Proteolytic cleavage of the A subunit is essential for maximal cytotoxicity of *Escherichia coli* O157:H7 Shiga-like toxin-1

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Members of the bacterial Shiga toxin family consist of a single A subunit that is non-covalently associated with a pentamer of B subunits. These toxins bind to receptors on susceptible mammalian cells and enter the cells by endocytic uptake. During cell entry, the 32 kDa A subunit is cleaved by the membrane-anchored protease furin to generate a catalytically active, 27·5 kDa A₁ fragment and a 4·5 kDa A₂ fragment. Previous studies have shown that mutating the furin site to prevent cleavage did not significantly affect toxin potency, suggesting that cleavage is not required for toxin activity. Here it is confirmed that preventing cleavage at the usual processing site does not prevent proteolytic processing of the *Escherichia coli* Shiga-like toxin-1 A subunit. However, simultaneous mutation of both the primary furin-recognition site and a nearby putative furin cleavage site did prevent intracellular processing of the A subunit. Comparison of the cytotoxicities of purified recombinant toxins to cultured mammalian cells demonstrated that even on prolonged incubation with toxin, the unprocessed mutant was 60-fold less toxic than the wild-type protein or other mutants still capable of being proteolytically processed during cell entry.

Keywords: Shiga-like toxin-1, proteolytic processing, toxin A subunit, toxin activation

INTRODUCTION

The Shiga toxin family includes Shiga toxin itself (ST, from *Shigella dysenteriae*) and Shiga-like toxins-1 and -2 (SLT-1 and SLT-2, from enterohaemorrhagic *Escherichia coli*) (Tesh & O'Brien, 1991). All of these toxins consist of an enzymic 32 kDa A subunit, which is an RNA N-glycosidase that removes a specific adenine residue from 28S rRNA (Endo *et al.*, 1988), non-covalently associated with a pentamer of 7·7 kDa B subunits, which bind the toxin to plasma-membrane globotriosyl ceramides present on susceptible mammalian cells (Lindberg *et al.*, 1987). The toxin A subunits contain two cysteines joined by a disulphide bond. The loop between the disulphide-linked cysteines contains the sequence Arg-X-X-Arg, which is readily cleaved *in vitro* by trypsin (Olsnes *et al.*, 1981) and, following toxin entry into mammalian cells, by the ubiquitous protease furin (Garred *et al.*, 1995a). Cleavage splits the A subunit

into a 27·5 kDa A₁ fragment, which has the RNA N-glycosidase activity, and a 4·5 kDa A₂ fragment. Several other bacterial toxins, including diphtheria toxin and *Pseudomonas* exotoxin A, must undergo an equivalent cleavage by furin during cell entry (Gordon & Leppla, 1994) before the catalytically active polypeptide fragment can be translocated into the cytosol, where the rRNA substrates are located.

It has been reported that proteolytic cleavage increases the enzymic activity of ST between 10- (Olsnes *et al.*, 1981) and 40-fold (Brown *et al.*, 1980), but proteolytic nicking of SLT-1 *in vitro* did not increase its toxicity to mammalian cells (Kongmuang *et al.*, 1988). It has also been shown that preventing cleavage at the furin-sensitive site, using site-directed mutagenesis to remove the Arg-X-X-Arg target sequence, has no significant effect on the toxicity of ST (Garred *et al.*, 1995b), SLT-1 (Burgess & Roberts, 1993) or SLT-2 (Samuel & Gordon, 1994) to mammalian cells after prolonged incubation times. However, the ST study (Garred *et al.*, 1995b) also found that cells were able to cleave both wild-type toxin and the mutants, although the cellular locations of these cleavage steps appeared to differ.

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Abbreviations: SLT, Shiga-like toxin; ST, Shiga toxin.

Apparently, ST can be cleaved at an alternative site close to the primary cleavage site for furin (Garred *et al.*, 1995b). On the basis of this observation, it seems possible that mutant SLTs might also undergo proteolytic cleavage at a second distinct site, and the conclusion that proteolytic processing is not essential for cytotoxicity (Garred *et al.*, 1995b; Burgess & Roberts, 1993; Samuel & Gordon, 1994) might not be valid. In order to test this, we have produced a recombinant SLT-1 mutant that does not undergo any apparent proteolytic cleavage during cell entry. Although this mutant retains some potency after prolonged incubation with cells, it is significantly less toxic than either wild-type toxin or the furin-site mutant, indicating that proteolytic processing to release a catalytically active fragment is required for optimal cytotoxicity.

METHODS

Bacterial strain, bacteriophage and plasmids. *E. coli* JM105 [*supE endA sbcB15 hsdR4 rps thi Δ(lac-proAB) F'(traD36 proAB⁺ lacI^qlacZΔM15)*] was used for all genetic manipulations and the expression of recombinant proteins. M13mp18 was used for the production of single-stranded DNA for sequencing and site-directed mutagenesis. The coding region of SLT-1, including the Shine-Dalgarno sequence, was amplified by PCR from *E. coli* O157:H7 cell paste using oligonucleotide primers designed to incorporate a *Pst*I site at the 5' end and an *Eco*RI site at the 3' end. The DNA oligonucleotides used were 5' GCTAGAATTCTCAACGAAAAATAACTT 3' and 5' CGGAAGTGCAGCAAGGAGTATTGTGTAATAT 3'. The resulting fragment was cloned into the *Pst*I/*Eco*RI sites of M13mp18. After mutagenesis, *Pst*I-*Eco*RI fragments encoding the entire wild-type SLT-1 operon or mutant A subunit containing the appropriate amino acid substitutions were cloned into the *Pst*I/*Eco*RI site of pUC19 to create the expression plasmids pSLTwt, pSLT1, pSLT2, pSLT3 and pSLT4. Amino acid substitutions in the various mutants are shown in Table 1.

Site-directed mutagenesis. The T7-Gen *in vitro* mutagenesis system (United States Biochemical) was used for the preparation of mutated M13 DNA. Mutagenic oligonucleotides,

which were synthesized on an Applied Biosystems DNA synthesizer, were designed to introduce the desired changes into the coding region of SLT-1 A subunit. Mutations were confirmed by sequencing using the dideoxy chain-termination method. The oligonucleotides used were: 5' ¹¹³⁰CATGCA-TCGGCAGTTGCCGGAATGGCA¹¹⁵⁶ 3' to generate mutant SLTP1, 5' ¹¹²⁵ATCATCATCGAGCGCGA¹¹⁴⁴ 3' and 5' ¹¹⁴⁷CAGAATGGGAGCTGATGAGT¹¹⁶⁶ 3' to generate mutant SLTP2, 5' ¹¹³¹ATGGAGCGGCAGTTGCCGGAATGGGA¹¹⁵⁶ 3' to generate mutant SLTP3, and 5' ¹⁰⁴⁶GAC-TCTGTTGGTGTAGGAGGAATTTCTT¹⁰⁷³ 3' to generate mutant SLTP4. Mismatched bases are underlined and the numbering denotes the position at which the primer anneals in the sequence published by Calderwood *et al.* (1987).

Purification of recombinant toxin. SLT-1 was prepared from periplasmic extracts of *E. coli* JM105 transformed with the wild-type expression plasmid pSLTwt or one of the mutant-encoding plasmids described above. Cells were grown in Luria broth supplemented with 100 µg ampicillin ml⁻¹ to an OD₆₀₀ of 0.6 (measured with a Shimadzu UV-160 spectrophotometer). Expression was induced by the addition of IPTG (1 mM final concentration) and allowed to proceed for 3 h. Cells were harvested by centrifugation (10 min at 7500 g), washed in 600 mM sucrose, 300 mM Tris/HCl (pH 8.0), 1 mM EDTA and 0.5 mM MgCl₂, and resuspended in 1 mM Tris/HCl, pH 7.5. After incubation on ice for 10 min, cell debris was removed by centrifugation (15 min at 15000 g). The supernatant was filter-sterilized by passage through a 0.2 µm filter and applied to a 6 ml globotriose-Sepharose affinity column (Muller *et al.*, 1998) equilibrated with 0.5 M NaCl in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). The column was washed with 40 vols 0.5 M NaCl in PBS prior to eluting SLT-1 with 6 M guanidine.HCl. Fractions of 1 ml were collected and immediately dialysed against PBS to remove the denaturant.

Quantification and activity of recombinant proteins. Purified recombinant toxins were quantified from their A₂₈₀ value; an absorption coefficient of 0.984 was calculated for SLT-1 from its primary sequence. Quantification was confirmed by comparison of known amounts of pure SLT-1 on silver-stained polyacrylamide gels. The activity of the recombinant proteins was determined by assessing their specific ability to depurinate 28S RNA of ribosomes isolated from untreated rabbit reticulocyte lysates (Promega). Toxins were incubated for 30 min at 30 °C with 30 µg purified ribosomes in a reaction volume of 30 µl. rRNA was isolated and the extent of depurination was determined by using the aniline assay as described previously (May *et al.*, 1989).

In vivo proteolytic cleavage of toxins. Recombinant SLT-1 and the SLT-1 mutants were labelled with ¹²⁵I by the Iodogen method to a specific activity of approximately 25000 c.p.m. ng⁻¹. Vero (African green monkey kidney) cells were grown in six-well plates in Dulbecco's modification of Eagle's medium (DMEM; Gibco-BRL) containing 5% foetal calf serum and 2 mM glutamine, to a density of approximately 5 × 10⁵ cells per well. After washing with PBS, 100 ng ¹²⁵I-labelled toxin ml⁻¹ was added to each well in 1.5 ml DMEM and the cells were incubated for various time periods. Following incubation, the cells were washed three times with PBS and lysed in cell-lysis buffer supplemented with protease inhibitors (1% Triton X-100, 150 mM NaCl, 50 mM Tris/HCl, pH 7.4, 210 µM leupeptin, 80 µM chymostatin, 6 µM elastinol, 1.6 µM pepstatin, 0.3 µM aprotinin, 1 mM PMSF and 1 mM EDTA). Cell debris was removed by centrifugation (5 min at 7500 g

Table 1. SLT-1 expression plasmids

Plasmid	Mutations introduced	Protein product
pSLTwt	None	SLT-1
pSLT1	Arg ₂₄₈ → Gly/Arg ₂₅₁ → Gly	SLTP1
pSLT2	Ala ₂₄₆ → Gly/Ser ₂₄₇ → Ala Ala ₂₅₃ → Gly/Ser ₂₅₄ → Ala	SLTP2
pSLT3	Arg ₂₄₈ → Gly/Arg ₂₅₁ → Gly Ala ₂₄₆ → Gly/Ser ₂₄₇ → Ala Ala ₂₅₃ → Gly/Ser ₂₅₄ → Ala	SLTP3
pSLT4	Arg ₂₄₈ → Gly/Arg ₂₅₁ → Gly Ala ₂₄₆ → Gly/Ser ₂₄₇ → Ala Ala ₂₅₃ → Gly/Ser ₂₅₄ → Ala Arg ₂₂₀ → Gly/Arg ₂₂₃ → Gly	SLTP4

and 4 °C) and protein was precipitated from the supernatant with acetone. The resulting protein was pelleted, dried and resuspended in SDS-PAGE loading buffer and electrophoresed on 15 % gels.

Cytotoxicity determination. Vero cells, grown in DMEM containing 5 % foetal calf serum and 2 mM glutamine, were plated out in 96-well plates at a density of approximately 2×10^4 cells per well. After removing the medium and washing with PBS, one of a range of toxin concentrations in 100 µl fresh medium was added to each well and the cells were incubated at 37 °C for the desired time. The cells were then washed with PBS, 1 µCi (3.7×10^3 Bq) [35 S]methionine in 100 µl PBS was added per well, and the plates were incubated at 37 °C for 30 min. Labelled protein was precipitated by three washes with ice-cold 5 % trichloroacetic acid and, after a further wash with PBS, solubilized in 0.1 M NaOH. The radioactivity incorporated into protein was determined by liquid scintillation counting. Each toxin concentration was assayed in quadruplicate.

SDS-PAGE. Electrophoresis of proteins in the presence of SDS was carried out according to the method of Laemmli (1970). [125 I]-labelled toxin was visualized by fixing the gels in 10 % acetic acid/40 % methanol followed by treatment with Amplify (Amersham) for 15 min. Preflashed Fuji X-ray film was exposed to dried gels at -70 °C.

RESULTS

Construction and expression of mutant toxins

The SLT-1 mutants generated during this study are listed in Table 1. In mutant SLTP1, Arg₂₄₈ and Arg₂₅₁ of the furin target site were each changed to Gly. An earlier report suggested that, based on Edman degradation, SLT-1 A subunit was nicked between Ala₂₅₃ and Ser₂₅₄, present in the disulphide loop, to generate the A₁ and A₂ fragments (Takao *et al.*, 1988). Accordingly, this Ala-Ser pair and a second pair also present in the disulphide loop, Ala₂₄₆ and Ser₂₄₇, were changed to Gly and Ala, respectively, to generate mutant SLTP2. Mutant SLTP3 contained a combination of the mutations present in SLTP1 and SLTP2. SLT-1 contains a second putative furin cleavage site (Arg₂₂₀-Val-Gly-Arg₂₂₃) outside the disulphide loop. Thus Arg₂₂₀ and Arg₂₂₃ were both changed to Gly in the background of mutant SLTP3 to generate mutant SLTP4.

Recombinant wild-type SLT-1 and the four mutants listed in Table 1 were expressed in *E. coli* and purified to homogeneity by affinity chromatography on a column of immobilized synthetic globotriose (Muller *et al.*, 1998). Fig. 1 shows the results of a typical purification.

Biological activity of recombinant toxins

The recombinant proteins were catalytically active. The N-glycosidase activity removes a specific adenine residue from a highly conserved loop close to the 3' end of 28S rRNA. Depurination renders isolated rRNA susceptible to amine-catalysed hydrolysis of the phosphodiester bonds on either side of the modification site. This cleavage generates a small RNA fragment of ~390

Fig. 1. Purification of recombinant SLT-1 and SLT-1 mutants. *E. coli* periplasmic fraction was passed down an immobilized globotriose column, unbound material was removed by washing and bound toxin was eluted with 6 M guanidine.HCl. Proteins in the various fractions were separated on a 15 % SDS-polyacrylamide gel and stained with Coomassie blue. Lanes: 1, molecular mass markers; 2, total periplasmic fraction from cells expressing wild-type SLT-1; 3, fraction of the periplasmic fraction that did not bind to the column; 4, purified wild-type SLT-1; 5, SLTP1; 6, SLTP2; 7, SLTP3; 8, SLTP4.

Fig. 2. 28S rRNA N-glycosidase activity of recombinant toxins. Rabbit reticulocyte ribosomes were incubated with toxin for 30 min at 30 °C. rRNA was isolated, treated with aniline, separated on a 1.2 % denaturing formamide gel and transferred to nitrocellulose. Northern blot analysis was performed using an end-labelled oligonucleotide probe complementary to a region of 28S rRNA downstream of the depurination site. The bottom arrow (aniline fragment) indicates the ~400 base RNA fragment diagnostic for depurination. Lanes: 1-3, RNA from ribosomes treated with 1000, 100 or 10 ng wild-type SLT-1; 4-6, 7-9, 10-12 and 13-15, RNA from ribosomes treated with the corresponding amounts of SLTP1, SLTP2, SLTP3 and SLTP4, respectively.

ribonucleotides from 28S rRNA that is diagnostic of SLT-1 N-glycosidase activity. Rabbit reticulocyte ribosomes were incubated with different amounts of the purified toxins and the rRNA was isolated and treated with acetic-aniline to hydrolyse depurinated rRNA before being separated on denaturing formamide gels. The presence of the short, diagnostic RNA fragment on gels of total rRNA from toxin-treated ribosomes was confirmed by Northern blotting using a radiolabelled DNA probe complementary to a sequence present in the released RNA fragment. As shown in Fig. 2, increasing the toxin concentration caused an increased release of

Fig. 3. *In vivo* proteolytic processing of recombinant toxins. ^{125}I -labelled wild-type SLT-1 or the mutants indicated, at a concentration of 100 ng ml^{-1} , were incubated with Vero cells at 37°C for 0, 0.5, 1, 2 or 6 h as shown. The cells were then lysed and centrifuged to remove cell debris and total protein was precipitated with acetone. Proteins were separated on 15% SDS-polyacrylamide gels and ^{125}I -labelled toxin was visualized by fluorography. The A fragment and the A₁ fragment resulting from proteolytic cleavage are indicated. Lane M: a ^{14}C -labelled, 31 kDa molecular mass marker.

the fragment with a concomitant decrease in the signal for unmodified 28S rRNA. Qualitatively, at least, the proteins appeared to be very similar in their abilities to depurinate rRNA. Subsequent work showed that the stability and properties of mutant SLTP2 were indistinguishable from those of wild-type SLT-1. SLTP2 was therefore not analysed further in the present study.

Proteolytic processing following entry into mammalian cells

In order to determine whether the various toxin mutants were proteolytically cleaved intracellularly, the purified toxins were labelled with ^{125}I and incubated with SLT-receptor-bearing Vero cells. After various incubation times, the cells were washed to remove extracellular toxin and lysed, total protein was precipitated and separated electrophoretically and toxin was visualized by fluorography. As shown in Fig. 3, wild-type SLT-1 and mutants SLTP1 and SLTP3 were all processed to generate the A₁ fragment, but mutant SLTP4 was apparently resistant to cellular proteolysis. The extent of cleavage of the A subunit to the A₁ fragment was quantified by scanning densitometry and is shown in Fig. 4. The resistance of mutant SLTP4 to proteolytic cleavage indicates that the cleavage seen here for mutants SLTP1 and SLTP3, and that reported elsewhere for a mutant in which the furin site in the disulphide loop had been disrupted (Garred *et al.*, 1995b), most likely occurs at the second putative furin site, Arg₂₂₀-Val-Gly-Arg₂₂₃.

Cytotoxicity to mammalian cells

The cytotoxicities of the wild-type and mutant SLTs were determined by measuring the capacity of Vero cells to synthesize protein after a 6 h incubation with a range

Fig. 4. Quantification of *in vivo* proteolytic processing. The bands shown in Fig. 3 were quantified by scanning densitometry and the proportion of total A subunit present as the A₁ fragment was plotted against length of incubation. ▲, SLT-1; ●, SLTP1; ▼, SLTP3; ■, SLTP4.

of concentrations of toxin (Fig. 5). Wild-type toxin and the mutants that could be cleaved proteolytically intracellularly were of comparable cytotoxicity, while mutant SLTP4 was clearly less toxic. The IC₅₀ (the toxin concentration that caused a 50% decrease in total protein synthesis in comparison with that of untreated control cells) for SLTP4 was 360 pg ml^{-1} , around 60-fold greater than that for wild-type SLT-1. When cells were incubated with toxin for 3 h, wild-type SLT appeared to be more toxic than mutants SLTP1 and SLTP3 (data not shown), indicating presumably that cleavage at the alternative site was less efficient than cleavage at the normal furin site. Incubating the cells with toxins for periods greater than 6 h gave toxicity profiles identical to those observed after 6 h (Fig. 5). The reason(s) for the

Fig. 5. Cytotoxicity of recombinant toxins. Various concentrations of wild-type SLT-1 and the mutants indicated were incubated with Vero cells for 6 h at 37 °C and [³⁵S]methionine incorporation into cellular protein was subsequently determined. All values are the means of four replicate samples and are expressed as percentages of protein synthesis by untreated, control cells. ▲, SLT-1; ●, SLTP1; ▼, SLTP3; ■, SLTP4.

reduced toxicity in the absence of proteolytic cleavage (mutant SLTP4) is unclear, particularly as the catalytic activity of mutant SLTP4 seems to be very similar to that of wild-type SLT-1 (Fig. 2, compare lanes 10–12 with lanes 1–3).

DISCUSSION

In this study, we have investigated whether proteolytic processing of the catalytic A subunit of members of the Shiga toxin family, exemplified here by SLT-1 from *E. coli*, is required for toxicity to mammalian cells. Previous work has clearly established that proteolytic processing of the A subunit into A₁ and A₂ fragments does occur following toxin entry into mammalian cells (Garred *et al.*, 1995b). Processing is achieved through the action of furin, which recognizes the sequence Arg-X-X-Arg (Molloy *et al.*, 1992), a sequence found within a disulphide loop in all members of the Shiga toxin family. Many bacterial toxins that modify target substrates catalytically in the cytosol of mammalian cells require proteolytic cleavage in order to act (Gordon & Leppla, 1994). Some of these toxins, such as diphtheria toxin (Drazin *et al.*, 1971) and *Pseudomonas* exotoxin A (Vasil *et al.*, 1977), are synthesized as catalytically inactive, non-toxic proprotein precursors in which the catalytic and cell-binding subunits are present initially as part of a single polypeptide. When the inactive, native proforms of either diphtheria toxin or *Pseudomonas* exotoxin A enter mammalian cells, proteolytic cleavage releases the A fragment from the cell-binding B fragment and thereby activates these toxins. Once again, it is now clear that furin is the cellular endoprotease responsible

for this activation. Both diphtheria toxin and *Pseudomonas* exotoxin A contain an Arg-X-X-Arg sequence within a disulphide loop and both are cleaved by furin *in vitro* (Chiron *et al.*, 1994). Mutating the cleavage site so that it is no longer recognized by furin abolished cytotoxicity (Williams *et al.*, 1990; Ogata *et al.*, 1990). It has also been shown that cells lacking furin are resistant to both diphtheria toxin and *Pseudomonas* exotoxin A, but sensitivity can be restored by transfecting a cDNA encoding furin (Moehring *et al.*, 1993; Tsuneoka *et al.*, 1993).

Clearly, toxins in which the catalytic and cell-binding polypeptides are present initially in a single precursor require proteolytic processing by furin to release and thereby activate the catalytic A fragment. The Shiga toxins are somewhat different, however, in that the A subunit is not covalently attached to the cell-binding peptides of the B-subunit pentamer. Cells lacking furin remain sensitive to ST, although transfecting the furin gene increased both the rate of cleavage of endocytosed toxin and sensitivity to it (Garred *et al.*, 1995a). Further, abolishing the furin cleavage site by mutation in the disulphide loops of ST (Garred *et al.*, 1995b), SLT-1 (Burgess & Roberts, 1993) and SLT-2 (Samuel & Gordon, 1994) does not abolish cytotoxicity. However, proteolytic processing may still be essential and, if a second cleavage site remains available, this could account for the toxicity of the mutants analysed thus far. In the present study, it has been shown that eliminating the primary furin-recognition site of SLT-1 (mutant SLTP1) does not prevent intracellular cleavage (Fig. 3), confirming an earlier observation made in the case of ST (Garred *et al.*, 1995b). On the basis of these data, mutant SLTP4 was produced, in which an additional putative furin cleavage site (Arg₂₂₀-Val-Gly-Arg₂₂₃) was mutated. It should be noted that the alternative processing site reported for ST by Garred *et al.* (1995b) lay within the disulphide loop, since reduction was required to release the A₁-sized fragment from the processed A subunit. Processing at this alternative site appeared to occur in the cytosol since, in contrast to the wild-type protein, it could be prevented by treating the cells with a membrane-permeable inhibitor of the cytosolic enzyme calpain (Garred *et al.*, 1995b). It is possible that this ST mutant could have been cleaved at one of the Ala-Ser pairs, present in the disulphide loop, which had earlier been implicated as potential processing sites (Takao *et al.*, 1988). In mutant SLTP3 produced in the present study, both Ala-Ser pairs as well as the primary furin site within the disulphide loop had been removed. This suggested that the processing observed for SLTP3 (Fig. 3) occurred at a site outside the disulphide loop, a contention supported by the finding that processing of mutant SLTP4 was abolished by an additional mutation introduced into the sequence outside the loop. Although mutant SLTP4 did not appear to be processed during cell entry (Figs 3 and 4) even when endocytosed toxin was incubated with cells for 6 h, it did remain cytotoxic upon prolonged (6 h) incubation with cells, albeit ~60-fold less toxic than wild-type SLT-1 (Fig. 5). At present, it is

not clear why some form of proteolytic processing is necessary for optimal cytotoxicity. It is possible that the uncleaved A₂ moiety reduces the efficiency of A subunit membrane translocation into the cytosol, or somehow partially destabilizes it or enhances the rate of its intracellular degradation, effectively reducing the concentration of functional toxin in the cytosol. However, the most likely explanation for the need for processing for optimal activity comes from the X-ray crystallographic structure of ST (Fraser *et al.*, 1994), which provides a clear structural basis for this requirement. In the uncleaved A subunit, residues 258–262 of A₂ lie adjacent to the active-site cleft of A₁ and the side chain of Met₂₆₀ protrudes into the active site itself. This structural feature may explain why removal of the A₂ portion of ST/SLT, including residue 260, results in increased catalytic activity. In conclusion, while it is clear that a form of SLT-1 that does not undergo any observable proteolytic cleavage during cell entry (mutant SLTP4) remains cytotoxic, cleavage at either the preferred furin site or an adjacent site is required for SLT-1 to exhibit maximal toxicity towards mammalian cells.

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