Phosphorylation of RhoGDI by p21-Activated Kinase 1

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Abstract

Rho GTPase activation is partially regulated at the level of guanine nucleotide dissociation inhibitors, or GDIs. The binding of Rho GTPases to GDIs has been shown to dramatically reduce the action of guanine nucleotide exchange factors (GEFs) to initiate Rho GTPase activation. The GDI–GTPase complex thus serves as a major point of regulation of Rho GTPase activity and function. It is likely that specific mechanisms exist to dissociate individual members of the Rho GTPase family from...
cytosolic Rho GDI complexes to facilitate the activation process. Such
dissociation would likely be tightly coupled to GEF-mediated guanine
nucleotide exchange and membrane association of the activated GTPase,
resulting in effector binding and functional responses.

Accumulating evidence suggests that the phosphorylation of either
the Rho GTPases themselves and/or phosphorylation of GDIs might serve
as a mechanism for regulating the formation and/or dissociation of Rho
GTPase–GDI complexes. Indeed, the selective release of Rac1 from
RhoGDI complexes induced by the p21-activated kinase-regulated phos-
phorylation of RhoGDI has been reported. We describe here methods for
the analysis of RhoGDI phosphorylation and regulation by p21-activated
kinase 1 (Pak1).

Introduction

The low-molecular weight Rho GTPases are involved in the regulation
of a variety of biological pathways. These GTPases function as molecular
switches, regulating cellular signaling by alternating between an inactive,
primarily cytosolic, GDP-bound state and an active GTP-bound state
usually associated with membranes where effector targets reside. Conver-
sion of inactive GTPases to an active form requires the action of guanine
nucleotide exchange factors (GEFs) that catalyze the exchange of bound
GDP for ambient GTP. GTPase inactivation involves the catalysis of GTP
hydrolysis, which is intrinsically slow, through the action of GAPs
(GTPase-activating proteins) that convert the GTPase to the inactive
GDP-bound state. An additional level of regulation exists for GTPases of
the Rho family because of their association with a third class of protein, the
GDP dissociation inhibitors (GDIs).

Three human Rho GDIs have been identified: the ubiquitously ex-
pressed RhoGDI (or GDIα/GDI1) (Fukumoto et al., 1990; Ueda et al.,
1990), the hematopoietic cell–selective Ly/D4GDI (or GDIβ/GDI2) (Lelias
et al., 1993; Scherle et al., 1993), and RhoGDIγ (or GDI3), specifically
expressed in lung, brain, and testis (Adra et al., 1997; Zalcman et al., 1996).
Both RhoGDI and D4GDI are cytosolic and form 1:1 complexes with Rho
family GTPases. Three distinct biochemical activities have been described
for Rho GDIs (DerMardirossian and Bokoch, 2005; Olofsson, 1999). First,
they inhibit the dissociation of GDP from Rho proteins, maintaining the
GTPase in an inactive form and preventing GTPase activation by GEFs.
Second, they are able to interact with the GTP-bound form of the
Rho GTPase to inhibit GTP hydrolysis, blocking both intrinsic and
GAP-catalyzed GTPase activity and preventing interactions with effector
targets. A third biochemical activity of GDIs is to modulate the cycling of Rho GTPases between cytosol and membranes. GDIs maintain Rho GTPases as soluble cytosolic proteins by forming high-affinity complexes in which the geranyl-geranyl membrane-targeting moiety present at the C terminus of the Rho GTPases is shielded from the solvent by its insertion into the hydrophobic pocket formed by the immunoglobulin-like β sandwich of GDI (Hoffman et al., 2000; Longenecker et al., 1999). When Rho proteins are released from GDIs, they are able to insert into the lipid bilayer of the plasma membrane through their isoprenylated C terminus. In this uncomplexed form, the Rho GTPases can then interact with, and are activated by, membrane-associated GEFs, thereby initiating the association with effector targets at the membrane. An extraction from the membrane because of the reassociation with GDI, possibly initiated by GTP hydrolysis, is postulated to induce recycling of the GTPase back into the cytosol (DerMardirossian and Bokoch, 2005; Olofsson, 1999).

The binding of Rho GTPases to GDI has been shown to dramatically reduce the action of GEFs, such as Dbl (Yaku et al., 1994) and Tiam1 (Robbe et al., 2003), to catalyze nucleotide exchange. The GDI–GTPase complex is thus a major point of regulation of Rho GTPase activity and function. It is likely that specific mechanisms exist to dissociate individual members of the Rho GTPase family from cytosolic RhoGDI complexes to facilitate the activation process. This dissociation would likely be tightly coupled to GEF-mediated guanine nucleotide exchange and membrane association of the activated GTPase, resulting in effector binding and functional responses.

DerMardirossian et al. (2004) recently described the binding and phosphorylation of RhoGDI, both in vitro and in vivo, by p21-activated kinase 1 (Pak1). Pak1 is a serine/threonine kinase whose activity is regulated by the binding of active Rac or Cdc42, as well as a number of other mediators (Bokoch, 2003). Phosphorylation was shown to occur on two sites (Ser101 and Ser174) in RhoGDI on the external surface of the hydrophobic cleft in which the GTPase prenyl group binds. Both of these sites lie adjacent to hydrophobic residues that directly line the RhoGDI geranylgeranyl-binding pocket. Phosphorylation of these two sites resulted in the selective release of Rac1, but not RhoA, from the GDI complex, leading to its subsequent activation by exchange factors. Rac1 dissociation from RhoGDI and subsequent Rac1 activation induced by the growth factors EGF and PDGF required phosphorylation of S101 and S174 by Pak1. The phosphorylation of RhoGDI by Pak1 might serve as a positive feed-forward mechanism to account for sustained Rac activation during processes such as cell motility (Fig. 1).
Materials and Methods

In Vitro Phosphorylation of RhoGDI by Pak1

To examine whether pure, recombinant Pak1 could directly phosphorylate RhoGDI, we performed an *in vitro* kinase assay using GST-Pak1 as a constitutively active form of Pak1 and recombinant RhoGDI as a substrate. The preparation of purified GST-Pak1 and RhoGDI have been described elsewhere in detail (Chuang *et al.*, 1993; King *et al.*, 2000a). We note that to have highly active GST-Pak1, it is better to retransform pGEX-GST-Pak1 DNA in *Escherichia coli* strain BL21–competent cells, plated on LB broth, Miller (Fisher) plus ampicillin, and incubate overnight at 30°. Do not incubate the plates at 37°, because spontaneous mutations in Pak1 may occur. Kinase reactions (King *et al.*, 2000b) contained recombinant GST-Pak1 (1 μg per reaction) in kinase buffer (50 mM HEPES/NaOH, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 0.2 mM DTT) in a 70-μl reaction mixture, with 2 μg of pure recombinant RhoGDI, 20 μM ATP
and 0.5 μCi/reaction radiolabeled [γ-32P]ATP (specific activity 4500 mCi/mmol, from ICN, Costa Mesa, CA). As a positive control, perform the kinase reaction in presence of 1 μg of myelin basic protein (Sigma M-1891). The reactions were incubated for 30 min at 30° and stopped by addition of 15 μl of 4× concentrated Laemmli’s SDS-PAGE sample buffer. Phosphorylation reactions were resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then gels were stained with Coomassie Brilliant Blue R-250, destained, dried, and subjected to autoradiography for 1–24 h (Fig. 2).

In Vivo Phosphorylation of RhoGDI by Pak1

HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) containing 8% heat-inactivated fetal bovine serum, 10 mM HEPES, pH 7.0, 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37° in 5% CO2. For transfection experiments, cells were seeded on 10-cm cell culture dishes at 50–70% confluency and transfected using appropriate concentrations of LipofectAmine (Life Technologies, Inc.) as a transfection agent. Cells were transfected with 1 μg His-tagged RhoGDI constructs (wt, S101A, S174A, S101/174A) and either 3 μg Myc-tagged Pak1 constructs (wt, constitutively active Pak1T423E,L107F or kinase-dead Pak1K299A) or 3 μg empty pCMV6-vector DNA control and 20 μl of LipofectAmine (Invitrogen, Carlsbad, CA) per dish. The transfection protocol was essentially followed according to the manufacturer’s guidelines. At 16 h after transfection, cells were gently washed once with phosphate-buffered saline (PBS 1×) and incubated at 37°, 5% CO2 for 1 h with phosphate-free DMEM; 0.4 mCi [32P] orthophosphate (ICN) was then added to the plates, and cells were incubated overnight at 37°, 5% CO2. Phosphate-buffered saline was used to wash once the plates (be very gentle, as cells may detach) and cells were

Fig. 2. Phosphorylation of RhoGDI by Pak1 in in vitro kinase assay. Purified recombinant RhoGDI (0.3 and 1 μg) were phosphorylated in an in vitro kinase assay with purified recombinant GST-Pak1, as described in the text. A concentration-dependent phosphorylation of RhoGDI by Pak1 was observed.
scraped into 0.5 ml of lysis buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 1% NP-40 supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml of aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 nM microcystein).

To verify protein expression, whole-cell lysates from nonradioactive transfected cells were scraped into 0.5 ml of lysis buffer. After 30 min on ice, the homogenate was spun down 10 min at 14,000 rpm at 4°C in a benchtop Eppendorf Microfuge to remove insoluble cells. A protein assay was carried out on the clarified homogenate using a BCA reagent protein assay (Pierce). Equivalent amounts of protein (25 μg) of the clarified homogenate were run on 12% polyacrylamide gels to resolve proteins. After electrophoresis, the proteins were transferred to nitrocellulose (Amersham) and probed with 9E10 anti-myc antibody (dilution 1/5,000, generated in-house) and with anti-His antibody (dilution 1/2,000, Babco) to detect the expression of Myc-tagged Pak1 and His-tagged RhoGDI proteins, respectively.

For precipitation experiments, His-tagged RhoGDI-expressing lysates were preincubated with equilibrated protein G-Sepharose (Amersham-Pharmacia) for 1 h at 4°C, then the beads were pelleted to reduce the background. Precleared lysates were then incubated with monoclonal anti-His antibody (4 μl/0.5 ml lysates, Babco) for 2 h at 4°C, followed by 2 h incubation at 4°C with equilibrated Protein G-sepharose beads (1:1 slurry/IP). The bead fraction was washed four times with lysis buffer and 4× concentrated Laemmli’s SDS-PAGE sample buffer was added to the beads, which were boiled 5 min at 110°C. Samples were resolved by 12% SDS-polyacrylamide gel electrophoresis and RhoGDI phosphorylation was detected by autoradiography, as described previously.

**Binding of Pak1 to RhoGDI**

To evaluate interactions between endogenous RhoGDI and Pak1, we carried out coimmunoprecipitation experiments. As described previously, HEK293T cells grown to approximately 70% confluence on 100-mm tissue culture dishes were transiently transfected with 20 μl LipofectAmine reagent and 3 μg of either empty vector control (pCMV6M) or Myc-tagged Pak1 (wt, Pak1K299A, Pak1T423E,L107F in pCMV6M vector) (King et al., 2000b). The cells were allowed to express the protein for 20 h after transfection and were then washed in PBS and scraped into 0.5 ml of lysis buffer. Clarified lysates were subjected to protein concentration determined by BCA assay, and protein expression analyzed by immunoblotting using enhanced chemiluminescence (Pierce). To immunoprecipitate endogenous
RhoGDI, we used 10 μl of RhoGDI antibody (Santa Cruz SC-360 A-20) per 0.5 ml of lysates. After overnight incubation at 4°C, 20 μl of pre-equilibrated protein A-Sepharose beads (Repligen, Waltham, MA) were added to the lysates and incubated for 2 h at 4°C. Precipitated proteins were washed four times with lysis buffer, suspended in SDS-PAGE sample buffer, boiled for 5 min, resolved by 12% SDS-PAGE, and transferred to nitrocellulose membranes. The immunoprecipitated proteins were analyzed by immunoblotting the membrane with either Myc (dilution 1/5,000) or RhoGDI (dilution 1/2,000) antibodies using enhanced chemiluminescence (Pierce). Because RhoGDI has the same molecular weight as the antibody light chain, to avoid any background on the immunoblot we use an anti-rabbit secondary antibody that does not recognize the light chain (Pierce #31463).

We observed substantial coimmunoprecipitation of the active form of Pak1 with RhoGDI. In contrast, there was only slight interaction of Pak1 wt with RhoGDI, whereas kinase-dead Pak1 failed to interact with RhoGDI. These data indicate the formation of a relatively stable complex between Pak1 and RhoGDI is dependent on Pak1 being in an active state capable of substrate binding and phosphorylation. To investigate the regions of Pak1 that were important for the interaction with RhoGDI, we transfected in HEK293T cells either the N-terminal regulatory domain (amino acids 1-205) or the C-terminal catalytic domain of Pak1 (amino acids 206-545) following the protocol described previously. Only the catalytically active C terminus, but not the N-terminal regulatory domain, of Pak1 binds RhoGDI.

**Analysis of Phosphorylation Sites on RhoGDI**

To analyze the phosphorylation sites on RhoGDI, we performed a phosphoamino acid analysis according to the procedure of Boyle et al. (1991) with minor modifications. HEK293T cells were transfected with His-tagged RhoGDI (wt S101A, S174A, S101/174A) and active Pak1 constructs, and cells were labeled with [32P] orthophosphate as described previously. Proteins were immunoprecipitated with anti-His mouse antibody and protein G-Sepharose, resolved by 12% SDS-PAGE, blotted onto nitrocellulose membrane, and phosphorylated RhoGDI proteins revealed by autoradiography. The radiolabeled band corresponding to RhoGDI was excised and blocked with 0.5% polyvinylpyrrolidone (PVP-10 Sigma) in 100 mM acetic acid for 30 min at 37°C. After extensive washes in H2O, the excised samples were incubated for 15 h at 37°C with 1.5 μg trypsin in 50 mM NH4HCO3, pH 8.3. Samples were then lyophilized, resuspended in 30 μl of 5.7 N HCl, and hydrolyzed at 110°C for 1 h. The hydrolysate was concentrated using a Speed-Vac concentrator, and the resulting pellet was washed...
twice in 200 μl of distilled water and finally resuspended in 3 μl of pH 1.9 electrophoresis buffer along with phosphoamino acid standards (phosphoserine, phosphotyrosine, phosphothreonine, at 1 mg/ml), and spotted onto cellulose-coated plates (EM Science 25 TLC plate 20 × 20 cm Cellulose OB124186). Separation of the proteolytic fragments was accomplished by electrophoresis in the first dimension for 20 min at 1500 V and by another electrophoresis in the second dimension (plates are turned 90 degrees counterclockwise) for 16 min at 1300 V in a Multiphor II horizontal electrophoresis unit (Amersham Pharmacia Biotech). The radioactive tryptic fragments were located by autoradiography. Phosphoamino acid standards were developed using 0.2% ninhydrin solution spray.

To determine the phosphorylated residues on RhoGDI, we performed two-dimensional phosphopeptide mapping of RhoGDI that had been phosphorylated in vivo by Pak1 as described previously. Proteolyzed samples were lyophilized and resuspended in 2 μl of pH 1.9 electrophoresis buffer (2.2% formic acid, 8% acetic acid), spotted onto 20-cm cellulose-coated plates (EM Science 25 TLC plate 20 × 20 cm Cellulose OB124186), and electrophoresed for 40 min at 1300 V in pH 1.9 electrophoresis buffer. Plates were air-dried and chromatographed in buffer containing 62.5% isobutyric acid, 1.9% n-butanol, 4.8% pyridine, and 2.9% glacial acetic acid for 14 h. The radioactive phosphopeptides were located by autoradiography (Boyle et al., 1991) (Fig. 3).

Analysis for Rac-RhoGDI Complexes In Vitro and In Vivo

In Vitro. To evaluate the effects of phosphorylation on GTPase–GDI complexation in vitro, we used an assay based on the binding of [35S]GTPγS to Rho GTPase. The basis for this assay lies in the fact that nucleotide binding only occurs to free GTPase, because nucleotide exchange is inhibited when the GTPase is bound with RhoGDI (Chuang et al., 1993; Ueda et al., 1990). Lipid-modified RhoA and Rac1 were purified from baculovirus membranes and preloaded with GDP as in Chuang et al. (1993) and Cerione et al. (1995). GST-GDP-Rac or RhoA immobilized on glutathione beads and precomplexed with RhoGDIwt or Rho GDI S101/174A mutant were obtained by incubating the GDP-RhoGTPase with 4–10-fold excess RhoGDI proteins overnight at 4°C. The resulting complexes were washed three times with 25 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM MgCl2, 1 mM EDTA, and 0.1% Chaps to remove free RhoGDI. We verified the formation of the complex by the lack of significant [35S]GTPγS binding in the absence of added detergent and a recovery of 100% of GTPase binding activity on the addition of 2% sodium cholate, which totally disrupts the complex and serves as a positive control. The
binding of $^{35}$S-GTP$\gamma$S to the RhoGTPases was monitored by filtration on BA85 nitrocellulose filters, using the method described in Chuang et al. (1993) and Knaus (1992); 50 nM of the complex was subjected to in vitro kinase assay with 1 mM unlabeled ATP for 20 min at 30$^\circ$C as described previously in the presence of 1 µg/ml of GST-Pak1 or 1 µg/ml of GST protein as a control. The sample was then pelleted and washed twice with 25 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM MgCl$_2$, 1 mM EDTA, and 0.1% Chaps. The binding of $^{35}$S-GTP$\gamma$S was determined by the filter binding method as described by Chuang et al. (1993) and Knaus (1992).

**In Vivo.** Growth factors such as PDGF and EGF activate Rac to modulate cell growth and cytoskeletal remodeling. We examined the role of Pak1 in hormone-initiated dissociation of Rac from RhoGDI complexes during cell activation. HeLa Tet-off cells (Gossen and Bujard, 1992) were stably transfected with pTRE2hyg-EGFP-tagged Pak PID (inhibits specifically Pak kinase activity) or pTRE2hyg-EGFP-tagged Pak PIDL107F (the corresponding inactive mutant) by transfection with lipofectAMINE (Invitrogen). Resistant clones were isolated in the presence of hygromycin (500 µg/ml), screened for inducible expression and then cultured in the presence of doxycycline (1 µg/ml) according to the manufacturer’s instructions. HeLa cells, which contained a Tet-off vector (Clontech), were maintained in DMEM supplemented with 10% fetal bovine serum, 10 mM

![Image of Figure 3](image_url)
HEPES pH 7.0, 2 mM glutamine, 100 µg/ml G-418, 100 µg/ml hygromycin B, and 1 µg/ml doxycycline at 37° in 5% CO₂. To induce EGFP-Pak-PID or EGFP-Pak-PIDL107F protein expression, doxycycline has to be removed from the medium for at least 30 h. Cells should be washed twice with PBS, trypsinized, reseeded on plates, and washed again twice with PBS after 3–12 h. Separate experiments verified that expression of PIDwt resulted in >90% inhibition of serum-induced Pak1 activity.

For stimulation experiments, the HeLa cells stably transfected with inducible EGFP-tagged Pak PID wt or EGFP-tagged Pak PID L107F were cultured in 6-well plates in normal serum without doxycycline. At confluence, the cells were serum-starved 18 h and then treated with or without human recombinant PDGF (10 ng/ml) or EGF (100 ng/ml) (Fisher Scientific, Tustin, CA) for 0, 5, and 10 min. For transient transfection, HeLa cells were seeded on 6-well culture dishes at 0.5 × 10⁶ cells and the next day were transfected using LipofectAMINE as described previously. At 18 h after transfection, cells were serum starved and treated with growth hormone as described. HeLa cells were then harvested in lysis buffer, and the whole-cell lysates were subjected to protein quantitation by BCA assay. For immunoprecipitation experiments, endogenous RhoGDI was immunoprecipitated from whole-cell lysates with RhoGDI antibody (Santa Cruz SC-360 A-20) overnight at 4°C, followed by 2 h incubation with protein-A Sepharose beads. The proteins were transferred to PVDF membranes, and the level of Rac or RhoA proteins, as well as the immunoprecipitated RhoGDI, was assessed by Western blotting analysis using anti-Rac1 (#23A8, #05–389, Upstate Biotechnology Inc., Lake Placid, NY), anti-RhoA (#119 Sc-179 or #26C4 Sc-418, Santa Cruz Biotechnology Inc., Santa Cruz, CA), or anti-RhoGDI antibodies (Sc-360 A-20, Santa Cruz Biotechnology Inc.), respectively. Densitometry analysis was used to compare the level of Rho GTPase binding to RhoGDI in the various samples, and results were averaged over a minimum of three experiments.

References


