Characterization of the cleavage site and function of resulting cleavage fragments after limited proteolysis of *Clostridium difficile* toxin B (TcdB) by host cells

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*Clostridium difficile* toxin B (TcdB) is a single-stranded protein consisting of a C-terminal domain responsible for binding to the host cell membrane, a middle part involved in internalization, and the N-terminal catalytic (toxic) part. This study shows that TcdB is processed by a single proteolytic step which cleaves TcdB10463 between Leu543 and Gly544 and the naturally occurring variant TcdB8864 between Leu544 and Gly545. The cleavage occurs at neutral pH and is catalysed by a pepstatin-sensitive protease localized in the cytoplasm and on the cytoplasmic face of intracellular membranes. The smaller N-terminal cleavage products [63 121 Da (TcdB10463) and 62 761 Da (TcdB8864)] harbour the cytotoxic and glucosyltransferase activities of the toxins. When microinjected into cultured Chinese hamster lung fibroblasts, the N-terminal cleavage fragment shows full cytotoxic activity shortly after injection whereas the holotoxin initially exhibits a very low activity which, however, increases with time. Twenty minutes after the start of internalization of TcdB, the larger cleavage products [206 609 Da (TcdB10463) and 206 245 Da (TcdB8864)] are found exclusively in a membrane fraction, whereas the N-terminal cleavage products appear mainly in the cytosol and associated with the membrane. This is in line with a proposed model according to which the longer, C-terminal, part of these toxins forms a channel allowing for the translocation of the toxic N-terminal part, which is subsequently cleaved off at the cytoplasmic face of an intracellular compartment, most likely endosomes.

**INTRODUCTION**

Toxins TcdA and TcdB are recognized as the main virulence factors responsible for intestinal damage during infection with *Clostridium difficile* (Johnson & Gerding, 1997). TcdA and TcdB belong to the group of large clostridial toxins (LCT) together with TcsL and TcsH from *Clostridium sordellii*, and TcnA from *Clostridium novyi*. These toxins are characterized by their large size (250–308 kDa), their cytotoxicity for cultured cells, and their mechanism of action (Eichel-Streiber et al., 1996; Thelestam et al., 1997; Thelestam & Chaves-Olarte, 2000).

TcdB acts intracellularly as a glucosyltransferase specific for the small GTPases Rho, Rac and Cdc42. The resulting inactivation of these GTPases causes disaggregation of the...
cytoskeleton and alterations of other cellular processes, which eventually lead to cell death (Thelestam & Chaves-Olarte, 2000; Qa’Dan et al., 2002).

Bacterial toxins with intracellular targets belong to the group of AB-toxins, which are characterized by their structural organization in that B-subunits or B-domains are responsible for their binding to host cells and their internalization, whereas A-subunits or A-domains are responsible for their toxic effects (Falnes & Sandvig, 2000). *C. difficile* TcdB, as well as other LCTs, are large single-stranded proteins with three functional domains. Their toxic activity is associated with the N-terminal part of the protein (Hofmann et al., 1997; Wagenknecht-Wiesner et al., 1997), whereas the C-terminal part with its short repetitive sequences is responsible for receptor binding (Eichel-Streiber et al., 1996; Frisch et al., 2002). The middle part represents the putative translocation domain (Eichel-Streiber et al., 1996) and was recently shown to be required for the internalization of the TcdA toxin (Frisch et al., 2002).

AB-toxins enter the host cell cytosol either from the endosomal compartment or by following the retrograde transport pathway to the ER, from where they become translocated into the cytoplasm. Regardless of the pathway used, generation of the active catalytic domain requires that AB-toxins become proteolytically processed. Cleavage can be due to bacterial proteases (e.g. clostridial neurotoxins) or host-cell proteases (e.g. anthrax toxin, diphtheria toxin, *Pseudomonas* toxin A) (Fryling et al., 1992; Gordon & Leppla, 1994; Gordon et al., 1995; Falnes & Sandvig, 2000).

Our knowledge of the internalization of LCTs is still far from complete. It is generally accepted that all LCTs are endocytosed after binding to poorly characterized cell receptors (Thelestam & Chaves-Olarte, 2000). Several lines of evidence suggest that LCTs use an endosomal pathway for translocation of their toxic domain. The involvement of an acidic compartment in the internalization of TcdB was suggested in early studies (Florin & Thelestam, 1983) and is in agreement with the more recent observation that the cytopathic effects of TcdB and TcsL are blocked by lysosomotropic inhibitors but not by Golgi-disrupting drugs (Qa’Dan et al., 2000, 2001). Recently it was also shown that at low pH TcdB can form ion channels, which is characteristic for toxins entering the cytoplasm from an endosomal compartment (Barth et al., 2001). The insertion of TcdB into the membrane may result from the fact that low pH triggers conformational changes of TcdB leading to increased hydrophobicity of the toxin (Qa’Dan et al., 2000).

Proteolytic processing of TcdB and other LCTs in host cells was suggested as an important step in internalization (Florin & Thelestam, 1986; Thelestam et al., 1997), and a cleavage of TcdB in Vero cells has indeed recently been shown (Pfeifer et al., 2003). However, details of the cleavage reaction are still lacking. Here we show for the first time how *C. difficile* TcdB is processed in the host cell by a specific proteolytic cleavage reaction which leads to the generation of an N-terminal cleavage product which is translocated from an intracellular compartment. As TcdB is an important virulence factor, knowledge of its intracellular processing contributes to a better understanding of pathogenesis of intoxication by LCTs. This may help to create new strategies for treatment as an alternative to or a complementation of the currently used antibiotic therapy.

**METHODS**

**Cell culture.** Vero cells (African green monkey kidney) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). DonWT cells (Chinese hamster lung fibroblasts, wild-type) and the DonQ mutant cell line resistant to *C. difficile* toxins (Chaves-Olarte et al., 1996) were grown in MEM supplemented with 10% FBS. Caco2 cells were maintained in DMEM supplemented with 10% FBS and nonessential amino acids (1 x ). All cell lines were grown in the presence of antibiotics (penicillin 100 U ml⁻¹ and streptomycin 100 µg ml⁻¹).

**Preparation of subcellular fractions.** Cells grown in Petri dishes (15 cm) were washed twice with phosphate-buffered saline (PBS) and harvested with a plastic scraper. After washing with SI buffer [250 mM sucrose, 3 mM imidazole, pH 7.5, supplemented with Pefabloc SC protease inhibitor (Biomol)], cells were homogenized by ten passages each through 23g and 27g needles. Post-nuclear supernatant (PNS) was obtained by centrifugation at 1000 g for 15 min. PNS was further spun for 45 min at 100,000 g. The 100,000 g supernatant was defined as ‘cytosolic fraction’. The 100,000 g pellet was resuspended in homogenization buffer and designated ‘membrane fraction’. Protein concentrations were 1–1.8 mg ml⁻¹ for the cytosolic fraction and 1–17 mg ml⁻¹ for the membrane fraction. Fractions were aliquoted and stored at −20°C.

**Labelling of toxins with Cy3.** *C. difficile* toxins TcdB10463 and TcdB8864 produced by the A⁺ B⁺ strain 8864 (CCUG 20309), respectively, were obtained from tgeBIOMICS. The toxins (300–500 µg) were labelled with Cy3 monofunctional reactive dye according to the manufacturer’s specifications (Amershams Biosciences). Cy3 dye dissolved in dimethyl formamide was incubated with the toxin for 1 h at 4°C. Unbound dye was removed by gel filtration. The molar dye/toxin labelling ratio ranged from 0.9 to 1.4. Labelled samples were aliquoted and stored at −80°C until further use.

**In vitro cleavage reaction.** The in vitro cleavage reaction was performed in a total volume of 10 µl consisting of 2 µl labelled toxin (40–100 ng), 2 µl cell fraction (cytosolic or membrane fraction) and homogenization buffer. It was stopped by addition of SDS-PAGE loading (Laemmli) buffer and heating at 95°C for 3 min. Different incubation times (20 min to 22 h) were tested. Unless specified otherwise, the reaction was incubated at room temperature overnight.

**SDS-PAGE, detection of Cy3-labelled toxin fragments, and immunoblotting.** After incubation, the in vitro cleavage reactions were processed by SDS-PAGE (8-5% gel) and fluorescent toxin bands were directly visualized by a Fuji Film Systems Image Reader LAS-1000 (Ray Test). Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes by semidy transfer in Tris/ glycine buffer [25 mM Tris/HCl, pH 8.3, 192 mM glycine, 20 % (v/v) methanol]. For immunodetection, a polyclonal anti-*C. difficile* serum (Wellcome) and the monoclonal antibody 2CV (Sauerborn et al., 1994) were used. The anti-*C. difficile* serum reacts with epitopes of the whole toxin molecule, whereas 2CV is specific for the receptor binding domain of TcdB (Sauerborn et al., 1994).
Effects of pH, divalent cations, EDTA and protease inhibitors on the cleavage reaction. To test pH dependence of the cleavage reaction, incubations were performed overnight in the presence of the following buffers (final concentration 0–2 mM each): sodium acetate (pH 5–6), MES (pH 6–7), Tris/HCl (pH 7–8, 8–0 and 9–0).

Potential effectors of the cleavage reaction were tested as above in the presence of the following components (final concentrations given): CaCl2 (1, 2, 5 and 10 mM), MgCl2 (5 and 10 mM), EDTA (2 mM), z-macroglobulin (0–2 U ml–1; Merck), pepstatin A (0–2 mg ml–1; Peptide Institute), leupeptin (0–2 mg ml–1) and apro tinin (0–2 mg ml–1) (both Roche Applied Science).

Separation of cleavage products by size-exclusion chromatography. The cleavage reaction (25 μl) used for purification of cleavage fragments contained 24 μg nonlabelled toxin and cytosolic fraction from Vero cells (5 μg protein) prepared as described above. After overnight incubation the cleavage products were separated on a Superdex 200 PC 3.2/30 column using a SMART System (Amersham Biosciences). The separation was performed in 20 mM HEPES/150 mM KCl (pH 7–4). Fractions (20 μl) were collected and analysed by SDS-PAGE and silver staining. The protein concentration of the purified 60 kDa fragment was estimated from silver-stained gels using BSA (10–70 ng per lane) as a standard. The concentration of proteins from the cytosolic fraction carried over into eluted fractions was below the detection limit of silver staining (see Fig. 1c). The peak fractions obtained by gel filtration, containing the 200 kDa fragment and the 60 kDa fragment, respectively, were further used for glucosylation and microinjection experiments, MALDI-TOF analysis (matrix-assisted laser desorption ionization time of flight-mass spectrometry), and N-terminal amino acid sequencing.

Protein sequencing. Coomassie-blue-stained bands corresponding to the 60 kDa proteolytic fragment were cut out from the gels and treated with trypsin as described by Shevchenko et al. (1996). Fingerprint analysis of the digest was performed under standard conditions with a MALDI-TOF mass spectrometer (Bruker Daltronics) as described by Hartmuth et al. (2002).

Fractions obtained by gel filtration and corresponding to the 200 kDa and 60 kDa proteolytic fragments were separated by SDS-PAGE, blotted onto PVDF membranes, and N-terminally sequenced by Edman degradation using a Procise cLC protein sequencer (Applied Biosystems). N-terminal protein sequencing was performed twice on two independent fragment preparations. Sequences obtained were compared with the known sequences of TcdB10463 (GenBank accession number X53138) and TcdB8864 (GenBank accession number AJ011301).

Glucosylation experiments. TcdB10463 and TcdB8864 holotoxins and their 60 kDa cleavage products were tested for glucosyltransferase activity using the small GTPases Rho or Rac and UDP-[14C]glucose (331 mCi mmol–1, 12.2 GBq mmol–1; Amersham Biosciences) as substrates. RhoA-GST and Rac-GST were expressed and purified and the glucosylation reaction was performed as described by Moos & Eichel-Streiber (2000). For testing substrate specificity of the toxins with respect to Rho and Rac the reaction (total volume 20 μl) contained either 200 ng of the holotoxins or 50–70 ng of the 60 kDa fragments. The specific enzyme activities of the holotoxins and the 60 kDa fragments were compared under the same conditions using Rac as a substrate. In these experiments the final concentration for both the holotoxin and the 60 kDa fragment was 1 nM. The assays were incubated for 45 min at 37°C, stopped by addition of Laemmli loading buffer and processed by 8–5% SDS-PAGE. Radioactive bands corresponding to Rho and Rac were detected with a Fuji Film BAS 2500 phosphoimager (Ray Test). Microinjection experiments. DonWT cells were seeded on 12 mm coverslips at a concentration of 0.5 × 10^6 cells ml–1 and incubated overnight in MEM supplemented with 10% FBS. Two to six hours prior to the experiment the medium was exchanged for the same medium without serum.

For the comparison of toxic activity, the 60 kDa cleavage products of TcdB10463 and TcdB8864 holotoxin were diluted with 20 mM HEPES/150 mM KCl buffer (pH 7–4) to a final concentration of 1 nM in the microinjection pipette (Eppendorf). The dilution buffer contained 5 mg ml–1 rhodamine or FITC-labelled dextran (RD-70 or FD-70) to detect microinjected cells. Control microinjections were performed with dilution buffer only. At least 30 cells per coverslip were injected using the Femtotjet microinjection system (Eppendorf). Three coverslips were used for each condition (60 kDa or holotoxin). Injection pulses of 20–50 hPa were applied for 0.5–1 s with compensation pressure of 1 hPa. Cells were analysed 30 min, 1 h and 2 h after the injection for changed morphology. The percentage of microinjected cells showing typical cytotoxic effects was determined.

Detection of cleavage products in toxin-treated cells. DonWT cells were grown in 15 cm Petri dishes in MEM supplemented with 10% FBS. After 15 min incubation in serum-free MEM on ice, cells were washed with ice-cold PBS and 5 μg TcdB10463 was added. For toxin binding without toxin internalization, the cells were incubated for 3 h on ice. They were then washed with ice-cold PBS and incubated at 37°C in pre-warmed medium for 20 min. The membrane and cytosolic fractions were prepared as described above with the following modification: the 100 000 g pellet (‘membrane fraction’) was resuspended in 100 mM Na2CO3 (pH 11) and incubated on ice for 10 minutes in order to disrupt the vesicles and extract the proteins not tightly bound to membranes. This was followed by centrifugation at 100 000 g for 30 min. The supernatant contained proteins extracted from subcellular compartments and proteins loosely attached to membranes, whereas the pellet contained transmembrane and tightly membrane-attached proteins. The pellet was resuspended in Laemmli loading buffer. Following heating for 2 min at 95°C all fractions were submitted to 8–5% SDS-PAGE and blotted onto nitrocellulose membranes. Toxins and toxin fragments were detected with anti-C. difficile polyclonal serum and a secondary horseradish-peroxidase-labelled monoclonal antibody using chemiluminescence (Western Lightning kit, Perkin Elmer Life Sciences, and Image Reader LAS-1000).

RESULTS

Limited proteolysis of C. difficile TcdB by subcellular fractions

When fluorescently labelled TcdB10463 was incubated with subcellular fractions, two cleavage fragments were obtained. The same cleavage fragments were obtained with the ‘cytosolic fraction’ and with the ‘membrane fraction’ (Fig. 1a). Cleavage was observed with ‘cytosolic’ and ‘membrane’ fractions of all tested cell lines (Vero, DonWT, DonQ, Caco2) (see supplementary Fig. S1 available with the online version of this paper). In all further experiments the ‘cytosolic fraction’ was used. The same results were observed with variant TcdB8864 (data not shown and Fig. 2). A significant processing of both toxins became visible already after 20 min incubation (see supplementary Fig. S2). On the other hand, even after overnight incubation not all holotoxin was cleaved (Figs 1 and 2). Cleavage fragments of both tested toxins, TcdB10463 and TcdB8864, had identical sizes on the gel (Fig. 2). According to SDS-PAGE the sizes
**Fig. 1.** Two fragments obtained after limited proteolysis of *C. difficile* TcdB<sub>10463</sub>. (a) Direct detection of fluorescent bands after incubation of Cy3-labelled TcdB<sub>10463</sub> with ‘cytosolic’ and ‘membrane’ fractions followed by SDS-PAGE. In addition to holotoxin two fluorescent cleavage fragments of about 200 kDa and 60 kDa are observed. No cleavage occurs in the absence of the cellular fractions (control). (b) Only the large 200 kDa fragment and holotoxin react with a monoclonal antibody specific for the receptor-binding C-terminal domain, whereas both cleavage products (200 kDa and 60 kDa) and holotoxin are recognized by a polyclonal antiserum specific for TcdB. (c) Separation of TcdB<sub>10463</sub> holotoxin and its 200 kDa and 60 kDa cleavage products by SMART-microchromatography over a Superdex 200 PC 3.2/30 column. The peak fractions were separated by SDS-PAGE and the gels were silver stained. Note that the 60 kDa band is completely separated from the other toxin fractions, whereas the 200 kDa fragment still contains some holotoxin.

**Fig. 2.** pH dependence of the cleavage reaction (left panels) and effects of divalent cations and protease inhibitors (right panels) with TcdB<sub>10463</sub> (upper panels) and TcdB<sub>8864</sub> (lower panels). The Cy3-labelled toxins were incubated with the ‘cytosolic fraction’ as described in Methods and afterwards processed by SDS-PAGE. Fluorograms are shown. Cleavage of both toxins exhibits the same pH dependence, with an optimum between pH 7 and 8. Note the decrease in total toxin protein at pH 9. The inhibition of the cleavage reaction by CaCl<sub>2</sub> (10 mM), MgCl<sub>2</sub> (10 mM) and pepstatin is weaker with TcdB<sub>8864</sub> than with TcdB<sub>10463</sub>.
of the fragments were approximately 200 kDa and 60 kDa, respectively. For simplicity we shall designate them as the 200 kDa and the 60 kDa fragments although we have determined their exact sizes (see below).

To confirm fluorescent fragments as specific holotoxin cleavage products their reactivity with antibodies was tested (Fig. 1b). Results are presented only for TcdB10463 but were identical for TcdB8864. The polyclonal antiserum specific for C. difficile toxins TcdA and TcdB recognized the holotoxins as well as their cleavage fragments (200 kDa and 60 kDa). In contrast, the monoclonal antibody 2CV, which is specific for the receptor binding domains of TcdB10463 and TcdB8864 (Sauerborn et al., 1994), recognized only the holotoxins and the 200 kDa fragments (Fig. 1b). These results suggest that the 200 kDa fragments represent the C-terminal binding/translocation domains of TcdBs and the 60 kDa fragments the N-terminal catalytic (toxic) domains.

In order to examine whether the same type of processing occurs also in intact cells, DonWT cells were incubated together with nonlabelled TcdB. After incubation, cells were washed, disrupted, subfractionated by centrifugation and analysed for the toxins and their cleavage products. After 20 min incubation the 60 kDa fragment but no 200 kDa fragment could be detected in the cytosolic fraction (see supplementary Fig. S3). However, the membrane fraction obtained after carbonate extraction contained not only the holotoxins and the 200 kDa fragments (Fig. 1b). These results suggest that the 200 kDa fragments represent the C-terminal binding/translocation domains of TcdBs and the 60 kDa fragments the N-terminal catalytic (toxic) domains.

**Properties of the in vitro cleavage reaction**

After showing that in vitro proteolytic processing corresponds to the events observed with intact cells, we further used our in vitro assay to study the properties of the cleavage reaction. The cleavage reaction was strongly pH dependent (Fig. 2). At pH 5-0 it was almost completely inhibited, whereas at pH 6-0 cleavage of the toxins occurred. The maximum was obtained at pH 7-0-8-0. At pH 9-0 the total amount of toxin fragments decreased slightly (Fig. 2), indicating that at this unphysiological pH value additional proteolytic processes come into play. When toxins were incubated at different pH values in the absence of the ‘cytosolic fraction’, cleavage did not occur (data not shown).

In order to get a hint as to the nature of the protease involved in the limited proteolysis of the TcdB toxins, various protease inhibitors were tested. Neither leupeptin (serine and cysteine protease inhibitor) nor aprotinin (serine protease inhibitor) (data not shown), nor EDTA (metalloprotease inhibitor, Fig. 2) had any inhibitory effect. Even the more general endoprotease inhibitor α-macroglobulin failed to show any inhibitory action (Fig. 2). In contrast pepstatin A, an aspartate protease inhibitor, clearly inhibited the proteolytic processing of the TcdB toxins (Fig. 2).

Some proteases responsible for toxin processing require divalent cations for full activity (Fryling et al., 1992; Molloy et al., 1992). However, in our system divalent cations inhibited the cleavage reaction. An inhibition was obtained with all tested concentrations of CaCl2 (1, 2, 5 and 10 mM) and 10 mM MgCl2 (Fig. 2), whereas 5 mM MgCl2 was only partially inhibitory.

**Characterization of cleavage fragments and determination of the cleavage site**

To further characterize the cleavage products they were subjected to N-terminal sequencing (200 kDa and 60 kDa fragments) and MALDI-TOF analysis after trypsin digestion of 60 kDa fragments. In accordance with the results obtained with different antibodies, the 60 kDa fragments of both TcdBs were confirmed by MALDI-TOF analysis as the N-terminal catalytic parts of the holotoxins (see supplementary Figs S4 and S5). Because of the large size the exact molecular mass of the whole 60 kDa fragments could not be determined by this method.

The sequence of the first 10 N-terminal amino acids of both the 60 kDa fragments showed identity with amino acids 2–11 of both holotoxins (SLVNRKQLEK). N-terminal sequencing of both 200 kDa fragments provided the position of the cleavage site (Fig. 3). The obtained N-terminal sequence of the 200 kDa fragments was identical in TcdB10463 [(G)E(D)(D)NL(D)F(S)Q] and TcdB8864 (GED-DNLDFSQNTVTD). The molecular masses calculated from the known toxin sequences and the identified cleavage sites (Table 1) are in good agreement with the molecular masses of the cleavage products estimated from SDS-PAGE (200 kDa and 60 kDa).

The cleavage site is located between Leu543 and Gly544 in TcdB10463. Since TcdB8864 has a single amino acid insertion before the cleavage site and a single amino acid deletion after the cleavage site when compared to TcdB10463, the position of the cleavage site in TcdB8864 is shifted by a single amino acid and is between Leu544 and Gly545 (Fig. 3). The cleavage site is located within a region which is highly conserved in TcdB10463 and TcdB8864 except for a single amino acid difference at position −2 (Fig. 3).

**Properties of the 60 kDa N-terminal fragment**

**Microinjection experiments.** As the catalytic (toxic) activity of TcdBs is located in the N-terminal part of the holotoxins (Hofmann et al., 1997; Wagenknecht-Wiesner et al., 1997) we tested the enzymic and cytotoxic activities of both 60 kDa fragments. After microinjection the 60 kDa fragments of both toxin variants were cytotoxic. The morphological changes of cultured cells caused by
holotoxin TcdB10463 differ from those caused by TcdB8864 (Eichel-Streiber et al., 1996; Chaves-Olarte et al., 2003). While cells intoxicated with TcdB10463 round up but still attach to the support with long protrusions, cells intoxicated with TcdB8864 round up without protrusions. The same toxin-specific cytopathic differences were observed for each 60 kDa fragment following microinjection (data not shown). When 60 kDa fragments were not microinjected but added to the culture medium no changes were detected within 2 h and only minor alterations became visible after more than 24 h. This suggests that the 60 kDa fragments cannot be effectively internalized by the cells.

If proteolytic processing contributed to TcdB toxicity, one would expect that microinjected holotoxin should have initially no or a very low cytotoxic activity which afterwards should increase due to proteolytic processing. The microinjected N-terminal 60 kDa peptide, on the other hand, should initially have a much higher cytotoxic activity than the holotoxin, which, however, should not further increase during prolonged incubation. This is exactly what we observed (Fig. 4): 30 min (the first time point taken) after microinjection the N-terminal fragment of TcdB10463 was about fivefold more toxic than the holotoxin, but the toxicity did not significantly change upon further incubation. The toxicity of the holotoxin on the other hand increased continuously over the time of the experiment (2 h). A similar trend was observed also for TcdB8864 and its 60 kDa cleavage product (results not shown).

The microinjection experiments were not performed with the 200 kDa fragment as this could not be separated from the holotoxin after size-exclusion chromatography (Fig. 1c).

**Glucosyltransferase activity**

Glucosyltransferase activities of the 60 kDa fragments were compared with those of the corresponding holotoxins.

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**Table 1. Molecular masses of the proteolytic cleavage products of TcdB10463 and TcdB8864 as estimated from the known toxin sequences and the cleavage site determined in this study**

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<th>TcdB10463</th>
<th>TcdB8864</th>
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<tbody>
<tr>
<td>Amino acids</td>
<td>Mol. mass (Da)</td>
<td>Amino acids</td>
</tr>
<tr>
<td>Holotoxin</td>
<td>1–2366</td>
<td>269 712</td>
</tr>
<tr>
<td>60 kDa fragment</td>
<td>1–543</td>
<td>63 121</td>
</tr>
<tr>
<td>200 kDa fragment</td>
<td>544–2366</td>
<td>206 609</td>
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in vitro using two different substrates, namely Rho and Rac. This was of interest as it had been reported that TcdB10463 and TcdB8864 differ in their substrate specificities in that both glucosylate Rac, but only TcdB10463 is active with Rho (Eichel-Streiber et al., 1996). This is in line with the results presented in Fig. 5(a), which show that the two 60 kDa fragments exhibit the same differences with respect to substrate specificity as the corresponding holotoxins.

At 1 nM concentration the 60 kDa fragments had the same or higher specific enzymic activities compared to the corresponding holotoxins (Fig. 5b). Additionally, at the same concentration (1 nM), TcdB8864 was not as effective as TcdB10463. At a concentration of 5 nM TcdB8864 had a similar enzymic activity to that of 1 nM TcdB10463 (Fig. 5b).

**DISCUSSION**

Proteolytic processing is an important step during the translocation of AB-toxins into the cytosol and was suggested also for *C. difficile* TcdB (Florin & Thelestam, 1986; Thelestam et al., 1997; Pfeifer et al., 2003). Here we present for the first time biochemical details concerning the proteolytic cleavage reaction and the cleavage site of *C. difficile* TcdB toxins. We have compared the processing of the ‘reference’ TcdB from strain VPI 10463 with that of variant TcdB from strain 8864 (TcdA-negative, TcdB-positive strain) (Borriello et al., 1992; Soehn et al., 1998). The two TcdBs differ slightly in sequence, in substrate specificity with respect to Rho and Rac, and in the morphology of cytopathic effects (Eichel-Streiber et al., 1996; Chaves-Olarte et al., 2003). We show here that both toxins are proteolytically processed at identical cleavage sites to yield active catalytic fragments. The same processing products were obtained in vitro using subcellular fractions and in situ with intact cells.

The proteolytic cleavage of TcdB results in two fragments: a 200 kDa C-terminal fragment which was shown by antibody reactivity and by N-terminal sequencing to include the receptor binding and the internalization domains, and a 60 kDa fragment representing the N-terminal catalytic domain. As reported here, the catalytic domains still have the same substrate specificity and show the same cytopathic effects as the corresponding holotoxins.

For several bacterial toxins it has been reported that proteolytic processing increases their toxicity (Kriegstein et al., 1997; Pfeifer et al., 2003). The 60 kDa fragments retain the same substrate specificities in vitro and in intact cells, and their enzymic activity is higher than that of the corresponding holotoxins. The 60 kDa fragments show the same differences in substrate specificities as do the corresponding holotoxins in vitro and in intact cells, and their enzymic activity is higher than that of the corresponding holotoxins.
The proteolytic step described here may not be an absolute requirement for toxicity of *C. difficile* TcdB, as TcdB holotoxins have glucosyltransferase activity (Fig. 5) and are cytotoxic after microinjection (Fig. 4). However, the specific glucosyltransferase activity of the active 60 kDa fragments is at least as high or higher in comparison with the corresponding holotoxins (Fig. 5). Moreover, in the microinjection experiments the 60 kDa fragment was fully active immediately after injection whereas equimolar amounts of injected holotoxin exhibited initially only a relatively low toxic activity, which increased with time (Fig. 4). Both results suggest that proteolytic processing of TcdB influences also the toxin activity. However, the processing is obviously needed for translocation of the catalytic fragment into the cytoplasm, where the toxin acts on small GTPases.

In our experiments the active 60 kDa cleavage product could be recovered shortly after the start of internalization mainly in the cytoplasm but also associated with the total membrane fraction, whereas the 200 kDa cleavage product was observed exclusively in the membrane fraction. Because of inhibition of cytotoxicity with lysosomotropic agents but not Golgi-disrupting drugs, TcdB is thought to translocate into the cytosol via an endosomal compartment. Several toxins characterized by translocation of the toxic subunit or domain from the endosomal compartment into the cytosol typically contain domains or subunits which can form transmembrane channels (Oh *et al.*, 1999; Olsnes *et al.*, 1999; Kariazova & Montal, 2003). These channels have been suggested to permit the translocation of the catalytic domain or subunit into the cytoplasm. Indeed, channel formation was also reported for *C. difficile* TcdB (Barth *et al.*, 2001).

For several AB-toxins it has been shown that after proteolytic cleavage A- and B-subunits or domains remain covalently linked by disulfide bridges during the translocation through the channel provided by the B-subunits or B-domains (diphtheria toxin, *Clostridium botulinum* neurotoxins: Olsnes *et al.*, 1999; Falnes & Sandvig, 2000). Disulfide bridges are cleaved after translocation by the reducing milieu in the cytoplasm (Falnes & Sandvig, 2000). We suggest here that TcdB represents a group of toxins where A- and B-domains remain linked by a peptide bond until the A-domain has become translocated to the cytosol. Similar to the cleavage of disulfide bonds typical of some other toxins, the cleavage of the peptide bond linking the A- and B-domains in TcdB occurs on the cytoplasmic site of the translocation compartment, most likely endosomes. This suggestion would be in line with the neutral pH optimum of the cleavage reaction and our observation that the proteolytic cleavage activity is found both in the cytosol and associated with membranes. We propose a model where the TcdB holotoxin undergoes a conformational change in the endosomal compartment due to low pH (Qa’Dan *et al.*, 2000). This conformational change favours insertion of the toxin into the endosomal membrane, where it forms a pore (Barth *et al.*, 2001). Still unnicked, the N-terminal A-domain translocates through the pore into the cytosol, where the proteolytic cleavage takes place.

The host cell protease catalysing the cleavage of *C. difficile* TcdB has not been identified yet. Although furin has been implicated in the processing of various bacterial toxins (Gordon & Leplla, 1994; Gordon *et al.*, 1995) this is excluded for the processing of TcdBs as described here, as (i) the observed cleavage site L/G differs strongly from the furin cleavage site RXR/X, and (ii) the main cleavage activity for TcdBs is found in the cytosol, whereas furin exists mainly in the trans-Golgi and the trans-Golgi network.

As the reaction could be inhibited only by pepstatin one might assume an aspartate protease to be responsible for the cleavage reaction. On the other hand known aspartate proteases have a significantly lower pH optimum than the cleavage reaction observed here. Pepstatin might also interfere with proteases of other classes (Lencer *et al.*, 1997); therefore another type of pepstatin-sensitive protease cannot be excluded.

As the cleavage site is the same for both toxins, TcdB10463 and TcdB8864, we assume that they are processed by the same protease, although the cleavage reaction with TcdB10463 proceeds more slowly than that with TcdB8864 (see Fig. S2). The nature of the amino acids close to the cleavage site can affect the protease reaction (Molloy *et al.*, 1992; Gordon *et al.*, 1995); therefore the difference at position −2 (Fig. 3) might be responsible for the different cleavage efficiencies with the two toxins.

The sequence is known for four out of five toxins within the LCT group, including *C. difficile* TcdA, *C. sordellii* TcsL and *C. novyi* TcnA. The cleavage site is changed in TcdA (GGSL/SEDN) and in TcnA (GRTL/NYED) when compared to TcdB (EGS(or A)L/GEDD). Those two toxins were not included in our study. However, the cleavage site in TcsL is identical to the sequence of TcdB8864 (EGAL/ GEDD). Indeed, TcsL (kindly provided by M. Popoff, Pasteur Institute, Paris, France) is cleaved under our conditions (data not shown), but further characterization of cleavage reaction was not done.

The results reported here may have practical implications, as processing of virulence factors, such as toxins or proteins, which are most important in pathogenesis of bacterial and viral infections, can be suitable therapeutic targets (Jean *et al.*, 2000). *Saccharomyces boulardii*, which has been used as a probiotic therapeutic for recurrent *C. difficile* infections, produces proteases which inactivate *C. difficile* toxins (Castagliuolo *et al.*, 1999). If it could be achieved, blockade of the protease responsible for the intracellular activation of *C. difficile* toxins might represent a much more potent therapeutic approach.
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