INFECTION AND IMMUNITY, Apr. 1995, p. 1421–1426 Vol. 63, No. 4
0019-9567/95/$04.00+0

Involvement of Ras-Related Rho Proteins in the Mechanisms of Action of *Clostridium difficile* Toxin A and Toxin B†

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Received 16 November 1994/Returned for modification 6 January 1995/Accepted 27 January 1995

Toxins A and B of *Clostridium difficile* are responsible for pseudomembranous colitis, a disease that afflicts a substantial number of hospitalized patients treated with antibiotics. A major effect of these proteins is the disruption of the actin cytoskeleton. Recently, I. Just, G. Fritz, K. Aktories, M. Giry, M. R. Popoff, P. Boquet, S. Hegenbarth, and C. von Eichel-Streiber (J. Biol. Chem. 269:10706–10712, 1994) implicated Rho proteins as cellular targets of *C. difficile* toxin B, since pretreatment of cells or purified Rho with toxin prevented subsequent ADP-ribosylation of Rho by exoenzyme C3. Moreover, they showed that overexpression of Rho proteins in cells suppressed cell rounding normally associated with exposure of cells to *C. difficile* toxin B. Here we expand these findings by showing directly that Rho proteins are covalently modified by both *C. difficile* toxins A and B. In addition, we demonstrate that the stability of toxin-modified Rho in NIH 3T3 cells is dramatically reduced. Finally, we show that *C. difficile* toxins A and B do not have similar effects on the closely related Rac and CDC42 GTP-binding proteins.

The intestinal pathogen *Clostridium difficile* is responsible for pseudomembranous colitis, a disease that afflicts a substantial number of hospitalized patients treated with antibiotics (13). Intestinal damage is mediated by two very similar protein exotoxins, toxin A and toxin B (1, 4). Toxin A has been shown to cause damage to the intestinal mucosa in situ (28), while both toxins are cytopathic for a variety of cell types in culture (15, 20, 27, 33). Intoxicated cells exhibit a retraction of cell processes and rounding of the cell body. This phenotype is a consequence of the disassembly of filamentous actin, as evidenced by a loss of F-actin and an increase in G-actin prior to the onset of cell rounding (15, 20). Since very few toxin molecules are necessary to produce cell rounding, it has been proposed that toxins A and B act enzymatically within target cells to modify proteins that regulate actin polymerization and fiber assembly (20).

The Rho family of GTP-binding proteins have recently been implicated in the regulation of actin filament assembly (8). This Ras-related family includes RhoA, RhoB, and RhoC; Rac 1 and 2; CDC42; TC-10; and RhoG. RhoA, RhoB, and RhoC are specific targets of the bacterial ADP-ribosyltransferase exoenzyme C3 (3, 11). ADP-ribosylation of Rho proteins by introduction of C3 into cells causes Rho inactivation and subsequent actin filament breakdown, cell retraction from the substratum, and rounding (19, 24). Moreover, microinjection of a constitutively activated form of Rho enhances the formation of actin stress fibers (21). Rac proteins also seem to regulate actin filaments, although these Rho family members preferentially influence cortical actin fibers associated with the ruffling of the plasma membrane (22).

In searching for potential targets of the *C. difficile* toxins, we reasoned that the Rho proteins would be good candidates because of their critical role in controlling actin and the precedent that they already were a target for a bacterial enzyme. In fact, one of us demonstrated several years ago that exoenzyme C3 could no longer ADP-ribosylate Rho proteins from cells pretreated with *C. difficile* toxin A or B (23). A similar finding for toxin B has recently been published by Just et al. (10). These results suggested that *C. difficile* toxins covalently modify Rho proteins. Moreover, this recent work showed that alteration of Rho was important, because overexpression of exogenous RhoA protected HEp-2 cells from toxin B-induced dissolution of actin filaments. Finally, a subsequent report showed that neither ADP-ribosylation nor phosphorylation of Rho was responsible for the inability of C3 to ADP-ribosylate *C. difficile*-treated Rho proteins (12).

Here we show directly that both *C. difficile* toxin A and *C. difficile* toxin B do in fact lead to the covalent modification of Rho proteins. Moreover, in NIH 3T3 cells, this alteration causes the destabilization of Rho proteins. Finally, similar toxin effects on the closely related Rac and CDC42 GTPases were not observed.

MATERIALS AND METHODS

Treatment of NIH 3T3 and COS-7 cells with purified toxin A and toxin B. NIH 3T3 cells were grown in Dulbecco's modified minimal medium supplemented with 10% bovine calf serum. The cells were transfected by Ca_2PO_4 precipitation of DNA, and the Myc-RhoA-expressing cell line was selected and maintained in medium containing G418 (400 μg/ml). COS-7 cells were grown in Dulbecco's modified minimal medium supplemented with 10% Fe^{2+}-enriched calf serum. COS-7 cells were transiently transfected by using DEAE-dextran. After 36 to 60 h, cells were treated with the specified reagent for the indicated time. Cells (~3×10^6) were gently removed from the dish with a rubber policeman, pelleted (1,000 × *g*; 5 min), snap frozen in a dry ice-ethyl alcohol (EtOH) bath, and stored at −80°C until use. Toxins A and B were purified from culture supernatants of toxigenic *C. difficile* VPI 10463 as described previously (7, 20). Typical stock solutions of toxin A used in these studies contained 1.0 mg/ml and were cytotoxic

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† This paper is dedicated to the memory of the late Michael Gill, who had remarkable insight into the connection between bacterial toxins and GTP-binding proteins.

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to IMR-90 fibroblasts at dilutions of 10^3 to 10^4. Toxin B stock solutions contained similar amounts of protein, but they were cytotoxic at a 10^6 dilution.

Plasmid constructions. The Myc-RhoA fusion protein was constructed by PCR using a 5′ primer encoding a *Bam*HI site followed by a 10-amino-acid epitope of the c-Myc protein (EQKLISEEDL) that is recognized by the mouse monoclonal antibody 9E10 (5, 17). The 3′ primer contained sequences distal to the cloning sites of pGEX2T (Pharmacia). PCR was performed on RhoA cloned into pGEX2T. The PCR product was cut with *Bam*HI and *Eco*RI so that it could be cloned into these sites in the polylinker of the mammalian expression vector pJ4. pJ4-Myc-RhoA was then cotransfected with pZIPneoSV(X) (which provided a selectable marker) into NIH 3T3 cells to produce cell lines stably expressing Myc-RhoA. For transient expression in COS-7 cells, the Myc-RhoA fusion was removed from pJ4 at the *Sal*I and *Eco*RI sites and then cloned into the same sites in pMT3. The pGEX2T-RhoA plasmid was obtained from Alan Hall (University of London).

Preparation of whole-cell extracts. Cell pellets were lysed in TB buffer (1% Triton X-100, 20 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 125 mM NaCl, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1 mM dithiothreitol) and assayed for protein content (14). No significant difference in protein content between untreated and toxin-treated cell extracts was detected. Sample buffer (2×; 100 mM Tris-HCl [pH 6.8], 3.0% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol) was then added to the samples (containing both TB-soluble and TB-insoluble fractions). They were then boiled for 5 min at 95°C and electrophoresed by SDS-polyacrylamide gel electrophoresis (PAGE).

Immunoblotting. Immunoblots were incubated with antibody (0.5 μg/ml) for 2.5 h at room temperature in 20 mM Tris-HCl (pH 7.5)–150 mM NaCl–0.05% Tween 20 (TBST) plus 5% dry milk. The blots were washed in TBST, incubated for 1 h with an anti-rabbit immunoglobulin G conjugated to horseradish peroxidase, and then developed by using enhanced chemiluminescence (Amersham). Anti-RhoA antibody was purchased from Santa Cruz, anti-Rac antibody was obtained from Gary Bokoch (Scripps Clinic, San Diego, Calif.), and anti-CDC42 antibody was obtained from Richard Cerione (Cornell University, Ithaca, N.Y.). Ras proteins were immunoprecipitated by using the monoclonal antibody YA6-259. Immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with polyclonal anti-Ras antibody (Upstate Biotechnology).

C3 ADP-ribosyltransferase assay. For ADP-ribosylation assays, purified C3 (10 to 40 μg/ml) (3a) was added in a reaction buffer (20 μl) (10 μM NAD, 0.25 μM [^{32}P]NAD^+ [250 Ci/mmol] [ICN], 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8.0], 2 mM $MgCl_2$, 1 mM dithiothreitol, 200 μM GTP, 10 mM thymidine, 15 mM isonicotinic acid hydrazide, 0.1% sodium deoxycholate) with 100 μg of whole-cell extract protein. This mixture was incubated for 1 h at 30°C. The reaction was stopped by adding 2× sample buffer and heating the mixture at 95°C for 5 min. Samples were then resolved by SDS-PAGE. The dried gel was exposed for autoradiography.

Precipitation of Rho family proteins, using GST-GDI. The binding of Rho proteins to glutathione *S*-transferase–GDP dissociation inhibitor (GST-GDI) agarose beads was adapted from a previously published protocol (25). Briefly, cells were lysed in TB on ice. Insoluble material was removed by centrifugation at 12,000 × *g* for 15 min at 4°C. Equivalent amounts (~4 μg of protein) of GST or GST-GDI (9) bound to Sepharose beads were added to each sample. The samples were incubated for 90 min with mixing at 4°C. The beads were then washed three times with TMNT buffer (0.5% Triton X-100, 20 mM Tris [pH 7.5], 1 mM $MgCl_2$, 100 mM NaCl), and the bound proteins were resolved by SDS-PAGE.

Microinjection of NIH 3T3 cells with RhoA protein. NIH 3T3 cells were grown to subconfluence on glass coverslips in Dulbecco's modified minimal medium supplemented with 10% fetal bovine serum. Recombinant RhoA was expressed as a GST fusion and purified as described previously (35). Recombinant Ras was purified as described previously (6). Purified recombinant proteins were microinjected into the cytosol of target cells, after which the cells were incubated at room temperature for 30 min, purified toxin B was then added and the cells were incubated for an additional 60 to 90 min. Cells were subsequently labelled with rhodamine phalloidin to stain filamentous actin and photographed at a magnification of ×1,000. Of the control cells treated with toxin, >90% displayed a rounded phenotype.

RESULTS

Specific loss of Rho proteins in *C. difficile* toxin A- and toxin B-treated NIH 3T3 cells. Previous reports have shown that pretreatment of several mammalian cell lines with *C. difficile* toxin B prevented subsequent ADP-ribosylation of Rho proteins by exoenzyme C3 (10). A similar finding is shown here for both toxin A- and toxin B-treated NIH 3T3 cells (Fig. 1B). Extracts of cells previously untreated or treated with toxin B were incubated with C3 plus [^{32}P]NAD^+. Radioactively labelled proteins were then separated by SDS-PAGE and detected by autoradiography. Treated cells (lane 4) contained less than 10% of the ADP-ribosylatable RhoA, RhoB, and RhoC proteins found in untreated cells (lane 3). Extracts treated with [^{32}P]NAD^+, but not C3, showed background ADP-ribosylation (lanes 1 and 2). Similar results were obtained with toxin A (data not shown).

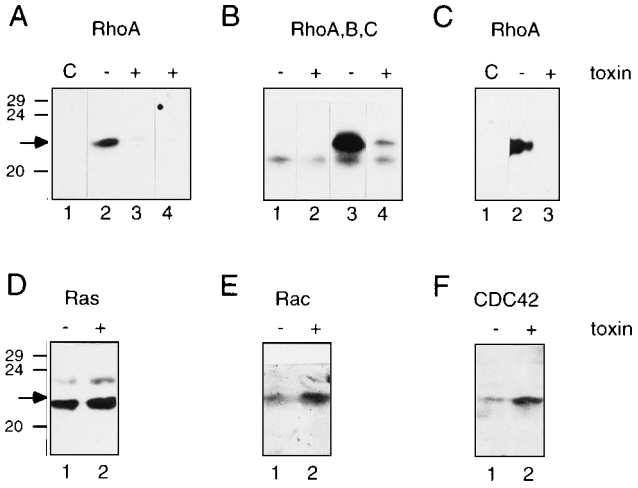

FIG. 1. Specific loss of Rho proteins in *C. difficile* toxin A- and toxin B-treated NIH 3T3 cells. Confluent monolayers of cells were treated with either buffer (−) or purified *C. difficile* toxin (+) (toxin A, 100 ng/ml; toxin B, 10 ng/ml). After 20 h, when >90% of the cells were rounded, cells were collected and assayed for total protein, and equivalent amounts were treated as described below. (A) Immunoblot of RhoA. Control immunoblotting with anti-RhoA immunoglobulin G in the presence of excess competing antigen (lane 1) and immunoblotting with anti-RhoA immunoglobulin G of lysates from untreated (lane 2), toxin A-treated (lane 3), and toxin B-treated (lane 4) cells were performed. (B) C3 ADP-ribosylation of Rho proteins. Control and toxin B-treated cell extracts incubated with [^{32}P]NAD^+ in the absence of exoenzyme C3 (lanes 1 and 2) and control and toxin B-treated cell extracts incubated with [^{32}P]NAD^+ and exoenzyme C3 (lanes 3 and 4) are shown. (C) Immunoblotting of Rho proteins precipitated first with Sepharose beads containing GST-GDI. Proteins precipitated with control GST-containing beads (lane 1) and proteins precipitated with GST-GDI-containing beads (lanes 2 and 3) are shown. (D) Immunoblot of Ras protein. (E) Immunoblot of Rac protein. (F) Immunoblot of CDC42 protein. (See Materials and Methods for details.)

However, the C3 assay does not discriminate between whether Rho proteins in cells are modified or whether the proteins are no longer present. To resolve this question, we performed immunoblotting with an anti-RhoA antibody (directed against amino acids 119 to 132) to test for the presence of the RhoA protein in these extracts. Whole-cell extracts were prepared in SDS-PAGE buffer from untreated and toxin A- or toxin B-treated NIH 3T3 cells. Equal amounts of protein were electrophoresed and then immunoblotted with anti-RhoA antibodies (Fig. 1A). A predominant band of the correct size (22 kDa) was observed when extracts from untreated cells were probed with anti-RhoA antibodies but not when they were probed with antibodies plus competing peptide antigen (Fig. 1A, lanes 1 and 2). Remarkably, when cells were first treated with either toxin A or toxin B for 24 h to allow toxin-induced cell rounding, the level of RhoA protein was reduced to barely detectable levels (lanes 3 and 4).

A third probe for Rho, Rho-GDI, also failed to detect RhoA in toxin-treated cells. Rho-GDI binds all Rho family members at their prenylated COOH termini (9, 30). As a fusion protein with GST, Rho-GDI can precipitate Rho, Rac, and CDC42 proteins from cell extracts (references 9 and 25 and data not shown). Thus, extracts from untreated cells or from cells treated with toxin B were incubated with Sepharose beads

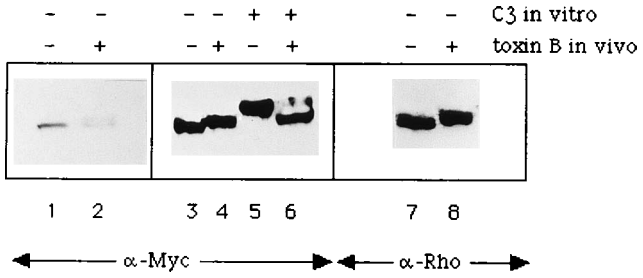

FIG. 2. Modification of a Myc-RhoA fusion protein by *C. difficile* toxin. Immunoblots of total cell lysates expressing the Myc-RhoA fusion protein are shown. Lanes 1 and 2 each contain 25 μg of whole-cell lysates of NIH 3T3 cells stably expressing Myc-RhoA. Lanes 3 through 8 contain whole-cell lysates of COS-7 cells transiently expressing Myc-RhoA. Lanes 3 through 6 contain 20 μg of protein, while lanes 7 and 8 contain 50 μg of protein. Cells were either untreated (lanes 1, 3, 5, and 7) or treated in vivo with toxin B (0.1 to 1 μg/ml) (lanes 2, 4, 6, and 8). An in vitro C3 assay was subsequently performed on the untreated and toxin B-treated whole-cell extracts (lanes 5 and 6). Lanes 1 through 6 were probed with anti-Myc (α-Myc) antibody. Lanes 7 and 8 were probed with anti-RhoA (α-Rho) immunoglobulin G. The top of the region of the gel shown represents proteins of 24 kDa, whereas the bottom of the region represents proteins of 20 kDa.

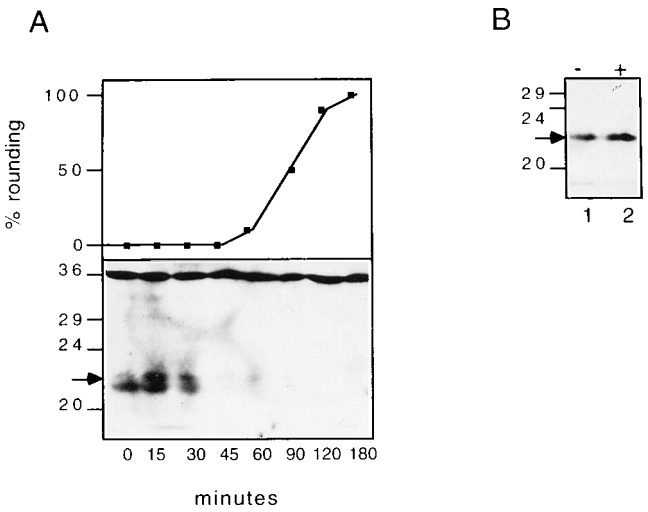

FIG. 3. The loss of Rho proteins precedes cell rounding and is not a result of actin filament breakdown. (A) Cells were exposed to *C. difficile* toxin B (100 ng/ml) for various times. (Top) The percentages of cells displaying a rounded phenotype were quantitated. (Bottom) RhoA protein was detected by immunoblotting. Similar results were obtained when Rho proteins were detected by C3 ADP-ribosylation (data not shown). (B) RhoA levels after actin filament breakdown by treatment with cytochalasin B. Cells were untreated (−) or treated with 2.5 μg of cytochalasin B per ml (+). After 20 h, when >90% of the cells were rounded, cell extracts were examined for RhoA protein by immunoblotting.

containing GST-GDI. The precipitated proteins were separated by SDS-PAGE, blotted onto nitrocellulose, and probed for the presence of Rho proteins with the previously described anti-RhoA antibodies (Fig. 1C). As expected, extracts treated with control GST-containing beads (lane 1) precipitated no detectable Rho protein; however, GST-GDI-containing beads precipitated Rho from untreated cell extracts (lane 2) but not from toxin-treated cell extracts (lane 3).

The loss of Rho proteins from toxin-treated cells was quite specific, since no significant loss of Ras proteins was observed (Fig. 1D). More remarkably, the levels of the closely related Rac and CDC42 proteins also did not decrease after toxin treatment (Fig. 1E and F). In fact, their levels seemed to rise. This increase was not unique to *C. difficile* toxin treatment, however, since it was also observed when actin filaments were disrupted by exposure of cells to cytochalasin B (data not shown).

Covalent modification of RhoA induced by *C. difficile* toxins. The simplest explanation for these results is that treatment of cells with either toxin A or B leads to the specific degradation of RhoA, RhoB, and RhoC. Given the known function of Rho proteins, this could contribute to the toxin-induced breakdown of the actin cytoskeleton. Alternatively, however, Rho could still have been present in an inactivated form in treated cells but modified such that it was no longer detectable by either anti-RhoA peptide antibodies in immunoblots or by exoenzyme C3.

To investigate this possibility further, we generated an additional detection site on RhoA by adding an epitope from the c-Myc protein to its amino terminus. Myc-RhoA was transfected into NIH 3T3 cells, and cells that expressed the tagged protein, as visualized by immunoblotting with a monoclonal antibody to the Myc sequences, were selected (Fig. 2, lane 1). These cells expressed Myc-RhoA at a level that was between 25 and 50% of that of endogenous RhoA (as assessed by C3 ADP-ribosylation). Surprisingly, toxin treatment did not result in a dramatic loss of Myc-RhoA, as seen for endogenous RhoA. Instead, a very small but detectable decrease in the electrophoretic mobility (equal to <1 kDa) of a fraction of the tagged protein was observed (Fig. 2, lane 2), indicating that *C. difficile* toxins lead to the covalent modification of RhoA.

To visualize the tagged protein more clearly, transient transfection of Myc-RhoA into COS-7 cells was performed (Fig. 2, lanes 3 through 8). Here again, the anti-Myc antibody detected no loss of Myc-RhoA protein after toxin treatment, but rather a toxin-induced decrease in the mobility of Myc-RhoA (compare lanes 3 and 4). This mobility shift was less than that observed when Myc-RhoA in cell lysates was ADP-ribosylated by exposure to exoenzyme C3 (lane 5). Interestingly, pretreatment of cells with toxin B reduced the amount of the more slowly migrating Myc-RhoA generated by C3 treatment (lane 6). Similar results were obtained when ADP-ribosylation of Myc-RhoA by C3 was detected by incorporation of [^{32}P]ADP-ribose (data not shown). Thus, the toxin-induced covalent modification of Rho prevented subsequent ADP-ribosylation by exoenzyme C3.

Did covalent modification also prevent RhoA from being recognized by anti-Rho peptide antibodies? The answer is no. The antibody against RhoA detected modified Myc-RhoA just as well as it detected unmodified Myc-RhoA in extracts of transiently transfected COS cells (Fig. 2, lanes 7 and 8). This finding proved that the absence of immunoreactive endogenous RhoA in toxin-treated NIH 3T3 cells (Fig. 1) truly represented the absence of endogenous RhoA protein. Apparently, the addition of the Myc epitope tag to the N terminus of RhoA allowed toxin-induced covalent modification but prevented subsequent degradation of Rho.

RhoA degradation precedes cell rounding induced by toxins. In order to determine the relevance of the degradation of the Rho proteins, we examined whether it occurred before or after the cells were visibly affected by the toxins. If the degradation of the modified Rho proteins occurs before the cells appear rounded, then it was possible that the actual degradation of the protein could cause the observed cytopathological effects and not the covalent modification. To assess directly when the loss of RhoA protein occurs, we performed time course immunoblotting with the anti-RhoA antibody (Fig. 3A). RhoA degradation preceded cell rounding, and therefore it does not appear to be a nonspecific effect of intoxicated cells. There is still

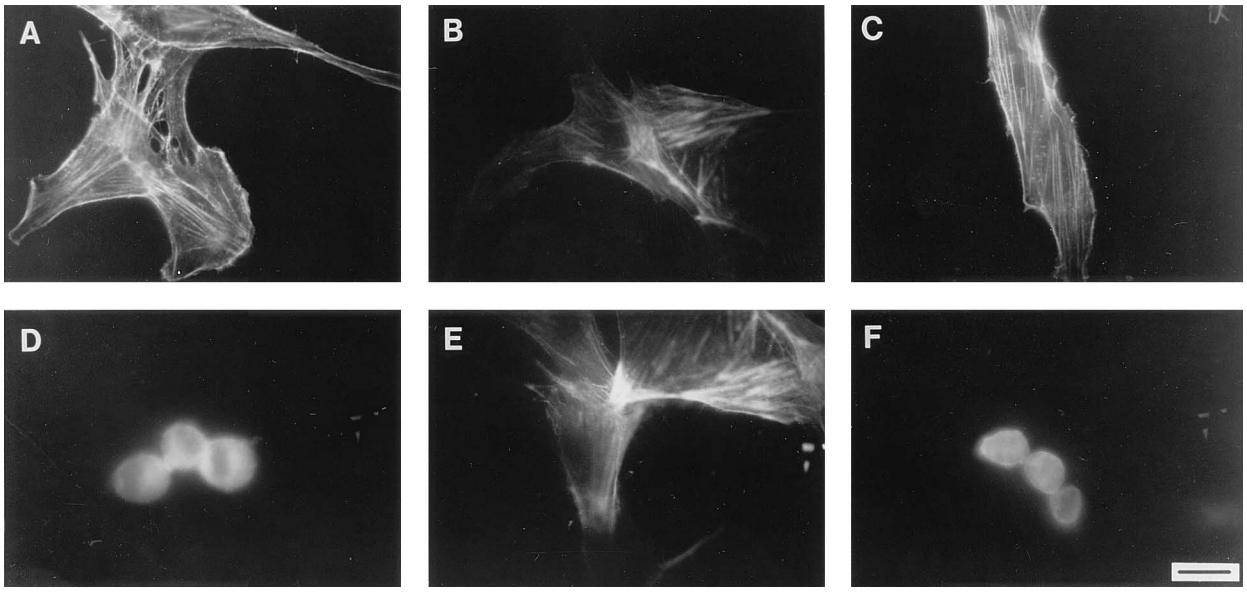

FIG. 4. Protection of cells from cytotoxic effects of *C. difficile* toxin B by microinjection of recombinant RhoA protein. (A through C) Cells not treated with toxin. (D through F) Cells treated with toxin B (1 to 5 μg/ml). (A and D) Cells microinjected with control buffer. (B and E) Cells microinjected with RhoA (150 μg/ml). (C and F) Cells microinjected with Ras (250 μg/ml). Bar, 20 μm.

the possibility that the observed degradation was merely a consequence of the breakdown of the actin filaments in NIH 3T3 cells. However, cytochalasin B-induced actin filament breakdown did not change the level of immunologically detectable RhoA protein for this cell line (Fig. 3B).

Microinjection of RhoA and not Ras protects cells from toxin-induced rounding. Finally, if the degradation of Rho proteins is primarily responsible for the phenotype induced by toxins A and B, then the introduction of excess Rho proteins would be predicted to confer resistance to cytotoxicity. In fact, this was observed. The microinjection of control buffer, RhoA, or Ras produced no visible effect on actin filaments (Fig. 4A through C). Cells microinjected with control buffer and then exposed to high levels of toxin B showed characteristic rounding within 60 min (Fig. 4D). However, >90% of the cells preinjected with recombinant RhoA were protected from toxin-induced cytotoxicity during the time course of this experiment (Fig. 4E). It was important to show that this protective effect was specific to the Rho proteins and could not be mimicked by another similar protein. Therefore, we also examined the ability of microinjected Ras protein to protect cells. The effect of RhoA was specific, since preinjection of similar amounts of Ras yielded no observable protective effect (Fig. 4F).

DISCUSSION

The experiments described in this paper extend the previous work reported by Just et al. (10, 12) in demonstrating that cytotoxicity induced by *C. difficile* toxins is mediated, at least in part, by the loss of functional Rho protein. In a manner consistent with that of previous publications (10, 23), we demonstrated that pretreatment of cells with these toxins modified Rho such that it was no longer a substrate for exoenzyme C3. This is the first study, however, to directly assay endogenous Rho in toxin-treated cells, and we find that the protein is highly destabilized. This mechanism could contribute to the loss of Rho function in toxin-treated cells. Although we placed the lysates rapidly into SDS sample buffer, we cannot exclude the possibility that the protein was rapidly degrading upon lysing of the cells.

Fortunately, we found that epitope-tagged Rho was stable in toxin-treated cells. This allowed us to visualize a toxin-induced reduction in the mobility of Rho in SDS gels, demonstrating convincingly that *C. difficile* toxins induce a covalent modification of Rho. Researchers who performed previous studies using Rho fused to GST could only infer a toxin-induced covalent modification from the inability of C3 to ADP-ribosylate toxin-treated Rho (10). Apparently, the large size of this Rho fusion protein made the subtle mobility change induced by toxin not detectable in the electrophoresis system used by these investigators. The stable, epitope-tagged version of Rho used here also allowed us to show that the anti-RhoA antibodies used in immunoblots described above retained the ability to detect toxin-modified Rho. This proved that the loss of immunoreactive endogenous Rho in toxin-treated cells truly reflected the loss of Rho protein.

Although Rho proteins were clearly modified by both *C. difficile* toxins, we could not detect any effects on the closely related Rac and CDC42 proteins. This apparent specificity is identical to that displayed by exoenzyme C3. In addition, modification of Rho by toxins A and B prevents subsequent ADP-ribosylation by exoenzyme C3. These findings suggest that *C. difficile* toxins A and B may modify Rho near the known C3 ADP-ribosylation site at Asn-41. This is in the effector domain known to be required for downstream signalling from Ras-related proteins. Thus, modification of Rho by toxins A and B may be inactivating even in the absence of subsequent degradation.

We have observed that the degradation of the modified Rho proteins was rapid and preceded cell rounding. However, the significance of this breakdown is not totally clear. The degradation of Rho was observed in vivo only, as we have not seen it occur in broken-cell extracts incubated with the toxins. The ADP-ribosylation of $G_{s\alpha}$ by cholera toxin has been demonstrated to lead to its specific degradation in vivo and in broken-cell extracts (2). However, the degradation of toxin-modified G

proteins is not a common phenomenon, since the modification of $G_{i\alpha}$ by pertussis toxin does not alter its stability.

Why is the Myc-RhoA protein stable and not the endogenous Rho protein? The most likely explanation is that the modified version of endogenous Rho is recognized as foreign and removed by endogenous protease systems. That the epitope-tagged Rho containing c-Myc sequences at its amino terminus resisted toxin-induced degradation is not particularly surprising. The amino termini of proteins have been shown to be critical determinants recognized by specific degradation pathways in cells (31).

This effect on Rho is critical for *C. difficile* toxicity, since toxin-induced actin breakdown was prevented by premicroinjection of RhoA protein and not Ras. A similar protecting effect of Rho was observed previously, although no negative control proteins were included in the study (10). This mechanism of toxicity is quite compatible with the observation that *C. difficile* toxins lead to the disassembly of the actin filament network and with the known requirement for Rho function in maintaining proper actin filament organization.

The chemical nature of the toxin-induced modification of Rho has not yet been elucidated. It has recently been shown by microinjection of a GST-RhoA fusion protein into *Xenopus* oocytes that the modification induced by toxin B is neither ADP-ribosylation nor phosphorylation (12). Using COS cells expressing Myc-RhoA, we have also found that the modification induced by toxin B is not ADP-ribosylation (data not shown). This sets *C. difficile* toxins apart from the vast majority of proteins that influence GTPases by ADP-ribosylation. These include cholera and pertussis toxins, which target heterotrimeric G proteins, diphtheria toxin, which inactivates translation factors, and exoenzyme C3, which inactivates Rho proteins (16).

It is remarkable how Rho is targeted by such a wide variety of bacterially produced proteins. Many bacterial strains produce C3-like ADP-ribosyltransferases specific for Rho, including various *Clostridium* strains (11, 24) and *Staphylococcus aureus* (26). Moreover, an *Escherichia coli*-produced toxin, cytotoxic necrotizing factor 2, has recently been shown to covalently modify Rho proteins (18) by an unknown mechanism. A decrease in electrophoretic mobility of Rho proteins was also observed after cytotoxic necrotizing factor 2 treatment. Like *C. difficile* toxins A and B, the *E. coli* protein does not appear to be an ADP-ribosyltransferase. However, the stability of the Rho protein was not altered. Moreover, treatment of cells with cytotoxic necrotizing factor 2 leads to an enrichment in actin filaments, suggesting that the toxin activates Rho.

We have demonstrated that toxins A and B possess similar activities towards Rho. Since most pathogenic *C. difficile* strains produce both toxins, it is interesting to consider why a microorganism would produce two very large exotoxins that possess similar activities. The answer most probably resides in the amino acid sequence differences that exist between the two toxins. Comparisons of the sequences of the two toxins showed that they are most divergent in the COOH-terminal thirds of the proteins (32). These regions contain amino acid repeat motifs of which similar types have been shown to bind to specific carbohydrates (34). It is known for toxin A that the COOH-terminal end of the protein is the part of the molecule that mediates binding to animal cells. For toxin A it has been directly demonstrated that the COOH-terminal end can bind to certain carbohydrate antigens that are found on the outside of human intestinal epithelial cells (29). The current model appears to be that toxins A and B bind and enter different cell types but that nevertheless they both carry out the same intracellular effect towards Rho.

Although current antibiotic therapies are reasonably effective for the treatment of *C. difficile* infection, novel strategies for blocking bacterial pathogenesis may be required to combat the rise in antibiotic-resistant bacterial strains. Future studies revealing how *C. difficile* toxins modify Rho proteins may suggest such strategies. In addition, these studies may reveal a novel protein modification. Finally, how this toxin-induced modification influences Rho activity may yield insight into this key regulator of the actin cytoskeleton.

ACKNOWLEDGMENTS

We acknowledge G. Bokoch for providing anti-Rac antiserum, R. Cerione for providing anti-CDC42 antiserum, J. Hancock for providing the GST-GDI expression vector, and A. Hall for providing the RhoA expression vector.

This work was support by NIH grants to L.A.F. and R.J.G. and an NIDDK GRASP Center grant.

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