

Activation of Rho GTPases by Cytotoxic Necrotizing Factor 1 Induces Macropinocytosis and Scavenging Activity in Epithelial Cells

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Macropinocytosis, a ruffling-driven process that allows the capture of large material, is an essential aspect of normal cell function. It can be either constitutive, as in professional phagocytes where it ends with the digestion of captured material, or induced, as in epithelial cells stimulated by growth factors. In this case, the internalized material recycles back to the cell surface. We herein show that activation of Rho GTPases by a bacterial protein toxin, the *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1), allowed epithelial cells to engulf and digest apoptotic cells in a manner similar to that of professional phagocytes. In particular, we have demonstrated that 1) the activation of all Rho, Rac, and Cdc42 by CNF1 was essential for the capture and internalization of apoptotic cells; and 2) such activation allowed the discharge of macropinosomal content into Rab7 and lysosomal associated membrane protein-1 acidic lysosomal vesicles where the ingested particles underwent degradation. Taken together, these findings indicate that CNF1-induced “switching on” of Rho GTPases may induce in epithelial cells a scavenging activity, comparable to that exerted by professional phagocytes. The activation of such activity in epithelial cells may be relevant, in mucosal tissues, in supporting or integrating the scavenging activity of resident macrophages.

INTRODUCTION

Macropinocytosis indicates a ruffling-driven phenomenon that leads to the ingestion of large particles into irregular primary endocytic vesicles called macropinosomes (Swanson and Watts, 1995). The formation of macropinosomes, which is the direct consequence of the closure of lamellipodia generated at ruffling membrane domains, occurs differently in different cell types. Whereas in macrophages or dendritic cells macropinosomes are constitutively formed (Swanson and Watts, 1995), in fibroblasts or epithelial cells their occurrence is dramatically but transiently stimulated by different stimuli, such as phorbol esters (Swanson, 1989) or growth factors (Racoosin and Swanson, 1992). The fate of macropinosomes also varies depending on the cell type, undergoing fusion with lysosomes in macrophages (Racoosin and Swanson, 1993) and recycling back to the cell surface in epidermal growth factor-stimulated A431 cells

(Hewlett *et al.*, 1994). From a functional point of view, macropinocytosis 1) may account for total nutrient supply in strains of the amoeba *Dictyostelium discoideum* (Hacker *et al.*, 1997); 2) may play a key role in antigen presentation by class II or even class I major histocompatibility complexes in dendritic cells (Sallusto *et al.*, 1995); 3) may be used by “professional phagocytes,” such as macrophages, to exert a “scavenging” activity (in addition to phagocytosis) (Swanson and Watts, 1995); and 4) can be exploited by several bacterial pathogens as a means of entry and survival in epithelial cells (Finlay and Cossart, 1997).

Among the different stimuli able to induce macropinocytosis in epithelial cells, we have previously described a protein toxin derived from pathogenic strains of *Escherichia coli* that favors the uptake of large material, such as latex beads or bacteria (Falzano *et al.*, 1993). This toxin, the cytotoxic necrotizing factor type 1 (CNF1) is a 110-kDa monomeric protein that has been shown to permanently activate Rho GTPases (Flatau *et al.*, 1997; Schmidt *et al.*, 1997). These GTPases encompass three groups of proteins (Rho, Rac, and Cdc42) that are differently involved in the actin cytoskeleton

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organization. Rho induces stress fibers assembly and Rac membrane ruffling activity, whereas Cdc42 is involved in filopodia formation (Hall, 1998). Rho, Rac, and Cdc42 are all activated by CNF1 through the deamidation of a pivotal glutamine residue in the switch 2 domain, which is involved in GTP hydrolysis (glutamine 63 in Rho [Flatau *et al.*, 1997; Schmidt *et al.*, 1997] or 61 in Cdc42 and Rac [Lerm *et al.*, 1999b]). Through the activation of Rho GTPases, CNF1 induces a number of actin-dependent phenomena, such as contractility, cell spreading, and the assembly of focal adhesion plaques (Fiorentini *et al.*, 1997; Lacerda *et al.*, 1997) as well as the formation of actin stress fibers and an intense and generalized ruffling activity (Falzano *et al.*, 1993; reviewed in Boquet and Fiorentini, 2000). CNF1-induced membrane ruffling is reminiscent of the ruffling elicited by invasive bacteria (Francis *et al.*, 1993; Hardt *et al.*, 1998) and is consistent with the ability of epithelial cells to exert macropinocytosis.

In this study we investigated 1) the specific role of Rho GTPases in the macropinocytosis induced by CNF1, and 2) the events after the engulfment of apoptotic cells by CNF1-stimulated human epithelial cells. We showed that the engulfment process in CNF1-treated epithelial cells was driven by the activation of Rho, Rac, and Cdc42 and was followed by a proper degradative pathway. This suggested that epithelial cells may be induced to share or compete with professional phagocytes in a crucial pathophysiological activity, such as the removal of apoptotic cells.

MATERIALS AND METHODS

Cell Cultures

HEp-2 cells (human larynx carcinoma cell line) and U937 cells (a monomyelocytic human cell line) were grown as previously described (Falzano *et al.*, 1993; Cossarizza *et al.*, 1995, respectively). Human monocytes-derived macrophages were obtained by plastic adherence from peripheral blood mononuclear cells as reported (Fais *et al.*, 1996). Apoptotic bodies were obtained from U937 as previously described (Cossarizza *et al.*, 1995).

CNF1 and CNF1 Mutant Recombinant Protein

Wild-type and mutant CNF1 were purified as previously described (Falzano *et al.*, 1993). To construct the CNF1 mutant, the *cnf1* gene together with the promoter region of the toxin was amplified by the human uropathogenic *E. coli* strain J96 (O4:K6) (Blum *et al.*, 1995). Plasmid pCR2.1 was used for cloning *cnf1* into *E. coli* INV α F' (TA Cloning kit; Amersham Pharmacia Biotech, Les Ulis, France). The *cnf1*-C866S mutant gene was obtained by site-directed mutagenesis with the use of the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Mutation of cysteine 866 into a serine abolished the catalytic activity of the toxin (Schmidt *et al.*, 1998). *cnf1*-C866S cloned in pCR2.1 plasmid was transformed into the *E. coli* XL1 blue strain from which the mutant protein was purified. The gel shift analysis was performed as previously described (Con-tamin *et al.*, 2000).

Cell Treatments

HEp-2 cells seeded at a concentration of 2×10^4 cells/ml, either in 24-well plate or in 35-mm Petri dishes, were exposed for 6, 18, 24, and 48 h to 10^{-10} M CNF1 and 4×10^{-8} M mutant CNF1. The mutant CNF1 used was 400 times more concentrated than the wild-type toxin, to obtain a complete competition for the binding step. Apoptotic cells (5×10^4) were next added to untreated, CNF1-, and CNF1 mutant-treated cells as well as to 7-d culture monocyte-

derived macrophages, for different time lengths (1, 5, 10, 20, 30 min, 3 h, and overnight). At the end of each treatment, cells were processed for light or electron microscopy. For inhibition of protein synthesis, HEp-2 cells were preincubated for 30 min with 50 μ g/ml cycloheximide (CHX) before exposure to CNF1. After 48 h of incubation, apoptotic cells were added as described above. All the experiments were performed at least four times with triplicate samples for each point. In each experiment at least 500 cells (randomly chosen) were counted.

Assay of Macropinocytosis

To define how bound but not ingested cells are distinguished from ingested cells we have determined the plane at which the apoptotic cell is laying with respect to the plane of epithelial cell nuclei. In fact, by varying the focal plane, it is possible to define which apoptotic cell lies at the same plane of the epithelial cell nucleus. That apoptotic cell was considered as internalized.

Transfection of HEp-2 Cells

Control HEp-2 cells were transfected either with 1 μ g/well of plasmid DNA encoding myc-tagged dominant positive forms of either Rho, Rac, or Cdc42 GTPases (RhoV14, RacV12, and Cdc42V12) or with 2.5 μ g/35-mm Petri dish of plasmid encoding RhoV14-GFP. HEp-2 cells treated with CNF1 for 24 h were transfected with 1 μ g of the negative forms of either Rho, Rac, or Cdc42 GTPases (RhoN19, RacN17, and Cdc42N17). Both control and CNF1-treated HEp-2 cells were transfected with 1 μ g of plasmids encoding Rab5-GFP and Rab7-GFP. DOTAP Liposomal Transfection Reagent kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to transfect cells according to the manufacturer's recommendations. Twenty-four hours after cell transfection, 5×10^4 apoptotic bodies were added to monolayers and, after different time points (1, 5, 10, 20, 30 min, 3 h, and overnight), cells were either fixed in paraformaldehyde and processed for fluorescence microscopy or observed with a phase contrast inverted microscope and monitored by microcinematography, as described below.

Rho-inhibiting Bacterial Protein Toxins

Clostridium difficile toxin B (CdB) and *Clostridium sordellii* lethal toxin (LT) were generously provided by M.R. Popoff (Paris, France). The chimeric toxin C3B, prepared as previously described (Aullo *et al.*, 1993), was a kind gift from P. Boquet (Nice, France). Epithelial cells were exposed for 3 h to CdB (0.5 μ g/ml) or LT (1 μ g/ml) or C3B (1 μ g/ml) before addition of CNF1. After further 48 h of incubation with CNF1, cells were overnight challenged with apoptotic cells, fixed, and processed for fluorescence microscopy as below described. All the experiments were performed at least four times with triplicate samples for each point.

Immunocytochemistry

HEp-2 cells were seeded on glass chamber slides (Nunc, Naperville, IL) at the concentration of 2×10^4 cells/ml. After treatment with CNF1 and the subsequent incubation with apoptotic bodies, chamber slides were fixed with ethanol (70%) or methanol (70%) for 5 min at 4°C for lysosomal associated membrane protein-1 (Lamp-1) detection. Cells were then stained by immunocytochemistry with the use of Dako EnVision System (Dako, Denmark) horseradish peroxidase, with the use of the peroxidase-antiperoxidase method. Rabbit polyclonal antibodies recognizing the cytosolic part of Lamp-1 were kindly provided by S. Méresse (Marseille, France). Terminal deoxynucleotidyl transferase dUTP nick-end labeling reaction (In Situ Cell Death Detection kit; Roche Molecular Biochemicals) (Negoescu *et al.*, 1996) was performed with the use of the peroxidase-antiperoxidase method or alkaline phosphatase antialkaline phosphatase (Dako) method, in single and double staining, as appropriate (Fais *et al.*, 1995).

Fluorescence Microscopy

Control and treated HEp-2 cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 10 min at room temperature and then permeabilized with 0.5% Triton X-100 in PBS (pH 7.4). Cells were stained with 1) the nuclear dye Hoechst 33258 (Sigma, St. Louis, MO) alone (for Rab-GFP transfected cells and for nontransfected cells); 2) Hoechst and anti-c-myc monoclonal antibody 9E10.3 (Chemicon International, Temecula, CA) (for cells transfected with dominant positive or negative forms of the Rho GTPases); 3) Hoechst and anti-early endosome antigen 1 (EEA1) goat polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). After 30 min at 37°C, cells were washed and in the cases 1) and 3) incubated with a fluorescein isothiocyanate-conjugated anti-mouse and anti-goat antibodies, respectively. Coverslips were mounted in glycerol/PBS (2:1) and analyzed with a Nikon Microphot fluorescence microscope. For acidic vesicle detection, both untreated and CNF1-treated living cells were incubated at 37°C for 10 min with 2.5 µg/ml acridine orange (Sigma). Finally, after washings, coverslips were mounted and analyzed as described above.

Video Microscopy

Time-lapse cinematography was obtained with the use of a phase contrast Nikon inverted microscope equipped with a Zeiss charge-coupled device camera and a JVC time lapse videotape recorder. Living HEp-2 cells treated for 48 h with CNF1 10^{-10} M or transfected with RhoV14-GFP, and then challenged with apoptotic cells, have been studied. Films were recorded under standard condition (5% CO₂ humid atmosphere at 37°C). The ingestion of apoptotic bodies by epithelial cells was clearly detected by decreasing tape speed at least 360 times (to obtain a quick-time movie after a 3-h recording).

Scanning Electron Microscopy

Control and treated cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min. After postfixation in 1% OsO₄ for 30 min, cells were dehydrated through graded ethanols, critical point dried in CO₂, and gold coated by sputtering. The samples were examined with a Cambridge 360 scanning electron microscope.

Transmission Electron Microscopy (TEM)

Cells grown in monolayer were fixed with 2.5% glutaraldehyde in buffer 0.2 M cacodylate (pH 7.4) for 30 min, washed, and postfixated for 1 h with 1% OsO₄ in the same buffer at 4°C. They were dehydrated in an alcohol gradient and embedded in epoxy resin (Agar 100 resin; Agar Scientific, Stansted, United Kingdom) by routine procedures. Ultrathin sections, obtained with an LKB Ultratome Nova, were stained with uranyl acetate and lead citrate and examined with a Philips 208 transmission electron microscope.

Statistical Analysis

The values in Table 1 and Figures 1 and 3–6 are the means ± SDs from four separate experiments. Student's *t* test for correlated samples was used. A *p* value of <0.01 was considered significant. Correlation has been evaluated by with the use of Statistics program for Macintosh by a specific paired correlation test.

RESULTS

CNF1 Induces Macropinocytosis of Apoptotic Cells by Human Epithelial Cells

We first investigated the ability of CNF1-activated epithelial HEp-2 cells to engulf U937 cells triggered to apoptosis. This

Table 1. Percentage of HEp-2 cells treated with CNF1 (for 48 h) effecting macropinocytosis: dependence on the time of exposure to apoptotic bodies

Time of exposure to apoptotic bodies (min)	Percentage of cells containing apoptotic bodies
15	2 ± 0.4
30	7 ± 0.8
60	15 ± 1.5
90	19 ± 2.0
120	25 ± 1.0
180	45 ± 1.4
Overnight	47 ± 1.6

The long incubation time with apoptotic bodies did not affect HEp-2 cells viability as viewed by trypan blue exclusion test (data not shown).

approach was chosen as representative of a specific cell scavenging activity. As observed by scanning electron microscopy, apoptotic cells did just adhere on the surface of unstimulated epithelial cells without being internalized (Figure 1a). In contrast, CNF1-activated epithelial cells first contacted the apoptotic cells via extension of filopodia and membrane ruffles (Figure 1b), which surrounded and subsequently wrapped around (Figure 1c) the apoptotic cells. These events led to the engulfment of apoptotic cells into the epithelial cells (Figure 1d). In cells exposed to CNF1, time course experiments showed that the percentage of cells containing apoptotic bodies increased with the length of exposure to the toxin, reaching a maximum at 48 h (Figure 1e). The internalization of apoptotic bodies did occur at different extents also depending on the time of their incubation with epithelial cells (Table 1). Moreover, while the percentage of internalized apoptotic bodies increased with the time of CNF1 treatment, apoptotic cells merely adhering to the epithelial cell surface progressively decreased (Figure 1f).

Macropinocytosis of apoptotic cells by CNF1-stimulated epithelial cells was also monitored by time-lapse video microscopy. A selected field describing the dynamics of such phenomenon is shown (Figure 2, a–d). The arrows point an apoptotic cell that was progressively internalized by a CNF1-stimulated epithelial cell. CNF1-activated epithelial cells were able to engulf apoptotic cells independently from the actual apoptotic state of the prey (because both early or late apoptotic cells were engulfed, as detected by annexin V staining) but were unable to ingest live cells (our unpublished results).

On the other hand, the long incubation time necessary for CNF1 to increase the apoptotic cell uptake (Figure 1e) suggests the occurrence of transcriptional changes. We have therefore carried out experiments with the use of the protein synthesis inhibitor CHX to verify whether the ex novo protein synthesis was required for the engulfment of apoptotic bodies. The results obtained, reported in Figure 3, showed that CHX was able to block the CNF1-induced macropinocytosis of apoptotic bodies, suggesting that cells needed to synthesize new proteins to exploit such function.

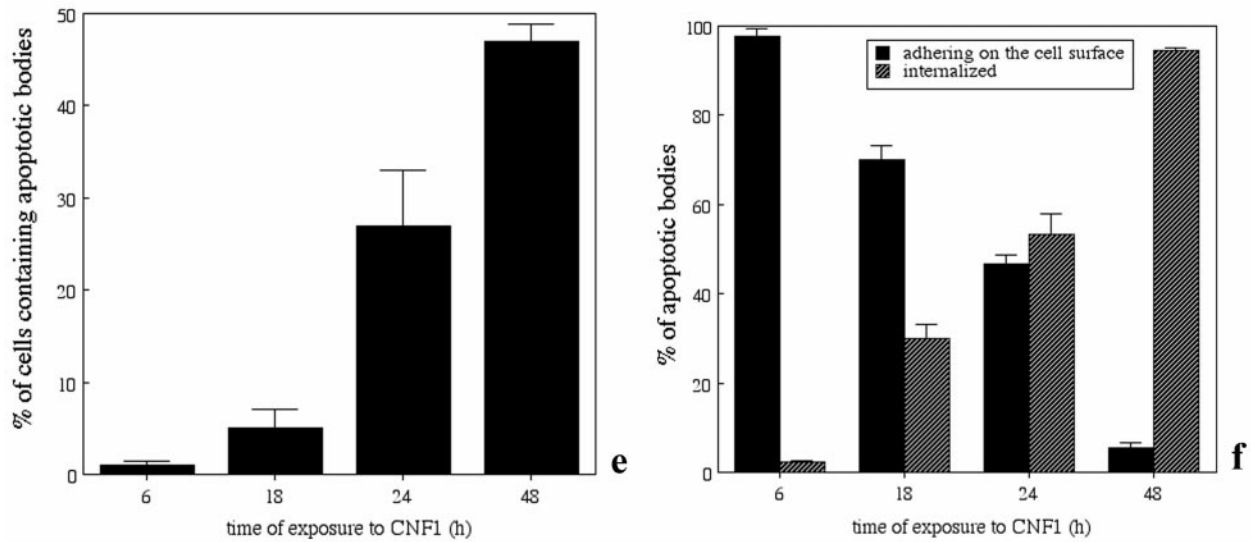
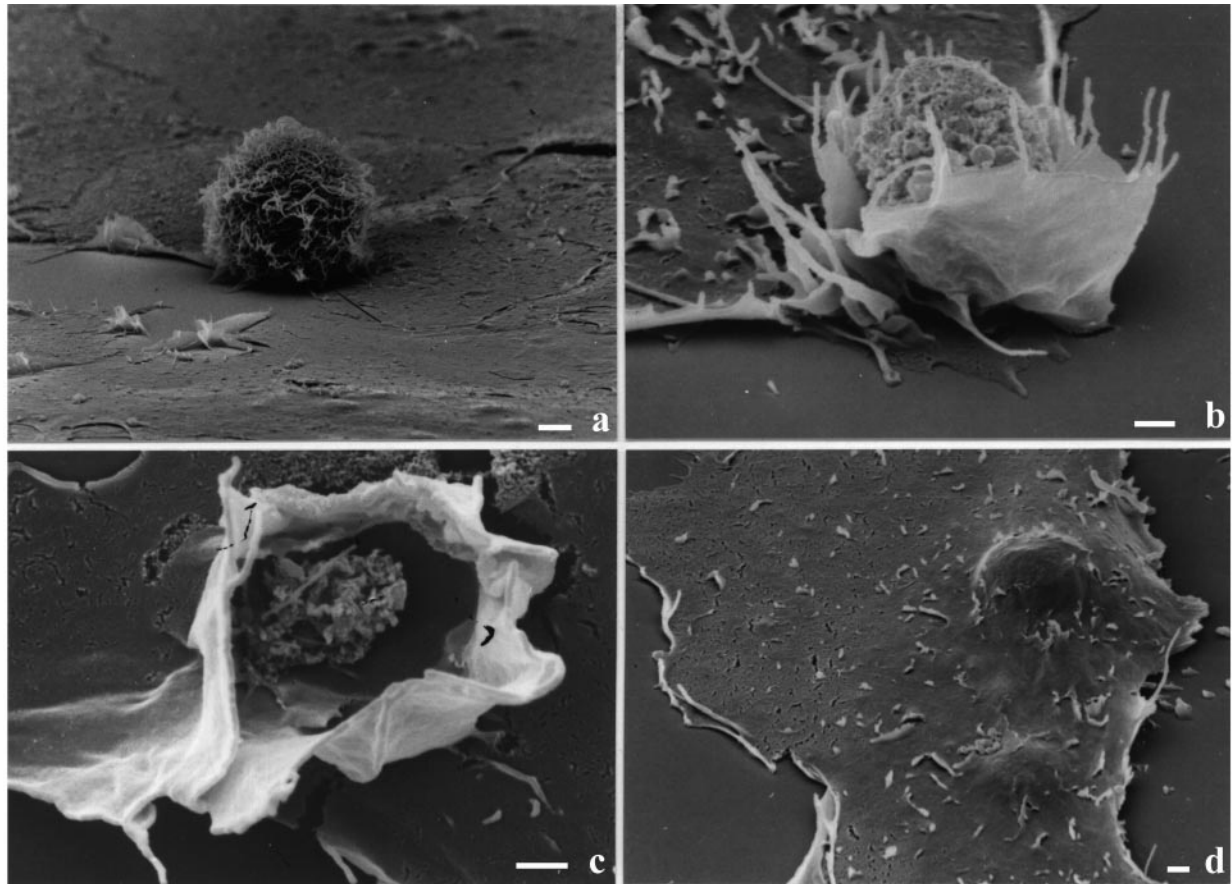


Figure 1. Macropinocytosis of apoptotic bodies induced by CNF1 in epithelial cells. Scanning electron microscopy analysis of unstimulated epithelial cells (a) and cells exposed to 10^{-10} M CNF1 for 48 h (b–d). After overnight interaction with epithelial cells apoptotic bodies are contacted by filopodia and small ruffles (b), surrounded and enveloped (c) by larger ruffles, and internalized into the cytoplasm (d). (e) Percentage of cells containing apoptotic bodies increases by time of treatment with 10^{-10} M CNF1. (f) Percentage of apoptotic cells present on the epithelial cell surface decreases over time, whereas the number of internalized apoptotic bodies concomitantly increases. The results, reported as percentages (\pm SDs), are from four different experiments in each of which at least 500 cells (randomly chosen) were counted. Bar in a–d, 1 μ m.

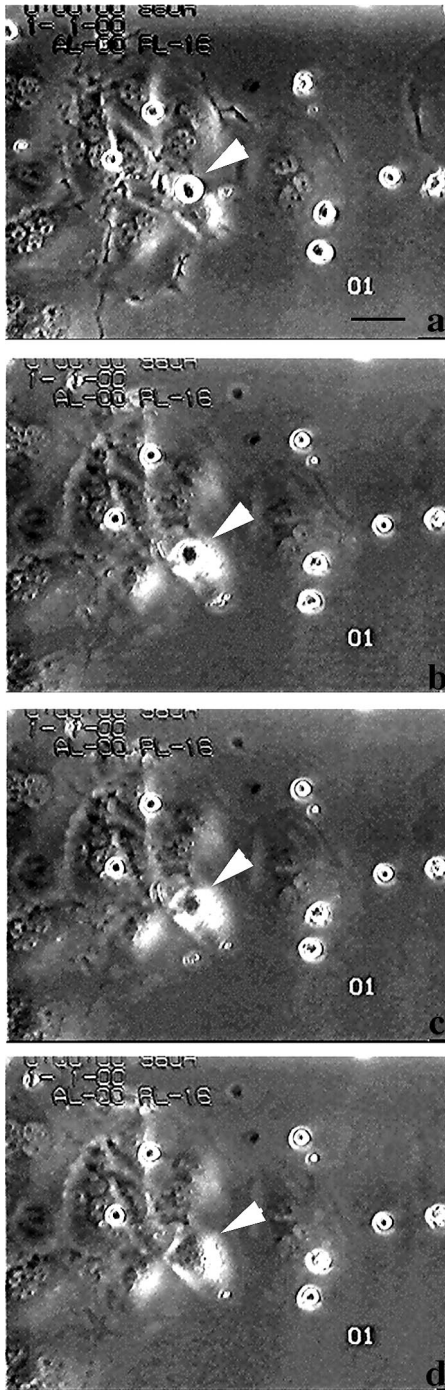


Figure 2. Microcinematography on apoptotic cell internalization by CNF1-treated epithelial cells. Cells were exposed to 10^{-10} M CNF1 for 48 h and then challenged with 5×10^4 apoptotic bodies. Such interaction was followed for 3 h and monitored by microcinematography. A selected field showing the dynamic of the capture and internalization processes was chosen (a–d). The selected micrographs were taken 0 min (a), 10 min (b), 20 min (c), and 1 h (d) after the addition of apoptotic cells to the culture medium. Arrowheads indicate an apoptotic cell that is progressively internalized by one epithelial cell. Bar, 10 μ m.

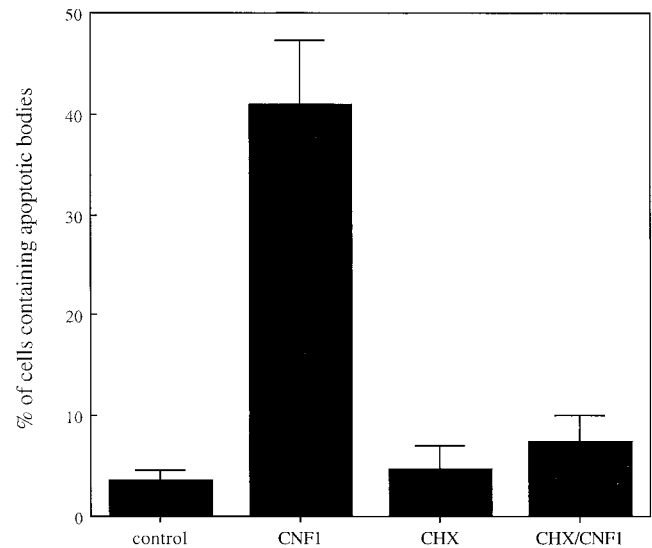


Figure 3. Graph showing the percentage of epithelial cells containing apoptotic bodies. Preexposure with 50 μ g/ml the protein synthesis inhibitor CHX clearly reduced the induction of the macropinocytotic activity of CNF1-treated HEP-2 cells. The results, reported as percentages (\pm SDs), are from four different experiments in each of which at least 500 cells (randomly chosen) were counted.

Rho GTPases Are Required for Macropinocytotic Activity Induced by CNF1 in Epithelial Cells

To analyze whether the enzymatic activity of CNF1 was necessary for the development of the toxin-induced macropinocytosis, a nontoxic mutant of CNF1 in which the catalytic cysteine residue (cys 866) (Figure 4a) was converted to serine was constructed. As already reported (Schmidt *et al.*, 1998), the CNF1 C866S completely lacks its enzymatic activity as demonstrated by the inability to upshift the apparent molecular weight of Rho (Figure 4b). Exposure to CNF1 C866S alone for 48 h rendered HEP-2 cells unable to capture apoptotic cells (Figure 4c). The simultaneous addition of the mutant together with the wild-type CNF1 led to the inhibition of the CNF1-induced macropinocytotic activity (Figure 4c), indicating a competition at the level of receptor binding (Contamin *et al.*, 2000). Together, these results demonstrate the need of the fully enzymatic activity of CNF1 to exert macropinocytosis.

We further investigated whether the CNF1-dependent macropinocytotic activity in epithelial cells was triggered by activated Rho GTPases by 1) blocking CNF1 activity with transfection of cells with the dominant negative forms of Rho GTPases or with the use of "classical" bacterial protein toxins as inhibitors of Rho proteins; and 2) activating epithelial cells with dominant positive forms of the Rho GTPases. CNF1-treated epithelial cells transfected with the dominant negative forms of Rac (RacN17) or Cdc42 (Cdc42N17) showed a reduced ability to ingest apoptotic bodies (49 ± 3 and $57 \pm 8\%$, respectively) with respect to CNF1-treated cells transfected with the control plasmid (pcDNA) (Figure 5c). Although being low the percentage of cells transfected with the dominant negative Rho N19 (our unpublished results), the results were consistent with those

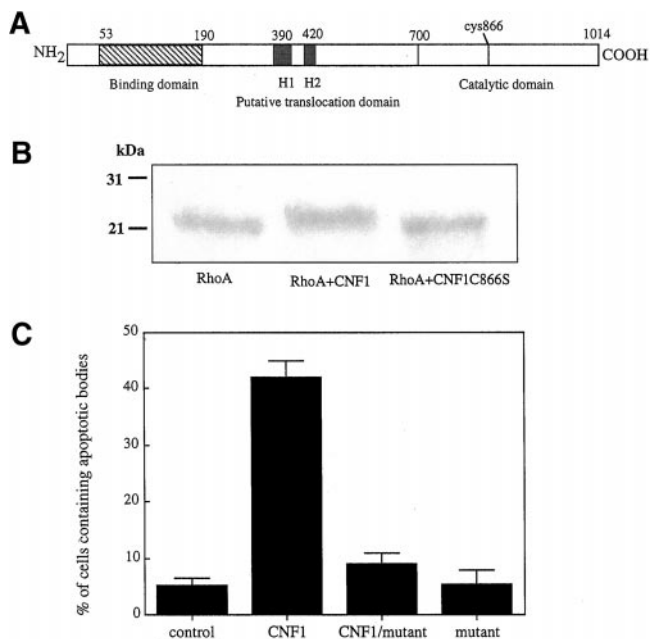


Figure 4. Rho deamidation is essential for macropinocytosis in epithelial cells. (a) Schematic representation of the CNF1 molecule. The position of the catalytic amino acid cys866, which has been changed into serine in the mutant CNF1, is evidenced. (b) Coomassie Brilliant Blue-stained gel of control recombinant Rho protein, Rho protein exposed to wild-type CNF1, or mutant CNF1 (CNF1 C866S). The molecular ratio of CNF1 to RhoA was 2:1. Note that the mutant CNF1 has lost the ability to cause the Rho upward shift typical of CNF1. (c) Graph showing the percentage of epithelial cells containing apoptotic bodies after 48 h of treatment with CNF1, CNF1 mutant, and CNF1 plus CNF1 mutant. The results, reported as percentages (\pm SDs) of epithelial cells containing apoptotic bodies after overnight exposure, are from four different experiments in each of which at least 500 cells (randomly chosen) were counted.

obtained after treatment with exoenzyme C3 (see below). We then used the after Rho inhibitors: 1) *Clostridium botulinum* exoenzyme C3, a selective inhibitor of Rho (Chardin *et al.*, 1989); 2) CdB, which inactivates Rho, Rac, and Cdc42 (Just *et al.*, 1995); and 3) LT, which inhibits Ras, Rap, and Rac but not Rho or Cdc42 (Popoff *et al.*, 1996). Besides blocking the typical CNF1-induced morphological changes, CdB and LT also impaired the engulfment of apoptotic bodies (Figure 5d). On the other hand, although unable to prevent the CNF1-induced phenotype (Fiorentini *et al.*, 1995), C3 also significantly decreased macropinocytosis of apoptotic bodies (Figure 5d). Consistently, when control epithelial cells were transfected with each of the dominant active form of RhoA, Rac, and Cdc42, a slight but significant ($p < 0.01$) increase in the phagocytic activity was observed compared with cells transfected with the control plasmid (Figure 6g). It is evident that DNA transfection per se induced a very modest uptake of apoptotic cells, which is nearly doubled in cells transfected with the GTPases (Figure 6g). Although the role of Rac as inducer of macropinocytosis is well defined (Dharmawardhane *et al.*, 2000; Nobes and Marsh, 2000), the finding that Rho may promote such process is still poorly investigated. Therefore, to further explore this last point, we

have used time-lapse video microscopy to follow the internalization of an apoptotic cell by epithelial cells transfected with the dominant active form of the Rho-GTPase (RhoV14-GFP). The results obtained showed that, similarly to what occurs in CNF1-treated epithelial cells, Rho activation induced the ingestion of apoptotic cells. Figure 6, a–f, clearly shows the progressive engulfment of an apoptotic cell by an adhering cell with spike-like protrusions. Together, these findings suggest that a coordinate activity of the Rho GTPases is required for mimicking the macropinocytotic activity triggered by CNF1

Macropinosomes in CNF1-activated Human Epithelial Cells May Discharge Their Contents into Lysosomal Degradative Compartments

We next followed the route undertaken by the captured apoptotic bodies in CNF1-activated epithelial cells. In particular, we studied the association between apoptotic body-containing vacuoles and the small GTPases Rab5 and Rab7, which define early and late endosomes, respectively (Bucci *et al.*, 1992; Vitelli *et al.*, 1997), and the lysosomal transmembrane protein Lamp-1 (de Saint-Vis *et al.*, 1998). Macropinosomes in CNF1-activated epithelial cells that contained the apoptotic bodies were always found negative for early endosomal markers such as Rab5 (Figure 7, a and b) or EEA1 (our unpublished results). In contrast, similarly to what occurs in late phagosomes of macrophages, apoptotic bodies were observed inside macropinosomal membranes expressing Rab7 (Figure 7, c and d). This finding strongly suggested that macropinosomes in human epithelial cells stimulated by CNF1 may undergo fusion with lysosomal-like structures. To explore this hypothesis, epithelial cells were stained with an antibody that recognizes the cytosolic component of Lamp-1, a transmembrane protein specifically associated with lysosomes (de Saint-Vis *et al.*, 1998). As shown in Figure 8, apoptotic bodies captured by CNF1-activated cells could be observed in vacuoles stained by the Lamp-1 antibody, a finding that suggests the occurrence of a lytic enzyme activity inside such vesicles. In CNF1-treated epithelial cells, the vesicles positive for Rab7 and Lamp-1 appeared very large irrespective of the presence inside of apoptotic bodies (Figures 7, c and d, and 8). This suggests that CNF1 is capable of affecting vesicle trafficking in accordance with the nature of macropinocytosis that is characterized by the continuous formation of ruffling-driven macropinosomes.

We therefore used TEM analysis to compare the degradative process afforded by CNF1-activated epithelial cells to that of classical primary human macrophages. Figure 9 clearly shows that the degradation of apoptotic bodies occurred progressively in a similar manner in macrophages (Figures 9, a–c) and CNF1-activated epithelial cells (Figure 9, d–f). Experiments aimed at evaluating acidification of vacuoles containing apoptotic bodies showed that such vacuoles (Figure 9g; phase contrast) assumed a strong orange-red staining with acridine orange (Figure 9h). Acridine orange is a metachromatic dye that has been used extensively to assess phagosome-lysosome fusion. The red staining inside vacuoles indicates an acidic pH and, as a consequence, acidic digestion of apoptotic bodies, whereas the apoptotic corps outside vacuoles were yellow-green. This

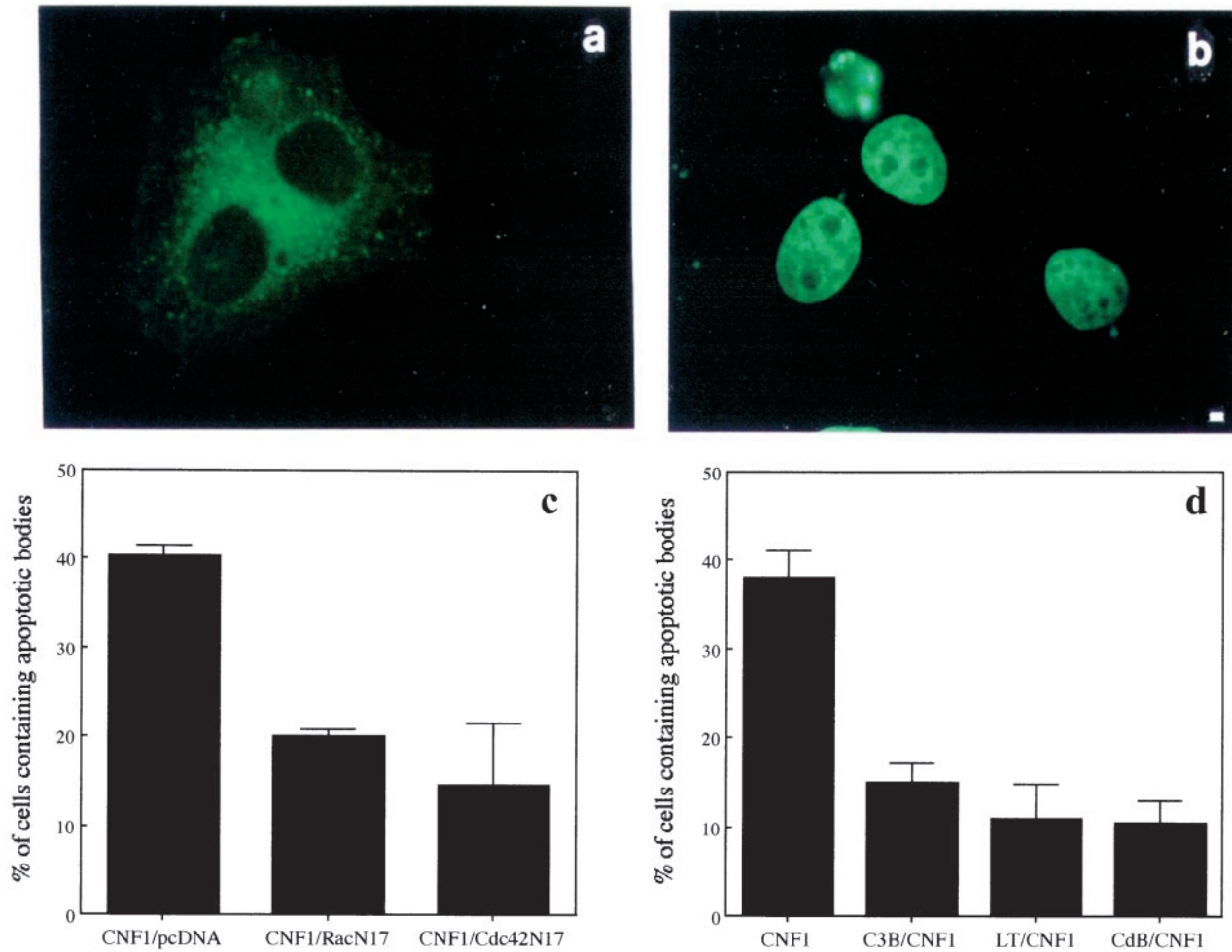


Figure 5. Rho GTPases drive macropinocytosis in epithelial cells. (a and b) As an example of a transfected cell with an apoptotic body inside we show the fluorescence micrograph of an epithelial cell exposed to 10^{-10} M CNF1 for 24 h and then transfected with $1 \mu\text{g}$ of RacN17 encoding plasmid. Apoptotic bodies were added for 3 h before fixation. Hoechst staining (b) clearly evidences the presence of an apoptotic body in a transfected cell (a). (c) Graph showing the percentage of CNF1-treated epithelial cells able to macropinocytose apoptotic bodies once transfected with dominant negative forms of the Rho GTPases. (d) Graph showing the inhibition of CNF1-induced macropinocytotic activity by exposure to the Rho-inhibiting bacterial toxins C3B ($1 \mu\text{g}/\text{ml}$), LT ($1 \mu\text{g}/\text{ml}$), and CdB ($0.5 \mu\text{g}/\text{ml}$) for 3 h. The results reported as percentages (\pm SDs) of epithelial cells containing apoptotic bodies are from four different experiments in each of which at least 100 cells were counted. Bar in a and b, $1 \mu\text{m}$.

last set of observations strongly supports our finding that, in CNF1-activated epithelial cells, the degradation of apoptotic bodies occurs in macropinosomes fused with lysosomal-like structures.

DISCUSSION

We herein show that CNF1 enables epithelial cells to perform a scavenging activity toward apoptotic cells via a mechanism that combines macropinocytosis and degradation and therefore is novel for epithelial cells. Macropinocytosis triggered by CNF1 was clearly dependent on the activation of regulatory proteins of the Rho family and started with the promotion of an intense membrane ruffling. Such

Rho-independent membrane activity drove the capture of apoptotic cells into macropinosomes lacking the early endocytic compartment marker Rab5. Such macropinosomes in CNF1-activated epithelial cells may then discharge their contents into Rab7 and Lamp-1 acidic compartments where the degradation of the ingested particles occurs.

Macropinocytosis by CNF1 in epithelial cells was a de novo-induced phenomenon that required synthesis of new proteins. Moreover, to promote macropinocytosis, CNF1 had to be enzymatically active because a mutation in its catalytic site blocked such ability. This suggests that Rho proteins, the targets of CNF1, may be pivotal in driving the macropinocytotic activity. In fact, epithelial cells exposed to CNF1 and then transfected with dominant negative forms of

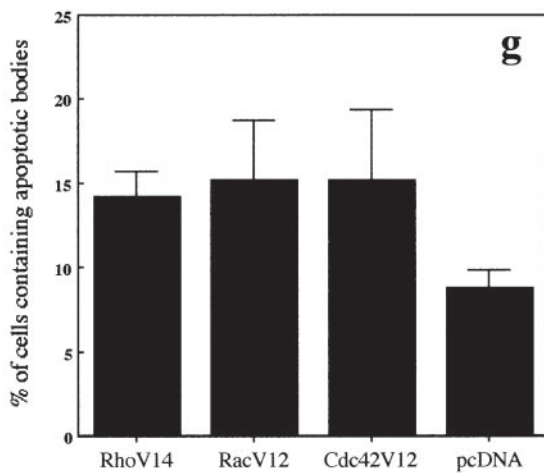
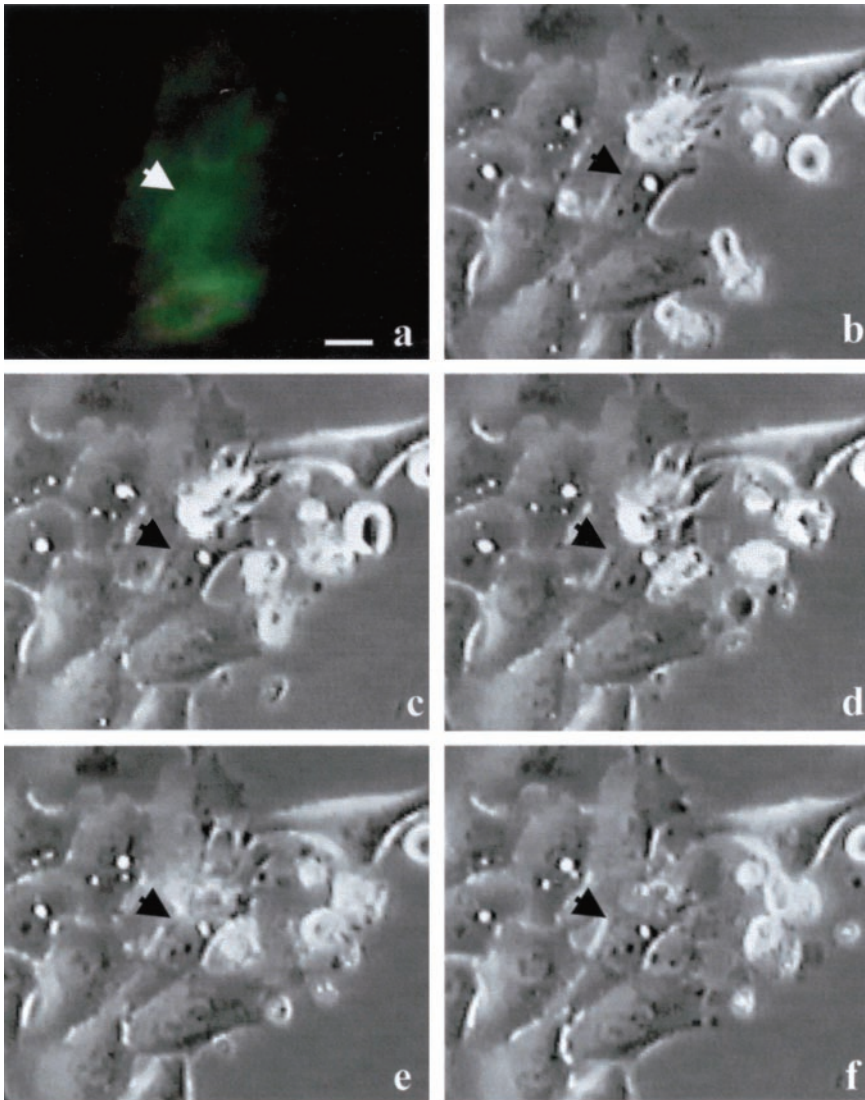


Figure 6. (a–f) Macropinocytosis of apoptotic bodies in HEP-2 cells transfected with RhoV14-GFP. Cells were transfected with 2.5 μg of RhoV14-GFP-encoding plasmid per Petri dish and then challenged with 5×10^4 apoptotic cells. The interaction between a HEP-2 transfected cell (a) and an apoptotic cell was monitored by microcinematography. A selected field showing the dynamics of the capture and internalization processes was chosen (b–f). The selected micrographs were taken 10 min (b), 20 min (c), 1 h (d), 2 h (e), and 3 h (f) after the addition of apoptotic cells to the culture medium. Arrowheads indicate a transfected epithelial cell, which contacts and progressively internalizes an apoptotic cell. (g) Graph showing the percentage of control HEP-2 cells able to macropinocytose apoptotic bodies once transfected with 1 μg of dominant positive forms of Rho GTPase (RhoV14-RacV12-Cdc42V12 myc-tagged). In these cells a slight but significant increase in the macropinocytotic activity is evident with respect to cells transfected with the control plasmid (pcDNA). The results, reported as percentages (\pm SDs) of transfected epithelial cells containing apoptotic bodies, are from four different experiments in each of which at least 100 transfected cells were counted. Bar, 10 μm .

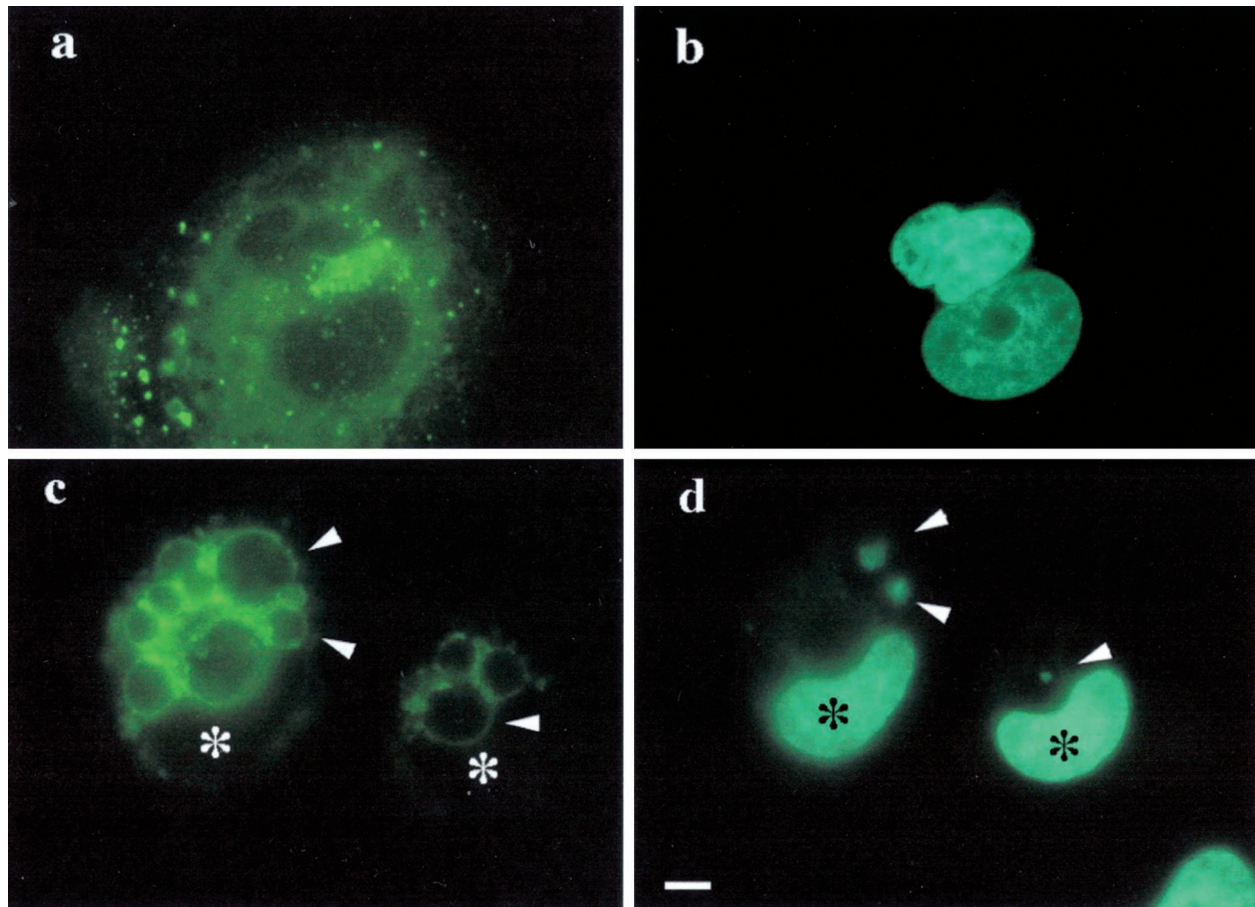


Figure 7. Intracellular trafficking of apoptotic bodies in Rho-activated epithelial cells. (a and c) Epithelial cells exposed to 10^{-10} M CNF1 for 24 h and then transfected with 1 μ g of Rab5-GFP- (a) or Rab7-GFP (c)-encoding plasmid. (b and d) Hoechst staining of the same fields as in a and c, respectively. Apoptotic bodies are constantly absent in Rab5-positive vesicles, independently from the time of interaction with epithelial cells. Apoptotic bodies evidenced by Hoechst staining are observable inside some Rab7-positive vesicles (arrowheads) (d), indicating a passage through such endosomal compartment. The results obtained with Rab5 and Rab7 are each from four separate experiments in each of which at least 100 cells were counted. Approximately 8% of Rab7-positive vesicles was found to contain apoptotic bodies. Asterisks indicate the position of nuclei in c and d. Bar in a–d, 1 μ m.

Rho GTP-binding proteins or treated with toxins inhibiting these GTPases, demonstrated a decrease in macropinocytotic activity. Conversely, transfection with each dominant positive Rho GTPase increased the percentage of epithelial cells able to capture apoptotic cells, although such ability was significantly lower than that observed with treatment of cells with CNF1. Thus, to be fully exploited, the CNF1-induced macropinocytotic activity needs the activation of Rho, Rac, and Cdc42 GTPases, being each of them probably involved in different phases of the process. Rho GTPases have been reported to be pivotal for induction of phagocytosis (Caron and Hall, 1998; Massol *et al.*, 1998) or macropinocytosis (Ridley *et al.*, 1992), but not always all three groups of proteins have been involved in such endocytic phenomena. In fact, it has been shown that the mechanism triggered by *Shigella* to invade epithelial host cells relies on the activation of Rho, Rac, and Cdc42 (Mounier *et al.*, 1999), whereas the mechanism induced by *Salmonella* requires the selective activation of Cdc42 and Rac but not Rho (Hardt *et al.*, 1998).

As regards phagocytosis, it has been reported that ingestion of particles bound by the Fc γ receptor on macrophages apparently requires Rac and Cdc42 activation (Caron and Hall, 1998), whereas phagocytosis of particles bound to the CR3 receptor involves Rho GTPase activation only (Caron and Hall, 1998). Note, the exoenzyme C3, although unable to prevent the morphological changes induced by CNF1 (Fiorentini *et al.*, 1995), was found to block the macropinocytotic activity. This might be due to the ability of C3 to impair the tyrosine phosphorylation induced by CNF1 (Lacerda *et al.*, 1997). Tyrosine phosphorylation is a critical step in the induction of phagocytosis by macrophages and, in fact, different bacterial pathogens exploit such signaling pathway to override phagocytosis and exert their pathogenicity (Goosney *et al.*, 1999). Together, these findings suggest that Rho, Rac, and Cdc42 may act in a coordinate manner in CNF1-treated epithelial cells to organize actin filaments, to build a functional membrane ruffle, and to allow macropinocytosis.

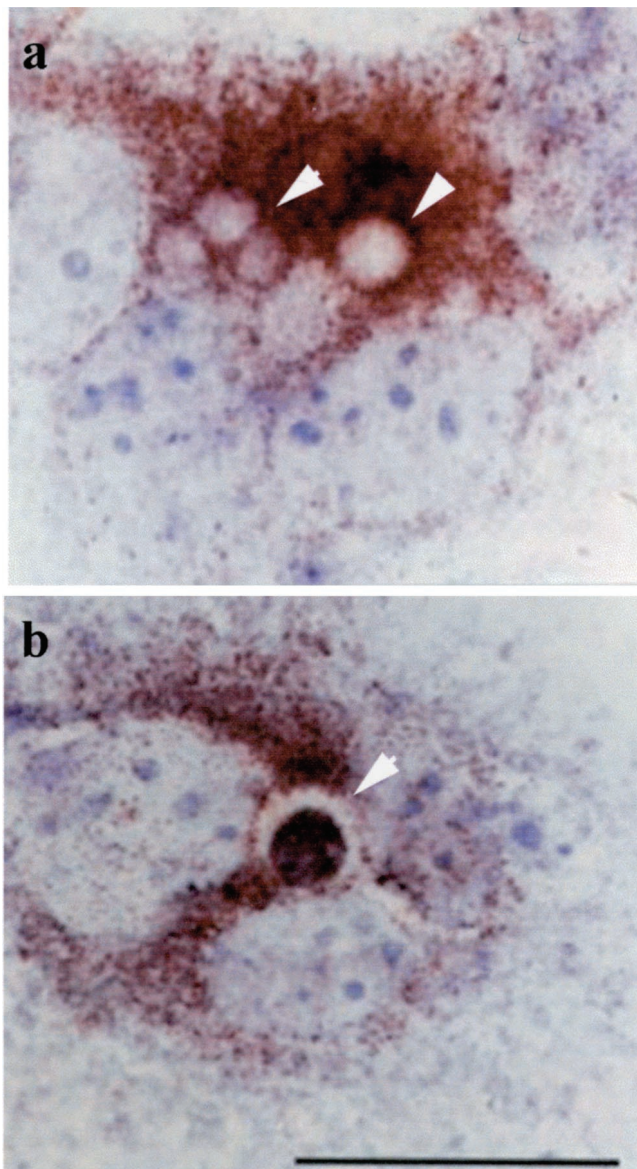


Figure 8. Intracellular distribution of Lamp-1 in CNF1-treated epithelial cells. Immunocytochemistry analysis of epithelial cells exposed to 10^{-10} M CNF1 for 48 h (a and b). Arrowheads in a and b indicate vesicles stained with Lamp-1. In b is well evident an apoptotic body (stained with the terminal deoxynucleotidyl transferase dUTP nick-end labeling reaction) inside a Lamp-1-positive vesicle. The results obtained with Lamp-1 are from four separate experiments in each of which 100 cells were counted. Approximately 10% of Lamp-1-positive vesicles was found to contain apoptotic bodies. Bar, 10 μ m.

In CNF1-activated epithelial cells, apoptotic bodies were always found in compartments negative for early endosomal markers such as Rab5 or EEA1. Rab5, a small GTPase that allows the mixing of endosome or early phagosome contents (Bucci *et al.*, 1992), and EEA1, an early endosome-associated antigen recently shown to be a Rab5 effector (Millis *et al.*, 1999), both function as regula-

tors of early endosome homotypic fusions. Thus, particles taken up by CNF1-activated cells are most likely engulfed by vesicles formed by the membrane ruffling activity as described when cells are transfected with the dominant positive form of Rac (Ridley *et al.*, 1992). Macropinocytosis stimulated by Rac-dependent ruffling in epithelial cells (as that induced by EGF [Hewlett *et al.*, 1994]), allows the capture and internalization of the material that is, however, rapidly recycled back to the cell surface (Racoosin and Swanson, 1993; Hewlett *et al.*, 1994), with a process called regurgitation (Veithen *et al.*, 1998). In the case of CNF1-treated epithelial cells (i.e., Rho, Rac, and Cdc42 contemporary stimulation), macropinosomes may undergo fusion with Rab7 and Lamp-1 positive vesicles (acidic degradative vesicles) where apoptotic cells are progressively degraded. Recent evidence suggests that the Rho GTPase family of signaling proteins is also implicated in the control of the endocytic traffic (Ellis and Mellor, 2000). For instance, RhoD has been reported to regulate early endosome dynamics and distribution by directing the rate of vesicular traffic along cytoskeletal tracks (Murphy *et al.*, 1996). RhoD, however, does not represent a target for CNF1. In fact, CNF1 specifically recognizes a short segment of the Rho, Rac, and Cdc42 switch 2 domain (Lerm *et al.*, 1999a; Flatau *et al.*, 2000) that is present but not strictly localized in the switch 2 domain in RhoD. One attractive alternative hypothesis to explain the fusion of macropinosomes with lysosomes in CNF1-treated cells is that RhoB (a substrate of CNF1), which localizes on the late endosomal/lysosomal compartment (Adamson *et al.*, 1992), may upon activation by CNF1 facilitate this fusion. Indeed, RhoB, through activation of its downstream effectors (Ellis and Mellor, 2000), has been implicated in the control of vesicles fusions (Ellis and Mellor, 2000). As shown above, apoptotic bodies engulfed by CNF1-activated cells reached both Rab7-positive and Lamp-1-positive acidic compartments. In addition, apoptotic bodies within these compartments exhibited classical features of progressive degradation identical to those observed in phagolysosomes. This observation strongly suggests that CNF1-activated epithelial cells behave as bona fide phagocytes, particularly in the last part of the scavenging process.

In conclusion, we have demonstrated that after CNF1 activation human mucosal epithelial cells may share the job of macrophages, in developing the ability to remove apoptotic cells by a novel mechanism driven by the activation of Rho GTPases. We can therefore hypothesize that such activity may be normally activated in mucosal epithelial cells in supporting or integrating the scavenging activity of resident macrophages during bacterial overgrowth. The possibility that Rho-activated epithelial cells might also have a role in processing and presenting self and foreign antigens to the mucosal immune system is currently under investigation.

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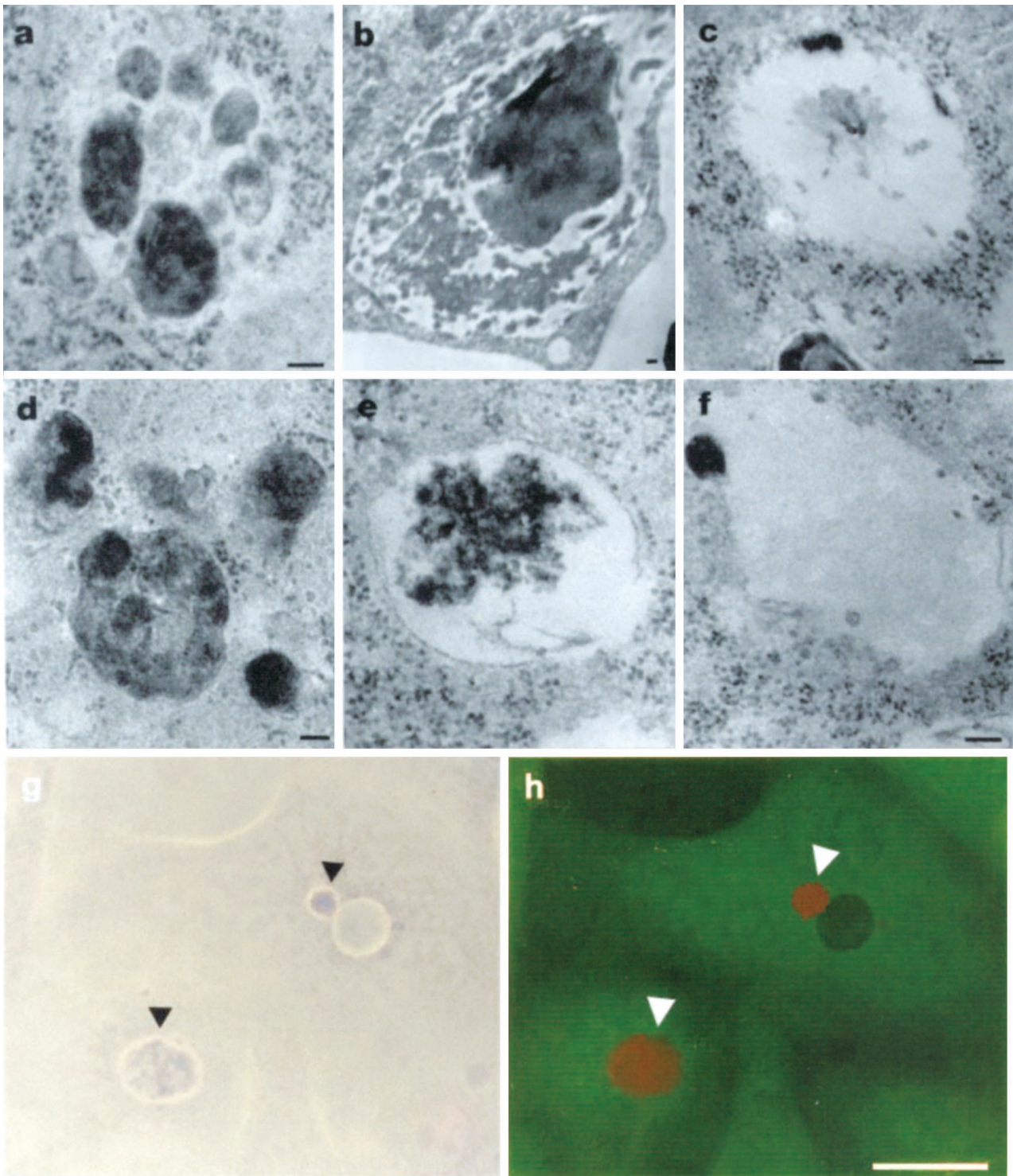


Figure 9. Phagocytotic activity in CNF1-treated epithelial cells. (a–f) TEM micrographs showing the engulfment and degradation of apoptotic cells in macrophages (a–c) and epithelial cells (d–f) treated with 10^{-10} M CNF1 for 48 h. In macrophages (a) and epithelial cells (d), the chromatinic structures of apoptotic cells are clearly visible inside the intracytoplasmic vacuoles. (b and e) Apoptotic bodies appear partially degraded in the vacuoles. (c and f) After overnight interaction of apoptotic bodies with macrophages (c) or epithelial cells (f), a small amount of chromatic debris is visible, suggesting a degradation activity inside the vacuoles. By acridine orange fluorescent staining, the acidic nature of the apoptotic body-containing vesicles (arrowheads) is well evident in h. (g) Respective brightfield image. Bar in a–f, $0.1 \mu\text{m}$ and in g and h, $10 \mu\text{m}$.

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