The Low Molecular Mass GTP-binding Protein Rho Is Affected by Toxin A from *Clostridium difficile*

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Abstract

Enterotoxin A is one of the major virulence factors of *Clostridium difficile*, and the causative agent of antibiotic-associated pseudomembranous colitis. In cell culture (NIH-3T3, rat basophilic leukemia cells) toxin A inhibits *Clostridium botulinum* ADP-ribosyltransferase C3 (C3)-catalyzed ADP-ribosylation of the low molecular mass GTP-binding Rho proteins. Rho participates in the regulation of the microfilament cytoskeleton. Decrease in ADP-ribosylation of Rho occurs in a time- and concentration-dependent manner and precedes the toxin A–induced destruction of the actin cytoskeleton. Action of toxin A is not due to proteolytical degradation of Rho or to an inherent ADP-ribosyltransferase activity of toxin A. Toxin A–induced decrease in ADP-ribosylation is observed also in cell lysates and with recombinant RhoA protein. A heat stable low molecular mass cytosolic factor is essential for the toxin effect on Rho. Thus, the enterotoxin (toxin A) resembles the effects of the *C. difficile* cytotoxin (toxin B) on Rho proteins (Just, I., G. Fritz, K. Aktories, M. Giry, M. R. Popoff, P. Boquet, S. Hegenbath, and C. Von Eichel-Streiber. 1994. *J. Biol. Chem.* 269:10706–10712). The data indicate that despite different in vivo effects, toxin A and toxin B act on the same cellular target protein Rho to elicit their toxic effects. (*J. Clin. Invest.* 1995. 95:1026–1031.) Key words: enterotoxin • cytoskeleton • ADP-ribosylation • actin filaments • cytotoxin

Introduction

Pathogenic strains of *Clostridium difficile* produce two potent exotoxins, the enterotoxin (ToxA)[1] and the cytotoxin (ToxB). These have been identified as the major virulence factors which cause antibiotic-associated diarrhea and its potentially fatal form, the pseudomembranous colitis (1, 2). ToxA causes hemorrhagic fluid secretion in the intestinal loop, mucosal inflammation, and necrosis of the intestinal tissue (3–5). In contrast to ToxA, ToxB exhibits no overt enterotoxicity. However, both toxins are lethal when injected parenterally into animals (6). After cloning and sequencing of both genes, the molecular masses of the toxins have been calculated to be 308 kD for ToxA and 270 kD for ToxB (7–9). ToxA and ToxB are encoded by two separate genes located in close vicinity. Both toxins are single-chained and show an amino acid identity of 49% (8). Their COOH terminus contains repetitive oligopeptide elements which are involved in the interaction of the toxin with the cellular glycoprotein receptor. In tissue culture, ToxA as well as ToxB are cytotoxic, thereby inducing cell shape changes but ToxB is about three orders of magnitude more potent. The toxins exert their cytopathic activity after receptor-mediated endocytosis (10, 11). The cellular receptor for ToxA has been characterized as a 163-kD glycoprotein (12). The morphological alterations induced by the toxins are accompanied by disaggregation of actin filaments, whereas microtubules and intermediate filaments are affected later or hardly at all (13, 14). The microfilament cytoskeleton is preferentially affected by these toxins, but actin itself is not the target (15). The precise molecular mode of action is still unknown (for review see 16, 17).

Another clostridial exotoxin, *Clostridium botulinum* ADP-ribosyltransferase C3 (C3) exoenzyme, also causes selective disaggregation of the microfilament cytoskeleton (18, 19). The features of the cytoskeletal changes are similar to those caused by *C. difficile* ToxA and B. The family of C3-like exoenzymes comprises exoenzymes from C3 (20–22), *Clostridium limosum* (23), *Bacillus cereus* (24) and *Staphylococcus aureus* epidermal differentiation inhibitor (25). These exoenzymes selectively ADP-ribosylate the low molecular mass GTP-binding Rho proteins. The Rho proteins are involved in the regulation of the actin cytoskeleton. They participate in the growth factor–stimulated formation of stress fibers and focal adhesions (26), cell motility (27, 28), and in the contraction of smooth muscle (29). The functional activity of the Rho proteins is regulated by exchange factors: Guanine nucleotide dissociation stimulator promotes the active GTP-bound form of Rho (30), whereas the GTPase-activating protein most likely mediates the inactivation (31, 32). The inactive GDP-bound form is trapped in complex with the guanine nucleotide dissociation inhibitor (33, 34). Rho proteins are ADP-ribosylated at amino acid Asn-41 (35), which is located in the putative effector domain (36). This modification renders Rho inactive resulting in depolymerization of actin filaments (37) by a so far unidentified mechanism.

Recently, ToxB has been reported to act on the Rho proteins to inhibit subsequent ADP-ribosylation by C3 (38). ToxB induces a time- and concentration-dependent decrease in the ADP-ribosylation of Rho both in intact cells and cell lysates. ToxB seems to modify Rho thereby rendering Rho functionally inactive. Here we studied whether the enterotoxin ToxA acts on the Rho proteins as ToxB does.

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1. *Abbreviations used in this paper:* C3, *Clostridium botulinum* ADP-ribosyltransferase C3; GST, glutathione S–transferase; RBL, rat basophilic leukemia; ToxA, *Clostridium difficile* enterotoxin A; ToxB, *C. difficile* cytotoxin B.

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Methods

Materials. [α-^{32}P]NAD was obtained from DuPont NEN Research Products (Dreieich, Germany). All other reagents were of analytical grade and purchased from commercial sources. C3 exoenzyme (20), *C. difficile* ToxA (39), *C. difficile* ToxB (39), and *C. botulinum* C2 toxin (40) were purified as described.

Cell culture. NIH-3T3 cells were grown in Dulbecco's medium supplemented with 10% fetal calf serum, 4 mM glutamine/penicillin/streptomycin. Rat basophilic leukemia (RBL) cells were grown in MEM Earle's medium supplemented with 18% heat-inactivated fetal calf serum, 3 mM glutamine/penicillin/streptomycin. After 24 h the medium was changed, and cells were incubated with either ToxA for the indicated concentrations and times or alternatively with *C. difficile* ToxB (0.5 ng/ml for 4 h), *C. botulinum* C2 toxin (C2I 100 ng/ml plus activated C2II 200 ng/ml), cytochalasin D (10 μM), or colchicine (30 μM) for 2 h.

Preparation of cell lysates. NIH-3T3 and RBL cells were rinsed with ice-cold PBS and were then mechanically removed from the dishes in the presence of lysis buffer (2 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol/0.3 mM PMSF/30 μg/ml leupeptin/50 mM triethanolamine-HCl, pH 7.4), sonicated five times on ice, and centrifuged for 10 min at 1,000 *g*. The supernatant was used as cell lysate. For ADP-ribosylation protein concentration was ~ 1 mg/ml and for preincubation with ToxA ~ 8 mg/ml.

Preparation of a crude fraction of the cytosolic cofactor. Cell lysate from RBL cells were ultacentrifuged (60 min at 100,000 *g*) and the cytosolic fraction was incubated for 15 min at 95°C. Denatured proteins were removed by centrifugation (15 min at 13,000 *g*) and the supernatant was passed through a filtration membrane (Ultrafiltration membranes YM3, 3,000 D cut off, Amicon Corp., Danvers, MA).

Correlation of cell rounding and ADP-ribosylation of Rho. NIH-3T3 cells (2×10^6 cells/well) were incubated with ToxA (500 ng/ml) for the indicated periods of time. Thereafter, the number of cells with normal morphology was determined from photographs taken from treated cells. Per time period 500 cells were counted. The cells were lysed as described above and the lysate was centrifuged for 60 min at 100,000 *g* to obtain the membrane fraction. These fractions were [^{32}P]-ADP-ribosylated with C3 as described. The SDS-gels were evaluated with Phosphorimager SF (Molecular Dynamics Inc., Sunnyvale, CA). The amount of ADP-ribosylation was calculated as percentage of the untreated control.

ADP-ribosylation reaction. Cell lysates or membrane fractions were incubated in buffer (3 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.3 mM PMSF, 50 mM triethanolamine-HCl [pH 7.4], 0.3 μM [0.3 μCi] [^{32}P]NAD) with C3 exoenzyme (1 μg/ml) for 15 min at 30°C. The reaction was terminated by addition of sample buffer.

Preparation of glutathione S-transferase (GST)-RhoA fusion protein. RhoA GST fusion protein was obtained by cloning of the PCR-generated product of RhoA into the bacterial expression vector pGEX-2T as described (38). GST-RhoA was isolated by affinity purification with glutathione-Sepharose beads (Pharmacia Laboratories Inc., Freiburg, Germany) and eluted with reduced glutathione (5 mM) in 50 mM Tris-HCl (pH 8.0).

Effect of ToxA in cell lysates. Lysates from RBL cells were incubated in the presence of GST-Rho fusion protein for 2 h at 37°C without or with ToxA (10 μg/ml) followed by 1:10 dilution and [^{32}P]ADP-ribosylation with C3. The samples were analyzed by SDS-PAGE and autoradiography. To test ADP-ribosyltransferase activity of ToxA, lysates from RBL cells were incubated for 2 h in the presence of [^{32}P]-NAD with ToxA (10 μg/ml) finally followed by 1:10 dilution. The diluted assay was divided into three samples: The first one was directly analyzed by SDS-PAGE and autoradiography. The second one was [^{32}P]ADP-ribosylated with C3 and the proteins were resolved on SDS-PAGE followed by autoradiography. The third one was directly electroblotted and probed with anti-RhoA.

Gel electrophoresis. Proteins were dissolved in sample buffer and subjected to 12.5% SDS-polyacrylamide gel electrophoresis (41) followed by autoradiography.

Nondenaturing gel electrophoresis was performed in a Mini-Protean II gel system (BioRad Laboratories, Munich, Germany) according to Safer (42). 4 μl of sample buffer (100 μM GDP/0.5 mM DTT/10 mM Tris-HCl, pH 8.0/glycerol 50% wt/wt and bromphenol blue) was added to the cytosol (20 μl) which was prepared from toxin-treated cells or ADP-ribosylated by C3. After incubation for 5 min at 21°C, the samples were centrifuged (10 min at 13,000 *g*), 6 μl of the supernatant were loaded onto the gel, and electrophoresis was performed for 50 min at 140 V.

Two-dimensional (2-D) gel electrophoresis was performed in a Mini-Protean II 2D cell (BioRad Laboratories) according to O'Farrell (43). Cell lysates from control and toxin-treated cells were loaded onto rod gels for isoelectric focusing in a pH gradient of 5–7. The second dimension was run on a 12.5% SDS slab gel.

Immunoblot. Immunoblotting was performed according to Towbin et al. (44) with anti-RhoA IgG (Santa Cruz Biotechnology, Santa Cruz, CA) (1:1,000) and peroxidase-coupled swine IgG to rabbit IgG as secondary antibody. Visualization was performed with the enhanced chemiluminescence system from Amersham Corp. (Arlington Heights, IL).

All experiments were performed at least three times.

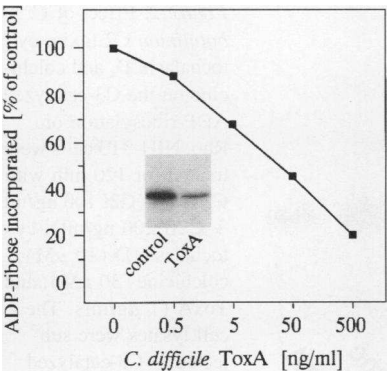

Figure 1. Effect of *C. difficile* ToxA on the C3-catalyzed ADP-ribosylation of Rho. NIH-3T3 cells were treated for 3 h with increasing concentrations of ToxA (0, 0.5, 5, 50, and 500 ng/ml). Thereafter, the cell lysates were [^{32}P]ADP-ribosylated with C3 and analyzed by SDS-PAGE. Evaluation of [^{32}P]ADP-ribosylated Rho was performed with the Phosphorimager and the amount of ADP-ribosylation was calculated as percentage of control (nontreated cells). The inset autoradiogram shows the ADP-ribosylation of Rho (22 kD) from control cells and cells treated with 500 ng/ml of ToxA.

Results

To test whether *C. difficile* ToxA acts on the Rho proteins, we used ADP-ribosyltransferase C3 as a tool for selective labeling of the GTP-binding proteins. NIH-3T3 cells were incubated with ToxA until the cells changed their shape and showed the characteristic morphology (retraction of the cell body and formation of long neurite-like arbors). The cells were lysed, ADP-ribosylated by C3 in the presence of [^{32}P]NAD followed by SDS-PAGE and autoradiography. ToxA treatment of the cells induced a significant, dose-dependent decrease in ADP-ribosylation of the 22-kD Rho proteins compared to untreated cells (Fig. 1). After an incubation time of 3 h, half-maximal effects of ToxA were observed at 25 ng/ml. Heat inactivation of ToxA completely abolished the toxin effect on both cell rounding and ADP-ribosylation of Rho. Inhibition in ADP-ribosylation was also detected when the ADP-ribosylation reaction of lysates was performed in the presence of high concentrations of NAD (data not shown), excluding alteration of NAD metabolism as the basis of inhibition. The inhibitory effect of ToxA on Rho

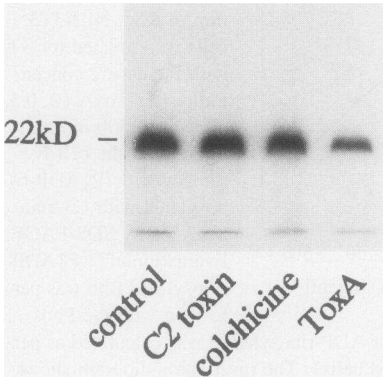

Figure 2. Effect of *C. botulinum* C2 toxin, cytochalasin D, and colchicine on the C3-catalyzed ADP-ribosylation of Rho. NIH-3T3 cells were treated for 120 min with C2 toxin (C2I 100 ng/ml + C2II 200 ng/ml), cytochalasin D (10 μM); colchicine (30 μM); and ToxA (1 μg/ml). The cell lysates were subjected to C3-catalyzed [^{32}P]ADP-ribosylation. Autoradiography of SDS-PAGE is shown.

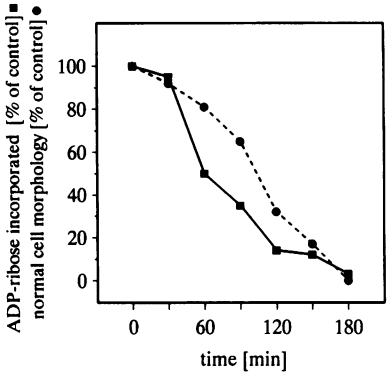

Figure 3. Influence of ToxA on cell rounding and on C3-catalyzed ADP-ribosylation of Rho. NIH-3T3 cells were treated with ToxA (500 ng/ml) for the indicated periods of time. Thereafter, the number of cells exhibiting normal morphology was determined, the cells were lysed, and the membrane fractions were [^{32}P]ADP-ribosylated by C3 as described under Methods. The amount of ADP-ribosylation and the amount of cells showing normal morphology was calculated as percentage of the untreated control. One representative experiment from three is shown.

ADP-ribosylation was reproduced with several cell lines, i.e., NIH-3T3 cells, RBL cells, chinese hamster ovary cells, mouse lung fibroblast L929 cells, mouse embryo teratocarcinoma F9 cells, and *Potoroo tridactylis* kidney (PTK2) cells (data not shown).

To clarify whether inhibition of ADP-ribosylation is merely a response to depolymerization of cytoskeletal elements, we tested the effect of *C. botulinum* C2 toxin and cytochalasin D, cytotoxins which induce gross changes in distribution of actin filaments. Furthermore, we studied the effect of the microtubule depolymerizing agent colchicine. Incubation of NIH-3T3 cells with C2 toxin (C2I 100 ng/ml and C2II 200 ng/ml, 120 min) or cytochalasin D (10 μM, 120 min) caused almost complete rounding of the cells whereas colchicine (30 μM, 120 min) induces no gross changes. Lysates taken from these cells showed the same amount of [^{32}P]ADP-ribosylated Rho as control cells, whereas ToxA induced a significant decrease in Rho modification (Fig. 2). Thus, decreasing ADP-ribosylation of Rho is ToxA-specific and not a mere response to depolymerization of actin filaments or microtubules.

To study whether toxin-induced inhibition of ADP-ribosylation and changes in cell morphology are related phenomena, NIH-3T3 cells were treated with ToxA (500 ng/ml). After increasing incubation times the percentage of cells with normal morphology was determined. Cells were then lysed and membrane fractions were subjected to C3-catalyzed [^{32}P]ADP-ribosylation. Increasing incubation times caused a decrease in ADP-ribosylation of Rho (Fig. 3). The portion of cells with normal morphology also decreased in a time-dependent manner reflecting the concomitant increase in the number of rounded cells. However, inhibition in ADP-ribosylation of Rho clearly preceded the morphological alterations, indicating a temporal relationship between the loss of the property of Rho to serve as substrate for C3 and toxin-induced depolymerization of filaments.

To exclude that ToxA-induced inhibition of ADP-ribosylation of Rho is simply caused by proteolytic degradation of the Rho proteins, the amount of Rho in cells was assessed by immunoblot analysis. RBL cells were used because they contain more immunoreactive Rho than NIH-3T3 cells and are similarly sensitive towards ToxA and ToxB. RBL cells were treated with ToxA or ToxB until all cells showed rounded morphology. The cell lysates were divided and subjected to either immunoblot analysis or C3-catalyzed [^{32}P]ADP-ribosylation. Immunoblot analysis with anti-RhoA antibody revealed no differences between control and toxin-treated cells in the amount of staining and no change in the apparent molecular mass (Fig. 4 *A*). ADP-ribosylation of Rho was almost completely inhibited after toxin treatment (Fig. 4 *B*). Thus, both ToxA- and ToxB-induced inhibition of Rho ADP-ribosylation is not caused by proteolytical degradation of the GTP-binding protein. 2-D gel electrophoresis of [^{32}P]ADP-ribosylated RBL-cell lysate revealed only one spot, which was assigned to RhoA, indicating that RBL cells exclusively contain RhoA. Therefore, application of the isotype-specific RhoA-antibody gives direct evidence about the total cellular amount of Rho protein.

To assess possible modification of Rho we applied 2-D gel electrophoresis of lysates from ToxA-treated RBL cells followed by immunoblot analysis with anti-RhoA (Fig. 5). There was no difference in the isoelectric point of RhoA after ToxA treatment and even combination of control lysate with that from toxin-treated cell did not reveal any change. However, on nondenaturing gel electrophoresis RhoA exhibited an altered migration behavior compared to control. This change in migration (decreased migration velocity) was opposite to Rho ADP-ribosylated by C3 (increase in migration velocity).

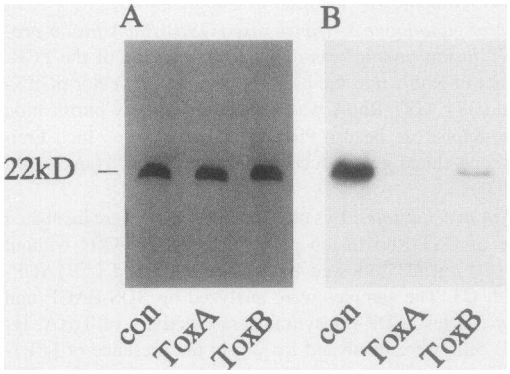

Figure 4. Immunoblot analysis of ToxA- and ToxB-treated RBL cells. RBL cells were treated without (*con*) or with either ToxA (100 ng/ml) or ToxB (0.5 ng/ml) for 4 h. The cell lysates were directly electroblotted (*A*) or [^{32}P]ADP-ribosylated with C3 (*B*). (*A*) Immunoblot was performed with anti-RhoA IgG and visualized with ECL. (*B*) Autoradiography.

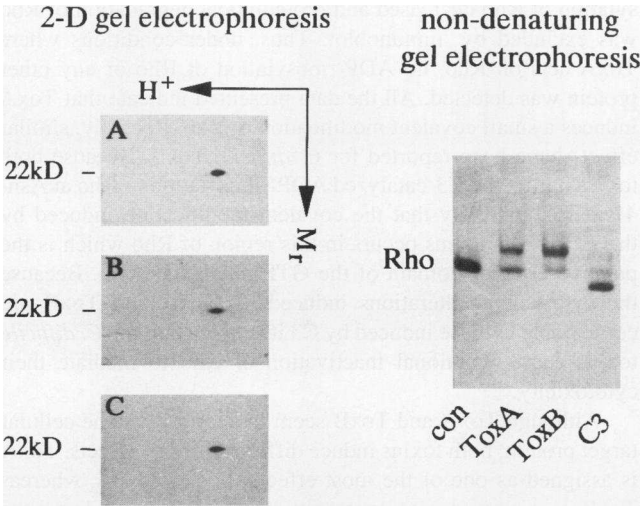

Figure 5. Immunoblot of cytosolic RhoA processed by either 2-D or nondenaturing gel electrophoresis. Lysates from RBL control cells (*A*), from ToxA-treated cells (*B*), and a combination of A and B were separated on 2-D gels. Lysates from ToxA and ToxB-treated RBL cells, or control lysate ADP-ribosylated with C3 were separated on nondenaturing gels. After electroblotting RhoA was detected with anti-RhoA.

When cell lysates from NIH-3T3 or RBL cells were incubated with ToxA (10 μg/ml for 2 h), subsequent ADP-ribosylation with C3 revealed a decrease in Rho modification (Fig. 6). This decrease was comparable to that obtained after toxin treatment of intact cells. Furthermore, GST-RhoA fusion protein (48 kD), which was added to the lysates, showed decreased ADP-ribosylation after ToxA incubation (Fig. 6). Even a 10-fold surplus of fusion protein compared to endogenous Rho (judged by ADP-ribosylation) was effected by ToxA. In the absence of cell lysate GST-RhoA was not affected by ToxA (not shown) indicating a cellular cofactor to be needed for ToxA activity. This cofactor is in the cytosolic fraction of the lysate and not membrane bound. Incubation of the cytosol for 15 min at 95°C did not abolish the ability of the cytosol to mediate ToxA-induced inhibition of ADP-ribosylation (data not shown). Furthermore, estimation of the molecular mass of the

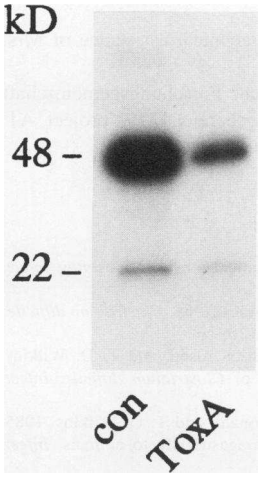

Figure 6. Effect of ToxA on endogenous and recombinant Rho proteins in cell lysates. Lysates from RBL cells were incubated in the presence of GST-Rho fusion protein for 2 h without (*con*) or with ToxA (10 μg/ml). Thereafter, the lysates were diluted 1:10 and subjected to [^{32}P]ADP-ribosylation with C3. Autoradiogram of SDS-PAGE is shown. GST-Rho (48 kD) and endogenous Rho (22 kD).

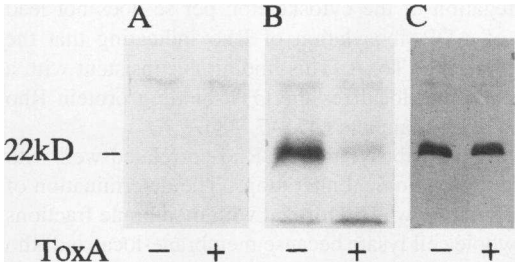

Figure 7. Effect of ToxA on Rho proteins in cell lysates. Lysates from RBL cells were incubated for 2 h in the presence of [^{32}P]NAD without or with ToxA (10 μg/ml). Thereafter, the proteins were divided into three samples. The first one was directly analyzed by SDS-PAGE followed by autoradiography (*A*, autoradiogram). The second sample was subjected to [^{32}P]ADP-ribosylation with C3 (*B*, autoradiogram). The third sample was electroblotted and probed with anti-RhoA (*C*, immunoblot).

factor by applying molecular sieves revealed a molecular mass below 3,000 D.

Because ToxA acted on Rho in cell lysates, we used this cell-free system to test whether ToxA exhibits an inherent ADP-ribosyltransferase activity. Therefore, lysates from RBL cells were incubated with ToxA for 2 h in the presence of [^{32}P]NAD. No [^{32}P]ADP-ribosylated proteins with molecular masses ranging from 15 to 35 kD were detected (Fig. 7 *A*). However, subsequent ADP-ribosylation of these samples with C3 showed a significant decrease in Rho modification (Fig. 7 *B*) indicating that the conditions were sufficient to exert ToxA effect on Rho. Concomitant immunoblot analysis with anti-RhoA showed no changes in staining or apparent molecular mass (Fig. 7 *C*). This experiment clearly demonstrates that ToxA does not possess ADP-ribosyltransferase activity to inhibit modification of Rho by C3.

Discussion

C3 exoenzyme catalyzes the selective ADP-ribosylation of the low molecular mass GTP-binding protein Rho, a modification which renders Rho functionally inactive resulting in dramatic redistribution of the microfilaments (18, 19, 26). In the present study, we used C3-catalyzed ADP-ribosylation as an indication for functional changes of Rho. We show that *C. difficile* ToxA treatment of NIH-3T3 and RBL cells induced a decrease in C3-catalyzed ADP-ribosylation of Rho in a time- and concentration-dependent manner. The effect of ToxA was observed with several cell lines (NIH-3T3, RBL, chinese hamster ovary, mouse embryo teratocarcinoma, mouse lung fibroblast, and *P. tridactylis* kidney cells) indicating that inhibition of ADP-ribosylation of Rho is a general feature of ToxA action.

Several cytotoxins affect the microfilaments via direct interaction with actin, e.g., *C. botulinum* C2 toxin and cytochalasin D. C2 toxin ADP-ribosylates monomeric G-actin, a modification inhibiting actin polymerization and trapping actin in the monomeric form (45). Cytochalasin D caps the barbed ends of filaments to block further elongation (46). Both toxins induce gross alterations in cellular F-actin and induce depolymerization of actin filaments. However, this dramatic disaggregation does not affect ADP-ribosylation of Rho. The same is true for the breakdown of the microtubule system induced by colchicine.

Thus, disaggregation of the cytoskeleton per se does not lead to inhibition of ADP-ribosylation of Rho indicating that the decrease is specific for ToxA. This finding is consistent with a signaling cascade that localizes the GTP-binding protein Rho upstream from actin filaments (37, 47, 48).

Decrease in ADP-ribosylation of Rho correlated well with ToxA-induced morphological alterations. The determination of Rho ADP-ribosylation was performed with membrane fractions and not with whole cell lysate because membrane-localized Rho is suggested to be functionally active whereas its cytosolic form was proposed to be the pool of inactive Rho (49, 50). Inhibition of ADP-ribosylation of membranous Rho clearly preceded the morphological changes, indicating that the loss of the property of Rho to serve as substrate for C3 and the ToxA-induced breakdown of filaments are temporally related. Decrease in ADP-ribosylation may reflect the change in the functional state of the GTP-binding protein which leads to actin depolymerization by a so far unidentified signal pathway.

Another group of potent clostridial toxins, the botulinum neurotoxins and the tetanus toxin, were shown to possess protease activity. They mediate their toxic effects by selective proteolytic cleavage of peptides, e.g., synaptobrevin involved in the secretion machinery of neuronal cells (51). Immunoblot analysis of ToxA-treated cells with a polyclonal anti-RhoA antibody reveals no changes in the amount of cellular Rho nor visible changes of the apparent molecular mass. The same holds true for ToxB, which also induces inhibition of ADP-ribosylation of Rho (38). These results definitely exclude proteolytic cleavage or degradation as basis for decreased ADP-ribosylation. Furthermore, ToxA does not seem to induce attachment of a bulky modification to Rho. In contrast, the altered migration behavior on nondenaturing gels gives evidence for a modification which changes the property of the Rho protein. 2-D gel electrophoresis which revealed no change in the isoelectric point suggests a modification with an uncharged moiety.

ToxA exhibited its effect on the ADP-ribosylation of Rho also in the cell-free system of lysates. Besides endogenous Rho, GST-RhoA fusion protein showed reduced ADP-ribosylation after incubation with ToxA. The finding that even a large surplus of fusion protein compared to endogenous Rho was effected by ToxA indicates a possible enzyme activity of the toxin and excludes a ToxA-induced interaction of Rho with Rho-regulating proteins as basis for decreased ADP-ribosylation. ToxA-induced decrease in Rho ADP-ribosylation in cell lysates worked only at an ~ 20-fold higher concentration than necessary for intact cells. This divergence in ToxA concentration may be due to failure to activate ToxA with regard to cell lysates, which, however, occurs when ToxA is endocytosed by intact cells. The toxin is most likely activated during the uptake mechanism in the postendosomes as reported for diphtheria, pertussis, or cholera toxin (52). However, this hypothesis has to be substantiated by additional experiments.

Both toxins ToxA and B need, for their activity on Rho, a cellular factor. This factor is remarkably heat stable, shows a molecular mass below 3,000 D, and is soluble in the cytosolic fraction. These properties of the low molecular cofactor or even cosubtrate argue against a secondary effect of the toxins on Rho.

Using the cell-free system (RBL cell lysate) we present evidence against ADP-ribosyltransferase activity of ToxA. In cell lysates, ToxA did not induce any ADP-ribosylation of cellular proteins. However, concomitantly C3-catalyzed ADP-ribosylation of Rho decreased and proteolytical degradation of Rho was excluded by immunoblot. Thus, under conditions where ToxA acts on Rho, no ADP-ribosylation of Rho or any other protein was detected. All the data presented indicate that ToxA induces a small covalent modification of Rho. Recently, similar effects have been reported for *C. difficile* ToxB. Because both toxins inhibit the C3-catalyzed ADP-ribosylation of Rho at Asn-41, it is thus likely that the covalent modification induced by the *C. difficile* toxins occurs in this region of Rho which is the putative effector domain of the GTP-binding protein. Because the cytoskeletal alterations induced by ToxA and ToxB are comparable to those induced by C3 it is likely that the *C. difficile* toxins cause functional inactivation of Rho to mediate their cytotoxicity.

Although ToxA and ToxB seem to act on the same cellular target protein, both toxins induce different in vivo effects. ToxA is assigned as one of the most effective enterotoxins, whereas ToxB is a very potent cytotoxin. One explanation for these divergent activities is that both toxins interact with different cell surface receptors, which determine the predominant localization of actin. Comparable features have been reported for the clostridial neurotoxins which cause flaccid paralysis in the case of botulinum neurotoxins and spastic paralysis in the case of tetanus toxin, respectively. However, at the molecular level both neurotoxin groups exhibit a common mode of action, i.e., zinc-protease activity against diverse synaptic proteins with subsequent inhibition of secretion of neurotransmitters.

In summary, *C. difficile* ToxA-induced depolymerization of microfilaments is accompanied by an inhibition in the ADP-ribosylation of the low molecular mass GTP-binding Rho proteins, which participate in the regulation of the actin cytoskeleton. Inhibited ADP-ribosylation is not caused by proteolytic degradation of Rho but most likely by a covalent modification, which does not alter the apparent molecular mass or the isoelectric point but cause changes in the migration behavior on nondenaturing gels. As reported by others and confirmed in our laboratory, ToxA does not possess ADP-ribosyltransferase activity (53). The loss of the property of Rho to serve as substrate for C3 precedes the toxin-induced breakdown of the filaments. Together, the data suggest that despite their different in vivo effects, ToxA and ToxB from *C. difficile* mediate their cytotoxic effects via functional inactivation of the GTP-binding protein Rho.

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