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Point of VIEW

Self-Cutting To Kill: New Insights into the Processing of *Clostridium difficile* Toxins

Klaus Aktories*

Institute for Experimental and Clinical Pharmacology and Toxicology, Albert-Ludwigs-University of Freiburg, D-79104 Freiburg, Germany

ABSTRACT *Clostridium difficile* toxins A and B are the major cause of antibiotic-associated diarrhea and pseudomembranous colitis. The toxins are one of the biggest protein toxins known, but only the N-terminal catalytic domains of the large proteins enter the cytosol. Now, a new study shows that the toxins are processed by autocatalytic cleavage, which depends on the presence of inositol hexaphosphate.

***Corresponding author, Klaus.Aktories@pharmakol.uni-freiburg.de.**

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Clostridium difficile toxins A and B are large proteins of 308 and 269 kDa (*1*) with similar structure and function. The toxins appear to consist of three major domains, an N-terminal enzyme domain possessing glucosyltransferase (GT) activity, a C-terminal cell receptor binding domain, and in the middle a putative translocation domain (*2*). Up to now, uptake and processing of the exotoxins remained largely enigmatic. The toxins bind to membrane receptors of target cells and are then endocytosed (Figure 1). They translocate from acidic endosomes into the cytosol. The toxins form pores, which are most likely involved in membrane crossing (*3*) (Figure 1). However, our knowledge about the precise structure and functions of the pores is still limited. Notably, not the complete toxin but only the N-terminal GT domain is translocated into the cytosol (*4*). Thus, a proteolytic activation of the toxin occurs in the course of its uptake and action. Cleavage of toxin B occurs after residue 543 (*5*). In line with this, microinjection of the N-terminal fragment of toxin B into target cells induces more rapid cytotoxic effects than microinjection of the holotoxin (*5*). Proteolytic activation of protein toxins is not unusual. For example, the binding component protective antigen (PA) of anthrax toxin is activated by proteolytic cleavage caused by a furin-like protease (*6*). But which protease is involved in the activation of the *C. difficile* toxins? A recent report by Reineke and coworkers published in *Nature* offers an unexpected answer to this question (*7*). It turned out that *C. difficile* toxin B is autocatalytically cleaved. The toxin seems to harbor its own protease. As described in the study, the way to this conclusion is a formidable scientific journey. The authors started with porcine splenocytes, which showed strong cleavage of the toxin in cell lysate. Purification, however, resulted in a non-proteinaceous factor, which facilitated autocleavage of the toxin. Further analysis identified inositol phosphates as crucial factors, with inositol hexaphosphate (InsP6) being the most active. Having an essential cofactor for cleavage at hand, the group set out to identify the protease activity in the structure of toxin B. For this purpose, they used 1,2-epoxy-3-(*p*-nitrophenoxy)propane, an aspartate protease inhibitor known to bind covalently to the active site. By mass spectrometric analysis, they succeeded in identifying the active site of the putative aspartate protease in toxin B as the motif DSG at position 1665. Thus, the authors suggested that toxin B is an aspartate protease having exclusively intramolecular protease activity, which acts only on the holotoxin.

As is often the case, exciting findings raise even more exciting questions. Autocleavage appears to be an activation mechanism of all members of the family of clostridial glucosylating toxins, including the *C. sordellii* lethal toxin and *C. novyi* α-toxin, in addition to *C. difficile* toxins A and B. However, *C. novyi* α-toxin does not contain the DSG motif that is predicted to be crucial for aspartate protease activity. What is the precise role of InsP6? It is a highly charged mol-

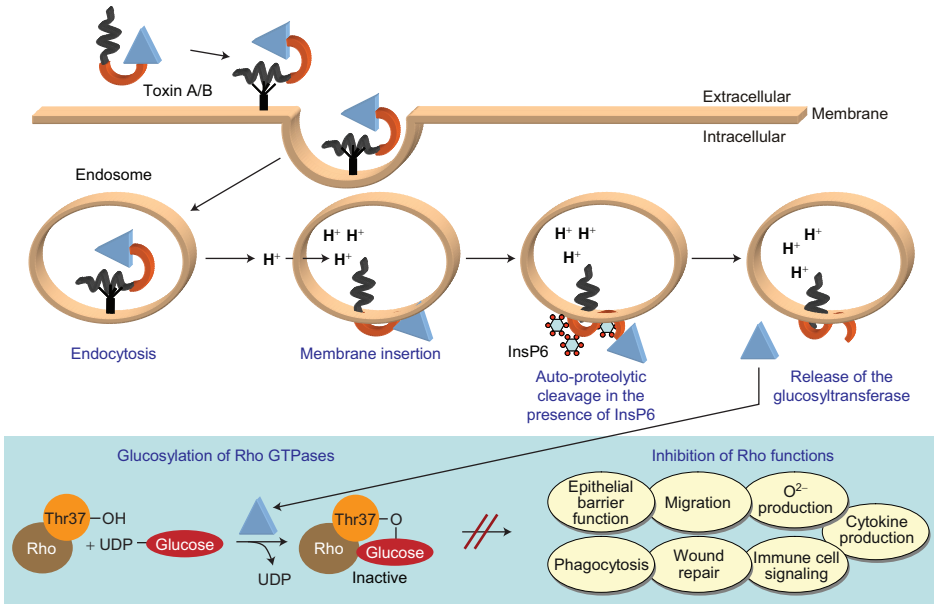

Figure 1. *C. difficile* toxins A and B bind to cell membrane receptors by their C-termini. The nature of toxin A and B receptors is not well defined. In the case of toxin A, carbohydrate structures may be involved. After binding, the toxins are endocytosed. Acidification of endosomes causes conformational changes, which allow insertion of the toxin into membranes and pore formation. According to the report by Reineke *et al.* (*7*), toxin B is processed by autocatalytic cleavage, which depends on the presence of InsP6 and an aspartate protease activity, which is located in the middle of toxin B. Only the GT domain of the N-terminus of the toxins is released into the cytosol. In the cytosol, Rho GTPases are glucosylated at Thr37 (*e.g.*, RhoA) or at Thr35 (*e.g.*, Rac or Cdc42). Glucosylation blocks the active conformation of Rho GTPases and inhibits downstream signaling events controlled by the GTPases. This may result in cell death by apoptosis.

ecule and appears to have diverse functions. It might be involved in stabilization of a toxin protein conformation, which is essential for protease activity, proper cleavage, or both. Many structural effects of InsP6 have been reported. Recently, InsP6 was shown to be essential for assembly for HIV type 1 Gag molecules (*8*). It would be important to know the precise interaction of InsP6 with toxin B. Why is toxin A much less sensitive toward InsP6, and why does it need 1000× higher concentrations (*e.g.*, 10 mM) for effective cleavage? We also want to know where and when the toxin is cleaved. Does cleavage have something to do with the membrane translocation, or is it independent?

Considering recent rapid progress in the analysis of the structure–function relationship of clostridial glucosylating toxins, I am convinced that we will get answers soon. Recently, crystal structure analyses of parts of the C-terminus of *C. difficile* toxin A have been presented. The C-terminus of the toxins is characterized by polypeptide repeats most likely involved in toxin binding to cellular receptors. Analysis of the crystal structure of a C-terminal fragment of 127 residues of toxin A revealed a β-solenoid fold with 32 short repeats of 15–21 residues and 7 long repeats consisting of 30 residues, with each repeat consisting of a β-hairpin followed by a loop (*9*). Moreover, cocrystallization of the polypeptide repeats with a synthetic carbohydrate structure derived from a putative receptor carbohydrate allowed the first insights into the interaction of the toxins with carbohydrate receptor structures (*10*). Recently, the crystal structure of the N-terminal enzyme domain, which is transported into the cytosol, was solved. It has a GT-A family glycosyltransferase structure of the catalytic core (*11*) similar to that of α-1,4-galactosyltransferase and bovine α-1,3-galactosyltransferase. These studies provided a new perspective into the structural requirements of the substrate specificity and the molecular mechanism of the retaining glucosylation reaction catalyzed by toxin B. Without question, the crystal structures of the remaining middle part and the holotoxin of *C. difficile* toxin are urgently needed.

In the cytosol of a target cell, the N-terminal enzyme domain glucosylates small GTPases of the Rho protein family (*12*). We know of ~20 members of this protein family. The best studies are RhoA, Rac, and Cdc42. They are involved in a wide array of cellular functions, including organization of the actin cytoskeleton, enzyme regulation, cell polarity, and control of transcription and proliferation (*13, 14*). The diverse roles of Rho GTPases in immune functions and host pathogen defense designate them as highly efficient targets of bacterial protein toxins (*15*). They control epithelial barrier function, signaling and motility of immune cells, superoxide anion production, or cytokine expression. Rho GTPases are molecular switch proteins, which are inactive in the GDP-bound form and are activated after nucleotide exchange caused by guanine nucleotide exchange factors. Toxins A and B from *C. difficile* glucosylate Rho GTPases at a specific threonine residue (*e.g.*, Thr37 in RhoA, B, and C and Thr35 in Rac or Cdc42), a modification essential for their switch functions (*12*). Toxin-catalyzed glucosylation prevents the structural change of Rho GTPases into their active conformation, thereby blocking all downstream signaling that is controlled by Rho GTPases (*1*).

Why do we need to know more about these toxins? Antibiotic-associated diarrhea and pseudomembranous colitis are major complications of antibiotic therapy. Toxins A and B produced by *C. difficile* are responsible for these diseases (*16*–*18*). Studies suggest that antibiotic-induced changes in the microflora of the gut allow *C. difficile* to proliferate and to produce potent cytotoxins, which cause damage to the mucosa. The incidence and severity of *C. difficile*-associated disease are dramatically increasing and culminated recently in endemic outbreaks of nosocomial infections with hypervirulent *C. difficile* strains, which produced a 10–20× higher amount of toxin and caused cumulative death rates of up to 16% (*19, 20*). Therefore, more knowledge about the toxins will help to develop efficient strategies against this pathogen and its toxins. Analyses of the toxin uptake and its processing are an important and essential prerequisite for this aim.

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