R-Ras Glucosylation and Transient RhoA Activation Determine the Cytopathic Effect Produced by Toxin B Variants from Toxin A-negative Strains of *Clostridium difficile**

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Clostridium difficile induces antibiotic-associated diarrhea through the production of toxin A and toxin B; the former toxin has been assumed to be responsible for the symptoms of the disease. Several toxin A-negative strains from *C. difficile* have recently been isolated from clinical cases and have been reported to produce toxin B variants eliciting an atypical cytopathic effect. Ultrastructural analysis indicated these toxins induce a rounding cytopathic effect and filopodia-like structures. Toxin B variants glucosylated R-Ras, and transfection with a constitutively active mutant of this GTPase protected cells against their cytopathic effect. Treatment of cells with toxin B variants induced detachment from the extracellular matrix and blockade of the epidermal growth factor-mediated phosphorylation of extracellular-regulated protein kinases, demonstrating a deleterious effect on the R-Ras-controlled avidity of integrins. Treatment with toxin B variants also induced a transient activation of RhoA probably because of inactivation of Rac1. Altogether, these data indicate that the cytopathic effect induced by toxin B variants is because of cell rounding and detachment mediated by R-Ras glucosylation, and the induction of filopodia-like structures is mediated by RhoA activation. Implications for the pathophysiology of *C. difficile*-induced diarrhea are discussed.

Clostridium difficile induces clinical entities like antibiotic-associated diarrhea and pseudomembranous colitis via the production of two toxins, toxin A (TcdA)[1] and toxin B (TcdB). TcdA is known as the enterotoxin, because when inoculated in animal models in purified state, it is able to reproduce the clinical manifestations induced by *C. difficile* (1). TcdB, on the other hand, is known as the cytotoxin because of its potent cytotoxic activity on cultivated cells. Although TcdA is also cytotoxic it is 1000 times less efficient than TcdB (2, 3). TcdB is not able to induce clinical symptoms in animal models unless inoculated together with sublethal amounts of TcdA (1). These two toxins belong to the large clostridial cytotoxin (LCT) family (~300 kDa) and are glucosyltransferases that modify GTPases of the RhoA subfamily by transferring the glucose moiety from UDP-glucose to threonine 35 (37 in RhoA) (4, 5). This amino acid is located in the so-called effector region, which transmits signals to downstream effector molecules. Thus, it is predictable that a modification in this domain will impair the signal transduction cascade controlled by the GTPase (6). Members of the RhoA subfamily are well known key controllers of the actin cytoskeleton (7), thus one of the main characteristics of cellular intoxication with LCTs is a collapse in this network structure.

In addition to TcdB and TcdA from the prototype strain VPI-10463, toxin variants of *C. difficile* toxin B, TcdB-1470 and TcdB-8864, have been isolated from TcdA-negative strains (8, 9). These variants have been reported to produce a somehow different cytopathic effect (CPE) than the classical neurite-like morphology induced in fibroblasts by TcdB-10463 (9, 10). Their role in pathogenic processes is uncertain although the appearance of TcdA-negative *C. difficile* strains producing gastrointestinal pathology (11, 12) has been reported. More recently, the prevalence of toxin A-negative strains producing *C. difficile*-associated diarrhea in 334 patients in France was found to be 2.7% (13). In all these cases toxin B was reported to produce an atypical CPE. Thus, cytotoxins from these strains might be relevant virulence factors that take over functions generally accepted to depend on TcdA. Of those variant cytotoxins, TcdB-1470 was shown to be a functional hybrid possessing the receptor-binding and internalization domain of TcdB-10463 and the glucosyltransferase domain of *Clostridium sordellii* TcsL (10). Because of these characteristics, TcdB-1470 and TcdB-10463 are the prototypes of the two different groups of LCTs

[1] The abbreviations used are: TcdA, *C. difficile* toxin A; TcdB, *C. difficile* toxin B; TcsL, *C. sordellii* lethal toxin; CNF, cytotoxic necrotizing factor from *E. coli*; LCT, large clostridial cytotoxin; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; RBD, rhotekin RhoA-binding domain; PBD, GTPase-binding domain of p21 activated kinase 1; TRITC, tetramethylrhodamine isothiocyanate; CPE, cytopathic effect; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.

divided according to substrate specificities as follows: (i) TcdA-10463 and TcdB-10463 modify RhoA, Rac1, and Cdc42 as common substrates; and (ii) TcdB-1470 (from *C. difficile* strain 1470) and TcsL (from *C. sordellii*) modify Rac1, Ral, Rap, and R-Ras (5, 10, 14, 15). R-Ras is a particularly interesting substrate, because it has a unique 30-amino acid sequence in its N terminus differentiating it from H-, K-, or N-*ras* oncogenes (16). Furthermore, whereas activated H-Ras has been shown to suppress the activation of certain integrins (17) R-Ras has the opposite activity and promotes integrin-dependent adhesion (18, 19). Thus, its inactivation could have dramatic consequences for cell morphology and adhesion.

The modification of RhoA has been shown to be the event responsible for the collapse of the actin cytoskeleton induced by the group of RhoA-modifying toxins (4, 20). The molecular consequences of this modification in terms of RhoA interaction with downstream effectors and with RhoA controlling proteins has been partially elucidated (21, 22). However, the sequence of events leading to induction of the early recognized neurite-like CPE (23), which is produced also by other RhoA-modifying toxins (including C3 from *Clostridium botulinum*), is barely understood (24). Presently, the relevant substrate for the induction of a CPE by toxins that do not modify RhoA is unknown.

In this study we have explored the molecular events leading to the CPE by toxin B variants from toxin A-negative strains. We demonstrate (i) that glucosylation of R-Ras induces cell detachment and rounding of the cell body by inactivation of integrins, and (ii) an imbalance in the activation state of RhoA via the inactivation of Rac1. The biological relevance of the two different types of CPE is discussed in the context of the pathology produced by *C. difficile*.

EXPERIMENTAL PROCEDURES

Materials—*C. difficile* toxins TcdB-10463, TcdB-1470, TcdB-8864, TcdA-10463, and *C. sordellii* toxin (TcsL) were kindly provided by Dr. Christoph von Eichel-Streiber (Johannes Gutenberg-Universität, Mainz, Germany). *Escherichia coli* cytotoxic necrotizing factor (CNF) 1 was prepared as described previously (25). Eucaryotic expression vectors containing *myc*-tagged small GTPase mutants were kindly provided by Dr. Alan Hall (University College London, London, England) and Dr. Patrice Boquet, (INSERM, Nice, France). Lipofectin was from Invitrogen. Fluorescein isothiocyanate-phalloidin and epidermal growth factor (EGF) were from Sigma. Anti-phospho ERK antibodies were from Cell Signaling, and anti-ERK antibodies were from Upstate Biotechnologies. The GST-R-Ras fusion protein construct was a generous gift from Adrienne Cox (University of North Carolina). The GST-tagged rhotekin RhoA-binding domain (RBD) was expressed from plasmid pGEX-2T-RBD (kindly provided by Dr. Martin Alexander Schwartz, Scripps Research Institute, La Jolla, CA). The GST-tagged GTPase-binding domain of p21-activated kinase 1 (PBD) was expressed from a derivative pGEX-2T plasmid (kindly provided by Dr. Gary Bokoch, Scripps Research Institute, La Jolla, CA). All plasmids were transformed into the *E. coli* BL-21 strain, and expression of fusion proteins was induced with isopropyl-1-thio-β-D-galactopyranoside. The recombinant proteins were purified with glutathione-Sepharose 4B (Amersham Biosciences). UDP-[^{14}C]glucose (specific activity, 318 mCi/mmol) was from PerkinElmer Life Sciences. All other reagents were of analytical grade and obtained from local commercial sources.

Glucosyltransferase Activity—5 μl of UDP-[^{14}C]glucose dissolved in ethanol were vacuum-dried, and 10 μl of reaction buffer (50 mM triethanolamine, 150 mM KCl, 2 mM MgCl$_2$, 0.5 mM GDP, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, pH 7.8) containing recombinant GST-R-Ras (2 μg) were added. The mixture was incubated for 1 h at 37 °C with 5 μg/ml of TcdB-10463, TcdB-1470, or TcdB-8864 or 100 μg/ml of TcdA-10463. The reaction was stopped by heating at 95 °C in Laemmli sample buffer. Proteins were separated by 12.5% SDS-PAGE. Radiolabeled R-Ras was detected by Phosphor-Imager analysis (Molecular Dynamics).

Cell Culture, Plasmids, and Transfection—Mouse BalbC 3T3 fibroblasts (ATCC CCL-163) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 5 mM L-glutamine,

penicillin (100 units/ml), and streptomycin (100 μg/ml). Human epithelial HeLa cells (ATCC CCL-2) were cultured in Eagle's minimal essential medium supplemented as indicated above. Both cell lines were incubated at 37 °C in a humid atmosphere containing 5% CO$_2$. Transfection of cells with plasmids encoding *myc*-tagged V12Rac1, V38R-Ras, V12Rap1, or V14RhoA was performed with Lipofectin according to the manufacturer's instructions. In brief, 50000 cells were seeded on 13-mm glass coverslips and cultivated for 24 h. Lipofectin was mixed with 1 μg of plasmid in Eagle's minimal essential medium without fetal bovine serum and antibiotics, and the complex was allowed to form for 15 min at room temperature. The mixture was then laid onto subconfluent HeLa cells, and the cells were incubated for 6 h at 37 °C. The original medium was changed to fully supplemented medium. Cells were intoxicated 48 h after this treatment at the conditions indicated in the figure legends.

Immunofluorescence—Cells cultivated on 13-mm glass coverslips and transfected as described above were intoxicated with clostridial toxins as indicated in the figure legends. Cells were then fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 in PBS. Cells were further incubated with anti-Myc 9E10 monoclonal antibody (Santa Cruz Biotechnology) for 30 min, followed by secondary TRITC anti-mouse antibody for an additional 30 min. For staining of the actin cytoskeleton, cells were incubated for 30 min with 0.5 μg/ml fluorescein isothiocyanate-phalloidin (Sigma). Fluorescence and phase contrast were visualized using an Olympus BH-2 microscope, and images were captured using a digital camera (MagnaFire). The percentage of cells protected from clostridial toxins was calculated as the fraction of cells showing no CPE as viewed by phase contrast among 300 cells positive for GTPase expression as determined by immunofluorescence using a monoclonal anti-Myc antibody.

Scanning Electron Microscopy—Subconfluent cells grown in 13-mm glass slides, intoxicated as indicated in the figure legends, were chemically immobilized with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C for 2 h and post-fixed in 1% osmium tetroxide in phosphate buffer for 30 min. Fixed cells were dehydrated in increasing concentrations of ethanol and frozen in *tert*-butyl alcohol. Cells were freeze-dried by sublimation and coated with 20 nm of gold-palladium (10 mAmp, 2 min). Cells were observed and photographed with an S-570 (Hitachi) scanning electron microscope operating at 15 kV.

Tyrosine Phosphorylation of ERKs—BalbC 3T3 cells growing in 24-well plates were serum-starved overnight and treated with clostridial toxins or left untreated as indicated in the figure legends. Cells were then stimulated with 100 ng/ml EGF for 5 min, washed twice with ice cold PBS, and lysed in Laemmli sample buffer. Protein concentration was determined by the Bio-Rad DC method according to the manufacturer's instructions, and equal amounts of protein (20 μg) were loaded onto a 12.5% SDS-polyacrylamide gel. Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with anti-phospho-ERK or anti-ERK antibodies. Membranes were further incubated with peroxidase-conjugated anti-mouse antibodies, and the detected bands were visualized with a chemiluminescence Western blotting kit (Pierce Super Signal WestDura).

Adhesion Assays—Cells cultivated on 24-well plates to 90% confluence were intoxicated or left untreated as indicated in the figure legends. After treatment, cells were washed three times with PBS, the remaining cells were lysed with SDS-PAGE sample buffer, and the protein concentration was quantified using bovine serum albumin as standard.

Quantification of GTP-RhoA, GTP-Rac1, and GTP-Cdc42—For precipitation steps, GTP-RhoA was quantified with GST-tagged RBD (26), and GTP-Rac1 and GTP-Cdc42 were quantified with GST-PBD (27). HeLa cells cultured in 6-well plates were intoxicated at the conditions indicated in the figure legends. After treatment, cells were washed with ice-cold PBS and lysed with 500 μl of ice-cold precipitation buffer (1% Triton X-100, 0.1% SDS, 0.3% Nonidet P-40, 500 mM NaCl, 10 mM MgCl$_2$, and 50 mM Tris, pH 7.2). Lysates were clarified by centrifugation at 14000 rpm for 1 min. Twenty μl of each lysate were saved as control for total GTPase content. GTP-loaded RhoA GTPases were precipitated with Sepharose beads coupled to either GST-PBD or GST-RBD protein. Samples were incubated for 30 min at 4 °C with shaking, washed with precipitation buffer, and resuspended in 25 μl of Laemmli sample buffer for analysis by SDS-polyacrylamide gel electrophoresis. Samples transferred to a PVDF membrane were probed with either rabbit antibodies against RhoA or Cdc42 (Santa Cruz Biotechnology) or with anti-Rac1 monoclonal antibody (Transduction Laboratories). Probing and developing were performed with peroxidase-labeled secondary antibodies and the chemiluminescence Western blotting kit (Pierce
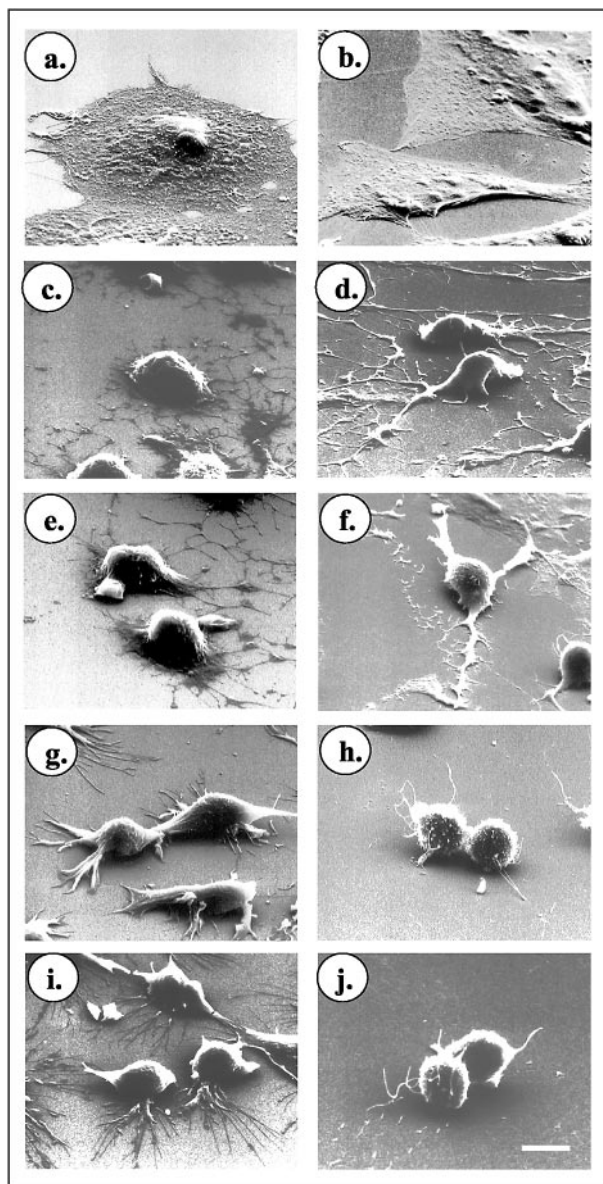
Cytotoxicity of C. difficile Toxin B Variants

FIG. 1. **Morphological effect induced by LCTs in epithelial cells and fibroblasts.** Epithelial HeLa cells (*A*, *C*, *E*, *G*, and *I*) and BalbC 3T3 fibroblasts (*B*, *D*, *F*, *H*, and *J*) were treated with TcdA-10463 (*C* and *D*), TcdB-10463 (*E* and *F*), TcdB-1470 (*G* and *H*) or TcdB-8864 (*I* and *J*) until a CPE was achieved in 100% of the cells. Control cells were left untreated (*A* and *B*). Cells were intoxicated for 1 h with all toxins at a concentration of 50 ng/ml except for the TcdA-10463 treatment, which was for 4 h at a concentration of 500 ng/ml. Cells were observed by scanning electron microscopy as described under "Experimental Procedures." *Bar*, 10 μm.

Super Signal WestDura), respectively. GTP-Cdc42, GTP-RhoA, and GTP-Rac1 levels were calculated using the Scion Image software for Windows and compared with control total levels of Cdc42, RhoA, and Rac1.

RESULTS

RhoA- and R-Ras-modifying LCTs Induce Distinguishable CPEs—The CPE induced by TcdA-10463, TcdB-10463, TcdB-1470, and TcdB-8864 was studied in 3T3 fibroblasts and epithelial HeLa cells by scanning electron microscopy. After intoxication with TcdA-10463 or TcdB-10463, 3T3 fibroblasts showed a neurite-like CPE characterized by the presence of long protrusions attached to the substratum (Fig. 1). HeLa cells treated with the same toxins developed a more rounded phenotype with few protrusions (Fig. 1); these protrusions are

barely seen under light microscopy (data not shown). After intoxication with TcdB-1470 or TcdB-8864, 3T3 fibroblasts presented a rounded phenotype. The cells appeared to be detaching from the substratum, and some presented blebs on their surface. A rounded CPE was also evident in HeLa cells, and filopodia-like structures emanating from the cell body were observed (Fig. 1). RhoA glucosylation is essential for the CPE induced by TcdB-10463 (4, 20); however, the relevant *in vivo* substrate for LCTs not affecting RhoA is not known. We tested the ability of TcdA-10463 and TcdB-8864 to glucosylate recombinant R-Ras *in vitro*. Because of its low enzymatic activity (2, 3) TcdA-10463 was used 100 times more concentrated than the negative control TcdB-10463. Under these conditions we did not detect any radioactive signal from R-Ras after TcdA-10463 treatment but detected a radioactive band after TcdB-8864 treatment, similar to the positive control, TcdB-1470 (Fig. 2*A*). This indicates that R-Ras is a substrate also for this toxin B variant. Thus, among LCTs there exists a strict correlation between RhoA glucosylation and induction of a neurite-like CPE and between R-Ras glucosylation and induction of a rounding CPE, suggesting R-Ras to be a crucial *in vivo* substrate for the latter group of LCTs. To test this hypothesis HeLa cells transiently transfected with constitutively active V38R-Ras were intoxicated with TcdB-1470. At conditions inducing 100% CPE by TcdB-1470 in control non-transfected cells, V38R-Ras-transfected cells showed no sign of CPE (Fig. 2*B*). On the other hand, V38R-Ras expression did not protect cells against TcdB-10463-induced CPE (Fig. 2*B*). The degree of protection obtained by transfection of activated GTPases was quantified. Almost no cells expressing V38R-Ras showed a detectable CPE after TcdB-1470 treatment whereas V12Rac1 and V12Rap1 expression conferred a lower degree of protection (Fig. 2*C*). Only ~10% of cells expressing V14RhoA (negative control) were protected against the TcdB-1470-induced CPE. Similar results were obtained with 3T3 fibroblasts (data not shown). Altogether these results indicate that R-Ras is a major *in vivo* substrate determining the CPE of one group of LCTs.

R-Ras-modifying Toxins Induce Cell Detachment—Cells treated with the R-Ras-modifying toxins TcdB-1470 or TcdB-8864 rounded up in less than 1 h and seemed loosely attached to the substratum, whereas TcdB-10463-treated cells still remained attached 24 h after intoxication (data not shown). These observations, together with the reported role of R-Ras in controlling the activation state of integrins (18), prompted us to investigate whether TcdB-1470 treatment induces cell detachment. 3T3 fibroblasts were intoxicated for 1 h with the different toxins, and after several washing steps, the remaining cells were quantified. TcdB-10463-treated cells remained attached to the substratum to the same extent as control non-intoxicated cells whereas TcdB-1470 induced a massive detachment of cells (Fig. 3*A*). The same result was obtained with TcdB-8864 and with HeLa epithelial cells (data not shown). Integrin engagement has been proven to be essential for EGF-mediated activation of ERK kinases (28); thus we tested whether TcdB-1470 treated cells were able to support this signaling pathway. Serum-starved cells intoxicated for 4 h with TcdB-10463, TcdB-1470, or TcdB-8864 were treated with EGF, and the tyrosine phosphorylation status of ERK kinases was investigated. ERK kinases were phosphorylated in cells intoxicated with TcdB-10463 to the same extent as in non-treated control cells upon stimulation with EGF. On the other hand, the EGF-induced phosphorylation of ERK kinases in TcdB-1470- or TcdB-8864-treated cells was significantly impaired when compared with the control cells (Fig. 3*B*). This result indicates that TcdB-10463-intoxicated cells have a biologically relevant adhesive
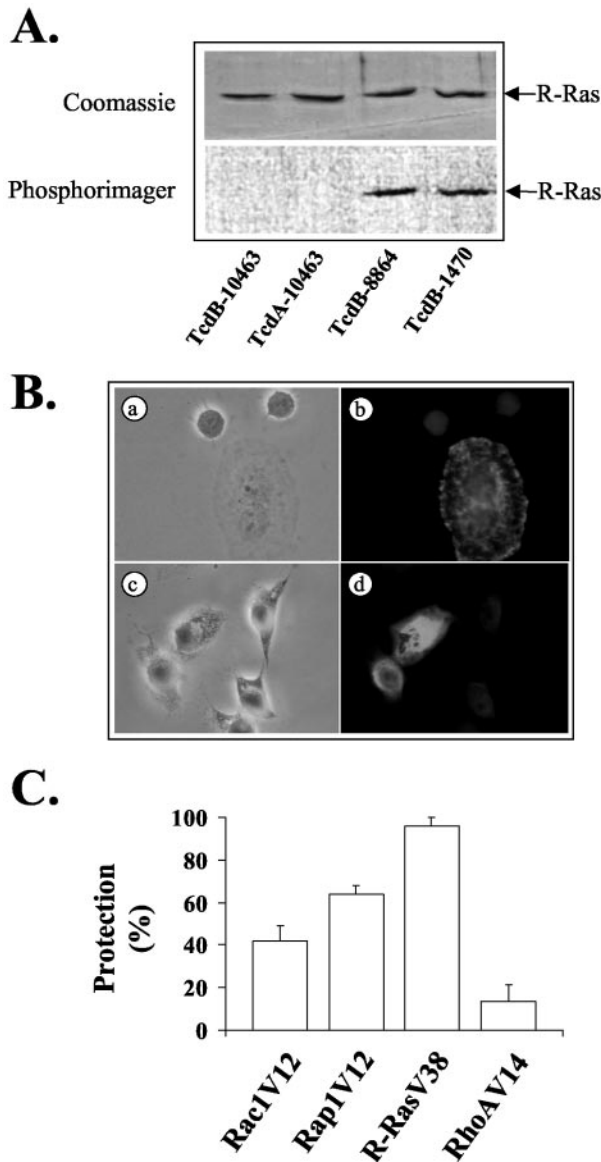
FIG. 2. **Relevance of R-Ras as substrate for TcdB-1470.** *A*, purified recombinant R-Ras (2 μg) in GST fusion form was incubated with TcdB-10463 (5 μg/ml), TcdA-10463 (100 μg/ml), TcdB-8864 (5 μg/ml), or TcdB-1470 (5 μg/ml) in the presence of UDP-[^{14}C]glucose (30 μM). After 1 h of incubation at 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. The Coomassie-stained gel and PhosphorImager analysis of the same gel are shown. *B*, HeLa cells transfected with *myc*-tagged constitutively active V38R-Ras were intoxicated with TcdB-1470 (*A* and *B*) or TcdB-10463 (*C* and *D*) for 2 h. R-Ras expressing cells were visualized by immunofluorescence using anti-Myc antibodies (*B* and *D*); the same fields were observed by phase-contrast microscopy (*A* and *C*). *C*, HeLa cells transfected with constitutively active *myc*-tagged V12Rac1, V12Rap1, V38R-Ras, or V14RhoA were intoxicated with TcdB-1470 for 2 h. The percentage of 300 successfully transfected cells (visualized as in *B*) protected from CPE is shown. Mean of triplicate transfections and S.D. from one representative experiment of three is shown.

phenotype whereas focal contacts are affected by intoxication with TcdB-1470 or TcdB-8864.

Dynamics in the Activation State of RhoA-GTPases after LCTs Intoxication—TcdB-8864 and TcdB-1470 (Fig. 1) induce filopodia-like structures in HeLa cells. These structures appear before the cells are rounded (~30 min), peak at 60 min, and then disappear after 3 h of intoxication. *C. sordellii* TcsL induces a similar phenomenon (14). We used the RhoA GTPase activator CNF1 from *E. coli* to assess the role of these GTPases
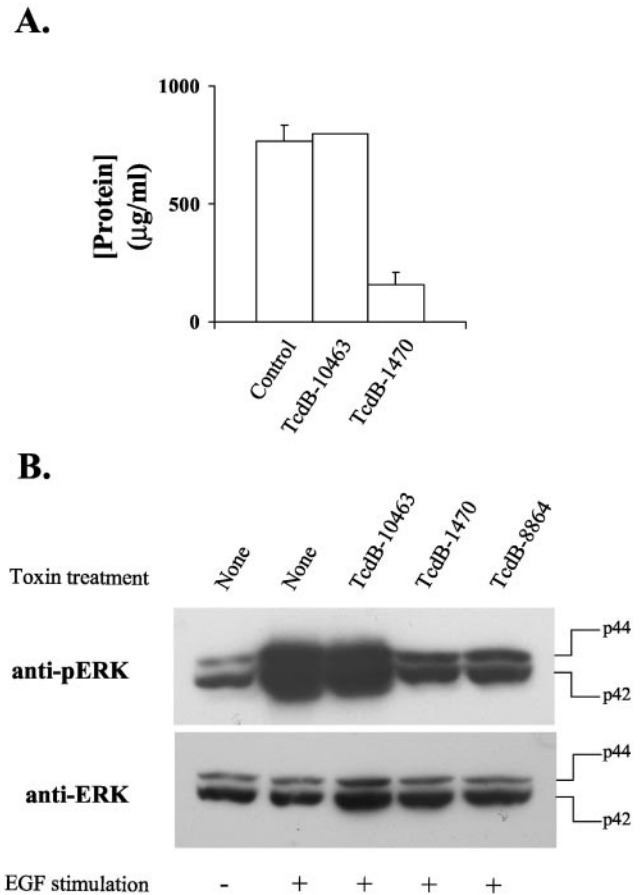

FIG. 3. **Effect of TcdB-1470 and TcdB-10463 on cell adhesion.** *A*, monolayers of BalbC 3T3 cells in 24-well plates were treated 1 h with TcdB-10463 or TcdB-1470 or left untreated (*Control*). After treatment the cultures were extensively washed with PBS, and cells remaining attached to the substrate were lysed by addition of SDS-containing buffer. Protein concentration (*[Protein]*), corresponding to adhered cells, was determined in lysates. Mean of triplicate samples and S.D. from one representative experiment of three is shown. *B*, BalbC 3T3 cells were serum-starved for 16 h. Cells were then intoxicated for 4 h with TcdB-10463, TcdB-1470, or TcdB-8864 or left untreated. After intoxication cells were stimulated with EGF for 5 min, washed with ice-cold PBS, and lysed in Laemmli sample buffer. Twenty μg of protein of each lysate were loaded on 12.5% SDS-polyacrylamide gels, proteins blotted on PVDF, and probed with antibodies against phospho-ERK (*upper panel*) or total ERK (*lower panel*) proteins. Immune complexes were visualized by the chemiluminescence reaction. ERK 1 (*p44*) and ERK 2 (*p42*) isoforms are shown.

in the induction of filopodia after TcdB-1470 intoxication. When HeLa cells were pre-treated with CNF1 for 2 h and further incubated with TcdB-1470, a strong promotion in both the number and length of filopodia was observed (Fig. 4). This result suggested an active involvement of some member(s) of the RhoA GTPase subfamily in the filopodia formation after toxin treatment. Thus, we monitored the activation state of individual RhoA GTPases after intoxication with TcdB-1470 and TcdB-10463 by pull-down assays (26, 29). A decrease in the level of Rac1-GTP by TcdB-1470 was evident as early as 30 min post-incubation (Fig. 5*A*). This decrease was followed at 60 min by disappearance of Rac1 from total cell lysates. Although this finding might suggest proteolytic degradation of Rac1 after glucosylation, this interpretation is not likely to be correct, because the anti-Rac1 antibody used in this study has been shown recently not to recognize glucosylated Rac1.[2] The same anti-Rac1 antibody recognized two isoforms of this protein

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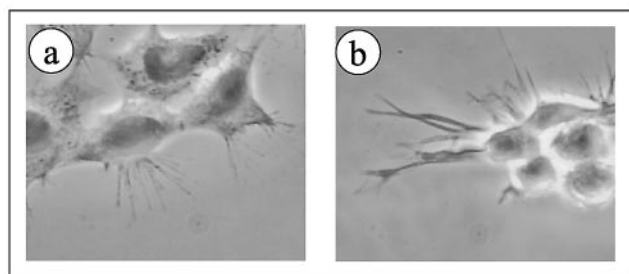
[2] I. Just, personal communication.

FIG. 4. **CNF1 pretreatment enhances filopodia-like structures induced by TcdB-1470.** HeLa cells were treated with CNF1 for 2 h (*B*) or left untreated (*A*). Then cells were intoxicated with TcdB-1470 (*A* and *B*) for an additional hour. Cells were visualized by phase-contrast microscopy.
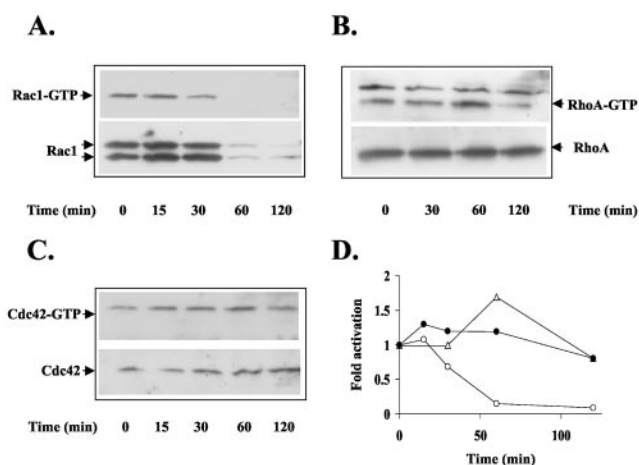

FIG. 5. **Effect of TcdB-1470 on the activation state of RhoA GTPases.** Confluent monolayers of HeLa cells in 6-well plates were intoxicated with TcdB-1470 for the indicated times. After treatment, cells were lysed, and 20 μl were separated as control for total amount of GTPases. Lysates were then incubated with RBD-GST (*B*, *upper panel*) or PBD-GST (*A* and *C*, *upper panel*)-Sepharose beads for 30 min. Active proteins were pulled down by centrifugation, resolved in 10% SDS-PAGE, and transferred to PVDF membranes. Small GTPases were detected using anti-Rac1 (*A*), anti-RhoA (*B*), or anti-Cdc42 (*C*) antibodies. *D*, the intensity of the bands corresponding to Rac1-GTP (*open circles*), RhoA-GTP (*open triangles*), and Cdc42-GTP (*closed circles*) was quantified and corrected according to the amount of total GTPase in the respective internal control (*A*, *B*, and *C*, *lower panel*). Each *time point* was expressed in relation to the amount of active GTPase found at 0 min of incubation with the toxin. A representative experiment of three is shown.

FIG. 6. **Effect of TcdB-10463 on the activation state of RhoA GTPases.** Confluent monolayers of HeLa cells in 6-well plates were intoxicated with TcdB-10463 for the indicated times. After treatment, cells were lysed, and 20 μl were separated as control for total amount of GTPases. Lysates were then incubated with RBD-GST (*B*, *upper panel*)- or PBD-GST (*A* and *C*, *upper panel*)-Sepharose beads for 30 min. Active proteins were pulled down by centrifugation, resolved in 10% SDS-PAGE, and transferred to PVDF membranes. Small GTPases were detected using anti-Rac1 (*A*), anti-RhoA (*B*), or anti-Cdc42 (*C*) antibodies. *D*, the intensity of the bands corresponding to Rac1-GTP (*open circles*), RhoA-GTP (*open triangles*), and Cdc42-GTP (*closed circles*) was quantified and corrected according to the amount of total GTPase in the respective internal control (*A*, *B*, and *C*, *lower panel*). Each *time point* was expressed in relation to the amount of active GTPase found at 0 min of incubation with the toxin. A representative experiment of three is shown.

whereas the PBD-Sepharose beads preferentially pulled down only one isoform (Fig. 5*A*). The level of RhoA-GTP exhibited a significant and consistent increase at 60 min post-intoxication (Fig. 5*B*), *i.e.* at the time when filopodia formation by TcdB-1470 was maximal (Fig. 4). After 120 min of incubation, RhoA-GTP returned to initial levels (Fig. 5*B*). The level of Cdc42-GTP remained essentially unaltered throughout intoxication (Fig. 5*C*). These results are consistent with the reported *in vitro* substrates for TcdB-1470 (10). When the same set of experiments was repeated with TcdB-10463, a decrease in Rac1-GTP, RhoA-GTP, and Cdc42-GTP levels was evident (Fig. 6). The inactivation kinetics of all three proteins followed a similar pattern with a significant decrease detected at 30 min. GTP-bound proteins reached undetectable levels at 60 min post-incubation. The Rac1 signal disappeared from total lysates 60 min after TcdB-10463 addition, mirroring the TcdB-1470-induced effect on this protein (Fig. 6*A*).

Altogether, these results suggest that glucosylation of other GTPases, probably Rac1, leads to transient RhoA activation, a reciprocal balance demonstrated previously on epithelial cells
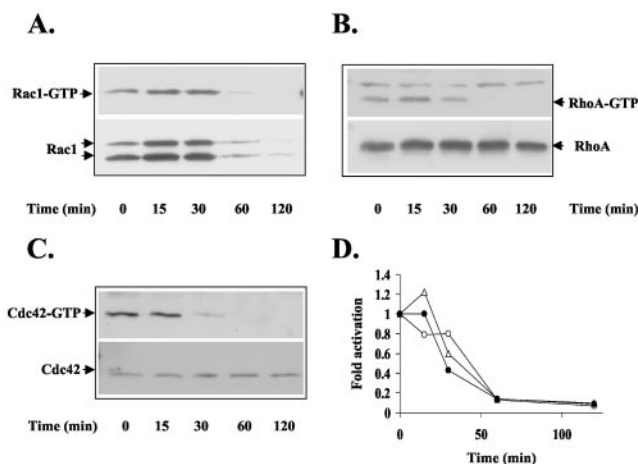
and fibroblasts (29, 30). To evaluate this hypothesis we studied the opposite scenario through the use of CNF1. The levels of RhoA-GTP, Rac1-GTP, and Cdc42-GTP increased after 30 min of incubation with CNF1 and peaked at 1 h. However, whereas Rac1-GTP and Cdc42-GTP levels remained augmented even after 24 h of incubation, the RhoA-GTP level showed a decrease at 4 h, and at 24 h had returned to basal levels (Fig. 7*C*). Thus, the reciprocal balance between Rac1 and RhoA is also observed upon toxin-induced activation of Rac1. To further explore this point, cells treated with CNF1 for 24 h were intoxicated with TcdB-1470, and the levels of RhoA-GTP were monitored. Under these conditions, an increase in the RhoA-GTP level was clearly detectable 60 min after TcdB-1470 addition, peaked at 120 min, and returned to basal level after 180 min (Fig. 8*A*). A parallel experiment determined that Rac1-GTP levels start to decline 60 min after TcdB-1470 addition to CNF-intoxicated cells strengthening the correlation between Rac1 glucosylation and transient activation of RhoA.

DISCUSSION

In this report we characterized in molecular terms the CPE induced by toxin B variants (TcdB-1470 and TcdB-8864) produced by toxin A-negative strains of *C. difficile* and compared it with the CPE induced by classic toxin B from strain VPI-10463.

R-Ras Modification Plays a Crucial Role for the Induction of a Rounding CPE by Toxin B Variants—We demonstrate that modification of R-Ras is a crucial event for induction of the CPE elicited by non-RhoA-modifying LCTs. This conclusion is based on several considerations. First, all R-Ras-modifying toxins tested elicit a complete rounding of the cell body accompanied by induction of filopodia-like structures in epithelial cells, whereas toxins that do not modify this GTPase induce a neurite-like CPE. R-Ras glucosylation seems to be dominant over RhoA modification in determining the resulting CPE, because (i) simultaneous treatment with TcdB-10463 and TcdB-1470 results in a rounding phenotype (data not shown), and (ii) a
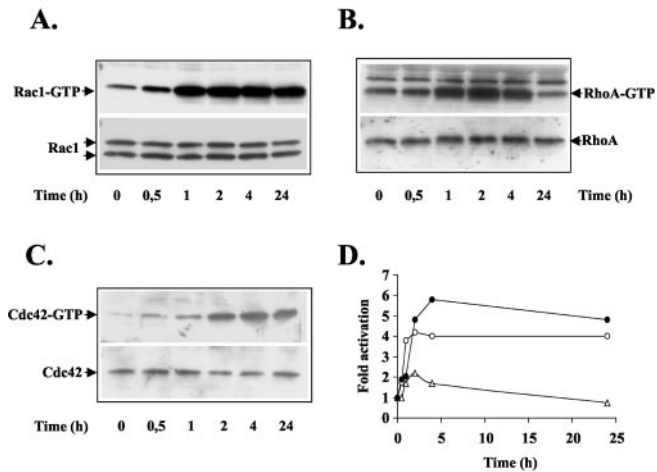
FIG. 7. **Effect of CNF1 on the activation state of RhoA GTPases.**
Confluent monolayers of HeLa cells in 6-well plates were intoxicated
with CNF1 for the indicated times. After treatment, cells were lysed,
and 20 μl were separated as control for total amount of GTPases.
Lysates were then incubated with RBD-GST (*B, upper panel*)- or PBD-
GST (*A and C, upper panel*)-Sepharose beads for 30 min. Active proteins
were pulled down by centrifugation, resolved in 10% SDS-PAGE, and
transferred to PVDF membranes. Small GTPases were detected using
anti-Rac1 (*A*), anti-RhoA (*B*), or anti-Cdc42 (*C*) antibodies. *D*, the in-
tensity of the bands corresponding to Rac1-GTP (*open circles*), RhoA-
GTP (*open triangles*), and Cdc42-GTP (*closed circles*) was quantified
and corrected according to the amount of total GTPase in the respective
internal control (*A, B,* and *C, lower panel*). Each time point was ex-
pressed in relation to the amount of active GTPase found at 0 min of
incubation with the toxin. A representative experiment from three is
shown.

variant toxin from *C. difficile* that has been reported recently to
glucosylate both RhoA and R-Ras induces a rounding pheno-
type (31). Second, analysis of *in vitro* modified substrates indi-
cates that R-Ras, Rac1, Rap, and Ral are four common sub-
strates for non-RhoA-modifying toxins. Of those, Ral is not
detected in lysates from the cells used in this study (10); it is
rather a more abundant protein in neuronal tissue. Rac1 and
Rap are not likely candidates, because they are also substrates
for toxins inducing a neurite-like CPE (2, 4, 5). Thus, R-Ras
remains the most likely candidate. We confirmed this hypoth-
esis, because transfection with constitutively activated R-Ras
protected nearly 100% of cells against TcdB-1470-induced CPE
whereas under the same conditions no protection was conferred
against TcdB-10463. Rac1 and Rap1 transfection conferred a
lower degree of protection, which can be attributed to substrate
competition (Rac1) or to the reported role (Rap1) in the control
of integrin-mediated cell adhesion (32, 33). Finally, the pheno-
type of TcdB-1470-intoxicated cells correlates with the reported
role of R-Ras in the control of cell adhesiveness through mod-
ulation of integrin avidity (18). Although TcdB-10463-intoxi-
cated cells did not detach from the substratum, TcdB-1470
induced a massive detachment of cells, indicating that glucosy-
lation of R-Ras, but not RhoA, alters the activation state of
integrins. Furthermore, EGF-mediated activation of ERK ki-
nases, an event requiring proper adhesion of the stimulated
cells (28), was inhibited by 4 h of treatment with TcdB-1470.
We have shown previously (10) that 1 h of treatment with this
toxin does not interfere with this signaling route even if under
those conditions the intracellular substrates are completely
modified. Thus, the interference we observe by prolonging the
treatment depends on the detachment of cells and not on the
modification of some GTPase(s) essential for this pathway such
as previously shown for the *C. sordellii* TcsL (14), which glu-
cosylates Ras.

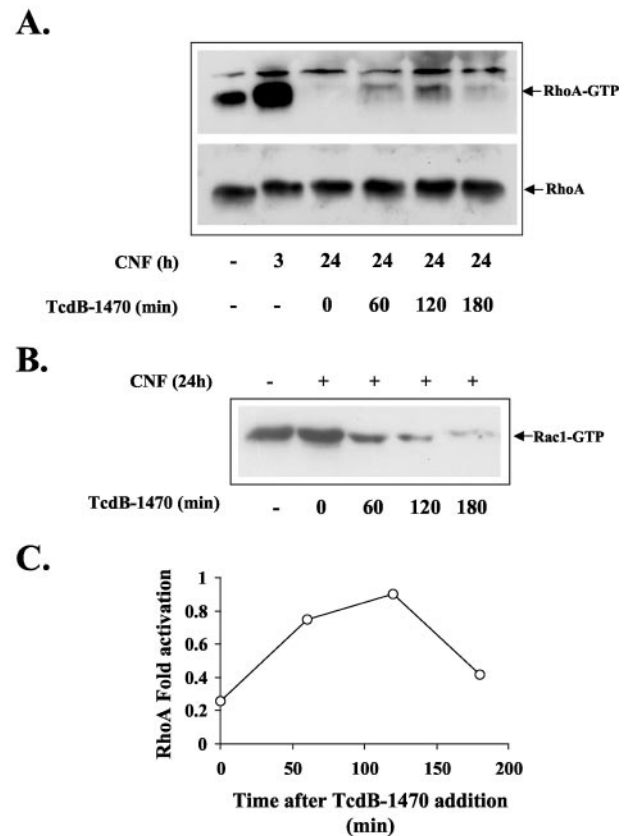What is the sequence of events occurring at the level of focal

FIG. 8. **TcdB-1470 treatment induces RhoA reactivation in
CNF1-treated cells.** *A*, confluent monolayers of HeLa cells in 6-well
plates were treated with CNF1 for 3 or 24 h or left untreated. Cells
treated with CNF1 for 24 h were further incubated with TcdB-1470 for
the indicated times. Cells were then lysed, and 20 μl were separated as
control for total amount of RhoA (*lower panel*). Lysates were incubated
with RBD-GST-Sepharose beads for 30 min. RhoA-GTP (*upper panel*)
was pulled down by centrifugation, resolved in 10% SDS-PAGE, trans-
ferred to PVDF membranes, and detected using anti-RhoA antibodies.
B, confluent monolayers of HeLa cells in 6-well plates were treated with
CNF1 for 24 h or left untreated. Cells were further incubated with
TcdB-1470 for the indicated times. Cells were then lysed, and lysates
were incubated with PBD-GST Sepharose beads for 30 min. Rac1-GTP
was pulled down by centrifugation, resolved in 10% SDS-PAGE, trans-
ferred to PVDF membranes, and detected using anti-Rac1 antibodies.
C, the intensity of the bands corresponding to RhoA-GTP on cells
treated with CNF1 for 24 h in *A* (*upper panel*) was quantified and
corrected according to the amount of total GTPase in their correspond-
ing internal control (*lower panel*). Each *time point* was expressed in
relation to the amount of active GTPase found in non-CNF1 non-TcdB-
1470-treated cells. A representative experiment of three is shown.

adhesions during cell intoxication by *C. difficile* cytotoxins?
Based on the present results and the facts that (i) RhoA has a
role in the induction of focal adhesions and stress fibers (34),
and (ii) vinculin disappears rapidly from focal adhesions upon
TcdB-10463 intoxication (10), we propose the following model:
RhoA inactivation by LCTs leads to disaggregation of adaptor
proteins located at the cytoplasmic side of the focal adhesion,
such as vinculin; this causes disassembly and in turn release of
the anchored stress fiber from the complex. There is not nec-
essarily a depolymerization of the F-actin into monomeric G-
actin, because the remaining protrusions still stain with phal-
loidin, which labels F-actin exclusively. Because TcdB-10463-
intoxicated cells retain their ability to adhere to extracellular
matrix proteins, we deduce that the integrin avidity to the
extracellular matrix is not altered by RhoA glucosylation. R-
Ras modification by toxin variants, on the other hand, induces
integrin inactivation that, in turn, will induce its release from
the extracellular matrix initiating a complete disassembly of

focal adhesions, including the stress fibers. These events will therefore culminate in a CPE differing from the one induced by RhoA-modifying toxins.

Toxin Variants Induce a Misbalance in the Activation State of Rho GTPases—Intoxication of HeLa cells with TcdB-1470 and TcdB-8864 induced the formation of filopodia-like structures closely resembling those induced by TcsL, which were shown previously (14) to contain fimbrin. Because filopodia formation is an active process modulated by RhoA GTPases (7) we analyzed their involvement in this particular feature induced by toxin variants. Pre-activation of RhoA GTPases by CNF1 treatment synergized with toxin variants to induce the formation of giant filopodia, suggesting an active role of some GTPase(s) in this phenomenon. Because some of the RhoA GTPases are substrates, at least *in vitro*, for the toxin variants, we analyzed the activation state of RhoA GTPases during early intoxication with toxin variants compared with the classic toxin TcdB-10463. As predicted from *in vitro* glucosylation assays with recombinant substrates (4), TcdB-10463 rapidly reduced the activity of RhoA, Rac1, and Cdc42. On the other hand, the TcdB-1470 treatment induced a rapid decrease in the activity of Rac1 and did not affect the activation state of Cdc42, again as predicted from *in vitro* studies (10). Interestingly, RhoA activity was transiently elicited, peaking at the same time as filopodia formation peaks after TcdB-1470 intoxication. This observation is in agreement with recent reports indicating the existence of a network of negative signaling between RhoA GTPases. It was observed that in different cell lines Rac1 modulates the activity of RhoA by inhibiting it (29, 30). In this context, it can be postulated that Rac1 inactivation by TcdB-1470, and in general toxin variants, releases the negative control on RhoA allowing its activity to increase. This of course does not occur upon TcdB-10463 intoxication, because RhoA itself is a substrate for this toxin (4). We could confirm this hypothesis by creating the opposite scenario through the use of CNF1, an activator of RhoA GTPases. Upon CNF1 intoxication the activity of RhoA, Rac1, and Cdc42 increased, but although the two latter remained permanently activated, RhoA-GTP rapidly returned to a basal level, even though it remained covalently modified as determined by the shift in molecular weight. Furthermore, we could release the CNF1-induced negative control of Rac1 on RhoA by TcdB-1470 treatment. These data indicate that Rac1 overactivation exerts a negative control over RhoA even though RhoA has lost its ability to interact with GTPase activating proteins because of CNF1 modification (35). The molecular details for this negative control of Rac1 on RhoA are currently unknown, but bacterial toxins like TcdB-1470 and CNF1 seem to be promising tools for its further elucidation. In conclusion, we have shown that treatment with *C. difficile* toxin variants induces a transient activation of RhoA, probably because of Rac1 inactivation, and resulting in the formation of filopodia-like structures characteristic of the CPE induced by these toxins.

Besides solving some molecular details behind the CPE induced by *C. difficile* toxin variants, this report contributes with significant findings concerning RhoA GTPase-modifying toxins. We determined that Rac1 is no longer detected in total cell lysates by Western blotting upon glucosylation by LCTs. Thus, the monoclonal antibody used in this study will become an important tool in the field of clostridial toxins to follow the dynamics of intoxication in intact cells avoiding previously used radioactive-dependent approaches. This report also illustrates the *in vivo* kinetics of inactivation of Rho GTPases upon treatment with LCTs and indicates a full agreement between the reported *in vitro* substrates for these toxins (4, 10) and their behavior *in vivo* with regard to activity state upon modification.

Our results with CNF1 also agree with the reported *in vitro* activation of the Rho GTPases and are consistent with recent reports concerning *in vivo* activation of Rho GTPases (36, 37). We did not observe any degradation *in vivo* of the CNF1-modified GTPases in the HeLa cells used in this study, contrasting the finding by Doye *et al.* (37) in a rat bladder carcinoma cell line and in primary endothelial HUVEC cells. These authors, however, report a decreased ubiquitinylation of Rac1 upon CNF1 modification in transformed epithelial cell lines consistent with our observations in HeLa cells (37). Furthermore, all GTPases, whether modified by LCTs or CNF1, showed similar kinetics in their activity responses, indicating that all the substrates are modified with the same efficiency by these bacterial toxins.

The long term aim of this kind of studies is to determine how toxin B variants from *C. difficile* TcdA-negative strains are able to induce clinical entities of their own, a finding contradicting the dogma that TcdA is responsible for the pathophysiological effects induced by this bacterium. One possible explanation is that R-Ras modification leads to more deleterious effects than RhoA modification via the induction of cell detachment, which for adherent cells is a potent inductor of apoptosis. Additionally, transient activation of RhoA could induce the release of chemical mediators by epithelial cells favoring the development of diarrhea. Thus, it is possible that R-Ras-modifying variants of toxin B are able to take over the diarrheagenic functions of TcdA in *C. difficile* disease whereas this does not seem possible for the RhoA-modifying TcdB-10463. Evaluation of this hypothesis by *in vivo* experiments will provide a deeper understanding of the relevant clinical entities such as antibiotic associated diarrhea and pseudomembranous colitis, which in turn would imply better treatment options for these diseases.

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R-Ras Glucosylation and Transient RhoA Activation Determine the Cytopathic Effect Produced by Toxin B Variants from Toxin A-negative Strains of *Clostridium difficile*

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