ADP-ribosylation of the GTP-binding protein RhoA blocks cytoplasmic division in human myelomonocytic cells

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To test the role of Rho GTP-binding proteins in growth regulation of human myelomonocytic tumour cells we used recombinant C3 exoenzyme of *Clostridium botulinum* to specifically ADP-ribosylate and inactivate Rho proteins *in situ*. In homogenates of HL60 cells, the C3 exoenzyme [³²P]ADP-ribosylated one protein that was identified as RhoA by immunoblot and two-dimensional gel electrophoresis. [³²P]ADP ribosylation of RhoA in HL60 homogenates *in vitro* was reduced to 10–20% when cells in culture were pretreated with C3 exoenzyme (10 µg, 24 h), indicating that 80–90% of RhoA could

be ADP-ribosylated *in situ*. The C3 exoenzyme inhibited HL60 cell proliferation by up to 80% and the degree of growth inhibition correlated with the amount of *in situ* ADP-ribosylated RhoA in a time- and dose-dependent manner. Cell cycle analysis demonstrated that the C3 exoenzyme-treated HL60 cells accumulated in mitosis, and nuclear staining revealed binucleated cells. These findings suggest that RhoA has a key role in human myelomonocytic tumour cell growth by regulating cytoplasmic division.

INTRODUCTION

The Rho-subgroup of Ras-related GTPases consists of RhoA, B, C and G, Rac1 and 2 as well as two CDC42Hs proteins and has been implicated in cytoskeletal organization [1]. Like all Ras-like proteins, Rho-GTPases bind and hydrolyse GTP and are thought to cycle between a GTP-bound active and a GDP-bound inactive state; they have been shown to be regulated by GTPase activating proteins (GAPs), guanine nucleotide dissociation-stimulators (GDS) or -inhibitors (GDI) [2]. RhoA, B and C are unique in that they can be ADP-ribosylated by the C3 exoenzyme from *Clostridium botulinum* [3–5]. The ADP-ribosylation takes place at an asparagine residue (Asn-41) in the putative effector domain and inactivates Rho, presumably by blocking the interaction with its downstream target [6]. When added in high concentrations (1–10 µg/ml) to cells in culture, the C3 exoenzyme can translocate to the cytosol and ADP-ribosylate and inactivate Rho-proteins *in situ* [3,7,8]. Using this technique it has become well established that Rho-GTPases organize the actin cytoskeleton in a variety of cells and thereby are involved in the regulation of cell-motility, -contraction, -shape and -adhesion [8–11].

Recent findings indicate that Rho-family proteins might also play a role in the proliferation and differentiation of human myelomonocytic cells. In Philadelphia chromosome positive leukaemias, fusion proteins of the *bcr* and the *c-abl* gene product cause oncogenic cell transformation by activating Ras [12,13]. A domain of the *bcr* gene product was shown to act as a GAP for Rac and CDC42Hs, albeit not for Rho [14]. Another previously identified protein, 3BP-1, binds to the SH3 domain of c-abl and serves as a GAP for Rho [15]. In addition, a recently cloned gene encodes a GDI for Rho-like GTPases that is preferentially expressed in developing haematopoietic tissues and is modulated in differentiating myelomonocytic HL60 and U937 cells [16]. In differentiating human monocytes and U937 cells, expression of the CDC42Hs protein is increased dramatically [17], although the function of this protein is still unknown.

Here we show that *in situ* ADP-ribosylation of the small GTP-binding protein, RhoA, in HL60, U937 and THP1 cells results in reduced cell proliferation due to a block in cell division. This suggests that RhoA is of fundamental importance for the growth of human myelomonocytic tumour cells.

MATERIALS AND METHODS

Materials

RPMI 1640, L-glutamine, fetal calf serum (FCS) and all other materials were from Sigma. U937 cells were a gift from Dr. C. Wilson (Children's Orthopedic Hospital, University of Washington, Seattle, WA, U.S.A.). THP1 and HL60 cells were purchased from ATCC (Rockville, MD, U.S.A.). Anti-RhoA and anti-RhoB antibodies were purchased from IC Chemikalien (Ismaning, Germany) and tested with recombinant glutathione S-transferase (GST) fusion protein of RhoA [18] on Western blots. To obtain platelet lysates, freshly drawn human blood, anticoagulated with 0.1% trisodium citrate, was centrifuged for 20 min at 180 g. Platelets were then pelleted by centrifugation of the supernatant (platelet-rich plasma) for 15 min at 800 g, resuspended in a buffer containing 20 mM Hepes (pH 7.4)/140 mM NaCl and sonicated twice for 10 s at 50 W with a Labsonic U (B. Braun, Melsungen, Germany).

Preparation of recombinant C3 exoenzyme

The C3 exoenzyme was expressed as a GST fusion protein in *Escherichia coli* strain NM522. The pGEX2T-C3 exoenzyme expression vector was a gift from Dr. L. Feig (Tufts University, Boston, MA, U.S.A.). Expression of the fusion protein was induced by isopropyl β-D-thiogalactoside (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

Abbreviations used: GAP, GTPase-activating protein; GDS, guanine nucleotide dissociation-stimulator; GDI, guanine nucleotide dissociation-inhibitor; GST, glutathione S-transferase; DTT, dithiothreitol; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum.

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Mannheim) as described in detail [18]. The purity of the C3 exoenzyme was tested on SDS/12% PAGE and protein concentration was determined using the BCA method (Pierce, Rockford, IL, U.S.A.) using BSA as a standard.

Cell culture and cell proliferation

HL60, U937 and THP1 cells were grown in RPMI 1640 with 2 mM L-glutamine and 10% FCS at 37 °C in a humidified 5% CO₂ atmosphere. Cultures were maintained for 96 h in 80 cm² culture flasks (Falcon, Becton Dickinson), centrifuged (150 g, 5 min) and resuspended at a density of 2×10^5 cells/ml in fresh medium. Cells were then seeded into 24-well plates (1 ml/well; Nunc, Roskilde, Denmark) or 96-well plates (200 µl/well; Nunc) for analysis of cell proliferation, ADP-ribosylation *in vitro* or cell cycle. Cell numbers were determined with a Coulter Counter Model ZM (Coulter Electronics, Krefeld, Germany) by counting at least 1×10^5 cells. Cell viability was determined by ethidium bromide/Acridine Orange fluorescence and was > 90% in the absence or presence of C3 exoenzyme or other substances.

ADP-ribosylation assay

Cells were pelleted by centrifugation (150 g, 5 min), washed once in PBS (pH 7.4), resuspended at 2×10^6 cells/ml in ice-cold buffer containing 250 mM sucrose, 1 mM EDTA, 5 mM MgCl₂, 10 mM Hepes (pH 7.4), 1 mM dithiothreitol (DTT), 0.1 mM GTP, 1 µg/ml of leupeptin, pepstatin, aprotinin and 0.2 mM phenylmethanesulphonyl fluoride and sonicated for 10 s at 50 W with a Labsonic U. A 100 µl volume of this homogenate was added to 50 µl of 150 mM Tris-HCl (pH 8), 30 mM thymidine, 3 mM DTT, 5 mM MgCl₂, 0.3 mM GTP and 0.5 µg of recombinant C3 exoenzyme on ice. The reaction was started by adding 1 µCi [³²P]NAD⁺ (800 Ci/mmol; New England Nuclear) to the sample; then it was incubated for 15 min in a 37 °C water-bath and precipitated for 45 min at -20 °C with 6 vols. of ice-cold acetone. Precipitates were analysed by SDS/12% PAGE (Mini-Protein II, Bio-Rad) and autoradiography using Kodak X-OMAT films. Bands were quantified by laser densitometry (Ultrosan XL, Pharmacia, Freiburg, Germany) using peak area integration according to the manufacturer's instructions (Pharmacia). Bands were only evaluated when their density was within the linear part of a standard curve obtained by [³²P]ADP-ribosylating serial dilutions of recombinant RhoA (100 ng, 30 ng, 10 ng, 1 ng and 0.3 ng). We also verified by Western blot that the total cellular amount of RhoA available for ADP-ribosylation was not changed by the C3 treatment. Values were expressed in arbitrary absorbance units. The percentage of *in situ* ADP-ribosylated substrate was calculated by subtracting the [³²P]ADP incorporation in C3 exoenzyme-pretreated cells from the [³²P]ADP incorporation in untreated controls.

Western blot and two-dimensional gel electrophoresis

After SDS/12% PAGE, proteins were electrophoretically transferred to Immobilon poly(vinylidene difluoride) membranes (Millipore, MA, U.S.A.) for 1 h at 80 V in 20 mM Tris-HCl/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% FCS; then they were incubated for 1 h with anti-RhoA or anti-RhoB antibody (both 1:2000). After incubation for 45 min with peroxidase-labelled donkey anti-rabbit IgG (1:5000; Amersham, U.K.) blots were developed using enhanced chemiluminescence (ECL; Amersham) and then exposed to X-Omat AR film (Kodak). For two-dimensional gel electrophoresis, acetone-precipitated proteins were dissolved

in 50 µl of 9 M urea/2% Nonidet P-40/2% mercaptoethanol/0.8% ampholytes (pH 3–10)/0.1% Bromophenyl Blue. Then they were subjected to isoelectric focusing on a 4% polyacrylamide gel containing 4.4% ampholytes, pH 3–10 and 0.7% ampholytes, pH 5–7 (Biolyte, Biorad), essentially as described by O'Farrell [19]. The second dimension was performed with SDS/12% PAGE using the Protean II xi system (Biorad).

Flow cytometric analysis

Cell cycle analysis was performed using the Cell Cycle Test Kit (Becton Dickinson). Cells (1.5×10^5) were washed with PBS and then treated with Triton X-100, trypsin and propidium iodide according to the manufacturer's instructions. Fluorescence was analysed using fluorescence-activated cell sorting (FACSscan; Becton Dickinson) using the CellFIT software to calculate percentage of cells in the G₀/G₁, S or G₂/M phase according to manufacturers instructions. For the analysis of differentiation markers, cells (2×10^5 in 200 µl) were incubated for 30 min at 4 °C in PBS with a 1:100 dilution of fluorescein isothiocyanate-conjugated monoclonal anti-CD11b, anti-CD14 or isotype control (Coulter). Samples were washed and analysed at 10^4 cells per sample in a FACSscan. Cells showing significant specific fluorescence intensity were defined as positive.

Cell staining

Control, cytochalasin D or C3 exoenzyme-treated cells were washed in PBS, resuspended at 1×10^6 cells/ml in 50% PBS/50% FCS and stained using Testsimplets according to the manufacturer's instructions (Boehringer Mannheim). Photographs were taken with an Olympus IMT-2 at 200-fold magnification.

RESULTS

Identification of ADP-ribosylation substrates in HL60, U937 and THP1 cells

To identify ADP-ribosylation substrates in HL60, U937 and THP1 cells, whole cell homogenates were incubated with [³²P]NAD⁺ and C3 exoenzyme from *C. botulinum*. SDS/PAGE and autoradiography revealed one [³²P]ADP-ribosylated band that migrated at 23 kDa in all three cell lines (Figure 1). A protein band of similar electrophoretic mobility was detected by anti-RhoA, but not by anti-RhoB antibody in Western blots

Figure 1 Autoradiography of the [³²P]ADP-ribosylation substrates and RhoA and RhoB immunoblots of HL60, U937 and THP1 cells

(a) Homogenates of 2×10^5 cells were incubated with [³²P]NAD⁺ and recombinant C3 exoenzyme of *C. botulinum* and then subjected to SDS/12.5% PAGE and autoradiography. Homogenates of 4×10^5 cells were subjected to SDS/12.5% PAGE and then immunoblotted using specific anti-RhoA antibody (b) or anti-RhoB antibody (c).

Figure 2 Two-dimensional PAGE of the [^{32}P]ADP-ribosylation substrates in HL60 cells and human platelets

(a) HL60 cells (1×10^6) were homogenized and incubated with [^{32}P]NAD $^{+}$ and recombinant C3 exoenzyme of *C. botulinum*. Samples were then subjected to two-dimensional gel electrophoresis and autoradiography as described in the Materials and methods section. (b) Same as (a) but with 100 μg of human platelet lysate as [^{32}P]ADP-ribosylation substrate.

(Figure 1). Two-dimensional electrophoresis of the HL60 cell homogenate showed one major and one minor [^{32}P]ADP-ribosylated protein spot with isoelectric points (pI) of 6.3 and 6.2 respectively (Figure 2a). These isoelectric points are close to the pI of RhoA (6.2, based on its amino acid sequence) but not of RhoC (6.8) or RhoB (5.3) [20]. In addition, two [^{32}P]ADP-ribosylated spots with identical isoelectric points and electrophoretic mobility are present in platelets (Figure 2b) which are known to contain RhoA as the sole ADP-ribosylation substrate [21]. These data strongly suggest that the ADP-ribosylated protein in HL60, U937 and THP1 cells is RhoA. At present we do not know the nature of the two [^{32}P]ADP-ribosylated protein spots that show slightly different pIs, but both react with anti-RhoA antibody (results not shown). It is possible that forms of RhoA exist that differ with regard to some kind of post-translational modification, for instance protein phosphorylation [22].

***In situ* ADP-ribosylation of RhoA is associated with reduced cell proliferation**

To test the effect of C3 exoenzyme on cell proliferation we added increasing concentrations to freshly diluted HL60 cells and determined cell numbers after 24, 48, 72 and 96 h. The C3 exoenzyme caused a time- and dose-dependent reduction in cell proliferation (Figure 3). After a 96 h incubation with 10 $\mu\text{g}/\text{ml}$ C3 exoenzyme, cell numbers were reduced to 20% of control ($n = 3$, results given in Figure 3 and two other experiments for which the results are not shown). To prove *in situ* ADP-ribosylation of RhoA, control and C3 exoenzyme (10 $\mu\text{g}/\text{ml}$) pretreated HL60 cells were homogenized after different time periods and incubated with [^{32}P]NAD $^{+}$ and C3 exoenzyme. Incorporation of [^{32}P]ADP into C3-treated RhoA was 19, 17, 12

Figure 3 Effect of C3 exoenzyme on proliferation of HL60, U937 and THP1 cells

Cells (a) HL60, (b) U937 and (c) THP1 were treated with *C. botulinum* C3 exoenzyme: ($\diamond$) 2.5 $\mu\text{g}/\text{ml}$, ($\circ$) 5 $\mu\text{g}/\text{ml}$, ($\triangle$) 10 $\mu\text{g}/\text{ml}$ or ($\oplus$) 20 $\mu\text{g}/\text{ml}$. Controls ($\square$) were left untreated. At indicated times the number of cells was determined (see Materials and methods section). The C3 exoenzyme seemed to be stable in culture for at least 72 h because we could reuse 72 h-old C3 exoenzyme-containing medium and inhibit cell proliferation in other cultures. Values are representative of three independent experiments.

and 14% of control at 24, 48, 72 and 96 h respectively ($n = 3$, results given in Figure 4 and two other experiments for which the results are not shown), suggesting that 80–90% of the RhoA substrate was ADP-ribosylated *in situ* at each time point. The C3 exoenzyme (96 h, 10 $\mu\text{g}/\text{ml}$) was less active in U937 and THP1 cells since it inhibited proliferation only by 65 and 21% respectively ($n = 3$, results given in Figure 3 and two other experiments for which the results are not shown). This most likely reflects less efficient uptake of C3 because only 70 and 60% respectively of RhoA became ADP-ribosylated *in situ* (results not shown). Treatment of THP1 cells with a higher C3 exoenzyme concentration (30 $\mu\text{g}/\text{ml}$) increased growth inhibition (Figure 3) and *in situ* ADP-ribosylation of RhoA (results not shown).

Figure 4 *In situ* ADP-ribosylation by C3 exoenzyme in HL60 cells

Cells were treated without (Control) or with 10 µg/ml C3 exoenzyme (C₃). At indicated times cells were homogenized, subjected to [³²P]ADP-ribosylation *in vitro* and analysed as described for Figure 1. One experiment representative of three is shown.

Figure 5 Dose-dependent effect of the C3 exoenzyme on cell proliferation and *in situ* ADP-ribosylation of RhoA

(a) HL60 cells were not treated (Control) or treated for 96 h with indicated concentrations of C3 exoenzyme. Cell numbers were determined (black bars) or cells were homogenized and subjected to [³²P]ADP-ribosylation *in vitro* as described for Figure 1. Incorporation of [³²P]ADP into protein bands (stippled bars) was expressed as a percentage of untreated controls. Each bar represents the mean ± S.D. of three independent experiments. (b) Shows a representative autoradiograph of the [³²P]ADP ribosylation reaction *in vitro*.

Because they were the most sensitive to the C3 exoenzyme we used the HL60 cells for all the following experiments. We next determined the relation between *in situ* ADP-ribosylation of RhoA and inhibition of HL-60 cell proliferation in a dose-response experiment. The results indicate good correlation between cell number and percentage of *in situ* ADP-ribosylated RhoA in HL60 cells treated for 96 h with 2.5, 5 or 10 µg/ml C3 exoenzyme (Figure 5).

In myelomonocytic cells the capacity to proliferate and the extent of cell differentiation are inversely related [23]. We therefore evaluated whether the C3 exoenzyme treatment affected expression of the granulocytic differentiation marker CD11b or the monocytic differentiation marker CD14. For this purpose we used native HL60 cells as well as cells differentiated along the

Table 1 Effect of C3 exoenzyme on differentiation markers in HL60 cells

HL60 cells were treated for 72 h with 1.25% dimethylsulphoxide, 1 µM retinoic acid or 10 nM 1,25-dihydroxycholecalciferol in the absence or presence of 10 µg/ml C3 exoenzyme. The percentage of cells staining positive for CD11b or CD14 was determined by FACS. n.d. indicates not determined. Each value represents the mean ± S.D. of three experiments performed in duplicate.

Cell treatment	Positive cells (%)	
	CD11b	CD14
Control	2 ± 0.1	2 ± 1
Control + C3	8 ± 1	5 ± 1
Dimethylsulphoxide	79 ± 3	n.d.
Dimethylsulphoxide + C3	84 ± 4	n.d.
Retinoic acid	70 ± 2	n.d.
Retinoic acid + C3	74 ± 4	n.d.
1,25-Dihydroxycholecalciferol	n.d.	57 ± 12
1,25-Dihydroxycholecalciferol + C3	n.d.	66 ± 3

Figure 6 The effect of C3 exoenzyme on cell cycle progression in HL60 cells

After 96 h in culture, HL60 cells were diluted with fresh medium to 2 × 10⁵ cells/ml and incubated for 24 h without (a) or with 10 µg/ml C3 exoenzyme (b). Cells were also incubated for 12 h with 1 µg/ml cytochalasin D (c) or 0.3 µg/ml nocodazol (d). The percentage of cells in the G₀/G₁, S or G₂/M phase of the cell cycle was determined by FACS analysis as described in the Materials and methods section. Representative FACS tracings of three independent experiments are shown.

granulocytic lineage by retinoic acid or dimethylsulphoxide, or along the monocytic lineage by 1,25-dihydroxycholecalciferol. We found that expression of CD11b and CD14 in the native and differentiated cells tended to be slightly higher in the presence of the C3 exoenzyme, but differences were minor and not significant (Table 1). This indicates that the effect of C3 exoenzyme on cell proliferation is not due to an enhancement of cell differentiation.

C3 exoenzyme-treated HL60 cells accumulate in the G₂/M-phase of the cell cycle

We tested whether the effect of C3 exoenzyme on the proliferation of HL60 cells might be due to alterations in cell cycle progression.

Figure 7 Time-course of accumulation in the G₂/M phase and *in situ* ADP-ribosylation of RhoA in C3 exoenzyme-treated HL60 cells

Cells were treated for the indicated time-periods with 10 μ g/ml C3 exoenzyme and the percentage of cells in the G₂/M phase (○) or the percentage of *in situ* ADP-ribosylated RhoA substrate (□) was determined as described in the Materials and methods section. Controls (△) were left untreated. Each value represents the mean $\pm$ S.D. of three independent experiments.

The fraction of cells in the G₀/G₁, S or G₂/M phase was determined by cellular DNA-quantification with FACS. After a 24 h treatment with C3 exoenzyme, 33% of HL60 cells accumulated in the G₂/M phase as compared with 10% of controls (Figure 6). Similarly, when cells were treated for 12 h with 1 μ g/ml cytochalasin D to disrupt actin filaments and inhibit cell division, 38% accumulated in the G₂/M phase. Higher concentrations of, or longer incubation times with, cytochalasin D, had no further effect and cytochalasin B (3 μ g/ml) produced identical results (not shown). A considerably higher percentage of cells (76%) arrested in the G₂/M phase when nocodazol was used to destabilize microtubules and inhibit nuclear division (Figure 6). The basis for these different (quantitative) effects of C3 exoenzyme or cytochalasin D and nocodazol are unclear but might be due to cell populations resistant to actin-perturbing agents. These data are consistent with the idea that the C3 exoenzyme inhibits cell proliferation by blocking cell division.

We investigated the relation between the C3 exoenzyme-induced block in mitosis and ADP-ribosylation of RhoA in a time-course experiment. The relative amount of *in situ* ADP-ribosylated RhoA was 20% after 1 h of C3 exoenzyme treatment and increased to a maximum of 80% after 12 h (Figure 7). In comparison, the percentage of HL60 cells in G₂/M was only slightly increased after 6 h of C3 exoenzyme treatment and reached a maximum after 24 h (Figure 7). Hence, it seems that ADP-ribosylation of RhoA precedes the accumulation of cells in mitosis, as would be expected for a causal relationship.

Cytoplasmic division but not nuclear division is blocked in C3 exoenzyme-treated cells

To show that the C3 exoenzyme blocked cell division and not nuclear division we stained nuclei in control and C3 exoenzyme-treated cells. As reference we used cells in which we blocked cell

Figure 8 Nuclear staining

Control (a), C3 exoenzyme-treated (24 h, 10 μ g/ml) (b) or cytochalasin D-treated (12 h, 1 μ g/ml) (c) HL60 cells were stained and photographed at 200-fold magnification (see Materials and methods section). The arrows point to binucleated cells. Photographs representative of three experiments are shown.

division by use of cytochalasin D. After treatment with either cytochalasin D or C3 exoenzyme, binucleate cells accumulated, indicating that nuclear division was intact but cytoplasmic division was inhibited (Figure 8). No binucleate cells could be detected in controls. The percentages of cells showing two nuclei were 30 and 25% after treatment with cytochalasin D or C3 exoenzyme respectively ($n = 3$, results not shown), which correlates well with the percentages of cells accumulated in the G₂/M phase (Figure 6).

DISCUSSION

The results of our study suggest that: (1) RhoA is the ADP-ribosylation substrate of *C. botulinum* C3 exoenzyme in human myelomonocytic HL60, U937 and THP1 cells; (2) *in situ* ADP-ribosylation of RhoA is associated with reduced cell proliferation; and (3) the decrease in cell proliferation is due to inhibition of cell division.

RhoA, B and C can all be ADP-ribosylated by clostridial toxins *in vitro* [3–5], but RhoA was identified as the sole ADP-ribosylation substrate in mouse fibroblasts [7], human blood platelets [21], lymphocytes [20] and myelomonocytic cell lines (this study). It is likely that RhoA and RhoC perform identical functions because their deduced amino acid sequences are 98% identical and both are exclusively located in the cytosol and the plasma membrane [24]. In contrast, RhoB is also present in endosomes [24], is only 88% identical with RhoA and C and is inducible by growth factors in quiescent fibroblasts [25]. So far, the function of RhoB has remained elusive but *in situ* ADP-ribosylation of RhoA was found to inhibit cellular functions that depended on a functional actin cytoskeleton, such as platelet aggregation [26], lymphocyte cytotoxicity [20] and fibroblast motility [9]. Micro-injection experiments indicated that Rho proteins mediate the formation of actin stress fibres and focal adhesion sites in quiescent fibroblasts stimulated with growth factors to enter the cell cycle [27]. Furthermore, ADP-ribosylation of RhoA *in situ* resulted in reduced proliferation of fibroblasts due to a block in G₁-S transition [7]. Such a role for RhoA is very unlikely in round, non-adherent cells growing in suspension. Recently it was shown that micro-injection of C3 exoenzyme or Rho GDI into developing *Xenopus* embryos blocked the formation of the actin-myosin-based cleavage furrow [28]. Here we have directly demonstrated that a Rho protein is involved in cell division in human myelomonocytic tumour cells and we have identified this protein as RhoA.

In view of a report that the C3 exoenzyme affects a particular filamentous actin pool in HL60 cells [29] it seems conceivable that RhoA may be involved in the formation of the contractile actin ring that divides myelomonocytic cells. Activated at the end of mitosis, RhoA might cycle between the cytosol and the cleavage furrow, transporting actin regulatory proteins. Besides investigating these molecular mechanisms it will be very interesting in the future to test whether Rho proteins are somehow involved in the genesis or the progression of abnormal myelomonocytic cell growth.

Recently, crosstalk between Ras- and Rho-dependent signalling pathways was suggested by the discovery of p190, a protein that forms a complex with Ras-GAP and contains Rho-GAP activity [30]. Considering the central role of Ras in malignant cell transformation it is tempting to speculate that Ras, via Rho, can control cell division and thus has another 'handle' to regulate cell proliferation.

We thank Barbara Böhlig for expert technical assistance, Ursula Wielert for help with the cell cycle assay, Franz Vauti for helpful discussions, Alan Hall for providing the pGEX2T-RhoA expression vector and Klaus Aktories for supplying some *Clostridium limosum* ADP-ribosyltransferase. We are particularly grateful to John Glomset for critically reading the manuscript and for continuous support. Some experiments in this study are part of the MD Thesis of M. E. at the University of Munich. Preliminary

experiments were supported by a postdoctoral grant from the Deutsche Forschungsgemeinschaft (Ae11/1-1) to M.A. The work in Munich was supported by August Lenz Stiftung.

REFERENCES

- Hall, A. (1990) *Science* **249**, 635–640
- Boguski, M. S. and McCormick, F. (1993) *Nature (London)* **366**, 643–654
- Chardin, P., Boquet, B., Madaule, P., Popoff, M. R., Rubin, E. J. and Gill, D. M. (1989) *EMBO J.* **8**, 1087–1092
- Braun, U., Habermann, B., Just, I., Aktories, K. and Vandekerckhove, K. (1989) *FEBS Lett.* **243**, 70–76
- Ogorochi, T., Nemoto, Y., Nakajima, M., Nakamura, E., Fujiwara, M. and Narumiya, S. (1989) *Biochem. Biophys. Res. Commun.* **163**, 1175–1181
- Sekine, A., Fujiwara, M. and Narumiya, S. (1989) *J. Biol. Chem.* **264**, 8602–8605
- Yamamoto, M., Marui, N., Sakai, T., Morii, N., Kozaki, S., Ikai, K., Imamura, S. and Narumiya, S. (1993) *Oncogene* **8**, 1449–1455
- Tominaga, T., Sugie, K., Hirata, M., Morii, N., Fukata, J., Uchida, A., Imura, H. and Narumiya, S. (1993) *J. Cell Biol.* **120**, 1529–1537
- Takaishi, K., Kikuchi, A., Kuroda, S., Kotani, K., Sasaki, T. and Takai, Y. (1993) *Mol. Cell. Biol.* **13**, 72–79
- Hirata, K., Kikuchi, A., Sasaki, T., Kuroda, S., Kaibuchi, K., Matsuura, Y., Seki, H., Saido, K. and Takai, Y. (1992) *J. Biol. Chem.* **267**, 8719–8722
- Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K. and Hall, A. (1990) *J. Cell. Biol.* **111**, 1001–1007
- Skorski, T., Kanakaraj, P., Ku, D.-H., Nieborowska-Skorska, M., Canaani, E., Zon, G., Perussia, B. and Calabretta, B. (1994) *J. Exp. Med.* **179**, 1855–1865
- Pendergast, A. M., Quilliam, L. A., Cripe, L. D., Bassing, C. H., Dai, Z., Li, N., Batzer, A., Rabun, K. M., Der, C. J., Schlessinger, J. and Gishizky, M. L. (1993) *Cell* **75**, 175–185
- Diekmann, D., Brill, S., Garrett, M. D., Totty, N., Hsuan, J., Monfries, C., Hall, Ch., Lim, L. and Hall, A. (1991) *Nature (London)* **351**, 400–402
- Cicchetti, P., Mayer, B. J., Thiel, G. and Baltimore, D. (1992) *Science* **257**, 803–806
- Lelias, J. M., Adra, C. N., Wulf, G. M., Guillemot, J. C., Khagad, M., Caput, D. and Lim, B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 1479–1483
- Aepfelbacher, M., Vauti, F., Weber, P. C. and Glomset, J. A. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4263–4267
- Smith, D. B. and Johnson, K. S. (1988) *Gene* **67**, 31–40
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4012
- Lang, P., Guizani, L., Vitté-Mony, I., Stancou, R., Dorseuil, O., Gacon, G. and Bertoglio, J. (1992) *J. Biol. Chem.* **267**, 11677–11680
- Nemoto, Y., Namba, T., Teru-uchi, T., Ushikubi, F., Morii, N. and Narumiya, S. (1992) *J. Biol. Chem.* **267**, 20916–20920
- Fritz, G. and Aktories, K. (1994) *Biochem. J.* **300**, 133–139
- Harris, P. and Ralph, P. (1985) *J. Leukocyte Biol.* **37**, 407–422
- Adamson, P., Paterson, H. F. and Hall, A. (1992) *J. Cell Biol.* **119**, 617–627.
- Jähner, D. and Hunter, T. (1991) *Mol. Cell. Biol.* **11**, 3682–3690
- Morii, N., Teru-uchi, T., Tominaga, T., Kumagai, N., Kozaki, S., Ushikubi, F. and Narumiya, S. (1992) *J. Biol. Chem.* **267**, 20921–20926
- Ridley, A. J., and Hall, A. (1992) *Cell* **70**, 389–399
- Kishi, K., Sasaki, T., Kuroda, S., Itoh, T. and Taka, Y. (1993) *J. Cell Biol.* **120**, 1187–1195
- Koch, G., Norgauer, J. and Aktories, K. (1994) *Biochem. J.* **299**, 775–779
- Settleman, J., Albright, C. F., Foster, L. C. and Weinberg, R. A. (1992) *Nature (London)* **359**, 153–154