

Autocatalytic cleavage of *Clostridium difficile* toxin B

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Clostridium difficile, the causative agent of nosocomial antibiotic-associated diarrhoea and pseudomembranous colitis, possesses two main virulence factors: the large clostridial cytotoxins A and B. It has been proposed that toxin B is cleaved by a cytosolic factor of the eukaryotic target cell during its cellular uptake. Here we report that cleavage of not only toxin B, but also all other large clostridial cytotoxins, is an autocatalytic process dependent on host cytosolic inositolphosphate cofactors. A covalent inhibitor of aspartate proteases, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, completely blocked toxin B function on cultured cells and was used to identify its catalytically active protease site. To our knowledge this is the first report on a bacterial toxin that uses eukaryotic signals for induced autoproteolysis to deliver its toxic domain into the cytosol of target cells. On the basis of our data, we present an integrated model for the uptake and inositolphosphate-induced activation of toxin B.

With the introduction of antibiotic therapy into clinical practice the lifespan of the population in the industrialized world increased and infections seemed to become manageable. However, this observation is deceptive, because many microbes possess or develop resistance mechanisms^{1,2}. Furthermore, antibiotics may cause prominent side effects, like the observed increase of pseudomembranous colitis when clindamycin was introduced in the 1970s^{3,4}. Today, we know that nearly all known antibiotics are capable of inducing antibiotic-associated diarrhoea or its fatal form, pseudomembranous colitis^{5,6}.

The factors inducing antibiotic-associated diarrhoea or pseudomembranous colitis are two toxins produced by *Clostridium difficile*, toxin A (TcdA) and toxin B (TcdB)^{7,8}, which are homologous to each other⁹, to TcsH and TcsL of *Clostridium sordellii*¹⁰ and to Tcn α of *Clostridium novyi*¹¹. Because of their sequence homology, similar domain structure and glycosyltransferase properties these toxins are designated 'large clostridial cytotoxins' (LCTs)^{12,13}.

Recently, *C. difficile* attracted great attention^{14,15} because hyper-virulent, ribotype 027 isolates, which are resistant to fluoroquinolones, were reported in Canada and the USA. These reports and some recent data of younger people encountering severe *C. difficile* disease without prior antibiotic treatment indicate a changing epidemiology and call for a shift in the perception that only elderly, immunocompromised people are at high risk of suffering from *C. difficile* infection, mainly following hospitalization¹⁶. There is the threat that such strains will spread worldwide and thus compromise antibiotic treatment in general^{17,18}. Accordingly, a better understanding of the mode of action of TcdA and TcdB is urgently needed.

TcdA and TcdB are single-chained and have a tripartite functional organization¹²: the carboxy-terminal domain is responsible for binding to the host cell membrane¹⁹; the hydrophobic middle part is supposed to be involved in translocation across cellular membranes²⁰; and the amino-terminal domain harbours the catalytically active glucosyltransferase site^{12,21}. Although knowledge about the uptake process is still fragmentary, it is generally accepted that on

binding to host cell receptors^{22,23} the toxins are endocytosed²⁴. The translocation of the toxins to the cytosol occurs from early endosomal compartments^{25,26}. Because TcdA forms pores in artificial membranes at low pH (ref. 27), it was suggested that in living cells the low endosomal pH triggers similar conformational changes and accordingly may propagate translocation via pore formation.

From early on, it was postulated that the toxin is activated through catalytic processing^{26,28}. Recently, it was shown that TcdB is cleaved between Leu 543 and Gly 544 of TcdB at neutral pH (ref. 29), generating fragments of 63 kDa (catalytic-DXD domain) and 207 kDa (ligand-translocation domain). Only the smaller N-terminal fragment harbouring the glucosyltransferase activity reaches the cytosol, whereas the 207 kDa C-terminal fragment remains in the membrane fraction^{20,29}. Microinjection experiments showed that the 63 kDa fragment exhibits full cytotoxic activity shortly after injection, whereas the holotoxin starts with low activity, which increases with time²⁹, indicating the biologic relevance of proteolytic processing for catalytic activity. The released cytotoxic fragment can then act on its cytosolic targets—the GTPases of the Rho/Rac family^{30,31}. Until now, the assumption was that this cleavage is catalysed by a pepstatin A-sensitive host cell protease^{20,29}.

Results

Identification of the host factor. To identify the host factor required for cleavage of TcdB, we used TcdB preparations³² that were Cy3-labelled as described in the literature²⁹. Cytosolic extracts of porcine splenocytes showed strong cleavage activity (Fig. 1a) and thus served as raw material for further analysis. Purification of cytosolic fractions of splenocyte extracts (Supplementary Fig. 1) led to the following inconsistencies with the thesis that we had purified a cellular protease: concentration by ammonium sulphate precipitation was not possible, activity was fully retained in heated extracts (96 °C, 30 min), effective TcdB cleavage was induced with the aqueous protein-free phase of phenol chloroform extractions and highly purified active

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extracts showed no significant absorption at 280 nm (Supplementary Fig. 1).

The active components were identified by nano-electrospray-ionization quadrupole time-of-flight (nanoESI-QTOF) mass spectrometry of purified active fractions (Fig. 1b). The comparison of fragment spectra of the active substance (Fig. 1c, d) with published spectra³³ revealed strong similarities to inositolphosphates (Fig. 1e). Several inositolphosphates (InsPs) were tested in the cleavage assay and inositolhexaphosphate (InsP₆) showed the highest cleavage activity (see Supplementary Table). Unlabelled TcdB visualized by zinc staining³⁴ showed activity of InsP₆ down to 1 μ M (Supplementary Fig. 2). Because the total cellular InsP₆ concentration is in the range of 15–100 μ M (ref. 35), concentrations of InsP₆ required for *in vitro* cleavage can be regarded as physiologically relevant. No cleavage activity has ever been observed or attributed to InsPs itself. Whereas activation of proteins by phosphorylation is a well known mechanism, inositolphosphates, in contrast to inositolpyrophosphates³⁶, do not contain high energy pyrophosphate bonds. It is therefore unlikely that inositolphosphates activate the toxin by phosphorylation.

Exclusion of contaminant proteases. In accordance with previous publications²⁹, cleavage was pepstatin A-sensitive, indicating that the TcdB preparation contained a latent, InsP_x-inducible aspartate protease (data not shown). A series of experiments was performed

to exclude the contribution of a contaminating InsP_x-inducible protease that might co-purify with TcdB. A putative unspecific action of our TcdB preparation on other proteins was excluded (Supplementary Fig. 3). Using monoclonal antibody 2CV (ref. 37), TcdB was additionally affinity purified, which yielded more than 98% pure TcdB as assessed by SDS–polyacrylamide gel electrophoresis (PAGE). The remaining contaminant proteins were identified by tandem mass spectrometry (MS/MS) analysis. All proteins matched to sequences from the recently published *C. difficile* genome³⁸ and showed no similarity to known endoproteases (data not shown). The affinity-purified TcdB retained the InsP₆-inducible cleavage activity. Further, cleavage of TcdB by a contaminant protease would be an intermolecular process, whereas autocatalysis is intramolecular. Because dilution favours intramolecular reactions in comparison to intermolecular reactions, we performed cleavage of TcdB at different concentrations and observed that in contrast to the partial cleavage at 5 μ M TcdB, cleavage is nearly completed after 30 min at a concentration of 50 nM TcdB (Fig. 2a). This result is inconsistent with the participation of a contaminating protease, which would show reduced activity at lower concentrations. Consequently, we postulated autocatalytic cleavage by an intrinsic TcdB aspartate protease.

Proof of autocatalytic TcdB cleavage. To prove further our model for autocatalytic TcdB activation, we decided to inactivate and identify the

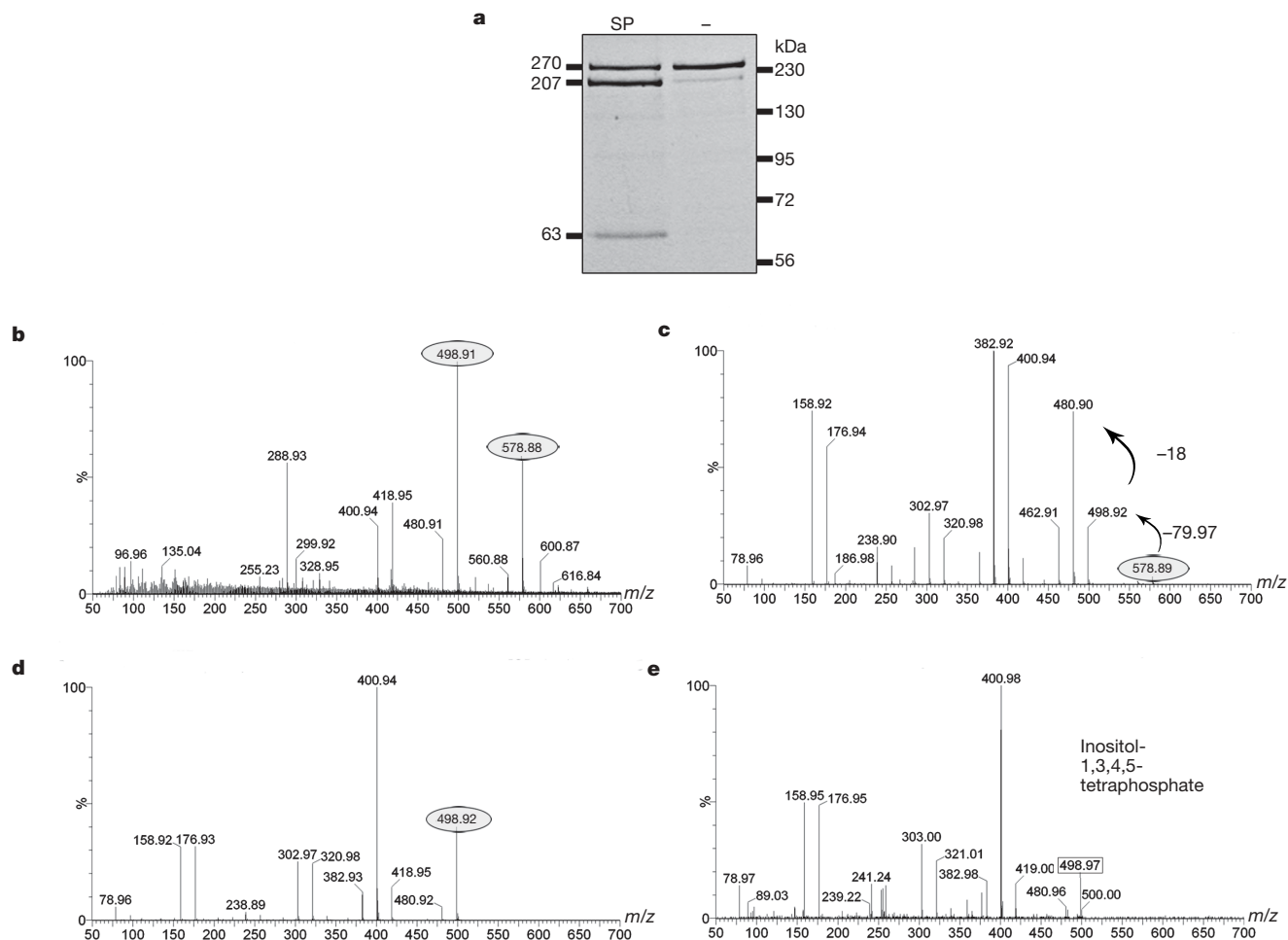


Figure 1 | Purified cytosolic splenocyte extract in the *in vitro* TcdB cleavage assay and mass spectrometry. **a**, *In vitro* cleavage reaction. Cleavage of fluorescently labelled Cy3-TcdB activated by splenocyte cytosolic extract (SP) and the same reaction without SP (–); visualization was by Cy3 fluorescence. **b–e**, Active fractions of the splenocyte extract purification (Supplementary Fig. 1) were analysed by mass spectrometry; representative spectra are shown. InsP_x-peaks from the purified porcine splenocyte extract are encircled. The y axis measures MS-intensity as a per cent of the most

intense peak. *m/z*, mass-to-charge ratio. **b**, Survey spectra from fraction C5 of S75 size exclusion chromatography (Supplementary Fig. 1f) were acquired over a mass range from 50–1,500 Da. **c**, **d**, From fraction C5 the peaks at 578.88/578.89 Da (encircled and shaded) and 498.91/498.92 Da (encircled and shaded) were manually selected for fragmentation in MS/MS mode (shown in **c** and **d**, respectively). **e**, The MS/MS fragment spectrum of commercially available inositol-1,3,4,5-tetraphosphate (boxed, with a peak at 498.97) served as a positive control.

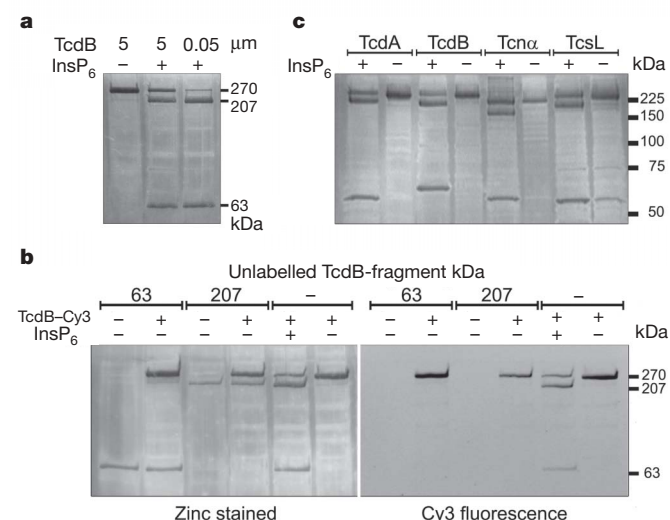


Figure 2 | In vitro cleavage of LCTs by purified splenocyte extracts or inositolhexaphosphate. **a**, Cleavage of native TcdB induced by the addition of InsP₆. Unlabelled toxin (16 μ g) was incubated with InsP₆ (at 100 μ M concentration) at different TcdB concentrations (5.0 or 0.05 μ M); following cleavage, the solutions were lyophilized, resuspended in water (approximately 1 μ g) subjected to SDS-PAGE and detected with zinc staining. **b**, Exclusion of a cascade reaction. Cy3-labelled TcdB was co-incubated with the isolated (pre-activated) 63 kDa catalytic-DXD or the 207 kDa ligand-translocation domain, respectively. Left panel, zinc staining; right panel, the same gel visualized by Cy3 fluorescence. **c**, Cleavage of LCTs from different *Clostridia*. Assay without (–) or with (+) activation by 10 mM InsP₆. LCTs were: TcdA and TcdB of *C. difficile* strain VPI10463; Tcn α of *C. novyi* strain 19402; and TcsL of *C. sordellii* strain IP-82. Visualization was by zinc staining.

active centre of the protease. EPNP (1,2-epoxy-3-(*p*-nitrophenoxy)propane) is a known inhibitor of aspartate proteases, which, in contrast to pepstatin A, covalently binds to the active site³⁹. To determine the TcdB intrinsic protease active site we thus generated an ‘EPNP-derivative’ of TcdB. Following addition of InsP₆, this modified TcdB was no longer cleaved (data not shown). This suggests that TcdB had lost its aspartate protease function. The active site of aspartate proteases is known to be situated in the motif DXG (where X is S or T)³⁹. According to database searches (see Supplementary Information), TcdB contains three of these motifs at positions 1665, 2001 and 2133. To exclude the possibility that EPNP modified a contaminating protease rather than TcdB, and to identify its active site, we analysed fragments generated by tryptic proteolysis of EPNP-treated and untreated TcdB by nanoESI-QTOF mass spectrometry. Only a single fragment with an EPNP-induced mass shift of 195 Da was detected. The triple charged fragment had an apparent mass of $m/z = 1075.49$ and was confirmed by MS/MS analysis to be amino acids 1652–1678 of TcdB (Supplementary Fig. 4). This fragment contains the aspartate protease consensus motif DSG at amino acid position 1665, confirming the predicted DSG(1665) to be the active site of the autocatalytic TcdB protease. Furthermore, EPNP pretreatment completely abrogated cellular toxicity when the toxin was added to the target cell culture (Fig. 3c). As expected, loss of cytotoxicity was not due to modification of the catalytic-DXD glucosyltransferase domain, because microinjection of EPNP-treated TcdB induced cell rounding (Fig. 3f).

To dissect further the autocatalytic cleavage mechanism, we isolated both N- and C-terminal fragments by anion exchange chromatography and tested their proteolytic activity. As indicated in Fig. 2b, neither the 63 kDa nor the 207 kDa fragment was able to cleave full-length Cy3-labelled TcdB in the absence of InsP₆.

Thus the cleavage reaction of TcdB is intramolecular and not followed by a cascade reaction catalysed by its cleavage fragments.

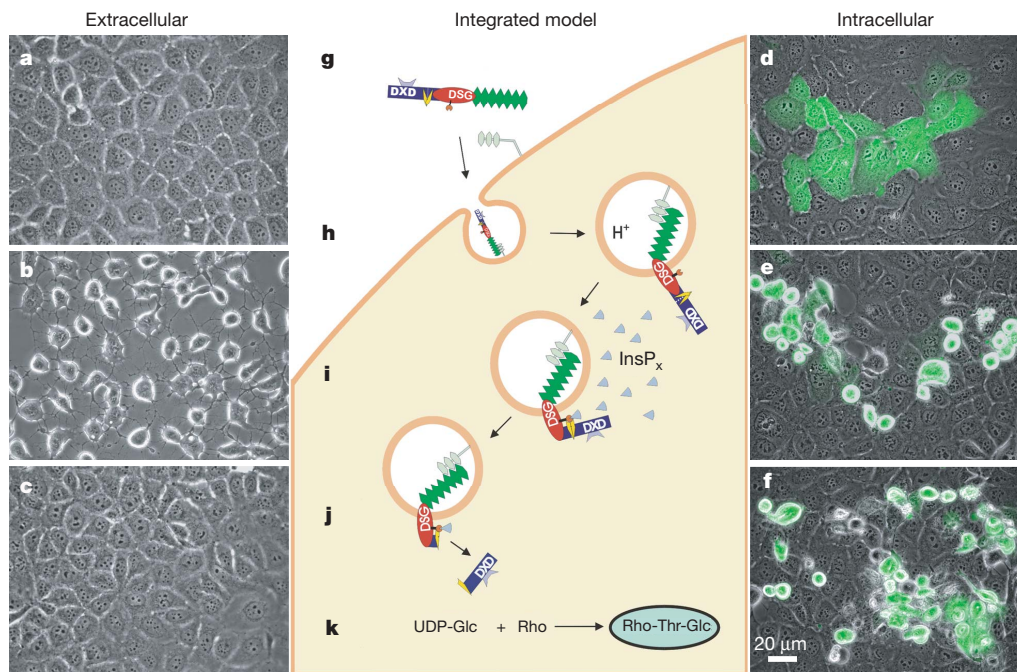


Figure 3 | An integrated model of events necessary for the cellular cytotoxic action of TcdB. **a–c**, Cytotoxic effects of TcdB (1 μ g ml^{–1}) applied to the supernatant of IHKE cells. **a**, Control buffer without TcdB. **b**, Unmodified TcdB. **c**, EPNP-modified TcdB. **d–f**, Effects of TcdB microinjected into the cytosol of IHKE cells. **d**, Control buffer (containing dextran-FITC) without TcdB. **e**, Unmodified TcdB. **f**, EPNP-modified TcdB. **g–k**, Schematic model of TcdB uptake and delivery of the catalytic-DXD domain into the eukaryotic cell. Colour code: dark green, the TcdB ligand domain; red, the TcdB translocation domain; dark blue, the TcdB catalytic-DXD domain; light grey-green, the

receptor for TcdB; brown, cellular membranes; and light blue, cytosolic inositolphosphates. **g**, Interaction with cell receptors induces receptor-mediated endocytosis. **h**, The acidic pH of the endosome triggers the first conformational change and results in pore formation of the ligand-translocation domain. **i**, Cytosolic InsP₆ interacts with TcdB and induces a second conformational change, activating the intrinsic protease function. **j**, Autocatalytic cleavage and release of the catalytic-DXD glucosyltransferase domain into the cytosol. **k**, Glucosylation of the cytosolic target GTPases, leading to disaggregation of the cytoskeleton and cell death.

Furthermore, Fig. 2b shows that TcdB loses its intrinsic protease function following cleavage.

Autocatalytic cleavage of other LCTs. Assuming autocatalytic activation, we expected that other members of the LCT family^{12,40} would show similar properties. An assay with InsP₆-activated *C. sordellii* TcsL, *C. novyi* Tcn α and *C. difficile* TcdA demonstrated cleavage like that observed with TcdB (Fig. 2c). Apparently, InsP_x-induced autocatalytic cleavage is a common property of all LCTs.

Model for the uptake and activation of toxin B. A model of the events occurring within the cells is presented in Fig. 3. On binding to host cell receptors^{22,23} (Fig. 3g) the toxins are endocytosed²⁴. The low pH in endosomes triggers the first conformational change of the toxin leading to membrane insertion^{26,28,41} (Fig. 3h), followed by translocation of the N-terminal catalytic-DXD domain into the cytosol^{20,29}.

The translocation-ligand domain remains associated with endosomal membranes and only the catalytic-DXD domain penetrates into the cytosol^{20,29}. We show here that cytosolic InsP_x is needed to induce autocatalytic cleavage of TcdB. It is known that in eukaryotic cells InsPs may induce conformational changes of proteins^{42,43}, and therefore we postulate that TcdB undergoes a second conformational change (Fig. 3i), which is induced by InsP_x and immediately leads to autocatalytic proteolysis and delivery of the catalytic-DXD domain into the cytosol (Fig. 3j). Because our experimental data show that the intramolecular reaction quickly follows the addition of InsP₆, cleavage of the glucosyltransferase portion could immediately occur on its translocation into the cytosol. Additionally, our data using EPNP-modified TcdB show that InsP_x-induced autocatalytic cleavage is absolutely required for the execution of TcdB's biological function. Once the toxin's aspartate protease function is blocked (Fig. 3c), the catalytic-DXD domain can no longer be cleaved off and thus, according to other researchers^{20,29}, the DXD domain can no longer reach the cytosol. Following its translocation and release, the catalytic-DXD fragment acts on its cytosolic targets (Fig. 3k), the GTPases of the Rho/Rac family, leading to the observed blockade of signal transduction processes and, consequently, the disaggregation of the cytoskeleton and cell death^{12,31,40}.

Discussion

In conclusion, we identified InsP_x as the host factor required for the autocatalytic proteolysis of TcdB. Cellular inositolphosphates are involved in the regulation of a large variety of cellular processes in the cytoplasm, the plasma membrane and the nucleus^{43,44}. Recently, it was reported that InsP₆ is required for the correct folding of ADAR2, involved in RNA editing⁴². However, InsP_x as a cofactor for activation of proteolytic activity has not been reported before, to the best of our knowledge. Furthermore, our data and some recent reports^{42,43} indicate that in eukaryotic cells InsPs serve more generally as cofactors for proper protein folding and function. The proof of InsP₆-induced cleavage of LCTs shown in Fig. 2c leads us to propose InsP_x-inducible proteolytic cleavage as an additional feature common to the LCT family. However, in contrast to TcdA, TcdB and TcsL, which have a DXG-motif at the same position, Tcn α has no DXG-site and therefore should possess a different protease function to the aspartate protease of the other LCTs.

Regarding the similar genomic organization of LCTs⁴⁵, it is not surprising that they carry their intramolecular protease with them because this combines complete mechanisms of regulated production, release of toxins and induced self-activation at the final site of action⁴⁶. During evolution, LCT-producing *Clostridia* have thus gained a very effective means of controlling the energy-demanding use of their toxins.

METHODS

Labelling of toxins with Cy3. The toxins TcdB and TcdA produced by the toxin high-producer strain VPI10463 were purchased from tgcBIOMICS (Mainz,

Germany). Aliquots of 200–400 μ g were transferred to NaHCO₃ buffer by gel filtration and labelled with Cy3 (GE Healthcare) according to the manufacturer's instructions. Unbound dye was removed by size exclusion chromatography using 10 mM Tris-HCl, pH 8.5, as running buffer. The molar dye/toxin-labelling ratio amounted to 0.8–1.6. Labelled toxins were aliquoted and stored at -80°C .

In vitro cleavage assay. The cleavage assay was performed as described previously²⁹, in Tris-HCl, pH 8.5, buffer at a volume of 10 μ l (unless stated otherwise) consisting of 500 ng of labelled toxin or 1 μ g of unlabelled toxin, 3 μ l of cell extract and purified fractions or commercially available inositol phosphates (purchased from Axxora, Merck, and Sigma, respectively). Following a 30 min incubation at 25°C , samples were separated by SDS-PAGE (10%), Cy3-labelled toxin and toxin fragments were visualized by fluorescence, unlabelled toxins were visualized by zinc staining and both were documented with a Gel Doc EQ System Image Reader (Bio-Rad).

Preparation of cytosolic porcine splenocyte extract. Freshly isolated pig spleens were prepared in phosphate buffered saline (PBS) and processed into single-cell suspensions. Erythrocytes were lysed by adding low-salt buffer (150 mM NH₄Cl, 1 mM KHCO₃ and 0.1 mM EDTA, pH 7.6). Splenocytes were washed twice with 10 mM Tris-HCl, pH 8.5, and the pellet was immediately frozen at -80°C . Cell extracts were obtained by diluting the splenocyte pellet 1:1 with 10 mM Tris-HCl, pH 8.5, followed by mechanical lysis in a Potter-Elvehjem homogenizer (20 strokes, 1,000 r.p.m.). The lysate was centrifuged for 1 h at $200,000 \times g$ at 4°C and the supernatant was promptly used for further experiments.

Purification of active fractions from porcine splenocyte extract. Details of purification are given in Supplementary Fig. 1.

Mass spectrometry. Lyophilized active fractions were dissolved in 50% acetonitrile/water (liquid-chromatography-MS grade, Merck). Samples (1–2 μ l) were applied to a gold-coated glass capillary (Type C nanoflow needle, Waters) and introduced into the nano-ESI source using a capillary voltage of 500–800 V, resulting in a spray of about 50 nl min⁻¹.

Mass detection was performed on a hybrid orthogonal acceleration QTOF MS/MS (Q-TOF Premier, Waters) equipped with an ESI interface and operated in a negative ionization mode. The mass spectrometer was operated in V-mode and tuned to a resolution of 10,000 full-width at half-maximum (FWHM) for singly charged ions. Prominent peaks in the survey mass spectrometry spectrum were manually selected for fragmentation in MS/MS mode using collision energies between 15 and 30 eV.

Affinity purification of TcdB. TcdB-specific monoclonal antibody 2CV (7 mg)³⁷ was coupled to a HiTrap NHS Sepharose column (GE Healthcare), according to the manufacturer's protocol. Approximately 4 mg of conventionally purified TcdB were applied to this column and washed with a buffer containing 50 mM Tris/HCl, pH 7.0 and 125 mM NaCl. TcdB was eluted with 0.1 M triethanolamine-HCl, pH 11, and immediately neutralized with a one-tenth volume of 1 M Tris-HCl, pH 7.5.

EPNP modification of TcdB. TcdB was transferred to a buffer containing 250 mM sodium acetate, 500 mM NaCl, 1 mM EDTA, 500 μ M DTT and 5% DMSO (pH 5.5). EPNP (10 mM; Acros Organics, Belgium) was added and incubated for 30 h at 500 r.p.m. on an orbital mixer at room temperature. Finally the buffer was changed (ZebaSpin desalting columns, Pierce) to HEPES 40 mM, pH 7.5.

Microinjection experiments. Microinjection experiments were performed with IHKE cells maintained in DMEM/HAM's F12-medium (1:1) supplemented with 10% FCS and 2 mM glutamine in the presence of 65 mg l⁻¹ penicillin-G and 100 mg l⁻¹ streptomycin. IHKE cells were seeded overnight on 35 mm Petri dishes with glass bottoms and then used in the experiments. Native or EPNP-modified TcdB were diluted in an intracellular buffer (145 mM potassium glutamate, 20 mM NaCl, pH 7.3) to a final concentration of 1 μ g ml⁻¹. The intracellular buffer contained fluorescein isothiocyanate (FITC)-labelled dextran (100 mg ml⁻¹, molecular mass approximately 19,500 Da; Sigma) to visualize successful microinjection. Microinjection was performed with an Eppendorf micromanipulator 5170 and microinjector 5242 system (Eppendorf AG). Injection pulses of 120 hPa were applied for 0.3 s. Images were taken at several time points (2 h, 7 h and 24 h) and acquired with an inverted Leitz DM-IRB phase-contrast microscope (Leica Microsystems) equipped with a VisiTron 'VisiChrome' High Speed Fluorescence Polychromatorsystem (VisiTron Systems). Excitation and emission wavelengths for fluorescence images were 490 and 525 nm.

For data acquisition, storage and processing we used Digital Camera Photometrics 'Coolsnap cf' (Roper Scientific) and Metafluor acquisition software (Universal Imaging Corporation).

Mass spectrometry of native and EPNP-modified TcdB. Details of the mass spectrometry are given in Supplementary Fig. 4.

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