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Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers

(bacterial pathogenesis/cytoskeleton/*Pasteurella multocida* toxin)

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ABSTRACT **Cytotoxic necrotizing factor type 2 (CNF2) produced by *Escherichia coli* strains isolated from intestinal and extraintestinal infections is a dermonecrotic toxin of 110 kDa. We cloned the CNF2 gene from a large plasmid carried by an *Escherichia coli* strain isolated from a lamb with septicemia. Hydropathy analysis of the deduced amino acid sequence revealed a largely hydrophilic protein with two potential hydrophobic transmembrane domains. The N-terminal half of CNF2 showed striking homology (27% identity and 80% conserved residues) to the N-terminal portion of *Pasteurella multocida* toxin. Methylamine protection experiments and immunofluorescence studies suggested that CNF2 enters the cytosol of the target cell through an acidic compartment and induces the reorganization of actin into stress fibers. Since the formation of stress fibers in eukaryotic cells involves Rho proteins, we radiolabeled these small GTP-binding proteins from CNF2-treated and control cells with a Rho-specific ADP-ribosyltransferase. The [^{32}P]ADP-ribosylated Rho proteins from CNF2-treated cells migrated slightly more slowly in SDS/PAGE than did the labeled proteins from the control cells. This shift in mobility of Rho proteins in SDS/PAGE was also observed when CNF2 and the RhoA protein were coexpressed in *E. coli*. We propose that Rho proteins are the targets of CNF2 in mammalian cells.**

Certain *Escherichia coli* strains isolated from humans or animals with diarrhea or extraintestinal infections produce a cytotoxic necrotizing factor (CNF) that causes multinucleation in tissue culture cells and necrosis in the rabbit skin (1–7). Two types of CNF have been described: CNF1, produced by *E. coli* strains isolated from humans and animals (1–6), and CNF2 (first named Vir cytotoxin), produced by *E. coli* strains isolated from cows and sheep (2, 4, 7). CNF1 and CNF2 are similar in size with an apparent molecular mass of 110–115 kDa in SDS/PAGE (8–10) and are immunologically related (2, 4, 10). However, CNF1 and CNF2 are distinguishable by (*i*) the specific necrotic activity of CNF2 in mouse footpads (11), (*ii*) the different responses of CNF1 and CNF2 in cross-neutralization assays (2, 4, 7), and (*iii*) the different serogroups and other virulence factors associated with the *E. coli* strains that produce these toxins (2, 12, 13). In addition, CNF1 is chromosomally encoded (14), whereas CNF2 is encoded by F-like plasmids (7) that were initially designated as Vir plasmids (15–17).

Several lines of evidence support a role for CNF in *E. coli* pathogenicity. First, 10–40% of *E. coli* strains isolated from humans and animals with diarrhea or extraintestinal infections produce CNF1 or CNF2 (1–6). Second, experimental infections of neonatal calves (18) and pigs (19) with CNF1- or CNF2-producing strains suggest that CNF1 and CNF2 contribute to the pathogenesis of colibacillosis. Third, CNFs are potent toxins that cause tissue damage and death of the animal host (20). Fourth, CNF1 can trigger the entry of noninvasive bacteria into HEp-2 human epidermoid carcinoma cells through phagosome-like organelles (21). This induction of phagocytic behavior in human epithelial cells is associated with the capacity of CNF1 to induce F-actin assembly (22).

In this paper, we present the cloning and sequence analysis of the gene encoding CNF2 and describe experiments which led to the identification of its target in the mammalian cell.††

MATERIALS AND METHODS

Cloning and Sequencing of *cnf2*. DNA manipulations were carried out by standard procedures (23). Vir plasmid DNA was purified from *E. coli* 711(pVir) (15), partially digested by *Sau*3A1, and ligated to *Bam*HI-digested pILL570 plasmid DNA (24). *E. coli* MC1061 (23) transformed with recombinant plasmids was screened for the production of CNF2 by the cell cytotoxic assay (4), the rabbit skin test (2), and the mouse footpad test (11). Tn*5* mutagenesis of pEOAL6 was conducted with the F′$_{ts}$114*lac*::Tn*5* plasmid (25). Subclones of pEOAL6 and pEOAL10 were constructed in plasmid vectors pBluescript II SK(+) (Stratagene) and pK184 (26). The nucleotide sequence of double-stranded template DNA was determined as described in the CircumVent thermal cycle dideoxy DNA sequencing kit (New England Biolabs). DNA and peptide sequence data were analyzed by the Genetics Computer Group software package (27). CNF2 secondary structure was predicted with the program developed by Burkhard and Sander (28).

Radiolabeling of Rho Proteins by ADP-ribosylation. Swiss 3T3 mouse cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After a 24-hr incubation with CNF2 extracts, cells were washed three times with cold phosphate-buffered saline, pooled by scraping, pelleted by centrifugation, and homogenized by

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Abbreviations: CNF, cytotoxic necrotizing factor; PMT, *Pasteurella multocida* toxin.

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††The sequence of *cnf2* presented in this paper has been deposited in the GenBank data base (accession no. U01097).

sonication (29). The proteins in the resulting homogenates were [^{32}P]ADP-ribosylated *in vitro* with pure *Staphylococcus aureus* epidermal cell differentiation inhibitor (EDIN) (30). The standard reaction mixture (100 μl) contained 10 μM [α-^{32}P]NAD (1000–3000 cpm/pmol), 40 ng of EDIN, and the cell lysate. After 20 min at 30°C, proteins were precipitated with 10% (wt/vol) trichloroacetic acid and analyzed by SDS/12% PAGE. The same techniques were used to ADP-ribosylate the RhoA protein present in sonic lysates of *E. coli* strains carrying pGC-RhoA and pK184 or pGC-RhoA and pEOSW30. The pGC-RhoA plasmid contains RhoA cDNA under the control of a tryptophan promoter (31). Samples of *E. coli* strains were prepared by sonication of the cells from a late-logarithmic-phase culture in Luria–Bertani (LB) medium.

RESULTS

Cloning of the DNA Region of pVir Required for CNF2 Production. A common region of 5.15 kb was identified by comparing the restriction maps of pEOAL6 and pEOAL10, two subclones from pVir that caused multinucleation of HEp-2 cells (Fig. 1). *E. coli* transformed with mutant plasmids containing Tn*5* insertions mapped in this common region did not produce CNF2 (Fig. 1). Additional evidence that this 5.15-kb region contained the gene(s) responsible for multinucleation was obtained by subcloning this region in a pBluescript II SK(+) vector. First, the 350-bp *Bgl* II fragment of pEOAL10 was ligated into *Bam*HI-digested pBluescript II SK(+). The resulting recombinant plasmid was digested with *Xba* I/*Bam*HI and ligated to the 4.8-kb *Xba* I–*Bam*HI fragment from pEOAL6. One such clone, pEOSW20, contained the correct 5.15-kb insert and expressed CNF2 (Fig. 1).

Identification and Sequence of the Open Reading Frame (ORF) Encoding CNF2. The region common to the CNF2-producing clones was sequenced. An open reading frame (ORF) of 3045 nucleotides was identified. This ORF was 85.7% identical to the recently sequenced gene encoding CNF1 (14). The identification of the 3045-bp ORF as *cnf2* was confirmed by subcloning the *Ban* I–*Hae* II DNA fragment encompassing the ORF and the 112-bp region upstream of the start codon. This 3332-bp *Ban* I–*Hae* II DNA fragment was digested with mung bean nuclease and ligated into *Sma* I-digested pK184. The resulting clone, pEOSW30 (Fig. 1), was transformed into *E. coli* XL1-blue; the lysate of *E. coli* XL1-blue(pEOSW30) induced multinucleation of HEp2 cells and caused necrosis in the mouse footpad and in the rabbit skin. By contrast, a similarly prepared lysate of control strain *E. coli* XL1-blue(pK184) was not toxic *in vitro* or *in vivo* (data not shown). Taken together, these results support our conclusion that the ORF cloned in pEOSW30 is *cnf2*.

Analysis of the Deduced Amino Acid Sequence of CNF2. *cnf2* encodes a protein of 1014 amino acids with a calculated molecular weight of 114,659. Analysis of the deduced amino acid sequence of CNF2 revealed an atypical codon usage for *E. coli* which reflects the low G+C content of *cnf2* (35.3%). The hydropathy profile predicted by the program of Kyte and Doolittle (32) showed that CNF2 was a relatively hydrophilic protein with two putative hydrophobic transmembrane domains (amino acids 333–372 and 386–409). These two domains were partially overlapped by two predicted α-helices (amino acids 327–342 and 391–410). Most notably, no classical signal sequence was found in the N-terminal 50 residues of CNF2. Beside the high homology between CNF2 and CNF1 (the two toxins shared 85% identical residues and 99% conserved residues over 1014 amino acids; Fig. 2), a comparison of the predicted amino acid sequence of CNF2 with sequences in GenBank (Release 80) revealed that the *Pasteurella multocida* toxin (PMT) shared 27% identical residues and 80% conserved residues over the N-terminal 528 amino acids of CNF2 (Fig. 3). Similar findings were noticed in the analysis of the deduced amino acid sequence of CNF1 (14). We also observed 77% homology between CNF2 amino acids 399–426 and the glucose-binding domain of hexokinase B from *Saccharomyces cerevisiae* (35) and 64% homology between CNF2 amino acids 67–319 and the N terminus of the epidermin-modifying enzyme EpiB of *Staphylococcus epidermidis* (33) (data not shown).

Methylamine Protection of HEp-2 Cells from CNF2 Toxicity. To study the mechanism by which CNF2 enters and reaches the cytoplasm in HEp-2 cells, various concentrations of methylamine chloride (pH 7.4) were added to the culture medium of subconfluent HEp-2 cells. Methylamine is an agent that increases endosomal and lysosomal pH and thereby inhibits the processing and translocation of many toxins into the cell cytoplasm (34). An *E. coli* XL1-

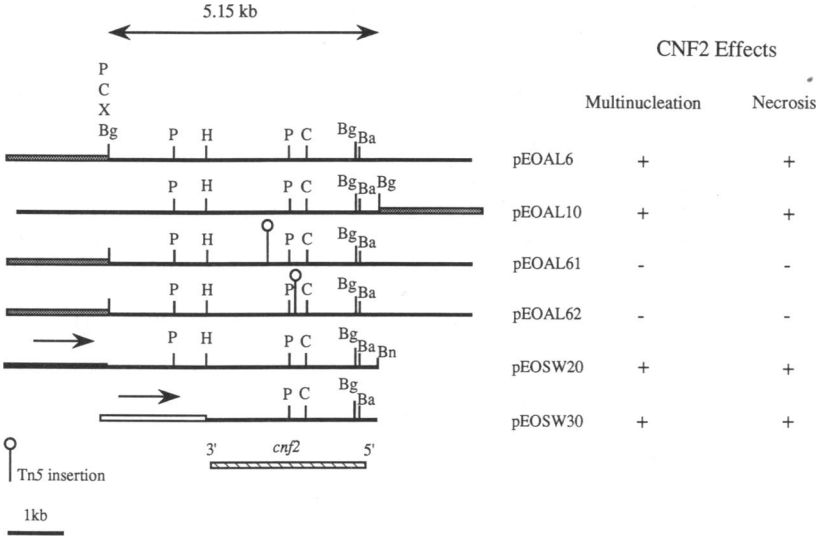

FIG. 1. Restriction maps of various subclones of the Vir plasmid that contain *cnf2*. The production of CNF2 after transformation into *E. coli* XL1-blue or MC1061 is shown at right. The cloning vector for pEOAL6, pEOAL61, pEOAL62, and pEOAL10 was pILL570. The cloning vector for pEOSW20 was pBluescript SK II(+). The cloning vector for pEOSW30 was pK184. Arrows indicate the 5′ → 3′ orientation of the *lacZ* gene in pBluescript SK II(+) and pK184. The restriction sites of relevant endonucleases are indicated: Ba, *Bam*HI; Bg, *Bgl* II; Bn, *Ban* I; C, *Cla* I; P, *Pst* I; H, *Hae* II; X, *Xba* I.

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CNF2    1 MNVQWQQQKYLLEYNELVSNFPSPERVVSDYIRRCFKTDLPWFSQVDPDNT 50
CNF1      -GN----------------------------KN----------RI----A

       51 YFIRFSQSRSNSRSYTGWDHLGKYKTGVLTLTQAALINIGYHFDVFDDAN 100
          ---C---N-----------------E-------------R--------

      101 ASAGIYKTSSADMFNEKNEEKMLPSEYLYFLKGCDFSGIYGRFLSDYWSK 150
          S-T-----K---V---E----------H--QK---A-V--KT-------

      151 YYDKFKLLLKNYYISSALYLYKNGEIDEYEYNFSISALNRRDNISLFFFD 200
          --------------------------L--R-----MN----S-----L---

      201 IYGYYSSDMFVAKNNERVMLFIPGAKKPFLFEKNIADLRISLKNLIKEND 250
          -----A--I------DK-------------K-------LT--E---DS-

      251 NKQLLSQHFSLYSRQDGITYAGVNSVLNAIENDGVFNESYFLYSNKRINN 300
          KQ--------------VS--------H------N----------TLS-

      301 KDVFDAVAFSVKKRSFSDGDIVIKSNSEAQRDYALTILQTILSMTPIFDV 350
          ------I-I-----------------------------------I

      351 AIPEVSVTLGLGIIASSMGISFDQLINGDTYEERRSAIPGLATNAALLGL 400
          VV-----P------T-----------------------------V----

      401 SFAIPFLISKAGTNQKILSRYTKHEIRTLNETNIDMFLEEYGINKNSISE 450
          -----L------I--EV--SVINN-G---------I--K----AED---S

      451 TKVLEVELKGSGQHVNIVKLSDEDNKIVAVKGNSLSGIYYEVDIETGYEI 500
          -NL-D-K--S---------------Q------S-----------------

      501 SSRRIYRTEYNDKIFWTRGGGLKGGQSFDFESLKLPIFFKDEPYSAVPGS 550
          L----------NE-L-----------P------NI-V----------T--

      551 SLSFINDDSSLLYPNSTPKLPQPTPEMEIVNYVKRAGNFGERLVTLMRGT 600
          P-------------DTN-------S--D------GS-S--D-F------A

      601 TEEEAWNIARYHTAGGSTEELHEILLGQGPQSSLGFTEYTSNINSADAAS 650
          ---------S-------------------------------V-------

      651 RRHFLVVIKVQVKYINNNNVSHVNHWAIPDEAPVEVLAVVDRRFNFPEPS 700
          ----------H----T-----Y-----------------------------

      701 TPPNISIIHKLLSLRYFKENIESTSRLNLQKLNRGNIDIFKGRGSISSTR 750
          ---D--T-R----------S-----KS-F---S-----VL----------

      751 QRAIYPYFESANADEQQPVFFYIKKNRFDDFGYDQYFYNSTVGLNGIPTL 800
          ---------A--------L------D---NH--------DN---------

      801 NTYTGEILSDASSLGSTYWKKYNLTNETSIIRVSNSARGANGIKIALEEV 850
          -------P--S-----------------------------------

      851 QEGKPVIIITSGNLSGCTTIVARKGGYLYKVHTGTTIPLAGFTSTTGVKKA 900
          ----------------------E--I--------KS-------------

      901 VEVFELLTNNPMPRVEGVMNNDFLVNYLAESFDESLITYSSSEQKIGSKI 950
          ---L----KE-I------S------D--S-N-ED---------K-PD-Q-

      951 TISRDNVSTFPYFLDNIPEKGFGTSVTILVRVDGNVIVKSLSESYSLNVE 1000
          --I------V----------H-----A-V--------V-R---------AD

     1001 NSNISVLHVFSKDF* 1015
          A-E----K----K--
```

FIG. 2. Similarity between the deduced amino acid sequences from *E. coli* CNF2 (this study) and *E. coli* CNF1 (14). Identical residues are indicated by dots, conserved residues by plain letters, and nonconserved residues by boldface letters.

```
  1 MNVQWQQQKYLLEYNELVSNFPSPERVVSDYIRRCFKTDLPWFSQVDPDNT 50
    ||:|:: ::  ::::: : |:| ::|:::|: :::::   ::::||:::|
 35 NNVKWKEVFINRFGQMMLDTPNPRKIVEKIINEGLEKQG--LKNIDPETT 82

 51 YFIRFSQSRSNSRSYTGWDHLGKYKTGVLTLTQAALINI--GYHFDVFDD 98
    ||  || | |:: :   :: |:    : : :|:|:|:||  : :|| :|
 83 YFNIFSSSDSSDGNVFHYNSLS----ESYRVTDACLMNIFVERYFDDWDL 128

 99 AN--ASAGIYKTSSADMFNEKNE----------EKMLPSEYLY-FLKG 133
      |  ||:|||:::::: : ::::            |::::|  :  :| :
129 LNSLASNGIYSVGKEGAYYPDHDYGPEYNPVWGPNEQIYHSRVIADILYA 178

134 CDFSGIYGRFLSDYWSKYYDKFKLLLKNYYISSALYLYKNGEIDEYEYNF 183
    :::: : ::: :|| || : :: :|:: ::: |:  |:::::: :: :
179 RSVWDEFKKYFMEYWQKYAQLYTEMLSDTFLAMAIQQYTRQTLTDEGFLM 228

184 SISA-LNRRDNISLFFFDIYGYYSSDMFVAKNNE----RVMLFIPGAKKP 228
    ::: :::::::: : ::|||||| |:|::: ::::    :|:|:|||:::|
229 VCNTYYGNKEEVQITLLDIYGYPSTDIICIEQKGLPTPKVILYIPGGTQP 278

229 FLFEKNIADLRISLKNLIKENDNKQLLSQHFSLYSRQDGITYAGVNSVLN 278
    |:   |::||: ::   :|:|::::  :::|||| ||:| |::|::::|:
279 FVEFLNTDDLKQWIAWHLKDNKHMVRFRKHFSLKQRQEGETFTGIDKALQ 328

279 AIENDGV--FNESYFLYSNKRINNKDVFDAVAFSVKKRSFSDGDIVIKSN 326
    |:::::   :::|:||: :::::::::|: :  :::::| ::|:|: |:||
329 YIAEESPEWPANKYILYNPTHLETENLFNIMMKRTEQRMLEDSDVQIRSN 378

327 SEAQRDYALTILQTILSMTPIFDVAIPEVSVTLGLGIIASSMGISFDQLI 376
    ||| |||||::|:|::|  : :|: :|:|:::::::: |:::|:| | ::
379 SEATRDYALSLLETFISQLSAIDMLVPAVGIPINFALSATALGLSSDIVV 428

377 NGDTYEERRSAIPGLATNAALLGLSFAIPFLISKAGTNQKILSRYTKHEI 426
    |||:||:|: :|:::|: :| : |::: || :||:::::  : :|| |:::|
429 NGDSYEKRKYGIGSLVQSALFTGINL-IP-VISETAEILSSFSR-TEEDI 475

427 RTL--NETNIDMFLEEYGINKNSISETKVLEVELKGSGQHVNIVKLSDED 474
    :::  :| :::  :|  : : :|||:::::     ::: :::::|:|::|:
476 PAFFTEEQALAQRFEIVEEELHSISPDDPPREITDENLHKIRLVRLNNEN 525

475 NKIVAVKGNSLSGIYYEVDIETGYEISSRRI--YRTEYNDKIFWTRGGGL 522
    :::|::::  : :: :: ::  |  ||::: :    :: ::|:::::::: |
526 QPLVVLRRLG-GNKFIRIEPITFQEIKGSLVSEVINPVTNKTYYVSNAKL 574

523 KGGQSF 528
    || ::
575 LGGSPY 580
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FIG. 3. Similarity between the deduced N-terminal amino acid sequence of *E. coli* CNF2 and the N terminus of PMT (43, 44). The GAP program of the Genetics Computer Group package was used to compare the deduced amino acid sequences of CNF2 (upper line) and PMT (lower line). Identical residues are indicated by vertical lines and conserved residues by colons.

blue(pEOSW30) lysate that contained a CNF2 activity estimated at 4 CD_{50} (4 times the median cytotoxic dose) was added to the HEp2 cells treated with methylamine. The percentage of multinucleated cells was measured after a 72-hr incubation. We observed that the addition of methylamine 1 hr prior to the addition of the toxic extract protected the HEp-2 cells from CNF2 toxicity in a dose-dependent manner. Indeed, 5 mM methylamine reduced to 40% the number of multinucleated cells as compared with untreated cells, and 10 mM methylamine completely inhibited the multinucleation effect induced by CNF2 (data not shown). In contrast, no protective effect was observed when the methylamine (up to 15 mM) was added 2 hr after CNF2 (data not shown). These findings suggest that acidification of endosomes is required for the expression of CNF2 cytotoxicity. Similar results have been observed with PMT (36).

CNF2-Induced Assembly of F-Actin. CNF1 mediates actin assembly in intoxicated cells (22). We speculated therefore that CNF2 might also cause the reorganization of F-actin, based on the similar effects of CNF1 and CNF2 in inducing multinucleation. To test this hypothesis, HEp-2 cells were incubated for 24 hr with a CNF2-containing extract from *E. coli* XL1-blue(pEOSW30) or, as a control, a similarly prepared extract from *E. coli* XL1-blue(pK184). Cellular F-actin from extract-treated cells was then stained with fluorescein isothiocyanate-conjugated phalloidin (21). Lysates of *E. coli* XL1-blue(pEOSW30) that contained CNF2 drastically modified the actin cytoskeleton of HEp-2 cells, whereas the control lysate of XL1-blue(pK184) had no such an effect (Fig. 4). The actin was reorganized into long thick filaments (classically called stress fibers) in the CNF2-treated cells, whereas the majority of F-actin remained diffuse in the control-treated cells or untreated cells (data not shown). The CNF2-induced actin filament reorganization preceded the multinucleation that was observed 48–72 hr after exposure of the HEp-2 cells to the toxin. We postulate, as has been proposed for CNF1 (22), that treatment of HEp-2 cells with CNF2 causes the reorganization of F-actin, which might block cytokinesis during mitosis and subsequently lead to the formation of multinucleated cells in culture.

CNF2-Modified Rho Proteins. It has been shown recently that a possible intracellular target of CNF1 in eukaryotic cells could be the small GTP-binding proteins of the Rho family (37). These proteins regulate the assembly of focal adhesion and stress fibers in eukaryotic cells (38, 39). To determine whether CNF2 mediates actin polymerization of intoxicated cells through these small GTP-binding proteins, we radiolabeled the Rho proteins from lysates of CNF2-treated Swiss 3T3 cells with a Rho-specific ADP-ribosyltransferase produced by *Staphylococcus aureus* (30). Nearly confluent Swiss 3T3 cell cultures were incubated for 24 hr with a CNF2-containing cell extract from *E. coli* XL1-blue(pEOSW30) or, as a control, a similarly prepared extract from *E. coli* XL1-blue(pK184). The radiolabeled Rho proteins from eukaryotic cells treated with CNF2 were shifted toward higher molecular weight as compared with those from control cells (Fig. 5*A*). To further demonstrate the role of CNF2 in the modification of Rho proteins in eukaryotic cells, we tried to directly reproduce the shift in Rho protein mobility observed in SDS/PAGE of RhoA protein expressed in *E. coli*. For this purpose, we transformed an *E. coli* strain carrying a recombinant clone that expresses RhoA with plasmid pK184 or pEOSW30 and tested the lysates for the presence of modified RhoA. Both *E. coli* C600(pK184; pGC-

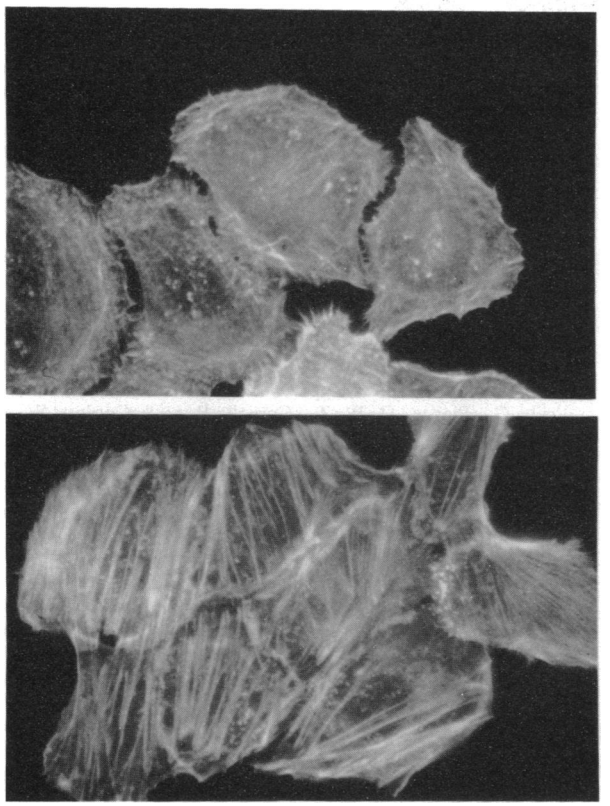

FIG. 4. Fluoresceinated phalloidin-stained actin in HEp2 cells after 24 hr of incubation with or without CNF2. (*Upper*) Control cells treated with an extract of *E. coli* XL1-blue(pK184). (*Lower*) Cells treated with an extract of *E. coli* XL1-blue(pEOSW30) that contained CNF2. (×500.)

RhoA) and C600(pEOSW30; pGC-RhoA) produced RhoA, but only the latter strain produced CNF2. In this strain, the radiolabeled RhoA protein showed an apparent, albeit small, increase in molecular weight as compared to control cells (Fig. 5*B*). However, this molecular weight shift was reproducibly larger in the eukaryotic cells (Fig. 5*A*) than in the prokaryotic cells (Fig. 5*B*). The biochemical basis of the mobility shift of CNF2-modified Rho proteins in SDS/PAGE remains to be determined. To test the possibility that CNF2 could be an ADP-ribosylating enzyme or an ATP-dependent protein kinase, we treated Swiss 3T3 cell homogenates with partially purified CNF2 extracts and either [^{32}P]NAD or [α-^{32}P]ATP. SDS/PAGE followed by autoradiography did not reveal any specific ^{32}P-labeled proteins in the Swiss 3T3 cell homogenates treated with CNF2 (data not shown). A

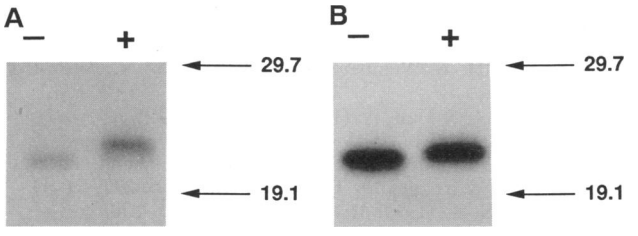

FIG. 5. Autoradiograms of the Rho proteins expressed by eukaryotic or prokaryotic cells in the presence (+) or absence (−) of CNF2. Lysates of eukaryotic or prokaryotic cells were prepared and analyzed. (*A*) Lysates of Swiss 3T3 cells treated with an extract of *E. coli* XL1-blue(pK184) (−) or with an extract of *E. coli* XL1-blue(pEOSW30) that contained CNF2 (+). (*B*) Lysates of control *E. coli* C600(pK184; pGC-RhoA) (−) and of *E. coli* C600(pEOSW30; pGC-RhoA) that contained CNF2 (+). Position and size (kDa) of the markers are shown at right.

similar finding was observed after microinjection of the toxin into *Xenopus* oocytes loaded with [^{32}P]NAD (P.B., unpublished data). These preliminary experiments show that the structural alteration of Rho proteins by CNF2 does not involve NAD-dependent ADP-ribosylation or ATP-dependent phosphorylation of Rho proteins.

DISCUSSION

Two major conclusions about CNF2 production and its biological properties can be derived from this study. First, CNF2 in *E. coli* is encoded by a single structural gene, *cnf2*, and the recombinant CNF2 is fully active in inducing multinucleation in cultured eukaryotic cell lines and in causing necrosis in both rabbit skin and mouse footpad. Second, CNF2 is a potent cytotoxin which drastically modifies the actin cytoskeleton of HEp-2 cells. The capacity of CNF2 to mediate polymerization of F-actin suggests that this toxin, like CNF1, may trigger the actin-dependent endocytosis of bacteria (21). Based on these results and on the incidence of CNF-producing strains in human and animal infections, we believe that CNF2 may be an important virulence determinant in *E. coli* strains that cause enteric and extraintestinal infections.

The actin cytoskeleton is a key player not only in the interactions of pathogenic bacteria with mammalian cells but also in many cellular processes, including cell division and growth control. Actin assembly in eukaryotic cells has been shown to involve the Rho proteins (38, 39). This family of Ras-related proteins is also involved in cell growth regulation (40) and in the signal transduction pathway linking growth factor receptors to the assembly of focal adhesions and the organization of actin into stress fibers (41). In the present study, we have shown that Rho proteins in CNF2-treated cells were shifted toward a higher molecular weight in SDS/PAGE. We were able to reproduce this shift when CNF2 and RhoA were coexpressed in *E. coli*. This result strongly supports a direct interaction between CNF2 and the Rho proteins and demonstrates that the modification of Rho proteins by CNF2 does not require prior C-terminal modification and processing by enzymes unique to eukaryotic cells. The effects of CNF2 on the actin cytoskeleton mimic those observed when a constitutively activated mutant of RhoA, [Val^{14}]RhoA, is microinjected into Swiss 3T3 cells (39). We propose that CNF2 covalently modifies the structure of Rho protein in such a way that the modified Rho protein is functionally hyperactive as compared with its native form. This hyperactive state may be a reflection of an altered interaction of activated Rho proteins with its target or with other accessory proteins such as the GTPase-activating proteins and GDP-dissociation inhibitors (42). Although we have not identified the mechanism of CNF2 action, the amino acid homology between CNF2 and EpiB, a protein involved in the posttranslational modification of epidermin (33), may provide some clues to the nature of the CNF2-induced modification of Rho proteins.

The mechanism by which CNF2 is delivered to the host cell during an infection remains unclear. Analysis of the deduced amino acid sequence of CNF2 showed the absence of a consensus signal sequence. This result was not surprising since most of the CNF2 activity was found in the cytoplasm of the bacteria after 24-hr shaking culture in tryptic soy broth (TSB) or LB broth (data not shown). However, since laboratory culture conditions do not necessarily reflect the *in vivo* environment of the host during an infection, we cannot rule out the possibility that the toxin is secreted through an inducible excretion machinery in the *in vivo* environment. Alternatively, lysis of some bacteria may release enough toxin to modify the cytoskeleton of the host cell without active secretion of CNF2. Indeed, picomolar concentrations

of CNF1 are sufficient to induce F-actin polymerization (21), and we have also found that the supernatant of a 24-hr TSB broth culture from a CNF2-producing strain contains enough toxin to modify the cytoskeleton of HEp-2 cells (data not shown).

Although there is no report of a multinucleating effect of PMT on cultivated cells, the homology observed between the N-terminal region of CNF2 and the 145-kDa PMT is intriguing for several reasons. First, PMT, which is the primary virulence determinant of strains of *P. multocida* that cause progressive rhinitis in pigs, has no significant homology to any protein except CNF2 and CNF1 (refs. 14, 43, and 44; this report). Second, like CNF2, PMT is a nonsecreted protein without a typical signal sequence (43, 44) that induces dermonecrotic lesions in animals and enters the cytoplasm of target cells via an acidic compartment (36). Third, PMT is a potent mitogen that appears to facilitate the coupling of heterotrimeric GTP-binding protein to phosphatidylinositol-specific phospholipase C which, in turn, stimulates the formation of inositol phosphate (45). This interaction of both PMT and CNF2 with a GTP-binding protein may indicate a link between the modes of action of PMT and CNF. Preliminary results indicate that CNF2, like PMT, can induce DNA synthesis in quiescent cells. Because PMT and CNF2 share several common characteristics, we propose that the CNF toxins and PMT are members of a heretofore unrecognized family of toxins which modify GTP-binding proteins in a novel way. We believe that PMT and CNF, and functionally similar proteins yet to be discovered, will provide unique molecular tools to elucidate cellular and molecular regulatory mechanisms of eukaryotic cells.

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