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Cytotoxicity of *Clostridium difficile* toxin B does not require cysteine protease-mediated autocleavage and release of the glucosyltransferase domain into the host cell cytosol

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Abstract

Clostridium difficile virulence requires secretion of two exotoxins, TcdA and TcdB. The precise mechanism of toxin uptake and delivery is undefined, but current models predict that the cysteine protease domain (CPD) mediated autocleavage and release of glucosyltransferase domain (GTD) is crucial for intoxication. To determine the importance of CPD mediated cleavage to TcdB cytotoxicity, we generated two mutant toxins TcdB-C698S and TcdB-H653A, and assayed their abilities to intoxicate cells. The CPD mutants include an intact GTD but lack the cysteine protease activity. The mutants had reduced potency in that their effect on cells was delayed and required higher concentrations than wild-type TcdB. They did eventually cause cell rounding, glucosylation of Rho GTPases, and apoptosis that was indistinguishable from that caused by TcdB. Although the mutant toxins caused a complete cell rounding, they failed to release their GTD into cytosol, whereas wild-type TcdB displayed significant autocleavage and release of GTD. We conclude that the cysteine protease-mediated autocleavage and release of GTD is not a prerequisite for the cytotoxic activity of TcdB, but rather limits the potency and speed of Rho GTPase glucosylation. Our findings revise and refine the current model for the mode of the action and cellular trafficking of TcdB.

Keywords

Clostridium difficile; toxin B (TcdB); cysteine protease; autocleavage; cytotoxicity

Introduction

The anaerobic Gram-positive bacterial species *Clostridium difficile* is the leading cause of infectious diarrhea, including antibiotic-associated pseudomembranous colitis, in hospitalized patients (Kelly & LaMont, 2008, Zilberberg, 2009). Pathogenic strains of *C. difficile* produce two potent exotoxins, TcdA and TcdB that induce mucosal inflammation and are largely responsible for the associated diarrhea (Voth & Ballard, 2005). TcdB, in

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particular, is critical for virulence and is found in all clinically isolated pathogenic strains (Lyras, *et al.*, 2009, Rupnik, *et al.*, 2009, Kuehne, *et al.*, 2010).

TcdA and TcdB are large polypeptides, 308 and 270 kDa, respectively, each with four functional domains (Jank & Aktories, 2008, Albesa-Jove, *et al.*, 2010, Pruitt, *et al.*, 2010). The amino-terminal end of each toxin contains the glucosyltransferase activity and is therefore called the GTD (glucosyltransferase domain). Immediately adjacent to the GTD is a domain that confers cysteine protease activity (CPD). In TcdB CPD-mediated cleavage occurs between amino acids 543 and 544 and results in release of the GTD (amino acids 1-543) into the cytosol (Rupnik, *et al.*, 2005). A catalytic triad consisting of C698, H653 and D587 is conserved in TcdB from multiple strains (Lanis, *et al.*, 2010) and the enzymatic activity of the CPD is allosterically regulated by the small molecule inositol hexakisphosphate (InsP6) (Pruitt, *et al.*, 2009). The CPD is flanked by the GTD, on the amino side and a large domain harboring a hydrophobic region termed the translocation domain (TD) on the carboxyl side. The extreme carboxyl end of the toxins includes a repetitive oligopeptide of the cell wall binding domain (RBD) (Figure 1 part A).

The precise mechanism of toxin uptake and GTD release into the eukaryotic cell cytosol remains to be elucidated; however, a model of domain activation has been developed (Giesemann, *et al.*, 2008). First the RBD binds to molecules on the cell surface resulting in receptor-mediated endocytosis of the toxin into an endosome (von Eichel-Streiber, *et al.*, 1992, Ho, *et al.*, 2005). Acidification of the endosomal compartment results in conformational changes (Qa'Dan, *et al.*, 2000) and insertion into the endosomal membrane to form a channel, through which the catalytic GTD and adjacent CPD translocate to the cytosolic side of the endosomal membrane (Barth, *et al.*, 2001, Pfeifer, *et al.*, 2003). Next a small cytosolic molecule InsP6 binds to CPD and triggers an auto-cleavage that releases the GTD into the cytosol (Reineke, *et al.*, 2007, Egerer, *et al.*, 2009). Finally, the cleaved GTD catalyzes the glucosylation of Rho-GTPases (small regulatory proteins of the eukaryote actin cell cytoskeleton, such as Rho, Rac 1 and Cdc42) resulting in cell rounding and apoptotic death (Just, *et al.*, 1995, Hofmann, *et al.*, 1997, Qa'Dan, *et al.*, 2002, Nottrott, *et al.*, 2007). According to this model, autocleavage of *C. difficile* toxins is crucial for the delivery of activity domains of GTD as part of the mechanism of action. An early report showed that unpurified recombinant C698S mutant TcdB in *E. coli* lysate has only 10-fold less cytotoxicity (Barroso, *et al.*, 1994), but more recent studies have showed that release of GTD into cytosol is crucial for TcdB-mediated host cell intoxication (Pfeifer, *et al.*, 2003, Rupnik, *et al.*, 2005). Whether GTD of TcdA is released into cytosol of host cells is not clear but the non-cleavable TcdA mutant has reduced cytotoxicity but is not devoid of it (Kreimeyer, *et al.*, 2011).

In this study we generated defined point mutations that inactivate the cleavage activity of the CPD in TcdB. Two mutants, TcdB-C698S and TcdB-H653A were unable to undergo autocleavage to release the GTD when incubated with InsP6, indicating a loss of cysteine protease activity. Surprisingly both mutants induced cell rounding in cultured cells. Wild-type TcdB was only more potent in causing cell rounding at lower concentrations but the cellular morphology induced by TcdB was identical to that induced by the mutant forms of the toxin. Both TcdB mutants catalyzed the glucosylation of Rac1 and induced apoptosis in cultured cells at levels similar to wild-type TcdB at higher concentrations. We conclude that cleavage to release the GTD by the CPD is not critical for TcdB-induced cytotoxicity and propose a revised model for TcdB action in host cells.

Results

Inactivation of the TcdB Cysteine Protease Domain (CPD)

Since the catalytic triad, C698, H653 and D587 is highly conserved we hypothesized that these amino acids were critical for the protease activity of the CPD and therefore constructed two mutant forms of TcdB, TcdB-C698S and TcdB-H653A each with a single amino acid substitution at one of the critical residues. We next used *B. megaterium* to over-produce TcdB-C698S and TcdB-H653A in a vector system that introduces a histidine tag to facilitate protein purification (see materials and methods (Yang, *et al.*, 2008)). Figure 1A includes a diagram indicating the location of each mutation in TcdB. The mutant proteins were indistinguishable from wild-type TcdB (270 kDa) when visualized with Coomassie blue on an SDS-polyacrylamide gel and both mutant proteins were recognized by Anti-TcdB specific antibody (Wang, *et al.*, 2012) in Western blots (data not shown).

As expected, TcdB-C698S and TcdB-H653A did not undergo autoproteolytic processing when incubated with inositol hexakisphosphate (InsP6) (Figure 1B). Unlike the CPD mutants wild-type TcdB did undergo autocleavage of GTD in the presence of InsP6 in a dose dependent manner (Figure 1B), as has been shown previously (Savidge, *et al.*, 2011). In contrast to their complete lack of autocleavage activity, TcdB-C698S and TcdB-H653A did show wild-type levels of glucosyltransferase activity (Figure 1C). In fact when we used an antibody specific for the non-glucosylated form of Rac1 to detect Rac1 in Vero cell lysates exposed to TcdB, TcdB-C698S and TcdB-H653A, all the toxins resulted in a similar loss of detection of non-glucosylated form of Rac1 (Figure 1C), indicating the same pattern of glucosylation of the Rho GTPase (Genth, *et al.*, 2006, Yang, *et al.*, 2008).

Effects of InsP6

In order to test the effect of extracellular autocleavage on cell rounding induced by TcdB and the mutant forms of the toxin, we pre-incubated each toxin with InsP6 for 20 minutes and then added the mixture to Vero cell monolayers for an overnight incubation. As shown in Figure 2A, pre-incubation of TcdB with InsP6 reduced its cytotoxic activity more than 1000 fold, whereas pre-incubation of TcdB-C698S and TcdB-H653A with InsP6 had no effect on their cytotoxicity. To exclude the possibility that the inability of InsP6 to reduce of cytotoxicity of CPD-mutants is due to inefficient binding of the molecule to the mutant CPD, we utilized a non-cleavable mutant TcdB-L543A which harbors an intact CPD (kindly provided by A. Shen). Due to the point mutation of leucine at 543 to alanine, this mutant form of TcdB fails to release the 63 kDa GTD in the presence of InsP6 (Figure 2B). Consistent with CPD-mutants, TcdB-L543A showed no reduction in its cytotoxic effect on Vero cells when it was pre-incubated with InsP6 (Figure 2C). We hypothesize that extracellular autocleavage of TcdB triggered by InsP6 destroyed TcdB cytotoxic activity. In contrast since TcdB-L543A, TcdB-C698S and TcdB-H653A are either not cleavable (TcdB-L543A) or lack the activity of the CPD (TcdB-C698S and TcdB-H653A) they were not affected by incubation with InsP6.

Induction of Cytotoxicity

Since cysteine protease-mediated autocleavage of TcdB is believed to be crucial for the delivery of effector domain and toxicity, we tested the cytotoxicity of the CPD-mutants on the cultured cells. As shown in Figure 3, both TcdB-C698S and TcdB-H653A induced cell rounding in both dose- and time-dependent manners (Figure 3A). Both TcdB-C698S and TcdB-H653A required a dose of 0.1–1 ng/ml to induce 50% cell rounding after 8 hr of exposure whereas TcdB had the same effect at concentration of 0.01 ng/ml (Figure 3A left panel). Therefore these mutations in the CPD reduced cytotoxicity approximately 10–100 fold from that of wild-type TcdB. At a given concentration, the two CPD-mutant toxins had

a slightly reduced speed in causing cell rounding as compared with wild-type TcdB but eventually caused the 100% cell rounding seen following exposure to wild-type TcdB (Figure 3A right panel). Morphologically, the cell rounding caused by the CPD mutant toxins was identical to that caused by TcdB (Figure 3B) when observed with a phase contract microscope. To ensure the cytotoxic activity of the mutant toxins was not cell specific, we evaluated their effects on other cell types including mouse intestinal epithelial cells CT26 and human colonic carcinoma cells HCT-8. Our results with these cell lines were identical to those observed in Vero cells (Figure 3C and data not shown). These data clearly demonstrate that the CPD mutants efficiently cause cytotoxic effects typical of exposure to wild-type TcdB in a variety of culture cell types.

Rho GTPase Glucosylation and Caspase Activation

Since the CPD mutant toxins exhibit potent cytotoxicity, we examine their ability to glucosylate Rho GTPases and induce caspase activation in host cells. To do this we exposed CT26 cells to TcdB or TcdB-C698S. TcdB-C698S caused a similar level of Rac1 glucosylation in CT26 cells when compared with wild type TcdB at concentrations of either 100 or 10 ng/ml (Figure 4A). To assess apoptosis induction in host cells by the toxins, we examined effector caspase activation after a 24-hr toxin exposure (Nottrott, *et al.*, 2007). As shown in Figure 4B, both mutant and wild-type toxins efficiently induced caspase activation in a dose-dependent manner. TcdB induced cleavage of pro-caspase at 1 ng/ml whereas TcdB-C698S required a concentration of 10 ng/ml to cause similar caspase cleavage. At higher toxin concentrations TcdB and TcdB-C698S induced similar levels of caspase-3 activation (Figure 4B).

Intracellular Release of the Glucosyltransferase Domain GTD

Since previous models predicted that the cysteine protease-mediated autocleavage and release of GTD into cytosol is a key step of intoxication of target cells, we assess whether CPD-mutant toxins TcdB-C698S and TcdB-H653A were able to release their functional GTD into cytosol. Vero cells were treated with TcdB and the CPD mutant toxins and then lysed and analyzed by immunoblotting with specific monoclonal antibodies against GTD or RBD. Cells treated with TcdB contained a significantly reduced level of full-length 270 kDa toxin as compared to cells treated with either TcdB-C698S or TcdB-H653A (Figure 5A and B). The 63-kDa GTD fragment was only detected in cells treated with TcdB and not those exposed to the CPD mutant toxins (Figure 5). Consistently, when the cell lysates were immunoblotted with antibody against C-terminal RBD, the 207 kDa fragment was ready to be detected in lysates from cells exposed to TcdB but not in lysates from the mutant toxins. Thus, neither TcdB-C698S nor TcdB-H653A undergoes autocleavage and release of GTD but both mutants are clearly capable of efficiently causing cell rounding, Rho GTPase glucosylation and apoptosis of host cells.

Next, we examined the kinetics of GTD release in cells intoxicated by wild-type TcdB. Toxin binding was synchronized by incubating on ice for 30 min followed by extensive washing to remove unbound toxin. Autocleavage and release of the GTD was evident after 20 min of incubation in fresh medium at 37°C and continued to increase as the incubation time increased (Figure 5C). Morphologically, cells started to round at 30 min of culture and complete rounding was observed after 40 min of exposure. Significantly at the 40 min time point a significant amount of TcdB remained uncleaved (Figure 5C).

Discussion

The current model of *C. difficile* toxin B activity predicts that cleavage by the CPD of TcdB between amino acids L543 and G544 (Rupnik, *et al.*, 2005, Jank & Aktories, 2008) to

release the GTD into the cytosol, is essential for cell intoxication (Genth, *et al.*, 2008, Giesemann, *et al.*, 2008). CPD activity is activated by a small molecule InsP6 (Shen, *et al.*, 2011) and there are three amino acids C698, H653 and D587, in TcdB that are homologous to those found in the cysteine protease catalytic site of *Vibrio cholerae* RTX toxins (Egerer, *et al.*, 2007, Sheahan, *et al.*, 2007). To determine the impact of CPD-mediated autoproteolytic cleavage on cytotoxicity of TcdB we mutated two of the three amino acids in the catalytic site and assayed the impact of the mutant toxins on cells.

We demonstrated that CPD mediated cleavage to release the GTD was not detectable in either mutant toxin, therefore TcdB-C698S and TcdB-H653A are unable to undergo autocleavage (Figure 1B) and therefore fail to release a detectable amount of GTD into cytosol *in vivo* (Figure 5). However, the mutant toxins caused a similar cell rounding, glucosylation of Rho GTPases, and apoptosis of host cells, indicating that the activity of the GTD may not be dependent on its release from the other domains of TcdB. This finding is in contrast to current models of TcdB action that predict that the activity of the GTD is dependent on cysteine protease-mediated autocleavage and its release into the cytosol following endocytosis.

The current model of TcdB action also predicts that cleavage of TcdB prior to cellular uptake would inhibit cytotoxicity as the GTD domain would be unable to enter the cell once it was separated from the other domains of the toxin. To test this prediction we determined the impact exposure to InsP6 had on the ability of both wild-type and mutant forms of TcdB to intoxicate cells. As indicated in Figure 2A, pre-incubation of TcdB with InsP6 prevented Vero cell rounding (1000 fold lower level), whereas pre-incubation of TcdB-C698S and TcdB-H653A with InsP6 had no effect on their activity. We propose that the lack of a reduction in cell rounding caused by the mutant toxins, following pre-exposure to InsP6, is a result of the inability of InsP6 to induce cleavage by the mutant CPDs. Our data indicates that in order for TcdB to intoxicate cells, autocleavage of GTD from TcdB must take place inside the cell. The finding may be able to be exploited therapeutically by using InsP6 or other substances that stimulate CPD mediated cleavage thus inactivate the toxin.

To study the role of autocleavage in the function of TcdB *in vivo*, we compared the cytotoxic effects of wild-type TcdB with those of the mutant toxins lacking the catalytic activity of the CPD. Our results demonstrate that wild-type TcdB is more potent (about 10-fold) than the non-cleavable forms of TcdB at low concentrations. We hypothesize that this difference in potency is due to difference in target access between the wild-type and mutant forms of TcdB. Cleavage of the wild-type TcdB results in the liberation of GTD into the cytosolic compartment and more rapid glucosylation of cytosolic Rho GTPases. In contrast, the GTD of the non-cleavable mutants is presumed to be tethered, by the rest of the toxin, to the cytosolic surface of the endosome membrane and therefore would only have access to a limited number of target Rho GTPases i.e. those membrane-associated or those come in contact with the endosome. Interestingly although the appearance of the cellular effects caused by the CPD mutant toxins is delayed as compared to those caused by wild-type TcdB, treated cells eventually show full cytopathic (complete cell rounding) and cytotoxic (cleavage of caspase-3) effects. We however cannot rule out that there is an undetectable amount of GTD release via process other than cysteine protease-mediate autocleavage. If that is the case, the trace amount of GTD liberated into host cytosol in those cells treated with CPD mutant toxins is sufficient to cause intoxication.

The TcdB expressed by hypervirulent strain NAP1, TcdB_{HV} is more cytotoxic than that found in laboratory strain VPI10463 (Stabler, *et al.*, 2008, Lanis, *et al.*, 2010). Recent characterization of CPD-mediated autocleavage in TcdB_{HV} indicates that cleavage is more efficient and mediated by lower doses of InP6 (Lanis, *et al.*, 2012) and it is tempting to

conclude that this increased efficiency of autocleavage results in the increased cytotoxicity of TcdB_{HY}. Similarly TcdA, which is 1000-fold less toxic to most culture cells than TcdB, exhibits significantly lower CPD activity and autocleavage when incubated with InP6 *in vitro* (Egerer, *et al.*, 2007, Reineke, *et al.*, 2007, Egerer, *et al.*, 2009). In addition a non-cleavable TcdA mutant (Kreimeyer, *et al.*) has reduced cytotoxicity but is not devoid of it. The CPD mutants of TcdB also retain a significant cytotoxicity (Savidge, *et al.*, 2011). All of these data support our conclusion that CPD-mediated cleavage of the GTD from TcdB accelerates the rate at which the GTD can find intracellular targets but is not critical for Rho GTPase glucosylation. In fact the data we present here as well as published data support a model in which the difference in the cytotoxicity observed between TcdB (more cytotoxic) and TcdA (less cytotoxic) can be partially explained by the differences in the activity of their respective CPDs. Autocleavage by the CPD of TcdA is less efficient *in vitro* than that of the TcdB CPD and this difference is correlated with their effects on cultured cells with TcdA being less cytotoxic than TcdB.

Materials and Methods

Cell lines, and toxins

The murine colonic epithelial cell line CT26 and the monkey kidney cell line Vero were obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate.

The full-length wild-type recombinant TcdB was purified from total crude extract of *Bacillus megaterium* as described previously (Yang, *et al.*, 2008). The highly purified recombinant toxins appeared as a single band on SDS-PAGE (data not shown).

Generation of mutant holotoxins

We previously cloned full-length *tcdB* into a shuttle vector pHis1525 (pHis-TcdB) and expressed the recombinant holotoxin in *B. megaterium* (Yang, *et al.*, 2008). Based this system, we synthesized the CPD with single mutation (C698S or H653A) and cloned these genes into pHis1525. The plasmid carrying a point mutation (L543A) of TcdB was a kind gift of Dr. Aimee Shen (University of Vermont). The constructs carrying mutant toxin genes were used to transform *B. megaterium* and mutant holotoxins were expressed and purified using the same methods described previously (Yang, *et al.*, 2008).

Glucosyltransferase activity of the toxins

The wild type and mutant toxins were assessed their glucosyltransferase activity by assaying glucosylation of the Rho GTPase Rac1, both in cell-based and cell-free assays. In a cell-based assay, Vero cells in 12-well plates were exposed to different concentrations of toxins for different time before harvesting (see figure legends of details). Cells were lysed with SDS sampling buffer. In cell-free assays, Vero cell pellets were resuspended in glucosylation buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 1 mM MnCl₂ and 2 mM MgCl₂) and lysed with a syringe (25G, 40 passes through the needle). After centrifugation (167,000g, 3 min), the supernatant was used as postnuclear cell lysate. To perform glucosylation assay, the cell lysate were incubated with different doses of TcdB, TcdB-C698S or -TcdB-H653A at 37 °C for 1 hr. The reaction was terminated by heating at 100 °C for 5 min in SDS-sample buffer. In both cell-free and *in vivo* assays Rac 1 glucosylation was detected using antibody that specifically recognizes the non-glucosylated form of Rac1 (clone 102, BD Bioscience) (Genth, *et al.*, 2006, Yang, *et al.*, 2008). Anti-β-actin (clone AC-40, Sigma) was used to detect B-actin and ensure that samples were loaded evenly on the SDS polyacrylamide gels.

Insp6-induced autocleavage of the toxins

All toxins were diluted in 10 mM Tris (pH 7.5) buffer to a concentration of 20 ng/ml in a final volume of 20 μ l. The reaction was initiated by addition of Insp6 at the indicated final concentrations. Following incubation at room temperature for the indicated time, the reactions were stopped by adding SDS sampling buffer and analyzed by Western blot using rabbit anti-TcdA or alpaca anti-TcdB polyclonal antibodies (generated in this laboratory) respectively.

Cytotoxic effects of toxins on cultured cells

Cytotoxic effects of the toxins on cultured cells were assessed by cell rounding assays. Vero, CT-26, or HCT-8 cells seeded in 96-well plates were treated with TcdB, TcdB-C698S, or TcdB-H543A. Cell rounding was visualized by phase-contrast microscopy. Approximately 100 cells from 5 random fields were counted and the percentage of rounded cells was determined (He, *et al.*, 2009). The experiments were repeated three times, and triplicate wells were assessed for cell rounding in each experiment.

Caspase activation

Vero Cells seeded in 12 well plates were exposed to the different concentrations of toxins for different time before harvesting. After incubation for 36h, cells were lysed with SDS sampling buffer. To immunodetect caspase 3, the lysates were separated on a 12% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. The membranes were then probed with antibodies that specifically recognize cleaved caspase-3 (Cleaved caspase-3 (Asp175) (5A1E) Rabbit mAb, Cell Signaling) and β -actin (clone AC-40, Sigma).

Intracellular cleavage of wild type and mutant toxins

Vero cells in 6-well plates were exposed to 1 μ g/ml of TcdB, TcdB-C698S, or TcdB-H653A for 1 hr before harvesting. Additionally, for time course experiments Vero cells were left untreated or exposed to 1 μ g/ml of TcdB on ice for 30 min before extensively washed. Cells were then incubated at 37°C at fresh medium for different time before harvesting via trypsinization. Western blotting was performed using monoclonal antibodies against GTD or RBD of TcdB (generated in this lab). β -actin was used as a loading control for total protein. To quantitate the relative cleavage of the individual toxins, gels were scanned using GBox-Chemi-XT4 (Synoptics, Frederick, MD) and the density of the specific bands was analyzed using GeneSnap acquisition software (Synoptics, Frederick, MD).

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Figure 1.

The diagram of the TcdB mutant toxins and their enzymatic activities. **(A)** The diagram shows the positions of mutations in TcdB-C698S and TcdB-H653A respectively. GTD: glucosyltransferase domain; CPD: cysteine protease domain; TD: translocation domain; RBD: receptor binding domain; **(B)** Autocatalytic activity of the toxin mutants. Wild type TcdB, TcdB-C698S and TcdB-H653A were exposed to the indicated amounts of InsP6 for 2 hr. The autocleavage and release of GTD fragment (63 kDa) were assessed by immunoblotting using monoclonal antibody against GTD; **(C)** Glucosyltransferase activity of the mutant toxins. Vero cell cytosolic fraction was collected and exposed to wild type and mutant toxins at the indicated concentrations for 1 hr. Rac1 glucosylation was analyzed by immunoblotting using monoclonal antibody (Clone 102) that only binds to non-glucosylated Rac1. β-actin was used as an equal loading control.

Figure 2.

Inhibitory effects of InsP6 on wild-type and mutant toxins. **(A)** Wild type TcdB or mutants TcdB-C698S or TcdB-H653A (Toxin alone) or preincubated with 100 μ M of InsP6 for 20 min were mixed with Vero cells. After overnight incubation, the percentage of cell rounding was determined by phase contrast microscopy; **(B)** TcdB (B) or TcdB-L543A (LA) were incubated with 100 μ M of InsP6 for the indicated time or overnight (O/N). The autocleavage and release of GTD was measured by western blotting using monoclonal antibody against GTD; **(C)** TcdB or TcdB-L543A were incubated with 100 μ M of InsP6 for 0, 5, 15, or 30 min before adding to Vero cells. After overnight incubation, the percentage of cell rounding was determined by phase contrast microscopy.

Figure 3.

Cell rounding induced by wild-type TcdB and CPD mutant toxins. **(A)** Vero cell monolayers were exposed to the indicated amount of toxin for 8 hr (left panel) or 10 ng/ml of the toxins for the indicated time (right panel); **(B)** Vero cells were exposed to the medium (cell control) or the indicated toxins (100 ng/ml) for 2 hr. The percentage of cells affected (cell rounding) were observed under a phase contrast microscope; **(C)** CT26 cells were exposed to the indicated amount of toxins overnight (left panel) or 10 ng/ml of the toxins for the indicated time (right panel). The data in A and B represents a pool the results from three independent experiments.

Figure 4.

Induction of glucosylation of Rac 1 and activation of caspase 3. **(A)** Vero cells were exposed to different doses of TcdB or CPD mutants. Cells were harvested 4 h later and western blot was performed using monoclonal antibody (Clone 102) that only binds to non-glucosylated Rac1. β-actin was used as an equal loading control. The right panel shows the relative band density of the non-glucosylated Rac1 to β-actin from three independent experiments; **(B)** Vero cells were exposed to the indicated doses of TcdB or TcdB-C698S for 24 hr. The activation of caspase 3 was assessed by antibody against cleaved caspase-3 (clone 5A1E). β-actin was used as an equal loading control. The right panel shows the relative band density of the non-glucosylated Rac1 to β-actin from three independent experiments (* indicated that $P < 0.05$ between TcdB and TcdB-C698S).

Figure 5.

Intracellular cleavage of wild-type and mutant toxins. **(A)** Vero cells were exposed to 1 μ g/ml of TcdB, TcdB-C698S, or TcdB-H653A for 1 hr before harvesting. Cells were extensively washed before lysing and Western blotting was performed using monoclonal antibodies against GTD or RBD of TcdB. The total toxins in the medium were measured for loading control of adding equal amount of toxins. β -actin was used as an equal loading of total proteins; **(B)** Graph analysis of three independent results in A. The relative density of 270, 63, or 207 kDa bands over beta actin is shown. The error bars show the SEM. The data was analyzed by ANOVA and * indicated $p < 0.01$; **(C)** Vero cells were untreated (Cells alone) or exposed to 1 μ g/ml of TcdB on ice for 30 min. Cells were then extensively washed before adding fresh medium and incubated for the indicated time before harvesting and TcdB autocleavage and release of GTD were determined by Western blotting using monoclonal antibody against GTD. β -actin was used as a control for equal loading of total protein.