

The Intracellular Journey of Shiga Toxins

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Abstract: The Shiga toxin family consists of Shiga toxin (Stx) that is produced as a virulence factor by *Shigella dysenteriae*, and the Shiga-like toxins produced by certain strains of enterohemorrhagic *E. coli* as well as by some other types of bacteria. Infection with bacteria producing these toxins is a threat to human health even in industrialized countries, as the initial diarrhea caused by the infection might be followed by a complication named hemolytic uremic syndrome. The Shiga toxins consist of a binding moiety that in most cases binds to the glycosphingolipid Gb3 on the surface of susceptible cells, and an A-moiety responsible for the toxic effect in the cytosol. In order to reach its cytosolic target, the toxin must be internalized and then transported via the retrograde pathway to the Golgi complex and further to the endoplasmic reticulum. From the endoplasmic reticulum the enzymatically active part of the A-moiety is translocated to the cytosol, and cellular protein synthesis is inhibited. Although the Shiga toxins are involved in disease, they may also be exploited for medical diagnosis and treatment. Interestingly, the toxin receptor, Gb3, has a limited expression in normal tissues, but is overexpressed in several types of cancer. Thus, the use of Shiga toxin, or the binding part of the toxin, has great potential in cancer diagnostics and treatment. Furthermore, studies of the various uptake mechanisms and intracellular transport pathways exploited by the toxins, provide important insight in basic cell biology processes.

Keywords: Shiga toxin, retrograde transport, endocytosis, Gb3, glycosphingolipids, trans-Golgi network.

INTRODUCTION

The Shiga toxins are AB₅-toxins consisting of a pentameric binding moiety (StxB) and an enzymatically active A-moiety (StxA) (Fig. 1). The 5 small B-chains (Mw 7.7 kD) interact non-covalently, and each B-chain contains three binding sites for the glycosphingolipid Gb3 [1]. Thus, the pentamer has in theory the ability to cluster up to 15 Gb3 molecules, which is important for toxin binding (see below). Only one type of Shiga-like toxin has been found to bind Gb4. The A-moiety, with a molecular mass of 32.2 kD, is non-covalently attached to the B-moiety [2] (Fig. 1), and contain a loop formed by a disulfide bond between cysteines 242 and 261.

An important step for intoxication with Shiga toxin is the cleavage of the A-chain by the protease furin, generating the enzymatically active A₁ fragment that is able to translocate into the cytosol [3]. Furin cycles between the TGN and the cell surface, and is involved in cleavage and activation of not only Shiga toxin, but also diphtheria toxin and *Pseudomonas* exotoxin A [4]. The pH-optimum for toxin cleavage varies with the substrate, and for Shiga toxin, furin-induced cleavage has a low pH-optimum [5], indicating that efficient cleavage can occur shortly after endocytosis. After cleavage, the A₁ fragment remains attached to the A₂ part via the internal disulfide bond [5], and the cleaved toxin is transported further via the Golgi complex to the endoplasmic reticulum (ER). From the ER, the A₁-fragment is

translocated to the cytosol and functions as a highly specific N-glycosidase that removes adenine from one particular adenosine residue in the 28S RNA of the 60S ribosomal subunit [6]. The depurinated subunit is unable to interact properly with elongation factors, and the protein synthesis is halted. This mechanism of action is identical for the Shiga toxins and several plant toxins, e.g. ricin and abrin [7], indicating that important features of these toxins are conserved.

The Shiga-like toxins are divided into two immunologically distinct groups. Shiga-like toxin 1 (Stx1) is virtually identical to Stx produced by *Shigella dysenteriae* and differs in only one amino acid in the A-chain. Shiga-like toxin 2 (Stx2) shares the overall toxin structure, but has lower amino acid similarity to Stx (~55%). There are several subtypes of Stx2, and some bacteria produce several types of Shiga-like toxins [8]. Although Stx1 is 5-10 fold more cytotoxic *in vitro* than Stx2 [9], Stx2 is most frequently associated with human disease, such as the hemolytic uremic syndrome (HUS). This condition is defined by acute renal failure, hemolytic anemia and thrombocytopenia, and most frequently affects children and elderly (reviewed in [10]). Potential sources of the toxin-producing bacteria are undercooked ground beef, unpasteurized milk and juice, raw vegetables, and contact with infected live animals.

In some cell types the Shiga toxins, as a long term effect, induce expression and secretion of pro-inflammatory cytokines, including TNF- α , IL-1, IL-6 and IL-8 [6, 11, 12]. Interestingly, the secreted cytokines can induce synthesis of Gb3 in other cell types, thereby sensitizing these cells to the toxin [13]. The Shiga toxins are also known to induce apoptosis either via the ribotoxic stress induced after transport of the A-chain into the cytosol, or, as shown in

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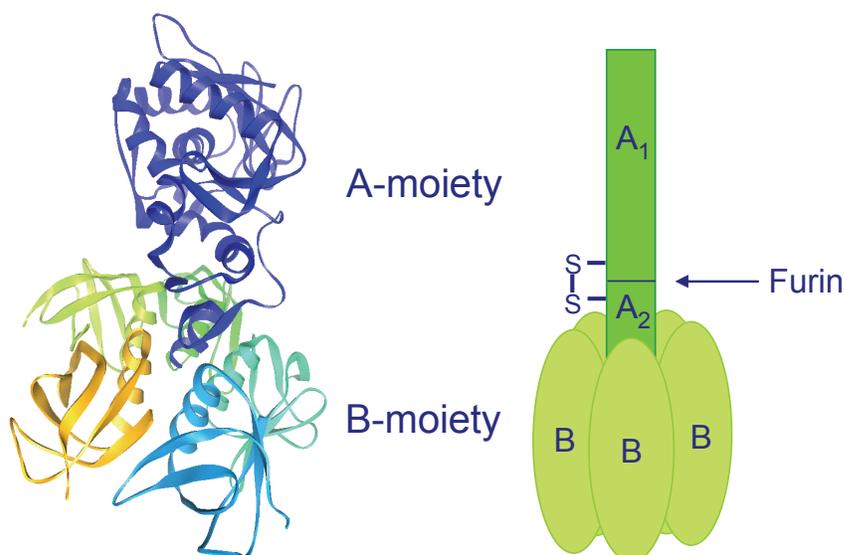


Fig. (1). The structure of Stx. Stx (PDB protein data bank: 1DMO) consists of an A-chain of ~32 kDa, non-covalently attached to the ring-shaped, pentameric B-moiety. The five B-chains of 7.7 kDa each, spontaneously assemble into the pentameric form. The A-chain is activated by proteolytic processing during the intracellular trafficking in the target cell. The protease furin has been shown to nick the A-chain into an enzymatically active A₁-fragment (~27 kDa) and a carboxyl terminal A₂-fragment (~4 kDa), which remain linked by a disulfide bond.

Burkitt lymphoma cells, by rapid signaling induced at the cell surface [11, 14-16].

BINDING OF STX TO CELLULAR MEMBRANES

All the members of the Shiga toxin family, except Stx2e, bind to the oligosaccharide part of the glycosphingolipid Gb3, with the structure Gal(α 1-4)Gal(β 1-4)GlcCer. Stx2e binds to Gb4 and is produced by bacteria that mainly infect pigs. Although Stx2 is most frequently associated with human disease, in most cases Stx1 has been shown to bind more strongly than Stx2 to Gb3 [17-20]. When the crystal structure of Stx2 and Stx is compared, some differences are found that might influence the ability of the toxins to interact with human cells and cause disease [21]. In contrast to Stx, the active site of the Stx2 A-moiety is accessible in the holotoxin, the orientation of the A-chain with respect to the B-pentamer differs in the two toxins, and one of the three Gb3 binding sites in the Stx2 B-chain has a different conformation than in Stx B-chain.

Although the toxin binds to the oligosaccharide part of Gb3, it is known that also the structure of the lipid moiety of the receptor is important for toxin binding, most likely by affecting the presentation of the oligosaccharide part. Both hydroxylation, the degree of unsaturation of the fatty acid, and the fatty acid chain length are important factors for optimal toxin binding [19, 22, 23]. The importance of the fatty-acid chain length of Gb3 for Shiga-like toxin binding has recently been found to differ for Stx1 and Stx2 [24]. In a model membrane system Stx1 bound selectively to C16, C22, and C24 Gb3-species, but not to C18 or C20 species. In contrast, Stx2 bound to all isoforms of Gb3, irrespective of chain length.

The structure of Gb3 is not the only parameter that affects Shiga toxin binding; also the local membrane environment surrounding Gb3, such as the phospholipid- and cholesterol-content of the membrane is important [9, 20, 23,

25]. Also the density of Gb3 in the membrane seems to affect Shiga toxin binding, as it was recently found that mutant Vero cells with a lower density of Gb3 were unable to bind Stx1 [26]. Notably, at least in HeLa cells, toxin binding seems to induce recruitment of the toxin/receptor complex to lipid microdomains, or rafts, in the plasma membrane [27], and this localization has been reported to be important for correct retrograde trafficking and toxicity (see below). Recently, it was demonstrated that Stx1 and Stx2 (also called Verotoxin 1 and 2) localized to both shared and distinct microdomains on the cell surface, and Stx1 was more resistant to detergent extraction than Stx2 both at the cell surface and during intracellular transport [9]. From this it seems that the toxin homologs bind different Gb3 lipid assemblies. Moreover, during their retrograde transport, the two toxins showed both overlapping and distinct localization, and also the kinetics of their retrograde transport differed [9]. These factors might contribute to the differential effects on target cells that these toxins display.

Interestingly, it has been shown that Stx binding rapidly activates several kinases, such as the Src kinases Yes [28, 29] and Lyn [30], the tyrosine kinase Syk [30, 31], the serine/threonine kinase PKC δ [32], and the MAP kinase p38 α [33]. The activity of some of these kinases is important for Stx entry and intracellular transport, suggesting that Stx is able to induce its own transport (see below).

ENDOCYTOSIS OF STX

Stx is able to exploit several endocytic mechanisms to gain entry into the cell, and the toxin was the first lipid-binding ligand shown to utilize clathrin-dependent endocytosis [34]. The Gb3 molecules does not seem to be constitutively present in clathrin-coated pits, rather, the toxin/receptor complex is recruited into coated pits and internalized [34]. Interestingly, Stx seems to be able to stimulate its own clathrin-mediated uptake. The B-moiety is sufficient to activate Syk and induce phosphorylation of

clathrin and uptake by this pathway [31], however, the efficiency of clathrin-mediated toxin uptake is dependent on the concentration of cell surface-bound toxin molecules and the presence of the toxin A-chain [35]. The stimulated uptake seen at high concentrations of intact toxin, might be explained by increased aggregation of toxin-receptor-complexes into large clusters that somehow enhance the recruitment to clathrin-coated pits. Also, the toxin-receptor complex might interact with other plasma membrane proteins recruited to clathrin-coated pits. In fact, Stx has been shown to associate with surface-proteins in Vero and CaCo-2 cells [36], although the identity of these proteins remains unknown.

Interestingly, both the holotoxin and the B-moiety without an A-chain, can induce rapid tyrosine phosphorylation of several proteins, including the tyrosine kinase Syk and clathrin heavy-chain [31, 37]. Upon stimulation with Stx/StxB a complex between Syk and clathrin heavy-chain was detected, and in Ramos cells both the phosphorylation of clathrin and the complex-formation seemed to require Src family kinase activity [37]. Moreover, depletion or inhibition of Syk reduced Stx uptake, suggesting a link between Stx-induced signaling and endocytosis. Subsequently, Stx binding has been shown to activate other key kinases that have roles in its transport, namely PKC δ [32] and p38 α [33]. These kinases do not seem to regulate the uptake of the toxin, but rather to be involved in the endosome-to-Golgi transport step (see below). Moreover, Stx has been shown to stimulate microtubule assembly in ACHN cells [38] and in Vero cells [39], and both microtubules and dynein were found to be required for transport of Stx to the Golgi [39]. Notably, it was shown that the Stx-induced activation of microtubuli assembly was not mediated via Syk, suggesting that multiple signaling pathways are induced by Stx.

Clathrin-mediated endocytosis can be responsible for a large fraction of the endocytic uptake of Stx [40], but also other, clathrin-independent, mechanisms have been shown to contribute to toxin internalization in different cells [41-45]. These clathrin-independent uptake mechanisms show different requirements for dynamin and lipid rafts. In HeLa cells, we find that ~50% of Stx uptake is independent of dynamin [40], however, it is important to bear in mind that inhibition of one pathway might lead to upregulation of another [46]. It is therefore difficult to determine the exact contribution from each pathway in toxin uptake. Recently, a novel clathrin-independent uptake mechanism was reported for StxB, where the toxin was shown to induce tubular membrane invaginations mediating its uptake [45]. It was postulated that clustering of up to 15 Gb3 molecules by StxB binding generates cluster domains that naturally induce negative curvature on the membrane, and that this leads to invagination and generation of tubules [45]. The formation of the tubules was shown to be energy-independent, but dynamin was required for proper fission. To which extent this process is important for intoxication is not yet known.

ENDOSOME-TO-GOLGI TRANSPORT OF STX

Retrograde transport from endosomes to the TGN and further to the Golgi is important for retrieval and correct localization of several endogenous proteins, e.g. Golgi-

resident proteins such as TGN46, mannose 6-phosphate receptors (MPRs) and furin (for review see [47]). Some pathogen-produced proteins, such as the Shiga toxins, cholera toxin, ricin and *Pseudomonas* exotoxin A, are able to exploit this pathway in order to reach their intracellular targets. After uptake into early endosomes, a key sorting process that determines the fate of the toxin takes place. The toxin/receptor complex might be sorted into the degradative pathway to the lysosomes or into the retrograde pathway to the TGN. Although, the sorting mechanism is currently not completely understood, several components that seem to play a role in this process have been revealed (Fig. 2).

Two main retrograde transport routes to the TGN have been identified; a direct pathway from early endosomes or the recycling compartment, and an indirect pathway via late endosomes [48-51]. Stx has been shown to utilize the direct pathway to the TGN, and not the Rab9-dependent pathway via late endosomes that transports amongst others, furin and a fraction of *Pseudomonas* exotoxin A [48, 52-55]. However, based on the differential requirements for retrograde transport of Stx, ricin and TGN38, it seems that more than one parallel pathway between early endosomes and the TGN may exist [32, 33, 56-62].

Several Rab- or Rab-associated proteins have been implicated in the transport of StxB from early endosomes to the TGN. Rab11 [60], Rab6a' [59, 63], and Rab6IP2 [64] have all been reported to positively regulate Stx transport, while Rab9 does not seem to be required for transport of either ricin or Stx [55, 56]. In a screen for potential Rab GTPase activating proteins (RabGAPs) involved in transport of Stx to the Golgi, the Golgi-localized Rab43 and its RabGAP RN-tre were found to be required [65]. Additional 5 RabGAPs were identified as regulators of Stx transport to the Golgi, however, their target Rabs remain unknown.

Interestingly, both clathrin and dynamin play an important role not only in Stx uptake, but also in Stx transport from early endosomes to the Golgi [40, 44, 66]. The clathrin adaptor epsinR was found to be required for transport of Stx to the Golgi, while AP-1 did not seem to be involved [44]. Moreover, coat-like components other than clathrin, such as the retromer complex, has been shown to mediate transport of Stx from early endosomes to the Golgi [67-69] (Fig. 2). The retromer consists of the components hVps26, hVps29, hVps35, SNX1, and possibly SNX2 (for review see [70, 71]), and this complex has been shown to mediate retrieval of MPRs [51, 72] and several other cargo proteins to the Golgi [70]. Importantly, the retromer components hVps26 and SNX1, and also SNX2, are required for endosome-to-Golgi transport of Stx [67-69]. Sorting nexins contain a Phox homology (PX) domain that binds to certain phosphoinositides, such as PI(3)P, and a BAR (Bin/Amphiphysin/Rvs) domain that senses or induces membrane curvature. The presence of PI(3)P and the activity of the PI(3) kinase hVps34 is important for efficient transport of both Stx and ricin to the TGN [68, 73]. The retromer-interacting proteins EHD1 (Eps15 homology domain-containing protein 1) and EHD3 are needed for proper localization of SNX1 and SNX2 to endosomal tubules [74], and both EHD3 and one of its interaction partners, the Rab4/5 effector rabenosyn-5, are required for endosome-to-Golgi transport of Stx [74]. How can we explain the

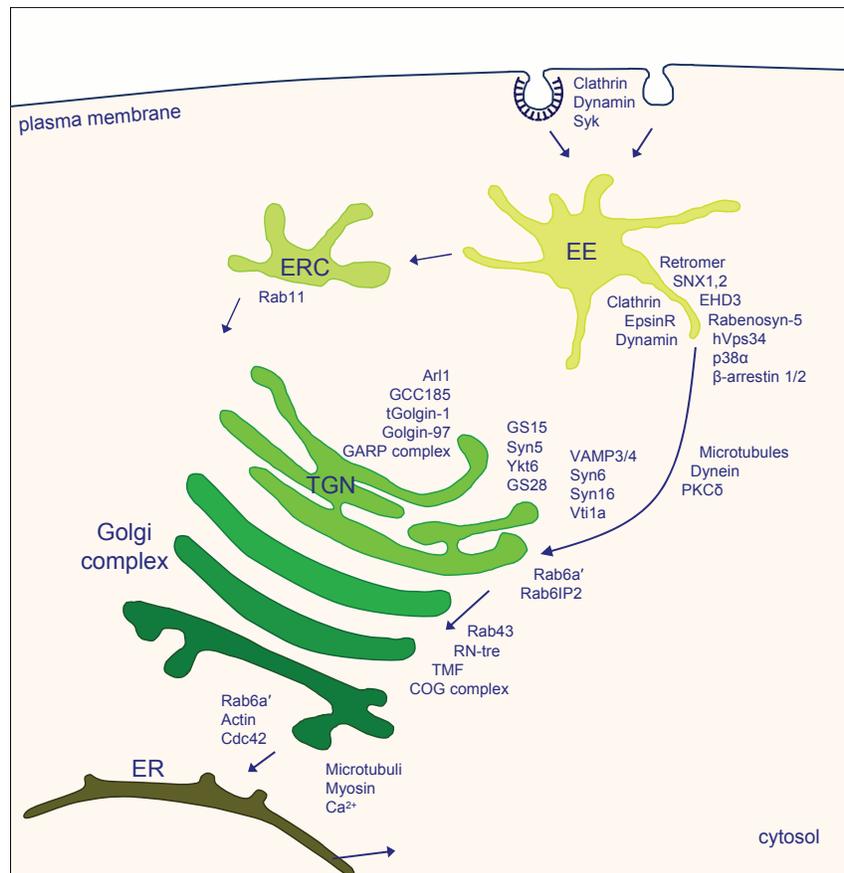


Fig. (2). Overview of components involved in the retrograde trafficking of Stx. Endocytosis of Stx is mediated by several uptake mechanisms, with different requirements for components such as clathrin, dynamin, and Syk. From early endosomes (EE) Stx is transported to the trans-Golgi network (TGN), either directly and/or via the endocytic recycling compartment (ERC). Several components have been implicated in the exit from EE and in the further retrograde transport. These are discussed in detail in the text.

requirement for both clathrin and the retromer in transport of Stx to the Golgi? One likely explanation is that the clathrin coat and the retromer complex are not independent of each other, but rather regulate the retrograde transport from endosomes sequentially.

Several tethering factors seem to be involved in retrograde transport of Stx. tGolgin-1 [75], Golgin-97 and its effector ARL1 [76, 77], the conserved oligomeric Golgi (COG)-complex [78], GCC185 [79], GARP (Golgi-associated retrograde protein) complex [80], and Rab6-binding TATA element modulatory factor (TMF) [81] have all been implicated in Stx transport, presumably by targeting the Stx-containing vesicles to the TGN/Golgi. Moreover, two distinct SNARE complexes seem to be involved in the fusion of Stx containing vesicles with the TGN. One consists of the v-SNARE VAMP3/4 recognizing the t-SNARE complex syntaxin-6/syntaxin-16/Vti1a [59, 77] and the other fusion complex consists of the v-SNARE GS15 and the t-SNARE complex syntaxin-5/Ykt6/GS28 [82]. It has been speculated that ARL1 is functionally connected to the t-SNARE complex, possibly by regulating the localization or function of the components Vti1a and syntaxin-6 [77].

In several cell-types, exit from early endosomes into the retrograde pathway of both cholera toxin and Stx has been shown to depend on lipid raft integrity [83-89]. It is

postulated that these toxins exploit an endogenous pathway for recycling of raft glycolipids and that the lipids mediate toxin transport all the way from the plasma membrane to the ER [87, 89]. In agreement with this, disruption of lipid rafts or reduction of cellular cholesterol by drugs such as m β CD or filipin, was found to strongly reduce transport of Stx to the Golgi [55, 87]. However, not only lipid raft integrity, but also specific targeting of the Stx/Gb3 complex to these domains seems to be important for retrograde transport [87]. This was indicated by comparing Stx transport in toxin sensitive HeLa cells vs. toxin insensitive monocyte-derived cells [87]. The toxin was internalized in both cell types, but was only targeted to the Golgi in HeLa cells, corresponding to raft localization of Gb3 in these cells and not in the monocyte-derived cells. A correlation between raft localization and toxicity has also been shown by comparing cholera toxin and *E. coli* heat-labile enterotoxin IIb [89]. Both toxins were internalized, but only raft-localized toxin-receptor complexes were targeted to the Golgi and displayed cytotoxicity [89]. Moreover, bovine intestinal epithelial cells express Gb3, but are insensitive to Shiga-like toxins [90]. In these cells the internalized toxin is routed to lysosomes for degradation, like in monocyte-derived cells. Because of the insensitivity towards Shiga-like toxins, cattle function as reservoirs of Shiga-like toxin-producing bacteria [90].

Targeting of the Stx/Gb3 complex from endosomes to the Golgi appears to depend on the fatty acid chain length of Gb3 [62, 91-93]. Sensitization of cells towards Stx by treatment with butyric acid is known to cause a change in Gb3 fatty acid chain length, and Gb3 species containing C16 fatty acids may favor endosome-to-Golgi transport of Stx [62, 91, 93, 94]. Recently, it was shown in HEp-2 cells that inhibition of glycolipid synthesis with PDMP or fumonisin B₁ results in loss of the glycolipid species at different rates [62]. Gb3 molecules with C16 fatty acids were degraded faster and to a larger extent than C24:1 Gb3 molecules. The

endosome-to-Golgi transport was inhibited and the toxin seemed to end up in endosomes or transport vesicles unable to fuse with the Golgi complex. Neither treatment with PDMP [62] nor fumonisin B₁ (Fig. 3) resulted in toxin transport to LAMP1-positive late endosomes/lysosomes. PDMP treatment changed the cellular localization of SNX1 and SNX2 from the Golgi area to an endosomal localization. A similar phenotype was previously shown for SNX1 after EHD3 knockdown [74], and also in these cells StxB accumulated in endosomes.

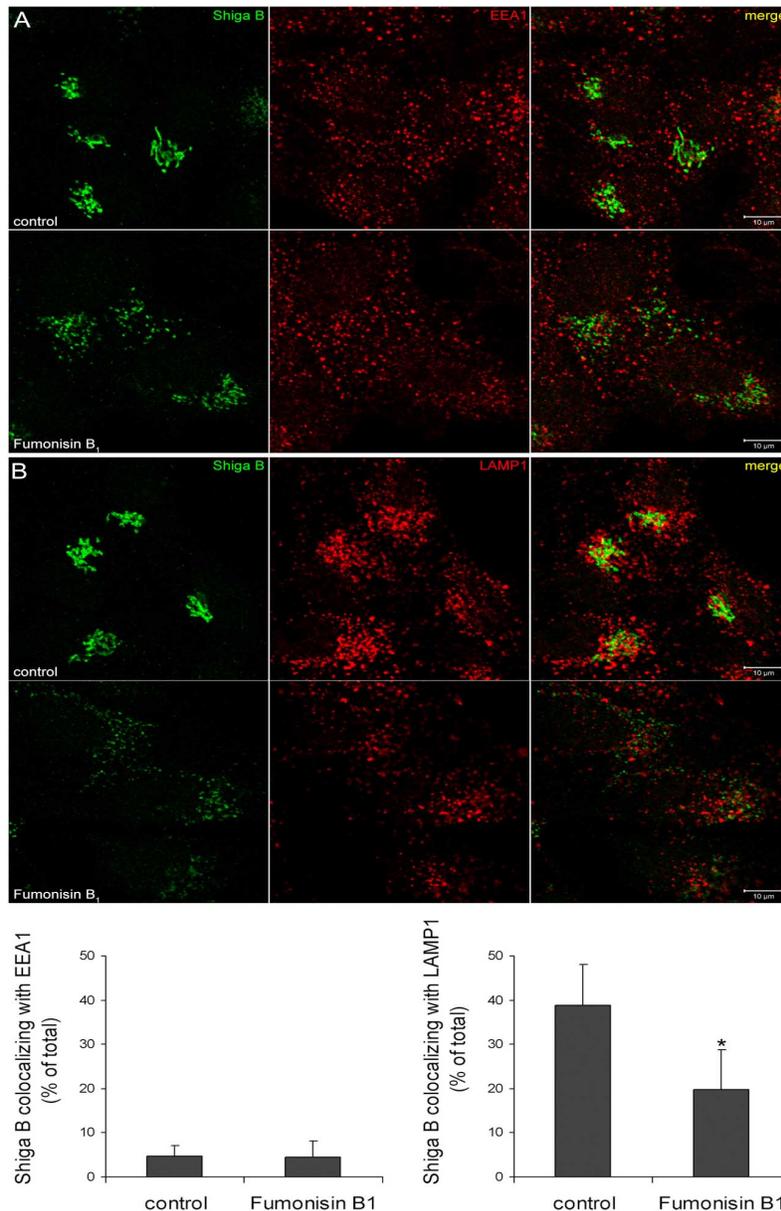


Fig. (3). StxB colocalization with lysosomes is not increased upon inhibition of Golgi transport with fumonisin B₁. The amount of receptor-bound Stx transported to the Golgi apparatus is reduced in HEp-2 cells pre-treated with 10 μM fumonisin B₁ for 48 hours. After 45 minutes of StxB incubation, the colocalization of StxB with early endosomal marker EEA1 (A) and lysosomal marker LAMP1 (B) does not increase in fumonisin B₁-treated cells compared to control cells. In the case of LAMP1 the colocalization rather decreases. ((A) n = 27 for both control and fumonisin B₁, (B) n = 21 for control and n = 14 for fumonisin B₁, *P < 0.05, unpaired Student's t-test). This suggests that in cells treated with fumonisin B₁, StxB is retained in endosomes or transport vesicles unable to fuse with the Golgi apparatus rather than in EEA1- or LAMP1-positive endosomes or lysosomes.

Inhibition of Stx trafficking and accumulation of the toxin in endosomes has also been shown when PKC δ [32] or p38 α [33] are inhibited or depleted from cells. Interestingly, both kinases are rapidly activated by Stx binding, further strengthening the hypothesis that Stx is able to stimulate its own trafficking by inducing signaling cascades. Activated p38 α was shown to translocate to endosomes upon Stx stimulation. Recently, potential modulators of the p38 α -dependent pathway were identified, the β -arrestins 1 and 2 [95]. Like p38 α , the β -arrestins were shown to translocate to endosomes upon Stx stimulation. However, the β -arrestins seem to be negative regulators of retrograde transport, as knockdown of these proteins increased the retrograde transport of both Stx and the MPRs [95]. It was proposed that since β -arrestins can form a complex with p38 they may sense the activated p38 and attenuate its signaling.

GOLGI-TO-ER TRANSPORT

ER-resident proteins that are missorted to the Golgi complex by anterograde transport, are normally retrieved by a retrograde transport system through the Golgi cisternae and back to the ER. The classical eukaryotic signal for retention in the ER lumen is the KDEL-motif, and proteins containing this motif are retrieved in COPI-coated vesicles by KDEL receptors [96, 97]. The *Pseudomonas* exotoxin A contains a KDEL-like sequence and exploits this pathway [98]. Also the cholera toxin A-chain contains a KDEL-motif, however, this motif is not strictly required for cholera toxin function, but rather improves the efficiency of intoxication [99]. Importantly, even the cholera toxin B-moiety can move retrogradely to the ER, demonstrating that the GM1-bound toxin can be transported independently of the A-chain containing the KDEL motif. Studies on Stx transport provided the first evidence that a bacterial protein was able to move retrogradely all the way from the plasma membrane to the ER [91], and a few years later this was also demonstrated to be the case for cholera toxin [100]. Interestingly, Stx does not have a KDEL motif and reaches the ER in a COPI-independent pathway [101]. Golgi-to-ER transport of Stx is dependent on Rab6a', actin, Cdc42, microtubuli and calcium [63, 102-105], and the vesicle trafficking seems to be mediated by myosin motors [106].

TRANSLOCATION TO THE CYTOSOL

In order to inhibit protein synthesis, the catalytically active A₁-chain of Stx must enter the cytosol, where the 28S rRNA substrate is located. Several lines of evidence indicate that StxA₁, like many other ER-targeted AB or AB₅ toxins (e.g. ricin, cholera toxin, and *Pseudomonas* exotoxin A) translocates to the cytosol from the ER. ER-to-cytosol translocation is presumably achieved by taking advantage of a process known as ER-associated degradation (ERAD), which normally functions to dislocate misfolded proteins from the ER to the cytosol for immediate ubiquitylation and proteasomal degradation [107]. Stx, and other ER-directed toxins, avoid massive ubiquitylation and degradation at least in part due to the extremely low content of lysine residues in their A-moieties [108].

In cells containing furin, the Stx A-chain is already proteolytically cleaved before ER arrival [3], so that the A₁ fragment is connected to the A₂ fragment (which again is bound to the pentameric StxB moiety) only by a disulfide

bridge between cysteines 242 and 261. For full catalytic activity, the A₁ fragment must presumably dissociate from A₂, since residues 258-262 of A₂ lie adjacent to the active site cleft, and the side chain of Met₂₆₀ protrudes into the active site itself [109]. Removal of the disulfide bridge is sufficient for the A₁ fragment to dissociate from the A₂-StxB-Gb3 complex [5]. How the disulfide bridge is reduced is not known, but in analogy with cholera toxin, whose active A₁ fragment also needs to be cleaved from its disulfide-linked A₂ fragment, it is possible that the reduction also of StxA is carried out by the ER-resident enzyme protein disulfide isomerase [110, 111]. In Vero cells (which contain furin) it was demonstrated that the vast majority of StxA in the cells was not reduced and only the A₁ chain could be detected in the cytosol [112]. However, it can not be excluded that also the whole A-chain can be translocated from the ER to the cytosol. Indeed, it has been reported that *in vitro* translated Stx A-chain is able to translocate from the lumen of isolated microsomal membranes [113]. Moreover, in Vero cells a Stx-mutant that lacks the furin cleavage site was shown to be processed in a manner that was sensitive to inhibitors of the cytosolic enzyme calpain [114], indicating that also *in vivo*, the whole A-chain may translocate to the cytosol. Given that the A-chain stays attached to the pentameric B-moiety (which again is most likely still bound to Gb3) all the way to the ER [112], it remains to be demonstrated how this intoxication process occurs.

The molecular mechanism responsible for StxA₁-translocation from the ER to the cytosol is not well defined. In yeast, it was shown that the stretch consisting of the 12 very last C-terminal amino acid residues in A₁ (240-251) is required for its ER-to-cytosol translocation [115]. Moreover, A₁ contains a hydrophobic region (residues 224-242) close to its C-terminus, which may be recognized by ER-resident chaperones mediating ER quality control [108], but experimental data to support this idea are lacking. It seems evident, however, that both the A- and B-chains of Stx can interact with ER-resident chaperones. *In vitro* translated StxA was shown to interact with the ER-resident Hsp40 chaperone HEDJ/ERdj3 inside the lumen of microsomes isolated from Vero cells [113], and interestingly the ER-resident Hsp70 chaperone BiP, which is likely to play a major role in ERAD substrate selection [107], was found to coimmunoprecipitate with StxA and HEDJ/ERdj3. More recently, a functional interplay between HEDJ/ERdj3 and BiP has been uncovered [116, 117]. Intriguingly, it appears that not only StxA, but also StxB is able to interact with BiP, since StxB has been reported to both colocalize [87] and coimmunoprecipitate with BiP [118]. Interestingly, BiP seems to play a central role not only in ERAD substrate selection, but possibly also in the targeting of ERAD substrates to an ER retrotranslocon channel [107]. It remains to be determined whether BiP actually both recognizes and drives StxA/StxA₁ to an ER retrotranslocon channel. Furthermore, the nature of the retrotranslocon channel(s) used by StxA/StxA₁ remains to be defined. One possibility is that Stx uses the same retrotranslocon (Sec61) as is apparently being exploited by cholera toxin and ricin to gain access to the cytosol [119, 120]. However, it is unclear whether StxA can interact with Sec61 or not [112, 113]. Alternatively, Stx might use other putative ER

retrotranslocon channels, such as those constituted by the derlin family.

EXPLOITATION OF STX IN MEDICINE

The use of Shiga toxin, or the binding part of the toxin, has great potential in cancer diagnostics and treatment (for reviews see [7, 121-123]). The Stx receptor Gb3 has a limited expression in normal human tissues, being mostly restricted to endothelial cells, kidney epithelium and some antigen presenting cells, such as subsets of dendritic cells and B cells [11]. This facilitates the use of StxB as a vector for peptide delivery to the MHC class I pathway for the development of vaccines against specific cancer epitopes (for reviews see [121, 123]). It has been shown that exogenous antigens coupled to StxB is targeted to the MHC class I pathway and then presented on the surface of dendritic cells, facilitating activation of cytotoxic T-cells and antitumor immunity [124-126]. In this way StxB might function as a non-live, non-toxic vaccine delivery system in cancer therapy.

Interestingly, Gb3 is overexpressed in several cancer types, such as B-cell lymphomas, and cancers of the ovary, breast, testis, and colon (reviewed in [123]). For diagnostic purposes and selective imaging of these Gb3-expressing cells, visualizing agents, such as radioactive isotopes, contrast agents or fluorescent dyes, might be coupled to StxB. It has been demonstrated that labeled StxB administered systemically in mouse models accumulates in tumor regions overexpressing Gb3, thus confirming that cancer cells can be targeted *in vivo* by StxB [124, 127]. Although not yet tested *in vivo*, the coupling of chemotherapeutic drugs to StxB may increase the selectivity of chemotherapy, and thereby reduce side-effects. When a topoisomerase I inhibitor prodrug was coupled to StxB, the complex was targeted to the biosynthetic/secretory pathway (most likely the ER) and exhibited cytotoxicity only in Gb3-positive cells [128].

There is also a potential use of the holotoxin in cancer treatment. It has been shown that ovarian-derived tumor cells were effectively killed by Stx1, and interestingly, multidrug resistant variants of these tumor cells expressed higher levels of Gb3 and were more sensitive to Stx1 [129]. Gb3 was expressed also in tumor vasculature, suggesting a potential role for Stx as an antiangiogenic agent [130]. Also some brain tumor cells, such as astrocytoma cells, are sensitive to Stx1 and are killed by apoptosis [131]. Moreover, Stx1 has proven useful as an *ex-vivo* purging agent to eradicate malignant cells from autologous stem cell grafts [132]. The Stx B-moiety has been demonstrated to reduce tumor growth of colon carcinoma cells in a mouse model [133].

CONCLUDING REMARKS

The fact that many protein toxins follow long intracellular transport routes in order to reach the cytosol, makes them invaluable tools in the study of cellular pathways. The investigations of toxin uptake and intracellular transport have given important insight in basic cell biology processes and led to the discovery of several transport pathways. Clearly, detailed knowledge of the regulation and the specific intracellular trafficking mechanisms used by the different toxins is crucial both to

improve the prevention and treatment of toxin-induced diseases, and also to exploit the toxins as tools in medicine.

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