Communication

αPix Stimulates p21-activated Kinase Activity through Exchange Factor-dependent and -independent Mechanisms*

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Activation of p21-activated kinases (Paks) is achieved through binding of the GTPases Rac or Cdc42 to a conserved domain in the N-terminal regulatory region of Pak. Additional signaling components are also likely to be important in regulating Pak activity. Recently, a family of Pak-interacting guanine nucleotide exchange factors (Pix) have been identified and which are good candidates for regulating Pak activity. Using an active, truncated form of αPix (amino acids 155–545), we observe stimulation of Pak1 kinase activity when αPix155–545 is co-expressed with Cdc42 and wild-type Pak1 in COS-1 cells. This activation does not occur when we co-express a Pak1 mutant unable to bind αPix. The activation of wild-type Pak1 by αPix155–545 also requires that αPix155–545 retain functional exchange factor activity. However, the Pak1180,186mutant that does not bind Rac or Cdc42 is activated in the absence of GTPase by αPix155–545 and by a mutant of αPix155–545 that no longer has exchange factor activity. Pak1 activity stimulated in vitro using GTPγS-loaded Cdc42 was also enhanced by recombinant αPix155–545 in a binding-dependent manner. These data suggest that Pak activity can be modulated by physical interaction with αPix and that this specific effect involves both exchange factor-dependent and -independent mechanisms.

Ras-related small GTPases have been the subject of intense investigation over recent years. These molecular switches cycle between GTP-bound “on” and GDP-bound “off” states. In their GTP-bound form they are able to specifically interact with and modulate the activity of effector molecules (1). The nucleotide state of low molecular weight GTPases is thus a key factor influencing their ability to effectively transduce signals, and it is therefore a tightly regulated process. The proteins that regulate the nucleotide bound state of small GTPases have been relatively well characterized, and are fall into three main categories: those that accelerate the intrinsic hydrolysis of GTP to GDP, hence switching off the G-protein (GTPase activating proteins, GAPs); those that stabilize the protein in the GDP-bound form in the cytoplasm awaiting the appropriate signals (GDP dissociation inhibitors, GDIs); and finally, guanine nucleotide exchange factors (GEFs).1 GEFs catalyze the exchange of GDP for GTP on the GTPase, thus switching the molecule to the “on” state (2).

Rho-family GTPases are among the best characterized members of the Ras superfamily and have been shown to regulate dynamic changes in the actin cytoskeleton that occur during many different cellular processes and in response to a wide variety of extracellular signals (3–5). Members of the Rho GTPase family (Rho, Rac, and Cdc42) interact with a diverse array of downstream effectors that may regulate specific processes depending on the extracellular environment or stimuli. It is still not clear what mechanisms are responsible for determining how Rac, Rho, or Cdc42 selectively interact with their different effectors and hence trigger specific intracellular pathways. Recent studies have suggested that guanine nucleotide exchange factors may be important determinants of the selectivity of small G-protein signaling (6). This concept has arisen for several reasons. First, the number of Rho-family GEFs (~40) already exceeds that of the Rho family members (~10), thus increasing the potential diversity of responses. Second, many of the GEFs identified contain multiple protein-protein interaction domains and are therefore good candidates for enabling coordinated, compartmentalized signaling events to occur (7–9). Finally, and most intriguingly, several recently identified GEFs for small GTPases have been shown to bind both the GTPase and its downstream effector, thus bringing the two key signaling elements in close proximity and suggesting a possible mechanism through which GEFs may generate the selectivity of GTPase-effector interaction (10–11).

p21-activated kinases (Paks) were first identified as targets of Rac and Cdc42 and have subsequently been implicated in various cellular processes regulated by Rac and Cdc42 (12–15). Studies in our laboratory and others have suggested that Pak may play a key role in regulating cytoskeletal dynamics (16–18). Recently a guanine nucleotide exchange factor termed Pix (p21-interacting exchange factor) that binds specifically to Pak via interaction of a Pix-SH3 domain with the fourth proline-rich region in Pak was identified (11). This region has also been implicated as playing a role in Pak-dependent regulation of neurite outgrowth and cytoskeletal activity (17, 19). Because of the reported exchange factor specificity of Pix toward Rac and Cdc42, it was suggested Pix might regulate GTPase-mediated activation of Pak (11). However, the only study to specifically address this possibility to date demonstrated that one isoform of Pix, αPix, appears to down-regulate Pak activation by upstream signals (20). The effect of the αPix isoform on Pak activity has not yet been investigated, nor has it been determined whether Pak catalytic activity can be directly enhanced by any Pix isoform.

We demonstrate here that a functionally active form of αPix

*1 The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology domain; Pak, p21-activated kinase; Pak, interacting exchange factor; SH3, src homology 3 domain; GTPγS, guanosine 5′-3′-(thio)triphosphate; WT, wild type; PCR, polymerase chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBD, p21-binding domain; CMV, cytomegalovirus.

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is able to induce Pak1 activation when co-expressed with Pak1 and Cdc42 or Rac in COS-1 cells. This activation requires both an intact aPix Dbll domain and the binding of aPix to Pak1. Additionally, we report an exchange factor-independent enhancement of Pak1 activity induced by the binding of aPix.

**EXPERIMENTAL PROCEDURES**

**Plasmids—** cDNA expression plasmids containing full-length Pak1 and its various mutants in the pCMV6M vector (CMV promoter, N-terminal myc tag) have been described elsewhere (14). Pak1 R193A and P194A double mutations were made in full-length WT-Pak1 by overlapping PCR. Universal primers PT637-5' and PT686-3' were used as outer boundary primers, and we used overlapping primer pairs to introduce the desired mutations (forward primer 5'-GTATTGGTCTCAGCCTACAGACACA-3' and reverse primer 5'-TGGTGGTCTCGGCGTGGGACATC-3'). The Pak1A193,194 was then inserted into pCMV6M at the BamHI/EcoRI site.

Wild-type full-length aPix (cDNA accession number D25304) was a gift of N. Nomura (Kazusa DNA Research Institute, Japan). Truncated aPix155-545 was amplified from the full-length aPix and cloned into pGEX 4T-1 (Amersham Pharmacia Biotech) by engineering an EcoRI site on the 5'- oligonucleotide (5'-GGCAATTCTAGGCGAAGT-3') and an Xhol site on the 3' oligonucleotide (5'-GCCTTGATTCTCGGCGTGGGACATC-3'). For mutant expression, aPix155-545 was sub-cloned into pRK5-myc by engineering a HindIII site at the 3' end and cloning into the BamHI/HindIII sites of pRK5-myc. The aPix155-545 point mutations in the Dbl homology domain (DH-anPix155-545) were introduced by generating sense and antisense oligonucleotides containing two base pair changes in the codons for amino acids Leu-383 and Leu-384 and utilizing the "Quick Change" kit (Stratagene). Full-length cDNAs encoding wild-type and mutations of Cdc42 (WT and Q61L) were subcloned into the pRK5 expression plasmid containing an N-terminal myc tag via BamHI and EcoRI sites generated by PCR using oligonucleotides flanking the cDNA coding sequence.

The glutathione S-transferase (GST) was fused to amino acids 67 to 150 of human Pak1 as follows. The WT-Pak1 region was amplified by PCR using primers OP1167-5' (5'-CGGGATTCCATGACGAAGGACGCGA-3') and OP1/150-3' (AGGAAAAGACGCGCTGGCTGACCTCAACTGACTTATCTGATGACC-3') using pGEX6p-6M as a template. The BamHI/SalI cut PCR product was inserted into BamHI/SalI cut pGEX-4T3 to give pGEX-hpak1-67-150.

**Transfection of COS-1 Cells—** COS-1 cells grown to 75% confluence on 10-cm tissue culture dishes were transiently transfected using the Lipofectamine transfection protocol (Life Technologies, Inc.) with a total of 7.5 μg of either pRK5M or pCMV6M expression vectors containing various myc-tagged constructs. 2.5 μg of each construct was used, and if the total number of constructs added was less than 7.5 μg, the concentration of DNA was normalized by adding the appropriate amount of empty vector. The cells were allowed to express the protein for 40 h post-transfection and were then washed in phosphate-buffered saline and scraped into 250 μl of lysis buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl2, 1 mM DTT, 5% glycerol, 1% Nonidet P-40) and incubated for 4°C. The bead pellet was then washed three times with 25 mM Tris-HCl, pH 7.5, 1 mM DTT, 30 mM MgCl2, 40 mM NaCl, 0.5% Nonidet P-40, and incubated for 1 h at 4°C. The bead pellet was then washed three times with 25 mM Tris-HCl, pH 7.5, 1 mM DTT, 30 mM MgCl2, 40 mM NaCl, 1% Nonidet P-40 and twice with the same buffer without Nonidet P-40. The bead pellet was finally suspended in 40 μl of Laemmli sample buffer. Proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-myc 9E10 antibody.

**RESULTS AND DISCUSSION**

We initiated studies to evaluate the effects of the recently described Pak-interacting protein aPix on kinase activity of Pak1. Co-expression of full-length aPix with Pak1 and WT-Cdc42 had no effect on Pak1 kinase activity in vivo (Fig. 1). Indeed, overexpression of full-length aPix with an activated mutant of Cdc42 (Q61L) inhibited the autophosphorylation of Pak and its ability to phosphorylate exogenous substrate (data not shown). In a recent study on the aPix isoform (COOL1), which itself has two alternatively spliced isoforms, a similar inhibition of stimulated-Pak3 kinase activity was seen (20). It is known with other Dbl-homology domain-containing GEFs that the full-length molecules often do not exhibit effective guanine nucleotide exchange activity. This probably reflects the requirement for additional, unidentified signals to allow appropriate conformational changes in the GEF leading to activation. Truncation of other Dbl-domain-containing Rho family GEFs, such as Cst, Osp, p115 Rho-GEF, and Dbl, removes internal inhibitory constraints and allows full activity to be observed (22–25). Manser et al. (11) demonstrated that

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effective exchange factor activity was only detected in vitro when αPix was truncated to amino acids 155–545, encompassing the SH3, DH, and PH domains of the protein. Therefore, we constructed an identical version of αPix and cloned it into mammalian and bacterial expression vectors (Fig. 1A). Coexpression of either full-length αPix or αPix155–545 with WT-Pak1 had no effect on Pak activity (data not shown). However, when WT-Cdc42 was overexpressed along with WT-Pak1 and αPix155–545 in COS-1 cells, we detected the formation of slower migrating species of Pak1 indicative of autopseudophosphorylation and activation (Fig. 1B). This activity was not seen in the absence of αPix155–545 or when full-length αPix was coexpressed. The increase in Pak1 activity was confirmed when the transfected proteins were immunoprecipitated from the lysates and subjected to an in vitro kinase assay with histone H4 as exogenous substrate (Fig. 1B). Expression of Pak1 and WT-Cdc42 alone or Pak1, full-length αPix, and WT-Cdc42 caused no significant substrate phosphorylation (Fig. 1B). These data suggested that the truncated active version of αPix was able to promote nucleotide exchange on WT-Cdc42 in vivo and hence stimulate Pak kinase activity (see Fig. 3 below).

We therefore investigated whether increased activation of Pak by Cdc42 in the presence of αPix155–545 was dependent on an intact DH domain in αPix. DHβ exchange activity has been shown to be dependent on two conserved leucine residues in the DH-domain (26). Mutation of these residues in αPix abolished GEF activity (11). We mutated the corresponding residues in αPix155–545 (L383R, L384S) and observed that when this construct was coexpressed with Pak1 and WT-Cdc42 in COS-1 cells, there was an ~60% decrease in the enhancement of Pak1 kinase activity observed with the active protein (Fig. 2A). The (DH-) αPix155–545 bound to Pak to the same extent as the unmutated construct (data not shown). This strongly impli-
Pak/Pix interaction, Pak1A193,194, was generated. This construct was no longer able to bind to aPix155–545 (Fig. 2B) but retained fully the ability to be activated in vitro by GTPγS-loaded Cdc42, indicating its intrinsic kinase activity was unaffected by mutating the Pix binding site (Fig. 2C). When Pak1A193,194 was co-transfected with the truncated aPix and WT-Cdc42 into COS-1 cells, no enhancement of Pak kinase activity was observed (Fig. 2C). This indicates that a direct binding between the exchange factor and Pak was required to enhance Pak activation in our system and that Pix exchange activity alone is not sufficient to promote the Cdc42-dependent activation of Pak1.

Because the enhancement of Cdc42-dependent activation of Pak kinase activity by aPix155–545 required direct interaction with Pak, two possibilities were raised: 1) aPix may influence the ability of Pak to be activated independently of its exchange factor function, and 2) efficient exchange factor activity of aPix155–545 requires Pak binding. To evaluate these two possibilities, we first tested whether the increased activation of Pak we observed was a consequence of an exchange factor-independent effect of aPix155–545 on Pak catalytic activity. We utilized a mutant of Pak that no longer binds Rac or Cdc42 but has a low level of intrinsic kinase activity in the absence of activated GTase, Pak1H183,360L (14, 27). Pak1H183,360L was co-expressed with aPix155–545 in COS-1 cells, immunoprecipitated, and subjected to an in vitro kinase assay. As Fig. 3A shows, the activity of Pak1H183,360L is significantly increased when co-expressed with aPix155–545. This is in contrast to WT-Pak, which was not activated by aPix155–545 unless Cdc42 was also overexpressed in the same cells (Fig. 1B). Because Pak1H183,360L does not bind GTases at all, these data suggest that aPix exerts an exchange factor-independent influence on Pak activity. This was further confirmed when the aPix155–545 mutated at residues Leu-383,384 ((DH-) aPix155–545) was also able to enhance the kinase activity of Pak1H183,360L (Fig. 3A). Additionally, we established that Pak1H183,360L activity was enhanced in vitro using a recombinant GST-fusion construct of aPix155–545 (Fig. 3A). This confirms that the Pak-Pix interaction alone in the absence of other cellular co-factors is sufficient to increase Pak activity.

To further demonstrate that Pix utilizes an exchange factor-independent mechanism to increase Pak1 activity, we measured the effect of aPix155–545 on Pak1 kinase activity in a standard in vitro kinase assay using Cdc42 loaded with GTPγS. As Fig. 3B shows, Cdc42-GTPγS activation of WT-Pak is increased 2- to 3-fold when recombinant GST-aPix155–545 is added to the reaction. This effect is consistent and was not seen when we used the mutant of Pak1 (Pak1A193, 194), unable to bind to aPix.

To test the second possibility, i.e. that aPix155–545 exchange activity was modulated by Pak1 binding, we utilized an assay to determine the level of GTP-bound Cdc42 in vitro. Co-expression of Cdc42 with aPix155–545 resulted in an increased production of GTP-bound Cdc42 that was dependent on a functional aPix DH domain (Fig. 3C). GTase activation was substantially enhanced when WT-Pak1 was also expressed with aPix155–545 and Cdc42 but not when Pak1A193,194 was expressed instead of WT-Pak1 (Fig. 3C). This suggests that in addition to the effect of aPix155–545 on Pak kinase activity, Pak can stimulate the exchange factor activity of aPix. This effect is entirely dependent on the binding of Pak to Pix, although the mechanism by which this occurs is not yet understood at a molecular level. Interestingly, Manser et al. (11) observed that co-expression of aPix with membrane-targeted Pak resulted in an enhanced Pix exchange factor activity, suggesting that Pak can also modulate the GEF activity of the aPix isoform.

The data we have presented establish that the Pak interacting exchange factor aPix can significantly enhance Cdc42-stimulated Pak1 kinase activity. Additionally, we have observed similar enhancement of Rac-stimulated Pak1 activity in vitro and in vivo (data not shown). Both functional exchange factor activity and the specific binding interaction between Pak1 and aPix is required for the optimal enhancement of Pak catalytic activity. Taken together, these results suggest a model in which direct protein-protein interaction between a GTase effector kinase (Pak) and a GTase exchange factor (aPix) can modulate the activities of both proteins. This provides a mechanism by which Pak can be more effectively activated in an environment where Pix is present and able to bind to Pak. This could achieve specificity in localized signaling and may also explain differences in Pak activity in various cell types that may reflect differences in Pix expression. Interactions with Pix may also account for differences in Pak activation/inactivation kinetics observed between intact cells versus in vitro. We are currently investigating these possibilities.

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