

Phosphorylation of RhoGDI by Pak1 Mediates Dissociation of Rac GTPase

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Summary

Selective activation of Rac GTPase signaling pathways requires the specific release of Rac from RhoGDI complexes. We identified a RhoGDI kinase from bovine brain as p21-activated kinase (Pak). Pak1 binds and phosphorylates RhoGDI both in vitro and in vivo at Ser101 and Ser174. This resulted in dissociation of Rac1-RhoGDI, but not RhoA-RhoGDI, complexes, as determined by in vitro assays of complexation and in vivo by coimmunoprecipitation analysis. We observed that Cdc42-induced Rac1 activation is inhibited by expression of Pak1 autoinhibitory domain. The dissociation of Rac1 from RhoGDI and its subsequent activation stimulated by PDGF or EGF is also attenuated by Pak1 autoinhibitory domain, and this is dependent on the ability of RhoGDI to be phosphorylated at Ser101/174. These results support a role for Pak1-mediated RhoGDI phosphorylation as a mechanism for Cdc42-mediated Rac activation, and suggest the possibility of Rac-induced positive feed-forward regulation of Rac activity.

Introduction

The Rho GTPases regulate cellular activities that include growth and differentiation, vesicular transport, reactive oxygen production, apoptosis, axonal guidance, and various other aspects of cytoskeletal dynamics and motility (Bishop and Hall, 2000; Van Aelst and D'Souza-Schorey, 1997). Rho GTPases function as molecular switches in cell signaling, alternating between inactive GDP-bound states maintained as cytosolic complexes with GDP dissociation inhibitors (GDIs), and active GTP-bound states usually associated with membranes where effector targets reside. Complexation of Rho, Rac, or Cdc42 with GDIs inhibits GDP dissociation and localizes GTPases to the cytosol in inert forms unable to interact with GEFs (guanine nucleotide exchange factors), GAPs (GTPase activating proteins), or effector targets (Van Aelst and D'Souza-Schorey, 1997). Indeed, there is substantial biochemical (Kikuchi et al., 1992; Yaku et al., 1994) and structural (Hoffman et al., 2000) evidence that Rho GEFs cannot act on Rho GTPases when the GTPases are complexed with RhoGDI. The GDI-GTPase complex is thus a major point of regulation of Rho GTPase activity and function.

The ability of hormonal stimuli to specifically activate

individual members of the Rho GTPase family is well documented. For example, the specific activation of Rac by bombesin, Cdc42 by bradykinin, and Rho by lysophosphatidic acid in various fibroblast-like cell lines has been established (Ridley and Hall, 1992; Ridley et al., 1992; Kozma et al., 1995). Such observations strongly indicate that specific mechanisms exist to dissociate individual members of the Rho GTPase family from existing cytosolic RhoGDI complexes. This dissociation is likely tightly coupled to GEF-mediate guanine nucleotide exchange and membrane association of the activated GTPase, resulting in effector binding and functional responses (Bokoch et al., 1994). Relatively little is known about the molecular basis for specific Rho GTPase release from RhoGDI.

Three human GDIs have been identified: the ubiquitously expressed RhoGDI (or GDI α) (Fukumoto et al., 1990; Ueda et al., 1990), the hematopoietic cell-selective Ly/D4GDI (or GDI β) (Lelias et al., 1993; Scherle et al., 1993), and RhoGDI γ , specifically expressed in lung, brain, and testis (Adra et al., 1997). Both RhoGDI and D4GDI are cytosolic and have a broad range of activity toward the Rho family GTPases. Both GDIs maintain Rho GTPases as soluble cytosolic proteins by forming high-affinity complexes that mask the C-terminal geranylgeranyl membrane-targeting moiety within a hydrophobic pocket formed by the immunoglobulin-like domain of RhoGDI (Gosser et al., 1997; Keep et al., 1997; Hoffman et al., 2000). When Rho proteins are released from GDIs, they insert into the lipid bilayer through their isoprenylated C terminus to be activated by membrane-associated GEFs, initiating the association with effector targets at the membrane. A reassociation with GDI, possibly initiated by GTP hydrolysis, is postulated to induce recycling of the GTPase to the cytosol.

The signaling mechanisms and protein components that modulate this GTPase-RhoGDI regulatory cycle remain to be defined. Proteins regulating the dissociation of Rab GTPases from Rab GDI are an integral part of the Rab regulatory cycle (Sivars et al., 2003). The analogous action of proteins able to stimulate GTPase dissociation from RhoGDI was suggested by the protease sensitivity of this process in studies using human neutrophil fractions (Bokoch et al., 1994). RhoGDI displacement activity resident in the ERM proteins (Takahashi et al., 1997) or the p75 neurotrophin receptor (Yamashita and Tohyama, 2003) has been reported to initiate the specific release of Rho GTPase. Phosphorylation of RhoGDI and/or Rho GTPases may represent another mode of complex regulation. Lang et al. (1996) demonstrated that the phosphorylation of RhoA at Ser188 by protein kinase A (PKA) enhanced the binding of RhoA to RhoGDI. This effect of PKA on RhoA was confirmed and extended to Cdc42 (Forget et al., 2002). Significantly, Rac lacks the relevant C-terminal PKA phosphorylation site and does not appear to be regulated by this mechanism. Bourmeyster and Vignais (1996) reported that RhoGDI from neutrophils was phosphorylated, and that treatment of the phosphorylated RhoA-RhoGDI complex with alkaline phosphatase induced complex dissociation. Rho

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activation initiated by the endothelial cell thrombin receptor required protein kinase C α (PKC α), and activation was accompanied by PKC α -mediated RhoGDI phosphorylation (Mehta et al., 2001). Consistent with the existence of phosphorylated forms of GDI, several differentially charged species of RhoGDI (Bourmeyster et al., 1994) and D4GDI (Gorvel et al., 1998) have been detected in cells by two-dimensional gel analysis.

The p21-activated kinases (Paks) are serine/threonine kinases whose activity is regulated by the binding of activated Rac or Cdc42 GTPases (Manser et al., 1994). Paks consist of a highly conserved C-terminal catalytic domain and an N-terminal regulatory domain. The latter contains binding sites for the adaptor proteins Nck and Grb2 (Bokoch et al., 1996; Galisteo et al., 1996; Puto et al., 2003), as well as for the Cdc42/Rac exchange factor Pix (Manser et al., 1998), in addition to the Cdc42/Rac binding CRIB domain, and an overlapping autoinhibitory domain (Chong et al., 2001). The binding of Cdc42-GTP or Rac-GTP to Pak1 relieves autoinhibition and induces an increase in kinase activity (Zenke et al., 1999; Lei et al., 2000). Paks are implicated in the regulation of multiple cellular activities, including cytoskeletal dynamics (Bokoch, 2003).

Here we provide evidence that Pak1 acts as a RhoGDI kinase *in vitro* and *in vivo*, and demonstrate a specific effect of phosphorylation on Rac/RhoGDI association. The interaction of Pak1 with RhoGDI appears to represent a novel mechanism for the regulation of Rac GTPase signaling.

Results

Identification of a RhoGDI Kinase

In preliminary studies, we observed that the addition of pure RhoGDI to *in vitro* kinase assays containing cytosol from either fMetLeuPhe (fMLP)-stimulated human neutrophils or from bovine brain resulted in the incorporation of [³²P]label into RhoGDI (data not shown). Phosphoamino acid analysis revealed phosphorylation of serine and threonine residues (neutrophils and brain) and tyrosine residues (neutrophils). In order to identify the kinase(s) that phosphorylate RhoGDI, we purified RhoGDI kinase activity from bovine brain cytosol using sequential DEAE anion exchange chromatography and size exclusion chromatography (Figures 1A and 1B). A single major peak of RhoGDI kinase activity was observed after DEAE fractionation, and this remained as a single peak on the subsequent gel filtration step. Attempts to purify the RhoGDI kinase to homogeneity via additional chromatographies led to a substantial loss of kinase activity (due to an ultimate separation of Rac and Cdc42 from Pak1; see following). We therefore characterized the biochemical properties of the RhoGDI kinase eluted from the Aca54 column. RhoGDI phosphorylated by the partially purified RhoGDI kinase (Aca54 fractions 16–25) was subjected to phospho-amino acid analysis and was found to contain phosphate exclusively on serine residues (data not shown).

In order to analyze the pharmacological properties of RhoGDI kinase, we studied its activity toward recombinant RhoGDI in an *in vitro* kinase assay in the absence or presence of various serine/threonine kinase inhibitors.

Compared to the untreated control, protein kinase A (PKA) inhibitors H89 and H7, the protein kinase C (PKC) inhibitors H7 and BIM, the calmodulin kinase inhibitor KN93, and staurosporine were essentially without effect at concentrations well over their respective K_is (Figure 2A). Interestingly, the addition of PBDID^{H83L}, a non-GTPase binding peptide containing the autoinhibitory domain derived from p21-activated kinase 1 (Pak1) that specifically inhibits activity of Paks 1–3, completely blocked RhoGDI phosphorylation by the brain kinase. This appeared to be a specific effect, as the PBDID^{L107F} mutant deficient in autoinhibitory activity caused no inhibition. This data strongly implicated that the bovine brain RhoGDI kinase is a form of Pak.

The addition of GTP γ S-loaded Cdc42 to the partially purified RhoGDI kinase substantially increased the level of RhoGDI phosphorylation (Figure 2B). In a separate experiment, we took advantage of the high-affinity interaction between Pak and Pix (aa 155–545 of Pix, containing the Pak binding SH3 domain), to deplete Pak from the RhoGDI kinase pool. After one round of affinity precipitation with the Pix fragment, the soluble RhoGDI kinase activity was substantially reduced (Figure 2C). However, the pull-down now exhibited a strong capacity to phosphorylate RhoGDI. Finally, we examined whether pure, recombinant Pak1 could directly phosphorylate RhoGDI. We observed a clear concentration-dependent phosphorylation of RhoGDI by Pak1 (Figure 2D). Subsequent examination of the DEAE and Aca54 column fractions by immunoblotting revealed the presence of both Pak1 and Cdc42 in fractions containing RhoGDI kinase activity (Figures 1C and 1D). Overall, these data strongly indicate that an activated Pak, probably Pak1, is the bovine brain RhoGDI kinase that we isolated.

Pak1 Phosphorylates RhoGDI in Intact Cells and Binds to RhoGDI via Its Kinase Domain

We performed metabolic labeling experiments to assess RhoGDI phosphorylation by Pak1 *in vivo*. Figure 3A shows that phosphorylation of RhoGDI was induced by the catalytically active Pak1 kinase, but not by the inactive kinase-dead Pak1 or Pak1 wt. We verified the phosphorylation of endogenous RhoGDI as well by 2D chromatography and immunoblot (Figure 3B). A shift in the mobility of RhoGDI was observed that was consistent with the addition of phosphate groups in the presence of active Pak1^{T423E,L107F}, but not in vector controls or upon expression of equal amounts of kinase-dead Pak1^{K299A}. These data indicate that Pak1 functions as a RhoGDI kinase when expressed in intact 293T or HeLa cells.

Possible *in vivo* interactions between endogenous RhoGDI and Pak1 were evaluated by coimmunoprecipitation. Figure 4A shows that there is substantial coimmunoprecipitation of the active form of Pak1 with RhoGDI. In contrast, there was only slight interaction of Pak1 wt with RhoGDI, while kinase-dead Pak1 failed to interact with RhoGDI. This data indicates the formation of a relatively stable complex between Pak1 and RhoGDI that is dependent upon Pak1 being in an active state capable of substrate binding and phosphorylation. Interestingly, only the catalytically active C terminus, but not the N-terminal regulatory domain, of Pak1 binds RhoGDI (Figures 4B and 4C). Taken together, these results dem-

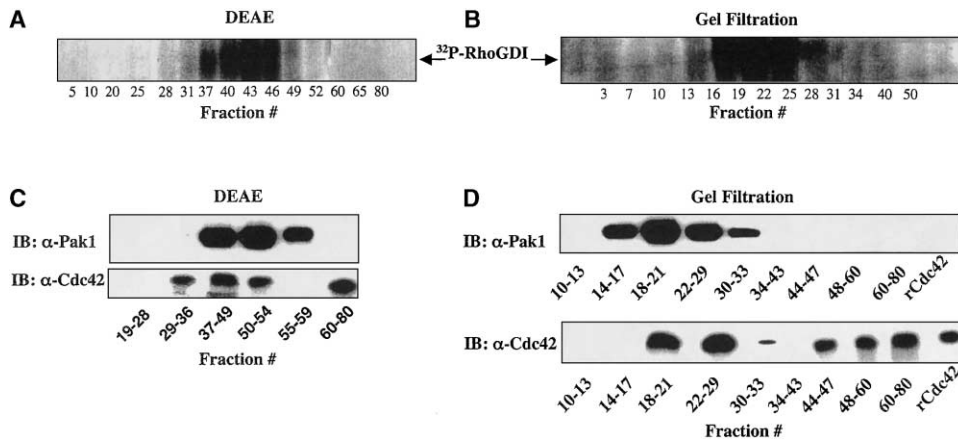


Figure 1. Partial Purification of RhoGDI-Kinase from Bovine Brain Cytosol

Bovine brain cytosol was fractionated by chromatography and fractions tested for RhoGDI phosphorylation by in vitro kinase assay, as in Experimental Procedures.

(A) GST-RhoGDI substrate was resolved by SDS-PAGE and phosphorylation by DEAE fractions detected by autoradiography.

(B) Pooled DEAE fractions containing RhoGDI kinase activity (Fr. 37–46) were analyzed via AcA54 chromatography, and kinase activity determined.

(C and D) Immunoblot analysis of DEAE and AcA54 column fractions with anti-Pak antibody R2124 and anti-Cdc42 antibody, respectively.

onstrate RhoGDI binds to active Pak1, and that amino-terminal regulatory elements, including the Rac/Cdc42 binding p21 binding domain of Pak1, are not necessary for Pak1-RhoGDI interaction. These data thus indicate that Pak1 does not bind RhoGDI through an indirect interaction mediated by the binding of PAK1 to Rac- or Cdc42-GTPase associated with endogenous RhoGDI.

Mapping of the Pak1 Phosphorylation Sites on RhoGDI

We next analyzed the serine residue(s) phosphorylated in RhoGDI by Pak1. 293T cells expressing active Pak1

and RhoGDI wild-type were metabolically labeled with [γ - 32 P] orthophosphoric acid and RhoGDI immunoprecipitated from the whole-cell lysates subjected to phosphopeptide mapping. Two distinct radiolabeled phosphopeptide spots were detected (Figure 5A). A similar pattern was detected with RhoGDI phosphorylated by Pak1 in vitro (data not shown). RhoGDI contains consensus Pak phosphorylation sites at Ser101 (KKQS¹⁰¹F) and at Ser174 (ARGS¹⁷⁴Y) (Bokoch, 2003). To investigate whether either site served as an actual Pak phosphorylation site, we generated the RhoGDI mutants S101A and S174A and assessed their phosphorylation by active

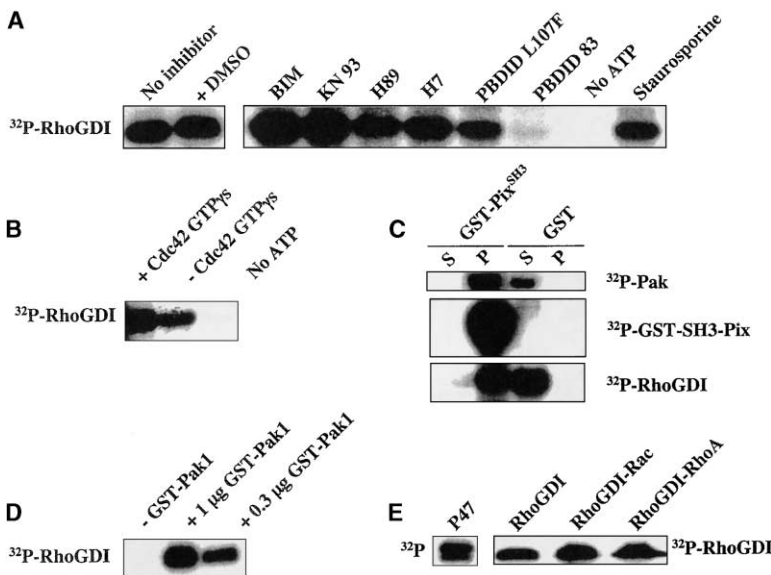


Figure 2. Effect of Various Serine/Threonine Kinase Inhibitors on RhoGDI Kinase Activity

(A) Purified recombinant GST-RhoGDI immobilized on glutathione beads was subjected to in vitro kinase reaction with partially purified RhoGDI kinase (AcA54 Fr. 18–21) in the presence or absence of various kinase inhibitors, as follows: bisindolylmaleimide (BIM) (0.5 mM), KN 93 (10 μ M), H89 (10 μ M), H7 (500 μ M), PBDID 83 (1 μ g), the autoinhibitory domain of Pak1 mutated at aa. 83 to eliminate GTPase binding capability, and PBDID 107 (1 μ g), in which the autoinhibitory effect is destroyed by the mutation L107F, and Staurosporine (10 μ M) were tested. RhoGDI phosphorylation was analyzed by SDS-PAGE/autoradiography. Results shown are representative of at least two independent experiments.

(B) GST-RhoGDI was subjected to in vitro kinase assay using partially purified RhoGDI kinase in the presence or absence of 1 μ g GTP γ S-loaded Cdc42.

(C) An active AcA54 column fraction was immunodepleted of p21-activated kinase with

GST- α Pix¹⁵⁵⁻⁵⁴⁵ (30 μ g) immobilized on glutathione beads. The immunodepleted fraction and the immunoprecipitate were subjected to in vitro kinase assay in the presence of 2 μ g of RhoGDI and Cdc42-GTP γ S.

(D) RhoGDI phosphorylation was assessed by in vitro kinase assay with 0.3 or 1 μ g of purified recombinant GST-Pak1.

(E) Recombinant free RhoGDI (50 pmol) or in preformed complex with Rac1 or RhoA GTPase (50 pmol) was subjected to an in vitro kinase assay using 1 μ g of GST-Pak1. The known Pak1 substrate p47 (1 μ g) was used as an assay control. Complexation was verified by [35 S]GTP γ S binding, as in Experimental Procedures. Results are representative of at least two independent experiments.

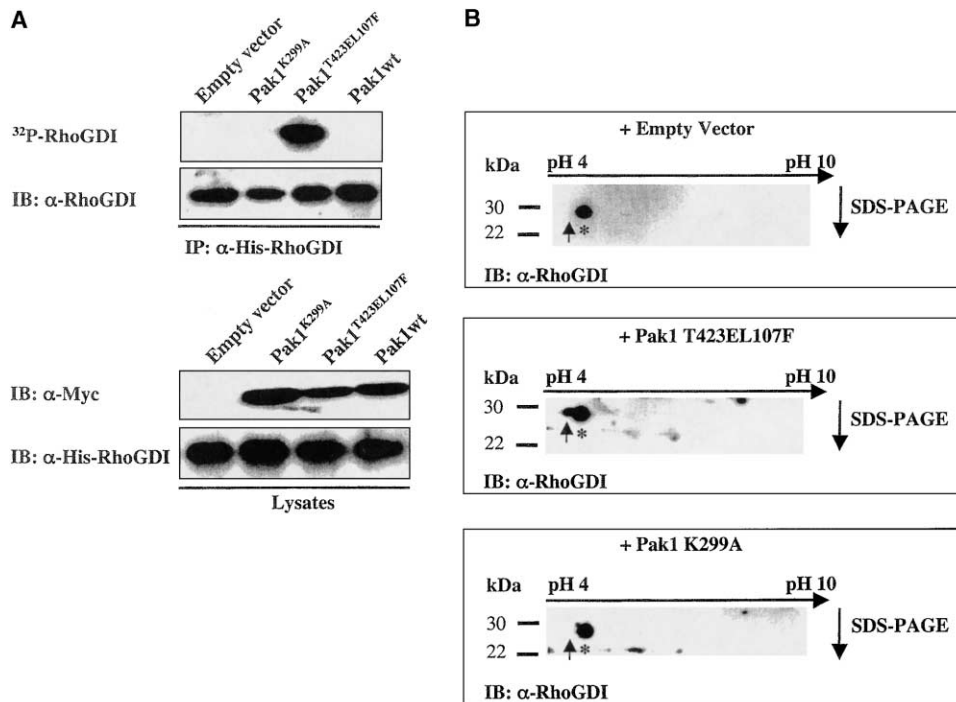


Figure 3. In Vivo Phosphorylation of RhoGDI by Pak1

(A) The indicated Myc-tagged Pak1 constructs were cotransfected with His-tagged RhoGDI wt into [³²P] orthophosphoric acid-labeled 293T cells. Immunoprecipitated RhoGDI was separated by SDS-PAGE and phosphorylation determined by autoradiography and RhoGDI content by Western blot (upper panel). The relative expression of the Pak1 and RhoGDI constructs in lysates were determined by Western blot with Myc 9E10 and His antibodies, respectively (bottom panel). The data are representative of three independent experiments.

(B) 2D gel electrophoresis. Lysates prepared from HeLa cells transfected with empty vector control, Myc-tagged Pak1^{T423E,L107L}, or Myc-tagged Pak1^{K299A} (30 μg protein) were resolved by 2D gel electrophoresis. RhoGDI was detected by Western blot (indicated by star; phosphorylated form indicated by arrowhead). Data are representative of two independent experiments.

Pak in ³²P-labeled cells (Figure 5A). 2D mapping performed on each single mutant showed the loss of the upper phosphopeptide spot for the mutant RhoGDI^{S101A} and the lower phosphopeptide spot for the mutant RhoGDI^{S174A}. Mutation of both sites to Ala abolished phosphate incorporation into RhoGDI and resulted in the loss of both phosphopeptide spots (data not shown), indicating that these residues are the only sites on RhoGDI phosphorylated by Pak1 in vivo. Identical results were obtained in vitro when we analyzed RhoGDI phosphorylated with purified recombinant GST-Pak1.

We noted that the sequences surrounding Ser101 and Ser174 were also consistent with protein kinase A (PKA) phosphorylation motifs. We observed that RhoGDI was phosphorylated by pure, active PKA in vitro (see Supplemental Figure S1 at <http://www.molecule.org/cgi/content/full/15/1/117/DC1>) and by coexpressed PKA in intact cells (data not shown). 2D phosphopeptide analysis of RhoGDI revealed a single PKA-phosphorylated site, which we identified as Ser174. However, our in vitro and in vivo (see Figure 6D) analyses indicate that phosphorylation at Ser174 by PKA is itself insufficient to induce release of Rac GTPase from RhoGDI complexes (see following and Discussion).

Phosphorylation of RhoGDI by Pak1 Decreases Its Affinity for Rac GTPase

Since a primary function of RhoGDI is to bind Rho GTPases, we wanted to compare the Rac binding prop-

erties of unphosphorylated RhoGDI versus Pak1-phosphorylated RhoGDI. We first verified that RhoGDI in preformed complex with Rac1 or RhoA GTPase was effectively phosphorylated by Pak1 (Figure 2E). This is consistent with the location of Ser101 and Ser174 at solvent-exposed sites in RhoGDI-GTPase complexes. In order to evaluate the effects of phosphorylation on GTPase-GDI complexes, we utilized an assay based upon the binding of [³⁵S]GTPγS to Rho GTPase. Binding only occurs to free GTPase, as nucleotide exchange is inhibited when the GTPase is bound with RhoGDI (Ueda et al., 1990; Chuang et al., 1993b). We prepared complexes of recombinant Rac1-RhoGDI and RhoA-RhoGDI in vitro, verifying complexation by the lack of [³⁵S]GTPγS binding in the absence of added detergent. As shown in Figure 5B, we observed that upon phosphorylation of the Rac1-RhoGDI complex, ~25% of the previously complexed Rac1 was now able to bind [³⁵S]GTPγS, while binding was not detected in the absence of Pak1. This correlated with a stoichiometry of RhoGDI phosphorylation in vitro of 0.25 mol/mol obtained under our standard phosphorylation conditions. In marked contrast, using the S101/174A RhoGDI mutant in which the Pak1 phosphorylation sites were inactivated, Pak1 now induced very little dissociation of the Rac1-RhoGDI complex and no significant increase in [³⁵S]GTPγS binding over background. Interestingly, we also observed no effect of RhoGDI phosphorylation by Pak1 to induce release of RhoA from RhoA-RhoGDI complex. To verify this result,

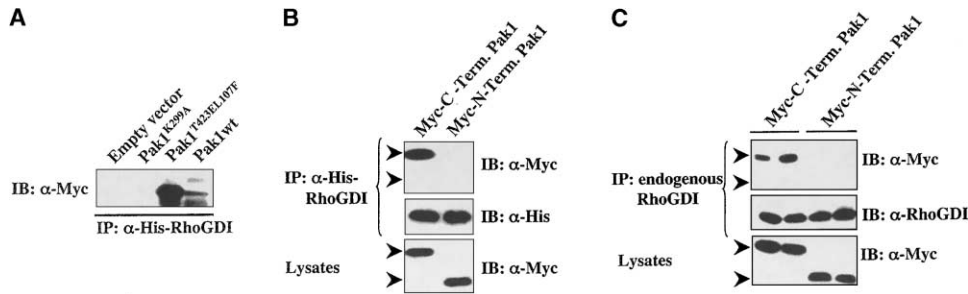


Figure 4. Physical Interaction of RhoGDI and Pak1

(A) The indicated Myc-Pak1 constructs were cotransfected with His-tagged RhoGDI into 293T cells. RhoGDI was immunoprecipitated using His antibody, separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-Myc antibody for coimmunoprecipitated Pak1. (B) The Myc-tagged Pak1 C-terminal kinase domain (aa 206–545) and the Myc-tagged Pak1 N terminus (aa 1–205) were coexpressed with His-tagged RhoGDI in 293T cells. The binding of truncated versions of Pak1 to RhoGDI (upper panel) and the immunoprecipitated RhoGDI (middle panel), as well as the level of protein expression in whole-cell lysates (bottom panel), were analyzed by Western blotting with anti-Myc and anti-His antibodies. (C) Association of endogenous RhoGDI with the kinase domain of Pak1. 293T cells were transfected with the indicated constructs of Pak1, and endogenous RhoGDI was immunoprecipitated and probed with Myc 9E10 antibody for coimmunoprecipitated Pak1 proteins. Immunoprecipitated endogenous RhoGDI (middle panel) and expression of truncated Pak1 proteins (bottom panel) were verified by probing with RhoGDI and Myc 9E10 antibodies, respectively.

we generated recombinant RhoGDI in which Ser101 and Ser174 were replaced with Glu as a phospho-mimetic substitution. RhoGDI^{S101A/S174A} binds to Rac1 and RhoA as efficiently as wild-type RhoGDI in an in vitro binding assay. However, Rac1 binding was reduced by ~40% in RhoGDI^{S101E/S174E} (data not shown). It is possible that the replacement of serine residues with glutamic acid may not fully mimic the phosphorylation state of the

protein. Nevertheless, these data support the in vitro phosphorylation data indicating that the modification of these two serine residues by Pak1 modulates the binding of Rac1 to RhoGDI.

In order to verify the in vitro results, we examined Rac1 binding to RhoGDI in intact cells in which RhoGDI was phosphorylated by Pak1. Figure 6A shows that in vector-transfected cells, Rac1 efficiently coprecipitates

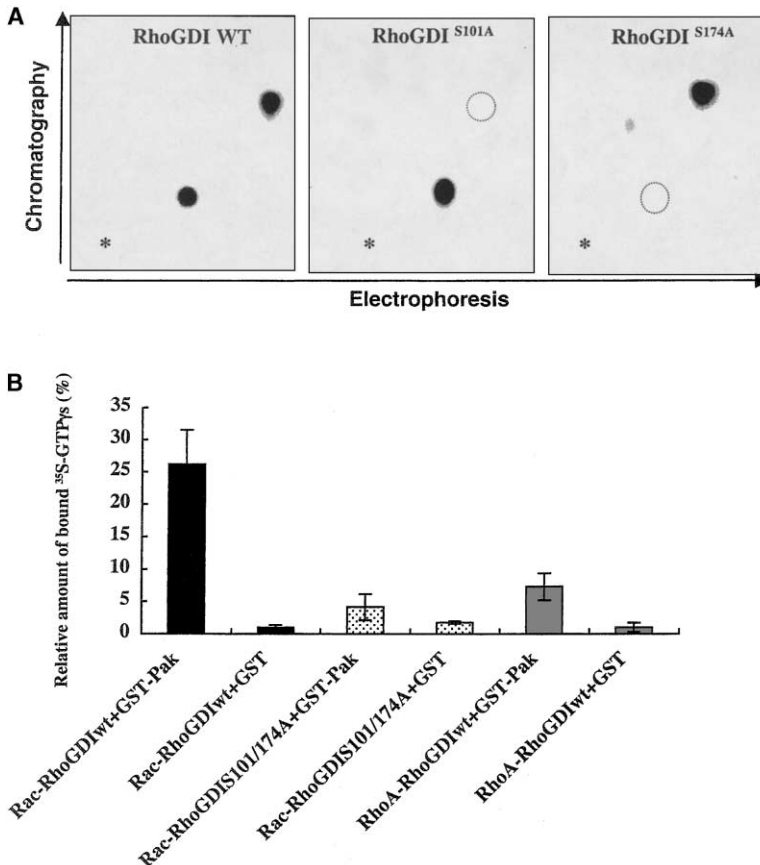
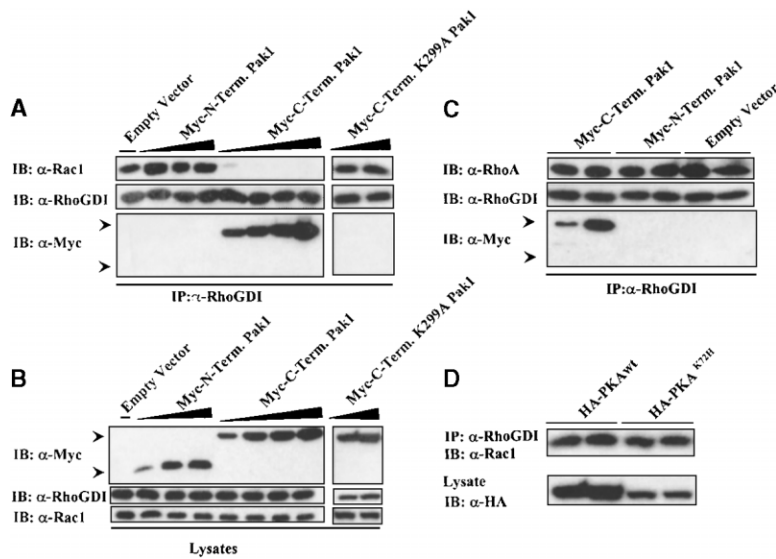


Figure 5. Analysis of Pak1 Phosphorylation Sites on RhoGDI

(A) 293T cells expressing His-tagged RhoGDI constructs and Myc-tagged PAK1^{T423E/L107F} were metabolically labeled with [³²P] orthophosphoric acid, and immunoprecipitated RhoGDI was subjected to 2D gel electrophoresis. The RhoGDI constructs are indicated at the top of each map; the sample application point is indicated by an asterisk, and an arrow shows the direction of electrophoresis and chromatographic migration. Phosphopeptides missing in RhoGDI mutants are circled by dots. On the electrophoresis axis, the cathode is on the right. The results shown are representative of at least two independent experiments.

(B) Recombinant RhoGDIwt or RhoGDIS101/174A in preformed complexes (50 nM) with Rac1 or RhoA GTPase were subjected to in vitro kinase assay in the presence of 1 μM GST-Pak1 or 1 μM GST control for 20 min at 30°C, per Experimental Procedures. The binding of [³⁵S]GTPγS to GDP-Rac or GDP-RhoA was determined by filtration assay. The results shown are the average ± SEM of four separate experiments. The amount of [³⁵S]GTPγS binding activity observed in the presence of 1% cholate (total free GTPase) was set to 100%.



with RhoGDI. However, in cells expressing the catalytically active Pak1 C terminus, we observed a dramatic decrease in RhoGDI-associated Rac1. This decrease was not observed in cells expressing the Pak1 N-terminal fragment or the catalytically inactive Pak1 C terminus. The expression of PKA was also not sufficient to induce the dissociation of endogenous Rac1 from RhoGDI (Figure 6D). No change in expression of endogenous Rac1 or RhoGDI was observed in cells expressing the Pak1 constructs (Figure 6B).

In contrast to the effect of Pak1 on the complexation of RhoGDI with Rac1, the association of endogenous RhoA with RhoGDI was not affected by Pak1 (Figure 6C). Similarly, we could detect only a slight effect of Pak1 on the complexation of endogenous Cdc42 with RhoGDI (data not shown). These results suggest that Pak1-mediated RhoGDI phosphorylation preferentially triggers the dissociation of Rac1 from RhoGDI.

Cdc42-Mediated Activation of Rac1 through Pak1 Kinase-Dependent Mechanisms in HeLa Cells

The release of Rho GTPases from RhoGDI is a key permissive step for subsequent GTPase activation. Pak1 is known to be a downstream target of both Rac and Cdc42 GTPases. In a number of studies, activation of Cdc42 has been shown to initiate a sequential downstream activation of Rac1 through an undefined mechanism (e.g., Kozma et al., 1995; Nobes and Hall, 1995). We postulated that the activation of Pak1 by Cdc42 might contribute to the subsequent induction of Rac activity via its ability to phosphorylate RhoGDI and induce Rac release. To test this hypothesis, we analyzed the activation of Rac after expressing constitutively active Cdc42^{Q61L} in inducible stable HeLa cell lines expressing either the Pak1 (auto)inhibitory domain (PID wt) or the noninhibitory PID^{L107F} mutant (Zenke et al., 1999). Equal expression of Cdc42 and PID constructs were verified by Western blotting and immunostaining. Cells

Figure 6. Pak1 Specifically Dissociates Rac from RhoGDI in 293T Cells

293T cells were transfected with either Myc-tagged N-terminal of Pak1 (1, 2.5, and 4 μ g DNA), Myc-tagged C-terminal kinase domain of Pak1 (0.5, 1, 2, and 3 μ g DNA), Myc-tagged inactive C-terminal kinase domain of Pak1 (C-termK299A 3 and 5 μ g DNA), or empty vector control. Precipitated endogenous RhoGDI was probed with anti-Rac1 (upper panel), RhoGDI (middle panel), and Myc 9E10 antibodies (bottom panel). Expression of Pak1 constructs and the level of endogenous Rac1 and RhoGDI expression in whole-cell lysates were assessed by Western blotting with respective antibodies (B). Precipitated RhoGDI was also probed with anti-RhoA antibody (C). In (D), HeLa cells were transfected with either HA-tagged active PKA wt (1 and 2 μ g DNA) or HA-tagged kinase dead PKA^{K72H} (2 and 3 μ g DNA). Endogenous RhoGDI precipitates were probed with anti-Rac1 antibody (D). Expression of PKA constructs in whole-cell lysates was assessed by Western blotting with HA antibody (bottom panel, [D]). Data shown are representative of four independent experiments.

expressing Cdc42^{Q61L} with inactive PID^{L107F} exhibited a significant increase in Rac1-GTP levels (Figure 7A). This Rac1 activation was not observed in vector controls, or cells expressing inactive Cdc42^{T17N} or Cdc42wt. We verified that only Cdc42^{Q61L} expression stimulated Pak1 activity (data not shown). The ability of Cdc42^{Q61L} to induce Rac1 activation was inhibited by more than 90% upon coexpression of PID wt, but not by the inactive PID^{L107F}. In contrast, Rac activation induced by expression of the guanine nucleotide exchange factor Vav2 was not inhibited effectively by the active Pak1 PID (Figure 7A, right-most panel). We confirmed in separate experiments that Pak1 activity was inhibited when Pak1 PID, but not Pak1 PID^{L107F}, was expressed in these cells (see Supplemental Figure S2). These results indicate that the activation of Rac1 by Cdc42^{Q61L} is at least partially dependent on Pak1 kinase activity, consistent with the ability of Pak1 to phosphorylate RhoGDI and induce Rac1 dissociation. A similar PID-sensitive Rac activation was obtained using a non-GDI binding Cdc42^{G12V,R66E} mutant (Gibson and Wilson-Delfosse, 2001).

Growth Factor-Induced Rac Dissociation from RhoGDI Requires Pak Activity

Growth factors such as PDGF and EGF activate Rac in order to modulate cell growth and cytoskeletal remodeling. We examined the role of Pak in hormone-initiated dissociation of Rac from RhoGDI complexes during cell activation. As shown in Figure 7B (upper panels), treatment of HeLa cells with either 10 ng/ml PDGF or 100 ng/ml EGF induced a dissociation of Rac from RhoGDI with a time course consistent with Rac activation and induced membrane ruffling. The time course and extent of release was similar in control cells (data not shown) as in cells stably expressing the inactive PID^{L107F} mutant. In contrast, Rac release was essentially blocked in HeLa cells expressing the active Pak1 PID. RhoA release from RhoGDI was not stimulated by either growth factor (Fig-

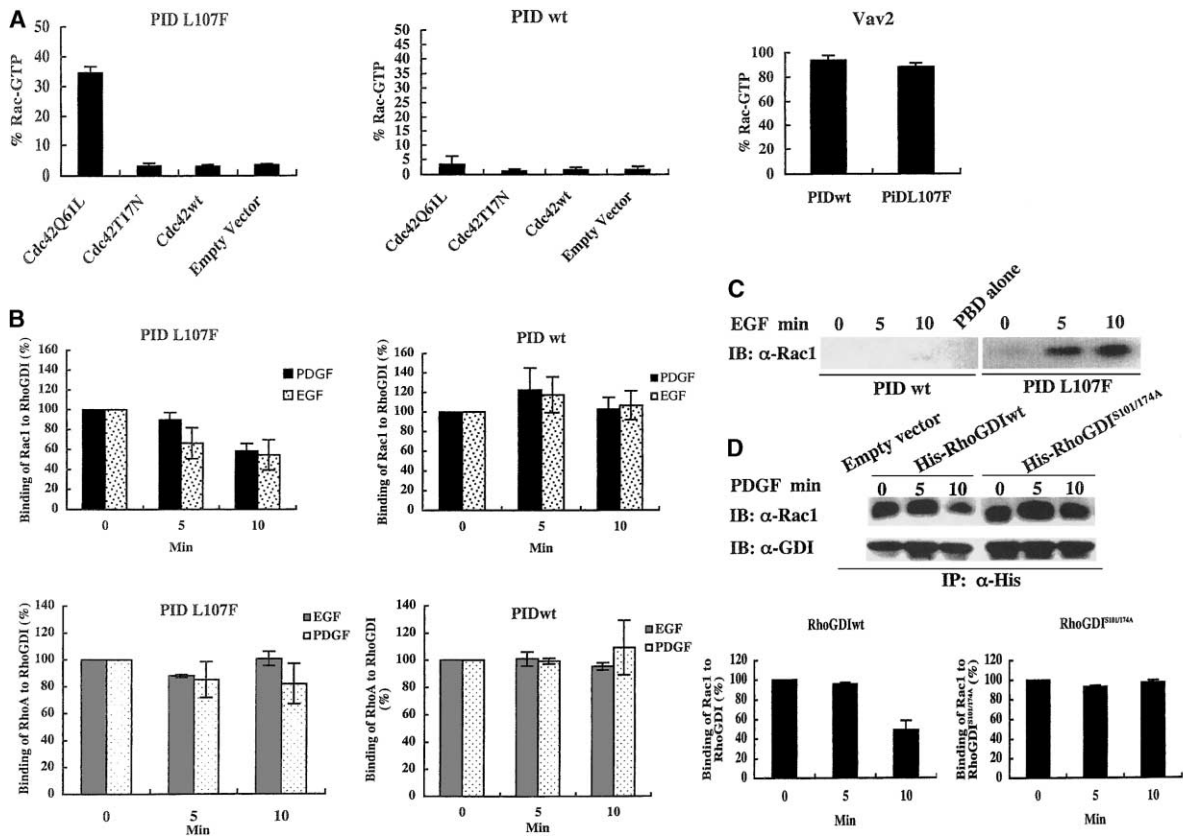


Figure 7. Pak Is Required for Cellular Activation of Rac1 Initiated by Cdc42^{O61L} or Growth Factors

(A) Cdc42-induced Rac activation requires Pak activity. Stable HeLa cell lines inducibly expressing EGFP-tagged PID L107F or EGFP-tagged PIDwt were transiently transfected with either empty vector, Myc-tagged Cdc42^{O61L}, Myc-tagged Cdc42^{T17N}, Myc-tagged Cdc42wt, or Myc-tagged Vav2. GTP-bound Rac1 was precipitated with GST-PBD immobilized to glutathione beads, and bound GTP-Rac1 was determined. Lysates loaded with GTP- γ S (100%) or GDP (0%) were analyzed as controls. Data shown are the results of three independent experiments.

(B) Dissociation and activation of Rac1 from RhoGDI induced by growth factors requires Pak activity. Stable HeLa cell lines inducibly expressing EGFP-Pak PID L107F (left panels) or EGFP-Pak PID wt (right panels) were treated with 10 ng/ml of PDGF or 100 ng/ml of EGF for the indicated times. Lysates were immunoprecipitated using anti-RhoGDI antibody and subjected to Western blot analysis using anti-Rac1 antibody (top panels) or RhoA antibody (bottom panels). Densitometry was used to quantitative the levels of Rac1 and RhoA present. Data are the average \pm SEM of three independent experiments.

(C) Rac activation by EGF requires Pak activity. Stable HeLa cell lines expressing EGFP-Pak PID wt or EGFP-Pak PID L107F were starved overnight and treated with 100 ng/ml of EGF for the indicated times. Active Rac1 was determined by GST-PBD assay.

(D) PDGF-induced dissociation of Rac from RhoGDI requires phosphorylation of serines 101/174. HeLa cells were transiently transfected with His-tagged RhoGDI wt or His-tagged RhoGDI^{S101A/S174A} and the cells treated with 10 ng/ml of PDGF for the indicated times. RhoGDI was immunoprecipitated using anti-His antibody and Rac1 detected with anti-Rac1 antibody. Densitometry was used to determine the level of Rac1 complexed with RhoGDI. Data shown are the result of three independent experiments.

ure 7B, lower panels). We verified that the dissociation of Rac from RhoGDI induced by EGF (and for PDGF, data not shown) was accompanied by Rac activation, and this activation was similarly blocked by expression of active, but not inactive, PID (Figure 7C). These results indicate that the mechanism utilized by PDGF and EGF to induce specific release of Rac from RhoGDI is dependent on Pak activity.

While RhoGDI was phosphorylated by Pak1 under the conditions used for these experiments (see Figure 3B), in order to test that Pak was inhibiting Rac release from RhoGDI via the phosphorylation of RhoGDI, we analyzed HeLa cells transfected with either wild-type RhoGDI or RhoGDI mutated to Ala at the Pak phosphorylation sites, Ser101 and Ser174. The ability of PDGF to induce release of Rac from these GDI constructs was then compared. As shown in Figure 7D, while PDGF effectively

induced the release of Rac from wild-type RhoGDI, there was essentially no dissociation of Rac from the mutant RhoGDI. This strongly supports the hypothesis that the Pak-induced phosphorylation of one or more of these sites is absolutely required for hormone-induced release and subsequent activation of Rac GTPase.

Discussion

We purified Pak1 from bovine brain cytosol as a serine kinase capable of phosphorylating recombinant human RhoGDI. Activated Pak1 not only phosphorylated endogenous RhoGDI in 293T and HeLa cells but also formed a precipitable complex with RhoGDI. Complex formation was observed with both active full-length Pak1 and the isolated C-terminal kinase domain, but not the Pak1 N terminus. This data indicates that the

interaction of Pak1 with RhoGDI does not require, nor is it mediated by, the interaction of Pak1 with GTPase, since the Pak1 C terminus lacks the GTPase binding domain. While binding of Pak1 to RhoGDI does not appear to be sufficient to induce the dissociation of Rac, as evidenced by the ineffectiveness of kinase-dead Pak1 or kinase-dead Pak1 C terminus (Figure 6A), we do not rule out that this interaction contributes to the dissociation reaction. Pak1 phosphorylated RhoGDI on two sites, Ser101 and Ser174, both in vitro and in vivo (Figure 5A). Mutation of these two residues to Ala resulted in the loss of RhoGDI phosphorylation by Pak1, indicating they are the only major phosphorylation sites.

Both Ser101 and Ser174 border the hydrophobic prenyl binding cleft of RhoGDI on its solvent-exposed surface (Hoffman et al., 2000). These residues are within 9 Å of each other and are both ~11 Å away from the C-terminal prenyl group of the GTPase in the binding pocket. We speculate that the presence of negative charges at these sites as a result of phosphorylation induces a conformational change that destabilizes binding of the prenyl group, thereby decreasing the affinity of RhoGDI for GTPase. This change in the prenyl binding hydrophobic pocket may result from mutual repulsion of the closely opposed negatively charged Ser101 and Ser174 residues. We note that phosphorylation of both of these sites appears to be required for Rac dissociation, as PKA-induced phosphorylation of Ser174 alone was insufficient to cause GTPase release (Figure 6D).

In accord with this hypothesis, we observed that phosphorylation of RhoGDI in preformed Rac1-RhoGDI complexes induced the dissociation of Rac1 from the complex to an extent consistent with the level of phosphorylated RhoGDI, as determined by induced binding of [³⁵S]GTP γ S (Figure 5B). Dissociation was dependent upon phosphorylation of RhoGDI at the Ser101/174 sites, as it did not occur when these sites were mutated to alanines. Interestingly, phosphorylation by Pak1 did not stimulate the release of RhoA from preformed RhoGDI complexes. Similarly, in the RhoGDI^{S101E,S174E} mutant, where glutamic acid residues were substituted for serine in an attempt to mimic phosphorylation, the binding of Rac1 GTPase was significantly reduced. In corroboration of these data, we also observed that RhoGDI was released into the supernatant from prebound complexes with GST-Rac1 on glutathione beads in the presence of Pak1 plus ATP, but not in the absence of ATP (data not shown). When we induced phosphorylation of endogenous RhoGDI by expression of Pak1 in vivo, we observed almost complete loss of Rac1 from endogenous RhoGDI complexes (Figures 6A and 6B). Again, the Pak1-mediated phosphorylation of endogenous RhoGDI had no effect on the complexation of RhoGDI with RhoA (Figures 6B and 6C) and only slight effect on Cdc42-RhoGDI complexes (data not shown). These data indicate that phosphorylation of RhoGDI by Pak1 is a specific mechanism for release of Rac GTPase. The molecular basis for the selective release of Rac versus Rho (and Cdc42) from RhoGDI complexes induced by Pak1-mediated phosphorylation of Ser101 and Ser174 is not evident. We speculate it might relate to differences in the interaction of specific residues in each GTPase with the hydrophobic pocket near the sites of phosphorylation. An indirect effect of Pak1 mediated through the interaction

with, or phosphorylation of, PIX appears unlikely, as (1) the phosphorylation of PIX by Pak1 has been reported to have no effect on its intrinsic GEF activity toward Rac (Koh et al., 2001); (2) we observe dissociation of Rac1 from endogenous RhoGDI complexes upon expression of the non-PIX-interacting Pak1 C terminus or a non-PIX binding full-length Pak1; and (3) dissociation of Rac1 from RhoGDI stimulated by growth factors was dependent upon the presence of phosphorylatable Ser101/174 sites in RhoGDI (Figure 7D).

Activation of Cdc42 can stimulate downstream activation of Rac, indicating that Cdc42 can induce the release of Rac from resting complexes with RhoGDI. We observed that expression of Cdc42^{Q61L} in HeLa cells stimulated significant formation of Rac1-GTP (Figure 7A). This stimulatory effect of Cdc42^{Q61L} was blocked (>90% decrease) by the expression of the Pak1 autoinhibitory domain (PID), but was not affected when an inactive version of the inhibitory domain (PID^{L107F}) was expressed. Similar results were obtained with an activated but non-RhoGDI binding Cdc42^{G12V,R66E} mutant (see Supplemental Figure S3), indicating that our results were not due to the interaction of the expressed Cdc42^{Q61L} protein with RhoGDI. These data indicate that at least part of the action of Cdc42 to stimulate Rac activation is due to a Pak kinase-dependent mechanism, consistent with phosphorylation-induced release from RhoGDI.

Similarly, growth factor stimulation of Rac activity by PDGF and EGF has been demonstrated. We observed significant dissociation of the Rac1-RhoGDI complex in response to these growth factors which was substantially inhibited when endogenous Pak activity was blocked by the expression of the Pak1 autoinhibitory domain, as was the subsequent conversion of Rac to the GTP form (Figures 7B–7D). Pak was required for phosphorylation of RhoGDI, as release of Rac did not occur when cells were transfected with the RhoGDI^{S101A,S174A} mutant (Figure 7D). Both PDGF (Dharmawardhane, et al., 1997) and EGF (Galisteo, et al., 1996) have been shown to stimulate Pak1 activation.

A Model for the Action of Pak1 in Cellular Rac Activation

We propose that, depending upon the GTPase stimulating Pak1 activation, the Pak1-mediated regulation of Rac-RhoGDI complexation would act either as an initiator of Rac signaling (in the case of activation by Cdc42) or as a positive feedback mechanism to enhance Rac activation once an initial Rac (and Pak1) activation has occurred, possibly through the growth factor-induced activation of a small pool of preexisting free (non-GDI-complexed) Rac. Alternatively, we have shown that growth factors can stimulate Pak1 activity through the release of sphingosine and related lipids (Bokoch, et al., 1998; King, et al., 2000), perhaps initiating Rac release from RhoGDI. The existence of such feed-forward mechanisms for Rac activation may be important in Rac-mediated activities such as formation and maintenance of the leading edge in motile cells, etc. The action of Pak1 provides a selective mechanism for regulation of Rac GTPase activity, and supports the view that distinct mechanisms are involved in the stimulus-induced release, and activation, of individual Rho family GTPases.

We do not mean to suggest that this is the only mechanism for inducing Rac release from RhoGDI, nor that all stimuli capable of activating Rac use this mechanism. However, based on our current results, as well as previous work describing phosphorylation-based regulation of GTPase-GDI complexes, we suggest that the action of distinct kinases acting on specific sites in Rho GTPases or RhoGDI itself may be an important means of controlling Rho GTPase signaling in response to diverse receptor-mediated stimuli.

Experimental Procedures

Reagents

Cell culture medium, fetal bovine serum, and supplements were from Invitrogen (Carlsbad, CA). [γ - 32 P] ATP (specific activity 4500 mCi/mmol) was from ICN (Costa Mesa, CA). Plasmids for transfection were purified using the Qiagen Qiafilter system (Chatsworth, CA). The following reagents were purchased as indicated: ECL reagents from Pierce (Rockford, IL), protein G- or protein A-Sepharose beads from Amersham (Piscataway, NJ), and Repligen (Waltham, MA), respectively; recombinant human EGF or PDGF from Fisher Scientific (Tustin, CA) and Upstate Biotechnology Inc. (Lake Placid, NY), respectively; rabbit polyclonal antibody specific to RhoGDI from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); monoclonal anti-histidine from Babco (Richmond, CA); monoclonal EGFP 3E6 antibody from Molecular Probes (Eugene, OR); monoclonal Rac1 antibody from Upstate Biotechnology; and polyclonal RhoA and Cdc42 antibodies from Santa Cruz Biotechnology. Rabbit polyclonal Pak antibody R2124 is equivalent to R627, described in (Knaus et al., 1995); expression and purification of RhoGDI and Pak1 from *E. coli* was as in (Chuang et al., 1993b) and (Sells et al., 1997), respectively.

Plasmid DNA and Construction

cDNA expression plasmids containing full-length Pak1 and its various mutants in the pCMV6M vector, and constitutively active Cdc42Q61L or inactive Cdc42T17N in the PRK5M vector have been described (Sells et al., 1997). EGFP-tagged Pak PID or PIDL107F were excised from the vector pEGFP-C1 Pak PID with NheI and Sall and inserted into the same restriction sites of pTRE2hyg (Clontech) harboring a Tet-responsive element and an internal hygromycin selection element.

All RhoGDI constructs (human RhoGDI S101A, S174A, S101/174A, S101/174E) were inserted into pCMV6 with a C-terminal His epitope, and mutants were prepared by site-directed mutagenesis using QuickChange kit (Statagene). Human RhoGDI wild-type was cloned into pGEX4T3 vector at the BamHI/EcoRI site.

Purification of RhoGDI Kinase

Bovine brain cytosol was prepared as described in (Sternweis et al., 1981). Kinase purification was as follows.

Step 1: Anion Exchange Chromatography

400 ml of cytosol was concentrated to 40 ml at 4°C and applied to DEAE Sepharose preequilibrated with buffer A (50 mM Tris, pH 8.0, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μ g/ml aprotinin) and washed with 5 vol of buffer A. Proteins were eluted from the column using a two-step linear NaCl gradient as indicated by the dotted line in Figure 1. The linear gradient was from 0 to 0.25 M NaCl (3 ml fractions were collected) and from 0.25 to 0.4 M NaCl (1 ml fractions were collected). A final elution with 1 M NaCl was performed (3 ml fractions were collected). Fractions were subjected to RhoGDI kinase assay in vitro as described below, and RhoGDI phosphorylation was detected by autoradiography after SDS-PAGE. Fractions containing the maximum RhoGDI kinase activity (eluted between 0.3 and 0.35 M NaCl) were pooled and concentrated.

Step 2: Size Exclusion Chromatography

An AcA 54 column was preequilibrated in buffer A supplemented with 150 mM NaCl. The concentrated DEAE fractions were applied to the AcA54 column at a flow rate of 0.5 ml/min and 1 ml fractions were collected. RhoGDI kinase activity eluted at an apparent size of ~90 kDa, based on known protein standards.

Kinase Assays

Kinase activity was determined as described in Knaus et al. (1995) using pure recombinant bead-immobilized GST-RhoGDI as a substrate at 2 μ g/reaction. Reactions (30 min at 30°C) were started by the addition of ATP (final concentration = 20 μ M and 0.5 μ Ci [γ - 32 P]ATP/reaction). The effect of kinase inhibitors on RhoGDI phosphorylation was determined using the same assay with partially purified RhoGDI kinase in the presence or absence of inhibitors: KN93 (10 μ M); staurosporine (10 μ M); H7 (100 μ M); H89 (10 μ M); bisindolylmaleimide (1 μ M); PBDID 83 (1 μ g), aa 83–149 of Pak1 containing the autoinhibitory domain mutated at aa 83 to eliminate GTPase binding capability; and PBDID 107 (1 μ g), the same Pak1 autoinhibitory domain construct inactivated by mutation at aa 107.

For in vitro kinase reaction with recombinant Pak1, the assay was performed as above with the exception that GST-Pak1 was used as a kinase at 1 μ g per reaction, and pure recombinant RhoGDI with no GST tag was used at 2 μ g per reaction. For 2D phosphopeptide mapping and phosphoamino acid analysis experiments, 3 μ Ci [γ - 32 P]ATP/reaction was used (Boyle et al., 1991).

Cell Transfection

Human embryonic kidney HEK293T cells were grown in Dulbecco's modified Eagle's Medium (DMEM; Life Technologies, Inc.) supplemented with 8% fetal bovine serum, 10 mM HEPES, pH 7.0, 2 mM glutamine, 100 U/ml penicillin G, and 100 μ g/ml streptomycin (GIBCO-BRL, Gaithersburg, MD) at 37°C in 5% CO₂. HeLa Tet-off cells (Clontech) were maintained in DMEM supplemented with 10% fetal bovine serum and 100 ng of G-418 (Invitrogen) per ml at 37°C in the presence of 5% CO₂.

For transfection experiments, 293T cells grown to approximately 70% confluence on 100 mm tissue culture dishes were transiently transfected with 20 μ l LipofectAmine reagent (GIBCO-BRL) and 1–3 μ g of either pCMV6 vector containing various Pak1 or RhoGDI constructs, or PRK5M containing Cdc42 constructs. HeLa cells stably transfected with EGFP-tagged Pak PID wt or EGFP-tagged Pak PID L107F were cultured in 6-well plates in normal serum without doxycycline. HeLa cells were seeded on 6-well culture dishes at 0.5×10^6 cells and the next day transfected using 20 ng of plasmid RhoGDI wt or plasmid RhoGDI S101A/S174A and 5 μ l of LipofectAmine with 2 μ l of plus reagent per dish. Immunoprecipitations were performed and analyzed essentially as described (King et al., 2000).

Metabolic Labeling in 293T Cells

293T cells were grown to 70% confluency in 100 mm cell culture plates and were transfected with 4 μ g vector DNA or 1 μ g RhoGDI constructs (wt, S101A, S174A, S101/174A) and 3 μ g Pak1 constructs using 20 μ l of LipofectAmine. At 24 hr posttransfection, cells were washed and incubated at 37°C and 5% CO₂ for 45 min with phosphate-free DMEM plus 0.4 mCi [32 P]orthophosphate. Washed cells were incubated overnight at 37°C, lysed, and His-tagged RhoGDI immunoprecipitated with anti-His monoclonal antibody for autoradiographic analysis. Phosphopeptide analysis and 2D electrophoresis were performed per standard procedures, as described (King et al., 2000).

In Vitro Nucleotide Exchange Assay

Prenyl-modified RhoA and Rac were purified from baculovirus membranes (Chuang et al., 1993b). GST-GDP-Rac or RhoA immobilized on glutathione beads complexed with RhoGDIwt or RhoGDI S101/174A were obtained by incubating GDP-RhoGTPase with 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, 0.1% Chaps to remove free RhoGDI. 50 nM of the complex was subjected to in vitro kinase assay with 0.5 mM unlabeled ATP for 20 min at 30°C in the presence of 1 μ M of GST-Pak1 or 1 μ M of GST protein. The sample was then washed twice with 25 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM MgCl₂, 1 mM EDTA, 0.1% Chaps, and [35 S]GTP γ S binding determined as described (Knaus et al., 1992; Chuang et al., 1993b).

GTPase Activation Assay

HeLa cells stably transfected with EGFP-tagged Pak PID wt or EGFP-tagged Pak PID L107F were cultured on 10 cm dishes and

transiently transfected with either Myc-tagged Cdc42wt, Cdc42^{T17N}, Cdc42^{Q61L}, Vav2, or empty vector control. After 24 hr of transfection, cells lysates were depleted of >80% Cdc42 with Myc 9E10 antibody. 0.4 mg of lysates were mixed with excess (20 μ g) GST-PBD immobilized to glutathione-Sepharose beads in binding buffer and Rac1 activation determined as in (Benard et al., 1999).

Growth Hormone Stimulation

HeLa cells stably transfected with EGFP-tagged Pak PID wt or EGFP-tagged Pak PID L107F were cultured in 6-well plates in normal serum without doxycycline. At confluency, the cells were serum starved 18 hr and treated with or without human recombinant PDGF (10 ng/ml) or EGF (100 ng/ml) at the indicated times. For transient transfection, HeLa cells were seeded on 6-well culture dishes at 0.5×10^6 cells and were transfected using LipofectAmine, as above. At 24 hr posttransfection, cells were serum starved and treated with growth hormone as described, then harvested in lysis buffer and subjected to protein quantification by BCA assay. For immunoprecipitation experiments, endogenous RhoGDI was precipitated from cell lysates with RhoGDI antibody (Santa Cruz) overnight at 4°C. His-tagged RhoGDI proteins were precipitated using His monoclonal antibody for 1 hr. The proteins were transferred to PVDF membranes and the level of Rac or RhoA proteins as well as the immunoprecipitated RhoGDI assessed by Western blot using anti-Rac1, anti-RhoA, or anti-RhoGDI antibodies, respectively. The level of Rho GTPase binding to RhoGDI in the various cell lines was compared by densitometry.

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