The A1 Subunit of Shiga Toxin 2 Has Higher Affinity for Ribosomes and Higher Catalytic Activity than the A1 Subunit of Shiga Toxin 1

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Shiga toxin (Stx)-producing *Escherichia coli* (STEC) infections can lead to life-threatening complications, including hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS), which is the most common cause of acute renal failure in children in the United States. Stx1 and Stx2 are AB₅ toxins consisting of an enzymatically active A subunit associated with a pentamer of receptor binding B subunits. Epidemiological evidence suggests that Stx2-producing *E. coli* strains are more frequently associated with HUS than Stx1-producing strains. Several studies suggest that the B subunit plays a role in mediating toxicity. However, the role of the A subunits in the increased potency of Stx2 has not been fully investigated. Here, using purified A1 subunits, we show that Stx2A1 has a higher affinity for yeast and mammalian ribosomes than Stx1A1. Biacore analysis indicated that Stx2A1 has faster association and dissociation with ribosomes than Stx1A1. Analysis of ribosome depurination kinetics demonstrated that Stx2A1 depurinates yeast and mammalian ribosomes and an RNA stem-loop mimic of the sarcin/ricin loop (SRL) at a higher catalytic rate and is a more efficient enzyme than Stx1A1. Stx2A1 depurinated ribosomes at a higher level *in vivo* and was more cytotoxic than Stx1A1 in *Saccharomyces cerevisiae*. Stx2A1 depurinated ribosomes and inhibited translation at a significantly higher level than Stx1A1 in human cells. These results provide the first direct evidence that the higher affinity for ribosomes in combination with higher catalytic activity toward the SRL allows Stx2A1 to depurinate ribosomes, inhibit translation, and exhibit cytotoxicity at a significantly higher level than Stx1A1.

Shiga toxin-producing *Escherichia coli* (STEC) is an emerging foodborne and waterborne pathogen. STEC infections can lead to life-threatening complications, including hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS), with potentially lethal consequences (1). Due to a very low infectious dose and ease of person-to-person spread, STEC infection is the leading cause of death from foodborne bacterial infection in children (2). Presently, there are no postexposure therapeutics or vaccines available for STEC infection. Due to recent outbreaks of *E. coli* O157:H7 in the United States and the emergence of highly virulent new strains, such as *E. coli* O104:H4, which caused the deadliest HUS outbreak in Germany in 2011, STEC remains a major challenge for food safety and public health (3–6).

The primary virulence factors of STEC, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), are AB₅ toxins consisting of an enzymatically active A subunit associated with a pentamer of receptor binding B subunits and are known as type II ribosome-inactivating proteins (RIPs). The A subunits of Stx1 and Stx2 consist of the catalytically active A1 (residues 1 to 251 in Stx1 and 1 to 250 in Stx2) and A2 (residues 252 to 293 in Stx1 and 251 to 297 in Stx2) chains, which are cleaved by the protease furin and kept together by a disulfide bond (7). The B subunits bind to a common receptor, globotriaosylceramide (Gb3 or CD77), and allow the toxin to enter mammalian cells by endocytosis. Stx holotoxin traffics in a retrograde manner from the endosome to the Golgi network and reaches the endoplasmic reticulum (ER). The A1 chain is released from the A2-B5 complex by cleavage of the disulfide bond in the ER (8). The A1 chain is proposed to be recognized as a misfolded protein by the ER chaperones and targeted for retrotranslocation across the ER membrane (9). The A1 chain is thought to refold into an active conformation in the cytosol to exert its cytotoxic effects (8). The A1 chain of Shiga toxin (Stx) produced by *Shigella dysenteriae* and the A1 chains of Stx1 and Stx2 produced by STEC

are N-glycosidases that remove a specific adenine from the highly conserved α -sarcin/ricin loop (SRL) in the large rRNA (10). Irreversible modification of the SRL blocks elongation factor 1 (EF-1)- and EF-2-dependent GTPase activity and renders the ribosome unable to bind EF-2, thereby blocking translation (11, 12).

Stx1 and Stx2 share only 55% and 57% amino acid sequence identities on the A and B subunits, respectively, and are immunologically distinct (13). The X-ray structures of Shiga toxin from *Shigella*, which differs from Stx1 in only one residue, and Stx2 are different (14–16). The active site of the A subunit is blocked by the A2 chain in the *Shigella* Stx holotoxin, while the active site is accessible to a small substrate in the Stx2 holotoxin (14, 15). The A subunit is in a different orientation with respect to the B subunit in Stx2 compared to Stx1 (15). The structures of the A1 subunits without the A2 and B subunits have not been determined for *Shigella* Stx or *E. coli* Stx1 or Stx2.

Although the molecular structures and functions of Stx1 and Stx2 are similar, their toxicities are different (17). STEC strains producing Stx2 are more commonly associated with HUS than

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those producing Stx1 (18–20). There is a strong correlation between the presence of the Stx2 gene and the severity of disease for human isolates from different serotypes (1). Stx2 has one prototype (Stx2a) and seven subtypes (Stx2b to Stx2h), which display high levels of sequence similarity but significantly differ in toxicity (21, 22). STEC strains producing Stx2a, Stx2c, or Stx2d are more commonly associated with HUS in humans than those producing Stx1a (21, 22). The 50% lethal dose (LD_{50}) of Stx2a is >100-fold lower than that of Stx1a in a mouse model (23, 24). However, the molecular basis for the higher potency of Stx2a is unknown. Although Stx2a was more toxic in animal models, Stx1a was more toxic to Vero cells (24). Stx1a had a higher affinity for the Gb3 receptor in Vero cells (23, 24). Surface plasmon resonance (SPR) analysis showed that Stx1a bound to the Gb3 receptor analog better than Stx2a and had higher association and dissociation rates (25), suggesting that differences in the cell binding properties of the B subunits could account for the higher toxicity of Stx1a to Vero cells (26).

To understand the contribution of the A and the B subunits to the higher toxicity of Stx2a, chimeric toxins were created by interchanging the A and B subunits of the two toxins (23). However, the chimeric toxins were usually found to be less stable than the holotoxins due to incorrect folding (23). In some cases, they showed equivalent cytotoxicity (27) or did not produce a functional chimera (28). Therefore, clear conclusions regarding the role of each subunit in toxicity could not be deduced. A recent study used the A2 subunit along with the B subunit to increase the stability of the chimeric toxin (29). It was concluded that the toxicity of the chimeric toxins to Vero and HCT-8 cells depended on the origin of the B subunit. However, Vero or HCT-8 cell-specific activities of the chimeric toxins differed by at least 50% (29). Lethality to mice correlated with the B subunit in one of the hybrids, but the other hybrid was not lethal, potentially due to instability (29). This study highlighted the importance of the B subunits in the differential toxicity of Stx1a and Stx2a. However, in none of the reported studies has the B subunit alone accounted entirely for the differential toxicity. Therefore, a potential role of the A1 subunit in the higher toxicity of Stx2a than of Stx1a has not been ruled out.

Previous studies demonstrated the importance of the ribosomal proteins and the P-protein stalk structure for the depurination activity of RIPs (30–35). The P-protein stalk is a lateral protruberance of the large subunit of the ribosome, which is responsible for recruitment of the translation factors to the ribosome and stimulation of GTP hydrolysis (36). The eukaryotic stalk consists of P0, which anchors two copies of the P1/P2 heterodimers organized together in a pentameric structure (37). All stalk P proteins contain a highly conserved motif at their C termini, which is involved in the recruitment of external factors to the ribosome (37). We showed that the P proteins of the ribosomal stalk are essential for the cytotoxicity of ricin toxin A chain (RTA) and that the ribosomal stalk is the docking site where RTA interacts with the ribosome (31, 32, 38). The catalytic A1 subunit of Stx1 was shown to interact with the conserved C-terminal motif of the P proteins (33). The interaction of Stx1A1 with the conserved peptide located at the C terminus of all three eukaryotic ribosomal stalk proteins is mediated by cationic and hydrophobic docking surfaces on the A1 subunit (33).

Since the B subunit of the RIPs is required for endocytosis and retrograde trafficking, it has not been possible to study the enzymatic activity of the A subunits in the absence of the B subunits *in vivo*.

Moreover, the role of the A1 subunits in differential toxicity was not investigated further because Stx1a and Stx2a showed similar translation-inhibitory activities in a cell-free system (24). We have developed the yeast *Saccharomyces cerevisiae* as a powerful tool to examine the toxicity and the ribosome interactions of the A subunits of Stx1a and Stx2a independent of the B subunits *in vivo* and have identified amino acids critical for toxicity (39). The depurination activity of the A subunits of Stx1a and Stx2a was reduced in the yeast P-protein mutants, and Stx1a and Stx2a differed in their requirements for the stalk proteins *in vivo* (40). The interaction of the A1 subunits of Shiga toxins with ribosomes or their catalytic activity on ribosomes has not been previously examined. In this study, we examined the interaction of the A1 subunits of Stx1a and Stx2a with yeast and mammalian ribosomes and used a highly sensitive assay to measure the kinetics of ribosome depurination. We compared the activities of the A1 subunits in yeast and in human cells. Our results provide the first direct evidence that Stx2A1 has a higher affinity for ribosomes and has higher catalytic activity than Stx1A1 and depurinates ribosomes and inhibits translation in cells to a greater extent than Stx1A1.

MATERIALS AND METHODS

Yeast strains and plasmids. *S. cerevisiae* strain W303 (*MATa ade2-1 trp1-1 ura3-1 leu2-3,112 his-3-11,15 can1-100*) was grown in either yeast extract-peptone-dextrose (YPD) or minimal medium supplemented with 2% glucose. Mature Stx1A1 (K1 to R251) (plasmid NT1419) and Stx2A1 (R1 to R250) (plasmid NT1429) were first cloned into the pYES2.1/V5-His-TOPO vector and then cloned into a low-copy-number vector, pRS416, containing the *URA3* marker at the NotI-XhoI sites with V5 and 6×His epitopes at their C termini.

Yeast cell viability assay. W303 cells carrying Stx1A1 (NT1419) and Stx2A1 (NT1429) under the control of the *GAL1* promoter were grown overnight at 30°C in synthetic dropout (SD)-glucose medium and were then transferred to SD-galactose medium. Cells were collected at 0 and 10 h postinduction, and serial dilutions of cells at an optical density at 600 nm (OD_{600}) of 0.1 were plated onto SD-glucose plates. The cells were then grown at 30°C for 2 to 3 days.

Total protein extraction and immunoblot analysis. Yeast (W303) cells carrying Stx1A1 and Stx2A1 were grown in SD-glucose medium at 30°C overnight with continuous shaking and were then transferred to SD-galactose medium for Stx1A1 expression. Cells were collected at 2, 4, and 6 h postinduction. Total protein was extracted from yeast cells at an OD_{600} of 5 as described previously (41), and cells were resuspended in 2× Laemmli buffer. Total protein was separated on a 12% SDS-PAGE gel, and monoclonal antibodies against V5 (Invitrogen, Carlsbad, CA) were used to detect Stx1A1 and Stx2A1 expression. Monoclonal antibodies against 3-phosphoglycerate kinase (Pgk1p) (Life Technologies, Grand Island, NY) were used as a loading control.

Purification of 10×His-tagged and untagged Stx1A1 and Stx2A1. His-tagged and untagged Stx1A1 and Stx2A1 proteins were purified by Karen Chave at the Northeast Biodefense Center protein expression core facility using the Impact protein expression system (New England Biolabs, Ipswich, MA). DNAs encoding Stx1A1 (K1 to R251) and Stx2A1 (R1 to R250) were PCR amplified using primers to incorporate a 10×His tag at their N termini, using primers pTXB1_His_Stx1A1_F (5′-GGTGGTC ATATGCCACCATCACCATCACCATCACCATCACCATAAGGAATTT ACCTTAGACTTC-3′) and pTXB1_Stx1A1_R (5′-GGTGGTTGCTCTT CCGCATCTGGCAACTCGCGATGC-3′) to generate NT1570 (Stx1A1) and primers pTXB1_His_Stx2A1_F (5′-GGTGGTCATATGCACCATCA CCATCACCATCACCATCACCATCGGGAGTTTACGATAGACTTTT CG-3′) and pTXB1_Stx2A1_R (5′-GGTGGTTGCTCTTCCGCAGCGAA CAGAACGCGCCC-3′) to generate NT1567 (Stx2A1). NdeI and SapI re-

striction sites were incorporated into the forward and reverse primers, respectively, to facilitate in-frame cloning of the PCR fragments into the polylinker of the pTXB1 vector (New England BioLabs, Ipswich, MA), resulting in a C-terminal fusion of the *Mycobacterium xenopi* intein tag and a chitin binding domain. For untagged-protein purification, primers pTXB1_Stx1A₁_F (5'-GGTGGTCATATGAAGGAATTTACCTTAGACTTC-3') and pTXB1_Stx2A₁_F (5'-GGTGGTCATATGCGGGAGTTTACGATAGACTTTTCG-3') were used. The restriction sites NdeI and SapI were introduced to clone untagged Stx1A1 (NT1576) and Stx2A1 (NT1577) in frame into the polylinker of the pTXB1 vector as described above. Constructs were expressed in *E. coli* strain Rosetta2(DE3)pLysS in 2× yeast extract tryptone (YT) medium overnight at 16°C. The fusion proteins were purified from *E. coli* lysates by using chitin beads, and thiol-induced cleavage of the intein was used to release the target proteins from the chitin beads.

Analysis of depurination. Yeast cells containing Stx1A1 and Stx2A1 plasmids were grown in minimal medium with 2% glucose and then switched to minimal medium with 2% galactose to induce toxin expression. Cells were collected 0, 1, 2, and 3 h after induction. Total RNA was extracted by using the RNeasy minikit (Qiagen, Valencia, CA) with on-column DNase treatment, and a reverse transcription-quantitative PCR (qRT-PCR) assay was used to quantify depurination (42). RNA was converted to cDNA by using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY), and depurination was detected by a quantitative real-time PCR method using the StepOnePlus real-time PCR system (Applied Biosystems, Grand Island, NY). The level of the 25S reference rRNA was measured by using the primers 5'-AGA CCG TCG CTT GCT ACA AT-3' and 5'-ATG ACG AGG CAT TTG GCT AC-3'. The depurinated rRNA was detected by using the forward primer 5'-CTA TCG ATC CTT TAG TCC CTC-3' and the reverse primer 5'-CCG AAT GAA CTG TTC CAC A-3'. The $\Delta\Delta C_T$ method was used to calculate the depurination levels, and data were expressed as the fold change of depurination in Stx-treated RNA over depurination in control nontreated RNA, as described previously (42).

Monomeric ribosomes (7 pmol) were incubated with different concentrations of Stx1A1 and Stx2A1 (0.08 nM, 0.25 nM, and 0.75 nM) in a final volume of 100 μ l in 1× RIP buffer (60 mM KCl, 10 mM Tris-HCl [pH 7.4], 10 mM MgCl₂) at 30°C for 5 min. One hundred microliters of 2× extraction buffer (120 mM NaCl, 25 mM Tris-HCl [pH 8.8], 10 mM EDTA, 1% SDS) was added to this mixture, and the mixture was vortexed. RNA was extracted from this mixture (43), and depurination was determined by using qRT-PCR (42).

Total RNA was extracted from cultures of yeast cells grown overnight by using the RNeasy minikit (Qiagen, Valencia, CA). Total RNA (1 μ g) was incubated with different concentrations of Stx1A1 and Stx2A1 (62.5 nM, 125 nM, and 250 nM) in a final volume of 20 μ l in 20 mM citrate buffer (pH 5) at 37°C for 15 min. RNA was purified (43), and the depurination of rRNA was quantified by using a qRT-PCR assay (42). The $\Delta\Delta C_T$ method was used to calculate the depurination level, and data were expressed as the fold change of depurination in Stx-treated RNA over depurination in nontreated RNA, as described previously (40, 42).

Isolation of yeast monomeric ribosomes. Monomeric ribosomes were isolated from W303 cells as described previously (40), with the following modifications. The cell-free supernatant was incubated with 1% Triton X-100 for 30 min at 4°C with gentle shaking to increase the ribosome yield. The supernatant was loaded onto a buffer C cushion to pellet the ribosome by centrifugation at 200,000 × *g* for 2 h. The following steps were performed as previously described (40).

Isolation of rat liver ribosomes. Livers were dissected rapidly after CO₂ knockdown and decapitation of rats; rinsed in cold buffer A (20 mM HEPES-KOH [pH 7.6], 5 mM magnesium acetate [MgOAc], 50 mM KCl, 10% glycerol) with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and a protease inhibitor cocktail for mammalian tissue culture (Sigma-Aldrich, St. Louis, MO); and frozen in liquid N₂. Thawed livers were homogenized and disrupted by 10 strokes of a Dounce

homogenizer in cold buffer A plus 1 mM PMSF, 1 mM DTT, and a protease inhibitor cocktail for mammalian tissue culture (Sigma-Aldrich, St. Louis, MO), and cell debris was removed by centrifugation at 20,000 × *g* for 20 min. Sodium deoxycholate was added to 1%. After 10 min of stirring in ice, ribosomes were sedimented at 150,000 × *g* for 90 min. The pellet was rinsed twice in buffer B (20 mM HEPES-KOH [pH 7.6], 20 mM MgOAc, 0.5 M KCl, 10% glycerol) with 1 mM PMSF and 1 mM DTT and stored overnight at 4°C in a small amount of the same buffer. The ribosomes were resuspended with a Dounce homogenizer in buffer B with 1 mM DTT and 1 mM PMSF and incubated for 30 min at 30°C with 1 mM puromycin and 1 mM GTP. The supernatant was clarified by centrifugation at 20,000 × *g* for 15 min and layered over a cushion of buffer B with the glycerol concentration increased to 25%. Ribosomes were sedimented at 150,000 × *g* for 2 h. The pellets were rinsed in buffer C (50 mM HEPES-KOH [pH 7.6], 5 mM MgOAc, 50 mM NH₄Cl, 0.1 mM DTT, and 25% glycerol), resuspended in 5 ml buffer C, and stored -80°C. A further purification step was performed with hydroxylapatite essentially as described previously by Hoffman and Ilan (44), except that a column was used rather than batch elution. All animal experiments were conducted with the approval of the Rutgers University Animal Care and Use Committee.

Interaction of Stx1A1 and Stx2A1 with the ribosome and the ribosomal stalk complex. Interactions were measured with a Biacore T200 instrument (GE Healthcare Bio-Sciences, Pittsburgh, PA), using either untagged or 10×His-tagged A1 subunits. The untagged toxins were immobilized on a CM5 chip by amine coupling, and the amount of immobilized toxin was monitored in real time by using the Biacore T200 instrument. The amounts of toxin immobilized were 1,692 resonance units (RU) for untagged Stx1A1 and 1,672 RU for untagged Stx2A1. Flow cell 1 was activated and blocked as the reference channel. A single-cycle kinetic method was used. Ribosomes at different concentrations were passed over the surface at 40 μ l/min for 2 min. A dissociation step was performed for 5 min. Running buffer contained 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM MgOAc, 50 μ M EDTA, and 0.003% surfactant P20. The surface was regenerated by two 1-min injections of 3 M NaCl. The 10×His-tagged Stx1A1 and Stx2A1 proteins were captured on a nitrilotriacetic acid (NTA) chip. The amounts of captured toxin monitored in real time with the Biacore T200 instrument were 2,300 and 2,230 RU for 10×His-Stx1A1 and 10×His-Stx2A1, respectively. Ribosomes were passed over each surface at different concentrations. The interaction conditions were the same as those described above for the untagged A1 subunits except that the surface was freshly captured at each cycle. For the interaction of the A1 subunits with the isolated ribosomal stalk pentamer, 10×His-tagged Stx1A1 and Stx2A1 were captured on an NTA chip at ~1,000 RU, and the same amount of 10×His-tagged enhanced green fluorescent protein (EGFP) was captured on the reference channel. The yeast stalk pentamer was passed over the surface at 70 μ l/min for 5 min, and a final dissociation step was performed for 10 min.

Ribosome depurination kinetics assay. Yeast monomeric ribosomes were isolated as previously described (32). Prior to use, the yeast ribosomes were passed through a 0.5-ml Zeba spin desalting column (Life Technologies, Grand Island, NY) to reduce AMP and ATP contamination, as described previously (45). In the same step, the buffer was changed to ribosome depurination buffer (20 mM Tris-HCl [pH 7.4], 25 mM KCl, 5 mM MgCl₂). Depurination was measured by a continuous luminescence assay as described previously by Sturm and Schramm (45). The toxin concentrations used were 4 pM for Stx1A1 and 3 pM for Stx2A1 when yeast ribosomes were used as the substrate and 4 pM for both Stx1A1 and Stx2A1 when rat ribosomes were used as a substrate. The reaction was set up in a 96-well plate in a total reaction mixture volume of 50 μ l. The reaction was started by adding toxin to the reaction mixture, and the luminescence intensity was recorded continuously for 30 min by using a BioTek Synergy 4 microplate reader (BioTek, Winooski, VT, USA). The rates were determined from the linear region of the luminescence intensity. The rate of Stx-independent adenine generation was sub-

tracted. Adenine standards covering the range of depurination were measured on the same plate and at the same time. The initial rate of adenine formation was calculated by converting the luminescence rate (lumens per second) to the enzymatic rate (picomoles of adenine per minute per picomole of enzyme) by using the adenine standard curve. Kinetic parameters (k_{cat} and K_m) were calculated by fitting initial rates to the Michaelis-Menten equation using Sigma Enzyme Kinetics Module 1.3 (Systat Software, Inc., San Jose, CA, USA).

SRL depurination kinetics assay. Stem-loop depurination was performed by using a synthetic 10-mer SRL oligonucleotide (5'-rCrGrCrGrArGrArGrCrG-3', where r stands for ribose) purchased from Integrated DNA Technologies (San Diego, CA). A discontinuous luminescence assay developed previously by Sturm and Schramm (45) was used, with modifications of both temperature and pH. Although linear depurination was achieved with ricin at 37°C at pH 4.0 (43), these conditions rapidly inactivated Stx1A1 and Stx2A1. Decreasing the temperature to 20°C and increasing the pH to 4.5 slowed both the reaction and inactivation such that linearity could be achieved for at least 5 min. Dilutions of the stem-loop RNA in 30 μ l 10 mM potassium citrate-KOH and 1 mM EDTA were equilibrated at 20°C. Each A1 subunit was preincubated at a 2 $\times$ final concentration in the same buffer for 5 min at 20°C prior to the addition to RNA to start the reaction. Samples (9 μ l) were withdrawn at timed intervals up to 5 min and added to 9 μ l of 2 $\times$ coupling buffer (45) in a chilled 96-well white luminescence plate. At the termination of the assay, the plate was warmed to 30°C for 10 min to initiate the coupled reaction. Thirty-five microliters of ATPlite (PerkinElmer, Waltham, MA) reagent was added per well. Adenine standards of 0.5 to 2 pmol prepared in pH 4.5 buffer give a linear response under these conditions. Luminescence intensity was measured by using a BioTek Synergy 4 microplate reader (BioTek, Winooski, VT, USA) at high sensitivity after incubation of the plate for 10 min in the dark. Parameters were calculated for Michaelis-Menten kinetics by using Sigma Enzyme Kinetics Module 1.3 (Systat Software, Inc., San Jose, CA, USA).

Transfection of HEK293T cells with the A1 subunits. The mature Stx1A1 (K1 to R251) (NT1776) and Stx2A1 (R1 to R250) (NT1777) constructs with V5 and 10 $\times$ His tags were cloned into the mammalian expression vector pCAGGS at the SacI-XhoI sites. HEK293T cells were cotransfected with these constructs and an EGFP expression plasmid, also in pCAGGS, by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were plated in Dulbecco's modified Eagle's medium (DMEM) (phenol red free) and 10% fetal calf serum at 5×10^4 cells/ml in black, clear-bottom, 96-well plates. After 24 h, with cells at $\sim$ 90% confluence, the medium was removed and replaced with 50 μ l medium lacking calf serum, which had been preincubated for 10 min with 50 ng of each DNA and 0.3 μ l Lipofectamine. After 2 h of incubation at 37°C in 5% CO₂, the transfecting solution was removed and replaced with 100 μ l medium containing 10% fetal calf serum. EGFP fluorescence was read at 22 h post-DNA exposure in a BioTek plate reader from the bottom of plate with a 485/20-nm bandpass excitation filter and a 530/25-nm bandpass emission filter. Assays were performed in quadruplicate. The fluorescence measured for cells cotransfected with EGFP and an empty vector was considered 100%, and fluorescence in controls lacking the EGFP plasmid was considered background (46).

Analysis of ribosome depurination in HEK293T cells. For *in vivo* depurination, HEK293T cells containing mature Stx1A1 and Stx2A1 plasmids were first grown as described above. Cell samples were collected 23 h after DNA exposure. Total RNA was extracted by using the RNeasy mini-kit (Qiagen, Valencia, CA) with on-column DNase treatment. A qRT-PCR assay was used to quantify depurination (47, 48).

Statistical analysis. Statistical analyses were conducted by using SAS 9.4 (SAS Institute, Inc., Cary, NC). Statistical analysis of data in Fig. 1B and 6A and B was performed by using PROC GLM (Procedure for General Linear Models). Statistical analyses of data in Fig. 1D and 2C and D were conducted by using SAS 9.4 (SAS Institute, Inc., Cary, NC). Data were analyzed by generalized mixed linear models using PROC GLIMMIX

(Procedure for Generalized Linear Mixed Models) to test for statistical differences between treatments. PROC GLIMMIX permits the inclusion of a random effect (PCR experiment as a block) in the generalized mixed linear model. All treatments were considered fixed effects, and blocks (separate qRT-PCR plates) were considered random effects (49). Least-square means were calculated, and specific preplanned contrasts (50) were computed to compare treatment means between Stx1A1 and Stx2A1 for each treatment.

RESULTS

Stx2A1 is more toxic and shows higher depurination than Stx1A1 in yeast. To compare the cytotoxicities of the A1 subunits of Stx1 and Stx2 *in vivo*, the mature A1 subunits were expressed in the yeast *S. cerevisiae*, with the V5 and 10 $\times$ His epitope tags under the tightly controlled *GAL1* promoter. Previously reported results indicated that the V5 and 10 $\times$ His epitope tags do not affect the activity of the A subunits (39, 40). Irreversible growth inhibition by Stx1A1 and Stx2A1 was determined by plating cells onto medium containing 2% dextrose after galactose induction for the indicated times in liquid medium. Cells transformed with an empty vector were used as a control. As shown in Fig. 1A and B, Stx1A1 and Stx2A1 reduced the viability of cells by 10-fold and 100-fold, respectively, compared to the vector control. Yeast cells expressing Stx2A1 grew >10 -fold less than those expressing Stx1A1, indicating that Stx2A1 is more toxic than Stx1A1.

To examine the expression of the A1 subunits in yeast, whole-cell lysates were analyzed by immunoblotting with monoclonal antibodies against the V5 epitope. Monoclonal antibodies against phosphoglycerate kinase 1 (Pgk1) were used as a loading control (Fig. 1C). Cells harboring Stx1A1 and Stx2A1 showed gradual increases in expression levels with time. Stx1A1 was expressed at a higher level than Stx2A1. These results were consistent with previously reported observations that showed that the expression level of the toxins is correlated inversely with their toxicity (43). A lower level of protein expression was detected for Stx2A1, consistent with our finding that Stx2A1 was more toxic than Stx1A1.

To determine if the higher toxicity of Stx2A1 than of Stx1A1 is due to a higher level of ribosome depurination in yeast, total RNA was isolated from yeast at different times after induction, and relative levels of depurination over time were quantified by using qRT-PCR. In this assay, two pairs of primers are used to amplify the target amplicon (depurinated SRL) and the reference amplicon (25S rRNA), and the data are analyzed by the comparative threshold cycle (C_T) ($\Delta\Delta C_T$) method relative to yeast harboring the empty vector (42). Data obtained by using 3 independent colonies showed that cells expressing Stx2A1 exhibited 1.7- to 2.4-fold-higher levels of depurination than cells expressing Stx1A1 (Fig. 1D). This difference was statistically significant by PROC GLIMMIX (50), which permits the inclusion of a random effect (separate qRT-PCR plates) into the generalized mixed linear model (see Table S1 in the supplemental material). Collectively, these data indicate that Stx2A1 depurinates ribosomes at a higher level and is more toxic to yeast than Stx1A1.

Stx2A1 depurinates yeast ribosomes and RNA at a higher level than Stx1A1. To examine ribosome depurination by Stx1A1 and Stx2A1 *in vitro*, 10 $\times$ His-tagged and untagged Stx1A1 and Stx2A1 were expressed in *E. coli* and purified to homogeneity. Equal amounts of purified toxins were analyzed by SDS-PAGE (Fig. 2A) and by immunoblot analysis (Fig. 2B). Each toxin migrated on SDS-PAGE gels as expected for its size. The 10 $\times$ His-tagged proteins migrated slower on SDS-PAGE gels, as expected

FIG 1 (A) Viability of yeast cells expressing Stx1A1 or Stx2A1. Yeast cells transformed with a plasmid carrying Stx1A1 or Stx2A1 and yeast cells carrying the empty vector (VC) were grown in SD medium supplemented with 2% glucose and then transferred to SD medium supplemented with 2% galactose. At 0 and 10 h postinduction, a series of 10-fold dilutions were plated onto medium containing 2% glucose, and cells were grown at 30°C for 2 to 3 days. (B) CFU per milliliter were calculated at 10 h postinduction from at least 3 independent transformants. Error bars represent standard errors ($n = 3$ independent experiments). Means for Stx2A1 and Stx1A1 were significantly different as determined by using a one-tailed t test ($***, P < 0.001$). (C) Immunoblot analysis of yeast cells transformed with Stx1A1 and Stx2A1. Total protein from cells at an OD₆₀₀ of 5 isolated at 2, 4, and 6 h postinduction was separated on an SDS-PAGE gel and probed with anti-V5. Anti-Pgk1 was used as a loading control. (D) Depurination of ribosomes in yeast. Total RNA (375 ng) isolated from cells at an OD₆₀₀ of 1 expressing Stx1A1 or Stx2A1 collected at 0, 1, 2, and 3 h postinduction was used to quantify the relative level of depurination by using qRT-PCR. The analysis was repeated three times, and data from one representative experiment are shown. The y axis shows the fold change in depurination of toxin-treated samples over the control samples without toxin (VC). Error bars represent standard errors ($n = 3$ replicates). Means for Stx2A1 and Stx1A1 were significantly different at 1 h, 2 h, and 3 h ($P < 0.01$) as determined by using PROC GLIMMIX.

(Fig. 2A), due to their higher molecular masses (28.9 kDa and 29.2 kDa for tagged Stx1A1 and Stx2A1, respectively, compared to 27.6 kDa and 27.9 kDa for untagged Stx1A1 and Stx2A1, respectively). A protein thermal shift assay was used to examine the relative stability of untagged Stx1A1 and Stx2A1 at neutral pH, and the results showed that purified Stx1A1 is more stable than Stx2A1 (see Fig. S1 in the supplemental material).

The depurination activity of untagged Stx2A1 and Stx1A1 on yeast ribosomes was measured by qRT-PCR (Fig. 2C). Yeast ribosomes were treated with 0.08 nM to 0.75 nM Stx1A1 and Stx2A1. Untreated ribosomes were used as a control. The experiment was repeated at least three times. The depurination level of Stx2A1 was 1.4- to 1.9-fold higher than the depurination level of Stx1A1 at each toxin concentration tested. This difference was statistically significant (see Table S2 in the supplemental material).

Since ribosome depurination is determined by toxin-ribosome interactions and catalytic activity once the toxin is bound, to determine whether the observed difference in ribosome depurination is due to a difference in the catalytic activity, we compared the depurination activities of Stx1A1 and Stx2A1 on free RNA. Total RNA from yeast cells was isolated and treated with different concentrations (62.5, 125, and 250 nM) of Stx1A1 and Stx2A1. The relative levels of depurination were quantified by using qRT-PCR. Stx2A1 depurinated RNA at a 1.6- to 4.3-fold-higher level than Stx1A1 (Fig. 2D). This difference was statistically significant (see Table S3 in the supplemental material). These results demonstrate

that Stx2A1 depurinates yeast ribosomes and RNA at a higher level than Stx1A1 *in vitro*.

Stx2A1 has higher affinity for yeast and mammalian ribosomes than Stx1A1. Since ribosome binding is critical for ribosome depurination, we examined the direct interaction of A1 subunits with ribosomes using surface plasmon resonance with a Biacore instrument. To compare the interaction of Stx1A1 and Stx2A1 with the ribosome, the untagged A1 subunits were immobilized on a CM5 chip of the Biacore T200 instrument at 1,692 RU and 1,672 RU, respectively, using amine coupling, and their interaction with yeast ribosomes was measured by single-cycle kinetic analysis by passing ribosomes over the surface at different concentrations. Since the surface is not regenerated between injections in the single-cycle analysis, multiple samples can be analyzed in a shorter period of time, allowing comparison of levels of binding under identical conditions (38). As shown in Fig. 3A, the binding levels of Stx2A1 were 12-fold higher than those of Stx1A1 when yeast ribosomes were used at 2.5 nM and 5-fold higher than those of Stx1A1 when yeast ribosomes were used at 40 nM. Since binding did not reach equilibrium and did not fit the 1:1 model, resonance units at the end of each ribosome concentration were used to calculate the dissociation constants (K_D) (Table 1). The apparent K_D for untagged Stx2A1 for yeast ribosomes was 6.6-fold lower than the apparent K_D for Stx1A1, and this difference was statistically significant. The level of binding of 10×His-tagged Stx2A1 to yeast ribosomes was 2.7- to 4-fold higher than the level of binding

FIG 2 (A) Coomassie blue staining of purified 10×His-tagged and untagged Stx1A1 and Stx2A1. Equal amounts (1 μg) of purified Stx1A1 (S1) and Stx2A1 (S2) were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie blue. (B) Immunoblot analysis of purified 10×His-tagged and untagged Stx1A1 and Stx2A1. Equal amounts (100 ng) of purified Stx1A1 and Stx2A1 were separated on a 12% SDS-polyacrylamide gel. The 10×His-tagged Stx1A1 and Stx2A1 proteins were detected with monoclonal anti-His antibody. Untagged Stx1A1 and Stx2A1 were detected with polyclonal anti-Stx1 or anti-Stx2 antibody. (C) Depurination of yeast ribosomes by purified Stx1A1 and Stx2A1. Yeast ribosomes (7 pmol) were incubated with different concentrations of Stx1A1 or Stx2A1 at 30°C for 5 min. The rRNA (375 ng) was used to quantify the relative levels of depurination by qRT-PCR (42). The y axis shows the fold change in depurination of toxin-treated samples over the control samples without toxin treatment (NT). The analysis was repeated three times, and data from one representative experiment are shown. Error bars represent standard errors (*n* = 3 replicates). Means for Stx2A1 and Stx1A1 were significantly different at 0.08 nM (*P* < 0.001), 0.25 nM (*P* < 0.0001), and 0.75 nM (*P* < 0.0001) as determined by using PROC GLIMMIX. (D) Depurination of total RNA from yeast by purified Stx1A1 or Stx2A1. Total RNA (1 μg) was incubated with different amounts of Stx1A1 or Stx2A1 at 37°C for 15 min. The relative levels of depurination were determined by using qRT-PCR (42). The y axis shows the fold change in depurination over the control samples without toxin treatment (NT). The analysis was repeated three times. Error bars represent standard errors (*n* = 3 replicates). Means for Stx2A1 and Stx1A1 were significantly different at 125 nM (*P* < 0.01) and 250 nM (*P* < 0.0001) as determined by using PROC GLIMMIX.

of Stx1A1 (Fig. 3B). The 10×His-tagged Stx2A1 protein had a 2-fold-lower *K_D* for yeast ribosomes than 10×His-tagged Stx1A1 (Table 1). However, this difference did not achieve statistical significance. The major difference in the interaction of Stx1A1 and Stx2A1 with ribosomes was in their interaction patterns. Untagged and 10×His-tagged Stx2A1 had a much faster association-and-dissociation pattern than untagged and 10×His-tagged

FIG 3 (A) Interaction of Stx1A1 and Stx2A1 with yeast ribosomes. Untagged Stx1A1 and untagged Stx2A1 were captured on a CM5 chip at 1,692 RU and 1,672 RU, respectively. Different concentrations of ribosomes were passed over the surface as analyte, as shown. (B) Interaction of 10×His-tagged Stx1A1 and Stx2A1 with yeast ribosomes. The 10×His-Stx1A1 and 10×His-Stx2A1 proteins were captured on an NTA chip. Different concentrations of ribosomes were passed over the surface as analyte, as shown. (C) Interaction of Stx1A1 and Stx2A1 with the isolated yeast ribosomal stalk pentamer. The 10×His-Stx1A1 or 10×His-Stx2A1 protein was captured on an NTA chip at 1,000 RU, and the same amount of EGFP was captured on the reference channel. Different concentrations of the stalk pentamer were passed over the surface as analyte, as shown.

Stx1A1 (Fig. 3A and B). This difference is not reflected by the *K_D* value. The association rate (*k_{on}*) and the dissociation rate (*k_{off}*) could not be accurately determined because the interaction did not fit a 1:1 interaction model.

Toxin	Mean apparent <i>K_D</i> (M) ± SD ^a	
	Yeast ribosomes	Rat liver ribosomes
Stx1A1	16.8 × 10 ⁻⁸ ± 2.0 × 10 ⁻⁸ A	8.6 × 10 ⁻⁸ ± 1.6 × 10 ⁻⁸ C
Stx2A1	2.5 × 10 ⁻⁸ ± 0.3 × 10 ⁻⁸ B	2.8 × 10 ⁻⁸ ± 0.4 × 10 ⁻⁸ D
10×His-Stx1A1	4.6 × 10 ⁻⁸ ± 2.2 × 10 ⁻⁸ E	3.0 × 10 ⁻⁸ ± 1.1 × 10 ⁻⁸ E
10×His-Stx2A1	2.2 × 10 ⁻⁸ ± 0.4 × 10 ⁻⁸ F	3.6 × 10 ⁻⁸ ± 1.0 × 10 ⁻⁸ F

^a Letters indicate statistical comparisons, where means were significantly different between A and B (*P* < 0.01) and between C and D (*P* < 0.05) and means were not significantly between E and F, as determined by using a one-tailed *t* test.

TABLE 2 Stalk interaction parameters^a

Toxin	Mean k_{on} ($M^{-1} s^{-1}$) $\pm$ SD	Mean k_{off} (s^{-1}) $\pm$ SD	Mean K_D (M) $\pm$ SD
Stx1A1	$1.6 \times 10^6 \pm 0.6 \times 10^6$ A	$4.1 \times 10^{-4} \pm 1.7 \times 10^{-4}$ C	$3.0 \times 10^{-10} \pm 2.2 \times 10^{-10}$ E
Stx2A1	$1.8 \times 10^6 \pm 0.9 \times 10^6$ B	$2.2 \times 10^{-4} \pm 1.0 \times 10^{-4}$ D	$1.6 \times 10^{-10} \pm 1.4 \times 10^{-10}$ F

^a Letters indicate statistical comparisons, where means were not significantly different between A and B, between C and D, and between E and F, as determined by using a one-tailed *t* test.

Previous studies showed that the P proteins of the ribosomal stalk are important for ribosome depurination by Stx1 and Stx2 (40). The A1 chain of Stx1 interacts with the conserved C termini of P proteins (33, 34). To determine if the higher level of binding of Stx2A1 to the ribosome is due to a higher level of binding to the ribosomal P-protein stalk complex, we examined the interaction of the A1 subunits with the pentameric stalk complex from yeast (38). We captured the 10 $\times$ His-tagged Stx1A1 and Stx2A1 proteins on an NTA chip at the same level and passed the purified stalk pentamer complex over the surface. As shown in Fig. 3C, the binding level of Stx2A1 was slightly higher than that of Stx1A1. The interaction of the A1 subunits with the stalk pentamer fit a 1:1 interaction model (Table 2). The 10 $\times$ His-tagged Stx2A1 protein showed slightly slower dissociation and had a slightly higher affinity for the stalk than 10 $\times$ His-tagged Stx1A1 (Table 2). However, the differences in the affinities (K_D) and the association (k_{on}) and dissociation (k_{off}) rates of Stx1A1 and Stx2A1 for the stalk complex were not significant. Therefore, the observed differences in the binding affinities of Stx1A1 and Stx2A1 for yeast ribosomes and the differences in ribosome depurination are not due to differences in their binding to the P-protein stalk.

To examine the relative affinity of Stx2A1 and Stx1A1 for mammalian ribosomes, the interaction of untagged Stx1A1 and Stx2A1 with rat liver ribosomes was analyzed with a Biacore instrument. The untagged A1 chains were immobilized on a CM5 chip using amine coupling at the same ligand level, and rat liver ribosomes were passed over the surface at different concentrations with single-cycle kinetics. As shown in Fig. 4A, rat ribosomes bound Stx2A1 at a considerably higher level than Stx1A1. The binding level of Stx2A1 was $\sim$ 14-fold higher than that of Stx1A1 at a ribosome concentration of 80 nM. We calculated the apparent K_D using the RU values at the end of the injection of each ribosome concentration. The apparent K_D of Stx2A1 for rat liver ribosomes was significantly (3-fold) lower than the apparent K_D of Stx1A1 for rat liver ribosomes (Table 1). Differences in binding levels were observed when 10 $\times$ His-tagged Stx1A1 and Stx2A1 were used (Fig. 4B). Although there were clear differences between the interaction patterns of 10 $\times$ His-tagged Stx2A1 and 10 $\times$ His-tagged Stx1A1, we did not see a significant difference in the apparent K_D values (Table 1).

Stx2A1 has higher catalytic activity on yeast and mammalian ribosomes. To examine the correlation between ribosome interaction and depurination, we measured the depurination kinetics of Stx1A1 and Stx2A1 toward monomeric yeast and rat liver ribosomes using a continuous enzymatically coupled luminescence assay with sufficient sensitivity to continuously measure single-adenine release from nanomolar concentrations of intact eukaryotic ribosomes (45). This assay can detect femtomoles of ricin in minutes using adenine phosphoribosyl transferase (APRTase) to convert adenine to AMP and then to ATP with pyruvate phosphate dikinase (PPDK). ATP generates light via luciferase, and the regenerated AMP is converted to ATP by PPDK (45). As shown in

Fig. 5A, the initial rates of Stx2A1 and Stx1A1 depurination were dependent on the concentration of yeast ribosomes and were fitted to the Michaelis-Menten equation. Stx2A1 showed a k_{cat} of $3,406 \text{ min}^{-1}$ and a catalytic efficiency (k_{cat}/K_m) of $5.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, while Stx1A1 showed a k_{cat} of $2,257 \text{ min}^{-1}$ and a k_{cat}/K_m value of $3.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ toward yeast ribosomes. The differences in k_{cat} and k_{cat}/K_m values between Stx1A1 and Stx2A1 for yeast ribosomes were statistically significant. However, Stx1A1 and Stx2A1 showed similar K_m values ($\sim 1 \mu\text{M}$) (Fig. 5A and Table 3). These results indicate that Stx2A1 depurinates yeast ribosomes at a significantly (1.5-fold) higher rate and significantly (1.4-fold) more efficiently than Stx1A1.

To determine if Stx2A1 has higher catalytic activity toward mammalian ribosomes, the enzymatically coupled assay was used to examine the depurination kinetics of Stx1A1 and Stx2A1 by using rat liver ribosomes. The purification of rat liver ribosomes required an additional purification step compared to yeast ribosomes to reduce the background in the enzymatically coupled assay. As shown in Fig. 5B, greater differences were observed for the catalytic rate and catalytic efficiency for rat liver ribosomes than for yeast ribosomes. Stx2A1 had a k_{cat} of $1,098 \text{ min}^{-1}$, com-

FIG 4 (A) Interaction of Stx1A1 and Stx2A1 with ribosomes from rat liver. The same conditions as those used for interactions with yeast ribosomes were used. Different concentrations of ribosomes were passed over the surface as analyte, as shown. (B) Interaction of 10 $\times$ His-Stx1A1 and 10 $\times$ His-Stx2A1 with ribosomes from rat liver. The 10 $\times$ His-Stx1A1 and 10 $\times$ His-Stx2A1 proteins were captured on an NTA chip. The same conditions as those used for interactions with yeast ribosomes were used. Different concentrations of ribosomes were passed over the surface as analyte, as shown.

FIG 5 (A) Michaelis-Menten fits of yeast ribosome depurination performed with a continuous luminescence assay. The Stx-independent rate of adenine generation was subtracted. Stx1A1 was used at 4 pM, and Stx2A1 was used at 3 pM. Adenine standards covering the range of depurination were measured for each toxin. Error bars represent standard errors ($n = 3$ replicates). (B) Michaelis-Menten fits of rat liver ribosome depurination performed with the continuous assay. The Stx-independent rate of adenine generation was subtracted. Stx1A1 and Stx2A1 were used at 4 pM for depurination. Adenine standards covering the range of depurination were measured for each toxin. Error bars represent standard errors ($n = 3$ replicates). (C) Michaelis-Menten fits of stem-loop RNA depurination performed with the discontinuous luminescence assay. Stx1A1 was used at 3 nM, and Stx2A1 was used at 2 nM. The Stx-independent rate of adenine generation was subtracted. Adenine standards covering the range of depurination were measured for each toxin. Error bars represent standard errors ($n = 3$ replicates).

pared to a k_{cat} of 404 min⁻¹ for Stx1A1, toward rat liver ribosomes. Stx2A1 depurinated rat liver ribosomes with a k_{cat}/K_m of 5.1×10^7 M⁻¹ s⁻¹, compared to 2.2×10^7 M⁻¹ s⁻¹ for Stx1A1 (Fig. 5B and Table 3). The differences in k_{cat} and k_{cat}/K_m values between Stx1A1

and Stx2A1 for rat liver ribosomes were statistically significant. There was no difference in the K_m for rat ribosomes ($\sim 0.3 \mu\text{M}$). These results demonstrated that Stx2A1 depurinated rat liver ribosomes at a significantly (2.7-fold) higher rate and significantly (2.3-fold) more efficiently than Stx1A1.

Since ribosome depurination requires the interaction of the toxin with the P-protein stalk to reach the SRL, we measured the depurination kinetics toward a 10-mer stem-loop RNA mimic of the SRL by using a discontinuous luminescence assay (45). Previous results indicated that RTA depurinates stem-loop RNA substrates at pH 4.0 but not at pH 7.0 (45). Similarly, the A1 subunits of Shiga toxins could not depurinate the stem-loop RNA at neutral pH but depurinated it at pH 4.5. Since the adenine-to-ATP conversion requires a neutral pH, the assay was done with the discontinuous format by quenching to neutral pH at timed intervals. The amount of adenine released was determined by a luminescence assay. The results (Fig. 5C and Table 3) showed that Stx2A1 depurinated the stem-loop RNA at a significantly higher (3-fold) initial rate (k_{cat} of 62.6 nM) than Stx1A1 (k_{cat} of 21.5 nM). However, Stx1A1 had a significantly lower K_m (0.93 μM) for the stem-loop RNA than Stx2A1 (2.46 μM). Consequently, there was no difference in the depurination efficiencies of Stx1A1 and Stx2A1 toward the stem-loop RNA.

Stx2A1 inhibits protein expression in human cells to a greater extent than Stx1A1. In order to examine the relative activity of Stx1A1 and Stx2A1 in mammalian cells, independent of B-chain influence, an EGFP transfection assay was used (46, 47). Changes in ribosome depurination correlate with the extent of inhibition of EGFP expression in this assay (46, 47). Human embryonic kidney cells (HEK293T) were cotransfected with equal amounts of an EGFP reporter plasmid and the Stx1A1 or Stx2A1 expression plasmid, and the EGFP signal was measured 22 h after transfection as a readout of translation activity. Since the expression levels of the A1 subunits were below the limit of detection by immunoblot analysis, mRNA levels were measured by qRT-PCR. Expression levels of Stx1A1 and Stx2A1 were comparable after transfection (see Fig. S2 in the supplemental material). The base fluorescence in cells transfected with Stx1A1 and Stx2A1 without EGFP was about the same as that in cells transfected with the empty vector. Stx2A1 showed a significantly higher (40-fold) level of inhibition of EGFP expression did Stx1A1 (Fig. 6A).

To determine if translation inhibition by endogenously expressed Stx2A1 correlated with depurination, total RNA from HEK293T cells was analyzed for depurination by qRT-PCR (47,

TABLE 3 Kinetic parameters of the A1 subunits with ribosomes and with stem-loop RNA^a

Substrate	Toxin	Mean K_m (μM) $\pm$ SD	Mean k_{cat} (min ⁻¹) $\pm$ SD	Mean k_{cat}/K_m (M ⁻¹ s ⁻¹) $\pm$ SD
Ribosome ^b	Yeast	Stx1A1	2,257 $\pm$ 202C	$3.6 \times 10^7 \pm 0.1 \times 10^7\text{E}$
		Stx2A1	3,406 $\pm$ 133D	$5.2 \times 10^7 \pm 0.5 \times 10^7\text{F}$
	Rat	Stx1A1	401 $\pm$ 13.1I	$2.2 \times 10^7 \pm 0.1 \times 10^7\text{K}$
		Stx2A1	1,098 $\pm$ 85.0J	$5.1 \times 10^7 \pm 0.7 \times 10^7\text{L}$
Synthetic SRL ^c	Stx1A1	0.93 $\pm$ 0.22M	21.5 $\pm$ 0.6O	$4.0 \times 10^5 \pm 0.9 \times 10^5\text{Q}$
	Stx2A1	2.46 $\pm$ 0.55N	62.6 $\pm$ 22.3P	$4.4 \times 10^5 \pm 1.6 \times 10^5\text{R}$

^a Letters indicate statistical comparisons, where means were not significantly different between A and B, between G and H, and between Q and R; means were significantly different between C and D, between I and J, and between M and N with a P value of <0.01 ; and means were significantly different between E and F, between K and L, and between O and P with a P value of <0.05 , as determined by using a one-tailed t test.

^b The ribosome substrate was assayed in a continuous format.

^c The synthetic SRL was assayed in a discontinuous format.

FIG 6 (A) Translation inhibition and ribosome depurination in mammalian cells expressing Stx1A1 or Stx2A1. HEK293T cells were cotransfected with Stx1A1 or Stx2A1 and EGFP. Cells carrying the empty vector (VC) were used as controls. EGFP fluorescence was read at 22 h posttransfection. Fluorescence measured in cells cotransfected with EGFP and the empty vector was considered 100%, and fluorescence in controls lacking the EGFP plasmid was considered background. Statistical analyses were conducted by using SAS 9.4 (SAS Institute, Inc.). Data were analyzed by general linear models using PROC GLM to test for statistical differences between treatments, and Tukey's test was used to perform pairwise comparisons (***, $P < 0.001$; NS, not significant). Error bars represent standard errors ($n = 3$ replicates). (B) Depurination of ribosomes from mammalian cells by Stx1A1 and Stx2A1. Total RNA (375 ng) from HEK293T cells expressing Stx1A1 or Stx2A1 collected at 23 h post-DNA exposure was used to quantify the relative levels of depurination by using qRT-PCR (42). The y axis shows the fold change in depurination over the control samples (VC). The table shows the fold change in depurination levels in cells transfected with Stx1A1 and Stx2A1 relative to cells transfected with the empty vector. Data were analyzed with PROC GLM to test for statistical differences between treatments, and Tukey's test was used to perform pairwise comparisons (*, $P < 0.05$). Error bars represent standard errors ($n = 3$ independent experiments).

48). As shown in Fig. 6B, ribosomes were depurinated in cells transfected with Stx1A1 and Stx2A1 relative to cells transfected with the empty vector (Fig. 6B), indicating that both proteins were expressed. Stx2A1 depurinated ribosomes at a significantly higher (10-fold) level than Stx1A1 in HEK293T cells. These results demonstrate that Stx2A1 is significantly more active in depurinating ribosomes and inhibiting translation in human cells than Stx1A1.

DISCUSSION

The A1 subunit of Stx2 is more toxic and has higher activity in yeast and in mammalian cells. We expressed the mature A1 subunits of Stx1A1 and Stx2A1 in yeast and in mammalian cells to compare their cytotoxicity and activity directly without the B subunits. Our results showed that Stx2A1 is 10-fold more toxic to yeast than Stx1A1 (Fig. 1A and B). The expression level of Stx1A1 was higher than that of Stx2A1 at all time points (Fig. 1C), consistent with our previously reported results which showed that RTA mutants with reduced toxicity are expressed at higher levels (43,

51). Using a highly sensitive qRT-PCR assay, we showed that Stx2A1 depurinates yeast ribosomes at a significantly higher level than Stx1A1 during the first 3 h of toxin induction (Fig. 1D). To confirm the *in vivo* results, we purified mature Stx1A1 and Stx2A1 from *E. coli* and showed that Stx2A1 depurinated yeast ribosomes (Fig. 2C) and yeast RNA (Fig. 2D) at a significantly higher level than Stx1A1 *in vitro*. Although the difference in the depurination levels of Stx1A1 and Stx2A1 was 2- to 3-fold, it gave rise to a 10-fold difference in toxicity, indicating that small changes in depurination activity could have much larger consequences for toxicity in cells.

We demonstrated that Stx2A1 is significantly more active in depurinating ribosomes and inhibiting protein synthesis than Stx1A1 in human cells. Larger differences were observed in the activities of Stx1A1 and Stx2A1 in human cells than in yeast. A 10-fold increase in depurination gave rise to a 40-fold-higher level of translation inhibition by Stx2A1 than by Stx1A1 in human cells. It is unlikely that the observed differences were due to a lower stability of Stx1A1, since purified Stx1A1 was more stable than Stx2A1 by protein thermal shift analysis (see Fig. S1 in the supplemental material) and since each endogenously expressed A1 subunit would be folded in the cytosol as the holotoxin-derived A1 subunit after retrotranslocation from the ER to the cytosol. Although A1 subunits were expressed in human cells below levels detectable by immunoblot analysis, Stx1A1 depurinated ribosomes in human cells at a 16-fold-higher level than in cells transfected with the empty vector (Fig. 6B), indicating that it was expressed in the cells.

In previous studies, differences were not observed for the protein synthesis inhibition abilities of Stx1 and Stx2 by using cell-free translation assays (23, 24, 52). While in some of these studies, the holotoxin was used (24), in others, the holotoxin was activated by digestion with trypsin to release the A1 chain from the A2-B5 complex or by chemical treatment with urea or DTT to break the disulfide bond between the A1 and A2 chains (23, 24, 53, 54). The amount of activated protein may vary after these treatments, precluding accurate comparison of the enzymatic activities of the trypsin- and DTT-activated toxins (40). Moreover, since translation inhibition is a downstream effect of depurination, cell-free translation assays measure toxin activity indirectly and do not quantify the catalytic activity of Stx1A1 and Stx2A1 on the ribosome. Consistent with data from previous reports, we did not see any difference between the translation-inhibitory activities of recombinant Stx1A1 and Stx2A1 in the reticulocyte lysate cell-free translation system.

To understand the basis for the higher activity of Stx2A1 than of Stx1A1, we measured their depurination kinetics with an enzymatically coupled luminescence assay using yeast ribosomes, rat liver ribosomes, and RNA as the substrates. The results showed that Stx2A1 had a higher k_{cat} than Stx1A1 when either yeast or rat liver ribosomes were used as a substrate. Similarly, Stx2A1 had a higher k_{cat} than Stx1A1 when RNA was used as a substrate. These results indicated that Stx2A1 has higher catalytic activity than Stx1A1 and were consistent with the *in vivo* data.

Ribosomes were better substrates than the stem-loop RNA and were depurinated optimally at physiological pH. However, Stx1A1 and Stx2A1 could not depurinate the stem-loop RNA at neutral pH. Even at acidic pH, RNA was depurinated at a very low rate. Stx1A1 and Stx2A1 depurinated the stem-loop RNA at pH 4.5 with catalytic efficiencies (k_{cat}/K_m) that were 55-fold and 110-

FIG 7 Crystallographic structures of Stx1A1 and Stx2A1 showing the electrostatic charge distribution. The A1 subunits of Shiga toxin and Shiga toxin 2 were modeled from the structure reported under Protein Data Bank accession numbers **1DM0** (Shiga toxin) and **1RP4** (Shiga toxin 2), as described previously (39). The solvent-accessible molecular surfaces are colored according to electrostatic charge, with blue indicating a positive charge, red indicating a negative charge, and white indicating a neutral charge. (A) Stx1A1 has a negatively charged (red) crease running diagonally across the front of the active site. A rotation about the y axis of $\sim 180^\circ$ reveals a positive zone (blue) in Stx1A1 that is considerably larger and more open to the outside than Stx2A1. (B) The active site in Stx1A1 has little negative charge, with most of the surface being neutral. A rotation about the y axis of $\sim 180^\circ$ reveals a smaller positive (blue) zone at the center.

fold lower, respectively, than those for the SRL on rat liver ribosomes at pH 7.4 (Table 3). The increase in catalytic efficiency was due to an improved k_{cat} . Previously reported results indicated that Shiga toxins bind to the P-protein stalk to depurinate the SRL (33, 34, 40). Therefore, the interaction of the A1 subunits with the stalk proteins and with other binding sites on the ribosome appears to have a profound effect on the catalytic efficiency of Shiga toxins toward the SRL.

Structural differences result in higher affinity of Stx2A1 for ribosomes. Previously reported results indicated that Stx1A1 interacts with peptides corresponding to the conserved C termini of the ribosomal P-protein stalk (33, 34). However, the interaction of Stx1 and Stx2 with either the ribosome or the ribosomal stalk complex has not been investigated. To investigate the basis for the higher activity of Stx2A1, we compared the interactions of the A1 subunits with yeast ribosomes and with the isolated ribosomal P-protein stalk pentamer from yeast (38). Our previously reported results indicated that the interaction of RTA with ribosomes did not fit a simple 1:1 interaction model (32). We showed that the ribosomal P-protein stalk binding surface of RTA is on the opposite face of the active site and proposed a model to explain how RTA depurinates ribosomes (38). According to this model, electrostatic interactions between RTA and the ribosome concen-

trate RTA molecules around the ribosome and allow their diffusion toward the P-protein stalk. Specific electrostatic interactions with the P-protein stalk stimulate the catalysis of ribosome depurination by orienting the active site of RTA toward the SRL and deliver the properly oriented RTA to the SRL through a conformational change of the hinge region of the P proteins (38). As observed with RTA, the interaction of Stx1A1 and Stx2A1 with either yeast or rat ribosomes did not fit a simple 1:1 interaction model. The interaction had an initial fast association-and-dissociation phase followed by a slow association-and-dissociation phase similarly to the interaction of RTA with ribosomes (43). This biphasic interaction curve was more obvious for the interaction of Stx2A1 with yeast and mammalian ribosomes (Fig. 3 and 4). Due to the structural similarity between RTA and Shiga toxins, the ribosome interaction model proposed for RTA may be applicable to Stx1A1 and Stx2A1.

Because of the size of the ribosome relative to the A1 subunits, not every interaction between Stx1A1 and Stx2A1 and the ribosome will result in depurination of the SRL. Therefore, on and off rates (k_{on} and k_{off}) are more important than affinity (K_D) since they determine how fast the depurination reaction will take place. Although we could not determine the on and off rates of the interaction between the A1 subunits and ribosomes, Stx2A1 had a

much faster association-and-dissociation pattern than did Stx1A1 (Fig. 3 and 4). We have previously shown that the high on and off rates reflect the interaction of RTA with the ribosomal P-protein stalk on yeast (32) and human (48) ribosomes. The interaction of RTA with the ribosomal stalk is determined by the local concentration of RTA around the ribosome (43). Since we did not observe a significant difference in the interaction of the A1 subunits with the purified P-protein complex (Fig. 3C) but observed large differences in their interactions with ribosomes, the differences in the interactions of Stx1A1 and Stx2A1 with ribosomes might be due to differences in the toxin concentrations around the ribosome. The higher local concentration around the ribosome should stimulate the interaction of Stx2A1 with the stalk complex on the ribosome, as reflected by the initial fast association-and-dissociation phase of the interaction between Stx2A1 and ribosomes (Fig. 3 and 4).

Stx1A and Stx2A have 55% amino acid sequence identity. Although the structures of Stx and Stx2 are similar, the sequence divergence between Stx2A1 and Stx1A1 has some influence on the structure. In the reported structure of holotoxins, some of the loops in the A1 subunits were missing. We have reconstructed the missing loops in the A1 subunits of Stx and Stx2 in the holotoxins, as previously described (39), and calculated the electrostatic surface charge with the Adaptive Poisson-Boltzmann Solver (APBS) (55, 56).

The electrostatic surfaces of Stx1A1 and Stx2A1 show a number of differences, and the molecular surfaces, on which the electrostatic charges are shown, also have somewhat different shapes (Fig. 7). Stx1A1 has a negatively charged crease running diagonally across the front (Fig. 7A), which is missing in Stx2A1 (Fig. 7B). The active site is masked by the left end of the crease in Stx1A1 and by the positive area that extends up to this region in both proteins. The negatively charged crease in Stx1A1 would decrease the ability of the protein to interact initially with the negatively charged surface of the ribosome.

Rotation of the molecules by $\sim 180^\circ$ along the y axis (Fig. 7A and B) brings a large, intense, positively charged zone into view. The Stx1A1 zone (Fig. 7A) is considerably larger and more open to the outside than in Stx2A1 (Fig. 7B). This positively charged region is on the opposite side of the molecule from the active site. Its interaction with the negatively charged RNA may reduce the availability of the active-site residues to perform catalysis. Previously reported results showed that mutations of residues at the active sites of Stx1A and Stx2A affect cytotoxicity and catalytic activity differently (39). The residues previously shown to be involved in the depurination reaction in Stx1 and Stx2 (E167 and R170) are more exposed in Stx2A1 than in Stx1A1 (39). Further studies are needed to understand if these differences are responsible for the affinity of the Shiga toxins for the RNA and their catalytic activity.

Stx2A1 is catalytically more efficient than Stx1A1 due to higher catalytic activity toward the SRL and higher affinity for the ribosome. Although Stx2A1 has a higher apparent affinity for both yeast and rat ribosomes than Stx1A1, Stx1A1 and Stx2A1 have similar K_m values for yeast (1 μM) and rat (0.3 μM) ribosomes (Table 3). The similar K_m values of Stx1A1 and Stx2A1 indicate that while ribosome interaction is a necessary step, it is not the only step in depurination. The K_m values were higher than the apparent K_D values for both Stx1A1 and Stx2A1. The high K_m value reflects multiple interactions that occur between the A1 sub-

units and the ribosome and suggests that the ribosome binds multiple A1 subunits to trigger depurination.

Since depurination of the stem-loop RNA is used to measure the catalytic activity in the absence of the ribosomal proteins, we examined the activity of Stx1A1 and Stx2A1 on the stem-loop RNA. Stx2A1 had 3-fold-higher catalytic activity (k_{cat}) toward the stem-loop RNA than Stx1A1. However, Stx1A1 had a lower K_m for the stem-loop RNA than Stx2A1, resulting in a similar catalytic efficiency for depurination of the SRL (Table 3). The higher K_m reflects the lower affinity of Stx2A1 for the stem-loop RNA at a lower pH. However, Stx2A1 has a higher affinity for the ribosome due to its interaction with the ribosomal proteins at physiological pH. Therefore, the higher affinity of Stx2A1 for the ribosome, in combination with its higher catalytic activity toward the SRL, allows Stx2A1 to depurinate the ribosome more efficiently than Stx1A1.

We examine here the differences in the interactions of the A1 subunits of Stx1a and Stx2a with ribosomes, depurination of the SRL, and the resulting translational arrest and show for the first time that in the absence of the B subunits, the catalytic subunits of Shiga toxins interact differently with ribosomes, depurinate the ribosome and the SRL at different catalytic rates, and cause different levels of inhibition of translation in mammalian cells. Our results indicate that small differences in depurination activity lead to much larger differences in translation inhibition and toxicity in cells and are likely to lead to even larger differences in animals. The A subunit influences potency in animal models (57–59). We conclude that differences in the A1 subunits together with the previously defined differences in the B subunits (29) contribute to the differential toxicities of Stx1a and Stx2a. Further investigations on the importance of the A1 subunit for the higher toxicity of Stx2a will identify mechanistic differences in the actions of Stxs on ribosomes and will provide a major step toward understanding the mechanism of catalysis and how to block their activity.

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REFERENCES

- Boerlin P, McEwen SA, Boerlin-Petzold F, Wilson JB, Johnson RP, Gyles CL. 1999. Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *J Clin Microbiol* 37: 497–503.
- Siegler R, Oakes R. 2005. Hemolytic uremic syndrome; pathogenesis, treatment, and outcome. *Curr Opin Pediatr* 17:200–204. <http://dx.doi.org/10.1097/01.mop.0000152997.66070.e9>.
- Frank C, Werber D, Cramer JP, Askar M, Faber M, an der Heiden M, Bernard H, Fruth A, Prager R, Spode A. 2011. Epidemic profile of

- Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. *N Engl J Med* 365:1771–1780. <http://dx.doi.org/10.1056/NEJMoa1106483>.
4. Kaper J, O'Brien A. 2014. Overview and historical perspectives. *Microbiol Spectr* 2(6):EHEC–0028–2014. <http://dx.doi.org/10.1128/microbiolspec.EHEC-0028-2014>.
 5. Karch H, Denamur E, Dobrindt U, Finlay BB, Hengge R, Johannes L, Ron EZ, Tønjum T, Sansonetti PJ, Vicente M. 2012. The enemy within us: lessons from the 2011 European *Escherichia coli* O104:H4 outbreak. *EMBO Mol Med* 4:841–848. <http://dx.doi.org/10.1002/emmm.201201662>.
 6. Bielaszewska M, Mellmann A, Zhang W, Köck R, Fruth A, Bauwens A, Peters G, Karch H. 2011. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *Lancet Infect Dis* 11:671–676. [http://dx.doi.org/10.1016/S1473-3099\(11\)70165-7](http://dx.doi.org/10.1016/S1473-3099(11)70165-7).
 7. Bergan J, Dyve Lingelem AB, Simm R, Skotland T, Sandvig K. 2012. Shiga toxins. *Toxicon* 60:1085–1107. <http://dx.doi.org/10.1016/j.toxicon.2012.07.016>.
 8. Sandvig K, van Deurs B. 2005. Delivery into cells: lessons learned from plant and bacterial toxins. *Gene Ther* 12:865–872. <http://dx.doi.org/10.1038/sj.gt.3302525>.
 9. Yu M, Haslam DB. 2005. Shiga toxin is transported from the endoplasmic reticulum following interaction with the luminal chaperone HEDJ/ERdj3. *Infect Immun* 73:2524–2532. <http://dx.doi.org/10.1128/IAI.73.4.2524-2532.2005>.
 10. Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K. 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. *Eur J Biochem* 171:45–50.
 11. Clementi N, Chirkova A, Puffer B, Micura R, Polacek N. 2010. Atomic mutagenesis reveals A2660 of 23S ribosomal RNA as key to EF-G GTPase activation. *Nat Chem Biol* 6:344–351. <http://dx.doi.org/10.1038/nchembio.341>.
 12. Shi X, Khade PK, Sanbonmatsu KY, Joseph S. 2012. Functional role of the sarcin-ricin loop of the 23S rRNA in the elongation cycle of protein synthesis. *J Mol Biol* 419:125–138. <http://dx.doi.org/10.1016/j.jmb.2012.03.016>.
 13. Tesh VL, O'Brien AD. 1991. The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. *Mol Microbiol* 5:1817–1822. <http://dx.doi.org/10.1111/j.1365-2958.1991.tb00805.x>.
 14. Fraser M, Chernaia M, Kozlov Y, James M. 1994. Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 Å resolution. *Nat Struct Biol* 1:59. <http://dx.doi.org/10.1038/nsb0194-59>.
 15. Fraser ME, Fujinaga M, Cherney MM, Melton-Celsa AR, Twiddy EM, O'Brien AD, James MN. 2004. Structure of Shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. *J Biol Chem* 279:27511–27517. <http://dx.doi.org/10.1074/jbc.M401939200>.
 16. Fraser ME, Cherney MM, Marcato P, Mulvey GL, Armstrong GD, James MN. 2006. Binding of adenine to Stx2, the protein toxin from *Escherichia coli* O157:H7. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 62:627–630. <http://dx.doi.org/10.1107/S1744309106021968>.
 17. Basu D, Tumer NE. 2015. Do the A subunits contribute to the differences in the toxicity of Shiga toxin 1 and Shiga toxin 2? *Toxins (Basel)* 7:1467–1485. <http://dx.doi.org/10.3390/toxins7051467>.
 18. Nataro JP, Kaper JB. 1998. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 11:142–201.
 19. Pickering L, Obrig T, Stapleton F. 1994. Hemolytic-uremic syndrome and enterohemorrhagic *Escherichia coli*. *Pediatr Infect Dis J* 13:459–475. <http://dx.doi.org/10.1097/00006454-199406000-00001>.
 20. Manning SD, Motiwala AS, Springman AC, Qi W, Lacher DW, Ouellette LM, Mladonicky JM, Somsel P, Rudrik JT, Dietrich SE. 2008. Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. *Proc Natl Acad Sci U S A* 105:4868–4873. <http://dx.doi.org/10.1073/pnas.0710834105>.
 21. Fuller CA, Pellino A, Flagler MJ, Strasser JE, Weiss AA. 2011. Shiga toxin subtypes display dramatic differences in potency. *Infect Immun* 79:1329–1337. <http://dx.doi.org/10.1128/IAI.01182-10>.
 22. Scheutz F, Teel LD, Beutin L, Pierard D, Buvens G, Karch H, Mellmann A, Caprioli A, Tozzoli R, Morabito S, Strockbine NA, Melton-Celsa AR, Sanchez M, Persson S, O'Brien AD. 2012. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *J Clin Microbiol* 50:2951–2963. <http://dx.doi.org/10.1128/JCM.00860-12>.
 23. Head S, Karmali M, Lingwood C. 1991. Preparation of VT1 and VT2 hybrid toxins from their purified dissociated subunits. Evidence for B subunit modulation of a subunit function. *J Biol Chem* 266:3617–3621.
 24. Tesh VL, Burris J, Owens J, Gordon V, Wadolkowski E, O'Brien A, Samuel J. 1993. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect Immun* 61:3392–3402.
 25. Nakajima H, Kiyokawa N, Katagiri YU, Taguchi T, Suzuki T, Sekino T, Mimori K, Ebata T, Saito M, Nakao H. 2001. Kinetic analysis of binding between Shiga toxin and receptor glycolipid Gb3Cer by surface plasmon resonance. *J Biol Chem* 276:42915–42922. <http://dx.doi.org/10.1074/jbc.M106015200>.
 26. Flagler MJ, Mahajan SS, Kulkarni AA, Iyer SS, Weiss AA. 2010. Comparison of binding platforms yields insights into receptor binding differences between Shiga toxins 1 and 2. *Biochemistry* 49:1649–1657. <http://dx.doi.org/10.1021/bi902084y>.
 27. Ito H, Yutsudo T, Hirayama T, Takeda Y. 1988. Isolation and some properties of A and B subunits of Vero toxin 2 and in vitro formation of hybrid toxins between subunits of Vero toxin 1 and Vero toxin 2 from *Escherichia coli* O157:H7. *Microb Pathog* 5:189–195. [http://dx.doi.org/10.1016/0882-4010\(88\)90021-6](http://dx.doi.org/10.1016/0882-4010(88)90021-6).
 28. Weinstein DL, Jackson MP, Perera LP, Holmes RK, O'Brien AD. 1989. *In vivo* formation of hybrid toxins comprising Shiga toxin and the Shiga-like toxins and role of the B subunit in localization and cytotoxic activity. *Infect Immun* 57:3743–3750.
 29. Russo LM, Melton-Celsa AR, Smith MJ, O'Brien AD. 2014. Comparisons of native Shiga toxins (Stxs) type 1 and 2 with chimeric toxins indicate that the source of the binding subunit dictates degree of toxicity. *PLoS One* 9:e93463. <http://dx.doi.org/10.1371/journal.pone.0093463>.
 30. Chan DS, Chu L-O, Lee K-M, Too PH, Ma K-W, Sze K-H, Zhu G, Shaw P-C, Wong K-B. 2007. Interaction between trichosanthin, a ribosome-inactivating protein, and the ribosomal stalk protein P2 by chemical shift perturbation and mutagenesis analyses. *Nucleic Acids Res* 35:1660–1672. <http://dx.doi.org/10.1093/nar/gkm065>.
 31. Chiou JC, Li XP, Remacha M, Ballesta JP, Tumer NE. 2008. The ribosomal stalk is required for ribosome binding, depurination of the rRNA and cytotoxicity of ricin A chain in *Saccharomyces cerevisiae*. *Mol Microbiol* 70:1441–1452. <http://dx.doi.org/10.1111/j.1365-2958.2008.06492.x>.
 32. Li X-P, Chiou J-C, Remacha M, Ballesta JP, Tumer NE. 2009. A two-step binding model proposed for the electrostatic interactions of ricin A chain with ribosomes. *Biochemistry* 48:3853–3863. <http://dx.doi.org/10.1021/bi802371h>.
 33. McCluskey AJ, Bolewska-Pedyczak E, Jarvik N, Chen G, Sidhu SS, Gariépy J. 2012. Charged and hydrophobic surfaces on the A chain of Shiga-like toxin 1 recognize the C-terminal domain of ribosomal stalk proteins. *PLoS One* 7:e31191. <http://dx.doi.org/10.1371/journal.pone.0031191>.
 34. McCluskey AJ, Poon GM, Bolewska-Pedyczak E, Srikumar T, Jeram SM, Raught B, Gariépy J. 2008. The catalytic subunit of Shiga-like toxin 1 interacts with ribosomal stalk proteins and is inhibited by their conserved C-terminal domain. *J Mol Biol* 378:375–386. <http://dx.doi.org/10.1016/j.jmb.2008.02.014>.
 35. Too PH-M, Ma MK-W, Mak AN-S, Wong Y-T, Tung CK-C, Zhu G, Au SW-N, Wong K-B, Shaw P-C. 2009. The C-terminal fragment of the ribosomal P protein complexed to trichosanthin reveals the interaction between the ribosome-inactivating protein and the ribosome. *Nucleic Acids Res* 37:602–610. <http://dx.doi.org/10.1093/nar/gkn922>.
 36. Gonzalo P, Reboud JP. 2003. The puzzling lateral flexible stalk of the ribosome. *Biol Cell* 95:179–193. [http://dx.doi.org/10.1016/S0248-4900\(03\)00034-0](http://dx.doi.org/10.1016/S0248-4900(03)00034-0).
 37. Tchorzewski M. 2002. The acidic ribosomal P proteins. *Int J Biochem Cell Biol* 34:911–915. [http://dx.doi.org/10.1016/S1357-2725\(02\)00012-2](http://dx.doi.org/10.1016/S1357-2725(02)00012-2).
 38. Li X-P, Grela P, Krokowski D, Tchorzewski M, Tumer NE. 2010. Pentameric organization of the ribosomal stalk accelerates recruitment of ricin A chain to the ribosome for depurination. *J Biol Chem* 285:41463–41471. <http://dx.doi.org/10.1074/jbc.M110.171793>.
 39. Di R, Kyu E, Shete V, Saidasan H, Kahn P, Tumer N. 2011. Identification of amino acids critical for the cytotoxicity of Shiga toxin 1 and 2 in *Saccharomyces cerevisiae*. *Toxicon* 57:525–539. <http://dx.doi.org/10.1016/j.toxicon.2010.12.006>.
 40. Chiou J-C, Li X-P, Remacha M, Ballesta JP, Tumer NE. 2011. Shiga toxin 1 is more dependent on the P proteins of the ribosomal stalk for

- depurination activity than Shiga toxin 2. *Int J Biochem Cell Biol* 43:1792–1801. <http://dx.doi.org/10.1016/j.biocel.2011.08.018>.
41. Zhang T, Lei J, Yang H, Xu K, Wang R, Zhang Z. 2011. An improved method for whole protein extraction from yeast *Saccharomyces cerevisiae*. *Yeast* 28:795–798. <http://dx.doi.org/10.1002/yea.1905>.
 42. Pierce M, Kahn JN, Chiou J, Tumer NE. 2011. Development of a quantitative RT-PCR assay to examine the kinetics of ribosome depurination by ribosome inactivating proteins using *Saccharomyces cerevisiae* as a model. *RNA* 17:201–210. <http://dx.doi.org/10.1261/rna.2375411>.
 43. Li X-P, Kahn PC, Kahn JN, Grela P, Tumer NE. 2013. Arginine residues on the opposite side of the active site stimulate the catalysis of ribosome depurination by ricin A chain by interacting with the P-protein stalk. *J Biol Chem* 288:30270–30284. <http://dx.doi.org/10.1074/jbc.M113.510966>.
 44. Hoffman WL, Ilan J. 1974. Purification on hydroxyapatite of liver ribosomes and polysomes from unfasted mice. *Biochim Biophys Acta* 366:199–214. [http://dx.doi.org/10.1016/0005-2787\(74\)90334-7](http://dx.doi.org/10.1016/0005-2787(74)90334-7).
 45. Sturm MB, Schramm VL. 2009. Detecting ricin: sensitive luminescent assay for ricin A-chain ribosome depurination kinetics. *Anal Chem* 81:2847–2853. <http://dx.doi.org/10.1021/ac8026433>.
 46. Redmann V, Oresic K, Tortorella LL, Cook JP, Lord M, Tortorella D. 2011. Dislocation of ricin toxin A chains in human cells utilizes selective cellular factors. *J Biol Chem* 286:21231–21238. <http://dx.doi.org/10.1074/jbc.M111.234708>.
 47. Jetzt AE, Cheng JS, Li XP, Tumer NE, Cohick WS. 2012. A relatively low level of ribosome depurination by mutant forms of ricin toxin A chain can trigger protein synthesis inhibition, cell signaling and apoptosis in mammalian cells. *Int J Biochem Cell Biol* 44:2204–2211. <http://dx.doi.org/10.1016/j.biocel.2012.09.004>.
 48. May KL, Li XP, Martinez-Azorin F, Ballesta JP, Grela P, Tchorzewski M, Tumer NE. 2012. The P1/P2 proteins of the human ribosomal stalk are required for ribosome binding and depurination by ricin in human cells. *FEBS J* 279:3925–3936. <http://dx.doi.org/10.1111/j.1742-4658.2012.08752.x>.
 49. Rieu I, Powers SJ. 2009. Real-time quantitative RT-PCR: design, calculations, and statistics. *Plant Cell* 21:1031–1033. <http://dx.doi.org/10.1105/tpc.109.066001>.
 50. Steel RGD, Torrie JH. 1980. Principles and procedures of statistics: a biometrical approach, 2nd ed. McGraw-Hill, New York, NY.
 51. Yan Q, Li X-P, Tumer NE. 2014. Wild type RTA and less toxic variants have distinct requirements for Pngl for their depurination activity and toxicity in *Saccharomyces cerevisiae*. *PLoS One* 9:e113719. <http://dx.doi.org/10.1371/journal.pone.0113719>.
 52. Brigotti M, Carnicelli D, Alvergnà P, Mazzaracchio R, Sperti S, Montanaro L. 1997. The RNA-N-glycosidase activity of Shiga-like toxin I: kinetic parameters of the native and activated toxin. *Toxicon* 35:1431–1437. [http://dx.doi.org/10.1016/S0041-0101\(96\)00225-5](http://dx.doi.org/10.1016/S0041-0101(96)00225-5).
 53. Siegler RL, Obrig TG, Pysher TJ, Tesh VL, Denkers ND, Taylor FB. 2003. Response to Shiga toxin 1 and 2 in a baboon model of hemolytic uremic syndrome. *Pediatr Nephrol* 18:92–96.
 54. Kozlov YV, Chernaiia MM, Fraser ME, James MN. 1993. Purification and crystallization of Shiga toxin from *Shigella dysenteriae*. *J Mol Biol* 232:704–706. <http://dx.doi.org/10.1006/jmbi.1993.1421>.
 55. Holst MJ, Saied F. 1995. Numerical solution of the nonlinear Poisson-Boltzmann equation: developing more robust and efficient methods. *J Comp Chem* 16:337–364. <http://dx.doi.org/10.1002/jcc.540160308>.
 56. Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA. 2001. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci U S A* 98:10037–10041. <http://dx.doi.org/10.1073/pnas.181342398>.
 57. Russo LM, Melton-Celsa AR, Smith MA, Smith MJ, O'Brien AD. 2014. Oral intoxication of mice with Shiga toxin type 2a (Stx2a) and protection by anti-Stx2a monoclonal antibody 11E10. *Infect Immun* 82:1213–1221. <http://dx.doi.org/10.1128/IAI.01264-13>.
 58. Sauter KA, Melton-Celsa AR, Larkin K, Troxell ML, O'Brien AD, Magun BE. 2008. Mouse model of hemolytic-uremic syndrome caused by endotoxin-free Shiga toxin 2 (Stx2) and protection from lethal outcome by anti-Stx2 antibody. *Infect Immun* 76:4469–4478. <http://dx.doi.org/10.1128/IAI.00592-08>.
 59. Stone SM, Thorpe CM, Ahluwalia A, Rogers AB, Obata F, Vozenilek A, Kolling GL, Kane AV, Magun BE, Jandhyala DM. 2012. Shiga toxin 2-induced intestinal pathology in infant rabbits is A-subunit dependent and responsive to the tyrosine kinase and potential ZAK inhibitor imatinib. *Front Cell Infect Microbiol* 2:135. <http://dx.doi.org/10.3389/fcimb.2012.00135>.