Killing of Rat Basophilic Leukemia Cells by Lethal Toxin from *Clostridium sordellii*: Critical Role of Phosphatidylinositide 3′-OH Kinase/Akt Signaling[†]

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ABSTRACT: *Clostridium sordellii* lethal toxin (TcsL) belongs to the family of clostridial glucosylating toxins. TcsL exhibits glucosyltransferase activity to inactivate Rho and Ras proteins. On cultured cells, TcsL causes actin reorganization ("cytopathic effect") and apoptotic cell death ("cytotoxic effect"). This study is based on the concept that the cytotoxic effects of TcsL depend on the glucosylation of critical substrate proteins rather than on the glucosyltransferase activity *per se*. The cytotoxic effects of TcsL depend on the glucosyltransferase activity of TcsL, as neither chemically inactivated TcsL nor a glucosyltransferase-deficient mutant version of TcsL caused it. The TcsL homologous toxin B from *Clostridium difficile* serotype F strain 1470 (TcdBF) also failed to cause cytotoxic effects. Correlation of the toxins' respective protein substrate specificities highlighted (H/K/N)Ras as critical substrate proteins for the cytotoxic effects. (H/K/N)Ras are critical upstream regulators of phosphatidylinositide 3′-OH kinase (PI3K)/Akt survival signaling. Tauroursodeoxycholic acid (TUDCA) classified to activate PI3K/Akt signaling downstream of apoptosis-inducing stimuli prevented the cytotoxic effects of TcsL. In conclusion, (H/K/N)Ras glucosylation and subsequent inhibition of PI3K/Akt signaling are critical for the cytotoxic effects of TcsL.

Clostridium sordellii is a Gram-positive, spore-forming, anaerobic bacterium, which causes rapidly progressing myonecrosis in obstetric patients and necrotizing fasciitis in injection drug users, in severe cases accompanied by a fulminant toxic shock syndrome (*1, 2*). The severity of *C. sordellii*-associated disease is due to the release of soluble virulence factors, the most toxic (in terms of the LD_{50}) among them the lethal toxin (TcsL)[1] and the hemorrhagic toxin (TcsH) (*3*). In an animal model, TcsL causes increased lung vascular permeability without major signs of inflammation (*4*). In cell culture models, TcsL induces reorganization of the actin cytoskeleton ("cytopathic effect") and apoptotic cell death ("cytotoxic effect") (*5, 6*).

TcsL is a single-chained protein with a molecular mass of about 270 kDa. It exhibits an AB toxin-like structure with an N-terminal glucosyltransferase domain and a C-terminal delivery domain, with the latter allowing target cell entry by receptor-mediated endocytosis (*7*). The glucosyltransferase domain (covering amino acids 1−543) causes monoglucosylation of low molecular weight GTP-binding proteins of the Rho and Ras subfamily. The glucose moiety is covalently linked to a pivotal threonine residue (Thr-35 in (H/K/N)Ras or Rac1) within their effector region (*8, 9*). Glucosylation uncouples the Rho/Ras proteins from their effector and regulatory proteins and causes their functional inactivation (*10*−*13*).

(H/K/N)Ras regulate cellular survival through a network of signal transduction pathways, including phosphatidylinositide 3′-OH kinase (PI3K)/Akt, RalGEF/Ral, and Raf/ERK (*14*). These pathways predominantly lead to activation or inhibition of transcription factors (e.g., NFkappaB, Elk-1, AFX) that regulate expression of both pro- and antiapoptotic proteins. One example is the proapoptotic GTP-binding protein RhoB that is suppressed by (H/K/N)Ras in a PI3K/Akt-dependent manner (*15*). Inactivation of (H/K/N)Ras by either farnesyltransferase inhibitors or TcsL results in "desuppression", i.e., upregulation of RhoB (*16, 17*).

Currently, there are two models suggested on how TcsL induces cytotoxic effects: (i) TcsL-catalyzed Ras glucosylation blocks critical cellular survival pathways such as the Ras/PI3K/Akt/GSKbeta pathway (*18*); (ii) TcsL (as well as the related toxin B from *Clostridium difficile* (TcdB)) causes apoptosis by a not yet identified toxin activity on mitochondria, independent of the glucosyltransferase activity (*6, 19*).

In this study, we provide evidence that the glucosyltransferase activity of TcsL is required for the cytotoxic effect. In contrast to the related toxin A from *Clostridium difficile* (TcdA), glucosyltransferase-deficient mutants of full-length

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[1] Abbreviations: CS546, glucosyltransferase domain (covering amino acids 1−546) of lethal toxin from *Clostridium sordellii* strain 6018; dial-TcsL, lethal toxin from *C. sordellii* alkylated with UDP-2′,3′-dialdehyde; ExoS, exoenzyme S from *Pseudomonas aeruginosa*; LatB, latrunculin B; RBL cells, rat basophilic leukemia cells; SLO, streptolysin O; STS, staurosporine; TcdA, toxin A from *Clostridium difficile* strain VPI10463; TcdB, toxin B from *C. difficile* strain VPI10463; TcdBF, toxin B from *C. difficile* serotype F strain 1470; TcsH, hemorrhagic toxin from *C. sordellii*; TcsL, lethal toxin from *C. sordellii*; TUDCA, tauroursodeoxycholic acid.

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TcsL are not available (*20*). This lack of mutant TcsL is circumvented here by two methods: (i) transport of the glucosyltransferase domain of TcsL (CS546, encompassing amino acids 1−546) and the respective glucosyltransferase-deficient mutant CS546-D286A-D288A into RBL cells using the streptolysin O delivery system (*21*); (ii) treatment of (full-length) TcsL with UDP-2′,3′-dialdehyde (dial-TcsL), an alkylating derivative of the TcsL cosubstrate UDP-glucose (*22*). Correlation of the biological activities of TcsL and the homologous TcdBF revealed that glucosylation of (H/K/N)Ras is required for the cytotoxic effects.

The endogenous hydrophilic bile acid ursodeoxycholic acid (UDCA) and its taurine conjugate tauroursodeoxycholic acid (TUDCA) act as antiapoptotic agents. Although their exact mode of action remains to be evaluated, there is good evidence that TUDCA exerts its antiapoptotic activity through a PI3K-dependent mechanism (*23, 24*). Recent data have implicated PI3K in promoting survival downstream of apoptosis-inducing stimuli. Applying TUDCA, an activator of PI3K/Akt signaling, we provide evidence on the critical role of PI3K/Akt signaling in the cytotoxic effects of TcsL.

MATERIALS AND METHODS

Materials. Commercially obtained reagents: caspase inhibitor I (Z-VAD(OMe)-FMK), caspase-3 inhibitor (Ac-DMQD-CHO), LY294002, latrunculin B, staurosporine (Calbiochem); WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate sodium salt) (Roche); Annexin V-Alexa Fluor 488 (Invitrogen); streptolysin O, tauroursodeoxycholic acid (TUDCA) (Sigma); anti-Rac1 (clone 102; BD Transduction Laboratories); anti-Rac1 (clone 23A8; Upstate Technologies); anti-caspase-3 (Acris); anti-β-actin (clone AC-40; Sigma); anti-RhoB (sc-8048; SantaCruz); anti-phospho-Akt (Ser-473) (Cell Signaling Technologies); horseradish peroxidase conjugated secondary antibodies mouse or rabbit (Rockland).

Toxin Purification. TcdBF, TcsL-6018, and TcsL-9048 were purified from the *C. difficile* serotype F strain 1470, the *C. sordellii* strain 6018 (identical to strain IP-82), and the *C. sordellii* strain 9048, respectively, as described (*25*). In brief, a dialysis bag containing 900 mL of 0.9% NaCl in a total volume of 4 L of brain heart infusion (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 eluted with a NaCl gradient ranging from 0 to 800 mM and subsequently dialyzed against buffer (50 mM Tris-HCl, pH 7.5, 15 mM NaCl). While the *C. sordellii* strain 6018 solely produces TcsL, the *C. sordellii* strain VPI9048 produces both the Rac/Ras-glucosylating TcsL and the Rho/Rac/Cdc42-glucosylating TcsH-9048 (*26*). No glucosylation of Rho(A/B/C) was detected, confirming that TcsL-9048 was free from TcsH-9048.

Preparation of Recombinant CS546 and CS546-AxA. The recombinant glucosyltransferase domain of TcsL (CS546) and its respective glucosyltransferase-deficient mutant CS546-D286A-D288A (CS546-AxA) were expressed as GST fusion proteins in *Escherichia coli* and purified by affinity chromatography using glutathione−Sepharose. CS546 and CS546-AxA were obtained by thrombin cleavage.

Preparation of Recombinant Rho/Ras Proteins. Vectors encoding for human Rho(A,B,C,D,E,G), TC10, Cdc42, (H,K,N,M,R)Ras, Rap(1,2), and Ral(A,B) were obtained from the Guthrie cDNA Resource Center. Fragments containing the coding regions of each of these genes were generated by PCR and subcloned into the expression vector pGEX-2T. The GST-tagged fusion proteins were isolated by affinity purification with glutathione−Sepharose (Amersham).

In Vitro Glucosylation of Rho/Ras Proteins. Recombinant GTP-binding proteins (1 μM) in glucosylation buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 100 mM KCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 100 μg/mL BSA) were incubated with TcsL-6018, TcsL-9048, or TcdBF (1 μg/mL) in the presence of UDP-[^{14}C]glucose (10 μM). After incubation at 37 °C for 20 min, the reaction was terminated by the addition of Laemmli sample buffer, and incorporated [^{14}C]glucose was detected by phosphorimager analysis.

Generation of Alkylated TcsL (Dial-TcsL). Alkylated TcsL was generated by treatment of TcsL with 0.3 mM UDP-2′,3′-dialdehyde at 37 °C for 24 h in alkylation buffer (50 mM Tris-HCl, pH 7.5, 15 mM NaCl, 2 mM $MnCl_2$), as described for the related TcdB (*22*). Dial-TcsL was subsequently dialyzed against dialysis buffer (50 mM Tris-HCl, pH 7.5, 15 mM NaCl) to remove excess of UDP-dialdehyde. The residual activity of dial-TcsL was analyzed in terms of Rac1 glucosylation in RBL cells (see below) (*25*). The glucosyltransferase activity of dial-TcsL was reduced by 2−3 orders of magnitude compared to TcsL.

Cell Culture. Rat basophilic leukemia (RBL-2H3) cells that express (H/K)Ras but not N-Ras (*27*) were grown as adherent monolayers on tissue culture flasks in minimum essential medium plus Earle's salts (MEM plus Earle's; Biochrom) supplemented with 15% heat-inactivated fetal calf serum, 100 μg/mL penicillin, 100 units/mL streptomycin, and 1 mM sodium pyruvate. Cells were maintained in 5% CO_2 at 37 °C. Upon confluence, cells were passaged.

Synchronization of Cells. Cells were synchronized by the thymidine double-block technique as described before (*28*). Exponentially dividing cells were incubated in medium containing 2 mM 2′-deoxythymidine for 19 h at 37 °C and 5% CO_2. The medium was removed, and a growth phase of 9 h in thymidine-free medium at 37 °C and 5% CO_2 was conducted. The cells were synchronized at the next G1/S boundary by incubating in medium containing 2 mM 2′-deoxythymidine for an additional 16 h. After a growth phase of 1 h in thymidine-free medium at 37 °C, cells were treated with the toxins or drugs as indicated and subsequently harvested.

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 sonified 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 chemoluminescence reaction, ECL Femto (Pierce) was used. All signals were analyzed densitometrically using the KODAK 1D software.

Evaluation of Equipotent Toxin Concentration. The glucosylating toxins differ in their kinetics of cellular uptake and substrate glucosylation. To compare the biological effect of glucosylating toxins, their equipotent toxin concentrations have to be evaluated (*28*). Therefore, Rac1 glucosylation and the cytopathic effect were concentration-dependently analyzed in RBL cells. Cellular Rac1 was glucosylated in response to either toxin, as detected in terms of apparently decreasing Rac1 level using the glucosylation-sensitive Rac1-mAb(102) (*25*). The cytopathic effect that was quantified in terms of rounded per total cells increased in RBL cells treated with either toxin. TcsL was by about 1 order of magnitude more potent than TcdBF regarding the cytopathic effect as well as Rac1 glucosylation.

Reversible Permeabilization with Streptolysin O. Cells were incubated in 500 μL of HBSS (Hank's buffered salt solution) without Ca^{2+} containing 30 mM Hepes, pH 7.2, for 15 min. Streptolysin O (SLO; 0.1 μg/mL) was then added together with recombinant toxin fragments as indicated for 15 min at 37 °C and 5% CO_2. To reseal, 1.5 mL of ice-cold HBSS containing 30 mM Hepes and 2 mM Ca^{2+}, pH 7.2, was added. After incubation for 1 h at 37 °C, HBSS was replaced by full growth medium.

Detection of Phosphatidylserine Exposure by Annexin V Staining. Cells were exposed to the toxins or drugs as indicated. Annexin V labeled with Alexa Fluor 488 (Invitrogen) was added directly into the medium, according to the manufacturer's instructions. After incubation at 37 °C for 15 min, apoptotic cells were visualized by fluorescence microscopy using a Zeiss Axiovert 200 M (Annexin V-Alexa Fluor 488: excitation 470 nm; emission 515 nm).

Caspase-3 Activity. Caspase-3 activity was assessed using Caspase-3 colorimetric assay (R&D Systems), according to the manufacturer's instructions. Cells were exposed to the toxins and drugs as indicated. Cells were lysed and subsequently incubated with the colorimetric substrate DEVD-pNA at 37 °C for 3 h. The caspase-3-dependent release of the reporter molecule pNA was quantified by photometry using a scanning multiwell spectrophotometer at 405 nm.

WST-1 Test. The activity of cellular dehydrogenases was assessed using WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate (Roche)), according to the manufacturer's instructions. Cells were exposed to the toxins or drugs as indicated. WST-1 was added directly into the medium. The reduction of WST-1 to formazan by mitochondrial dehydrogenases was quantified by photometry using a scanning multiwell spectrophotometer at 450 nm.

RESULTS

The Cytotoxic Effect of TcsL Depends on Its Glucosyltransferase Activity. Unlike other myeloid cell lines, adherently growing RBL cells exhibit a spindle-shaped morphology including dendrite-like extrusions (Figure 1A). TcsL-6018 and TcsL-9048 induced rounding of S-phase synchronized RBL cells ("cytopathic effect") (Figure 1A). Either toxin further caused phosphatidylserine exposure in almost the complete population of cells (Figure 1). Both TcsL isoforms were nearly as efficient as staurosporine (STS), a broad-spectrum inhibitor of serine/threonine protein kinases (Figure 1). In contrast, the actin-depolymerizing marine toxin latrunculin B (LatB) did not cause phosphatidylserine exposure (Figure 1), excluding actin reorganization as trigger (Figure 1). Only a subpopulation of nonsynchronous RBL cells (most likely the S-phase population) was sensitive to the cytotoxic effect of TcsL (Figure 1), in line with previous reports (*6, 29*). Regardless of synchronization, the complete population of cells was susceptible to the cytotoxic effect of staurosporine (Figure 1).

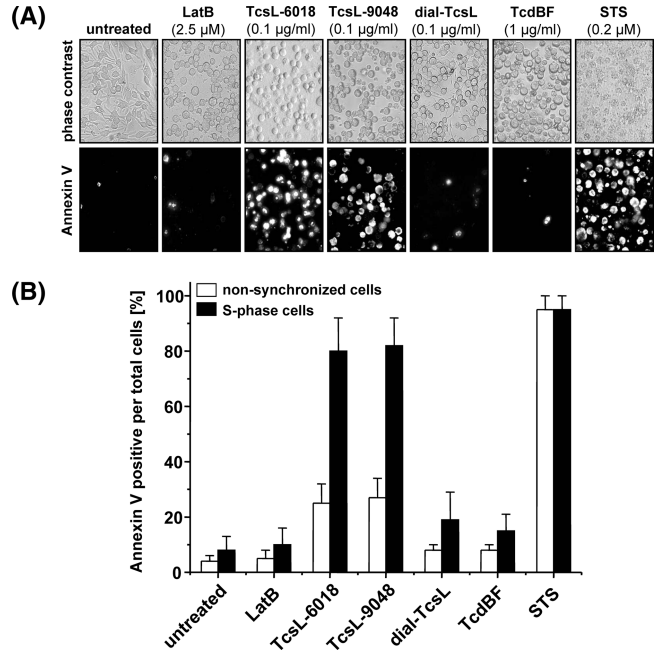

FIGURE 1: The cytotoxic effect of TcsL. (A) The cytotoxic effect of TcsL on S-phase RBL cells. S-phase synchronized RBL cells were incubated with TcsL-6018 (0.1 μg/mL), TcsL-9048 (0.1 μg/mL), dial-TcsL-6018 (0.1 μg/mL), TcdBF (1 μg/mL), latrunculin B (LatB, 2.5 μM), or staurosporine (STS, 0.2 μM) or left untreated for 24 h. All toxins caused morphological changes in RBL cells, as shown by the phase contrast microcopy (upper panel). TcsL-6018, TcsL-9048, and staurosporine induced apoptosis, as visualized by Annexin V staining of extracellular phosphatidylserine (lower panel). (B) Difference in the cytotoxic effect of TcsL in S-phase and nonsynchronized RBL cells. The cytotoxtic effect of TcsL was quantified as Annexin V stained per total cells. RBL cells were treated with the toxins for 24 h as indicated in panel A. Apoptosis was more pronounced in S-phase synchronized (filled bars) than in nonsynchronized (empty bars) RBL cells. Results are the mean $\pm$ SD of 100 cells for each condition ($n = 3$).

The level of the active form of executioner caspase-3 was analyzed in TcsL-treated RBL cells. TcsL-6018 (and TcsL-9048, data not shown) caused cleavage of caspase-3, as the level of its active form (17/19 kDa) increased in a TcsL concentration-dependent manner (Figure 2A). In RBL cells treated with stauroporine, the increase in the active form of caspase-3 was accompanied by a decrease of the pro form (data not shown). In contrast, the level of the pro-caspase-3 remained unchanged in TcsL-treated cells (Figure 2A), which may be based on protein *de novo* synthesis of pro-caspase-3. Caspase-3 activation was further analyzed in terms of the cleavage of its colorimetric substrate DEVD-pNA. TcsL-6018 and staurosporine strongly increased caspase-3 activity, whereas caspase-3 activation by latrunculin B was less

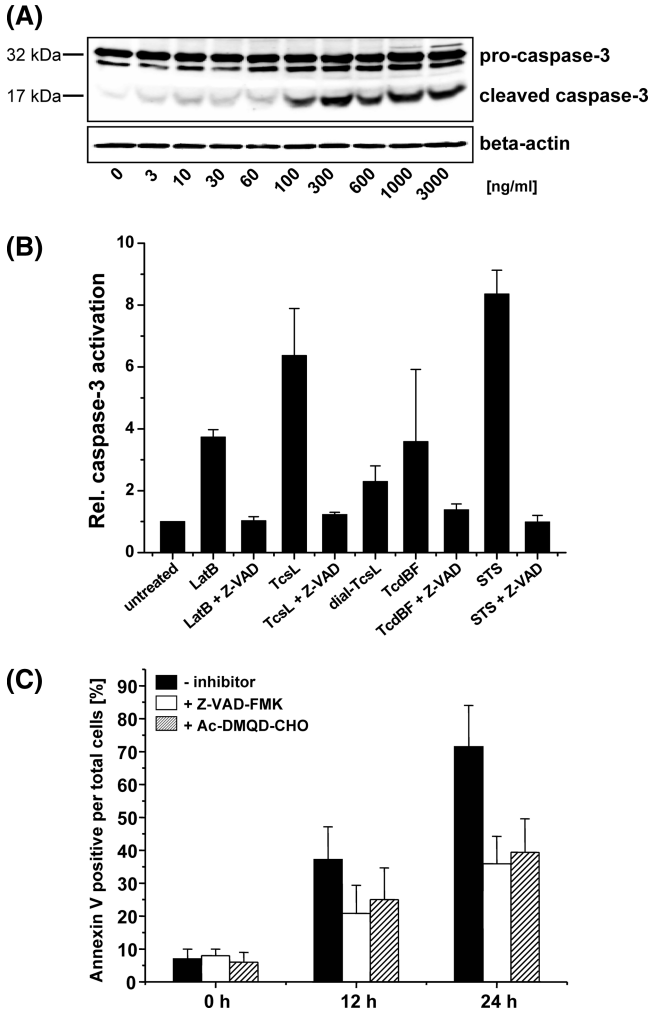

FIGURE 2: Involvement of caspase-3 in the cytotoxic effect of TcsL. (A) Caspase-3 cleavage induced by TcsL-6018 in S-phase RBL cells. S-phase synchronized RBL cells were exposed to the indicated concentrations of TcsL-6018 for 3 h. The cellular levels of pro-caspase-3 (32 kDa, inactive), caspase-3 (17 kDa, active), and β-actin were analyzed by Western blot analysis. Western blots from representative experiments are shown ($n = 3$). (B) Caspase-3 activation induced by TcsL-6018 in RBL cells. RBL cells were treated with Z-VAD(OMe)-FMK, a broad spectrum inhibitor of caspases (pan-caspase inhibitor, 20 μM) or left untreated for 1 h. Latrunculin B (LatB, 2.5 μM), TcsL-6018 (0.1 μg/mL), dial-TcsL (0.1 μg/mL), TcdBF (1 μg/mL), or staurosporine (STS, 0.2 μM) was then added as indicated and incubation continued for a further 6 h. Cells were then lysed and submitted to the colorimetric caspase-3 activity assay using the caspase-3 substrate DEVD-pNA. The caspase-3 activity of untreated cells was set to 1. Results are the mean $\pm$ SD ($n = 3$). (C) Inhibition of TcsL-6018-induced phosphatidylserine exposure by caspase inhibitors. S-phase synchronized RBL cells were pretreated with either Z-VAD(OMe)-FMK, a broad spectrum inhibitor of caspases (20 μM, empty bars) or Ac-DMQD-CHO, a caspase-3-specific inhibitor (20 μM, striped bars) or left untreated (filled bars) for 1 h. Then, TcsL-6018 (0.1 μg/mL) was added, and incubation was continued. After 12 and 24 h, phosphatidylserine exposure was visualized by Annexin V staining. The cytotoxic effect was quantified as the ratio of Annexin V positive per total cells. Results are the mean $\pm$ SD of 100 cells for each condition ($n = 3$).

pronounced (Figure 2B). TcdBF induced activation of caspase-3 to some extent (Figure 2B), which did not result in phosphatidylserine exposure (Figure 1). Thus, caspase-3 cleavage by TcsL-6018 was reflected by caspase-3 activation. Caspase-3 activation induced by either toxin or drug was completely suppressed by Z-VAD(OMe)-FMK, a broad-spectrum inhibitor of caspases. TcsL-induced phosphatidylserine exposure was responsive to inhibition by either Z-VAD(OMe)-FMK or Ac-DMQD-CHO, a specific caspase-3 inhibitor (Figure 2C). Thus, TcsL-6018-induced phosphatidylserine exposure was caspase-dependent. Furthermore, TcsL-6018 and TcsL-9048 reduced the viability of TcsL-treated RBL cells by about 50%, as analyzed using the WST-1 test (Figure 3). Staurosporine-treated RBL cells were not viable. In contrast, latrunculin B had no effect on viability (Figure 3). Actin reorganization was thus not the trigger of the cytotoxic effect of TcsL, in line with a former report (*6*).

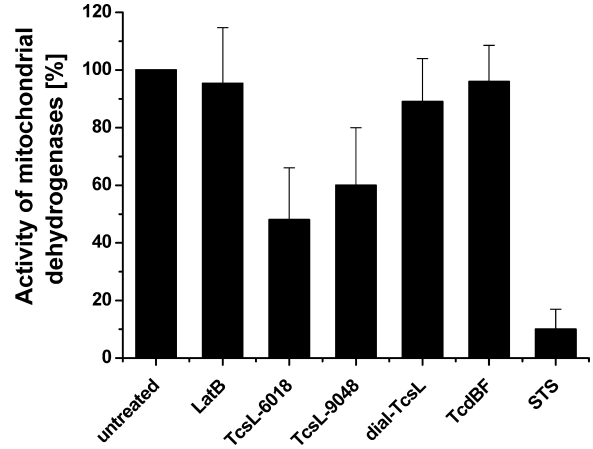

FIGURE 3: Viability of TcsL-treated RBL cells. S-phase synchronized RBL cells were exposed to TcsL-6018 (0.1 μg/mL), TcsL-9048 (0.1 μg/mL), dial-TcsL-6018 (0.1 μg/mL), TcdBF (1 μg/mL), latrunculin B (LatB, 2.5 μM), or staurosporine (STS, 0.2 μM) or left untreated for 24 h. Cell viability was assessed by the WST-1 assay. The viability of untreated cells was set 100%. Results are the mean $\pm$ SD ($n = 3$).

It is under debate whether the cytotoxic effect of TcsL depends on its glucosyltransferase activity. A glucosyltransferase-deficient TcsL mutant is not available. Therefore, the glucosyltransferase domain of TcsL was chemically inactivated using UDP-2′,3′-dialdehyde (dial-TcsL), an alkylating derivative of its cosubstrate UDP-glucose (*22*). It is conceivable that dial-TcsL binds to its (not yet identified) cell surface receptor, as presented for the related TcdB (*22*). The glucosyltransferase activity of dial-TcsL was reduced by about 2−3 orders of magnitude (data not shown). Due to its residual activity, dial-TcsL caused morphological changes of RBL cells (Figure 1A). Dial-TcsL caused neither phosphatidylserine exposure (Figure 1) nor a pronounced caspase-3 activation (Figure 2B) nor a reduction of cell viability (Figure 3). These findings (i) exclude that TcsL induces its cytotoxic effects through binding to its cell surface receptor and (ii) strongly suggest that the cytotoxic effect depends on the glucosyltransferase activity of TcsL. To confirm this hypothesis, the glucosyltransferase domain of TcsL-6018 (CS546, covering amino acids 1−546) was transported into RBL cells using the pore-forming toxin streptolysin O (SLO) as a delivery system (*21*). CS546 induced the cytopathic effect in S-phase synchronized RBL cells (Figure 4) comparable to full-length TcsL-6018 (Figure 1A). Mutation of the DxD motif results in glucosyltransferase-deficient CS546-D286A-D288A (*30*). Application of CS546-D286A-D288A to RBL cells induced (minor) morphological changes, showing a residual activity of CS546-D286A-D288A (Figure 4). Application of CS546 (but not mutant CS546-D286A-

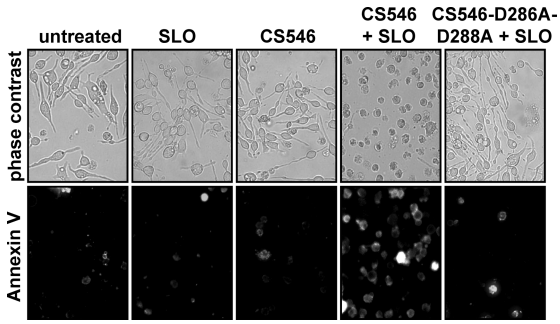

FIGURE 4: The cytotoxic effect of the glucosyltransferase domain of TcsL-6018 (CS546). CS546 was applied to S-phase synchronized RBL cells using the streptolysin O (SLO) delivery system. Cells were incubated with CS546 (0.1 μg/mL), SLO (0.1 μg/mL), or a combination of either CS546 (0.1 μg/mL) or CS546-D286A-D288A (0.1 μg/mL) with SLO (0.1 μg/mL) or left untreated for 24 h. Either CS546 or CS546-D286A-D288A delivered to RBL cells by SLO caused morphological changes in RBL cells, as shown by phase contrast microscopy (upper panel). CS546 (not CS546-D286A-D288A) delivered to RBL by SLO induced phosphatidylserine exposure, as visualized by Annexin V staining (lower panel).

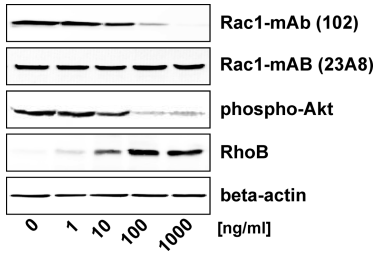

FIGURE 5: Effects of TcsL-6018 on PI3K/Akt signaling. S-phase synchronized RBL cells were exposed to the indicated concentrations of TcsL-6018. After 3 h, cells were lyzed and analyzed for the levels of Rac1, phospho-Akt, RhoB, and β-actin using Western blot analysis. Western blots from representative experiments are shown ($n = 3$).

D288A) induced phosphatidylserine exposure (Figure 4). Cells treated with either SLO or CS546 alone exhibited no increase of Annexin V positive cells (Figure 4). These findings suggest that the glucosyltransferase activity of TcsL was sufficient for the cytotoxic effect of TcsL.

Inhibition of PI3K/Akt Signaling in TcsL-Treated RBL Cells. TcsL glucosylates low molecular weight GTP-binding proteins of the Rho and Ras subfamilies. To confirm substrate glucosylation by TcsL in RBL cells, we analyzed Rac1 glucosylation using the glucosylation-sensitive Rac1-mAb (clone 102) (*25*). TcsL concentration-dependently glucosylated Rac1, as evidenced by the apparently decreasing Rac1 level (Figure 5). This decreased level was due to Rac1 glucosylation but not due to Rac1 degradation, as the level of total Rac1 (analyzed by Rac1-mAb (clone 23A8)) was unchanged in TcsL-treated RBL cells (Figure 5).

Akt is a serine/threonine kinase that mediates (H/K/N)Ras-dependent survival signaling. Its activity was analyzed in terms of phosphorylation (indicative of activation). The level of phosphorylated Akt decreased in TcsL-treated RBL cells in a concentration-dependent manner (Figure 5), corroborating recent findings (*18*).

A downstream target of PI3K/Akt signaling is the immediate-early gene product RhoB (*15*). RhoB, a member of the Rho subfamily of GTP-binding proteins, is classified as a tumor suppressor protein due to its proapoptotic activity (*31, 32*). The *rhoB* promoter is suppressed by (H/K/N)Ras in a PI3K/Akt-dependent manner (*15, 16*). RhoB was "desuppressed", i.e., strongly upregulated in response to increasing concentrations of TcsL (Figure 7B), further indicating inhibited PI3K/Akt signaling in TcsL-treated cells.

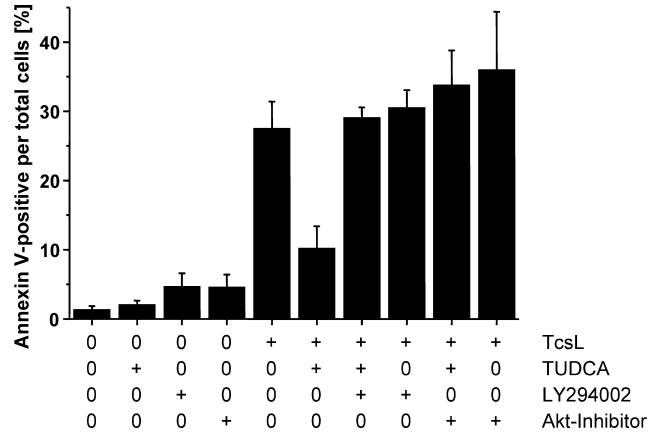

FIGURE 6: Critical role of PI3K/Akt signaling in the cytotoxic effect of TcsL. S-phase synchronized RBL cells were pretreated with TUDCA (300 μM) or left untreated for 1 h. LY294002 (10 μM) or Akt inhibitor I (10 μM) was then added and incubation continued for 1 h. Subsequently, cells were exposed to TcsL-6018 (0.1 μg/mL) or left untreated for 12 h. Phosphatidylserine exposure was visualized by Annexin V staining; the cytotoxic effect was expressed as the ratio of Annexin V positive per total cells. Results are the mean $\pm$ SD of 100 cells for each condition ($n = 3$).

Preservation of the Cytotoxic Effect of TcsL by TUDCA. Voth and Ballard have hypothesized that inhibition of Ras/PI3K/Akt/GSKbeta signaling is critical for the cytotoxic effect of TcsL (*18*). If this hypothesis was true, preservation of PI3K/Akt signaling by TUDCA, an activator of PI3K/Akt signaling, should prevent the cytotoxic effect of TcsL. The cytotoxic effect of TcsL was analyzed in RBL cells pretreated with TUDCA or left untreated. TUDCA prevented the cytotoxic effects of TcsL, as analyzed in terms of phosphatidylserine exposure (Figure 6). This protective effect of TUDCA depended on PI3K activation, as it was blocked by LY294002, a specific inhibitor of PI3K (Figure 6). The protective effect of TUDCA was further responsive to inhibition by a 3-(hydroxymethyl)-bearing phosphatidylinositol analogue that specifically inhibits Akt (Akt inhibitor) (*33*). Thus, TUDCA acted through activation of PI3K/Akt. Inhibition of PI3K/Akt signaling is most likely critical for the cytotoxic effect of TcsL.

Differences in the Biological Activity of TcsL and TcdBF. To further confirm that Ras/PI3K/Akt signaling is critical for the cytotoxic effect of TcsL, we made use of toxin B from the variant *C. difficile* serotype F strain 1470 (TcdBF). TcdBF has been characterized as a functional hybrid between TcdB and TcsL: TcdBF shares the cell surface receptor of TcdB but supposedly glucosylates an identical panel of Rho/Ras substrate proteins as TcsL (*34, 35*). Both TcsL and TcdBF exhibited the cytopathic effect (Figure 1A) but differed in their cytotoxic effect: In contrast to TcsL-6018, TcdBF (applied with a concentration of 1 μg/mL, equipotent to 100 ng/mL TcsL) caused neither phosphatidylserine exposure (Figure 1) nor pronounced caspase-3 activation (Figure 2B) nor a reduction of cell viability (Figure 3). TcdBF thus failed to cause cytotoxic effects even in highly sensitive S-phase cells. These findings show that glucosyltransferase activity *per se* is not sufficient to cause the

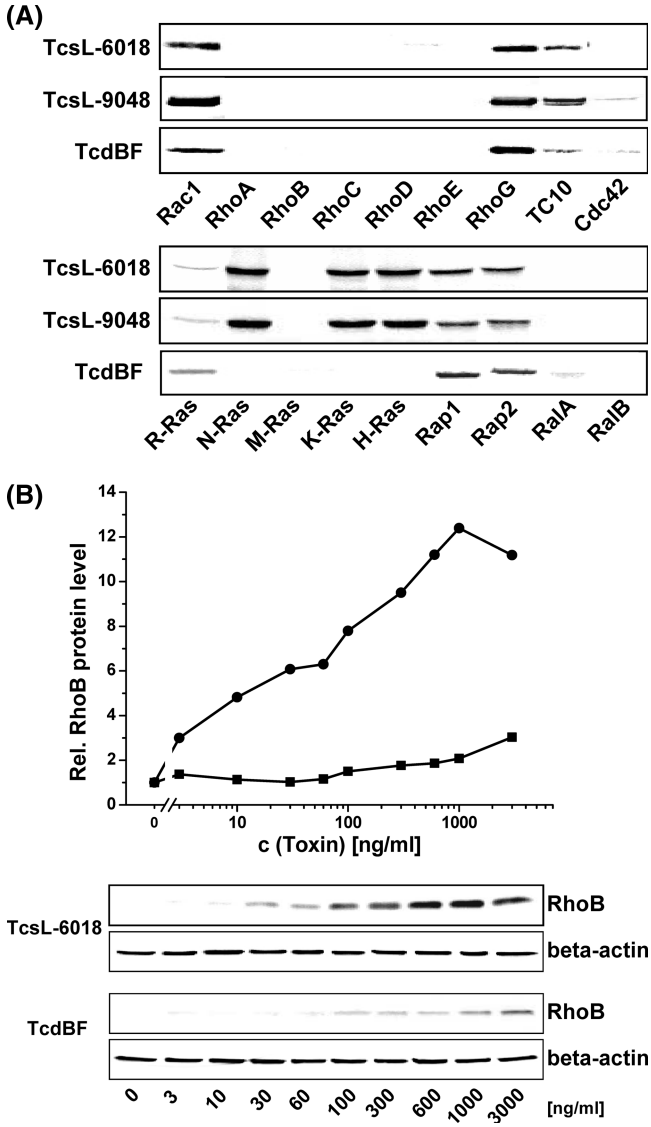

FIGURE 7: Comparison of the effects of TcsL and TcdBF. (A) Glucosylation of recombinant Rho/Ras proteins by TcsL-6018, TcsL-9048, and TcdBF. Recombinant Rho and Ras proteins (1 μM each) were incubated with the indicated toxins (5 μg/mL) in the presence of UDP-[^{14}C]glucose (10 μM) for 30 min. [^{14}C]Glucosylated proteins were detected by phosphorimager analysis after separation on SDS−PAGE. (B) RhoB level in toxin-treated cells. S-phase RBL cells were exposed to the indicated concentrations of TcsL-6018 (●) or TcdBF (■) for 3 h. The cellular level of RhoB and β-actin was analyzed by Western blot analysis. Western blots from representative experiments are shown. Signal intensities were recorded densitometrically. For quantification, the RhoB signal was normalized to the β-actin signal. The RhoB level of untreated cells was set to 1. Data shown represent the mean of three independent experiments.

cytotoxic effects. The glucosylation of critical Rho/Ras proteins (targeted by TcsL but not by TcdBF) may be required for the cytotoxic effect. (H/K/N)Ras are appropriate candidates, as they are the upstream activators of PI3K/Akt signaling. We thus expected that TcsL but not TcdBF glucosylates (H/K/N)Ras. The protein substrate specificities of the toxins were reanalyzed exploiting their *in vitro* glucosyltransferase activity applying recombinant Rho and Ras proteins. Both TcsL-6018 and TcsL-9048 glucosylated the Rho proteins Rac1, RhoG, and TC10, as well as the Ras proteins (H/K/N/R)Ras and Rap(1,2) (Figure 7A). Reanalysis of the TcdBF substrate specificity revealed that TcdBF glucosylated Rac1, RhoG, and TC10 and the Ras proteins R-Ras and Rap(1,2) (Figure 7A). In contrast to TcsL, TcdBF did not glucosylate (H/K/N)Ras, those Ras isoforms positively regulating PI3K/Akt signaling. To show that this difference in (H/K/N)Ras glucosylation affected PI3K/Akt signaling, desuppression, i.e., upregulation of its downstream target RhoB was analyzed. RhoB upregulation was more pronounced in TcsL-treated RBL cells compared to TcdBF-treated RBL cells (Figure 7B). This finding suggests that (H/K)Ras glucosylation and subsequent inhibition of PI3K/Akt signaling were more efficient in TcsL- than in TcdBF-treated cells.

DISCUSSION

The cytotoxic properties of the glucosylating toxins correlate with the pathogenicity of the producing strains (*28*). We recently showed that TcdB (from *C. difficile* strain VPI10463) but not isomeric TcdBF (from *C. difficile* strain 1470) causes cytotoxic effects (*28*). The most important outcome of this study was the finding that the glucosylation of critical substrate proteins (most likely RhoA in the case of TcdB) rather than the glucosyltransferase activity *per se* is required for the cytotoxic effects (*28*).

Among the TcsL isoforms, TcsL-9048 has been suggested not to cause cytotoxic effects, although it exhibits glucosyltransferase activity (*6*). In our hands, however, TcsL-9048 and TcsL-6018 exhibited comparable cytotoxic effects. TcdBF has formerly been characterized as a functional hybrid between TcdB and TcsL (*34, 35*). Correlation of the biological effects of the TcsL isoforms and TcdBF highlights (H/K/N)Ras as the critical substrate required for the cytotoxic effects of TcsL.

It would be helpful to inhibit the cytotoxic effect of TcsL through ectopic expression of constitutively active Ras proteins. Ectopic expression of constitutively active Rho proteins preserves Rho activity in cells treated with the glucosylating toxins. Applying this experimental approach, we most recently showed that the cytopathic effect of glucosylating toxins is prevented by active Rac1 (*36*). For the cytotoxic effects prolonged incubation and higher toxin concentrations were required (*28*). Unfortunately, constitutively active Rho/Ras proteins were glucosylated and inactivated, if cytotoxic concentrations of TcdB or TcsL were applied (unpublished observation), precluding this approach.

Our view that (H/K/N)Ras may represent critical substrates for the cytotoxic effect of TcsL meets the recent hypothesis by Voth and Ballard stating that inhibition of Ras/PI3K/Akt/GSKβ signaling is critical for TcsL-induced apoptosis (*18*). We here show that PI3K/Akt signaling is preserved in TcsL-treated cells by applying the antiapoptotic bile acid TUDCA. TUDCA activates PI3K/Akt independently of (H/K/N)Ras and thereby efficiently protects RBL cells from the cytotoxic effects of TcsL. The protective effect of TUDCA was responsive to inhibitors of either PI3K or Akt, confirming that TUDCA acts in a PI3K/Akt-dependent manner. Furthermore, TUDCA prevented TcsL-induced Akt dephosphorylation and RhoB upregulation (data not shown).

Former studies have pointed out the mitochondrial effects of TcsL: (i) TcsL associates with mitochondria (*6*); (ii) the mitochondrial proteins Bcl-xL and Bid have been shown to be processed in TcsL-treated cells (*18*); (iii) ectopic expres-

sion of (antiapoptotic) Bcl-2 prevents TcsL-induced mitochondrial alterations (*6*). The mitochondrial proteins Bcl-xL and Bid both are regulated by Akt, providing a link between Ras signaling and the mitochondrial cell death pathway (*37*). Our findings on the critical role of PI3K/Akt signaling in the cytotoxic effects of TcsL complement the former studies. Based on the recent findings on toxin uptake and proteolytic processing of the glucosylating toxins, it is highly conceivable that the glucosyltransferase domain of TcsL is targeted to the outer mitochondrial membrane (*6, 7*). K-Ras has recently been shown to translocate from the plasma membrane to the outer mitochondrial membrane (*38*). That glucosylation of mitochondria-associated Ras proteins contributes to mitochondrial cell death, however, remains to be evaluated.

Ras proteins are the cellular targets of further bacterial pathogenicity factors, among them the exoenzyme S (ExoS), a type III secretion effector protein from *Pseudomonas aeruginosa* (*39*). Comparable to (H/K/N)Ras glucosylation by TcsL, (H/K/N)Ras ADP-ribosylation by ExoS results in Akt inactivation and apoptotic cell death of phagocytotic cells (*40*). ExoS-induced cell death has been interpreted by means of defending *P. aeruginosa* against eukaryotic phagocytosis (*40*). *C. sordellii* bacteraemia is a rare event with a mortality rate of 67% (*41*). In some cases, *C. sordellii* remains localized to the site of infection and a shock-like death occurs (*2*). It is conceivable, however, that phagocytotic cells are targeted by TcsL to prevent phagocytosis of *C. sordellii*.

In conclusion, the glucosyltransferase activity is required for the cytotoxic effect of TcsL. It is most conceivable that inhibited PI3K/Akt-dependent survival signaling in TcsL-treated cells is based on (H/K/N)Ras glucosylation. Inhibited PI3K/Akt signaling is causative for the cytotoxic effects of TcsL. This study on the cytotoxic effects of TcsL supports our concept that the cytotoxic effects of the glucosylating toxins depend on the glucosylation of critical substrate proteins rather than on the glucosyltransferase activity *per se*.

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REFERENCES

1. Kimura, A. C., Higa, J. I., Levin, R. M., Simpson, G., Vargas, Y., and Vugia, D. J. (2004) Outbreak of necrotizing fasciitis due to Clostridium sordellii among black-tar heroin users. *Clin. Infect. Dis. 38*, e87−e91.
2. Sinave, C., Le, T. G., Blouin, D., Leveille, F., and Deland, E. (2002) Toxic shock syndrome due to *Clostridium sordellii*: a dramatic postpartum and postabortion disease. *Clin. Infect. Dis. 35*, 1441–1443.
3. Voth, D. E., Martinez, O. V., and Ballard, J. D. (2006) Variations in lethal toxin and cholesterol-dependent cytolysin production correspond to differences in cytotoxicity among strains of *Clostridium sordellii*. *FEMS Microbiol. Lett. 259*, 295–302.
4. Geny, B., Khun, H., Fitting, C., Zarantonelli, L., Mazuet, C., Cayet, N., Szatanik, M., Prevost, M. C., Cavaillon, J. M., Huerre, M., and Popoff, M. R. (2007) *Clostridium sordellii* lethal toxin kills mice by inducing a major increase in lung vascular permeability. *Am. J. Pathol. 170*, 1003–1017.
5. Fiorentini, C., Fabbri, A., Falzano, L., Fattorossi, A., Matarrese, P., Rivabene, R., and Donelli, G. (1998) *Clostridium difficile* toxin B induces apoptosis in intestinal cultured cells. *Infect. Immun. 66*, 2660–2665.
6. Petit, P., Bréard, J., Montalescot, V., El Hadj, N. B., Levade, T., Popoff, M., and Geny, B. (2003) Lethal toxin from *Clostridium sordellii* induces apoptotic cell death by disruption of mitochondrial homeostasis in HL-60 cells. *Cell. Microbiol. 5*, 761–771.
7. Reineke, J., Tenzer, S., Rupnik, M., Koschinski, A., Hasselmayer, O., Schrattenholz, A., Schild, H., and Von Eichel-Streiber, C. (2007) Autocatalytic cleavage of *Clostridium difficile* toxin B. *Nature 446*, 415–419.
8. Just, I., Selzer, J., Hofmann, F., Green, G. A., and Aktories, K. (1996) Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. *J. Biol. Chem. 271*, 10149–10153.
9. Popoff, M. R., Chaves, O. E., Lemichez, E., Von Eichel-Streiber, C., Thelestam, M., Chardin, P., Cussac, D., Chavrier, P., Flatau, G., Giry, M., Gunzburg, J., and Boquet, P. (1996) Ras, Rap, and Rac small GTP-binding proteins are targets for *Clostridium sordellii* lethal toxin glucosylation. *J. Biol. Chem. 271*, 10217–10224.
10. Genth, H., Aktories, K., and Just, I. (1999) Monoglucosylation of RhoA at threonine-37 blocks cytosol-membrane cycling. *J. Biol. Chem. 274*, 29050–29056.
11. Vetter, I. R., Hofmann, F., Wohlgemuth, S., Herrmann, C., and Just, I. (2000) Structural consequences of mono-glucosylation of Ha-Ras by *Clostridium sordellii* lethal toxin. *J. Mol. Biol. 301*, 1091–1095.
12. Sehr, P., Joseph, G., Genth, H., Just, I., Pick, E., and Aktories, K. (1998) Glucosylation and ADP-ribosylation of Rho proteins—Effects on nucleotide binding, GTPase activity, and effector-coupling. *Biochemistry 37*, 5296–5304.
13. Herrmann, C., Ahmadian, M. R., Hofmann, F., and Just, I. (1998) Functional consequences of monoglucosylation of H-Ras at effector domain amino acid threonine-35. *J. Biol. Chem. 273*, 16134–16139.
14. Cox, A. D., and Der, C. J. (2002) Ras family signaling: therapeutic targeting. *Cancer Biol. Ther. 1*, 599–606.
15. Jiang, K., Sun, J., Cheng, J., Djeu, J. Y., Wei, S., and Sebti, S. (2004) Akt mediates Ras downregulation of RhoB, a suppressor of transformation, invasion, and metastasis. *Mol. Cell. Biol. 24*, 5565–5576.
16. Huelsenbeck, J., Dreger, S. C., Gerhard, R., Fritz, G., Just, I., and Genth, H. (2007) Upregulation of the immediate early gene product RhoB by exoenzyme C3 from *Clostridium limosum* and toxin B from *Clostridium difficile*. *Biochemistry 46*, 4923–4931.
17. Delarue, F. L., Adnane, J., Joshi, B., Blaskovich, M. A., Wang, D. A., Hawker, J., Bizouarn, F., Ohkanda, J., Zhu, K., Hamilton, A. D., Chellappan, S., and Sebti, S. M. (2007) Farnesyltransferase and geranylgeranyltransferase I inhibitors upregulate RhoB expression by HDAC1 dissociation, HAT association and histone acetylation of the RhoB promoter. *Oncogene 26*, 633–640.
18. Voth, D. E., and Ballard, J. D. (2007) Critical intermediate steps in *Clostridium sordellii* lethal toxin-induced apoptosis. *Biochem. Biophys. Res. Commun. 363*, 959–964.
19. Matarrese, P., Falzano, L., Fabbri, A., Gambardella, L., Frank, C., Geny, B., Popoff, M. R., Malorni, W., and Fiorentini, C. (2007) *Clostridium difficile* toxin B causes apoptosis in epithelial cells by thrilling mitochondria. Involvement of ATP-sensitive mitochondrial potassium channels. *J. Biol. Chem. 282*, 9029–9041.
20. Nottrott, S., Schoentaube, J., Genth, H., Just, I., and Gerhard, R. (2007) *Clostridium difficile* toxin A-induced apoptosis is p53-independent but depends on glucosylation of Rho GTPases. *Apoptosis 12*, 1443–1453.
21. Walev, I., Bhakdi, S. C., Hofmann, F., Djonder, N., Valeva, A., Aktories, K., and Bhakdi, S. (2001) Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. *Proc. Natl. Acad. Sci. U.S.A. 98*, 3185–3190.
22. Genth, H., Selzer, J., Busch, C., Dumbach, J., Hofmann, F., Aktories, K., and Just, I. (2000) New method to generate enzymatically deficient *Clostridium difficile* toxin B as an antigen for immunization. *Infect. Immun. 68*, 1094–1101.
23. Sola, S., Castro, R. E., Laires, P. A., Steer, C. J., and Rodrigues, C. M. (2003) Tauroursodeoxycholic acid prevents amyloid-beta peptide-induced neuronal death via a phosphatidylinositol 3-kinase-dependent signaling pathway. *Mol. Med. 9*, 226–234.
24. Beuers, U., Denk, G. U., Soroka, C. J., Wimmer, R., Rust, C., Paumgartner, G., and Boyer, J. L. (2003) Taurolithocholic acid exerts cholestatic effects via phosphatidylinositol 3-kinase-dependent mechanisms in perfused rat livers and rat hepatocyte couplets. *J. Biol. Chem. 278*, 17810–17818.

25. Genth, H., Huelsenbeck, J., Hartmann, B., Hofmann, F., Just, I., and Gerhard, R. (2006) Cellular stability of Rho-GTPases glucosylated by *Clostridium difficile* toxin B. *FEBS Lett. 580*, 3565–3569.
26. Genth, H., Hofmann, F., Selzer, J., Rex, G., Aktories, K., and Just, I. (1996) Difference in protein substrate specificity between hemorrhagic toxin and lethal toxin from *Clostridium sordellii*. *Biochem. Biophys. Res. Commun. 229*, 370–374.
27. Graham, T. E., Pfeiffer, J. R., Lee, R. J., Kusewitt, D. F., Martinez, A. M., Foutz, T., Wilson, B. S., and Oliver, J. M. (1998) MEK and ERK activation in ras-disabled RBL-2H3 mast cells and novel roles for geranylgeranylated and farnesylated proteins in Fc epsilonRI-mediated signaling. *J. Immunol. 161*, 6733–6744.
28. Huelsenbeck, J., Dreger, S., Gerhard, R., Barth, H., Just, I., and Genth, H. (2007) Difference in the cytotoxic effects of toxin B from *Clostridium difficile* strain VPI 10463 and toxin B from variant *Clostridium difficile* strain 1470. *Infect. Immun. 75*, 801–809.
29. Fiorentini, C., Fabbri, A., Falzano, L., Fattorossi, A., Matarrese, P., Rivabene, R., and Donelli, G. (1998) *Clostridium difficile* toxin B induces apoptosis in intestinal cultured cells. *Infect. Immun. 66*, 2660–2665.
30. Busch, C., Hofmann, F., Selzer, J., Munro, J., Jeckel, D., and Aktories, K. (1998) A common motif of eukaryotic glycosyltransferases is essential for the enzyme activity of large clostridial cytotoxins. *J. Biol. Chem. 273*, 19566–19572.
31. Fritz, G., and Kaina, B. (2000) Ras-related GTPase RhoB forces alkylation-induced apoptotic cell death. *Biochem. Biophys. Res. Commun. 268*, 784–789.
32. Liu, A., Cerniglia, G. J., Bernhard, E. J., and Prendergast, G. C. (2001) RhoB is required to mediate apoptosis in neoplastically transformed cells after DNA damage. *Proc. Natl. Acad. Sci. U.S.A. 98*, 6192–6197.
33. Hu, Y., Qiao, L., Wang, S., Rong, S. B., Meuillet, E. J., Berggren, M., Gallegos, A., Powis, G., and Kozikowski, A. P. (2000) 3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth. *J. Med. Chem. 43*, 3045–3051.
34. Chaves-Olarte, E., Löw, P., Freer, E., Norlin, T., Weidmann, M., Von Eichel-Streiber, C., and Thelestam, M. (1999) A novel cytotoxin from *Clostridium difficile* serogroup F is a functional hybrid between two other large clostridial cytotoxins. *J. Biol. Chem. 274*, 11046–11052.
35. Chaves-Olarte, E., Freer, E., Parra, A., Guzmán-Verri, C., Moreno, E., and Thelestam, M. (2003) R-Ras glucosylation and transient RhoA activation determine the cytopathic effect produced by toxin B variants from toxin A-negative strains of *Clostridium difficile*. *J. Biol. Chem. 278*, 7956–7963.
36. Halabi-Cabezon, I., Huelsenbeck, J., May, M., Ladwein, M., Rottner, K., Just, I., and Genth, H. (2008) Prevention of the cytopathic effect induced by *Clostridium difficile* toxin B by active Rac1. *FEBS Lett. 582*, 3751–3756.
37. Downward, J. (2004) PI 3-kinase, Akt and cell survival. *Semin. Cell Dev. Biol. 15*, 177–182.
38. Bivona, T. G., Quatela, S. E., Bodemann, B. O., Ahearn, I. M., Soskis, M. J., Mor, A., Miura, J., Wiener, H. H., Wright, L., Saba, S. G., Yim, D., Fein, A., Perez, d. C. I., Li, C., Thompson, C. B., Cox, A. D., and Philips, M. R. (2006) PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its association with Bcl-XL on mitochondria and induces apoptosis. *Mol. Cell 21*, 481–493.
39. Aktories, K., and Barbieri, J. T. (2005) Bacterial cytotoxins: Targeting eukaryotic switches. *Nat. Rev. Microbiol. 3*, 397–410.
40. Jansson, A. L., Yasmin, L., Warne, P., Downward, J., Palmer, R. H., and Hallberg, B. (2006) Exoenzyme S of *Pseudomonas aeruginosa* is not able to induce apoptosis when cells express activated proteins, such as Ras or protein kinase B/Akt. *Cell. Microbiol. 8*, 815–822.
41. Abdulla, A., and Yee, L. (2000) The clinical spectrum of *Clostridium sordellii* bacteraemia: two case reports and a review of the literature. *J. Clin. Pathol. 53*, 709–712.

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