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ArhGAP15, a Rac-specific GTPase-activating Protein, Plays a Dual Role in Inhibiting Small GTPase Signaling*

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Background: p21-activated kinases are effectors of Rac1. How Paks are inactivated is not well understood.
Results: We found that the Rac GAP ArhGAP15 binds to and inhibits Pak2. Conversely, Pak2 binds to, phosphorylates, and inhibits ArhGAP15.
Conclusion: ArhGAP15 and Pak2 are mutual inhibitors
Significance: These results suggest that ArhGAP15 acts at two levels: through Rac GTP hydrolysis and direct interaction with Pak.

Signaling from small GTPases is a tightly regulated process. In this work we used a protein microarray screen to identify the Rac-specific GAP, ArhGAP15, as a substrate of the Rac effectors Pak1 and Pak2. In addition to serving as a substrate of Pak1/2, we found that ArhGAP15, via its PH domain, bound to these kinases. The association of ArhGAP15 to Pak1/2 resulted in mutual inhibition of GAP and kinase catalytic activity, respectively. Knock-down of ArhGAP15 resulted in activation of Pak1/2, both indirectly, as a result of Rac activation, and directly, as a result of disruption of the ArhGAP15/Pak complex. Our data suggest that ArhGAP15 plays a dual negative role in regulating small GTPase signaling, by acting at the level of the GTPase itself, as well interacting with its effector, Pak kinase.

The Rho family of small GTPases (Cdc42, Rac, and Rho) have been shown to play critical roles in a wide variety of key cellular activities, including cell proliferation, apoptosis, gene expression, maintenance of proper cell architecture, attachment, and motility (1–4). The activities of these GTPases are tightly controlled and coordinated by a series of regulatory proteins, including guanine nucleotide exchange factors (GEFs),[3] GTPase-activating proteins (GAPs), and guanine-nucleotide disassociation inhibitors (GDIs) (5–8). The activities of these regulators are in turn governed by a variety of mechanisms, most prominently protein phosphorylation and ubiquitination (9–11). The proper coordination of Rho-family GTPase activity is vital to phenomena such as cell motility, in which spatial and temporal regulation of Cdc42, Rac, and Rho are required to choreograph the distinct properties of the leading and trailing edges of motile cells (12, 13).

p21-activated kinases (Paks) are downstream effectors of Rac and Cdc42, but not Rho. While the total signaling burden borne by Paks remains uncertain, expression of activated Pak recapitulates several activities of Rac and Cdc42, including enhanced cell motility, proliferation, and resistance to apoptosis (14–16). In addition to its role as an effector for Rac and Cdc42, the group A Paks (Pak1, -2, and -3) also can affect the activity of these GTPases. For example, group A Paks interact with, and phosphorylate, the Rac GEF PIX, the Rho GAPs GEF-H1, Net, (17, 18), as well as Rho-GDI (19). These activities indicate that Paks can act both upstream and downstream of Rho-family GTPases, and suggest that Paks are involved in coordinating the activities of these enzymes.

In this work, we used a protein microarray screen to identify potential new Pak substrates. In addition to previously identified Pak substrates, we also identified several novel proteins that are possible substrates and interactors of Pak, including ArhGAP15, a Rac1 specific GTPase. Here, we show that ArhGAP15 is a direct substrate of Pak1 and Pak2 proteins and that ArhGAP15 inhibits Pak activity independent of its GAP activity on Rac. These findings suggest that ArhGAP15 plays a dual negative role in Rac signaling, acting at the level of the GTPase and its major effector, Pak.

EXPERIMENTAL PROCEDURES

Human Protein Microarrays—Kinase substrate identification ProtoArrays (KSI version 4.0 arrays, containing 8,274 full-length GST fusion proteins, provided by Invitrogen) were processed as recommended by the manufacturer. Recombinant His_6-Pak2 protein was expressed in *Escherichia coli* and was used as the exogenous kinase. Bacterial produced Pak2 has been shown previously to be constitutively active (20) excluding the requirement for activation with Rac/Cdc42-GTP. "ATP only" and "ATP + Pak2" slides were identically processed in parallel. Radiographic images of the slides were obtained using a Fuji phosphorimager, and spots were identified using GenePix Pro (Molecular Devices). Data analysis was conducted in ProtoArray

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[3] The abbreviations used are: GEF, guanine-nucleotide exchange factor; GAP, GTPase-activating protein; GDI, guanine nucleotide disassociation inhibitor; MBP, myelin basic protein; Pak, p21-activated kinase; PBD, p21-binding domain.

Prospector version 2.0 software as recommended (Invitrogen). For overlay images, spots were pseudocolored in Adobe Photoshop.

Cloning of ArhGAP15 and Recombinant Protein Expression in E. coli—Full-length and different domains of ArhGAP15 DNA were cloned in pGEXT-2T and pEBG vectors using BamH1 and SmaI restriction sites. The following primers were used: Full-length ArhGAP15 forward: 5′ CGG GAT CCA TGC AGA AAT CTA CAA AAT C 3′; Full- length ArhGAP15 reverse: 5′-TCC CCC GGG CAT CAA GAC AGA TGT G-3′; NPH domain forward: 5′-TCC CCC GGG CAT CAA GAC AGA TGT G-3′; PH domain reverse: CAC CCG GGG ATA GCG TGG AAC CA-3′; NPH+constant domain forward: 5′-TCC CCC GGG CAT CAA GAC AGA TGT G-3′ NPH+constant domain reverse: 5′-GGC CCG GGA GAG CCA AAA ATT TG-3′; GAP domain forward: 5′-CCG GAT CCG TGT GTG AAC GTG AA-3′; GAP domain reverse: 5′-TCC CCC GGG CAT CAA GAC AGA TGT G-3′.

DNA plasmids expressing different GST-ArhGAP15 fragments were transformed in BL21 *E. coli* for protein expression. Bacteria were inoculated in a starter culture of 5 ml LB media with antibiotic overnight. The starter culture was added to 200 ml of LB media with antibiotic and incubated on a shaker until OD_{600} reached 0.8. Protein expression was induced with 1 mM IPTG and carried on at 16 °C for 16 h. Protein expression was verified on Coomassie-stained gels.

RNA Purification and RT-PCR—RNA was purified using a RNA extraction kit following manufacturer (Invitrogen, Grand Island, NY) protocol. One microgram of total RNA was reverse transcribed using Advantage RT-for-PCR kit (Clontech Laboratory, Inc.) following the manufacturer's instructions. GAPDH primers were supplied with the cDNA synthesis kit.

Cell Culture, Treatments, Transfection, Immunoblot, GST-pull-down, and Immunoprecipitation—HEK293 cells were maintained in DMEM medium supplemented with 20% FBS, 10% glutamine, and antibiotics. HEK293 cells were transfected with Lipofectamine 2000 (Invitrogen) following manufacturer recommendation. For MAPK signaling analysis the cells were starved overnight in serum-free DMEM and stimulated with 10 ng/ml EGF for 10 min before total protein lysate was collected. The Rac1 inhibitor NSC23766 (Millipore, Billerica, MA) was added at a concentration of 100 μM for 6 h. Pak inhibitors Frax597 (a generous gift from Afraxis) and PF3758309 (a generous gift from Pfizer) were added to the cells for 30 min.

Immunoblot, GST pull-down, and immunoprecipitation were described previously (21). Briefly, HEK293 cells were lysed in RIPA buffer, and total protein concentration was quantified by Bradford method. Equal amounts of total protein were loaded on 12% gels and transferred to PVDF membrane. Immunoblot analysis was carried with the following antibodies: Pak1, Pak2, P-199/204 Pak1,2, P-20-Pak2, P-Erk1,2 P-Akt, GAPDH, and GST, Rac1, Myc tag antibodies (Cell Signal Technologies; Pickerington, ON). For GST pull down GST proteins were incubated with GST agarose beads (GE Healthcare, Pittsburgh, PA) for 20 min. The beads were washed three times with ice-cold wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated with the recombinant proteins or the HEK293 cell lysates for 6 h at 4 °C. Pulled down complexes were analyzed by immunoblot.

For immunoprecipitation experiments, 4 μg of primary antibody was incubated with 100–500 μg of total protein from cell lysate at 4 °C overnight. 20 μl of protein A/G beads (Thermo Fisher Scientific, Rockford, IL) were added to the antibody-protein mix and incubated for 3 h at 4 °C. The beads were washed with ice-cold lysis buffer, and bound proteins were analyzed by Western blot. For GTP-Rac1 immunoprecipitation, we used antibodies that recognize only active, GTP-bound Rac1 protein (New East Biosciences, Malvern, PA).

In Vitro Kinase Assay—*In vitro* kinase assay using Pak1 and Pak2 recombinant proteins was carried out as described previously (22). Briefly, recombinant WT Pak1 or Pak2 (100 ng) was incubated in phosphobuffer with 5 μCi of [γ-^{32}P]ATP (Perkin Elmer, Waltham, MA) and recombinant NPH domain, GAP domain, or full-length ArhGAP15 (1 μg). Reactions were incubated at 30 °C for 45 min. Reactions were terminated by boiling for 5 min in an equal volume of 2× SDS-PAGE sample buffer. The reactants were then separated by SDS-PAGE run, transferred to PDVF membranes, and the membrane was exposed to a radiographic film for 1 h. Alternatively, Pak2 was dephosphorylated with 100 units of λ-phosphatase (New England Biolabs, Ipswich, MA) at 30 °C for 1 h. λ-Phosphatase was inactivated with 2 mM Na_3Vo_4 and 1 M β-glycerophosphate at room temperature for 30 min. Dephosphorylated protein was purified using Amicon Ultra Centrifugal columns (Millipore, Billerica, MA) and used in the kinase assay as described.

GAP Assay—Rac1 (30 ng) protein was incubated at 30 °C for 10 min in 20 μl of nucleotide exchange buffer: 10 μCi of [λ-^{32}P]GTP (Perkin Elmer Waltham MA), 20 mM Tris-HCl, pH 7.5, 4 mM EDTA, 0.1 mM DDT, 25 mM NaCl). The reaction was stopped by adding $MgCl_2$ to a final concentration of 20 mM. The GAP assay was carried out at 25 °C. 3 μl of Rac1-GTP mixture were added to 27 μl of GAP assay buffer (25 mM Tris-HCl, 1.25 mM $MgCl_2$, 1.25 mM DTT). For specified samples, GST-ArhGAP15 (1 μg) and Pak2 (200 ng) recombinant proteins were added. At each time point 50 μl of the mixture was pipetted out, and the reaction was stopped by adding 1 μl of 5 M NaCl. 5 μl of the reaction was spotted on nitrocellulose membrane. The membrane was washed twice for 10 min in ice-cold wash buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 50 mM NaCl) and exposed to a radiographic film.

LC-MS Analysis—NPH domain, GAP domain, and full-length ArhGAP15 proteins were phosphorylated *in vitro* as described above. The phosphoproteins from the kinase assay were digested and enriched using PolyMAC resin to isolate the phosphopeptides as described previously (23). Mass spectrometry analysis was performed with a LTQ-Orbitrap Velos device (Thermo Fisher). Localization of phosphorylation sites by mass spectra was determined by the PhosphoRS algorithm within the Proteome Discoverer 1.4 software.

RESULTS

Pak2 Phosphorylates ArhGAP15 at One or More Serine Residues—We have previously used a high-density protein microarray to identify Erk3 as a Pak2 substrate (24). In this screen, active, recombinant Pak2 was incubated with the spotted microarray

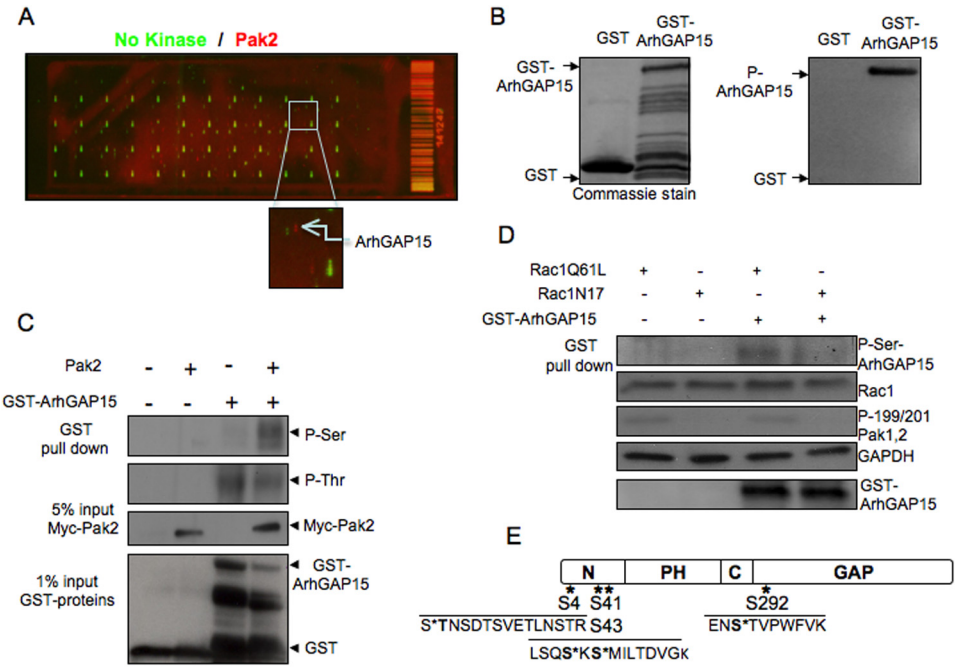

FIGURE 1. **Pak2 phosphorylates ArhGAP15 *in vitro* and in HEK293 cells.** *A*, protoarray slides were incubated with or without recombinant Pak2 kinase. Substrate proteins are spotted in duplicate. The ^{33}P signal from the no kinase array was pseudocolored *green*, and the ^{33}P signal from the array incubated with Pak2 was pseudocolored *red*. The green and red signals were overlaid, and only proteins that produced a red signal after overlay were considered as true Pak2 substrates. ArhGAP15 red spots are shown in the magnified *inset*. *B*, radiogram showing ArhGAP15 phosphorylation by Pak2. Recombinant GST and GST-ArhGAP15 proteins were incubated with Pak2 and [^{32}P]ATP in kinase buffer. The kinase reaction was run on a polyacrylamide gel that was exposed to radiographic film. *C*, HEK293 cells were co-transfected with GST or GST-ArhGAP15 coding plasmids and a plasmid that codes for Myc-Pak2. Protein lysates were applied to a glutathione-agarose column, and bound proteins were analyzed with anti-serine and anti-threonine antibodies. *D*, HEK293 cells were transfected with GST or GST-ArhGAP15 coding plasmids and plasmids coding constitutive active Rac1Q61L or dominant negative Rac1N17. Protein lysates were applied to a glutathione-agarose column and bound proteins were analyzed with anti-serine and anti- threonine antibodies. *E*, mass spectrometry analysis mapped four serine residues as targets of Pak2 phosphorylation. Peptides and phospho-serine residues are presented relative to their location within the full-length ArhGAP15 protein.

in the presence or absence of [γ-^{32}P]ATP. ArhGAP15, a protein known to possess Rac-GAP activity (25), also appeared among the proteins that became phosphorylated only in the presence of Pak2 and [γ-^{32}P]ATP (Fig. 1*A*).

To confirm these findings, we cloned full-length ArhGAP15 in bacterial and mammalian expression vectors as GST-ArhGAP15 fusion proteins. Bacterially produced GST or GST-ArhGAP15 was incubated with recombinant Pak2 and [γ-^{32}P]ATP in an *in vitro* kinase assay. The resulting autoradiogram showed significant phosphorylation of GST-ArhGAP15 but no phosphorylation of GST (Fig. 1*B*). This result confirms that Pak2 directly phosphorylates ArhGAP15 *in vitro*.

To test whether ArhGAP15 is a potential Pak2 substrate in cells, we overexpressed GST-ArhGAP15 and Myc-Pak2 in HEK293 cells. GST-ArhGAP15 protein was pulled down with glutathione-agarose beads, and the protein complexes were subjected to immunoblot analysis with anti-phosphoserine and anti-phosphothreonine antibodies. We found that ArhGAP15 was constitutively phosphorylated at threonine residues, but was specifically phosphorylated at serine residues only in cells that overexpress Pak2 (Fig. 1*C*). To examine the ability of endogenous Pak to phosphorylate ArhGAP15, we overexpressed constitutively active Rac1Q61L or dominant negative Rac1N17 along with GST-ArhGAP15. As expected, only Rac1Q61L was able to activate Pak, while overexpression of Rac1N17 did not result in increase of active Pak levels. Only cells that expressed active Rac1 showed increased levels of phospho-ArhGAP15, consistent with the idea that active Pak phosphorylates ArhGAP15 (Fig. 1*D*).

To further characterize ArhGAP15 phosphorylation, we employed LC-MS analysis. Full-length ArhGAP15 recombinant protein was phosphorylated *in vitro* as described above. Phosphorylated ArhGAP15 was then trypsin digested and phosphopeptides enriched with PolyMAC resin (26). Mass spectrometry analysis of the phosphorylated peptides showed that Pak2 phosphorylates ArhGAP15 at several sites both in the N-terminal domain and the GAP domain. The N-terminal domain was phosphorylated by Pak2 at Ser-4, -41, and -43, whereas the GAP domain was phosphorylated at Ser-292 (Fig. 1*E*).

Pak2 Inhibits ArhGAP15 Activity—ArhGAP15 has been described as a GTPase specific for Rac1 (25). Since phosphorylation events often result in modulation of the activity or stability of the affected substrate, we determined the effect of Pak2 phosphorylation on ArhGAP15 GAP activity. We first employed an *in vitro* GTPase assay to assess Rac1 activity in the presence or absence of ArhGAP15. In this assay, the Rac1-GTP half-life was ~15 min. When ArhGAP15 was added, the Rac1-GTP half-life fell below 5 min, reflecting the GAP activity of ArhGAP15 (Fig. 2*A*). To determine if Pak2 interferes with ArhGAP15 activity, we added recombinant Pak2 to the GTPase assay mixture. While the addition of Pak2 alone had no effect on Rac GTPase activity, Pak2 strongly reduced the inhibitory effect of ArhGAP15 on Rac1 GTPase activity (Fig. 2*B*). These

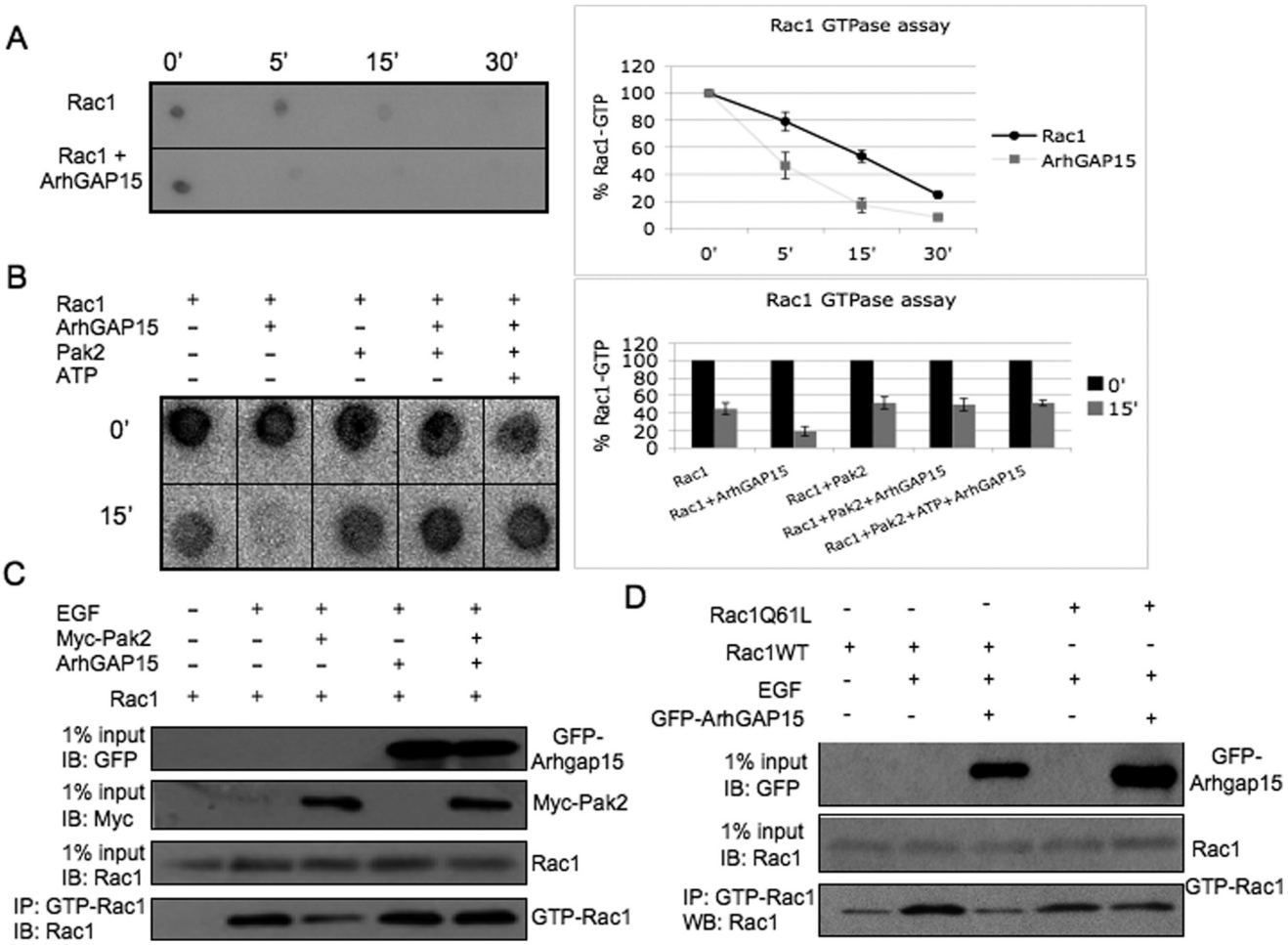

FIGURE 2. **ArhGAP15 Rac1 GTPase activity is reversed by Pak2.** *A*, GAP assay using recombinant ^{32}P GTP-Rac1 and ArhGAP15 proteins. Aliquots were spotted on a nitrocellulose membrane that was exposed to radiographic film. *B*, recombinant Pak2 was incubated with ArhGAP15 with or without ATP. Phosphorylated or unphosphorylated ArhGAP15 was used in a radioactive GAP assay toward Rac1. *C*, HEK293 cells were co- transfected with Rac1, GFP-ArhGAP15, and Myc-Pak2 coding plasmids. Cells were starved overnight and stimulated with EGF for 10 min. Protein lysates were immunoprecipitated with antibodies that recognize only GTP-bound Rac1. Immunoprecipitated complexes were analyzed by immunoblot with anti-Rac1 antibodies. *D*, HEK293 cells were co- transfected with wild type Rac1 or constitutively active Rac1 Q61L and GFP-ArhGAP15. Cells were starved overnight and stimulated with EGF for 10 min. Protein lysates were immunoprecipitated with antibodies that recognize only GTP-bound Rac1 (New Eastwick Biosciences). Immunoprecipitated complexes were analyzed by immunoblot with anti-Rac1 antibodies.

results were not dependent of phosphorylation, as identical results were seen in the presence or absence of ATP (Fig. 2*B*, *columns 4* and *5*).

We further tested the influence of ArhGAP15 and Pak2 on Rac1 GTPase activity by co-transfecting HEK293 cells with Rac1, ArhGAP15, and Pak2. We pulled down GTP-bound Rac1 using GST-PBD beads and used total Rac1 antibodies to detect active Rac1. As expected, HEK293 cells transfected with ArhGAP15 showed significantly lower levels of GTP-Rac1 compared with cells transfected with Rac1 alone (Fig. 2*C*, compare *lane 2 to lane 3*). In contrast, HEK293 cells co-transfected with Rac1, ArhGAP15, and Pak2 had similar levels of active Rac1 compared with the cells overexpressed with Rac1 alone (Fig. 2*C*, compare *lane 2 to lane 4*). ArhGAP15 has been shown to be a Rho GTPase specific for Rac1 (25). Transfection of ArhGAP15 along with wild type Rac1 resulted in a decrease of GTP-Rac1 levels, as measured by immunoprecipitation of Rac-GTP using anti-active Rac antibodies, followed by immunoblot with anti-total Rac1 antibodies (Fig. 2*D*, *lanes 2* and *3*). Constitutively active Rac1 Q61L was not affected by ArhGAP15 (Fig. 2*D*, *lanes 4* and *5*).

ArhGAP15 Binds Pak2 through Its N-PH-C Domains—Many phosphorylated proteins form transient or stable interactions with their cognate kinases (28, 29). To determine if ArhGAP15 associates with Pak1/2, we performed pull-down assays using recombinant GST-ArhGAP15 and His-Pak1 or His-Pak2. Following incubation of purified His-Pak1/2 with GST-ArhGAP15, and capture of complexes on glutathione-agarose beads, the beads were analyzed by immunoblot with Pak1 and Pak2 antibodies. We found that Pak2 readily bound ArhGAP15 in this *in vitro* setting (Fig. 3*A*).

To further test the interaction between ArhGAP15 and Pak proteins, we overexpressed GST-ArhGAP15 and either Myc-Pak1 or Myc-Pak2 in HEK293 cells. Protein lysates were purified using gluthatione-agarose beads and bound proteins were analyzed with Myc antibodies. Both Pak1 and Pak2 bound ArhGAP15 in HEK293 cells, with Pak2 having a significantly stronger interaction (Fig. 3, *B* and *C*).

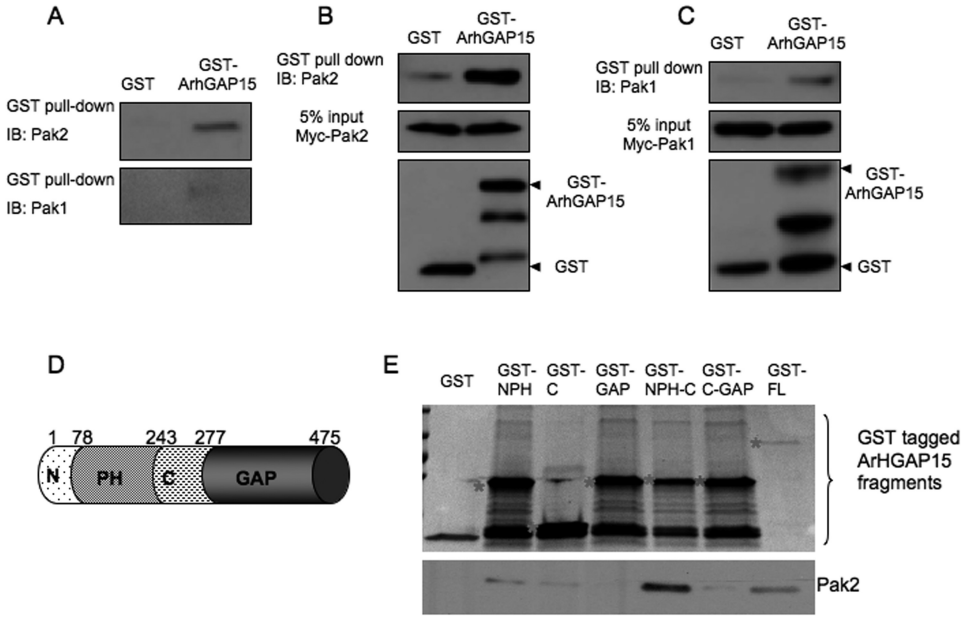

FIGURE 3. **Pak proteins bind the PH domain of ArhGAP15.** *A*, recombinant Pak1 and Pak2 protein was incubated with GST-ArhGAP15 protein on glutathione agarose beads. Bound complexes were analyzed with anti Pak1 and Pak2 antibody. *B* and *C*, Hek293 cells were co-transfected with Myc-Pak2 (*B*) or Myc-Pak1 (*C*) and a plasmid coding for GST-ArhGAP15. Protein lysates were run on a glutathione agarose column and bound complexes were analyzed with anti Pak1 or Pak2 antibodies. *D*, full-length ArhGAP15 comprises a PH domain, a domain conserved between its close relatives ArhGAP9 and ArhGAP12 (25) and a GAP domain. *E*, HEK293 cells were co-transfected with a Myc-Pak2 plasmid and plasmids encoding for GST-fused domains of ArhGAP15. Protein lysates were purified using a glutathione-agarose column, and bound complexes were analyzed with anti-Pak2 antibodies.

It has been previously shown that ArhGAP15 binds Rac1 through its conserved domain (25). We expressed different domains of ArhGAP15 protein (Fig. 3*D*) along with Myc-Pak2 in HEK293 cells and studied the binding to Pak2. Following pull-down of GST fused domains of ArhGAP15 with agarose beads, the interacting proteins were analyzed with antibodies directed against Myc tag. We found that the Arh-GAP15 N-terminal and PH domains were able to bind Pak2. The binding was further strengthened when both the N-PH and the conserved domain were both present (Fig. 3*E*). The ArhGAP15 conserved domain alone was able to bind Pak2 but with weaker affinity compared with the fused N-PH-C domains (Fig. 3*E*, compare *lane 3 to lane 5*).

ArhGAP15 Binds Pak2 in a Kinase-independent Manner—As group A Paks undergo marked conformational shifts upon activation by small GTPases (30), we asked if the activity state of this kinase affected its ability to bind ArhGAP15. First, we made recombinant Pak2 in *E. coli* and fully dephosphorylated the recombinant protein using λ phosphatase. Both phosphorylated and dephosphorylated Pak2 bound ArhGAP15 with similar affinity (Fig. 4*A*). Second, we co-expressed wild-type Pak2, inactive Pak2 mutant (K278R), or constitutive active Pak2 (T402E) with GST-ArhGAP15 in HEK293 cells. We found that all Pak2 mutants bound ArhGAP15 with similar affinity (Fig. 4*B*).

Since Pak2 activity did not affect its ability to bind ArhGAP15, we speculated that the binding motif on Pak2 for ArhGAP15 might reside outside the protein kinase domain. We tested a Pak2 mutant, $Pak2^{H82,85L}$, bearing mutations in the p21-binding domain (PBD), which cannot associate with Rac1 (31) (Fig. 4*C*). Interestingly, this mutant also failed to associate with full-length ArhGAP15 (Fig. 4*C*). These results suggest that Pak2 uses the PBD to bind both ArhGAP15 and its specific GTPase, Rac1.

ArhGAP15 Decreases Pak2 Kinase Activity—Since Pak2 kinase is a downstream effector for Rac1, it is expected that ArhGAP15 expression will decrease Pak2 activity by inactivating Rac. However, the direct binding of ArhGAP15 to Pak2 suggested that other regulatory mechanisms could also contribute to controlling Pak2 activity. We found that the addition of ArhGAP15 to Pak2 resulted in lower kinase activity toward an exogenous substrate, MBP (Fig. 5*A*). These results show that ArhGAP15 inhibits Pak2 activity *in vitro*.

We further tested Pak2 kinase activity toward MBP in a kinase assay that contained GST-ArhGAP15 fragments expressed in HEK293 cells. These assays showed that ArhGAP15 expression diminished Pak2 activity in cells (Fig. 5*B*). In addition, only the ArhGAP15 fragments that are able to bind Pak2 *in vitro* had an inhibitory negative effect on Pak2 kinase activity toward an exogenous substrate (Fig. 5*B*). These same ArhGAP15 fragments also altered Pak2 auto phosphorylation, suggesting that ArhGAP15 binding alters Pak2 activity *per se*.

Next, we down-regulated ArhGAP15 levels in cells using a pool of shRNA directed against ArhGAP15. ArhGAP15 mRNA levels were significantly reduced in HEK293 cells transfected with the ArhGAP15 shRNA pool (Fig. 5*C*). Cells with reduced ArhGAP15 levels showed markedly increased levels of phosphorylated Pak1,2 proteins (Fig. 5*D*, compare *columns 1* and *3*). These results suggest that ArhGAP15 negatively regulates Pak1,2 phosphorylation and activity.

As loss of ArhGAP15 is expected to activate Rac, which would in turn activate Pak, we sought to distinguish direct *versus* indirect effects of ArhGAP15 on Pak activity by suppressing

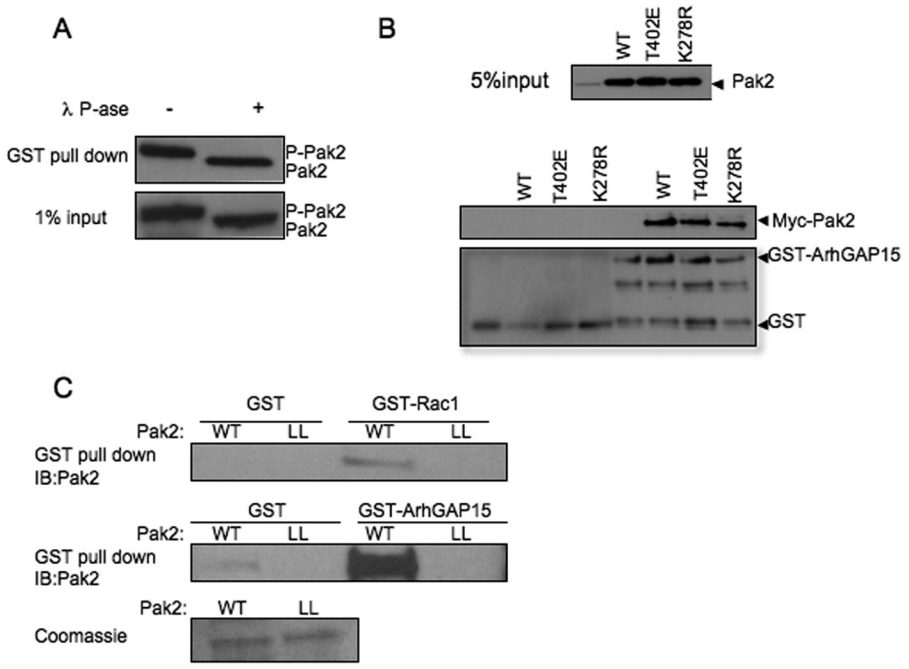

FIGURE 4. **ArhGAP15 binds PBD domain of fully activated Pak proteins.** *A*, GST-ArhGAP15 was incubated with Pak2 or de-phosphorylated Pak2 on agarose beads. Bound complexes were analyzed with Pak2 antibody. *B*, HEK293 cells were co-transfected with GST-ArhGAP15 plasmid and different Myc-Pak2 mutants. Cell lysates were fractionated on a glutathione-agarose column, and bound complexes were analyzed with anti-Pak2 antibodies. *C*, recombinant Pak2 wild type or Pak2 LL mutant was used in pull down assays with GST-ArhGAP15 or GST-Rac1 proteins. Bound complexes were analyzed with anti-Pak2 antibodies.

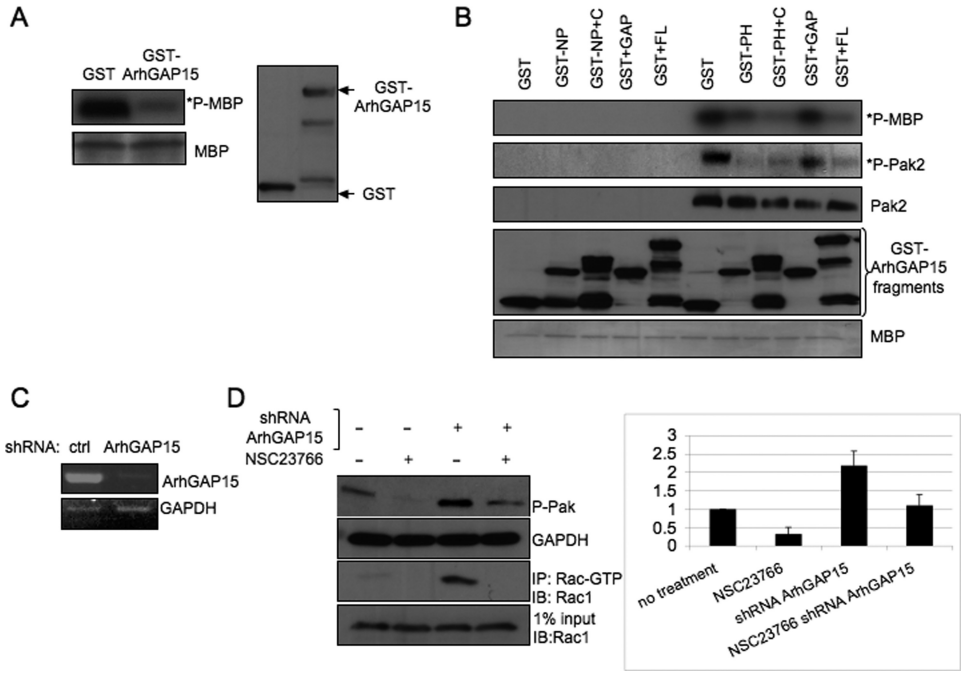

FIGURE 5. **ArhGAP15 decreases Pak2 kinase activity.** *A*, GST or GST-ArhGAP15 recombinant proteins were added to an *in vitro* kinase assay containing Pak2 and MBP in the presence of [^{32}P]ATP. Radiogram shows radioactive MBP. *B*, *in vitro* kinase assay using Pak2 and MBP in the presence of various GST-linked domains of ArhGAP15. Radiogram shows radioactive MBP and Pak2. *C*, down-regulation of ArhGAP15 mRNA levels using an shRNA mixture directed against ArhGAP15. *D*, HEK293 cells transfected or not with ArhGAP15 shRNA were treated with Rac1 inhibitor NSC23766 for 6 h. Protein lysates from HEK293 transfected with ArhGAP15 shRNA or control shRNA were analyzed with the indicated antibodies. Quantitation of the anti-P199/204 Pak1/2 signal is presented in the panel on the *right*.

Rac1 with a small molecule inhibitor. We therefore used the Rac inhibitor NSC23766 to ask if the activation of Pak in cells transfected with ArhGAP15 shRNA could occur even in the absence of activated Rac. As expected, expression of ArhGAP15 shRNA was associated with elevated Rac1 activity, and this elevation was blocked by the Rac1 inhibitor NSC23766 (Fig. 5*D*, compare *columns 3* and *4*). Whereas Rac inhibition by NSC23766 was also associated with decreased P-Pak1,2 levels in control cells (Fig. 5*D*, compare *lanes 1* and *2*), this decrease was not apparent in cells expressing ArhGAP15 shRNA (*lanes 3*

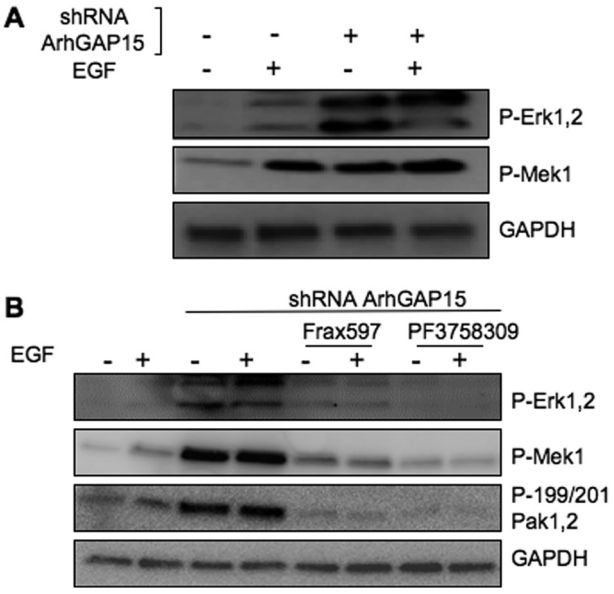

FIGURE 6. **Down-regulation of ArhGAP15 levels results in altered MAPK signaling.** *A* and *B*, HEK293 transfected with control shRNA or ArhGAP15 shRNAs were stimulated with 10 nM EGF for 10 min. Alternatively, (*B*) the cells also underwent treatment with Pak inhibitors Frax597 and PF3758309 for 30 min. Protein lysate was analyzed by immunoblot with antibodies against P-Pak, P-Erk, P-Akt, and P-Mek.

and *4*). These data suggest that ArhGAP15 has a direct effect on P-Pak1,2 levels, independent of Rac1.

ArhGAP15 Modulates Pak2 Activity and Interferes with Erk Signaling—Down-regulation of ArhGAP15 levels results in activation of Pak (Fig. 5*D*), and modulation of Pak activity results in altered Erk pathway. We therefore tested Erk signaling activity in HEK293 cells transfected with control or ArhGAP15 shRNA. Serum-starved HEK293 cells were stimulated with EGF for 10 min and Erk activity was assessed by immunoblot analysis. Cells transfected with ArhGAP15 shRNA exhibited higher levels of P-Erk1,2, P-Akt, and P-Mek1, suggesting that ArhGAP15 represses Erk (Fig. 6*A*). We inhibited Pak activity in shRNA ArhGAP15 transfected HEK293 cells with the small molecules Pak inhibitors PF3758309 (32) or Frax597 (33). In cells expressing ArhGAP15 shRNA, both Pak phosphorylation and Erk activity were inhibited by the drug treatment, reverting to levels similar to those seen in wild type cells that express ArhGAP15.

DISCUSSION

Signaling from Rho-family GTPases is tightly controlled by factors that affect the rate of binding and hydrolysis of GTP. These enzymes, including GEFs, GAPs, and GDIs, are themselves regulated by a variety of mechanisms, including phosphorylation, ubiquitination, lipid and protein binding, and proteolytic cleavage (11, 34–36). Likewise, Rho GTPase effectors are highly regulated. In this report, we present evidence in support of a bidirectional inhibitory circuit involving the Rac-GAP Arh-GAP15 and the Rac effectors Pak1/2 (Fig. 7).

ArhGAP15 was discovered in a screen for GAPs specific for Rac1 (25). By sequence, it is closely related to two other Rac-GAPs, ArhGAP9 and ArhGAP12. While there is limited research studying the role of ArhGAP15 in cellular functions, this protein has been shown to play roles in adjusting actin stress fiber architecture (25) and also as a negative regulator of neutrophil functions (37). Here, we report the identification of ArhGAP15 as a Pak1/2 substrate in a high-density protein microarray (24). We then confirmed that ArhGAP15 is directly phosphorylated by Pak2 at least at four sites. The Pak consensus phosphorylation motif has been previously determined as favoring Arg residues at positions −5 to −1 and large hydrophobic residues at positions +1 to +3 (38). All four identified sites follow this pattern. Three of the phosphorylation sites are located in the N- terminal domain that is required for Pak binding, whereas one site (Ser-292) is located in the functional GAP domain. The significance of these phosphorylation events is not clear, as we did not find a direct effect of these modifications on Rac GAP activity (Fig. 2*B*) or the localization of ArhGAP15 in cells (data not shown). Interestingly, another group also reported the *in vitro* phosphorylation of ArhGAP15 by Pak1, though no functional studies were discussed (25).

While the role of ArhGAP15 phosphorylation remains at present speculative, we found that the association of Pak2 with ArhGAP15 inhibited the GAP activity of the latter protein. The association of these two proteins required the Pak2 PBD and either the PH or the conserved domain of ArhGAP15. However, a mutant form of ArhGAP15 that contains residues 1–277 (PH domain plus conserved domain) bound Pak2 with higher affinity than the PH domain or conserved domain alone (Fig. 3*E*). As ArhGAP15 binds Rac1 through its conserved domain (residues 240–332) (25), it is likely that ArhGAP15 cannot simultaneously engage both Rac and Pak, and that Pak-bound ArhGAP15 is therefore unavailable to deactivate Rac1. By the same token, Pak appears to use identical or overlapping motifs (the PBD domain) to engage both Rac and ArhGAP15. These data suggest that Pak cannot bind ArhGAP15 and Rac at the same time. It is unclear how inactive Pak dissociates from ArhGAP15 in order to bind Rac1 and later be activated. It is possible that yet another factor is involved in the dissociation between inactive Pak and ArhGAP15. It is also possible that active Rac competes out ArhGAP15 for binding Pak PBD domain (Fig. 7).

Overexpression of ArhGAP15 in HEK293 cells decreased Rac GTPase activity (Fig. 2*C*). Down-regulation of endogenous ArhGAP15 levels resulted in higher levels of phosphorylated Pak (Fig. 5*D*) and activation of the Erk pathway (Fig. 6, *A* and *B*). We hypothesize that ArhGAP15 binds Rac-GTP after Pak is activated and released by Rac. ArhGAP15 binds Rac1 independent of its GTP- or GDP-bound state (25). It is possible that upon GTP hydrolysis ArhGAP15 remains bound to Rac-GDP until a sufficient active Pak will displace it from the GTPase. Pak binding to ArhGAP15 leads to inactivation of its kinase activity (Figs. 5, *A* and *B* and 7).

The mutual inhibitory interaction of ArhGAP15 and Pak resembles the model described for the tumor suppressor protein Merlin and Pak. In the latter case, disruption of the Merlin/Pak complex by activation of Pak or by mutations in the Merlin is thought to result in the activation of multiple oncogenic signaling pathways (39, 40).

In summary, in this report we describe two mechanisms by which ArhGAP15 inhibits Pak kinase activity. First, ArhGAP15 acts as a GAP for Rac1, resulting in decreased GTPase activity.

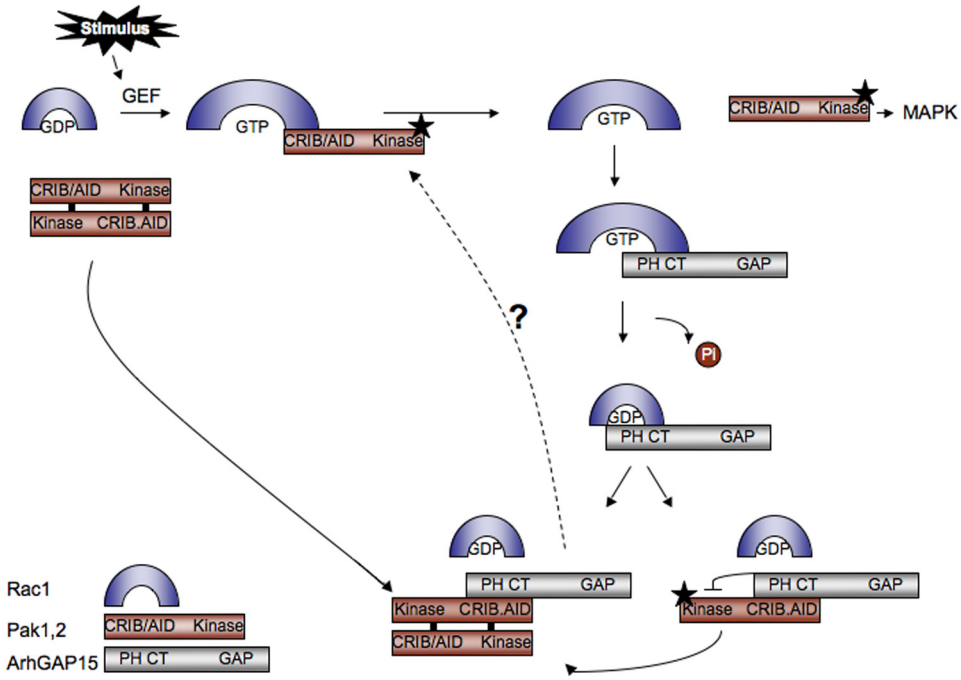

FIGURE 7. **Model for dual role of ArhGAP15 in regulating Rac1 signaling.** In the proposed model, Pak binds Rac1-GTP, then dissociates from it when fully activated. Upon Pak dissociation from Rac1, ArhGAP15 is able to bind and inactivate Rac1 signaling by augmenting its GTPase activity. In this model, ArhGAP15 remains bound to Rac1-GDP until sufficient activated Pak binds ArhGAP15 and removes it from Rac1-GDP. The direct binding of ArhGAP15 to Pak inhibits the activity of this kinase, providing an independent mechanism for down-regulating Rac signaling.

This event alone would be expected to decrease Pak activity (Figs. 6*C* and 7). Second, ArhGAP15 appears to have a direct, independent inhibitory effect on Pak, as knock down of ArhGAP15 is associated with elevated Pak activity, even in the absence of Rac activation (Fig. 5*D*). These findings suggest that negative regulators of signaling such as ArhGAP15 use multiple mechanisms to impede pathway activation.

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