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# Phosphorylation states of Cdc42 and RhoA regulate their interactions with Rho GDP dissociation inhibitor and their extraction from biological membranes

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The Rho GDP dissociation inhibitor (RhoGDI) regulates the activation–inactivation cycle of Rho small GTPases, such as Cdc42 and RhoA, by extracting them from the membrane. To study the roles of $Mg^{2+}$, phosphatidylinositol 4,5-bisphosphate ($PIP_2$), ionic strength and phosphorylation on the interactions of RhoGDI with Cdc42 and RhoA, we developed a new, efficient and reliable method to produce prenylated Rho proteins using the yeast *Saccharomyces cerevisiae*. It has been previously reported that protein kinase A (PKA)-treatment of isolated membranes increased RhoA extraction from membranes by RhoGDI [Lang, Gesbert, Delespine-Carmagnat, Stancou, Pouchelet and Bertoglio (1996) EMBO J. **16**, 510–519]. In the present study, we used an *in vitro* affinity chromatography system to show that phosphorylation of RhoA and Cdc42 significantly increased their interaction with RhoGDI under physiological conditions of ionic strength. This increase was independent of the nucleotide (GDP or guanosine 5′-[γ-thio]-triphosphate) loaded on to the Rho proteins, as well as of $Mg^{2+}$ and $PIP_2$. Moreover, dephosphorylation of rat brain membranes by alkaline phosphatase significantly decreased the extraction of RhoA and Cdc42 by RhoGDI. Subsequent re-phosphorylation by PKA restored the extraction levels, indicating the reversibility of this process. These results clearly demonstrate that the phosphorylation states of Cdc42 and RhoA regulate their interactions with RhoGDI and, consequently, their extraction from rat brain membranes. We therefore suggest that phosphorylation is a mechanism of regulation of Cdc42 and RhoA activity that is independent of GDP–GTP cycling.

Key words: ionic strength, rho, small GTP-binding proteins, GDI.

## INTRODUCTION

The Rho family of small GTP-binding proteins contains several members, including RhoA, RhoB, RhoE, Cdc42 and Rac1. Rho GTPases function as tightly regulated molecular switches that govern a series of cellular functions [1]. Activated Rho proteins are able to initiate signalling pathways controlling reorganization of the actin cytoskeleton during growth, division, motility, adhesion and transformation of cells [2]. They also interact with downstream effectors that regulate diverse cellular functions, including oxidant generation, apoptosis, membrane trafficking, cell cycle control and gene expression [1,3]. Moreover, Rho proteins are essential to the induction of malignant transformation by Ras GTPases [4].

Rho proteins interact with Rho GDP dissociation inhibitor (RhoGDI) protein and its homologues D4/LyGDI and RhoGDIγ. In addition to being an inhibitor of nucleotide dissociation, RhoGDI plays a crucial role in the shuttling of Rho GTPases between the cytoplasm and membranes [5]. During the course of cell activation, Rho proteins complexed with RhoGDI in the cytosol translocate to the membranes by a mechanism that is spatially and temporally determined [6]. The involvement of two groups of proteins (the GDP dissociation stimulators and the ezrin/radixin/moesin family) in the recruitment of Rho proteins to the membranes has been suggested [7,8]. However, the precise sequence of events leading to the release of Rho proteins from RhoGDI followed by nucleotide exchange and attachment to the membrane remains to be determined.

Aside from its role in the translocation of cytosolic Rho toward membranes, RhoGDI also extracts Rho, Cdc42 and Rac from the membranes and forms inactive cytosolic complexes with them. Characterization of the cellular events leading to membrane extraction of Rho GTPases will be an important step in understanding the regulation of Rho protein activity. Early studies showed that the interaction with RhoGDI was dependent on the nucleotide state of Rho proteins. Sasaki et al. [9] reported that GDP-bound RhoA and Rac1 had 10-fold higher affinities for RhoGDI than the GTP-bound forms. However, RhoGDI extracts GDP- as well as GTP-bound Cdc42 from membranes [10]. Fluorescence spectroscopy showed that the apparent $K_d$ values for the binding of RhoGDI to GDP- or GTP-bound Cdc42 are almost identical (30 nM) [11]. Regardless of their nucleotide state, RhoGDI extracts only a fraction of RhoA and Cdc42 from the membranes [12], even if sequential extractions are performed [13]. These studies show clearly that other factors are involved in the regulation of the interaction between RhoGDI and Rho proteins. For example, phosphatidylinositol 4-phosphate or phosphatidylinositol 4,5-bisphosphate ($PIP_2$) increased nucleotide exchange in the Rac1–RhoGDI complex [14]. More recently, Faure et al. [15] demonstrated that phosphoinositides partially open RhoGDI–RhoA complexes, sufficient to allow nucleotide exchange to occur even if RhoA is still bound to RhoGDI. This suggests that phospholipids might modify the interaction between RhoGDI and Rho proteins.

The phosphorylation–dephosphorylation cycle controls the activity of a large number of proteins, including various GTPases

Abbreviations used: DTT, dithiothreitol; [$^3$H]GDP, [8,5′-$^3$H]GDP trisodium salt; GST, glutathione S-transferase; GTP[S], guanosine 5′-[γ-thio]triphosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKAc, catalytic subunit of PKA; PKC, protein kinase C; RhoGDI, Rho GDP dissociation inhibitor; ROK, Rho coiled-coiled kinase; TBS, Tris-buffered saline; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone.

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of the Ras superfamily. For instance, protein kinase A (PKA) phosphorylates Ser[180] of Rap1 and Ser[179] of Rap1B in platelets and neutrophils, causing their translocation from membranes to the cytosol [16,17]. Phosphorylation of a serine residue (Ser[196]) also modifies the cellular location of other small GTPases, like Rab1A and Rab4, which are translocated to the cytosol [18,19]. Whether RabGDI is involved in this translocation is not yet known. In addition, Martin et al. [20] recently reported that PKA exerts an important regulatory role in the recruitment of ADP-ribosylation factor 1 into Golgi membranes. K-Ras can be phosphorylated by PKA or protein kinase C (PKC), *in vitro* and *in vivo*, on Ser[181] in the C-terminal domain [21]. Moreover, phosphorylation of RhoA by PKA increased RhoGDI extraction of GTP-bound RhoA from natural killer lymphocyte membranes [22]. This suggests that the phosphorylation state of Rho GTPases could modulate their interactions with their regulatory proteins and effectors. Consistent with this, phosphorylation of RhoA at Ser[188] by PKA was shown to decrease its binding to the effector Rho coiled-coiled kinase (ROK)$\alpha$ and to inhibit the activity of this kinase, which is involved in the regulation of actin polymerization [23]. Another downstream effector of RhoA, phospholipase D, was inhibited by phosphorylation of RhoA [24]. The effect of phosphorylation on Cdc42 or Rac1 binding to RhoGDI has never been reported. However, the phosphorylation of Rac1 at Ser[71], by Akt protein kinase, inhibits Rac1 GTP-binding capacity and the inhibition of Akt activity modulates Rac1 downstream signalling, causing an up-regulation of c-Jun N-terminal kinase/stress-activated protein kinase activity [25].

To improve our understanding of the activation–inactivation cycle of RhoA and Cdc42, we investigated the roles of various cellular factors that may control the interaction of these proteins with RhoGDI. Since prenylation of Rho proteins is an essential prerequisite for their binding with RhoGDI [26,27], we report in the present study the development of a eukaryotic expression system to produce post-translationally modified proteins using the yeast *Saccharomyces cerevisiae*. This method is faster and simpler then those previously published employing the purification of Rho proteins from bovine brain or their expression using the baculovirus system [27,28]. Moreover, it provided sufficient amounts of purified prenylated Cdc42 and RhoA to study their *in vitro* interaction with RhoGDI under highly controlled conditions.

A previous report by Lang et al. [22] showed that RhoA phosphorylation by the catalytic subunit of PKA (PKAc) increased its extraction from membranes by RhoGDI, in a hypotonic phosphorylation buffer. In the present study, we investigated the roles of nucleotides, $Mg^{2+}$, $PIP_2$, ionic strength, PKA-dependent phosphorylation and combinations of these factors in the formation of complexes between RhoGDI and Cdc42 or RhoA, using affinity chromatography columns. In addition, we studied the roles of ionic strength, as well as of phosphorylation and dephosphorylation, on the extraction of Rho proteins from rat brain membranes. We showed that phosphorylation of the membranes with PKAc increased the extraction of RhoA and Cdc42 by RhoGDI, whereas dephosphorylation by alkaline phosphatase decreased it significantly. Taken together, our results strongly suggest that the phosphorylation states of Cdc42 and RhoA regulate their interaction with RhoGDI and thus play a key role in their activation–inactivation and in the control of the actin cytoskeleton organization. Moreover, the interaction between phosphorylated Cdc42 or RhoA and RhoGDI was increased in the presence of physiological ionic strength and became independent of the nucleotide {GDP or guanosine 5′-[$\gamma$-thio]triphosphate (GTP[S])} loaded on to the Rho proteins. Thus these data suggest that phosphorylation is an alternative mechanism to terminate RhoA and Cdc42 activity independently of the nucleotide state.

## EXPERIMENTAL

### Materials

Zymolyase 20T was obtained from ICN (Costa Mesa, CA, U.S.A.) and benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK) was purchased from Bachem (Torrance, CA, U.S.A.). The following affinity-purified antibodies were obtained. Anti-RhoA (monoclonal antibody), anti-Cdc42 (polyclonal antibody), anti-RhoE (Rho8 polyclonal antibody) and anti-RhoGDI (polyclonal antibody) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), anti-Rac1 (monoclonal antibody) was from Transduction Laboratories (Lexington, KY, U.S.A.), and anti-phosphoserine (polyclonal antibody) was from Research Diagnostics (Flanders, NJ, U.S.A.). [8,5′-$^{3}$H]GDP trisodium salt ([$^{3}$H]GDP) was obtained from NEN Life Science Products (Boston, MA, U.S.A.). GSH, glutathione S-transferase (GST), GSH–Sepharose 4B beads, thrombin and [$\gamma$-$^{32}$P]ATP (5000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Montreal, QC, Canada). PKAc, protein kinase inhibitor type II and alkaline phosphatase were purchased from Sigma–Aldrich (Oakville, ON, Canada). Male Sprague–Dawley rats (250–300 g) were obtained from Charles River (St-Constant, QC, Canada).

### Construction and transformation of yeast expression vectors

cDNAs encoding Cdc42, RhoA and RhoE were subcloned into the pYEX 4T-2 expression vector and the resulting constructs were inserted into *S. cerevisiae* strain DY150 using the lithium acetate protocol [29]. Transformants were selected by plating cells on to uracil-deficient plates.

### Yeast culture and membrane preparation

One colony of yeast expressing GST–Cdc42, GST–RhoA or GST–RhoE was transferred into 10 ml of uracil-deficient culture medium [30] and placed in an incubator/shaker (New Brunswick Scientific Co., Edison, NJ, U.S.A.) for 18 h at 30 °C, at 200 rev./min. An aliquot of this culture (1 ml) was added to 1 litre of the same medium and incubated for a further 18 h. Yeast cultures were then centrifuged at 1500 ***g*** for 5 min at 4 °C. Pellets were resuspended in 1 vol. of ice-cold water and re-centrifuged. The supernatants were discarded, and then the pellets were re-suspended in zymolyase buffer (50 mM Tris, 10 mM $MgCl_2$ and 1 M sorbitol, pH 7.5) containing 30 mM dithiothreitol (DTT) and incubated for 15 min at 22 °C to disrupt disulphide bonds in the yeast cell walls. Following further centrifugation (1500 ***g*** for 5 min at 4 °C), the pellets were resuspended in zymolyase buffer containing 1 mM DTT and 200 units of zymolyase/g of pellet, and the yeast proteoglycan cell walls were digested for 1 h at 30 °C with agitation. Samples were centrifuged as described above and the pellets were washed with zymolyase buffer containing 1 mM DTT. Pellets were then resuspended in lysis buffer (50 mM Tris, 10 mM $MgSO_4$, 1 mM EDTA, 10 mM potassium acetate and 1 mM DTT, pH 7.5) containing a protease inhibitor cocktail (100 $\mu$M Pefabloc, 2 $\mu$M E-64, 100 $\mu$M benzamidine, 300 nM antipain, 2 $\mu$M leupeptin and 1.5 $\mu$M pepstatin A) and washed once. Yeast cell lysis was achieved by two cycles of nitrous cavitation at 1500 lbf/in$^2$ (1 lbf/in$^2$ = 6.9 kPa) for 15 min on ice. Cell lysates were then centrifuged at 1500 ***g*** for 10 min at 4 °C to remove unbroken cells and nuclei. Pellets were discarded and supernatants were centrifuged at 100000 ***g*** for 1 h. The final pellets of crude membranes were resuspended

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in 20 mM Hepes/Tris buffer (pH 7.0) containing 10 mM $MgCl_2$. Protein content was determined with the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.) using BSA as a standard. Membranes containing prenylated GST–Cdc42, GST–RhoA or GST–RhoE were then divided into aliquots and frozen at $-80$ °C until use. The *S. cerevisiae* system overexpressing GST–Cdc42 or GST–RhoA used in the present study produced approx. 500 $\mu$g of Rho proteins/litre of culture. Since the cytosol contains a mixture of prenylated and non-prenylated Rho GTPases, we chose to keep only the membrane fraction containing approx. 25 $\mu$g of prenylated Cdc42 or RhoA/litre of yeast culture.

### Expression of recombinant RhoGDI, RhoA and Cdc42 in *Escherichia coli*

Expression of GST–RhoGDI, GST–RhoA and GST–Cdc42 fusion proteins using a recombinant pGEX-2T vector in transformed *Escherichia coli* was carried out as previously described [31]. Fusion proteins were isolated by affinity chromatography on GSH–Sepharose beads. When necessary, the GST tag was cut by thrombin following the manufacturer's instructions (Amersham Pharmacia Biotech). SDS/PAGE and Western-blot analysis were used to assess the purity and identity of the proteins.

### Purification of prenylated GST–Cdc42 and GST–RhoA

To extract prenylated GST–RhoA or GST–Cdc42 from membranes, 500 $\mu$g of yeast membranes was incubated with 20 $\mu$g of RhoGDI in 20 mM Hepes/Tris buffer (pH 7.0) containing 10 $\mu$M Z-VAD-FMK, for 30 min at 25 °C. Samples were then centrifuged at 40000 ***g*** for 30 min at 4 °C in order to separate supernatants containing prenylated Rho proteins complexed with RhoGDI. CHAPS was added to the supernatants at a final concentration of 1 % (w/v) and they were incubated for 15 min on ice to break the small GTPase–RhoGDI complexes. The supernatant from 1 mg of membranes, containing approx. 350 ng of fusion protein, was then loaded on to each column containing 150 $\mu$l of GSH–Sepharose beads. Columns were washed five times with 600 $\mu$l of buffer containing 20 mM Hepes/Tris (pH 7.0), 10 mM $MgCl_2$ and 1 % (w/v) CHAPS to remove all of the RhoGDI and other possible contaminants. Washing buffer [20 mM Hepes/Tris (pH 7.0), 10 mM $MgCl_2$ and 0.1 % CHAPS] was added to decrease the amount of detergent on the column ($3 \times 600$ $\mu$l).

### Loading Rho proteins with nucleotides

Nucleotide loading was performed on the columns, following a protocol adapted from Self and Hall [32] and tested by the addition of 5 $\mu$Ci of [$^3$H]GDP (10 mM). Briefly, columns were incubated for 10 min with 20 mM Hepes/Tris, 10 mM EDTA and 10 mM GDP or GTP[S] at pH 7.0. EDTA was then removed with washing buffer ($3 \times 600$ $\mu$l). Beads were incubated for another 10 min with 20 mM Hepes/Tris, 10 mM $MgCl_2$ and 10 mM GDP or GTP[S] at pH 7.0. Excess nucleotides were removed with washing buffer ($3 \times 600$ $\mu$l), with or without $MgCl_2$, depending on the conditions required for each sample.

### Analysis of interactions between Rho GTPases and RhoGDI

Following the completion of the purification and the nucleotide exchange on prenylated Rho proteins, the study of the effect of various factors that might affect the interaction between RhoGDI and prenylated Rho proteins was performed. RhoGDI (10 $\mu$g) was added to each column containing GST–Cdc42 or GST–RhoA in the presence of 20 mM Hepes/Tris (pH 7.0) with or without 10 mM $MgCl_2$ and/or 50 $\mu$M $PIP_2$ and/or 150 mM KCl. Prior to its addition on to the columns, $PIP_2$ was resuspended in water and sonicated. Proteins were allowed to form complexes for 30 min at 22 °C. Following incubation, columns were rinsed five times with 600 $\mu$l of washing buffer to remove excess unbound RhoGDI. The flowthrough of the last wash was collected and analysed by SDS/PAGE and Western blotting, in order to confirm that all excess RhoGDI had been washed out. To dissociate the Rho GTPase–RhoGDI complexes from the beads, Laemmli sample buffer [33] was added and the beads were heated at 100 °C. SDS/PAGE and Western-blot analysis were performed to determine the amount of GST–RhoA or GST–Cdc42 and RhoGDI that had been complexed. Recombinant proteins purified from *E. coli* were used as standards on each gel to determine the amount (in pmol) of the particular proteins loaded into each well of the gels.

### Phosphorylation of prenylated Rho proteins on GSH–Sepharose columns and interaction with RhoGDI

After GST–Cdc42 or GST–RhoA had been extracted from *S. cerevisiae* membranes by RhoGDI, and purified on GSH–Sepharose columns, nucleotide exchange was performed as described above. Columns were then rinsed four times with 600 $\mu$l of washing buffer. Phosphorylation of GST–RhoA or GST–Cdc42 bound to GSH–Sepharose beads was carried out with PKAc (20 units) in 50 mM Tris (pH 7.5) containing 20 $\mu$M ATP and 10 mM $MgCl_2$ for 1 h at 37 °C. PKAc was removed by rinsing with the washing buffer and the columns were then incubated with RhoGDI (10 $\mu$g) in 20 mM Hepes/Tris (pH 7.0) containing 10 mM $MgCl_2$ and 15 $\mu$g of protein kinase inhibitor type II, with or without 150 mM KCl, for 30 min at 22 °C. The inhibitor used is specific for PKAc and has been previously used in a similar system [22]. Columns were then extensively washed. Beads were heated in Laemmli sample buffer [33] and phosphorylated GST–RhoA and GST–Cdc42 were analysed by SDS/PAGE and Western blotting. In order to show that PKAc directly phosphorylates Cdc42 and RhoA, experiments were also performed in the presence of 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (5000 $\mu$Ci/mmol) and the phosphorylation state was monitored by SDS/PAGE and autoradiography.

### Preparation of rat brain membranes

Rats were anaesthetized with $CO_2$ and decapitated. The brains were then quickly removed and chilled in ice-cold homogenization buffer (10 mM Hepes/Tris, 250 mM sucrose and 10 mM $MgCl_2$, pH 7.4). Brains were minced and homogenized using a Polytron homogenizer (Brinkmann Instruments, Mississauga, ON, Canada). Crude homogenates were centrifuged at 3000 ***g*** for 10 min at 4 °C to remove unbroken cells and nuclei. The resulting supernatant was centrifuged at 100000 ***g*** for 1 h to obtain a pellet of crude membranes that was resuspended in the same buffer. The protein concentration was determined as described above.

### Extraction of Rho proteins from rat brain membranes by RhoGDI, following treatment with alkaline phosphatase and PKA

Rat brain membranes (400 $\mu$g) were incubated with 1 unit of alkaline phosphatase in 20 mM 2-(*N*-cyclohexylamino)ethanesulphonic acid ('Ches'; pH 9.5) containing 10 mM $MgCl_2$ and a cocktail of protease inhibitors, for 30 min at 25 °C. The reaction

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was stopped by addition of ice-cold 20 mM Hepes/Tris (pH 7.0) containing 10 mM $MgCl_2$, 10 mM NaF and the protease inhibitor cocktail. Samples were then centrifuged at 40000 ***g*** for 30 min at 4 °C. Supernatants were discarded and the pellets were washed twice with the same buffer. The final pellet was resuspended and the protein concentration was determined as described above. Alkaline phosphatase-treated membranes (20 $\mu$g) were incubated for 10 min at 30 °C with PKAc (4 units) in the presence of 20 mM Hepes/Tris (pH 7.0), 20 $\mu$M ATP, 10 mM $MgCl_2$, 10 mM NaF and the cocktail of protease inhibitors. The reaction was stopped by the addition of 3 $\mu$g of protein kinase inhibitor type II and cooling on ice. The extraction of Rho proteins from the membranes was performed by addition of RhoGDI (1 $\mu$g) with or without 150 mM KCl, for 30 min at 25 °C. Separation of the solubilized proteins from membranes was performed by centrifugation at 40000 ***g*** for 30 min at 4 °C. All the fractions were resolved by SDS/PAGE, and the released Cdc42, RhoA and Rac1 were assessed by Western-blot analysis.

### SDS/PAGE and Western-blot analysis

SDS/PAGE was performed according to the method of Laemmli [33] with a Mini-Protean II apparatus (Bio-Rad Laboratories, Mississauga, ON, Canada). Samples were heated at 100 °C in Laemmli sample buffer and loaded on to 12.5% (w/v) polyacrylamide gels. The proteins were electroblotted in a semi-dry apparatus (Millipore, Bedford, MA, U.S.A.) on to 0.45 $\mu$m pore size PVDF membranes (Immobilon-P; Millipore) in transfer buffer [96 mM glycine, 10 mM Tris and 10% (v/v) methanol] at 80 mA/gel for 90 min. The blots were blocked overnight at 4 °C in Tris-buffered saline [TBS; 20 mM Tris (pH 7.5)/150 mM NaCl] containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dried milk powder. In order to immunodetect phosphoserine residues following electroblotting, the PVDF membranes were dried and immunodetected following the non-block technique proposed by Sadra et al. [34]. Usually, blots were incubated with a 1:2000 dilution of anti-RhoA, anti-Cdc42, anti-Rac1 and anti-RhoGDI antibodies (1:250 dilution for anti-phosphoserine antibodies) in TBS containing 0.1% Tween, 3% (w/v) BSA and 0.02% $NaN_3$ for 1 h at 22 °C. The Rho8 polyclonal antibody was used for the detection of RhoE [35]. This step was followed by a 1 h incubation with a 1:5000 dilution of donkey anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) in TBS containing 0.1% Tween and 5% (w/v) non-fat dried milk powder [or 5% (w/v) BSA for phosphoserine detection]. Immunoreactive bands were detected with the ECL® Western blotting kit as described in the manufacturer's instructions (Amersham Pharmacia Biotech). Blots were exposed to Fuji films and the autoradiograms were scanned with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

### Quantification of Rho proteins binding to RhoGDI

The interaction between RhoGDI and GST–Cdc42 or GST–RhoA bound to GSH–Sepharose columns was assessed by immunoblotting. Standard curves were established using known amounts of bacterially expressed recombinant Rho proteins and RhoGDI in order to determine the linear parts of the curves. Furthermore, recombinant proteins were loaded on to each gel as standards and were used in order to estimate the amounts (in pmol) of immunodetected Rho proteins bound to RhoGDI. Following each experiment, the PVDF membranes were stained with Coomassie Brilliant Blue to confirm that a constant amount of protein was loaded in each well.

### Statistical analysis

Data obtained from the densitometric analyses were expressed as the ratios of RhoGDI (pmol) to Rho proteins (pmol) ± S.E.M. or as the percentages of extraction from membranes by RhoGDI (± S.E.M.). Statistical analysis was performed using either the Student's *t* test or by one-way ANOVA with a post-hoc Bonferroni/Dunn test. Data from Figure 4 (below) were analysed using a two-way ANOVA where the nucleotide loaded on the Rho proteins and the conditions of incubation with RhoGDI were the variables. The ANOVA was followed by a post-hoc Tukey's HSD ('Honestly Significant Difference') test ($P < 0.05$ was considered significant).

## RESULTS

### Expression and purification of prenylated Rho proteins

*In vitro* studies of protein–protein interactions provide a unique opportunity to assess the effects of different factors on these interactions under highly controlled experimental conditions. To produce prenylated Cdc42 and RhoA, we developed a method of expression of these proteins in the yeast *S. cerevisiae* using a GST tag. This permitted study of the conditions controlling their interaction with RhoGDI. Following yeast culture, membranes containing prenylated GST–Cdc42, GST–RhoA or GST–RhoE were prepared. They led to the purification of approx. 25 $\mu$g of prenylated Cdc42, RhoA or RhoE/litre of *S. cerevisiae* culture. Since only 350 ng of proteins were necessary per affinity chromatography column, the efficiency of production attained was very satisfactory.

The efficiency of RhoGDI at extracting prenylated Rho proteins from yeast membranes was compared with that of other compounds that solubilize membrane proteins. DMSO was poor at releasing Cdc42 or RhoA from the *S. cerevisiae* membranes (Figure 1, left-hand panel). The addition of 1% (v/v) Triton X-100, 1% (w/v) CHAPS or 1% (w/v) sodium cholate led to extraction of Cdc42 and RhoA from membranes, but also to a significant proteolysis of GTPases, as shown by a reduction in the recovery of intact proteins, as well as the generation of immunodetectable proteolytic fragments for RhoA (Figure 1, left-hand panel). GST–RhoA was particularly affected by complete degradation when released from the membranes by 1% (w/v) CHAPS. Interestingly, RhoGDI was very efficient at extracting both GST–Cdc42 and GST–RhoA from membranes with 76 and 94% extraction respectively (Figure 1, left-hand panel). Furthermore, Coomassie Brilliant Blue staining of the PVDF membranes showed that the extraction by RhoGDI was highly selective, whereas a large number of proteins, in addition to Rho proteins, appeared in the soluble fractions following solubilization by detergents (results not shown). Incubation of the membranes with detergents probably released and/or activated endogenous proteases, thus causing the proteolytic degradation of GST–Cdc42 and GST–RhoA. This was not the case when a specific extraction was carried out using RhoGDI.

The extractability of Cdc42 and RhoA from *S. cerevisiae* membranes was established by comparing GST–RhoGDI, RhoGDI and denatured RhoGDI, which had been heated at 100 °C for 5 min (Figure 1, right-hand panel). There was no significant effect of the GST tag on the extraction activity of RhoGDI, whereas its thermal denaturation abolished GST–Cdc42 and GST–RhoA extraction, thus showing the specificity of the RhoGDI–Rho protein interaction. GST–RhoE served as a negative control, since RhoE is primarily membrane-bound, suggesting that it does not associate with RhoGDI in cells [35].

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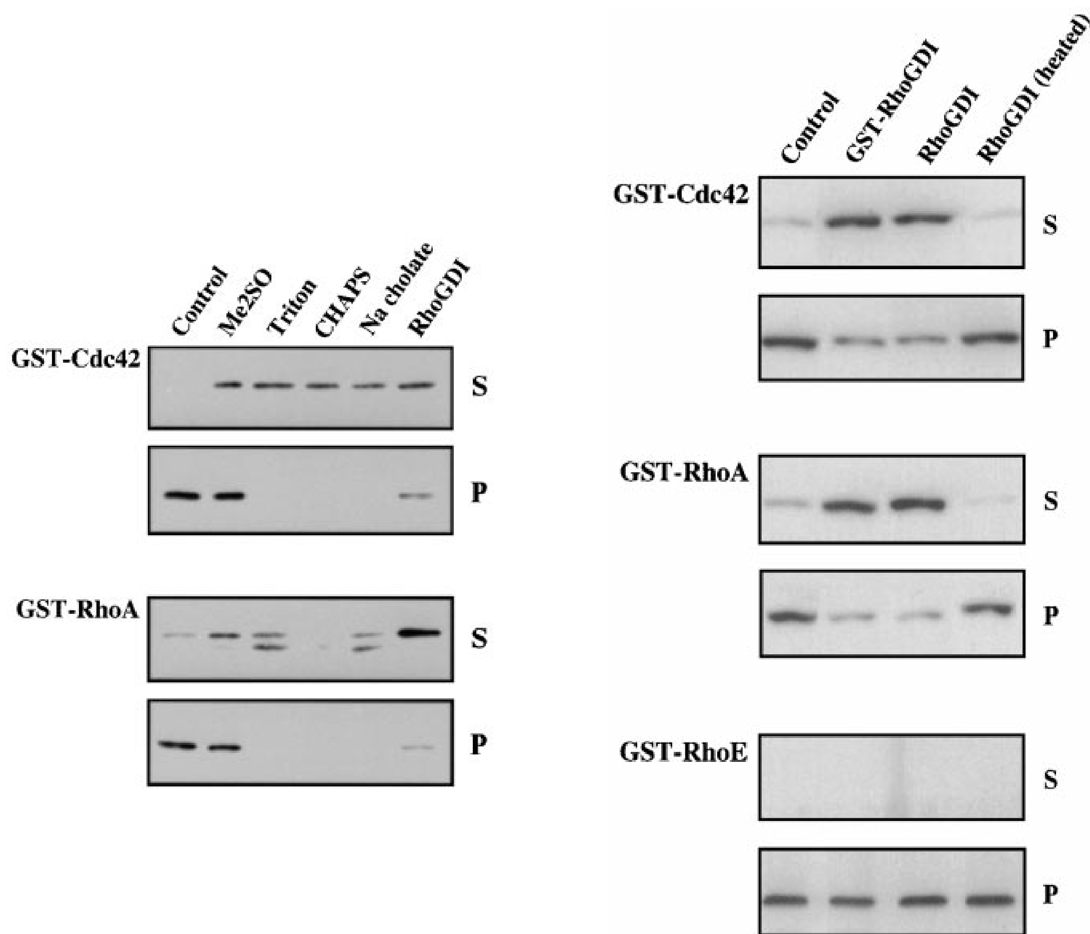


**Figure 1 Extraction of prenylated GST–Cdc42, GST–RhoA and GST–RhoE from *S. cerevisiae* membranes**

Left-hand panel: *S. cerevisiae* membranes (50 $\mu$g of protein) containing overexpressed Rho proteins were incubated with either 1 % (v/v) DMSO (Me2SO), 1 % (v/v) Triton X-100, 1 % (w/v) CHAPS, 1 % (w/v) sodium cholate or 2 $\mu$g of recombinant RhoGDI. Right-hand panel: membranes (50 $\mu$g of protein) were incubated with GST–RhoGDI (4 $\mu$g), RhoGDI (2 $\mu$g) or RhoGDI (2 $\mu$g) denatured at 100 °C for 5 min. Separation of soluble proteins (S) from pelleted membranes (P) was performed by centrifugation at 40000 ***g*** at 4 °C for 30 min. Laemmli sample buffer was added to all fractions and proteins were separated by SDS/PAGE. Cdc42, RhoA and RhoE were detected by Western-blot analysis. These results are representative of two experiments performed in duplicate.

As expected, RhoGDI was unable to extract GST–RhoE from membranes (Figure 1, right-hand panel).

Following extraction, the complexes formed between Rho proteins and RhoGDI were broken by the addition of 1 % (w/v) CHAPS, and the purification of GST–Cdc42 and GST–RhoA was performed on GSH–Sepharose columns. The attachment to the beads was efficient, since the amount of Rho proteins lost in the flowthrough was minimal (Figure 2). The presence of 1 % (w/v) CHAPS had no effect on this attachment. The RhoGDI used for membrane extraction was easily washed out from the columns with a buffer containing 1 % (w/v) CHAPS. The percentage of detergent was then decreased to 0.1 % to allow subsequent interactions between Rho GTPases and RhoGDI while avoiding precipitation of the prenylated Cdc42 or RhoA. Purified proteins can be cleaved from the GST tag by incubation with thrombin and can then be eluted from the columns. However, for the sake of simplicity, all the subsequent assays involving purified prenylated Cdc42 and RhoA were carried out directly in the GSH–Sepharose columns, using Rho proteins freshly extracted from the membranes and purified. Our data show that, using transfected yeast cells, it is possible to obtain purified prenylated Rho proteins *in vitro*, which opens new possibilities for the study of protein–protein interactions under highly controlled conditions.

#### Nucleotide loading of Rho proteins on to the GSH–Sepharose columns

To control the nucleotide state of prenylated GST–Cdc42 and GST–RhoA, nucleotide loading was performed while the proteins were attached to the GSH–Sepharose columns. [$^3$H]GDP was rapidly loaded on GST–Cdc42 as well as GST–RhoA, reaching a plateau after 5–10 min of incubation with the nucleotide, and columns loaded with GST alone were used as a control (results not shown). The kinetics of [$^3$H]GDP binding to GST–Cdc42 and GST–RhoA were similar to previously reported results for nucleotide exchange in solution [32]. Therefore the prenylated Rho proteins purified from *S.cerevisiae* were functional, since they could be loaded with nucleotides.

#### Prenylation of Cdc42 and RhoA is essential to their interaction with RhoGDI

The first step in studying *in vitro* interactions between RhoGDI and either Cdc42 or RhoA was to assess the effect of prenylation

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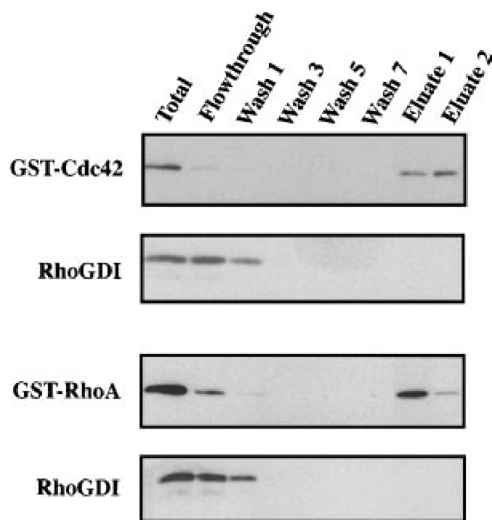


**Figure 2 Purification of prenylated GST–Cdc42 and GST–RhoA**

*S. cerevisiae* membranes (1 mg of protein) were incubated with RhoGDI (40 $\mu$g) at 25 °C for 30 min, and then centrifuged at 40000 ***g*** for 30 min. The soluble fraction was adjusted to 1 % (w/v) CHAPS and incubated for 15 min on ice. Samples were then loaded on to GSH–Sepharose columns and washed with 20 mM Hepes/Tris buffer containing 1 % (w/v) CHAPS and 10 mM $MgCl_2$ (washes 1–5). The columns were subsequently equilibrated with Hepes/Tris buffer containing 0.1 % CHAPS and 10 mM $MgCl_2$ (washes 6–8). Elution of fusion proteins was performed by addition of an excess of GSH (100 mM) in the presence of 0.1 % CHAPS. To verify the purity of fusion proteins, fractions collected from the columns were analysed by SDS/PAGE and Western blotting. These results are representative of three experiments.

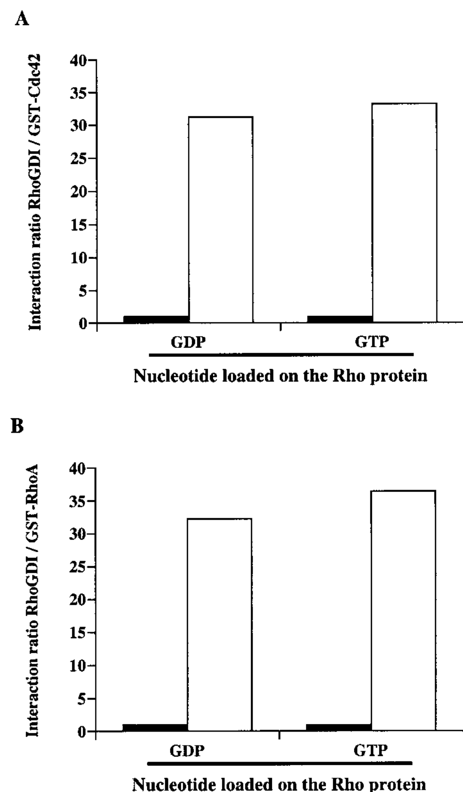


**Figure 3 Analysis of the role of prenylation in the interaction between RhoGDI and either GST–Cdc42 or GST–RhoA isolated from *E. coli* or *S. cerevisiae***

Membranes from *S. cerevisiae* (1 mg of protein) containing either prenylated GST–Cdc42 or prenylated GST–RhoA were incubated with RhoGDI (40 $\mu$g), and then centrifuged at 40000 ***g*** for 30 min. Recombinant GTPases were purified on GSH–Sepharose columns as described in the Experimental section. For the columns with *E. coli* unprenylated proteins, an equivalent amount of purified GST–Cdc42 or GST–RhoA was loaded. Small Rho GTPases were then incubated with RhoGDI (10 $\mu$g) for 30 min. After extensive washing, beads from the columns were mixed with Laemmli sample buffer and analysed by SDS/PAGE and Western Blotting for RhoGDI and Cdc42 (**A**) or RhoA (**B**). Standards of GST–Cdc42, GST–RhoA and RhoGDI were loaded on to the gels, in order to establish the absolute quantities of Rho protein and RhoGDI present. Data were quantified by laser densitometry and expressed as the ratio of RhoGDI to Rho proteins from *E. coli* (black bars) or from *S. cerevisiae* (white bars). The ratios obtained for *E. coli* proteins loaded with GDP were set at 1.

in our system. Therefore the formation of complexes between RhoGDI and either prenylated Rho proteins from *S. cerevisiae* or unprenylated Rho proteins from *E. coli* were compared. The presence of a prenyl group on Cdc42 or RhoA induced a significant increase in the amount of complexes formed with RhoGDI (Figures 3A and 3B). Using recombinant GST–Cdc42, GST–RhoA and RhoGDI as standards on each gel, densitometric analysis of immunodetected proteins allowed quantification of the proteins involved in the complexes. When values obtained for unprenylated proteins loaded with GDP were set at 1, the presence of the prenyl group led to a 31-fold increase in the interaction of GST–Cdc42 with RhoGDI when this protein was loaded with GDP, and to a 33-fold increase when loaded with GTP[S] (Figure 3A). The increases were 32- and 38-fold respectively in the case of GST–RhoA (Figure 3B). These data demonstrate that prenylation is essential for establishing a stable interaction between RhoGDI and either Cdc42 or RhoA. In addition, our results found no significant effect associated with the identity of the nucleotide bound to the small GTP-binding protein on the efficiency of the interaction with RhoGDI.

### Effects of nucleotides, $Mg^{2+}$, $PIP_2$ and ionic strength on the formation of RhoGDI complexes with Rho GTPases

The literature contains contradictory results on the mechanisms regulating the interaction between Rho proteins and RhoGDI, probably because different experimental models were used and because only certain factors were taken into account [1,5]. Having developed a functional system to study the conditions controlling the interaction between prenylated Rho proteins and their regulator RhoGDI, we systematically studied the influences of compounds that probably affect this interaction. For instance, nucleotides regulate the conformation of Rho proteins, the magnesium ion stabilizes the conformation of Rho GTPases, and $PIP_2$ has been postulated to partially open RhoGDI–Rho protein complexes, while solution ionic strength could modulate the bonds linking RhoGDI and Rho GTPases [13,14,22]. Consequently, recombinant prenylated Rho proteins, on GSH–Sepharose columns, were loaded with GDP or GTP[S] and then incubated with RhoGDI in the presence of 10 mM $Mg^{2+}$ and/or 50 $\mu$M $PIP_2$ and/or 150 mM KCl. The effects of these agents on the formation of complexes between RhoGDI and either Cdc42 or RhoA were analysed by Western blotting and quantified by densitometry to determine the stoichiometry of these interactions. Association of RhoGDI with each GTPase varied similarly under these conditions although the level of complexes between RhoGDI and GST–Cdc42 was usually larger than with GST–RhoA (Figures 4A and 4B). At low ionic strength (0 mM KCl), $Mg^{2+}$ promoted a high interaction between RhoGDI and the Rho proteins (0.4–0.7 pmol/pmol of RhoGDI; $P < 0.01$). However, in the presence of a physiological ionic strength (150 mM

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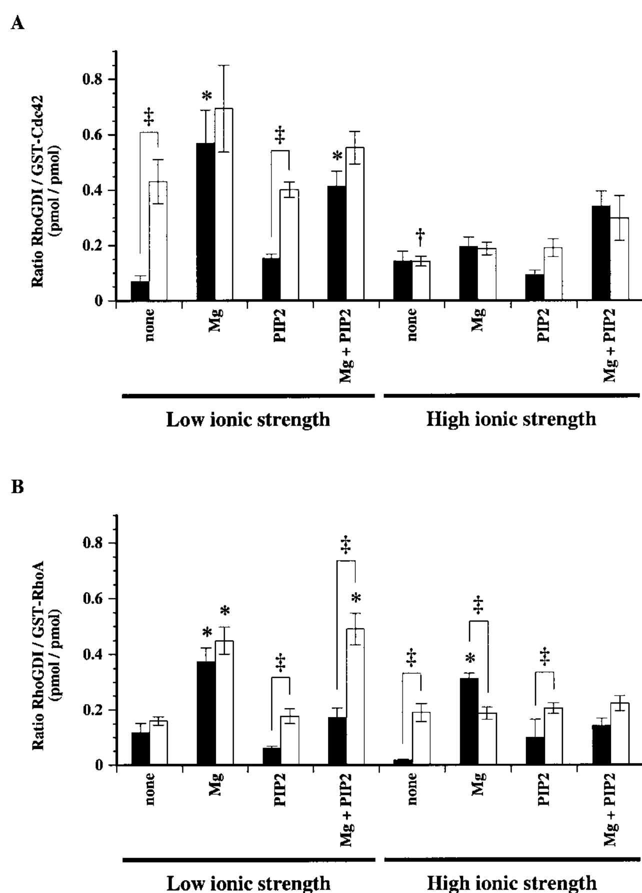


**Figure 4 Interaction of RhoGDI with prenylated GST–Cdc42 or GST–RhoA preloaded with GTP[S] or GDP**

Prenylated GST–Cdc42 or GST–RhoA was extracted from *S. cerevisiae* membranes and purified on GSH–Sepharose columns as described in the Experimental section. Nucleotide exchange was performed to load the Rho proteins with GTP[S] (black bars) or GDP (white bars). Rho proteins on the columns were then incubated with RhoGDI (10 $\mu$g) in the presence of 10 mM $MgCl_2$ and/or 50 $\mu$M $PIP_2$ with either high ionic strength (150 mM KCl) or low ionic strength (0 mM KCl) medium. After extensive washing, RhoGDI–GST–Cdc42 and RhoGDI–GST–RhoA complexes bound to beads on the columns were solubilized with Laemmli sample buffer and analysed by SDS/PAGE. Cdc42 (**A**), RhoA (**B**) and RhoGDI present in the complexes were detected by Western-blot analysis. Data were quantified by laser densitometry and expressed as the ratio of RhoGDI to Rho proteins (pmol/pmol) using standards of recombinant proteins that were loaded on to each gel. Each value is the average from three to four experiments ($\pm$S.E.M.). For Cdc42, a two-way ANOVA with the nucleotide loaded on the Rho protein and the conditions of incubation ($Mg^{2+}$, $PIP_2$ and ionic strength) with RhoGDI as the variables, indicated a significant nucleotide effect [$F(1,64) = 12.90$, $P = 0.0006$], a significant conditions effect [$F(7,64) = 14.56$, $P < 0.0001$] and a significant nucleotide × conditions effect [$F(7,64) = 2.32$, $P = 0.0355$]. Similarly for RhoA, a significant nucleotide effect [$F(1,64) = 33.77$, $P = 0.0001$], a significant conditions effect [$F(7,64) = 21.63$, $P < 0.0001$] and a significant nucleotide × conditions effect [$F(7,64) = 6.88$, $P = 0.0001$] were indicated. Simple main effect followed by post-hoc Tukey's HSD ('Honestly Significant Difference') test showed significant differences as indicated: *different from control with low ionic strength at $P < 0.01$; †different from control with low ionic strength at $P < 0.05$. ‡indicates a significant effect of the nucleotide at $P < 0.05$.

KCl), $Mg^{2+}$ had no significant effect, except when RhoA was loaded with GTP[S], where a significant increase in the amount of complexes (0.3 pmol/pmol of RhoGDI) was observed ($P < 0.01$), as compared with the control (0.1 pmol/pmol of RhoGDI) (Figure 4B). These observations are of interest, since previous studies on the effect of nucleotides and $Mg^{2+}$ on the interaction of Rho proteins with RhoGDI were performed under low ionic strength conditions [9–11].

Since $PIP_2$ has been reported to loosen previously formed complexes between RhoGDI and RhoA [15], the effect of this phospholipid on the formation of the complexes was also investigated. Alone, $PIP_2$ had no significant effect on the formation of these complexes as compared with the control at low ionic strength (Figures 4A and 4B). Neither did $PIP_2$ modify the interaction between RhoGDI and either GST–Cdc42 or GST–RhoA in 150 mM KCl. When incubated in the presence of physiological ionic strength and $PIP_2$, the amount of complexes formed was also independent of $Mg^{2+}$. Although postulated to weaken the interaction between RhoGDI and RhoA, $PIP_2$ did not prevent the binding of RhoGDI to either Cdc42 or RhoA.

The identity of the nucleotide loaded on Rho proteins prior to incubation with RhoGDI affects the formation of the complexes. For GST–RhoA, GDP favoured the interaction with RhoGDI as compared with GTP[S], in pmol/pmol, when the complexes were formed in the presence of either $PIP_2$, $Mg^{2+} + PIP_2$, KCl or $PIP_2 + KCl$ ($P < 0.05$) (Figure 4B). However, the interaction with GST–RhoA was slightly lower for GDP versus GTP[S] when the incubation was performed with $Mg^{2+} + KCl$ ($P < 0.05$) (Figure 4B). An effect due to the nucleotide loaded on GST–Cdc42 was also observed, but only at low ionic strength. For instance, GDP increased the amount of complexes formed in the presence of buffer alone (control conditions) or with added $PIP_2$, compared with GTP[S] ($P < 0.05$) (Figure 4A). These observations agree with previous studies indicating that RhoA is more sensitive than Cdc42 to the nucleotide state (GDP or GTP) with regard to the formation of complexes with RhoGDI [10,11,36,37]. Taken together, our results confirm that Rho protein prenylation is critical for the interaction with RhoGDI. Moreover, the formation of complexes between RhoGDI and either GST–Cdc42 or GST–RhoA is enhanced by the presence of $Mg^{2+}$, but is not significantly regulated by $PIP_2$. Finally, when incubated under conditions of physiological ionic strength, the interaction between RhoGDI and prenylated GST–RhoA is strongly dependent on the specific nucleotide bound to it.

### GST–Cdc42 and GST–RhoA are phosphorylated by PKAc and this regulates the formation of complexes with RhoGDI

Phosphorylation–dephosphorylation cycling controls the activities and the cellular locations of various proteins of the Ras superfamily. We investigated the effect of Rho phosphorylation as another factor potentially regulating these protein–protein interactions. Lang et al. [22] demonstrated that phosphorylation of RhoA increased its extraction from membranes, but that study was limited to RhoA and was performed in the absence of ionic strength, whereas the results in Figure 4 of the present paper suggest that 150 mM KCl could affect the interaction between RhoGDI and either Cdc42 or RhoA. Therefore prenylated GST–Cdc42 or GST–RhoA were purified on GSH–Sepharose columns, loaded with GDP or GTP[S] and then phosphorylated by incubation with PKAc. Before the addition of RhoGDI (to form complexes with phosphorylated Rho proteins) PKAc was washed out extensively. RhoGDI contains potential phosphorylation sites for PKA and PKC [38,39], and the phosphorylation of RhoGDI has been reported during Fas-induced apoptosis in Jurkat T-cells [40]. Therefore the protein kinase inhibitor type II, a specific inhibitor of PKAc [22], was added in the RhoGDI solution to prevent its phosphorylation by any residual PKAc activity on the columns.

Using [$\gamma$-$^{32}$P]ATP, autoradiography analysis showed that GST–Cdc42 and GST–RhoA were both phosphorylated by PKAc on the GSH–Sepharose columns while GST was not labelled, indicating that the phosphorylation occurred on the

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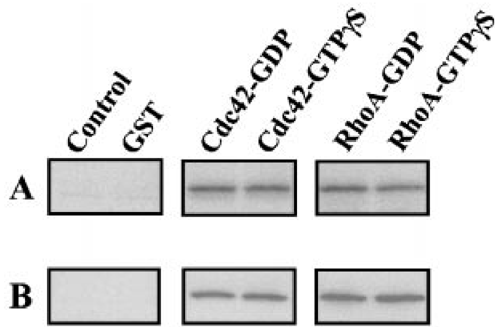


**Figure 5 Phosphorylation of GST–Cdc42 and GST–RhoA by PKA**

Prenylated GST–Cdc42 and GST–RhoA were purified on GSH–Sepharose columns, loaded with GDP or GTP[S] (GTPγS) and incubated with PKA in the presence of [$\gamma$-$^{32}$P]ATP. Proteins bounds to the beads were then mixed with Laemmli sample buffer and separated by SDS/PAGE. Autoradiography (**A**) and Western-blot (**B**) detection of Cdc42 and RhoA were performed. These data are representative of three experiments.

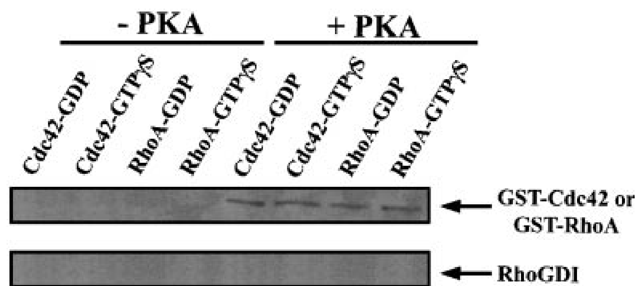


**Figure 6 PKA phosphorylates prenylated GST–Cdc42 or GST–RhoA on a serine residue**

GST–Cdc42 or GST–RhoA were extracted from *S. cerevisiae* membranes and purified on GSH–Sepharose columns as described in the Experimental section. Rho proteins on the columns were then preloaded with GDP or GTP[S] (GTPγS) and incubated with (+PKA) or without (−PKA) PKAc. RhoGDI was then added to the columns to form complexes with Rho proteins. After extensive washing, proteins bound to the GSH–Sepharose beads were mixed with Laemmli sample buffer and analysed by SDS/PAGE. Phosphoserine residues were detected by Western blotting.

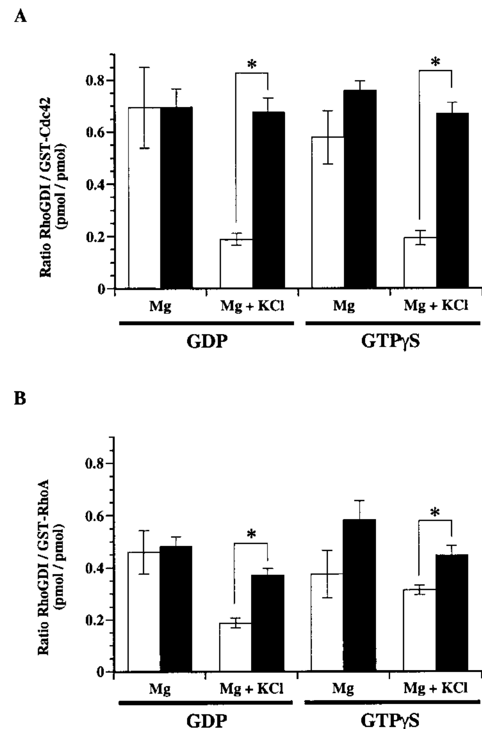


**Figure 7 Phosphorylation induced by PKA up-regulates the interaction between GST–Cdc42 or GST–RhoA and RhoGDI**

RhoGDI was used to extract prenylated GST–Cdc42 (**A**) or GST–RhoA (**B**) from *S. cerevisiae* membranes. The Rho proteins were purified on GSH–Sepharose columns, loaded with GDP or GTP[S] (GTPγS) then phosphorylated by PKAc as described in the Experimental section. RhoGDI was subsequently added to the columns with or without 150 mM KCl. Excess unbound RhoGDI was washed out and the RhoGDI–Rho protein complexes bound to the beads were analysed by SDS/PAGE and Western blotting. Data were quantified by laser densitometry and expressed as the ratio of RhoGDI to Rho proteins (pmol/pmol) incubated in the presence (black bars) or absence (white bars) of PKAc, using recombinant proteins as standards to quantify their levels in complexes. Results are the averages from three experiments (±S.E.M.). A one-way ANOVA indicated a significant increase of the RhoGDI/Rho protein ratio following treatment with PKAc for GST–Cdc42 ($P < 0.0001$) and GST–RhoA ($P < 0.002$). Post-hoc Bonferroni/Dunn's tests showed a significant difference as indicated: *different from data in the absence of PKAc at $P < 0.05$.

Rho portion of the fusion proteins (Figure 5A). Indeed, the signal obtained by autoradiography co-localized with the immunodetected bands of Cdc42 and RhoA (Figure 5B). Next, the phosphorylation state of the Cdc42, RhoA and RhoGDI bound to the beads was also analysed by SDS/PAGE and Western blotting, following the formation of RhoGDI–Rho GTPases complexes. The immunodetection with an anti-phosphoserine antibody showed that the incubation with PKAc resulted in phosphorylation of a serine residue located on RhoA and on Cdc42. The nucleotide (GDP or GTP[S]) bound to GST–Cdc42 and GST–RhoA did not affect their levels of phosphorylation by PKAc as measured by autoradiography or by immunodetection with the anti-phosphoserine antibody (Figures 5 and 6). In the absence of PKAc, no basal level of phosphorylation was detectable on Rho proteins purified from *S. cerevisiae*. As expected, no phosphorylation was observed on RhoGDI in any of the conditions of the present study (Figure 6), since PKAc was washed out or inhibited by the specific inhibitor used.

When $Mg^{2+}$ was added under low ionic strength conditions, the interaction of Rho GTPases with RhoGDI was high (0.5–0.8 pmol/pmol) and was not affected by treatment with PKAc (Figures 7A and 7B). In the presence of a high ionic strength, the RhoGDI–Rho GTPase interactions were initially lower (0.2–0.3 pmol/pmol) but were significantly increased ($P < 0.05$) by phosphorylation (to 0.7 pmol/pmol for Cdc42 and to 0.4 pmol/pmol for RhoA). Major increases in the amounts of complexes formed were observed following phosphorylation of GST–Cdc42 and GST–RhoA in the presence of $Mg^{2+}$ with either nucleotide, GDP or GTP[S], at high ionic strength (Figures 7A and 7B). Interestingly, phosphorylation of GST–RhoA and GST–Cdc42, loaded with either GDP or GTP[S], resulted in complex formation at similar levels under both low and high ionic strength conditions. These data suggest that, in the presence of physiological ionic strength, phosphorylation of Cdc42 and RhoA could promote interaction with RhoGDI regardless of the nucleotide state.

### RhoGDI extraction of Cdc42 and RhoA from rat brain membranes depends on the phosphorylation state of the Rho proteins

Based on the effects of Cdc42 and RhoA phosphorylation on the interactions with RhoGDI in the *in vitro* system described above,

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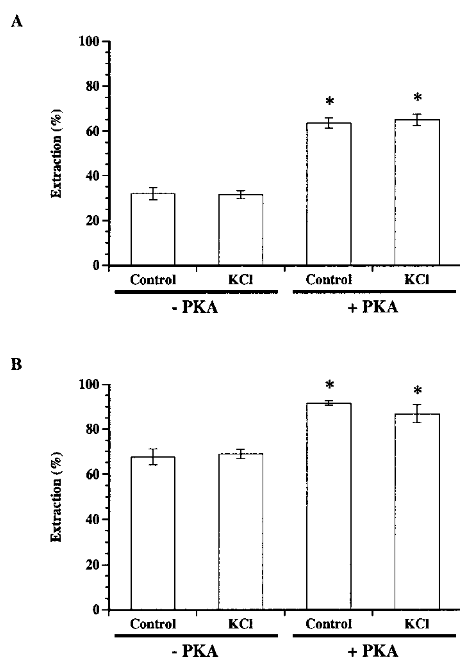


**Figure 8 Ability of RhoGDI to extract Cdc42 and RhoA from rat brain membranes following pre-treatment with PKA**

Rat brain membranes (20 $\mu$g of protein) were incubated with PKAc (4 units). The reaction was stopped by addition of protein kinase inhibitor type II and cooling on ice. RhoGDI (10 $\mu$g) was then added with or without 150 mM KCl for a further incubation of 30 min. Soluble and particulate fractions were separated by centrifugation and proteins were analysed by SDS/PAGE. Cdc42 and RhoA were immunodetected and the intensity of the bands was measured by densitometric analyses as described in the Experimental section. Data are expressed as the percentage of extraction, by RhoGDI, of Cdc42 (**A**) or RhoA (**B**) ($\pm$S.E.M.) and are the averages from three experiments performed in duplicate. $^*P < 0.01$, compared with the control in the absence of PKAc.

we next analysed the role of phosphorylation on membrane extraction of Rho proteins by RhoGDI. Rat brain served as a source of membranes, since we previously found that they contain a large amount of Rho proteins and weak proteolytic activity [13]. Rat brain membranes were phosphorylated by PKAc and then incubated with RhoGDI. In order to avoid possible phosphorylation of RhoGDI by PKAc, protein kinase inhibitor type II was added to the membrane preparations prior to addition of RhoGDI. As shown in Figures 8(A) and 8(B), phosphorylation of endogenous Cdc42 and RhoA by PKAc significantly increased extraction by RhoGDI (to 65 and 90 % respectively) from brain membranes ($P < 0.01$) both at low and physiological ionic strengths.

In order to confirm the role of phosphorylation on the extraction of Rho proteins from biological membranes, the membranes were also treated with alkaline phosphatase to study the effect of dephosphorylation on their extraction by RhoGDI. Treatment with alkaline phosphatase caused a significant reduction in the extraction of Cdc42 and RhoA, from rat brain membranes, in the presence of physiological ionic strength ($P < 0.01$) (Figures 9A and 9B). As expected, the levels of extracted Cdc42 and RhoA were similar if the membranes were incubated with PKAc only or with alkaline phosphatase followed by PKAc (Figure 9). In all cases, phosphorylation significantly increased the extraction of Cdc42 and RhoA ($P < 0.01$). Similar results were obtained at low or high ionic strength conditions (results not shown). This suggests not only that RhoA and Cdc42

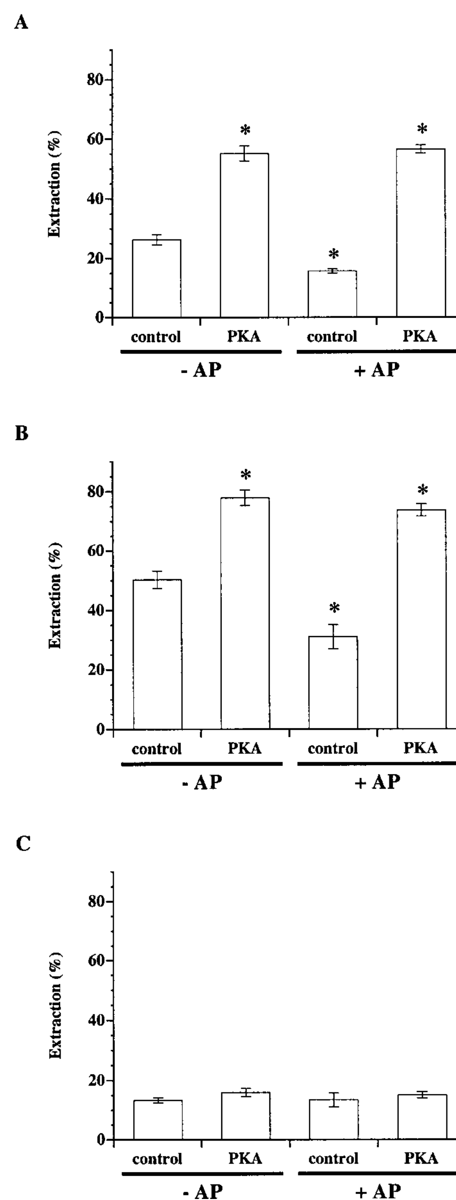


**Figure 9 Effect of pre-treatments with alkaline phosphatase and PKA on RhoGDI extraction of Cdc42, RhoA and Rac 1 from rat brain membranes**

Rat brain membranes (400 $\mu$g of protein) were incubated with 1 unit of alkaline phosphatase (+AP) or without alkaline phosphatase (−AP). The reaction was stopped by the addition of ice-cold buffer and 10 mM NaF. Soluble proteins were separated from pelleted membranes by centrifugation at 40000 ***g*** for 30 min. Pelleted membranes were resuspended in the same buffer and washed twice. Membranes (20 $\mu$g) in the final pellet were incubated with PKAc as described in the Experimental section and Rho proteins were extracted by RhoGDI, in the presence of 150 mM KCl. Soluble and membrane fractions were separated by centrifugation and analysed by SDS/PAGE and Western blotting. The intensity of the immunodetected proteins was determined by laser densitometry. Data are expressed as the percentage of extraction, by RhoGDI, of Cdc42 (**A**), RhoA (**B**) or Rac1 (**C**) ($\pm$S.E.M.) and are the averages from three experiments performed in duplicate. $^*P < 0.01$, compared with the control in the absence of PKAc.

can be phosphorylated but that this is a dynamic process modulating their *in vivo* interaction with RhoGDI.

It has been reported that RhoA can be phosphorylated on Ser$^{188}$ by PKAc [22,24]. A serine residue is also present in Cdc42 at a similar position (185), but is absent from the Rac1 protein. Since phosphorylation of Cdc42 and RhoA occurred on a serine residue (Figure 6), the affinity of Rac1 for RhoGDI should not

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be affected by treatment with alkaline phosphatase or PKAc. Figure 9(C) shows that, as expected, Rac1 extraction by RhoGDI was not modified by incubation with alkaline phosphatase and/or PKAc. Thus phosphorylation of Cdc42 and RhoA augments interaction with RhoGDI and increases the proportions of these two proteins extracted from membranes by RhoGDI. Taken together, these results suggest that phosphorylation could be a molecular mechanism regulating the extraction of Cdc42 and RhoA from biological membranes. Since Rho proteins complexed with RhoGDI are inactive [5], the phosphorylation state could also be an essential step in the regulation of Cdc42 and RhoA activity.

## DISCUSSION

This study showed that the phosphorylation states of Cdc42 and RhoA regulate their interaction with RhoGDI. Using an *in vitro* system, where prenylated GST–Cdc42 and GST–RhoA are bound to GSH–Sepharose beads, we demonstrated that phosphorylation by PKAc significantly increased the interaction between RhoGDI and these Rho proteins in the presence of a physiological ionic strength. The increased affinity of RhoGDI for phosphorylated Rho GTPases is nucleotide-independent (GDP or GTP[S]). Furthermore, this effect of phosphorylation was confirmed using a more relevant physiological system (rat brain membranes). Dephosphorylation of these membranes by treatment with alkaline phosphatase significantly decreases RhoA and Cdc42 extraction by RhoGDI, while subsequent rephosphorylation by PKAc restores the extraction ability, illustrating the reversibility of this process. We therefore suggest that phosphorylation is able to terminate Cdc42 and RhoA activity independent of GDP–GTP cycling by augmenting the interaction with RhoGDI.

Since prenylation of Cdc42 and RhoA is essential for membrane attachment and interaction with RhoGDI, we developed a simple and efficient method that produces prenylated Rho proteins using the yeast *S. cerevisiae*. Following culture of the yeast and membrane isolation, Rho GTPases are purified in a single step using GSH–Sepharose beads. Since the purification process from frozen membranes takes less than 90 min, prenylated GST–Cdc42 or GST–RhoA can be freshly made for each experiment. This method, as compared with those involving purification of Rho proteins from mammalian tissues or the baculovirus system, avoids multiple chromatographic steps and is less time consuming. An *in vitro* processing method using a mixture of reticulocyte lysate and mevalonolactone has also been proposed, but this method is hindered by difficulties in controlling the degree of prenylation and the elimination of unprocessed proteins [27,28,41]. Furthermore, lipid-modified Rho proteins are difficult to handle and tend to precipitate. To overcome this difficulty, one group used unprenylated Rac1 possessing a C-terminal truncation mixed with a peptide which corresponded to the C-terminal end of Rac1 but containing a farnesyl group instead of a geranylgeranyl group [42]. This combination should mimic geranylgeranylated Rac1 and form a complex with RhoGDI. It has been shown that farnesylated V14RhoA, the constitutively active analogue of RhoA where the glycine in position 14 has been replaced with a valine residue, is able to interact with RhoGDI [43], and that the presence of a farnesyl group instead of a geranylgeranyl group on Cdc42 or Rac1 does not affect the binding with RhoGDI [44]. However, the effect of this farnesylation on the extraction of Rho proteins from membranes was not studied. The absence of extraction from yeast membranes of farnesylated RhoE observed in Figure 1 (right-hand panel) suggests that the specific prenyl moiety, farnesyl or geranylgeranyl, could affect the extractability by RhoGDI, particularly since the formation of complexes involves the close insertion of Rho prenyl groups into a hydrophobic pocket located in the C-terminal portion of RhoGDI [45,46]. The method developed in the present study overcomes difficulties for the solubilization of prenylated Rho proteins by using one brief purification step before each experiment. In fact, our method produces sufficient purified prenylated RhoA and Cdc42 for *in vitro* studies of the biochemical factors regulating the interaction of RhoA and Cdc42 with RhoGDI in a highly controlled environment.

Both membrane insertion and GDI recognition depend on the presence of the geranylgeranyl group on Rho proteins [26,47]. Therefore the Rho proteins purified from *S. cerevisiae* must already be prenylated, since they were isolated from the membrane fraction and could be extracted by RhoGDI. Our results show that RhoGDI extraction of GST–Cdc42 and GST–RhoA from yeast membranes is efficient and is more specific than extraction with detergents, therefore protecting the Rho proteins from proteolytic degradation (Figure 1, left-hand panel). When compared with unprenylated Rho proteins produced in *E. coli*, the prenylated Cdc42 and RhoA purified from yeast exhibited an important increase (more than 30-fold) in the level of complexes formed with RhoGDI (Figure 3).

Another finding from our study is that $Mg^{2+}$ significantly increased the interaction between GST–Cdc42 or GST–RhoA and RhoGDI (Figure 4). The importance of the $Mg^{2+}$ ion in the structural integrity of GTP-binding proteins has already been pointed out in crystallographic studies performed by several groups [48,49]. $Mg^{2+}$ may stabilize the conformation of Rho proteins, making them more accessible to RhoGDI. When $Mg^{2+}$ is added in the presence of physiological ionic conditions (150 mM KCl), the amount of complexes formed between GST–Cdc42 or GST–RhoA and RhoGDI is not enhanced and is relatively low compared with the values obtained with $Mg^{2+}$ at low ionic strength. This suggests that a physiological ionic strength reduces Rho GTPase structural plasticity more than $Mg^{2+}$. In addition, these data underline the importance of investigating the interactions of Rho GTPases with their regulators and effectors using an ionic strength which mimics physiological conditions.

A major finding of our study is that phosphorylation of Rho proteins promotes the interaction with RhoGDI under a physiological KCl concentration, reaching the levels obtained for incubation with $Mg^{2+}$ at low ionic strength (Figure 7). The prenylated Rho proteins purified from *S. cerevisiae* have no detectable basal phosphorylation (Figure 6), strongly suggesting that the observed increase in the interaction between RhoGDI and Cdc42 or RhoA is due to the phosphorylation by PKAc. This is in agreement with previous reports showing that treatment with PKAc increased RhoGDI extraction of RhoA from membranes [22,24]. Moreover, our results show that the levels of phosphorylation of Cdc42 and RhoA are similar and are not affected by the nucleotide (GDP or GTP[S]) loaded on the Rho proteins prior to the addition of PKAc (Figure 5). In our study, unphosphorylated GST–Cdc42 and GST–RhoA were almost totally extracted by RhoGDI from *S. cerevisiae* membranes during the purification step. This is probably due to the hydrophilic GST tag which might weaken the attachment of Rho proteins to the membranes, as well as to the overexpression that might affect the yeast membranes themselves. The almost complete extraction of fusion Rho proteins by RhoGDI from yeast membranes supports this view, since it has been reported that the percentage of extraction of endogenous Rho GTPases is usually about 50 % [12,13]. In fact, results from our laboratory previously

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suggested that a complementary factor was required to extract the remaining Rho proteins from membranes [13]. Phosphorylation may well be this factor. It has also been reported that ionic strength had no effect on the interaction between GSH–Sepharose bead–immobilized GST–RhoGDI and Rho proteins solubilized from rat kidney brush border membranes, but that it inhibited Rho extraction from the same membranes [13]. This apparent inhibitory effect of KCl could be explained by the conditions of incubation used in these experiments, in the absence of phosphatase inhibitors, which might have promoted the dephosphorylation of RhoA and Cdc42 and therefore decreased the interaction with RhoGDI.

A crucial step in our study was the demonstration of the role of phosphorylation in Rho protein interaction with RhoGDI, as suggested by the results on GSH–Sepharose columns. Therefore we extended these experiments to the ability of RhoGDI to extract Rho GTPases from biological membranes. Brain tissue contains a large amount of RhoA [50] and a low level of proteolytic activity [13], and it was thus used as a source of membranes. The phosphorylation of rat brain membranes by PKAc significantly increased the extraction, by RhoGDI, of Cdc42 and RhoA (Figures 8A and 8B). Physiological ionic strength had no effect on the extraction. Continuing this approach, brain membranes were dephosphorylated using alkaline phosphatase. Although the buffer used during the dephosphorylation, at pH 9.5, slightly reduced the percentage of extraction by RhoGDI in control samples (compare Figures 8 and 9), dephosphorylation significantly reduced the extraction of Cdc42 and RhoA (Figures 9A and 9B). Membrane-bound Rho proteins treated with alkaline phosphatase can subsequently be re-phosphorylated by PKAc and efficiently extracted by RhoGDI, demonstrating the reversibility of this process and suggesting that interaction with RhoGDI depends upon the phosphorylation states of Cdc42 and RhoA, and that phosphorylation is a dynamic process *in vivo*.

It has been demonstrated that RhoA is phosphorylated on $Ser^{188}$, in the C-terminal domain, using a mutant of RhoA with an alanine residue in position 188 or by phosphorylation in the presence of [$\gamma$-$^{32}$P]ATP [22–24]. Furthermore, the replacement of $Ser^{188}$ of RhoA with an alanine residue abolished the inhibitory effect of PKA on ROK$\alpha$, a downstream effector of RhoA. A serine residue is found in a similar position on Cdc42 ($Ser^{185}$) but is absent from the Rac1 sequence. The treatment of brain membranes with alkaline phosphatase and/or PKAc did not modify the extraction of Rac1 by RhoGDI (Figure 9C), strongly supporting our hypothesis that the phosphorylation of $Ser^{188}$ in RhoA or $Ser^{185}$ in Cdc42 is responsible for the modifications observed in their interaction with RhoGDI. Modulation of the interaction of Rac1 with RhoGDI must involve a different factor, possibly arachidonic acid or phospholipids, such as $PIP_2$ or phosphatidic acid [14,51], or phosphorylation by another protein kinase (such as Akt) on a different residue [25]. The phosphorylated serine residue of Cdc42 and RhoA is located between the polybasic domain in the C-terminal region and the prenylated cysteine residue at the C-terminal end. These two domains are required for membrane targeting and binding [52,53]. Thus phosphorylation of the serine residue between the polybasic domain and the lipidated cysteine could decrease the affinity of Cdc42 and RhoA for the hydrophobic membrane environment by introducing a negative charge or may induce a conformational change that facilitates the interaction with RhoGDI.

The present data provide evidence that the phosphorylation states of Rho GTPases regulate their interaction with RhoGDI under physiological conditions of KCl, therefore controlling their inactivation. Although $Ser^{188}$ of RhoA is a consensus site for PKA phosphorylation [22], the actions of the kinase on Rho proteins *in vivo* could be different from the results *in vitro*. Sauzeau et al. [54] reported that, in cultured vascular myocytes, RhoA can be phosphorylated on $Ser^{188}$ by the cGMP-dependent protein kinase, leading to the translocation of RhoA from the membrane to the cytosol. The transformation of HeLa cells with constitutively active cGMP-dependent protein kinase blocked the formation of RhoA-induced stress fibres [55]. Moreover, the phosphorylation of RhoA decreased its binding with ROK$\alpha$, a kinase promoting the formation of actin stress fibres and focal adhesion complexes [23], therefore directly affecting the cytoskeleton. Phospholipase D, another downstream effector of RhoA (that catalyses the hydrolysis of phosphatidylcholine), is inhibited following the phosphorylation of RhoA, modifying phospholipid metabolism [24]. Finally, PKA inhibits nucleotide exchange on RhoA, as well as chemoattractant-triggered integrin-dependent leucocyte adhesion, a pathway requiring active RhoA [56]. Taken together, these results and our findings emphasize the physiological implications of phosphorylation as a crucial element in the regulation of Cdc42 and RhoA activities. The inactivation of these proteins through phosphorylation will affect all of the downstream pathways controlled by Rho GTPases, including the organization of the actin cytoskeleton, the control of the cell cycle, cell migration and malignant transformation. Phosphorylation could therefore regulate the inactivation of Cdc42 and RhoA by attachment to RhoGDI, thus inhibiting the multiple activities of Rho GTPases elicited through their effectors. Finally, our results support the existence of phosphorylation as a mechanism regulating Cdc42 and RhoA activity independent of GDP–GTP cycling.

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