ADP-ribosylation of Rho proteins by *Clostridium botulinum* exoenzyme C3 is influenced by phosphorylation of Rho-associated factors

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Specific [32 P]ADP-ribosylation by *Clostridium botulinum* exoenzyme C3 was used to study the involvement of phosphorylation in the regulation of the low-molecular-mass GTP-binding protein Rho. Dephosphorylation of CHO cell extracts by alkaline phosphatase treatment resulted in a 80–90 % reduction in the C3-catalysed [32 P]ADP-ribosylation of Rho proteins in both cytosolic and membrane fractions. Similar results were obtained after dephosphorylation with protein phosphatase type-1 from bovine retina, whereas type-2B and type-2C phosphatases had no effect on the level of subsequent [32 P]ADP-ribosylation of Rho by C3. Incubation of CHO cell lysate under phosphorylation conditions increased the subsequent C3-mediated [32 P]ADP-ribosylation of Rho proteins. The protein kinase inhibitors H7 and H9 had no effect on [32 P]ADP-ribosylation at concentrations which are specific for inhibition of protein kinase A or C. Recombinant

glutathione S-transferase–RhoA fusion protein (GST–RhoA) was phosphorylated by protein kinase A; however, the phosphorylation had no stimulatory effect on the ADP-ribosylation of GST–RhoA by C3. An approx. 48 kDa phosphoprotein was identified which bound specifically to recombinant GST–RhoA fusion protein. By gel-permeation chromatography, Rho-containing complexes of approx. 50 kDa and 130–170 kDa were detected. The ADP-ribosylation of Rho in the 130–170 kDa complex was reduced by alkaline phosphatase pretreatment. The data suggest that Rho activity is influenced by phosphorylation of Rho-associated regulatory factors. Phosphorylation/dephosphorylation of these Rho-regulating factors appears to alter the ability of Rho to serve as a substrate for C3-induced [32 P]ADP-ribosylation.

INTRODUCTION

The highly related low-molecular-mass (~ 21 kDa) GTP-binding proteins RhoA, B and C have been shown to be specifically ADP-ribosylated by the exoenzyme C3 [1–8] from C and D strains of *Clostridium botulinum* [6–8]. As well as C3, bacterial ADP-ribosyltransferases produced by *Bacillus cereus* [9], *Clostridium limosum* [10] and *Staphylococcus aureus* [11–13] have been described which also modify Rho proteins. Morphological changes observed after treatment of cells with C3 or after microinjection of Rho proteins suggest the participation of Rho proteins in the organization of the microfilamental cytoskeleton [3,14,15]. Cell motility [16,17] and smooth muscle contraction [18] have also been proposed to be regulated by Rho proteins. Like other low-molecular-mass GTP-binding proteins, Rho is active in the GTP-bound form and inactive when GDP is bound. Various regulatory proteins such as GTPase-activating proteins (GAPs) and guanine nucleotide dissociation-stimulating (GDS) and dissociation-inhibiting (GDI) proteins have been described [19–23], which control the activity state of Rho. So far, little is known about the regulation of these GTPase-cycle-controlling proteins.

Because phosphorylation plays a central role in cellular regulation (for reviews see [24,25]) the involvement of phosphorylation in the regulation of small GTP-binding proteins of the Rho family seems conceivable. It is known that Ras–GAP can be phosphorylated on tyrosine residues [26]. The Ras–GAP-associated protein p190, which acts as a Rho/Rac–GAP *in vitro*, has been shown to be phosphorylated on tyrosine [27]. Moreover, on the basis of the finding that Rho proteins are involved in

cellular adhesion [28] and from the identification of an integrin-associated focal adhesion kinase [29,30], one might presume an influence of phosphorylation in the regulation of Rho. However, no convincing evidence for this hypothesis has been presented to date. It has been shown that the extent of C3-mediated ADP-ribosylation of Rho proteins is influenced by the guanine nucleotide bound [9,31] and by the binding of Rho to Rho–GDI [32]. Thus it appears that the C3-catalysed ADP-ribosylation of Rho proteins is useful as an indicator of regulatory changes occurring to Rho proteins. Here we have studied whether, in addition to guanine nucleotides and Rho–GDI, the phosphorylation state of Rho or of Rho-associated proteins influences the [32 P]ADP-ribosylation of Rho by C3. Changes in the C3-mediated [32 P]ADP-ribosylation of Rho were used as an indication of the involvement of protein phosphorylation in the regulation of Rho proteins.

MATERIALS AND METHODS

Materials

The protein kinase inhibitors H7 and H9 were obtained from Research Biochemicals Inc. Cell culture materials were obtained from Sarstedt (Nümbrecht, Germany) and Biochrom (Berlin, Germany) respectively. Calf intestine phosphatase (CIP) was obtained from Biolabs (Schwalbach/Taunus, Germany). Protein phosphatases type-1 and type-2C (purified from bovine retina) were obtained from Dr. S. Klumpp (Tübingen, Germany); phosphatase type-2B was from Sigma (C-1907; from bovine brain). The catalytic subunit of protein kinase A was provided by Dr. W. Nastainczyk (Homburg, Germany), and the anti-Rho

Abbreviations used: GAP, GTPase-activating protein; GDS protein, guanine nucleotide dissociation-stimulating protein; GDI protein, guanine nucleotide dissociation-inhibiting protein; CIP, calf intestinal phosphatase; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; GST, glutathione S-transferase.

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GDI antibody JK-5 by Dr. Y. Takai (Kobe, Japan). Radioactive material ($[^{32}\text{P}]\text{NAD}^+$, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $\text{L}\text{-}[^{35}\text{S}]\text{methionine}$) was from NEN (Bad Homburg, Germany).

Cell culture

CHO (hamster ovary cells) and NIH 3T3 cells (mouse fibroblasts) were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10 % heat-inactivated fetal calf serum. Medium was additionally supplemented with L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). For treatment with H7 and H9, the drugs were added directly to the medium.

ADP-ribosylation

After harvesting, cells were disrupted by sonication in a buffer containing 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1.5 mM MgCl_2 and 0.1 mM phenylmethanesulphonyl fluoride (PMSF). After centrifugation (10 min, 600 g , 4 °C), the supernatant was used for protein determination according to Bradford [33]. ADP-ribosylation of Rho proteins by C3 was performed with 10–20 μg of the total cell extract. Incubation was for 30 min at 37 °C in a volume of 50 μl [reaction buffer: 20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM MgCl_2 , 1 mM dithiothreitol (DTT), 10 mM thymidine, 0.2 μM NAD^+ , 0.1 μg of C3 and 0.5 μCi of $[^{32}\text{P}]\text{NAD}^+$]. Labelled proteins were analysed by SDS/PAGE according to Laemmli [34] followed by exposure to Kodak X-OMAT films.

When cytosolic or membrane fractions were used, the total extract was centrifuged (10000 g) for 20 min at 4 °C.

Isolation of recombinant RhoA protein

The complete RhoA coding sequence was amplified from a RhoA plasmid ([20]; a gift from Dr. A. Hall, Institute of Cancer Research, Chester Beatty Laboratories, London, U.K.) by the PCR technique. The oligonucleotides used for RhoA amplification contained additional *Bam*HI and *Eco*RI linker sequences. Using these restriction sites, the PCR product was cloned into a pGEX-2T expression plasmid (Pharmacia). The ~ 50 kDa glutathione S-transferase (GST)–RhoA fusion protein was isolated according to the manufacturer's protocol. The fusion protein was shown to bind GTP and was ADP-ribosylated by C3. The correct reading frame was also confirmed by sequencing. To screen for cellular proteins which interfere with the ADP-ribosylation of the GST–RhoA fusion protein, total cell extract (or BSA as a control) was incubated with GST–RhoA for 30 min on ice. Subsequently ADP-ribosylation was performed.

Phosphatase treatment

Cellular protein (10–20 μg) was incubated in the presence of 5–10 units of CIP for 30–90 min at 37 °C in a total volume of 20 μl [CIP reaction buffer: 50 mM Tris/HCl (pH 9.0), 10 mM MgCl_2 , 0.1 mM ZnCl_2]. After the incubation period, C3-induced ADP-ribosylation was performed in a total volume of 50 μl as described. Changes in the ADP-ribosylation were referred to control conditions (preincubation of the cell extract in the presence of CIP buffer without enzyme). Dephosphorylation reactions with phosphatases type-1, type-2C (purified from bovine retina) and type-2B (Sigma; C-1907) were performed in a

total volume of 10 μl for 2 h at 37 °C (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 2 mM MgCl_2 , 0.1 mM PMSF, 0.5 mg/ml BSA, 0.1 % 2-mercaptoethanol, 10–15 ng of phosphatase/assay). The buffer additionally contained 20 mM MgCl_2 and 1 mM CaCl_2 for dephosphorylations by protein phosphatase type-1 and type-2B respectively.

Phosphorylation of cell extracts

For phosphorylation reactions, cells were sonicated in buffer A (20 mM Tris/HCl, pH 7.4, 10 mM NaCl, 1 mM EDTA, 1 mM MgCl_2 , 1 mM DTT, 5 % glycerol and 0.5 mM PMSF). Afterwards, 20–30 μg of total cell extract (600 g supernatant) was incubated for 60 min at 37 °C in a total volume of 20 μl (phosphorylation buffer: 20 mM Tris/HCl, pH 7.4, 10 mM MgCl_2 , 0.3 mM CaCl_2 , 2 μM cyclic AMP, 50 μM ATP, 2 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ /assay). Subsequently either ADP-ribosylation was performed or the binding of phosphoproteins to GST–RhoA was analysed.

Binding analysis

To identify proteins which bind to RhoA, $\text{L}\text{-}[^{35}\text{S}]\text{methionine}$ -labelled total cell extracts or phosphorylated cell extracts were incubated in the presence of the recombinant GST–RhoA protein (bound to GSH–Sepharose beads) and 0.5 % Triton X-100 for 15 min on ice. After pelleting of the Sepharose-bound GST–RhoA by centrifugation (1 min, 2000 g , room temperature), unspecifically bound proteins were removed by washing twice at room temperature (washing buffer: 20 mM Tris/HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl_2). After the last washing step, SDS sample buffer was added to the pellet and the proteins bound to Sepharose–GST–RhoA were identified by SDS/PAGE [34] and subsequent autoradiography. Unspecific binding was determined by incubation of cell extracts with Sepharose–GST.

Metabolic labelling

For metabolic labelling, cells were grown overnight in minimal essential medium (without L-methionine). Subsequently, minimum essential medium was replaced by complete medium (DMEM). $\text{L}\text{-}[^{35}\text{S}]\text{Methionine}$ (25 $\mu\text{Ci}/\text{ml}$) was added and the cells were further incubated for 6 h at 37 °C. After the incubation, cells were washed with PBS, trypsinized, washed again and frozen at –80 °C. ^{35}S -labelled cells were disrupted by thawing and freezing in buffer A.

Gel-permeation chromatography

To determine the molecular mass of protein complexes containing Rho proteins, cells were disrupted by sonication in buffer A and the extract further incubated in the presence of 1 % Triton X-100 for 30 min on ice. After centrifugation (20 min, 10000 g , 4 °C) the supernatant was loaded on to a Superdex 75 or Superose 12 column (Pharmacia; bed volume 120 ml). The running buffer consisted of 50 mM triethanolamine (pH 7.4), 100 mM NaCl and 1 mM MgCl_2 . Fractions of 300–500 μl were collected and 30 μl of each fraction was subjected to ADP-ribosylation.

Western blotting

After separation of proteins by SDS/PAGE (12.5 % gel), proteins were blotted on to nitrocellulose by semi-dry blotting (Bio-Rad equipment). The filter was blocked with PBS/1 % gelatin for 2 h at room temperature. Hybridization with the monoclonal anti-

Rho-GDI antibody JK-5 (provided by Y. Takai, Kobe, Japan) was carried out overnight in PBS/1% gelatin (antibody dilution 1:200). Rho-GDI proteins were detected after incubation with peroxidase-coupled goat anti-mouse IgG by chemiluminescence.

RESULTS

Phosphorylation increases the C3-mediated [32 P]ADP-ribosylation of Rho proteins

In order to study whether phosphorylation of Rho or Rho-associated proteins might interfere with the extent of C3-mediated [32 P]ADP-ribosylation of Rho proteins, we performed ADP-ribosylation experiments after dephosphorylation of CHO extracts by alkaline phosphatase treatment. As shown in Figure 1, preincubation of CHO cell extracts with alkaline phosphatase resulted in a 80–90% reduction of the C3-catalysed [32 P]ADP-ribosylation of Rho proteins in both the cytosolic and membrane fractions. The decrease in 32 P label incorporation was not

Figure 1 Dephosphorylation of CHO cell extracts by alkaline phosphatase and protein phosphatase type-1 causes a decrease in the C3-catalysed [32 P]ADP-ribosylation of Rho proteins

(a) After centrifugation of 20 μ g of total CHO cell extract (20 min, 10000 g, 4 °C), supernatant (S) and pellet (P) fractions were treated with 10 units or 5 units respectively of CIP for 30–90 min at 37 °C. Subsequently [32 P]ADP-ribosylation was performed as described in the Materials and methods section. Lane C, [32 P]ADP-ribosylation after 90 min of incubation at 37 °C in the presence of CIP buffer without alkaline phosphatase (control). Shown is autoradiography after SDS/PAGE. (b) Densitometric analysis of the autoradiography shown in (a). The ADP-ribosylation of the control (C) is set to 1.0. ●, Supernatant; ■, pellet. (c) Cytosolic CHO extracts (10 μ g) were dephosphorylated by protein phosphatases type-1 (lane 2), type-2B (lane 4) and type-2C (lane 6) for 2 h at 37 °C as described in the Materials and methods section. [32 P]ADP-ribosylation of Rho by C3 was then performed. Reaction products were separated by SDS/PAGE and detected by autoradiography. Lanes 1, 3 and 5, [32 P]ADP-ribosylation of Rho after 2 h of incubation at 37 °C without the appropriate phosphatase (controls).

Figure 2 Stimulation of the C3-catalysed [32 P]ADP-ribosylation of endogenous Rho proteins after phosphorylation of CHO cell extracts

(a) Total cell extracts (20 μ g) from CHO cells were phosphorylated as described in the Materials and methods section. Thereafter, 300 ng of recombinant GST-RhoA fusion protein was added and the C3-catalysed ADP-ribosylation reaction with [32 P]NAD $^{+}$ was performed. Labelled proteins were analysed by SDS/PAGE followed by autoradiography. —ATP, ADP-ribosylation without preceding Mg^{2+} /ATP-dependent phosphorylation reaction; +ATP, ADP-ribosylation after Mg^{2+} /ATP-dependent phosphorylation reaction; +PKA, ADP-ribosylation after phosphorylation reaction in the presence of the catalytic subunit of protein kinase A (0.1 μ g). Controls = ADP-ribosylation of GST-RhoA (0.5 μ g) in the presence of 20 μ g of BSA instead of cell extract without (C1) or in the presence of (C2) phosphorylation buffer. (b) Quantitative densitometric analysis of (a). The [32 P]ADP-ribosylation of the endogenous Rho proteins and of the recombinant GST-RhoA fusion protein respectively were set to 1.0. (c) Protein kinase A (PKA; 0.1 μ g of the catalytic subunit)-induced [32 P]phosphorylation of recombinant GST-RhoA fusion protein (0.5 μ g; lanes 2–4). Phosphorylation was performed in a total volume of 20 μ l for 60 min at 37 °C. For subsequent [32 P]ADP-ribosylation, the phosphorylation reaction was performed in the absence of [γ - 32 P]ATP. Lane 1 contains GST only.

observed with heat-inactivated CIP, and was not influenced by the addition of GDP or guanosine 5-[β -thio]diphosphate (0.1–1.0 mM) to the dephosphorylation reaction (results not shown).

In order to further identify the involvement of cellular phosphatases in Rho regulation, CIP was replaced by protein phosphatase type-1, type-2B or type-2C. As shown in Figure 2(c), only dephosphorylation with bovine retinal protein phosphatase type-1 caused an approx. 50% reduction in 32 P labelling of Rho; type-2B and type-2C had no effect on the level of subsequent ADP-ribosylation of Rho. The results suggested

that phosphoproteins influence the ability of Rho proteins to serve as substrates for [32 P]ADP-ribosylation by C3.

In order to substantiate this conclusion, we studied the influence of phosphorylation on the ADP-ribosylation of Rho. As shown in Figures 2(a) and 2(b), incubation of CHO extracts under phosphorylation conditions induced an approx. 5-fold increase in the ADP-ribosylation of endogenous Rho proteins. The increase in ADP-ribosylation was not caused by the phosphorylation buffer, because ADP-ribosylation of recombinant GST-RhoA fusion protein added after completion of the phosphorylation reaction was not enhanced. The increase in the ADP-ribosylation of endogenous Rho proteins does not seem to be due to phosphorylation of Rho itself, because addition of recombinant RhoA protein to the phosphorylation reaction did not result in 32 P labelling of the recombinant protein (results not shown). An *in vitro* phosphorylation of the recombinant GST-RhoA fusion protein was observed only with the purified catalytic subunit of protein kinase A, but this phosphorylation had no stimulatory effect on subsequent [32 P]ADP-ribosylation of GST-RhoA by C3 (Figure 2c). In agreement with this finding, supplementation of the phosphorylation reaction of CHO extracts with the catalytic domain of protein kinase A did not further stimulate the [32 P]ADP-ribosylation of Rho by C3 (Figures 2a and 2b). Thus we conclude that the observed stimulation of the [32 P]ADP-ribosylation of endogenous Rho proteins after phosphorylation is not due to protein kinase A-dependent phosphorylation of Rho itself.

In order to further elucidate the involvement of protein kinases in regulation of Rho, CHO cells were treated with the protein kinase inhibitors H7 and H9 [35]. As shown in Figure 3, an approx. 30 % reduction in the ADP-ribosylation of membranous Rho proteins was observed 24 h after the addition of H7/H9 (each at 40 μ M) to the medium. No significant (> 10 %) change in Rho ADP-ribosylation was detected in the crude cytosolic fraction. The same results were obtained after 12 h of treatment with H7/H9 (results not shown). However, the inhibitory effect of H7/H9 on the ADP-ribosylation of Rho was not observed at concentrations lower than 40 μ M (Figure 3). This finding indicates that the decrease in ADP-ribosylation caused by H7/H9 is most likely not due to the specific inhibition of protein kinases A or C.

Because growth factors have been shown to induce stress fibre formation [29] and, furthermore, to cause tyrosine phosphorylation of cytoskeleton-associated factors [36], we next investigated the involvement of tyrosine phosphorylation in the C3-catalysed [32 P]ADP-ribosylation of Rho. For this purpose, serum-deprived NIH 3T3 cells (24 h, 0 % fetal calf serum) were induced with medium containing 20 % fetal calf serum. However, no change in the C3-mediated [32 P]ADP-ribosylation of Rho proteins was detected in extracts from cells serum-induced for 2–60 min (results not shown). Furthermore, immunoprecipitation experiments with monoclonal anti-phosphotyrosine antibody (PT66; Sigma) followed by C3-mediated ADP-ribosylation failed to demonstrate a specific co-precipitation of Rho proteins (results not shown). Thus an influence of tyrosine phosphorylation in the observed changes in Rho ADP-ribosylation seems unlikely.

Specific binding of an ~ 48 kDa phosphoprotein to recombinant RhoA protein

The data presented so far suggest that phosphorylation of Rho-associated proteins enhances the ability of Rho to serve as substrate for C3-mediated [32 P]ADP-ribosylation. This hypothesis suggests an association of Rho with other phosphoproteins.

Figure 3 Influence of protein kinase inhibitors H7 and H9 on the C3-catalysed [32 P]ADP-ribosylation of Rho proteins

(a) Exponentially growing CHO cells were treated with H7 or H9 (10–40 μ M). At 24 h after treatment, cells were harvested and cytosolic (S) and membrane (P) fractions were [32 P]ADP-ribosylated by C3 as described in the Materials and methods section. Shown is the autoradiography after SDS/PAGE. Lanes C, untreated cells. (b) Quantitative densitometric analysis of (a). The [32 P]ADP-ribosylation of untreated control cells is set to 1.0.

Figure 4 Specific binding of cellular proteins to recombinant GST-RhoA fusion protein

(a) Total extracts from L-[35 S]methionine-labelled CHO and NIH 3T3 cells were incubated with 1 μ g of the recombinant GST-RhoA fusion protein (bound on GSH-Sepharose beads) for 30 min on ice. After washing, GST-RhoA-bound proteins were electrophoretically analysed by SDS/PAGE and detected by autoradiography. GST bound to GSH-Sepharose beads was used as control to identify unspecific binding. (b) Extracts from CHO cells were [32 P]phosphorylated as described in the Materials and methods section. GST-RhoA-binding phosphoproteins were detected and analysed as in (a). The arrow indicates an ~ 48 kDa 35 S- or 32 P-labelled protein which was specifically bound to the recombinant GST-RhoA fusion protein.

Figure 5 Analysis of Rho-associated proteins by gel-permeation chromatography

(a) A Triton extract of CHO cells (2 mg) was separated by gel-permeation chromatography on a Superdex 75 column; a 30 μ l sample of each fraction collected was analysed by C3-mediated [32 P]ADP-ribosylation as described in the Materials and methods section. In order to identify Rho-binding proteins which influence the level of Rho ADP-ribosylation, 30 μ l of each fraction was preincubated for 15 min on ice with the recombinant GST-RhoA fusion protein. [32 P]ADP-ribosylated proteins were separated by SDS/PAGE and identified by autoradiography. (b) Quantitative densitometric analysis of (a). The levels of [32 P]ADP-ribosylation of the recombinant GST-RhoA and the endogenous Rho proteins from fraction no. 2 were set to 1. (c) Influence of detergent (SDS; 0.01 %) and phospholipid (phosphatidylinositol phosphate; PIP; 0.1 mg/ml) on the [32 P]ADP-ribosylation of Rho proteins in fractions containing high-molecular-mass (no. 5) and low-molecular-mass (no. 14) proteins. A 30 μ l sample of the given fraction was used for ADP-ribosylation in the presence of SDS and PIP respectively. Shown is autoradiography after SDS/PAGE. C, [32 P]ADP-ribosylation without SDS or PIP (control). (d) Proteins from 100 μ l samples of the fractions 6–18 were precipitated with trichloroacetic acid (10%) and subsequently separated by SDS/PAGE. Rho-GDI proteins were detected by Western analysis with a monoclonal anti-Rho-GDI antibody (JK-5; diluted 1:200) as described in the Materials and methods section.

Therefore we investigated whether cellular proteins can specifically bind to recombinant GST-RhoA fusion protein. Cells were metabolically labelled with L-[35 S]methionine and the cell extract was treated with GST-RhoA fusion protein immobilized to Sepharose beads. Thereafter, Rho-bound 35 S-labelled proteins were isolated by precipitation and identified by SDS/PAGE. As a control, immobilized GST was used. As shown in Figure 4(a), several putative RhoA-associated proteins were isolated. When the identical procedure was performed with [32 P]phosphorylated extracts from CHO cells, GST-RhoA beads precipitated a phosphorylated protein of approx. 48 kDa. A RhoA-binding protein of the same size was also detected in the binding studies with 35 S-labelled extracts (Figure 4a).

Rho proteins are complexed with different factors

In order to obtain support for the hypothesis of an association of Rho proteins with regulatory (phospho)proteins, we separated

total extracts from CHO cells by gel-permeation chromatography on Superdex 75. When the various fractions eluted were [32 P]ADP-ribosylated, the 32 P label was mainly detected in elution fractions of molecular mass > 60 kDa (Figures 5a and 5b). The molecular mass of a second, poorly [32 P]ADP-ribosylated, protein complex was estimated to be 40–50 kDa (Figures 5a and 5b). The molecular mass of the larger complex was determined more exactly by gel-permeation chromatography on Superose 12, revealing a molecular mass of 130–170 kDa (results not shown). Alkaline phosphatase pretreatment of the high-molecular-mass fractions collected from the Superdex 75 column prevented subsequent [32 P]ADP-ribosylation of Rho proteins (results not shown). No [32 P]ADP-ribosylation was observed in fractions containing 20–25 kDa proteins (Figure 5a), indicating that Rho proteins are quantitatively complexed. Also, after size fractionation of CHO extracts by centrifugation on Centricon 30 (Amicon), no < 30 kDa proteins were detectable which were [32 P]ADP-ribosylated by C3 (results not shown).

In order to detect cellular proteins which stimulate the C3-mediated [32 P]ADP-ribosylation of Rho proteins by their binding to Rho, the recombinant GST-RhoA fusion protein was preincubated with aliquots from the elution fractions (Figure 5a). As shown in Figures 5(a) and 5(b), enhancement of the [32 P]ADP-ribosylation of GST-RhoA was observed with aliquots from the high-molecular-mass fractions. Thus these data suggest the existence of at least one factor of molecular mass > 70 kDa which stimulated the C3-catalysed [32 P]ADP-ribosylation of Rho.

Next we studied the influence of SDS and of phosphatidylinositol phosphate on the C3-catalysed ADP-ribosylation of Rho in fractions eluted from gel-permeation chromatography. SDS and phosphatidylinositol phosphates have been shown to release Rho from the complex with Rho-GDI [32], thereby greatly enhancing its ADP-ribosylation by C3. The ADP-ribosylation of Rho in the low-molecular-mass (40–50 kDa) complex was dramatically (> 30-fold) increased in the presence of both agents (Figure 5c), indicating an association of Rho with Rho-GDI. In contrast, neither phosphatidylinositol phosphate nor SDS influenced the ADP-ribosylation of Rho in the high-molecular-mass complex (Figure 5c), indicating that Rho-GDI is not involved in the formation of this complex. These data were confirmed by Western analysis, where only the 40–50 kDa ADP-ribosylated complex contained detectable amounts of Rho-GDI (Figure 5c). In total, the data indicate the association of Rho proteins with at least two different kinds of proteins. One of these proteins was identified as Rho-GDI. The other Rho-binding protein is assumed to be a phosphoprotein whose phosphorylation state influences the extent of the C3-mediated [32 P]ADP-ribosylation of Rho proteins.

DISCUSSION

We have studied the possible involvement of phosphorylation reactions in the regulation of Rho proteins. As an indicator of changes of Rho functions caused by phosphorylation, we used the specific ADP-ribosylation of Rho proteins by the exoenzyme C3 from *Clostridium botulinum*. The C3-catalysed [32 P]ADP-ribosylation of Rho has already been shown to be influenced by guanine nucleotide binding and the binding of Rho-GDI [9,31,32]. Thus we speculated that the ability of Rho to serve as a substrate for C3-mediated ADP-ribosylation might also be influenced by phosphorylation of Rho or Rho-associated proteins. In fact, dephosphorylation of cytosolic and membrane fractions with alkaline phosphatase resulted in an almost complete inhibition of the C3-mediated [32 P]ADP-ribosylation of Rho. Dephosphorylation experiments with protein phosphatases type-1, -2b and -2c revealed a decrease in C3-mediated [32 P]ADP-ribosylation of Rho only after treatment with the first of these enzymes. Interestingly, a specific disassembly of the actin microfilament network by protein phosphatase type-1 has been described [37]. On the other hand, phosphorylation of total cell extracts prior to ADP-ribosylation caused an increase in the [32 P] labelling of Rho proteins. These findings indicate the involvement of phosphorylation in the regulation of Rho.

In view of observations that actin is depolymerized by protein kinase A-induced phosphorylation [38–40] and that Rho proteins may play a central role in the organization of the cytoskeleton [3,14,15], we tested recombinant GST-RhoA fusion protein as substrate for protein kinase A. Although no typical Arg-Arg-Xaa-Ser consensus sequence for protein kinase A was detectable in the amino acid sequence of RhoA, the recombinant fusion protein was accepted as a substrate. However, phosphorylation of GST-RhoA by protein kinase A induced no stimulation of

ADP-ribosylation by C3, indicating that the observed increase in Rho ADP-ribosylation upon phosphorylation is independent from direct Rho phosphorylation by protein kinase A. No phosphorylation of recombinant RhoA protein was observed with extracts from CHO and NIH 3T3 cells (exponentially growing, serum-starved and serum-induced) or after microinjection of recombinant RhoA protein together with [γ - 32 P]ATP into frog oocytes (I. Just and K. Aktories, unpublished work).

Treatment of CHO cells with inhibitors of protein kinase A and protein kinase C reduced the [32 P]ADP-ribosylation of Rho in membrane fractions. However, this effect depended on very high concentrations of H7 and H9 (each 40 μ M). Given the K_i values for H7 and H9 (H7, 3.0 μ M and 6.0 μ M for protein kinases A and C respectively; H9, 1.9 μ M and 18 μ M for protein kinases A and C respectively), the observed decrease in [32 P] labelling of Rho is probably not mediated by a specific inhibition of protein kinase A or C. Furthermore, induction of tyrosine phosphorylation by serum stimulation of serum-deprived NIH 3T3 cells followed by immunoprecipitation with an anti-phosphotyrosine antibody revealed no co-precipitation of Rho proteins, excluding a tight association of Rho with phosphotyrosine proteins. The data suggest that phosphorylation events stimulate the C3-catalysed [32 P]ADP-ribosylation of Rho proteins independently from protein kinase A, protein kinase C and tyrosine kinases. Thus serine/threonine kinases other than protein kinases A or C affect Rho ADP-ribosylation.

Although phosphorylation of endogenous Rho proteins under specific conditions seems possible, our data indicate that the phosphorylation not of Rho itself, but rather of Rho-associated factors, mediates the observed enhancement in the [32 P]ADP-ribosylation of Rho by C3. In agreement with this hypothesis, we observed an approx. 48 kDa phosphoprotein which, after phosphorylation of CHO extracts and subsequent SDS/PAGE, was identified to bind specifically to the recombinant GST-RhoA fusion protein. It is already known that Ras-GAP proteins are regulated by phosphorylation [26]. Accordingly, regulation of Rho-GAP proteins by phosphorylation is also feasible. Considering the apparent molecular mass of the Rho-binding phosphoprotein (~ 48 kDa) one might speculate that the phosphoprotein detected is a Rho-GAP. Rho-GAPs of 28 kDa [41,42], 50 kDa, 150 kDa [43] and 190 kDa [27] have been described. It is noteworthy that Manser et al. [43] exclusively detected 50 kDa and 150 kDa Rho-GAP by using an overlay assay, but did not detect a 28 kDa Rho-GAP which was isolated by purification from bovine adrenal gland and from human spleen [41,42].

The association of GST-RhoA with an ~ 48 kDa phosphoprotein was detected after precipitation with the immobilized fusion protein and subsequent SDS/PAGE. In order to support the idea of complex formation of Rho with other proteins, and in order to identify such Rho-binding proteins in their native state, we analysed cellular proteins by gel-permeation chromatography. In agreement with several authors [22,32] we found an approx. 46 kDa complex which most likely consists of Rho and Rho-GDI. This was concluded from (i) the large increase in the [32 P]ADP-ribosylation of Rho in the presence of SDS and phosphatidylinositol phosphate, which have been shown to release Rho from Rho-GDI [32], and (ii) the immunological detection of Rho-GDI in the ~ 46 kDa complex by Western blotting. The observed Rho-containing complex of > 60 kDa (130–170 kDa as determined by Superose 12 chromatography) has not been described so far. This complex does not contain Rho-GDI and its [32 P]ADP-ribosylation is not affected by SDS or phosphatidylinositol phosphates. Because [32 P]ADP-ribosylation of the 130–170 kDa complex was prevented by alkaline

phosphatase pretreatment, we speculate that under native conditions phosphoproteins of 110–150 kDa are associated with Rho. This Rho protein complex appears to be a favoured substrate for C3-catalysed ADP-ribosylation as compared with uncomplexed or Rho-GDI-complexed Rho proteins. Accordingly, ADP-ribosylation of the recombinant GST-RhoA protein was greatly increased in the presence of aliquots from the > 60 kDa fractions. An influence of high-molecular-mass protein complexes on the ADP-ribosylation of purified Rho proteins has been described previously by Ohtsuka et al. [44]. Two observations suggest that the high-molecular-mass Rho [32 P]ADP-ribosylated complex contains Rho and Rho-GAP proteins. First, the high-molecular-mass Rho-containing fraction co-eluted with Rho-GAP activity (60% increase compared with BSA control) and, furthermore, a direct correlation of Rho-GAP activity with the extent of Rho [32 P]ADP-ribosylation has been observed in various tissues (G. Fritz and K. Aktories, unpublished work). Further studies are under way to investigate the influence of phosphorylation on Rho-GAP.

In summary, our results show that Rho proteins are complexed with different factors. Two of them seem to be Rho-GDI and Rho-GAP. Furthermore, it seems likely that the phosphorylation/dephosphorylation of Rho-associated factors regulates the biological activity of Rho, as indicated by a change in the ability of Rho to serve as a substrate for C3-catalysed [32 P]ADP-ribosylation.

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