ADP-ribosylation of Rho enhances adhesion of U937 cells to fibronectin via the $\alpha5\beta1$ integrin receptor

Martin Aepfelbacher*

Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Ludwig-Maximilians Universität München, Pettenkoferstr. 9, 80336 München, Germany

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Abstract To examine the role of Rho GTP binding proteins in the adhesion of monocytic cells to fibronectin we used the C3 exoenzyme of *Clostridium botulinum* which ADP-ribosylates and inactivates Rho proteins in situ. Treatment of human monocytic U937 cells with C3 exoenzyme (10 μg/ml, 24 h) increased adhesion to fibronectin 2-fold but had no effect on adhesion to collagen or human serum albumin. The increase in fibronectin adhesion was prevented by antibodies against the α5 and β1 integrin subunits, but surface expression of β1 and α5 was not altered. These results suggest that Rho proteins regulate the interaction of the monocyte α5β1 integrin receptor with fibronectin by post receptor mechanisms.

Key words: ADP-ribosylation; GTP binding protein; Rho; Monocyte; Fibronectin; Integrin

1. Introduction

Ras-related Rho GTP binding proteins have been implicated in the organisation of the actin cytoskeleton and in the assembly and activity of integrin receptors [1]. These functions of Rho proteins have been established mainly by use of the C3 exoenzyme of *Clostridium botulinum* which specifically ADP-ribosylates and thereby inactivates Rho (A, B and C) in intact cells, presumably by blocking its interaction with downstream targets [2–5]. Evidence that Rho is involved in integrin function comes from the finding that C3 exoenzyme blocked formation of focal adhesion sites in quiescent fibroblasts stimulated by the growth factors PDGF and lysophosphatidic acid and that, vice versa, microinjection of activated Rho stimulated focal adhesion formation [6]. At focal adhesion sites, intracellular actin fibers are connected to extracellular integrin receptors via multimolecular regulatory complexes at the plasma membrane [7]. Further reports implicating Rho in integrin function showed that ADP-ribosylation of Rho inhibited LFA-1-dependent lymphocyte aggregation [8] as well as thrombin-induced platelet aggregation mediated by the glycoprotein IIb–IIIa [9].

Here the effect of ADP-ribosylation of Rho on fibronectin receptor function was examined. For this purpose we took advantage of the human myelomonocytic U937 cell line which adheres to fibronectin through the $\alpha5\beta1$ and $\alpha4\beta1$ integrin receptors [10], but does not adhere to a variety of other extracellular matrix proteins tested ([11], and this study). We demonstrate that ADP-ribosylation of Rho in monocytic U937 cells results in an enhanced adhesion to fibronectin which is mediated by the $\alpha5\beta1$ fibronectin receptor.

2. Materials and methods

2.1. Materials

The pGEX2T-C3 exoenzyme expression vector was a generous gift from Dr. L. Feig (Tufts University, Boston, USA). Rabbit polyclonal anti-RhoA was purchased from IC Chemikalien (Ismaning, Germany) and tested with the recombinant glutathione *S*-transferase fusion protein of RhoA, Rac1 and CDC42Hs [12], on Western blot. Mouse monoclonal anti-α5 (clone P1D6) and β1 (clone P4C10) were from Gibco (MD, USA), FITC-conjugated rat anti-mouse IgG was from Serotec (Oxford, UK) and horseradish peroxidase conjugated anti-rabbit IgG was from Amersham. RPMI 1640, L-glutamine, fetal calf serum and all other materials were from Sigma.

2.2. Preparation of recombinant C3 exoenzyme

The C3 exoenzyme was expressed as a glutathione *S*-transferase (GST) fusion protein in *E. coli* strain NM522. Expression of the fusion protein was induced by isopropylthiogalactopyranoside (Boehringer, Mannheim, Germany), bacteria were lysed by sonication and the fusion protein was purified with glutathione-Sepharose 4B beads (Pharmacia, Sweden) and cleaved with thrombin (Boehringer, Mannheim, Germany) as described in detail [13]. Purity of the C3 exoenzyme was tested on SDS/12% PAGE and protein concentration was determined with the BCA method (Pierce, Rockford, IL, USA) using BSA as the standard.

2.3. Cell culture and C3 exoenzyme treatment

U937 cells, a gift from Dr. C. Wilson (Children's Orthopedic Hospital, University of Washington, Seattle, WA), were grown in RPMI 1640 with 2 mM L-glutamine and 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. Cultures were maintained for 72 h in 80 cm^2 culture flasks (Falcon, Becton Dickinson), centrifuged (150 × *g*, 5 min) and resuspended at a density of 2×10^5 cells/ml in fresh medium with or without C3 exoenzyme. Cell viability was determined by ethidium bromide/Acridine orange fluorescence and was > 90% in the absence or presence of C3 exoenzyme.

2.4. [^{32}P]ADP-ribosylation and SDS-PAGE

Cells were pelleted by centrifugation (150 × *g*, 5 min), washed once in PBS, resuspended at 2×10^6/ml in ice-cold buffer containing 250 mM sucrose, 1 mM EDTA, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 1 mM dithiothreitol, 0.1 mM GTP; 1 μg/ml of leupeptin, pepstatin, aprotinin, and 2 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated for 10 s at 50 W with a Labsonic U (B. Braun, Melsungen, Germany). 100 μl of this homogenate was added to 50 μl of 150 mM Tris-HCl (pH 8), 30 mM thymidine, 3 mM dithiothreitol, 5 mM $MgCl_2$, 0.3 mM GTP and 0.5 μg of recombinant C3 exoenzyme on ice. The reaction was started by adding 1 μCi [^{32}P] NAD (800 Ci/mmol, NEN) to the sample; then it was incubated for 15 min in a 37°C waterbath and precipitated for 45 min at −20°C with 6 vols. of ice-cold acetone. Precipitates were analyzed by SDS-12% PAGE (Mini-Protean II; Bio-Rad) and autoradiography using Kodak X-OMAT films. Bands were quantified by laser densitometry (Ultroscan XL; Pharmacia, Freiburg, Germany) using peak area integration according to manufacturers instructions (Pharmacia). Bands were only evaluated when their density was within the

*Corresponding author. Fax: (49) (895) 160 4382.

Abbreviations: LFA, lymphocyte function antigen; C3, C3 exoenzyme from *Clostridium botulinum*; MFI, mean fluorescence intensity.

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linear part of a standard curve obtained by [^{32}P]ADP-ribosylating serial dilutions of recombinant RhoA (100 ng, 30 ng, 10 ng, 1 ng and 0.3 ng).

2.5. Western blot

After SDS-12% PAGE (Mini Protean II; Bio-Rad), proteins were electrophoretically transfered to Immobilon PVDF membranes (Millipore, MA, USA) for 1 h at 80 V in 20 mM Tris, 192 mM glycine. Membranes were blocked for 1 h in a solution containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.3% Tween, 10% calf serum; then they were incubated for 1 h with anti-RhoA antibody (1:2000). After incubation for 45 min with peroxidase-labeled anti-rabbit IgG (1:5000), blots were developed using ECL (Amersham) and then exposed to X-Omat AR film (Kodak).

2.6. Cell adhesion assay

Cells were washed in PBS, resuspended at 10^6 cells/ml in RPMI medium containing 1% human serum albumin and 100 μl per well was added to a 96-well microtiter plate (Falcon) that had been precoated overnight at room temperature with 100 μl of 10 μg/ml fibronectin from human plasma, 100 μg/ml collagen G (90% collagen I, 10% collagen III; Biochrom, Berlin, Germany) or 1 mg/ml human serum albumin. Cells were incubated for 1 h at 37°C and non-adherent cells were removed by centrifugation of the inverted microtiter plate for 3 min at 200 × *g*. Pretreatment with antibodies was performed for 1 h at 4°C using 10 μl/ml anti-α5 or 10 μl/ml anti-β1 antibodies. For quantification of adherent cells 150 μl of RPMI and 15 μl of MTT (5 mg/ml) was added, incubated for 4h at 37°C, and cells were lysed with 100 μl of 15% SDS in 15 mM HCl per well. After overnight incubation at room temperature, plates were read at 570 nm in an ELISA reader and the number of adherent cells was calculated using calibration curves obtained with known amounts of cells.

2.7. FACS-analysis

Cells (2×10^5 in 200 μl) were incubated for 30 min at 4°C in phosphate-buffered saline with 10 μl/ml anti-α5 or IgG_3 isotype control or 10 μl/ml anti-β1 or IgG_1 isotype control, washed in PBS and then incubated for 1 h at 4°C with a 1:40 dilution of FITC-conjugated rat anti-mouse IgG. Samples were washed and analyzed at 10^4 cells per sample in a FACScan (Becton Dickinson). ΔMFI is defined as the difference in the mean fluorescence intensities of signals obtained with anti-integrin antibodies and their isotype controls.

3. Results and discussion

To detect the Rho ADP-ribosylation substrate in U937 cells we incubated whole cell homogenate with C3 ADP-ribosyltransferase from *Clostridium botulinum* and [^{32}P]NAD. SDS-PAGE and autoradiography revealed one [^{32}P]ADP-ribosylated band at 23 kDa that co-migrated with RhoA on a

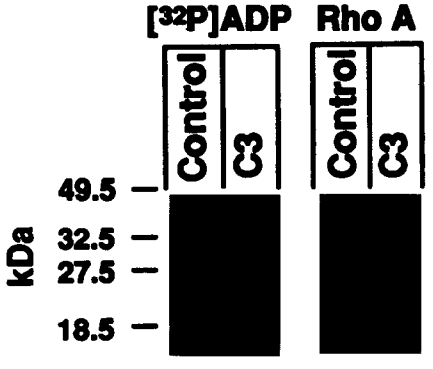

Fig. 1. Autoradiography of the [^{32}P]ADP-ribosylation substrate and RhoA immunoblot in U937 cells. Control and C3-pretreated (24h, 10 μg/ml) U937 cells were homogenised, incubated with [^{32}P]NAD and recombinant C3 exoenzyme and subjected to SDS-12.5% PAGE and either autoradiography (left panel) or immunoblot using specific anti-RhoA antibody (right panel).

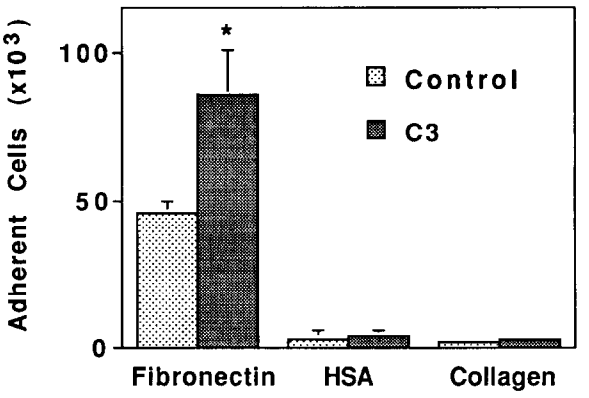

Fig. 2. Effect of C3 exoenzyme on fibronectin adhesion of U937 cells. Cells were pretreated without (Control) or with C3 exoenzyme (24 h, 10 μg/ml) and then adhesion to fibronectin, human serum albumin (HSA) or collagen was tested (section 2). Each bar represents the mean ± S.D. of three experiments. *Indicates $P < 0.01$.

Western blot (Fig. 1, Controls). When intact U937 cells were pretreated with C3 (10 μg, 24 h), in vitro incorporation of [^{32}P]ADP into the substrate was diminished to 20–30% of control (Fig. 1, left panel), suggesting that 70–80% of Rho was ADP-ribosylated in situ. The total cellular amount of the Rho ADP-ribosylation substrate was not altered by the C3 treatment as demonstrated by anti-RhoA Western blot (Fig. 1, right panel). No protein band was found with anti-RhoB antibody (data not shown). The ADP-ribosylation substrate in U937 cells most likely is RhoA: (i) on a two-dimensional gel it co-migrated with RhoA detected by Western blot (Aepfelbacher et al., unpublished) and (ii) it co-migrated with the ADP-ribosylation substrate in platelets that was identified as RhoA [14]. Consistent with these findings, RhoA seems to be the major ADP-ribosylation substrate in most cells investigated so far, including mouse fibroblasts [15], human blood platelets [14], lymphocytes [16], neuronal cells [17], and myelomonocytic HL60 cells (Aepfelbacher et al., submitted). Taken together these data suggest that the C3 from *Clostridium botulinum* ADP-ribosylates a protein in intact U937 cells that most likely is RhoA.

To test the effect of ADP-ribosylation of Rho on fibronectin adhesion of U937 cells we incubated control- and C3-treated cells in fibronectin-coated culture dishes (1 h, 37°C) and removed non-adherent cells by centrifugation. The percentage of cells that adhered to fibronectin increased from approximately

Table 1
Effect of C3 exoenzyme on surface expression of the α5 and β1 integrin subunits

Integrin subunit	ΔMFI	
	Control	C3
α5	87 ± 13	84 ± 2
β1	81 ± 8	91 ± 7

U937 cells were treated for 24 h without (control) or with 10 μg/ml C3 exoenzyme. ΔMFI (difference in mean fluorescence intensity) was determined for each integrin subunit by FACS analysis as described in section 2. Each value represents the mean ± S.D. of three experiments performed in duplicate.

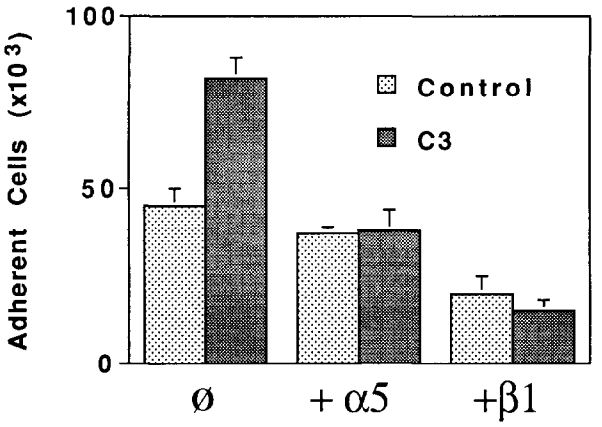

Fig. 3. Effect of anti-α5 and -β1 antibodies on fibronectin adhesion of control and C3-treated U937 cells. Cells were treated without (Control) or with C3 exoenzyme (24 h, 10 μg/ml) preincubated (1 h, 4°C) with vehicle (Ø) or indicated antibodies and tested for adhesion to fibronectin (section 2). Each bar represents the mean ± S.D. of three experiments.

45% in controls to 90% after C3 treatment (Fig. 2). Less than 5% of cells adhered to collagen or human serum albumin with or without C3 treatment. These data confirm the observation that U937 cells only adhere to fibronectin and not to a variety of other extracellular matrix proteins [11] and suggest that the increased adhesion of C3-treated U937 cells is fibronectin receptor dependent.

U937 cells adhere to fibronectin mainly via the α5β1 and to a lesser extent via the α4β1 receptor [10]. To identify the integrin receptors that mediated the enhanced adhesion to fibronectin in C3-treated cells, we preincubated cells with blocking antibodies against the α5 and β1 subunits. The anti-α5 antibody P1D6 completely prevented the increase in fibronectin adhesion in C3-treated cells and slightly reduced the percentage of control cells adhering to fibronectin (Fig. 3). In comparison, the anti-β1 antibody P4C10 also prevented the C3 induced increase in fibronectin adhesion but markedly reduced the percentage of control cells adhering to fibronectin (Fig. 3). Hence, the increased fibronectin adhesion in C3-treated cells seems to be mediated entirely by the α5β1 integrin whereas basal fibronectin adhesion seems to involve an additional α subunit, most likely α4.

To investigate the possibility that the increased fibronectin adhesion in C3 treated cells was due to up-regulation of the α5β1 fibronectin receptor, we performed FACS analysis using specific anti-α5 and anti-β1 antibodies. Surface expression of neither integrin subunit was affected by the C3 treatment, as evidenced by the unchanged differences in the mean fluorescence intensities (ΔMFI) between control and C3-treated cells (Table 1).

In summary, our data provide evidence that ADP-ribosylation of Rho GTP binding proteins increases adhesion of human monocytic U937 cells to fibronectin via the α5β1 integrin. Recent studies using different cell types suggested that blocking of Rho proteins by ADP-ribosylation resulted in inhibition rather than stimulation of integrin function [6,8,14]. However, the functions of Rho proteins might be cell type specific. For example, in neuronal cells ADP-ribosylation of Rho leads to cell flattening and neurite outgrowth [17], whereas in Vero and 3T3 cells it causes cell rounding and loss of contact with the extracellular matrix [1,2].

A recent study demonstrated that in myelomonocytic cells ADP-ribosylation of Rho resulted in *enhanced* actin polymerisation after cell stimulation [18]. Considering that actin polymerisation has been implicated in integrin dependent adhesion of leukocytic cells to the extracellular matrix [19], it is possible that this or a related phenomenon may be responsible for the increased fibronectin adhesion of C3-treated U937 cells observed in our study.

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