Upregulation of the Immediate Early Gene Product RhoB by Exoenzyme C3 from *Clostridium limosum* and Toxin B from *Clostridium difficile*†

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ABSTRACT: ADP-ribosylation of Rho(A,B,C) by the family of exoenzyme C3-like transferases induces reorganization of the actin cytoskeleton based on inactivation of RhoA. No data are available on the role of RhoB in C3-treated cells. In murine fibroblasts treated with the cell-permeable exoenzyme C3 from *Clostridium limosum* (C3), an increase in the level of RhoB was observed. This upregulation of RhoB was based on transcriptional activation, as it was responsive to inhibition by actinomycin D and accompanied by activation of the *rhoB* promoter. Upregulation of RhoB was not observed in cells treated with either the actin ADP-ribosylating C2 toxin from *Clostridium botulinum* or latrunculin B, suggesting that inactivation of Rho but not actin reorganization was required for the upregulation of RhoB. This notion was confirmed, as the Rho/Rac/Cdc42-glucosylating toxin B from *Clostridium difficile* (TcdB) but not the Rac/R-Ras-glucosylating variant toxin B from *C. difficile* strain 1470 serotype F (TcdBF) induced a strong upregulation of RhoB. Upregulation of RhoB was further observed in response to the Rac/(H-,K-,N-,R-)Ras-glucosylating lethal toxin from *Clostridium sordellii*. The level of active, GTP-bound RhoB was increased in TcdB-treated cells compared to untreated cells (as determined by Rhotekin pull-down assay). In contrast, no active RhoB was found in C3-treated cells. RhoB-GTP was required for the TcdB-induced apoptosis (cytotoxic effect), as this effect was responsive to inhibition by C3. In conclusion, RhoB was upregulated by Rho-/Ras-inactivating toxins, as a consequence of the inactivation of either Rho(A,B,C) or (H-,K-,N-)Ras. In TcdB-treated cells, RhoB escaped its inactivation and was required for the cytotoxic effect.

Rho proteins are involved in the control of actin dynamics, cell-cycle progression, gene transcription, and vesicle trafficking (*1*−*3*). They are also targets of clostridial glucosylating toxins: *Clostridium difficile* toxin A (TcdA)[1] and toxin B (TcdB) glucosylate Rho, Rac, and Cdc42 (*4*). Although variant toxin B from *C. difficile* strain 1470 serotype F (TcdBF) exhibits an identity of about 93% to TcdB at the amino acid level, TcdBF glucosylates Rac and R-Ras but neither Rho nor Cdc42. Lethal toxin from *Clostridium sordellii* (TcsL) shares its substrates with TcdBF but additionally glucosylates (H-,K-,N-)Ras (*5*, *6*). The glucose moiety transferred is covalently linked to a pivotal threonine residue within the effector region of the Rho/Ras proteins, resulting in impairment of effector and regulator coupling (*7*−*9*).

C3-like transferases ADP-ribosylate Rho(A,B,C); the acceptor amino acid asparagine-41 is located within the effector domain of the Rho proteins (*10*). ADP-ribosylated RhoA is inactive because of its high affinity to the inhibitory regulator guanine nucleotide dissociation inhibitor-1 (GDI-1) (*11*, *12*). Inactivation of Rho-GTPases by glucosylating toxins results in reorganization of the actin cytoskeleton ("cytopathic effect") and in cell death ("cytotoxic effect") (*13*−*15*). Although it is well-established that inactivation of RhoA (either by Rho-inactivating toxins or by ectopic expression of dominant-negative RhoA) results in the cytotoxic effect, other reports show that Rho-inactivating toxins suppress (e.g., Fas-induced) apoptosis (*16*−*18*); thus, the Rho-inactivating toxins exert a dual role as inducers and inhibitors of apoptosis.

RhoA and RhoB exhibit an identity of 88% at the amino acid level; therefore, it has been suggested that RhoA and RhoB share similar biological activities: Both Rho proteins govern the dynamic of the actin cytoskeleton and both promote transactivation of the serum response element of the c-fos promoter and potentiate the transforming activity of oncogenic Ras (*1*, *19*). In contrast to RhoA, the level of RhoB is low in the majority of cell lines due to suppression by either (active) RhoA or (H-,K-,N-)Ras (*20*−*22*).

We recently reported on the upregulation of RhoB by the Rho/Rac/Cdc42-inactivating TcdA and speculated that inactivation of Rac1 triggers upregulation of RhoB (*23*). In

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[1] Abbreviations: Act D, actinomycin D; C2, C2 toxin from *Clostridium botulinum*; C3, exoenzyme C3 from *Clostridium limosum* fused to the C2 toxin; CAT, chloramphenicol acetyltransferase; LatB, latrunculin B; TcdA, toxin A from *Clostridium difficile* strain VPI10463; TcdB, toxin B from *C. difficile* strain VPI10463; TcdBF, toxin B from *C. difficile* strain 1470 serotype F; TcsL, lethal toxin from *Clostridium sordellii* strain 6018.

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the present study, we provide evidence that the upregulation of RhoB is based on the inactivation of either Rho(A,B,C) or (H-,K-,N-)Ras but neither on the reorganization of the actin cytoskeleton nor the inactivation of Rac1. Furthermore, RhoB is shown to be required for the cytotoxic effect of TcdB.

EXPERIMENTAL PROCEDURES

Materials. The GST-C21 vector construct was a kind gift of Dr. John Collard (Amsterdam). Commercially obtained reagents: latrunculin B, actinomycin D, Z-VAD(OMe)-FMK (Calbiochem); cycloheximide (Sigma); antibodies RhoA (26C4, SantaCruz), RhoB (BL927, Bethyl Laboratories), and Rac1 (Mab102) (BD Transduction Laboratories); β-actin (AC-40, Sigma); horseradish peroxidase conjugated secondary antibodies, mouse/rabbit (Rockland).

Toxin Purification. TcdA, TcdB, TcdBF, and TcsL were purified from the respective *C. difficile* strains VPI 10463 and 1470 or the *C. sordellii* strain 6018 (*24*). Briefly, a dialysis bag containing 900 mL of 0.9% NaCl in a total volume of 4 L of brain heart infusion medium (Difco) was inoculated with 100 mL of an overnight culture of *C. difficile* (or *C. sordellii*) and grown under microaerophilic conditions at 37 °C for 72 h. Proteins were precipitated from the culture supernatant by ammonium sulfate at 70% saturation. The precipitates were dialyzed against Tris-HCl buffer, pH 7.5, overnight, and loaded onto a MonoQ column (Amersham Biosciences). The toxins were subsequently eluted with Tris-HCl buffer, pH 7.5, containing 500 mM NaCl. *Clostridium botulinum* C2 toxin, exoenzyme C3, and C3 fusion toxin were expressed in *Escherichia coli* using the pGEX-2T vector system and purified with GSH−Sepharose beads (AP Biotech) as described (*25*, *26*).

Cell Culture. NIH3T3 fibroblasts were cultivated in Dulbecco's modified essential medium (Biochrom, +10% FCS, 100 μg/mL penicillin, 100 units/mL streptomycin, and 1 mM sodium pyruvate) at 37 °C and 5% CO_2. Upon confluence, cells were passaged.

Synchronization of Cells. The thymidine double block was applied to synchronize NIH3T3 fibroblasts as described (*27*). Exponentially growing cells were exposed to 2 mM 2′-deoxythymidine in full-growth medium for 16 h at 37 °C and 5% CO_2. The medium was then removed and replaced by full-growth medium without thymidine. After 9 h at 37 °C and 5% CO_2, this medium was again replaced by full-growth medium containing 2 mM 2′-deoxythymidine. Cells were incubated for 16 h at 37 °C and 5% CO_2. Subsequently, the medium was again replaced by thymidine-free full-growth medium for 1 h. Cells were then synchronized in the S-phase, as confirmed by FACS analysis for DNA content.

Treatment of Cells. Subconfluent cells were treated with TcdB, TcdBF, TcsL, C2 toxin, or C3 fusion toxin as indicated. The agents used were applied with the following concentrations: actinomycin D (1 μg/mL), latrunculin B (1 μg/mL), and caspase inhibitor I (20 μM).

Transfection Experiments and Analysis of rhoB Promoter Activity. For transient transfection experiments, fibroblasts were seeded and transfected 24 h later with 1.5 μg of the 3.5 kb *rhoB* promoter CAT construct for 14 h (*20*). Cells were treated with the toxins and harvested. The protein concentration of the lysates was normalized using the method by Bradford. The level of CAT expression was analyzed using an enzyme-linked immunosorbent assay (CAT-Elisa kit, Roche) according to the manufacturer's instructions. Data obtained from CAT analysis of the *rhoB* promoter were reproduced three times.

Cell Lysis for Western Blot Analysis. After toxin treatment, cells were washed and scraped into Laemmli sample buffer. The obtained suspension was shaken at 37 °C for 10 min and subsequently sonicated on ice. The lysate was then incubated at 95 °C for 10 min and submitted to SDS−PAGE.

Western Blot Analysis. Complete lysate proteins were separated using SDS−PAGE and subsequently transferred onto nitrocellulose membranes by a tank blot system. The membranes were blocked with 5% (w/v) nonfat dried milk for 60 min; incubation with primary antibody was conducted overnight at 4 °C and treatment with the secondary antibody at room temperature for 1 h. For the chemiluminescence reaction, ECL Femto (Pierce) was used. All signals were analyzed densitometrically using the KODAK 1D software and normalized to β-actin signals.

Cell Lysis and Sequential ADP-Ribosylation. Cells treated with either TcdB or buffer were washed and lysed in a buffer containing NaCl (150 mM), Tris-HCl (50 mM, pH 7.2), $MgCl_2$ (5 mM), PMSF (1 mM), and NP-40 (1%). After sonication, the soluble fraction was prepared by centrifugation (10 min at 16000*g*). Soluble fractions were incubated with *C. botulinum* C3 exoenzyme in the presence of 1 μM NAD at 37 °C for 30 min. The reaction was terminated by addition of Laemmli sample buffer. Subsequently, the samples were separated by SDS−PAGE and subjected to Western blot analysis.

RhoB Activity Assay. Fibroblasts treated with the toxins were lysed in lysis buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM phenylmethanesulfonyl fluoride, 5 mM dithiothreitol). The soluble fraction was obtained by centrifugation (10000*g* for 5 min). It was then incubated with glutathione−Sepharose-bound GST-C21 at 4 °C for 1 h. After the beads were washed, the protein was mobilized by incubation with Laemmli sample buffer at 95 °C for 10 min. Samples were submitted to SDS−PAGE and subsequently to Western blotting.

Glucosylation of Recombinant Rho and Ras Proteins. Rho-(A,B,C) and N-Ras (50 μg/mL each) purified from their parent GST-fusion proteins were incubated with the toxins as indicated in glucosylation buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 100 mM KCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 100 μg/mL BSA, 20 μM UDP-[^{14}C]glucose) at 37 °C for the indicated time periods. The reaction was terminated by addition of Laemmli sample buffer followed by SDS−PAGE and analysis by autoradiography.

Analysis of Apoptosis. After toxin treatment, annexin V labeled with Alexa Fluor 488 (Cambrex) was added directly into the medium (1:50) to visualize phosphatidylserine exposure. Cells were analyzed by fluorescence microscopy using a Zeiss Axiovert 200 M (Alexa Fluor 488; excitation, 470 nm; emission, 515 nm).

RESULTS

Upregulation of RhoB by Exoenzyme C3. Ectopic expression of either dominant-negative RhoA or the Rho-inhibitory

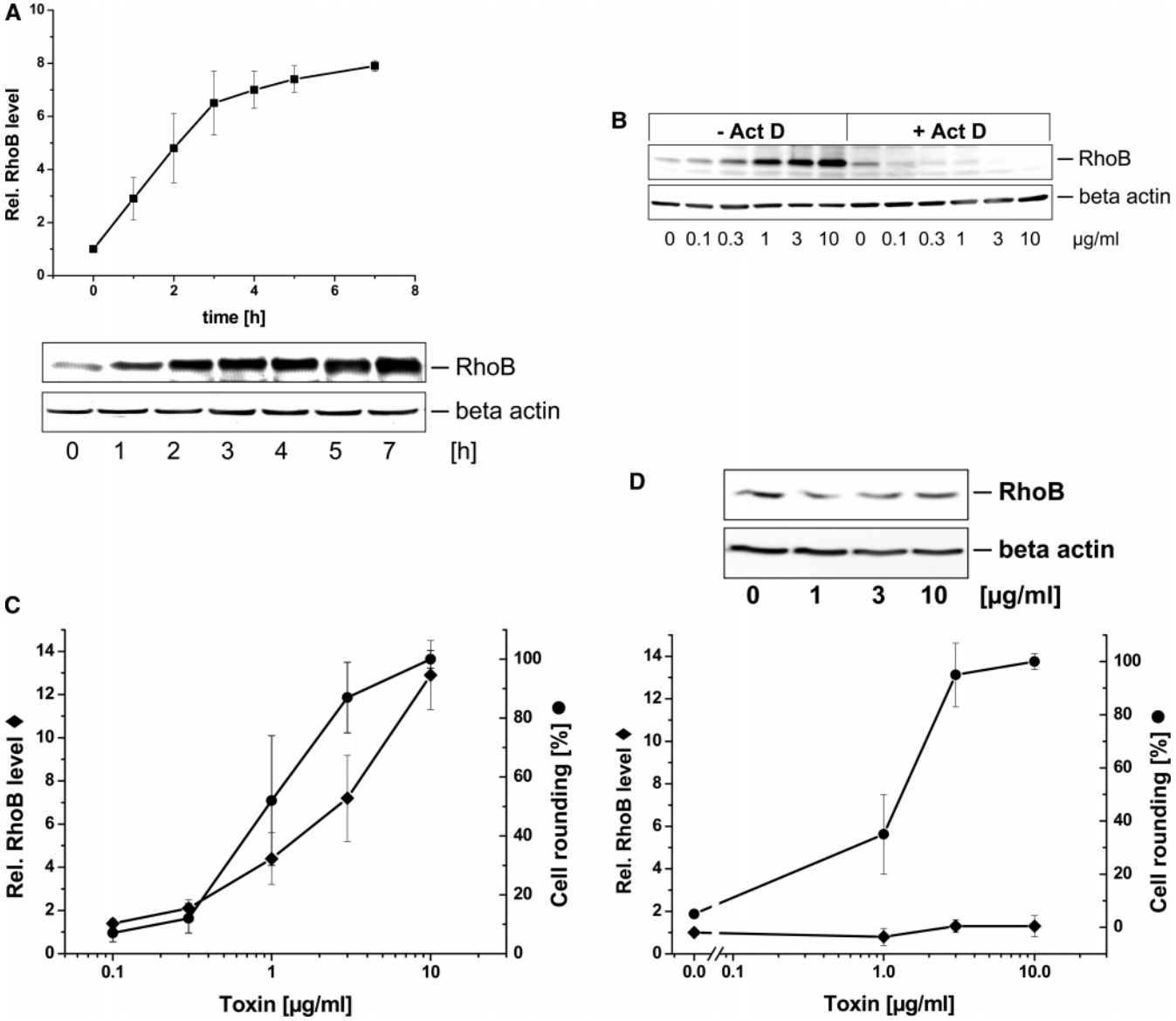

FIGURE 1: (A) Time-dependent upregulation of RhoB after treatment with C3. Fibroblasts were treated with C3 (1 μg/mL) for the indicated periods. Cells were harvested and analyzed for RhoB and β-actin using Western blotting. Signal intensities were recorded densitometrically; the RhoB signal was normalized to the β-actin signal. Data shown represent the mean of three independent experiments. (B) C3 concentration-dependent upregulation of RhoB. Fibroblasts were treated with increasing concentrations of C3 in the absence or the presence of actinomycin D for 4 h. Cells were harvested and analyzed for RhoB and β-actin using Western blotting. (C) Correlation of the upregulation of RhoB and actin reorganization. Fibroblasts were treated with increasing concentrations of C3 for 4 h. Actin reorganization was quantified in terms of rounded per total cells. Cells were harvested and analyzed for RhoB and β-actin using Western blotting. Signal intensities of the upregulation of RhoB (Figure 1B) were recorded densitometrically; the RhoB signal was normalized to the β-actin signal (●, actin reorganization; ◆, relative RhoB level). Data shown represent the mean of three independent experiments. (D) RhoB is not upregulated in response to actin reorganization. Fibroblasts were treated with increasing concentrations of C2 toxin as indicated for 4 h. Actin reorganization was quantified in terms of rounded per total cells. Cells were harvested and analyzed for RhoB and β-actin using Western blotting. Signal intensities were recorded densitometrically; the RhoB signal was normalized to the β-actin signal (●, actin reorganization; ◆, relative RhoB level). Data shown represent the mean of three independent experiments.

regulating protein guanine nucleotide dissociation inhibitor-1 (GDI-1) has been reported to activate the *rhoB* promoter (*20*−*22*). In this line, treatment of cells with the Rho-inactivating exoenzyme C3 may activate the *rhoB* promoter and cause upregulation of RhoB. To challenge this hypothesis, fibroblasts were treated with the cell-permeable exoenzyme C3 from *Clostridium limosum* (C3). C3 specifically ADP-ribosylates (inactivates) Rho(A,B,C) (*28*). C3 was delivered into the cell, exploiting the uptake mechanism of the C2 toxin (*26*). RhoB was time-dependently upregulated; the level of RhoB increased 8-fold compared to the level in untreated cells after 7 h (Figure 1A). Upregulated RhoB exhibited a shift to apparent higher molecular weight compared to RhoB from untreated cells (Figure 1A); this suggested that upregulated RhoB was ADP-ribosylated, i.e., inactive. The level of β-actin was not changed by C3 (Figure 1A). Furthermore, RhoB was upregulated in a C3 concentration-dependent manner (Figure 1B). Actinomycin D completely inhibited C3-induced upregulation of RhoB protein, suggesting that upregulation of RhoB was based on transcriptional activation (Figure 1B). Upregulation of RhoB correlated with actin reorganization as quantified in terms of rounded per total cells (Figure 1C).

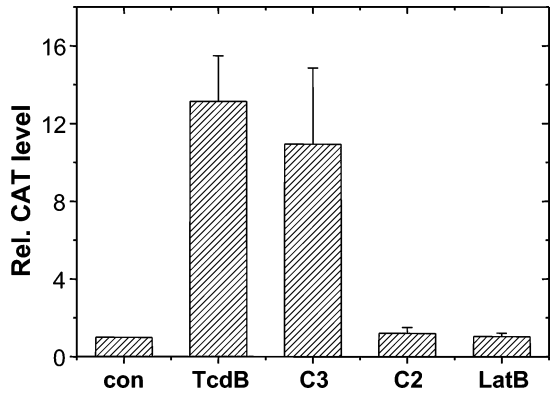

FIGURE 2: Activation of the *rhoB* promoter by toxin B (TcdB) and C3. Fibroblasts were transfected with 1.5 μg of the 3.5 kb *rhoB* promoter CAT construct followed by treatment of the cells with TcdB (1 ng/mL), C3 (1 μg/mL), C2 toxin (10 μg/mL), and latrunculin B (2.5 μM) for 24 h. Cells were harvested for analysis of CAT protein. Data shown represent the mean of three independent experiments.

Fibroblasts were further treated with increasing concentrations of the actin−ADP-ribosylating C2 toxin (*25*). C2 toxin induced actin reorganization in a concentration-dependent manner. The level of RhoB, however, was unchanged in C2-treated cells (Figure 1D). The latter finding shows that upregulation of RhoB is based on inactivation of Rho(A,B,C) but neither on the uptake of the components of the C2 delivery system nor on reorganization of the actin cytoskeleton.

Activation of the rhoB Promoter. To directly show that C3 treatment activates the *rhoB* promoter, fibroblasts were transfected with the 3.5 kb promoter construct using CAT expression as reporter (*20*). As shown in Figure 2, C3 caused an ~10-fold increase of CAT expression compared to untreated cells, suggesting that inactivation of Rho(A,B,C) is sufficient for the activation of the *rhoB* promoter. The Rho(A,B,C)/Rac/Cdc42-glucosylating TcdB has most recently been shown to upregulate RhoB in rat basophilic leukemia cells (*27*). Accordingly, TcdB caused an ~12-fold increase of *rhoB* promoter-driven CAT expression compared to untreated fibroblasts. Neither latrunculin B nor the actin−ADP-ribosylating C2 toxin increased the activity of the *rhoB* promoter (Figure 2), showing that the activation of the *rhoB* promoter is based on impaired Rho signaling.

TcdB but Not TcdBF Induced RhoB Upregulation. To further confirm that upregulation of RhoB is due to inactivation of Rho(A,B,C), we analyzed upregulation of RhoB in cells treated with either the Rho/Rac/Cdc42-glucosylating TcdB or the Rac/R-Ras-glucosylating, "variant" toxin B from strain 1470 serotype F (TcdBF) and the Rac/(H-,K-,N-,R-)-glucosylating lethal toxin from *C. sordellii* (TcsL) (*5*, *6*). If upregulation of RhoB was due to inactivation of Rho(A,B,C), one must expect that TcdB but neither TcdBF nor TcsL induce upregulation of RhoB. TcdB, TcdBF, and TcsL induced actin reorganization in fibroblasts, with TcdB being between 1 and 2 orders of magnitude more potent than TcdBF (Figure 3A). TcsL turned out to be between 1 and 2 orders less potent than TcdBF (Figure 3A). A comparison of equipotent concentrations of TcdB, TcdBF, and TcsL revealed that RhoB was strongly upregulated by TcdB and TcsL, while TcdBF hardly did so (Figure 3B). Accordingly, the *rhoB* promoter was strongly activated by TcdB and TcsL but less pronounced by TcdBF (Figure 3C). Comparison of the data on TcdB and TcdBF confirms that upregulation of RhoB is based on inactivation of Rho(A,B,C). The unexpected activation of *rhoB* and the upregulation of RhoB by TcsL were not due to inactivation of Rho(A,B,C) but most likely based on the inactivation of Ras proteins. At the highest concentration (100 ng/mL) of TcdBF, the *rhoB* promoter was activated to some extent (Figure 3C), which may be based on the fact that TcdBF loses its reported specificity for R-Ras at high concentrations, resulting in the inactivation of further Ras proteins involved in the regulation of *rhoB*. This notion turned out to be true, as partial glucosylation of exemplarily N-Ras by TcdBF was observed, when the glucosylation of recombinant N-Ras was analyzed in a toxin concentration-dependent manner (Figure 3D). In contrast, N-Ras was efficiently glucosylated by TcsL (Figure 3D). In line with published data, TcdB neither glucosylated Ras proteins nor did TcsL or TcdBF glucosylate Rho(A,B,C) (data not shown) (*5*, *6*). Thus, the partial activation of *rhoB* observed at high concentrations of TcdBF correlated with the partial glucosylation of N-Ras, whose inactivation reportedly triggers the activation of the *rhoB* promoter (*21*).

State of RhoB Activity. The state of RhoB activity in C3- and TcdB-treated fibroblasts was assessed by the Rhotekin (C21) pull-down assay (*29*). Although RhoB was upregulated, no active RhoB was found in C3-treated fibroblasts (Figure 4A); note that upregulated RhoB exhibited an apparent higher molecular weight (upper panel), indicating ADP-ribosylation, i.e., inactivation of RhoB (Figure 4A). In contrast, the level of active RhoB was elevated about 7-fold in TcdB-treated cells compared to untreated cells; thus, TcdB did not inactivate RhoB (Figure 4A). The pull-down assays were further analyzed for RhoA; it was completely inactive in both C3- and TcdB-treated cells (data not shown). The difference in the activity of RhoA and RhoB in TcdB-treated cells may be due to distinct glucosylation kinetics of RhoA and RhoB. To check this notion, recombinant RhoA, RhoB, and RhoC were subjected to [^{14}C]glucosylation by TcdB. RhoB was in fact glucosylated to a minor extent compared to RhoA and RhoC (Figure 4B). The presence of nonglucosylated RhoB in TcdB-treated cells was directly shown, exploiting the fact that unmodified RhoB was the substrate for C3, while glucosylated RhoB was not. As shown in Figure 4C, the cellular level of RhoB was elevated in TcdB-treated cells. RhoB from TcdB-treated cells was responsive to ADP-ribosylation by C3, shown by a shift to apparent higher molecular weight (Figure 4C). The glucosylation of Rac1 by TcdB was detected as an apparent decrease of the cellular Rac1 level applying anti-Rac1 (Mab102) (Figure 4C), an antibody that does exclusively react to nonglucosylated Rac1 (*30*). We recently showed that glucosylated RhoA was more efficiently degraded by the proteasome than nonmodified RhoA (*30*, *31*). The cellular level of RhoA was reduced in TcdB-treated cells, indicating glucosylation of RhoA (Figure 4C). Thus, the presence of active RhoB and the absence of active RhoA in TcdB-treated cells were most likely based on distinct kinetics of glucosylation.

Requirement of RhoB for the Cytotoxic Effect of TcdB. RhoB is involved in the initiation of apoptosis (cytotoxic effect) (*32*, *33*). Assuming that active RhoB is required for the cytotoxic effect, the cytotoxic effect may occur in TcdB-

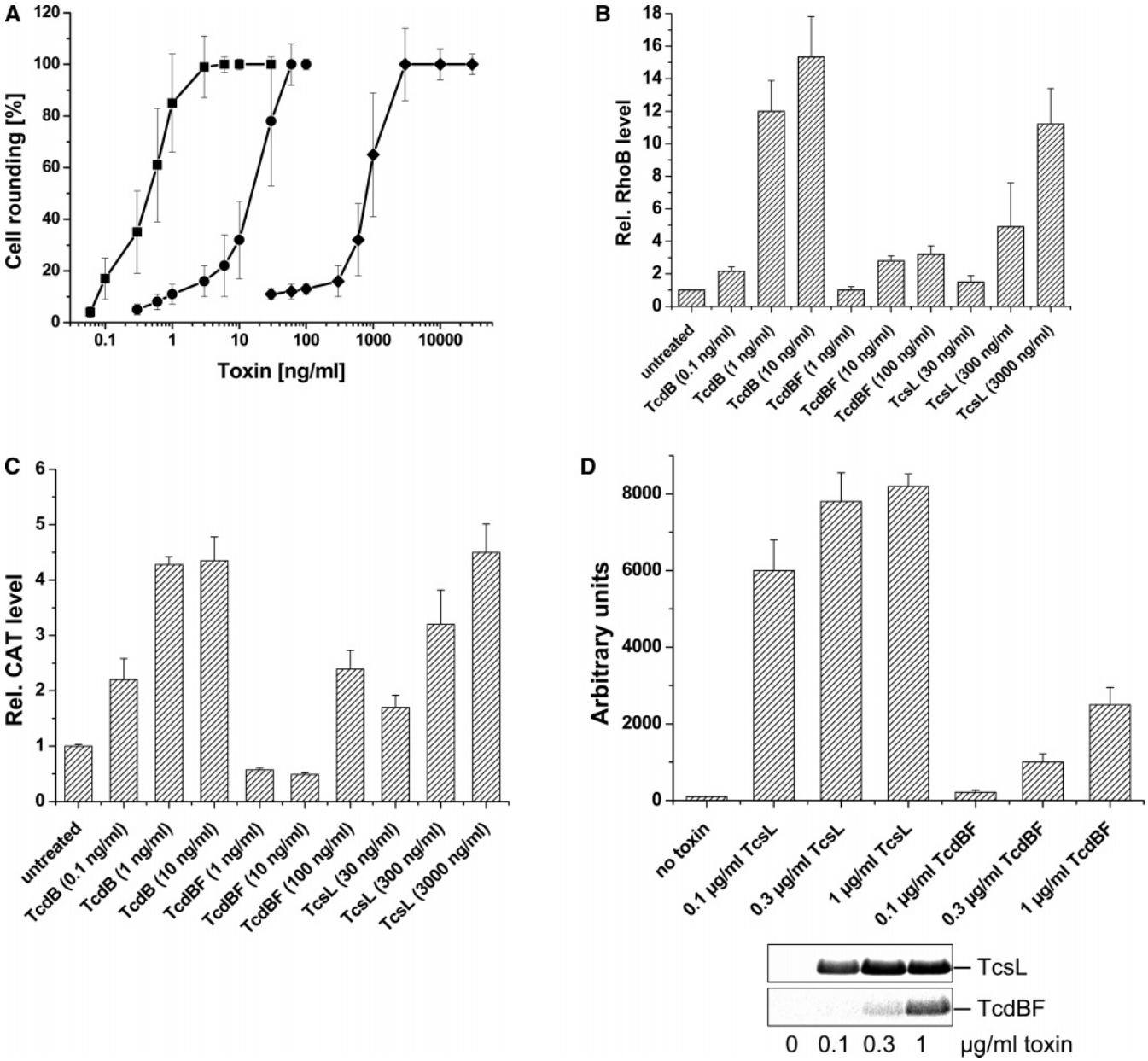

FIGURE 3: (A) Distinct kinetics of the action reorganization induced by toxin B (TcdB), variant toxin B (TcdBF), and lethal toxin (TcsL). Fibroblasts were exposed to increasing concentrations of either toxin for 4 h. Actin reorganization was quantified as the ratio of rounded per total cells (TcdB, ■; TcdBF, ●; TcsL, ◆). Data shown represent the mean of three independent experiments. (B) Variant toxin B (TcdBF) hardly induces upregulation of RhoB. Fibroblasts were exposed to increasing concentrations of TcdB, TcdBF, and TcsL for 4 h. The cells were harvested and analyzed for RhoB and β-actin using Western blotting. Signal intensities were recorded densitometrically; the RhoB signal was normalized to the β-actin signal. Results displayed represent the mean of three independent experiments. (C) The *rhoB* promoter is activated by TcdB and TcsL. Fibroblasts were transfected with 1.5 μg of the 3.5 kb *rhoB* promoter CAT construct followed by treatment of the cells with the indicated concentrations of TcdB, TcdBF, or TcsL for 7 h. Cells were harvested for analysis of CAT protein. Data shown represent the mean of three independent experiments. (D) Partial glucosylation of N-Ras by TcdBF. Recombinant N-Ras (50 μg/mL) was incubated with the indicated concentrations of TcsL or TcdBF in the presence of 20 μM UDP-[^{14}C]glucose for 20 min at 37 °C. The reaction was terminated by addition of Laemmli sample buffer followed by SDS−PAGE and analysis by autoradiography. Signal intensities were recorded densitometrically. Results displayed represent the mean of three independent experiments.

treated but not in C3-treated cells. The cytotoxic effect was analyzed in synchronized fibroblasts in the S-phase that are most susceptible to the cytotoxic effect of TcdB (*27*). TcdB concentration-dependently induced the cytotoxic effect as analyzed in terms of phosphatidylserine exposure (Figure 5A). Phosphatidylserine exposure was due to apoptotic cell death, as it was responsive to inhibition by Z-VAD(OMe)-FMK, a pan-caspase inhibitor (Figure 5A). Z-VAD(OMe)-FMK alone did not elevate the level of annexin V-positive cells (Figure 5A). In contrast to TcdB, C3 did not induce the cytotoxic effect (Figure 5B), even though it induced actin reorganization (data not shown). To provide direct evidence on the requirement of RhoB for the cytotoxic effect, S-phase fibroblasts were cotreated with TcdB and C3, the inhibitor of cellular RhoB (Figure 4A). C3 was added either 1 h before or 1 h after TcdB treatment to avoid any interference with the uptake of the two toxins (data not shown); the cytotoxic effect of TcdB was suppressed by C3 in either case (Figure 5B), suggesting that active RhoB was required for the cytotoxic effect of TcdB.

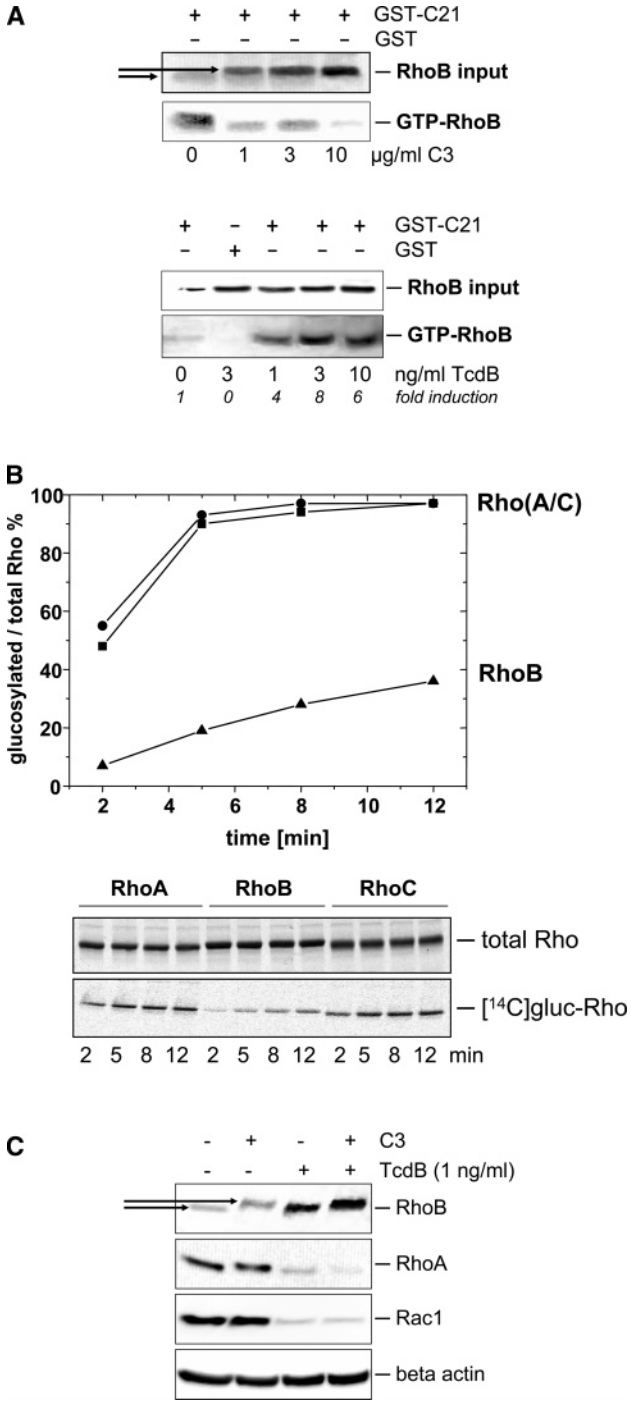

FIGURE 4: (A) Presence of active RhoB in TcdB-treated fibroblasts. Fibroblasts were treated with increasing concentrations of cell-permeable C3 or TcdB for 4 h. Cells were then lysed and the lysates submitted to the Rhotekin pull-down assay to assess RhoB activity. Bound RhoB was analyzed using Western blotting. (B) Distinct kinetics of the glucosylation of recombinant Rho proteins by toxin B (TcdB). Recombinant Rho proteins (50 μg/mL) were incubated with TcdB (1 μg/mL) in the presence of 20 μM UDP-[^{14}C]glucose for the indicated periods. The autoradiography (lower panel) of the SDS−PAGE (upper panel) is presented. Signal intensities were recorded densitometrically. Results displayed represent the mean of three independent experiments (RhoA, ■; RhoB, ▲; RhoC, ●). (C) Presence of nonglucosylated RhoB in TcdB-treated fibroblasts. Fibroblasts were incubated with or without TcdB (1 ng/mL) for 4 h. Cells were then lysed and the lysates incubated with or without *C. botulinum* C3 exoenzyme in the presence of 1 μM NAD for 30 min. The reaction was terminated by addition of Laemmli sample buffer. RhoB, RhoA, Rac1, and β-actin were analyzed by Western blotting.

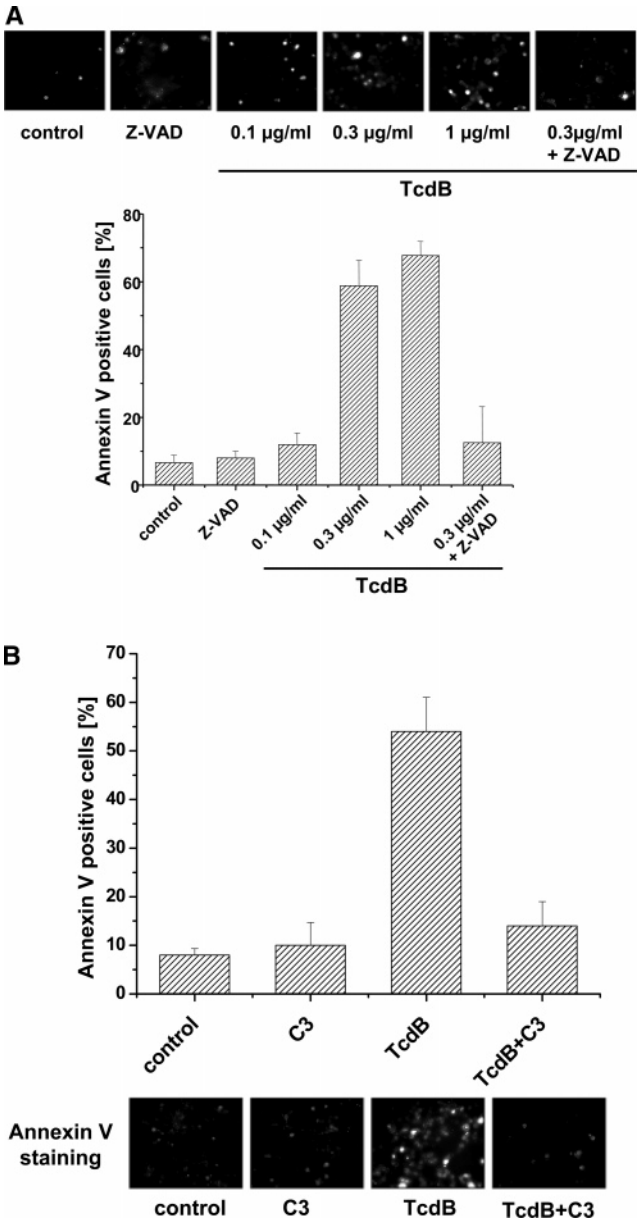

FIGURE 5: (A) The cytotoxic effect of TcdB is responsive to inhibition of caspase activity. Synchronized S-phase fibroblasts were exposed to increasing concentrations of TcdB as indicated for 12 h. The pan-caspase inhibitor [Z-VAD(OMe)-FMK] was added to the cells 1 h before the toxin treatment. Phosphatidylserine exposure was assessed by annexin V staining. The cytotoxic effect was quantified as the ratio of annexin V-positive per total cells. Results displayed represent the mean of three independent experiments. (B) C3 inhibits the cytotoxic effect of TcdB. Synchronized S-phase fibroblasts were treated with C3 (10 μg/mL) and/or TcdB (0.3 μg/mL) as indicated for 12 h. Phosphatidylserine exposure was then assessed by annexin V staining. The cytotoxic effect was quantified as the ratio of annexin V-positive per total cells. Results displayed represent the mean of three independent experiments.

DISCUSSION

The cellular level of RhoB is low in the majority of cell lines due to suppression of the activity of the *rhoB* promoter through (active) RhoA and Ras proteins, as constitutively active RhoA as well as constitutively active (H-,K-,N-)Ras suppresses the activity of the *rhoB* promoter and upregulation of RhoB (*20*−*22*). In this study, we show that activation of the *rhoB* promoter and upregulation of RhoB occurs once the suppression of the *rhoB* promoter was lifted by inactiva-

tion of RhoA or (H-,K-,N-)Ras by Rho-/Ras-inactivating toxins: (i) the Rho-ADP-ribosylating exoenzyme C3; (ii) the Rho-glucosylating TcdB; (iii) the (H-,K-,N-)Ras-glucosylating TcsL. The Rac/R-Ras-glucosylating TcdBF that did neither inactivate RhoA nor (H-,K-,N-)Ras failed to activate the *rhoB* promoter and to upregulate RhoB (*5*, *6*). At high concentrations, however, TcdBF lost its reported specificity for R-Ras, and further Ras proteins (exemplarily shown for N-Ras) involved in the regulation of *rhoB* were glucosylated. This partial glucosylation of Ras proteins most likely results in a less pronounced activation of *rhoB* and a faint upregulation of RhoB. Our experimental approach using exoenzyme C3 does not allow delineating if (besides the inactivation of RhoA) the inactivation of RhoB and RhoC further contributes to the activation of *rhoB*.

rhoB belongs to the family of immediate−early genes, some of which (e.g., the connective tissue growth factor, CTGF) are activated in response to changes of actin dynamics induced by actin depolymerizing agents (*34*, *35*). We observed neither activation of the *rhoB* promoter nor upregulation of RhoB protein in cells treated with C2 toxin or latrunculin B, confirming that RhoB is upregulated by a Rho/Ras-dependent mechanism rather than by actin reorganization. Furthermore, TcdB-induced upregulation of RhoB was not responsive to latrunculin B (unpublished observation), suggesting that upregulation of RhoB was independent of the cellular G-actin/F-actin ratio. In this respect, upregulation of RhoB can be classified as a specific stress response.

The activity of Rho proteins is intrinsically connected to the regulation of the actin cytoskeleton. Inactivation of Rho proteins by TcdB and C3 is reflected by actin reorganization ("cell rounding"). As upregulation of RhoB is also governed by inactivation of Rho(A,B,C), one must expect actin reorganization and upregulation of RhoB to exhibit comparable kinetics, which was in fact observed. Thus, both actin reorganization and upregulation of RhoB independently reflected the inactivation of Rho proteins in TcdB- or C3-treated cells.

Even though C3 strongly upregulated RhoB, no active RhoB was precipitated from C3-treated cells using the Rhotekin pull-down assay, confirming former notions that C3 is an efficacious inhibitor of both RhoA and RhoB. In contrast, an increased amount of active RhoB was precipitated from cells treated with the Rho-glucosylating toxins TcdB (this study) and TcdA (*23*). This observation was unexpected, as we and others found that recombinant Rho(A,B,C) are comparable substrates of the recombinant catalytic domain of TcdB (encompassing amino acids 1−546) (data not shown) (*36*). Applying TcdB purified from *C. difficile*, we found that RhoB was less efficiently glucosylated compared to RhoA and RhoC. Thus, TcdB purified from *C. difficile* and the recombinant catalytic domain of TcdB differ in their kinetics of RhoB glucosylation with RhoB being a poor substrate for TcdB purified from *C. difficile*.

An increased level of unmodified RhoB is most likely not active per se. The simultaneous and complete inactivation of RhoA, however, may allow the highly homologous RhoB to interact with orphaned RhoA guanine nucleotide exchange factors (GEFs).

We observed no effects of RhoB on the actin cytoskeleton. Thus, we focused on a possible role of RhoB in the regulation of toxin-induced apoptosis (cytotoxic effect). The cytotoxic effect of TcdB and C3 has been attributed to the inactivation of RhoA, based on reports showing that dominant-negative RhoA induced apoptosis in several cell lines (*15*, *17*, *37*, *38*). This notion is strongly supported by our recent data showing that the Rho-glucosylating TcdB but not the Rac/R-Ras-glucosylating TcdBF induced the cytotoxic effect (*27*). The notion that inactivation of RhoA induced apoptosis in several cell lines likely includes a role of RhoB, as dominant-negative RhoA reportedly activates *rhoB* (*20*−*22*). A role of RhoB in the regulation of apoptosis is well established in the cancer field (*32*, *39*). The Rho effector protein mDia is thereby a potential RhoB death effector, based on the role of mDia in coordinating actin−microtubule organization (*40*). RhoB is further reported to suppress NFκB and the survival kinase Akt in a PRK/PDK1-dependent manner, which may further contribute to the initiation of apoptosis (*21*, *41*, *42*). Finally, a direct interaction of RhoB with caspase 2 has been suggested (*43*).

The cytotoxic effect of C3 and TcdB was analyzed in synchronized fibroblasts in the S-phase (*27*). RhoB is physiologically upregulated during the S-phase, indicating that RhoB-dependent signaling is most likely crucial in this phase of the cell cycle (*44*). We observed a strong cytotoxic effect of TcdB, responsive to inhibition by a pan-caspase inhibitor, in line with reported data (*27*). In contrast, no apoptosis was observed in cells treated with C3. The initiation of the cytotoxic effect correlated with the presence of active RhoB (TcdB, active RhoB; C3, no active RhoB) leading to the assumption that active RhoB is required for the cytotoxic effect. This turned out to be true, as treatment of cells with C3, an inhibitor of RhoB, blocked the cytotoxic effect of TcdB. Accordingly, the cytotoxic effect of TcdA was also responsive to inhibition by C3 (unpublished observation). Thus, RhoB upregulated in TcdA/TcdB-treated cells escapes its inactivation and is required for the cytotoxic effect.

Fibroblasts are not sensitive to C3 directly applied to the medium. Therefore, C3 was delivered into fibroblasts, exploiting the uptake mechanism of the C2 toxin (*26*). Applying this C3/C2 toxin fusion construct, we and others did not observe the cytotoxic effect of C3 in fibroblasts (this study) or in a macrophage-like cell line (Heine and Barth, unpublished observation). In contrast, other researchers report on C3-induced apoptosis if C3 is either directly applied to endothelial cells (*15*, *45*), ectopically expressed in EL4 lymphoma cells (*17*), or delivered to myoblasts as HIV Tat fusion protein (*46*). It would be interesting to analyze if the cytotoxic effect of C3 (observed under the condition of an alternative route of uptake) depends on active RhoB. The different routes of the uptake may result in different intracellular localization of C3, which may influence the ADP-ribosylation of Rho(A,B,C).

In conclusion, RhoB is upregulated in response to Rho-/Ras-inactivating toxins. Upregulated RhoB may immediately be inactivated, if it is a substrate of the respective toxin (as shown for C3). If RhoB escapes from its inactivation (as shown for TcdA and TcdB), RhoB contributes to the death of the target cell (this study) or represses NFκB signaling (e.g., in macrophages), an effect that may reduce host defense, allowing the survival of the producing microbe (*41*, *47*).

There is increasing evidence that upregulation of RhoB is a general response of target cells to Rho-/Ras-inactivating pathogenicity factors. Besides the Rho-/Ras-inactivating clostridial toxins, pathogenic *Yersinia enterocolitica* have been shown to upregulate *rhoB* mRNA in macrophages, most likely triggered by the Rho-inactivating Cys protease *Y. enterocolitica* outer protein T (YopT) (*47*−*49*).

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