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Pore formation by the CDTb component of the *Clostridioides difficile* binary toxin is Ca²⁺-dependent

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Clostridioides difficile infection (CDI) is one of the five most urgent bacterial threats in the United States. Furthermore, hypervirulent CDI strains express a third toxin termed the *C. difficile* binary toxin (CDT), and its molecular mechanism for entering host cells is not fully elucidated. Like other AB-type binary toxins, CDT enters host cells via endosomes. Here we show via surface plasmon resonance and electrochemical impedance spectroscopy that the cell-binding component of CDT, termed CDTb, binds and form pores in lipid bilayers in the absence of its enzymatic component, CDTa. This occurs upon lowering free Ca²⁺ ion concentration, and not by decreasing pH, as found for other binary toxins (i.e., anthrax). Cryogenic electron microscopy (CryoEM), X-ray crystallography, and nuclear magnetic resonance (NMR) studies show that dissociation of Ca²⁺ from a single site in receptor binding domain 1 (RBD1) of CDTb triggers conformational exchange in CDTb. These and structure/function studies of a Ca²⁺-binding double mutant targeting RBD1 (i.e., D623A/D734A) support a model in which dissociation of Ca²⁺ from RBD1 induces dynamic properties in CDTb that enable it to bind and form pores in lipid bilayers.

The Centers for Disease Control and Prevention (CDCP) identified *Clostridioides difficile* infection (CDI) as one of the five most urgent bacterial threats in the United States. *Clostridioides difficile* (*C. difficile*) is a grampositive anaerobic bacterial pathogen responsible for high-risk nosocomial disease prevalent among patients undergoing prolonged antibiotic treatments and/or cancer therapy¹. Reduced levels of symbiotic gut microbiota allow for a dominance of *C. difficile* bacteria that cause severe diarrhea and pseudomembranous colitis². Treatments of CDI are available that target the large clostridial toxins, termed TcdA (Toxin A) and TcdB (Toxin B)^{3–5}. However, CDIs associated with hypervirulence and high recurrence rates often contain a third binary toxin that harbors ADP-ribosyltrasferase enzymatic activity (CDT)⁶. Targeting the CDT binary toxin in addition to the large toxins is supported by in vivo data, which demonstrates that inhibition of both the binary toxin and the large clostridial toxins (TcdA, TcdB) provides the highest level of protection from morbidity associated with CDT-containing strains of CDI⁷.

An endosomal delivery mechanism for the CDT binary toxin (i.e., CDTa/CDTb) into host cells was thought to be like that of other "AB"-type binary toxins including *Clostridium perfringens*, *Clostridium spiroforme*, *Clostridium botulinum*, and *Bacillus anthracis*⁸⁻¹¹. However, the molecular mechanism for how CDT enters host cells requires further study. Upon host cell entry, the CDTa ribosyltransferase component of CDT, termed CDTa, is known to modify intracellular actin, resulting in actin depolymerization, cytoskeleton destruction, and rapid host cell death¹¹. CDTa is composed of an N-terminal domain that binds to CDTb and a C-terminal catalytic domain that is enzymatically toxic to cells¹². Pro-CDTb (99 kDa) undergoes proteolytic cleavage by trypsin/chymotrypsin to form active CDTb subunits (75 kDa). Under soluble conditions and at millimolar Ca²⁺ ion

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EIS data were collected in the (A) absence or (B) presence of the Ca^{2+} ion chelator EGTA (see section "Methods").

concentrations in the extracellular environment, CDTb subunits can oligomerize into stable "dumbbell-shaped" dimer of heptamer complexes¹³. This includes two distinct CDTb diheptamer structures, a symmetric (^{Sym}CDTb) and an asymmetric (^{Asym}CDTb) form of CDTb¹³. ^{Asym}CDTb has one heptamer unit that forms an extended β-barrel originating from its heptameric core while ^{Sym}CDTb lacks the β-barrel extension and displays two identical heptamer units. The diheptamer interfaces for both AsymCDTb and ^{Sym}CDTb involve a unique "donut-like" assembly comprised of receptor binding domain 2 (RBD2). ^{Sym}CDTb is organized into an "open" pore state, whereas ^{Asym}CDTb is in a "closed" state^{13,14}. The open/closed terminology describes the inner diameter of what is called the φ -gate, which is present in numerous AB-type binary toxins. In both the open and closed forms of CDTb, the φ -gate is composed of side chains from phenylalanine residues (F455) originating from the heptamerization domain 2 (HD2) of each subunit^{13,14}. Although present in cryoEM-derived structure determinations, the biological relevance of diheptamer assemblies is somewhat controversial. While the relevance of the diheptamers of CDTb requires conformation in vivo, it is possible that individual subunits (i.e., monomers), biologically relevant heptamers, and diheptamers of CDTb are in dynamic equilibrium, as they are all observed in vitro¹²⁻¹⁷.

As with other AB-type binary toxins^{3,17,18}, the toxic CDTa component of CDT traverses from endosomes into the host cytosol via the cell-binding component, CDTb. Such a mechanism of toxin entry would require CDTb conformational transition(s) for CDTa to translocate through the φ -gate observed in structures of CDTb. However, the molecular mechanism for how CDTb engages and delivers CDTa into host cells is not fully elucidated despite the availability of several high-resolution structures for CDTa, CDTb, and CDT complexes under varying conditions^{12,13,15-17}. Based on mechanisms of host cell entry established for anthrax toxin¹⁹, it was anticipated that CDTb, CDTa, and/or the CDT complex would undergo conformational change(s) as facilitated by entering the acidic pH environment found in endosomal compartments. Whether conformational changes, unfolding process(es), and/or molten globule states of CDTb are required for CDT toxicity complex remains a question for consideration²⁰. In this study, we found that lowering pH alone was not sufficient for CDTb to bind membrane or form pores, as found for the cell-binding component of anthrax, termed the protective antigen (PA), so the structural requirements for lipid bilayer and poreforming functions of CDTb were examined here at atomic resolution.

Results and discussion

There is a >1000-fold drop in free Ca²⁺ ion concentration from around 2.3 mM in the extracellular environment to <2 μ M upon entering endosomes^{3,21}, which could affect conformational, biophysical, and functional properties of CDTb. Of interest, Ca²⁺-dissociation from a single site in

the CDTb receptor binding domain 1 (RBD1) was found to induce conformational exchange dynamics throughout RBD1. Studies reported here show that Ca^{2+} depletion triggers CDTb binding to lipid bilayers and promotes pore formation (Fig. 1). These findings for CDT were of additional interest as the cell binding and pore-forming component of the toxin, CDTb, was found to activate K⁺ efflux dependent cellular inflammation on its own, in the absence of CDTa²². Thus, structure/function studies of the CDTb cell-binding component of the binary toxin alone, in the absence of CDTa, are relevant towards understanding how it contributes to cellular inflammation, as reported²², in addition to clarifying its role for CDTa delivery into host cells.

${\rm Ca}^{2+}$ dissociation triggers lipid bilayer binding and pore formation by CDTb in the absence of CDTa

A downward pH gradient to acidic levels is required for the endosomal delivery of several members of the AB binary toxin family, including the protective antigen (PA) of anthrax^{23,24}. We tested whether CDTb could bind lipid bilayers and form pores at acidic pH values. Such data was collected using a custom-built instrument^{25,26} to simultaneously measure (i) CDTb adsorption/desorption via surface plasmon resonance (SPR) and (ii) pore formation via electrochemical impedance spectroscopy (EIS). The SPR data collection involves exciting gold surface plasmons, and the same gold serves as a working electrode for EIS^{27,28}. Unlike observations for other binary toxins^{19,29,30}, and as verified here for anthrax PA (Fig. S1)³¹⁻³³, CDTb did not bind or form pores in lipid bilayers upon lowering pH (Fig. 1).

A role for Ca²⁺ was examined by SPR/EIS at millimolar Ca²⁺ ion concentrations where negligible changes in SPR and EIS signal were observed (Fig. 1A). These data indicate that neither membrane binding nor pore-formation occurs at millimolar Ca²⁺ concentrations, such as found in an extracellular environment. As free Ca²⁺ ion concentration in endosomes is roughly 3 orders of magnitude lower (<2 μ M) than in the extracellular environment (2.3 mM), studies at much lower Ca²⁺ concentration were examined²¹. For these studies, Ca²⁺ concentration was depleted via dialysis of CDTb against EDTA/EGTA containing buffers followed by SPR/EIS data collection (Fig. 1B). As demonstrated by simultaneous increases in SPR signal and electrical conductance by EIS, Ca²⁺-depleted CDTb (CDTb^(-Ca)) was found to bind lipid bilayers and form pores (Fig. 1B). Of importance, the SPR/EIS measurements demonstrated that CDTb lipid binding and CDTbdependent pore formation occurred simultaneously (Fig. 1B), versus other possible kinetic mechanisms²⁷. Together, the results implicate changes in free Ca²⁺ concentration upon endosomal delivery as another change in conditions, in addition to lowering pH, to be considered for CDT endosomal delivery into host cells. Likewise, other binary toxins may respond to pH change and/or Ca²⁺-depletion upon entering endosomes, but this requires



Fig. 2 | Local resolution in electron density maps for two classes of Ca^{2+} -depleted CDTb conformations detected by cryoEM (termed CDTb^(-Ca)). (Top) The RBD2 distorted structure (class 1) and (Bottom) the heptamer structure with the RBD2 domains intact (class 2). The difference in the degree of RBD2 resolution is distinguishable by side and top projections of each class.

further study. The SPR/EIS data were intriguing for CDTb considering that Ca²⁺ ion dissociation from a single site within the RBD1 domain induced extensive conformational exchange, as shown previously by NMR¹³.

CryoEM structures of CDTb in a Ca2+-depleted state

Studies of CDTb constructs by NMR, X-ray crystallography, and cryoEM showed previously that three well-defined Ca^{2+} -binding sites are occupied at mM free Ca^{2+} ion concentrations, as is found in the extracellular environment¹³. Two of the three CDTb Ca^{2+} ion binding sites are high-affinity and located in the N-terminal heptamerization domain (HD1). Both HD1 sites are conserved in anthrax toxin PA and iota toxin Ib and are essential for CDTb binding site is lower affinity and resides in the RBD1 domain of CDTb^{13,36}. This Ca^{2+} -binding site in RBD1 was of interest, as extensive conformational exchange in the millisecond to microsecond timeframes was observed by NMR throughout this domain as Ca^{2+} levels were depleted¹³.

To understand structural consequences of Ca^{2+} dissociation from RBD1, the structure of full-length wild-type CDTb was solved in a Ca^{2+} -depleted state using single-particle cryo-electron microscopy (cryoEM; Figs. 2 and S2). As with the EIS experiments, the CDTb sample used for cryoEM was dialyzed versus an EGTA/EDTA buffer. This procedure removed Ca^{2+} from the weaker RBD1 site but retained binding to the higher-affinity Ca^{2+} ion binding sites in the HD1 (Fig. S3). That Ca^{2+} binding to HD1 of CDTb was retained with this preparation (Fig. S3) is consistent with reports indicating that harsh denaturing conditions are required to dissociate Ca^{2+} from these high-affinity HD1 sites³⁷. Thus, as anticipated, removal of Ca^{2+} from RBD1 via chelation did not affect Ca^{2+} binding to the two HD1 sites, and the overall structural integrity of the heptamerization core of CDTb remained intact (Fig. 2).

Initial 2D classification of Ca^{2+} -depleted CDTb particles by cryoEM revealed the presence of a single heptamer unit. Subsequent 3D refinement of this structure found two distinct structural moieties derived from the "pore-forming" conformation of CDTb (Fig. 2). The difference between the two structures was the degree of flexibility observed for the RBD2 domain. An RBD2 disordered structure (class 1) was solved at a resolution of 3.06 Å and the heptamer structure with RBD2 intact (class 2) was solved at 3.28 Å resolution (Figs. 2 and S2). While the fully elongated β -barrel was not observed in either structure, partial β -barrel formation was detected. As



Fig. 3 | Mutation of the Ca²⁺-coordinating residues D623A and D734A of RBD1 abolish Ca²⁺ binding. Ca²⁺ binding to RBD1^{WT} (red filled circles) and RBD1^{D623A/} D^{734A} (blue filled circles) was examined by fluorescence spectroscopy at 350 nM (*n* = 3). The fluorescence emission intensity of the single tryptophan residue in each RBD1 construct (Trp632) was monitored as a function of increasing Ca²⁺ concentration. Ca²⁺ binding was observed only for RBD1^{WT} (^{Ca}K_D = 40 ± 10 µM) upon fitting a model with noncooperative binding of Ca²⁺ to a single site in the RBD1^{WT}. No Ca²⁺ binding was detected up to 10 mM Ca²⁺ for RBD1^{D623A/D734A} (^{Ca}K_D > 10 mM).

expected, the RBD1 domains in both structures were mobile as is consistent with NMR data upon Ca²⁺ removal from isolated RBD1¹³. Structural comparisons of Ca²⁺-depleted CDTb (Figs. 2 and S2) to other published CDTb structures in the pore-forming state (\pm CDTa) show high overall similarity with alignments of their structural regions giving RMSD values ranging from 0.27 to 1.41 Å (Table S1)^{13,15,17}. It is interesting that the two Ca²⁺-depleted structures of CDTb solved here (classes 1, 2) somewhat resemble what is termed a "prepore state" for CDTb¹⁶ or to a lesser degree what is termed the "pre-insertion state" of CDTb when CDTa is bound¹⁵ (Figs. 2 and S2; Table S1).

Structure/function studies of a Ca²⁺-binding mutant of CDTb

Two Ca²⁺-coordinating residues in RBD1 of CDTb were mutated (D623A/ D734A) to examine the structure/function relationship of the weak RBD1 Ca²⁺ binding site. To ensure Ca²⁺-binding to the RBD1 domain was abolished by the double mutation, the fluorescence of the single tryptophan residue (W632) in RBD1 was monitored in side-by-side Ca2+ titrations with $RBD1^{\rm WT}$ and $RBD1^{\rm D623A/D734A}$ (Fig. 3). $Ca^{2+}\mbox{-binding}$ was detected for $RBD1^{WT}$ (^{Ca}K_D = 40 ± 10 µM; Fig. 3) but not for $RBD1^{D623A/D734A}$, even at 10 mM Ca^{2+} concentration (^{Ca}K_D > 10 mM; Fig. 3). Likewise, no change in the fluorescence emission profile (Fig. S4A) was observed for RBD1^{D623A/} ^{D734A} (±Ca²⁺), providing further evidence that Ca²⁺ binding was abolished for RBD1^{D623A/D734A} (Figs. 3 and S4B). RBD1^{WT} showed a pronounced change in its fluorescence emission profile upon Ca²⁺ addition that is indicative of structural differences (Fig. S4B). The lack of Ca²⁺ binding to RBD1^{D623A/D734A} was confirmed via NMR studies, which did not show significant chemical shift perturbations (CSPs) after addition of Ca²⁺ at pH 5.0 or at pH 7.0 (Figs. S5 and S6) even at 10 mM Ca^{2+} concentration ($^{Ca}K_D > 10$ mM).

An NMR 2D¹H⁻¹⁵N HSQC spectrum for RBD1^{D623A/D734A} was shown to mimic that of RBD1^{WT} in a Ca²⁺-depleted state (Fig. S7). This includes exhibiting severe exchange broadening at both acidic and neutral pH values (pH 5.0 and 7.0; Figs. S5 and S6). However, unlike RBD1^{WT} at varying pH values (pH 5–7.4; Figs. 4, S7, S8)¹³ RBD1^{D623A/D734A} showed little or no effect on the NMR spectra at any pH values with or without 10 mM Ca²⁺ addition (Figs. S5, S6, S9). Whereas, Ca²⁺ titrations into RBD1^{WT} eliminated conformational exchange and provided highly dispersed and narrowed ¹H-¹⁵N correlations consistent with a single structure regardless of pH, as is typical of a binding and folding event (Figs. 4, 5, S7, and S8)^{13,38}. Whether Mg²⁺ affected NMR spectra of RBD1^{WT} or RBD1^{D623A/D734A} was examined, as it is Fig. 4 | In the presence of Ca²⁺, RBD1^{WT} retains a single structure regardless of pH. A Overlay of NMR data for RBD1^{WT} at neutral (black) and acidic (red) pH values in 10 mM Ca²⁺. ¹⁵N-HSQC NMR data were collected using 0.1 mM RBD1WT at 25 °C and pH 7.0 (black) or pH 5.0 (red). Residues of RBD1^{WT} were assigned sequence-specifically (residues 616-748), and those marked by blue asterisks are associated with the His-tag. The contour marked "X" shifted significantly but could not be assigned unambiguously as correlations to this H^N were very weak in 3D NMR data sets¹³. The contour labeled G645^{*} appeared in the noise at pH 7.0 and grew stronger at pH 5.0. Correlations connected by horizontal lines correspond to sidechain NH₂ groups. B Graphed chemical shift perturbations (CSPs) of Ca²⁺-bound RBD1^{WT} residues upon lowering pH from 7.0 to 5.0. The average CSP for all residues is indicated by a red dashed line. The solid red line indicates one standard deviation $(1\sigma; 0.03)$ above the average CSP, and residues are indicated that were above the average CSP. Blue bars are shown above residues that are in beta-strand regions of RBD1 as determined by X-ray crystallography and NMR chemical shift values. White bars represent random coils.



not uncommon for Mg²⁺ to occupy Ca²⁺ sites³⁸. However, unlike what was found for Ca²⁺ (Fig. S7), Mg²⁺ addition had no effect on NMR spectra for RBD1^{WT} (Fig. S10). Whether Mg²⁺ bound to RBD1^{WT} or RBD1^{D623A/D734A} was also examined by tryptophan fluorescence (Fig. S11A) and CD methods (Fig. S11B), but again, no changes were detected in the fluorescence or CD spectra upon Mg²⁺ addition for either RBD1 construct. Together, fluorescence, NMR, and CD data confirmed that the divalent metal ion binding site in RBD1^{WT} is specific for Ca²⁺ versus Mg²⁺.

Structure/function data of RBDWT and RBD1D623A/D734A outlined above indicate that D623A + D734A double mutation is a good model for examining the Ca²⁺-depleted RBD1 domain of CDTb. As Ca²⁺ depletion was required for CDTb binding to lipid bilayers and forming pores (Fig. 1), it was important to show that the D623A + D734A double mutation could be engineered into full-length CDTb (i.e., CDTb D623A/D734A) and retain its ability to deliver CDTa into Vero cells, as hypothesized. A site-directed mutagenesis approach was needed for these cytotoxicity studies versus Ca2+ depletion because the cellular biology of Vero cells is drastically impacted by Ca²⁺ removal¹⁶, and controlling Ca²⁺ levels for cellular cytotoxicity studies is challenging. Therefore, side-by-side Vero cell toxicity assays were performed upon addition of CDT having either full-length CDTbWT or CDTb^{D623Â/D734A}. The CDTa:CDTb ratio was maintained at 1:7 throughout the assays for both constructs of CDTb, as described³⁹. As hypothesized, potent cytotoxicity was detected upon CDTb^{D623A/D734A} addition at pM concentration (TC₅₀ = 560 \pm 60 pM) consistent with CDTa delivery into the cellular host via CDTb^{D623A/D734A}. It was not surprising that some loss in cytotoxicity was observed for CDTbD623A/D734A versus CDTbWT $(TC_{50} = 70 \pm 20 \text{ pM}; \sim 8\text{-fold loss; Fig. S12})$ as the thermodynamic stability for the double mutation was lowered ($T_M = 53.6$ °C) versus wild-type CDTb $(T_M = 56.9 \,^{\circ}C;$ Fig. S4). Such a lowering of its stability could make CDTb^{D623A/D734A} more susceptible to cellular proteases and/or be affected in some other way versus CDTb^{WT}.

Structural studies by single particle cryoEM for CDTb^{D623A/D734A} were done, so it could be compared to Ca²⁺-depleted CDTb^{WT} (Table S1). Much like Ca^{2+} -depleted CDTb^{WT} (Fig. 2), the structure of CDTb^{D623A/D734A}, solved at 3.56 Å resolution, was found to be a single heptamer (Fig. 6). Unlike the Ca²⁺depleted state, where 2 classes of structures were solved, only a single class of heptamers was identified for CDTb^{D623A/D734A}. As anticipated, alignment of the Ca²⁺-binding mutant of CDTb to other previously deposited CDTb structures showed relatively low RMSD values for structured regions, which ranged from 0.54 to 1.13 Å (Table S1). Of all the structural comparisons made (Table S1), the structure of the CDTb^{D623A/D734A} mutant most resembled the Ca²⁺-depleted CDTb structures (RMSD: Class 1; 0.55, Class 2; 0.54; Table S1, Fig. S13). Like that found for Ca2+-depleted CDTbWT, the structure of $CDTb^{D623A/D734A}$ also lacks clear density within its RBD1 domains. The lack of clear density observed in these full-length CDTb structures is consistent with NMR studies of apo-RBD1 and apo-RBD1^{D623A/D734A}, which exhibit significant conformational exchange on chemical shift timescales.

The observed heptameric structure of CDTb^{D623A/D734A} resembles a single 'pore-forming state' with an elongated β -barrel assembly (Figs. 6 and S14). Likewise, the structure of CDTb^{D623A/D734A} and Ca²⁺-depleted CDTb^{WT} illustrates the existence of a single heptamer unit in soluble conditions, without detergents or lipid membrane-like support (Figs. 2, 6, S2 and S14, Table 1). That isolated RBD2 is monomeric in solution and crystallizes as a monomer further demonstrates that the donut-shaped RBD2 structure found in the diheptamer oligomerization state is assisted by heptamerization and that the RBD1 domains may stabilize the overall protein framework at millimolar Ca²⁺ levels¹³. Nonetheless, it remains clear that the β -barrel domain of CDTb^{D623A/D734A} (residues 298–401) originates from the heptameric core and elongates to form a "stem-like" structure that is essential for membrane insertion when no Ca²⁺ is bound to RBD1. The CDTb^{D623A/D734A} structure depicted weaker density at the end of the β -barrel and therefore residues ranging from 332 to 362 could not be modeled



Fig. 5 | Ca^{2+} is necessary to stabilize RBD1 from a molten globule state to single stable structure. Overlay of 2D ¹⁵N-edited HSQC NMR spectra of RBD1^{D623A/D734A} (black) and RBD1^{WT} (red) after incubation with excess Ca²⁺ (10 mM Ca²⁺). Residues of RBD1^{WT} were assigned sequence-specifically (residues 616-748), and those marked by blue asterisks are associated with the His-tag. A correlation for G645 appeared in the noise of the RBD1^{WT} sample and is indicated by a box. The spectrum

for RBD1^{D623A/D734A} remained in conformational exchange even with Ca²⁺ added, so the remaining few detectable correlations (in black) could not be sequence-specifically assigned. Correlations connected by horizontal lines correspond to sidechain NH₂ groups. RBD1^{WT} data were collected with 0.25 mM RBD1^{WT} at 25 °C and pH 7.0; RBD1^{D623A/D734A} data were collected with 0.1 mM RBD1^{D623A/D734A} at 25 °C and pH 7.0.

Fig. 6 | Comparisons of the structural architecture for CDTb^{WT} and CDTb^{D623A/D734A}. A Side and top projections of the diheptamer structure in the "asymmetric form" of CDTb (PDB: 6UWR). The heptamer with the β-barrel extension is illustrated in different colors; heptamerization domain 1 (HD1; residues 212-297) in red, β-barrel domain (βBD; residues 298-401) in green, heptamerization domain 2 (HD2; residues 402-486) in violet, linker region 1 (L1; residues 487-513) in gray, heptamerization domain 3 (HD3; residues 514-615) in yellow, receptor binding domain 1 (RBD1; residues 616-748) in blue, linker region 2 (L2; residues 745-756) in gray, and receptor binding domain 2 (RBD2; residues 757-876) in cyan. Dual Ca2+ ions are bound in HD1 and the single Ca2+ ion bound in RBD1 are shown as green spheres. B The single heptamer extracted from CDTb is superimposed with the electron mesh map of the CDTb^{D623A/D734A} structure. C The electron density map of $\text{CDTb}^{\text{D623A/D734A}}$ is colored by local resolution with C7 symmetry imposed. Increased flexibility is observed in the outer regions of the core heptamer, and it is most pronounced for the RBD1 domain and the tip of the β-barrel extension.



(Figs. 6 and S14). This could be due to partial unfolding of the tip of the β barrel in the absence of lipid bilayers or membrane and/or from some other dynamic features associated with the lack of Ca²⁺ bound to RBD1 and/or from the double mutation itself. Importantly, the orientation of Phe455 remained intact in this structure, such that this key residue for the " ϕ -gate" assembly is arranged to form a 6 Å pore (Fig. S15). This 6 Å opening more closely represents a "closed" versus "open" pore state in CDTb, which under some conditions can approach 12.5 Å¹³. Thus, Ca²⁺-depletion alone from

Table 1 | CryoEM data collection, processing, refinement, and variation statistics

	CDTb ^{D623A/D734A}	CDTb ^(-Ca) (Class 1) EMDB-	CDTb ^(-Ca) (Class 2) EMDB-	
	28207/8EKM	28206/8EKL	28205/8EKK	
Data collection and processing				
Magnification	56,240	56,240	56,240	
Voltage (kV)	200	200	200	
Electron exposure (e ⁻ /Ų)	45.3	59.5	59.5	
Defocus range (µm)	0.5–2.7	0.5–2.7	0.5–2.7	
Pixel size (Å)	0.889	0.889	0.889	
Symmetry imposed	C7	C7	C7	
Initial particle images (no.)	993,305	1,109,860	1,109,860	
Final particle images (no.)	78,587	33,531	36,395	
Map resolution (Å)	3.56	3.06	3.28	
FSC threshold	0.143	0.143	0.143	
Map resolution range (Å)	3.56–4.0	3.06-4.0	3.28–4.0	
Refinement				
Initial model used (PDB code)	6UWR	6UWR	6UWR	
Model resolution (Å)	3.56	3.06	3.28	
FSC threshold	0.143	0.143	0.143	
Model resolution range (Å)	3.56-4.0	3.06-4.0	3.28-4.0	
Map sharpening <i>B</i> factor (Ų)	-172.9	-116.2	-117.1	
Model composition				
Non-hydrogen atoms	21,340	19,320	29,610	
Protein residues	2807	2485	3731	
Ligands	-	-	-	
B factors (Å2)				
Protein	86.7	64.9	45.79	
Ligand	-	-	-	
R.m.s. deviations				
Bond lengths (Å)	0.013	0.003	0.003	
Bond angles (°)	1.056	0.681	0.509	
Validation				
MolProbity score	2.38	1.58	1.67	
Clashscore	12.7	5.36	6.39	
Poor rotamers (%)	0	0.32	0.27	
Ramachandran plot				
Favored (%)	77.3%	95.81	95.38	
Allowed (%)	22.7%	4.19	4.62	
Disallowed (%)	0	0	0	

RBD1 is not sufficient to open the pore fully in CDTb. This CDTb alone conformation, without Ca²⁺ occupying RBD1, and prior to binding to lipid bilayers (Fig. 1) would not exist in an extracellular environment, where Ca²⁺ concentrations are at the millimolar level, as the Ca²⁺-binding site within RBD1 site (^{Ca}K_D = 40 ± 10 μ M; Fig. 3) would be fully occupied under these conditions¹⁴.

The receptor binding domains (RBD1, RBD2) were relatively disordered in CDTb^{D623A/D734A}, as compared to wild-type CDTb with Ca²⁺ present^{13,17}. When the CDTb^{D623A/D734A} map was examined with C1 symmetry, the occurrence of stronger RBD1 density was noticed in one or two of the protomers versus others (Fig. S14); whereas application of C7 symmetry averaged out density in all the RBD1 protomers among the seven subunits. Thus, the absence of a bound Ca²⁺ ion in the RBD1 domain of CDTb^{D623A/}D^{734A} led to a less defined orientation of the RBD1 and RBD2 domains than in Ca²⁺-bound wild-type CDTb. Increased flexibility of RBD1 in CDTb^{D623A/}D^{734A} was also found to impact a stable orientation of the RBD2 domains and prevent observation of the assembly of RBD2 "donut" structures observed in the wild type diheptamer CDTb structures^{13,17}.

Secondary structural elements are retained for Ca $^{2+}\text{-depleted}$ RBD1 $^{\text{WT}}$ and RBD1 $^{\text{D623A/D734A}}$

In the Ca²⁺-bound state, cryoEM and X-ray crystal structures of CDTb^{WT} showed that the RBD1 domain is dominated by beta-strand/sheet secondary structure¹³. The X-ray crystal structure of Ca²⁺-bound RBD1^{WT} (residues 616–748) was determined here at 2.3 Å (PDB ID: 9MUI; Fig. 7A, Table 2), and it retained the beta-strand/sheet secondary and closely matched the RBD1 fold observed in full-length CDTb^{WT}, when fully Ca²⁺-loaded (Table S1). As these structures closely align, the isolated RBD1 domain is a good construct for examining structure/function relationships involving the role of RBD1 in the full-length CDTb.

On the other hand, X-ray structures could not be solved for Ca²⁺depleted CDTb^{WT}, Ca²⁺-depleted RBD1^{WT}, or for the Ca²⁺-binding mutant constructs, CDTb^{D623A/D734A} and RBD1^{D623A/D734A}, as crystals could not be obtained. NMR structure determinations were also not feasible for RBD1^{WT} or RBD1 $^{\rm D623A/D734A}$, as resonances were extremely broad in the absence of Ca²⁺ bound (Figs. 5 and S5–S10). The broad NMR resonances, the inability to grow crystals, and the lack of cryoEM density in RBD1 are all consistent with conformational exchange occurring in RBD1 in the absence of bound Ca²⁺. However, no evidence of fast timescale motions was observed by NMR or circular dichroism (CD), indicative of an intrinsically disordered domain (IDDs), for either RBD1^{WT} (\pm Ca²⁺) or RBD1^{D623A/D734A} (\pm Ca²⁺) unless 8 M urea was added (Figs. 7, S16 and S17). Thus, whether secondary structure elements persisted in the Ca2+-depleted state of RBD1^{WT} or for RBD1^{D623A/} $^{\text{D734A}}$ (±Ca^{2+}) remained a question. To address this, we analyzed circular dichroism (CD) data for both RBD1^{WT} (±Ca²⁺) (Fig. 7B) and RBD1^{D623A/} ^{D734A} (±Ca²⁺) (Fig. 7C). Interestingly, CD spectra for RBD1^{WT} (±Ca²⁺) and $(\pm Ca^{-})$ (Fig. ($\Delta)^{-1}$ intercomp.), $\Delta = \frac{1}{r}$ and r ($\pm Ca^{2+}$) were all consistent with having significant betasheet secondary structure whether Ca²⁺ was present or not. These data showed that RBD1^{WT} retained beta-strand features (Fig. 7B, Table S2) under multiple conditions, including in the presence and absence of mM levels of Ca²⁺. Likewise, RBD1^{D623A/D734A}, despite its inability to bind Ca²⁺, also retained beta-strand features ($\pm Ca^{2+}$; Fig. 7C, Table S2) as was found for $\mathrm{Ca}^{2+}\text{-depleted}\ \mathrm{RBD1}^{\mathrm{WT}}.$ In both samples, the beta-strand content was comparable to that of the X-ray crystal structure of Ca²⁺-bound RBD1^{WT} (Fig. 7A; Table S2). The CD data showing beta-sheet structure together with the conformational exchange found by NMR and the weak cryoEM density are all consistent with RBD1^{WT} and RBD1^{D623A/D734A} existing in a dynamic state, in which secondary structure is retained in the absence of Ca²⁺. Such a state for a protein or protein domain is termed a "molten globule" or a "foldon" state, respectively, as reviewed^{20,40-43}. These results are consistent with a model in which Ca²⁺ dissociation from RBD1, or mutation of this RBD1 Ca²⁺-binding site, provides a dynamic molten globule state in RBD1 that is important for CDTb to bind lipid bilayers, form pores, and deliver CDTa-dependent cellular toxicity at pM levels (Figs. 1 and S12).

Ca²⁺-depletion and partial unfolding of RBD1 in CDTb enables lipid bilayer binding and pore formation

From our findings, a mechanism is presented for host cell engagement by the cell-binding and toxin delivery component of the binary toxin, CDTb, in the absence of CDTa and a host-cell receptor. Specifically, Ca^{2+} -dissociation from a relatively weak RBD1 Ca^{2+} binding site is sufficient for the CDTb component of CDT to bind and pierce lipid bilayers on its own. This is unlike what is reported for other AB toxins, such as anthrax PA, for which



Fig. 7 | Ca^{2+} stabilizes the global fold of RBD1. A X-ray crystal structure of Ca^{2+} bound RBD1^{WT} (residues 616-748; 2.3 Å; PDB = 9MUI) with β -sheets 1–10, and N and C-termini labeled. The inset shows backbone and sidechain atoms of residues in the Ca^{2+} -binding site of RBD1^{WT}. Ca^{2+} is represented by a green sphere. B CD spectra of RBD1^{WT} without Ca^{2+} (black), in the presence of Ca^{2+} (2.4 mM; red, 10 mM; blue),

and in 8 M urea (without Ca^{2+} ; gray, with 2.4 mM Ca^{2+} ; dark gray). C CD spectra of RBD1^{D623A/D734A} without Ca^{2+} (black) and in the presence of Ca^{2+} (2.4 mM, red; 10 mM, blue), and 8 M urea (without Ca^{2+} ; gray, 2.4 mM Ca^{2+} ; dark gray). All CD experiments were performed in triplicate (n = 3).

lowering pH is the mechanism for triggering membrane binding (Fig. S1). Structure/function studies completed here show that removal of this relatively weak RBD1 Ca2+ binding site by mutation provided a functional CDTb toxin at pM concentrations, albeit with 8-fold decreased toxicity (Fig. S12). One of several reasons for the decrease in mutant toxicity compared to wild type may be related to a decrease in the thermal stability of the CDTb mutant ($T_M = 53.5$ °C for CDTb^{D623A/D734A} versus $T_M = 56.9$ °C for CDTb^{WT}; with 1 mM Ca²⁺) (Fig. S4C). This loss of thermal stability would be particularly important in the extracellular space where wild-type CDTb will have RBD1 fully occupied with Ca2+ at approximately 2 mM concentration, whereas no Ca^{2+} binding is possible for the double mutant (T_M = 40.0 °C for RBD1^{D623A/D734A} versus $T_M = 47.5$ °C for RBD1^{WT}; with 10 mM Ca²⁺) (Fig. S4D). However, other explanations are possible. Of secondary interest is that Ca2+-depletion from CDTb and the CDTb^{D623A/D734A} mutation predominantly revealed a heptamer structure in its "pre-pore" state, versus diheptamer oligomerization states, as detected by cryoEM. The physiological relevance of the diheptamer in vivo for wild-type CDTb remains somewhat controversial as a single mutation of CDTb (F774L), reported previously¹⁷, also promotes a diheptamer to heptamer transition.

Nonetheless, a mechanism of action for CDTb binding and forming pores in lipid bilayers, in the absence of CDTa, is reported here. Such CDTb-dependent pore formation is triggered by Ca^{2+} dissociation from a physiologically relevant site in RBD1. It is possible that the ancestral translocase from which both CDTb and anthrax PA evolved did not employ any direct mechanism of favorable membrane insertion upon endosome maturation, and these two branches of this protein family evolved independently to respond to either higher acidity and/or calcium depletion upon endosome delivery into host cells and to direct toxin function.

Mechanistic insights are provided by examining structural and dynamic properties of CDTb and RBD1 at the molecular level by CD, NMR, and cryoEM. The main finding from these biophysical and structural studies was that Ca²⁺ dissociation from RBD1 of CDTb induces conformational dynamics in RBD1 consistent with it transitioning from a folded state into what is termed a "molten globule" and/or a "partially unfolded state"^{42,44–48}. As determined by circular dichroism (CD), RBD1 was found to retain its beta-strand elements of secondary structure upon Ca²⁺ depletion or when the site was doubly mutated (Fig. 7). However, Ca²⁺-depleted RBD1^{WT} and RBD1^{D623A/D734A} both were found to have millisecond (ms) to microsecond

 Table 2 | Data collection and refinement statistics (molecular replacement) for Ca²⁺-bound RBD1^{WT}

	$\textbf{RBDI}^{\textbf{WT}} + \textbf{Ca}^{\textbf{2}+}$	
PDB	9MUI	
Data collection		
Space group	R3	
Cell dimensions		
a, b, c (Å)	119.7, 119.7, 125.1	
α, β, γ (°)	90, 90, 120	
Resolution (Å)	39.91–2.28 (2.36–2.28)	
R _{sym} or R _{merge}	0.259 (2.156)	
l / σl	4.4 (1)	
Completeness (%)	95.9 (67.8)	
Redundancy	7.2 (5.3)	
Refinement		
Resolution (Å)	39.91–2.28 (2.36–2.28)	
No. reflections	210321 (11032)	
R _{work} /R _{free}	0.2016 / 0.2337	
No. atoms	4260	
Protein	1020	
Ligand/ion	4	
Water	170	
B-factors	45.2 (28.99–90.24)	
Protein	45 (30.8-83.3)	
Ligand/ion	41.25 (37.4–46.3)	
Water	50.20 (32.1-83.2)	
R.m.s. deviations		
Bond lengths (Å)	0.007	
Bond angles (°)	0.898	

Values in parentheses are for the highest resolution shell.

Fig. 8 | Model for membrane association and poreformation by CDTb. The change in orientation of the β-barrel domain (purple) within a single monomer of the CDTb heptamer is presented as CDTb transitions from an extracellular (A) to an early endosomal environment (B) and mature endosomal environment (C). In the extracellular environment, Ca²⁺ is bound to RBD1 and HD1. In a Ca2+-bound state, CDTb alone is unable to bind lipid bilayers or form pores. Following its uptake, dissociation of Ca2+ from the RBD1 domain of CDTb ($^{Ca}K_D = 40 \pm 10 \,\mu\text{M}$) occurs as Ca^{2+} is exported out of the endosome, resulting in the destabilization of the RBD1 domain as it enters conformational exchange. At this point (i), residues associated with the β-barrel domain begin extending into a "prepore" conformation of heptameric CDTb. (ii) The βbarrel domain extends fully, creating pores through the endosomal membrane at low micromolar [Ca2+] (i.e., <40 µM). (iii) Pore formation can begin to serve as channels for CDTa to enter the cytosol, leading to cytotoxic effects and the disruption of the intestinal epithelium that facilitate the increased severity of CDT-associated CDI. However, a more complete mechanism of action for CDTa delivery by CDTb requires further examination.

(us) dynamic properties throughout with nearly all its NMR resonances broadening below baseline (Figs. S16 and S17), again consistent being in a molten globule state. We further confirmed this conclusion by showing directly that neither Ca²⁺-free RBD1 nor RBD1^{D623A/D734A} were intrinsically disordered and having fast timescale dynamics and very narrow linewidth values unless they were examined by NMR under fully denaturing conditions (Figs. S16 and S17). CryoEM data supports the CD and NMR studies. These data also show that Ca²⁺ depletion and/or mutation of the RBD1 Ca²⁺ site provided a heptamer state of full-length CDTb. They also show that RBD1 and additionally RBD2 have dynamic properties that diminish the ability to map cryoEM data at atomic resolution (Figs. 2, 6, S2, S14 and Table 1). Together, the CD, NMR, cryoEM, and X-ray studies are consistent with a mechanism of action in which conformational exchange occurs at lower Ca²⁺ ion concentrations encountered upon entering endosomes, where free Ca^{2+} is at the 1–2 μ M level versus >2 mM outside the cell. This mechanism is further supported based on the ^{Ca}K_D value for the weak Ca²⁺ site in RBD1 (Fig. 3; $^{Ca}K_D = 40 \pm 10 \mu M$). The dynamic properties of CDTa in the CDTa/CDTb complex are also important, as described previously for CDTa and iota toxin^{17,18}, make the findings intriguing considering that we show that conformational dynamics occur in CDTb when RBD1 is Ca²⁺-free.

The structures solved here provide insight about why lowering pH alone, as observed for anthrax toxin, does not likely trigger pore formation alone by CDTb (Figs. 1 and S1). In this regard, we superimposed what are termed the prepore and pore states of CDTb and anthrax PA (Fig. S18). While the heptamerization core is well aligned in both proteins, a key difference in the β -barrel forming domain was detected in the prepore state overlay (Fig. S18). A direct link of β-barrel domain (Y345) and RBD1 (S708) in CDTb restricts mobility of the β -loop and stabilizes the prepore assembly. Therefore, Ca²⁺ removal sacrifices the stability of RBD1, triggering a conformational rearrangement in the CDTb membrane insertion β-loop to transition from prepore state to pore state. Thus, the overall mechanism proposed for RBD1 Ca²⁺-sensing is that the stable Ca²⁺-bound conformation of RBD1 forms direct interactions with the β-barrel domain, locking it into an orientation that is folded into the heptamerization domain. Ca²⁺ depletion, as found in maturing endosomes, would unfold RBD1 into a molten globule state, thus freeing the β-barrel domain to reassemble into the heptameric membrane-binding and pore-forming conformation (Fig. 8).



The mechanistic insights provided here should be considered for how bacterial translocases of the AB binary toxin class are triggered for delivery into the host cell cytoplasm from within maturing endosomes. This includes a mechanism involving a (i) lowering of pH, as found for anthrax, (ii) a lowering of free Ca²⁺ ion concentration, and/or (iii) a combination of both relevant mechanisms. Physiologically, these findings are consistent with a model in which entry of CDTb into early to late endosomes is sufficient for Ca²⁺-dissociation from RBD1 followed by CDTb membrane binding and pore formation, even in the absence of CDTa. The findings here also provide definitive evidence that changes in Ca²⁺ ion concentration, versus changes in pH dominate the conformational exchange properties within the RBD1 domain of CDTb. These protein dynamic properties do not involve a fully intrinsically disordered domain, but rather one that retains secondary structure, as detected by CD. Such a dynamic state is often termed a "molten globule" and/or a "foldon" state when folding/unfolding conformational exchange equilibria are considered^{20,42,44-48}. That this "foldon" or "molten globule" state of conformational exchange in RBD1 was important for CDTb function was supported by structure/function findings, which demonstrate that the double mutant Ca²⁺ construct remained highly toxic to Vero cells at pM levels when combined with CDTa.

Conclusions

The levels of free Ca²⁺ must be considered carefully when interpreting structural, dynamics and/or functional data involving the CDT binary toxin⁴⁹. Studies of CDTb alone show that Ca²⁺ depletion from RBD1 induced significant conformational exchange. Of functional importance, Ca^{2+} dissociation from RBD1 ($^{Ca}K_D = 40 \pm 10 \mu M$) triggered concurrent CDTb binding to lipid bilayers and pore formation (Fig. 1). These results indicate that such a Ca²⁺-dependent unfolding transition observed upon Ca²⁺ depletion would occur upon entering endosomes, where Ca²⁺ levels drop by more than three orders of magnitude. This finding can now be incorporated into mechanistic studies involving how other physiologically important changes such as cellular pH change(s), host cell receptor binding, CDTa binding, and/or a combination of these events contribute to the structure and host cell toxicity from CDT. They are also highly relevant to how CDTb alone affects cellular inflammation, as reported by Lacy and colleagues²². Studies of other evolutionarily conserved AB toxins may also require control of free Ca²⁺ to understand fully their mechanisms of action, so this should be considered in future studies. In such studies, and for practical purposes, free Ca²⁺ levels need to be considered carefully when adding detergents that emulate membrane-like conditions. As these and perhaps other reagents used may contribute to depleting free Ca²⁺ levels that could artificially obscure functionally relevant Ca2+ sites that may be present in other AB binary toxins.

Materials and methods

Protein expression and purification

Active CDTa^{C2A} and CDTb^{WT} were expressed and purified as previously described^{7,13}. To remove Ca²⁺ from CDTb^{WT}, the purified protein was dialyzed into 15 mM HEPES buffer, pH 7.0, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM triethylene glycol diamine tetraacetic acid (EGTA) at 4 °C overnight. To remove EDTA and EGTA from Ca²⁺-depleted CDTb (CDTb^(-Ca)), dialysis into 15 mM HEPES buffer, pH 7.0, and 150 mM NaCl was performed overnight at 4 °C. To test whether residual EDTA affected membrane binding and pore formation, a fraction of CDTb^{WT} was passed through a Sephedex G25 column equilibrated with 20 mM HEPES buffer, pH 7.0, and 50 mM NaCl to eliminate residual EDTA. No difference was observed when membrane binding and pore formation for CDTb^{WT} were compared before and after Sephadex G25 size exclusion chromatography (SEC), indicating that Ca²⁺-depleted CDTb in this manner was able to retain its membrane binding and pore formation activities.

The D623A + D734A CDTb Ca^{2+} -binding double mutant (CDTb^{D623A/D734A}) was engineered into pET21a (Amp^R) DNA expression plasmid, codon optimized by TOPGene Technologies, and engineered with

an N-terminal 6xHis-tag for purification. Purification of the double mutant construct followed procedures analogous to those of wild-type CDTb^{WT7,13}, with minor modifications. Briefly, proCDTb^{D623A/D734A} expression was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) addition once cell cultures reached an OD_{600} of 0.6, at which point they were incubated at 37 °C for an additional 4 h. Cells were harvested by 15 min centrifugation at 5000 rpm at 4 °C, and the cell pellet was resuspended in lysis buffer containing 20 mM Tris at pH 8.0, 300 mM NaCl, 5 mM βmercaptoethanol (BME), and 200 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit cellular proteases. Cell lysis involved 3 cycles of sonication while cooled, and cell debris were removed by centrifugation at 15000 rpm for 45 min at 4 °C. A filtered clear supernatant was loaded onto a HiPrep 16/ 60 IMAC column and purified (>95%). Pure fractions of proCDTb^{D623A/D734A} were pooled and dialyzed into 15 mM HEPES buffer, pH 7.0, 150 mM NaCl, and 10% glycerol prior to activation. Active CDTb^{D623A/D734A} was obtained by proteolytic cleavage of the N-terminal activation domain with a slight modification from that used for wild-type CDTb^{7,13}. Specifically, for active CDTb^{D623A/D734A} preparations, the cleavage reaction was achieved by overnight incubation with chymotrypsin at 20 °C. After >90% of cleavage was confirmed by SDS-PAGE, activated CDTb^{D623A/D734A} protein was injected onto a Superdex S200 size exclusion column equilibrated 15 mM HEPES, pH 7.0, 150 mM NaCl, and 0.5 mM dithiothreitol (DTT) with the purified protein elution (~70 kDa; >95%) pooled, concentrated, aliquoted, and stored at -80 °C.

Wild-type or Ca²⁺-mutant plasmids of RBD1 (residues 616-748), RBD1^{WT} and RBD1^{D623A/D734A}, respectively, were engineered into pET21a (Amp^R) DNA expression plasmids, codon optimized by TOPGene Technologies, and engineered with an N-terminal 6xHis-tag for purification. Protein production occurred in [¹⁵N]-labeled minimal MOPS media or LB media for ¹⁵N-labeled and unlabeled expression, respectively. Overexpression was induced with 0.5 mM IPTG when cultures reached OD₆₀₀ values of ~0.6, followed by continued induction at 25 °C for 18 h. Cells were harvested by centrifugation at 4000 rpm for 30 min at 4 °C, resuspended in buffer containing 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 10 mM β ME, and lysed as described^{6,11}. Purification was achieved via Ni Sepharose[™] 6 Fast Flow or Ni NTA-agarose columns and fractions containing RBD1^{WT} or RBD1^{D623A/D734A} (~16.4 kDa) were pooled and dialyzed into 15 mM HEPES, pH 7.0, 150 mM NaCl, and 0.5 mM TCEP before being concentrated, aliquoted, and stored at -80 °C.

Circular dichroism (CD) spectroscopy

Purified RBD1^{WT} and RBD1^{D623A/D734A} were monitored with far-UV CD spectroscopy from 200 to 260 nm using an AppliedPhotophysics Chirascan V100 spectropolarimeter. The spectra were measured for protein concentration of 0.05 mg/mL (2.45μ M) in 1 mM Tris-Base (pH 7.4) containing 150 mM NaCl, with or without CaCl₂ or MgCl₂ at concentrations of 2.4 mM or 10 mM. CD experiments were performed in a 1.0 cm quartz cuvette at 25 °C. Denaturing experiments were carried out for RBD1^{WT} and RBD1^{D623A/D734A} using 8 M urea in the absence or presence of 2.4 mM CaCl₂. Samples with urea were heated to 95 °C for 5 min and cooled to room temperature shortly before running. Spectral acquisitions were done using a time-per-point of 5.0 s, a spectral bandwidth of 1 nm, and a step size of 1 nm. CD conditions were used for secondary structure determination and fold recognition from RBD1^{WT} and RBD1^{D623A/D734A} circular dichroism spectra^{50,51}.

Cryo-EM sample preparation, data acquisition, and processing

A 3 μ L aliquot of purified CDTb^{D623A/D734A} protein sample at a concentration of 30 μ M in buffer (15 mM HEPES (pH 7.0) and 150 mM NaCl) was applied to a glow discharged QUANTIFOIL® copper R 1.2/1.3 grid with a 2 nm thin extra carbon layer (QUANTIFOIL, GMBH). For calcium-depleted CDTb samples, the same buffer was used and samples were applied to a glowdischarged QUANTIFOIL® copper R 2/2 grid with a 2 nm thin extra carbon layer (QUANTIFOIL® Copper R 2/2 grid with a 2 nm thin extra carbon layer (QUANTIFOIL, GMBH). Grids were double blotted for 2.5 s using a FEI Vitrobot IV with a waiting time of 0 s, in 100% humidity. The sample grids were plunged into liquid ethane for vitrification. Frozen grids were stored in liquid nitrogen until data collection.

Motion-corrected micrographs were produced, and contrast transfer function estimates were obtained in cryoSPARC^{52,53}. For CDTb^{D623A/D734A}, a total of 993,305 particle images were picked by template picker, and several rounds of 2D classifications were performed in cryoSPARC to select the most resolved particles. A total of 126,516 particles with different orientations were re-extracted to build the initial model of CDTb^{D623A/D734A}. The selected particles were used for ab initio model generation with 15 classes to clean out the particle stack. The classes with distinguishable initial volumes were selected for further 3D refinement and CTF refinement. Final 3D homogeneous refinement of 78,587 particles at C1 symmetry yielded a highresolution density map at 4.04 Å. C7 symmetry refinement of the structure resulted in a density map with an improved resolution at 3.56 Å. Resolutions were estimated based on the Fourier shell correlation (FSC).

For Ca²⁺ depleted CDTb, a total of 1,109,860 particles were picked by template picker, and multiple rounds of 2D classifications were performed in cryoSPARC to screen the particles with high resolution. A total of 105,207 particles with different orientations were re-extracted to generate ab-initio models. Out of the 10 ab-initio models generated, 8 classes consisting of 91,077 particles were selected for another round of ab-initio modeling. The classes generated revealed two distinguishable initial models including a "flower-on-a-stem" form with and without the RBD2 ring at the bottom. The volumes with and without the RBD2 electron density consisting of 36,395 and 33,531 particles, respectively, were used for 3D refinement separately. Non-uniform refinement in cryoSPARC using the two initial models yielded two high-resolution density maps, at 3.71 and 3.61 Å resolution. C7 symmetry refinements of the two structures were carried out in cryoSPARC and yielded density maps for both heptamers with and without the RBD2 ring with improved resolutions, at 3.28 Å and 3.06 Å resolution, respectively. Resolutions were estimated based on the Fourier Shell Correlation (FSC) with 0.143 as cutoff (Table 1).

Model building and refinement To build the models for $CDTb^{D623A/D734A}$ and $CDTb^{(-Ca)}$, a single heptamer assembly of the asymmetric CDTb structure (PDB: 6UWR) with the βbarrel was used as a starting model and fitted into the electron density maps of CDTb variants using UCSF Chimera⁵⁴. Then, the models were manually adjusted in COOT and refined using real space refinement in PHENIX^{55,56}.

X-Ray crystallography

Robotic sparse-matrix screening was performed to obtain initial crystallization conditions for isolated Ca²⁺-bound RBD1^{WT} (residues 616-748). These were further optimized to obtain X-ray quality crystals. Large crystals grew over 1-2 weeks with mother liquor consisting of 0.2 M sodium chloride, 1.0 M sodium citrate, and 0.1 M Tris (pH 7.0). Crystals were cryoprotected by transferring them into 1.6 M sodium citrate, flash-cooled in liquid nitrogen, and stored for data collection. X-ray data were collected at SSRL beamline 12-2 and processed using MOSFLM and AIMLESS. Molecular replacement using PHASER with the receptor binding domain 1 domain derived from the X-ray structure of full-length AsymCDTb structure (PDB: 6UWI) as a model produced high quality electron density maps that required only minor manual rebuilding and refinement. Refinement statistics are shown in the SI Appendix, Table 2.

Protein-lipid interaction by SPR/EIS

To prepare the tethered bilayer lipid membrane (tBLM) on the gold-coated sapphire chip, a thiol lipid mimic 1,2-di-O-myristyl-3-[x-mercaptohexa(ethylene oxide) glycerol] (WC14) was used (synthesized by Dr. David Vanderah) as the tethering molecule⁵⁷. Distilled 2-Mercaptoethanol (βME) (purity >99%) was used as a space-filling component between WC14 molecules. Powder of the lipid (purity >99%) 1-palmitoyl-2-oleoyl-snglycero-3-phosphocholine (POPC, Avanti Polar Lipids) was dissolved in anhydrous ethanol (purity >99.5%), to a final concentration of 10 mM. The gold metal deposition on a sapphire chip for the SPR-EIS experiment was performed by magnetron sputtering on a Denton Vacuum Discovery 550 Sputtering System at the NIST Center for Nanoscale Science and Technology cleanroom in Gaithersburg, MD, USA. Substrate was then incubated overnight in a 0.2 mM ethanol solution of WC14/BME with a molar ratio of 25:75 to form a mixed self-assembled monolayer (SAM) of WC14/BME. After rinsing with ethanol and drying in a nitrogen stream, the SAM-coated chip was installed onto the home-built SPR/EIS setup with the cylindrical SPR/EIS cell (1 mL volume) equipped with a perfluoro elastomer Kalrez O-ring (i.d. = 6 mm) against Au/SAM surface. To ensure optical contact between sapphire prism and sapphire chip a few drops of 1-iodonaphthalene ($\eta = 1.70$) was used when mounting the chip to the instrument. A tBLM was formed with the fast solvent exchange method⁵⁸. For the tBLM, 40 µL of a phospholipid (PL) solution was transferred into the SPR/EIS cell with the SAM-coated chip. After 1 min of incubation, 20 µL of PL solution was removed carefully and after a total of 2 min incubation, it was rigorously rinsed with 1 mL of 20 mM HEPES buffer at pH 7.0 and 50 mM NaCl for 10 repetitive cycles. Formation of PL molecules was confirmed by EIS measurements. The PL overlayers were eliminated from the tBLM by incubation in a 10-20% ethanol solution in water for 10 min. Ethanol solution was replaced with water in the EIS/SPR cell followed by rinsing with 950 μL of buffer (during washing cycles 50 μL was left in the cell to avoid the tBLM contact with air). Buffer replacement was repeated 7-8 times and left undisturbed for ~1 h to ensure a homogenous stable bilayer on the tBLM before protein introduction. Then, pH 7 buffer was replaced by 20 mM NaOAc buffer at pH 5.5 and 50 mM NaCl and left undisturbed for ~40 min to ensure a homogenous stable bilayer on the tBLM before protein introduction. Initial formation of a homogenous stable tBLM was ensured at the physiological pH of 7.0. Buffer exchange to low pH buffer increases the electrical resistance of the lipid bilayer because of the protonation and the decrease in charge of the phospholipid phosphate groups. Hence, the electrical conductance decreases. In addition, low pH results in a decrease in SPR signal of phospholipid bilayers. After successful formulation and stability of a tBLM was confirmed by EIS measurements, 1 µM of protein was introduced. Protein adsorption was monitored by SPR and EIS measurements. Successful formulation and stability of a tBLM was confirmed by EIS measurements. The characteristic small semicircle in the EIS spectrum confirmed the bilayer association. EIS data fitting was conducted using the ZView software (Scribner Associates, Inc.) to derive capacitance and resistivity values of the bilayer behavior. While the change in the SPR signal was recorded every 2 s for the duration of the experiment, EIS measurements were recorded every 5 min intervals for >1 h and resistance were obtained by fitting to an equivalent circuit model. Adsorption of the CDTb⁽⁻ ^(a) to the phospholipid membrane at pH 5.5 generates an influx of ions due to pore insertion. The difference in SPR reading at the beginning of protein adsorption and at the end of protein desorption provides the net change. A positive net change signified channel formation in the lipid bilayer. The same procedure was repeated with the CDTb alone. Before each new experiment, the SPR/EIS cell with the O-ring was rinsed with detergent followed by 20-30 mL of miliQ water. Then it was sonicated in ethanol and dried under a stream of nitrogen. The experiments were repeated three times to ensure reproducibility. All measurements were conducted at 25.0 ± 0.3 °C. Care was taken to avoid any data that results from the rupture of lipid bilayers, which can occur with antibacterial peptides, but typically does not occur for toxins. In this regard, lipid bilayer rupture is readily detected by an abrupt decrease in electrical resistance, nearly to zero, accompanied by a sharp drop in the SPR signal. Additionally, there will be a tenfold increase in capacitance, all indicative of lipid bilayer disintegration. As toxin molecules typically do not cause lipid bilayer rupture, it was not surprising that no rupture of the lipid bilayers was detected in studies completed here with CDTb. Rather, CDTb was found to bind lipid bilayers, as detected by SPR, and form pores, as detected via EIS, but such SPR/EIS data was obtained only when Ca²⁺ was depleted from the sample via chelation (Fig. 1).

NMR spectroscopy

2D [1H, 15N]-HSOC NMR experiments of 0.1 mM RBD1WT in 15 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, 0.2 mM TPEN, and 20% D₂O from pH 5.0 to 7.0 at 25 °C with and without 10 mM CaCl₂ were performed on an 800 MHz Bruker Avance NMR spectrometer. A 2D [1H, 15N]-HSQC (heteronuclear single quantum coherence) NMR experiment of 0.25 mM RBD1^{WT} in 15 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, 0.2 mM TPEN, 10 mM CaCl₂, and 20% D₂O at pH 7.0 and 25 °C was performed on an 800 MHz Bruker Avance NMR spectrometer. A 2D [1H, 15N]-HSQC NMR experiment of 0.1 mM RBD1^{D623A/D734A} in 15 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, 0.2 mM TPEN, 10 mM CaCl₂, and 20% D₂O at pH 7.0 and 25 °C was performed on a 600 MHz Bruker Avance III NMR spectrometer. 2D [1 H, 15 N]-HSQC NMR experiments of 0.1 mM RBD1 $^{\hat{D}623A/D734A}$ in 15 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, 0.2 mM TPEN, and 20% D2O at 25 °C with and without 10 mM CaCl2 at pH 5.0 or 7.0 and 25 °C were performed on a 600 MHz Bruker Avance III NMR spectrometer. 2D [1H, ¹⁵N]-HSQC NMR experiments of 0.25 mM of RBD1^{WT} in 15 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, 0.2 mM TPEN, and 20% D₂O at pH 7.4 and 25 °C without or with 2.5 µM, 25 µM, 250 µM, 2.4 mM, and 25 mM CaCl₂ were performed on an 800 MHz Bruker Avance NMR spectrometer. 2D [¹H, ¹⁵N]-HSQC NMR experiments of 0.1 mM RBD1^{WT} or RBD1^{D623A/D734A} in 15 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, 0.2 mM TPEN, and 20% D₂O at 25 °C with and without 10 mM MgCl₂ at pH 7.0 and 25 °C were performed on an 800 MHz Bruker Avance NMR spectrometer. A 2D [1H, 15 N]-HSQC NMR experiment of 0.1 mM RBD1 in 15 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, 0.2 mM TPEN, 10 mM MgCl₂, and 20% D₂O at pH 7.0 and 25 °C was performed on an 800 MHz Bruker Avance NMR spectrometer. 10 mM CaCl₂ was then added and 2D [¹H, ¹⁵N]-HSQCs were collected under the same conditions over a 20-h period. 2D [1H, 15N]-HSQC NMR experiments of 0.1 mM RBD1^{D623A/D734A} in 15 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, 0.2 mM TPEN, 10 mM CaCl₂, and 20% D₂O at 25 °C from pH 5.0 to 7.0 were performed on an 800 MHz Bruker Avance NMR spectrometer.

2D [¹H, ¹⁵N]-HSQC experiments monitoring denaturation of 0.1 mM RBD1^{WT} and RBD1^{D623A/D734A} (in 15 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, 0.2 mM TPEN, and 20% D₂O at pH 7.4 and 25 °C) were done using 8 M urea in the absence and presence of 2.4 mM CaCl₂. Samples with 8 M urea were heated to 95 °C for 5 min and cooled to room temperature shortly before running on an 800 MHz Bruker Avance NMR spectrometer. To ensure adequate binding, RBD1 was incubated O/N with CaCl₂ for all NMR experiments. All NMR experiments were processed through NMRPipe and analyzed using CcpNmr Analysis^{59,60}. Chemical shift perturbations (CSPs) were calculated as described by Willamson⁶¹.

Ca²⁺ and Mg²⁺-binding monitored by fluorescence spectroscopy Ca²⁺ titrations of RBD1^{WT} and RBD1^{D623A/D734A} were carried out using a Varian Cary Eclipse fluorometer at 25 °C to monitor RBD1^{WT} Ca²⁺-binding via fluorescence emission62. Samples contained 25 µM RBD1WT or RBD1^{D623A/D734A}, 15 mM HEPES (pH 7.0), 150 mM NaCl, 0.5 mM TCEP, and increasing concentrations of CaCl₂ (0 µM, 2 µM, 5 µM, 10 µM, 25 µM, $50 \,\mu\text{M}, 100 \,\mu\text{M}, 150 \,\mu\text{M}, 200 \,\mu\text{M}, 375 \,\mu\text{M}, 500 \,\mu\text{M}, 750 \,\mu\text{M}, 1 \,\text{mM}, 5 \,\text{mM},$ and 10 mM, respectively). Free Ca²⁺ concentrations were estimated via Quin-2, and samples were incubated overnight to achieve equilibrium. Ca^{2+} -binding was monitored via the change in tryptophan emission (W632) at 350 nm upon excitation at 295 nm. The slit width for excitation and emission was 5 nm. Fluorescence Ca2+-binding experiments for the RBD1 constructs were done in triplicate, and data were fit to a log(agonist) vs. response (three parameters) non-linear regression curve ($\log_{10}([Ca^{2+}])$ vs. fluorescence emission intensity) with a 90% confidence interval (CI) to calculate the binding affinity of RBD1 constructs for Ca²⁺. The titration of MgCl₂ into RBD1^{WT} was carried out and analyzed similarly. The fluorescence profiles of RBD1^{WT} and RBD1^{D623A/D734A} were generated from samples containing 25 µM RBD1^{WT} or RBD1^{D623A/D734A}, 15 mM HEPES (pH 7.0), 150 mM NaCl, and 0.5 mM TCEP with and without 10 mM CaCl2 at 25 °C. Samples were excited at 280 nm and fluorescence emissions from 300 to 450 nm were collected. Experiments are presented as the normalized average emission intensity of five scans.

Vero cell toxicity assays of CDT

Vero cell cytotoxicity assays are established as a reliable protocol for examining the cytotoxicity of binary toxins and their inhibition⁶³. The assay is robust and produces linear results across serial dilutions and can be analyzed quantitatively, including in a high-throughput mode³⁹. As Vero cell cytotoxicity assays are commonly used, the results here can be evaluated relative to binary toxins from various sources and/or from different laboratories^{39,63}. The Vero cells used here were obtained from American Type Culture Collection (ATCC-CCL-81), cultured in complete media EMEM (Corning Cellgro 10-009-CV) with 10% FBS. A cell density of 4000 cells/well was maintained in 200 µL complete media placed in a 96-well plate (Corning #3904) by overnight incubation in a humidifier with 5% CO₂ at 37 °C. To formulate the binary toxin complex, purified CDTa was combined with active CDTb^{D623A/D734A} or CDTb in complete media at the optimal molar ratio of 1:7, CDTa:CDTb. A final volume of 100 µL from each dilution was added into each well in 96-well plate with existing media to test at toxin concentrations up to 1 nM (1 nM CDTa: 7 nM CDTb). Vero cells were incubated with binary toxin at 5% CO2 and 37 °C in a dose-dependent manner. After 24 h=, plates were centrifuged at 1000 rpm for 1 min. The supernatant was removed, and cells were fixed with $1 \times$ PBS with 3.7% formaldehyde for 15 min at room temperature. Cells were washed in 1× PBS and then incubated in 1× PBS with 1% Triton-X-100 for 5 min at room temperature, to permeabilize the cells. Cells were then washed again with 1× PBS and incubated in the dark with 0.016 μ M Alexa Fluor 488 Phalloidin in 1× PBS and 1% BSA for 1 h at room temperature. For imaging, cells were washed and incubated with 1× PBS. Fluorescence emission of Alexa Fluor 488 Phalloidin at 520 nm was recorded in well-scanning mode using the BMG PHERAstar FS with an excitation wavelength of 495 nm. Vero cell toxicity assay data were plotted and a TC50 was calculated via regression analyses. The Vero cell data presented are representative of three biological replicates.

Differential scanning fluorimetry (DSF)

The wild-type and D623A + D734A mutant constructs of CDTb were incubated at an initial concentration of 2 µM in buffer (15 mM HEPES at pH 7.0, 150 mM NaCl, and 0.5 mM DTT), with an overall 1000-fold dilution of the 5000X stock provided to give a 5X aqueous working solution of SYPRO^{\circ} Orange in the final sample. The wild-type and D623A + D734A double mutant RBD1 constructs were incubated at an initial concentration of 10 µM in buffer (15 mM HEPES at pH 7.0, 150 mM NaCl, and 0.5 mM TCEP) with a 100-fold dilution of the 5000X stock to give a 50X aqueous working solution of SYPRO[®] Orange in the final sample. Incubation was done in a 364-well reaction plate for 30 min. The final concentration of the CDTb constructs was 1 µM in 5× SYPRO® Orange in each sample well at a final volume of 20 μ L. The final concentration of RBD1 constructs was 5 μ M in 50× SYPRO° Orange in each sample well to a final volume of 20 µL. To prepare samples with calcium, CaCl₂ was added to a final concentration of 1 mM for CDTb constructs and 10 mM for RBD1 constructs and incubated overnight prior to incubation with SYPRO® Orange. The 364-well reaction plate was sealed with optical adhesive (Applied Biosystems) and spun down using a PCR plate spinner (VWR) to force the volume to the bottom of each well. The plate was inserted into an RT-PCR machine (ViiA 7) by Life Technologies, Applied Biosystems) to record the fluorescence intensities upon protein thermal unfolding. Experiments were performed from 4 to 90 °C with a heating rate of 0.02 °C/s using the ViiA 7 RUO software. The melting temperature (T_M) was evaluated using the Boltzmann fitting and first derivative analysis, and the analyses for each construct were from the average of four replicates.

Statistics and reproducibility

GraphPad Prism 9 or 10 were used to plot and analyze data. The number of replicates (n = 3-4 depending on experiment) is described in the

corresponding methods and figure legends. CryoEM and X-ray crystallography refinement and data validation statistics were reported from cyroSPARC and PHENIX and are outlined in Table 1 and Table 2, respectively.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Cryoelectron microscopy density maps were deposited in the Electron Microscopy Data Bank (EMDB), https://www.ebi.ac.uk/pdbe/emdb/ (accession nos. EMD-28207 for CDTb^{D623A/D734A}, EMD-28206 for CDTb^(-Ca) class 1, and EMD-28205 for CDTb^(-Ca) class 2). Model coordinates were deposited in the Protein Data Bank (PDB), https://www.wwpdb.org/ (PDB ID codes 8EKM for CDTb^{D623A/D734A}, 8EKL for CDTb^(-Ca) class 1, 8EKK for CDTb^(-Ca) class 2, and 9MUI for RBD1^{WT}). Source data for results presented in the main article are provided in Supplementary Data 1. All other data are available from the corresponding authors upon request.

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Author contributions

Conceptualization: E.P., A.d.G., D.J.W. Methodology: D.L.A., S.E.S., K.A.A., R.G-R., K.M.V., I.F.N., A.X.S., R.R.R., V.I.S., E.P., D.J.W. Investigation: D.L.A., S.E.S., K.A.A., R.G-R., K.M.V., I.F.N., A.X.S., R.R.R., V.I.S., D.H., A.d.G., E.P., D.J.W. Funding acquisition: E.P., A.d.G., D.J.W., S.E.S. Supervision: E.P., K.M.V., V.I.S., D.J.W. Writing—original draft: S.E.S., D.L.A., K.A.A., R.G-R., K.M.V., R.R.R., V.I.S., M.E.C., E.P., D.J.W. Writing review and editing: S.E.S., K.M.V., D.L.A., K.A.A., R.G-R., R.R.R., V.I.S., A.d.G., M.E.C., E.P., D.J.W.

Competing interests

The authors declare no competing interests.

Additional information

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