Direct Interaction of p21-Activated Kinase 4 with PDZ-RhoGEF, a G Protein-linked Rho Guanine Exchange Factor*

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Rho GTPases regulate a wide variety of cellular processes, ranging from actin cytoskeleton remodeling to cell cycle progression and gene expression. Cell surface receptors act through a complex regulatory molecular network that includes guanine exchange factors (GEFs), GTPase activating proteins, and guanine dissociation inhibitors to achieve the coordinated activation and deactivation of Rho proteins, thereby controlling cell motility and ultimately cell fate. Here we found that a member of the RGL-containing family of Rho guanine exchange factors, PDZ RhoGEF, which, together with LARG and p115RhoGEF, links the G12/13 family of heterotrimeric Gi proteins to Rho activation, binds through its C-terminal region to the serine-threonine kinase p21-activated kinase 4 (PAK4), an effector for Cdc42. This interaction results in the phosphorylation of PDZ RhoGEF and abolishes its ability to mediate the accumulation of Rho-GTP by Goαi. Moreover, when overexpressed, active PAK4 was able to dramatically decrease Rho-GTP loading in vivo and the formation of actin stress fibers in response to serum or LPA stimulation. Together, these results provide evidence that PAK4 can negatively regulate the activation of Rho through a direct protein-protein interaction with G protein-linked Rho GEFs, thus providing a novel potential mechanism for cross-talk among Rho GTPases.

Rho GTPases, including Rho, Rac, and Cdc42, play a key role in the regulation of cellular processes that involve actin cytoskeleton remodeling, such as cell migration, polarity, morphogenesis, and axon guidance (1, 2), as well as in the control of gene expression and cell cycle progression (3–5). Although these GTPases are often studied in diverse biochemical and biological systems individually, emerging evidence suggests that Rho proteins act as part of a complex and highly coordinated signaling network. Indeed, in certain cellular systems Cdc42 can cause the hierarchical activation of Rac and Rho (6). Most often, however, the activation of one Rho GTPase can prevent the activation or the effector function of another Rho GTPase. For example, in neuronal cells, Cdc42 antagonizes the effects of Rho in growth cone chemo-repulsion (7), and in motile cells, Rac prevents the phosphorylation of myosin light chain through its effector PAK1 by phosphorylating myosin light chain kinase, thus decreasing the contractile effect exerted by Rho (8). This particular process balances the protrusive forces, generated by Rac and Cdc42, and the contractile forces, promoted by Rho, a critical requirement for directional cell movement (9). In addition to counteracting the downstream effectors of Rho, Rac can lead to a decrease of active GTP-bound Rho by the generation of reactive oxygen species, which act as diffusible second messengers by promoting the inhibition of a low molecular weight protein tyrosine phosphatase (10). That results in the prolonged phosphorylation and activity of p190 RhoGAP, thus inhibiting Rho by promoting the hydrolysis of GTP bound to Rho (10). Together, these recent findings revealed the existence of a complex interplay among Rho GTPases, whose underlying molecular mechanisms are just beginning to be understood.

The activity of Rho GTPases is tightly regulated by guanine exchange factors (GEFs)1 that promote GDP release, leading to GTP loading and activation of Rho proteins, and by GTPase activating proteins and GDP-dissociation inhibitors that act as negative regulators by either accelerating the intrinsic GTPase activity or by preventing their release of GDP, respectively (11, 12). Rho GEFs are characterized by the presence of a highly conserved Dbl homology (DH) and pleckstrin homology (PH) module that is responsible for the exchange activity toward Rho proteins (12). These GEFs include a number of distinct functional domains that enable them to interact with and be regulated by a diverse array of cell surface and cytoplasmic molecules, thus mediating the activation of Rho GTPases in response to external or intracellular signals (12). Of interest, PDZ RhoGEF (PRG), LARG, and p115 RhoGEF (p115) represent a distinct GEF family that is characterized by the presence of a regulator of G protein signaling-like (RGL) domain by which they bind the α-subunit of heterotrimeric Gi proteins of the Gi2/α3 protein family and thereby increasing their GEF activity toward Rho (13, 14). PRG and LARG can also associate through their peptide-binding domain Z (PDZ) domains with the insulin-like growth factor receptor (15) and Plexin B (16, 17), an axon-guiding semaphorin receptor, thus suggesting

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1 The abbreviations used are: GEF, guanine exchange factors; DH, Dbl homology; PH, pleckstrin homology; PRG, PDZ RhoGEF; p115, p115 RhoGEF; RGL, regulator of G protein signaling-like; PDZ, PSD-95/Dlg/AZO-1 binding domain; PAK4, p21 activated kinase 4; PAK4-C, C-terminal part of PAK4; HA, hemagglutinin; GST, glutathione S-transferase; GST-RBD, GTP-dependent binding domain of rhoetokin; wt, wild type; RBD, rhoetokin binding domain; LPA, 1-α-lysophosphatidic acid; PBS, phosphate buffered saline; AD, GAL4 transactivation domain; X-α-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; CRIB, Cdc42/Rac interactive binding domain; MBP, myelin basic protein.

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that their GEF activity can be regulated by complex protein-protein interactions. However, the molecular mechanisms controlling the ability of these Rho GEFs to induce Rho activation are still poorly defined.

Recently, we have observed that the C-terminal region of PRG, LARG, and p115 mediates their ability to homo- and hetero-oligomerize (18). Furthermore, deletion of their C-terminal region abolished their ability of these GEFs to activate Rho, and unleashes their high transforming potential, thus supporting the view that the C-terminal region represses the function of these GEFs in vivo (18). In this study, we found that this C-terminal inhibitory region of PRG associates specifically with p21 activated kinase 4 (PAK4), a Cdc42 effector, which leads to a decreased activation of Rho in vivo by Go13, and its coupled receptors. These findings suggest the existence of a novel biochemical route by which PAKs can regulate the activity of Rho, thus providing a direct mechanism for the cross-talk among small GTPases of the Rho family.

**MATERIALS AND METHODS**

**Yeats Two-hybrid Screening**—A human brain cDNA library was screened using the Matchmaker system III (Clontech, CA) following the manufacturer’s instructions. The C-terminal region of PRG (amino acids 1081–1523) was cloned in-frame with the Gal4 DNA-binding domain into pGBK7T vector and used as the bait. Transformants were grown under high stringency conditions using growth media lacking adenine, histidine, leucine, and tryptophan (Clontech, CA). Specificity of the interaction was confirmed by using expression plasmids p5ADT7-T and pGBK7-53 as control prey and control bait, respectively. An X-o-gal assay was performed as per manufacturer’s instructions.

**Constructs**—The C-terminal part of PAK4 (amino acids 252–597), PAK4-C, was subcloned from the library expression plasmid pACT2 into the eukaryotic expression vector pCMV myc (Clontech, CA) as an EcoRI-BglII fragment. Full-length human PAK4 was amplified by PCR from expressed sequence tag cDNA (IMAGE clone ID 5178669, Resgen, Inc.) and polyclonal antibody against (R/K)XpS(Hyd)(R/K) motif was obtained from Cell Signaling Technology, Inc (antibody 2261). Goat anti-rabbit and anti-mouse secondary antibodies coupled to horsedarsh peroxidase were purchased from Cappel, ICN Biomedicals. Proteins were visualized by using enhanced chemiluminescence reagent (Amer sham Biosciences) according to the manufacturer’s instructions.

**GST Fusion Protein Pull-down in Vitro Kinase Assays and in Vivo Rho Activation Assays**—For GST fusion protein pull-down experiments, cell lysates from the 293T cells transfected with HA PAK4 445N were incubated by rocking for 1 h at 4 °C with 12 μg of a GST fusion protein pre-coupled to glutathione-Sepharose™ 4B (Amersham Biosciences) beads. Sepharose beads were collected by centrifugation and washed three times with the lysis buffer. Bound proteins were either resolved by SDS-PAGE and detected by Western blotting using anti-HA antibody, or they were exposed to a solid-phase kinase reaction in the presence of 1 μCi [32P]ATP per reaction and 20 μl unlabelled ATP as described (21); reaction products were analyzed by SDS-gel electrophoresis. For in vitro PAK4 kinase assay, 293T cells were transfected with HA-PAK4 wild type (wt), HA PAK4 445N, or control expression vector; PAK4 autophosphorylating and MBP phosphorylating kinase activity was assessed in immunocomplex kinase assays, essentially as described previously for extracellular signal-regulated kinase kinase assays (4). Rho activity was assessed by a modified method as described (24), using purified GST-RBD previously bound to glutathione-Sepharose™ 4B (Amersham Pharmacia, Sweden) beads to affinity-precipitated GTP-bound Rho. Western blot analysis of total and active Rho was performed by using a monoclonal antibody against RhoA (26C4, Santa Cruz Biotechnology).

**Fluorescence Microscopy**—Swiss 3T3 cells were grown in six-well plates on cover-slips and transfected with pCEFL HA PAK4 445N, pCEFL HA PAK4 350 m, or the control expression vector. Cells were serum-starved for 36 h and then stimulated with 0.5 μM LPA (Sigma) or serum (10%) for 20 min, washed twice with phosphate buffered saline (PBS), fixed with 3.7% paraformaldehyde in PBS, and then permeabilized with PBS containing 0.5% Triton X-100. Cover-slips were washed with PBS three times, blocked with PBS containing 1% bovine serum albumin and incubated with mouse anti-HA antibody (1:200) for one hour. After incubation, cover-slips were washed with PBS three times and then incubated with fluorescein isothiocyanate-conjugated anti-mouse- IgG (Jackson ImmunoResearch) for one hour. Cells were then washed with PBS and stained with Texas Red-X phallloidin (Molecular Probes, Inc.) following the manufacturer’s instructions. Coverslips were mounted by using Vectashield mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA) and visualized by using an Axiosplan2 microscope (Zeiss).

**RESULTS**

As the C-terminal region of PRG has an important role in the regulation of its in vivo GEF activity, we searched for potential interacting proteins by a yeast two-hybrid approach, using the C-terminal 341 amino acids of PRG (Fig. 1A, PRG-C) fused in-frame with the GAL4 DNA-binding domain as bait to screen a human brain GAL4 transactivation domain (AD) fusion cDNA library. Upon screening 0.8 × 10^8 independent transformants, we identified only few clones that exhibited efficient growth under high stringency conditions, such as growth in the absence of adenine, histidine, leucine, and tryptophan (−AHLT). Of interest, one clone encoded for the entire C-terminal kinase domain of the serine-threonine p21-activated kinase 4 (Fig. 1A, PAK4-C). The specificity of this interaction was first verified in yeasts where expression plasmids for PAK4-C and SV40 large T antigen fused to GAL4 AD were cotransformed with either PRG-C or p53 fused to GAL4 DNA-binding domain and tested for their ability to grow on −AHLT plates in the presence of X-a-Gal. As shown in Fig. 1A, only transformants expressing a known interacting pair, p53 and the large T antigen, or expressing the PRG-C together with PAK4-C were able to grow at high stringency conditions and turn the yeast colonies blue in the presence of X-a-Gal, indicating the expression of the α-galactosidase promoter. To investigate whether PAK4-C and PRG-C can also interact in eukaryotic cells, we transfected expression plasmids for a myc-tagged PAK4-C and HA-tagged PRG-C in 293T cells. As shown in Fig. 1B, these proteins were readily detectable by Western blotting with the corresponding anti-tag antibodies, and HA PAK4-C co-immunoprecipitated with myc PAK4-C from co-
transfected cells but not from control lysates. In line with these observations, this interaction was also observed when using the corresponding tagged full-length proteins, HA PAK4 and AU1 PRG (Fig. 1C). Furthermore, when AU1 PRG was expressed in 293T cells, it also interacted with endogenous PAK4 protein (Fig. 1D).
PAK4 is a member of the p21-activated kinase family that consists of at least six members, which can be divided into two groups based on their structural features and sequence similarities (Fig. 2A). Group I includes PAK1, PAK2, and PAK3; they share a Cdc42/Rac binding domain (PBD), an autoinhibitory domain, and a kinase domain; PAK4, PAK5, and PAK6 represent a recently described group II of PAKs that shares a Cdc42/Rac interactive binding domain (CRIB) domain.

**Fig. 2. The interaction between PAK4 and PRG is specific and requires an intact PRG C terminus.** A, analysis of the primary sequence relationship among human PAKs and specificity of the interaction of PRG with hPAK4. Upper panel, phylogenetic tree of human PAK family members. Lower panel, tagged PAK proteins (myc PAK1, V5 PAK2, and HA PAK4) were transfected in 293T cells together with AU1 PRG. Total cell lysates or anti-AU1 immunoprecipitates were analyzed by Western blotting with the anti-myc, anti-V5, and anti-HA antibodies, as indicated, to detect the presence of tagged PAKs. B, PAK4 interaction with RhoGEFs. Cell lysates from 293T cells co-transfected with HA PAK4 and AU1-tagged PRG, p115, or LARG were immunoprecipitated with anti-AU1 and anti-HA antibodies followed by Western blotting using anti-HA and anti-AU1 antibodies, respectively. C, the C terminus of PRG is required and sufficient for PAK4 binding. Left panel, structure of PRG and its deletion mutants. Right upper panels, 293T cells were co-transfected with myc-tagged PAK4 together with the indicated AU1/HA-tagged wild type, Δ702, ΔDH-PH, ΔC, DH-PH, or C-terminal region of PRG or control expression vector. Expression of each tagged protein was examined by immunoblotting with anti-AU1, anti-HA, or anti-myc antibodies. Right lower panel, anti-myc immunoprecipitates were Western blotted with anti-AU1 and anti-HA antibodies.
and a related kinase domain but lacks an autoinhibitory region (25). Whereas PAK2 and PAK4 are widely expressed, other members of this group exhibit a tissue-restricted distribution (25). To examine whether PRG can also interact with members of group I of the PAKs, we co-expressed in 293T cells AU1 PRG together with myc-tagged PAK1 or V5-tagged PAK2 and HA-tagged PAK4 as a control. After anti-AU1 immunoprecipitation and Western blotting with the specific anti-tag antibodies, we observed that only PAK4 associated with PRG (Fig. 2A). Next, we tested whether other RGL-containing GEFs, LARG and p115, can bind PAK4 (Fig. 2B). As judged by co-immunoprecipitation experiments, PAK4 was able to bind strongly only to PRG, whereas it showed much weaker binding to p115 and no interaction with LARG. Collectively, these experiments supported the preferential interaction between PAK4 and PRG.

To map more precisely the domains intervening in the binding of PAK4 to PRG, we co-expressed myc-tagged PAK4 together with the AU1- or HA-tagged deletion mutants of PRG indicated in Fig. 2C. Myc-PAK4 was able to bind to full-length PRG, PRG lacking its N-terminal 702 amino acids (Fig. 2C, Δ702), or its DH-PH domains (Fig. 2C, ΔDH-PH), but its ability to bind PRG was abolished when the C-terminal portion of PRG was deleted (Fig. 2C, ΔC). Furthermore, PRG C terminus alone (Fig. 2C, PRG-C) was able to bind to myc PAK4 when co-expressed in 293T cells, whereas the DH-PH domain (DH-PH) could not be detected in the anti-myc immunoprecipitates. Together, these results indicated that the C-terminal region of PRG is required and sufficient to bind PAK4.

To assess the functional significance of PAK4-PRG interaction, we engineered a constitutively active mutant of PAK4 (HA PAK4 S445N). This mutation has been reported previously to stabilize the catalytic loop and elevate strongly the kinase activity of PAK4 toward His-4 (26). Indeed, when HA-tagged versions of the wild type and active PAK4 were expressed in 293T cells and tested for kinase activity, the activated form of PAK4 showed much higher MBP and auto-phosphorylating activity than wt PAK4 (Fig. 3A). We also tested the activity of a kinase inactive PAK4 mutant (PAK4 350 m) that contains a methionine residue in place of a conserved lysine in subdomain II, which disrupts the ability of PAK4 to bind ATP and, thus, its kinase activity. Consistent with previous reports (27), PAK4 350 m was unable to autophosphorylate and had low MBP phosphorylating activity. Next, we examined whether activated PAK4 was able to bind PRG protein in vitro. As the most C-terminal amino acids of PRG participate in the association with PAK4, this region from PRG, and LARG as a control, were expressed in bacteria as GST fusion proteins (GST PRG-C and GST LARG-C), purified and coupled to Sepharose beads. Using these purified proteins, we observed that PRG-C, but not similar amounts of LARG-C (Fig. 3B, Comassie stain), was able to affinity-purify HA PAK4 445N (Fig. 3B). Furthermore, the “pulled down” PAK4 strongly phosphorylated PRG-C when kinase reactions were performed on the precipitates (Fig. 3B). These results suggest that the C-terminal part of PRG binds PAK4 and serves as a PAK4 substrate.

We then co-transfected full-length AU1 PRG with HA PAK4 445N in 293T cells to examine whether active PAK4 kinase can phosphorylate PRG in vivo. As an approach, we used both a polyclonal anti-phosphothreonine antibody that recognizes phosphorylated threonines independently of the neighboring amino acids, and an anti-phosphoserine antiserum that was raised against a consensus (R/K/KxX(phosphoS)(Hyd)/(R/K)) motif. This motif shows high homology to the amino acid sequence within the activation loop of PAK4 (RKSVL, amino acids 472–476) that has been shown to be a high affinity in vitro substrate for the kinase (28) and thus could represent a likely PAK4 phosphorylation motif; this possibility is suggested by the observation that the anti-R/K/KxXHyd/VK antiserum recognized the autophosphorylated PAK4 (not shown). When anti-AU1 precipitates were probed with anti-phosphothreonine and anti-phosphoserine (Fig. 3C), we observed a strong increase in threonine phosphorylation on PRG in the presence of active PAK4, which was even more remarkable for serine phosphorylation within that consensus site. Together, these results suggested that PRG may represent an in vivo substrate for PAK4 kinase.

As an approach to address the consequences of PAK4 interaction with RGL-containing GEFs, we tested whether PAK4 may interfere with the signaling ability of receptors and G protein α-subunits that utilize these GEFs to stimulate Rho. One of the hallmarks of Rho activation is the rapid formation of actin stress fibers as a result of LPA or serum stimulation (6), a process that is dependent upon the activation of the G12/13 family of heterotrimeric G proteins and the subsequent stimulation of RGL-containing RhoGEFs (29). To explore whether PAK4 affects LPA-induced Rho activation, we treated control-transfected HA PAK4 445N and HA PAK4 350 m-transfected Swiss 3T3 fibroblasts with LPA and examined the cytoskeletal changes by staining polymerized actin. As expected, LPA and serum treatment of control-transfected fibroblasts led to a rapid change in cell shape and appearance of stress fibers (Fig. 4A and data not shown). These events were abolished in cells transfected with HA PAK4 445N. However, transfection of PAK4 350 m did not affect LPA-induced morphological changes, indicating that the kinase activity of PAK4 is required for the inhibition of stress fiber formation. To test whether the inhibition of stress fiber formation occurs upstream or downstream to Rho, we examined LPA-induced accumulation of endogenous Rho-GTP in vivo in 293T cells in the presence or absence of activated PAK4. As shown in Fig. 4B, using a Rhokinin pull-down assay (30), we observed that LPA stimulation led to a strong increase in Rho GTP-loading. Interestingly, Rho-GTP levels were dramatically reduced upon expression of activated PAK4 in a dose-dependent manner, suggesting that PAK4 can, indeed, interfere with the ability of LPA to induce Rho activation. A similar inhibitory effect of active PAK4 on Rho activation was also observed in response to serum (Fig. 4C). To examine more directly whether PAK4 interferes with the ability of G12/13 proteins to stimulate the guanine exchange activity toward Rho, we measured the generation of Rho-GTP levels in 293T cells co-transfected with active mutant α-subunit of G13 heterotrimeric protein (HA G13QL) together with increasing concentrations of HA PAK4 445N (Fig. 4D). Although transfection of G13QL led to a strong increase in Rho-GTP accumulation, the expression of HA PAK4 445N led to a dramatic decrease in the level of Rho-GTP. This effect was even more dramatic considering that increasing concentrations of HA PAK4 445N can consistently promote higher expression of HA-G13QL by unknown mechanisms. Together, these experiments provide evidence that PAK4 can act as a negative regulator of the Rho activation pathways induced by G13QL, as well as by LPA and serum, which stimulate G12/13-dependent pathways.

**Discussion**

The nature of the molecular mechanisms underlying the complex interplay among small GTPases of the Rho family is still poorly understood. In search of molecules regulating the activity of a recently described Rho GEF, PRG, we found that PAK4 can bind specifically to its inhibitory C-terminal region. PAK4 can also phosphorylate the PRC C terminus in vitro, and it promotes the phosphorylation of full-length PRG in vivo. Furthermore, we provide evidence that PAK4 can inhibit the stimulation of Rho induced by G12/13-coupled receptors and by the activated forms of G13QL. Thus, the interaction between
PAK4 and Rho GEF may represent a direct mechanism by which small GTP-binding proteins acting on PAK4, such as Cdc42 and its related GTPases, may interfere with Rho activation, resulting in the inhibition of stress fiber formation.

The precise mechanism by which PAK4 inhibits signal transmission from G protein-coupled receptors and Gα13 proteins to Rho through PRG is still unknown. Accumulating evidence suggests that the in vitro activity of RGL-containing Rho GEFs does not fully reflect their likely complex regulation in vivo. For example, these Rho GEFs are poorly activated in vitro by Gα12, despite the strong evidence that Gα12 interacts and activates the GEF activity of this class of Rho GEFs in vivo (13, 31, 32).

Fig. 3. PAK4 phosphorylates PRG. A, PAK4 445N is autophosphorylated and phosphorylates MBP. 293T cells were transfected with HA-tagged wt PAK4, constitutively active HA-PAK4 445N, kinase inactive HA-PAK4 350 M, or the expression vector; in vitro kinase reactions were performed as described under “Materials and Methods.” Autoradiography of PAK4 autophosphorylation and MBP phosphorylation are shown. Similar expression levels of wt PAK4, PAK4 445N, and 350 M mutants were confirmed by immunoblotting total cell lysates with anti-HA antibody. B, PRG-C serves as an in vitro substrate for PAK4 kinase. The last 100 amino acids of PRG and LARG were purified as GST-fusion proteins (FP) and coupled to Sepharose beads. Cell lysates of 293T cells transfected with HA PAK4 445N or control expression vector were incubated with control Sepharose beads or with Sepharose beads bound to GST PRG-C or GST LARG-C. Affinity purified (AP) proteins were divided into two aliquots and either Western blotted (WB) with anti-HA antibodies or incubated with [32P]ATP in the in vitro kinase buffer (kinase reaction). Autoradiogram of the phosphorylated GST-fusion proteins is shown. Equal expression of active PAK4 was confirmed by anti-HA immunoblot of total cell lysates, and Coomassie stain of GST-fusion proteins indicated equal recovery of the GST-fusion proteins. C, PAK4 phosphorylates PRG on serine and threonine residues. Cell lysates from 293T cells co-transfected with AU1 PRG and HA PAK4 445N or control vectors were immunoprecipitated (IP) with anti-AU1 antibody and resolved on SDS-PAGE. Membranes were Western blotted using polyclonal anti-phospho-threonine antibody (anti P-Thr) or the rabbit anti-phosphoserine antibodies that were raised against the R/KxS/HydR/K motif (anti P-S). Total cell lysates were probed with anti-AU1 and anti-HA antibodies (upper panels).
PAK4 Interacts with RhoGEFs

Similarly, the removal of the potent inhibitory activity exerted by the C-terminal region of p115, PRG, and LARG in vitro does not result in the enhanced ability of these GEFs to stimulate nucleotide exchange on Rho in vitro (18). In line with the latter findings, phosphorylation of PRG by PAK4 did not affect the in vitro GEF activity of PRG (data not shown). Similarly, we did not detect any changes in the ability of Ga13 to bind PRG upon co-expression with active PAK4, nor did this kinase affect the overall cellular distribution of PRG in the resting state or that in response to Ga13-QL expression. However, we cannot rule out at this time whether PAK4 interferes with the targeting of PRG by activated Ga13 to a particular subcellular fraction or membrane microdomain. On the other hand, PAK4 did not phosphorylate the DH-PH module (data not shown); thus, it is also possible that PAK4 phosphorylation may promote the association of PRG to a yet to be identified negative regulatory molecule, which, for example, may hinder the ability of the catalytic DH-PH domain to interact with Rho in vivo. These, as well as additional possibilities, are under current intense investigation.

The observation that PAK4 binds PRG may have important implications for the PAK family of serine-threonine protein kinases, which are key mediators of Rac and Cdc42 function. Of interest, PAK4 is widely expressed and has been shown to interact with Cdc42 and to participate in its cytoskeletal effects by inducing sustained filopodia formation (27). Active PAK4 also induces a decrease in actin stress fibers and focal adhesions, and may participate in tumor invasion and metastasis (26–28), but its downstream effectors are still poorly defined (33). In this regard, our current results indicate that PRG may represent a direct target for PAK4. These findings may help explain how PAK4 inhibits Rho function, as this kinase may limit the accumulation of GTP-bound Rho by inhibiting the activation of Rho GEFs. This unexpected finding raises the possibility that other PAK family members may similarly regulate other Rho GEFs, thereby providing a direct mechanism by which one small GTP-binding protein may affect, either positively or negatively, the activation of another Rho family member.

In summary, our observations suggest that signaling pathways regulating PAK4, such as those promoting the activation of Cdc42 and its related GTPases, may interfere directly with the local activation of Rho by inhibiting the activity of a GEF that links heterotrimeric G proteins and their coupled receptors to Rho. This potential molecular mechanism of cross-talk among Rho GTPases, together with recently published reports (10), support the emerging notion that the activation of Rho labeled phalloidin. Examples of transfected cells are depicted. B, PAK4 445N inhibits LPA-induced Rho-GTP loading. 293T cells transfected with the control vector or with increasing concentrations of PAK4 445N were serum-starved and either left untreated (-LPA) or stimulated with LPA (+LPA) for 3 min. Cell lysates were affinity-precipitated (AP) with GST-rhotekin Rho-binding domain (GST-RBD) coupled to Sepharose beads, followed by Western blotting with anti-RhoA antibody (bottom panel). Total cell lysates were probed with anti-HA and anti-RhoA antibodies to detect the levels of the transfected HA PAK4 445N and total RhoA, respectively. C, PAK4 445N inhibits serum-induced Rho activation. Left panel, 293T cells transfected with increasing concentrations of PAK4 445N were serum-starved and then left untreated or stimulated with serum for 3 min. In vivo Rho-GTP loading and protein expression were analyzed as in A. Right panel, quantification of the RhoA in GST-RBD precipitates were Western blotted with anti-RhoA antibody. Data are averages ± S.E. from three independent experiments, quantified by densitometric analysis using NIH Image and expressed as a % of active RhoA with respect to that present in serum-stimulated control cells. D, Ga13-QL activation of Rho is prevented by PAK4 445N. 293T cells were co-transfected with Ga13-QL together with increasing concentrations of HA PAK4 445N and examined for in vivo Rho GTP-loading as in B.

Fig. 4. Activated PAK4 inhibits Rho activation in vivo. A, PAK4 445N prevents LPA-induced stress fiber formation. Swiss 3T3 cells were transfected with HA-PAK4 445N, HA-PAK4 350 M (bottom panels), or expression plasmids (vector, top panels). Serum-starved cells were either left untreated (-LPA) or treated with LPA (+LPA) for 20 min, fixed, and immunostained with anti-HA followed by rhodamine-
GTPases is a highly spatial and temporally coordinated process, which can be achieved by the complex interplay between the downstream targets of one Rho protein and the molecules that regulate the state of activation of another GTPase.

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