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

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Carla Fiorentini‡§, Alessia Fabbrini‡, Gilles Flatau¶, Gianfranco Donelli¶, Paola Matarrese‡, Emmanuel Lemichez‡, Loredana Falzano‡, and Patrice Boquet¶

From the ‡Department of Ultrastructures, Istituto Superiore di Sanitá, Viale Regina Elena 299, 00161, Rome, Italy and the ¶INSERM U452, Faculté de Médecine, Av de Valombrose, 06107 Nice, cedex 2, France

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 (PIP2) nor the phosphatidylinositol 3,4-bisphosphate (PI3,4-P2) 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** (CdaA and CdB, respectively) indirectly disrupt F-actin structures by activating 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 CdaA 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 CdaA 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 (PtdIns-4-P 5-kinase), whose product is PtdIns-4,5-P2, has been shown to be activated by Rho GTP (22). Several lines of evidence point out that the elevation of PIP2 can lead to increased actin polymerization. It has been reported, in fact, that PIP2 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, PIP2 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 PIP2. 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 musc.
Concentration has been used: 10^{-5} M. Purification of CNF1 from bacterial extracts was achieved by reiteration in vivo of a cytoskeleton-associated PtdIns-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 actin-myosin pattern previously described by Mitchison and co-worker (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). Parts of cells were added to the cell culture medium at a concentration of 10^{-5} M and incubated for 4 h in the labeling medium. Cells were then washed twice with PBS, reseeds were then stopped by adding ice-cold HCl (2.4 M), and cells were recovered by scraping. Lipids were then extracted as described previously (28) and separated by thin layer chromatography, and radiolabeled PIP2 was recovered by scraping the appropriate bands. Separation and analysis of decylated products by high pressure liquid chromatography were done as described (34).

Fluorescence and Scanning Electron Microscopy—HEp-2 or Hela cells were grown on square glass coverslips in separate wells (5 cm diameter) and then labeled with 250 μCi/ml [32P]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^{-5} M and incubated 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 described (34). HEp-2 cells were then incubated with BDM 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.

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.
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 electrophoretic 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 close 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. 3d). 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 preincubated with CNF1 and then exposed to LT (Fig. 3f).

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

**CNF1 Activates a Cytoskeleton-associated PtdIns-4-P 5-kinase but Does Not Increase the PIP2 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 PtdIns-4-P 5-kinase (22), we measured the PtdIns-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- and dose-dependent manner a PtdIns-4-P 5-kinase activity associated with the HEp-2 cells cytoskeleton.

We next examined whether CNF1, by activating a PtdIns-4-P 5-kinase activity, could selectively increase the PIP2 content or eventually the amount of other phosphorylated phosphoinositides. HEp-2 cells were metabolically labeled with [32P]orthophosphate and stimulated with CNF1 for 8 h. After lipid extraction, phosphoinositides content was determined as described previously (28). As shown in Table I, no increase of PIP2, PI 3,4-P2, or PIP3 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 PtdIns-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. 7b). F-actin staining of control cells showed a few stress fibers and retraction fibers (Fig. 7a). 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 μM did not modify the F-actin and myosin immunofluorescent cell patterns, compared with control preparations (Fig. 7, a and b). BDM did block CNF1-
induced stress fibers, myosin 2 relocalization (Fig. 8, e and f), however, being without effects on CNF1-induced retraction fibers (Fig. 8). 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. 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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### Table I

| Lipid Type | Control | CNF1 | CNF1 + LY294002 |
|------------|---------|------|-----------------|
| PtdIns-3P  | 780 ± 36| 920 ± 43| 492 ± 22 |
| PtdIns-4P  | 34564 ± 1352| 26443 ± 1031| 11379 ± 443 |
| PtdIns 3,4-P2| Traces | Traces | Traces |
| PIP2       | 66886 ± 1358| 54268 ± 1090| 26436 ± 528 |
| PIP3       | 0       | 0    | 0   |

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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.
Upon interaction with PIP2, vinculin unmasks cryptic of the major proteins of focal contact points (38), to be activated probably harmful or lethal since this phosphoinositide is used for U364, Nice, France) for help in the PtdIns-4-P 5-kinase assay and

Contrast, the PI 3-kinase activity in CNF1-treated HEp-2 cells, an increase in the activity of PtdIns-4-P 5-kinase, this level in response to adhesion (22). CNF1 induced, in HEp-2 cells together with our results obtained with BDM are in favor of controlling many different regulatory and enzymatic proteins.

Recently, it has been shown that PIP2 allowed vinculin, one of the major proteins of focal contact points (38), to be activated (24). Upon interaction with PIP2, vinculin unmasking 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 PIP2 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.