CLOSTRIDIAL GLUCOSYLATING TOXINS: INOSITOL HEXAKISPHOSPHATE-DEPENDENT PROCESSING OF CLOSTRIDIUM SORDELLII LETHAL TOXIN AND CLOSTRIDIUM NOVYI α-TOXIN

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Clostridium sordellii lethal toxin and C. novyi α-toxin, which are virulence factors involved in toxic shock syndrome or gas gangrene syndrome, are members of the family of clostridial glucosylating toxins. The toxins inactivate Rho/Ras proteins by glucosylation or attachment of GlcNAc (α-toxin). Here we studied the activation of the auto-proteolytic processing of the toxins by inositol hexakisphosphate (InsP6) and compared it with the processing of C. difficile toxin B. In the presence of low concentrations of InsP6 (<1 µM), toxin fragments, consisting of the N-terminal glucosyltransferase (or GlcNAc transferase) domain and the cysteine protease domains of C. sordellii lethal toxin, C. novyi α-toxin and C. difficile toxin B were autocatalytically processed. The cleavage sites of lethal toxin (leucine-543) and α-toxin (leucine-548) as well as the catalytic cysteine residues (cysteine-698 of lethal toxin and cysteine 707 of α-toxin) were identified. Affinity of the cysteine protease domains to bind InsP6 was determined by isothermal calorimetry. In contrast to full length toxin B and α-toxin, auto-catalytic cleavage and InsP6-binding of full length lethal toxin depended on low pH (pH 5) conditions. The data indicate that C. sordellii lethal toxin and C. novyi α-toxin are InsP6-dependently processed. However, full length lethal toxin but not its short toxin fragments, consisting of the glucosyltransferase domain and the cysteine protease domain, require a pH sensitive conformational change to allow binding of InsP6 and subsequent processing of the toxin.

Various pathogens of the genus Clostridium produce highly potent glucosylating toxins (clostridial glucosylating toxins, CGTs), which act on host target cells by modification and inactivation of Rho and Ras GTPases (1-4) (5). This group of toxins, comprising Clostridium difficile toxin A and B, C. novyi α-toxin, C. sordellii lethal and hemorrhagic toxin are major virulence factors (6). Whereas C. difficile toxins A and B cause antibiotic-associated diarrhea and pseudomembranous colitis (7), C. sordellii lethal toxin is implicated in toxic-shock syndrome (e.g., after medical-induced abortion) (8) and, like C. novyi α-toxin, plays a pathogenetic role in gas gangrene syndrome (9,10).

CGTs are single-chain proteins subdivided into at least four functional domains (11). Binding of the toxins to cell surface receptors is mediated by a C-terminal region, including a series of repetitive oligopeptides (12). After uptake of the toxins by clathrin-dependent endocytosis (13), a central region, harboring a pattern of hydrophobic amino acids, is suggested to mediate pore formation and translocation from endosomes into the cytosol (12,14). The glucosyltransferase domain is located at the N-terminal end of the toxins (15). As shown for toxins A and B, this domain is released into the cytosol by auto-catalytic cleavage induced by an adjacent cysteine protease domain (CPD) (16-19).

Previous studies revealed that auto-catalytic processing of CGTs depends on inositol hexakisphosphate (InsP6) (17,18). It was shown for C. difficile toxins A and B that InsP6 can bind to the intrinsic cysteine protease domain (20,21). Binding of InsP6 causes conformational changes of the catalytic center of the CPD, resulting in activation of the protease, cleavage of the toxin molecule between the glucosyltransferase and the...
cysteine protease domain and release of the glucosyltransferase into the cytosol (21-23).

The present study deals with the biochemical characterization of the InsP6-induced autocatalytic cleavage of C. sordellii lethal toxin and C. novyi α-toxin. For this purpose exclusively recombinant toxins were used. Although no major differences could be observed in the binding affinity of InsP6 to the CPDs of all CGTs, significant differences between CGTs were observed in InsP6-induced auto-catalytic processing of the holotoxins. In particular, we report on the activation of the InsP6-induced autoproteolytic cleavage of C. sordellii lethal toxin, which suggests differences in the modular organization and function of the toxin domains of the various members of the CGT family.

**Experimental Procedures**

*Cultivation of mammalian and bacterial cells-* Vero cells were grown at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Biochrom AG, Berlin, Germany), supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids (NEA; PAA, Pasching, Austria), 4 mM penicillin/streptomycin (PAN Biotech, Aidenach, Germany) and 1% sodium pyruvate (Biochrom, Berlin, Germany). *Bacillus megaterium* (strain WH320; MoBiTec, Göttingen, Germany) bacteria were cultivated in Luria Bertani (LB) medium at 37°C.

*Constructs, cloning and mutagenesis-* For cloning of *C. novyi* α-toxin into the *B. megaterium* expression vector pHIS1522 (MoBiTec, Göttingen, Germany), the gene was amplified by PCR from genomic DNA (*C. novyi* strain 6018) in two parts. First, the upstream sequence of an intrinsic SpeI restriction site at base pair position 2965 was amplified with oligonucleotides introducing a 5´-BsrGI restriction site, and second, the downstream sequence of the SpeI restriction site was amplified by omitting the stop codon and by introducing a 3´-KpnI restriction site. Subsequent ligation of the two DNA fragments into BsrGI/KpnI-digested pHis1522 resulted in α-toxin-6His. *C. difficile* toxin B and *C. sordellii* lethal toxin were cloned into the same expression vector essentially as described previously (24), (13). For the generation of His-tagged constructs of α-toxin, lethal toxin and toxin B, comprising only the glucosyltransferase domain (toxin B₁₋₅₄₆), the cysteine protease domain (CPD) or the glucosyltransferase domain and the cysteine protease domain (GD-CPD) in pHIS1522, the corresponding sequences were amplified with oligonucleotides introducing a 5´-BsrGI and a 3´-KpnI restriction site and by using the full length pHIS1522 constructs as template. Then, the ligation of BsrGI/KpnI-digested pHis1522 vector and PCR products was performed. Eventually, mutations were introduced by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA, USA).

*Expression and purification of recombinant proteins-* Full length versions or only parts of toxin B, lethal toxin and α-toxin that were cloned into the pHis1522 vector, were recombinantly expressed in *Bacillus megaterium* and purified as His-tagged proteins by Ni-affinity chromatography. Briefly, *B. megaterium* protoplasts were first transformed with plasmids according to the manufacturer’s instructions (MoBiTec, Göttingen, Germany). Then, the cells were grown at 37°C in LB medium until an OD₆₀₀nm of 0.3. Subsequently, protein expression was induced by addition of 5% xylose and by further incubation at 37°C for 3 – 6 h or at 29°C overnight. The cells were then harvested by centrifugation and the pellet was suspended in lysis buffer (20 mM Tris-HCl/pH 8.0, 300 mM NaCl, 20 mM imidazole, 10% glycerol and 500 µM EDTA) supplemented with Complete protease inhibitor cocktail (Roche, Mannheim, Germany). The bacteria were lysed by the use of a microfluidizer (Microfluidics, Newton, MA, USA) at 15000 psi and cell debris was removed by centrifugation at 164.000 g for 1 h at 4°C. The supernatant was then passed through a pre-equilibrated, nickel-charged HisTrap column (GE Healthcare, Munich, Germany). The column was washed with buffer, containing 20 mM Tris-HCl/pH 8.0, 300 mM NaCl, 50 mM imidazole and 10% glycerol, and bound His-tagged proteins were eluted with 20 mM Tris-HCl/pH 8.0, 300 mM NaCl, 500 mM imidazole and 10% glycerol. In addition, the eluted proteins were further purified by size-exclusion chromatography on a Superdex 200 column (GE Healthcare, Munich, Germany), equilibrated with the buffer, containing 20 mM Tris-HCl/pH 7.4, 150 mM NaCl and 10% glycerol. Rac1, Ha-Ras and RhoA were cloned as GST-tagged versions and purified from *Escherichia coli* BL21(DE3) essentially as described elsewhere (25).
Intoxication of cultured Vero cells- Cultured Vero cells were seeded in 24-well plates and cultivated in DMEM to form semi-confluent monolayers, prior to washing with phosphate-buffered saline (PBS) and intoxication by addition of toxins in DMEM at indicated concentrations. Upon onset of intoxication characteristics (cell rounding), images were taken by using an inverted microscope (Axiovert 25; Carl Zeiss Jena, Germany).

In vitro glucosylation assay- At indicated concentrations, full length toxins or toxin fragments were incubated in glucosylation buffer (50 mM Hepes/pH 7.5, 100 mM KCl, 2 mM MgCl₂, 1 mM MnCl₂ and 100 µg/ml bovine serum albumin) in the presence of 10 µM UDP-[¹⁴C]-glucose or UDP-[¹⁴C]-N-acetyl-glucosamine (α-toxin) and 5 µM recombinant Rac1, Ha-Ras or RhoA, respectively, for 20 – 45 min at 37°C. The reaction was stopped by the addition of Laemmli buffer and boiling for 5 min at 95°C. Radiolabeled proteins were separated by SDS-PAGE and visualized by autoradiography using a phosphorimager (GE Healthcare, Freiburg, Germany).

In vitro cleavage assay - The in vitro cleavage assay was performed with 2 µg of full length toxins or toxin fragments in either neutral cleavage buffer (100 mM Tris- HCl/pH 7.4) or at acidic conditions (100 mM Na-acetate/pH 5.0). Cleavage was initiated by the addition of indicated concentrations of InsP6 and the reaction was incubated at 37°C for the indicated time points. The reaction was stopped by addition of Laemmli buffer and boiling for 5 min at 95°C. Cleavage products were visualized as Coomassie-stained protein bands after SDS-PAGE.

Filter binding assay with radiolabeled inositol hexakisphosphate- 1 µM of full length toxins or toxin fragments were incubated with a mixture of 4.8 µM inositol hexakisphosphate (InsP6) and 0.2 µM [³²P]-InsP6 in binding buffer containing 100 mM Tris-HCl/pH 7.4 or 100 mM Na-acetate/pH 5.0, each supplemented with 1 mg/ml bovine serum albumin, for 10 min at 4°C. Then, the reactions were terminated by rapid vacuum filtration through a pre-wetted 0.45 µm nitrocellulose membrane (Whatman GmbH, Dassel, Germany), followed by washing with 3 ml binding buffer to separate free and bound [³²P]-InsP6. Membrane-bound radioactivity was determined by liquid scintillation counting.

Isothermal titration calorimetry-The Kₐ-values of inositol hexakisphosphate binding to the cysteine protease domains of toxin B, lethal toxin and α-toxin were determined by isothermal titration calorimetry (ITC200; GE Healthcare, Freiburg, Germany) at 25°C and at neutral pH conditions in buffer, containing 20 mM Tris-HCl/pH 7.4, 150 mM NaCl and 10% glycerol, or at acidic conditions in buffer, containing 20 mM Na-acetate/pH 5, 150 mM NaCl and 10% glycerol. The reaction cell was filled with 50 – 100 µM of protein and inositol hexakisphosphate was injected at 10-fold higher concentrations. As a control, InsP6 was injected alone into the cell filled with buffer only. Data were evaluated with the manufacturer’s software.

RESULTS

The cysteine protease domain of C. sordellii lethal toxin and C. novyi α-toxin is responsible for the auto-catalytic processing. Studies by Pruitt et al. delimited the CPD of toxin A to amino acids 543-809 (corresponding to amino acids 544-807 in toxin B). Sequence comparison of toxins A and B with lethal toxin and α-toxin revealed a putative CPD of lethal toxin and α-toxin to residues 543-807 and 548-813. To study the auto-catalytic cleavage of the toxins, we therefore expressed toxin fragments consisting of the glucosyltransferase domains (GD) and the putative cysteine protease domains (CPD) from various CGTs (toxin B1-807, lethal toxin 1-807, and α-toxin1-813). Then, an in vitro cleavage assay was performed by incubation of increasing concentrations of InsP6 with the respective GD-CPD fragments of toxins. As shown in Fig. 1A, InsP6-induced auto-catalytic processing was detected with each toxin fragment at concentrations of 0.1-1 µM InsP6. Thus, auto-catalytic processing of all CGT members occurred independently of any domains that are located beyond the intrinsic cysteine protease domain.

Recently, the cleavage site of the CPD in toxin A and B, as well as amino acids that are crucial for the activity of the protease, were characterized (17,19,21). In order to identify the cleavage site and the catalytically active cysteine residue of the lethal toxin and the α-toxin, a sequence comparison of all CGTs was performed. The multiple alignments of CGT sequences surrounding the cleavage site and the catalytically active cysteine residue of toxin A and B is depicted in Figure 1B. This
analysis revealed the cleavage sites leucine-543 and leucine-548 for lethal toxin and \( \alpha \)-toxin, respectively. Furthermore, by sequence comparison, we identified cysteine-698 and cysteine-707 as catalytic cysteine residues of the CPD of lethal toxin and \( \alpha \)-toxin, respectively. The \textit{in silico} data were confirmed by mutagenesis studies. Change of the proposed catalytic cysteine residues of the three toxin fragments (C698A-toxin B1-807, C698A-lethal toxin1-807, C707A-\( \alpha \)-toxin 1-813) to alanine inhibited auto-catalytic cleavage in the presence of 100 \( \mu \)M InsP6. Moreover, we corroborated the proposed cleavage sites by mutation. Again, all cleavage site mutants (L543A/G543A-toxin B1-807, L543A/G543A-lethal toxin1-807, L548A/N549A-\( \alpha \)-toxin1-813) were stable towards auto-catalytic cleavage. \textit{In vitro} glucosylation of Rac1 in the presence of radiolabeled UDP-glucose (or UDP-GlcNAc for \( \alpha \)-toxin) was performed with each GD-CPD mutant, showing that the recombinant proteins were correctly folded, because Rac1 was modified in comparable levels by the mutants as compared with wild-type GD-CPD fragments (Fig. 1D).

A prerequisite of the auto-proteolytic cleavage of the CGTs is the binding of InsP6 to the cysteine protease domain. We compared the binding affinity of InsP6 to the cysteine protease domains by isothermal titration calorimetry at pH 7.4 (Fig. 2A-C). The studies were performed with the recombinant CPD domains. Titrations were carried out twice and yielded \( K_d \) values of 4.4 and 5.1 \( \mu \)M for toxin B344-807, 2.1 and 2.8 \( \mu \)M for lethal toxin544-807 and 6.7 and 8.9 \( \mu \)M for \( \alpha \)-toxin549-813, respectively. These results indicated that InsP6 has a similar affinity for binding to the CPD of each CGT. The stoichiometry of the complex was 0.81 and 0.85 (toxin B344-807), 0.64 and 0.84 (lethal toxin544-807) or 0.74 and 0.77 (\( \alpha \)-toxin549-813), respectively, indicating that InsP6 binds to all CPDs of the toxins at a ratio of 1:1.

Full-length \textit{C. sordellii} lethal toxin differs in its requirement for \textit{InsP6}-induced auto-catalytic processing - For biochemical characterization of the auto-catalytic cleavage of the full-length clostridial glucosylating toxins, we expressed the proteins in \textit{Bacillus megaterium}. Fig. 3A shows the SDS-PAGE of the purified recombinant proteins. To test the glucosyltransferase activity of the toxins, \textit{in vitro} glucosylation assays were performed with Rac1, Ha-Ras and RhoA in the presence of radiolabeled UDP-[\( ^{14} \)C]glucose (or UDP-[\( ^{14} \)C]GlcNAc for \( \alpha \)-toxin) as a donor substrate. As expected, incorporation of radiolabeled glucose (or GlcNAc) was detected only in Rac1 and RhoA with toxin B or \( \alpha \)-toxin and only in Rac1 and Ha-Ras with lethal toxin (Fig. 3B), confirming same substrate specificity as described for the native toxins (3,26). Moreover, addition of recombinant CGTs to cultured Vero cells induced phenotypic alterations in cell morphology (e.g. cell rounding) that is typical for cytotoxicity (Fig. 3C). This finding implies the proper folding of domains of the recombinant CGTs implicated in the uptake of the proteins into endosomal compartments and the release of the glucosyltransferase domain into the cytosol.

Next, the auto-catalytic processing of the recombinant full length CGTs was studied in \textit{in vitro} cleavage assays at pH 7.4 with increasing concentrations of InsP6. Surprisingly, significant auto-catalytic cleavage of CGTs at physiological concentrations of InsP6 (\( \leq 100 \) \( \mu \)M) was observed only for toxin B and \( \alpha \)-toxin (Fig. 3D). In contrast, lethal toxin exhibited much less auto-catalytic processing at up to 10 mM of InsP6. Also the time course of InsP6 (100 \( \mu \)M)-induced autocatalysis revealed that full-length lethal toxin was much less processed (even after overnight incubation) under the conditions used (Fig. 3E). Obviously, in contrast to CGT fragments, consisting of the glucosyltransferase and the cysteine protease domain, full-length CGTs differed in their susceptibility towards InsP6-induced auto-catalytic processing.

A functional cysteine protease domain is a prerequisite for intoxication of Vero cells by lethal toxin - Since full length lethal toxin showed no auto-catalytic cleavage upon incubation with InsP6 under \textit{in vitro} conditions, we aimed to determine the biological significance of auto-catalytic processing by performing intoxication assays on cultured Vero cells with lethal toxin carrying an inactive cysteine protease domain (C698A-lethal toxin). Accordingly, CPD-inactive toxin mutants were also synthesized for toxin B (C698A-toxin B) and \( \alpha \)-toxin (C707A-\( \alpha \)-toxin). Interestingly, a lack of cytotoxicity compared to wild-type toxins was not only observed for the CPD-inactive toxin B and \( \alpha \)-toxin mutant, but also for the CPD mutant of lethal toxin (Fig. 4A). Therefore, CPD-dependent processing represents an essential step in complete intoxication not only
by toxin B and α-toxin, but also by lethal toxin. The inability of the CDP-inactive toxin mutants to undergo auto-catalytic processing was confirmed in vitro by incubation with InsP6 (Fig. 4B). In addition, to exclude the possibility that the lethal toxin and α-toxin mutants lack cytotoxicity due to general misfolding, a time-dependent in vitro glucosylation assay with recombinant Rac1 was performed. Similar $k_{cat}$ values for wildtype and mutant lethal toxin (912.6 h$^{-1}$, SD ±37.1 h$^{-1}$ vs. 1098 h$^{-1}$, SD ±84.9 h$^{-1}$) and α-toxin (120.1 h$^{-1}$, SD ±29.8 h$^{-1}$ vs. 187.8 h$^{-1}$, SD ±54.6 h$^{-1}$), respectively, were calculated from three time points in the linear range (5, 10 and 15 min), confirming that the CDP-inactive mutants are fully active in glucosyltransferase or GlcNAc-transferase activity (Fig. 4C). The importance of the auto-catalytic cleavage in the intoxication process was demonstrated for toxin A and toxin B previously (17,27).

**InsP6-induced auto-catalytic processing of full-length lethal toxin requires an acidic pH** - Because auto-catalytic processing of full-length lethal toxin was not induced by incubation with InsP6, we wondered whether steric hindrance may prevent binding of InsP6 to the cysteine protease domain. Therefore, we studied the direct interaction of InsP6 with the full-length CGT. Because isothermal titration calorimetry is hampered by the large size of full length toxins (note, very large amounts of toxins would be necessary), we employed a filter binding assay. To this end, recombinant holotoxins were preincubated with radiolabeled $[^{3}H]$InsP6 at neutral pH and non-bound InsP6 was removed by filtration through nitrocellulose filters. These binding studies revealed that significant binding of InsP6 was observed only for full-length variants of toxin B and α-toxin, but not for lethal toxin (Fig. 5A).

CGTs are clathrin-dependently endocytosed to reach low pH endosomes (13). It is widely accepted that the low pH triggers conformational changes within CGTs, thereby enabling the insertion of the toxins into endosomal membranes (28). We speculated that conformational changes induced by low pH may be a prerequisite for InsP6-binding to the cysteine protease domain of lethal toxin. Therefore, we studied the auto-catalytic cleavage of full-length lethal toxin with increasing concentrations of InsP6 at pH 5 and compared the results with the processing of the short toxin fragments (GD-CPD), which consisted of the glucosyltransferase domain and the cysteine protease domain. Interestingly, full-length and GD-CPD fragments of the lethal toxin were processed equally at physiological concentrations of InsP6 (10-100 µM) at low pH (Fig. 5B).

Appropriately, low pH-induced rearrangements within full length lethal toxin enabled binding of InsP6, activation of the cysteine protease domain and subsequent auto-catalytic cleavage. Accordingly, a filter binding assay, performed with InsP6 and full-length lethal toxin at pH 5, revealed binding of InsP6 at low pH conditions (Fig. 5C). Direct comparison of InsP6-binding of lethal toxin with toxin B showed that the latter toxin exhibited maximal binding at pH 7.4 and reduced binding at pH 5 (Fig. 5D). We next asked whether the binding of InsP6 to full-length lethal toxin is reversibly controlled by the pH of the incubation medium. For this purpose, lethal toxin was first incubated in buffer with pH 7.4. The samples were then acidified to pH 5 and, thereafter, the mixture was again brought to pH 7.4, which was followed by the binding assay with radiolabeled InsP6. As shown in Fig. 5E, InsP6 did not bind to full-length lethal toxin after readjustment of the medium from acidic pH to pH 7.4. In contrast, toxin B, which was tested in parallel, did partially bind InsP6 after preincubation in acidic buffer and re-adjustment to neutral pH (Fig. 5E). Moreover, we determined the influence of acidification on binding of InsP6 to the CPD fragment of lethal toxin by isothermal titration calorimetry. This study revealed that at low pH (pH 5) the CPD of lethal toxin exhibited an ~10-fold reduced affinity for InsP6 ($K_d$ values 33.0 µM and 40.4 µM), when compared with results obtained at neutral pH (Figure 5F and 2C). These data indicated that the low pH-shift facilitated binding of InsP6 only to the full length lethal toxin but not to the toxin GD-CPD fragment, supporting the notion that the holotoxin undergoes a major conformational change at low pH, which allows InsP6 binding.

**DISCUSSION**

Here we studied the InsP6-dependent auto-catalytic processing of 3 clostridial glucosylating toxins. Initial studies with short fragments of the toxins, covering the glucosyltransferase (GlcNAc transferase of α-toxin) domain and the cysteine protease
domain, revealed that all toxins needed InsP6 for cleavage. This is not surprising, because the cysteine protease domain of lethal toxin and α-toxin is 78% and 34% identical to the cysteine protease domain of toxin B and the proposed catalytic triad (toxin B: D587/H653/C698) as well as the InsP6-binding sites are highly conserved among the toxins. Accordingly, we determined very similar $K_d$ values for the binding of InsP6 to the toxins’ protease domains. Moreover, the concentration range of InsP6 needed for cleavage was similar, indicating that the mechanism of InsP6-induced activation of the protease activity is similar among all these toxins. In addition, we predicted and confirmed the cleavage sites of all three toxins by mutagenesis studies, indicating that the toxins are split after a conserved leucine residue.

Whereas the short fragments, covering the glucosyltransferase (GlcNAc transferase) and cysteine protease domains of the toxins, exhibited similar properties in respect with InsP6-binding and activation, full length toxins differed significantly. While full length toxin B and α-toxin exhibited InsP6 sensitivity at 1 and 100 µM, respectively, lethal toxin was much more resistant against auto-catalytic cleavage even at high concentrations ($\geq$ 1 mM) of InsP6. Moreover, we were not able to detect significant binding of InsP6 to lethal toxin in filter binding assays, whereas toxin B and α-toxin exhibited InsP6-binding under these conditions. This discrepancy was not caused by misfolding of recombinant Bacillus megaterium expressed proteins, because glucosyltransferase activity as well as cytotoxicity were confirmed, indicating proper folding of the proteins.

To study the impact of toxin processing on cytotoxicity of full length toxins, we changed the catalytic cysteine residues of the CPD to alanine. This resulted in inhibition of cytotoxicity of all three toxins, including lethal toxin, which was largely resistant towards in vitro processing even in the presence of a high concentration of InsP6. These findings indicated that also processing of lethal toxin is essential for full cytotoxicity.

As CGTs translocate from acidic endosomal compartments into the cytosol (11,28,29), we wondered whether incubation at low pH might affect the susceptibility of the toxins towards InsP6. We observed that the lowering of the pH strongly increased the auto-catalytic cleavage of full length lethal toxin in the presence of InsP6. In line with this finding, we observed that the binding of InsP6 to full-length lethal toxin was largely increased at low pH. This effect was most likely caused by conformational changes of the overall structure of lethal toxin and not only by charge effects at the binding site. A decrease in the pH should rather reduce the binding of the negatively charged InsP6 to the toxin. This was confirmed in a direct comparison of the InsP6-binding of full length lethal toxin with full length toxin B at different pH values. Whereas the binding of InsP6 to lethal toxin increased at low pH, the reverse was true for toxin B.

The notion that at low pH the binding of InsP6 to its binding pocket at the cysteine protease is reduced was confirmed by isothermal titration calorimetry with the isolated protease domain of lethal toxin, showing a ~10-fold reduced binding affinity for InsP6. Thus, all these findings indicate that the pH shift causes major conformational changes of the overall structure of lethal toxin, resulting in exposure and/or structural changes of the cysteine protease domain, allowing InsP6-binding.

Major pH-dependent structural changes of lethal toxin have been reported before. Ballard and coworkers showed that the cytotoxicity of C. sordellii lethal toxin is increased in a pH-dependent manner (30). A short pH pulse strongly increased the velocity of cytotoxic effects. His group showed major conformational changes of lethal toxin by fluorescence methods at low pH, which were reversible (30). These data are in agreement with our studies, indicating that at low pH a major conformational change of the toxin exposes the InsP6-binding site, allows binding of InsP6 and subsequent auto-catalytic processing. As processing is important for activity, low pH exposure of the toxin results in increase in cytotoxicity.

More recently, it has been proposed that lethal toxin forms high molecular complexes, which dissociate at low pH and re-associate after increase in pH (31). We suggest that these studies performed with native lethal toxin purified from C. sordellii might be biased by pH dependent impurities, which could affect complex formation. We used recombinant toxins expressed in B. megaterium. By gel filtration of the recombinant full length lethal toxin, we could confirm a monodispers preparation of monomers. Therefore, formation...
of multimeric toxin complexes most likely did not play a major role in our studies. However, we also observed reversibility of the pH–dependent effect. After readjustment of the low pH to pH 7.4, the binding of InsP6 and the processing of the toxin was again inhibited. This finding indicates that the proposed pH-dependent conformational change, which allows InsP6-binding is largely reversible.

Recent studies solved the crystal structure of the glucosyltransferase domains of C. difficile toxin B (32), C. novyi α-toxin and C. sordellii lethal toxin (33), the cysteine protease domain of toxin A (21) and parts of the C-terminal repetitive oligopeptide domain (34,35). However, the structure of the holotoxin is still enigmatic. Related CPD structures are available not only from toxin A but also from Vibrio cholerae MARTX toxin. Notably, both CPDs share conserved lysine residues, which are involved in InsP6 binding. However, the conformation of the bound allosteric activator is largely different in the CPD of toxin A and of MARTX (21,22,36). Because the InsP6-binding properties of the isolated CPD domains of toxin B, α-toxin and lethal toxin is very similar, we do not suggest that major structural differences exists in the CPDs of these toxins. Therefore, structural features located distantly to the CPD may affect the conformation and binding of InsP6 to the CPD in the full length lethal toxin. A recent study with negative stain electron microscopy revealed a model of the holotoxin A and B and proposed conformational changes, occurring at low pH of endosomes (37). In this model the large extension of the C-terminal domain is remarkable, not even excluding interaction with N-terminal structures of the toxins. Especially the C-terminal polypeptide repeat domain, which has an extended solenoid-like structure differs between the various CGTs. Therefore, it remains to be studied whether the C-terminal domain is involved in the different susceptibility of holotoxins towards InsP6.

What is the physiological reason for the unique, low pH-dependent InsP6-binding properties of the lethal toxin? One might speculate that C. sordellii has adopted to environments that frequently exhibit high concentrations of extracellular InsP6. The disintegration of cells in wounds and injured tissues (frequent sites of C. sordellii infection) would allow cytosolic InsP6 to reach extracellular compartments. Thereby, unique structural characteristics of the lethal toxin at neutral pH conditions prevent activation of the cysteine protease domain by InsP6 binding prior to cell entry. Such prerequisites might not be necessary for e.g. C. difficile toxin B, because phytase enzymes are present in the small intestine that hydrolyze extracellular, luminal InsP6 efficiently (38). After endocytosis, however, all clostridial glucosylating toxins are exposed to endosomal acidification, resulting in partial unfolding and/or activation of domains involved in membrane insertion and delivery of the enzyme portions across the endosomal membrane. Eventually, the cysteine protease domain is accessible for InsP6-driven activation, leading to the release of the glucosyltransferase into the cytosol. However, one has to keep in mind that activation of the CPD and release of the glucosyltransferase domain should not occur before entering the cytosol. Otherwise the delivery of the biologically active part is altered. Therefore, it makes sense that the affinity of toxin B for InsP6 is reduced at low pH (e.g., which is observed in endosomes).

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**FOOTNOTES**

We greatly appreciate the excellent technical assistance of Otilia Wunderlich and Sven Hornei. This work was supported by grants from the Deutsche Forschungsgemeinschaft (AK6/16-3).

**FIGURE LEGENDS**

*Fig. 1.* Comparative analysis of CGT fragments, comprising the glucosyltransferase and the putative cysteine protease domain. (A) InsP6-induced *in vitro* cleavage of CGT fragments, comprising the glucosyltransferase domain and the adjacent cysteine protease domain. The toxin fragments were expressed as recombinant proteins in *B. megaterium* and purified. Processing of CGT fragments (2 µg) was induced with increasing concentrations of InsP6 as indicated and incubation for 1 h at 37°C. (B) Alignments of primary sequences of the CGTs representing the putative auto-catalytic cleavage site (↓) and the conserved catalytic cysteine residue of the cysteine protease homology region (shown in bold). (C) Amino acids highlighted in (B) were substituted for each CGT fragment in alanine residues...
and analyzed for auto-catalytic processing in the presence or absence of 100 μM InsP6 and incubation for 1 h at 37°C. (D) *In vitro* glucosylation of Rac1 with CGT fragments and mutants shown in (C), in the presence of radiolabeled UDP-glucose or UDP-N-acetyl-glucosamine (α-toxin), respectively. Samples were subjected to SDS-PAGE and modified Rac1 was visualized by autoradiography.

**Fig. 2.** Binding affinity of InsP6 to the CGTs cysteine protease domain determined by isothermal calorimetry. The respective cysteine protease domains of the CGTs were expressed as recombinant proteins in *B. megaterium* and purified, prior to analysis of the binding of InsP6 at pH 7.4 by isothermal calorimetry. (A) toxin B\textsubscript{544-807}, (B) lethal toxin\textsubscript{544-807} and (C) α-toxin\textsubscript{549-813}.

**Fig. 3.** Functional characterization of recombinant CGTs. (A) *C. difficile* toxin B, *C. sordelli* lethal toxin and *C. novyi* α-toxin were purified from *B. megaterium*, subjected to SDS-PAGE and separated proteins were stained with Coomassie. Bands annotated with arrows represent the respective purified toxins. (B) *In vitro* glucosylation of Rac1, Ha-Ras and RhoA with recombinant CGTs. (C) Intoxication of Vero cells with recombinant CGTs. Processing of CGTs (2 μg) was induced with increasing concentrations of InsP6 as indicated and incubation for 1 h at 37°C. (E) Processing of CGTs (2 μg) was induced with 100 μM InsP6, followed by an incubation period as indicated at 37°C.

**Fig. 4.** Intoxication of cultured Vero cells with CPD-inactive toxin mutants. (A) Cultured Vero cells were incubated with wildtype toxins (toxin B [100 pM], lethal toxin [1 nM] and α-toxin [100 pM]) or CPD-inactive versions (C698A-toxin B [100 pM], C698A-lethal toxin [1 nM] and C707A-α-toxin [100 pM]) and images were obtained after 1 h (toxin B/C698A-toxin B) and 6 h (lethal toxin/C698A-lethal toxin and α-toxin/C707A-α-toxin), respectively. (B) *In vitro* cleavage assay. Wildtype and mutant toxins (2 μg each) shown in (A) were incubated for 1 h at 37°C with 100 μM (toxin B/C698A-toxin B, lethal toxin/C698A-lethal toxin) or 1 mM (α-toxin/C707A-α-toxin) InsP6, followed by SDS-PAGE and visualization of proteins by Coomassie-staining. (C) Time-dependent *in vitro* glucosylation of Rac1. 10 nM of lethal toxin/C698A-lethal toxin or 10 nM α-toxin/C707A-α-toxin was incubated with 5 μM Rac1 in the presence of radiolabeled UDP-glucose or UDP-N-acetyl-glucosamine (α-toxin), respectively. Samples were taken at indicated time points, subjected to SDS-PAGE and modified Rac1 was visualized by autoradiography.

**Fig. 5.** Influence of pH in binding of InsP6 to lethal toxin for induction of auto-catalytic processing. (A) Filter-binding assay. 1 μM recombinant CGT protein was incubated with \[^{3}H\]-InsP6 for 10 min at 4°C. Thereafter, the mixture was given onto nitrocellulose filters and after extensive washing, bound radioactivity was measured by liquid scintillation counting. The glucosyltransferase domain of toxin B (toxin B\textsubscript{1-546}) was included in the assay as a negative control. (B) InsP6-induced *in vitro* cleavage of lethal toxin at low pH. Processing of full length toxin and GD-CPD fragments of lethal toxin was induced at pH 5 with increasing concentrations of InsP6 as indicated and incubation for 1 h at 37°C. (C) Filter \[^{3}H\]-InsP6-binding assay shown in (A) was repeated with lethal toxin and toxin B\textsubscript{1-546} (negative control) at pH 5. (D) Filter binding assay shown in (A) was performed with full length toxin B and full length lethal toxin at various pH conditions as indicated. The highest InsP6-binding values for each toxin were set to 100%. (E) Filter-binding assay in the presence of \[^{3}H\]-InsP6 with lethal toxin and toxin B at pH 7.4, after lowering the pH of the mixture to pH 5 and after incubation of the toxins at pH 5 for 10 min followed by readjustment of the pH from pH 5 to pH 7.4. Bound radioactivity is given in percent of maximal binding (100%). (F) Isothermal calorimetry of the cysteine protease domain of lethal toxin and InsP6 at pH 5. Error bars depicted in (A), (C), (D) and (E) represent standard deviation values (n≥3).
Figure 1

**A**

- InsP$_6$ toxin B$_{1-807}$
- InsP$_6$ toxin B$_{1-543}$
- InsP$_6$ lethal toxin$_{1-807}$
- InsP$_6$ lethal toxin$_{1-543}$
- InsP$_6$ α-toxin$_{1-813}$
- InsP$_6$ α-toxin$_{1-548}$

**B**

- toxin A
- toxin B
- lethal toxin
- α-toxin

**C**

- InsP$_6$ (100µM) toxin B$_{1-807}$
- InsP$_6$ toxin B$_{1-543}$
- InsP$_6$ lethal toxin$_{1-807}$
- InsP$_6$ lethal toxin$_{1-543}$
- InsP$_6$ α-toxin$_{1-813}$
- InsP$_6$ α-toxin$_{1-548}$

**D**

- [C$^{14}$]-Rac1 toxin B$_{1-807}$
- [C$^{14}$]-Rac1 lethal toxin$_{1-807}$
- [C$^{14}$]-Rac1 α-toxin$_{1-813}$
Figure 2
Figure 3

A

B

C

D

E
Figure 4

A

B

C

Figure 4
Figure 5

(A) pH 7.4

(B) pH 5

(C) pH 7.4 pH 5

(D) pH 5 6 7 7.4

(E) pH 7.4 pH 5 pH 6 pH 5 pH 7.4 pH 6

(F) Time (min) pH 5

relative bound InsP6 (% from highest)

relative bound InsP6 (% from highest)

relative bound InsP6 (% from highest)

KCal/Mole of Injectant

Molar Ratio
Clostridial glucosylating toxins: inositol hexakisphosphate-dependent processing of Clostridium sordellii lethal toxin and Clostridium novyi α-toxin
Gregor Guttenberg, Panagiotis Papatheodorou, Selda Genisyuerek, Wei Lü, Thomas Jank, Oliver Einsle and Klaus Aktories

J. Biol. Chem. published online March 8, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.200691

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