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Receptor binding mechanisms of *Clostridioides difficile* toxin B and implications for therapeutics development

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Abstract

Clostridioides difficile is classified as an urgent antibiotic resistance threats by the CDC. *C. difficile* infection (CDI) is mainly caused by the *C. difficile* exotoxin TcdB, which invades host cells via receptor-mediated endocytosis. However, many natural variants of TcdB have been identified including some from the hypervirulent strains, which pose significant challenges for developing effective CDI therapies. Here, we review the recent research progress on the molecular mechanisms by which TcdB recognize Frizzed proteins (FZDs) and chondroitin sulfate proteoglycan 4 (CSPG4) as two major host receptors. We suggest that the receptor-binding sites and several previously identified neutralizing epitopes on TcdB are ideal targets for the development of broad-spectrum inhibitors to protect against diverse TcdB variants.

Keywords

Clostridioides difficile ; *Clostridioides difficile* infection (CDI); bacterial toxin; virulence factor; host receptor; host-pathogen interaction; receptor mimicry; antibody; nanobody; single-domain antibody

Introduction

Clostridioides difficile (formerly *Clostridium difficile*, or *C. difficile*) is a family of Gram-positive, spore-forming anaerobic bacteria, whose spores are widespread and can colonize human colons when normal gut microbiota is disrupted due to antibiotic treatment or other nutritional and medical conditions. *C. difficile* infection (CDI) is the most frequent cause of healthcare-acquired gastrointestinal infections and death in developed countries [1, 2]. Two homologous large exotoxin, TcdA and TcdB, are the major virulence factors of *C. difficile*, among which TcdB alone is capable of causing the full-spectrum of diseases associated with CDI in humans [3-5].

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Author contributions

P.C. prepared the figures and P.C. and R.J. wrote the manuscript.

Competing interests

A patent application entitled “A Broadly Neutralizing Molecule Against Clostridium Difficile Toxin B” has been filed by The Regents of the University of California with P.C. and R.J. as inventors.

However, the development of therapeutics, vaccines, or diagnostic agents against TcdB has met great challenges in the face of recent findings that TcdB has greatly diversified throughout its entire primary sequence during evolution [6-8]. For example, a variant of TcdB (TcdB2) expressed by some hypervirulent strains displays a ~8% sequence variation in comparison to the prototype TcdB from the reference strain (TcdB1) [7-10]. The sequence variations have profound impacts on TcdB activity and pathogenicity. For example, many clinically important TcdB variants, represented by TcdB2, showed altered receptor binding capabilities [6, 8, 11-14]. Bezlotoxumab, the only FDA approved anti-TcdB antibody, showed ~200-fold lower potency on neutralizing TcdB2 than TcdB1 [15-18]. As additional TcdB variants are expected to continuously emerge, there is an urgent need for novel approaches to develop broad-spectrum therapeutics. In this review, we focus on the recent advances in understanding the receptor recognition strategies for a broad range of TcdB variants and the identification of several key neutralizing epitopes on TcdB, which should provide novel insights into therapeutic development against CDI.

Structural basis for recognition of FZDs and CSPG4 by TcdB

Each TcdB molecule (~270 kDa) is composed of four structurally and functionally distinct modules, including an N-terminal glucosyltransferase domain (GTD), a cysteine protease domain (CPD), a delivery and receptor-binding domain (DRBD), and a large C-terminal combined repetitive oligopeptides domain (CROPs) [19]. The disease symptoms of CDI are mainly caused by the GTD, which is delivered by TcdB to the cytosol of infected cells. GTD modifies and inhibits the functions of Rho family small GTPases, leading to actin cytoskeleton damage and ultimately cell death [3, 20]. However, it is the host receptors that offer a cellular “doorknob” for TcdB to enter cells. More specifically, TcdB exploits the Wnt receptor Frizzled (FZD) members 1, 2, and 7 and chondroitin sulfate proteoglycan 4 (CSPG4) as high-affinity receptors for binding and entering host cells [18, 21-24]. Therefore, it is crucial to understand how TcdB recognizes FZD and CSPG4, which will inform new strategies to neutralize TcdB by blocking it from entering cells.

We reported the crystal structure of a fragment of TcdB1 in complex with the extracellular cysteine-rich domain (CRD) of FZD2 in 2018 (Figure 1A) [25], which was also discussed in one of our recent reviews [26]. The FZD-binding site is located in the middle of the DRBD of TcdB. But besides the traditional protein-protein interactions, TcdB exploits an endogenous fatty acid as a co-receptor, which is completely buried between FZD2 and TcdB through extensive hydrophobic interactions with both proteins, to enhance binding affinity and specificity toward FZD1, 2, and 7 (Figure 1A) [25]. This lipid is bound in a hydrophobic groove on the CRD, which is also the docking site for a palmitoleic acid (PAM) lipid modification of Wnt [27]. TcdB binding would lock this endogenous lipid in the PAM-binding pocket on the CRD, which is therefore unavailable to engage the PAM of Wnt. Using this strategy, TcdB not only gains access to host cells via FZDs, but also inhibits Wnt signaling that may contribute to pathogenesis of CDI.

More recently, we determined the structure of TcdB1 in complex with CSPG4 using cryogenic electron microscopy (cryo-EM) (Figure 1B) [18]. CSPG4 is a large glycosylated single transmembrane protein (~251 kDa). Nevertheless, our structure reveals that only the

first CSPG repeat of CSPG4 (termed Repeat1, residues 410–551) is mainly responsible for TcdB binding, which was also confirmed by a crosslinking mass spectrometry (XL-MS) study [18]. In spite of its small size, the Repeat1 of CSPG4 binds at a strategic location on TcdB where multiple TcdB domains converge, and it direct interacts with TcdB amino acids spreading across the CPD, DRBD, CROPs, and a hinge region connecting the DRBD and CROPs. Since TcdB holotoxin has a flexible conformation especially its CROPs [19, 28], the multiple structural units of TcdB would need to properly organize in order to form the composite binding site to accommodate CSPG4, which is supported by the real-time binding studies showing a relatively slow binding on-rate. Nevertheless, once Repeat1 is engaged with TcdB, the complex is very stable as evidenced by their slow binding off-rate.

On the context of TcdB holotoxin, the binding sites for FZDs and CSPG4 are completely separated and ~78 Å away from each other, which would permit simultaneous binding of both if they are expressed on the same cell surface (Figure 1C). However, either FZDs or CSPG4 is sufficient to mediate cell entry, as our structure-based mutagenesis studies demonstrate that FZDs and CSPG4 act as independent receptors for TcdB, which is consistent with results from Gerhard and colleagues [13]. Since CSPG4 and FZDs have different tissue distribution *in vivo* [21, 29, 30], the receptor binding strategy of a particular TcdB variant that is capable of targeting FZDs, CSPG4, or both would partly determine the pathogenicity of the *C. difficile* strain that produces it.

CSPG4 is a conserved receptor for diverse TcdB variants

Based on the structure of the TcdB–CRD2 complex, we found that a single mutation at F1597 of TcdB1, a key residue that simultaneously binds CRD2 and the lipid co-receptor, is sufficient to abolish FZD binding [25], which was later confirmed by other groups [12, 13, 31]. Interestingly, based on sequence analysis of 206 unique TcdB variants, we found that ~22% of TcdB variants including TcdB2 have this Phe residue replaced by a Ser residue, suggesting they have lost FZD binding capability during evolution (Figure 2A and 2C) [6, 8, 11-14]. Therefore, these TcdB variants will have to rely on another receptor for cell entry.

In contrast to sequence variations observed at the FZD-binding site, the CSPG4-binding site is highly conserved among most known TcdB variants (Figure 2B) [8, 18]. Notable variations are only observed at four CSPG4-binding residues (e.g., I1809, D1812, V1816, and N1850 of TcdB1) at frequencies between 11.7% and 27.7%. Among these four sites, residues I1809, V1816, and N1850 are likely more tolerable for amino acid substitutions, because TcdB2 has variations at all three site (I1809L, V1816I, and N1850K), but is still able to bind CSPG4 with a high affinity [18]. However, residue D1812 of TcdB is crucial for CSPG4 binding as a D1812G mutation was sufficient to abolish TcdB1 binding to CSPG4 [18]. Sequence analysis showed that 14 of the 206 known TcdB variants (~7%) have the D1812G substitution, indicating that these TcdB variants may have lost the CSPG4 binding ability (Figure 2D). Since these 14 TcdB variants all have a well-preserved FZD-binding site, we believe they may only use FZDs as their receptor. Intriguingly, we found that ~5% of TcdB variants have D1812H or D1812K substitutions, and these TcdB are predicted to have lost FZD binding due to mutations at their FZD-binding sites (Figure 2E). We also notice that these TcdB variants have other residue substitutions on or around

the CSPG4-binding interface, although those residues are not directly involved in CSPG4 binding based on the structure of the TcdB1–CSPG4 complex. We envision that some of these TcdB variants may have evolved additional amino acid changes besides D1812H/K to partly compensate for the loss of D1812 toward CSPG4 binding. If not, some of these TcdB variants may have dramatically decreased toxicity due to the loss of both receptors, or have to evolve to bind a yet unknown host receptor.

In summary, our structure and sequence analyses suggest that ~71% of TcdB variants could bind to both FZD and CSPG4; ~17% and ~7% could only use CSPG4 or FZD as their receptor, respectively; while the receptor-binding strategies for the rest ~5% of TcdB variants remain unknown (Figure 2E). Interestingly, *C. sordellii* lethal toxin (TcsL), another member in the family of large clostridial glucosylating toxins (LCGTs) with ~76% sequence identity to TcdB1, does not bind FZD or CSPG4, but exploits semaphorins (SEMA6A and SEMA6B) as its receptors. Moreover, the semaphorins-binding interface on TcsL is structurally homologous to the FZD-binding site on TcdB1 [32, 33]. Will a *C. difficile* strain evolve a TcdB variant that targets a yet unknown host receptor, and if so how will that affect the pathogenesis of CDI? These intriguing questions await to be uncovered in future research.

Sites of vulnerability on TcdB are ideal therapeutic targets

Clearly, the receptor-binding sites on TcdB discussed above are among the weakest spots on TcdB, which could be blocked by biologics or small molecules to stop TcdB from cell entry. For example, TcdB binding to FZDs could be directly inhibited by the CRD of FZD2 [21] or a DARPin (designed ankyrin repeat protein) that occupies the FZD-binding site on TcdB [34]. However, it would be more complicated to pharmacologically block CSPG4 binding due to the unique configuration of the composite CSPG4-binding site on TcdB. In fact, while bezlotoxumab could inhibit CSPG4 binding, its epitopes are completely separated from the CSPG4-binding site (Figure 3A and 3B) [16, 17]. In the context of TcdB holotoxin, only one of the two bezlotoxumab epitopes located on the CROPs is fully accessible for bezlotoxumab, while the other one is partially masked by the GTD and the DRBD [16-19]. In order to occupy both epitopes, bezlotoxumab will force the CROPs domain to reorient, which will subsequently abolish the composite CSPG4-binding site [18]. We envision that CSPG4 binding can be disturbed by alteration of TcdB conformation by other steric interference. In contrast to the allosteric inhibition displayed by bezlotoxumab, two DARPins were found to directly compete with CSPG4 by occupying sites on TcdB that overlap with the CSPG4-binding pocket (Figure 3C) [34, 35].

In the last few years, several TcdB neutralizing antibodies and camelid single-domain antibodies (a.k.a. VHHs or nanobodies) that target regions outside the receptor-binding sites have been reported. For example, VHH-5D binds to the pore forming region on TcdB, prevents the pore formation of TcdB under acidic condition, and therefore inhibits the delivery of the GTD to the cytosol; and VHH-7F binds TcdB at the connecting region between the GTD and the CPD and inhibits the InsP6-induced auto cleavage (Figure 3D) [19]. Furthermore, three distinct neutralizing epitopes were identified in the GTD, which are targeted by VHH-E3, mAb PA41, and mAbs ViF087-A10 and SH1429-B1, respectively

(Figure 3B and 3D) [19, 36, 37]. Based on epitope mapping by peptide array, the core binding epitopes for mAbs ViF087-A10 and SH1429-B1 were identified in a region on the GTD that is involved in recognizing Rho GTPases [37, 38]. Therefore, these two mAbs likely neutralize TcdB by inhibiting substrate binding to the GTD. Interestingly, VHH-E3 and mAb PA41 do not directly inhibit the glucosyltransferase activity of the GTD, nor do they interfere with GTD binding to Rho GTPases [19, 36, 38]. While the detailed mechanisms are not fully understood, it is believed that VHH-E3 that binds to the N-terminal 4-helix bundle on the GTD may disrupt the plasma membrane association of the GTD, and PA41 may block the translocation of the GTD across the endosomal membrane [19, 36]. We expect more neutralizing epitopes on TcdB will be identified in the near future as many neutralizing mAbs against TcdB have been reported and their epitopes have been mapped to various domains of TcdB [39]. We suggest that these neutralizing epitopes, especially the ones that are highly conserved among TcdB variants, and the FZD/CSPG4-binding sites on TcdB are among the most vulnerable parts of the toxin and therefore the ideal therapeutic targets to disarm TcdB.

Future prospects

Administration of bezlotoxumab as a form of passive immunity could reduce the recurrence rate of CDI in patients [40], which underscores the importance of TcdB in causing the symptoms of CDI. However, bezlotoxumab-escaping mutations have been observed in many TcdB variants including some from the hypervirulent strains [6-8, 18], which significantly decrease the neutralization efficacy of bezlotoxumab toward these TcdB variants [8, 15, 16]. The DARPins that block TcdB binding to CSPG4 also see amino acid substitutions in their epitopes among different TcdB variants [34, 35]. These findings suggest that the sequence diversification of TcdB is very hard to be fully addressed by conventional antibody development considering that new TcdB variants will continuously emerge.

We believe that a more efficient strategy to achieve a broad-spectrum protection is to target the receptor-binding sites on TcdB, as receptor binding is an indispensable step during the cell entry of toxin. The advantage of this strategy is evidenced by the fact that ~95% of all known TcdB variants use either CSPG4, FZD, or both as receptors, and that the CSPG4-binding site is highly conserved in at least 88% of all known TcdB variants. In our recent studies, we found that the Repeat1 as a CSPG4 mimic could potently neutralize both TcdB1 and TcdB2 in a cell-based assay and protect mice from both TcdB1 and TcdB2 [18]. As ~71% of the known TcdB variants could target both CSPG4 and FZDs, we further suggest that the Repeat1 of CSPG4 and the CRD of FZDs could be combined into a hybrid molecule, which should have higher neutralizing potency against TcdB due to synergistic binding of both components (Figure 3E). This bi-specific decoy receptor would be difficult for TcdB to escape, and if it does happen, the new TcdB variants will unlikely be able to recognize CSPG4 or FZD for cell entry. One could envision that such a decoy receptor would have high specificity and affinity, and broad protection activities against diverse *C. difficile* strains.

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Abbreviations

CDI	*Clostridioides difficile* infection
CPD	cysteine protease domain
CRD	cysteine-rich domain
CROPs	combined repetitive oligopeptides domain
CSPG4	chondroitin sulfate proteoglycan 4
DRBD	delivery and receptor-binding domain
FZD	frizzled
GTD	glucosyltransferase domain
PAM	palmitoleic acid
TcdA	*C. difficile* toxin A
TcdB	*C. difficile* toxin B
VHH	variable domain (VH) of a heavy-chain antibody

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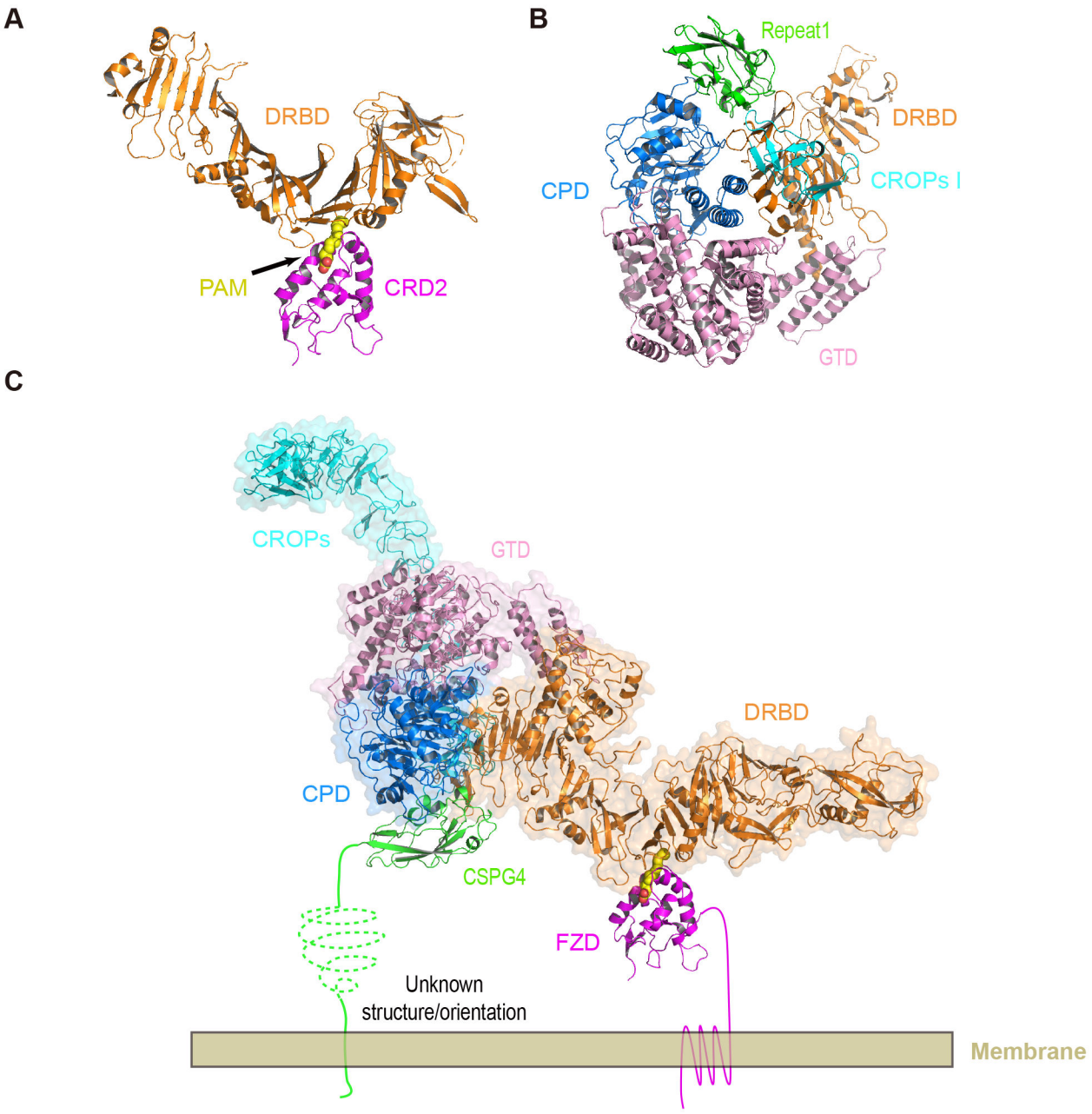

Fig. 1. FZDs and CSPG4 are two independent receptors for TcdB.

(A) Overall structure of a fragment of the DRBD of TcdB1 (orange) in complex with the CRD of FZD2 (CRD2, pink) (PDB:6C0B). The co-receptor PAM is shown as yellow spheres. (B) The cryo-EM structure of TcdB1 (GTD, pink; CPD, blue; DRBD, orange; CROPs I cyan) in complex with the Repeat1 (green) of CSPG4 (PDB:7ML7). (C) A structure model of simultaneous binding of FZD and CSPG4 to TcdB. The model was made by superimposing the structures of the TcdB–PAM–CRD2 complex and the TcdB–Repeat1 complex to TcdB1 holotoxin (PDB:6OQ5).

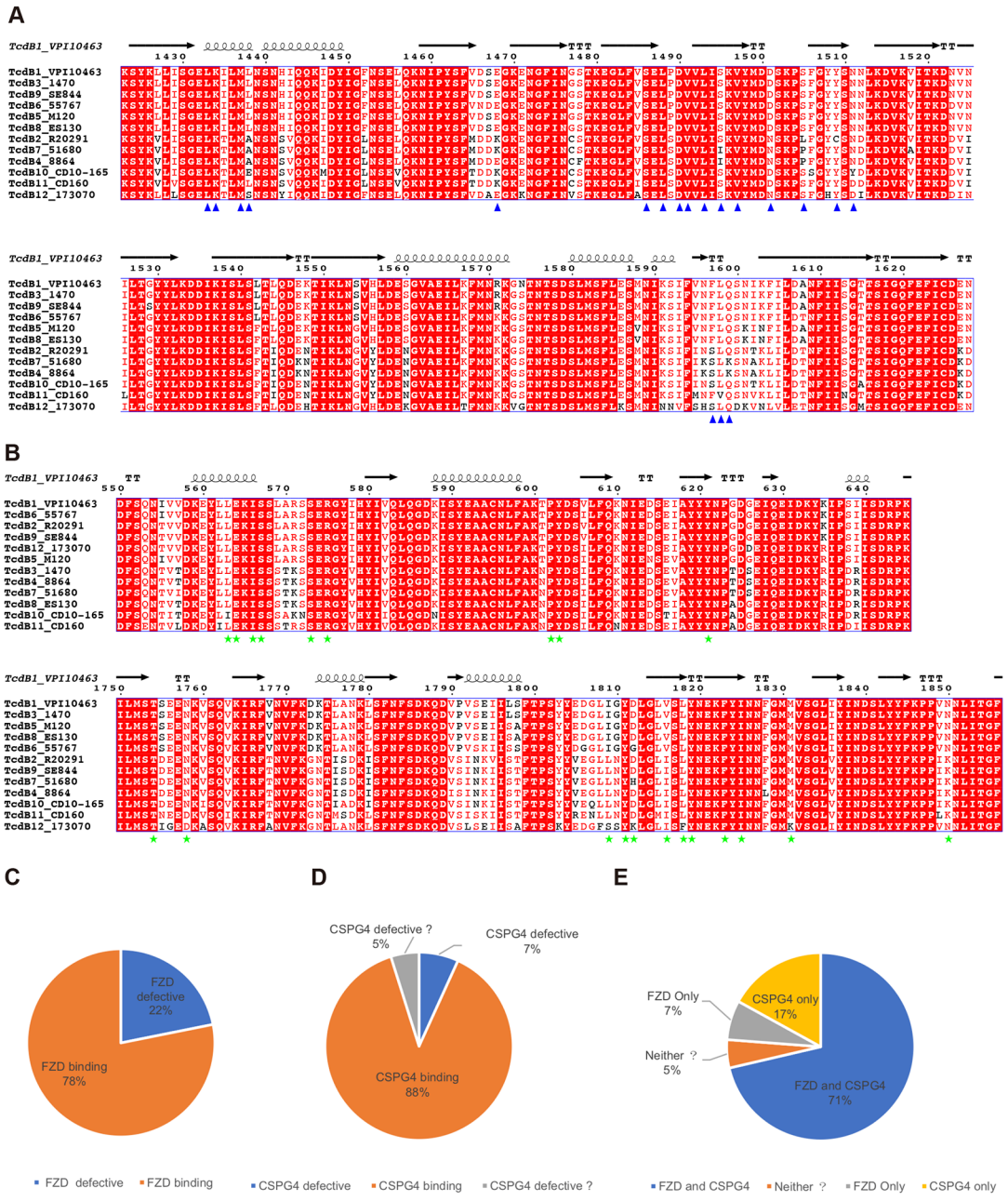

Fig. 2. The conservation of the FZD- and CSPG4-binding sites on diverse TcdB variants.

Sequence alignments among 12 major TcdB subfamilies focusing on the FZD-binding site (A) and the CSPG4-binding site (B). A representative sequence from each of the 12 TcdB subfamilies was used for analysis. The blue triangles in (A) indicate the FZD-binding residues, and the green stars in (B) indicate the CSPG4-binding sites. (C-E) Summaries of the frequencies of TcdB using different receptors based on analysis of 206 TcdB variants available in DiffBase [8]

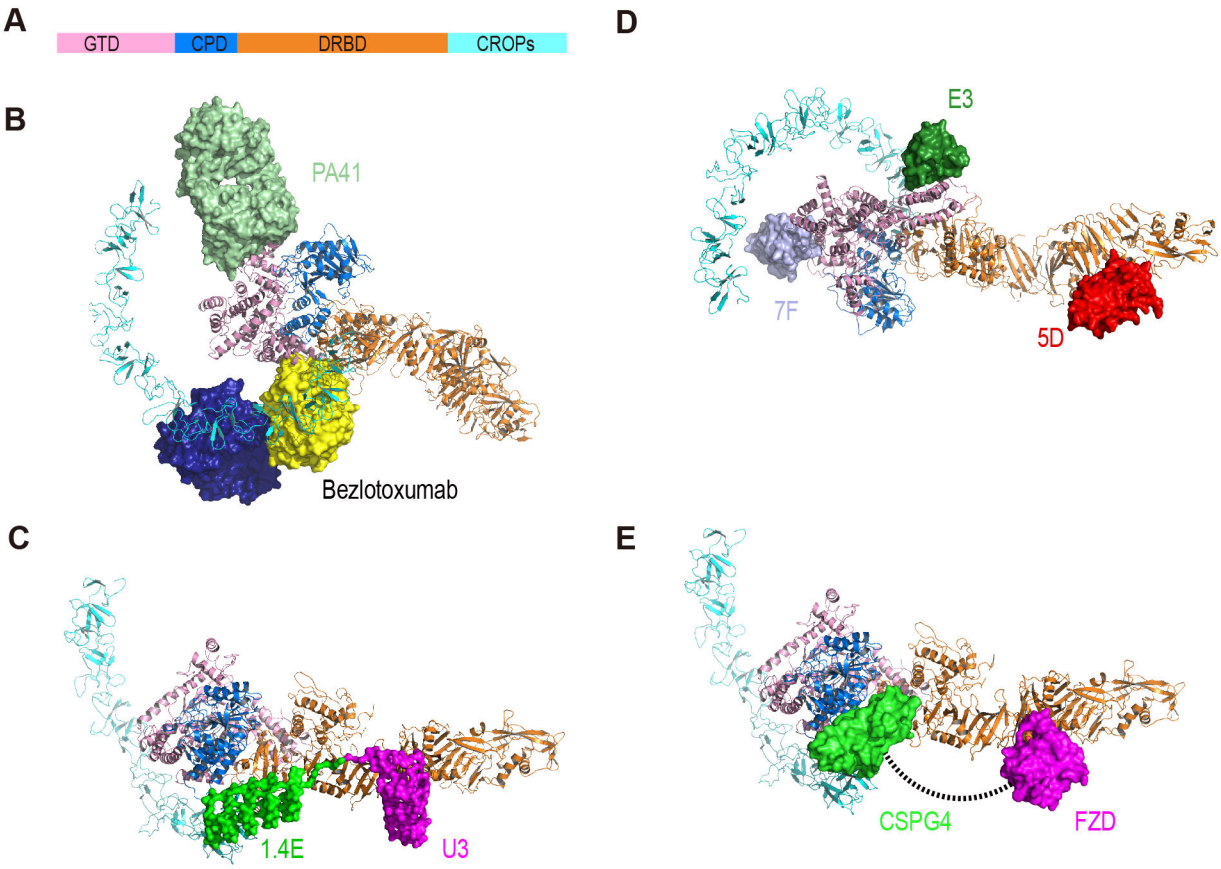

Fig. 3. Major neutralizing epitopes on TcdB revealed by structural studies.

(A) A schematic diagram showing the domain organization of TcdB holotoxin. (B) Bezlotoxumab (PDB: 4NP4) binds to two neighboring epitopes on the CROPs with the two Fab fragments colored in blue and yellow. A Fab fragment of PA41 (green, PDB: 5VQM) binds to the GTD. (C) DARPin U3 (pink) binds to the DRBD, while 1.4E (green) interacts with the CPD, DRBD, and CROPs (PDB: 6AR6). (D) VHH-5D (red) binds to the DRBD, VHH-E3 (green) and -7F (gray) both bind to the GTD at two different sites. (E) CSPG4 (green) and FZD (pink) bind to two separated sites on TcdB, which could be designed into one fusion protein with a proper peptide linkage (dashed line).