

Pak1 Kinase Homodimers Are Autoinhibited in *trans* and Dissociated upon Activation by Cdc42 and Rac1

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Summary

Pak1, a serine/threonine kinase that regulates the actin cytoskeleton, is an effector of the Rho family GTPases Cdc42 and Rac1. The crystal structure of Pak1 revealed an autoinhibited dimer that must dissociate upon GTPase binding. We show that Pak1 forms homodimers in vivo and that its dimerization is regulated by the intracellular level of GTP-Cdc42 or GTP-Rac1. The dimerized Pak1 adopts a *trans*-inhibited conformation: the N-terminal inhibitory portion of one Pak1 molecule in the dimer binds and inhibits the catalytic domain of the other. One GTPase interaction can result in activation of both partners. Another ligand, β PIX, can stably associate with dimerized Pak1. Dimerization does not facilitate Pak1 *trans*-phosphorylation. We conclude that the functional significance of dimerization is to allow *trans*-inhibition.

Introduction

Pak1 is a member of the Pak/Ste20 family of serine/threonine kinases, which comprises six mammalian isoforms (Dan et al., 2001; Knaus and Bokoch, 1998). Pak proteins participate in regulating the actin cytoskeleton, focal adhesion contacts (Frost et al., 1998; Manser et al., 1997; Sells et al., 1997; Zhao et al., 1998), and cell motility (Sells et al., 1999). Paks have also been implicated in other cellular functions, such as activation of MAP kinase pathways (Brown et al., 1996; Frost et al., 1996; Zhang et al., 1995), apoptotic/survival signaling (Lee et al., 1997; Rudel and Bokoch, 1997; Tang et al., 2000), and HIV pathogenesis (Renkema et al., 1999).

Paks are downstream effectors of the small GTPases Cdc42 and Rac, which are well-known regulators of the actin cytoskeleton (Bishop and Hall, 2000). Binding of active (GTP-loaded) Cdc42 or Rac results in increased Pak1 kinase activity and autophosphorylation at multiple sites (Manser et al., 1997). Other Pak1 binding partners have been identified that contribute to the intricate regulation of this multifunctional kinase. It has been proposed that members of a recently discovered family of Pak-binding proteins, named PIX (Manser et al., 1998) or Cool (Bagrodia et al., 1998), modulate Pak1 directly by regulating its kinase activity (Bagrodia et al., 1998;

Daniels et al., 1999) or indirectly by recruiting it to focal adhesion contacts (Manser et al., 1998). Studies in mammalian cells and *Drosophila* indicate that the Nck SH2/SH3 adaptor regulates Pak1 by plasma membrane recruitment (Hing et al., 1999; McCarty, 1998). In addition, certain sphingolipids activate Pak1, suggesting that Paks may transduce lipid signals (Bokoch et al., 1998). Finally, recent reports indicate that phosphorylation by kinases such as p35/cyclin-dependent kinase 5 (Nikolic et al., 1998) or 3-phosphoinositide-dependent kinase-1 (PDK1) (King et al., 2000) may also regulate Paks.

Pak molecules have a C-terminal kinase domain and an N-terminal regulatory region, which contains distinct binding sites for Cdc42/Rac, Nck, and PIX. What was originally defined as the GTPase binding domain (GBD) (aa 70–113), also found in several other Cdc42/Rac effectors (Burbelo et al., 1995; Symons et al., 1996), partially overlaps a region (aa 70–150) with autoinhibitory features (Brown et al., 1996; Frost et al., 1998; Tu and Wigler, 1999; Zenke et al., 1999; Zhao et al., 1998). The autoinhibitory region can inhibit Paks in *trans* in vitro and in vivo, and various mutations in this segment activate Pak1 kinase activity.

The recently determined X-ray crystal structure of Pak1 in the autoinhibited conformation (Lei et al., 2000) showed that the core of this inhibitory region is an “inhibitory switch” (IS) domain, whose conformation is contingent on its interactions (Figures 1A and 1B). In the autoinhibited state, the IS domain is a bundle of three α helices with a short N-terminal β hairpin, enclosing a hydrophobic core. Through interactions with the large lobe of the kinase domain, the IS domain positions its C-terminal extension, the kinase inhibitor segment (KI) (aa 136–150), directly into the catalytic cleft of the kinase domain. The N-terminal extension of the IS domain is a segment (Di) (aa 81–87) that forms an antiparallel β ribbon dimer interface with the Di of a second Pak1 molecule. Binding of Cdc42/Rac to the N-terminal part of the Di/IS segment is expected to rearrange part of the IS domain and unfold the rest, leading to the active-state conformation in which the KI is removed from the catalytic site (Figure 1B).

One of the most unexpected features in the Pak1 X-ray crystal structure was the dimer interaction. In vitro biophysical studies, including gel filtration and analytical ultracentrifugation, indeed confirmed that the majority of recombinant purified Pak1 is a dimer in solution (Lei et al., 2000; Buchwald et al., 2001). The dimer in the crystal structure is, however, composed of four polypeptide chains: two N-terminal regulatory segments and two kinase domains. Therefore, it was not possible to determine whether the inhibitory contacts between the IS and catalytic domains would normally occur in *cis* or in *trans*.

How do these structural discoveries relate to Pak1 regulation in vivo? We describe here experiments on the mechanism of Pak1 autoinhibition in cells. We find that the majority of Pak1 is dimeric in vivo, and we provide strong evidence for inhibition in *trans*. Moreover, we demonstrate that activation by Cdc42/Rac1 leads to

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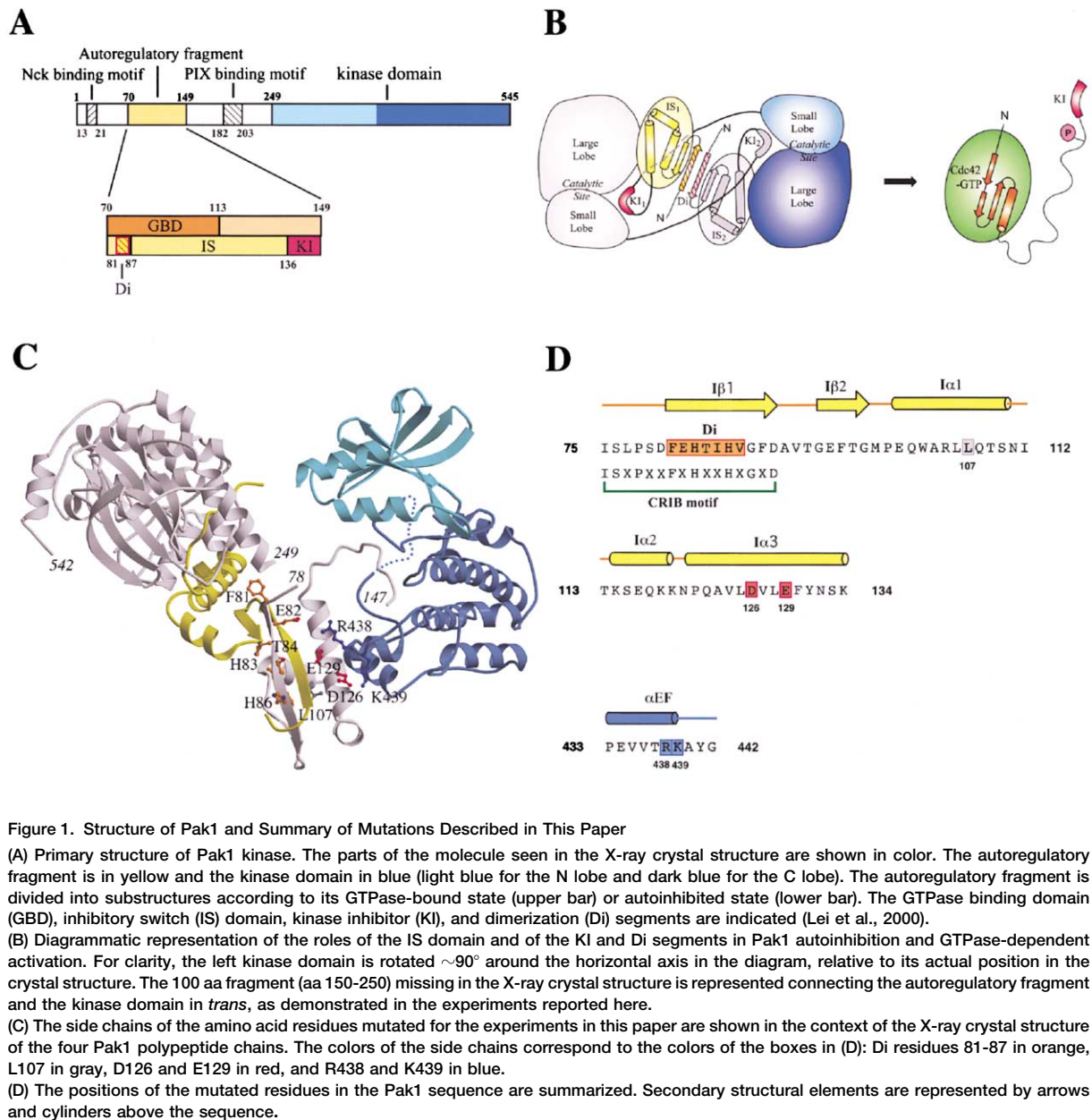


Figure 1. Structure of Pak1 and Summary of Mutations Described in This Paper

(A) Primary structure of Pak1 kinase. The parts of the molecule seen in the X-ray crystal structure are shown in color. The autoregulatory fragment is in yellow and the kinase domain in blue (light blue for the N lobe and dark blue for the C lobe). The autoregulatory fragment is divided into substructures according to its GTPase-bound state (upper bar) or autoinhibited state (lower bar). The GTPase binding domain (GBD), inhibitory switch (IS) domain, kinase inhibitor (KI), and dimerization (Di) segments are indicated (Lei et al., 2000).

(B) Diagrammatic representation of the roles of the IS domain and of the KI and Di segments in Pak1 autoinhibition and GTPase-dependent activation. For clarity, the left kinase domain is rotated $\sim 90^\circ$ around the horizontal axis in the diagram, relative to its actual position in the crystal structure. The 100 aa fragment (aa 150-250) missing in the X-ray crystal structure is represented connecting the autoregulatory fragment and the kinase domain in *trans*, as demonstrated in the experiments reported here.

(C) The side chains of the amino acid residues mutated for the experiments in this paper are shown in the context of the X-ray crystal structure of the four Pak1 polypeptide chains. The colors of the side chains correspond to the colors of the boxes in (D): Di residues 81-87 in orange, L107 in gray, D126 and E129 in red, and R438 and K439 in blue.

(D) The positions of the mutated residues in the Pak1 sequence are summarized. Secondary structural elements are represented by arrows and cylinders above the sequence.

dimer dissociation. This work reveals previously unsuspected levels of Pak1 regulation; it may illustrate a novel mechanism of kinase autoinhibition by dimerization.

Results

Pak1 Self-Associates In Vivo

To explore the biological relevance of the Pak1 dimerization observed *in vitro*, we investigated whether Pak1 molecules can associate with each other *in vivo*. Human 293T cells were cotransfected with plasmids expressing two Pak1 constructs, one tagged with the Myc epitope and a second bearing the HA epitope. Pak1-Myc was specifically detected in anti-HA immunoprecipitates, and Pak1-HA was likewise found in anti-Myc immuno-

precipitates (Figure 2A), demonstrating that Pak1 molecules can stably associate with each other in cells. By calibrated size exclusion chromatography of transfected and untransfected 293T cell lysates, we found that the majority of both endogenous and overexpressed Pak1 has an apparent molecular weight of ~ 130 – 160 kDa (Figure 2B and data not shown); this is consistent with a dimer, as Pak1 has a monomer molecular weight of 61 kDa. We also calculated the percentage of self-associated Pak1 *in vivo* by quantitative analysis of coprecipitation experiments (see Experimental Procedures). In multiple experiments the percentage of dimerization was estimated to be 25%–50% for wild-type Pak1 and 50%–100% for the kinase-inactive form. Considering the likelihood that some existing complexes dissociate during the extensive washes following immunoprecipitation,

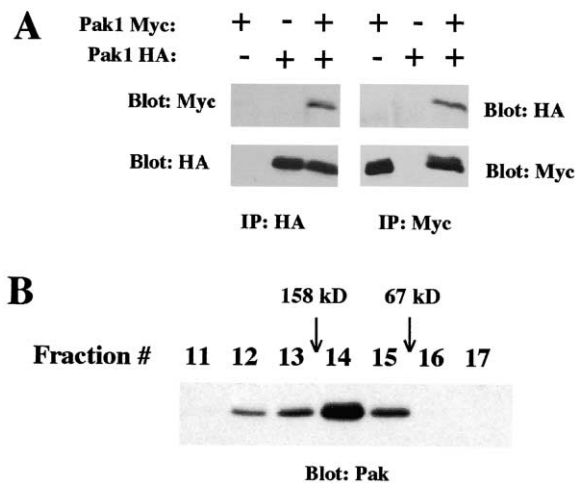


Figure 2. Pak1 Self-Association In Vivo

(A) Coimmunoprecipitation. 293T cells were transfected with plasmids encoding Pak1-HA, Pak1-Myc, or both. Lysates were immunoprecipitated with polyclonal anti-HA or anti-Myc antibodies and immunoblotted with monoclonal anti-HA and anti-Myc antibodies as indicated.

(B) Gel filtration. Untransfected 293T cell lysates were subjected to Superdex 200 column chromatography, and the fractions were analyzed by anti-Pak Western blot. Elution positions of 158 and 67 kDa markers are indicated.

we conclude that a large fraction, if not all, of the overexpressed Pak1 is in dimerized form in unstimulated cells.

Structural Determinants for Dimerization: Pak1 Dimers Are Autoinhibited in *trans*

In order to identify the structural determinants necessary for dimer formation in vivo, we introduced a series of mutations rationally designed from the crystal structure (Lei et al., 2000) and summarized in Figures 1C and 1D. We first considered the dimerization interface (Di) segment, which bridges two Pak1 molecules through seven hydrogen bonds and through salt bridges between Glu 82 on one strand and His 86 on the other. With the aim of disrupting the polar interactions, His 83 and 86 were substituted with Glu (H83/86E). In addition, we introduced a proline at position 85 (I85P) to interrupt the continuity of the β strands (this mutation also mimics the β bulge formed upon binding of Cdc42-GTP) (Morreale et al., 2000). Finally, the entire Di segment from residues 81-87 was substituted with the linker Ala-Ser-Ala-Ala-Ser-Ala-Ala (link81-87) or completely deleted (del81-87).

The three mutants H83/86E, I85P, and link81-87 dimerized as well as the wt molecule (Figure 3A). The del81-87 mutation could not easily be tested for dimerization in the context of the wt kinase domain because the resulting protein was poorly expressed, a behavior that was consistently observed with constitutively activated Pak1 mutants. Expression of the K299R kinase-dead (KD) version (del81-87 KD) was normal, however. The dimerization capacity of Pak1 del81-87 KD was strongly reduced compared to Pak1 KD, but it was still clearly detectable (Figure 3B). These results indicate that while the Di segment may contribute to stabilization

of dimers, it cannot be the major determinant for dimerization of full-length Pak1 in vivo.

Because residues 150 to 250 of Pak1 were not present in the protein used for crystallization, it was impossible to determine from the X-ray structure whether the N-terminal regulatory domain of one Pak1 molecule associates with and inhibits its own kinase domain or that of the dimer partner—that is, whether inhibition is in *cis* or in *trans*. Contacts between the IS domain and the large lobe of the kinase domain would form a stable dimer interface (buried surface 1300 Å²) and the isolated regulatory and catalytic domains indeed associate strongly in vitro (Lei et al., 2000; Zenke et al., 1999). A mutation in the IS domain, L107F, is known to prevent the interaction between N-terminal regulatory portion and catalytic domain, leading to kinase activation (Brown et al., 1996; Zenke et al., 1999). We tested the L107F mutant in a kinase-inactive background (L107F KD) to bypass the problem of low expression or downregulation associated with Pak1 activation and found that the L107F mutation completely eliminated dimerization (Figure 3B). This result strongly suggested a *trans*-interaction between the IS domain and the large lobe of the kinase, but we could not rule out an indirect effect on the Di β ribbon through destabilization and unfolding of the IS domain.

In order to dissect the roles of IS and Di in dimerization, we used the crystal structure to design mutations that target the IS domain/kinase domain interface more specifically than L107F. E129 of I α 3 in the IS domain makes a salt bridge with R438 of α EF in the kinase domain (Lei et al., 2000). Mutation of the neighboring residue D126, whose side chain is only 3.8 Å from K439, weakly activates Pak1 (Zhao et al., 1998). We chose to perturb these ionic contacts by introducing the charge substitutions D126K/E129R (DE→KR) and R438E/K439D (RK→ED), individually or in combination, in a kinase-dead Pak1 background. We then tested the capacity of mutants carrying the charge substitutions to dimerize (Figure 3C); the expected effects of the mutations on dimerization in *cis* or in *trans* are depicted schematically in Figure 3D.

Both the DE→KR (lane 6) and RK→ED (lane 7) mutants displayed reduced dimerization, with the latter mutation having a stronger effect. These mutations are not expected to affect *cis* dimerization mediated only by the Di interface (Figure 3D), and we can conclude that the IS/kinase interaction must help drive dimerization.

Assuming that the *trans*-dimerization model is correct, we can make some predictions. First, cotransfection of the DE→KR and RK→ED mutants should lead to increased apparent dimerization. This result is expected because neither can efficiently homodimerize, but heterodimerization is possible because the repulsive charge interactions are compensated in *trans*. What is really measured by coimmunoprecipitation is the amount of heterotagged complexes (that is, two proteins bearing different epitope tags), while homodimers (two proteins bearing the same epitope tag) cannot be detected. Therefore, when homodimerization and heterodimerization are equally likely, then only ~50% of the dimers will be detectable heterotagged dimers. When homodimerization is less favorable than heterodimerization, however, a greater fraction will heterodimerize, and the apparent signal will increase up to 2-fold. As shown in

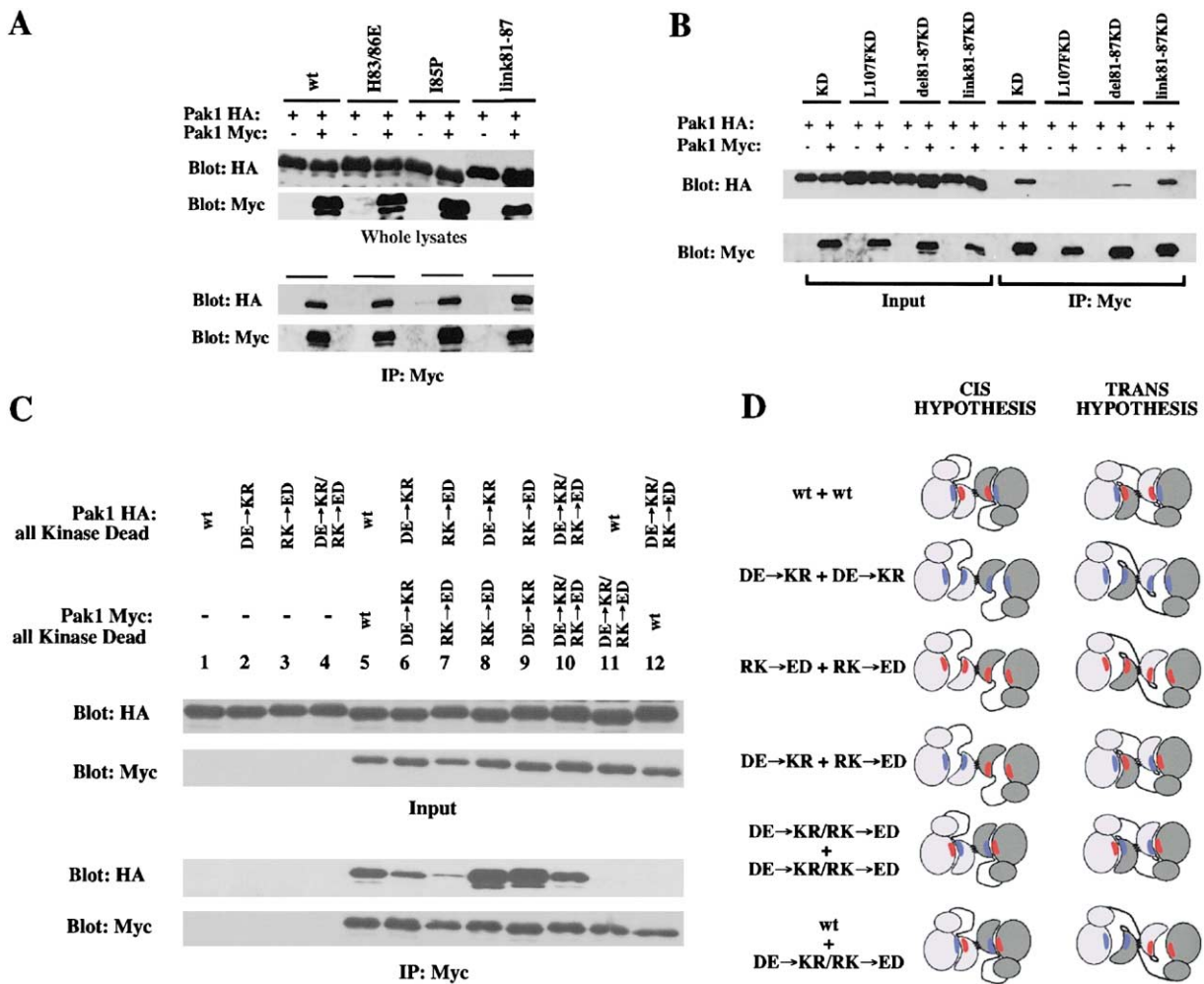


Figure 3. Pak1 Regions Involved in Dimerization In Vivo

(A) Di/CRIB mutants. 293T cells were transfected with plasmids expressing HA- or Myc-tagged Pak1 wt or various mutants and analyzed by immunoprecipitation and immunoblotting. The upper panel shows the expression levels of the various Pak1 forms in the whole lysates. (B) L107F and del81-87 mutants. All Pak constructs in this panel carry the K299R (KD) mutation that inactivates the kinase domain. The left part shows the input total lysates. (C) IS/large lobe ionic interaction mutants. The KD versions of DE→KR, RK→ED, and DE→KR/RK→ED mutants were used in various combinations as indicated. (D) The possible predicted interactions of the mutants used above are depicted according to the *cis*-inhibited or *trans*-inhibited dimer models. The blue shading represents the two basic residues (RK), and the red shading represents the two acidic residues (DE) at the IS/large lobe interface. The two zigzag lines represent the two interacting Di strands.

lanes 8 and 9, heterodimerization of the DE→KR and RK→ED mutants in fact increases to a level greater than that seen with wt Pak1 KD. In contrast, we expect the quadruple DE→KR/RK→ED mutant to form homodimers and heterodimers with equal efficiency, like wt Pak. As shown in lane 10, the extent of dimerization for this mutant was indeed essentially the same as that of wt Pak1 KD.

When nonmutated Pak1 KD was coexpressed with the quadruple DE→KR/RK→ED mutant (lanes 11 and 12), no heterodimers at all could be detected. We know that both wt Pak1 and DE→KR/RK→ED can form homodimers efficiently, while heterodimers are predicted to form inefficiently because of repulsive charge interactions. Therefore, in this situation the equilibrium would be shifted toward homodimers, which cannot be detected because they bear the same epitope tags. The

absence of heterodimerization between nonmutated Pak1 KD and DE→KR/RK→ED strongly argues against models of *cis* regulation, because in a *cis*-inhibited heterodimer there should be no charge repulsion and dimerization should be detected (Figure 3D).

These results demonstrate the crucial role of the IS/large lobe interface in dimerization and prove that the Di segment is not sufficient for stable dimerization of full-length Pak1. These results also suggest that Pak1 autoinhibition can occur in *trans* and that in our overexpression conditions, the majority, if not all, of Pak1 molecules adopt a *trans*-inhibited conformation.

GTP-Loaded Cdc42 and Rac1 Disrupt Dimerization
Cdc42 and Rac1 in their GTP-bound states are known to bind the GBD of Pak1, thereby relieving inhibition of its kinase activity (Knaus and Bokoch, 1998). The crystal

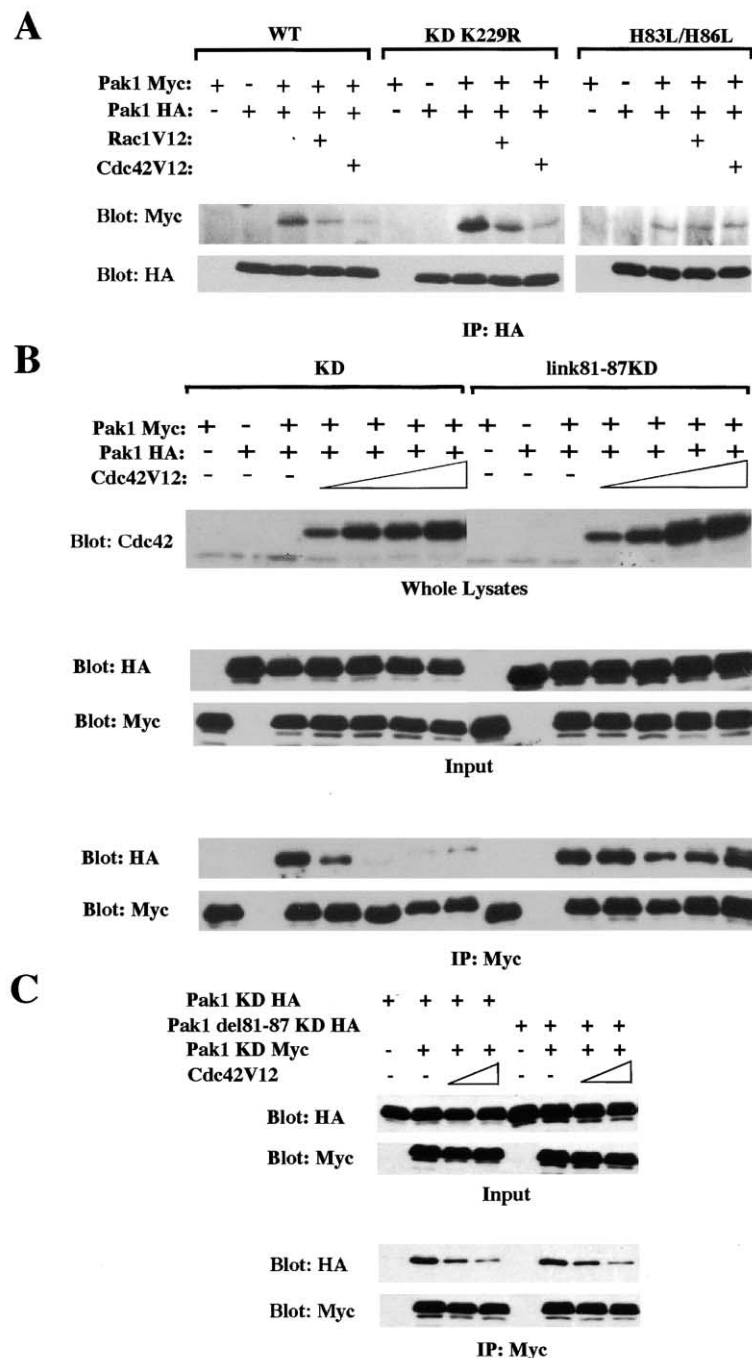


Figure 4. Effect of Cdc42 and Rac1 on Pak1 Dimerization

(A) Activated Cdc42 and Rac1 disrupt dimerization of wt and kinase-dead mutant Pak1. 293T cells were transfected with plasmids expressing HA- or Myc-tagged Pak1 wt, K229R (kinase-dead), or H83L/H86L (cannot bind Cdc42/Rac). Where indicated, plasmid encoding Flag-Cdc42V12 or Flag-Rac1V12 were cotransfected. Lysates were immunoprecipitated with anti-HA antibodies and immunoblotted as indicated.

(B) Active Cdc42 affects Pak1 KD dimers in a dose-dependent manner, while it does not affect the Di/CRIB mutant dimer. 293T cells were transfected with plasmids expressing HA- or Myc-tagged Pak1 KD or link81-87 KD mutant and increasing amounts of pCMV5-Cdc42V12 (0.1, 0.25, 0.5, or 1 μ g). Immunoblotting with polyclonal anti-Cdc42 was performed on whole lysates. Lysates were immunoprecipitated with anti-Myc antibodies and immunoblotted as indicated.

(C) Pak1 dimer dissociation by active Cdc42 does not require two Di segments. 293T cells were transfected as indicated with plasmids expressing HA- or Myc-tagged Pak1 KD or del81-87 KD mutant and two amounts of pCMV5-Cdc42V12 (0.3 and 0.6 μ g).

structure of Pak1 and the NMR structures of Cdc42 complexed with the IS region of N-WASP and Pak1 suggest that dimerization and binding of Cdc42 or Rac are mutually exclusive because Cdc42 binding involves the β strand at the dimerization interface and also partially unfolds the IS domain (Kim et al., 2000; Lei et al., 2000; Morreale et al., 2000) (Figure 1B). We therefore examined the effect of high levels of GTP-bound Cdc42 and Rac1 on the dimerization of Pak1 in vivo. The data in Figure 4 show that coexpression of either Cdc42 or Rac1 (locked into the active, GTP-bound form by mutation of Gly12 to Val) strongly decreases Pak dimerization. Dimers of the kinase-dead, K229R mutant are equally sensitive to

active Cdc42/Rac1 (Figure 4A), showing that disruption of the homodimer is not a consequence of inter- or intramolecular phosphorylation mediated by the catalytic domain. In contrast, overexpression of active Cdc42 or Rac1 does not disrupt dimerization of a mutant Pak1 (H83L/H86L) that cannot bind the small G proteins (Figure 4A). Disruption thus depends on the binding of GTP-Cdc42/Rac1 and is not merely an indirect consequence of high levels of active Cdc42/Rac1.

We used the KD mutant for a dose-response study because activation of wt Pak1 by Cdc42/Rac1 leads to downstream effects that could complicate interpretation, including Pak1 autophosphorylation and downreg-

ulation. Because the crucial sequence for GTPase binding, the 16-residue CRIB (Cdc42 Rac interactive binding) motif (Burbelo et al., 1995), encompasses the Di segment, we used our Di/CRIB mutant link81-87 KD as negative control after verifying by overlay assay that it could not bind active Cdc42 (data not shown). As shown in Figure 4B, disruption of Pak1 KD homodimers was roughly proportional to the amount of Cdc42V12 expressed in the cells, while no effect was observed on Pak1 link81-87 KD homodimers. These results, together with the structural considerations, further demonstrate that binding of Cdc42/Rac1-GTP leads to Pak1 dimer dissociation.

We also analyzed the effect of Cdc42V12 on the KD + del81-87 KD Pak1 heterodimer, which contains only one Di strand, in comparison with the KD Pak1 homodimer (Figure 4C). Deletion of one of the two Di segments in the dimer interface does not produce any detectable effect on the ability of Cdc42V12 to dissociate dimers, suggesting that one functional Cdc42 binding segment per dimer is sufficient for full Cdc42 dissociation activity. The same result was obtained with the KD/link81-87 KD heterodimer (data not shown). These experiments suggest that one Cdc42 molecule can lead to the dissociation (and presumably activation) of two Pak1 molecules.

Effect of β PIX on Pak1 Dimerization

The PIX/Cool family of putative guanine nucleotide exchangers is implicated in regulating Pak activity (Bagrodia et al., 1998; Daniels et al., 1999; Manser et al., 1998). These proteins interact via their SH3 domain with a proline-rich sequence within the flexible segment connecting the IS domain with the kinase domain (Manser et al., 1998), and they have been reported to dimerize as well via their leucine-zipper motif (Kim et al., 2001). We coexpressed full-length human β PIX with Pak1 KD and found that, although β PIX was abundant in the immunoprecipitates, the extent of Pak1 dimerization was unchanged (Figure 5). The introduction in Pak1 KD of a point mutation, R193A, that abolishes binding to β PIX (Manser et al., 1998) (Figure 5) had no effect on Pak1 dimerization, indicating that Pak1/Pak1 association is not driven by PIX/PIX association. These results suggest the formation of multiprotein complexes including dimerized Pak1 and β PIX molecules. This interpretation is consistent with the presence of a population of endogenous Pak1 molecules found in high molecular weight aggregates in the gel filtration experiment (Figure 2B).

Dimerization Does Not Facilitate *trans*-Activation

As with most protein kinases, increased Pak1 catalytic activity is associated with autophosphorylation of key residues, including one site (T423) in the so-called "activation loop." The proximity of two Pak1 molecules in the dimer might therefore facilitate activation by promoting efficient *trans*-phosphorylation as the dimer dissociates. To test this notion directly, we constructed a "long" version of Pak1 by adding six Myc tags to its N terminus. "Long Pak1" was easily distinguishable by size from "short Pak1" bearing a single HA tag. We isolated by coimmunoprecipitation with anti-Myc antibody long/short Pak1 heterodimers (Figure 6A) in which one of the

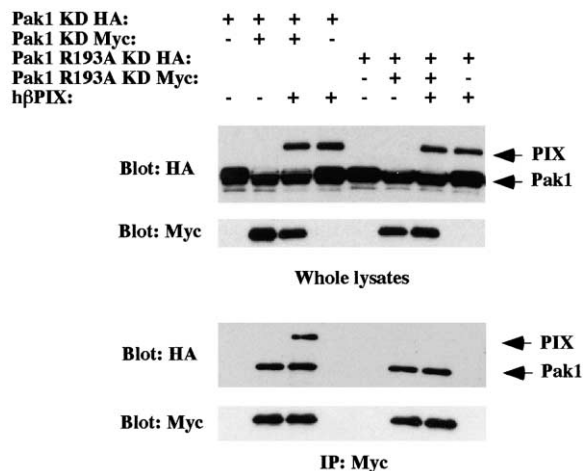


Figure 5. β PIX Stably Associates with Pak1 Dimers

293T cells were transfected with plasmids expressing HA- or Myc-tagged kinase-dead Pak1 or Pak1R193A (cannot bind PIX) and h β PIX-HA. Both HA-tagged Pak1 and PIX are detected by anti-HA antibody. Lysates were immunoprecipitated with anti-Myc antibodies and immunoblotted as indicated.

two catalytic domains was mutationally inactivated. We then added Cdc42V12-GTP γ S to activate the immunoprecipitated Pak molecules *in vitro* and measured the incorporation of phosphate from labeled ATP (Figure 6B), Pak gel mobility shift induced by phosphorylation (Figure 6C), and the phosphorylation of T423 by immunoblotting with an antibody that specifically recognizes T423-phosphorylated Pak1 (King et al., 2000) (Figure 6D).

Kinase-active short Pak1 could phosphorylate itself, but not its dimer partner, long Pak1 KD, even though the latter was present in slight excess (Figures 6B and 6C, lane 1). The short Pak1 autophosphorylated *in cis* on serines but not on T423, as the Pak band shifted completely but could not be detected at all by the phosphospecific antibody (Figure 6D, lane 1). In the complementary treatment, long Pak1 wt phosphorylated itself efficiently but only very weakly phosphorylated its short Pak1 KD partner (Figures 6B and 6C, lane 3). Unlike the short version, long Pak1 autophosphorylated on T423 (Figure 6D, lane 3). When both Pak1 dimer partners were wt (lane 4), a small fraction of short wt molecules displayed a further shift (compare lanes 1 and 4 in Figure 6C) that reflected a partial phosphorylation of T423 (compare lanes 1 and 4 in Figure 6D). These results can be explained assuming that T423 phosphorylation occurs in *trans* among activated monomers. The long Pak1 is immobilized on the beads through its epitope tag even after dissociation of dimers, inducing a very high local concentration that probably promotes *trans*-phosphorylation. In contrast, the short Pak1 is free to diffuse from the bead after dissociation by Cdc42 and phosphate is therefore transferred only *in cis*.

Although these data suggest that there is a contribution in *trans* in Pak1 autophosphorylation, particularly for the crucial T423 residue, they argue against the hypothesis that intradimer *trans*-phosphorylation occurs as dimers are being disrupted by Cdc42—that is, against the notion that dimerization facilitates *trans*-phosphory-

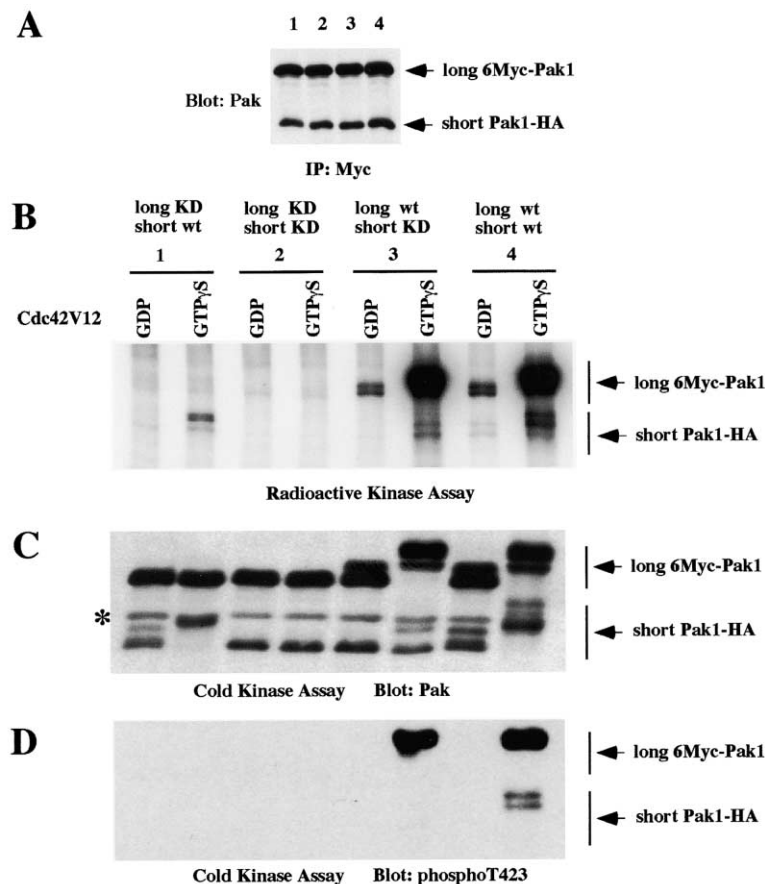


Figure 6. In Vitro Phosphorylation of Dimerized Pak1 upon Cdc42 Stimulation

(A) Long/short Pak1 dimers. 293T cells were transfected with various combinations of long Pak1 bearing six tandem Myc tags or short singly HA-tagged Pak1 in either kinase-active (wt) or kinase-dead (KD) forms: long KD and short wt (1), long KD and short KD (2), long wt and short KD (3), long wt and short wt (4). Lysates were immunoprecipitated with anti-Myc antibodies and immunoblotted with polyclonal anti-Pak antibodies.

(B) Radioactive kinase assay. Aliquots of immunoprecipitates were incubated with either Cdc42V12-GDP or Cdc42V12-GTP γ S in the presence of [γ - ^{32} P]-ATP, and incorporation of radioactive phosphate was directly visualized by autoradiography.

(C) Gel mobility shift. Aliquots of immunoprecipitates were incubated with either Cdc42V12-GDP or Cdc42V12-GTP γ S in the presence of cold ATP, and gel mobility shift induced by phosphorylation was detected by anti-Pak Western blot. The band indicated with the asterisk is a contaminant protein present in the Cdc42 preparation and nonspecifically recognized by anti-Pak antibodies.

(D) T423 phosphorylation. Kinase assays were performed with unlabeled ATP as above, and phosphorylation of T423 was followed by immunoblotting with antibodies specific for T423-phosphorylated Pak.

lation. If this were the case, we would expect essentially all the long KD molecules in lane 1 and the short KD molecules in lane 3 to be phosphorylated to some extent because they were engaged in dimerization with wt molecules. The *trans*-phosphorylation of a small fraction of short molecules observed in lanes 3 and 4 is explained by occasional phosphorylation in *trans* among already dissociated monomers. Thus, these results are consistent with a model in which Pak1 autophosphorylation occurs either intramolecularly in *cis* or in *trans* among activated monomers. Moreover, our data suggest that T423 is accessible for phosphorylation only in *trans* (extradimer) as previously proposed by another group (King et al., 2000).

Discussion

Pak1 Dimerization In Vivo

Purified Pak1 kinase is a dimer in crystals, as shown by the X-ray structure, and also in solution, as shown by a combination of biophysical techniques (analytical ultracentrifugation, gel filtration, and light scattering) (Lei et al., 2000; Buchwald et al., 2001). The results reported here provide several lines of evidence that Pak1, in its basal inactive state, is also a dimer in vivo. (1) A large fraction of overexpressed full-length Pak1 is coassociated in mammalian cells. (2) Gel filtration shows that the hydrodynamic properties of intracellular Pak molecules, endogenous and overexpressed, are consistent with a

dimer. (3) Targeted point mutations in Pak1 are sufficient to weaken or eliminate coassociation. (4) The activators Cdc42-GTP and Rac1-GTP disrupt coassociation.

The sensitivity of intracellular dimerization of Pak1 to directed mutation in the protein (Figure 3) shows that the *in vivo* dimer depends on a stable interface between the IS domain of one partner and the large lobe of the kinase domain of the other. By contrast, the antiparallel β strand dimerization interface (Di) seen in the crystal structure is not essential for dimerization in vivo because mutations designed to perturb or delete it do not completely eliminate the observed self-association. Three observations suggest, however, that the Di interface does participate in the Pak/Pak interaction. (1) Complete deletion of the Di segment reduces dimerization in vivo. (2) In the recombinant protein used for crystallization, Di is the only possible dimerization interface, because the Di/IS and kinase regions are on distinct polypeptide chains and the four chains indeed associate in solution. (3) Heterodimerization is not completely abolished in the DE $\rightarrow$ KR and RK $\rightarrow$ ED mutants, despite strong predicted electrostatic repulsion between the IS and catalytic domains. The Di segment may contribute to Pak1 dimerization directly by providing its seven main chain intermolecular hydrogen bonds and/or indirectly by stabilizing the folding of the IS domain. It is possible that the antiparallel β ribbon persists in some of the mutants, with altered side chain conformations or altered packing against the IS domain.

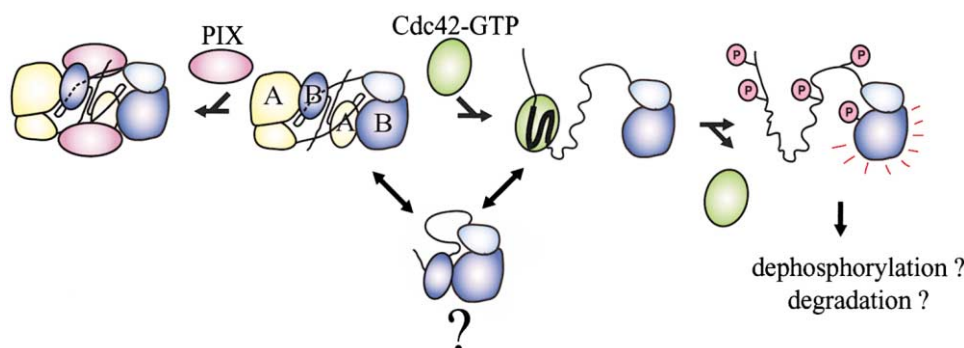


Figure 7. Model for Ligand-Regulated Intermolecular Autoinhibition of Pak1 Dimers

(Center) Pak1 kinase in the basal-inhibited state is a dimer in which the kinase domain of one molecule (A) is inhibited by the regulatory domain of the other molecule (B), and vice versa.

(Right) Binding of activated GTPases, such as Cdc42-GTP, breaks the dimer and removes the *trans*-inhibition. Upon dimer dissociation, Pak1 autophosphorylates in *cis*; dissociated monomers can also be phosphorylated in *trans* (Figure 6). The modified residues include the T423 in the catalytic site and several serine residues in the regulatory portion (Chong et al., 2001; Manser et al., 1997). When the activator dissociates, it leaves a fully autophosphorylated and activated Pak1. Phosphorylation on serine in the regulatory portion, such as on S144, may block IS domain/kinase domain reassociation (Chong et al., 2001). Activated Pak1 is targeted for dephosphorylation or degradation.

(Left) βPIX can stably bind to a Pak1 dimer, forming a four-protein complex, which may bind other proteins as well.

(Lower part) Our data cannot completely rule out the presence of a small fraction of Pak1 monomer in a *cis*-inhibited conformation.

Because the IS domain/kinase domain interaction is essential for dimerization and the Di segment is not, we can conclude that the IS domain of one Pak1 molecule in the dimer must interact with the kinase domain of the other. Therefore, autoinhibition of Pak1 is indeed a *trans*-inhibition: the kinase inhibitory segment locking the catalytic site of one Pak1 molecule in the inactive conformation originates from the second Pak1 molecule. We note that, among the Di mutants we made, the del81-87 is the only one with decreased dimerization and is also the only one that is activated (data not shown). This correlation of activation and loss of association suggests that *trans*-dimerization is indeed important for inhibition. While our data clearly show the prevalence of a *trans*-inhibited conformation in vivo, we cannot completely rule out the presence of a small fraction of Pak1 in a *cis*-inhibited, monomeric state (Figure 7). The linker segment between the inhibitory and catalytic domains is about 100 residues long, and therefore it may permit both *cis*- and *trans*-inhibited conformations. Dimerization will also depend on local concentration. Gel filtration analysis on endogenous Pak suggests that a large percentage of Pak1 is dimerized even at its physiological concentration (Figure 2B).

Cdc42 and Rac1 Dissociate and Activate Pak1 Dimers

High levels of active Cdc42 or Rac1 inhibit Pak1 dimerization in vivo. We propose a molecular mechanism for how Cdc42 and Rac1 dissociate and activate Pak1 dimers (termed here for clarity molecule A and molecule B) (Figure 7), based on the results described in this paper.

The X-ray crystal structure shows that the Pak1 homodimer is asymmetric. One of the two GBDs in the Pak1 homodimer is more accessible to Cdc42/Rac than the other. In solution, it is likely that the dimer flips back and forth between the two alternative, asymmetric states, but with unknown kinetics. Consistent with the asymmetry of the dimer, we find that only one Di seg-

ment is sufficient for Cdc42 to break Pak dimers. Part of the polypeptide N-terminal to Di is disordered in the Pak1 dimer but ordered when bound on the surface of Cdc42. This element could serve as an entry point for G protein invasion, followed by disruption of the dimer interaction through dissociation of the β ribbon and unfolding of the IS domain. The on rate for binding of GTP-Cdc42 or Rac to the Pak GBD is relatively slow, consistent with a requirement for conformational change in the IS region (Buchwald et al., 2001; Thompson et al., 1998). In the model shown in Figure 7, disruption and partial refolding of the IS domain (A) leads to the loss of the IS (A)/kinase domain (B) intermolecular interaction, displacement of the kinase inhibitor segment (Ki) of (A) from the catalytic site (B), and activation of the partner Pak1 molecule (B). Cdc42/Rac1 also competes directly for the Di interface. Sequestration by Cdc42/Rac1 of one Di segment (A) thus further weakens dimer contacts and destabilizes IS domain (B), leading to the activation of the second kinase domain (A). Alternatively, assuming that the initially activated molecule (B) could autophosphorylate intramolecularly within the dimer, phosphorylation of the IS (B) domain could weaken the IS (B)/kinase domain (A) interface activating the second molecule (A). Although a recent report (Buchwald et al., 2001) suggested that Cdc42-induced phosphorylation may be necessary to dissociate the regulatory and catalytic domains in vitro, our results with full-length kinase-dead Pak1 clearly show that phosphorylation is not strictly required to dissociate dimers in vivo (Figure 4). We support the view that the crucial factor for the Cdc42/Rac1-regulated, intermolecular inhibition of Pak1 is the structural plasticity of the IS domain.

Why Dimerization?

The observation that Pak1 is a *trans*-inhibited homodimer in vivo compels us to consider the question of the biological role of dimerization. At first glance it would appear more straightforward for autoinhibition to occur

in *cis*, instead of requiring interaction with a second Pak molecule. In this regard, we note that for Pak1, Pak2, and Pak3, the IS domain sequence is highly conserved, suggesting these three Pak family members all dimerize. Recombinant Pak2 is also dimeric (A. Hoelz and J. Kuriyan, personal communication), and we find that Pak1 and Pak2 can form heterodimers regulated by Cdc42/Rac1 when overexpressed in 293T cells (data not shown). On the contrary, Pak4, Pak5, and Pak6, which lack most of the IS-domain sequence, would not be expected to dimerize, but this expectation remains to be proved experimentally.

We considered the possibility that the proximity of two molecules in the dimer might facilitate activation of Pak by *trans*-phosphorylation. Our *in vitro* data show, however, that efficient *trans*-phosphorylation between dimer partners does not occur upon activation (Figure 6), arguing against a positive role for dimerization in *trans*-activation. Immunoprecipitation from mammalian cells followed by *in vitro* kinase assay, although extensively used to characterize Pak activities, has technical limitations, which became particularly evident while analyzing phosphate transfer from the new perspective of Pak dimerization. The complexity of the Pak1 activation mechanism warrants more detailed analysis in the future.

While many other possible rationales for dimerization can be envisioned, only three will be discussed below. One possibility is that dimerization may increase the efficiency of inhibition. Pak1 is a member of the group of "intrinsically active" kinases, which can catalyze phosphorylation even in the absence of activators (Chen et al., 2000). Therefore its active site must be tightly regulated. Interactions between the inhibitory segment (KI) and the catalytic domain may be disrupted occasionally by "breathing" of the structure, allowing momentary release of the active-site blockade. In this context, dimerization may serve to prevent phosphorylation of Pak on sites such as Ser 144 in the KI segment, which is involved in activation (Chong et al., 2001). In a *trans*-inhibited dimer, the KI segment of the transiently activated molecule may be protected by its association with the catalytic domain of the (inactive) dimer partner. Because we have shown that intradimer *trans*-phosphorylation does not occur, neither of the N-terminal regulatory regions in the dimer would be phosphorylated as a result of KI release. In a *cis*-inhibited monomer, however, the N terminus would be freely accessible for phosphorylation by the catalytic domain, and thus stable activation could occur spontaneously.

For the receptor class of tyrosine kinases, it is well known that ligand-induced dimerization leads to *trans*-phosphorylation and *trans*-activation. Our data demonstrate that Pak1 is regulated by an almost diametrically opposed mechanism: dimerization allows *trans*-inhibition, while ligand-induced dimer dissociation leads to activation. Autoinhibition in *trans* may be a robust mechanism for repression of intrinsically active kinases, while activation in *trans* may be more appropriate for intrinsically inactive kinases.

A second possible function for dimerization is to double the response to a single activated GTPase. We have shown that a single GTP-bound Cdc42 or Rac will dissociate autoinhibited Pak1, producing two active kinases. Finally, it is possible that dimerization allows Pak to be

a more efficient nucleator of signaling complexes. Pak binds a variety of molecules, each of which has other partners. For example, Nck binds tyrosine phosphorylated proteins and other SH3-binding proteins such as Abl, Cbl, and N-WASP (McCarty, 1998), while PIX binds focal adhesion proteins such as PKL and paxillin (Turner et al., 1999). A dimer of Pak would therefore contain multiple interaction sites, increasing the avidity of interactions and the potential complexity of signaling capacity.

Experimental Procedures

DNA Constructs

The wt and mutant full-length Pak1 genes were cloned in the pEBB expression vector (Tanaka et al., 1995) with a C-terminal HA tag or Myc tag (Lu et al., 1997). The various Pak1 mutants were constructed by standard DNA recombinant techniques and mutagenic PCR. We found that all our Pak1 constructs, as well as Pak1 plasmids obtained from other laboratories, contain at codon 516 ATA (Ile) instead of CTA (Leu) as posted in the GenBank (U24152 and U51120). To bypass the toxicity in *E. coli* of plasmids expressing active Pak1, we used the strain JM109, grew plates and liquid cultures at 30°C, amplified DNA in small volume liquid cultures, and always verified sequences.

The N-terminal 6Myc-tagged long Pak1 wt and KD constructs were prepared by ligating into the BamHI and NotI sites of pCS2MBN (Gupta and Mayer, 1998) the BamHI-Asp718 Pak1 inserts together with an Asp718-NotI linker containing STOP codons. The pCS2MBN-Pak1 wt and KD plasmids express 6Myc-Pak1 proteins with an extra 4-amino-acid C-terminal tail (RSGT). To express the C-terminally HA-tagged full-length human β PIX, the gene was amplified by PCR and then cloned into the BamHI and Asp718 sites of pEBB-HA (C-ter). Rac1V12 and Cdc42V12 in pCMV5, Flag-tagged at the N terminus, were provided by Ajay Rana.

Cell Cultures and Immunoprecipitations

Human kidney 293T cells were transiently transfected in 10 cm dishes by the calcium phosphate method (Tanaka et al., 1995) with Pak1-HA and Pak1-Myc plasmids (1–3 μ g each). Two days after transfection the cells were lysed in 1 ml KLB buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM β -glycerophosphate, and 10% glycerol), supplemented with 1 mM PMSF, 1% aprotinin solution (Sigma A-6279), and 1 mM DTT.

For immunoprecipitation, 50–200 μ l of transfected cell lysates (final volume kept constant at 200 μ l by adding nontransfected cell lysates) were precleared by 1 hr incubation with 20 μ l protein G-coupled beads (Pierce) and then spun in a microfuge for 10 min. The supernatant was incubated with rabbit polyclonal antibodies anti-Myc or anti-HA (Santa Cruz, 1 μ g affinity-purified) for 1–2 hr and then for a further 1–2 hr after addition of 20 μ l protein G-coupled beads. The immobilized immunocomplexes were washed three times with 1 ml KLB buffer, boiled in gel loading buffer, electrophoresed on SDS-PAGE gels, transferred to PVDF membranes, probed with monoclonal anti-HA or anti-Myc antibodies (Babco) and horseradish peroxidase-conjugated secondary antibodies (Pierce), and finally detected by the ECL chemiluminescence method (Amersham). Membranes were always probed first for the heterotagged binding partner, then stripped and reprobed for the protein specifically immunoprecipitated to verify normalization.

To estimate the percentage of Pak in dimer form, we first verified that the Pak1-HA and Pak1-Myc proteins were expressed at roughly equal levels in doubly transfected cells. We did this by determining the absolute amount of Pak corresponding to a particular anti-HA or anti-Myc signal by immunoblotting lysates of cells expressing only HA-tagged or Myc-tagged Pak with antibodies to Pak, Myc, and HA. The fraction of each Pak species immunoprecipitated was measured in multiple experiments (such as the one shown in Figure 3B) by scanning the blots and quantitating using the NIH Image software. Assuming that Pak can form nondetectable homotag dimers (i.e., Myc/Myc and HA/HA) as efficiently as heterotag dimers

(Myc/HA), then the maximum ratio of Pak-HA:Pak-Myc in a Myc immunoprecipitate (or of Pak-Myc:Pak-HA in an HA immunoprecipitate) would be 1:2 if 100% of Pak molecules were dimerized. For example, if 10% of total Myc-tagged Pak is immunoprecipitated with anti-Myc, then detection of 5% of total HA-tagged Pak in anti-Myc immunoprecipitates would indicate that 100% of Pak was in dimer form.

Size Exclusion Chromatography and Kinase Assay

For gel filtration, 200 μ l of untransfected or Pak1 KD transfected cell lysates was passed through a Superdex 200 column equilibrated with 25 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 5 mM EDTA, with or without 1% Triton X-100. The column was calibrated with molecular weight markers in the same buffers. Aliquots of the collected fractions were analyzed by Western blot with polyclonal antibodies raised against Pak1 that could in principle crossreact with other Pak family proteins (Lu et al., 1997). The Pak elution profiles were essentially identical for endogenous or overexpressed Pak and in the presence or absence of nonionic detergent.

For the kinase assay, Cdc42V12 was purified as a GST fusion from *E. coli* and cleaved from GST. Nucleotide exchange was performed before each assay by incubating 20 μ M Cdc42V12 with 200 μ M GDP or GTP γ S for 10 min at 30°C in 25 mM Tris [pH 7.4], 100 mM NaCl, 5 mM EDTA, and 1 mM DTT. After stopping the exchange reaction by adding MgCl₂ to 10 mM, the GDP- or GTP γ S-loaded Cdc42V12 was mixed with ATP (for labeling experiments with [γ -³²P]-ATP). The immunoprecipitated Pak1 kinase was washed twice in 1 ml 50 mM Hepes (pH 7.4) and 10 mM MgCl₂. 10 μ l of the Cdc42V12/ATP mixture was added to 10 μ l of the immunoprecipitated Pak1 kinase and incubated for 30 min at 30°C. The final concentrations were 10 μ M GDP- or GTP γ S-loaded Cdc42V12, 30 μ M ATP; in labeling experiments the specific activity was $\sim$ 33 μ Ci/nmol, $\sim$ 20 μ Ci/reaction. The reaction was stopped by adding 5 $\times$ gel loading buffer. Products were resolved by SDS-PAGE and visualized by Western blots with either anti-Pak1 polyclonal antibodies (Lu et al., 1997) or phospho-specific polyclonal antibodies that recognize Pak1 only when phosphorylated at T423 in the activation loop (King et al., 2000).

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