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Escherichia coli Cytotoxic Necrotizing Factor 1 (CNF1), a Toxin That Activates the Rho GTPase*

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Cytotoxic necrotizing factor 1 (CNF1), a 110-kDa protein toxin from pathogenic *Escherichia coli* induces actin reorganization into stress fibers and retraction fibers in human epithelial cultured cells allowing them to spread. CNF1 is acting in the cytosol since microinjection of the toxin into HEp-2 cells mimics the effects of the externally applied CNF1. Incubation *in vitro* of CNF1 with recombinant small GTPases induces a modification of Rho (but not of Rac, Cdc42, Ras, or Rab6) as demonstrated by a discrete increase in the apparent molecular weight of the molecule. Preincubation of cells with CNF1 impairs the cytotoxic effects of *Clostridium difficile* toxin B, which inactivates Rho but not those of *Clostridium sordellii* LT toxin, which inhibits Ras and Rac. As shown for Rho-GTP, CNF1 activates, in a time- and dose-dependent manner, a cytoskeleton-associated phosphatidylinositol 4-phosphate 5-kinase. However, neither the phosphatidylinositol 4,5-bisphosphate (PIP_2) nor the phosphatidylinositol 3,4-bisphosphate (PI 3,4-P_2) or 3,4,5-trisphosphate (PIP_3) cellular content were found increased in CNF1 treated HEp-2 cells. Cellular effects of CNF1 were not blocked by LY294002, a stable inhibitor of the phosphoinositide 3-kinase. Incubation of HEp-2 cells with CNF1 induces relocalization of myosin 2 in stress fibers but not in retraction fibers. Altogether, our data indicate that CNF1 is a toxin that selectively activates the Rho GTP-binding protein, thus inducing contractility and cell spreading.

Actin filaments are common targets for several bacterial protein toxins that exert their activities by either directly or indirectly breaking the actin cytoskeleton. Toxins such as *Clostridium botulinum* C2 or iota from *Clostridium perfringens* directly modify globular actin by ADP-ribosylating arginine 177 (1). Others toxins, such as *C. botulinum* C and D exoenzyme C3, or toxins A and B from *Clostridium difficile* (CdA and CdB, respectively)[1] indirectly disrupt F-actin structures by inactivating small GTP-binding proteins of the Rho family. To this family belong proteins (Rho, Rac, and Cdc42), known to be involved in the regulation of the actin cytoskeleton (2). RhoA and RhoC are constitutively produced, whereas RhoB is an early growth factor-induced gene (3, 4). RhoA, B, and C have apparently identical activities on actin polymerization consisting in the formation of actin stress fibers (5). Rac controls membrane ruffling (6) but also the NADPH-oxidase activity in neutrophils (7). Cdc42 has been shown to regulate the formation of F-actin filaments in filopodia (8). Exoenzyme C3 ADP-ribosylates RhoA, B, and C at asparagine 41 (9, 10), whereas CdA and CdB are glucosyltransferases, which modify Rho, Rac, and Cdc42 by covalently linking a glucose moiety from UDP-glucose at threonine 37 of such GTP-binding proteins (11, 12). *Clostridium sordellii* lethal toxin (LT) is, like CdA or CdB, a glucosyltransferase that also induces actin reorganization (13) by selectively modifying Ras, Rap, and Rac at threonine 35 (corresponding to threonine 37 of Rho), but not Rho or Cdc42 (14). Although most toxins active on the cytoskeleton cause the distruption of F-actin structures, newly studied toxins named cytotoxic necrotizing factors (CNF1 and CNF2) have been described to induce a dose-dependent increase in membrane ruffling and stress fibers (15). CNF1 and CNF2, produced by a number of pathogenic *Escherichia coli* strains (16), also interact with small GTP-binding proteins of the Rho family (17, 18) by probably causing a permanent activation of Rho (19). Very recently, *Bordetella bronchiseptica* dermonecrotic toxin, which shares some sequence homology with CNFs (20), has been shown to induce actin reorganization and to interact with Rho (21). Thus, small GTP-binding proteins of the Rho family often serve as intracellular targets for bacterial protein toxins.

A certain number of cellular proteins have been suggested to control or to be controlled by the Rho family of small GTP-binding proteins. For instance, phosphoinositide 5-kinase (PdtIns-4-P 5-kinase), whose product is PtdIns-4,5-P_2, has been shown to be activated by Rho GTP (22). Several lines of evidence point out that the elevation of PIP_2 can lead to increased actin polymerization. It has been reported, in fact, that PIP_2 is able to interact with actin-binding proteins such as profilin and gelsolin, inhibiting their interaction with microfilaments (23), and also to activate molecules implicated in F-actin binding such as vinculin (24) or ezrin (25), stimulating the assembly of focal adhesions. Furthermore, PIP_2 has been shown to uncap the barbed ends of actin filaments, provoking bursts of actin polymerization (26). The Rho family of small GTPases may thus control the actin cytoskeleton by regulating the local concentration of PIP_2. Recently, it has been shown that Rho GTP, by stimulating the Rho kinase (27), induces phosphorylation of the myosin light chain (MLC) phosphatase 130-kDa subunit (28). This phosphorylation provokes, by inhibiting the MLC phosphatase activity, the calcium sensitization of smooth mus-

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[1] The abbreviations used are: CdA and CdB, *Clostridium difficile* toxins A and B, respectively; LT, lethal toxin; CNF, cytotoxic necrotizing factor;PdtIns-4-P 5-kinase, phosphoinositide 5-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PI 3,4-P_2, phosphatidylinositol 3,4-bisphosphate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BDM, butanedione monoxime.

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cle contraction (29). Interestingly, Rho-stimulated contractility has been proposed to drive the formation of stress fibers and focal adhesions (30).

In the present work, we demonstrated that CNF1 i) is a cytosolic acting toxin, the microinjection of it into cells triggering the formation of actin stress fibers; ii) activates the Rho GTP-binding protein since it modifies Rho *in vitro*; and iii) provokes an increase *in vivo* of a cytoskeleton-associated PdtIns-4-P 5-kinase activity, which is known to be stimulated by Rho activation (22). In addition, we have shown that CNF1 promoted, in HEp-2 cells, contractility and cell spreading, two Rho-dependent phenomena (2), since it induced the same actin-myosin pattern previously described by Mitchison and coworker (31) in postmitotic spreading cells. This was characterized by the relocalization of myosin 2 within stress fibers but not within retraction fibers, the last being long, thin, actin-rich fibers where the spreading edges move outward over (31). Taken altogether, our results suggest that CNF1 activates Rho, which in turn induces contractility and cell spreading.

EXPERIMENTAL PROCEDURES

Materials—Recombinant small GTP-binding proteins were produced in *E. coli* under GST-fusion proteins and then processed by thrombin as described previously (14).

Cell Cultures—HEp-2 and Vero cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (Flow Laboratories, Irvine, UK), 1% non-essential amino acids, 5 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). The subcultures were serially propagated after harvesting with 10 mM EDTA and 0.25% trypsin in phosphate buffer solution (PBS, pH 7.4).

Toxins—CNF1 was purified from *E. coli* TG1 in which the CNF1 gene was cloned. Purification of CNF1 from bacterial extracts was achieved by ammonium sulfate precipitation, DEAE ion exchange chromatography, hydroxylapatite chromatography, and finally ion exchange chromatography on a Mono-Q column (Pharmacia, Orsay, France).[2] *C. sordellii* LT was purified as reported previously (33). Purified CdB was a gift of Dr. C. von Eichel-Streiber (Mainz, Germany). Twenty-four h after seeding on glass coverslips in 24-well plates (initial inoculum 10^4 cells/ml), HEp-2 cells were treated with toxins. For each toxin, the following concentrations have been used: 10^{-9} M for CNF1, 10^{-9} M for *C. difficile* toxin B, and 10^{-7} M for *C. sordellii* LT.

Microinjection Experiments—CNF1 (2.5 μg/ml) diluted in 50 mM Tris-HCl buffer, pH 7,5, 5 mM $MgCl_2$, 150 mM KCl, 0,1 mM dithiothreitol (microinjection buffer) containing 0.5 mg/ml non-immune rabbit antibodies (to localize microinjected cells) was microinjected into Vero cells with a Transjector 5246 system (Eppendorf, Germany). Microinjections were performed in DMEM medium containing 25 mM Hepes buffer to maintain a pH of 7.3 and rabbit anti-CNF1 antibodies to neutralize possible leakages of CNF1 outside the microinjected cells. The microinjection step lasted for 5 min and then cells were incubated at 37 °C for 20 min. Cells were fixed with paraformaldehyde and stained with both FITC-phalloidin for F-actin detection and rhodamine-labeled anti-rabbit antibodies (Amersham Life Science, Inc.) for detecting the microinjected cells. Samples were examined and photographed with a fluorescence equipped photomicroscope.

PtdIns-4-P 5-Kinase Assay—PtdIns-4-P 5-kinase activity associated to the cytoskeleton was assayed as described (34). Cells were grown to 10^5 cells/cm^2 in 10 cm-diameter dishes whether or not treated with CNF1. After incubation, monolayers were rinsed twice with PBS and then cytoskeleton was prepared by extraction with 1 ml of 0.5% Triton X-100 in 20 mM Hepes buffer, pH 7.4, 50 mM NaCl, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, and 100 mM sodium orthovanadate. After 15 min at 4 °C, cytoskeleton extracts were scraped off the dishes with a rubber policeman and pelleted in Eppendorf tubes at 12000 × *g* for 1 min at 4 °C. Cytoskeleton extracts were washed twice at 4 °C in 0.5 ml of the same buffer but without Triton X-100 and finally resuspended in 0.1 ml of 50 mM Tris-HCl buffer, pH 7.4, assayed for protein concentration, and immediately used for enzymatic assay. The lipid kinase assay was performed in a final volume of 200 μl, containing 50 mM Tris-HCl buffer, pH 7.4, 10 mM $MgCl_2$, and 25 μM ATP, 50 μg of phosphatidylinositol 4-P (Sigma), 100 μg phosphatidylserine (Sigma, l'Isle d'Abeau, France) (before addition to the reaction, these lipids were dried under N_2 stream resuspended with 50 μl of Tris-HCl buffer, pH 7.4, and sonicated 3 times for 10 s on ice), 20 μCi of [γ-^{32}P]ATP (Amersham, France), and 100 μg of each cytoskeleton preparation. Samples were incubated for 15 min at 37 °C under gentle agitation. Reactions were stopped by adding 0.4 ml of chloroform:methanol (1:1). Before extraction, each sample was acidified by adding HCl to a final concentration of 0.4 M. After extraction, the lower organic layers were collected, dried under nitrogen, then resuspended in a minimum volume of chloroform to collect all the radioactivity, and spotted onto silica plates. Plates were developped in chloroform, methanol, 4.3 M ammonium hydroxide (90:70:20) (v:v:v), as solvent. Unlabeled phospholipid standards were visualized by iodine vapors, and radiolabeled lipids were by autoradiography. Radioactivity associated with PIP_2 was measured by extracting the spots from the plate and counting them by Serenkov radiation.

Determination of Cellular Phosphoinositides Content—Cellular amounts of PI 3,4-P_2, PIP_3, and PIP_2 was assayed as follows. HEp-2 (1×10^6) cells were seeded in 75 cm^2 flasks and grown at 37 °C in 5% CO_2 for 24 h. Cells were then transferred in DMEM containing 0.5% fetal calf serum for 24 h and labeled with 250 μCi/ml [^{32}P]orthophosphate (Amersham) in DMEM without phosphate (ICN, France) supplemented with glutamine and 0.5% fetal calf serum. After 2 h of labeling, CNF1 was added to the cell culture medium at a concentration of 10^{-9} M and incubated for 8 h in the labeling medium. Cells were then washed twice with cold PBS, reactions were stopped by adding ice-cold HCl (2.4 N), and cells were recovered by scraping. Lipids were then extracted as described previously (28) and separated by thin layer chromatography, and radiolabeled PI 3,4-P_2, PIP_2, and PIP_3 were recovered by scraping the appropriate bands. Separation and analysis of deacylated products by high pressure liquid chromatography were done as described (34).

Butanedione Monoxime Experiments—Butanedione Monoxime (Sigma) was diluted in dimethyl sulfoxide (Me_2SO) at the concentration of 1 M. Since BDM is stable for only 1 h in tissue culture media, HEp-2 cells were incubated with CNF1 (10^{-8} M) for 2 h, and then BDM (10 mM) was added for 40 min. Cells were then fixed and processed for immunofluorescence for F-actin and myosin 2 as described below. The reversibility of BDM activity on CNF1-induced actin reorganization was studied as follows. HEp-2 cells were incubated with CNF1 (10^{-8} M) for 2 h, and then BDM (10 mM) was added for 40 min. Cell medium was removed, monolayers were washed once with DMEM, and cells were reincubated with fresh DMEM supplemented with fetal calf serum at 37 °C for an additional 1 h. Cells were then fixed and processed for immunofluorescence for actin and myosin 2 as described below.

Fluorescence and Scanning Electron Microscopy—HEp-2 or Hela cells were grown on square glass coverslips in separate wells (5×10^4 cells/well). Following toxin treatments, cells were washed 3 times with PBS and fixed with 3.7% paraformaldehyde prepared in the same buffer for 20 min. After being washed three times with 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 with 0.5% Triton X-100 in PBS for 5 min at room temperature. For F-actin detection, cells were incubated with FITC-phalloidin (Sigma) at 37 °C for 30 min. For microinjection experiments, cells already stained for F-actin detection were incubated with the first appropriate anti-rabbit antibodies for 30 min at 37 °C. This primary antibody binding was detected by 30 min of incubation at room temperature with Texas Red-conjugated sheep anti-mouse antibody (Amersham). For myosin 2 detection, the monoclonal antibody CC212 (35) was used undiluted on cells fixed with cold methanol. Incubation of cells with monoclonal antibody CC212 first and then with the Texas Red anti-mouse antibody as well as the washing procedure were performed as described above. Finally after washing, coverslips were mounted with Moviol (Calbiochem, La Jolla, Ca), observed, and photographed with a fluorescence microscope. For scanning electron microscopy, control and CNF1-treated cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 20 min. Following postfixation in 1% OsO_4 for 30 min, cells were dehydrated through graded ethanols, critical point dried in CO_2, and gold coated by sputtering. The samples were examined with a Cambridge 360 scanning electron microscope.

RESULTS

CNF1 Selectively Modifies the Rho GTPase in Vitro—In addition to the increase in actin stress fibers and to the promotion of cell spreading, CNF1 induced, in HEp-2 cells, the formation of actin-rich retraction fibers (Fig. 1, *e* and *f*, *arrows*). All these actin structures (stress fibers, spreading-dependent membrane

[2] G. Flatau and P. Boquet, manuscript in preparation.

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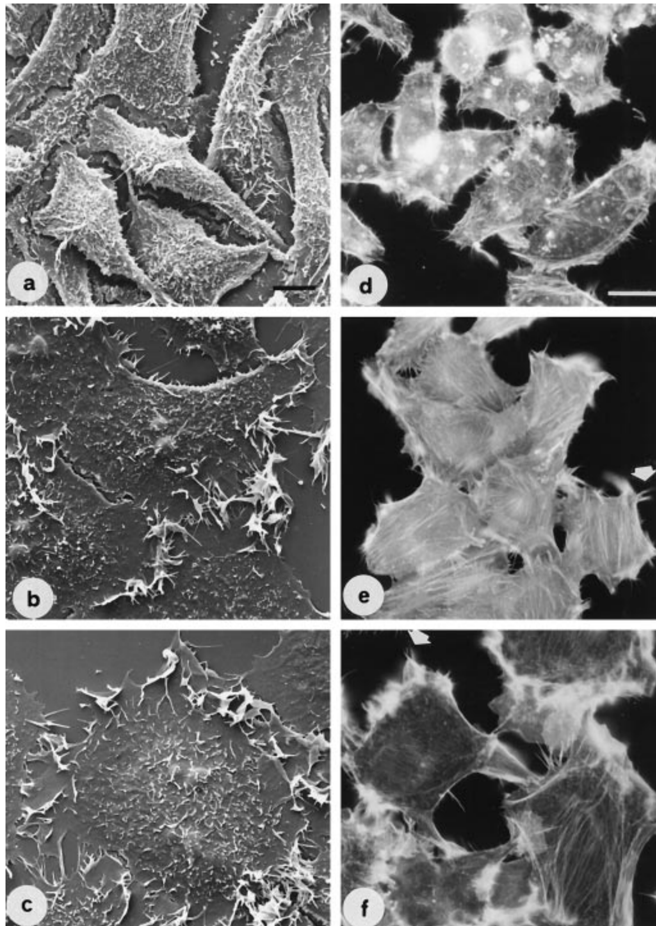

FIG. 1. **Induction of cell spreading and actin stress fibers by CNF1 as a function of time.** Scanning electron micrographs (*a-c*) and fluorescent staining for F-actin by FITC-phalloidin (*d-f*) of HEp-2 cells. Control cells (*a* and *d*) and cells treated with CNF1 for 3 (*b* and *e*) and 18 h (*c* and *f*). *Arrows* indicate CNF1-induced actin retraction fibers. *Bars* represent 10 μm.

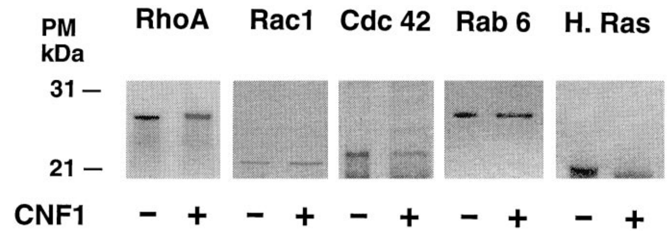

FIG. 2. **Effects of CNF1 on small GTPases *in vitro*.** CNF1 and the small GTPases were incubated for 2 h at 37 °C at a ratio of 1 molecule of CNF1 for 5 molecules of each GTPase in 20 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM EDTA. Incubation (total volume of 20 μl) was terminated by adding 5 ml of sample electrophoresis buffer containing 2.5% SDS. Samples were then boiled for 1 min. Electrophoresis was performed on 15% SDS gels that were then stained by Coomassie Blue.

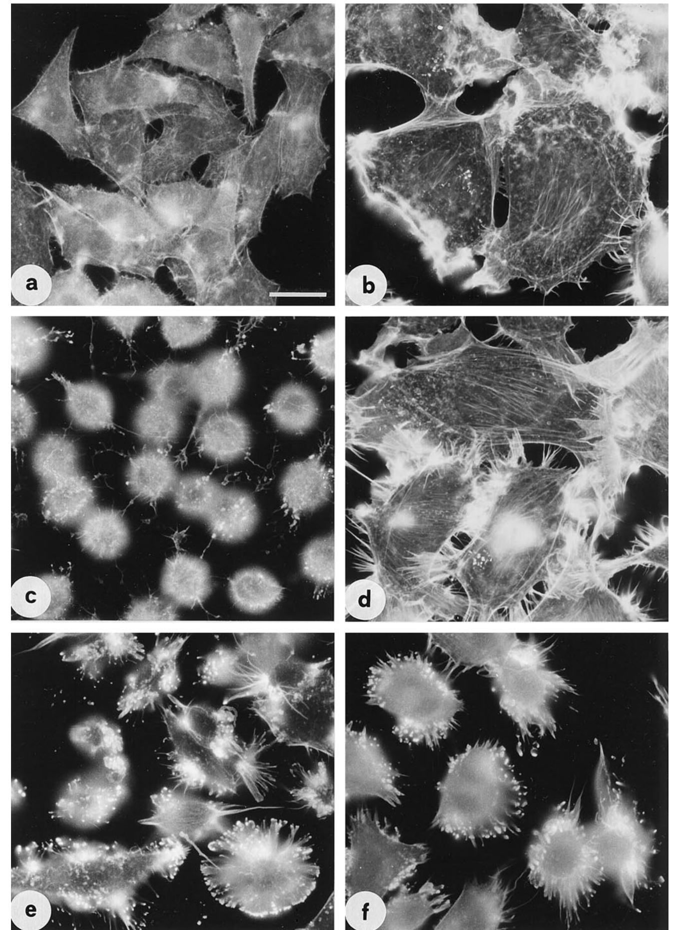

FIG. 3. **Effects of CNF1 preincubation on CdB or LT cytotoxicity in HEp-2 cells.** Shown are fluorescence micrographs of HEp-2 cells stained by FITC-phalloidin for F-actin detection. Control cells (*a*), cells incubated with CNF1 for 18 h (*b*), cells exposed to CdB for 4 h (*c*), cells incubated with CNF1 and exposed to CdB (*d*), cells treated with LT for 4 h (*e*), and cells incubated with CNF1 and then exposed to LT (*f*). *Bar* represents 10 μm.

folding, and retraction fibers) are clearly visible, observing cells by scanning electron and fluorescence microscopy (Fig. 1). Prolonging the time of exposure to CNF1 (micrographs in Fig. 1 were taken at 3 and 18 h), all the above described actin structures became more evident.

We have previously shown that incubation of HEp-2 cells with CNF1 or CNF2 induces an increase in the apparent molecular weight of Rho as visualized, after exoenzyme C3 ADP-ribosylation, by a shift in the electrophetic mobility of the GTPase (17, 18). We have thus tested whether an *in vitro* incubation of Rho, Rac, Cdc42, Ras, or Rab6 with purified CNF1 was able to induce a change in the electrophoretic mobility of one or more of these GTPases. As shown in Fig. 2, incubation of CNF1 for 2 h at 37 °C with the recombinant GTPases induced a shift of the electrophoretic mobility of the Rho GTPase only.

CNF1 Blocks the Effects of C. difficile Toxin B, but Does Not Block the Effects of C. sordellii LT in HEp-2 Cells—CNF1 can protect cells against CdB activity (19). *C. sordellii* LT is a toxin immunologically and structurally closed to CdB. LT glucosylates at threonine 35 (identical to threonine 37 of Rho) of the small GTP binding proteins Ras, Rap, and Rac (14). Thus, to test whether CNF1 activity was specific on Rho, HEp-2 cells were first incubated with CNF1 for 18 h and then challenged with either CdB or LT (Fig. 3). As expected from our previous results (19), effects of CdB on HEp-2 cells were prevented by preincubation of cells with CNF1 (Fig. 3*d*). LT induces in HEp-2 cells a strong cytopathogenic effect, consisting in the rounding up of cell bodies together with the formation of filopodia (13). The same effects were detectable also in cells pretreated with CNF1 (Fig. 3*f*), thus probably excluding Ras, Rap, and Rac as possible direct substrates for CNF1.

Microinjection of CNF1 into Cells Induces Production of Actin Stress Fibers—Microinjection of CNF1 into cells was performed to demonstrate that this toxin acts on Rho from inside the cytosol. When microinjected into cells, CNF1 induced a massive formation of stress fibers (Fig. 4*b*), comparable with

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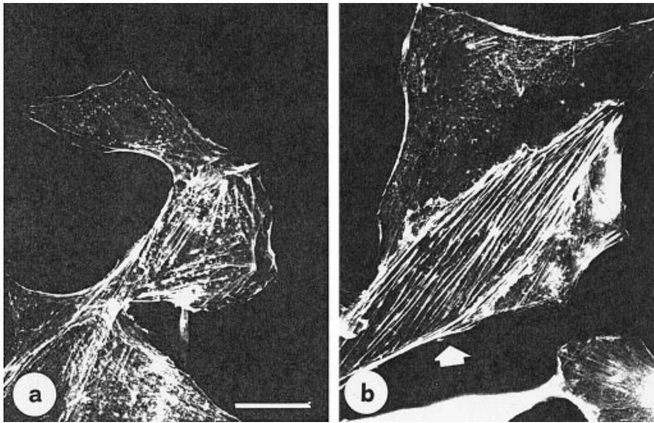

FIG. 4. **Microinjection into Vero cells of CNF1 induces accumulation of F-actin structures.** Shown are control cells microinjected with buffer and non-immune rabbit antibodies (*a*) and cells microinjected with CNF1 (*b*). *Arrow* indicates a microinjected cell. *Bar* represents 10 μm.

that caused by external added CNF1. Control microinjection with buffer and rabbit non-immune antibodies alone did not induce actin reorganization (Fig. 4*a*). This result clearly indicates that CNF1 acts on Rho in the cytosol and not via a transmembrane signaling mechanism.

CNF1 Activates a Cytoskeleton-associated PdtIns-4-P 5-kinase but Does Not Increase the PIP_2 Content of CNF1-treated HEp-2 Cells—Pretreatment of HEp-2 cells with 40 μM LY294002, a stable inhibitor of the PI 3-kinase (36), for 10 min followed by incubation of cells with 40 μM LY294002 together with CNF1 could not inhibit the cytoskeletal effects induced by the toxin (Fig. 5). This finding indicates that CNF1 does not trigger its effects on the actin cytoskeleton via an activation of the PI 3-kinase. Since it has been shown that Rho-GTP activates the PdtIns-4-P 5-kinase (22), we measured the PdtIns-4-P 5-kinase activity associated with the cytoskeleton of CNF1-treated HEp-2 cells. As shown in Fig. 6, CNF1 was able to increase in a time- (Fig. 6*a*) and dose-dependent (Fig. 6*b*) manner a PdtIns-4-P 5-kinase activity associated with the HEp-2 cells cytoskeleton.

We next examined whether CNF1, by activating a PdtIns-4-P 5-kinase activity, could selectively increase the PIP_2 content or eventually the amount of other phosphorylated phosphoinositides. HEp-2 cells were metabolically labeled with [^{32}P]orthophosphate and stimulated with CNF1 for 8 h. After lipid extraction, phosphoinosites content was determined as described previously (28). As shown in Table I, no increase of PIP_2, PI 3,4-P_2, or PIP_3 was observed in CNF1-treated HEp-2 cells compared with control preparation. It is worthy to note that LY294002 at 40 μM not only reduced the PI 3-kinase activity but also had some noticeable effects on the PdtIns-4-P 5-kinase activity (Table I).

CNF1 Induces Myosin 2 Relocalization into Stress Fibers but Not in Retraction Fibers—Myosins type 2 are activated through phosphorylation of their light chains (MLCs). MLCs phosphorylation leads to myosin 2 interaction with actin filaments and development of contractility. In control HEp-2 cells, myosin 2 was observed as diffuse throughout the cytosol but also concentrated around cell edges (Fig. 7*b*). F-actin staining of control cells showed a few stress fibers and retraction fibers (Fig. 7*a*). After 3 h of incubation of HEp-2 cells with CNF1, relocalization of myosin 2 was detectable in stress fibers but not in retraction fibers (Fig. 7, *c* and *d*).

BDM, an Inhibitor of the Myosin ATPase, Blocks Cell Spreading and Formation of Stress Fibers but Not of Retraction Fibers in Cells Exposed to CNF1—BDM, an inhibitor of skeletal myosin ATPase (32) has been used to demonstrate that Rho-stimulated contractility drives the formation of actin stress fibers (30). BDM also has been utilized to show the involvement of myosins in cell spreading (31). We, therefore, used this compound to study the effects of CNF1 on both the formation of actin stress fibers and on the induction of cell spreading. As shown in Fig. 8, *a* and *b*, BDM incubated with HEp-2 cells for 40 min at a concentration of 10 mM did not modify the F-actin and myosin immunoflorescent cell patterns, compared with control preparations (Fig. 7, *a* and *b*). BDM did block CNF1-

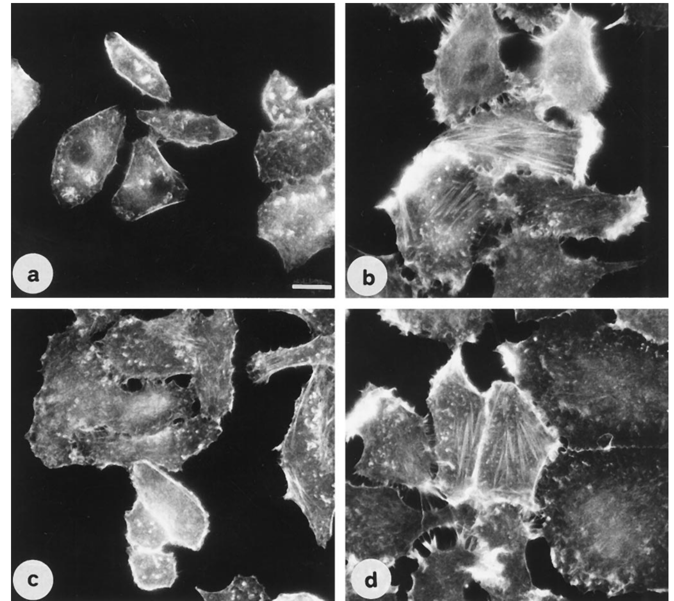

FIG. 5. **CNF1-induced effects in HEp-2 cells are not blocked by the PI 3-kinase inhibitor LY294002.** Fluorescence micrographs of HEp-2 cells stained with FITC-phalloidin for F-actin detection. Control cells (*a*), cells treated with CNF1 for 18 h (*b*), cells treated with 40 μM LY294002 (*c*), and cells treated with LY294002 and then incubated with CNF1 (*d*). *Bar* represents 10 μm.

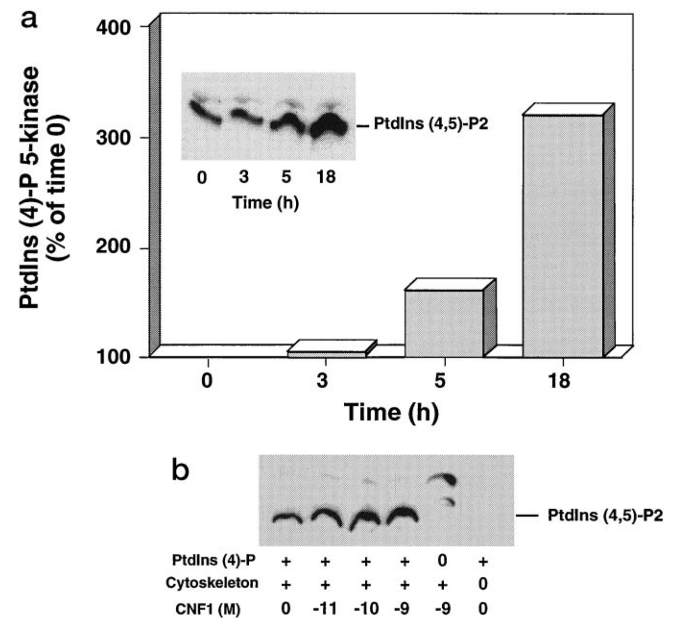

FIG. 6. **Effects of CNF1 on a cytoskeleton associated PtdIns-4-P 5-kinase activity in HEp-2 cells.** Shown is the PtdIns-4-P 5-kinase activity in cytoskeleton extracts from CNF1-treated HEp-2 cells: PtdIns-4-P 5-kinase activity as a function of time upon cell treatment with 10^{-9} M CNF1 for 0, 3, 5, and 18 h (*a*); and PtdIns-4-P 5-kinase activity in cells incubated for 18 h with concentrations of 10^{-9}, 10^{-10}, and 10^{-11} M CNF1 (*b*).

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TABLE I
Effect of CNF1 on the level of ^{32}P-labeled inositol lipids in HEp-2 cells

Cells prelabeled with ^{32}P for 2 h were incubated with 10^{-9} M CNF1 or with 10^{-9} M CNF1 in the presence of 40 μM LY294002 for 8 h at 37 °C. Lipids were extracted and analyzed as described (28). Values are in dpm.

	PtdIns-3P	PtdIns-4P	PtdIns 3,4-P2	PIP_2	PIP_3
Control	780 ± 36	34564 ± 1352	Traces	66886 ± 1358	0
+CNF1	920 ± 43	26443 ± 1031	Traces	54268 ± 1090	0
+CNF1+LY294002	492 ± 22	11379 ± 443	Traces	26436 ± 528	0

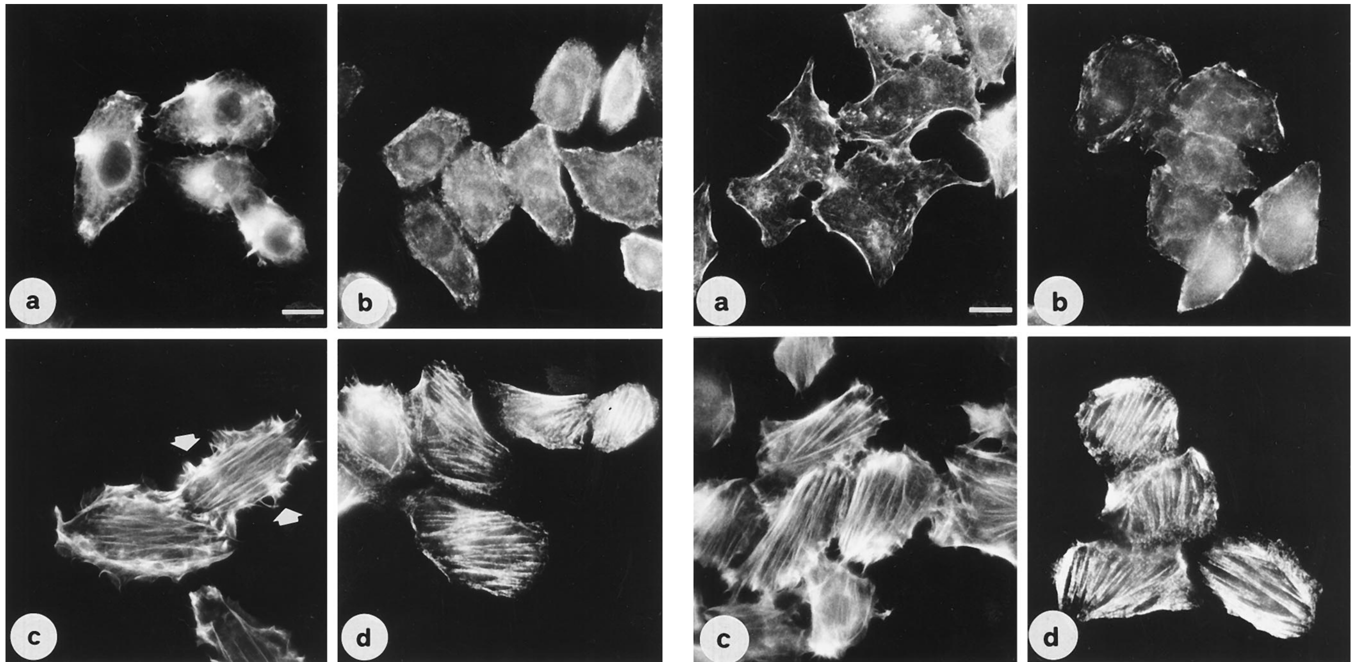

FIG. 7. **CNF1 induces myosin 2 relocalization into stress fibers but not in retraction fibers in HEp-2 cells.** F-actin detection in control cells (*a*) and in cells exposed to CNF1 for 18 h (*c*). Myosin 2 localization in control cells (*b*) and cells treated with CNF1 for 18 h (*d*). *Arrows* indicate actin retraction fibers. Since the fixation procedures were different whether cells were stained for F-actin or myosin 2 detection, *panels a-d* do not represent the same field. *Bar* represents 10 μm.

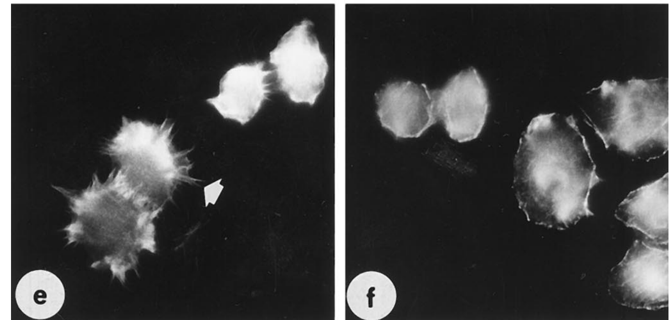

FIG. 8. **BDM inhibits cell spreading and the formation of stress fibers but not of retraction fibers in CNF1-treated HEp-2 cells.** F-actin detection by FITC-phalloidin (*a*, *c*, and *e*) and myosin 2 staining (*b*, *d*, and *f*) in BDM-treated cells (*a* and *b*), cells exposed to CNF1 (*c* and *d*), and cells challenged with BDM before CNF1 (*e* and *f*). *Arrow* indicates actin retraction fibers. Since the fixation procedures were different whether cells were stained for F-actin or myosin 2 detection, *a-f* do not represent the same field. *Bar* represents 10 μm.

induced stress fibers, myosin 2 relocalization (Fig. 8, *e* and *f*), however, being without effects on CNF1-induced retraction fibers (Fig. 8*e*). The effects of BDM on CNF1-induced formation of stress fibers, relocalization of myosin 2, and cell spreading were totally reverted within a few minutes by replacing the cell culture medium, containing BDM, with fresh medium (data not shown).

DISCUSSION

In the present study, by further investigating the activity of CNF1 at the molecular level, we have shown that CNF1 can provoke *in vitro* a modification of Rho, inducing an increase in the molecular weight of the GTPase. This activity was probably specific on the Rho protein since Rac, Cdc42, Ras, and Rab6 did not show any alteration in their molecular weights after incubation with CNF1. However, we cannot rule out the possibility that the gel electrophoresis system used to analyze the mobility of the different GTPases after exposure to CNF1 treatment could be unable to resolve the modified forms of Rac, Cdc42, Ras, and Rab.

Other findings supporting the specific activity of CNF1 on Rho came from studies carried out with bacterial toxins acting on p21 Ras-like proteins. As shown previously (19), CNF1 blocks most of the cytopathogenic effects of CdB on cells. CdB is a toxin known to monoglucosylate Rho (but also Rac and Cdc42) at threonine 37 (11). However, we have shown that the CNF1-induced modification of Rho *in vitro* does not impair the CdB glucosylation of this GTPase also when performed *in vitro*.[2] It has been shown that Rho in the GTP-bound form is a weak substrate for CdB glucosylation (11). One possibility is that *in vivo* the CNF1-modified Rho has lost its ability to hydrolyze GTP, therefore being permanently bound to GTP and thus protected from the enzymatic activity of CdB. By contrast, CNF1 does not protect cells against *C. sordellii* LT, which acts specifically on Ras, Rac, and Rap (by adding a glucose moiety at threonine 35 which corresponds to threonine 37 of Rho), but not on Rho (14).

Accordingly, we have recently observed that, upon microinjection, CNF1-activated RhoA could induce the CNF1-pheno-

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type.[3] This finding, together with the results obtained by microinjecting CNF1 directly into cells, clearly indicates that, inside the cells, CNF1 is able to trigger the same accumulation of actin stress fibers that occurs when cells are incubated with the toxin. Thus, CNF1 is a cytosolic-acting toxin and not a molecule inducing a cellular response through activation of an external membrane receptor, and Rho is, therefore, its cytosolic target.

Several previous studies have suggested pathways by which Rho may affect the cytoskeletal organization (for review, see Ref. 2). In particular, Rho has been shown to stimulate the activity of PdtIns-4-P 5-kinase, leading to an increase in PIP_2 level in response to adhesion (22). CNF1 induced, in HEp-2 cells, an increase in the activity of PdtIns-4-P 5-kinase, this result indicating that the Rho protein was in its GTP-bound form or in a GTP-bound form-like upon activation by CNF1. In contrast, the PI 3-kinase activity in CNF1-treated HEp-2 cells resulted in being unaffected since i) the toxin-induced effects were not blocked by LY294002, a potent inhibitor of the PI 3-kinase, and ii) PI 3,4-P_2 and PIP_3 amounts in treated cells were not increased. These findings indicate that the cascade of signaling molecules modulating the activity of the PI 3-kinase is not used by CNF1 to trigger its effects on the actin cytoskeleton. It is curious, however, that the increase in the *in vitro* measured activity of the PdtIns-4-P 5-kinase enzymatic activity associated with the cytoskeleton of CNF1-treated cells did not lead to a measurable accumulation of PIP_2 into treated cells. It has been previously reported that serum-starved Swiss 3T3 cells incubated with lysophosphatidic acid, a specific ligand activator of the Rho GTP-binding protein (2), did not accumulate PIP_2 (37). It is, therefore, possible that PIP_2 syntesized under the control of Rho-GTP can be very rapidly hydrolyzed by a phospholipase. Accumulation of PIP_2 in cells is probably harmful or lethal since this phosphoinositide is used for controlling of many different regulatory and enzymatic proteins.

Recently, it has been shown that PIP_2 allowed vinculin, one of the major proteins of focal contact points (38), to be activated (24). Upon interaction with PIP_2, vinculin unmasks cryptic binding sites for talin and F-actin, thereby linking the cytoplasmic domain of integrins to stress fibers (38). Our data concerning myosin 2 relocalization into stress fibers in CNF1-treated cells together with our results obtained with BDM are in favor of a mechanism in which activation of Rho by CNF1 may induce actin reorganization by the mechanism of acto-myosin tension. We cannot rule out, however, the possibility that in cells exposed to CNF1, a rapid transient increase in PIP_2 does occur, thus adding to the above reported contractility mechanism, the one driven by vinculin activation proposed by Chrzanowska-Wodnicka and Burridge (30).

Finally, we would like to stress that CNF1, by activating Rho, might be a useful new toxin in studying this GTP-binding protein.

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[3] G. Flatau and P. Boquet, submitted for publication.

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Escherichia coli Cytotoxic Necrotizing Factor 1 (CNF1), a Toxin That Activates the Rho GTPase

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