Processing of *Clostridium difficile* toxins

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The pathogenicity of *Clostridium difficile* depends on the large clostridial glucosylating toxins A and B (TcdA and TcdB). The proteins accomplish their own uptake by a modular structure comprising a catalytic and a binding/translocation domain. Based on a proteolytic processing step solely the catalytic domain reaches the cytosol. Within the cells, the glucosyltransferases inactivate small GTPases by mono-O-glucosylation. Here, a short overview is given regarding latest insights into the intramolecular processing, which is mediated by an intrinsic protease activity.

**Toxins A and B possess glucosyltransferase activity and inactivate Rho GTPases**

Intracellular targets of the bacterial glucosyltransferases are small GTPases of the Rho family (Just et al., 1995), which comprise a family of about 20 GTP-binding proteins. Rho proteins function as molecular switches and are involved in multiple cellular signalling processes, including regulation of the actin cytoskeleton, adhesion, migration and cell polarity. They control enzyme activities, gene transcription, cell cycle progression and apoptosis (Etienne-Manneville & Hall, 2002). The toxins catalyse the mono-O-glucosylation of the Rho GTPases at a threonine residue (Thr35/37), which is essential for the switch function of the GTPases (Just et al., 1995). Glucosylation blocks the activation of Rho GTPases by their activators (guanine nucleotide exchange factors, GEFs), inhibits interaction with their effectors (e.g. protein kinases and adaptor proteins), blocks their membrane–cytosol cycling and favours membrane binding. The structural basis of the inhibiting effects on
Rho functions is probably a blockade of the active conformation of Rho GTPases by glucosylation (Sehr et al., 1998; Vetter et al., 2000; Geyer et al., 2003). This leads, amongst others, to the depolymerization of the actin cytoskeleton, cell rounding and finally apoptosis (Just & Gerhard, 2004; Voth & Ballard, 2005).

**N-terminus: the catalytic centre**

The biologically active domain, which is delivered into the cytosol, comprises the first 543 aa (Rupnik et al., 2005). The recently solved 3D-structure of this fragment revealed that it was closely related to other bacterial glucosyltransferases belonging to the GT-A family (Reinert et al., 2005). The catalytic core consists of 234 aa and is formed by a mixed α/β-fold with mostly parallel β-strands as the central part. The more than 300 additional residues are mainly helices, of which the first four N-terminal helices are most probably involved in membrane association, therefore assuring close proximity of the enzyme with its substrates. Characteristic for GT-A family members is the DXD motif involved in complexation of manganese ions, UDP and glucose. Mutation of these essential aspartate residues leads to inactivation of the toxin (Busch et al., 1998). The cosubstrate for the bacterial glucosyltransferases is UDP-glucose; only α-toxin from Clostridium novyi utilizes UDP-N-acetylgalcosamine (UDP-GlcNAc) (Selzer et al., 1996). This difference in cosubstrate specificity is based on sterical hindrance by bulky amino acids (e.g. Ile385/Gln385 in toxin B) blocking the catalytic pocket for the larger UDP-GlcNAc. In α-toxin, small serine and alanine residues at the corresponding positions allow UDP-GlcNAc to enter the catalytic cleft (Jank et al., 2005). Little is known so far about the molecular/structural determinants underlying the differences in substrate recognition by different glucosylating toxins. Based on crystallographic and biochemical data, a preliminary docking model has been proposed where the GT-Pases bind to the glucosyltrans-ferases with the same consensus region and in a comparable manner to how they normally bind to effector molecules (Dvorsky & Ahmadian, 2004; Jank et al., 2007a).

The C-terminal region mediates receptor binding

The C-terminus of the ‘B’ domain consists of clostridial repetitive oligopeptides, which are involved in receptor binding. The nature of the receptor has still not been solved, but there are hints for a role of carbohydrate structures in toxin binding. In the case of toxin A, binding to, for example, a galactose- and N-acetylgalcosamine glucoprotein, a membranous saccharide-isomaltase glucoprotein and Galα1-3Galβ1-4GlcNAc in different animal model systems has been reported (Rolfe & Song, 1993; Pothoulakis et al., 1996; Krivan et al., 1986; Tucker & Wilkins, 1991). However, since these structures are absent in a wide variety of sensitive cells and also α-anomeric galactose bonds are absent in human tissue (Larsen et al., 1990), these carbohydrate structures are unlikely to be or cannot be part of the intestinal receptor in humans. Nevertheless, the proposed role of carbohydrates was supported by the recently solved structure of two C-terminal fragments of toxin A and the co-crystallization of toxin A with an artificial trisaccharide (Ho et al., 2005; Greco et al., 2006). These data showed that the C-terminus possesses a solenoid-like structure, consisting of 7 large repeats with 30 residues and 32 small repeats with 15–21 residues. The large and small peptide repeats have single β-hairpin structures with antiparallel β-strands of 5–6 residues. The β-hairpins are connected by loops of 7–10 residues in short repeats, and by 18 residues in long repeats. Each hairpin is rotated by 120°, resulting in a screw-like structure. However, since the identified amino acid residues participating in carbohydrate binding are not conserved in other clostridial glucosylating toxins, the receptor(s) still remains to be identified.

The central translocation domain

The large middle part of the protein toxins makes up more than 50 % of the total size, but little is known about its exact functions. It is characterized by a hydrophobic stretch which is most probably responsible for membrane penetration (transmembrane prediction) (von Eichel-Streiber et al., 1992). Therefore, this region is referred to as the ‘translocation domain’. Deletion studies proved the importance of the hydrophobic region for toxin activity (Barroso et al., 1994). The same report also indicated a large impact of specific residues located inside the translocation domain but outside the hydrophobic region on the cytotoxic activity of the protein. For example, exchange of cysteine 698 to serine or histidine 653 to glutamine in toxin B reduced the cytotoxic titre by about 90 or 99%, respectively. At that time, no molecular explanation for these observations was available, but it was already proposed that these residues may be involved in the uptake and processing of the toxins.

**Uptake of clostridial glucosylating toxins**

Clostridial glucosylating toxins enter eukaryotic target cells according to the ‘short trip model’ of bacterial exotoxin uptake (Sandvig et al., 2004). Following receptor-mediated endocytosis, the acidification of early endosomes by the vesicular H+-ATPase induces a conformational change characterized by an increase in hydrophobicity (Florin & Thelestatm, 1983; Barth et al., 2001; Qa’Dan et al., 2000). This is probably due to a surface exposure of the hydrophobic region, which then enables the corresponding part of the toxin to insert into the membrane and to build a pore through which the catalytic domain can translocate into the cytosol. Pore formation under acidic conditions has been demonstrated for C. difficile toxin A and toxin B (Barth et al., 2001; Giesemann et al., 2006). As mentioned above, solely the N-terminal catalytic domain (aa 1–543) is then released from the early endosomes and reaches the cytosol of eukaryotic cells. This translocation of the ‘A’
domain across the cellular membrane and the release into the cytosol still remains enigmatic. One essential step is the secession of the first 543 aa from the protein under controlled conditions. Where this separation takes place is not clear, neither is the exact nature of the proteolytic activity involved in this process. Just recently, it was demonstrated that this cutting may be accomplished by an intrinsic activity of the toxin itself. Two independent studies identified autoproteolysis activated by dithiothreitol (DTT) and/or myo-inositol hexakisphosphate (InsP₆). One of these studies ascribed the function to a putative aspartate protease domain located in the C-terminal part of the translocation domain (Reineke et al., 2007). The second study reports that the proteolytic activity is based on an intrinsic cysteine protease domain (CPD) located adjacent to the autocleavage site in the N-terminal part of the translocation domain (Egerer et al., 2007).

Identification and biochemical characterization of an intrinsic CPD

The primary sequence of toxin B aa 544–955, a fragment bordered by the N-terminal glucosyltransferase domain (‘GT’) and the hydrophobic, putative transmembrane region (‘HR’, aa 956–1128; see Fig. 1, upper panel) displays a striking sequence similarity to repeat in toxin (RTX) protein toxins and autotransporter adhesins from, for example, *Vibrio cholerae* and *Vibrio vulnificus/Vibrio splendidus*. Although overall similarity is relatively low, ranging from 23 to 25%, the sequence identities concentrate in specific clusters resembling a putative catalytic triad of a cysteine protease (see Fig. 1, lower panel; D₅⁸⁷H₆⁵³C₆⁹₈). This assumption is strengthened by the recent identification and characterization of the corresponding intrinsic CPD within *V. cholerae* RTX (Sheahan et al., 2007).

According to the prediction of an intrinsic proteolytic activity, the degradation of the holotoxin into GT domain and binding/translocation domain can be induced by factors and/or conditions as found in the cytosol. For example, under reducing conditions, toxin A and toxin B show a split product at about 63 kDa, a size corresponding to the isolated N-terminal glucosyltransferase domain. MALDI-TOF analysis confirmed the expected degradation of the holotoxin into the corresponding domains. The onset of autoproteolysis under the influence of DTT points to an intramolecular disulfide bond within toxin B544–955 implicated in the regulation of the CPD.

Data that clearly indicate an essential role of cysteine residues in this process come from the utilization of *N*-ethylmaleimide (NEM), a common inhibitor of cysteine proteases. When NEM is added after onset of proteolytic cleavage achieved by low DTT concentrations, further degradation is inhibited.

InsP₆ induces autoproteolysis of toxin B in a comparable manner, but InsP₆ is more efficient than DTT. Proteolysis starts at lower concentrations and is faster compared to proteolysis with DTT. Interestingly, when varying concen-

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**Fig. 1.** Clostridial glycosylating toxins: primary structure and partial sequence alignment of the catalytic triad of the cysteine protease domain. Upper panel: model of the primary structure of *C. difficile* toxin B with the N-terminal glucosyltransferase domain (GT), the newly identified intrinsic cysteine protease domain (CPD), the central translocation domain, including a hydrophobic region (HR), and the N-terminal receptor binding domain, consisting of clostridial repetitive oligopeptides (CROPs). Due to the multifunctionality of the toxins, which is mirrored by the multidomain structure, we suggest amending the classical denomination ‘AB toxins’ to ‘ABCD toxins’, with ‘A’ for biological activity, ‘B’ for binding, ‘C’ for cutting and ‘D’ for delivery. Lower panel: partial sequence alignment of the putative catalytic triad of toxin B CPD with RTX toxin and autotransporter adhesin from *Vibrio* spp. (as indicated). The alignment is based on a BLAST search with a fragment of toxin B (aa 544–955). Depicted here are typical consensus sequences representing the catalytic triad of a cysteine protease.
trations of InsP_6 are combined with low DTT concentrations, a synergistic effect on the proteolysis is observable.

The exchange of single residues of the putative catalytic triad (D_587H_653C_698) in a fragment of toxin B encompassing aa 1–955 proved the importance of each of these residues for autoproteolysis. When the corresponding ^35_S-labelled polypeptides are produced by in vitro transcription/translation, wild-type toxin B 1–955 undergoes autoproteolysis during or right after translation. In contrast, the point mutants D_587N, H_653A and C_698A are stabilized. Of these, toxin B1–955 C_698A and H_653A are stabilized. Exchange of C_595 has no stabilizing effect and mutation of the autocleavage site (toxin B 1–955 L_543A/G_544A) also results in a stabilized protein. (d) Impact of autoproteolysis on cytotoxicity. Recombinant toxin B and the point mutant C_698A were produced as recombinant glutathione S-transferase fusion proteins as described by Egerer et al. (2007). Wild-type recombinant toxin B displays limited proteolysis during purification, which is absent in the C_698A mutant (see Coomassie-stained SDS-PAGE gels, upper panel). For cell intoxication, HeLa cells were cultivated in Dulbecco’s minimal Eagle’s medium in 12-well cell culture dishes. Recombinant toxin B (rec. toxin B) and toxin B point mutant (C_698A) (1 mg/ml) were applied for 4–6 h at 37 °C. Wild-type toxin B induced complete cell rounding within 4–5 h. The C_698A mutant showed a significantly diminished cytotoxic potential.
show no degradation at all, whereas toxin B\textsuperscript{1–955} D587N is not completely insensitive to degradation. This is probably based on other aspartate and glutamine residues neighbouring D587 which can in part substitute D587 in the catalytic triad. Notably, exchange of an unrelated cysteine (C595) has no stabilizing effect and the mutation of the autocleavage site (L\textsuperscript{442}G\textsuperscript{444}) results in a stabilized protein comparable to the catalytic triad point mutants.

Autoproteolysis is essential for the cytotoxic potential of toxin B. When the catalytic cysteine 698 is mutated in recombinant holotoxin, the corresponding toxin variant is stabilized (comparable to toxin B\textsuperscript{1–955} C698A) and loses its cytotoxicity almost completely. This effect is in line with former observations concerning toxin B mutagenesis (Barroso et al., 1994).

These data (summarized in Fig. 2; see also Egerer et al., 2007) indicate that the translocation domain of clostridial glucosylating toxins comprises a CPD. This intrinsic activity is responsible for the autocalytic processing of the toxins. The proteolysis, which is essential for cytotoxic activity, is activated by reducing conditions and/or InsP\textsubscript{6}.

**Conclusions**

*C. difficile* toxins are large multidomain proteins. Their action depends on a complex uptake mechanism including proteolytic processing (Aktories, 2007). Although the theoretical model of toxin uptake (schematically outlined in Fig. 3) is generally accepted (Sandvig et al., 2004), the precise molecular mechanisms have not been well characterized. However, a major step forward was made with the finding of an intrinsic proteolytic activity of the toxins. This autoproteolytic activity is induced by InsP\textsubscript{6} and/or DTT and is responsible for the separation of the catalytic domain from the holotoxin (Reineke et al., 2007; Egerer et al., 2007). These findings are in line with reports on RTX toxin from *V. cholerae*, which also undergoes autocalytic processing during uptake (Sheahan et al., 2007). The striking similarity between clostridial glucosylating toxins and RTX toxins is limited to the CPD of RTX and concentrates around the putative catalytic residues, e.g. D\textsuperscript{587}H\textsuperscript{655}C\textsuperscript{698} of toxin B. The importance of these residues for autocalytic processing was shown by site-directed mutagenesis. Notably, these residues are conserved in all clostridial glucosylating toxins. Processing does not require the holotoxin, but is also detectable with fragments comprising only the first 955 aa. This suggests against the hypothesis of an intrinsic aspartate protease domain located around a DXG motif at position D\textsuperscript{465} in close vicinity to the C-terminal polypeptide repeats domain (Reineke et al., 2007). In this context, it is noteworthy that the DXG motif is absent in *C. novyi* α-toxin, although this toxin is also autocalytically cleaved under the same conditions. Furthermore, the localization of the CPD in toxin B adjacent to the GT domain is comparable to the CPD flanking the actin-cross-linking domain in RTX toxins. Since, in the case of toxin B, InsP\textsubscript{6} seems to be the physiologically relevant inducer of autoproteolysis, this close proximity of the CPD and GT domain makes sense with regard to the uptake process (see Fig. 3). Here, a cotranslocation of the GT domain and the CPD would guarantee access of the CPD to cytosolic InsP\textsubscript{6} and therefore a controlled onset of proteolysis after complete translocation. The exact role of InsP\textsubscript{6} in this process is still unclear, but since this highly charged, multifunctional molecule seems to have diverse structural effects, an impact on the stability by modulation of toxin conformation seems to be a plausible assumption (Shears, 2001).

Considering the fast propagation of severe nosocomial infections with hypervirulent strains of *C. difficile*, new strategies for therapy are needed (Bartlett & Perl, 2005). Since the pathogenicity of *C. difficile* depends on its glucosylating protein toxins and severity seems to correlate with toxin amount, these molecules may be targets for
therapeutic strategies. Development of glycan-mimicking compounds blocking the receptor-binding domain is a potential approach (Greco et al., 2006). Moreover, the 3D-structure of the catalytic domain of various glucosyltransferases may allow the design of membrane-permeable inhibitors targeting the catalytic domain (Reinert et al., 2005). In any case, further progress in understanding the molecular mechanisms involved in the actions of *C. difficile* toxins will certainly provide new perspectives for development of new strategies against the pathogen and its toxins.

References


