Clostridium difficile Toxin A Induces Expression of the Stress-induced Early Gene Product RhoB*

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Ralf Gerhard†‡, Helma Tatgé†, Harald Genth, Thomas Thumb, Jürgen Borlak†, Gerhard Fritz†, and Ingo Just‡

From the †Institute of Toxicology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany, ‡Institute of Toxicology and Experimental Medicine, Fraunhofer Gesellschaft, Nikolai-Fuchs-Strasse 1, 30625 Hannover, Germany, and ¶Institute of Toxicology, Johannes-Gutenberg-University Mainz, Obere Zahlbacher Strasse 67, D-55131 Mainz, Germany

Clostridium difficile toxin A monoglucosylates the Rho family GTPases Rho, Rac, and Cdc42. Glucosylation leads to the functional inactivation of Rho GTPases and causes disruption of the actin cytoskeleton. A cDNA microarray revealed the immediate early gene rhoB as the gene that was predominantly up-regulated in colonic CaCo-2 cells after treatment with toxin A. This toxin A effect was also detectable in epithelial cells such as HT29 and Madin-Darby canine kidney cells, as well as NIH 3T3 fibroblasts. The expression of RhoB was time-dependent and correlated with the morphological changes of cells. The up-regulation of RhoB was approximately 15-fold and was based on the de novo synthesis of the GTPase because cycloheximide completely inhibited the toxin A effect. After 8 h, a steady state was reached, with no further increase in RhoB. The p38 MAPK inhibitor SB202190 reduced the expression of RhoB, indicating a participation of the p38 MAPK in this stress response. Surprisingly, newly formed RhoB protein was only partially glucosylated by toxin A, sparing a pool of potentially active RhoB, as checked by sequential C3ʻ′/κ Catalyzed ADP-ribosylation. A pull-down assay in fact revealed a significant amount of active RhoB in toxin A-treated cells that was not present in control cells. We demonstrate for the first time that toxin A has not only the property to inactivate the GTPases RhoA, Rac1, and Cdc42 by glucosylation, but it also has the property to generate active RhoB that likely contributes to the overall picture of toxin treatment.

Toxins A and B from Clostridium difficile are the major pathogenicity factors of antibiotic-associated diarrhea and are responsible for the monoglucosylation and thereby inactivation of the Rho GTPases (1, 2). The Rho GTPases Rho, Rac, and Cdc42 are involved in a variety of cellular functions, such as the dynamic regulation of the actin cytoskeleton (3), regulation of cell cycle progression (4), cellular transformation, and apoptosis (5–8). In addition, they are implemented in signal cascades involving mitogen-activated protein kinases (MAPK)1 (9, 10). The activation of MAPK results from cellular or genotoxic stress and involves Rho GTPases independently of their role as regulators of the actin cytoskeleton (11). Rac and Cdc42 are the predominant Rho GTPases that are involved in the regulation of the different MAPK, i.e. extracellular-regulated kinase, c-Jun N-terminal kinase, and p38 MAPK (12). Rho GTPases are involved positively in the signal transduction because dominant-negative mutant forms of Rac and Cdc42 inhibit these pathways. Accordingly, the inhibition of Rho family GTPases by C. difficile toxin B also blocks c-Jun N-terminal kinase activation in gentamycin-treated auditory hair cells (13) and interleukin-1-induced activation of c-Jun N-terminal kinase, p38 MAPK, and NFκB in murine EL-4 thymoma cells (14).

The RhoB GTPase also belongs to the Rho GTPase family and shows an identity to RhoA of 86% at the amino acid level. RhoB, which is constitutively poorly expressed, has been reported as an immediate early inducible gene product formed in response to genotoxic stress caused by UV irradiation or alkylating agents (15) and in response to growth factors (16). RhoB is exclusively membrane bound and is localized to the plasma membrane and early endosomes (17–19). It contributes to transforming and apoptotic processes presumably by regulating the intracellular transport of cell surface receptors (20). DNA damage-induced apoptosis especially requires RhoB (21, 22), although RhoB does not directly mediate apoptosis. Early observations (16, 18) showed that RhoB positively regulates cell proliferation. More recent studies (23, 24), however, have reported that RhoB is more of a regulator than an inducer of apoptosis. Its role as a cellular target for farnesyl transferase inhibitors makes RhoB a promising target for anticaner therapy.

Only a few signal pathways involving RhoB have been reported in more detail. RhoB reduces the NFκB activity induced by tumor necrosis factor-α or genotoxic stress by decreasing the degradation of the inhibitory κB-α (25). Furthermore, RhoB plays a role in the Akt survival pathway in endothelial cells (26). In RhoB-depleted cells, Akt was excluded from the nucleus and was degraded in a proteosome-dependent manner. Taken together, RhoB is induced by cellular stress, especially genotoxic stress, and is involved in various pathways regulating apoptotic processes. In this context, the induction of RhoB by C. difficile toxin A is most remarkable and offers new insight into the cellular response to Rho-modifying toxins. The dilemma of RhoB, that it can be both induced by toxin A and serve as substrate for toxin A at the same time, was the focus of this study.

<ref>endothelial cell; GST, glutathione S-transferase; GTP-γS, guanosine 5′-O-(thiotriphosphate); PI3K, phosphatidylinositol 3-kinase.</ref>
**Toxin A Induces RhoB Expression**

**EXPERIMENTAL PROCEDURES**

Materials—The RNeasy® kit, Oligotex mRNA mini kit, and QIAquick™ from Qiagen. RNAsin was from Promega. Superscript™II enzyme, First Strand buffer, RNaseH, and endothelial serum-free basal growth medium were obtained from Invitrogen. FluoroLink™ Cy3/5-dCTP from Amersham Biosciences. Bacillus megaterium protoplasts were from MoBiTec, Göttingen, Germany. All cell culture media were from Biochrome AG, Berlin, Germany. G418 was purchased from Sigma. Thrombin was from Roche Applied Science. [32P]Nicotinamide adenine dinucleotide was from PerkinElmer Life Sciences. Monoclonal anti-RhoB (C-5) and monoclonal anti-p38 MAPK (A-12) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Monoclonal antibody against double phosphorylated (Thr-180/Tyr-182) p38 MAPK (clone 28B10) was from Cell Signaling.

cDNA Array—cDNA arrays were obtained from Memerec Biotec GmbH (Cologne, Germany). According to the manufacturer’s information, the defined 200–400-bp fragments of selected cDNAs were generated by RT-PCR using Superscript™II enzyme, sequence-specific primers, and RNA derived from appropriate human tissue and cell lines. All amplified inserts were transferred to a 384-well plate and double spotted on treated glass slides using a customized ink jet spotter. The cDNA microarray was performed as described in detail by Borlak and Bosio (27).

Total RNA was isolated from cultured CaCo-2 cells (107 cells) using an RNeasy® kit according to the manufacturer’s recommendations. Total RNA (40 μg) was combined with a control RNA consisting of an in vitro transcribed genomic DNA fragment carrying a 30-nucleotide poly(A) tail (EC17), and mRNA was isolated using an Oligotex mRNA mini kit. 0.8 μg of mRNA or 2 μg of amplified RNA was diluted to 17 μl and combined with 2 μl of a second control RNA, a mixture of the three different transcripts EC7, EC20, and EC21. mRNA and amplified RNA were reverse-transcribed by adding a mixture consisting of 8 μl of 5 × first strand buffer (Superscript™II) 2 μl of primers mix (oligo(dT)), randomers for mRNA or randomers only for amplified RNA, 2 μl of low C dNTPs (10 mM dATP, 10 mM dGTP, 10 mM dCTP, 4 mM dTTP), 2 μl of FluoroLink™ Cy3/5-dCTP, 4 μl of 0.1 M dithiothreitol, and 1 μl of RNasin (20–40 units). 1 μl (200 units) of Superscript™II enzyme was added and incubated at 42 °C for 30 min. This step was repeated once. 0.5 μl of RNaseH was added and incubated at 37 °C for 20 min to hydrolyse RNA. Cy3- and Cy5-labeled samples were combined and cleaned using QIAquick™.

Image capture and signal quantification of hybridized PIQOR™ cDNA arrays were carried out with the ScanArray3000 (GSI Lumonics) and ImaGene software version 2.0 (BioDiscovery). The local background was subtracted from the signal to obtain the net signal intensity and the ratio of Cy5/Cy3. Subsequently, the mean of the ratios of the two corresponding spots representing the same cDNA was computed. The mean ratios were normalized to the median ratio using mean ratios of control slides. For each channel, four slides were hybridized to obtain a ratio of protein each were added to each sample and rotated at 4 °C for 5 min followed by centrifugation at 14,000 rpm for 10 min. The supernatant (10 μg of protein) was used for pull-down experiments. 20 μl of beads slurry consisting of GST-Rhotekin (C21) containing 20 μg of protein each were added to each sample and rotated at 4 °C for 30 min. The beads were collected by centrifugation at 10,000 rpm and washed twice with lysis buffer. Pull-down experiments with recombinant RhoB were performed under the same conditions using lysis buffer, which was supplemented with 1 mg/ml bovine serum albumin to prevent unspecific binding of RhoB to Sepharose beads. The exchange of nucleotides was performed as described previously by Sehr et al. (33). Quantitative glucosylation of recombinant RhoB was checked by matrix-assisted laser desorption ionization time-of-flight analysis.

Expression of GST Fusion Proteins RhoB and Clostridium botulinum Exoenzyme C3—RhoB and the exoenzyme C3 were expressed as GST fusion proteins in E. coli (31). Affinity purification was performed using glutathione-Sepharose. The GST fusion proteins were cleaved by thrombin. Thrombin was removed from the RhoB or C3-containing supernatant by precipitation with benzamidine beads.

**ADP-ribosylation Assay**—After toxin treatment the cells were washed once with ice-cold phosphate-buffered saline. The cells were lysed in ribosylation buffer (50 mM HEPES (pH 7.4), 10 mM thymidine, 5 mM MgCl2, 2.5 mM dithiothreitol, and 2.5 μM NAD, 0.1% SDS). C3ad (2 μg/ml) and [32P]NAD (0.5 μM) were added to start the ADP-ribosylation reaction. The ADP-ribosylation assay was stopped after 15 min by the addition of Laemmli buffer. The proteins were separated by 15% SDS-PAGE followed by filmless autoradiography (Packard). For the gel shift assay, ADP-ribosylation was performed in the absence of radiolabeled NAD. Western blot analysis for the detection of ADP-ribosylated and non-ADP-ribosylated RhoB was performed using RhoB-specific antibody.

**Pull-down Assay**—Pull-down experiments were performed as described previously by Reid et al. (32). After toxin treatment, the culture medium was removed, and cells were rinsed twice with ice-cold phosphate-buffered saline. Cells were lysed by the addition of 500 μl of ice-cold lysis buffer (50 mM NaCl, 20 mM Tris-HCl (pH 7.4), 3 mM MgCl2, 1% w/v Nonidet P-40, 0.25% w/v Triton X-100, 5 mM dithiothreitol, 100 mM phenylmethylsulfonyl fluoride) at 4 °C for 5 min followed by centrifugation at 14,000 rpm for 10 min. The supernatant (10 mg of protein) was used for pull-down experiments. 20 μl of bead slurry consisting of GST-Rhotekin (C21) containing 20 μg of protein each were added to each sample and rotated at 4 °C for 30 min. The beads were collected by centrifugation at 10,000 rpm and washed twice with lysis buffer. Pull-down experiments with recombinant RhoB were performed under the same conditions using lysis buffer, which was supplemented with 1 mg/ml bovine serum albumin to prevent unspecific binding of RhoB to Sepharose beads. The exchange of nucleotides was performed as described previously by Sehr et al. (33). Quantitative glucosylation of recombinant RhoB was checked by matrix-assisted laser desorption ionization time-of-flight analysis.

**RESULTS**

In this study we investigated the expression of the immediately early gene product RhoB in response to the toxin treatment of cells with C. difficile toxin A. The first evidence of an effect of toxin A was the cDNA array. The mRNA coding for RhoB increased by approximately 15-fold in toxin-treated cells compared with untreated cells. RhoB was the predominant gene that was up-regulated in CaCo-2 cells after 4 h of treatment with 1 μg/ml toxin A (Fig. 1). Surprisingly, the expression of only a few other genes was altered significantly, and these genes will be the topic of a separate study.

To prove directly the increase of RhoB at the protein level, we confirmed RhoB expression by Western blot analysis. The immunoblot showed a time-dependent expression of the RhoB GTPase starting at 2 h after toxin treatment (Fig. 2A). The
maximum level of RhoB was observed approximately 8 h after the addition of the toxin and continued for at least 24 h. After 8 h of toxin treatment, a steady state of RhoB expression was reached. The up-regulation of RhoB correlated well with the morphological changes of cells (Fig. 2B), indicating a relationship with the cytosolic action of toxin A. However, the inhibition of RhoB synthesis by cycloheximide did not prevent or delay rounding up of cells, excluding a major role of RhoB in cytoskeleton reorganization (data not shown). Several cell lines were tested and found to respond positively to toxin A with expression of RhoB (Fig. 2C). Among them were epithelial cell lines, such as CaCo-2, HT29, and MDCK cells, and NIH 3T3 fibroblasts. RhoB was detected neither in HMC-1 nor in HUVEC after toxin A treatment. However, we cannot exclude very low induction escaping Western blot detection.

To get further insight into the pathway involved in RhoB expression, we studied signal pathways that might possibly be involved. In fact, toxin A induced an increase in p38 MAPK phosphorylation as shown by Western blot against the double phosphorylated form (Thr-185/Tyr-187) (Fig. 3A). The activation of p38 MAPK started approximately 1 h after the addition of toxin A. As a positive control, osmotic shock by 500 mM sorbitol was applied. This signal was likely to reflect the maximum stimulation of p38 MAPK. Interestingly, p38 MAPK activation correlated well with the increase in RhoB expression. To test whether there was a causative correlation, toxin A-induced RhoB up-regulation was performed in the presence of the p38 MAPK-specific inhibitor SB202190 (10 μM). The inhibitor decreased toxin A-induced RhoB protein expression (Fig. 3B). SB202190 itself, however, did not induce RhoB expression to a detectable level.

Because p38 MAPK are regulated positively by Rho GT-Pases, toxin A-catalyzed glucosylation of the GTPases should have resulted in the inhibition of p38 MAPK rather than its activation, as observed in this study. Therefore, we tested whether toxin A stimulated p38 MAPK in a Rho-independent way, i.e., through a ligand-like interaction with membrane receptors. If this was the case, inhibition of cytoplasmic delivery of toxin A should not prevent RhoB up-regulation. To test this, the entry of toxin A into the cytoplasm was blocked by bafilomycin A1, which entraps the receptor-bound toxin within the endosomes by preventing acidification. Bafilomycin (2 μM) completely prevented the up-regulation of RhoB by toxin A.

FIG. 2. Toxin A induces up-regulation of the RhoB GTPase. A, treatment of CaCo-2 cells with 500 ng/ml C. difficile toxin A led to a time-dependent increase of RhoB. Western blot analysis of cell lysates with anti-RhoB is shown. B, the morphological changes of cells correlated with the up-regulation of RhoB. Round up of cells started approximately 2 h after toxin treatment and was significant after 4 h. Shown are micrographs of CaCo-2 cells treated with toxin A for 0, 2, and 4 h. C, toxin A-induced expression (+) of RhoB was detected by immunoblot in epithelial cells CaCo-2, HT29, and MDCK and in NIH 3T3 fibroblasts. HMC-1 and HUVEC showed no detectable up-regulation of RhoB in response to toxin A (−).

FIG. 3. p38 MAPK regulates the expression of RhoB. A, Western blot using specific antibody against the phosphorylated form of p38 MAPK (upper panel) exhibited a sustained activation when cells were treated with toxin A. Sorbitol served as a positive control, showing a maximum stimulation of p38 MAPK. The lower panel shows the amount of total p38 MAPK in cell lysates. B, the p38 MAPK inhibitor SB202190 (10 μM) completely prevented the toxin A-induced expression of RhoB. C, to check for the receptor-mediated effects of toxin A, cells were treated with bafilomycin A1, an endosomal ATPase inhibitor that inhibits translocation of toxin A from the endosomes into the cytosol. Bafilomycin (2 μM) completely prevented the up-regulation of RhoB by toxin A.
Toxin A induces RhoB expression.

A, CaCo-2 cells were preincubated with toxin A for 8 h to increase the cellular RhoB levels. At this time point (8 h), p38 MAPK inhibitor SB202190 (10 \( \mu \text{M} \)), cycloheximide (CHX) (10 \( \mu \text{M} \)), or MeSO as control was applied, and the RhoB content was followed for another 2 and 8 h, respectively. The immunoblot (IB) (left panels) was densitometrically analyzed, and the area determined was given as the means \( \pm \) S.D. (\( n = 4 \)). The RhoB content of cells treated with SB202190 for 8 h decreased to \( \geq 70 \% \) compared with control cells (A) (88 \( \pm \) 7\%) in the presence of toxin A. RhoB was decreased even more strongly in cycloheximide-treated cells (B) to \( \leq 27 \% \). These results showed that RhoB is still induced 16 h after the addition of toxin A. B, toxin A-dependent RhoB expression was inhibited in the presence of the translation inhibitor cycloheximide (10 \( \mu \text{M} \)).

approximately 70 \( \pm \) 25% after 8 h. In cycloheximide-treated cells, the RhoB level decreased to 45 \( \pm \) 27% after 2 h and remained at this level for another 6 h (42 \( \pm \) 12%). This experiment showed a long lasting induction of RhoB protein expression rather than an inhibition of RhoB degradation. The treatment of CaCo-2 cells with cycloheximide prior to the addition of toxin A completely prevented the increase in the amount of RhoB (Fig. 4B). Thus, the up-regulation of cellular RhoB by toxin A was achieved by continuous de novo synthesis of the GTPase.

RhoA, Rac, and Cdc42 have been reported to be the major cellular substrates of toxin A. To test whether RhoB is also an intracellular substrate of toxin A, NIH 3T3 fibroblasts stably transfected with His-RhoB were used (25). His-RhoB reveals two advantages. First, because of its higher molecular mass, radiolabeled His-RhoB can be easily detected separately from radiolabeled endogenous RhoA by phosphorimaging. Second, His-RhoB did not underlie an up-regulation induced by toxin A. The glucosylation of RhoB was first verified by sequential exoenzyme C3\( ^{\text{bot}} \)-catalyzed ADP-ribosylation. Cell lysates of controls and of cells treated with recombinant toxin A were used for in vitro \( ^{32} \text{P} \)ADP-ribosylation by C3\( ^{\text{bot}} \) in those cells treated with toxin A, the 23-kDa signal of \( ^{32} \text{P} \)ADP-ribosylated GTPases was decreased strongly compared with the control (Fig. 5), indicating a previous glucosylation of part of the Rho GTPase pool by toxin A in the intact cell. Furthermore, the weakly expressed His-tagged RhoB (28 kDa) of toxin A-treated cells was not the substrate for C3\( ^{\text{bot}} \), verifying cellular RhoB as the substrate for toxin A. The 35-kDa-labeled band represented endogenous ADP-ribosylation and served as a marker to show identical protein concentration. Thus, endogenous RhoB is considered to be the substrate for toxin A. Two pools of RhoB were present within the toxin A-treated cell: (i) potentially active RhoB supplied by continuous de novo expression and (ii) inactive RhoB supplied by continuous modification catalyzed by toxin A. To estimate the pool of potentially active RhoB, i.e. non-glucosylated, we performed ADP-ribosylation of lysates from toxin A-treated NIH 3T3 fibroblasts. The ADP-ribosylation of previously non-glucosylated RhoB could be followed by a gel shift assay combined with a subsequent Western blot analysis. In control cells, no RhoB was detectable (Fig. 6A, upper panel), whereas a strong signal of RhoB was detectable in toxin A-treated cells. Non-glucosylated RhoB subjected to ADP-ribosylation shifted to a higher apparent molecular weight. This pool of RhoB was counted for potentially active RhoB. The amount of shifted RhoB (approximately 40%) revealed a surplus of unmodified and therefore potentially active RhoB.

To prove directly the presence of active RhoB, we performed a pull-down assay using the Rho-binding domain C21 of Rhotekin. A study of the binding of GTP\( ^{\gamma} \)S-loaded RhoB to the Rho-binding domain of Rhotekin was described by Reid et al. (32), whereas Gampel and Mellor (34) applied the Rhotekin-based pull-down assay for activation studies of RhoB. We first excluded nucleotide-independent binding of recombinant glucosylated RhoB in a pull-down assay. RhoB bound only to GST-C21 beads in the GTP-bound form (Fig. 6B). Glucosylated RhoB did not bind to GST-C21, GTP\( ^{\gamma} \)S, or GDP. The validated pull-down assay was then applied to cell lysates. Active GTP-bound RhoB was precipitated from toxin A-treated NIH 3T3 fibroblasts but not from control cells because of the missing RhoB amount (Fig. 6C). In contrast to RhoB, active GTP-bound RhoA was precipitated from control cells but was hardly precipitated from toxin A-treated cells. The blot showed less RhoA in toxin A-treated lysates, which was caused by the high affinity of inactive glucosylated RhoA to the particulate fraction (35). This pull-down assay confirmed the presence of active GTP-bound RhoB in toxin A-treated cells.

**DISCUSSION**

*Clostridium difficile* toxin A is an intracellular acting exotoxin that inactivates the Rho-GTPases Rho, Rac, and Cdc42 by monoglucosylation. The most prominent outcome of glucosylation that allows easy detection is the reorganization of the actin cytoskeleton accompanied by morphological changes. The inactivation
Toxin A Induces RhoB Expression

In the present study, toxin A was shown to cause a sustained up-regulation of RhoB protein. The up-regulation of rhoB mRNA (detected by DNA microarray) reflected in fact the expression of RhoB, as shown by immunoblot. The amount of RhoB in cells increased for approximately 8 h and then reached a steady state level, which was maintained for more than 24 h. Because the reported half-life of RhoB is approximately 2 h, the long lasting up-regulation may have been caused by the inhibition of RhoB degradation. If this is the case, the inhibition of de novo protein synthesis with cycloheximide should have been only slightly influenced by the amount of RhoB. However, cycloheximide completely prevented the formation of RhoB, and when it was added to the steady state phase, a rapid decline of RhoB concentration resulted. Thus, in the presence of toxin A, a strong long lasting de novo protein synthesis was induced, which reached an equilibrium with degradation after 8 h. Under this condition, RhoB showed a high protein turnover.

Because RhoB is the substrate for toxin A, its immediate inactivation by glucosylation should have resulted in an increased concentration of inactive glucosylated RhoB in the target cell. Unexpectedly, the strongly increased amount of RhoB, up to 15-fold, was only partially glucosylated, remaining approximately 40% of the unmodified RhoB. Using the pull-down assay, it was clearly obvious that the unmodified RhoB or at least part of it was active, i.e. it was GTP-bound and therefore was capable of downstream signaling. In contrast, the toxin substrate RhoA that was constitutively expressed in much higher concentrations was almost completely inactive.

The failure of glucosylated GTPases to bind to their effector proteins is based generally on structural constraints caused by the glucose moiety (33, 36–38). The same constraints are also valid for the homologous RhoB. Thus, the pull-down assay really reflects active, signaling-competent RhoB. What are the signals leading to activation of RhoB? The high homology of RhoB with RhoA in the N-terminal half strongly suggested the ability to interact with guanine nucleotide exchange factors, GTPase-activating proteins, and effector proteins of the RhoA signaling pathways. Different intracellular localizations of RhoA (cytosol and plasma membranes) and RhoB (endosomes and plasma membranes) under normal cellular conditions separate RhoA from RhoB signaling. However, the majority of newly synthesized RhoB in toxin A-treated cells was located to the plasma membrane but not to endosomes. The plasma membrane-bound RhoB did find orphaned regulatory proteins of the RhoA signaling pathways, which activated RhoB.

The most prominent and dramatic effect of toxin A was the reorganization of the actin cytoskeleton, detected as morphological changes. Because the inhibition of RhoB synthesis did not change the kinetics of cell rounding, the idea that newly synthesized RhoB is responsible for the F-actin reorganization can be excluded. Instead, it is conceivable that this reorganization may trigger RhoB up-regulation. The molecular mechanism and signaling proteins that are involved in the perception of cytoskeletal breakdown can only be hypothesized. It was also reported that the interruption of the Ras-driven PI3K/Akt pathway results in increased RhoB expression (39). A further member in the PI3K/Akt signaling pathway of Rho, Rac, and Cdc42 is thought to be highly specific. This notion has led to the establishment of the toxins as widely used tools in studying cell biology.

In fact, not only toxin A but also toxin B from C. difficile as well as latrunculin B induced an up-regulation of RhoB (data not shown). In a separate study, toxin B was also reported to induce a weak up-regulation of RhoB (41). However, in our...
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In conclusion, C. difficile toxin A induces a long lasting, strong up-regulation of the RhoB-GTPase through the activation of p38 MAPK. Newly expressed RhoB is only partially inactivated by glucosylation, so a significant portion of RhoB is active and capable of downstream signaling. This is the first report showing that toxin A is not an exclusive inhibitor of Rho GTPases but that it also causes the activation of Rho GTPases that likely contributes to the overall picture of toxin treatment, especially to the proinflammatory in vivo effects of the C. difficile toxins, which as yet is not well understood.

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