Molecular Cloning of a New Member of the p21-Cdc42/Rac-activated Kinase (PAK) Family*

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A number of "target" proteins for the Rho family of small GTP-binding proteins have now been identified, including the protein kinases ACK and p65PAK (Manser, E., Leung, T., Salihuddin, H., Zhao, Z-S., and Lim, L. (1994) Nature 367, 40-46). The purified serine/threonine kinase p65PAK has been shown to be directly activated by GTP-Rac1 or GTP-Cdc42. Here we report the cDNA sequence encoding a new brain-enriched PAK isoform β-PAK, which shares 79% amino acid identity with the previously described α-PAK-isoform. Their mRNAs are differentially expressed in the brain, with α-PAK mRNA being particularly abundant in motor-associated regions. In vitro translation products of the α- and β-PAK cDNAs exhibited relative molecular masses of 68,000 and 65,000, respectively, by SDS-polyacrylamide analysis. A specific β-PAK peptide sequence was obtained from rat brain-derived CNS-PAKs exhibited an increase in kinase activity mediated by GTP-p21 induced autophosphorylation. Cdc42 was a more potent activator in vitro of α-PAK kinase, and the fully-activated enzyme is 300 times more active than the unphosphorylated form. Interestingly the down-regulation in the binding of p21s to recombinant β-PAK and brain p65PAK, which is observed upon kinase activation does not occur with recombinant α-PAK.

Morphological roles for the most common members of the mammalian Rho family of small GTP-binding proteins, Rac1, RhoA, and Cdc42, have been established in fibroblasts (Ridley et al., 1992; Ridley and Hall, 1992; Kozma et al., 1995). Cdc42 in Saccharomyces cerevisiae is required for cell budding and may provide the polarization signal at this site (Ziman et al., 1993); in fibroblasts filopodial formation is dependent on the closely related mammalian homologue of Cdc42 (Kozma et al., 1995, 1996; Nobs and Hall, 1995). Although an increasing number of p21 Rho GTPase-activating proteins (GAPs) have been identified (for review, see Lamarche and Hall (1994)), there is as yet no evidence that they are able to exhibit effector function. These proteins can be identified by sequence homology to the GAP domain and by their activity in overlay assays (Manser et al., 1992). The most closely related RhoGAPs comprise the chimaerin family (Hall et al., 1993; Leung et al., 1993, 1994), acting on Rac and whose activity is regulated through a protein kinase C-like cysteine-rich domain (Ahmed et al., 1993). Although many RhoGAPs are somewhat promiscuous in vitro, they appear to show distinct p21 specificities in vivo (Ridley et al., 1993).

The prototype small GTP-binding protein p21-Ras is an oncogene that has effector targets which include Raf kinases (Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993) and phosphatidylinositol 3-kinase (Rodriguez-Viciana et al., 1994) and RasGAP itself (Schweighoffer et al., 1992). The use of the p21 GTP/GDP cycle is exemplified by the role of Ras in growth factor signal transduction, where GTP-Ras functions to activate proteins of the “mitogen-activated protein kinase cascade” through the serine/threonine kinase Raf (Warne et al., 1993), and MEK kinase (Lange-Carter and J ohnson, 1994). It seems probable that part of p21 Rho family signaling also occurs through associated kinases for which the prototypes are the activated Cdc42-associated tyrosine kinase p120-ACK (Manser et al., 1993) and the Cdc42- and Rac1-activated kinase p65-PAK (Manser et al., 1994). Both ACK and PAK inhibit intrinsic as well as GAP-stimulated GTPase activity of the p21s. PAK belongs to a family of kinases that includes the S. cerevisiae STE20 gene product (Leberer et al., 1992; Ramer and Davis, 1993) which acts upstream of the pheromone response mitogen-activated protein kinase cascade (Ammerer, 1994). Two other related S. cerevisiae kinases Cla4p2 and a putative gene product present in the yeast genome we designate as Sc-PAK show homology to PAK in their putative kinase and Cdc42-binding domains.

The use of a [γ-32P]GTP-p21 overlay technique has allowed us to identify at least eight mammalian candidate target proteins for Rac1, Cdc42, and RhoA (Manser et al., 1994). The brain-enriched p65PAK co-purified with a number of kinases of similar size also identified in [γ-32P]-GTP-Cdc42 overlays. A human PAK designated hPAK 65 has been reported to be ubiquitously expressed (Martin et al., 1995) and probably represents the human homologue of the ubiquitous rat p62 Cdc42/Rac1 binding protein. Thus although PAK kinases are most abundant in the brain, they appear to be a common target for Cdc42 and Rac "molecular switches" in all mammalian cells. Two mammalian Cdc42 isoforms have been identified

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1 The abbreviations used are: GAP, GTPase-activating protein; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PAK, p21 (Cdc42/Rac1)-activated protein kinase; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; MBP, myelin basic protein; GTP-γS, guanosine 5′-O-(thiotriphosphate).
We describe a novel PAK cDNA (designated $b$-PAK) encoding a protein which is closely related to our previously published sequence (now termed $a$-PAK) and also to the hPAK65 cDNA (Martin et al., 1995). The putative protein products exhibit remarkable conservation of amino acid residues in their kinase and p21-binding domains. Despite this similarity, it has been possible to establish the relationship between the $a$- and $b$-cDNAs and PAK species found in the brain based on differences in their biochemical properties. In vitro translated $a$- and $b$-PAK exhibit relative molecular masses of 68,000 and 65,000 Da, respectively. A peptide sequence derived from purified p65PAK has been found to be specific for the $b$-PAK isoform. We show that, in a manner similar to purified p65PAK, binding of Cdc42 to recombinant $b$-PAK kinase, but not to $a$-PAK, is down-regulated upon kinase activation.

MATERIALS AND METHODS

Isolation of Rat Brain $b$-PAK cDNAs—A subclone containing bovine PAK cDNA derived from polymerase chain reaction (PCR) with degenerate primers and encoding part of the kinase domain (Manser et al., 1994) was used as a probe to screen a rat brain cortex cDNA library (LZAP, Stratagene). Two filters (Hybond N, Amersham Corp.) containing DNA from $3 \times 10^5$ bacteriophage plaques were hybridized overnight in $0.5 \times $ sodium phosphate (pH 6.8), $7\%$ SDS, $10\%$ formamide at $55^\circ$C and washed in $1 \times $ SSC at $55^\circ$C. Positive plaques were taken through further rounds of purification and excised in vivo as plasmids (in pBluescript SK) according to the manufacturer's protocol. Three similar clones were identified whose restriction maps did not correspond to $a$-PAK. One of these clones contained the complete coding sequence of a predicted 61-kDa protein ($b$-PAK). The region from this clone shown in Fig. 1 was sequenced completely on both strands using restriction subclones and specific oligonucleotide primers. Because of the presence of nonconservative residues at $a$-H73, C92, and N258 in our published sequence (Manser et al., 1994), we resequenced these regions of the $a$-PAK cDNA with oligonucleotide primers to resolve apparent sequencing ambiguities. The cDNA was confirmed to encode proline at position 73, valine at position 92, and serine at position 258 (Fig. 2); the GenBank entry has been amended appropriately.

Determinations of the localization of $a$- and $b$-PAK mRNAs in rat brain were performed by in situ hybridization using 35S-labeled oligonucleotide probes.}

**Fig. 1.** Nucleotide sequence of rat $b$-PAK. The numerals on the left and right side of the sequence indicate the nucleotide and predicted amino acid positions, respectively. Regions encompassing the p21-binding and kinase domains have been marked in bold. The N-terminal sequence of a 34-kDa cyanogen bromide-generated peptide derived from purified p65PAK is underlined.
with horseradish peroxidase-anti rabbit antibodies (3,3'-diaminobenzidine) according to standard protocols.

Extract Preparation and [γ-32P]GTP-Cdc42 Overlays—Tissue extracts were prepared by dounce homogenization of fresh tissue in 10 mM Tris (pH 8.0), 0.5 mM dithiothreitol, with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotonin, pepstatin, and leupeptin, Sigma). The supernatant fractions after 100,000 × g 30 min spin were stored at −70 °C with 5% added glycerol. Rho-p21-binding proteins were detected as described previously (Manser et al., 1994).

Expression and Purification of Recombinant Proteins—Recombinant glutathione S-transferase (GST)/α-PAK was expressed from pGEX-2T plasmid (BamHI/EcoRI cut) containing α-PAK cDNA sequence that includes the initiator methionine. At the 5' end (N terminus) a PCR-engineered BamHI linker sequence (5' GGA TCC ACA ATG-3') was inserted, and at the 3' end an EcoRI cloning site was used. GST/α-PAK 1-251 was subsequently created by removing the cDNA coding sequence from the internal EcoRI site to the 3' EcoRI site and ligating the flushed ends. Recombinant β-PAK was expressed from pGEX-4T-2 (Smal/XhoI cut) containing cDNA sequence having at the 5' end a PCR-engineered Smal linker sequence (CCC GGG AAA ATG-3') and at the 3' utilizing the 5'-plasmid-derived XhoI site. PCR-derived sequences were in all cases checked by sequencing. These constructs were introduced into the BL21 Escherichia coli strain for protein production. Cells grown to OD600 (1 cm) of 0.6 in 50 μg/ml ampicillin were induced for 4–6 h at 25 °C in the presence of 0.5 mM isopropl-1-thio-β-D-galactopyranoside. Fusion proteins of recombinant kinases and p21s were purified on glutathione-Sepharose (Pharmacia-LKB) as described previously (Manser et al., 1992).

Antibody Purification—Recombinant GST/α-PAK (200 μg) was used as an immunogen in rabbits at 4-week intervals. Serum was collected and affinity-purified on 2 mg/ml GST/α-PAK coupled to cyanogen bromide-activated Sepharose (Sigma) and eluted with 100 mM glycine-HCl (pH 2.5), 0.05% Triton X-100. The first 2 column volumes were neutralized with Tris(HCl) (pH 8.5) and used at 1:500 dilution for Western blots.

Western Blots and Immunoprecipitation—Filters were blocked for 1 h in 3% skimmed milk prior to incubation with primary anti-PAK antibodies (1:500) in phosphate-buffered saline, 1% bovine serum albumin, 0.1% Triton X-100 for 2 h at room temperature. Filters were incubated with 1:4000 dilution of horseradish peroxidase-coupled second antibodies (DAKO) for 1 h. Bands were visualized with hyphenin in the presence of luminol (Amer sham). For immunoprecipitation the rabbit reticulocyte lysate was diluted to 4 mg/ml in tissue extraction buffer. Extracts (100 μl) were incubated with 10 μl of antibody-purified antibody for 1 h at 4 °C collected on 50 μl of protein A-Sepharose (Sigma), washed with 200 μl of extraction buffer, then 400 μl of phosphate-buffered saline + 1% Triton X-100 and eluted in SDS sample buffer.

Protein Kinase Assays—Kinase reactions were carried out in buffer containing 50 mM HEPES (pH 7.3), 10 mM MgCl2, 2 mM MnCl2, 1 μM dithiothreitol, and 0.05% Triton X-100 with 20 μM [γ-32P]ATP. For “in-gel” kinase reactions standard 9% polyacrylamide gels were cast containing 0.1 mg/ml myelin basic protein and processed as described (Kameshita et al., 1989). In-gel phosphorylation was performed in kinase buffer containing 10 μM CaCl2 of 10 μM [γ-32P]ATP for 30 min, then gels washed in phosphate-buffered saline (2 × 10 min), stained with Coomassie Blue dye, and dried for autoradiography.

RESULTS

Isolation of cDNAs Encoding a New p21-activated Kinase—Overlays of SDS-polyacrylamide electrophoresed tissue extracts with [γ-32P]GTP-labeled Cdc42 have indicated that there are a number of different proteins of M, 62,000-88,000 with similar p21 binding characteristics as the purified p65-PAK (Manser et al., 1994). Of these, the p62 protein is apparently present at similar concentrations in all tissues, whereas the larger species are more highly expressed in the central nervous system. A rat brain cortex cDNA library was screened with the 400-base pair bovine PCR-generated cDNA corresponding to...
bovine α-PAK kinase domain sequence. In addition to the clone reported previously (Manser et al., 1994), three clones from a total of 30 “positives” in this screen were found by restriction mapping to consist of overlapping sequences derived from mRNA of a related kinase. One encompassed the entire coding region of this kinase, and a region of 2247 nucleotides was sequenced on both strands (Fig. 1). The entire open reading frame of 544 amino acids, from nucleotide 61 to 1693, shows a
striking similarity to the rat PAK we described previously and hPAK65 (Martin et al., 1995) as shown in Fig. 2. We term the related kinases we have isolated α- and β-PAK. The α- and β-PAKs show 74% identity at the DNA level and 79% identity at the amino acid level. The hPAK65 amino acid sequence is equally related to α- and β-PAKs and is almost identical to a rat isoform we have designated as γ-PAK. All three mammalian PAKs have conserved p21-binding and kinase domains which they share with the three yeast PAK-like kinases (Fig. 2). The mammalian PAKs also show conservation outside of these regions; the greatest sequence divergence between them is in the “linker” sequence of −100 amino acids that lies between the p21-binding and kinase domains. The N-terminal hPAK65 sequence appears truncated relative to the rat α- and β-isosforms (Fig. 2; see “Discussion”).

Expression Pattern of α- and β-PAK mRNAs—Labeled divergent cDNA sequences of α- and β-PAK were used to probe Northern blots containing mRNA isolated from various rat tissues (Fig. 3). The 4-kilobase α-PAK mRNA was expressed predominantly in the brain and at lower levels in the spleen. Surprisingly β-PAK (with a 1.7-kilobase open reading frame) appears to be translated from a 9-kilobase mRNA only detected in brain and at low level in testis (the relative weakness of the β-PAK signal was consistent with in situ hybridization results).

We performed in situ hybridization with specific oligonucleotides to determine the regional expression pattern of the two kinases (Fig. 4). α-PAK mRNA was more abundant than β-PAK mRNA, and exposure times were chosen to determine brain regional distribution of α- and β-mRNAs, rather than to compare their absolute levels. Equivalent sections hybridized to α- and β-specific probes are shown side by side (Fig. 4). In both cases sense oligonucleotide probes were used to determine “background” signals (data not shown).

α-PAK mRNA was expressed in the cortex with highest levels of hybridization over cell layers IV and V, in limbic regions of the cortex, and in piriform cortex (Fig. 4A). β-PAK mRNA was also relatively high in piriform cortex while in the cortex enriched in layers II–III and V. Both mRNAs were expressed in the hippocampal formation (Fig. 4B) in both the CA1 and dentate gyrus. α-PAK mRNA was highly expressed in subiculum (Fig. 4C) and showed some of its highest expression in the ventral tier nuclei of the thalamus. Here β-PAK mRNA exhibited only low expression, but showed greater enrichment in the hypothalamus; in medial preoptic (Fig. 4A), ventromedial and arcuate nuclei (Fig. 4B). Relatively high levels of β-PAK mRNA were present in the monoaminergic dorsal raphe nucleus (Fig. 4C) and locus coeruleus.

Interestingly, α-PAK mRNA was highly expressed in a number of neuronal groups associated with motor function, including the pontine nucleus, reticulotegmental, external cuneate, and lateral reticular nuclei, which send mossy fiber input to the cerebellum (Fig. 4D and E). α-PAK mRNA was also highly expressed in scattered neurons in the pontine and medullary reticular formation, and in patches of cells comprising the linear nucleus of the medulla, with moderate expression in the inferior olivary nucleus which provides climbing fiber input to the cerebellum (see Fig. 5 for detailed comparisons with protein expression). Both α- and β-PAK mRNAs were highly enriched in the embryonic central nervous system (Fig. 4F) with relatively little expression elsewhere, confirming the specificity of the probes.

Cellular Localization of α-PAK Expression in the Central Nervous System—Within the nervous system we sought to establish which type of cells might be responsible for the strong expression of α-PAK. Fig. 5 demonstrates that in a mid portion of the medulla there was co-localization of the in situ hybridization signal and anti-α-PAK immunoreactivity to the linear nuclei. High powered photomicrographs of these regions showed large neurons with high densities of silver grains adjacent to smaller unlabeled cells (which only show counter-
treatment in SDS sample buffer to disrupt complexes. As controls, expression of immunoreactive. In the cerebellum, there were high levels of both the cell bodies and processes were strongly staining. Within large neurons of the pontine reticular formation both the cell bodies and processes were strongly staining. At higher magnification could be localized to granule cells, basket cells, and deep cerebellar nuclei (data not shown).

The β-PAK cDNA encodes p65PAK—β-PAK antibody immunoprecipitated both α- and β-PAK in vitro translated proteins derived from the two cDNAs (utilizing the endogenous ATG start codons Fig. 6B). The relative masses of the translation products were 68,000 and 65,000 Da, respectively. This is consistent with the presence of three distinct bands in brain extracts, representing the different PAK isoforms, as detected by Cdc42 overlay (Fig. 6A). Heat treatment of the in vitro products in SDS buffer to release the immunoprecipitates caused some streaking but did not alter the relative mobility of the kinases compared with the unheated proteins (Fig. 6B, first two lanes). The mobility of the in vitro translated proteins suggests that α-PAK corresponds to the p68 Cdc42-binding protein detected in brain extracts (Manser et al., 1994), and β-PAK to a brain protein that elutes last from the Cdc42 affinity column during protein purification (the previously described p65PAK).

Tryptic peptides obtained previously from within the kinase domain of affinity-purified bovine PAK corresponded to regions of α- and β-PAK which are identical in sequence. However confirmatory evidence for the presence of β-PAK in affinity-purified rat brain PAK (Fig. 6C, lane 3) was obtained from a cyanogen bromide digest of the protein. The peptides were subjected to SDS-PAGE, transferred to PVDF membrane, and overlaid with [γ-32P]GTP-Cdc42. Lanes 1 and 2, with 45 and 5 μg of digested protein(s), contains a Cdc42-binding polypeptide with apparent mass of 34 kDa which was subjected to N-terminal amino acid sequencing. The obtained sequence (M)APEEXNXAXLXXIFPGGG was not informative at every position but corresponds to the predicted β-PAK product fol-
lowing cleavage at M37 (underlined in Fig. 1). Since this region of \( \beta \)-PAK is divergent from both \( \alpha \)-PAK and hPAK65 sequences, it is highly likely that the peptide derived from the \( \beta \)-PAK gene product. Although a mass of \( >30 \) kDa for a CNBr-generated peptide of \( \beta \)-PAK is not consistent with its C terminus being derived from cleavage at \( \beta \)-M138, there are many documented cases of methionines which show inefficient CNBr cleavage, and lower bands were also detected by \( \gamma^{32P} \)GTP-Cdc42 (Fig. 6C). We cannot estimate the stoichiometry of \( \alpha \) and \( \beta \)-PAK by CNBr digestion and overlay because the \( \alpha \)-PAK p21-binding domain is probably destroyed by cleavage at an internal methionine (\( \omega \)-M99), a position occupied by a conservative isoleucine in \( \beta \)-PAK (I94).

Tissue Distribution of PAK Using Anti-\( \alpha \)-PAK Antibodies—Affinity-purified antibodies to \( \alpha \)-PAK residues 1–251 showed strong immunoreactivity to proteins of 65–68 kDa on Western blots of brain extracts (Fig. 7A). Similar sized proteins were recognized by the antisera in spleen also expressing the \( \alpha \)-PAK mRNA. In the testis an immunoreactive band was detected at longer exposure times whose mobility was greater than the broad band detected in the spleen (Fig. 7B), consistent with the presence of testicular \( \beta \)-PAK mRNA. It is probable that the presence of multiple bands on Western blots is also the result of different phosphorylation states of the PAKs in vivo. All of these immunoreactive bands corresponded to proteins larger than the ubiquitous p62 band detected by \( \gamma^{32P} \)GTP-Cdc42 overlay (Fig. 7C). As discussed later, the p62 is also a PAK isoform we designate as \( \gamma \)-PAK, but which is not recognized by the antisera.

Cdc42 and Rac1 Activate Recombinant PAK—We have expressed full-length \( \alpha \)- and \( \beta \)-PAK as \( \sim 95 \) kDa GST fusion proteins in E. coli to study kinase activation using homogeneous preparations. Because the \( \beta \)-PAK sequence contained additional polylinker-derived sequence, it has a lower mobility than recombinant \( \alpha \)-PAK. Thrombin cleavage of this GST/PAK fusion protein caused some internal cleavage of the kinase and thus gave truncated products. We therefore used the GST fusion protein, since it showed similar p21 activation to the “native” purified protein (Manser et al., 1994). Fig. 8A shows the activities of glutathione-Sepharose-purified GST/PAK in the absence and presence of GST/Cdc42. Autophosphorylation of recombinant \( \alpha \)- and \( \beta \)-PAK and concomitant phosphorylation of myelin basic protein (MBP) substrate were significantly activated in vitro by adding purified recombinant \( \gamma^{32P} \)GTPS-Cdc42. This was observed previously with affinity-purified native brain PAK, which from data described in this paper corresponds to \( \beta \)-PAK gene product. The recombinant \( \beta \)-PAK exhibited higher basal activity, which might be related to its phosphorylation state in E. coli. Since the behavior of the p68 shows \( 32P \)-phosphorylated proteins. B, effect of various p21s on GST/PAK activity; Rho-p21s (1 \( \mu \)g) were preloaded with the appropriate nucleotide (0.5 mM) and incubated with 0.5 \( \mu \)g of GST/PAK fused to 0.1 mg/ml MBP. Following the denaturation/renaturing steps (see “Materials and Methods”), \( \gamma^{32P} \)ATP was added to the gel in kinase buffer to detect MBP kinase activity (2-h exposure). D, activity of inactive and Cdc42-activated \( \alpha \)-PAK (A); 2 \( \mu \)g of recombinant kinase was dialyzed for 2 h against kinase buffer and incubated at 32°C with 20 \( \mu \)g of MBP (total volume, 100 \( \mu \)l) with 50 \( \mu \)M \( \gamma^{32P} \)ATP. At the times shown 25-\( \mu \)l aliquots were removed, quenched by the addition of SDS buffer and fractionated on a 12% gel. Radioactivity associated with MBP is in arbitrary units (an averaged value of three determinations is shown); errors for the unactivated PAK (■) were within the values covered by the symbol.

![Fig. 8. Recombinant PAK kinases are p21-activated.](http://www.jbc.org/Downloaded from http://www.jbc.org)
α-PAK has not been described previously, we examined its activation in more detail. Activation of recombinant α-PAK occurred only with GTPγS-Rac and GTPγS-Cdc42, but not GTPγS-RhoA (Fig. 8B). In their GDP forms all p21s were inactive (exchange of GDP into GTPase-deficient GTP-Cdc42 binding protein probably being incomplete during the standard 4-min period). Quantification of the MBP phosphorylation (indicated as relative activity) showed that GTP-Cdc42 was more effective than GTP-Rac1 under these assay conditions.

Following complete activation (1 h in the presence of GTPγS-Rac or GTPγS-Cdc42), α-PAK exhibited slower mobility under SDS-polyacrylamide electrophoresis (Fig. 8C), as seen with the purified protein (Manser et al., 1994) and many other protein kinases. The hyperphosphorylated kinase exhibited some size heterogeneity in its activated state. After separation from the GTPγS-p21s by SDS-PAGE, α-PAK was still active as determined by an in-gel kinase assay against MBP (Fig. 8C). No labeling was seen in the absence of MBP in the gel (data not shown). This supports the model we presented previously in which the autophosphorylated kinase, after dissociation of p21, would remain active in the absence of dephosphorylation. The ability of this active kinase to phosphorylate MBP was tested (under similar conditions to the co-activation assay in Fig. 8B). Based on the initial rates of phosphorylation (Fig. 8D), the activity of the phosphorylated form was found to be more than 300 times higher than the unphosphorylated α-PAK.

Activation of β-PAK Leads to a Decrease in p21 Binding—We had observed previously that purified p65βPAK upon activation exhibited lower affinity for both GTP-Rac1 and GTP-Cdc42 in overlay assays (Manser et al., 1994). Similar experiments were conducted with recombinant α- and β-PAK proteins. Fig. 9A shows the binding of [γ-32P]GTP-Cdc42 to normal and activated kinase in a typical overlay experiment. Averaged data from three independent experiments are shown below. The data clearly demonstrated that only β-PAK exhibits down-regulation of p21 binding upon activation. Since this effect is probably related to the phosphorylation state of the kinase, we sought to mimic the effect using the N-terminal of β-PAK (and of α-PAK both expressed as a GST fusion proteins cf Fig. 9C) exogenously phosphorylated by full-length β-PAK. Although phosphorylation to completion could be observed by monitoring the upward mobility shift of the N-terminal fusion proteins, similar to that seen with the whole kinase (Fig. 9B), this did not result in any significant change in affinity for the [γ-32P]GTP-Cdc42 by β-PAK. It therefore seems that the down-regulation of Cdc42 binding either requires the whole β-kinase (conformational effects) or a specific intramembrane phosphorylation site is involved. Interestingly when the recombinant α-PAK kinase domain alone was expressed in E. coli, the fusion protein exhibited no kinase activity, nor could it be phosphorylated by full-length PAKs (data not shown).

The Rac1/Cdc42-binding domain of PAK shows sequence homology to the Cdc42-specific binding domain of the tyrosine kinase AEC (Manser et al., 1993). This region is highly conserved in α- and β-PAK (Fig. 2). Although PAK binding to Rac1 appeared significantly weaker than to Cdc42, their ability to activate the autophosphorylation and MBP kinase activity of purified p65-PAK depended on the assay conditions (Manser et al., 1994). As illustrated in Fig. 9C, by normalizing the labeling of the p21s using excess (cold) GTP in the "exchange" reaction, the amount of [γ-32P]GTP-Cdc42 or [γ-32P]GTP-Rac1 bound to the N-terminal region of α- and β-PAK in overlays was found to be the same (±10%). Note there is no potential autophosphorylation of the construct. The labeling of Rac1 with [γ-32P]GTP is normally poor, probably because of its high intrinsic GTPase activity during the exchange reaction in low magnesium buffer (Menard et al., 1992).

**DISCUSSION**

The heterogeneity in Cdc42-binding proteins with apparent molecular mass between 60 and 70 kDa in different tissues...
suggested these kinases to be expressed from a number of related genes. The identity and relationship of two of these kinases (α-PAK and β-PAK) have now been established through isolation of their cDNAs. Amino acid sequence comparison between the two mammalian PAKs described here and hPAK65 (Martin et al., 1995) reveals functionally important regions of the protein. In particular the p21-binding domain showed almost no sequence divergence. PAK kinases also contain polyacetic and proline-rich sequences between the p21-binding and kinase domains. The use of purified recombinant proteins has enabled us to confirm that addition of GTPγS-p21 was sufficient to activate the kinase in the absence of any factor that might have co-purified with brain-derived native PAK. This activation by Rac1 or Cdc42 was achieved through the p21-mediated autophosphorylation of the kinase. Recombinant (p68) α-PAK showed robust activity toward MBP in the presence of GTPγS-p21, as we have described for purified p65βPAK (β-PAK). Here we show with recombinant α-PAK protein that there is a 300-fold increase in activity following p21-mediated autophosphorylation.

While α-PAK was expressed predominantly in the brain, both mRNA and protein were detected in the spleen. Within the immune system it appears that PAK-related kinases are relatively abundant; the report that neutrophil p62 and p68 Cdc42/Rac-binding proteins are not reactive to antibodies to both mRNA and protein were detected in the spleen. Within matomammotropin, Seeburg (Martin Cdc42/Rac-binding proteins are not reactive to antibodies to both mRNA and protein were detected in the spleen. Within matomammotropin, Seeburg

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