ADP-ribosylation of the GTP-binding protein Rho by *Clostridium limosum* exoenzyme affects basal, but not *N*-formyl-peptide-stimulated, actin polymerization in human myeloid leukaemic (HL60) cells

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Treatment of human myeloid leukaemic (HL60) cells with *Clostridium limosum* exoenzyme, which inactivates the small GTP-binding protein Rho by ADP-ribosylation, decreased the basal F-actin content. Inhibition of F-actin occurred after long-term treatment (24 h) of intact HL60 cells or after introduction of the toxin by electroporation in a toxin-concentration-dependent manner. Concomitantly with the decrease in the basal F-actin content, the GTP-binding protein Rho was ADP-ribosylated in intact cells. However, *Clostridium limosum* toxin had no inhibitory effect on *N*-formyl-peptide-induced actin

polymerization. Moreover, the relative *N*-formyl-peptide-stimulated polymerization was substantially enhanced in cells treated with *Clostridium limosum* transferase. In contrast with *Clostridium limosum* exoenzyme, component C2I of the *Clostridium botulinum* C2 toxin, which ADP-ribosylates G-actin, depolymerized basal F-actin and inhibited *N*-formyl-peptide-induced actin polymerization in electroporated HL60 cells. These findings indicate that Rho proteins are involved in the basal, but not the ligand-evoked, actin polymerization in HL60 cells.

INTRODUCTION

Activation of neutrophil leucocytes by chemoattractants induces a series of physiological responses such as shape change, chemotaxis, adhesion, secretion and phagocytosis [1,2]. All of these events appear to involve redistribution of the actin cytoskeleton [2–5]. It is known that activation of neutrophils by chemoattractants induces a rapid polymerization of actin, followed by depolymerization of the filaments to basal actin levels [3,5,6]. Stimulated polymerization has been suggested to be regulated by interactions of phosphoinositides and actin-binding proteins such as gelsolin and profilin [7,8]. The regulation mechanism of the basal F-actin content in resting leucocytes is poorly understood.

Recently, the specific ADP-ribosylation of actin by *Clostridium botulinum* C2 toxin was used to study these phenomena [4,5,9–11]. ADP-ribosylated G-actin loses its ability to polymerize [12] and behaves like a capping protein which binds to the fast-growing ends of actin filaments [13]. By using the *Clostridium botulinum* C2 toxin as a tool, it was shown that ADP-ribosylation at first inhibits the ligand-evoked actin polymerization. Thereafter, even the basal F-actin level was decreased and, finally, F-actin was completely depolymerized [5].

More recently, it has been shown that small GTP-binding proteins of the Rho family are basically involved in the organization of the actin cytoskeleton [14–16]. ADP-ribosylation of Rho proteins by *Clostridium botulinum* C3 ADP-ribosyltransferase [17–19], which functionally inactivates the GTP-binding protein, causes depolymerization of actin filaments in various cell types [14–16,20]. Moreover, it was shown that Rho protein participates in the growth-hormone-receptor-mediated stress-fibre formation in 3T3 cells [16].

Here we investigated whether the Rho protein is involved in the regulation of the actin network in HL60 cells. For these

studies we used the C3-like transferase from *Clostridium limosum* [21], which inactivates Rho by ADP-ribosylation on asparagine-41 like *Clostridium botulinum* C3 exoenzyme [22].

MATERIALS AND METHODS

Materials

Component I of *Clostridium botulinum* C2 toxin [23] and *Clostridium limosum* exoenzyme [21] were purified as described. *N*-formyl-peptide (*N*-formyl-Met-Leu-Phe) was obtained from Saxon Biochemicals (Hannover, Germany). Fluorescein isothiocyanate-phalloidin (FITC-phalloidin) and dimethyl sulphoxide (DMSO) were from Sigma (Deisenhofen, Germany); RPMI 1640 medium, fetal-calf serum, non-essential amino acids, pyruvate, L-glutamine, penicillin and streptomycin were from Biochrom (Berlin, Germany). [³²P]NAD⁺ was purchased from NEN (Dreieich, Germany) and all nucleotides were from Boehringer (Mannheim, Germany). Human promyelocytic leukaemia cells (HL60) were kindly given by Dr. K. H. Jakobs (Essen, Germany). All other materials were from commercial sources.

Cell culture

HL60 cells [24] were maintained in suspension culture in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal-calf serum, 1% (v/v) non-essential amino acids, 11 mM glucose, 24 mM NaHCO₃, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 µg/ml streptomycin in an atmosphere of air/CO₂ (19:1) at an approximate density of 1 × 10⁶ cells/ml. Differentiation into mature myelocytic cells was induced by addition of 1.25% (v/v) DMSO for 2–3 days [25].

Abbreviations used: DMSO, dimethyl sulphoxide; *N*-formyl-peptide, *N*-formyl-Met-Leu-Phe (FMLP); FITC-phalloidin, fluorescein isothiocyanate-phalloidin.

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Electropermeabilization of HL60 cells

HL60 cells were electropermeabilized essentially as described [26]. Differentiated HL60 cells were centrifuged (5 min, 300 *g*) and resuspended in permeabilization buffer (140 mM KCl, 1 mM MgCl₂, 10 mM glucose, 1 mM ATP, 1 mM EGTA, 0.193 mM CaCl₂ and 10 mM Hepes, pH 7.0), to give a final concentration of 2×10^7 cells/ml. The cell suspension was kept on ice and electropermeabilized in the absence or presence of component I of *Clostridium botulinum* C2 toxin (in 50 mM triethanolamine) or *Clostridium limosum* exoenzyme (in RPMI 1640 culture medium without fetal-calf serum) in the given concentrations. The proportion of exoenzyme solution did not exceed 20% of the final volume. Samples (0.2 ml) in a 0.2 cm Bio-Rad Pulser cuvette were exposed to five discharges of 1.25 kV/cm from a 25 μ F capacitor. Cells were incubated for 1 h at 37 °C, centrifuged (3 min, 300 *g*), resuspended in 0.4 ml of permeabilization buffer and stimulated with *N*-formyl-peptide.

To test the specificity of the effect of *Clostridium limosum* exoenzyme, various concentrations of the exoenzyme and buffer were preincubated for 1 h before electropermeabilization at 4 °C without and with an antiserum raised in rabbits with purified *Clostridium limosum* exoenzyme as antigen [21]. Thereafter, antiserum-treated and untreated exoenzyme was added to the cell suspensions, which were subsequently electropermeabilized and prepared for *N*-formyl-peptide stimulation as described above.

F-actin measurement

F-actin content of HL60 cells was determined by staining with FITC-phalloidin and subsequent flow-cytometric analysis as described [27]. Briefly, samples of the cell suspension (90 μ l) with either vehicle (DMSO in permeabilization buffer in a final dilution of 1:10000) or *N*-formyl-peptide (0.1 or 1 μ M) were stimulated for 20 s at 37 °C if not otherwise stated. The reaction was terminated by addition of ice-cold staining solution [0.33 μ M FITC-phalloidin, 0.06% saponin, 7.2% formalin (all v/v) in permeabilization buffer]. The fluorescence was analysed with a flow cytometer (FACScan; Becton-Dickinson, Heidelberg, Germany). A total of 10000 cells per sample were measured. The log of the green fluorescence of the peak channel was determined and converted into a linear scale according to the manufacturer's instructions. The relative fluorescence was determined by calculating the ratio of fluorescence peak channels of *N*-formyl-peptide-stimulated and unstimulated cells. The extent of *N*-formyl-peptide-induced stimulation of actin polymerization varied with different batches of DMSO-differentiated HL60 cells; however, the effects of *C. limosum* exoenzyme were identical. Experiments were performed in duplicates or as indicated, with intra-assay variation of usually less than 5% of the means, and were repeated at least three times with comparable results.

ADP-ribosylation of HL60-cell lysate

DMSO-differentiated HL60 cells resuspended in permeabilization buffer were electropermeabilized in the presence of *Clostridium limosum* exoenzyme (60000, 600 or 6 ng/ml). As controls, cells were either electroporized in the absence of toxin or did not receive electropermeabilization and were kept on ice in the absence or presence of *Clostridium limosum* exoenzyme. Cells were incubated for 10 min at 37 °C and washed twice. The cell pellet was resuspended in 35 μ l of lysis buffer (10 mM triethanolamine/1 mM phenylmethanesulphonyl fluoride, pH 7.5) and snap-frozen in liquid nitrogen. After thawing, 30 μ l

of cell lysate was used for ADP-ribosylation as described [21]. The reaction medium contained the cell lysates, *Clostridium limosum* exoenzyme (250 ng/ml), [α -³²P]NAD⁺ (0.1 μ M, 0.2 μ Ci/ml) and ADP-ribosylation buffer (50 mM triethanolamine/HCl, pH 7.5), 1 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride, 1 mM EDTA, 2 mM MgCl₂ and 30 μ M GDP). Incubation (20 min, 30 °C) was terminated by addition of Laemmli sample buffer. After SDS/PAGE, gels were stained and destained and subjected to autoradiography.

RESULTS

Since *Clostridium limosum* exoenzyme hardly enters intact cells [21], DMSO-differentiated HL60 cells were electropermeabilized in the absence and presence of increasing concentrations of the *Clostridium limosum* transferase. After permeabilization, cells were further incubated for 1 h at 37 °C, and thereafter the cells were stimulated with *N*-formyl-peptide. The cells were fixed and the F-actin content was determined. As shown in Figure 1, the *Clostridium limosum* transferase decreased the fluorescence intensity of stained F-actin in electropermeabilized resting HL60 cells in a concentration-dependent manner; a maximal decrease in F-actin occurred at about 60 ng/ml. *N*-formyl-peptide (1 μ M) increased the F-actin content (FITC-phalloidin fluorescence) of electropermeabilized control cells by about 90–100% within 20 s. In contrast with the basal F-actin content, *Clostridium limosum* toxin did not decrease *N*-formyl-peptide-stimulated actin polymerization (Figure 1). The relative stimulated actin polymerization was much increased (up to ~7-fold) with respect to the basal F-actin content.

Next we studied the time course of *N*-formyl-peptide-induced actin polymerization in electropermeabilized HL60 cells treated without and with *Clostridium limosum* exoenzyme. Under control conditions, the F-actin content was maximally increased by *N*-formyl-peptide after 10 s (Figure 2) and the F-actin content returned to basal levels within 60 s. In *Clostridium limosum* transferase-treated cells, basal F-actin content was decreased

Figure 1 Influence of *Clostridium limosum* ADP-ribosyltransferase on basal and *N*-formyl-peptide-stimulated F-actin content of HL60 cells

DMSO-differentiated HL60 cells were electropermeabilized without and with increasing concentrations of *Clostridium limosum* transferase (*C. lim.* toxin). Cells were incubated for 60 min at 37 °C and then stimulated with 1 μ M *N*-formyl-peptide for 20 s. Thereafter, the F-actin content of unstimulated (○) and stimulated (●) cells was analysed by determination of the FITC-phalloidin fluorescence as described in the text. Data are means $\pm$ S.E.M. from 4 independent experiments performed in duplicates and are given as percentage of control. The control F-actin fluorescence of each experiment was taken as 100%.

Figure 2 Time course of *N*-formyl-peptide-stimulated actin polymerization in HL60 cells

DMSO-differentiated HL60 cells were electroporated in the absence (○) and presence (●) of 60 µg/ml *Clostridium limosum* ADP-ribosyltransferase. Cells were further incubated for 1 h at 37 °C and then stimulated by 1 µM *N*-formyl-peptide. At the indicated time periods the relative F-actin content compared with unstimulated cells was determined as described in the text. Data are given as means from one experiment performed in duplicate, repeated three times with comparable results.

Figure 3 Effects of anti-C3 antibody on *Clostridium limosum* ADP-ribosyltransferase induced decrease in F-actin

HL60 cells were electroporated at the indicated concentrations of *Clostridium limosum* ADP-ribosyltransferase (*C. lim. toxin*) pretreated for 1 h without (○) and with (●) polyclonal rabbit anti-C3 antibody, which inactivates the *Clostridium limosum* transferase. After 1 h of incubation at 37 °C, the FITC-phalloidin fluorescence was determined. Fluorescence is presented as means ± S.D. from a representative experiment repeated three times.

Table 1 Influence of *Clostridium limosum* ADP-ribosyltransferase on F-actin content of HL60 cells after 24 h incubation

DMSO-differentiated HL60 cells were incubated without and with 100 µg/ml *Clostridium limosum* ADP-ribosyltransferase for 24 h. Thereafter, the cells were stimulated without (Basal) or with 1 µg/ml *N*-formyl-peptide (FMLP) and the FITC-phalloidin fluorescence was determined. Data are given as means ± S.D. ($n = 4$).

Additions	Fluorescence (mean channel number)
Control	
Basal	417 ± 15
FMLP	607 ± 3.5
<i>C. limosum</i> toxin	
Basal	300 ± 23
FMLP	550 ± 10

Figure 4 ADP-ribosylation of Rho protein by *Clostridium limosum* ADP-ribosyltransferase

DMSO-differentiated HL60 cells were electroporated (Permeab.) in the presence of the indicated concentrations of *Clostridium limosum* ADP-ribosyltransferase (*C. lim. toxin*). Thereafter, the cells were incubated for 10 min at 37 °C, washed twice and lysed. The lysates were ADP-ribosylated in the presence of 0.1 µM [32 P]NAD⁺ and 250 ng/ml *Clostridium limosum* transferase for 20 min at 30 °C. The labelled proteins were analysed by SDS/PAGE and autoradiography, as shown.

(results not shown), but the relative stimulated F-actin polymerization was enhanced. However, the time course of actin polymerization induced by *N*-formyl-peptide was similar in toxin-treated and control cells.

To test the specificity of the effects induced by *Clostridium limosum* exoenzyme, we used a polyclonal antibody raised against *Clostridium botulinum* C3 ADP-ribosyltransferase, which cross-reacts with *Clostridium limosum* transferase and inhibits its activity [21]. Figure 3 shows that this anti-C3 antibody effectively prevented the *Clostridium limosum* toxin effect on basal F-actin at a toxin concentration of 60 ng/ml. At higher toxin concentration (600 ng/ml) the antibody was partially inhibitory. Furthermore, similar effects of *Clostridium limosum* toxin on basal and stimulated F-actin of HL60 cells were obtained after long-term treatment of the cells (24 h) with high concentration of the toxin (100 µg/ml) (Table 1).

In order to study the efficiency of Rho ADP-ribosylation after toxin electroporation, HL60 cells were loaded with *Clostridium limosum* toxin by high-voltage discharge, and thereafter the cells were incubated for a short period (10 min), washed, lysed, and a second ADP-ribosylation with the transferase was performed in the presence of [32 P]NAD⁺ in the cell lysate. Figure 4 shows that electroporation of HL60 cells with the toxin effectively decreased the labelling of Rho in the cell lysate, indicating the modification of Rho by the first toxin treatment in intact cells. In contrast, in unpermeabilized cells no decrease in the labelling of Rho proteins occurred even at rather high concentrations (60 µg/ml) of *Clostridium limosum* ADP-ribosyltransferase.

Finally, we compared the effects of the Rho-ADP-ribosylating *Clostridium limosum* toxin with those of actin-ADP-ribosylating *Clostridium botulinum* C2 toxin. C2 toxin is a binary toxin which consists of an enzyme component possessing transferase activity (C2I) and a binding component (C2II) which is responsible for binding to the cell surface and the transfer of the enzyme component into the cell [28,29]. To compare both toxins and to test the effectiveness of the electroporation further, we used only the enzyme component (C2I) of C2 toxin, which is not able to enter intact cells. Figure 5 shows that electroporation of HL60

Figure 5 Influence of *Clostridium botulinum* C2 toxin on F-actin content of HL60 cells

DMSO-differentiated HL60 cells were electroporated in the absence and presence of the indicated concentrations of *Clostridium botulinum* C2 toxin. Cells were incubated for 1 h at 37 °C, and thereafter stimulated by *N*-formyl-peptide (100 ng/ml). The FITC-phalloidin fluorescence was determined from stimulated (●) and unstimulated (○) cells as described in the text. Data are given as means $\pm$ S.D. from a representative experiment repeated three times.

cells in the presence of C2I effectively decreased basal F-actin content in a concentration-dependent manner. In contrast with *Clostridium limosum* toxin, C2I was even more effective at inhibiting the *N*-formyl-peptide-stimulated actin polymerization. Finally, C2I completely prevented polymerization of actin, and depolymerized pre-existing actin filaments. Similar effects of C2 toxin were observed in the presence of both toxin components (C2I plus C2II); however, 10-fold lower toxin concentrations were sufficient (results not shown).

DISCUSSION

Here we studied the influence of *Clostridium limosum* ADP-ribosyltransferase on basal and stimulated F-actin content in HL60 cells. In a manner similar to *Clostridium botulinum* C3 transferase, the exoenzyme from *Clostridium limosum* ADP-ribosylates low-molecular-mass GTP-binding proteins of the Rho family [21], which were suggested to be involved in actin regulation [14–16,20]. ADP-ribosylation of Rho by these transferases occurs in asparagine-41 [22] and results in the functional inactivation of the protein [14]. As shown here, treatment of HL60 cells with *Clostridium limosum* exoenzyme decreased the basal F-actin content, but did not inhibit stimulation of actin polymerization induced by the chemoattractant *N*-formyl-peptide. In contrast, the percentage stimulation was largely increased after toxin treatment. Several controls were performed to validate the methods used and to exclude the possibility that the effects observed were caused by the electroporation procedure. First, we observed identical effects on HL60 cells after long-term treatment of the cells with high concentrations of the *Clostridium limosum* exoenzyme. Second, we show that Rho protein was effectively ADP-ribosylated after introduction of *Clostridium limosum* exoenzyme by electroporation. Third, we tested the efficiency of electroporation with C2 toxins. For this purpose, only the exoenzyme component (C2I) was used, but not the binding and transfer component (C2II). Finally, the specificity of the *Clostridium limosum* transferase effect was shown by the finding that the addition of anti-C3 antibody blocked the *Clostridium limosum* effects on HL60 cells. The polyclonal antibody was raised against the homologous C3 transferase [21].

The antibody cross-reacts with the *Clostridium limosum* transferase and inhibits its action.

All findings reported in this paper show that the ADP-ribosylation of Rho, a modification which causes inactivation of the GTP-binding protein, has no inhibitory effect on *N*-formyl-peptide-stimulated F-actin formation. These observations indicate that most probably Rho protein is not involved in F-actin formation induced by the chemoattractant *N*-formyl-peptide. However, Rho proteins regulate the basal actin content in leucocytes. In contrast, C2 toxin, which ADP-ribosylates G-actin [12], thereby inhibiting actin polymerization, prevented ligand-evoked F-actin formation and decreased the F-actin content in HL60 cells. Similar effects were reported for the C2 toxin action on human polymorphonuclear leucocytes [4,5,9,10].

It has been shown recently that treatment of polymorphonuclear leucocytes by C3 inhibits chemoattractant-induced migration, but has no effect on secretion or superoxide-anion production [30]. Most likely, the reason for these virtually contradictory results is based on the existence of various actin pools which are differently regulated. Recent findings suggest that Rho proteins are involved in the actin polymerization occurring at the focal adhesion sites [16]. It is generally accepted that actin-based motility is closely associated with the reorganization of the actin cytoskeleton at the plasma membrane and focal adhesions [31]. Similarly, the cortical actin, which most probably contributes a large portion to 'basal' F-actin in HL60 cells, is associated with these structures. Thus inactivation of Rho protein causes destruction of the basal actin cytoskeleton and inhibition of migration. In contrast, *N*-formyl-peptide-induced F-actin formation appears to be regulated by different mechanisms, probably involving actin-binding proteins such as gelsolin, profilin and thymosin β_4 [7,32]. As demonstrated here, *N*-formyl-peptide-evoked actin polymerization, which involves signal transduction via G-proteins [25,33,34], was not inhibited by the Rho-ADP-ribosylating *Clostridium limosum* toxin. In contrast, the relative F-actin content stimulated by *N*-formyl-peptide was rather increased after treatment with *Clostridium limosum* toxin. Thus it appears that the G-actin pool which is available for ligand-evoked polymerization increased after Rho ADP-ribosylation.

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