Another surprise in receptor binding of *C. difficile* toxins

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Received: March 31, 2022; Accepted: May 15, 2022; Published Online: May 18, 2022; https://doi.org/10.1016/j.xinn.2022.100261

Citation: Aktories K. (2022). Another surprise in receptor binding of *C. difficile* toxins. The Innovation **3(4)**, 100261.

Clostridioides difficile (formerly named *Clostridium difficile*) is the dominant causative agent of a spectrum of illnesses, which frequently occur as the consequence of antibiotic treatment of patients. *C. difficile* infections (CDIs) cause mild to severe diarrhea (so-called antibiotic-associated diarrhea) but also pseudomembranous enterocolitis with complications like toxic megacolon, bowel perforation, and death. Three protein toxins are involved in the pathology of CDIs, toxin A (TcdA) and toxin B (TcdB), which are the prototypes of large clostridial glucosylating toxins, and *C. difficile* ADP-ribosyltransferase CDT. TcdB is most likely the driving toxin responsible for the major pathology of the infection.

Recent studies showed that *C. difficile* exhibits considerable genome diversity, requiring the classification into different *C. difficile* clades (at least 5 major clades), which may have different impact on infections. Beside others, *C. difficile* clade 2 is of special interest because it contains strains like ribotype 027 (NAP1), which caused major clinical outbreaks with high morbidity and mortality. The enormous diversity in *C. difficile* strains leads to the question of diversity in toxins. Indeed, numerous subtypes of TcdB were described, which appear to differ in cell targeting and toxicity. At least 8 toxin subtypes (TcdB1–8) with >200 different members have been reported for TcdBs. All toxins (including TcdBs and all other "large clostridial glucosylating toxins") share a very similar overall structure and consist of 4 major domains: a glucosyltransferase domain (GTD) at the N terminus, a cysteine protease domain (CPD), a delivery/receptor-binding domain (DRBD), and a so-called combined repetitive oligopeptides (CROPs) domain at the C terminus (Figure 1). The GTDs of all toxins modify small GTPases of the Rho/Ras family, mainly resulting in inhibition of Rho-dependent signaling.[1] However, it is now clear that the biological activities of the various toxins are not identical, a fact that is most important for therapy of CDIs.

Up to now, several receptors have been described for TcdB toxins, including chondroitin sulfate proteoglycan 4 (CSPG4) and the heptahelical Frizzled receptors (FZD 1, 2, and 7).[2] For both receptor types, which bind independently from each other, the pathophysiological relevance is evident. In addition, nectin 3 (also called PVRL3) and low-density lipoprotein receptor-related protein 1 (LRP1) have been described as potential receptors, but their roles in toxin functions are not clear. Recently, in a mouse model, it was shown that various clade 2 *C. difficile* strains including the hypervirulent ribotype 027 strains cause severe

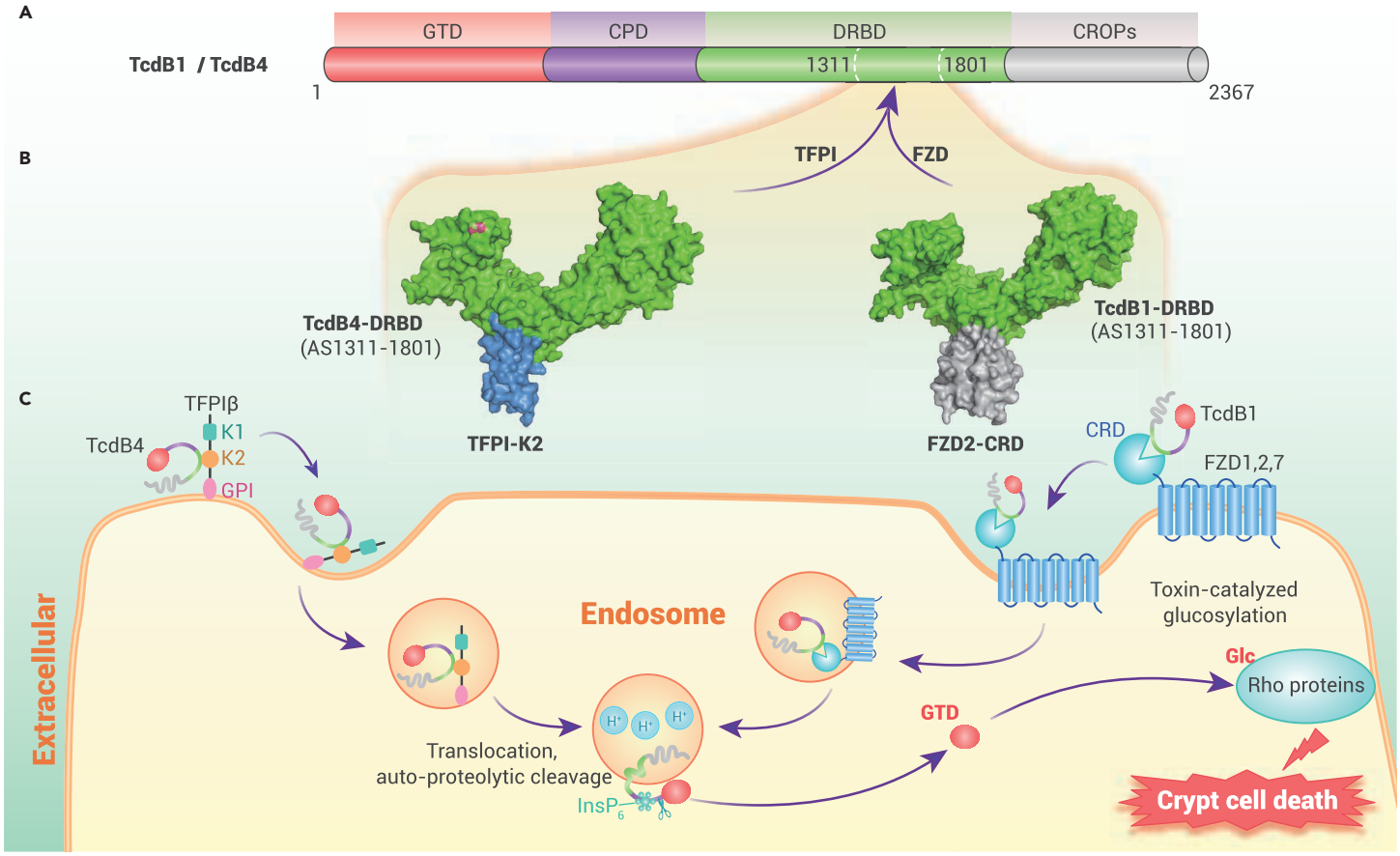

Figure 1. Receptor binding and action of *C. difficile* TcdB1 and TcdB4 (A) Cartoon of the structure of TcdB1 and TcdB4. Both toxins consist of 4 major parts: the glucosyltransferase domain (GTD), the cysteine-protease domain (CPD), the delivery/binding domain (DRBD), and the C-terminal combined repetitive oligopeptides (CROPs) domain. The interaction of TcdB1 and TcdB4 with their receptor proteins Frizzled (FZD) and tissue factor pathway inhibitor (TFPI), respectively, occurs at the identical protein region around residues 1,431–1,606. (B) The structures show the complexes of the toxin regions covering residues 1,311–1,801 (AS 1,311–1,801) of TcdB4 with Kunitz-2 domain of TFPI (TFPI-K2) and of TcdB1 with the CRD of FZD2. Pictures were designed from PDB: 6C0B and 7V1N by PyMol. The interaction sites of both toxins with their specific receptors are practically identical. (C) Scheme of the action of TcdB1 and TcdB4 on target cells. TcdB4 binds to the K2 domain of TFPIβ. TFPIβ is attached to the cell membrane by a GPI anchor. TcdB1 binds to the extracellular CRD domain of the heptahelical receptors of the FZD family (FZD1, 2, and 7). After binding, both toxin-receptor complexes are endocytosed. At low pH of endosomes, the toxins insert into endosomal membranes and translocate their GTD and CPD domains into the cytosol. Here, the CPDs are activated by inositol hexakisphosphate (InsP6) and release GTD. The GTDs of both toxins glucosylate (Glc) mainly small GTPases of the Rho family, which results in inhibition of numerous signal pathways, which depend on Rho GTPases, thereby causing, for example, destruction of the actin cytoskeleton and eventually cell death.

colonic damage with massive stem cell injury by subtype toxins, which do not bind to FZD receptors.[3] So far, FZD binding and inhibition of FZD-dependent Wnt signaling has been attributed to stem cell damage. By contrast, CSPG4, the other well-known toxin receptor, is not expressed in colon epithelium but rather in subepithelial myofibroblasts and is probably not involved in stem cell damage, which might be of major impact for the pathology of CDIs. What, then, is the relevant toxin receptor?

Now, using CRISPR-Cas9-dependent genome-wide screening, Liang Tao, Yanyan Li, and co-workers show that tissue factor pathway inhibitor (TFPI) is a colonic crypt receptor for TcdBs from hypervirulent clade 2 *C. difficile* strains.[4] Especially TcdB4, another clade 2 toxin, which is ~85% identical with the prototype TcdB1, depends totally on the TFPI receptor. The essential role of TFPI in binding and action of clade 2 toxins was convincingly verified by various knockout cell and animal models.

TFPI is well known for its role as an anticoagulant protein produced primarily by endothelium and megakaryocytes. It inhibits coagulation factor Xa, the function of the TF-FVIIa complex and the initial prothrombinase complex.[5] TFPI occurs in two alternatively spliced isoforms, TFPIα and TFPIβ. TFPIα is secreted and found in plasma and on cell membranes. It consists of three multivalent Kunitz domains (K1–3) and a basic C terminus and is most likely responsible for membrane attachment. TFPIβ has only K1,2 domains but possesses a GPI anchor for membrane insertion. The K1 domain of TFPI binds FVIIa, and the K2 domain ($TFPI^{K2}$) binds and inhibits FXa. The researchers found that TcdB binds both TFPI isoforms at their K2 domain (and blocks interaction with FXa). TFPIβ might be particularly important as a receptor for TcdB because the CRISPR-Cas9-dependent genome-wide screenings found, in addition to TFPI, numerous enzymes involved in GPI-anchor formation. Using cryoelectron microscopy (cryo-EM), the interaction of full-length TcdB4 with TFPI at high resolution (3.1–3.7 Å) was analyzed, revealing that the $TFPI^{K2}$ binds to a convex region of the delivery domain of TcdB4, forming a receptor-binding interface (RBI) that covers residues 1,431–1,606 of TcdB4. This part is identical with the region for binding of the FZD receptor by toxin subtype TcdB1. They identified amino acids that favor binding to TFPI and block binding to FZD, and vice versa. Moreover, phylogenetic analysis of various TcdBs revealed 2 major toxin classes, with class I RBIs common in TcdB1, TcdB3, and TcdB5 and class II interfaces for binding of TcdB2, TcdB4, TcdB6, and TcdB7, which mainly represent clade 2 *C. difficile* toxins. Knockout of TFPI resulted in increased resistance of the cells toward TcdB2, TcdB4, TcdB6, and TcdB7. Worth mentioning is that an equivalent, almost identical region of the related TcsL toxin from *Paeniclostridium sordellii* (formerly *Clostridium sordellii*), sharing ~76% identity with TcdB1, is involved in binding to Semaphorin A and B, the receptors of TcsL.

In human intestine, TFPI is highly expressed in endothelial and colon crypt cells. In wild-type mice, TcdB4 caused acute kidney damage and death after intraperitoneal (i.p.) injection. By contrast, in Tfpiβ-knockout (KO) mice, TcdB4 was much less toxic and showed normal kidneys. Further studies revealed the important role of TFPI in TcdB2 intoxication. While co-injection of TcdB4 with the construct $TFPI^{K2}$-Fc in ligated colon segments efficiently prevented colon crypts damage, the decoy-based inhibition of TcdB2 was suboptimal, suggesting that TcdB2 could damage gut tissue via its CSPG4 receptor. Therefore, a fusion construct consisting of domain K2 of TFPI and the first repeat of CSPG4 (residues 410–551), which is involved in toxin binding, was employed and showed enhanced protection against colon damage. Thus, it is speculated that such constructs might be of therapeutic value in clade 2 CDIs.

Taken together, the recent findings are a significant step forward in the understanding of the pathology of CDIs and give answers to important questions about the diversity and evolutionary development of *C. difficile* toxins and the variability of toxin effects. The new results may open novel perspectives for treatment of CDI. Moreover, CRISPR-Cas9-dependent screenings and cryo-EM analyses of toxin-receptor complexes may hopefully lead to further "surprising" results on receptor binding of other subtypes of TcdBs and related large clostridial glycosylating toxins.

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DECLARATION OF INTERESTS

The author declares no competing interests.