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# Correlation between Shiga toxin B-subunit stability and antigen crosspresentation: A mutational analysis

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**Abstract The homopentameric B-subunit of Shiga toxin (STxB) is used as a tool to deliver antigenic peptides and proteins to the cytosolic compartment of dendritic cells (DCs). In this study, a series of interface mutants of STxB has been constructed. All mutants retained their overall conformation, while a loss in thermal stability was observed. This effect was even more pronounced in trifluoroethanol solutions that mimic the membrane environment. Despite this, all mutants were equally efficient at delivering a model antigenic protein into the MHC class I-restricted antigen presentation pathway of mouse DCs, suggesting that the structural stability of STxB is not a key factor in the membrane translocation process.**


*Keywords:* Shiga toxin B-subunit; Retrograde transport; Membrane translocation; Crosspresentation; Antigen presenting cell

## 1. Introduction

Like verotoxins and cholera toxin, Shiga toxin belongs to the $AB_5$ family of bacterial toxins [1]. Structurally, these toxins consist of two non-covalently associated subunits, a monomeric catalytic A-subunit, and a pentameric B-subunit that is responsible for receptor binding and intracellular trafficking of the holotoxins [2,3]. In the case of Shiga toxin, the A-subunit has ribosomal RNA *N*-glycosidase activity, leading to protein biosynthesis inhibition, while the B-subunit (STxB) binds specifically to the glycosphingolipid $Gb_3$ [4]. The crystal structures of STxB and holotoxin have been solved [5–7]. STxB is a homopentamer composed of monomers of 69 residues (7.7 kDa). Each monomer has two three-stranded antiparallel β-sheets and an α-helix (see Fig. 1). The pentamer forms a ring-like structure with a central pore whose diameter is about 11 Å, and that is delimited by the five α-helices and surrounded by β-sheets from pairs of adjacent monomers. Recent structural and mutational studies suggest that STxB has three $Gb_3$ binding sites per monomer [8,9].

The toxin–receptor complex is internalized via clathrin-dependent and -independent endocytosis, and the toxin is then transported from the early endosome to the endoplasmic reticulum (ER), via the *trans*-Golgi network and Golgi apparatus, a pathway known as the retrograde route [10,11]. Transport to the ER is believed to be required for translocation of the A-subunit to the cytosol where its molecular target – ribosomal RNA – resides [12]. STxB has been used as a tool for the characterization of the retrograde route [13,14], and in biomedical research as a delivery tool for immunotherapy [3,15] and cancer targeting [16,17]. It was shown that STxB delivers exogenous peptides into the MHC class I and II pathways of human and mouse dendritic cells (DCs) and induces humoral and cell-mediated immune responses that protect mice from tumor growth [18–22]. Functional TAP and proteasome activities are required for STxB-induced MHC class I-restricted antigen presentation, strongly suggesting that STxB delivers exogenous antigenic proteins and peptides across membranes into the cytosolic compartment [19]. The mechanism and exact intracellular site of this membrane translocation event have not yet been identified, but intracellular trafficking studies on human monocyte-derived DCs suggested that the retrograde route might not be involved [23].

The interaction of STxB with membranes has been analyzed in some detail. The two-dimensional structure of STxB on $Gb_3$-containing bilayer stacks overlaps perfectly with the projection map of its solution structure [24], and the binding of soluble $Gb_3$ analogues does not induce structural changes at the atomic level of resolution [8]. In agreement with these data, no significant conformational changes occurred upon interaction of STxB with large unilamellar vesicles [25]. In the latter report, a fluorescence technique allowed us to monitor the conformational state of STxB due to the presence of tryptophan residues at the protein/membrane interface. However, when trifluoroethanol (TFE) was used to mimic a hydrophobic environment similar to that of membranes, a

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*Abbreviations:* STxB, Shiga toxin B-subunit; CD, circular dichroism; DC, dendritic cell; ER, endoplasmic reticulum; TFE, trifluoroethanol

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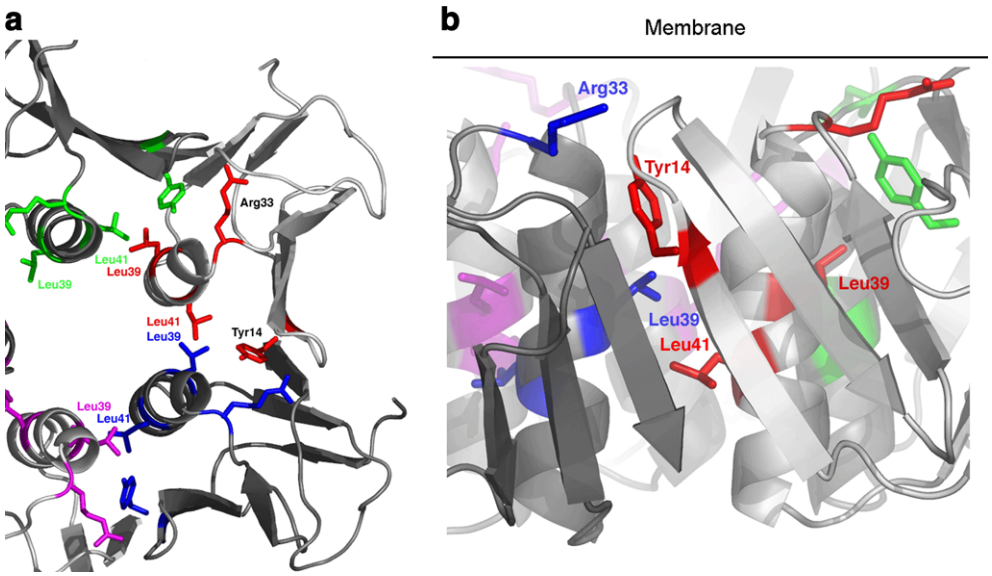


Fig. 1. Location of mutated residues in the three-dimensional structure of STxB.Structural elements shown in light grey or dark grey correspond to different monomers. Residues 39 and 41 are located in the α-helix, whereas residue 14 is in the β2 strand, interacting with the β6 strand of the adjacent monomer. Residue 33 is at the protein–membrane interface.

monomeric intermediate was observed [26], which could not be detected when STxB was analyzed in the absence of solvent [27].

Here, a series of single-residue mutants at the interface between STxB monomers was constructed. We demonstrate that STxB pentamer stability is compromised to variable extents in these mutants, and in some cases, this effect is particularly pronounced in membrane-mimicking conditions. However, translocation to the cytosol does not appear to be favored, as assessed by MHC class I-restricted antigen presentation.

## 2. Materials and methods

### 2.1. Cells and materials

HeLa cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose (Life Technologies Inc.) supplemented with 10% fetal calf serum (Life Technologies Inc.), 0.01% penicillin/streptomycin, 4 mM glutamine, and 5 mM pyruvate in a 5% $CO_2$ incubator. D1 cells were cultured in Iscove's Modified Dulbecco's Medium (Sigma), supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5 mM sodium pyruvate (all Invitrogen), and 30% conditioned medium from GM-CSF-producing NIH-3T3 cells, as previously described [28].

### 2.2. Plasmid construction

The pSU108 plasmid was used for the construction of STxB mutants, as described [29]. Primers encoding the desired point mutations and specific primers Shiga-AtpE and Shiga-fd were used to produce fragments that were cloned into the SphI and SalI restriction sites of pSU108. For the construction of $OVA_{257–264}$ fusion proteins, the same strategy was used, starting with a plasmid encoding wild-type STxB fused to $OVA_{257–264}$ peptide including three amino-terminal flanking amino acids (QLESIINFEKL). Sequences derived by polymerase chain reaction were verified by dideoxy sequencing.

### 2.3. Expression, purification, and size-exclusion chromatography of recombinant STxB and mutants

Purification of recombinant STxB was essentially done as described elsewhere [30]. After preparation of periplasmic extracts, these were loaded on a QFF column (Pharmacia) and eluted by a linear NaCl gradient in 20 mM Tris/HCl, pH 7.5. Depending on the construction, recombinant STxB eluted between 120 and 400 mM. STxB-containing fractions were dialyzed against 20 mM Tris/HCl, pH 7.5, reloaded on a Mono Q column (Pharmacia), and eluted as before. The resulting proteins, estimated to be 95% pure by SDS–PAGE, were stored at −80 °C until use. Protein concentration was estimated by spectrophotometry using an extinction coefficient of 9100 $M^{-1}$ for STxB wild-type and R33A, 8700 for Y14A, and 14500 for L39W and L41W. For size-exclusion chromatography experiments, samples were loaded on a 30 ml Sephadex 75-column (GE Healthcare).

### 2.4. Circular dichroism spectroscopy

Far-UV (190–250 nm) circular dichroism (CD) spectra were recorded with a Jasco J-715 spectropolarimeter coupled to a Peltier thermal control unit. Spectra are the average of five accumulations that all were background corrected and smoothed using the noise reduction software of the instrument. Data were converted to molar ellipticity per mean residue: $[\Theta] = M_{res}\Theta_{obs}l^{-1}p^{-1}$, where $M_{res} = 111.5$ is the mean residue molar mass, $\Theta_{obs}$ the measured ellipticity in millidegrees, $l$ the optical path length of the cell in millimetres, and $p$ is the protein concentration (mg/ml). Experiments were performed in a 1-mm pathlength cuvette and sample concentration of about 0.2 mg/ml. Thermal stability curves were collected at the fixed wavelength of 225 nm, and between 40 and 90 °C, with a constant heating rate of 1 °C/min.

### 2.5. Immunofluorescence

Immunofluorescence was performed as previously described [11]. Briefly, cells were fixed at room temperature for 10 min in 3% paraformaldehyde, quenched with ammonium chloride, permeabilized with 0.05% saponin, incubated with the indicated primary or secondary antibodies, mounted, and viewed by confocal microscopy (Leica Microsystems, Mannheim, Germany).

### 2.6. Scatchard analysis

$1 \times 10^5$ cells were placed on ice and washed once with ice-cold PBS. The indicated concentrations of recombinant iodinated wild-type or mutant STxB (5.000 cpm/ng; concentrations from 0.05 to 2 μM) were added in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. After 90 min incubation on ice in presence of 10 mM HEPES, pH 7.2, the cells were washed three times with ice-cold PBS. Cells were lysed in 0.1 M KOH, cell-associated radioactivity and radioactivity in the culture medium and wash solutions were counted, and binding data were obtained as published [29].