Identification of a Central Phosphorylation Site in p21-activated Kinase Regulating Autoinhibition and Kinase Activity*

(Received for publication, June 3, 1999, and in revised form, August 18, 1999)

Frank T. Zenke‡, Charles C. King§, Benjamin P. Bohl, and Gary M. Bokoch¶

From the Departments of Immunology and Cell Biology, The Scripps Research Institute, La Jolla, California 92037

p21-activated kinases (Pak)/Ste20 kinases are regulated in vitro and in vivo by the small GTP-binding proteins Rac and Cdc42 and lipids, such as sphingosine, which stimulate autophosphorylation and phosphorylation of exogenous substrates. The mechanism of Pak activation by these agents remains unclear. We investigated Pak kinase activation in more detail to gain insight into the interplay between the GTPase/sphingosine binding, an intramolecular inhibitory interaction, and autophosphorylation. We present biochemical evidence that an autoinhibitory domain (ID) contained within amino acid residues 67–150 of Pak1 interacts with the carboxyl-terminal kinase domain and that this interaction is regulated in a GTPase-dependent fashion. Cdc42- and sphingosine-stimulated Pak1 activity can be inhibited in trans by recombinant ID peptide, indicating similarities in their mode of activation. However, Pak1, which was autophosphorylated in response to either GTPase or sphingosine, is highly active and is insensitive to inhibition by the ID peptide. We identified phospho-acceptor site threonine 423 in the kinase activation loop as a critical determinant for the sensitivity to autoinhibition and enzymatic activity. Phosphorylation studies suggested that the stimulatory effect of both GTPase and sphingosine results in exposure of the activation loop, making it accessible for intermolecular phosphorylation.

Localized regulation of protein kinase activity is an essential means to ensure spatial and temporal control of signaling events in a cellular environment. Hormonal or other stimuli are usually necessary to switch a kinase into a catalytically competent state, allowing phosphorylation of substrates to take place. An emerging regulatory theme is that inhibitory mechanisms exist to keep protein kinases in an inactive state (1, 2), and that relief of such inhibition allows activation to occur. Kinases often act autocatalytically to phosphorylate key amino acid residues that relieve autoinhibition and enhance catalytic efficiency. Alternatively, exogenous kinases may also serve this role. However, activation must be reversed in the absence of the stimulus, and dephosphorylation by protein phosphatases is thought to mediate switching the active kinase back to an inactive or basally activated state.

* This work was supported in part by National Institutes of Health Grants GM 39434 and AG15430 (to G. M. B.). This is publication 12404-IMM from the Scripps Research Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by an EMBO postdoctoral fellowship.
§ Recipient of a postdoctoral fellowship from the National Arthritis Foundation.
¶ To whom correspondence should be addressed: Immunobiology-IMM14, Scripps Research Inst., 10550 N. Torrey Pines Rd., La Jolla, CA 92037.

---

p21-activated kinases (Paks) are a growing family of serine/threonine kinases involved in the control of various cellular processes, including the cell cycle, dynamics of the cytoskeleton, apoptosis, and transcription (3). Pak kinase activity is regulated by members of the Rho family of GTPases, specifically Cdc42 and Rac. These GTPases bind to Pak kinase solely in their active forms, i.e. the GTP-bound state, resulting in stimulation of the kinase activity both in vitro and in vivo. The molecular details of how the GTPases exert their effect on the kinase to induce its activation remain unclear, however. Several lines of evidence suggested that the amino-terminal non-kinase region of Pak, in which the Cdc42/Rac-binding site is located, is crucial for the regulation of kinase activity. It has been shown by several groups that removal of the NH2-terminal portion of Pak by protease digestion leads to activation of the kinase fragment (4, 5). A physiologically relevant example is known for the 62-kDa isoform Pak2, which has been shown to be cleaved and activated by the cysteine protease caspase-3 in response to apoptosis-inducing stimuli (6–8).

Zhao et al. (9) have recently used mutational analysis to characterize a region in the NH2-terminal regulatory domain of Pak adjacent to the p21-binding domain that is important for the inhibition of kinase activity. Using a plasmid injection approach, they showed that cellular effects that depend on Pak kinase activity, including dissolution of actin stress fibers and focal adhesions, can be blocked by coexpression of the autoinhibitory domain. Similar conclusions were reached using a genetic analysis of Schizosaccharomyces pombe Pak1 (10). We undertook a biochemical approach to characterize an interaction between the Pak1 kinase domain and the regulatory amino terminus. We localized the interacting site in the NH2 terminus to the same area as the autoinhibitory domain (ID) characterized by Zhao et al. (9). Our data suggest that the interaction observed is responsible for maintaining the kinase in an inactive state. Cdc42 was able to disrupt the interaction, but only in the GTP-bound active form. Using characterized mutations in Pak1 that abolish GTPase binding or inactivate the inhibitory function, we could show that the p21-binding and autoinhibitory domains are separable but overlapping. Recently, we have demonstrated that sphingosine is an activator of human Pak1 in vitro and in vivo (11), although the activation by the lipid in vivo is controversial (12). We observed that, at non-saturating concentrations, sphingosine-induced Pak1 activation was also sensitive to the autoinhibitory peptide, suggesting that the lipid mediates activation of Pak1 via a similar mechanism, i.e. relief of autoinhibition by the NH2-terminal ID domain. We demonstrate that one major phosphorylation event on threonine 423 within the COOH-terminal kinase
domain renders the kinase activity independent of the autoinhibitory module and simultaneously increases its specific activity. Both Cdc42 and sphingosine act on Pak1 kinase to expose threonine 423, which in turn becomes accessible for cross-phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture medium, fetal bovine serum and supplements were from Life Technologies, Inc. (γ-32P-ATP (specific activity 4500 Ci/mmol) was from ICN, Costa Mesa CA. Plasmids for transfection were purified using the Qiafilter purification system of Qiagen, Chatsworth CA. The T7-tag monoclonal antibody was purchased from Novagen, Madison WI. Thrombin and sphingosine were purchased from Sigma; GTP-γ-S and [35S]GTPγS were from NEN Life Science Products. For polymerase chain reactions, the Expand High Fidelity PCR system from Roche Molecular Biochemicals was used. The PKCβ1 antibody (originally raised against a subdomain VIII phosphopeptide of the protein kinase C isoform β1, kindly provided by Eric Dutil and Alexandra Newton, University of California, San Diego, CA) was used to detect the threonine 423 phosphorylated subdomain VIII activation loop of Pak1.

**Plasmid DNA and Constructions—**pGEX-KG/rpaki (233–544) was kindly provided by Melanie H. Cobb (University of Texas Southwestern Medical Center, Dallas, TX). pCMV6M is a myc tag-containing derivative of pCMV5 and has been described elsewhere (13). All Pak1 variants (human Pak1 wild type, Pak1-T423E, Pak1-T423A, Pak1-K299A) were inserted into pCMV6M and contain a myc epitope at the amino terminus for detection. The threonine to alanine exchange at position 423 in hPak1 was performed using oligonucleotides ([5′-CAGGACCAAGGACGCGCATTG-GTAAAGACC-3′] and the complementary oligonucleotide) and the Quikchange Site-directed mutagenesis kit of Stratagene, La Jolla, CA.

The NH2-terminal fragments of hpak1 (amino acid 1–234: hpak1-(1–234), aa 67–150: hpak1-(67–150) and mutated derivative H83L,H86L) were subcloned in pET28a (Novagen, Madison). The wild type and H83L,H86L mutation of hpak1-(1–234) were amplified by PCR from the corresponding pCMV6M/hPak1 derivatives using primers pT637 and OP1/254–3′ (5′-CCG GAA TTC TTA AGC ATC TGG TGG AGT GGT GTC GAC TTA AGC TGA CTT ATC TGT GTG TTC-3′), cut with BamHI and EcoRI, and inserted into BamHI/EcoRI-cut pET28a. The p21-binding domain (PBD) wild type fragment (amino acid 66–170) was produced by PCR using primers OP1/67–5′ (5′-GGC GGA TCC AAG AAA GAG AAA GAG CGG-3′) and reverse primer 5′-TTA GAT TTT GTA TAC TGG TGG AGT GGT GTC GAC TTA AGC TGA CTT ATC TGT GTG TTC-3′, cut with BamHI and EcoRI, and inserted into BamHI/EcoRI-cut pET28a. The COOH-terminal kinase domains of human Pak1 (amino acids 233–544) was amplified by PCR (primers for hpak1-(233–544): 5′-GCC GCC TCC CCA GAT GCT TTG ACC CGG-3′ and reverse primer 5′-G CCG GCC TGG CTA AAG ACC CAC ACA ATC CTA GTC GTT GTT -3′) using primers for hpak1-(233–544) in vector pET28a were expressed in BL21/DE3 (pLysS). The recombinant kinase fragment (hpak1-(233–545)) was purified following the standard batch purification protocol under native conditions (Qiagen QIExpressionist, March 1997). The histidine-tagged fusion proteins, hpak1-(1–234) and hpak1-(1–67–150), were purified under denaturing conditions according to the batch purification method by Qiagen. After elution the proteins were dialyzed twice against 2 liters of buffer (50 mM Hepes/NaOH, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 5% glycerol) and stored frozen at −70 °C.

**Binding Assay—**Rat Pak1 kinase fragment (amino acids 233–544) fused to glutathione S-transferase (GST-rpaki-(233–544)) was purified and used for in vitro binding studies. The rat Pak1 COOH-terminal kinase domain does not differ in amino acid sequence from the human homologue (sequence of human Pak1 revealed that amino acid 503 in human Pak1 is glutamic acid, codon GAT, and not aspartic acid, codon GAC, as in the GenBank data base sequence). 1–5 μg of NH2-terminal protein fragments in binding buffer (50 mM Hepes/NaOH, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 1 mM DTT, 1% Nonidet P-40) in a volume of 200–500 μl. To immobilize the GST protein, glutathione-agarose beads equilibrated in binding buffer were added to the reaction and incubated for 30 min to 1 h at 4 °C under constant agitation. Binding reactions were washed four times with 1 ml of binding buffer each. Samples were boiled in SDS-sample buffer and separated on SDS-PAGE gels for immunoblotting or Coomassie staining.

In Cdc42 competition experiments, the binding reactions were mixed with binding buffer to remove unbound hpak1-(1–234) and Cdc42 was added to the complex for 15 min on ice or for 15 min at 30 °C as indicated in the figure legends. The bead fraction was washed again four times with binding buffer and prepared for SDS-PAGE.

**Transfections and Immunoprecipitations—**Cos-1 or HeLa cells were seeded on 10 cm cell culture dishes at 50–70% confluency and transfected using LipofectAMINE (Life Technologies, Inc.) as a transfection agent. 5 μg of plasmid DNA and 15 μl of LipofectAMINE were used per dish, and the transfection protocol was essentially followed according to the manufacturer’s guidelines (Life Technologies, Inc.). After 48 h the dishes were washed once with 1× Hank’s buffered saline solution (Life Technologies, Inc.) and lysed in 0.5 ml of lysis buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). The expression of proteins in the lysates was analyzed by immunoblotting, dissolved in 20 μl of Tris/His, pH 7.5, at 3–4 mM concentration. Before addition of sphingosine to the kinase reaction, the lipid solution was sonicated for 30–60 s.

**In Vitro Kinase Assays—**Kinase reactions with purified recombinant or immunoprecipitated Pak were performed in kinase buffer (50 mM Hepes/NaOH, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 0.2 mM DTT) in a volume of 60 μl with 250 μM ATP except for the experiment shown in Fig. 1D, where 50 μM ATP was used. Radiolabeled ATP was used at 10 μCi/reaction. The reactions were incubated for 30 min at 30 °C and stopped by addition of sample buffer. Myelin basic protein (MBP) was used as a substrate at 2–4 μg/reaction.

To prephosphorylate Pak prior to a kinase reaction, the protein G-bound hpak1 was incubated with 250 μM unlabeled ATP and an activator (0.5–2 μM of GTPγS-loaded Cdc42 or 0.4 μM sphingosine) for 30 min at 30 °C. The reactions were washed three times with kinase buffer and twice with kinase buffer and directly used in kinase assays as described above.

**RESULTS**

Interaction between the Pak1 Kinase Domain and a NH2-terminal Region—Pak kinases consist of a NH2-terminal regulatory domain and a COOH-terminal kinase domain having many of the conserved features of all known serine/threonine kinases. Several lines of evidence have suggested that the NH2-terminal region down-regulates the enzymatic activity of the COOH-terminal kinase. We tested whether we could detect...
a physical interaction between these domains that might be responsible for inhibiting Pak kinase activity.

As seen in Fig. 1A, the purified hpak1-(1–234) fragment efficiently complexed with the bead-coupled GST-rpak1-(233–544) COOH-terminal piece containing the entire kinase domain. Binding was specific to the GST-rpak1-(233–544) fusion since the amino terminus was not precipitated with GST alone. Incubating the preformed GST-rpak1-(233–544)/NH2 terminus complex with GTP-loaded Cdc42 reduced the amount of the hpak1-(1–234) in the pull-down fraction (Fig. 1A, lane 4). This reduction was specific for GTP-loaded Cdc42, as a GDP-loaded or a non-loaded Cdc42 was not able to compete (Fig. 1B). Addition of GTP-loaded Cdc42 decreased complex formation in a concentration-dependent manner (Fig. 1C). A hpak1-(1–234) fragment in which the Cdc42-binding site was mutated (H83L,H86L) bound as efficiently as the wild type fragment to the GST-rpak1-(233–544) fusion. As seen in Fig. 1A, the NH2 terminus was not precipitated with GST alone. However, in this case the GTP-loaded Cdc42 was not able to disrupt the interaction, indicating that binding of the GTPase to the NH2-terminal fragment is required for the observed competitive effect. In vitro kinase assays showed hpak1-(1–234) is capable to inhibit GST-rpak1-(233–544) kinase activity (Fig. 1D, lanes 3–6). With increasing amounts of the NH2 terminus, autophosphorylation and phosphorylation of myelin basic protein were decreased. However, hpak1-(1–234) itself as a good substrate for GST-rpak1-(233–544) (data not shown) and this, due to competitive substrate phosphorylation, made it difficult to determine the $K_i$ value.

Cdc42 binds Pak1 in a minimal region consisting of amino acids 75–89 (14). However, amino acids surrounding this sequence contribute to the efficiency of the interaction (9). We hypothesized, based on the Cdc42 competition experiments (Fig. 1), that the domain interacting with the kinase core might be in close vicinity or overlap with the Cdc42 binding site. To localize the carboxy-terminal interacting and autoinhibitory domain in the NH2 terminus, we expressed smaller regions of human Pak1 as recombinant His6/T7-tagged proteins in Escherichia coli and tested these in pull-down assays with GST-rpak1-(233–544) immobilized on glutathione-agarose. We detected a very efficient interaction with a His6/T7-tagged fusion of the wild type hpak1-(67–150) peptide (Fig. 2A, lane 4). The amino-terinal fragment did not bind to the GST protein (Fig. 2A, lane 3), demonstrating the specificity of this interaction. Other amino-terminal Pak fragments (amino acids 1–74 and 174–306) were not pulled down by the COOH-terminal fragment (data not shown). The region encompassing amino acids 67–150 contains the previously characterized GTPase/p21-binding domain and confirmed our hypothesis that the interaction site is in close proximity to the p21 binding domain. To further refine the interacting region, we constructed smaller peptides of hpak1 (aa 67–89, 67–108, and 109–150). However, we could not detect an interaction of these peptides with the Pak1 kinase domain using the pull-down assay (data not shown).

We employed already characterized mutations within hpak1-(67–150) to further analyze the function of this region. Mutation of both conserved histidine residues to leucine (H83L,H86L) has been shown to disrupt the interaction with small GTPases, accompanied by a moderate increase in kinase activity (13, 15). Mutating leucine 107 to phenylalanine, on the other hand, leads to strong activation of full-length Pak1, and activity is independent of GTPases (16), even though binding is maintained. We analyzed how these mutations influence the interaction with the GST-rpak1-(233–544) fusion. As seen in Fig. 2A (lanes 5–12), the H83L and H86L single and double mutants bound as efficiently to the kinase domain as did the wild type peptide. In contrast, the L107F mutation exhibited drastically reduced binding to the kinase domain.
The purified hpak1-(67–150) fragments had different abilities to inhibit GST-rpak1-(233–544) kinase (Fig. 2B). The wild type hpak1-(67–150) was most potent in inhibiting kinase activity, whereas the L107F mutated peptide was strongly reduced in its inhibitory potency, and the H83L/H86L double mutant was moderately reduced in inhibiting GST-rpak1-(233–544) kinase activity. The hpak1-(67–150) fragments, unlike hpak1-(1–234), were not phosphorylated substantially. We termed the hpak1-(67–150) region PBD/ID (p21 binding domain/inhibitory domain).

**Influence of hpak1-(67–150) Peptides on Cdc42-stimulated hPak1 Kinase Activity**—Using GST-rpak1-(233–544) kinase to evaluate the inhibitory potential of the PBD/ID region (aa 67–150) had the advantage that the isolated COOH terminus is constitutively active, independently of GTPases. However, we asked whether Cdc42-stimulated hPak1 activity could also be inhibited by the purified peptides. Since a functional p21 binding site is contained within the PBD/ID fragment, it was expected that inhibition of kinase activity could in part be due to sequestering of the GTPases. The ability of the PBD/ID fragments to bind activated Cdc42 was tested (Fig. 3). Only the wild type fragment and the L107F mutated form could bind Cdc42 in overlay assays, although the latter bound with somewhat reduced affinity (about 50% of wild type). Mutation of histidines 83 and 86 either separately or together led to a complete loss of binding to Cdc42. Any kinase inhibition by these Cdc42 binding-deficient fragments would therefore not be due to titration of GTPases.

We were able to show that not only the Cdc42-binding proficient, but also the binding-deficient versions were able to inhibit full-length hPak1 activity. As Fig. 4A shows, both autophosphorylation and substrate phosphorylation are reduced by hpak1-(67–150) peptides. The wild type and L107F mutant peptides strongly reduce kinase activity. The inhibition by the L107F peptide is most likely due to sequestration of the activated Cdc42. Of the GTPase binding-deficient versions, the H83L mutant peptide was most potent in its kinase-inhibitory effect. Mutation of position 86 significantly reduced the inhibitory effect to a similar extent as did the H83L/H86L double mutant. All peptides showed concentration-dependent inhibitory effects. We titrated the H83L peptide to determine the half-maximal inhibitory concentration using a constant amount of immunoprecipitated hpak1 stimulated with Cdc42 (Fig. 4B). The purified peptides were not significantly phosphorylated and therefore did not interfere with the $K_i$ determination. We calculated the apparent $K_i$ as 1.2 $\mu$M for MBP phosphorylation (Fig. 4C). Overall, the peptide inhibition data obtained with p21-activated Pak1 fit well with those using GST-rpak1-(233–544) (Fig. 2C). Taken together, these data demonstrate that the functional domains for p21 binding and autoinhibition of kinase activity overlap in part within the PBD/ID. Both activities can, however, be separated from each other, demonstrating that the PBD and the ID have distinct structural determinants for function.

**Effect of Autophosphorylation on Inhibition by the ID Domain**—Pak kinases autophosphorylate after stimulation with activated Cdc42 and remain activated after removal of the GTPase (17). In light of our above results, this could indicate that phosphorylation events antagonize the inhibitory effect of the ID domain by decreasing its interaction with the kinase domain. Full-length hPak1 was activated by autophosphorylation for 30 min in the presence of excess unlabeled ATP and Cdc42-GTP$\gamma$S; these were then removed by washing (Fig. 5A).

As shown in lanes 3–7, in a subsequent kinase reaction with labeled ATP the incorporation of label into hPak1 was drastically decreased due to an efficient incorporation of unlabeled ATP in the initial reaction. Autophosphorylated hPak1 was highly active toward substrate even without further addition of activated Cdc42. The hpak1-(67–150) wild type peptide and all the mutated versions (data not shown) did not affect substrate phosphorylation activity of the autophosphorylated Pak1 at levels sufficient to efficiently block Cdc42-stimulated hPak1. Addition of activated GTPase to prephosphorylated hPak1 did not restore the sensitivity toward ID-mediated in-

---

**Fig. 2**: Interaction of the GST-rpak1-(233–544) kinase domain with hpak1-(67–150) and inhibition of kinase activity. A, upper panel, the His/T7-tagged amino-terminal fragments hpak1-(67–150) (wild type [wt] and mutants indicated by amino acid change introduced and its position) were purified by denaturing chromatography on Ni-NTA. As a control, an eluate from control vector (pET28a) expressing E. coli was purified and used in binding experiments and kinase reactions (lane 1). Shown are the electrophoresed peptides (normalized for protein) used in the binding assay below. Lower panel, 5 $\mu$g of GST or 2.5 $\mu$g of GST-rpak1-(233–544) was incubated with 1.2 $\mu$g of the indicated hpak1-(67–150) proteins and glutathione-agarose beads for 1 h at 4°C and washed four times with binding buffer. Binding reactions were resolved on a 13% SDS-polyacrylamide gel. The Ponceau S-stained GST and GST-rpak1-(233–544) are shown in the upper panels. The hpak1-(67–150) and mutated fragments recovered in the bead fraction were detected with the T7 tag antibody. B, a kinase assay was performed using GST-rpak1-(233–544) (0.5 $\mu$g/reaction) in the presence of the indicated hpak1-(67–150) peptides. The purified peptides were used at 1 and 2 $\mu$M concentration, respectively, in the kinase assay. The phosphorylated GST-rpak1-(233–544) and MBP are labeled on the autoradiograph.

**Fig. 3**: Binding of activated Cdc42 to hpak1-(67–150) and its mutated derivatives. The His/T7-tagged amino-terminal peptides (aa 67–150) were normalized and separated by SDS-PAGE on a 13% polyacrylamide gel. The upper panel shows the Ponceau S-stained hpak1 peptides blotted onto the membrane. The blotted proteins were subjected to a GTPase-overlay assay as has been described (20). The immobilized proteins were incubated with $[^{35}$S]GTP$\gamma$S-labeled Cdc42 and the bound GTPases were visualized by autoradiography (lower panel).
2. Cdc42-mediated activation was completely insensitive to the wild type hpak1-(67–150) peptide (Fig. 7). The concentration (400 μM) used in the assay resulted in a highly activated hPak1 in which auto- and substrate phosphorylation was comparable to the Cdc42-stimulated enzyme (Fig. 7A). Under these conditions, sphingosine-mediated activation was completely insensitive to the wild type PB DI fragment. However, we observed that activation of hPak1 with non-saturating concentrations (50 μM) of the lipid was reduced by the wild type hpak1-(67–150) peptide (Fig. 7B).

3. Prephosphorylation of hPak1 in the presence of sphingosine led (as shown for activation by Cdc42) to an increased activity in the absence of the primary stimulus (Fig. 7A, lane 9). Sphin-gosine-mediated autophosphorylation is therefore sufficient to maintain Pak1 activity. In this case, where lipid was washed out after the initial incubation, the autophosphorylated activated species could clearly be shown to be unresponsive to high concentrations of the purified PB DI fragment (Fig. 7A, lane 10).

4. Activator-induced Opening of the Kinase Results in Exposure and Subsequent Phosphorylation of Threonine 423—Autophosphorylation of hPak1 by sphingosine or GTPases results in increased kinase activity that is maintained even in the absence of the primary stimulus (Fig. 7A). This raised the question whether phosphorylation of hPak1 by another active Pak1 molecule (intermolecular phosphorylation) could be sufficient for activation in the absence of stimulators (GTPase, lipids).
A Central Phosphorylation Site in Pak

FIG. 5. Prephosphorylated hPak1 and hPak1-(T423E) are not inhibited by the PBD/ID peptides. Upper panel, immunoprecipitated wild type hPak1 was prephosphorylated in the presence of GTPγS-loaded Cdc42 (lanes 3 and 7) and used in kinase reactions in comparison to untreated hPak1 (lanes 1 and 2). The amount of immunoprecipitated hPak1 was kept constant in all reactions. 1.5 μg of Cdc42 was added to the kinase (lane 2) and prephosphorylation reactions (lanes 3–7). Control Ni-NTA eluate (buffer control, co) or wild type hPak1-(67–150) (2 and 4 μM, ut) was added to the reactions. hPak1 and MBP signals are indicated. Lower panel, a constant amount of immunoprecipitated hPak1-(T423E) was used per kinase reaction. Control Ni-NTA eluate and wild type hPak1-(67–150) peptide (2 and 4 μM) were added as indicated.

We used recombinant His$_6$/T7-tagged hpak1-(232–545) kinase to phosphorylate wild type human Pak1. The hpak1-(232–545) kinase fragment phosphorylated the full-length form of hPak1 overexpressed in Cos-1 cells (Fig. 8A, middle panel). Although the recombinant kinase fragment was highly active toward substrates (MBP, full-length hPak1), we were not able to see it autophosphorylate (Fig. 8, left panel), possibly due to prior autophosphorylation in E. coli (data not shown). However, intermolecular phosphorylation of full-length hPak1 did not result in, and therefore might not be sufficient for, activation (Fig. 8A, right panel).

Further evidence suggested that the key phosphorylation site threonine 423 is protected from intermolecular phosphorylation when full-length Pak1 is in an inactive conformation. A phosphospecific antiserum specifically detecting the threonine 423-phosphorylated form of the subdomain VIII activation loop showed that threonine 423 was not phosphorylated by the recombinant hpak1-(232–545) kinase (Fig. 8B, lane 7), whereas this antiserum efficiently detects the threonine 423-phosphorylated species of wild type hPak1 stimulated with Cdc42 or sphingosine (Fig. 8B, lanes 2 and 3). In this experiment the kinase-inactive hpak1-(K299A) was used to investigate the role of activator binding on threonine 423 phosphorylation (Fig. 7C, lanes 4–9). Indeed, if GTPγS-loaded Cdc42 or sphingosine were added together with the recombinant pak1-(233–544) kinase to hpak1-(K299A), then threonine 423 was phosphorylated.

These data suggest that the conformational status of Pak1 determines the accessibility of threonine 423 for intermolecular phosphorylation. In the closed conformation (absence of activators), this residue is not accessible. Opening of the Pak1 structure by activators eliminates these conformational restraints and leads to exposure of threonine 423 for phosphorylation. The fact that both activators qualitatively give the same results again argues that sphingosine action on the Pak1 molecule is virtually equivalent to that of Cdc42.

**Mechanistic Model of Pak Activation**—The data presented here suggest the following model for the regulation of Pak kinase activity (Fig. 9A). A key feature in the control of Pak enzymatic activity is a regulated interaction between an amino-terminal inhibitory domain and the catalytic kinase domain. Cdc42 and Rac GTPases that are in the active GTP-loaded conformation bind to the amino-terminal PBD and disrupt this interaction, thereby relieving initial inhibition of the kinase. Binding of the GTPase to Pak is a reversible event, i.e. when the GTPase drops off, the inhibitory mechanism can engage again and silence the kinase as long as no autophosphorylation occurs.

Phosphorylation of the kinase domain at threonine 423 in the activation loop in subdomain VIII renders the enzymatic activity insensitive to the autoinhibitory module, possibly reflecting the inability of the inhibitory module to interact with the kinase domain. At this stage, the dissociation of the GTPase from Pak is no longer sufficient to inhibit enzymatic activity; instead, dephosphorylation has to occur to enable Pak to refold and engage the inhibitory module.

However, our data indicate that phosphorylation of threonine 423 serves a dual purpose, as it is also required to achieve full kinase activity. This appears to be similar to the mechanism described for protein kinase A, in which phosphorylation of the equivalent threonine positions the activation loop to allow better substrate access in the groove between the two lobes of the enzyme structure (19).
Pak Activation by GTPases/Lipids—How does GTPase binding interfere with the intramolecular interaction within the Pak molecule? In our studies, we could localize the interacting and autoinhibitory domain in the amino terminus to amino acids 67–150 of human Pak1. This region contains also the p21-binding domain. The minimal requirement for GTPase binding that has been defined by Burbelo et al. (14) spans amino acids 75–89 of human Pak1. However, it has been shown that amino acids both amino- and carboxyl-terminal of the minimal site enhance the binding affinity to the GTPases.

![FIG. 7. Influence of sphingosine on PBD/ID-mediated inhibition.](image)

Wild type hPak1 was used at a constant amount in all kinase reactions. A, hPak1 was prephosphorylated with unlabeled ATP in the presence of 1 μg of GTPγS-loaded Cdc42 (lanes 4 and 5) or 400 μM sphingosine (lanes 9 and 10). The same concentration of activators was used in the kinase reactions with untreated hPak1 as indicated. The hpak1-(67–150) peptide was used at 4 μM final concentration (lanes 3, 5, 8, and 10). The autorad is presented in the upper panel; the PhosphorImager quantification is shown below. Note, that due to traces of thrombin-activity in the Cdc42 preparation, a fraction of MBP was cleaved into two smaller fragments in lane 2 (labeled with “*”). Phosphate incorporation into these cleaved fragments was included in the PhosphorImager quantification. Phosphorylation of MBP in lane 2 was set to 100%. B, Kinase assay using wild type hPak1 stimulated with the indicated concentrations of sphingosine or 1 μg of Cdc42 in the presence and absence of 4 μM wild type hpak1-(67–150).

![FIG. 8. Intermolecular phosphorylation of hPak1.](image)

A, a kinase reaction was performed using recombinant and kinase active hpak1-(232–545) to phosphorylate full-length human Pak1 in Cos1 lysates. Left panel, kinase assay with hpak1-(232–545) in the presence and absence of MBP. Middle panel, a kinase reaction was performed using lysates from Cos-1 cells expressing control vector or wild type hPak1. The hpak1-(232–545) kinase fragment was added to the reactions as indicated. Right panel, immunoprecipitated wild type hPak1 was prephosphorylated in the presence and absence of hpak1-(232–545), was washed, and subjected to a kinase assay using MBP as a substrate. The activity toward MBP of the amount of hpak1-(232–545) used for cross-phosphorylation is shown on the right. B, phosphorylation of threonine 423 by hpak1-(232–545). Immunoprecipitated wild type and kinase inactive hPak1-(K299A) were incubated with 1 μg of GTPγS-loaded Cdc42 and 400 μM sphingosine as indicated. hpak1-(232–545) was added to the reactions as indicated. 250 μM ATP was present in all reactions. The reactions were incubated for 30 min at 30 °C, then separated on a 12% SDS-polyacrylamide gel and blotted for immunodetection using an antiserum recognizing the threonine 423-phosphorylated activation loop of hPak1. Upper panel, the Ponceau S-stained hPak1 proteins are shown. Wild type hPak1 showed significant decrease in mobility after phosphorylation with GTPγS-loaded Cdc42 or sphingosine. Lower panel, immunodetection of threonine 423 phosphorylation using the alkaline phosphatase color reaction.
A Central Phosphorylation Site in Pak

**FIG. 9. Model for the regulation of p21-activated kinase.**

**A,** *left upper panel,* Pak kinases exist in a closed conformation in the absence of an activator (GTPase, lipid) due to an interaction between the ID and the kinase domain (KD). The conformationally closed Pak has a basal level of kinase activity. *Right upper panel,* binding of activators (GTPase, sphingosine) leads to a conformational change to open the Pak structure, relieving the initial inhibitory constraints. (Our data suggest that binding of the lipid occurs within the PBD/ID region, but the binding requirements have not been defined yet). However, binding of the activators disrupts the ID-kinase domain interaction due to an overlap of their binding sites. The opened and unphosphorylated Pak has increased activity toward substrate. *Right lower panel,* as a consequence of increased kinase activity, Pak autophosphorylates at several residues, including threonine 423, which leads to a maximal increase in the enzymatic activity. *Left lower panel,* even if the activator dissociates from its binding site, Pak kinase activity remains high, since phosphorylation of threonine 423 and possibly serine/threonine residues in the inhibitory domain keep the kinase in an open and highly active conformation. Dephosphorylation has to occur to switch Pak back to the closed and inactive conformation. *B,* opening of Pak leads to intra- and intermolecular autophosphorylation.

Zhao *et al.* (9) have recently described an autoinhibitory region by *in vitro* kinase assays in rat alpha Pak, which is the rat homologue of human Pak1. Rat and human Pak1 are more than 98% identical and differ only at seven amino acid positions located in the NH₂-terminal regulatory region. Consistent with the autoinhibitory function described, we characterized the inhibitory domain in biochemical interaction assays using recombinant proteins. Zhao *et al.* (9) identified an autoinhibitory region from amino acid 83 to 149 that is contained within our minimal inhibitory region spanning amino acids 67–150 (the numbering of amino acids between rat and human Pak1 is identical up to amino acid 178, where human Pak1 contains an additional aspartic acid residue). Using mutagenesis, they identified additional amino acids (conserved in hPak1) essential for the inhibitory function. These amino acids are located within the COOH-terminal portion of the PBD/ID region (glutamic acid 116/glutamine 117 and aspartic acid 126). As we demonstrated for leucine 107, these mutations do not disrupt GTPase binding but abolish the inhibitory function, supporting the idea that both functions have different determinants within this region. A more indirect approach using the yeast two-hybrid system was used to demonstrate that the equivalent regulatory and kinase domains of the yeast *S. pombe* Pak1 interact in a Cdc42-sensitive fashion (10).

Most of the Pak-homologous kinases, in addition to the minimal GTPase binding domain, exhibit sequence homologies in not to affect the interaction with the kinase domain, as indicated by our GST pull-down assay, histidine 86 does make a functionally important contact required for inhibition of the Pak kinase core. Histidine 86 thus defines an overlapping site encompassing the end of the GTPase binding domain and the start of the autoinhibitory domain. The close proximity between the GTPase-binding and autoinhibitory domains suggests a simple model for the opening of the Pak structure. Binding of the GTPase to the PBD/ID interferes with functionally important regions of the autoinhibitory module and consequently separates the intramolecular interaction.

We propose that stimulating lipids, like sphingosine, induce Pak kinase activation in a similar fashion as small GTPases. Indeed, previous observations (11) suggested that the lipid interaction domain is also located (at least in part) within the characterized PBD/ID region. Sphingosine action on hPak1 leads to phosphorylation of threonine 423, as does activated GTPase. We therefore believe that sphingosine interferes with the autoinhibitory module leading to opening of the Pak structure. We have been unable to show that sphingosine disrupts the interaction in our established pull-down assay, however, probably due to the need to use non ionic detergents to prevent unspecific binding of the amino-terminal fragments (hpak1-(1–234), hpak1-(67–150)) to glutathione-agarose. The presence of the detergents destroys lipid vesicle formation necessary for sphingosine to act properly.
the immediate carboxyl-terminal region where determinants for the autoinhibitory module are located, suggesting that the described mechanism of kinase inhibition/activation is conserved within the Pak kinase family. Interestingly, a recently isolated and characterized Pak isoform, Pak4, shows constitutive kinase activity, independent of the binding of GTPases. Pak4 contains a p21-binding domain, but does not show homology in the carboxyl-terminal portion of this region where determinants for the inhibitory domain are located (21).

**Threonine 423 in the Activation Loop as a Key Regulatory Site**—Phosphorylation of threonine 423 seems to be a key event for full activation of Pak1 and maintaining the kinase in a catalytically competent state. Studies on Pak65 (4) suggested that autophosphorylation of the kinase domain results from inter- and not intramolecular phosphorylation events. For protein kinase A, it has been proposed that phosphorylation within the activation loop might occur through an intermolecular mechanism (19). Due to its position and proximity to the catalytic center, we consider it unlikely that threonine 423 can be accessible for intermolecular phosphorylation. Whether autophosphorylation of the kinase domain results from phosphorylation or whether it induces a structural change when activated GTPase or sphingosine were added, indicating that opening of the Pak structure is required to make threonine 423 accessible to intermolecular phosphorylation. Our intermolecular phosphorylation studies showed that threonine 423 is not accessible in the closed Pak structure (Fig. 9B). Threonine 423 was only phosphorylated in Pak1-(K299A) when activated GTPase or sphingosine were added, indicating that opening of the Pak structure is required to make threonine 423 accessible for intermolecular phosphorylation. Whether the autoinhibitory module physically masks threonine 423 from phosphorylation or whether it induces a structural change in the kinase domain as depicted in the model in Fig. 9B is not clear.

Intermolecular phosphorylation only occurs if the Pak molecules are bound to activators. We envision this as a mechanism to spatially regulate Pak activation inside the cell, for example to areas where GTPases are localized. Inactive Pak molecules not interacting with GTPases/lipids cannot be activated by intermolecular phosphorylation, thereby preventing an undesirable chain reaction of Pak activation.

**Acknowledgments**—We thank Erica M. Dutil and Alexandra C. Newton for their excellent phosphospecific anti-PKC\(b\)I antiserum and Melanie H. Cobb for providing the pGEX-KG/pak1 construct. We thank Toni Lestelle for expert secretarial assistance.

**REFERENCES**

1. Taylor, S. S., and Radzio-Andzelm, E. (1997) Curr. Opin. Chem. Biol. 1, 219–226
2. Kemp, B. E., and Pearson, R. B. (1991) Biochim. Biophys. Acta 1094, 67–76
3. Sells, M. A., and Chernoff, J. (1997) Trends Cell Biol. 7, 162–167
4. Benner, G. E., Dennis, P. B., and Masaracchia, R. A. (1995) J. Biol. Chem. 270, 21212–21219
5. Yu, J. S., Chen, W. J., Ni, M. H., Chan, W. H., and Yang, S. D. (1998) Biochem. J. 334, 121–131
6. Lee, N., MacDonald, H., Reinhard, C., Halenbeck, R., Roulston, A., Shi, T., and Williams, L. T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13642–13647
7. Rudel, T., and Bokoch, G. M. (1997) Science 276, 1571–1574
8. Walter, B. N., Huang, Z., Jakobi, R., Tuazon, P. T., Ahemri, E. S., Litwack, G., and Traup, J. A. (1998) J. Biol. Chem. 273, 28733–28739
9. Zhao, Z. S., Manser, E., Chen, Z. Q., Chung, C., Leung, T., and Lim, L. (1998) Mol. Cell. Biol. 18, 2153–2163
10. Tu, H., and Wigler, M. (1999) Mol. Cell. Biol. 19, 602–611
11. Bokoch, G. M., Reilly, A. M., Daniels, R. H., King, C. C., Oliviera, A., Spiegel, S., and Knaus, U. G. (1998) J. Biol. Chem. 273, 8137–8144
12. Lian, J. P., Huang, R., Robinson, D., and Budwey, J. A. (1998) J. Immunol. 161, 4375–4381
13. Sells, M. A., Knaus, U. G., Bagrodia, S., Ambrose, D. M., Bokoch, G. M., and Chernoff, J. (1997) Curr. Biol. 7, 202–210
14. Burbelo, P. D., Dreschel, D., and Hall, A. (1995) J. Biol. Chem. 270, 29971–29974
15. Daniels, R. H., Zenke, F. T., and Bokoch, G. M. (1999) J. Biol. Chem. 274, 6047–6050
16. Brown, J. L., Stowers, L., Baer, M., Trejo, J., Coughlin, S., and Chant, J. (1996) Curr. Biol. 6, 598–605
17. Martin, G. A., Bollag, G., McCormick, F., and Abe, A. (1995) EMBO J. 14, 1970–1978
18. Manser, E., Huang, H. Y., Lu, T. H., Chen, X. Q., Dong, J. M., Leung, T., and Lim, L. (1997) Mol. Cell. Biol. 17, 1129–1143
19. Steinberg, R. A., Coughlin, S. R., Symcox, M. M., and Shuntoh, H. (1993) Mol. Cell. Biol. 13, 2332–2341
20. Knaus, U. G., Wang, Y., Reilly, A. M., Warnock, D., and Jackson, J. H. (1998) J. Biol. Chem. 273, 21512–21518
21. Abo, A., Qu, J., Cammarano, M. S., Dan, C., Fritsch, A., Baud, V., Belisle, B., and Minden, A. (1998) EMBO J. 17, 6527–6540