx1 and Stx2 are categorized as AB_5 toxins (30). The A subunit of the toxin is the enzymatically active section of the toxin and the B subunit binds to the cell surface (Fig. 2). The A1 and A2 subunits are non-covalently bonded by a trypsin sensitive disulfide bridge between two cystines (6,7,8). The A complex is responsible for toxicity and has enzymatic activity inside the host cell. The B subunits form a pentameric ring which is capable of binding to a receptor on the surface of the host cell. The receptor for the B subunits is glycolipid receptor globotriaosylceramide (Gb3).

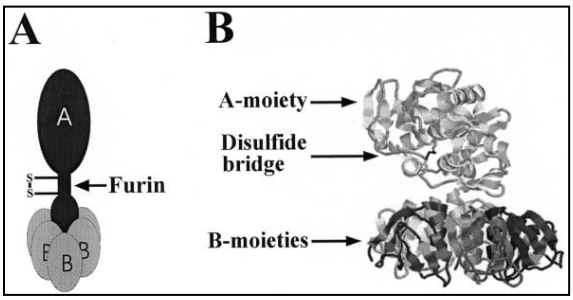

Fig. 2. Schematic (A) and crystal structure of Stx (B) from Protein Data Bank 1DM0 (6,30). The enzymatic A subunit is bonded to a pentamer of B subunits that binds to specific glycolipids on the host cell

Shiga toxins can be classified into different groups. Shiga toxin (Stx) which is produced by *Shigella dysenteriae* differs from Shiga-like toxin 1 (Stx1) by one amino acid. Shiga-like toxin 2 (Stx2) only shares 56% sequence homology with Stx1 (26). The A subunit of Stx and Stx1 are comprised of 293 amino acids, and Stx2 A subunit is 297 amino acids in length. The B subunits of all toxins are composed of 89 amino acids (30). The holo-toxin is approximately 70 kDa (26). Shiga toxin binds to the glycolipid Gb3. One variant of the toxin, Stx2e, can bind to Gb4 which are present in pigs (30). High levels of Gb3 are found in human kidneys (25). Binding of Shiga toxin to cells and sensitivity to the toxin are affected by many different factors. Changes in signal transduction with cAMP can affect binding and sensitivity, and different cytokines can induce Gb3 synthesis in different cell types (25).

Shiga toxin enters the cell through receptor mediated endocytosis (13, 22, 30). Clathrin-coated pits are formed on the cell surface and are pinched off to form a vesicle (Fig. 3). Retrograde trafficking of the toxin then occurs allowing the toxin to enter the trans-Golgi network, Golgi stacks, endoplasmic reticulum and nuclear envelope (24).

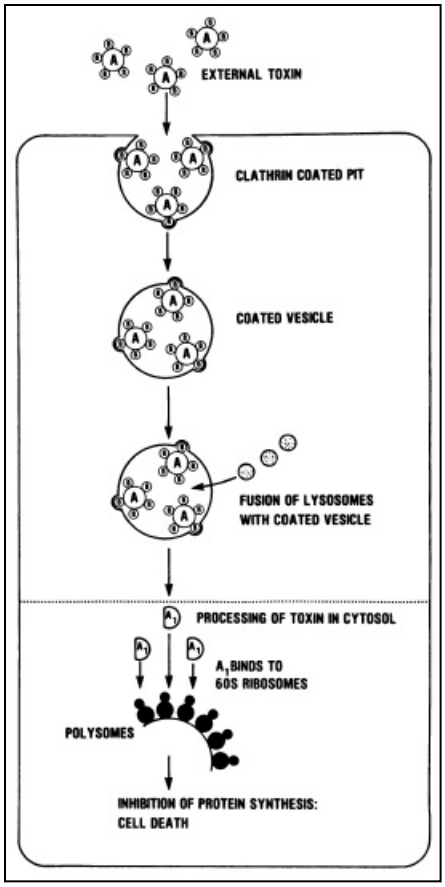

Fig. 3. Model for the receptor-mediated endocytic entry of Shiga toxin and processing of Shiga toxin. The B subunit of the toxin binds to the mammalian cell receptor and begins invagination of the cell wall. Clathrin-coated pits are formed on the cell surface and are pinched off to form a vesicle (22).

In Shiga toxins, there is an internal disulfide bond between the A1 and A2 fragments. This link is sensitive to cleavage by trypsin. Cleavage of the A subunit increases enzymatic activity (24). Furin, which recognizes the Arg-X-X-Arg sequence, can also cleave the toxin inside the cell (30).

The A subunit of Shiga toxin has RNA N-glycosidase activity. It removes a specific adenine residue (A4324) from the conserved α- sarcin ricin loop (SRL) in the 28S subunit of the eukaryotic rRNA (Fig. 4). This inhibits binding of the amino-acyl-tRNA to the 60s ribosomal subunit, therefore inhibiting protein synthesis. Shiga toxin can also induce cell lysis and other characteristics of programmed cell death through depurination and translation inhibition (30).

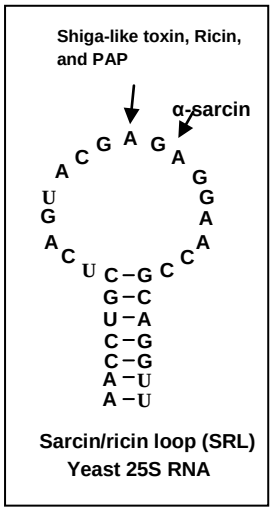

Fig. 4. Representation of sarcin/ricin loop (SRL) of yeast 25S RNA. The arrows indicate the site of target for ribosome inactivating proteins and ribotoxin α-sarcin.

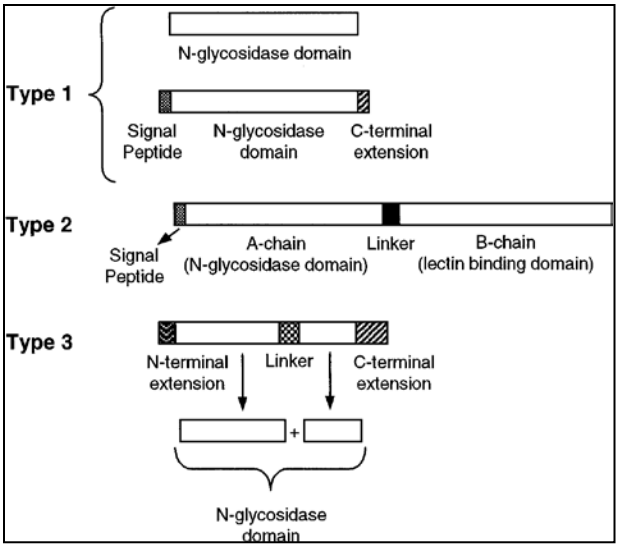

Fig. 5. Alignment and comparison of different RIP structures (21).

Stx belongs to a class of proteins called Ribosome Inactivating Proteins (RIPs). RIPs are grouped in three different types based on structure (Fig. 5). Other RIPs include ricin and PAP. Stx is a Type II RIP, as stated earlier contains two domains, the enzymatic A chain, and a receptor binding B chain. Ricin also has a similar structure. The sequence of the A1 fragment of Stx is similar to the A chain of ricin; this is the fragment with N-glycosidase activity (6,7,8). Most Type I RIPs are single chain proteins similar to the A chain in Type II RIPs. PAP and saporin are Type I RIPs. It has been suggested that Type II RIPs evolved from a fusion of Type I RIPs and a lectin binding protein (3). Type I RIPs lacking the presence of a binding B chain do not exhibit optimal binding to the cell surface and are considerably less toxic than Type II RIPs. Type III RIPs, which are much less prevalent than Type I and II RIPs, have been characterized only from maize and barley. They require proteolytic processing to occur between amino acids to be active and exhibit enzymatic activity (21).

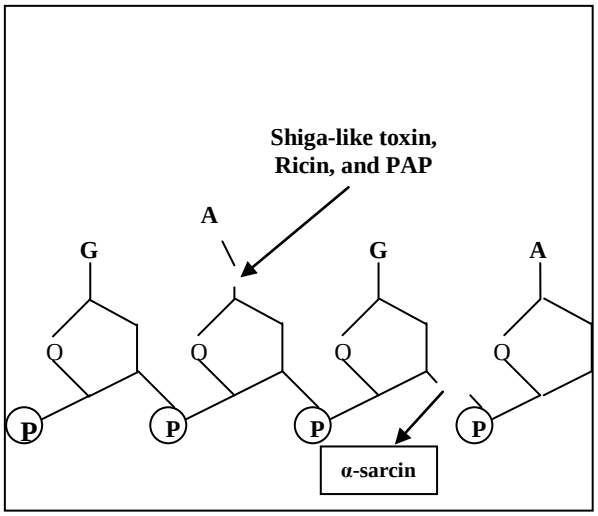

Fig. 6. The sites of attack for Ribosome Inactivating proteins and α-sarcin from GAGA sequence in the sarcin/ricin loop.

Ribosome inactivating proteins attack the adenine site while α-sarcin cleaves the bond between adjacent guanine and adenine residues (Fig. 6). Pokeweed antiviral protein (PAP), ricin, and Stx remove the adenine base.

These toxins are found in different plants and bacteria, but exhibit several common characteristics and mechanism of action. Evolution of these toxins is unknown, although it is hypothesized that RIPs originated as a mechanism for plant defense. Most RIPs share similar structures, indicating a close evolutionary background. One of the most highly conserved regions for these RIPs is the active site. The active site for PAP, ricin, and Stx this is the region of E167 to R170; which is composed of Glu-X-X-Arg (10,12,16).

Despite similar structure and mechanisms of the A subunit, Stx1 and Stx2 cause different severity of infection and different tissue damage. If a patient is infected with *E. coli* O157:H7 that mainly produces Stx2, they will more likely develop HUS compared to infection by a strain producing Stx1 (8).

There are other factors that may play a role in the pathogenicity of Stx. There is greater accessibility to the substrate in the crystal structure of the Stx2 active site than in

Stx (7,8). There are differences in the carboxyl terminus of the Stx1 A and Stx2 A-subunits. In Stx2, this region forms an ordered structure, a short 2-turn α-helix, through the B pentamer. The Stx A subunit shows disorder in this same region (6,8). One of the receptor binding sites in the Stx2 B-pentamer has a different conformation compared to Stx. In addition, the C-terminus of Stx2A interacts with one of the receptor binding sites; in Stx the site is unoccupied (6,8). These differences in structure may affect pathogenicity, and affinity of substrate and receptor binding of the toxin.

It is important to understand the mechanism of action of Stx1 and Stx2 on a molecular level in order to find efficient treatment for the diseases caused by STEC. There is indirect evidence that human vaccination against STEC may be effective in preventing illness, but there are no vaccines or therapeutics for human STEC infections. STEC strains are generally susceptible to a variety of antibiotics, but their use exacerbates the symptoms of infection. One hypothesis is that the use of antibiotics lyses the bacteria, releasing more toxin or the bacteria produces more toxin in response to antibiotics. This study attempts to understand the mechanism of action of Shiga-like toxins at the molecular level, which is an important step towards developing treatment methods against STEC.

In the following sections I will discuss the comparative analysis for mutations in homologous regions between Stx1A and Stx2A and interpret why Stx2 is generally more toxic. In addition, I will discuss the results of some Stx2A mutants by random mutagenesis, which demonstrates that Stx2A toxicity is not a direct result of ribosome depurination or translation inhibition alone.

Materials and Methods

The full-length cDNAs of Stx1A and Stx2A was cloned into pGEMT-easy (Promega) vector by PCR using DNA isolated from *E. coli* O157:H7 strain ATCC43895 which produces both Stx1 and Stx2. The cloning was performed by Dr. Rong Di (Biotech Center, Rutgers). Stx1A cDNA includes the N-terminal 22-residue signal peptide and the 293-residue A1/A2 coding sequence and the Stx2A cDNA contains the N-terminal 22-residue signal peptide and the 297-residue A1/A2 coding sequence. The Stx1A and Stx2A cDNA was then cloned into the yeast expression vector pYES2.1/V5-His-Topo (Invitrogen) downstream of the galactose-inducible *GAL1* promoter, resulting in NT890 for Stx1A and NT901 for Stx2A. They contain a V5 and Histidine tag at the 3' end of the genes. NT890 and NT901 plasmids were transformed into *Saccharomyces cerevisiae*, yeast strain W303 (MAT a, ade2-1 trp1-1 ura3-1 leu2-3, 112 his3-11, 15 can1-1000)

Random mutagenesis

The NT809 and NT901 plasmids were incubated with 7% hydroxylamine for 20 hr at 37°C. Hydroxylamine is a mutagen which produces point mutants randomly along the entire length of the plasmid. The plasmid was cleaned by precipitation using phenol-chloroform and ethanol, and then was transformed into yeast cells. Yeast cells were plated onto synthetic SD-Ura dropout media with 2% glucose. Approximately 150 colonies were grown and replicated onto SD-Ura with 2% galactose. Plasmids were isolated from colonies which were able to grow in the presence of galactose. Growth on galactose indicates a reduction of toxicity. No growth on galactose plates indicate the

mutation is still toxic, as in the case of wild-type Stx1A and Stx2A. These plasmids which could grow on galactose containing media were selected for further characterization. The plasmids were transformed into *E. coli* (DH5α) cells by electroporation transformation. Electroporation is a method of transforming *E. coli* with efficiencies greater than chemical methods. The surviving cells have a higher percentage of containing the plasmid. To prepare competent bacterial cells for electroporation, fresh cells were treated with ice cold water and 10% glycerol. These cells were then transformed in sterile cuvettes along with the DNA plasmid obtained from the yeast colonies as stated above. A Gene Pulser apparatus set at 1.8 kv sends pulse through the mixture. The mixture is plated onto Luria-Bertani (LB) plates containing ampicilin (100 μg/mL). The bacterial colonies were grown to isolate plasmids which were retransformed into yeast to confirm that the resistance was due to the plasmid. Subsequently the plasmids were sequenced to determine its mutation.

Site-directed mutagenesis

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate point mutations in NT890 and NT901 according to manufacturer instructions with mutated primers.

Structural analysis of Stx

Coordinates from the crystal structures from the Protein Data Bank of Stx (1DM0), and Stx2 (14RP) were analyzed using Protein Explorer software.

Growth rate analysis

Yeast cells were grown in SD-Ura containing 2% glucose to an OD of 0.3 at A_{600}. They were then transferred to SD-Ura containing 2% galactose to induce Stx expression. The OD was measured every 2 hours up to the 12 hour time point. Doubling time was calculated based on these time points using the following formula.

$$T_d = (t_2 - t_1) * \frac{\log(2)}{\log(\frac{q_2}{q_1})}$$

(T_d=doubling time; t_1=initial time; t_2=final time; q_1= initial OD; q_2=final OD)

Cell viability assay

Yeast cells expressing wild type and mutant Stx1A and Stx2A were grown in SD-Ura containing 2% glucose to an OD of 0.3 at A_{600} and then transferred to SD-Ura containing 2% galactose. 10 µL of serially diluted cells were plated on SD-Ura plates containing 2% glucose at 0, 4, 6 and 10h post induction. The cell growth was compared to wild type Stx1A or Stx2A.

Protein extraction from yeast cells

Yeast cells were grown for protein isolation using the same growing conditions as above and induced for 8 hours. Cells were resuspended in 1 × low salt buffer (20 mM HEPES-KOH, pH 7.6, 0.1 M potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) and lysed using glass beads. Samples were centrifuged briefly at 2300 rpm to remove cell debris and glass beads. The supernatant was transferred to a new tube and centrifuged at 15,000 rpm for

15 min to separate cell membranes and cytosolic fraction. After removing the cytosolic fraction, the membrane pellet was resuspended in 10 µl of 1× low salt buffer.

rRNA depurination assay

Dual primer extension analysis was used to quantify rRNA depurination. 2 µg of total yeast RNA from cells expressing wild type Stx1A and Stx2A and mutants were hybridized with 10^6 cpm of γ-ATP end-labeled depurination primer (5′-AGCGGATGGTGCTTCGCGGCAATG-3′) downstream of the depurination site. The second primer hybridized upstream of the depurination site close to the 5′ end of the 25S rRNA (25S). The labeled 25S control primer was diluted 1:4 with unlabeled 25S control primer. Superscript II reverse transcriptase was used in the primer extension assay. Extension fragments (100 nt and 73 nt, respectively) were separated by electrophoresis on a urea-5% polyacrylamide denaturing gel and quantified by a PhosphoImager.

Translation inhibition

Translation inhibition was measured by *in vivo* [^{35}S]-methionine incorporation. Yeast cells were grown to an A_{600} of 0.3 in SD-Ura-Met containing 2% glucose. Cells were then resuspended in SD-Ura-Met containing 2% galactose for 6 h to induce the expression of either wild-type pre-RTA or the mutant forms. At time zero, [^{35}S]-methionine was added to induced cells. [^{35}S]-methionine was added at 0 min, 15 min, 30 min, and 45 min, 400 µl of yeast cells was removed for growth measurements, and additional aliquots of 400 µl were assayed for methionine incorporation in duplicate. The cpm was normalized to the A_{600} reading, and rates of translation were determined as

cpm/A_{600}/minute. Final results were displayed as percentages of total translation in yeast harboring the empty vector.

Results

Random and site-directed mutagenesis

The Stx1A and Stx2A genes were cloned into the pYES2.1/V5-His-Topo yeast expression vector (Invitrogen) downstream of the *GAL1* promoter. This resulted in NT890, the plasmid containing Stx1A and NT901, the plasmid containing Stx2A. NT890 and NT901 plasmids were randomly mutagenized by hydroxylamine and transformed into yeast. Colonies that were observed growing on SD-Ura medium with 2% glucose were then picked and replica plated on SD-Ura plates containing 2% galactose. Colonies growing on galactose indicate a reduction of toxicity, whereas wild-type Stx1A and Stx2A do not grow on galactose-containing plates.

82 of these Stx2A random mutations were selected for further characterization. Of these 82 mutations, 61 were unique. 19 contained single amino acid changes, 27 has early stop codons, 12 contained two amino acid changes, and the rest consisted of wild type, frame shifts, and three or more amino acid changes.

The random mutations were separated into three groups for classification purposes. Group I contains 21 single amino acid mutants. Group II contains mutations with stop codons, 10 in total. Group III contains 13 double mutants. Table 1.1 and 1.2 summarizes the data for all the mutants analyzed.

An additional set of 14 mutants were created, for both Stx1A and Stx2A, through site directed mutagenesis. Site-directed mutants are labeled “s.d.”. The mutations were made based on previous works in our lab with other RIPs, pokeweed antiviral protein (PAP) and ricin A subunit (RTA) (11,16). Point mutations were made corresponding to PAP point mutants that abolished cytotoxicity. Alignment of the amino acid sequence of

Stx2A against PAP and RTA show conserved domains in the active site and C-terminal domain and a region critical for substrate binding (Fig. 7). Mutations were made in these conserved regions. N75 and Y77 residues are related to substrate binding; residues between E167 and R170 are in the active site; C-terminal truncation mutants were made by introducing stop codons starting at the Cys residue in the A1 fragment which forms the disulfide bond to the Cys in the A2 fragment. The residues changed to stop codons are from positions V236 to C242 in Stx1A, and the homologous region V235 to C241 in Stx2A. A summary of the analysis of the site-directed and random Stx1A and Stx2A mutants are listed in two tables at the end of the results section.

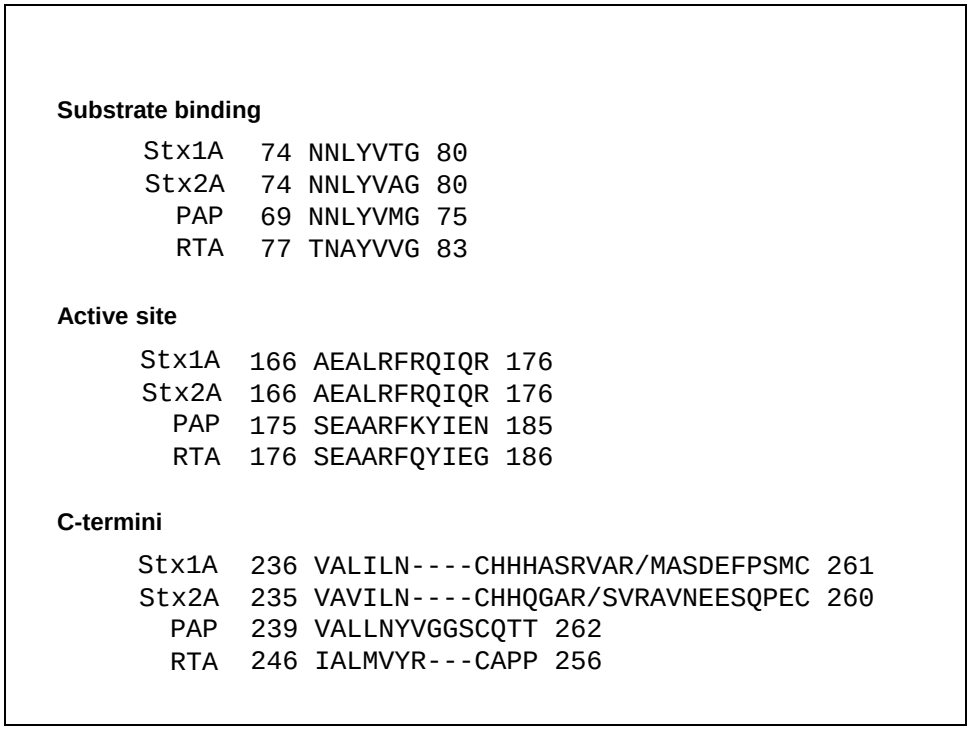

Fig. 7 Amino acid alignment of conserved regions in Stx1A, Stx2A, PAP, and RTA

Comparison of Stx1A and Stx2A substrate-binding domain and active site mutants

Cytotoxicity of Stx1A and Stx2A mutants were greatly reduced or abolished. The growth inhibition of yeast cells expressing Stx1A or Stx2A mutants were determined by viability assay (16). Viability assay involved induction of cells with galactose containing medium, this induced the protein expression. After 10 hours of induction cells were plated on to SD-Ura plates containing glucose. The amount of surviving cells was visually compared to the cells at 0 hour induction and the vector control after the same treatment. Mutants given a "++" rating showed similar amount of survival as the wild, indicating that mutant had approximately the same toxicity as the wild type. Mutants rated "-" had similar amounts of survival as the vector control, meaning expression of that mutant was non-toxic. Mutants rated "+" had greatly reduced toxicity (Table 1). The viability assays were repeated in triplicate and the representative colonies were shown (Fig. 8).

On visual inspection yeast cells expressing Stx1A showed survival to the 10^{-3} dilution and had one colony growing at the 10^{-4} dilution. In contrast, after induction of Stx2A in the same conditions there was a sharp decrease of surviving cells at the corresponding dilutions of Stx1A. This is a general indication that Stx2A is more toxic in yeast cells than Stx1A (Fig. 8).

The Asn and Tyr residues (N75 and Y77) are conserved among all four RIPs (PAP, ricin, Stx1, Stx2) at the substrate binding domain. In both Stx1A and Stx2A, the N75A mutation greatly reduced the cytotoxicity. The Stx1A Y77A mutation showed reduced cytotoxicity as well, but Stx2A Y77A was slightly more toxic (Fig. 7). This suggests Stx1A-Y77 plays a more critical role in toxicity than Stx2A-Y77A.

E167 and R170 are part of the active site in Stx1 and Stx2 (6, 8, 10). The E167K/R176K double mutant in Stx2A was originally selected through the random screening. This mutant was completely non-toxic. This prompted us to make the double mutation in Stx1A and single mutations of each amino acid individually in Stx1A and Stx2A. The allowed us to determine if either the E167 or the R176 position played a more critical role in toxicity or they worked in conjunction. The single mutations; E167A, R170A, R176K and the double mutations; E176A/R170A, E167K/R176K all reduced toxicity of both Stx1A and Stx2A. The double mutants together have a greater effect and are completely non-toxic. The behavior of E167 in Stx2A correlates well with the same mutation in Stx1A (10). This suggests that E167K and R176K have an additive effect of abolishing toxicity and shows R176 may play a role in the active site. This demonstrates for first time that R176 plays an important role in toxicity. Stx2-R170A shows less survival than the same mutations in Stx1A, indicating a difference in the active sites of Stx1A and Stx2A (Fig. 8).

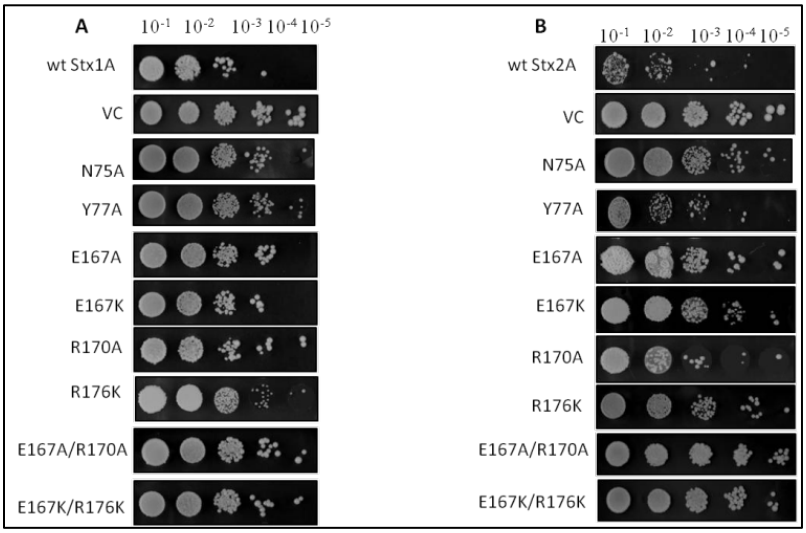

Fig. 8. Viability of yeast cells expressing Stx1A (A) and Stx2A (B) substrate-binding domain and active site mutants. Mutants or the empty vector were induced for 10 hours on SD-Ura galactose media and were then plated as serial dilutions onto SD-Ura glucose plates.

The doubling time of Stx1A and Stx2A and substrate-binding domain and active site mutants are lower than Stx1A and Stx2A. The growth rate of each of the mutants was calculated by their doubling time after induction with galactose. The doubling time for Stx1A is 13.3 hours, slightly shorter than Stx2A. The doubling time for the cells expressing wild type Stx2A is 14.7 hours and the vector control is 6.0 hours (Table 1). Those mutants that were non-toxic or have greatly reduced toxicity have a doubling time between 6 and 10 hours. Doubling time correlated well with viability assay.

Proteins for wild-type Stx1A and Stx2A and substrate-binding domain and active site mutants are expressed and accumulate in yeast membrane. All mutants were induced for 6 hours with galactose containing SD-Ura medium in order to examine the protein expression. The Stx1A and Stx2A genes were cloned to express a V5 tag at the C-terminal end. Anti-V5 antibody was used to probe the blots for protein expression. The blot was then reprobed with antibody against dolichol-phosphate mannose synthase (Dpm1p) as a loading control. Both constructs of Stx1A (NT890) and Stx2A (NT901) contained an N-terminal 22-residue signal peptide that directed the toxin to the ER membrane. The molecular weight of Stx1A and Stx2A are approximately 32 kDa. All of the substrate-binding domain and active site mutants showed expression and had equivalent molecular weight to wild type Stx1A and Stx2A (Fig. 9). Stx2A-Y77A shows greater expression levels than Stx1A-Y77A. Those mutations were created through site-directed mutagenesis. There should be no other mutations in the plasmid. It will be shown later, this higher level in expression does not affect depurination or translation

inhibition in Stx2-Y77A. These results show that the loss of cytotoxicity was not due to the loss of protein expression.

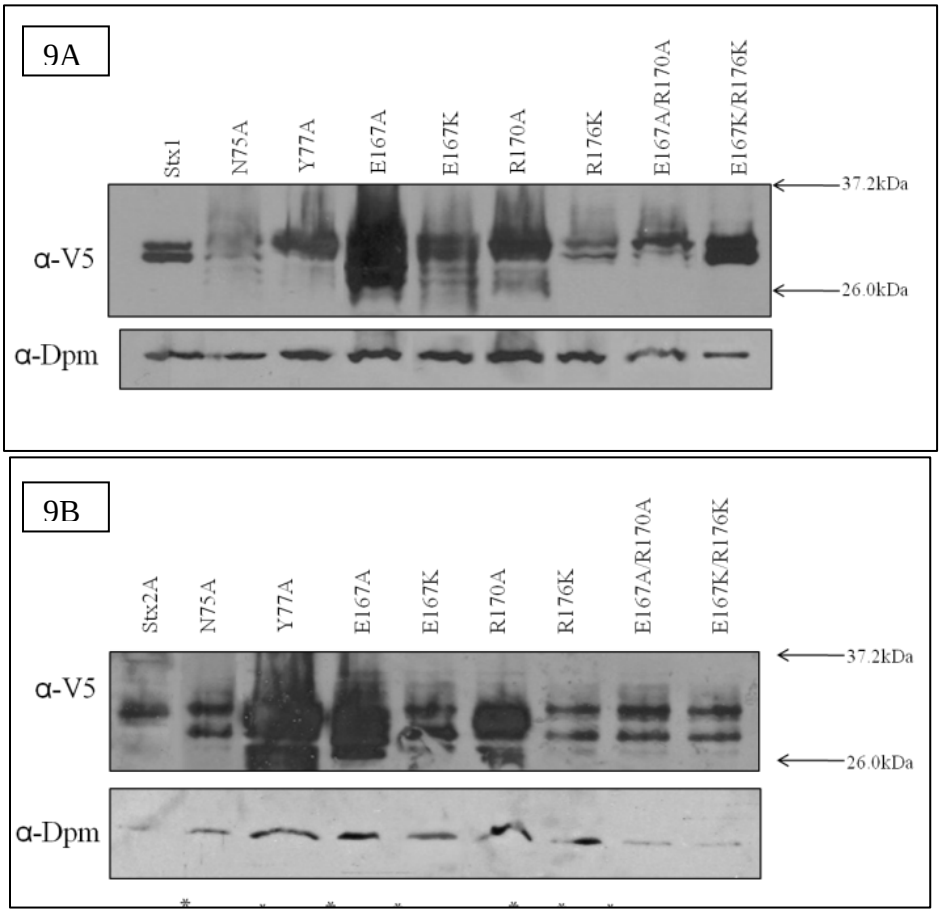

Fig. 9. Immunoblot analysis of yeast cells expressing Stx1A (A) and Stx2A (B) mutants. Membrane fractions (20-30 μg) isolated from cells expressing mutants containing a premature termination codon, mutations were separated on a 12% SDS-polyacrylamide gel and probed with monoclonal anti-V5(1:5,000). The blots were stripped and probed with the ER membrane marker Dpm1p as a loading control.

Wild-type Stx1A and Stx2A and substrate-binding domain and active site mutants depurinate rRNA. Total RNA was isolated from each mutant after 6 hours of induction and examined for depurination of rRNA by dual primer extension analysis. Ribosomes were depurinated in cells expressing Stx1A and Stx2A. The percentage of rRNA depurination of each mutant was calculated and averaged from several independent experiments compared to wild type Stx1A and Stx2A (Table 1). Some of the mutants were either non-toxic or had reduced toxicity but were able to depurinate rRNA (Fig. 10). This phenomenon has also been observed in PAP and ricin, indicating that depurination is not the only factor responsible for cell death for Stx1A and Stx2A (11,16).

In Stx1A, the N75A and Y77A mutation had depurination activity equal to the wild type, but in Stx2A depurination was significantly decreased (Fig. 10). X-ray crystal structure analysis has shown Y77 forms different conformations to the catalytic site involving Stx1A and Stx2A and affects the affinity for binding the substrate (7,8). This indicates that this site is more important for depurination in Stx2A because an alteration at this site almost completely abolished depurination even though it did not abolish toxicity (Fig. 8). As shown in the viability assay this site may be why Stx2 is more toxic. N75 and Y77 seem to be more critical for depurination in Stx2 than Stx1. Although depurination of Stx2-N75A and Y77A are abolished, its toxicity is not. This provides further evidence that toxicity can be separated from depurination of the SRL.

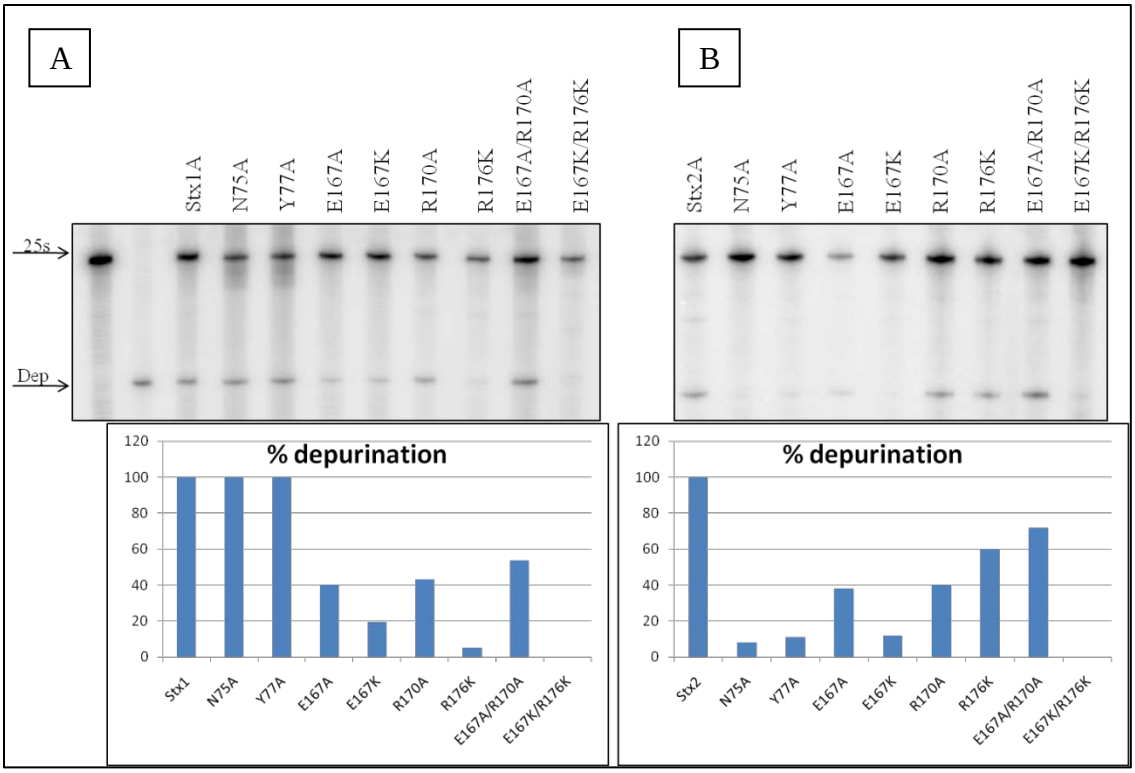

Fig. 10. Ribosome depurination in yeast expressing Stx1A (A) and Stx2A (B) substrate-binding domain and active site mutants. Total RNA isolated from the Stx1A and Stx2A yeast mutants after 6hr of growth on galactose was analyzed by dual primer extension. The depurination bands and 25S bands were quantified and graphed as a ratio of depurination:25S.

The active site single and double mutations at E167, R170, and R176 reduced depurination activity of Stx1A and Stx2A (Fig. 10). The E167K/R176K mutation showed no depurination activity in both toxins. Based on depurination activity of the active site mutants, Stx1A-R176 and Stx2A-E167 appeared most critical for reducing depurination. R176 may play a role in the active site of both toxins. This shows that there are differences in the active sites of both toxins.

Ribosome depurination of Stx1A and Stx2A in several substrate binding domain and active site mutants did not correlate with translation inhibition. Total protein translation in cells expressing Stx1A and Stx2A was examined by [^{35}S]-methionine incorporation. The percentage translation was compared to vector control cells. Translation was reduced to 42% in cells expressing Stx1A and 45% in cells expressing Stx2A (Fig. 11). In general the substrate -binding domain and active site mutants had higher translation rates than the wild type toxins. The R170A mutation in both toxins did not have an effect on inhibition of translation. Whereas Stx1A-E167K and Stx1A-E167K/R176K did not inhibit translation, these same mutations in Stx2A showed translation at 70% of the vector control. This is another indication that Stx2A is more toxic than Stx1A. R176K showed very little depurination in Stx1A (10% of wt Stx1A), but inhibited translation (50.1% of vector). This indicates that depurination does not correlate with translation inhibition for Stx1A-R176K. While E176A/R170A showed reduced depurination (50% of wt Stx1A), translation inhibition is also reduced, 81.6% of vector compared to 43.4% of wt Stx1A indicating a correlation. Also, it shows R176K decreased translation inhibition and may be involved in the active site. A variation of depurination activity and translation inhibition between the active site of Stx1A and Stx2A demonstrates there are differences in the active site between the two toxins. This data suggest that depurination is not the only mechanism for Stx1A and Stx2A to cause cell death. Translation inhibition may also play a role in decreased viability of cells expressing Stx1A and Stx2A.

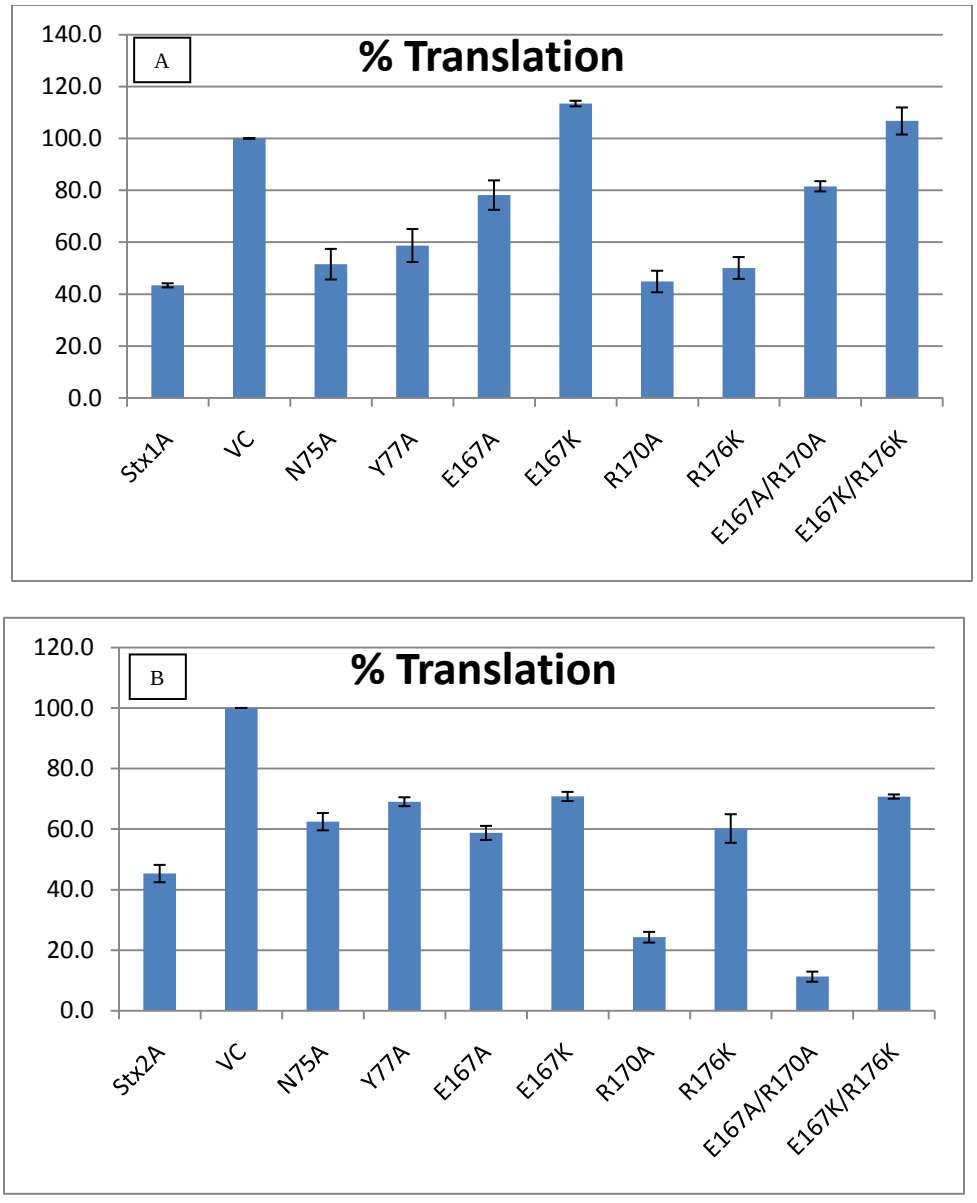

Fig. 11. Translation rate of yeast expressing Stx1A (A) and Stx2A (B) site-directed mutants. Yeast cells were grown to an A_{600} of 0.3 in SD-Leu-Met containing 2% glucose. Cells were then resuspended in SD-Ura-Met containing 2% galactose for 6 h to induce the expression of either wild-type pre-RTA or the mutant forms. At time zero, [^{35}S]-methionine was added to the induced cells. After 30 min, 400 µl of yeast cells was removed for growth measurements, and additional aliquots of 400 µl were assayed for methionine incorporation in duplicate as previously described. The cpm was normalized to the A_{600} reading, and rates of translation were determined as cpm/A_{600}/minute. Final results were displayed as percentages of total translation in yeast harboring the empty vector.

Comparison of Stx1A and Stx2A C-terminal mutants

Cytotoxicity of Stx1A and Stx2A C-terminal mutants were greatly reduced or abolished. In Stx1A, C242* and N241* appeared as toxic as the wild-type. L240* is where toxicity is lost. All other deletions towards the N-terminus are non-toxic. The mutations in Stx2A are slightly different. In Stx2A toxicity is lost at I238* and all other deletions towards the N-terminus are non-toxic. Stx1A-L240* is still toxic but the homologous mutation Stx2A-L239* is not toxic (Fig. 12). Again this indicates Stx2A is more toxic than Stx1A because Stx2A needs a truncation of an additional residue to be non-toxic. Stx1A-L240* and Stx2A-I238* are critical residues where toxicity is lost.

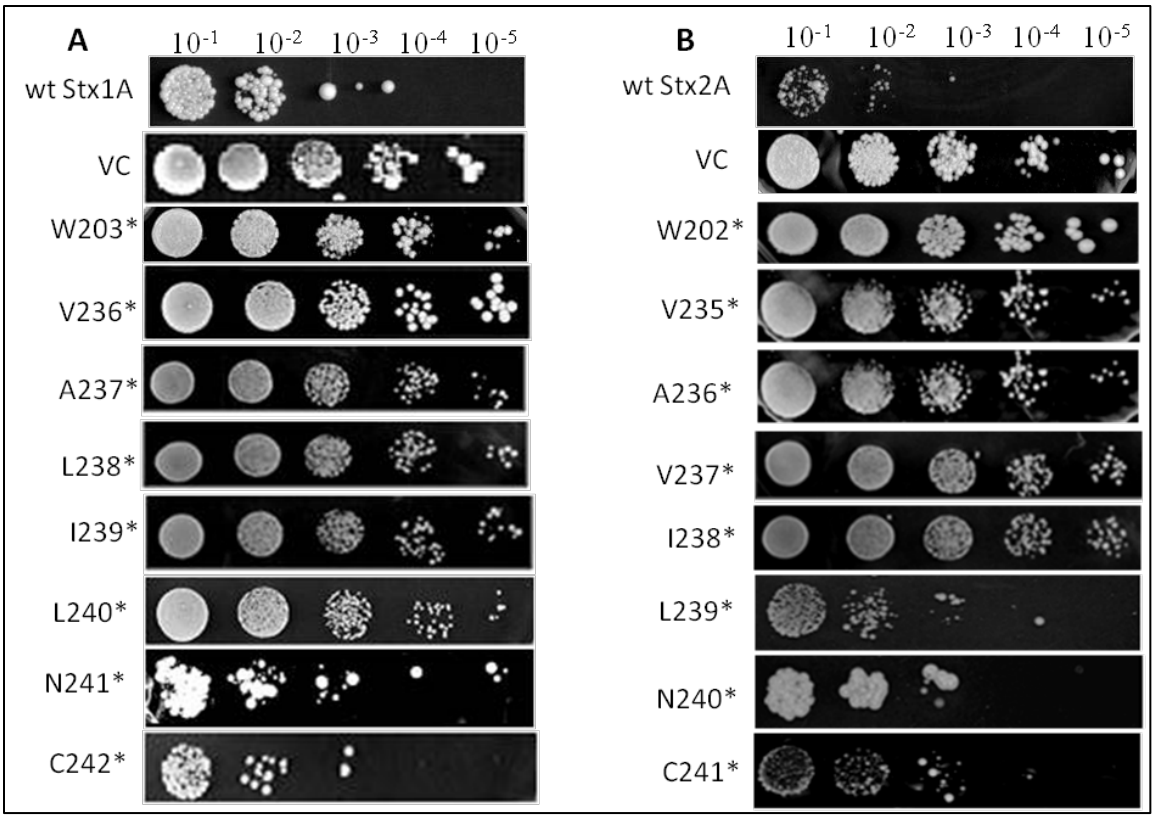

Fig. 12. Viability of yeast cells expressing Stx1A (A) and Stx2A (B) C-terminal mutants. Yeast cells expressing Stx1A or Stx2A C-terminal mutants induced for 10 hours in SD-Ura galactose media and were then plated as serial dilutions onto SD-Ura glucose plates.

Non-toxic Stx1A and Stx2A C-terminal mutants grow faster than wild-type toxin. Non-toxic Stx1A mutants had doubling time between 4.4 to 12.6 hours. Non-toxic Stx2A mutants had doubling time between 6.3 to 9.2 hours. The doubling time for the C-terminal mutants correlate with viability assay (Table 1).

Stx1A and Stx2A C-terminal mutants result in unstable protein. The protein sizes of the C-terminal mutants for both toxins appear smaller than the wild type (Fig. 13). Each additional truncation towards the N-terminus resulted in a slightly smaller toxin. A majority of the C-terminal mutants show additionally smaller bands or smearing, indicating possible effects of protein aggregation and breakdown.

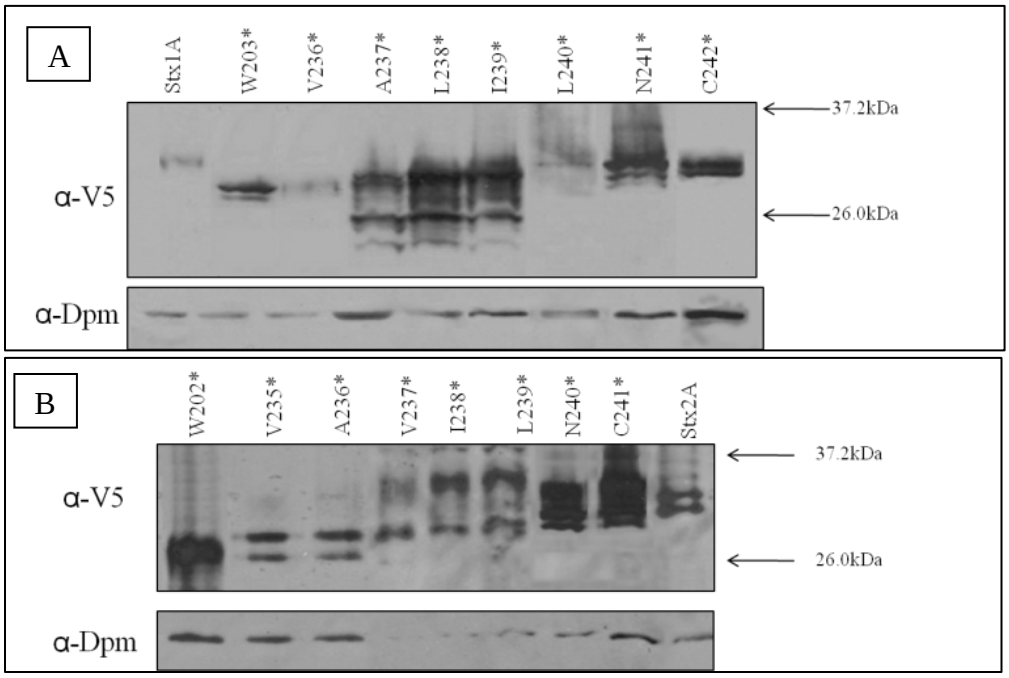

Fig. 13. Immunoblot analysis of yeast cells expressing Stx1A (A) and Stx2A (B) C-terminal mutants. Membrane fractions (20-30 μg) isolated from cells expressing mutants containing a premature termination codon, mutations were separated on a 12% SDS-polyacrylamide gel and probed with monoclonal anti-V5(1:5,000). Blots were stripped and probed with the ER membrane marker Dpm1p as a loading control.

Stx2A C-terminal mutants show more depurination activity than Stx1A mutants. Both toxins exhibit a similar trend seen in the viability assay. The toxins lose depurination activity with each successive deletion (Fig. 14). In Stx1A there is a large decrease in depurination from N241*(100%) to L240*(53%). L240* is also the same mutation where it appears non-toxic in viability assay. In Stx2A a similar drop in depurination activity occurs from V237*(80%) to A236*(20%). Stx2A is non-toxic at I238*. This is further evidence showing non-toxic mutants (Stx2A-V237* and I238*) are still capable of depurination at approximately the same levels as the wild-type. Stx2A mutants showed more depurination activity than the homologous Stx1A mutants. Stx1A-V236* had no depurination activity, while Stx2A-V235* had 20%. These data again indicate that depurination is not solely responsible for cell death caused by Stx1A and Stx2A.

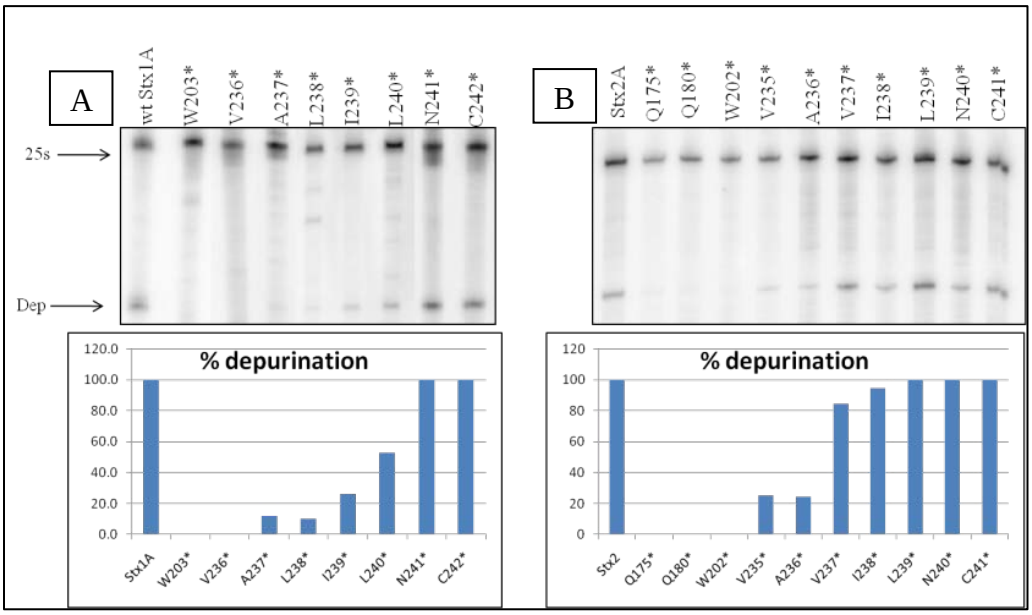

Fig. 14. Ribosome depurination in yeast expressing Stx1A (A) and Stx2A (B) C-terminal mutations. Total RNA isolated from the Stx1A and Stx2A yeast mutants after 6hr of growth on galactose was analyzed by dual primer extension. The depurination bands and 25S bands were quantified and graphed as a ratio of depurination:25S.

Non-toxic Stx1A and Stx2A C-terminal mutants can inhibit translation. Stx1A-L240* and Stx2A-I238* are critical residues where toxicity is lost. Stx1A-L240* had a translation rate of 47.3%, while Stx2A-I238* had a translation rate of 72.4%. Stx1A-L240* is non-toxic but inhibits translation similar to the wild-type (43.4%). The ability of Stx2A-I238* to inhibit translation was greatly compromised in comparison to its wild-type (45.3%). The homologous mutation, Stx1A-I239*, did not inhibit translation. The mutations Stx1A-W203*, V236*, A237*,L238* , I239* and Stx2A-W202*, V235*, A236*, V237*, all lost their ability to inhibit translation (Fig. 15).

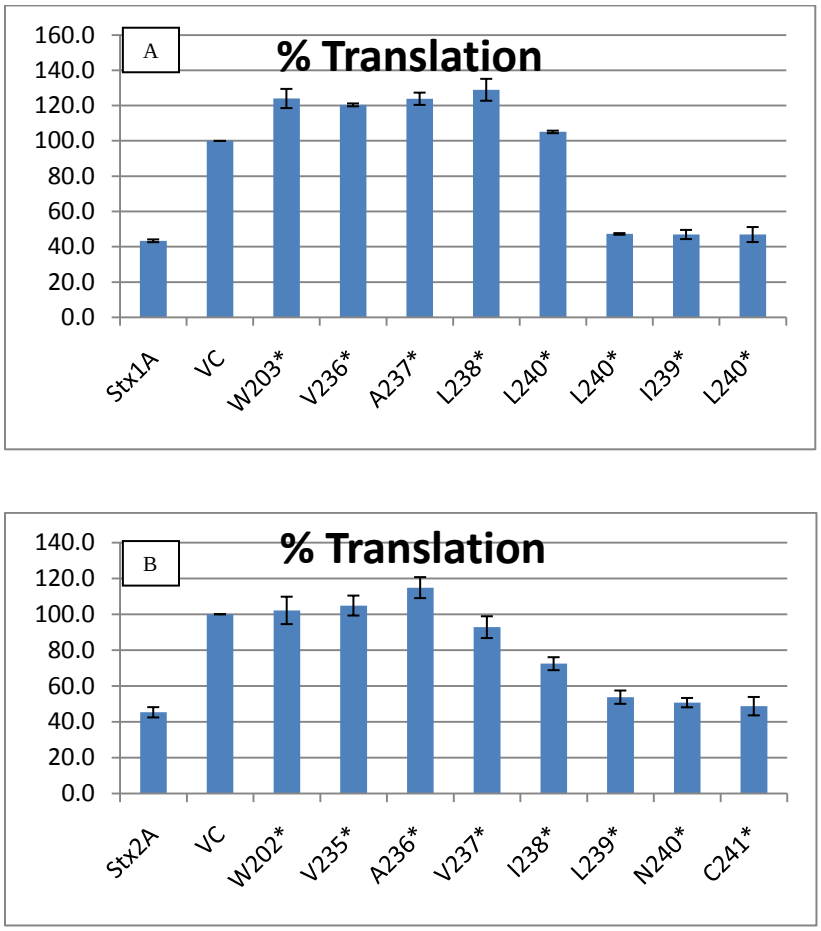

Fig. 15. Translation rate of yeast expressing Stx1A (A) and Stx2A (B) C-terminal deletion mutants.

Analysis of other Stx2A random mutants

The majority of Stx2A random mutants are still toxic. In this section I will discuss about the other random mutations of Stx2A. Some random mutations have been previously analyzed in the comparison of Stx1A and Stx2A mutants. Only 10 out of the 30 random mutations showed reduced or abolished toxicity.

In the Group I single mutations, cytotoxicity of R11W, G80E, R119C, R179H, and R179C was greatly reduced (Fig. 16A). All of the Group II stop mutations were non-toxic, Q175*, Q180*, and W202*. W202* was previously analyzed in comparison with mutations in Stx1A. Any additional truncations upstream of W202* are assumed to be non-toxic. In the Group III double mutations, V-4I/G80R and E167K/R176K showed reduced toxicity (Fig. 16C). V-4I/G80R has a mutation in the signal sequence. This may disrupt its signaling towards the ER or effect how it is processed through retrograde transport. In general, mutations outside the 3 previously mentioned domains did not affect toxicity.

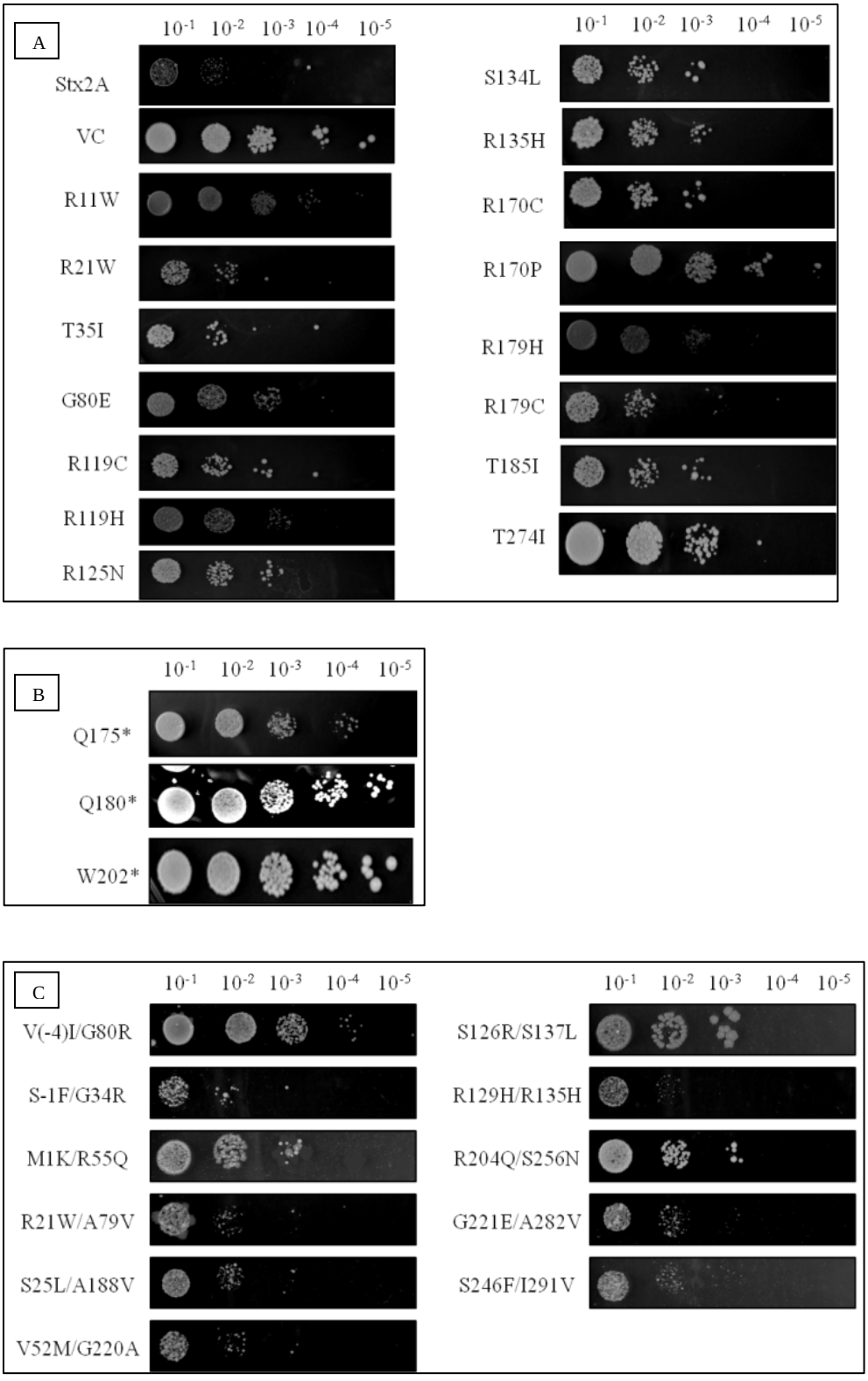

Fig. 16. Viability of yeast cells expressing Stx2A random mutants. (A) Group I single mutants. (B) Group II stop mutants. (C) Group III double mutants

The doubling times of Stx2A random mutants are equivalent to wild-type Stx2A. The doubling times of the random mutants correlated with the viability assay. The toxic mutants had doubling times between 10 and 15 hours. The mutants with reduced toxicity from the viability assay had doubling times between 6 and 8 hours (Table 2). The same assumption from the viability can be made, mutations outside of the 3 previously mentioned domains have little effect on doubling time.

Stx2A random mutants are expressed and accumulate in yeast membrane. All of the Stx2A random mutants showed detectable levels of expression (Fig. 17). The Group II C-terminal mutants ran further as expected compared to the wild type Stx2A. A majority of the Group III double mutants contained larger and smaller bands, indicating possible effects of protein aggregation and breakdown. These results show that the loss of cytotoxicity was not due to the loss of protein expression.

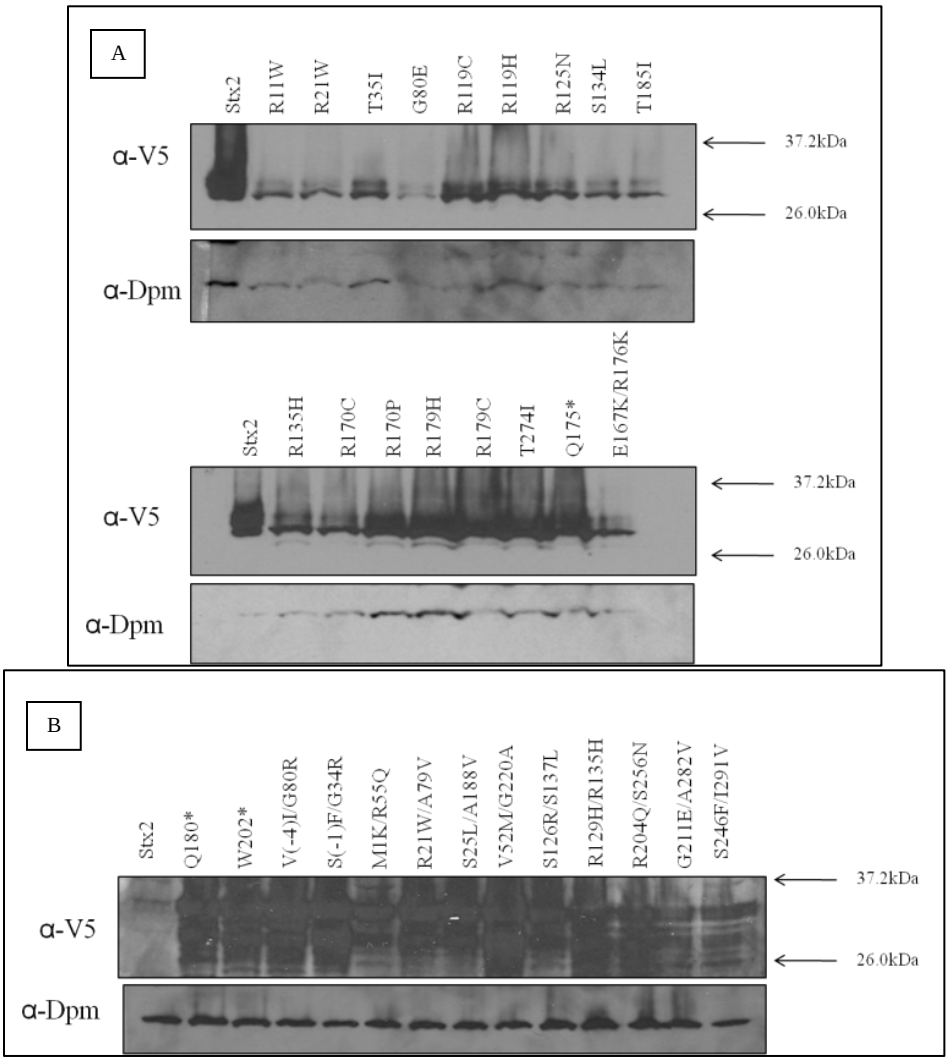

Fig. 17. Immunoblot analysis of yeast cells expressing Stx2A random mutants. Membrane fractions (20-30 μg) were isolated analyzed by the same methods previously described in the comparison of Stx1A and Stx2A mutants. (A) Group I Mutants and Q175* from Group II. (B) Group III mutants and Q180* and W202* from Group II.

Stx2A random mutants depurinate rRNA. All of the Group I mutants were still able to depurinate rRNA (Fig. 18). Mutations R21W (24%), R119H (26%), and R179H (10%) showed the greatest reduction in depurination activity. According to the viability assay, these mutantions only slightly reduced toxicity. This shows mutants can still be toxic even though their depurination activity has been greatly reduced (Table 2). All of the Group II mutants did not depurinate. Based on the site directed C-terminal mutations, any truncations upstream of W202* should not depurinate (Table 2). Only one mutant in Group III showed significant reduction in depurination,V(-4)I/G80R (23%). As previously mentioned, the signaling towards the ER or how it processed through retrograde transport may be affected. Therefore, accessibility to the rRNA may be reduced.

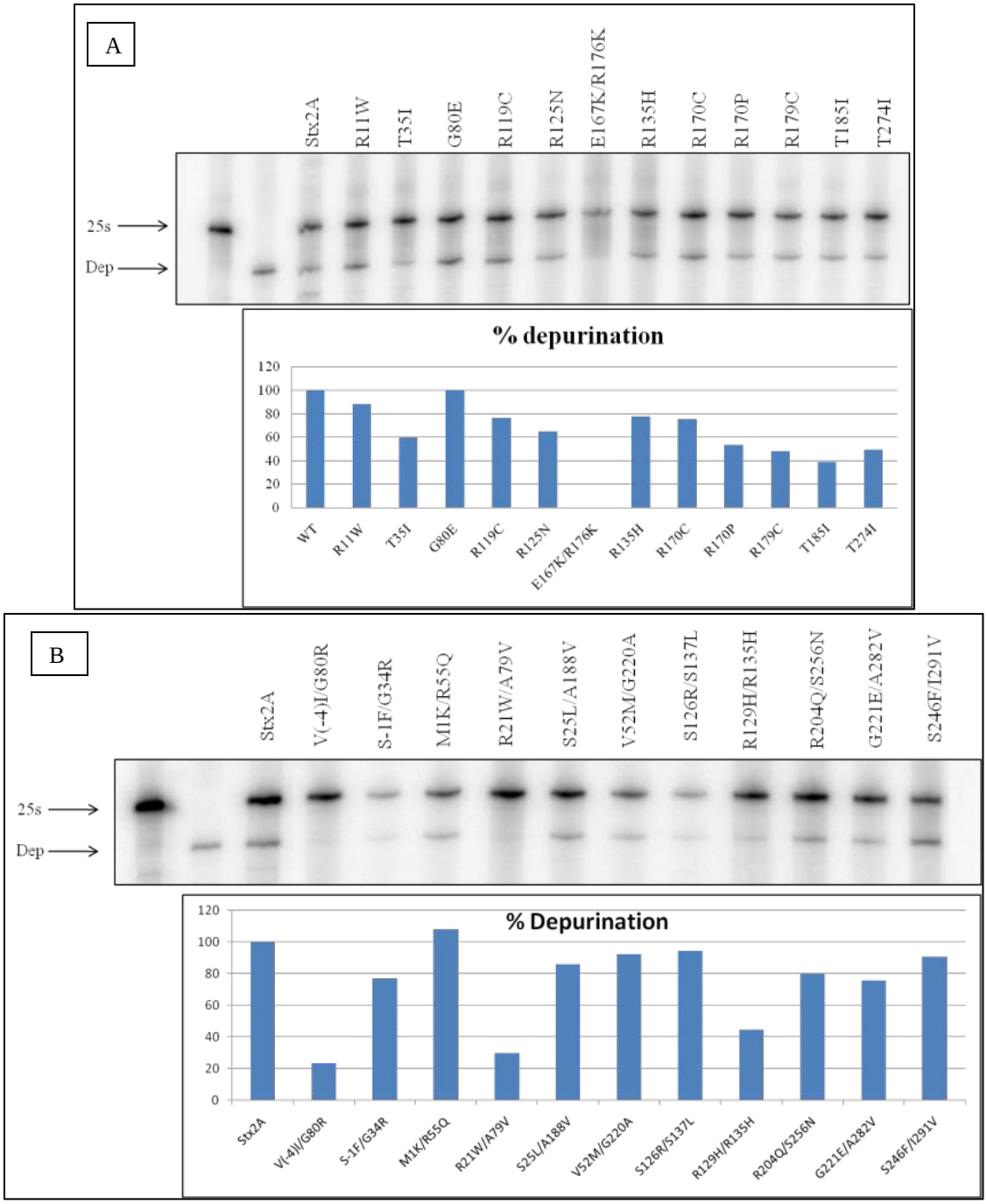

Figure 18. Ribosome depurination in yeast expressing Stx2A random mutations. Total RNA isolated from the Stx1A and Stx2A yeast mutants after 6hr of growth on galactose was analyzed by dual primer extension. The depurination bands and 25S bands were quantified and graphed as a ratio of depurination:25S. (A) Group I muntants. (B) Group III mutants. Group II mutants were shown previously in Figure 14.

Stx2A random mutants inhibit translation. Two mutants in Group I did not significantly inhibit translation, R21W (95%) and R119H (88%). The Group II mutants did not inhibit translation. The Group III mutants inhibited translation from 50% to 80%. Translation inhibition of Stx2A random mutants correlated with depurination activity, similar to the site-directed mutants. Fig. 19 summarizes translation rate for Stx2A random mutants.

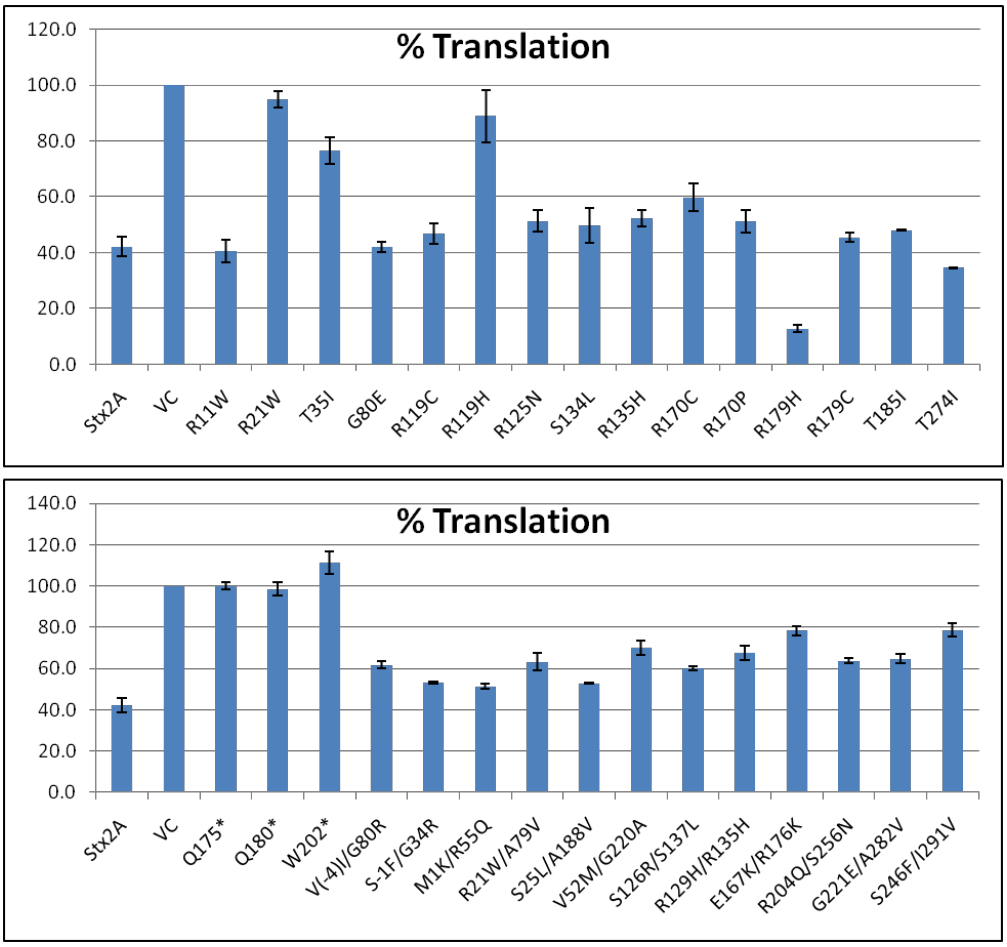

Fig. 19. Translation rate of yeast expressing Stx2A random mutants. Yeast cells were grown to an A600 of 0.3 in SD-Leu-Met containing 2% glucose. Cells were then resuspended in SD-Ura-Met containing 2% galactose for 6 h to induce the expression of either wild-type pre-RTA or the mutant forms. At time zero, [35]-methionine was added to induced cells. After 30 min, 400 µl of yeast cells was removed for growth measurements, and additional aliquots of 400 µl were assayed for methionine incorporation in duplicate as previously described.

Table 1 Characterization and comparison of Stx1A and Stx2A mutants

Yeast cell lines **Stx1A mutants**	**Mutation**	**Cytotoxicity**	**Depurination (% of wt)**	**Doubling time (hr)**	**Translation (% of VC)**
NT890	wild type (wt)	++	100	13.3	43.4
VC		-	0	4.5	100
NT2034 (s.d.)	N75A	+	100	6.3	51.6
NT2014 (s.d.)	Y77A	+	100	6	58.7
NT998 (s.d.)	E167A	+	50	6.5	78.2
NT2028	E167K	+	19	7.8	113.4
NT2041 (s.d)	R170A	+	53	10.3	44.9
NT2052 (s.d.)	R176K	+	5.3	6.2	50.1
NT2039 (s.d.)	E167A/R170A	-	50	5.8	81.6
NT2048 (s.d.)	E167K/R176K	-	0	4.7	106.7
NT2010	W203*	-	0	9.6	124
NT2032 (s.d.)	V236*	-	0	12.6	120.4
NT2049 (s.d.)	A237*	-	12	5.7	123.9
NT2050 (s.d.)	L238*	-	10	4.4	129
NT2051 (s.d.)	I239*	-	26	9.4	105.1
NT2040 (s.d.)	L240*	-	53	8.8	47.3
NT2042 (s.d.)	N241*	++	100	12.3	47
NT2033 (s.d.)	C242*	++	100	14.7	47
Stx2A mutants					
NT901	wild type (wt)	++	100	14.7	45.3
VC		-	0	6	100
NT2106 (s.d.)	N75A	+	8	5.3	62.4
NT2109 (s.d.)	Y77A	+	11	4.8	69
NT1057 (s.d.)	E167A	+	38	8.3	58.8
NT2142 (s.d.)	E167K	+	12	8.6	70.8
NT2108 (s.d.)	R170A	+	40	17.9	24.3
NT2143 (s.d.)	R176K	+	60	11.7	60.2
NT2125 (s.d.)	E167A/R170A	-	72	6.6	11.3
NT2114	E167K/R176K	-	0	7	70.7
NT2149	W202*	-	0	6.3	102.1
NT2115 (s.d.)	V235*	-	20.9	7.5	104.8
NT2128 (s.d.)	A236*	-	20.9	9.2	114.8
NT2129 (s.d.)	V237*	-	80	7	92.8
NT2130 (s.d.)	I238*	-	93.6	7.6	72.4
NT2131 (s.d.)	L239*	++	100	8.1	53.7
NT2124 (s.d.)	N240*	++	100	11	50.7
NT2113 (s.d.)	C241*	++	100	12.4	48.7

Table 2 Characterization of more Stx2A random mutants

Stx2 mutants	AA change	Cytotoxicity	Doubling time Hours	Depurination (% of wild-type)	% Translation Rate at (30 min)
wild type (wt)	Stx2A	++	14.7	100.0	42.1
Vector	VC	-	6.0	0.0	100.0
	Group I				
NT2127	R11W	-	9.3	87.9	40.5
NT2104	R21W	++	8.2	24.0	95.0
NT2131	T35I	++	8.5	60.0	76.5
NT2132	G80E	+	9.3	100.0	41.9
NT2110	R119C	+	9.6	76.7	46.8
NT2133	R119H	++	10.4	26.0	88.9
NT2134	R125N	++	10.6	65.2	51.3
NT2126	S134L	++	11.1	82.3	49.7
NT2135	R135H	++	11.4	77.8	52.4
NT2118	R170C	++	10.8	75.5	59.8
NT2136	R170P	++	13.3	53.3	51.2
NT2137	R179H	+	7.3	10.0	12.7
NT2138	R179C	+	7.7	48.0	45.4
NT2139	T185I	++	13.5	39.2	48.0
NT2140	T274I	++	10.0	49.6	34.5
	Group II				
NT2141	Q175*	-	6.2	5.0	100.0
NT2148	Q180*	-	6.8	47.6	98.4
NT2149	W202*	-	6.3	18.1	111.4
	Group III				
NT2150	V(-4)I/G80R	-	7.3	23.2	61.8
NT2151	S-1F/G34R	++	10.7	76.8	53.0
NT2152	M1K/R55Q	++	15.6	100.0	51.3
NT2153	R21W/A79V	++	13.7	29.7	63.2
NT2102	S25L/A188V	++	10.4	85.6	52.8
NT2154	V52M/G220A	++	13.8	92.0	70.0
NT2155	S126R/S137L	++	11.6	94.1	60.1
NT2156	R129H/R135H	++	12.0	44.5	67.6
NT2114	E167K/R176K	-	7.0	0.0	78.2
NT2157	R204Q/S256N	++	15.4	79.7	63.6
NT2123	G221E/A282V	++	12.1	75.5	64.7
NT2107	S246F/I291V	++	12.8	90.6	78.6

Discussion

Site directed and large scale random mutagenesis was carried out using 7% hydroxylamine on Stx1A and Stx2A wild type plasmid DNA. *Saccharomyces cerevisiae* was used as a simple and genetically tractable system to investigate the biological activity of Stx toxins and demonstrated that expression of Stx1A and Stx2A is lethal to yeast. Site-directed mutagenesis was performed based on conserved sequence alignment with other RIPs and previous works with PAP and ricin. These conserved regions are the substrate binding domain, active site, and C-terminal domain. The site directed mutants of Stx1A and Stx2A were compared to determine what factors play a role in the different levels of toxicity of each toxin. Analysis of the random mutants was performed to discover any other regions or residues are critical for toxicity.

Mutations that significantly reduced toxicity occurred in conserved domains; the substrate binding domain, the active site, and the C-terminus. The mutations in the substrate binding domain are N75A and Y77A, in both Stx1A and Stx2A. The main difference for the N75 and Y77 mutations is depurination activity in Stx1A, which is largely unaffected but in Stx2A activity is nearly abolished. In Stx1A, Y77 interacts with P258 of the A2 subunit. In Stx2A, Y77 binds the substrate by stacking with the adenine (7). Structural analysis of both toxins show a mutation at N75 may indirectly affect the positioning and interaction of Y77 with adenine (Fig. 20). The mutation at N75 may slightly alter the conformation or folding of the beta sheet Y77 is in, thus affecting binding for Stx2A. There is also a significant difference in the positioning of the Y77 with respect to the active site in both toxins (Fig. 22). Stx2-Y77 appears to position the adenine towards the active site. The crystal structure of Stx has not been resolved with any substrate in the region around the substrate binding and active site. Stx-Y77 may

have less involvement in binding for the substrate because of its positioning. This maybe why N75 and Y77 are more critical for binding the adenine substrate in Stx2A compared to Stx1A.

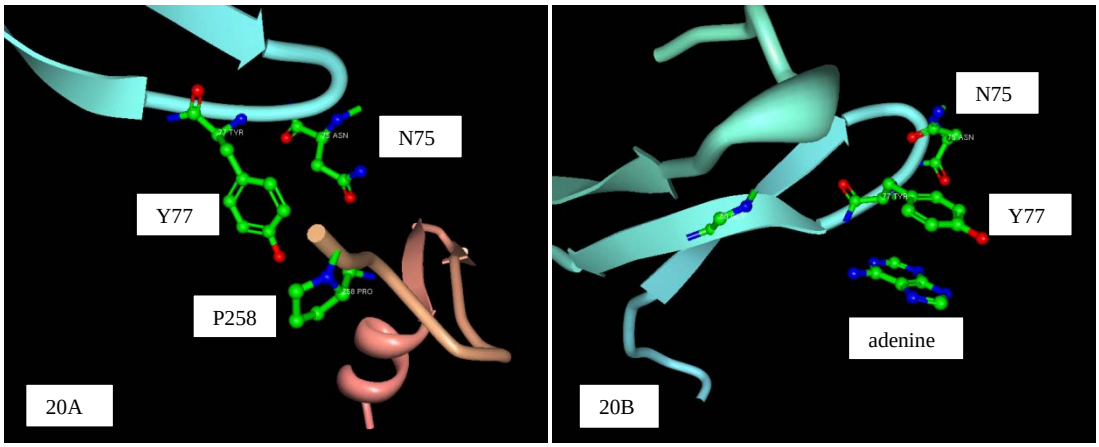

Fig. 20. Structural interaction of Y77 in Stx (A) and Stx2 (B). Protein data bank of Stx (1DM0), Stx2 (2GA4) (19)

As previously mentioned, Stx2A-N75A and Stx2A-Y77A may not be able to interact with the adenine in the SRL. The other main characteristics of these mutants in Stx1A and Stx2A are similar; cytotoxicity and translation inhibition. There were higher amounts of expression detected in Stx2A-Y77A than in Stx1A-Y77A. There was no depurination activity in Stx2A-Y77A even though there was more protein expression. This suggests that Stx2A-N75A and Stx2A-Y77A cause translation inhibition through another means besides depurination. One theory may be those mutations are still able to bind the ribosome even though they have lost depurination activity. This could prevent other elongation factors from interacting with the ribosome, thus inhibiting translation.

The mutations at the active site, E167, R170, and R176 behave roughly the same in both toxins. The decrease in toxicity of E167K is probably due to the negatively charged glutamic acid is being replaced with the positively charged lysine. E167K has less depurination activity and higher translation than E167A. R170A reduced

depurination to ~40% in both toxins. Alanine has a much shorter polar side chain than arginine. The interaction with the substrate is either reduced or abolished at R170 (Fig. 21). R176 is located on an alpha helix adjacent to the active site. Interestingly double mutations of E167K/R176K completely abolished depurination activity in both toxins.

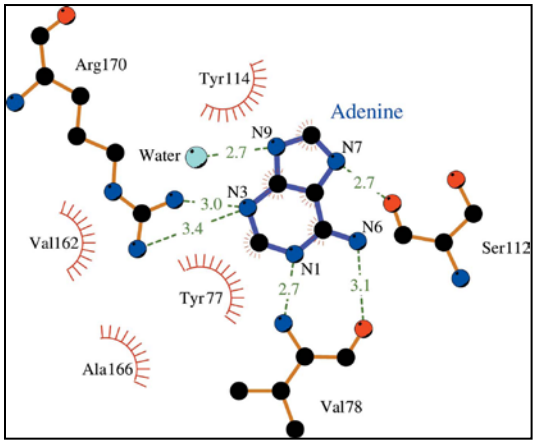

Fig. 21. Interaction of adenine in the active site of Stx2 (7)

We present the first evidence that R176 interacts with the active site in contributing toxicity, depurination activity, and translation inhibition. Again, these same mutants of Stx2A show more toxicity than those of Stx1A. There are differences in the conserved region of the active site in Stx1A and Stx2A based on differences seen in cytotoxicity, depurination activity, and translation inhibition.

Structurally, E167, R170, and R176 appear to have similar conformations in both toxins (Fig. 22). R176 may be more exposed on the surface of the toxin. A mutation at R176 may alter the active site confirmation to the adenine and substrate binding site. R176K did not significantly affect translation inhibition in both toxins but it is more critical in reducing depurination in Stx1A. It appears to abolish toxicity there needs to be a mutation at both E167 and R176, not R176 alone.

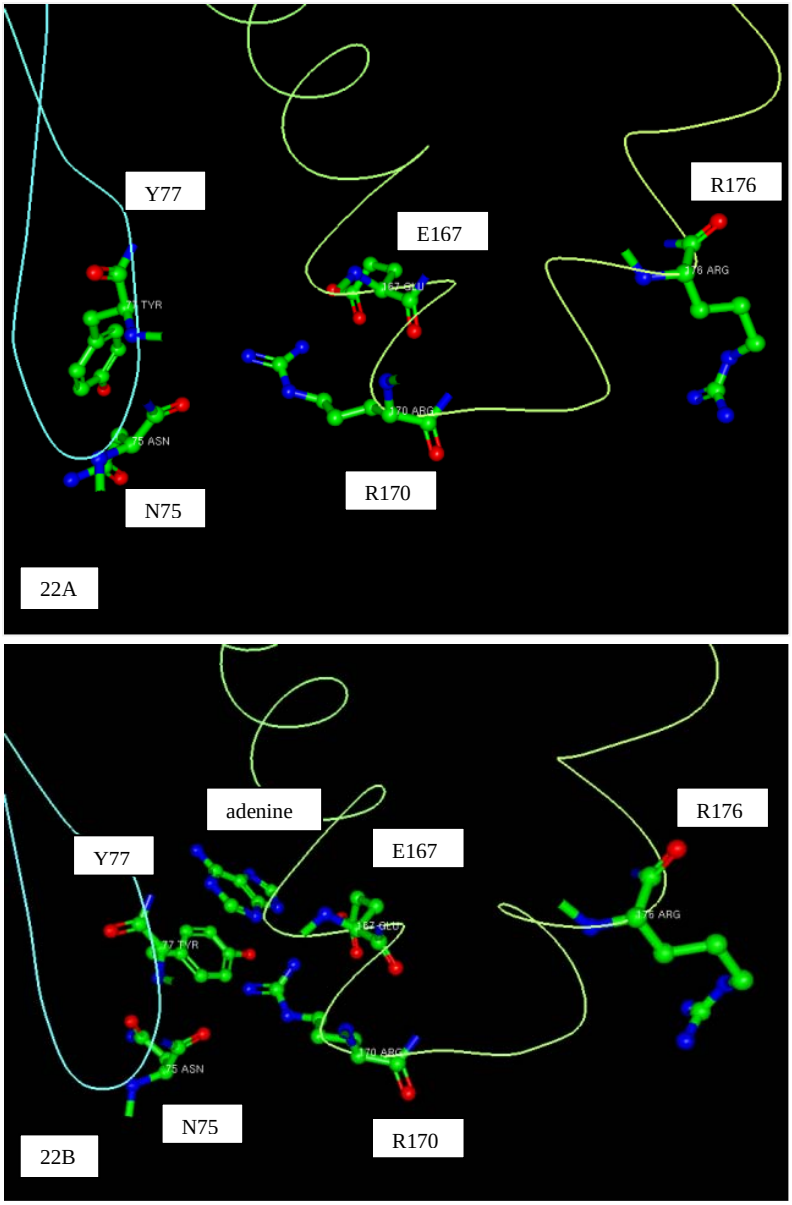

Fig. 22. Structural of active site in Stx (A) and Stx2 (B). Protein data bank of Stx (1DM0), Stx2 (2GA4) (19)

The C-terminus of the shiga toxins is necessary for cytotoxicity. It has been shown in several RIPs C-terminus is necessary for protein translocation in the cell, primarily by containing a hydrophobic membrane spanning domain (9,11,12). The stop codon mutations in Stx1A, V236 to C242, and in counterparts of Stx2A, V235 to C241, are in an important transmembrane domain (34). After binding to the target cell, the Stx is internalized by endocytosis and transported to the trans-Golgi network. It then undergoes retrograde transport and enters the endoplasmic reticulum. The C-terminal region is critical for transport of other RIPs like ricin and PAP into the cytosol (16,31). The C-terminal deletions in Stx1A and Stx2A show similar characteristics to homologus mutations in ricin and PAP suggesting retrotranslocation of Stx from the ER to the cytosol may also be inhibited. A key observation in these deletion mutations is that toxicity is lost before depurination activity. This is shown in Stx1A-L240* and Stx2A-I238*. Both of these mutants appear non-toxic, but they still depurinate. Stx2A loses toxicity at an earlier homologous residue than Stx1A. There is a good correlation between the deletion mutations and depurination activity. As more of the toxin is truncated, the depurination activities decreases and at the same time the amount of protein translation increases to the level of the vector control.

A majority of random mutants, not including any of the C-terminal deletions, in Stx2A were still toxic. R170P was the only random mutation to show less toxicity in the viability assay. It is assumed that proline completely alters the active site by changing the conformation. Interestingly, the mutation R170C was still toxic. This indicates not only the position of the mutation affects cytotoxicity but also which residue it is changed to. The mutation R21W had the lowest depurination activity. Additionally, all the

random double mutations were toxic in the viability assay. The double mutant R21W/A79V also had low depurination activity which matches with the single mutation of R21W. Random mutations besides the previously mentioned conserved domains did not affect cytotoxicity, depurination, and translation inhibition as dramatically. The differences seen in the toxicity of Stx1A and Stx2A can be attributed to the differences in their structure.

Some mutants of both Stx1A and Stx2A show data where translation inhibition and depurination do not correlate. The mutations Stx1-R176K, Stx2A-N75A, Stx2A-Y77A, and Stx2A-E167A show significant reduction in depurinaion activity but translation is still inhibited. As mentioned previously, these mutations may have lost catalytic activity to depurinate the ribosome, but they may still be able to bind the ribosome, thus blocking any elongation factors from interacting with the ribosome and inhibiting translation.

The mutations Stx1A-I239* and Stx2A-V237* still are able to depurinate but there is no translation inhibition. This phenomenon where mutations still depurinate but there is no translation inhibition is also seen in PAP (11). Those mutations may depurinate in cis. Ribosomes are only depurinated while there is active translation. Depurination is dependent on the amount of protein translation. While there is no translation inhibition, these mutants show depurination activity.

Ribosome depurination and translation inhibition do not account entirely for the cytotoxicity of Stx1A and Stx2A; there must be other mechanisms employed by Stx toxins to induce cell death. It has been shown that ricin inhibits the unfolded protein response (UPR) (25). UPR is activated in response to an accumulation of unfolded or

misfolded proteins in the ER. UPR halts protein translation and activates signaling pathways to increase the production of molecular chaperones involved in protein folding. In addition UPR can activate components of the Endoplasmic Reticulum Associated Protein Degradation pathway (ERAD). ERAD targets misfolded proteins to the ER for ubiquitination and subsequent degradation by the proteasome. ERAD retrotranslocates misfolded proteins from the ER to the cytosol for destruction by the ubiquitin proteasome pathway (25). Stx undergoes conformational changes in the ER such as reduction of disulfide bonds and transporter binding (13,31). Stx must evade degradation to reach ribosomes in the cytosol. Further studies can investigate if Stx can inhibit UPR allowing it to escape degradation.

In summary, depurination and translation inhibition are not the only factors that contribute to cell death. The mutations that lie in the conserved regions are more critical for reduction of cytotoxocity, depurination, and translation inhibition. Cytotoxicity, depurination and translation inhibition of random mutations outside the substrate binding, active site, and C-terminal were not reduced to the same degree as mutations in those conserved regions. There are differences on the substrate binding and active site of Stx1A and Stx2A. This is shown by the differences in cytotoxicity, depurination activity, and levels of translation inhibition. Residue R176 has been shown for the first time to play a role in the active site of Stxs. The double mutation E167K/R176K completely abolished cytotoxicity, depurination, and cytotoxicity in both toxins.

It is important to understand the mechanism of action of Stx. Our results have identified specific mutations of Stx1A and Stx2A that can potentially be used as targets for the generation of treatments of Stx intoxication. Stx mutants that are able to

depurinate ribosomes but are not toxic to yeast, such as Stx1A Y77A and Stx2A V237*, have a functional active site, but are non-toxic to yeast cells. These types of mutants may be good candidates for vaccine development as the active site can still initiate an immune response, but the toxin may not induce cell death.

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