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Transient Expression of RhoA, -B, and -C GTPases in HeLa Cells Potentiates Resistance to *Clostridium difficile* Toxins A and B but Not to *Clostridium sordellii* Lethal Toxin

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The bacterial pathogen *Clostridium difficile* synthesizes two high-molecular-weight toxins (A and B), which exhibit toxic effects in vivo and in vitro. Here, we present evidence that the major intracellular targets of these two toxins are the Rho GTPases. Overexpression of RhoA, RhoB, or RhoC GTPases in transfected HeLa cells conferred an increased resistance to toxins A and B, indicating that these toxins cause their cytopathic effects primarily by affecting Rho proteins. In addition, toxin A and B treatment appeared to result in modification of Rho, since Rho isolated from toxin-treated cells had a decreased ability to be ADP-ribosylated by *Clostridium botulinum* C3 exoenzyme. In contrast, the lethal toxin (LT) of *Clostridium sordellii*, although structurally and immunologically related to *C. difficile* toxin B, appeared to induce cytopathic effects independently of the Rho GTPases. Overexpression of RhoA in transfected HeLa cells did not protect them from the effect of LT, and Rho isolated from lysates of LT-treated cells was not resistant to modification by C3. Immunofluorescence studies showed that LT treatment caused a cytopathic effect that was very different from those described for *C. difficile* toxins A and B, resulting in an increase in cortical F-actin, with a concomitant decrease in the number of stress fibers, and in the formation of numerous microvilli containing the actin-bundling protein fimbrin/plastin.

Clostridium difficile produces two high-molecular-weight toxins, called A and B, which are the cause of antibiotic-associated pseudomembranous colitis (5, 6, 45). Toxins A and B are also cytotoxic for cultured cells but have different potencies, with toxin B being 1,000-fold more active than toxin A. Both *C. difficile* toxins induce similar morphological changes in cultured cells, including rounding of cell bodies (9, 14) and formation of membrane arborization (15). The N termini of toxins A (300 kDa) and B (250 kDa) show extensive similarities (50). This similarity may indicate that these two toxins share a common intracellular activity, since the enzymatic domain (effectomer) in many bacterial toxins is located near the amino terminus of the protein (18).

Toxins A and B from *C. difficile* act on cultured cells primarily by disrupting the F-actin cytoskeleton network without interfering directly with F-actin formation (35, 46, 47). Recently, it was shown that *C. difficile* toxin B may affect actin microfilaments by causing a covalent modification of the Rho GTPase, which presumably inactivates this regulatory protein (23, 24).

It is now clear that the Ras-like Rho protein family, which in mammalian cells includes RhoA, RhoB, RhoC, Cdc42Hs, RhoG, Rac1, and Rac2 (11, 13, 30, 34, 44, 48, 52) is involved mainly in the regulation of the F-actin cytoskeleton (reviewed in reference 20). Direct evidence that Rho molecules are involved in the assembly of F-actin structures such as stress fibers (10) was obtained by treating cells with a specific rho ADP-ribosylating bacterial enzyme (exoenzyme C3), isolated from the culture supernatants of *C. botulinum* C and D (3, 43). This observation was confirmed by microinjection into serum-starved mouse Swiss 3T3 cells of the constitutively activated form of RhoA GTPase (RhoA Val14) which resulted in the formation of a dense network of actin stress fibers (36). The role of Rho proteins was further characterized by Ridley and Hall, who demonstrated that Rho GTPases regulate formation of stress fibers in response to growth factor-stimulated signal transduction pathways (40, 42). Recent observations have implicated Rho GTPases in the regulation of actin assembly via activation of different enzymes such as phosphatidylinositol 3-phosphate kinase (53), a kinase which phosphorylates the focal adhesion kinase protein (p125FAK) (26), a tyrosine kinase involved in the phosphorylation of certain adhesion plaque proteins (41), and phospholipase D (7). It is therefore of interest to demonstrate that *C. difficile* cytotoxins A and B could act directly on the Rho protein family, since these toxins can provide valuable tools for cell biologists to study how these small GTPases are involved in the control of F-actin assembly and disassembly.

In the present work, we have developed an assay to test the possibility that *C. difficile* toxins A and B were active in vivo either on a specific form of Rho or on different Rho members of this family of small GTPases. The rationale of our experiments was that if toxins A and B act on Rho proteins (by an enzymatic covalent modification), one should be able to potentiate the cellular resistance to these toxins by greatly increasing the intracellular concentration of Rho GTPases. We have transfected cells with RhoA, RhoB, and RhoC cDNAs and shown that such cells have an increased resistance to *C. difficile* A and B toxins. In contrast, *C. sordellii* lethal toxin (LT), which is immunologically and structurally related to *C. difficile* toxin B (37), did not act on Rho GTPases, since cells transfected with the cDNAs of these proteins did not show any resistance to LT toxin. Instead, LT toxin induced the formation of numerous microvillus structures on the cell surface. These structures contained the actin-binding and microfilament-bundling protein fimbrin/plastin (8).

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MATERIALS AND METHODS

Materials. *Escherichia coli* TG1 was used as the host of recombinant plasmids. Human RhoA cDNA was kindly provided by P. Madaule, Institut Pasteur, Paris, France, and human RhoB and RhoC cDNAs were kindly provided by P. Chardin, Institut de Pharmacologie Moléculaire et Cellulaire, Sophia Antipolis, France. As vectors, we used pUC19, M13mp18, pKC3 (a eukaryotic expression vector derived from the parental vector pSV-neo) containing the simian virus 40 early promoter, and pCB6 (a eukaryotic expression vector containing the cytomegalovirus promoter). PCR amplifications were performed with *Taq* polymerase (Beckman, Paris, France). DNA sequencing was carried out with the Sequenase DNA-sequencing kit (version 2.0; U.S. Biochemical Co., Cleveland, Ohio) as specified by the manufacturer.

Cell culture. HeLa (human epitheloid carcinoma) cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Eragny, France), supplemented with 5% fetal calf serum (Gibco BRL), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (Gibco BRL), at 37°C under a 5% CO_2 atmosphere. LLCPK1 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Antibodies and toxins. Monoclonal antibody P5D4 was raised against the 11-amino-acid peptide (YTDIEMNRLGK) contained in the vesicular stomatitis virus glycoprotein G carboxy terminus (25). *C. botulinum* type D (strain 1873) exoenzyme C3 (43), *C. difficile* toxins A and B (49), C2-like toxin from *C. spiroforme* (strain NCTC 11493 [Rhône-Mérieux collection no. 15991]) (38), and *C. sordellii* LT from strain IP82 (37) were purified as previously described.

Constructions of epitope-tagged Rho proteins. Rho proteins were tagged with oligonucleotides encoding 11 amino acids of the vesicular stomatitis virus (VSV) glycoprotein G epitope (25). The epitope tag was fused to the N termini of the Rho proteins. RhoA cDNA was subcloned into pUC19 digested with *Xma*I and *Xba*I. Complementary oligonucleotides encoding the epitope tag were synthesized with an *Eco*RI 5′-flanking site and a *Kpn*I 3′-flanking site. The linker, formed by annealing the two oligonucleotides, was introduced in frame upstream of RhoA into pUC19-RhoA. The *Eco*RI-*Pst*I fragment, encoding VSV G-RhoA, was subcloned into the eukaryotic expression vector pKC3, and the insert was verified by sequencing. RhoB cDNA was amplified by PCR to create a *Kpn*I site upstream of the initiation codon and a *Bam*HI site downstream of the stop codon. PCR products were digested with *Kpn*I and *Bam*HI and cloned into the eukaryotic expression vector pCB6 (previously digested with the same enzymes). Complementary oligonucleotides encoding the tag were synthesized with a *Bgl*II 5′-flanking site and a *Kpn*I 3′-flanking site, and the corresponding linker was introduced in frame upstream of RhoB into pCB6-RhoB. The *Bgl*II-*Bam*HI fragment encoding VSV G-RhoB was subcloned into pUC19 vector to allow the subcloning of a larger *Eco*RI-*Xba*I fragment from pUC19 into the eukaryotic expression vector pKC3. The sequence of this fragment was verified. The *Eco*RI-*Bam*HI fragment containing RhoC cDNA (subcloned into the M13mp18 vector) was cloned into pCB6, and a linker encoding the VSV G epitope tag was introduced in frame directly upstream of RhoC into pCB6-RhoC. This linker shares a *Kpn*I 5′-flanking site and an *Eco*RI 3′-flanking site. The subsequent recombinant plasmid pCB6-VSV G-RhoC was digested. The *Bgl*II-*Bam*HI DNA fragment present in pCB6-VSV G-RhoC was subcloned into pUC19 to allow the subcloning of a larger *Sac*I-*Pst*I DNA fragment into the eukaryotic expression vector pKC3.

Transfection of HeLa cells and assay of toxin activity. Exponentially growing HeLa cells were seeded on coverslips at 10^5 cells per 35-mm culture dish 24 h before transfection. DNA transfection was done by the classical calcium phosphate technique. The calcium phosphate-DNA complex was allowed to form gradually for 18 h. Cells were then washed twice with Dulbecco's modified Eagle's medium, refed, and incubated for an additional period of 24 h before treatment with toxins. Preliminary experiments were performed with 96-well tissue culture plates to determine the concentration of each toxin yielding 100% of cells rounding within 3 h at 37°C. The doses found were 16 μg of *C. difficile* toxin A per ml, 500 pg of *C. difficile* toxin B per ml, 15 μg of *C. sordellii* LT per ml with addition of 100 μM dithiothreitol (37), and 100 ng of component Sa plus 200 ng of component Sb of *C. spiroforme* toxin per ml. The concentration of cytochalasin D used was 0.625 μg/ml. Transfected cells were washed twice with Dulbecco's modified Eagle's medium and incubated for 3 h with the indicated toxins before fixation.

Immunofluorescence. The presence of the expressed epitope-tagged proteins was determined by indirect immunofluorescence with monoclonal antibody P5D4 directed against the VSV G epitope. After fixation by 4% paraformaldehyde, monolayers were washed three times with phosphate-buffered saline (PBS), free aldehyde groups were quenched by incubation with 50 mM NH_4Cl for 10 min, and the monolayers were washed three times in PBS. Cells were then permeabilized for 5 min at room temperature in PBS containing 0.2% Triton X-100 and 0.2% bovine serum albumin (BSA) (buffer A). Monolayers were incubated for 30 min at room temperature with the first appropriate antibody. The coverslips were washed extensively in buffer A, and the primary antibody binding was detected by a 30-min incubation at room temperature with Texas red-conjugated sheep anti-mouse antibody (Amersham, Les Ulis, France), together with fluorescein isothiocyanate (FITC)-phalloidin (1 μg/ml; Sigma Chemical Co., L'Isle d'Abeau, France) to visualize F-actin. After three washes in buffer A followed by three washes in PBS, the coverslips were mounted in Mowiol (Calbiochem, La Jolla, Calif.) and observed for fluorescence.

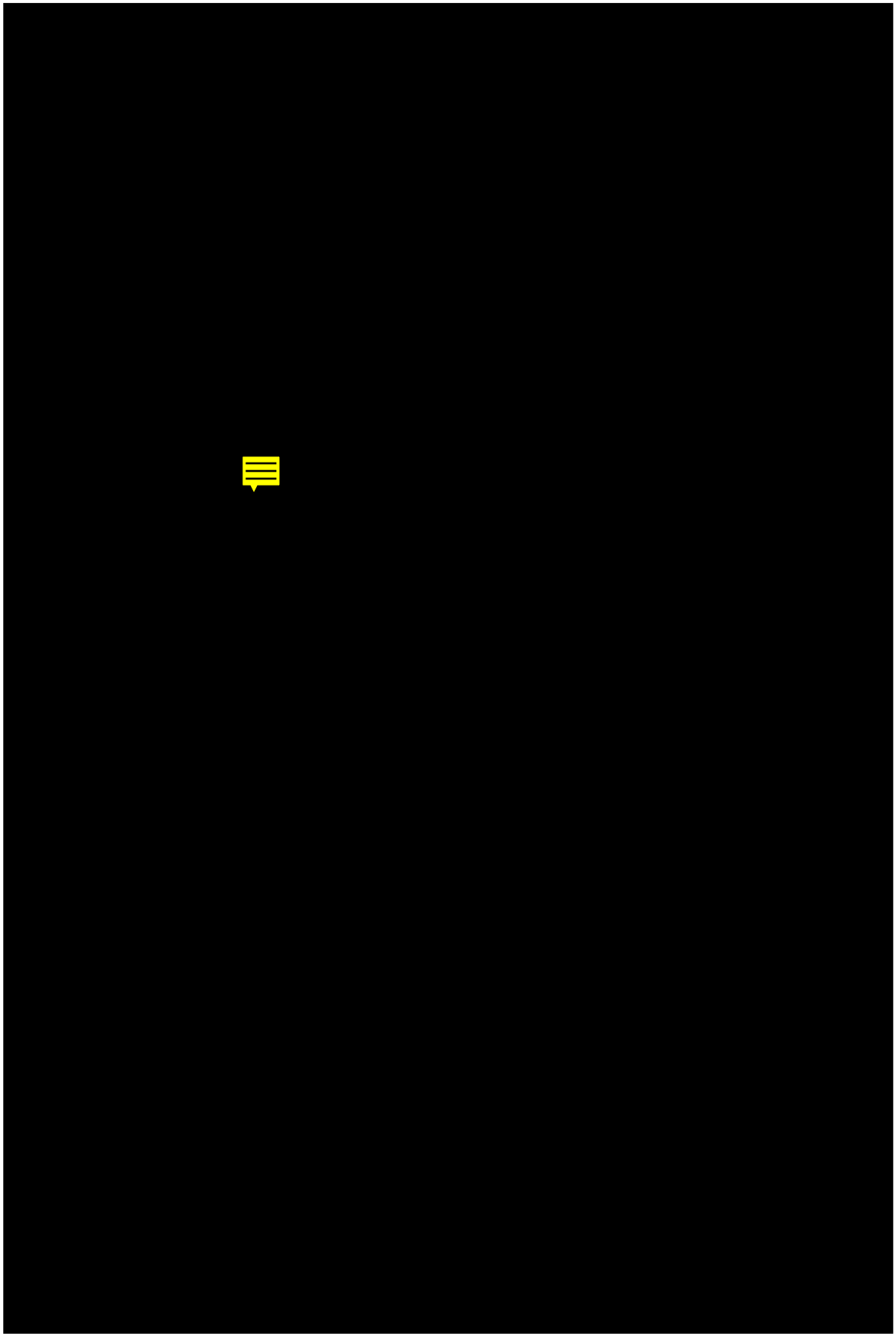
ADP-ribosylation. Exponentially growing HeLa cells were seeded at 2×10^5 cells per 35-mm tissue culture dish 24 h prior to treatment with toxins. After incubation with different concentrations of toxins, leading to different stages of morphological changes, the cells were washed twice with PBS and detached from their support with a rubber policeman in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 8.0) in the presence of protease inhibitors (10 μM leupeptin, 1 μM pepstatin, 250 μM phenylmethylsulfonyl fluoride). Harvested cells were lysed by three cycles of freezing and thawing and centrifuged at 10,000 × *g* for 5 min at 4°C. The concentrations of the cytosolic proteins were determined by the Bradford assay (Bio-Rad, Paris, France) with BSA as the standard. ADP-ribosylation was carried out with samples containing 20 μg of total proteins in a final volume of 15 μl in 20 μM HEPES buffer (pH 8.0), incubated for 1 h at 37°C with 5 μl of a radioactive solution containing 2.5 μM [^{32}P]NAD (20,000 d.p.m./pmol; NEN-Du Pont de Nemours, Dreieich, Germany), 2 mM AMP, and 4 μg of exoenzyme C3 per ml. The reaction was terminated by addition of 5 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. After being boiled for 5 min, the samples were subjected to electrophoresis on a 15% polyacrylamide gel. The gel was stained with Coomassie brilliant blue and dried. The results of [^{32}P]ADP-ribosylation were evaluated by autoradiography.

RESULTS

Effects of *C. difficile* toxin B on transfected RhoA, RhoB, and RhoC HeLa cells. Microinjection of an activated form of RhoA (RhoA Val14) into subconfluent mouse Swiss 3T3 cells induces both a change in cell morphology and the formation of a dense microfilament network (36). The intracellular localization of RhoA, RhoB, and RhoC in Rat-2 and MDCK cells has been studied by a nuclear microinjection approach with eukaryotic expression vectors containing c-*myc* epitope-tagged RhoA, RhoB, and RhoC cDNAs (1). From this work, it was demonstrated that the majority of RhoA and RhoC was cytosolic but that RhoB was associated with intracellular compartments identified as early and late endosomes (36). In the present work, we have used the VSV G epitope to label RhoA, RhoB, and RhoC and have introduced, by calcium phosphate transfection, eukaryotic expression vectors containing the different VSV G-tagged Rho cDNAs. We found that the choice of the eukaryotic vector was important to obtain transfected cells with acceptable morphology. For instance, vectors in which the Rho cDNAs were under the control of a very strong promoter such as the cytomegalovirus promoter did not result in transfected cells with interpretable morphologies (18a).

There is good evidence suggesting that *C. difficile* toxin B inhibits Rho GTPases (23, 24). We therefore tested the effects of *C. difficile* toxin B on HeLa cells transfected with cDNAs of the different Rho GTPases. A 3-h incubation with 125 pg of toxin B per ml induced a cytopathic effect of toxin B in nontransfected HeLa cells (or HeLa cells transfected with the parent vector pKC3) (Fig. 1A and B). In contrast, cells transfected with VSV G-RhoA were unaffected by treatment with 500 pg of toxin B per ml for the same period. Companion nontransfected RhoA cells, however, exhibited the classical

FIG. 1. Effects of *C. difficile* A and B toxins on HeLa cells transfected with RhoA. HeLa cells were transfected with RhoA cDNA, as described in Materials and Methods. (A and C) Cells were stained for F-actin with FITC-phalloidin. (B and D) Cells corresponding to those in panels A and C, respectively, were stained with the monoclonal antibody P5D4 followed by Texas red-labeled anti-mouse antibody. (A and B) Effects of 500 pg of toxin B per ml for 3 h on RhoA-transfected and nontransfected HeLa cells. (C and D) Effects of 16 μg of toxin A per ml on RhoA-transfected HeLa cells. Large arrows show the transfected cells which are resistant to toxins A and B; small arrows show toxin-affected nontransfected cells; arrowheads show stress fibers in toxin-resistant cells. Thin arrows in panels C and D show a cell transfected with RhoA expressing a small amount of the GTPase (D) and sensitive to toxin A (C).



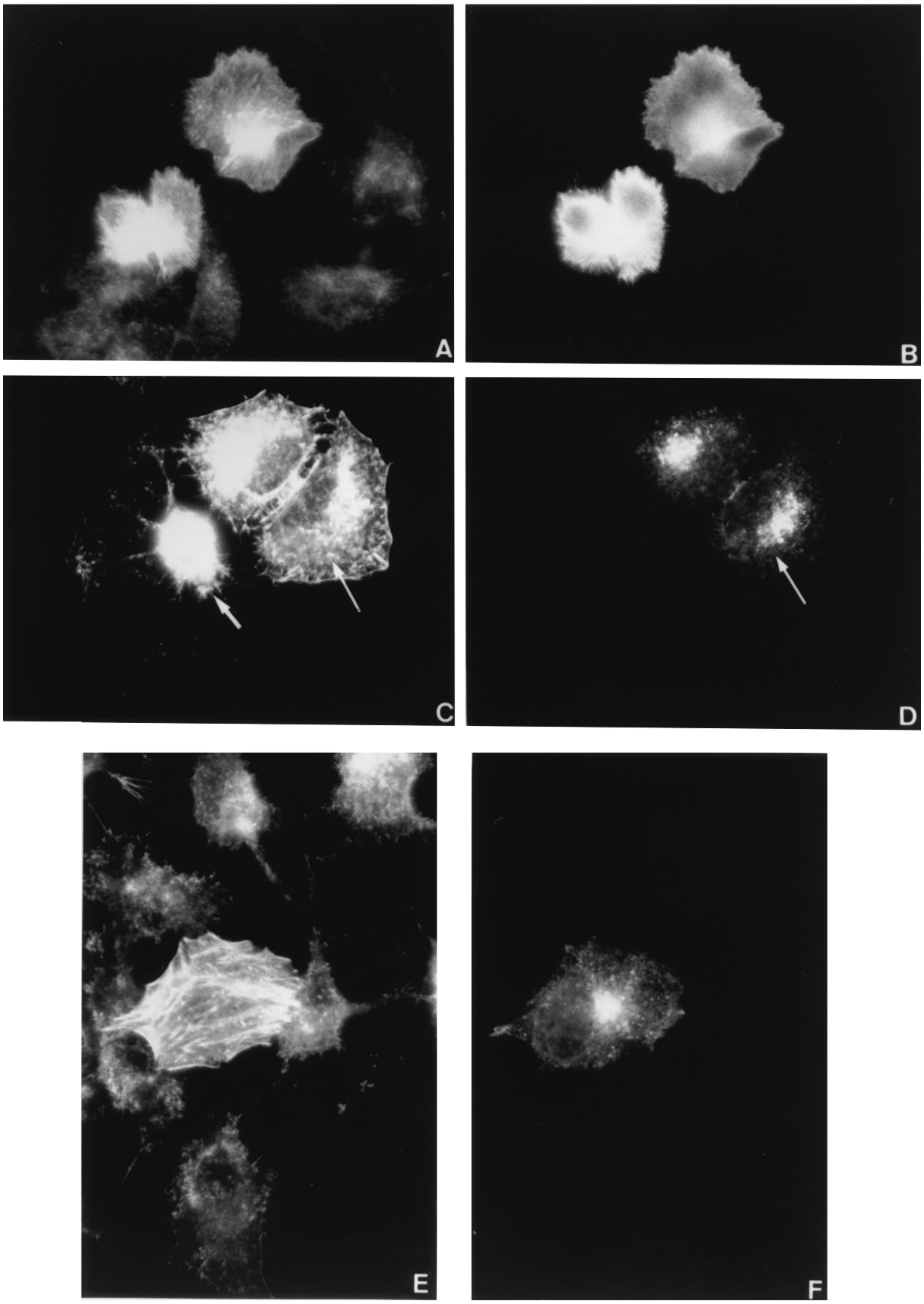

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FIG. 2. Effects of *C. difficile* toxin B on HeLa cells transfected with RhoB or RhoC. HeLa cells were transfected with the RhoB and RhoC cDNAs as described in Materials and Methods. (A, C, and E) Cells were stained for F-actin with FITC-phalloidin. (B, D, and F) Cells were stained with monoclonal antibody P5D4 and then with Texas red-labeled anti-mouse antibody. (A and B) Cells transfected with RhoB cDNA treated with 500 pg of toxin B per ml for 3 h. (C and D) Cells transfected with RhoB cDNA with a moderate expression of the GTPase (thin arrows). The small arrow in panel C shows a nontransfected cell affected by toxin B, stained for F-actin. Indeed, in the same cell, no staining can be seen with the anti-VSV G monoclonal antibody (D). (E and F) RhoC toxin-resistant transfected cells treated with 500 pg of toxin B per ml for 3 h.

morphologies of toxin B-treated cells. An identical result was found for cells transfected with RhoB and RhoC (Fig. 2). We must point out that cells transfected with the Rho GTPases were not "resistant" to toxin B. Indeed, after a prolonged incubation in the presence of toxin B (usually 18 h with 500 pg of toxin B per ml), cells transfected with the different Rho GTPases were finally affected by toxin B. Also, a low level of the GTPase expression in transfected cells did not fully protect them against toxin B, as shown in Fig. 2C and D. Therefore, transfection of Rho GTPases into HeLa cells decreased the sensitivity of these cells to toxin B, as expected if Rho was the intracellular target of this bacterial protein.

Toxin B treatment of cells appears to result in an unidentified covalent modification of Rho proteins. Lysates of HeLa cells treated with increasing amounts of toxin B contained Rho proteins that were increasingly resistant to ADP-ribosylation by exoenzyme C3 (Fig. 3).

Effects of *C. difficile* toxin A on transfected RhoA, RhoB, and RhoC HeLa cells. *C. difficile* toxin A induces morphological effects in cultured cells, and these effects are close to those caused by *C. difficile* toxin B (15). However, doses of toxin A required for cytotoxicity are much higher than those of toxin B (reviewed in reference 16). The lowest dose of toxin A found to be sufficient to induce a cytopathogenic effect on HeLa cells within 3 h was 4 μg/ml. In contrast, HeLa cells transfected with RhoA and subsequently incubated with 16 μg of *C. difficile* toxin A per ml did not show cytopathic effects (Fig. 1C and D). RhoB and RhoC were also effective in protecting HeLa cells against toxin A (data not shown). As shown in Fig. 1C and D, a low level of RhoA expression in HeLa cells did not induce any protection against toxin A. As observed for toxin B, prolonged incubation (18 h with 16 μg of toxin A per ml) did affect transfected Rho cells (data not shown).

As shown in Fig. 3, Rho proteins present in cytosolic extracts of toxin A-treated HeLa cells were ADP-ribosylated by exoenzyme C3 to a much lesser extent than were those present in extracts from untreated cells.

We then verified the effects of cytochalasin D and *C. spiroforme* toxin on the F-actin cytoskeleton (38) in rho-transfected cells. These two agents are known to disrupt the F-actin-associated cell structures by a Rho-independent mechanism. Cytochalasin D is a drug which caps the barbed ends of F-actin filaments, thus decreasing the rate of filament assembly (39), whereas *C. spiroforme* toxin, like *C. botulinum* C2 toxin, ADP-ribosylates nonmuscular G-actin, thereby blocking F-actin filament formation (2, 51). As shown in Fig. 4, Rho GTPases did not afford protection against the effects of cytochalasin D and *C. spiroforme* toxin.

Effects of *C. sordellii* LT on the HeLa cell cytoskeleton. *C. sordellii* produces two toxins, LT and hemorrhagic toxin (4), which have some similarities to the toxins elaborated by *C. difficile* (31). Like toxins A and B, LT induces rounding of cultured cells, with, however, a morphological aspect different from the one shown by *C. difficile* toxin B (37). It was therefore of interest to test the effects of LT on our system of transfected cells to determine a possible role of the Rho GTPases in the LT mechanism of action. As shown in Fig. 5, the cytopathic effect induced by LT in HeLa cells consists of the rounding of the cell body with reorganization of F-actin structures into numerous cell surface microvilli and a loss of F-actin stress fibers. Increasing the intracellular concentration of RhoA did not protect the cells from the cytopathic effects of LT (Fig. 5).

We wondered whether the microvilli induced by LT contain an actin-binding and bundling protein, fimbrin/plastin, which is normally found in these structures (8). Using a polyclonal rabbit antibody which recognizes the different isoforms of fimbrin/plastin, we observed that a considerable amount of fimbrin/plastin was present in microvilli induced by LT (Fig. 5).

Incubation of HeLa cells with concentrations of *C. sordellii* LT or cytochalasin D that induced cytopathic effects did not modify the subsequent in vitro ADP-ribosylation of Rho proteins (Fig. 3).

DISCUSSION

Transfection of HeLa cells with cDNAs encoding epitope-tagged RhoA, RhoB, and RhoC allowed us to establish an experimental system for the study of the putative role of these GTPases in the intracellular activities of several bacterial protein toxins.

Cells transfected with RhoA, RhoB, and RhoC GTPases were then treated with several large clostridial cytotoxins: *C. difficile* A and B toxins (47), *C. sordellii* LT (37), and *C. spiroforme* iota-like toxin (38) or cytochalasin D (12), known to act on the F-actin cytoskeleton. Cells transfected with expression vectors containing cDNAs coding for RhoA, RhoB, and RhoC were able to maintain F-actin structures in the presence of high doses of toxin A or B from *C. difficile*. Although we cannot totally rule out that the intracellular overexpression of the GTPases did not alter the ability of cells to take up toxin A or B by endocytosis, this seem unlikely since *C. spiroforme* C2-like binary toxin and *C. sordellii* LT were equally active on control

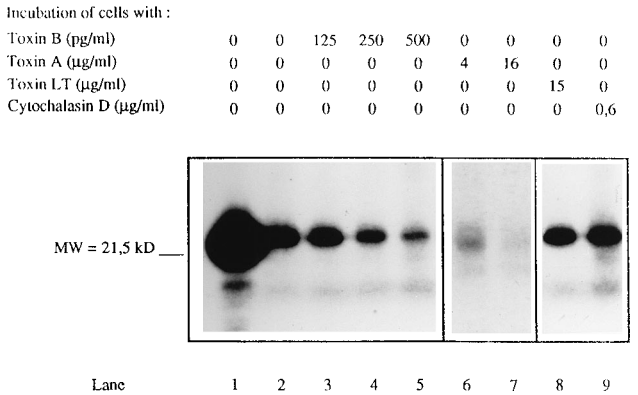

FIG. 3. Effect of *C. difficile* toxins A and B, *C. sordellii* LT, and cytochalasin D on ADP-ribosylation of Rho. HeLa cells were incubated for 3 h with the indicated amounts of the following toxins: control untreated cells (lane 2), toxin B (lanes 3 to 5), toxin A (lane 6 and 7), *C. sordellii* LT (lane 8), and cytochalasin D (lane 9). ADP-ribosylation of each cell lysate containing identical amounts of proteins was then performed as described in Materials and Methods. ADP-ribosylation of 100 pg of purified rhoC by exoenzyme C3 is shown in lane 1. Molecular masses are given in kilodaltons.

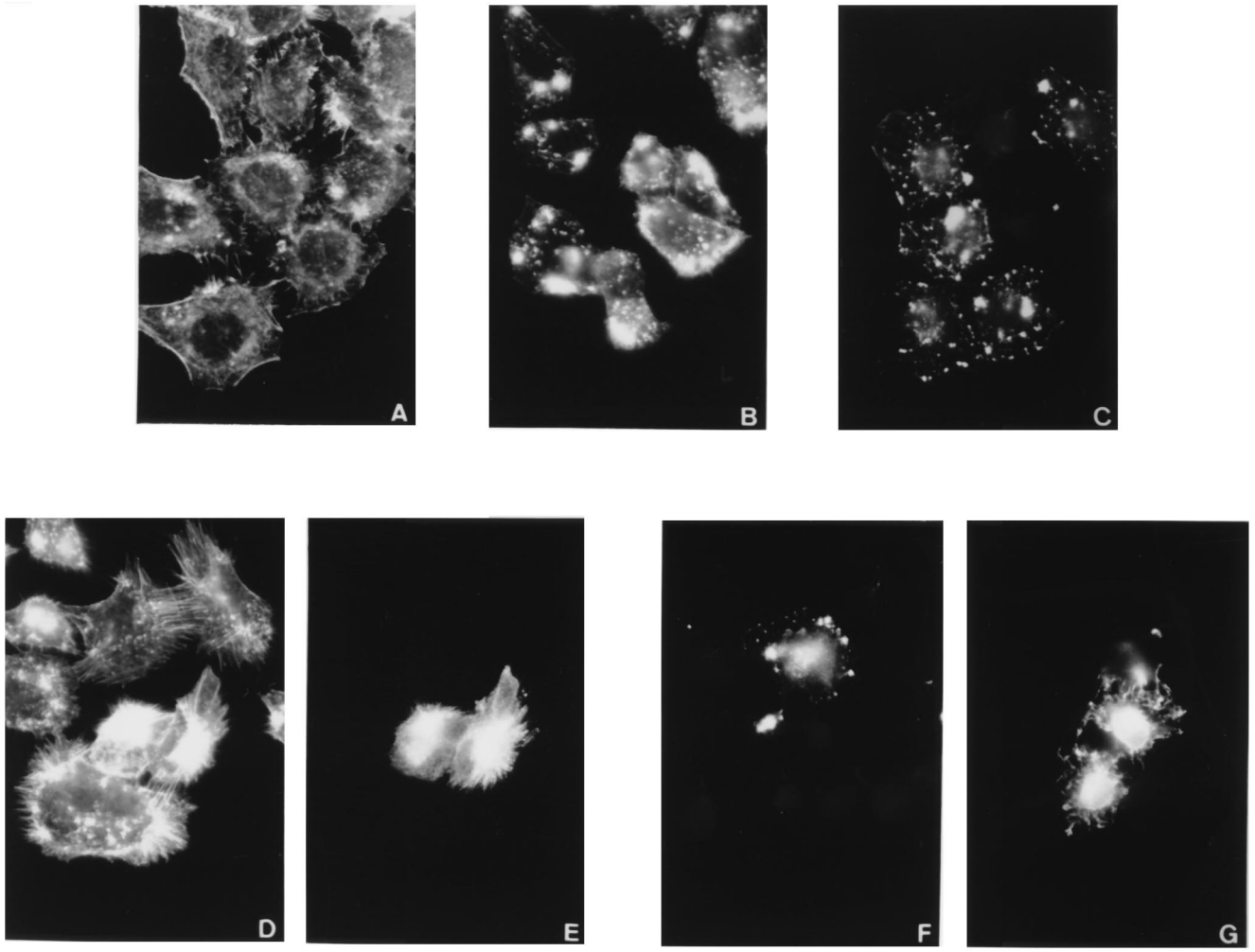

FIG. 4. Effects of *C. spiroforme* and cytochalasin D on HeLa cells transfected with RhoA. HeLa cells were transfected with RhoA cDNA as described in Materials and Methods. (A, B, C, D, and F) Cells were stained for F-actin with FITC-phalloidin. (E and G) The same cells were stained with monoclonal antibody P5D4 and then with Texas red-labeled anti-mouse antibody. (A) Control cells. (B and C) Effects of 0.625 μg of cytochalasin D per ml (B) or 100 ng of component 1 plus 200 ng of activated component 2 of *C. spiroforme* binary toxin per ml (C). (D and E) Effects of cytochalasin D (0.625 μg/ml) on HeLa cells transfected with RhoA. (F and G) Effects of *C. spiroforme* binary toxin (100 ng of component 1 per ml plus 200 ng of trypsin-activated component 2 per ml) on HeLa cells transfected with RhoA.

and Rho-transfected cells. The resistance of Rho-overexpressing HeLa cells to toxins A and B must therefore be attributed to the high intracellular concentration of their putative cytosolic targets. Our results indicate that RhoA, RhoB, and RhoC are substrates for both *C. difficile* toxins. It is well documented that *C. difficile* toxins A and B must enter the cytosol to induce F-actin cytoskeleton disruption (16, 17). Furthermore, direct microinjection of toxin B into cells has been shown to induce the same effects on F-actin cytoskeleton as does incubation of cells with the toxin (33).

To confirm that Rho GTPases are the targets of *C. difficile* toxins A and B, we have shown that in cell lysates of toxin A- or B-treated cells, Rho had a decreased ability to be ADP-ribosylated, as previously reported for toxin B (23).

Inhibition of exoenzyme C3-mediated ADP-ribosylation of rho has also been accomplished recently in vitro by incubating together cytosol, toxin B, and recombinant RhoA (23) or by microinjecting toxin B into *Xenopus laevis* oocytes (24). These results suggest that toxin B must posttranslationally modify Rho in such a way that the GTPase is no longer a substrate for exoenzyme C3. Incubation of cells with *C. difficile* toxins A and B results in the delocalization of the focal adhesion plaque proteins vinculin, α-actinin, and talin (15, 35), suggesting that both of the *C. difficile* toxins act on focal adhesion structures. The idea that Rho GTPases are modified and probably inactivated by *C. difficile* A and B toxins therefore fits with the role of Rho in the control of focal adhesion proteins (20).

According to our results, both toxins A and B must act intracellularly. It has been suggested that toxin A and B share a cytotoxic subunit (47), and this hypothesis is supported by amino acid sequence similarity in the amino-terminal regions of the two toxins (50). Epithelial intestinal differentiated cells such as T84 cells are much more sensitive to *C. difficile* toxin A than to toxin B (21, 22). The cytopathic effects induced by the two toxins on these cells are, however, strictly similar (21, 22). The difference in activities of toxins A and B on cultured cells is therefore probably due to a small number of specific receptors for toxin A rather than to a difference in the intracellular activities of these toxins.

Intracellular activities of toxins A and B consist of the inactivation of Rho induced by a covalent modification, different from ADP-ribosylation (23, 39).

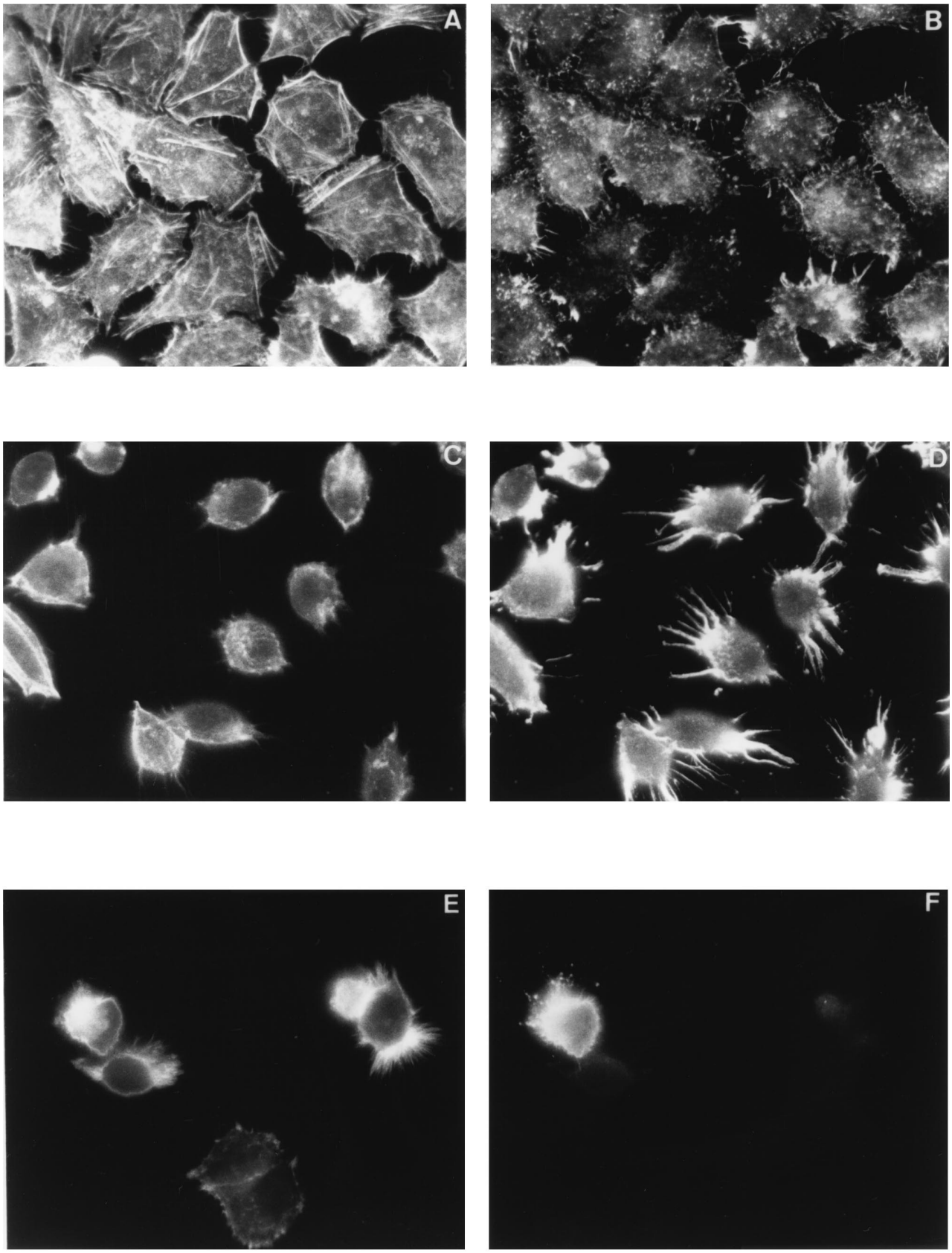

FIG. 5. Effects of *C. sordellii* LT on HeLa cells transfected with RhoA. Effects of LT on fimbrin are shown. HeLa cells were transfected with RhoA cDNA as described in Materials and Methods. (A, C, and E) Cells were stained for F-actin with FITC-phalloidin and with the polyclonal anti-fimbrin/plastin antibody (B and D) or with monoclonal antibody P504 (F) and then with Texas red-labeled anti-mouse antibody. (A and B) Control cells. (C and D) Effects of 15 μg of *C. sordellii* LT per ml in a 3-h incubation period on the F-actin content (C) or on the fimbrin/plastin content (D) of HeLa cells. (E and F) Effects of the same treatment by LT on RhoA-transfected cells.

Induction of diarrhea by toxin A has been shown to be the result of an increase in intestinal permeability which is followed by Cl^- secretion (32). Toxins A and B of *C. difficile* were shown to alter the permeability of epithelial T84 cell monolayers grown on collagen filters (21, 22). It was concluded from these experiments that toxin-induced changes of T84 monolayer permeability was due to modifications of the actin cytoskeleton, resulting in the opening of tight junctions without cell alterations (21, 22). Tight junctions are belt-like structures that seal epithelial cells together and act as a diffusion barrier (19). Tight junction permeability is a highly dynamic process and depends on the F-actin cytoskeleton (27–29). The effects of *C. difficile* toxins A and B on Rho indicate an important role for these GTPases in the regulation of the dynamics of tight junction structures and assembly.

The following is a speculative model for the mechanism of *C. difficile* A and B toxins on intestinal epithelial cells. Toxins produced by bacteria will diffuse and bind to their respective membrane receptors. After receptor-mediated endocytosis (17), toxins are released into the cytosol, where they then posttranslationally modify Rho GTPases (12, 13), thereby opening tight junctions (21, 22), resulting in Cl^- secretion (32) and diarrhea.

C. sordellii LT is comparable to *C. difficile* toxin B in molecular weight, amino-terminal sequence, and immunological cross-reactivity (31, 37). Despite these similarities, LT induced a totally different cytopathic effect on cultured cells from that induced by *C. difficile* toxin B. The LT cytopathic effect consisted of the disruption of actin cables (but not of cortical actin) and a dramatic accumulation of fimbrin into microvilli which radiated from toxin-treated cells. Transfection of the Rho GTPase-cDNAs into HeLa cells caused no effect on LT-induced toxicity. This observation demonstrates that LT toxin does not, in spite of its similarity to *C. difficile* toxin B, act on Rho GTPases. Perhaps LT modifies another small p21 GTP-binding protein of the Ras or Rho families.

C. sordellii LT caused recruitment of a large amount of fimbrin/plastin inside microvilli. This toxin activity probably does not reflect a direct relationship of LT with fimbrin/plastin but, rather, could induce nucleation of specific cortical actin filaments (forming the core of the toxin induced microvilli), which then allow the binding of this actin-bundling molecule.

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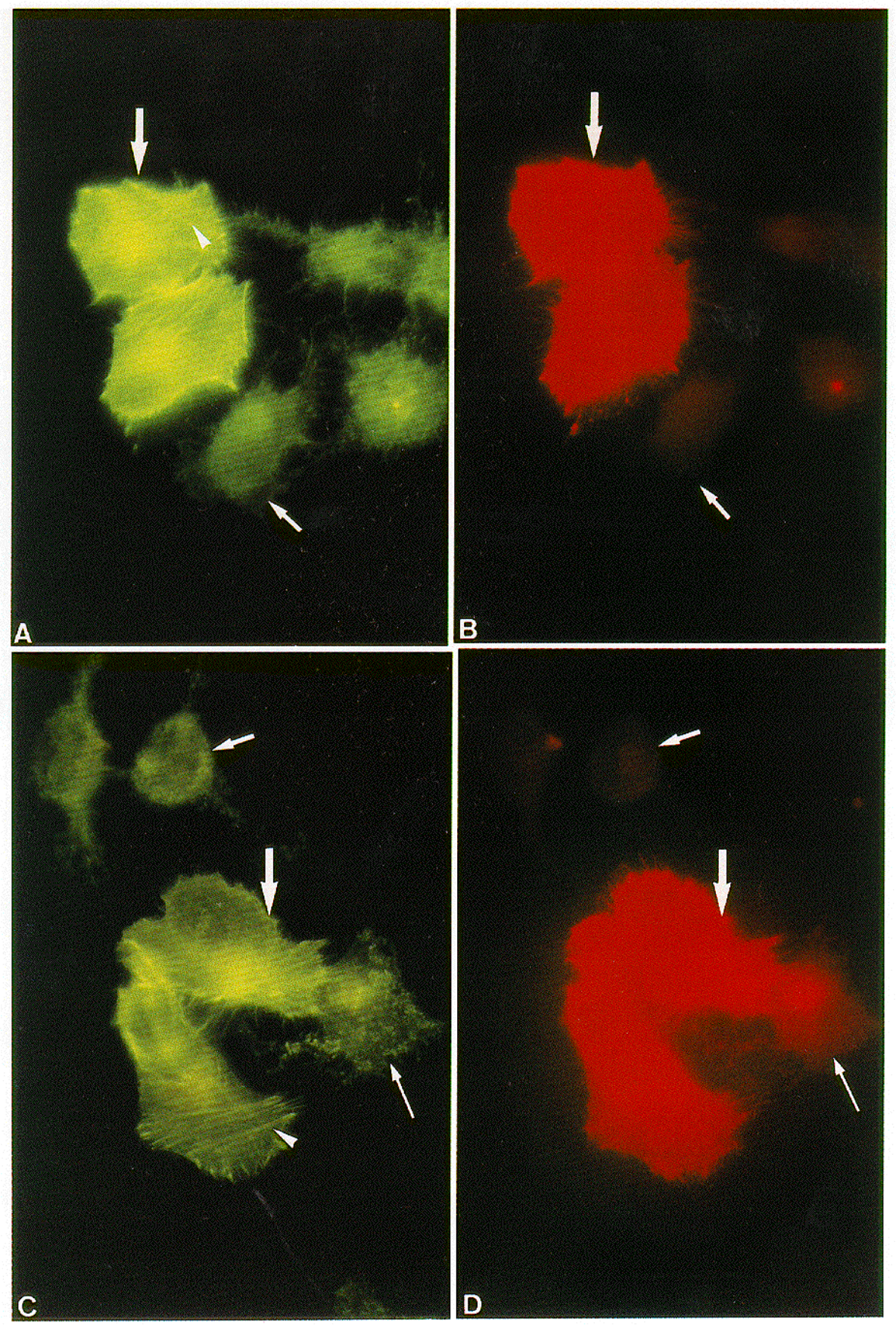
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