

# Analysis of Small GTPase Signaling Pathways Using p21-activated Kinase Mutants That Selectively Couple to Cdc42\*

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**p21-activated kinase 1 (Pak1) is an effector for the small GTPases Cdc42 and Rac. Because Pak1 binds to and is activated by both these GTPases, it has been difficult to precisely delineate the signaling pathways that link extracellular stimuli to Pak1 activation. To separate activation of Pak1 by Cdc42 versus activation by Rac, we devised a genetic screen in yeast that enabled us to create and identify Pak1 mutants that selectively couple to Cdc42 but not Rac1. We recovered several such Pak1 mutants and found that the residues most often affected lie within the p21 binding domain, a region previously known to mediate Pak1 binding to GTPases, but that several mutations also map outside the borders of the p21 binding domain. Pak1 mutants that associate with Cdc42 but not Rac1 were also activated by Cdc42 but not Rac1. In rat 3Y1 cells expressing oncogenic Ha-Ras, the Pak1 mutants defective in Rac1 binding are not activated, suggesting that Ras signals through a GTPase other than Cdc42 to activate Pak1. Similar results were obtained when epidermal growth factor was used to activate Pak1. However, Pak1 mutants that are unable to bind Rac are nonetheless well activated by calf serum, implying that this stimulus may induce Pak activation independent of Rac.**

Cdc42 and Rac are small GTPases in the Rho family that play major roles in regulating the actin cytoskeleton as well as key cellular functions such as differentiation, cell cycle progression, transformation, apoptosis, motility, and adhesion (1–4). Although these two small GTPases are structurally similar and share certain biochemical properties, there is ample evidence that Rac and Cdc42 are differentially regulated and affect distinct signaling pathways. For example, Rac is activated by platelet-derived growth factor, induces formation of lamellipodia, and stimulates cell survival pathways, whereas Cdc42 is activated by tumor necrosis factor- $\alpha$ , induces the formation of filopodia, and when expressed at high level, is pro-apoptotic.

Like their distant cousin Ras, activated Cdc42 and Rac transmit signals by recruiting an array of effector proteins. Among these effectors, the p21-activated kinases (Paks)<sup>1</sup> have

attracted much attention. Paks are serine/threonine kinases that bind to and are activated by both Cdc42 and Rac (5–8). These kinases are highly conserved among eukaryotes and have been shown to affect actin reorganization (9–11), cell motility (12, 13), apoptosis (14, 15), and gene transcription (16). The ability of Pak1 to bind both Cdc42 and Rac makes it unusual among GTPase effectors as most known effectors are specific for one but not both of these GTPases. Because Pak1 binds to and is activated by both Cdc42 and Rac1, it is often difficult to determine the precise nature of the signaling mechanisms that stimulate this kinase. For example, transient expression of Ras activates Pak1 (17, 18); this could be mediated by a Rac-dependent pathway, a Cdc42-dependent pathway, or possibly both or neither. Similarly, growth factors and cell adhesion activate Pak1 (19–22) as do various other stimuli, such as exposure of cells to UV irradiation or alkylating agents (23). Although the signals from these stimuli are thought to be funneled through Rac1 or Cdc42, it is difficult to determine which of these GTPases is specifically involved. In principle, expression of dominant negative forms of Rac or Cdc42, which act by titrating out endogenous GEFs, might be used to dissect these signaling pathways. In practice, however, dominant negative approaches suffer many specificity problems that limit their utility (24). Alternate approaches could therefore be useful in analyzing the mechanisms of Pak1 activation.

Here we present an alternate method to analyze Pak1 signaling. To delineate the Pak1 pathways involving Rac1 versus those involving Cdc42, we devised a genetic screen in yeast to create and identify mutants of Pak1 that can specifically bind Cdc42 but can no longer effectively bind Rac1. Several such mutants were isolated, and their binding and activation properties were confirmed *in vitro* and *in vivo*. Unlike the one previously known example of a Pak1 Cdc42-specific binding mutant (25), several of the Pak1 mutants isolated in our screen retained normal activation by Cdc42. We have used these mutants to analyze the mechanism by which Ras and EGF activate Pak1 and determined that Ras and EGF activation of Pak1 is mediated by a GTPase other than Cdc42 but that Pak1 activation by calf serum is mediated by a GTPase other than Rac.

## EXPERIMENTAL PROCEDURES

**Dual-bait Two-hybrid Screen**—A cDNA encoding the N-terminal 186 amino acids of Pak1 was created by PCR and cloned as an *EcoRI/XhoI* fragment into the yeast two-hybrid activation domain vector pJG4–5 (26) to create pJG4–5 Pak1. The cDNA fragment was randomly mutated by error-prone PCR using a protocol based on the method of Fromant *et al.* (27). 1  $\mu$ g of pJG4–5 Pak1 was amplified by PCR in a

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<sup>1</sup> The abbreviations used are: Pak, p21-activated kinase; PBD, p21 binding domain; CRIB, Cdc42/Rac-interactive binding; MES, 4-morpho-

lineethanesulfonic acid; EGF, epidermal growth factor; GEF, guanine nucleotide exchange factor; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MBP, myelin basic protein; HA, hemagglutinin; WASP, Wiskott-Aldrich Syndrome Protein.

100- $\mu$ l mixture containing *Taq* polymerase buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM MnCl<sub>2</sub>, 200  $\mu$ M ATP and TTP, and 20  $\mu$ M CTP and GTP. The forward (sense) primer 5'-CGCCGATCCAGCTGACTGGCTGA-3' matched the sequence of pJG4-5 from position -128 to -105, 5' to the *Eco*RI cloning site, whereas the reverse (antisense) primer 5'-GTGT-CAACAACGTATCTACCAACG-3' matched the sequence of pJG4-5 from position +109 to +132, 3' to the *Xho*I cloning site. *Taq* polymerase was used to amplify the DNA using 20 cycles of PCR with each cycle consisting of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 120 s.

The resulting mutagenized mixture of PCR fragments was isolated on an agarose gel, excised, and purified using glass beads. The fragment was then co-transformed along with 0.5  $\mu$ g of *Eco*RI/*Xho*I-cut pJG4-5 vector into the yeast SKY48 strain (*MAT $\alpha$  ura3 his3 trp1 cIop-LYS2 lexAop-LEU2*), which had been previously transformed with the two-hybrid bait vectors pGKS-8-Rac1 L61(28), pEG202-Cdc42 L61(26), and the pSH18-34*lacZ* reporter vector. The transformants were isolated on Trp<sup>-</sup> (to select for pJG4-5), Ura<sup>-</sup> (to select for pSH18-34), His<sup>-</sup> (to select for pEG202), and Zeocin<sup>+</sup> (to select for pGKS-8) plates. Colonies were then replica-plated to Lys<sup>-</sup> (to select for Rac1 interactors) and Leu<sup>-</sup> (to select for Cdc42 interactors) media and assayed for  $\beta$ -glucuronidase (Rac1 interaction reporter) and  $\beta$ -galactosidase (Cdc42 interaction reporter) activity (28).

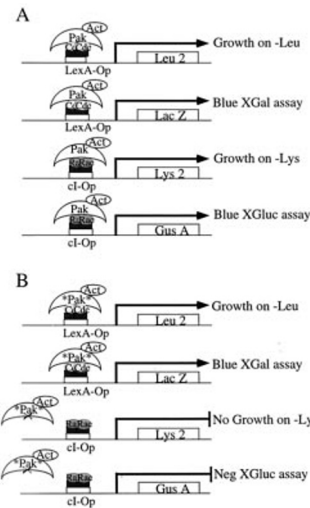
The Pak1 inserts from individual yeast colonies were retrieved by colony PCR using the same primers as for mutagenesis but normal PCR conditions. These inserts were purified and retransformed, along with *Eco*RI/*Xho*I-digested pJG4-5, into yeast to reconfirm the phenotype.

**Transfections and Lysis**—3Y1 rat fibroblasts, Swiss 3T3 mouse fibroblasts, or COS7 African green monkey epithelial cells maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum or calf serum were seeded at  $2 \times 10^5$  for 3Y1 or  $4 \times 10^5$  for COS and 3T3 cells per 60-mm plate and transfected with the LipofectAMINE Plus method (Life Technologies, Inc.) with a total of 1.5 to 2  $\mu$ g of DNA. Cells were harvested at 24 or 48 h post-transfection in Robert's lysis buffer (1% Nonidet P-40, 10% glycerol, 20 mM Tris, pH 8.0, 137 mM NaCl, 50 mM NaF, 10 mM  $\beta$ -glycerol phosphate, 10 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin, 10 mM sodium orthovanadate). The lysates were adjusted for equal protein content and then boiled with SDS-PAGE sample buffer for Western blotting and overlay assays or quick frozen on dry ice and stored at -80 °C until needed for immunoprecipitation.

**Co-immunoprecipitation**—3Y1 cells were transiently transfected with 1.0  $\mu$ g of various Myc-tagged Pak proteins along with 0.5  $\mu$ g of HA-tagged activated Rac1 or Cdc42. Cells were harvested at 24 h post-transfection in Robert's lysis buffer, equalized for protein content, and immunoprecipitated overnight with  $\alpha$ -Myc polyclonal antibodies (A-14, Santa Cruz, CA). The immune complexes were sedimented with 50% protein A-agarose beads (Pierce), washed with lysis buffer without protease inhibitors, and analyzed by Western blotting to detect Rac1 or Cdc42 proteins precipitated with the wild-type or mutant Pak1 proteins.

**Overlay Assays**—For Pak1 overlays, 3Y1 cells were transiently transfected to express various Myc-tagged Pak proteins. The cells were harvested, and the lysates were equalized for protein and separated by SDS-PAGE. The Pak proteins were immobilized on PVDF membrane and blocked in phosphate-buffered saline containing 1% bovine serum albumin, 0.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, and 5 mM dithiothreitol for 1 h at 4 °C. Wild-type Rac and Cdc42 were either made as GST-tagged proteins in bacteria or purchased as His-tagged proteins (Cytoskeleton) and were activated with 25  $\mu$ Ci of <sup>35</sup>S-labeled GTP $\gamma$ S in exchange buffer (25 mM MES, pH 6.5, 50 mM NaCl, 5 mM EDTA, 0.05% Triton X-100) for 10 min at 30 °C. The binding reaction was allowed to occur for 20 min at room temperature in binding buffer (25 mM MES, pH 6.5, 0.5 mM GTP, 5 mM MgCl<sub>2</sub>, 50 mM NaCl, 5 mM dithiothreitol). The membranes were then washed briefly in phosphate-buffered saline containing 25 mM MES, pH 6.5, 5 mM MgCl<sub>2</sub>, and 0.05% Triton X-100, and bound proteins were visualized and quantified by phosphorimaging (Fuji FLA 2000) and autoradiography.

**Kinase Assays**—COS7, 3Y1, or Swiss 3T3 cells were transiently transfected to express various Myc-tagged Pak proteins along with activated forms of Rac1, Cdc42, or Ras or were treated with growth factors. The cells were lysed in Robert's lysis buffer, and the lysate was equalized for protein content and immunoprecipitated overnight with anti-Myc polyclonal antibodies. The immune complexes were sedimented with 50% protein A-agarose beads and then washed with lysis buffer without protease inhibitors twice and once with kinase buffer (40 mM HEPES, pH 8.0, 10 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>). The immunoprecipitated Pak1 was then incubated with 1  $\mu$ Ci of [<sup>32</sup>P]ATP in kinase buffer containing 20  $\mu$ M ATP and 5  $\mu$ g of MBP (Sigma) or

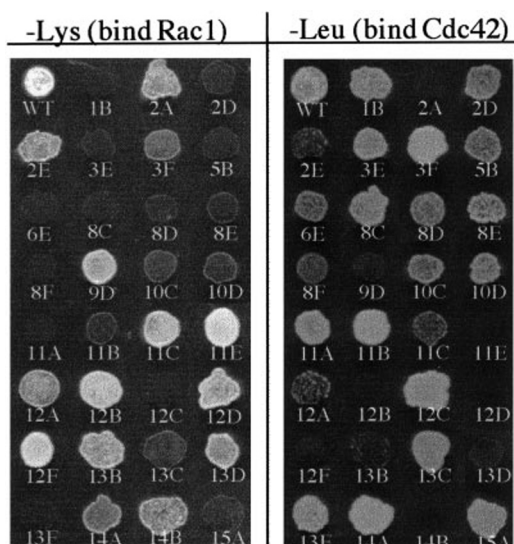


**FIG. 1. Schematic of dual-bait strategy.** Activated forms of Rac1 and Cdc42 are expressed in the yeast SKY48 strain as dual baits. Cdc42, fused to the DNA binding domain of LexA, serves as the first bait, whereas Rac1, fused to the DNA binding domain of the  $\lambda$  phage cI protein, serves as the second bait. The yeast, which also contain auxotrophic and chromogenic transcriptional reporters responding to the respective binding domains on the baits, are also transformed with wild-type or mutant Pak1 bound to an activation domain. Wild-type Pak1 binds both Rac1 and Cdc42, turning on all four reporters, thus resulting in growth on media lacking leucine or lysine, and this should induce a color change when assayed on media containing the appropriate substrates for  $\beta$ -galactosidase (*XGal*) and  $\beta$ -glucuronidase (*XGlu*) (A). A mutant Pak1 which, as in this example, could bind Cdc42 but not Rac1 would turn on only the Cdc42 responsive reporters, resulting in growth only on media lacking leucine and a color change only on media containing the substrate for  $\beta$ -galactosidase (B). By screening yeast expressing randomly mutagenized Pak1 in this assay, one can correlate growth on media lacking leucine but no growth on media lacking lysine as Pak mutants that specifically bind Cdc42 and not Rac1 and vice versa.

Histone H4 (Fluka) for 30 min. The reaction was stopped by the addition of SDS-PAGE sample buffer followed by incubation in a boiling bath for 5 min. The samples were separated by SDS-PAGE and then visualized and quantified by phosphorimaging (Fuji FLA 2000) and autoradiography.

## RESULTS

**Creation of Pak1 Mutants That Selectively Bind to Cdc42 Versus Rac1**—Although most CRIB-containing proteins bind to Cdc42 but not Rac, Paks 1, 2, and 3 associate with and are activated by both these GTPases (6). This unusual property makes it difficult to analyze the pathways by which upstream signals activate any of these Paks as these may be channeled through Cdc42, Rac, or both. We therefore attempted to create Pak1 mutants that could selectively couple to Rac1 or Cdc42 but not both. We used a dual-bait two-hybrid system in yeast in which it is possible to monitor the binding of a protein to two baits simultaneously (28). In this system, two baits are introduced into SKY48 yeast cells along with an interactor and two sets of auxotrophic and chromogenic transcriptional reporters. For the Pak1 screen, an activated allele of Cdc42, fused to the DNA binding domain of LexA, served as the first bait, whereas an activated allele of Rac1, fused to the DNA binding domain of the  $\lambda$  phage cI protein, served as the second bait. When wild-type Pak1 is co-expressed in these cells, all four reporters should be activated (Fig. 1A). The first set of reporters is controlled by the Lex-Cdc42-Pak1 interaction (*LexAop-LEU2* and *LexAop-lacZ*), whereas the second set is controlled by the cI-Rac1-Pak1 interaction (*cIop-LYS2* and *cIop-GusA*). Thus, such yeast should grow on media lacking both leucine and lysine and should induce a color change when assayed on media

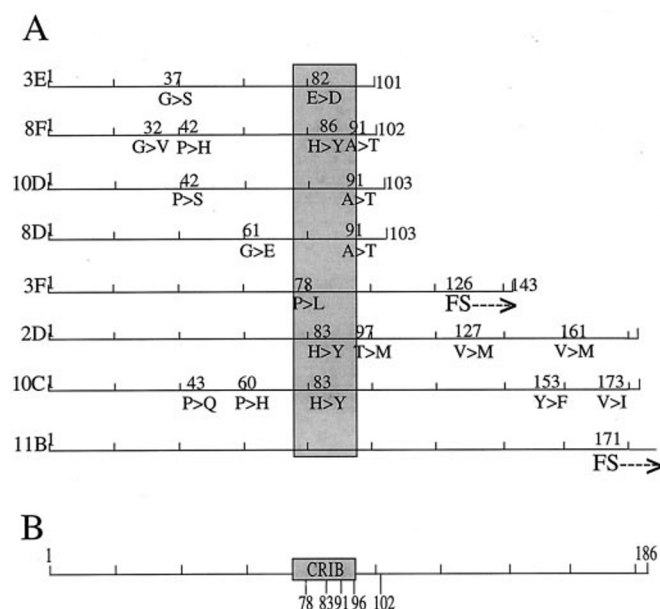


**FIG. 2. Results of dual-bait two-hybrid differential binding screen.** SKY48 yeast cells expressing both Rac1 and Cdc42 baits were transformed with pJG4-5 containing randomly mutagenized Pak1 fragment amino acids 1-186 and grown on complete media. The yeast colonies were replica-plated to media lacking lysine ( $\text{Lys}^-$ ) or lacking leucine ( $\text{Leu}^-$ ). Those colonies growing only on  $\text{Leu}^-$  media and not on  $\text{Lys}^-$  media correlate to Pak1 mutants that bind Cdc42 and not Rac1, and those growing on  $\text{Lys}^-$  media and not on  $\text{Leu}^-$  media correlate to Pak1 mutants that bind Rac1 and not Cdc42. *wt*, wild type.

containing the appropriate substrates for  $\beta$ -galactosidase and  $\beta$ -glucuronidase. A Pak1 protein that, for example, contains a mutation that disables binding to Rac1 but not Cdc42 would activate the *Leu2* and *LacZ* but not the *Lys2* and *GusA* reporters, resulting in cells that grow on medium lacking leucine and produce a color change in a  $\beta$ -galactosidase enzymatic assay but which fail to grow on medium lacking lysine and fail to produce a color change in a  $\beta$ -glucuronidase enzymatic assay (Fig. 1B).

To create and isolate Pak1 mutants that differentially bind Cdc42 and Rac1, we used an error-prone PCR protocol (27) to create a library of Pak1 mutants encoding the N-terminal segment (amino acids 1-186) of this protein. The 5' and 3' ends of the PCR fragments contained sequences derived from the pJG4-5 plasmid, allowing efficient *in vivo* recombination in yeast. Yeast expressing both GTPase baits were co-transformed with *EcoRI/XhoI*-digested pJG4-5 and the library of Pak1 mutants. *In vivo* recombination between the homologous ends of the pJG4-5 vector and the Pak1 PCR fragment results in the formation of intact pJG4-5-Pak1 plasmids in the yeast cells that confer growth on  $\text{Trp}^-$  media. The transformants were picked and tested for the ability to grow selectively on  $\text{Lys}^-$  or  $\text{Leu}^-$  media. As a secondary screen, the yeast in the colonies were also tested for the ability to cause a color change in a  $\beta$ -galactosidase or  $\beta$ -glucuronidase substrate assay. Following selection of positive colonies, plasmid DNA containing the mutated Pak1 was retrieved and retransformed into fresh yeast expressing both baits. The colonies were again screened for growth on  $\text{Leu}^-$  and  $\text{Lys}^-$  media. From a total of 2000 colonies screened, we recovered 20 colonies that showed selectivity in activating Cdc42-responsive reporters and 12 colonies that showed selectivity in activating Rac1-responsive reporters (Fig. 2). The phenotype of most of these colonies was stable and reproducible as multiple independent isolates of each clone behaved similarly upon retesting.

**Identification of Mutations Conferring Binding Selectivity**—We chose to focus on those colonies that showed strong



**FIG. 3. Pak1 mutants that specifically bind Cdc42.** A, schematic representation of mutations found in N-terminal Pak1 proteins that displayed relative specificity toward Cdc42 in both the yeast two-hybrid screen and in an *in vitro* binding assay. Most proteins contained multiple mutations, and a number of the mutations resulted in a truncated or frameshifted protein. Mutations are represented by the notation  $X>Y$ , and frameshifts are represented by  $\text{FS} \rightarrow$ . The CRIB motif of Pak is illustrated by a shaded box. B, schematic representation of point mutants created in the N-terminal Pak1 protein amino acids 1-186 in pJG4-5 by site-directed mutagenesis. These mutations were created based on sequence analysis of the Pak1 mutants that differentially bind Cdc42 in the two-hybrid screen and in the *in vitro* binding assay.

positive growth on  $\text{Leu}^-$  but not on  $\text{Lys}^-$  media (*i.e.* mutants of Pak1 that potentially bound Cdc42 but not Rac1). Most of these clones displayed relatively enhanced specificity for Cdc42 *versus* Rac1 binding in both the two-hybrid assay as well as in a GTPase overlay assay (data not shown), suggesting that the dual-bait screen is indeed able to select for GTPase binding mutants. We sequenced the Pak1 inserts to determine the basis for this binding selectivity. A schematic representation of the mutations is shown in Fig. 3A. Most of the clones contained more than one mutation. However, mutations at particular sites (*e.g.* Pro-42, Pro-78, His-83, Ala-91, stop codons at residues 101-103, and frameshifting mutations at residue 126 that result in a truncated protein) appeared in more than one clone. Many of these common mutations (*i.e.* Pro-78, His-83, and Ala-91) as well as one unique mutation (His-86) alter key residues within the CRIB motif, whereas other mutations map outside this region.

To determine which of these mutations account for the altered binding properties of Pak1, we used site-directed mutagenesis to create single amino acid changes or early stop codons in the Pak1 1-186 fragment contained in the yeast two-hybrid plasmid. We focused on those mutations that were common to more than one clone as well as those lesions that affected residues within the CRIB domain. We also made point mutations at Gln-102 as this site was frequently mutated in the Cdc42 binding selective clones. In addition to these mutations derived from the dual-bait two-hybrid screen, we mutated Pak1 Phe-96 (to alanine) as this residue is conserved in Paks 1, 2, and 3 but not in the CRIB domains of MRCK, Ack, or WASP, which bind Cdc42 but not Rac1. The Pak1 mutations are summarized in Fig. 3B. These point mutants were retested in the two-hybrid system against both GTPase baits (data not shown). Pak1-bearing point mutations at either Pro-78, His-83, Phe-96,

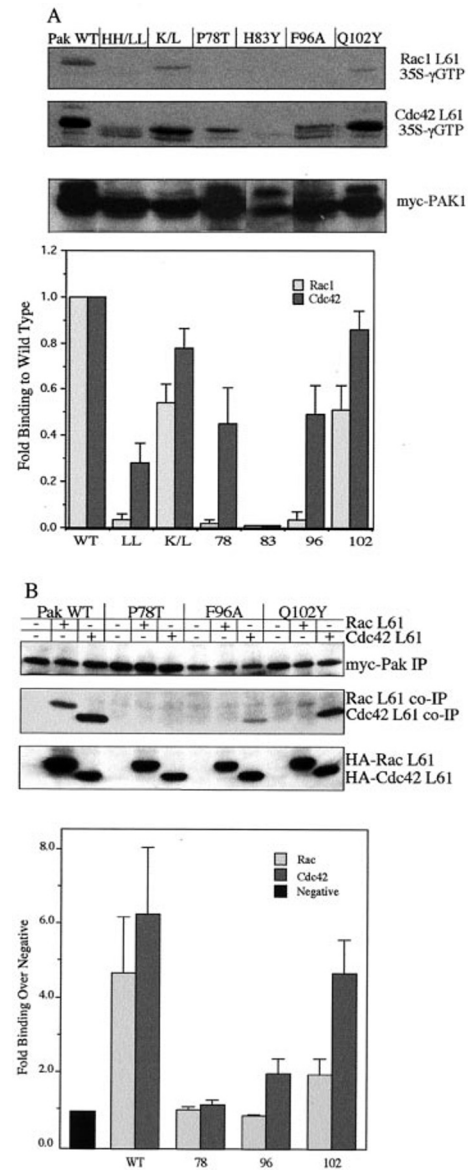
and Gln-102 bound to Cdc42 as well or better than did wild-type Pak1 but bound poorly or not at all to Rac1. Interestingly, certain early stops in the Pak1 protein, for example at residues 96 or 102, were associated with selective binding for Cdc42. These truncations were not studied further, however, as we wished to use the binding mutants to dissect upstream signals that activate Pak1, and this could not be done in molecules that lack the protein kinase catalytic domain.

**Binding Properties of Pak1 Binding Mutants**—We next studied the binding properties of these mutants in the context of full-length Pak1 in mammalian cells. Based on the two-hybrid and overlay data, we introduced single amino acid point mutations in Myc-tagged wild-type Pak1 in a mammalian expression vector. The mutant Paks were expressed in both COS7 (not shown) and 3Y1 cells, and the cell lysates were bound to PVDF and probed with radiolabeled GTP-loaded Rac1 or Cdc42 (Fig. 4A). As expected, wild-type Pak1 bound to both Rac1 and Cdc42 and the control Pak1 binding mutant H83L/H86L bound poorly to Cdc42 and not at all to Rac1. The P78T and F96A mutants, bound effectively to Cdc42 (45 and 49% compared with wild-type Pak1, respectively) but not at all to Rac1. The H83Y mutant did not bind either GTPase, whereas the Q102Y mutant bound to Cdc42 nearly as well as wild-type Pak1 (86%) and was partially compromised for Rac1 binding (55%). By contrast, the K66L/K67L/K68L Pak1 mutant, as reported by Knaus *et al.* (25), also displayed partially compromised binding to Rac1 (54%) and was only slightly defective in binding Cdc42 (78%), similar to the Q102Y mutant described here. Thus, when assayed in the context of the full-length Pak1 protein, the mutants uncovered by the dual-bait two-hybrid screen retained relative binding specificity for Cdc42.

To assess the binding of the full-length Pak1 mutants to Rac1 and Cdc42 in an *in vivo* environment, we carried out co-immunoprecipitation assays. Myc-tagged full-length wild-type Pak1 or the P78T, F96A, or Q102Y mutants were co-transfected into COS7 or 3Y1 cells with either vector control or HA-tagged activated Rac1 or Cdc42. Myc-Pak1 was then immunoprecipitated, and the immune complexes were assayed for the presence of Rac1 or Cdc42 by immunoblot (Fig. 4B). Wild-type Pak1 was able to precipitate both Rac1 and Cdc42. Pak1 Q102Y precipitated Cdc42 nearly as well as did wild-type Pak1 but precipitated Rac1 weakly, consistent with the overlay assays. Pak1 F96A precipitated Cdc42 weakly but did not detectably precipitate Rac1, whereas the P78T mutant precipitated neither GTPase. That the last Pak1 mutant failed to co-precipitate Cdc42 despite binding this GTPase in the overlay assay suggests that the native conformation of the full-length protein affects its ability to complex with Cdc42.

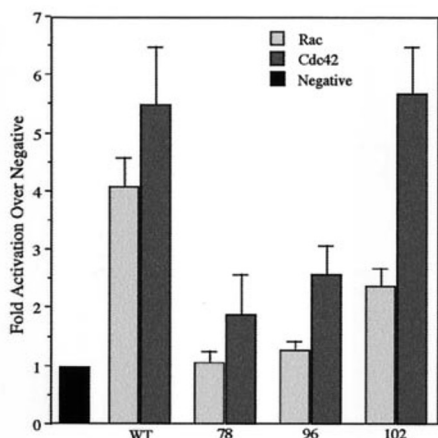
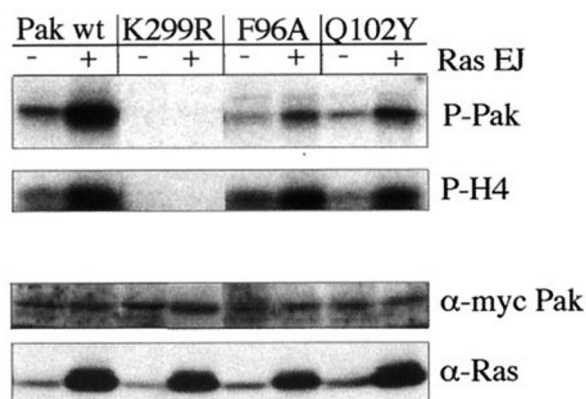
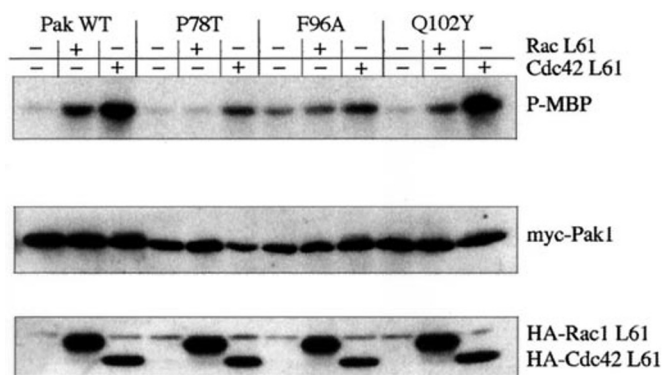
In summary, the binding data indicate that it is possible to create mutants of Pak1 that are relatively selective in their ability to bind Cdc42 *versus* Rac1. Two Pak1 mutants in particular, F96A and Q102Y, show good selectivity in the two-hybrid, GTPase overlay, and co-immunoprecipitation assays, suggesting their potential utility in analyzing the activation pathways for Pak1.

**Activation of Binding Mutants by Cdc42 and Rac1**—Because mutations in Pak1 that affect GTPase binding may also affect kinase activity (9–11, 25, 29), we compared the basal and GTPase-stimulated activity of wild-type Pak1 with that of the P78T, F96A, and Q102Y mutants. COS cells were co-transfected with these vectors plus or minus expression vectors bearing activated (Leu-61) Cdc42 or Rac1. Pak1 protein was immunoprecipitated with anti-Myc antibodies and tested for protein kinase activity (Fig. 5). Both active Rac1 and active Cdc42 strongly activated wild-type Pak1. Consistent with pre-



**FIG. 4. Binding properties of the Pak1 mutants.** *A*, *in vitro* binding of full-length Pak1 point mutants to Cdc42 and Rac1. Point mutations were created in full-length Pak1 contained in the mammalian expression vector pCMV6 by site-directed mutagenesis. These mutants, along with wild-type (*wt*) Pak1 and the negative control Pak1 H83L/H86L, were expressed in 3Y1 fibroblasts, and equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with recombinant wild-type Rac1 or Cdc42 loaded with 25  $\mu$ Ci of  $^{35}$ S-labeled GTP $\gamma$ S. The membranes were washed briefly to eliminate nonspecific binding, and bound labeled GTPase was visualized by autoradiography. *B*, *in vivo* interaction of full-length Pak1 point mutants with Cdc42 and Rac1. Myc-tagged Pak1 and mutants of Pak1 were co-transfected into 3Y1 fibroblasts with activated forms of Rac1 or Cdc42. The cells were harvested at 24 h post-transfection, and the lysates were immunoprecipitated (*IP*) with anti-Myc antibodies. The immune complexes were separated by SDS-PAGE and transferred to PVDF. HA-tagged Rac1 and Cdc42 were visualized by anti-HA immunoblotting. Both experiments were done at least three times, and the data shown are representative of a typical experiment. The bar graph shown represents the mean value with standard error.

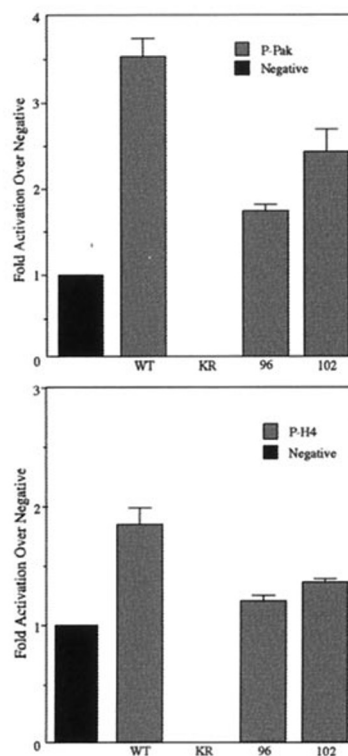
vious studies, Cdc42 activated Pak1 to a greater extent than did Rac1, as seen in the kinase assay. The Pak1 P78T mutant, which failed to co-immunoprecipitate either Rac1 or Cdc42, was not activated by either GTPase. Interestingly, the F96A and Q102Y mutants were fully activated by Cdc42 despite their partially compromised ability to bind to this GTPase. Similarly,



**FIG. 5. Activation of full-length Pak1 point mutants by Cdc42 and Rac1.** Full-length Myc-tagged Pak1 and mutants of Pak1 were co-transfected into COS-7 cells with activated forms of Rac1 or Cdc42. The cells were harvested at 36 h post-transfection, and the lysates were immunoprecipitated with anti-Myc antibodies. The immunoprecipitated Pak1 was then incubated with 1  $\mu$ Ci of [ $^{32}$ P]ATP in kinase buffer containing 20  $\mu$ M ATP and 5  $\mu$ g of MBP (Sigma). Phosphorylation of MBP by the Pak1 proteins was visualized by autoradiography. Protein expression of the tagged Pak1 proteins and Rac1 and Cdc42 was visualized by immunoblotting followed by autoradiography. The data shown are representative of at least three independent experiments. The bar graph shown represents the mean value with standard error. *wt*, wild type.

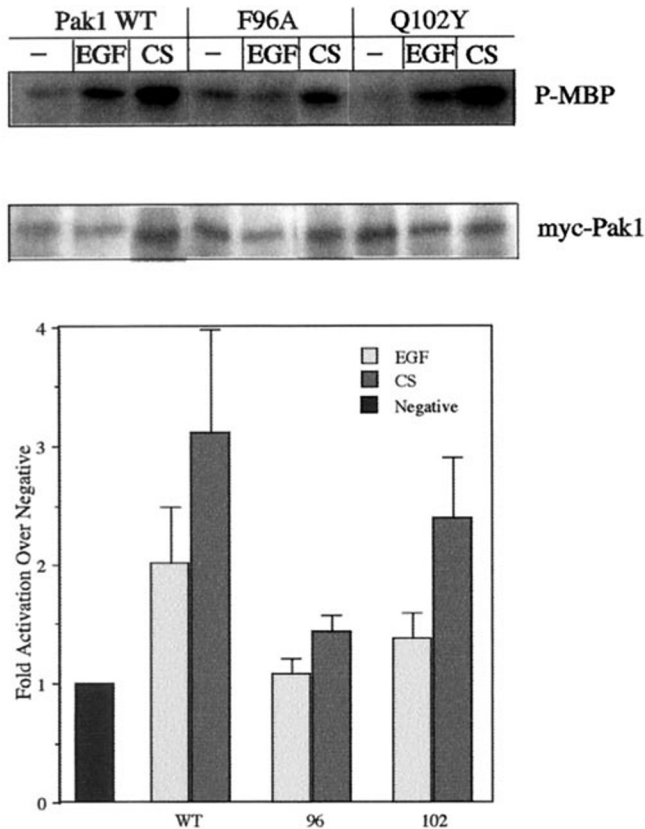
these mutants, which do not detectably co-precipitate with Rac1, were nevertheless partially activated by this GTPase (33 and 50% of wild-type value, respectively). These data suggest either that Pak1, even when overexpressed as a transgene, is limiting in cells or that weak binding to the GTPase is sufficient for activation. In either case, the fact that these two mutants retain full activation by Cdc42 but compromised activation by Rac1 means that in cells, the Pak1 F96A and Q102Y mutants, unlike the previously described Pak1 K66L/K67L/K68L mutant (25), can be used to assess the contributions of Cdc42 versus Rac1 to Pak1 activation.

**Pak1 Mutants Defective in Rac1 Binding Are Not Activated by Ras**—Previous published results suggest that Ras activates Pak1 mainly through a Rac or Cdc42 pathway (17). We used our Pak1 binding mutants to distinguish whether Ras operates primarily through Rac or Cdc42. If Ras activates Pak1 via Rac, then Ras should fail to fully activate mutants of Pak1 that cannot bind Rac, whereas the opposite would hold true whether Ras activates Pak1 via Cdc42. To distinguish these possibilities, we co-transfected 3Y1 cells with a control vector or an activated form of Ras plus either wild-type or mutant forms of Pak1. These mutant forms included a kinase inactive mutant



**FIG. 6. Activation of full-length Pak1 point mutants by Ras.** Full-length Myc-tagged Pak1 and mutants of Pak1 were transfected into 3Y1 cells alone or with an activated (V12) non-epitope-tagged form of Ras. The cells were harvested at 16 h post-transfection, and the lysates were immunoprecipitated with anti-Myc antibodies. The immunoprecipitated Pak1 was then incubated with 1  $\mu$ Ci of [ $^{32}$ P]ATP in kinase buffer containing 20  $\mu$ M ATP. Phosphorylation of Pak1 and Histone H4 in response to Ras EJ activation was visualized by autoradiography. Protein expression of Pak1 wild type (*wt*) and mutants, along with Ras expression, was visualized by immunoblotting followed by autoradiography. The data shown are representative of four independent assays. The bar graph shown represents the mean value with standard error.

(K299R) and the Cdc42 but not Rac1-activable mutants F96A and Q102Y. Myc-tagged Pak1 was immunoprecipitated and tested for the ability to autophosphorylate (Fig. 6). Ras was able to induce the activation of wild-type Pak1 4-fold, consistent with published reports (17, 18). The Pak1 K299R mutant was not activated by Ras, demonstrating that the kinase assays indeed are measuring Pak activity. The F96A mutant, which is fully activated by Cdc42 but poorly activated by Rac1, was not activated by Ras, whereas the Q102Y mutant, which is fully activated by Cdc42 but only about 50% by Rac1, was activated nearly 50% by Ras. Together, these data are most consistent



**FIG. 7. Activation of Pak1 mutants by growth factors.** Full-length Myc-tagged Pak1 and mutants of Pak1 were transfected into Swiss-3T3 cells. At 24 h post-transfection, the cells were serum-starved for 24 h and then treated for 5 min with either empty vehicle, 250 ng/ml EGF, or 10% calf serum (CS). Pak1 was immunoprecipitated and then incubated with 1  $\mu$ Ci of [ $^{32}$ P]ATP in kinase buffer containing 20  $\mu$ M ATP and 5  $\mu$ g of MBP (Sigma). Phosphorylation of MBP by the Pak1 proteins was visualized by autoradiography. Protein expression of the tagged Pak1 proteins and Rac1 and Cdc42 was visualized by immunoblotting followed by autoradiography. The data shown are representative of at least three independent experiments. The bar graph shown represents the mean value with standard error. *wt*, wild type.

with a Ras-Rac-Pak1, rather than a Ras-Cdc42-Pak1, signaling pathway.

**Analysis of Growth Factor Activation of Pak1**—We tested the ability of EGF and calf serum to stimulate wild-type and mutant Pak1. EGF treatment of cells expressing wild-type Pak1 resulted in about 5-fold stimulation of this kinase (Fig. 7). The F96A mutant, which is severely compromised in Rac binding, did not respond to EGF, whereas the Q102Y mutant, which retains about 50% Rac binding, was activated 2.2-fold. These data are most consistent with a model in which EGF signals are transmitted primarily through Rac. In contrast, calf serum caused an 8.5-fold activation of wild-type Pak1, but this activation was unaffected by the Q102Y mutation and was only partly lost in the F96A mutant. As these mutants bind Rac poorly, these data suggest that calf serum acts through other GTPases to activate Pak1.

#### DISCUSSION

Pak1 is an unusual GTPase effector in that it binds to and is activated by both Cdc42 and Rac. Most PBD-containing proteins, such as MRCK (30–32), WASP (33), and neural (N)-WASP (34), Pak4 (35), and the Borg (36) and SPEC (37) proteins, associate selectively with Cdc42-subfamily proteins only. The basis for these binding differences can be explained, at least in part, by differences within the PBD of Pak1 *versus* the

PBD of these other proteins. However, it is apparent from previous publications (25) as well as from the data presented here that mutations outside the Pak1 PBD can also affect its binding specificity to small GTPases.

What is the basis for the binding specificity of Pak1? The Pak1 protein can be divided into two functional domains, the catalytic domain, which contains the protein kinase function, and the regulatory domain, which contains the PBD, the auto-inhibitory domain (38), and binding sites for various signaling proteins including PIX (39, 40) and Nck (20, 41, 42). The functional binding domain for Rac and Cdc42 was originally described as a region from amino acids 67 to 149 (43) and later narrowed down to a 16-amino acid stretch variously referred to as the PBD (p21 binding domain), G-protein binding domain, or CRIB (cdc42/rac interactive binding) domain. This domain is present in all members of the Pak family and also in a number of other potential Rac/Cdc42 effector proteins and is required for GTPase binding (44). However, the smallest fragment of Pak1 to bind with high affinity to both Rac and Cdc42 is a fragment from amino acids 74 to 132 (45), although the 44-residue fragment 74 to 118 (45) and the 46-residue fragment 67 to 113 (46) also display measurable binding. These data indicate that Pak1 residues between 118 and 132 (C-terminal to the CRIB motif) contribute to stable binding to Rac and Cdc42. Recently, the structure of the Pak1 fragment 75–118 was established both in free form and in complex with Cdc42 along with the PBD structure of ACK and WASP, which bind specifically to Cdc42 and not to Rac (47). The N-terminal region of the Pak1, ACK, and WASP fragments (which contains the entire PBD) is quite homologous and interacts with Cdc42 in a similar manner, forming an intermolecular  $\beta$  sheet by binding to the  $\beta$ 2 strand of Cdc42. The structures of the C-terminal portions of the three fragments diverge; however, these differences are unlikely to explain their different binding properties because this region contacts the switch II region in Cdc42, which is identical to that of Rac. Instead, the ability of Pak1 to bind both GTPases may be explained by the relatively flexible interactions between the N-terminal segment of the Pak PBD with the C-terminal  $\alpha$ 5 helix in Rac and Cdc42. The orientation of Ile-75 in Pak1 is such that it is likely to be able to stack up against Leu-174 in Cdc42 and against the methylene group of Arg-174 in Rac1 (47). In contrast, the equivalent residues in ACK and WASP are unlikely to accommodate Arg-174 in Rac1. The notion of Pak1 flexibility *versus* ACK and WASP rigidity is reinforced by mutational analysis of Cdc42, which shows that mutation of amino acid 174 in the  $\alpha$ 5 helix of Cdc42 reduces the affinity of this GTPase to WASP and to ACK ~30-fold but to Pak1 by only 2.5-fold (48).

Past mutational analyses of Pak1 have been carried out to investigate the requirements for binding to small GTPases (9–11, 25, 38). However, few binding studies have compared the effects of Pak1 mutation on association to Cdc42 with Rac1. The sole exception of which we are aware is the study of Knaus *et al.* (25), in which mutation (K66L/K67L/K68L) in Pak1 was shown to abolish binding to Rac1 while compromising binding to Cdc42 to a lesser extent (this study). However, this mutation also rendered Pak1 unable to be activated by either GTPase. This property makes this mutant unsuitable for studies designed to evaluate the contributions of Rac *versus* Cdc42 in Pak1 activation.

The three most useful mutants uncovered in our screen were P78T, F96A, and Q102Y. These three mutations are located within the boundaries of the PBD. As the structure of the Pak1 PBD in complex with Cdc42 has been determined, it is possible to evaluate these point mutants in the context of atomic interactions. Pro-78 in Pak1 is highly conserved among PBD-con-

taining proteins. This residue packs against the Cdc42  $\alpha$ 1 helix and  $\beta$ 2 strand. As the  $\alpha$ 1 helix is identical in Cdc42 and Rac, the differential binding effects of the Pak1 P78T mutant are likely to be related to interactions with the  $\beta$ 2 strand of the two GTPases. The most striking difference in the  $\beta$ 2 strand of these two GTPases is at position 43, where Cdc42 has a Thr residue and Rac has an Asn. Presumably, the P78T mutation is structurally more easily tolerated by Cdc42 than by Rac. Interestingly, although the P78T mutation described here mildly reduces binding to Cdc42 but nearly abolishes binding to Rac, mutation of Pro-78 to Ala severely compromises binding to Cdc42 (38). That small structural changes (*i.e.* the substitution of Thr *versus* Ala at residue 78) can give rise to such large, unpredictable, and useful binding properties bespeaks the utility of the randomized, nonbiased genetic approach used in this study.

The differential binding effect of the Pak1 F96A and Q102Y mutations are more difficult to explain. The F96A mutation lies within a  $\beta$ -hairpin conformation in Pak1. This residue interacts with Leu-19 in the  $\alpha$ 1 helix of Cdc42. As the  $\alpha$ 1 helices are composed of identical residues in Cdc42 and Rac, it is unlikely that the differential binding we observed with the Pak1 F96A mutant can be explained on the basis of interactions with Leu-19 in either GTPase. Instead, we theorize that the substitution of the smaller Ala residue at this position distorts the  $\beta$ -hairpin structure in Pak1 and displaces the  $\alpha$  helices on either side. In the case of the Pak1 Q102Y mutation, Gln-102 is contained within a short helix of Pak1 that contacts Leu-67 and Leu-70 in the switch II region of Cdc42. As mentioned previously, the switch II regions are identical in Cdc42 and Rac, so any important changes in the structure of the short helix of Pak1 would be expected to affect binding to both Cdc42 and Rac. However, Gln-102 of Pak1 does not make direct contacts with residues in switch II of Cdc42. It is possible that the effects of mutating this residue are due to more distant conformational effects rather than alterations in the short helical structure in Pak1 that contacts switch II. Alternatively, binding to the GTPase switch II region may indeed be altered by the Q102Y mutation, but this interaction may be more critical for the stable binding of Pak1 to Rac than to Cdc42.

In transiently transformed rat1 cells, Ras has been reported to activate Pak1 (17, 18). Perhaps the most plausible model for Pak1 activation by Ras is as follows. Ras activates PI3 kinase, which in turn stimulates a guanine nucleotide exchange factor such as Tiam1, which then activates Rac and then Pak1. However, the role, if any, of Cdc42 in the activation of Pak1 by Ras has not been firmly established. In addition, other molecules, including Akt (18) and perhaps PKD1 (49), may play a role in this signaling pathway. The Pak1 mutants described in this study enable us to shed some light on these issues. The Pak1 mutants that are defective in Rac binding are defective in activation by Ras. These Pak1 mutants are fully activated by Cdc42, making it unlikely that Cdc42 plays a significant role in mediating the activation of Pak1 by Ras. Instead, the magnitude of activation of Pak1 by Ras correlates precisely with the degree to which Pak1 binds Rac. These data indicate that the primary connection between Ras and Pak1 in rat 3Y1 cells is via Rac, not Cdc42. Akt is unlikely to play a major role in this system, although we have not formally excluded this possibility.

In this study the Pak1 GTPase binding mutants were used to show that Ras and EGF appear to signal through Rac, whereas calf serum is not Rac-specific. It should be possible to use these Pak1 mutants to analyze additional signaling pathways. For example, the role and identity of small GTPases in mediating Pak1 activation by stressors such as UV irradiation and other

DNA damaging agents has not been determined. Similarly, the relative role of Cdc42 *versus* Rac in adhesion-mediated Pak1 activation is not clear. In addition, the method used here to generate and identify partner-specific Pak1 mutants could also be applied to other problems involving signaling proteins that associate with multiple partners. For example, the oncogenic forms of several GEFs, such as Db1, Lfc and Lbc, bind to and activate more than one GTPase (50). It would be of interest to use the dual-bait screening procedure described here to isolate GEF mutants that selectively activate only one GTPase and examine the signaling and transforming potential of such molecules.

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