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Structural determinants for membrane insertion, pore formation and translocation of *Clostridium difficile* toxin B

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Summary

Clostridium difficile **toxins A and B bind to eukaryotic target cells, are endocytosed and then deliver their N-terminal glucosyltransferase domain after processing into the cytosol. Whereas glucosyltransferase, autoprocessing and cell-binding domains are well defined, structural features involved in toxin delivery are unknown. Here, we studied structural determinants that define membrane insertion, pore formation and translocation of toxin B. Deletion analyses revealed that a large region, covering amino acids 1501–1753 of toxin B, is dispensable for cytotoxicity in Vero cells. Accordingly, a chimeric toxin, consisting of amino acids 1–1550 and the receptor-binding domain of diphtheria toxin, caused cytotoxic effects. A large N-terminal part of toxin B (amino acids 1–829) was not essential for pore formation (measured by $^{86}Rb^{+}$ release in mammalian cells). Studies using C-terminal truncation fragments of toxin B showed that amino acid residues 1–990 were still capable of inducing fluorescence dye release from large lipid vesicles and led to increased electrical conductance in black lipid membranes. Thereby, we define the minimal pore-forming region of toxin B within amino acid residues 830 and 990. Moreover, we identify within this region a crucial role of the amino acid pair glutamate-970 and glutamate-976 in pore formation of toxin B.**

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Introduction

Clostridium difficile toxins A and B are major virulence factors that are responsible for antibiotic-associated diarrhoea and pseudomembranous enterocolitis (Kelly and LaMont, 2008). The toxins belong to the family of clostridial glucosylating toxins, also including the haemorrhagic and lethal toxins from *Clostridium sordellii* and *C. novyi* α-toxin, which are involved in toxic shock syndrome and gas gangrene respectively (Von Eichel-Streiber *et al.*, 1996; Just and Gerhard, 2004; Schirmer and Aktories, 2004; Voth and Ballard, 2005). The toxins glucosylate small GTPases of Rho and Ras families at threonine 35/37, thereby inhibiting the signalling and regulatory functions of these switch proteins (Just *et al.*, 1995). All of these toxins are large proteins of 250–308 kDa, which are comprised of several functional domains. Recently, an ABCD model has been proposed for the structure–function relationship of the toxins (Jank and Aktories, 2008). The N-terminus harbours the glucosyltransferase activity and is the biologically active domain (A domain) (Hofmann *et al.*, 1997). Crystal structures of the glucosyltransferase domain have been solved, identifying the toxins as members of the GT-A family of glycosyltransferases (Reinert *et al.*, 2005; Ziegler *et al.*, 2008). The C-terminal part consists of the binding domain B, which appears to be involved in cell membrane binding (Dove *et al.*, 1990; Von Eichel-Streiber *et al.*, 1992). This part is characterized by polypeptide repeats, which have a solenoid-like structure in the related toxin A (Ho *et al.*, 2005; Greco *et al.*, 2006). The A domain is followed by the C domain, which possesses cysteine protease activity and is involved in processing of the toxin (Egerer *et al.*, 2007). The cysteine protease activity is regulated by inositol hexakisphosphate (InsP6) (Reineke *et al.*, 2007; Egerer *et al.*, 2009; Egerer and Satchell, 2010). Recently, the crystal structure of the cysteine protease domain from toxin A has been solved, showing a fold similar to that of the cysteine protease of *Vibrio cholerae* MARTX toxin (Pruitt *et al.*, 2009). Between the cysteine protease

domain and the C-terminal binding domain, a delivery domain (D) is suggested, which is responsible for toxin translocation across membranes and delivers the glucosyltransferase into the cytosol of target cells (Jank and Aktories, 2008).

The toxins bind to cell surface receptors by their B domain. Several putative receptors have been described for toxin A, including carbohydrates, glycophospholipids and proteins; however, the precise toxin receptor in humans has not been identified (Krivan *et al.*, 1986; Pothoulakis *et al.*, 1996; Na *et al.*, 2008). Even less is known for binding of toxin B and the receptor involved. After binding the toxins are endocytosed via clathrin- and dynamin-dependent pathways to reach acidic endosomal compartments from where the toxins are translocated into the cytosol (Papatheodorou *et al.*, 2010). Most likely in the cytosol, the cysteine protease domain is activated by binding of InsP6, resulting in autocleavage and release of the glucosyltransferase domain, which then targets Rho proteins.

The molecular mechanisms of the translocation of clostridial glucosylating toxins into the cytosol are not well understood. Recent studies showed that toxins A and B form pores in membranes at low pH (Barth *et al.*, 2001; Giesemann *et al.*, 2006). A pH-dependent increase in electrical conductance has been also identified in black lipid bilayer assays (Barth *et al.*, 2001; Giesemann *et al.*, 2006). Toxin A-induced pore formation occurs in a cholesterol-dependent manner (Giesemann *et al.*, 2006). A hydrophobic region within the D domain has been suggested to be involved in toxin translocation (Dove *et al.*, 1990; Von Eichel-Streiber *et al.*, 1992; Jank and Aktories, 2008). However, so far no experimental data have localized the regions that are involved in pore formation and or translocation. Here we identified the region between amino acids 830 and 990 of TcdB as being essential for pore formation in intact cells, lipid vesicles and black lipid membranes. Furthermore, we identified, within this region, glutamate-970 and -976 as potential amino acids, acting as pH sensors for membrane insertion. However, downstream regions are necessary for efficient translocation of proteins. We also present evidence for an additional domain of unknown function between the pore-forming/translocation domain and the C-terminal binding domain.

Results

Cytotoxicity studies of internal deletions of C. difficile *toxin B*

To get more insights into the structure–function relationships of *C. difficile* toxin B (TcdB), and to understand the proposed delivery function of the middle part of the toxin, we performed deletion studies. To this end the N-terminal glucosyltransferase domain and CPD as well as the C-terminal binding domain, consisting of polypeptide repeats, were left intact. We deleted parts from the middle part of the toxin, resulting in proteins in which amino acids 1501–1753 ($TcdB_{\Delta 1501–1753}$), 1401–1753 ($TcdB_{\Delta 1401–1753}$), 991–1753 ($TcdB_{\Delta 991–1753}$) and 848–1753 ($TcdB_{\Delta 848–1753}$) were deleted (Fig. 1A). The various deletion mutants were expressed in *Bacillus megaterium* and purified as His-tagged proteins. At first, we studied the cytotoxicity of the deletion toxins on Vero cells. As shown in Fig. 1B, only full-length TcdB and $TcdB_{\Delta 1501–1753}$ caused rounding of cells. To exclude that lack of cytotoxicity may be caused by improper folding of the proteins, we tested their glucosyltransferase and cysteine protease activities. All proteins efficiently glucosylated Rac1 and were able to induce autocatalytic cleavage in the presence of InsP6, indicating the intact structures of these enzyme domains (Fig. 1C and D and Fig. S1A).

Exchange of the C-terminal polypeptide repeats of toxin B with the receptor-binding domain of diphtheria toxin

Next, we wanted to know whether the cytotoxic effects observed were independent of the type of the C-terminal binding domain. Therefore, toxin chimeras were constructed that possessed the receptor-binding domain of diphtheria toxin (DTRD) instead of the polypeptide repeats (Fig. 2A). These proteins were then tested for cytotoxicity on Vero cells (Fig. 2B). Exchange of the C-terminal polypeptide domain with the DTRD reduced toxin activity. However, the chimeric toxin $TcdB_{1–1755}$-DTRD caused typical rounding of cells. Similar as with the deletion protein $TcdB_{\Delta 1501–1753}$, the chimeric protein $TcdB_{1–1550}$-DTRD, which lacks toxin B residues 1551–2366, also induced rounding of cells. In contrast the chimeras $TcdB_{1–1370}$-DTRD and $TcdB_{1–830}$-DTRD did not display cytotoxic effects, although they possessed glucosyltransferase and cysteine protease activities *in vitro* (Fig. 2C and D and Fig. S1B). These findings allow two conclusions. First, the C-terminal solenoid-like part (CROPs; combined repetitive oligopeptides) is not essential for the cytotoxic effects and can be substituted by a completely unrelated receptor-binding domain (e.g. the DTRD). Second, the region between residue 1501 and the CROPs is not essential for toxin translocation.

Uptake of chimeric proteins via diphtheria toxin receptor

To make sure that the chimeric proteins are specifically taken up via the diphtheria toxin (DT) receptor, we performed a competition between the chimeric protein $TcdB_{1–1550}$-DTRD and $TcdB_{1–830}$-DTRD. The toxic effects of the chimeric protein $TcdB_{1–1550}$-DTRD could be inhibited

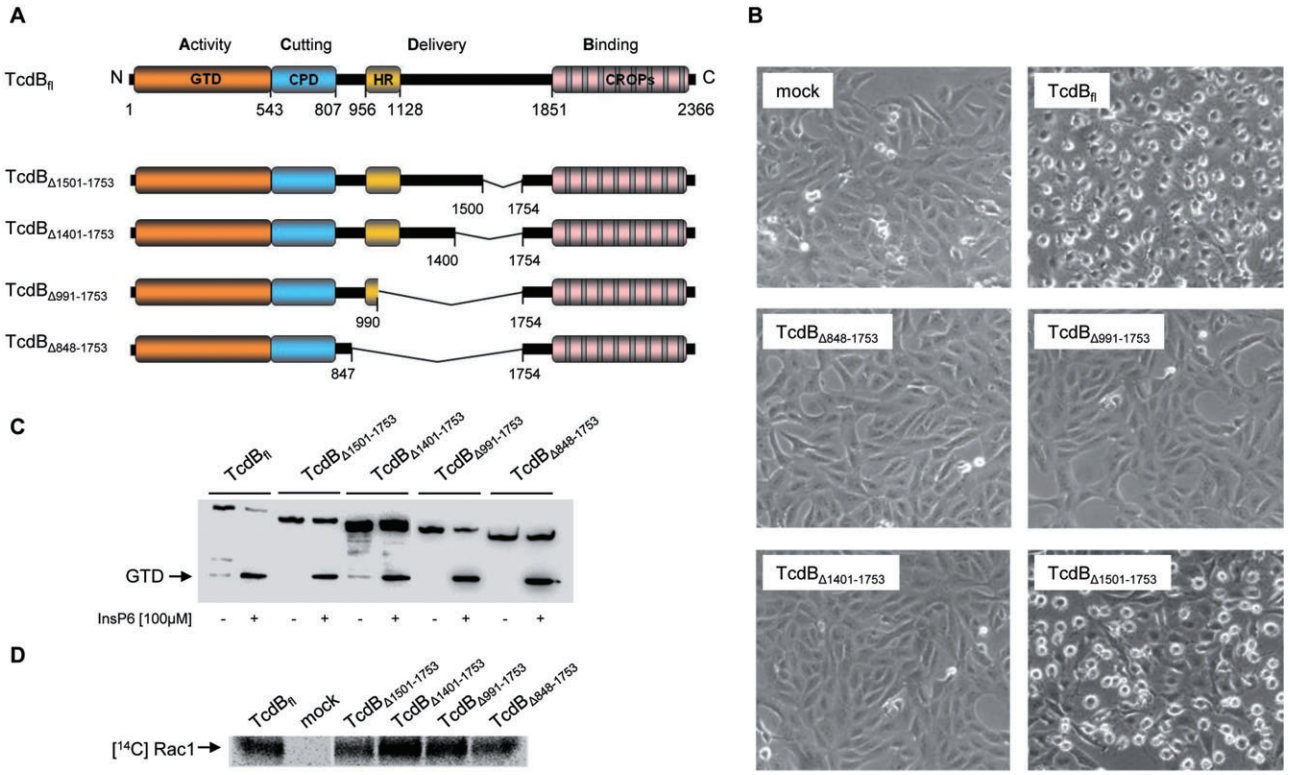

Fig. 1. Scheme of toxin B constructs with internal deletions and functional characterization of the toxin deletions.
A. ABCD model of full-length toxin B (TcdB) and internal deletions. GTD (glucosyltransferase domain) is responsible for the biological effects of the toxin (activity); CPD (cysteine protease domain) is responsible for autocatalytic cleavage (cutting); HR (hydrophobic region) is suggested to be involved in toxin translocation (delivery); CROPs (combined repetitive oligopeptides) are involved in receptor binding. Not well-defined toxin regions in the middle of the protein are suggested to be involved in toxin up-take (delivery). In $TcdB_{\Delta1501-1753}$, $TcdB_{\Delta1401-1753}$, $TcdB_{\Delta991-1753}$ and $TcdB_{\Delta848-1753}$ subscript amino acids are deleted.
B. Intoxication of cultured Vero cells. Semi-confluent cell monolayers were incubated with full-length TcdB (3 pM, 1 h) and TcdB deletions (30 nM, 5 h), respectively, or were left untreated (mock control).
C. Inositol hexakisphosphate (InsP6)-induced autoproteolytic processing. Full-length TcdB and toxin deletions (each 0.5 μg) were incubated with InsP6 (100 μM) for 1 h at 37°C. Cleavage of the GTD was detected by immunoblotting with anti-GTD antibody.
D. *In vitro* glucosylation of Rac1. Rac1 (10 μM) was incubated with TcdB deletion (each 10 nM), or without toxin (mock), in the presence of radiolabelled UDP-[^{14}C]glucose. Samples were subjected to SDS-PAGE and radiolabelled Rac1 was visualized by autoradiography.

by a 10-fold excess of the *in vivo* inactive $TcdB_{1-830}$-DTRD protein (Fig. S2A). This result indicates that the chimeric proteins are taken up by the diphtheria receptor and not by any non-specific mechanism.

N-terminal region of toxin B involved in pore formation

The delivery of the glucosyltransferase domain into the cytosol requires a specific part of toxin B to mediate pore formation in endosomal membranes. To get information about the N-terminal regions, which are involved in pore formation of toxin B, we studied the ability of N-terminal toxin deletions to release ^{86}rubidium ions from CHO-K1 cells upon binding to the cell surface and acidification of the medium to trigger insertion into the plasma membrane (Fig. 3A). As expected, these studies showed that the glucosyltransferase domain and the cysteine protease domain are not necessary for pore formation, because the fragments $TcdB_{544-2366}$ and $TcdB_{830-2366}$ induced ^{86}rubidium ions release similarly as observed with the full-length toxin. However, further N-terminal deletion of 20 amino acids ($TcdB_{850-2366}$) resulted in loss of rubidium release and this hold true for all other N-terminal deletions ($TcdB_{880-2366}$, $TcdB_{906-2366}$, $TcdB_{938-2366}$, $TcdB_{956-2366}$, $TcdB_{975-2366}$, $TcdB_{1129-2366}$). The possibility that the N-terminal toxin B truncations lack binding to the cell surface could be excluded, since $TcdB_{850-2366}$ was able to competitively inhibit intoxication of Vero cells by full-length toxin B (Fig. S2B). In addition, it is shown in Fig. S1C that differences between pore-forming $TcdB_{830-3266}$ and non-pore-forming $TcdB_{850-2366}$ in the ^{86}rubidium release assay do not originate from potential impurities in the protein preparations.

Obviously, the N-terminal delimitation of a region of toxin B that mediates pore formation resides within amino acids 830–850.

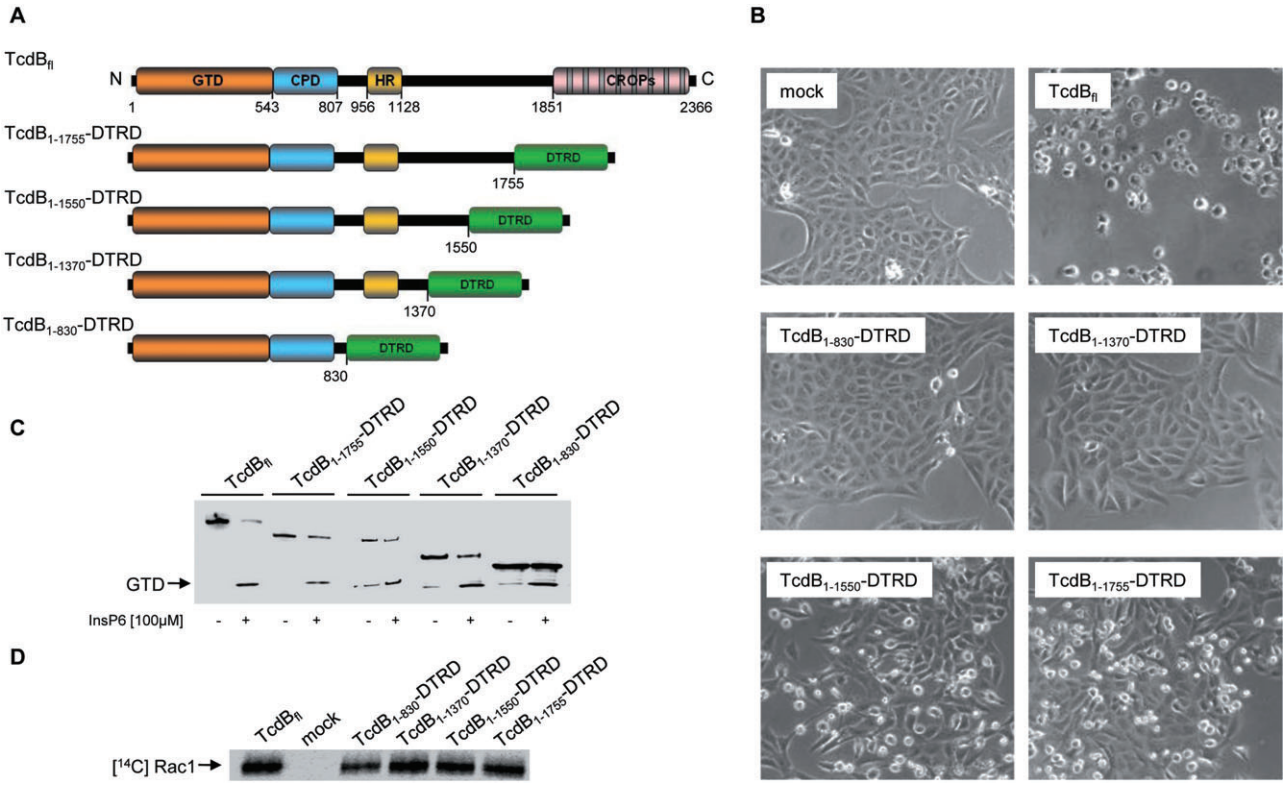

Fig. 2. C-terminal deletions and fusion toxins of toxin B.
A. Schematic representations of C-terminal deletions and fusion proteins. Model of full-length TcdB as in Fig. 1A with the glucosyltransferase domain (GTD), the cysteine protease domain (CPD), the hydrophobic region (HR) and the domain of repetitive polypeptides (CROPs). Fusion proteins of toxin B deletions ($TcdB_{1–1755}$-DTRD, $TcdB_{1–1550}$-DTRD, $TcdB_{1–1370}$-DTRD, $TcdB_{1–830}$-DTRD) with the diphtheria toxin receptor-binding domain (DTRD) indicate length of the N-terminal portion of TcdB in the constructs.
B. Intoxication of Vero cells by toxin constructs. Semi-confluent cell monolayers were incubated with full-length TcdB (1 pM, 1 h) or various TcdB/DTRD chimeric proteins as given in (A) (each 10 nM, 5 h), respectively, or were left untreated (mock control).
C and D. The TcdB/DTRD chimeric constructs (0.5 μg) were tested for autoproteolytic processing (C) and for glucosylation of Rac1 (D) as in Fig. 1B and D respectively.

C-terminal region of toxin B involved in pore formation

To define the C-terminal boundary of a region of toxin B that mediates pore formation, we synthesized several C-terminal truncations. Due to the fact that these toxin constructs lack at least the entire C-terminal receptor-binding domain, their use in a cell-based rubidium release assay is not feasible. We rather aimed to study pore formation of C-terminal toxin B deletion constructs in large unilamellar vesicles (LUVs). Since the lipid vesicles were prepared with lipids tagged with Ni-ions, the C-terminal truncations of toxin B, expressed as His-tagged proteins, could use the Ni-ions as an artificial receptor. Pore formation was monitored by the release of the fluorescent dye HPTS, which is quenched in the lipid vesicles by DPX. Release of HPTS/DPX from vesicles causes dilution of the compounds and increase in fluorescence by reduction of collision quenching. As shown in Fig. 3B, addition of full-length toxin followed by pH reduction induced pore formation and increase in fluorescence. Heat-inactivated full-length toxin B, BSA and liposomes without any protein were used as controls, and did not induce an increase of fluorescence after acidification. As expected, we also did not observe an increased fluorescence signal with the glucosyltransferase domain (fragment $TcdB_{1–543}$), with the fragment $TcdB_{1–807}$ that contains the glucosyltransferase and cysteine protease domains and with $TcdB_{1–955}$. However, pore formation occurred with the fragment $TcdB_{1–990}$ and all other fragments, which possessed additional C-terminal residues downstream of residue 990. Increase in C-terminal length caused increase in pore formation. However, full-length toxin B displayed less pore-forming ability than the respective C-terminal truncations. The reason for this findings is unclear. It might be that the CROPs domain reduces interaction with the liposome membrane. Correct folding of the various C-terminal toxin B fragments was confirmed by assaying their glucosyltransferase activity (Fig. S1D). In addition, cysteine protease activity was validated for non-pore-forming $TcdB_{1–955}$ and pore-forming $TcdB_{1–990}$ (Fig. S1E).

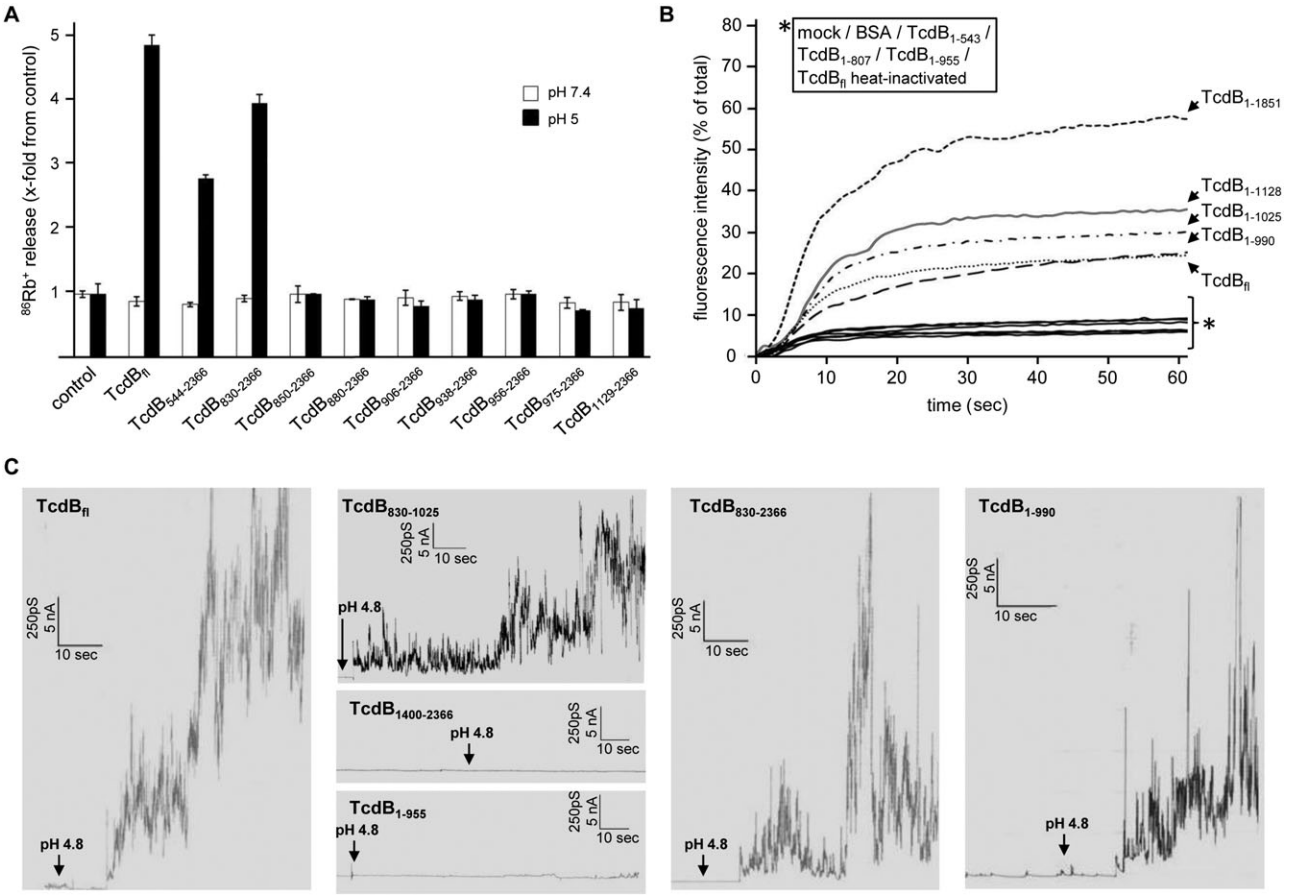

Fig. 3. Pore-forming ability of several N- and C-terminal truncated toxin B proteins.
A. $^{86}Rb^+$ release assay with N-terminally truncated TcdB constructs. Pore formation of toxins in $^{86}Rb^+$-preloaded CHO-K1 cell monolayer was induced by acidification of the medium (pH 5; black bars; control incubation at pH 7.4; white bars) in the presence of 0.5 nM full-length TcdB (TcdB$_{fl}$) or various N-terminal deletions (as indicated) and without toxin (control). Release of $^{86}Rb^+$ into the medium was determined by liquid scintillation counting. Data are given as *x*-fold release (mean values ± SD, $n = 3$) compared with the $^{86}Rb^+$ efflux of control cells.
B. Pore formation of C-terminally truncated TcdB proteins in liposomal membranes measured by toxin-induced dequenching of HPTS/DPX-loaded, large unilamellar vesicles (LUVs). Pore formation (increase in HPTS fluorescence over time) was induced by acidification (pH 5) in the presence of full-length TcdB (TcdB$_{fl}$, positive control), heat-inactivated TcdB$_{fl}$ (negative control), BSA (negative control) and various C-terminally truncated TcdB proteins (each 0.5 nM) respectively.
C. Pore formation of N- and/or C-terminal deletions of TcdB in black lipid bilayers. Single channel recordings of DiPhPC/n-decane membranes in the presence of indicated TcdB (each 3.5 nM) deletion proteins. Measurements were performed with 50 mV at room temperature in 1 M KCl buffer (pH 7.4). Pore formation by toxins was induced by acidification of the aqueous phase at the side of TcdB mutant addition, the *cis*-side (pH 4.8; arrows).

Taken together, apparently, an area between amino acids 955–990 constitutes the C-terminal boundary of the minimal pore-forming region of toxin B.

Black lipid membrane studies

The intriguing finding that the fragment TcdB$_{1–990}$ but not fragment TcdB$_{1–955}$ induced pore formation encouraged us to additionally investigate pore formation of these fragments in a black lipid membrane assay. As shown in Fig. 3C, full-length toxin B induced increase in membrane activity at low pH (pH 4.8). This was also observed with the N-terminal deletion TcdB$_{830–2366}$, the C-terminal deletion TcdB$_{1–990}$ and the N- and C-terminal deletion TcdB$_{830–1025}$ that mainly consists of the proposed pore-forming region (amino acids 830–990) with only 35 additional amino acid residues at its C-terminus (Fig. S1F provides information about the purity of this protein fragment, which was used due to high expression); however, the C-terminal deletion TcdB$_{1–955}$ and the N-terminal deletion TcdB$_{1400–2366}$ were without effect.

Residues involved in pH dependence of toxin up-take

The finding that the fragment TcdB$_{1–955}$ lacks pore-forming activity, whereas TcdB$_{1–990}$ causes pore formation, suggested crucial functional residues within the C-terminally extended region of TcdB$_{1–990}$ (amino acids 955–990). We

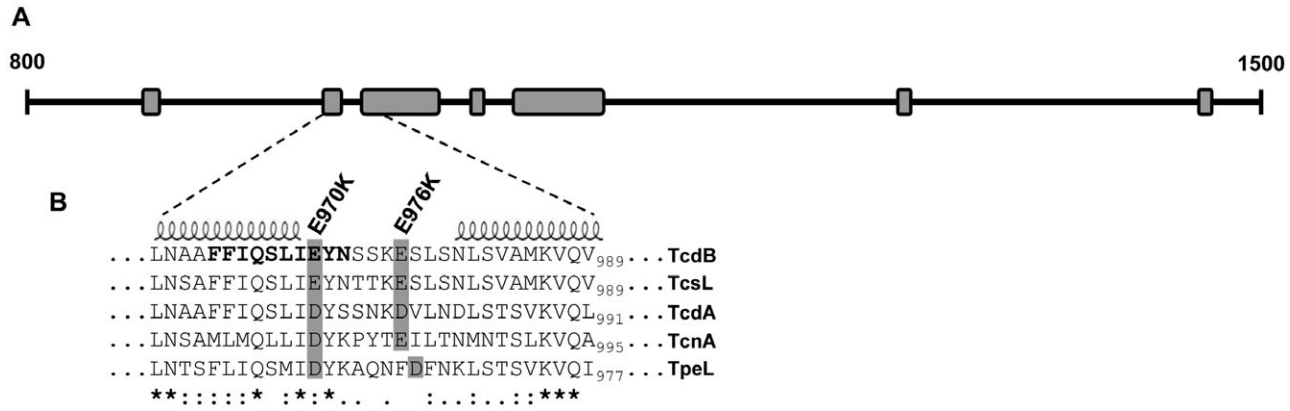

Fig. 4. *In silico* analysis of toxin B region 800–1500.
A. Alpha-helical regions of toxin B were predicted with three different algorithms (GOR4, NetSurfP, APSSP2) and hydrophobic stretches with the Kyte & Doolittle method (ProtScale). Hydrophobic sequences with a minimal length of five amino acids that putatively form alpha-helical structures (predicted by at least two algorithms) are highlighted with grey boxes.
B. Partial sequence comparison of toxin B covering the glutamate residues mutated in $TcdB_{E970K/E976K}$. A multiple alignment was calculated with CLUSTALW by using additional protein sequences of *C. difficile* toxin A (TcdA), *C. sordellii* lethal toxin (TcsL), *C. novyi* alpha-toxin (TcnA) and *C. perfringens* TpeL. The TcdB glutamate residues E970 and E976 are presumably located within a loop region that is flanked by hydrophobic, alpha-helical structures (predicted area in TcdB is indicated with a line drawing of an alpha-helix). A grey box underlines E970/E976 of TcdB, together with the respective amino acids of TcsL, TcdA, TcnA and TpeL. Note, bold residues in the sequence of toxin B indicate a peptide, which is significantly similar to a conserved motif (TKIESLKEHG) in diphtheria toxin suggested to be involved in toxin delivery to the cytosol. Stars indicate identical, colons and dots similar amino acids within the multiple alignment.

analysed this region in more detail. Secondary structure and hydrophobicity analyses of this region predicted alpha-helical structures matching together with hydrophobic clusters (Fig. 4A). We investigated acidic residues in between these hydrophobic clusters, assuming that acidic amino acids are crucial for the pre-insertion of pore-forming helices into the endosomal membrane (Silverman *et al*., 1994).

We suggested glutamate-970 and glutamate-976 as residues that might confer pH dependence for membrane insertion (Fig. 4B). Therefore, these residues were changed to lysine, because in contrast to glutamate this amino acid is not neutralized upon acidification. First, we tested the cytotoxicity of the mutants TcdB E970K, TcdB E976K and TcdB E970K/E976K. These studies showed reduced activity of the double mutant TcdB E970K/E976K, whereas the single-amino-acid mutations were similarly active as wild-type toxin B (data not shown). In contrast, the comparable double mutant TcdB E1050K/D1053K was toxic in the cell intoxication assay (Fig. 5A).

To test that the decrease in cytotoxicity of TcdB E970K/E976K was not caused by a general folding defect, we tested its glucosyltransferase and the cysteine protease activity *in vitro* (Fig. 5B and C). These studies showed no major differences between wild-type TcdB and the TcdB E970K/E976K mutant, indicating proper folding of the protein. Furthermore, we wanted to analyse whether the negative charge of the glutamate is essential for the activity of the protein. Therefore, we changed glutamate-970 and -976 to aspartate or alanine, resulting in the double mutant TcdB E970D/E976D and E970A/E976A respectively.

These double mutant proteins (the purity of all purified double mutant proteins is assayed in Fig. S1G) were tested in the ^{86}rubidium release assay. As compared with control, the release of rubidium ions was completely inhibited with TcdB E970K/E976K at low pH, indicating a role of glutamate-970 and -976 in pore formation (Fig. 5D). The finding that the pore-forming activity of the mutants TcdB E970D/E976D and TcdB E970A/E976A was the same as with wild-type TcdB indicates that both amino acids may be present in a loop region that requires neutralization of the negative charges upon acidification for proper insertion into the membrane. We also tested certain mutants in the transepithelial electrical resistance (TER) assay. The decrease in TER was largely reduced by the double mutant E970K/E976K as compared with wild-type toxin and control mutant E1050K/D1053K (Fig. 5E). To compare wild-type TcdB and TcdB E970K/E976K over a prolonged period with different concentrations, we used the electric cell-substrate impedance sensing (ECIS) method which allows the continuous detection of toxin-induced changes of the overall impedance of Caco-2 cell monolayers. These studies detected a ~100-fold reduction in the toxic potency of TcdB E970K/E976K compared with the wild-type TcdB protein (Fig. S3).

Discussion

After binding and endocytosis, the clostridial glucosylating toxins reach acidic endosomal compartments, from where they translocate into the cytosol (Barth *et al*., 2001; Just

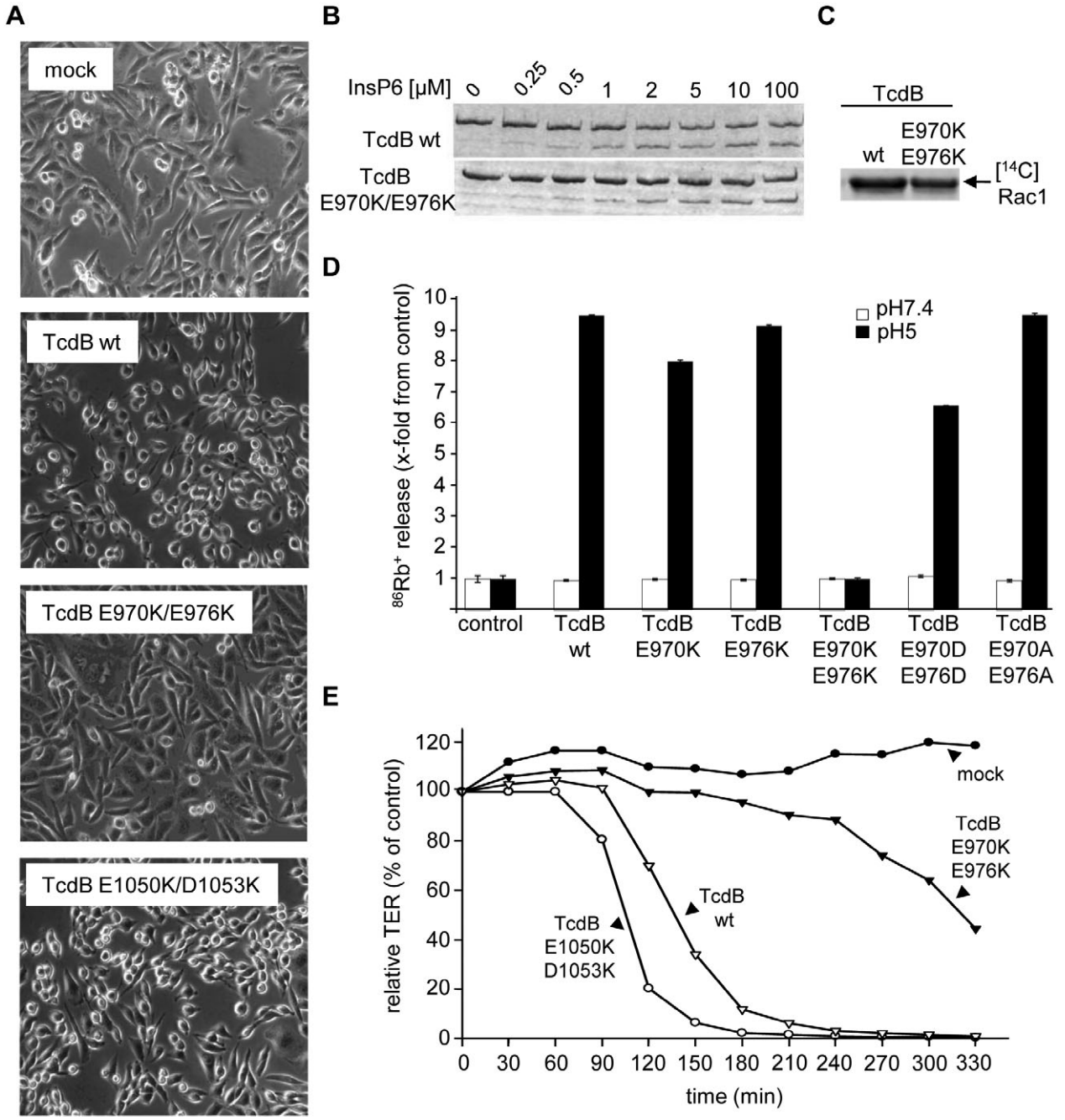

Fig. 5. Characterization of the role of glutamate-970 and glutamate-976 of toxin B by mutagenesis studies.
A. Cytotoxicity. Semi-confluent CHO-K1 cells were incubated for 1 h with wild-type TcdB and TcdB mutants (TcdB E970K/E976K or TcdB E1050K/D1053K) (each 1 pM) or left untreated (mock control). Microscopic pictures are shown.
B. InsP6-induced autoproteolytic processing. TcdB wild type and TcdB E970K/E976K (each 2 μg) were incubated with increasing concentration of InsP6 for 1 h at 37°C. Thereafter, proteins were analysed by SDS-PAGE and Coomassie staining (shown).
C. *In vitro* glucosylation of Rac1 by TcdB E970K/E976K. Rac1 (10 μM) was glucosylated by wild-type TcdB or TcdB E970K/E976K (each 10 nM) in the presence of radiolabelled UDP-[^{14}C]glucose. Samples were subjected to SDS-PAGE and modified Rac1 was visualized by autoradiography (shown).
D. $^{86}Rb^{+}$ release induced by toxin B mutant proteins. Pore formation of ^{86}Rb-pre-loaded CHO-K1 cells by wild-type TcdB (TcdB wt) or TcdB mutants (TcdB E970K, TcdB E976K, TcdB E970K/E976K, E970D/E976D, E970A/E976A, each 0.5 nM) was induced by acidification of the medium (pH 5; black bars; control pH 7.4, white bars) and incubation at 37°C for 5 min. Incubation proceeded for 1 h at 4°C and total efflux of $^{86}Rb^{+}$ into the medium was determined by liquid scintillation counting. Data are given as *x*-fold release of controls (mean values ± SD, $n = 3$).
E. Measurement of transepithelial electrical resistance (TER) of Caco-2 cells incubated with full-length TcdB or TcdB mutants (each 30 pM) over time. Toxins were added to the apical side of the cells. Given is the decrease in TER in per cent of controls of a representative experiment, which was repeated at least three times with similar results.

and Gerhard, 2004; Voth and Ballard, 2005; Papatheodorou *et al.*, 2010). Here we attempted to narrow down the region of toxin B, which is involved in this process. Besides the well-defined N-terminal glucosyltransferase domain (residues 1–543), the cysteine protease domain (residues 544–807) and the C-terminal oligopeptide repeat domain (residues 1851–2366), a region between amino acid residues 808 and 1850 is suggested to be involved in membrane translocation of the toxin or parts of the toxin (Jank and Aktories, 2008). Quite early, a hydrophobic area, covering residues 956–1128 of toxin B, has been deduced from the primary toxin structure and suggested to participate in membrane insertion and translocation (Von Eichel-Streiber *et al.*, 1992). Using various types of assays for studies on protein membrane insertion and pore formation, we show for the first time biochemical data that characterize this large delivery domain.

At first, we constructed internal toxin deletions, which still possessed the catalytic domain, the cysteine protease domain and the C-terminal receptor-binding domain. These studies revealed that the deletion mutant $TcdB_{\Delta 1501-1753}$ exhibited cytotoxicity. Although the cytotoxicity of this internally deleted toxin was reduced as compared with the wild-type toxin, the study indicates that only a part of the proposed delivery region, covering amino acids 808–1500, is sufficient for toxin delivery from acidic vesicles into the cytosol. This result was confirmed by applying a chimeric protein, which possessed the DTRD instead of the polypeptide repeats-binding domain of TcdB.

It has been shown that clostridial glucosylating toxins form pores, which are suggested to be functionally associated with the translocation process (Barth *et al.*, 2001; Giesemann *et al.*, 2006). We studied pore formation in lipid vesicles (by dye release), black lipid bilayers (by conductance measurement) and intact cells (by ^{86}rubidium release). N-terminal deletions, studied by rubidium release in intact cells, revealed that $TcdB_{830-2366}$ induced pore formation but not $TcdB_{850-2366}$, indicating the presence of essential structural features for pore formation between residues 830 and 850 of toxin B. Further C-terminal deletions narrowed down the region for pore formation in lipid vesicle and black lipid membranes to residue 990, indicating that most likely the region covered by amino acid residues 830–990 possesses the ability to insert into membranes and to form pores under acidic conditions. Although we failed to produce recombinant $TcdB_{830-990}$, we succeeded in producing $TcdB_{830-1025}$, a fragment mainly composed of the proposed pore-forming region (amino acid residues 830–990) with 35 additional amino acids at the C-terminus. This protein fragment was able to induce membrane activity in black lipid membranes upon pH reduction, finally proving our deduced localization of the pore-forming region of toxin B.

Interestingly, this region covers only a small part of the main hydrophobic region of toxin B. Originally, this hydrophobic region was assigned to residues 956–1128 from toxin B and residues 958–1130 from toxin A (Von Eichel-Streiber *et al.*, 1992). Thus, it appears that only a small part of the hydrophobic region (e.g. residues 956–990) is necessary for membrane insertion and pore formation in vesicles and black lipid membranes. However, it is important to note that this small region is not sufficient to translocate the glucosyltransferase domain into the cytosol.

The pH-dependent membrane insertion of toxins often depends on the presence of acidic residues, which are located in a loop, connecting two hydrophobic helices, which are crucial for initial membrane interaction and/or insertion. These residues are protonated at the low pH of endosomes. For example, this has been described for DT (Silverman *et al.*, 1994), the cytotoxic necrotizing factor (CNF) from *Escherichia coli* (Pei *et al.*, 2001) and *Pasteurella multocida* toxin (PMT) (Baldwin *et al.*, 2004). Residues glutamate-970 and glutamate-976 of toxin B may have a similar function. Exchange of these residues into lysine caused significant delay and decrease in the efficiency of intoxication determined by the reduced cytotoxicity. Moreover, these mutants exhibited reduced activity to form pores. Lysine residues are not neutralized at acidic pH. Therefore, the charge of these residues might prevent or delay membrane insertion. Notably, change of glutamate-970 and glutamate-976 to alanine did not block pore formation of the double mutant in the rubidium release assay. This supports the idea that low pH-triggered conformational changes of the delivery domain, accompanied by neutralization of negative charges in loop regions are prerequisites of efficient membrane insertion of the pore-forming region. Recently, Murphy and co-workers identified a conserved peptide motif (215-TKIESLKEHG-224) in the transmembrane helix 1 of DT, which is suggested to be involved in an early event of toxin delivery to the cytosol (Ratts *et al.*, 2005). Interestingly, we found a similar decapeptide (963-FFIQSLIEYN-972) by computational analysis (CLUSTALW) in the proposed large translocation region (residues 830 through 1850) of toxin B exactly at the position of glutamate-970. Murphy and co-workers suggested that the respective DT peptide is involved in interaction with a cytosolic translocation factor complex (Ratts *et al.*, 2005). It remains to be studied whether a similar role can be assigned to this motif in the delivery of the glucosyltransferase domain of toxin B to the cytosol.

To date the crystal structures of three major parts of *C. difficile* toxins are available: the glucosyltransferase domain (Reinert *et al.*, 2005; Ziegler *et al.*, 2008), the cysteine protease domain (Pruitt *et al.*, 2009) and parts of the C-terminal repeats domain (Ho *et al.*, 2005; Greco *et al.*, 2006). However, we still do not know the structure

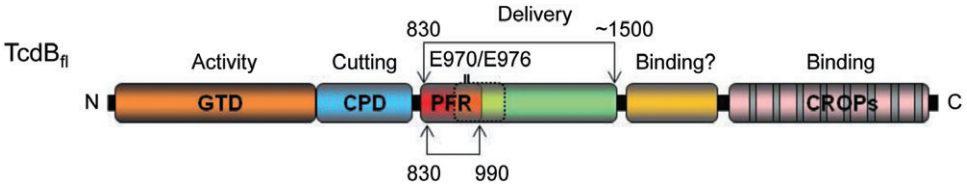

Fig. 6. Model of the domain structure of toxin B. The minimal pore-forming region (PFR) is located downstream of the N-terminal glucosyltransferase domain (GTD) and the cysteine protease domain (CPD) and covers residues 830–990. Glutamate-970 and glutamate-976, which are crucial for pH-dependent toxin up-take, are located in this area. The minimal pore-forming region overlaps with the previously described hydrophobic region of toxin B (dashed line). For toxin translocation the region between residues 830 and 1500 is necessary. It is suggested that the region between amino acids 1500 and 1851 forms an additional domain.

of main parts of the toxin covering ~1000 residues, which are between the cysteine protease domain and the domain of oligopeptide repeats. A first hint about the structure of the holotoxin came from small-angle X-ray scattering analysis of toxin B (Albesa-Jove *et al.*, 2010), supporting the model of the modular structure of the toxin. However, more informative and relevant for insights into toxin translocation mechanisms are very recent negative-stain electron microscopy studies by Lacy and co-workers (Pruitt *et al.*, 2010). These studies give a first view of structural changes occurring upon acidification and showing that a part of the toxin, which is most likely located between the C-terminal domain of polypeptide repeats and the cysteine protease domain, undergoes a major conformational change and forms a protein protrusion, which might be responsible for the initial membrane interaction and/or insertion. These preliminary structural insights also suggest that an additional domain might exist, covering the region of residues ~1500 through ~1850. Our data suggest that residues 1–~1500 are sufficient for toxin activity, including translocation, processing and intracellular toxicity, when a receptor-binding domain, e.g. the receptor-binding domain from DT, is added to the toxin B fragment (see Fig. 2). What is the function of the region between residues 1500 and 1850? One can speculate that the clostridial glucosylating toxins harbour a second receptor binding site, which is located in this region. Supporting information (Fig. S4) is in line with this notion, because a CROPs-deficient toxin B mutant (TcdB$_{1–1851}$), but not a truncation lacking the CROPs domain and the region 1501–1850 (TcdB$_{1–1500}$), was still able to intoxicate Vero cells. Preliminary data obtained with *C. difficile* toxin A (I. Just and R. Gerhard, MHH, Hannover, Germany, pers. comm.) support this view. Moreover, the recently discovered TpeL toxin from *Clostridium perfringens*, which shares the glucosyltransferase domain, the autocatalytic domain and major parts of the delivery domain with other clostridial glucosylating toxins, does not possess a C-terminal region of polypeptide repeats. Nevertheless, TpeL acts like an exotoxin and exhibits potent cytotoxicity towards mammalian target cells (Amimoto *et al.*, 2007).

In summary, we provide first experimental evidence that the pore-forming ability of TcdB is located in a region covering approximately residues 830 and 990. The amino acid pair glutamate-970 and glutamate-976, which is located within this area, plays a pivotal role in pore formation of toxin B and eventually in pore formation of other clostridial glucosylating toxins, since acidic residues are conserved at the same position. Additional structural elements until residue ~1500 are necessary for effective toxin translocation into the cytosol. This translocation module can act independently of the catalytic glucosyltransferase domain, the autocatalytic cysteine protease domain and the C-terminal oligopeptide repeats. Moreover, a not well-defined region between residues 1500 and 1851 is also not necessary for translocation. This region might represent an additional receptor binding site.

According to those findings, we present a new model of the modular composition of toxin B, which is most likely also valid for other clostridial glucosylating toxins (Fig. 6).

Experimental procedures

Cultivation of mammalian and bacterial cells

All mammalian cell lines were cultivated as monolayers in 75 cm^2 tissue culture flasks at 37°C and 5% CO_2. Three times a week, the cells were washed with PBS, trypsinized and reseeded in fresh medium.

CHO-K1 cells (Chinese hamster ovary cells) were cultivated in Ham's F-12/Dulbecco's modified Eagle's medium (DMEM; Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 2 mM L-glutamate, 100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (PAN Biotech, Aidenbach, Germany).

Caco-2 cells (human epithelial colorectal adenocarcinoma cells) were cultivated in DMEM supplemented with 10% fetal calf serum, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 1% Na^+-pyruvate (PAN Biotech, Aidenbach, Germany).

Vero cells (kidney epithelial cells from African green monkey) were cultivated in DMEM supplemented with 10% fetal calf serum, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 1% Na^+-pyruvate and 1% non-essential amino acids (NEA; PAN Biotech, Aidenbach, Germany).

Escherichia coli (strains XL1-Blue and TG1; Stratagene, La Jolla, CA) and *B. megaterium* (strain WH320; MoBiTec, Göt-

tingen, Germany) bacteria were cultivated in Luria–Bertani (LB) medium (Carl Roth, Karlsruhe, Germany) at 37°C unless otherwise indicated.

Cloning of toxin B constructs

For the generation of full-length and N- and/or C-terminal deletion constructs of toxin B (TcdB), the respective DNA sequences were amplified by PCR with oligonucleotides generating 5′-BsrGI and 3′-KpnI restriction sites for in-frame ligation into the *B. megaterium* expression vector pHIS1522 (MoBiTec, Göttingen). Genomic DNA from *C. difficile* (strain VPI 10463) was used as template. Eventually, mutations were introduced in pHIS1522/TcdB by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA, USA). Cloning of TcdB constructs with internal deletions was performed in the pHIS1522 vector backbone by first introducing a silent SpeI restriction site at bp position 2542 of the TcdB sequence with the QuikChange method. Then, since a second SpeI site is present in the TcdB sequence at bp position 5260, SpeI digestion and ligation of the plasmid resulted in pHIS1522/$TcdB_{\Delta 848–1753}$. Finally, additional TcdB constructs with internal deletions (Δ991–1753, Δ1401–1753 and Δ1501–1753) were generated by ligation of SpeI-flanked PCR products of TcdB sequences encoding amino acids 848–990, 848–1400 and 848–1500, respectively, into the SpeI-digested plasmid pHIS1522/$TcdB_{\Delta 848–1753}$. Chimeric constructs of C-terminally truncated TcdB fragments and the DTRD (amino acids 403–560) were cloned by using pHIS1522 as vector backbone. First, the respective DNA sequence encoding the DTRD was amplified by PCR from pET28a/DT (a gift from Dr Y. Belyi, Moscow, Russia) and by introducing 5′-SpeI and 3′-BamHI restriction sites for ligation into pHIS1522 to produce pHIS1522/DTRD. Then, a TcdB sequence, comprising amino acids 1–1755, obtained by digestion of pHIS1522/TcdB with BsrGI and SpeI, was introduced upstream of the DTRD sequence by ligation into BsrGI/SpeI-digested pHIS1522/DTRD to generate pHIS1522/$TcdB_{1–1755}$-DTRD. This plasmid served as template to generate pHIS1522/$TcdB_{1–1550}$-DTRD, pHIS1522/$TcdB_{1–1370}$-DTRD and pHIS1522/$TcdB_{1–830}$-DTRD, by applying the Phusion® site-directed mutagenesis method (New England Biolabs, Frankfurt, Germany) for deletion of the respective TcdB sequences upstream of the DTRD sequence.

Expression and purification of TcdB constructs

The transformation of *B. megaterium* protoplasts was performed according to the manufacturer's instructions (MoBiTec, Göttingen, Germany). All TcdB constructs were expressed as C-terminally His-tagged proteins in *B. megaterium* as described previously (Yang *et al*., 2008).

Purification of the His-tagged TcdB constructs was performed by Ni-affinity chromatography. Briefly, the bacterial pellet was resuspended in 5 ml of lysis buffer [300 mM NaCl, 20 mM Tris-HCl/pH 8.0, 500 μM EDTA, 20 mM imidazole and 10% glycerol, supplemented with Complete protease inhibitor cocktail (Roche, Mannheim, Germany)] per gram of bacterial pellet. Cells were lysed by the use of a microfluidizer (Microfluidics, Newton, MA, USA) at 15 000 psi and cell debris was removed by centrifugation at 48 000 *g* for 30 min at 4°C. The supernatant was then applied to pre-equilibrated Ni-IDA beads (Protino Ni-IDA Resin, Macherey-Nagel, Düren, Germany) and incubated 1 h at 4°C for binding. The beads were washed with buffer containing 300 mM NaCl, 20 mM Tris-HCl/pH 8.0, 20 mM imidazole and 10% glycerol, and bound proteins were eluted with 150 mM NaCl, 20 mM Tris-HCl/pH 7.4, 500 mM imidazole and 10% glycerol. Proteins were dialysed overnight against an imidazole-free buffer containing 150 mM NaCl, 20 mM Tris-HCl/pH 7.4 and 10% glycerol. For the purification of the TcdB and DT fusion proteins, additional 0.25% (v/v) Tween-20 was added into the lysis buffer, to avoid aggregation of the proteins.

Western blotting

Proteins were separated on a 3–12% gradient polyacrylamide gel by SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membrane with a semi-dry blotter (Bio-Rad, München, Germany). Detection of the catalytic domain of TcdB was performed by using a mouse-derived monoclonal anti-CDB1-546 antibody at a dilution of 1:100 000. As a second antibody, an anti-mouse IgG, coupled with horseradish peroxidase (1:3000; Biotrend, Köln, Germany), was used.

Microscopy

Cytotoxicity of TcdB constructs on different cell lines was analysed by using an inverted microscope (Axiovert 25, Carl Zeiss Jena, Germany) and images were obtained with an inbuilt digital camera (AxioCam HRc). The toxins were directly given into FCS-free cell culture medium at indicated concentrations.

In vitro *cleavage assay*

The *in vitro* cleavage assay was performed with 0.5–2 μg of protein of each TcdB construct in cleavage buffer (150 mM NaCl, 20 mM Tris pH 7.4). Cleavage was initiated by addition of 100 μM InsP6 and incubation for 1 h at 37°C. The reaction was stopped by addition of Laemmli buffer and boiling at 95°C for 2 min. The samples were then analysed by Coomassie staining or immunoblotting.

In vitro *glucosylation assay*

Rac1 (10 μM) was incubated with 10 nM of different TcdB fragments in the presence of 10 μM UDP-[^{14}C]glucose in glucosylation buffer (50 mM HEPES, 100 mM KCl; 2 mM $MgCl_2$ and 1 mM $MnCl_2$, pH 7.5), for 15 min at 30°C. The reaction was stopped by addition of Laemmli buffer and boiling at 95°C for 2 min. Radiolabelled proteins were separated by SDS-PAGE and visualized by autoradiography using a phosphorimager (GE Healthcare, Freiburg, Germany).

Black lipid bilayer experiments

Black lipid bilayer experiments were performed as described previously (Benz *et al*., 1978). Membranes were formed from

a 1% solution of diphytanoyl phosphatidylcholine (DiPhPC; Avanti Polar Lipids, Alabaster, AL, USA) in n-decane across a circular hole (0.5 mm diameter) in a wall separating two aqueous compartments. Toxins were added into the front bathing of the membrane cell (the *cis*-side). The pH drop to pH 4.8 was achieved by the addition of predetermined amounts of 0.1% HCl into the same aqueous compartment containing TcdB constructs.

Fluorophore leakage from lipid vesicles

Pore formation activity of TcdB constructs was measured by the leakage of a fluorophore from LUVs. The LUVs were prepared with egg phosphatidylcholine (EPC) (Lipoid, Ludwigshafen, Germany) and cholesterol (Chol) (ChB:66H0655, Sigma-Aldrich, München, Germany) in a molar ratio of EPC/Chol 3:1 and 0.8% 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DGS-NTA[Ni]) purchased from Avanti Polar Lipids. The preparation of the LUVs, loaded with the fluorophore mixture of 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTS) and *p*-xylene-bis(*N*-pyridinium bromide) (DPX); HPTS/DPX (Molecular Probes, Darmstadt, Germany), was described earlier (Peschka-Süss and Schubert, 2003). The leakage assay was performed in a 3 ml quartz glass cuvette, where the TcdB constructs were added at indicated concentrations to LUVs (~5 μM final liposome concentration in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4). The fluorescence of the mixture was monitored (excitation 403 nm, emission 510 nm) with continuous stirring at 25°C. The baseline of the fluorophore leakage was determined, and then the insertion of TcdB proteins into the liposomal membrane was initiated by addition of HCl to reach a pH of 5.0 in a final volume of 3 ml. The leakage of HPTS and DPX into the surrounding buffer leads to dequenching of HPTS due to dilution, resulting in an increase in fluorescence. To determine total HPTS fluorescence (set to 100%), Triton X-100 (0.3% final concentration) was added to the mixture, inducing complete release of the fluorophore.

Competition experiments

The chimera $TcdB_{1–830}$-DTRD (100 nM) was added to Vero cells in FCS-free medium and binding to the DT receptor occurred for 5 min at 37°C. Then, 10 nM of the chimeric toxin $TcdB_{1–1550}$-DTRD was added and intoxication was determined by monitoring cell rounding. The same procedure was applied for the competitive inhibition of $TcdB_{fl}$ (1 pM) with $TcdB_{850–2366}$ (1 nM).

$^{86}Rb^+$ release assay

CHO-K1 cells were seeded in 24-well plates in medium (Ham's F-12/DMEM with 5% fetal calf serum), supplemented with $^{86}Rb^+$ (1 μCi ml^{-1}), at a density of about 10^5 cells per well. Cells were then incubated at 37°C for 48 h prior to medium exchange with fresh growth medium supplemented with 100 μM bafilomycin A1 (Biotrend, Köln, Germany) and continued incubation for 20 min at 37°C. Next, cells were first chilled on ice and subsequently ice-cold, fresh medium containing TcdB constructs (each 0.5 nM) was added. Toxins were allowed to bind to the cell surface for 1 h at 4°C. Cells were then washed twice with ice-cold PBS to remove unbound toxin. Insertion into the plasma membrane of bound toxin was induced by treating cells with warm, acidified growth medium (37°C, pH 5 or pH 7.5) for 5 min at 37°C. After a further incubation on ice for 40 min, medium was removed and the amount of released $^{86}Rb^+$ was determined by liquid scintillation counting with a Liquid Scintillation Analyser (TRI-CARB 2900TR, PerkinElmer, Rodgau, Germany).

TER assay

Caco-2 cells were seeded on Falcon™ cell culture filters and incubated for 6–9 days to form confluent monolayers with medium exchange every 3 days. Assays were performed when TER values reached ~2–3 kΩ cm^{-2}. TER was determined using a resistance system for electrophysiological readings of filter cups (Endohm-12; World Precision Instruments, Sarasota, FL). Toxins (30 pM) were added to the apical site of cells and the decrease of the TER was monitored over a time period of up to 9 h.

Electric cell-substrate impedance sensing (ECIS)

Cell detachment after intoxication with TcdB constructs was measured with the ECIS system (Applied BioPhysics, Troy, NY, USA). Caco-2 cells were seeded on ECIS chips (8W10E, Applied BioPhysics, Troy, NY, USA) and grown to confluence. The TcdB constructs were applied directly into the medium and the decrease of impedance was measured over a time period of 18 h, at 2–4 kHz.

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