RESEARCH ARTICLE

Different roles of the C-terminal end of Stx1A and Stx2A for AB₅ complex integrity and retrograde transport of Stx in HeLa cells

Linn Kymre¹,², Roger Simm¹,²,†, Tore Skotland¹,² and Kirsten Sandvig¹,²,³,*

¹Department of Molecular Cell Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, 0379 Oslo, Norway, ²Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, 0379 Oslo, Norway and ³Department of Biosciences, University of Oslo, 0316 Oslo, Norway

*Corresponding author: Department of Molecular Cell Biology, Institute for Cancer Research, Oslo University Hospital – The Norwegian Radium Hospital, 0379 Oslo, Norway. Tel: +47-22-78-18-28; Fax: +47-22-78-17-45; E-mail: ksandvig@ibv.uio.no
†Present address: Norwegian Veterinary Institute, Oslo, Norway

One sentence summary: A study on Stx1 and Stx2 complex integrity and retrograde transport after C-terminal truncations in the A-subunit.

ABSTRACT

Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) differ regarding receptor affinity, cellular toxicity and clinical outcome. To this date, it is not clarified in detail why the subtypes display these differences. Even though the crystal structures of Stx1 and Stx2 share overall similarities, significant differences were found in the C-terminal end of the A-subunits. The aim of this study was to investigate the role of the C-terminal end of the A-subunit in complex stability and retrograde transport by generating truncated mutants where 2, 4, 6 and 8 amino acids were removed from the C-terminal end of Stx1A and Stx2A. The results obtained show that removal of 6 or 8 amino acids from the Stx1A C-terminus abolishes the AB₅ complex integrity, while removing up to 8 amino acids from Stx2A does not affect the complex in vivo (in the bacteria). We also present results showing different levels of A₁-subunit in HeLa cells after exposure to Stx1, Stx2 and their truncated mutants.

Keywords: Shiga toxin; Stx1; Stx2; A-subunit; endocytosis; retrograde transport

INTRODUCTION

Shiga toxins (Stx) are produced by the enteric pathogens Shigella dysenteriae and the Shiga toxin producing Escherichia coli. These toxins are involved in the pathogenesis of foodborne outbreaks of gastroenteritis, and can contribute to endemic enteric infections (O’Loughlin and Robins-Browne 2001). The different Shiga toxin variants are associated with differences in severity of disease, including the likelihood of developing haemolytic uremic syndrome. The ability to specifically target certain cells, and the cytotoxic effects of Shiga toxins, can however be exploited for research purposes (Bergan et al. 2012; Sandvig et al. 2014). There are also potential uses of Shiga toxins in targeted cancer therapy, as the receptor, the neutral glycosphingolipid Gb₃, is known to be overexpressed in certain cancer types (Engedal et al. 2011). To this date, it is not fully understood how structural differences between the Shiga toxin variants relate to receptor binding, endocytosis and toxin activity. Thus, it is important to understand how the structure–function relationship for Shiga toxin affects binding to the receptor, and how structural changes in the toxin may affect the intracellular transport.

The Shiga toxins belong to a family of protein toxins with a characteristic AB₅ structure, where five identical B-subunits, each of them ∼7.7 kDa, form a pentameric ring structure assembled with one A-subunit of ∼32.2 kDa. The B-pentamer...
interacts with Gb3 at the cell surface, which initiates endocytosis, transport to the Golgi apparatus and retrograde transport of the toxin to the ER (Sandvig et al. 1992). A protein loop in the A-moiety containing the sequence motif Arg-X-X-Arg, recognized by the protease furin, is cleaved in an early step of the retrograde transport, possibly in endosomes and/or in the trans-Golgi network (Garred, van Deurs and Sandvig 1995). The resulting A1- and A2-subunits (∼27.5 and ∼4.5 kDa, respectively) are held together by a disulfide bond (Bergan et al. 2012) until the toxin reaches the ER, where the disulfide bond is reduced, and the A2-subunit released and translocated to the cytosol. In the cytosol, the A3-subunit inhibits protein synthesis by acting as a specific RNA N-glycosidase able to remove a particular adenine from the 28S rRNA (Endo et al. 1988). The translocation from the ER to the cytosol is likely to occur via the cellular ER-associated protein degradation pathway, which possibly recognizes the hydrophobic C-terminal end of the A1-chain as a misfolded peptide (LaPointe, Wei and Gariépy 2005).

The subgroups of the Shiga toxin family produced by E. coli are designated as Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Among the variants within the subgroups, Stx1a is most similar to Shiga toxin produced by S. dysenteriae, as they have identical B-moieties and differ by only 1 amino acid residue in the A-subunit (De Grandis et al. 1987; Bergan et al. 2012). In contrast, the amino acid sequence identity between the A-moieties of Stx1a and Stx2a is 56% (Takao et al. 1988). The N-glycosidase activity has previously been reported to be indistinguishable between Stx1 and Stx2 in a cell-free system (Head, Karmali and Lingwood 1991); however, more recent investigations suggest that the role of the A-subunits in ribosome binding and depurination activity may differ between Stx1 and Stx2 due to different requirements for ribosomal stalk proteins (Chiou et al. 2011; Basu and Tumer 2015). Fraser et al. (1994, 2004) determined the crystal structure for Stx1 and Stx2, showing that the overall structure is similar, although some differences are found. The amino acids 279–286 of the Stx1A-moiety form an α-helical secondary structure in the C-terminus where it penetrates the pore formed by the five B-subunits. The amino acid residues Ser279, Ser280 and Thr281 lie above the Stx1B-pentamer. The residues Leu282–Met287 form a hydrophobic helical segment; however, the last six amino acids, Arg288–Ser293, are unstructured and therefore not seen in the crystal structure of Stx1 (Fraser et al. 1994). For Stx2, Ser278–Leu285 are located in the pore in an α-helical structure. Ser289 initiates the final C-terminal α-helix, which projects out at the base of the pore at an angle of ∼30°. The final two amino acids, Gly296 and Lys297, are not part of the α-helix. They are, however, well structured and appear to pack against one tryptophan amino acid at a Gb3 low-affinity site, and this amino acid has been shown to be crucial for receptor binding at this site. All potential binding-sites to Gb3 appear to be accessible in the crystal structure of Stx1 (Fraser et al. 2004). In this context, it is interesting that experiments performed in our lab have shown that Stx1 stimulates its own rapid uptake in several cell lines (Torgersen, Lauvrak and Sandvig 2005). Treating cells with increasing concentrations of unlabelled Stx1 increased internalization of TAG- and biotin-labelled Stx1 by a factor of ∼2 in Baby Hamster Kidney cells, Vero, HEp-2 and HeLa cells during 10 min of incubation. There was no effect on Stx1 uptake by addition of Cholera toxin, and increased endocytosis of Stx1 was only seen at very high concentrations of Stx2. Most intriguing was perhaps the finding that adding the StxB-pentamer alone did not have any stimulatory effect, showing that the A-subunit was required for the self-induced uptake of Stx1 (Torgersen, Lauvrak and Sandvig 2005). In this regard, it is striking that in a study performed by Haddad and Jackson (1993), removing 4 amino acids from the C-terminus of the Stx1A resulted in a 100-fold reduction in cytotoxicity without affecting complex stability. In the same study, they found that removing the 8 most C-terminal amino acids in Stx1A abolished the AB2 complex integrity, suggesting that this part of the A-subunit is required for association between Stx1A and the B-pentamer (Haddad and Jackson 1993). There are clearly unresolved issues regarding the role of the A-subunit in binding and/or uptake of Shiga toxins and the role this might have in pathogenicity.

In this study, we truncated the C-terminal end of Stx1A and Stx2A and studied the effects it had on AB2 complex integrity as well as the impact on retrograde transport. In order to study the role of the StxA-subunit in intracellular transport, 2, 4, 6 and 8 amino acids were removed from the C-terminal end of the A-subunit of Stx1 and Stx2, generating truncation mutants referred to as Stx1m-(T2–T8) and Stx2m-(T2–T8). Figure 1 shows the crystal structure of Stx1 and Stx2, and illustrates the difference in the location of the amino acids removed when truncating Stx1 and Stx2 from the C-terminal end by the same number of amino acids.

**MATERIALS AND METHODS**

**Plasmids and constructs**

The plasmids pSW09 and pNR100 encoding genetic toxoids of Stx1 and Stx2, respectively, were kind gifts from Professor Alison O’Brien (Uniformed services, University of the Health Sciences, Bethesda, Maryland, USA). Stx1A carries point mutations changing the codons tyrosine in position 77 to serine (T77S) and glutamate in position 167 to glutamine (E167Q) in the active site, rendering it non-toxic. The corresponding mutations in Stx2 are Y77S and E167Q. These toxoids will be referred to as Stx1m and Stx2m.

**DNA manipulation**

The Stx1m and Stx2m genes were subcloned into the expression plasmid pSETA, resulting in plasmids pSM101b and pSM201, which were used to construct plasmids pSM103 and pSM203 encoding the His6-tagged variants HisStx1m and HisStx2m, respectively. pSM103 and pSM203 were in turn used as templates to construct the plasmids expressing the truncated mutants HisStx1m-(T2–T8) and HisStx2m-(T2–T8), respectively. Deletion and insertion mutagenesis by overlap extension polymerase chain reaction (PCR) were performed by standard molecular biology techniques (Ho et al. 1989; Sambrook and Russell 2006). A list of the bacterial strains and plasmids used in this study can be found in Table 1. All plasmids were sequenced to confirm the integrity of the intended inserts and mutations. The sequences of oligonucleotide primers used for PCR are listed in Table 2.

**Protein expression and periplasmic extraction**

All reagents were from Sigma-Aldrich unless otherwise stated. Approximately 5 mL LB medium supplemented with 100 μg mL⁻¹ ampicillin (LB (amp)) was inoculated with BL21 (DE3) carrying the relevant plasmid, and incubated at 37°C, 220 rpm for 7–8 h. The bacteria were harvested at 500 g for 10 min. The pellet was resuspended in a hypertonic buffer consisting of 20% sucrose (w/v), 1 mM EDTA (Merck Millipore) and 20 mM Tris.
Figure 1. Structural features of Stx1, Stx2 and their truncated mutants. The A-subunit is shown in pink, and the B-pentamer is shown in yellow. The first amino acid of the region of the A-subunit that extends through the pore formed by the B-pentamer is indicated in green. The residues removed from the C-terminus of the A-subunit in the truncated mutants are indicated in different colours. (a) Crystal structure of Stx1, where the amino acids L286 and M287 are indicated in red. The last six amino acids of the C-terminal end of Stx1A are unstructured and missing from the crystal structure. (b) Alignment of the C-terminal primary structures of Stx1m and the truncated mutants, showing the amino acids S292 and S293 in blue, T290 and I291 in turquoise, R288 and R289 in orange and Q290 and F291 in red. (c) Crystal structure of Stx2, where the amino acids G296 and K297 are indicated in blue, T294 and T295 in turquoise, L292 and Y293 in orange and Q290 and F291 in red. (d) Alignment of the C-terminal primary structures of Stx2m and the truncated mutants. The colours of the amino acids highlighted in the crystal structures correspond to the same colours indicated in the primary structures of Stx1A and Stx2A. It should be noted that the protein sequence of Stx2A is longer than the protein sequence of Stx1A.

According to the crystal structure, the C-terminal end of Stx2A extends well through the pore and ends below the base of the B-pentamer, in contrast to Stx1A.

(pH 8.0). The resulting suspension was incubated for 20 min at room temperature, and the bacteria were pelleted by centrifugation at 5000 g for 10 min. The bacteria were carefully resuspended in ice-cold deionized water with Complete Protease Inhibitors (Roche Diagnostics), and incubated on ice for 10 min, before centrifugation at 13 000 g for 10 min, producing the periplasmic extract in the supernatant.

Detection of Stx1 and Stx2

The primary mouse anti-Stx1-antibody 3C10 and Stx2-antibody BB12 (both Toxin Technology) and mouse anti-His (Life Technologies) were diluted as recommended by the manufacturer. The secondary antibody used was donkey anti-mouse IRDye® 680RD (1:10 000, Li-Cor). The Odyssey® imager was used to scan the membranes at 700 nm, and the images were processed using the Odyssey® software (Li-Cor). According to the manufacturer, the Stx1-specific antibody 3C10 may detect both the A- and B-subunits in western blots; however, in our experiments it only detected the B-subunit. The need to detect the A-subunit motivated the cloning process, introducing a His₆-tag between the signal peptide and the first amino acid of the mature Stx constructs. The tagged proteins can be detected by immunoblotting using an anti-histidine antibody. To confirm that we were able to detect the A-chain of HisStx1m, the His-antibody was tested on a western blot with purified Stx1m in two dilutions. In the same experiment, the Stx1B-specific antibody 13C4, and the antibody 3C10 (Toxin Technology) presumably able to detect Stx1A, was tested. As demonstrated, the His₆-tag on the A-chain was detected by the His-antibody. None of the other antibodies detected the HisStx1m A-subunit (Fig. 2).

Protein precipitation from periplasmic and cytosolic fractions

For these experiments, cultures of the bacteria expressing the different protein variants were adjusted to the same optical density (OD). Periplasmic extracts (10 mL) were prepared by the method previously described, from 200 mL of the adjusted bacterial cultures. The remaining pellets were used to prepare cytoplasmic fractions. The pellets were suspended in 10 mL 20 mM Tris (pH 7.5) and the cells lysed by sonication (30 s × 6 repetitions) while kept on ice. The bacterial cell lysates were centrifuged to separate the cytoplasmic fraction (supernatant) from
the pelleted cellular debris. One millilitre aliquots of the cytoplasmic and periplasmic extracts were used to analyse the presence of Stx1m and Stx2m protein complexes. The HisStx1m constructs were immunoprecipitated via the B-subunit by using mouse anti-Stx1 antibody 3Cl0 immobilized on Protein A Sepharose (GE Healthcare). Ni-NTA agarose (QIAGEN) with affinity for the His6-tag was used to precipitate the HisStx1m and HisStx2m constructs via the A-subunit. The Protein A Sepharose and Ni-NTA agarose precipitates were washed with 0.35% (w/v) Triton X-100 in PBS and 20 mM imidazole in 20 mM Tris (pH 8.0), respectively. Proteins were released by boiling in 2 X SDS sample buffer (Bio-Rad Laboratories Inc) and separated by reducing SDS-PAGE on gradient gels (4-20%), and the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories Inc) was used to transfer the proteins to an Immobilon-FL polyvinylidene fluoride (PVDF) membrane.

**Protein purification**

The constructs that assembled into AB4 complexes were purified from the periplasm of BL21 (DE3). Periplasmic fractions were prepared as described, and the His6-tag was exploited for protein purification by affinity chromatography using Ni-NTA agarose. The periplasmic extracts were incubated with Ni-NTA agarose beads (QIAGEN) for 1 h (room temperature). Endogenous proteins were removed by washing with 20 mM Tris, 20 mM imidazole (pH 8.0) before eluting the proteins with 20 mM Tris, 500 mM imidazole (pH 8.0). Fractions containing Stx protein were pooled and the buffer was changed by ultrafiltration using Amicon Ultra centrifugal filters (Merck Millipore) with molecular weight cut-off of 3 kDa. The purity of the Stx protein preparations was confirmed by running samples on SDS-PAGE 4-20% polyacrylamide gel (Bio-Rad Laboratories Inc.), followed by Brilliant Blue G Coomassie staining. Only two bands corresponding in size to the A- and B-subunits of Stx were visible (data not shown). The protein concentration was determined using the Qubit® Protein Assay Kit containing pre-diluted BSA standards and measured on the Qubit® Fluorometer (Invitrogen).

**125I-labelling of Stx**

The IODO-GEN Iodination Reagent (Fierce Biotechnology) was used for 125I-labelling of the Stx constructs according to the manufacturer’s protocol using Na125I from PerkinElmer. The efficiency of iodine incorporation varied from construct to construct; the final concentrations of labelled protein used for experiments varied from 95 to 110 ng ml−1 for Stx1m constructs, and from 65 to 89 ng ml−1 for Stx2m constructs. For all constructs, more than 95% of the radioactivity was protein associated.

**Monitoring the integrity of 125I-labelled HisStx1m and HisStx2m constructs**

The 125I-labelling of the truncated mutants allowed us to study the complex stability in more detail in vitro. It was discovered that only freshly labelled complexes of truncated HisStx1m constructs could be used to test interaction with cells, as storage of the protein aliquots in the refrigerator or freezer over a time period of 2 weeks resulted in degradation of the truncated A-chain. This was visible when a sample from the protein batch was analysed by non-reducing SDS-PAGE. This did not occur to the same extent for 125I-HisStx1m with a full-length A-subunit (data not shown). Thus, to monitor the integrity of the A-subunit of 125I-labelled HisStx1m constructs, a sample was taken from the protein aliquot used for cell experiments and run on reducing and non-reducing SDS-PAGE the same day as the experiment was performed. Only results from experiments using truncated 125I-HisStx1m with essentially no degradation product are included in this paper. Similar problems were not observed for the 125I-labelled HisStx2m constructs.

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**Table 1. Bacterial strains and plasmids used in this study.**

| Bacterial strain | Relevant genotype or description | Source |
|------------------|--------------------------------|--------|
| E. coli DH10B    | F- endA1 recA1 galE15 galK16 mupG rpsL ΔlacX74 ΔPhi80lacZΔM15 araD139 Δ araLeu7697 mcrA λ(mrr-hsdRMS-mcrBC λ) | Life Technologies |
| E. coli BL21 (DE3) | F- ompT gal dcm lon hsdS(r− m80−) λ (DE3 lacIacUV5-T7 gene 1 ind1 sam7 nin5)) | Life Technologies |

| Plasmid | Relevant genotype or description | Protein | Source |
|---------|---------------------------------|---------|--------|
| pRSETA | Expression vector, T7-promoter, Amp | - | Invitrogen |
| pSM101b | pRSETA::Stx1A(T77S;E167Q)Stx1B | Stx1m | This study |
| pSM103 | pRSETA::His6-stx1A(T77S;E167Q)Stx1B | HisStx1m | This study |
| pSM103-T2 | pSM103ΔT2 | HisStx1m-T2 | This study |
| pSM103-T4 | pSM103ΔT4* | HisStx1m-T4 | This study |
| pSM103-T6 | pSM103ΔT6* | HisStx1m-T6 | This study |
| pSM103-T8 | pSM103ΔT8* | HisStx1m-T8 | This study |
| pSM203 | pRSETA::His6-stx2A(T77S;E167Q)Stx2B | Stx2m | This study |
| pSM203-T2 | pSM203ΔT2 | HisStx2m | This study |
| pSM203-T4 | pSM203ΔT4* | HisStx2m-T4 | This study |
| pSM203-T6 | pSM203ΔT6* | HisStx2m-T6 | This study |
| pSM203-T8 | pSM203ΔT8* | HisStx2m-T8 | This study |

*T2–T8 signify the codons encoding the most C-terminal amino acids, T2 meaning the last two codons and T4 the last four codons, etc. Consequently, ΔT2 denotes a deletion of the last two codons, ΔT4 a deletion of the last four codons, etc. *His- indicates that a nucleotide sequence encoding a His6-tag and three sequential restriction sites (pSM103: BamHI, XhoI and PstI, pSM203: BamHI, XhoI and KpnI) was inserted between the codons encoding the signal peptide and the first amino acid of the mature protein (i.e. after the first 66 nucleotides of the gene).
Table 2. Primers.

| Primer name          | Sequence                      |
|----------------------|-------------------------------|
| Stx1-pRSET-F         | ACTCCATATGAAAAAATAATTATTTTAGAGTG |
| pRSET SP-F           | AAGTGAGCTCTTCAAGGAAAAATACTCAGCTG |
| Stx1 Intro-His-F     | AAAGGGATCCCTCGAGTCGAGAAGATTACCTATAGTCAGCTC |
| Stx1 Intro-His-R     | CTTCAGCTCGGAGACTCCCTGCCACCACTTTAGGAAAG |
| Stx1A-313F           | GCATCATCACCATCACCAGATCTCCCTGAGCTCAG |
| Stx1A-313R           | CGTGGTAGATGTATCTGCTCCACCCACCATTAAGCTG |
| Stx1A-311F           | ACTATTGTAAAGGTAAATGAAAAAAATGCCAAGAGGCCTG |
| Stx1A-311R           | CATTTACCTCCTCATAAGTTCTCGGACTAGAATTTG |
| Stx1A-309F           | CAGATGGGAAATGAAAAAACGGAGCTG |
| Stx1A-309R           | CATTTATCCCTCCTCAGAATTGCCGCCAGAGAAC |
| Stx1A-307F           | TACGACTCAGATAGAGGGAGCTG |
| Stx1A-307R           | ACCACGGATCCCTCGAGTACCGAGTTGCTAGAGCTTTTCG |
| Stx2-pRSET-F-NdeI    | GTTATTGCTGAGCGGTG |
| Stx2 pRSETA-F        | CATTTTACGTTGTATATAAAAACTGTGAC |
| Stx2 Intro-His-R     | CAGTTTTTATATTAAAGGAGTTAAGCATGAAAG |
| Stx2 Truncation2 F   | TACGACTCACTATAGGA |
| Stx2 Truncation4 F   | ACCACGGATCCCTCGAGGTTACCGAGTTAGATAGACTTTTCG |
| Stx2 Truncation4 R   | GATCCGTGGTGATGGTGATGATGCCGGGAATAGGATACCGAAGAAAA |
| Stx2 Truncation6 F   | TACGACTCACTATAGGA |
| Stx2 Truncation6 R   | CAGTTTTTATATTAAAGGAGTTAAGCATGAAAG |
| Stx2 Truncation8 F   | TACGACTCACTATAGGA |
| Stx2 overlap ext. F  | GATCCGTGGTGATGGTGATGATGCCGGGAATAGGATACCGAAGAAAA |
| Stx2 overlap ext. R  | CAGTTTTTATATTAAAGGAGTTAAGCATGAAAG |

Figure 2. Western blot of purified HisStx1m, detected with antibodies 13C4, 3C10 and His-antibody. Purified HisStx1m was run on an SDS-PAGE in two dilutions, applying ~700 ng (1:1 dilution, left lane) and ~350 ng (1:2, right lane) toxin to the gel. The expected location of bands for the A- and B-subunits is indicated with arrows.

RESULTS AND DISCUSSION

Truncation of the C-terminal end of the A-subunit affects the integrity of the AB5 complex of Stx1 but not Stx2

We first investigated the AB5 complex integrity of the truncated Stx1m constructs. Since none of the commercially available antibodies tested was specific for the StxA1 subunit, we added His-tags to the N-terminus of the mature A-subunits of all Stx1m and Stx2m constructs. This allowed us to specifically detect the A-subunit of all constructs with a single antibody and enabled A-subunit-mediated precipitation of the complexes.
The resulting constructs HisStx1m, HisStx1m-T2, HisStx1m-T4, HisStx1m-T6, HisStx1m-T8 (collectively referred to as HisStx1m-(T2–T8)), and HisStx2m, HisStx2m-T2, HisStx2m-T4, HisStx2m-T6, HisStx2m-T8 (collectively referred to as HisStx2m-(T2–T8)) were used throughout this study. An antibody specific for the Stx1 B-subunit was used to immunoprecipitate the potential HisStx1m-(T2–T8) complexes from periplasmic and cytoplasmic fractions. It should be noted that the signal sequence is cleaved during export from the cytosol to the periplasm, and the mature toxin is therefore found in this location. The signal sequence consists of 22 amino acids, and the cleavage upon translocation accounts for the difference in size (~2 kDa) sometimes observed between the cytosolic and periplasmic fractions (Fig. 3).

By using the His-antibody in western blot analysis, the A-subunits that coprecipitated with the B-subunits in the immunoprecipitation experiments were detected. Thus, we could determine which of the truncated mutants that were able to form stable AB₅ complexes (Fig. 3a). The result of this experiment showed that in the periplasmic fraction HisStx1m-T2 and HisStx1m-T4 (will be referred to as HisStx1m-(T2–T4)) occurred as complexes, whereas HisStx1m-T6 and HisStx1m-T8 did not. The B-subunit of HisStx1m-T6 and HisStx1m-T8 was clearly expressed; however, the A-subunit was not associated with the B-subunits in the periplasmic fraction to a significant extent. In the cytosol, some stable HisStx1m-T6 complexes occurred, whereas very little or no stable complexes were detected for HisStx1m-T8. Since the results indicated that the A- and B-subunits of HisStx1m-T6 were to some extent able to associate, purification of this construct was attempted. We were however unable to purify HisStx1m-T6 as a stable AB₅ complex. Occurrence of stable complexes of the Stx1m truncation mutants was also investigated by precipitation using Ni-NTA agarose with affinity for the His₉ tag on the A-subunit (data not shown). The B-subunits that coprecipitated with the A-subunit were detected by western blotting using an Stx1 B-subunit-specific antibody, in addition to detecting the A-subunit using the His-antibody. In agreement with the results shown in Fig. 3a, the B-subunits of HisStx1m-(T2–T4) were detected, showing that these constructs assembled into stable complexes. A weak band was detected for the B-subunits of HisStx1m-T6, confirming that only a small fraction of the A-subunit was associated with B-subunits. This is probably due to a highly unstable AB₅ complex, shifting the equilibrium towards dissociation of the subunits. There were no B-subunits detected that coprecipitated with the A-subunit for HisStx1m-T8. Thus, the results of the Ni-NTA precipitations via the A-subunit were consistent with the results obtained by the immunoprecipitation via the B-subunits. The results demonstrate that the A-subunit was in principal not associated with the B-subunits in a stable manner after removing 6 and 8 amino acids from the C-terminal end of the A-chain of HisStx1m.

We then investigated the occurrence of AB₅ complexes of HisStx2m after truncation of the A-subunit. This was done by Ni-NTA precipitation of the A-subunit, followed by detection of the A- and B-subunits by western blotting using the His-antibody and an Stx2B-specific antibody, respectively (Fig. 3b). The result shows that the B-subunits were detected for all constructs, indicating that truncation of the C-terminal end of Stx2A by up to 8 amino acids did not affect the occurrence of stable complexes in the periplasm and/or cytoplasm. In this context, it is important to notice that Stx2A penetrates the whole pore and ends below the base of the B-pentamer, in contrast to Stx1A (see Fig. 1).

Concerning Stx1, our data are in agreement with the previous study (Haddad and Jackson 1993), and importantly provide further information about the amino acids required for complex integrity. In the study performed by Haddad and Jackson, site-directed mutagenesis was performed to introduce amber termination codons that shortened the Stx1A polypeptide from the C-terminus when expressed by MC4100. The authors concluded that deletion of the amino acids Met287–Ser293, removing 7 amino acids from the C-terminus of the A-chain, completely abolished AB₅-complex assembly of Stx1. This was based on reduced detection of the A-subunit in a receptor analogue enzyme linked immunosorbent assay (ELISA), and absence of
the A-subunit in autoradiographs following immunoprecipitation of radioactively labelled Stx1 via a B-subunit-specific antibody. Haddad and Jackson (1993) also found that the four most C-terminal amino acids were not required for subunit association. The data presented here confirm that assembly of the Stx1 AB₅ complex was possible for the mutants truncated by 2 or 4 amino acids in the C-terminus of the A-subunit, while a truncation of 6 and 8 amino acids reduced the occurrence of stable AB₅ complexes. Furthermore, the limit for how many of the C-terminal amino acids that can be removed from the A-subunit without impairing occurrence of stable Stx1 AB₅ complexes was more precisely determined to be 4 or 5. The fifth and sixth amino acids (counted from the C-terminus) are both arginines (Arg288 and Arg289), which are positively charged residues that according to the crystal structure of Stx1 are located approximately in the centre of the central pore of the B-subunit complex (Fraser et al. 1994).

Considering the importance of these positively charged amino acids for complex integrity, one might suspect that they could interact with negatively charged amino acids in the pore of the Stx1 B-subunit complex, and that these interactions would stabilize the AB₅ complex by electrostatic interactions. Figure 4a and b show where all negatively and positively charged amino acids are located in the crystal structure of the B-chains of Stx1 and Stx2, respectively, with the positively charged amino acids labelled blue and negatively charged amino acids labelled red. As demonstrated in Fig. 4a, there are no negatively charged amino acids in the central pore of the Stx1 B-subunit complex. In addition, Haddad and Jackson (1993) showed that arginine to glycine substitutions, R288G and R289G, caused a 10-fold reduction in cytotoxicity without having any effect on receptor binding and subsequent detection of the A-subunit in the ELISA setup they used to analyse complex integrity. Taken together, this indicated that the charge of the amino acids R288 and R289 is not essential for the stability of the AB₅ complex. Instead it appears that the length of the C-terminal tail of the A-subunit penetrating the pore of the B-subunit-pentamer determines the stability of the AB₅ complex.

It is interesting that the Stx1 truncated mutants appeared to be more sensitive to degradation in vitro, as the A-subunits of 125I-HisStx1m-(T2–T4) were cleaved and the amount was reduced upon storage over time. Why this is the case is not clear, but it is possible that conformational changes in the truncated mutants expose protease sensitive regions of the Stx1 A-chain and/or that A-subunits that dissociate from the AB₅ complexes may aggregate and/or be exposed to degradation. This is supported by a study performed by Kim et al., where it was shown that free Stx A-subunit could not exist in the periplasm and was more susceptible to periplasmic proteases in vitro. The authors suggested that the Stx B-pentamer is important for the stability of the A-subunit in the periplasm (Kim et al. 2011). The data shown here indicate that the C-terminal end of Stx1A is important for complex integrity in vivo, as well as for toxin stability in vitro over time.

Occurrence of stable complexes following C-terminal truncation of Stxa2 has to our knowledge not been previously investigated. The results presented here show that a C-terminal truncation of Stx2A by 8 amino acids did not affect the occurrence of stable AB₅ complexes, which is in contrast to the effects observed for the corresponding truncation of Stx1A. This could be explained by the apparent difference in the location of the amino acids removed, as suggested by the crystal structures (Fig. 1).

**Nicking of the Stx1 A-subunit is slightly reduced in the truncated constructs compared to the full-length protein**

After establishing which of the truncated Stx1 and Stx2 constructs that could form stable complexes, the intracellular transport was investigated. In order to investigate cell association and cellular nicking of the A-subunits into A₁ and A₂ fragments, HeLa cells were incubated with equal amounts of the 125I-labelled constructs for 5 h at 37°C. The cells were lysed and samples were detected by radiography following SDS-PAGE under reducing conditions (Fig. 5a and b). As shown in Fig. 5a–d, all constructs were efficiently cleaved into A₁ and A₂ fragments. The intensity of the bands corresponding to the A- and A₁-subunits of 125I-HisStx1m-(T2–T4) and 125I-HisStx2m-(T2–T8) was quantified and the amount of nicked A was determined by calculating the A₁ signal as a percentage of the total value for the A + A₂ signals. The values were normalized to the corresponding value for 125I-HisStx1m or 125I-HisStx2m. There was no significant difference in the total amount of cell-associated A-subunit in cells incubated with any of the truncated mutants compared to their respective full-length proteins (Fig. 5e and f). Binding and uptake in cells at earlier time points was also tested, giving similar results (data not shown). Based on crystallography, the C-terminus of Stx2 seems to pack against an amino acid residue in one of the receptor-binding sites (Fraser et al. 2004). Thus,
Figure 5. Detection of the A₁-subunit in HeLa cells following exposure of the cells to HisStx1m, HisStx2m or their truncated mutants. HisStx1m is shown in the left column and HisStx2m in the right column. HeLa cells were incubated with equal amounts of ¹²⁵I-labelled constructs for 5 h. The total amount of StxA₁ and the amount of free A₁ in the cells were determined by SDS-PAGE and radiography under reducing (+DTT; a, b) and non-reducing (−DTT; c, d) conditions, respectively. The intensity of the bands was quantified and the relative amount of A₁-subunit was calculated against the total cell-associated A-subunit (A + A₁). The signals from the truncated mutants were normalized to the corresponding ¹²⁵I-HisStx1m or ¹²⁵I-HisStx2m signals. Quantification of the total amount of A-subunit associated with the cells for Stx1m (e) and Stx2m (f). Quantification of the relative amount of A₁-subunit against total amount of A-subunit associated with the cells for Stx1m-(T2–T4) (g) and Stx2m-(T2–T8) (h) normalized against the relative amount A₁ in the controls; Stx1m and Stx2m, respectively. Relative amount of free A₁ compared to total amount of A-subunit associated with the cells for Stx1m-(T2–T4) (i) and Stx2m-(T2–T8) (j) normalized against the relative amount A₁ in the controls; Stx1m and Stx2m, respectively. The data represent the results from three independent experiments, and the error bars indicate the standard deviation; * indicates values that were significantly different from the control P < 0.05; two-tailed unpaired Students t-test.
since the C-terminus of Stx2A might obstruct one of the 15 available binding sites on the B-pentamer surface, one could speculate that removing these amino acids from Stx2A could possibly increase Stx2 binding to the Gβ3 receptor. However, the results presented show that no large effect, neither increase nor decrease, was observed on cell-associated Stx2A after removing the amino acids predicted to occlude one of the receptor-binding sites. Although most of the toxin added was nicked by the cells, there was a small reduction (10%–20%) in the amount of nicked A-subunit found in cells incubated with the truncated HisStx1m constructs. In contrast, no large difference was seen in A-subunit nicking in cells incubated with the HisStx2m constructs (Fig. 5g and h).

In conclusion, the binding and nicking of the A-subunit from 125I-HisStx1m-(T2–T4) and 125I-HisStx2m-(T2–T8) appear to be relatively similar to the respective full-length constructs, indicating only minor effects of the A-subunit truncations on the early steps of the retrograde transport.

**Less free A1 is found in cells incubated with truncated Stx1 and Stx2**

To study the amount of free A1 present in the cells after reduction of the inter-A1–A2-subunit disulphide bond in the endoplasmic reticulum, cells incubated with 125I-labelled constructs were lysed in sample buffer without DTT after 5 h incubation with the different constructs. The samples were analysed by non-reducing SDS-PAGE and radiography (Fig. 5c and d). The amount of free A1 was calculated as a percentage of the total value of the A + A1 signals, and normalized to the corresponding 125I-HisStx1m or 125I-HisStx2m signal. The level of free A1 found in cells incubated with 125I-HisStx1m-T2 and 125I-HisStx1m-T4 was reduced by approximately 50% (Fig. 5f). For 125I-HisStx2m-T2, on the other hand, there was a small increase in A1 release whereas there was a small reduction of –20% for 125I-HisStx2m-(T4–T8) (Fig. 5j). It has been suggested that optimal binding of Shiga toxin to the cell surface requires binding of Gβ3 to all 15 potential binding-sites of an AB5 complex. It is therefore possible that the small increase in A1 release observed for Stx2m-T2 is due to removal of the amino acids obstructing one of the 15 potential binding-sites of Stx2, whereas further truncations reduced the complex integrity in the HeLa cells, thereby causing the reduction in A-subunit processing. Overall, we found a relatively large reduction in the amount of free A1 in the cells treated with the truncated 125I-HisStx1m constructs in contrast to a small reduction of free A1 in cells treated with the 125I-HisStx2m-(T4–T8) truncated constructs. Since nicking of 125I-HisStx1m-(T2–T4) was reduced by 10–20% compared to that of 125I-HisStx1m, while the reduction in amount of free A1 was reduced by 50%, it appears that the truncations mainly affect the transport/processing of the truncated A-subunit between the early endosomes/trans-Golgi network and the ER. We speculate that the apparent reduction in stability of the truncated constructs might affect the endosomal sorting of Stx, resulting in decreased retrograde transport and release of the A1-subunit. Another possibility is that even though the truncated Stx1A-subunit may be transported to the ER to the same extent as Stx1A, it is less efficiently reduced or more efficiently degraded than the wild type. In this context, it is interesting that it has been shown that there is a strong reduction in cytotoxicity for an Stx1 mutant with an A-subunit truncated by 4 amino acids in the C-terminus (Haddad and Jackson 1993). This reduction in cytotoxicity and the data reported here demonstrate a role for the C-terminal end of the A-subunit in the stability and/or activity of Stx1 and Stx2 in eukaryotic cells.

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