

A Novel Cytotoxin from Clostridium difficile Serogroup F Is a Functional Hybrid between Two Other Large Clostridial Cytotoxins*

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The large clostridial cytotoxins (LCTs) constitute a group of high molecular weight clostridial cytotoxins that inactivate cellular small GTP-binding proteins. We demonstrate that a novel LCT (TcdB-1470) from Clostridium difficile strain 1470 is a functional hybrid between “reference” TcdB-10463 and Clostridium sordellii TcsL-1522. It bound to the same specific receptor as TcdB-10463 but glucosylated the same GTP-binding proteins as TcsL-1522. All three toxins had equal enzymatic potencies but were equally cytotoxic only when microinjected. When applied extracellularly TcdB-1470 and TcdB-10463 were considerably more potent cytotoxins than TcsL-1522. The small GTP-binding protein R-Ras was identified as a target for TcdB-1470 and also for TcsL-1522 but not for TcdB-10463. R-Ras is known to control integrin-extracellular matrix interactions from inside the cell. Its glucosylation may be a major determinant for the cell rounding and detachment induced by the two R-Ras-attacking toxins. In contrast, fibroblasts treated with TcdB-10463 were arborized and remained attached, with phosphotyrosine containing structures located at the cell-to-cell contacts and β2-integrin remaining at the tips of cellular protrusions. These components were absent from cells treated with the R-Ras-inactivating toxins. The novel hybrid toxin will broaden the utility of the LCTs for clarifying the functions of several small GTPases, now including also R-Ras.

Bacteria of the genus Clostridium produce a wide variety of toxins displaying different enzymatic activities. Among them, the large clostridial cytotoxins (LCTs) comprise the largest bacterial protein toxins known, ranging in size from 250 to 308 kDa (1). So far, five toxins belong to this group: the Clostridium difficile toxins A and B (TcdA and TcdB), the Clostridium sordellii hemorrhagic and lethal toxins (TcsH and TcsL) and α-toxin (TcnA) from Clostridium novyi. TcdA and TcdB are responsible for the symptoms in C. difficile-induced antibiotic-associated diarrhea and pseudomembranous colitis, whereas TcsH, TcsL, and TcnA are virulence factors in gas gangrene (1). In cultured cells the LCTs elicit cytopathic effects (CPEs) characterized by collapse of the actin cytoskeleton followed by arborization and/or rounding up of the cells. This effect is due to a glycosylation of different small GTP-binding proteins (2). The sugar moiety from either UDP-glucose or UDP-N-acetylglucosamine is transferred to a conserved threonine in the effector region of the target protein. The GTP-binding protein is inactivated, its proper interaction with immediate downstream effectors is impaired, and normal cell signaling is interrupted (3). The CPEs induced by the different LCTs depend on the targets attacked. In fibroblasts TcdA, TcdB, and TcnA induce an arborizing effect (1) reported to arise upon modification of Rho alone, although these toxins also affect Rac and Cdc42 (3, 4). TcsL modifies Ras, Rap, Ral, and Rac but not Rho (5–7) and induces a complete rounding of the cell body without arborization (1). It is not known which GTP-binding protein target(s) is crucial for the TcsL-induced morpholgy.

A new member of the LCT family was recently identified in a sero group F strain of C. difficile and named TcdB-1470 (the number indicates the strain from which it was isolated) (8, 9). The amino acid sequence of this toxin shows an overall identity of 93 and 75% with TcdB-10463 (“reference” TcdB) and TcsL-1522, respectively. Despite the high overall identity of TcdB-1470 with TcdB-10463, it is only 79% in the first 560 amino acids comprising the catalytic domain (Fig. 1). On the other hand, the identity is 99% in the carboxyl-terminal domain where the receptor-binding region is located (10). In contrast to this polarized identity of TcdB-1470 with TcdB-10463, the sequence comparison with TcsL reflects the overall identity in both domains (Fig. 1). In this work we have characterized TcdB-1470, in terms of its cell surface binding, cytotoxic and enzymatic potencies, substrate pattern, and type of CPE. Comparing these parameters with those of TcdB-10463 and TcsL-1522 established the hybrid character of TcdB-1470. Importantly, R-Ras was identified as a major substrate for TcdB-1470 hemorrhagic toxin; TcsL, C. sordellii lethal toxin; TcnA, C. novyi α-toxin; CPE, cytopathic effect; GST, glutathione S-transferase; EGF, epidermal growth factor; ERK, extracellularly regulated kinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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1 The abbreviations used are: LCTs, large clostridial cytotoxins; TcdA, C. difficile toxin A; TcdB, C. difficile toxin B; TcsH, C. sordellii

2 This paper is available on line at http://www.jbc.org
and TcsL-1522. The significance of this novel substrate for development of the TcsL-like CPE is discussed.

EXPERIMENTAL PROCEDURES

Materials—TcdB-10463, TcsL-1522, and TcdB-1470 were prepared as described (11). The toxin preparations showed >95% purity as assessed by SDS-PAGE. UDP-[14C]Glc (specific activity, 318 mCi/mmol) was from NEN Life Science Products and N-succinimidyl 3-(4-hydroxy, 5-[131I]iodophenyl)propionate (specific activity, 2000 Ci/mmol) from Amersham Pharmacia Biotech. Anti-vinculin antibody was from Sigma; and anti-ERK1 antibody and anti-β-integrin antibody were from Transduction Laboratories (Lexington, KY). Mouse antisera against TcdB was provided by Dr. M. R. Popoff (Institut Pasteur, Unite´ de Toxines Microbiennes, Paris, France). Recombinant proteins Rho, Rac, Cdc42, Ras, and Rap were provided by Dr. P. Boquet (INSERM, Nice, France). A GST-Ral fusion protein was made as follows: The rat RalA clone (accession number L19698, a gift from Gary M. Wildey, Cambridge Foundation, Paris, France) was a generous gift from Adrienne Cox (University of North Carolina). All recombinant proteins were purified with glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) and cleaved with the appropriate protease. Proteases were removed with benzamidine Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) and cleaved with the appropriate protease. Proteases were removed with benzamidine Sepharose 4B. All other reagents were of analytical grade and obtained from local commercial sources.

Amino Acid Sequence Analysis—Binary alignment was performed by the SIM software (ExPASy Molecular Biology Server) (12) after translation of DNA sequences with the following accession numbers: TcdB-10463, X53138; TcsL-1522, X82638; and TcdB-1470, 223277.

Cell Culture—Preparation of Lysates—Chinese hamster lung fibroblasts (Don cells, CCL-16; American Type Culture Collection, Rockville, MD) were cultured in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum, 5 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Life Technologies, Inc.). Mouse Swiss 3T3 fibroblasts were obtained from Dr. Crister Höög (Department of Cell and Molecular Biology, Karolinska Institutet, Sweden) and cultured in Dulbecco’s medium supplemented as above. Both cell lines were incubated at 37 °C in a humid atmosphere containing 5% CO₂. Cytotoxic activities were titrated on cells cultivated to 90% confluence in 96-well plates. The initial toxin concentration was 2 μg/ml, and subsequent 1:10 dilutions were made. Cytotoxicity was scored microscopically, and the results were expressed as percentages of affected cells. Cell lysates were prepared as described previously (11). Briefly, confluent cells in 75-cm² flasks were rinsed, mechanically removed, and washed twice with ice-cold Hanks’ balanced salt solution. Cell pellets were resuspended in 200 μl of lysis buffer (50 mM triethanolamine, 150 mM KCl, 2 mM MgCl₂, 0.5 mM GDP, 1 mM diithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, pH 7.8) and sonicated five times for 5 s. After centrifugation (14,000 g, 3 min), the supernatant was used as postnuclear cell lysate. The amount of protein in lysates was determined by Bio-Rad protein assay with bovine serum albumin as a standard.

Scanning Electron Microscopy—Subconfluent Don and Swiss 3T3 cells were treated with TcdB-10463 (50 ng/ml for 1 h), TcdB-1470 (50 ng/ml for 1 h), and TcsL-1522 (600 ng/ml for 6 h). Control cells were not treated. After treatment the cells were chemically immobilized with 2% glutaraldehyde in 0.1 M phosphate buffer, at 4 °C for 2 h and post-fixed in 1% osmium tetroxide (Agar Scientific Ltd., Cambridge, UK) in phosphate buffer for 30 min. The fixed cells were dehydrated in increasing concentrations of ethanol and frozen in tert-butyl alcohol (T. J. Baker, Inc., Phillipsburg, NJ). Cells were freeze-dried by sublimation and coated with 20 nm of gold/palladium (10 nAmp, 2 min). Cells were observed and photographed with a S-570 (Hitachi, Tokyo, Japan) Scanning Electron Microscope operating at 15 kV.

Microinjection Experiments—Don cells were cultivated on 13-mm slides for 48 h. Toxin antisera (1:100) was added to the medium to neutralize any toxin molecules leaking from the injection needle. Goat anti-TcdB-10463 was used for both TcdB-10463 and TcdB-1470 injections. Mouse anti-TcsL was used for TcsL-1522 injections. Semicellular cells were microinjected (Automatic Eppendorf Microinjector) with TcdB-10463, TcsL-1522, or TcdB-1470 diluted in 0.01 M phosphate-buffered saline (PBS) at the concentrations defined in figure legends. The solution contained 2% fluorescein dextran to localize microinjected cells. After microinjection, cells were incubated 2 h at 37 °C, washed with PBS, and fixed with 3.7% paraformaldehyde (Fisher). The CPE of microinjected cells was determined by phase contrast microscopy. Three different fields (400×) were analyzed per experiment, and the activity of the toxins was expressed as the percentage of affected cells/microinjected (fluorescent) cells.

Glucosyltransferase Activity—5 μl of UDP-[14C]Glc dissolved in ethanol were vacuum dried, and 10 μl of cell lysate (5 mg protein/ml) or 10 μl of lysis buffer containing recombining Rho, Rac, Cdc42, Rap1, Rap2, Ras, RaI, or R-Ras (1–3 μg) were added. The final concentration of UDP-[14C]Glc was 30 μM. The mixtures were incubated 1 h at 37 °C with TcdB-10463, TcsL-1522, or TcdB-1470 at the concentrations defined in figure legends. The reaction was stopped by heating at 95 °C in sample buffer. Proteins were separated by 12.5% SDS-PAGE (14) or by two-dimensional gel electrophoresis (15). Radiolabeled bands or spots were detected by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). The intensity of the bands was calculated using the ImageQuant software (Molecular Dynamics). The result was expressed as a percentage of the labeling obtained with the highest toxin concentration (5 μg/ml). For differential glucosylation experiments, cells were treated in vitro with the indicated toxins until 100% CPE was achieved. Cell lysates were then prepared and processed for glucosylation as described above.

Specific Binding and Competition with 125I-TcdB-10463—TcdB-10463 was labeled with 125I as described previously (16). Total binding of 125I-TcdB-10463 was determined by the addition of ice-cold 125I-TcdB-10463 (2 μg/ml) to precooled (15 min at 4 °C) Don cells in 96-well plates. After 2 h of incubation at 4 °C, cells were washed three times with PBS and lysed with 50 μl of 1% Nonidet P-40, and the radioactivity was measured in an automated γ counter. Competition experiments were performed following the same protocol but using a 50-fold excess of the indicated cold competitor.

EGF-induced ERK Phosphorylation—Experiments examining the effects of LCTs on EGF-stimulated phosphorylation and activation of ERK were performed as follows. Confluent Swiss 3T3 cells were serum-starved overnight in medium with 0.1% fetal bovine serum. These cells were treated with TcdB-10463 (50 ng/ml) or TcdB-1470 (50 ng/ml) for 30 min or with TcsL-1522 (600 ng/ml) for 6 h. When 100% of the cells showed a clear CPE, they were exposed to EGF (100 ng/ml) for 5 min. This step was omitted in control cells. Then cells were lysed with Laemmli sample buffer (14), and 20 μg of total protein/lane were loaded. Proteins were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes, which were then incubated with monoclonal anti-ERK1 antibodies (1:250). Finally, blots were developed with a horseradish peroxidase-labeled secondary antibody. The cross-reacting...
tion of the anti-ERK1 antibody with ERK2 (44 kDa) has been documented in rat cells (catalog of Transduction Laboratories) and was also observed in a previous study (5) with 3T3 fibroblasts.

Measurement of Toxin-induced Cell Detachment—Triplicate cultures of Swiss 3T3 fibroblasts in 24-well plates were incubated with TcdB-10463 or TcdB-1470 (50 ng/ml) 1 h at 37 °C. Control cells were not treated. Then the cells were washed three times with PBS to remove detached cells. Remaining cells were collected with 0.1% trypsin (10 min at 37 °C), suspended in 200 μl of PBS, recovered by centrifugation (3000 × g for 10 min), and lysed in 30 μl of 1% SDS. The protein concentration in lysates was taken as a measure of the number of cells remaining attached after toxin treatment.

Immunofluorescence Experiments—Swiss 3T3 cells on 13-mm glass coverslips were left untreated (control cells) or treated with 50 ng/ml of TcdB-10463 or TcdB-1470 for 30 min at 37 °C. Cells were fixed with 3.7% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS and incubated 1 h at room temperature with the antibodies specified in figure legends. Then the cells were washed three times in PBS/0.5% Triton X-100 and incubated for 30 min at 22 °C with rhodamine-conjugated antibody, followed by three washes with PBS. The cells were analyzed at 600 × using a confocal microscope (Molecular Dynamics).

RESULTS

Cytopathic Effect and Cell Detachment Induced by TcdB-1470—As viewed by scanning electron microscopy, TcdB-1470 induced 3T3 cell rounding with a mitotic-like morphology, indistinguishable from that induced by TcsL-1522 (Fig. 2). All cell-to-cell contacts disappeared after treatment with these toxins. In contrast, TcdB-10463 induced the characteristic arborizing effect (Fig. 2), and cell-to-cell contacts were retained. Similar CPEs were induced in Don cells (data not shown). Light microscopy indicated that fibroblasts treated with TcdB-1470 or TcsL-1522 detached easily from the plastic substrate, whereas cells treated with TcdB-10463 remained attached. Indeed, the protein concentration in lysates of TcdB-10463-treated cells (799 ± 21 μg/ml) was equal to control cells (765 ± 0.70 μg/ml), whereas it was greatly diminished in cultures exposed to TcdB-1470 (158 ± 51 μg/ml), reflecting a massive detachment of cells.

Cytopathic and Enzymatic Potency of TcdB-1470—The cytopathic potency of extracellularly applied TcdB-1470 was determined in Don cells by titration and compared with the potencies of TcdB-10463 and TcsL-1522. TcdB-1470 was almost as strongly cytotoxic as TcdB-10463, whereas TcsL-1522 showed a weak potency (Fig. 3a), in agreement with previous reports on other cell lines (17, 18). However, when the cytotoxic potencies were determined after intracellular application by microinjection, all three toxins showed similar activities (Fig. 3b). In the presence of UDP-[14C]Glc TcdB-1470 modified 20–30-kDa target proteins in cell lysates confirming its glucosyltransferase character. The activities of the toxins were compared in terms of the glucosyltransferase reaction. All three toxins exhibited similar enzymatic potency (see Fig. 5a). This result, together with the similar cytotoxic potencies observed after microinjection (Fig. 3b), indicates that the low cytotoxicity of TcsL-1522 is not a matter of a low enzymatic activity but rather due to an inefficient binding and/or internalization into the cell.

TcdB-10463 and TcdB-1470 Share a Specific Receptor on the Cell Surface—To test the possibility that TcdB-1470 uses the same receptor as TcdB-10463, competition experiments were performed. Pretreatment with TcdB-1470 reduced the binding of 125I-TcdB-10463 to Don cell surfaces at 4 °C, almost to the same extent as cold TcdB-10463 did (Fig. 4), suggesting that TcdB-10463 and TcdB-1470 share the same receptor on these cells. In contrast, TcsL-1522 did not compete with 125I-TcdB-10463 (Fig. 4) and thus binds to a different receptor on Don
TcdB-1470 and TcsL-1522 Glucosylate Similar Cellular Sub-
substrates—The substrate pattern of TcdB-1470, as analyzed by SDS-PAGE, was very similar to that of TcsL-1522, particularly with respect to a 30-kDa band typically seen after TcsL-1522 modifications (Fig. 5). Accordingly, the patterns obtained by two-dimensional electrophoresis showed great similarity between TcsL-1522 and TcdB-1470, whereas a different pattern of labeled GTPases was obtained with TcdB-10463 (Fig. 5a). The only observable major difference between TcdB-1470 and TcsL-1522 was an acidic spot of approximately 20 kDa labeled by the latter. To determine whether TcdB-1470 and TcsL-1522 modify the same substrates also in intact cells, a differential glucosylation experiment was performed. TcsL-1522 labeled its substrates in lysates from TcdB-10463-treated cells almost to the same extent as in lysates from nontreated cells (Fig. 5b). The GTPase, which is obvious in lane 4 but not labeled in lane 5, probably represents Rac, the only known common substrate of TcdB-10463 and TcsL-1522. In lysates from TcdB-1470-treated cells, however, TcsL-1522 was no longer able to glucosylate its substrates (Fig. 5b). On the other hand TcdB-10463 labeled its substrates in lysates from TcdB-1470-treated cells (Fig. 5b), demonstrating that TcdB-1470 in intact cells does not modify the substrates recognized by TcdB-10463. The actual substrates of TcdB-1470 were determined with a panel of recombinant small GTPases. Rac, Rap1, Rap2, and RaLα were labeled by TcdB-1470, whereas Cdc42 was modified to a lower extent (Fig. 6a). Interestingly, R-Ras, a small GTP-binding protein important in integrin activation (19), was glucosylated both by TcdB-1470 and TcsL-1522 but not by TcdB-10463 (Fig. 6b). Thus, inactivation of R-Ras might play a role in the cell rounding (Fig. 2) and detachment induced by TcdB-10463 and TcsL-1522.

TcdB-1470 Does Not Block EGF-induced Phosphorylation of ERKs—To determine the cellular effect of TcdB-1470 on activation of ERKs, serum-starved 3T3 cells were treated with toxin until 100% CPE was observed. After stimulation with EGF for 5 min, phosphorylation of ERKs was assessed as a shift in their molecular weights. This shift occurred in TcdB-1470- and TcdB-10463-treated cells to the same extent as in nontreated control cells (Fig. 7). As an additional control we included TcsL-1522, which is known to inhibit the EGF stimulation of ERK (5). We conclude that inactivation of R-Ras did not affect the Ras-dependent mitogen-activated protein kinase pathway.

Effect of TcdB-10463 and TcdB-1470 on Focal Adhesion Components—The different CPEs induced by TcdB-10463 and TcdB-1470 might reflect a differential effect of these toxins on focal adhesion complexes. Thus, toxin-treated 3T3 cells were probed with anti-vinculin, anti-β3-integrin, and anti-phosphotyrosine antibodies. Both TcdB-10463 and TcdB-1470 caused vinculin to disappear from focal adhesions after only 20 min (Fig. 8, a–c). However, β3-integrin-containing protrusions remained at the ruffling edges of the fibroblasts after treatment with TcdB-10463 (Fig. 8e), whereas TcdB-1470 completely disrupted these structures (Fig. 8f). Tyrosine-phosphorylated proteins remained at most cell-to-cell contacts (Fig. 8h) in TcdB-10463-treated cells, whereas TcdB-1470 completely disrupted also these structures. (Fig. 8i). Thus, TcdB-10463 and TcdB-1470 affect focal adhesion components differentially.

**DISCUSSION**

Some *C. difficile* sero group F strains do not elaborate TcdA (9), although they produce the cytotoxin TcdB-1470 (8), which differs somewhat from the classic cytotoxin TcdB-10463. Here we characterize the enzymatic properties of this novel toxin and compare its cytotoxic effects with those of the most closely related LCTs, i.e. TcdB-10463 and TcsL-1522. As discussed below TcdB-1470 turns out to be an interesting addition to the family of LCTs, behaving functionally as a TcsL-1522/TcdB-10463 hybrid, with the same cytotoxic potency as TcdB-10463 but inducing a TcsL-like CPE.

All three toxins had equal cytotoxic potencies upon microinjection, i.e. when the normal receptor binding and internalization routes were bypassed. In contrast, extracellularly applied
TcdB-1470 (5 \mu M). After 1 h of incubation at 37 °C, the reaction was stopped by the addition of Laemmli sample buffer. Proteins were resolved by 12.5% SDS-PAGE, and labeled bands were detected by PhosphorImager analysis. a, purified recombinant R-Ras in GST fusion form was incubated with TcdB-1470, TcsL-1522, or TcdB-1470 (5 \mu M) in the presence of UDP-[14C]Glc (30 \mu M) and processed following the same procedure as in A. Coomassie-stained gel and PhosphorImager analysis of the same gel is shown.

TcsL-1522 was remarkably less cytotoxic than TcdB-10463 or TcdB-1470. In view of the equal enzymatic potencies when tested in vitro, we propose that this difference is mainly determined by the receptor binding event of each toxin. The high cytotoxic potency of extracellularly applied TcdB-1470 probably depends on its specific binding to the same receptor as used by TcdB-10463. This was predicted from the high identity (99%) between the receptor-binding domains of TcdB-1470 and TcdB-10463 (8) and here substantiated by cell surface binding experiments, demonstrating a competition between TcdB-1470 and \(^{125}\)I-TcdB-10463. That this competition was somewhat lower than with TcdB-10463 could be due to a slightly lower affinity of TcdB-1470 for the receptor. In contrast, TcsL-1522 could not compete out \(^{125}\)I-TcdB-10463 at all, suggesting that it binds to a different receptor. The nature of the receptors is not known, but the existence of a specific receptor for TcdB-10463 on the fibroblast surface was recently demonstrated (16). In Don cells this TcdB-1470/TcdB-10463 receptor should be present in considerably higher numbers than the putative receptor for TcsL-1522. Alternatively TcsL-1522 might have a very weak affinity for its own receptor.

Despite the similar cytotoxic potencies of TcdB-10463 and TcdB-1470, they induce different types of CPE. TcdB-10463 induces a collapse of the actin cytoskeleton with retraction of the cell body. Because protrusions remain attached to the extracellular matrix, this morphology is designated arborizing, actinomorphic, or neurite-like (2). TcdB-1470, in contrast, induced a complete rounding of the fibroblast body, conferring a mitotic-like appearance indistinguishable from that induced by TcsL-1522. The respective CPEs should somehow correlate with the substrates modified. Indeed, Rho was previously shown to be the crucial substrate for the arborizing CPE, because transient coexpression with Rho could protect cells against TcdB-10463 but not TcsL-82 (18). The crucial substrate(s) for the rounding CPE by TcsL-1522 and TcdB-1470 is not known, but we hypothesized that these two toxins modify the same or similar substrates. This concept was substantiated by two-dimensional electrophoresis showing similar in vitro substrate patterns and by the differential glycoylation experiments (Fig. 5), which confirmed that the same substrates are also modified in vivo. Thus, the substrate pattern of TcdB-1470 is similar to that of TcsL-1522 with the only difference that TcsL-1522 is also able to modify Ras (5, 6).

The possibility of an additional substrate for TcdB-1470 and TcsL-1522 was considered because none of the known substrates could adequately explain the TcsL-like rounding CPE: (i) Rac is a common substrate for all LCTs, including those that induce the arborizing effect, (ii) Rap has been shown to be also modified by TcdA (16), which induces the arborizing effect, (iii) Ras is modified only by TcsL-1522 and not by TcdB-1470, and (iv) Ral is mainly present in neuronal tissue and was almost undetectable with specific polyclonal antibodies in the fibroblasts used here (data not shown). R-Ras, being a member of the Ras subfamily of small GTPases, shows a high sequence identity with Ras (20) but has partially differing downstream targets (21). Interestingly, R-Ras was found recently to control the activation of integrins from inside the cell (19). GTP-loaded R-Ras activates integrins, whereas GDP-loaded R-Ras inactivates them, thereby inducing detachment from the extracellular matrix (19). This new function of R-Ras suggested it could be involved in the rounding CPE induced by TcdB-1470 and TcsL-1522. Indeed, R-Ras turned out to be a major substrate for both toxins, whereas TcdB-10463 did not modify this GTPase (Fig. 6b). The molecular weight of the uppermost band labeled by TcdB-1470 and TcsL-1522 in Fig. 5 agrees with the reported molecular weight of R-Ras (20).

We propose that modification of R-Ras in fibroblasts treated with either TcdB-1470 or TcsL-1522 inactivates this small GTP-binding protein and that this in turn leads to inactivation of integrins from inside the cell. This would induce the loss of integrin adhesive interactions to extracellular matrix proteins, resulting in the observed rounding and detachment of cells. This hypothesis is supported by the TcdB-1470-induced efficient fibroblast detachment, whereas TcdB-10463-treated cells remained attached. Inactivation of integrins is also known to induce a disassembly of focal adhesion complexes. Indeed, cells treated with either toxin showed a disappearance of vinculin from focal adhesion complexes. However, TcdB-10463-treated cells showed two peculiarities that did not occur in TcdB-1470-treated cells: (i) \(\beta\)-integrin remained at the tip of the protrusions surrounding the cells and (ii) phosphoryrosine containing structures were detected at the cell-to-cell contacts.

Based on these findings we speculate that inactivation of
R-Ras in cells treated with TcdB-1470 or TcsL-1522 causes a collapse of the entire focal adhesion complexes. In contrast, the inactivation of Rho by TcdB-10463 probably induces only a partial disassembly of the focal adhesion complexes. The disappearance of vinculin may be initiated at a point “downstream” the cytoplasmic tail of integrins, closer to the actin filaments. Therefore, integrins remain in place and active, explaining the persisting attachment of TcdB-10463-treated cells to the substrate, and the remaining protrusions that confer the arborizing or neurite-like appearance. Furthermore, glucosylation of Rho apparently does not destroy the cell-to-cell contacts because TcdB-10463-treated cells retained phosphotyrosine-containing structures at these sites (Fig. 8).

Thanks to their highly specific mechanisms of action, bacterial toxins can be used as exquisite tools in cell biology. The addition of TcdB-1470 to the panel of already known LCTs broadens the possibilities to apply this family of toxins for elucidating the functions of several small GTPases, now including also R-Ras for two of the LCTs. TcdB-10463 and TcdB-1470 together form a particularly useful pair because they have the same enzymatic potency and bind the same (or a similar) receptor. Thus they enter cells by the same internalization pathway, implying that the only difference in cells treated with these toxins is the GTPase substrate attacked. These features allow accurate comparisons of the cellular consequences at various levels of inactivation of the different GTPases. The fact that these two toxins act rapidly is another advantage for their use as cell biology tools, because secondary pleiotropic effects due to long incubation times are avoided. Finally, we also want to emphasize the potential utility of TcdB-1470 for understanding the pathogenic significance of the various toxin domains. The use of this natural hybrid toxin might enable clarification

FIG. 8. Effects of TcdB-10463 and TcdB-1470 on the distribution of vinculin, β3-integrin, and tyrosine phosphorylated proteins. 3T3 cells growing on 13-mm round glass coverslips were treated 20 min with TcdB-10463 (b, e, and h) or TcdB-1470 (c, f, and i) (50 ng/ml for both toxins) or left untreated (a, d, and g). Cells were fixed with 3.5% paraformaldehyde and stained with anti-vinculin (a–c), anti-β3-integrin (d–f), or anti-phosphotyrosine (g–i) antibodies. β3-Integrin and phosphotyrosine accumulations are indicated with large (control cells) or small (TcdB-10463-treated cells) arrowheads.
of the respective roles of the binding and catalytic regions in the action of TcdB on host tissues, whether in the induction of colitis or in its lethal effect.

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REFERENCES
1. Eichel-Streiber, C. V., Boquet, P., Sauerborn, M., and Thelestam, M. (1996) Trends Microbiol. 4, 375–382
2. Thelestam, M., Florin, I., and Chaves-Olarte, E. (1997) in Bacterial Toxins: Tools in Cell Biology and Pharmacology (Aktories, K., ed), pp. 141–158, Chapman & Hall, Weinheim, Germany
3. Aktories, K., and Just, I. (1995) Trends Cell Biol. 5, 441–443
4. Just, I., Selzer, J., Wilm, M., Eichel-Streiber, C. V., Mann, M., and Aktories, K. (1995) Nature 375, 500–503
5. Popoff, M. R., Chaves-Olarte, E., Lemicher, E., Eichel-Streiber, C. V., Thelestam, M., Chardin, P., Cussac, D., Antennay, B., Chavrier, P., Flatau, G., Giry, M., Gunzburg, J., and Boquet, P. (1996) J. Biol. Chem. 271, 10217–10224
6. Just, I., Selzer, J., Hofmann, F., Green, G. A., and Aktories, K. (1996) J. Biol. Chem. 271, 10149–10153
7. Hofmann, F., Rex, G., Aktories, K., and Just, I. (1996) Biochem. Biophys. Res. Commun. 227, 77–81
8. Eichel-Streiber, C. V., Meyer, D., Habermann, E., and Sartingen, S. (1995) Mol. Microbiol. 17, 315–321
9. Depitre, C., Delmee, M., Avesani, V., Haridon, R. L., Roels, A., Popoff, M., and Corthier, G. (1993) J. Med. Microbiol. 38, 434–441
10. Sauerborn, M., Leukel, P., and Eichel-Streiber, Cv. (1997) FEMS Microbiol. Lett. 155, 45–54
11. Eichel-Streiber, C., Harperath, C., Bosse, D., and Hadding, U. (1987) Microbiol. Pathog. 2, 307–318
12. Huang, X., and Miller, W. (1991) Adv. Appl. Math. 12, 337–357
13. Chaves-Olarte, E., Florin, I., Boquet, P., Popoff, M., von Eichel-Streiber, C., and Thelestam, M. (1996) J. Biol. Chem. 271, 6925–6932
14. Laemmli, U. K. (1970) Nature 227, 680–685
15. O’Farrell, P. Z., Goodman, H. M., and O’Farrell, P. H. (1977) Methods Cell Biol. 16, 407–420
16. Chaves-Olarte, E., Weidmann, M., Eichel-Streiber, C. V., and Thelestam, M. (1997) J. Clin. Invest. 100, 1734–1741
17. Popoff, M. R. (1987) Infect. Immun. 55, 35–43
18. Giry, M., Popoff, M. R., Eichel-Streiber, C., and Boquet, P. (1995) Infect. Immun. 63, 4063–4071
19. Zhang, Z., Vuori, K., Wang, H. G., Reed, J. G., and Ruoslahti, E. (1996) Cell 83, 61–69
20. Lowe, D., Capon, D., Delwart, E., Sakaguchi, A., Naylor, S., and Goeddel, D. (1987) Cell 48, 137–146
21. Huff, S. Y., Quilliam, L. A., Cox, A. D., and Der, C. J. (1997) Oncogene 14, 133–143