**Clostridium difficile** Toxin B Acts on the GTP-binding Protein Rho*

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**Clostridium difficile** toxin B exhibits cytotoxic activity that is characterized by the disruption of the microfilamentous cytoskeleton. Here we studied whether the GTP-binding Rho protein, which reportedly participates in the regulation of the actin cytoskeleton, is involved in the toxin action. Toxin B treatment of Chinese hamster ovary cells reveals a time- and concentration-dependent decrease in the ADP-ribosylation of Rho by *Clostridium botulinum* C3 exoenzyme in the cell lysate. Disruption of the microfilament system induced by *C. botulinum* C2 toxin or cytchalasin D does not cause impaired ADP-ribosylation of Rho. Toxin B exhibits its effects on Rho not only in intact cells but also when added to cell lysates. Besides endogenous Rho, Rho-A-glutathione S-transferase (Rho-GST) fusion protein added to cell lysate showed decreased ADP-ribosylation after toxin B treatment. Immunoblot analysis reveals identical amounts of Rho-GST and no change in molecular mass after toxin B treatment compared with controls. ADP-ribosylation of Rho-GST purified from toxin B-treated cell lysate is inhibited, indicating a modification of Rho itself. Finally, transfection of rhoA DNA under the control of a strong promoter into cells protects them from the activity of toxin B. Altogether, the data indicate that *C. difficile* toxin B acts directly or indirectly on Rho proteins to inhibit ADP-ribosylation and suggest that the cytotoxic effect of toxin B involves Rho.

The anaerobic bacterium *Clostridium difficile* has been identified as the causative agent of the antibiotic-associated diarrhoea or its fatal form, the pseudomembranous colitis (1, 2). Only strains producing two toxins, the enterotoxin (toxin A) and the cytotoxin (toxin B), are etiologically linked with the disease. After molecular cloning and sequencing of both genes the molecular masses of the single-chained molecules have been estimated to be 308 kDa for toxin A and 270 kDa for toxin B (3). In animal studies, toxin A induces hemorrhagic fluid secretion in the intestine and causes necrosis of intestinal tissue. Toxin B lacks overt enterotoxicity but is an extremely potent cytotoxin. Compared with toxin B, toxin A is about 3 orders of magnitude less potent. Both toxins are intracellularly acting bacterial protein toxins that have to be internalized via receptor-mediated endocytosis to exert their cytotoxicity (4). The toxins need not to be activated because morphological changes of the cells are identical both after the addition of toxins to the culture medium and when microinjected into target cells (5). In *vitro* the cytotoxic effects of both toxins are characterized by rounding up and arborization of cells. These morphological changes are accompanied by disruption and dramatic redistribution of the microfilament network of cells, whereas microtubules and intermediate filaments are later or much less affected (6). Although it is generally accepted that the actin cytoskeleton is mainly affected by the toxins, the precise pathobiological mechanism and the cellular targets of the cytotoxins are still unknown (for review see Ref. 7).

Various clostridial exoenzymes have been reported to influence the cytoskeleton. Whereas *Clostridium botulinum* C2 toxin directly ADP-ribosylates actin, the so-called C3-like exoenzymes, which are produced by *C. botulinum* (8–11), *Clostridium limosum* (12), Bacillus cereus (13), and Staphylococcus aureus (14), ADP-ribosylate low molecular mass GTP-binding proteins of the Rho family (RhoA, B, C). Various findings reported recently indicate that Rho proteins are involved in the regulation of the microfilament cytoskeleton (15–18). ADP-ribosylation of Rho at asparagine 41 (19), which is suggested to block the Rho-effector coupling (16), causes depolymerization of the actin cytoskeleton (15, 17). Conversely, actin polymerization is mediated by Rho 

*Note added in proof* (16). The later effect is blocked by C3-catalyzed ADP-ribosylation (16). Furthermore, C3 blocks growth factor-stimulated formation of stress fibers and focal adhesions in fibroblasts (18). Rho shares several features with other GTP-binding proteins (20). It is active in the GTP-bound form and inactive when GDP is bound. Exchange factors might be involved in the regulation of Rho activity, such as the guanine nucleotide dissociation stimulator (21) and the guanine nucleotide dissociation inhibitor (GDI), which appears to trap the inactive Rho protein (22, 23). The active state of the GTP-bound form is most likely terminated by GTPase-activating protein (24–26).

Because Rho appears to be a downstream regulator of the signal transduction pathway controlling the microfilamentous system, we studied whether *C. difficile* toxin B acts on this GTP-binding protein. Here we report that *C. difficile* toxin B inhibits subsequent ADP-ribosylation of Rho by C3-like trans- 

*The abbreviations used are: GDI, GDP dissociation inhibitor; CHO cell, Chinese hamster ovary epithelial cell; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PIP, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-bisphosphate; FITC, fluorescein isothiocyanate; GTP(γS), guanosine-5'-O-(3-thiotriophosphate).*

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EXPERIMENTAL PROCEDURES

Materials—[32P]NAD and [3H]GTP were obtained from DuPont NEN (NEN, NEN, Germany). H7 hydroxyechinomycin was from Research Biochemicals Inc.; Go 6983 was kindly donated by Dr. C. Schachtele (Godecke AG, Freiburg, Germany). All other reagents were of analytical grade and purchased from commercial sources. C. botulinum C3 exoenzyme (27), C. botulinum C2 toxin (28) and C. difficile toxin B (5) were purified as described.

Cell Culture—Chinese hamster ovary epithelial (CHO) cells were grown in Ham’s F-12 and Dubcco’s modified Eagle’s medium (1:1) supplemented with 5% fetal calf serum, 4 mM glutamine/penicillin/streptomycin; fibroblast NIH-3T3 cells and mouse embryo teratocarcinoma F9 cells, mouse lung fibroblast L929 cells and HaKat keratinocyte cells were grown in Dulbecco’s medium supplemented with 10% fetal calf serum, 4 mM glutamine/penicillin/streptomycin. After 24 h, the medium was changed, and cells were incubated with either toxin B at the indicated concentrations and times or C. botulinum C2 toxin (C2I: 100 ng/ml plus activated C2II 200 ng/ml) or cytochalasin D (9-cm diameter) was performed with 100 pl of lysis buffer.

Preparation of RhoA Fusion Protein—The complete RhoA coding sequence was amplified from RhoA plasmid ((29), kindly donated by A. Hall, Institute of Cancer Research, Chetwynd Beatty Laboratories, London, U. K.) by the polymerase chain reaction technique. The oligonucleotides for amplifying the whole coding sequence of RhoA contained additional BamHI and EcoRI linker sequences. This restriction sites allow direct cloning into pGEX-2T expression plasmid (30). The RhoA glutathione S-transferase fusion protein (Rho-GST) was isolated by affinity purification with glutathione-Sepharose beads (Pharmacia, Freiburg, Germany). The fusion protein was eluted with reduced glutathione (5 mM) in 50 mM Tris-HCl, pH 8.0.

ADP-ribosylation Reaction—Cells lysates (50 µg) or the cytosolic fraction of RhoA (1.2 mg) in lysis buffer (5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol), 50 mM trisethanolamine-HCl, pH 7.5) containing 0.3 µM or (in the case of fusion protein) 5 µM [32P]NAD (0.5 µCi) for 15 min at 30 °C. For dissociation of the Rho-GDI complex, the cytosolic fraction was incubated for 5 min with 40 µg/ml phosphatidylinositol (crude extract, mixture of PIP and PIP2 equivalent to 20 µg/ml PIP2) or SDS (0.01%), w/v) followed by the ADP-ribosylation reaction.

Guanine Nucleotide Exchange—Cell lysate was incubated with a 300 µM concentration of either GDP, GTP, or GTPS in the presence of 5 µM EDTA for 10 min at 30 °C. After the addition of 7 µM MgCl2, the ADP-ribosylation reaction was performed as described above.

Toxin B Effects in Cell Lysates—Lysates of CHO cells (12 mg/ml of protein) and Rho-GST (200 µg/ml) were incubated for 120 min at 30 °C without or with toxin B (2 µg/ml). Samples were diluted 1:10 and precipitated with trichloroacetic acid (20%, v/v), either directly or after [32P]ADP-ribosylation by C3. The pellets (30 µg) were dissolved in Laemmli sample buffer and analyzed by 12.5% SDS-polyacrylamide gel electrophoresis. To isolate Rho protein from the toxin treatment, Rho-GST immobilized to glutathione-Sepharose beads (100 µg/ml) together with lysates of CHO cells (12 mg/ml of protein) were incubated under gentle agitation and with toxin B (2 µg/ml) for 180 min at 30 °C. Beads were recovered by centrifugation and successively washed with PBS, Diethylpyrocarbonate (500 µl), and PBS plus 1× NaCl. Washed beads were resuspended in 50 mM trisethanolamine-HCl, pH 7.5, 2 mM MgCl2, 100 µM GDP, and the ADP-ribosylation reaction was performed as described above. Thereafter, the supernatant was removed, and the beads were boiled in Laemmli sample buffer and applied to SDS-polyacrylamide gel electrophoresis (12.5%).

Gel Electrophoresis—Proteins were precipitated by 20% trichloroacetic acid, and the pellets were dissolved in sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (12.5%) followed by autoradiography.

Immunolabel—Immunohistochemistry was performed according to (32) with mouse anti-Rho-GST antiserum (1,500,000) and peroxidase-coupled swine IgG against mouse IgG as secondary antibody. Visualization was achieved with the enhanced chemiluminescence system from Amersham-Buchler (Brunschwde, Germany).

Binding of [32P]GTP to Rho—Rho-GST immobilized to glutathione-Sepharose was isolated from cell lysates after toxin B treatment as described above. Binding was performed in binding buffer (0.5 mM MgCl2, 2 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM trisethanolamine-HCl, pH 7.5, 0.1 mM GTP (32) [saturating serum albumin]) in the presence of 5 µM [32P]GTP for 10 min at 30 °C and terminated by the addition of 10 µM MgCl2. The beads were washed twice with binding buffer supplemented with 10 µM MgCl2. Rho was cleaved by thrombin treatment, and the beads containing GST were removed by centrifugation. A sample of the supernatant was run on SDS-polyacrylamide gels to determine protein concentration. The second sample was run on TLC (polyethyleneimine-cellulose) to determine the bound radioactivity.

DNA Constructs and Transfections of Epitope-tagged Rhoa into Cells—rhop was ligated into a double-stranded oligonucleotide encoding the peptide sequence YTDIEMNRLGK to create an epitope tag at the amino terminus (33). This sequence is an epitope for the anti-mesothelial stromatia virus glycoprotein monoclonal antibody PS1D5 (34). The epitope-tagged rhoa DNA was inserted into the polymer of the mammalian expression vector pCB6 (a kind gift of Dr. M. Roth, Southwestern Medical Center, Dallas, Texas) under the control of the cyto-megalovirus promoter. DNA of the rhoa construct, cloned in the pCB6 vector, was prepared by the clear lysate technique and banded on CaCl2 gradients. The calcium phosphate transfection method was used according to (35) using Hep-2 cells grown on coverslips in 3.3-cm dishes. About 2 µg of vector DNA was used per dish. Calcium phosphate DNA solution was supplemented dropwise to the cells and the mixture incubated for 3% CO2 for 18 h at 37 °C. Then, the medium was removed, and the cells were rinsed twice with growth medium, refed, and incubated under 5% CO2 at 17 °C for 36 h. Transfected cells were incubated with an appropriate dilution of C. difficile toxin B for 3 h. The cells were fixed by 4% paraformaldehyde for 10 min and then processed for indirect immunofluorescence.

Immunofluorescence Labeling—For indirect immunofluorescence staining, cells were rinsed three times with PBS after paraformaldehyde fixation and incubated for 10 min with PBS containing 30 mM NH4Cl and then for 20 min in PBS containing 6.5% bovine serum albumin and 0.65% saponin. Cells were incubated with mAb P5D4 (ascitic fluid) at 1:250 dilution for 30 min. After washing with PBS containing 0.5% bovine serum albumin and 0.5% saponin, the cells were incubated with a mixture of anti-mouse antibody labeled with Texas red (Amersham) diluted 1:500 and 0.1 mg of FITC-phallolidin (Sigma) for 30 min. The preparations were washed three times with PBS containing 0.5% bovine serum albumin and 0.5% saponin and mounted in moviol. Pictures were taken with a fluorescence microscope (Zeiss Axiohot).

RESULTS AND DISCUSSION

C. difficile toxin B causes rounding up, retraction of the perikaryon, and formation of neurite-like ramifications in several cell lines. Staining of the cytoskeleton reveals disassembly and clustered condensation of the microfilament cytoskeleton (5, 36, 37). Comparable selective destruction of the actin cytoskeleton of cultured cell lines is observed after treatment with C. botulinum C3 exoenzyme (10, 15, 17), which selectively ADP-ribosylates Rho proteins (8, 15). Therefore, we studied whether toxin B acts on the cytoskeleton by interfering with the microfilament. Treatment of CHO cells with toxin B and subsequent incubation of the cell lysates with [32P]NAD and C3 exoenzyme resulted in an inhibition of [32P]ADP-ribosylation of Rho. As shown in Fig. 1A, toxin B revealed a time-dependent effect on ADP-ribosylation of Rho. At a concentration of 500 µg/ml of toxin B, initial effects were observed after 3 h, and maximal inhibition of ADP-ribosylation occurred after
transf erase activity of toxin B in intact *Xenopus laevis* oocytes. The inhibitory effect of *C. difficile* toxin B on Rho ADP-ribosylation was reproduced with several cell lines, i.e. NIH-3T3 cells, mouse embryo teratocarcinoma F9 cells, mouse lung fibroblast L929 cells, and HaKat keratinocytes (data not shown), indicating that inhibition of ADP-ribosylation of Rho is a general feature of toxin B action.

To study whether the morphological changes induced by toxin B and toxin-induced decrease in ADP-ribosylation of Rho are related phenomena, NIH-3T3 cells were treated with increasing concentrations of toxin B (2–2000 pg/ml) for 3 h. Thereafter, the completely rounded cells were counted, and the amount of ADP-ribose incorporated into Rho was determined by C3-catalyzed [32P]ADP-ribosylation of the cell lysates. As shown in Fig. 1C, treatment of NIH-3T3 cells with increasing concentrations of toxin B showed an increased rounding up of cells. In a dose-dependent manner cell rounding was inversely related to ADP-ribosylation of Rho.

Several cytotoxins affect the microfilament cytoskeleton by interacting directly with actin. *C. botulinum* C2 toxin ADP-ribosylates actin and traps it in the monomeric form (39). Cytochalasin D acts as barbed end-capping protein to inhibit further elongation of the filament (40). Both toxins induce gross changes in the distribution of cellular actin and depolymerization of actin filaments. To exclude that the action of toxin B on Rho is secondary to the destruction of the microfilament cytoskeleton, we studied the effects of C2 toxin and cytochalasin D on ADP-ribosylation of Rho. Incubation of CHO cells with either C2 toxin (100 ng/ml C2I and 200 ng/ml C2II), cytochalasin D (10 µM), or toxin B (2 ng/ml) for 3 h induced almost complete rounding up in each case. However, lysates from C2 toxin- and cytochalasin D-treated cells showed the same amount of ADP-ribosylation of Rho as control lysates, whereas lysates from toxin B-treated cells revealed a clear decrease in ADP-ribosylation (Fig. 2). Thus, inhibition of ADP-ribosylation of Rho appears to be specific for toxin B and, most likely, is no response to depolymerization of actin filaments.

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Impaired ADP-ribosylation of Rho by toxin B treatment may reflect functional inactivation of the GTP-binding protein, an effect that is consistent with the toxin action on the actin cytoskeleton. Recently it has been reported that Rho is functionally blocked by Rho-GDI, which forms a high affinity 1:1 complex with Rho and inhibits modification by C3 (41). This complex is apparently cleaved by phosphatidylinositides or (PIP2, 40 mg/ml) caused an increase in ADP-ribosylation. EDTA-induced guanine nucleotide exchange was performed without Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography (shown).

clarified whether changes in phosphorylation are direct effects of the toxin or merely the response to the altered cytoskeleton. We therefore examined whether kinase inhibitors interfere with the toxin B effect on Rho. The kinase inhibitors were added either before (60 min) toxin treatment or simultaneously with the toxin. Neither H-7 (10, 30, or 100 μM) (44) nor the specific inhibitor for protein kinase C Gö 6983 (1, 10 μM) (Gö 6983 is named compound 23 in Ref. 45) influenced the effect of toxin B on cell morphology and on Rho modification by C3. Furthermore, the involvement of protein kinase A was studied by using cAMP/forskolin. When serum-starved NIH-3T3 cells were treated with dibutyryl cAMP (1 mM) plus forskolin (10 μM) for 24 h, the observed morphological effects were similar to those induced by toxin B. These agents, however, did not cause impairment of the ADP-ribosylation of Rho (data not shown). All of these findings largely exclude that toxin B does act on Rho via cGMP-, cAMP-dependent kinases or protein kinase C.
Fig. 6. Effects of the transfected RhoA protein in Hep-2 cells on cytotoxic activity of toxin B. Hep-2 cells were transfected with RhoA as described under “Experimental Procedures.” Panel 1: A, control Hep-2 cells stained with anti-vesicular stomatitis virus glycoprotein monoclonal antibody P5D4 or B, with FITC-phalloidin. C, Hep-2 cells transfected with epitope-tagged RhoA (17 h after transfection) and stained with P5D4.
The intact cell structure is not essential for the toxin B effect on Rho because the inhibitory effect on the ADP-ribosylation of Rho was also detected in cell lysates. Lysates from CHO cells (protein concentration 12 mg/ml) were incubated with toxin B (2 µg/ml) for 3 h, and subsequent ADP-ribosylation of Rho was decreased (not shown). Furthermore, RhoA-GST (Rho-GST) fusion protein (48 kDa), which was added to the lysates, was also affected by toxin B after incubation for 3 h at 30 °C (Fig. 4B). Increasing concentrations of the fusion protein did not prevent the toxin B-induced inhibition of the ADP-ribosylation of Rho and Rho-GST (not shown). However, to detect a decreased labeling comparable to that observed with endogenous Rho, the incubation time was prolonged up to 5 h. Thus, recombinant Rho fusion protein showed the same decreased ADP-ribosylation after toxin treatment as endogenous Rho. It is not clear whether the fusion protein interacts with cellular Rho-regulating proteins, but it behaves as endogenous Rho with respect to GTP binding, ADP-ribosylation and decreased ADP-ribosylation after toxin treatment. Therefore, the fusion protein is a valuable model to study the effects of toxin B on Rho. The finding that increasing concentrations of Rho fusion protein (up to a surplus compared with endogenous Rho) were affected by toxin B at a constant low concentration suggests that toxin B acts rather directly on Rho than on Rho-regulating proteins. Furthermore, this finding supports the notion that the toxin exhibits enzyme activity.

Recently clostridial neurotoxins were shown to mediate their toxic effect via proteolytic cleavage of neural peptides (46). To test whether toxin B affects Rho by proteolysis, immunoblot analysis was employed. In default of antibodies that recognize endogenous Rho, we studied the Rho fusion protein with anti-GST antibody. Cell lysates (12 mg/ml) plus Rho-GST fusion protein (100 µg/ml) were incubated with toxin B (2 µg/ml), diluted, subsequently electroblotted, and analyzed with anti-GST (Fig. 4A). This antisera was raised against Rho-GST and did not recognize endogenous Rho. Toxin-treated and untreated Rho-GST revealed no differences in staining and molecular mass (48 kDa of Rho-GST). In controls, ADP-ribosylation of Rho resulted in a slight increase in the apparent molecular mass (Fig. 4A). The corresponding autoradiography (Fig. 4B) shows the impaired [32P]ADP-ribosylation of Rho from toxin-treated cells. The results of the immunoblot analysis clearly showed that toxin B did not cause gross alterations such as proteolytic degradation or clipping of Rho nor a modification that visibly increases the molecular mass. In the absence of cell lysate, toxin B treatment caused neither differences in staining of Rho-GST (Fig. 4A) nor impaired ADP-ribosylation of Rho-GST (not shown). Thus, cell lysates were essential for the toxin effects on Rho, indicating that toxin B needs a cellular cofactor for its action.

Besides inhibition of ADP-ribosylation of endogenous Rho and Rho-GST in cell lysate, toxin B also affected fusion protein that was immobilized to glutathione-Sepharose beads. After incubation with toxin B, the beads containing Rho-GST were removed from the lysate and washed with buffer containing high salt and detergent. The ability of Rho-GST to serve as substrate for C3 was decreased after toxin treatment but remained unaltered in control preparations (Fig. 5). The Coomassie Blue-stained SDS-polyacrylamide gel revealed an almost homogenous Rho-GST protein band. No additional Rho-binding proteins possibly involved in inhibition of ADP-ribosylation were detectable. To exclude that toxin treatment induces denaturing of Rho-GST, [α-32P]GTP binding was studied. Therefore, isolated Rho-GST immobilized to beads was loaded with [α-32P]GTP. Rho was removed from the fusion protein by thrombin treatment, and the bound radioactivity of Rho was determined by TLC. A second sample was run on SDS-polyacrylamide gels to prove identical protein concentration. Toxin B-treated Rho-GST exhibited guanine nucleotide binding identical to that of control Rho (not shown), indicating intact protein structure of Rho after toxin treatment. These findings suggest that Rho is modified by a covalent-linked moiety or, at least, a tightly linked moiety that prevents C3-catalyzed ADP-ribosylation. Further studies are required to characterize the exact nature of the modification.

Finally, to demonstrate that Rho was directly implicated in the mechanism of C. difficile toxin B activity, we transfected the DNA of RhoA under the control of a strong promoter (cytomegalovirus) into Hep-2 cells. That way a large amount of RhoA will be produced inside the cells, making them less sensitive to toxin B, if this GTP-binding protein is the target of the cytotoxin. The vector (plasmid without Rho) itself showed absolutely no effect on the actin cytoskeleton. To improve immunofluorescent visualization of Rho we employed epitope-tagged RhoA as done by (47) except that the vesicular stomatitis virus glycoprotein epitope (34) was used instead of the myc peptide. After transfection and expression of the protein, cells were treated with toxin B (250 pg/ml for 3 h which gave 100% of round cells in control nontransfected Hep-2 cells). After fixation the cells were processed for immunofluorescence to detect both RhoA and F-actin. As shown in Fig. 6, Hep-2 cells transfected with RhoA (Fig. 6, panel 1C) contained a large amount of F-actin structures as already reported (16) (Fig. 6, panel 1D). When Hep-2 cells, transfected with RhoA, were incubated with toxin B, they clearly exhibited abundant F-actin structures (Fig. 6, panel 2D, large arrows), whereas nontransfected cells had their stress fibers completely destroyed (Fig. 6, panel 2, C and D, small arrows). This result indicates that overexpression of the RhoA protein affords protection against toxin B. This is probably because of the large quantity of the RhoA inside transfected cells.

In summary, we show that C. difficile toxin B acts on the Rho protein to inhibit C3-catalyzed ADP-ribosylation. The Rho protein has been shown to participate in the regulation of the actin cytoskeleton. Loss of the ability of Rho to serve as substrate for C3 correlates with the toxin-induced disarrangement of microfilaments. The effects of toxin B are correlated with a selective destruction of the actin filaments, but as shown by Lin and co-workers (48), the toxin does not interact directly with actin. Our findings indicate that a regulatory protein of the actin cytoskeleton is modified by toxin B. Furthermore, the data suggest that the cytotoxic effect of C. difficile toxin B is mediated via functional inactivation of the GTP-binding protein Rho, most likely by an enzymatic activity.

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