Identification and Molecular Cloning of a p21<sup>cdc42/rac1</sup>-activated Serine/Threonine Kinase That Is Rapidly Activated by Thrombin in Platelets*

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The brain-enriched p21<sup>cdc42/rac1</sup>-activated serine/threonine kinase, p62<sup>PAK</sup>, was identified and purified on the basis of overlays with [γ-<sup>32</sup>P]GTP-Cdc42 onto SDS-fractionated proteins (Manser, E., Leung, T., Salihuddin, H., Zhao, Z.-S., and Lim, L. (1994) Nature 367, 40–46). In this study, the ubiquitously expressed p21<sup>cdc42/rac1</sup>-binding protein with relative molecular weight of 62,000 was purified from rat testes and shown to contain peptides related to PAK. It has thus been designated as the γ-PAK isoform (α- and β-isoforms being brain enriched). Isolation of γ-PAK cDNAs show that the kinase is highly conserved with α-PAK in both the p21 binding and kinase domains. The purified protein exhibited kinase activity that was activated by GTP-Cdc42 or GTP-Rac1 in vitro. In platelets, a p62 in situ reenative kinase was recognized by antibodies raised against γ-PAK. This thrombin-activated protein kinase appears to coprecipitate with another kinase of M, 86,000, suggesting that PAK may be part of a thrombin-responsive signaling complex.

MATERIALS AND METHODS

Purification and Peptide Analysis of p62<sup>PAK</sup>—p62<sup>PAK</sup> was purified by column chromatography at 4°C using a modification of the method described previously (17). Briefly, adult rat testes were quickly frozen and stored at −70°C. The tissue (60 g) was thawed, minced, and resuspended in 300 ml of buffer Q (25 mM MES-NaOH, pH 6.5, 0.5 mM MgCl<sub>2</sub>, 0.05 mM ZnCl<sub>2</sub>, and 0.05% Triton X-100) containing 0.5 mM phenylmethylsulfonyl fluoride and 1 μg/ml each of leupeptin, peptatin, and apronitin. The tissue was homogenized twice, 10 strokes each, with a Dounce glass homogenizer and centrifuged at 100,000 × g for 1 h. The supernatant was loaded onto a 100-ml S-Sepharose column in Q buffer, in which the column was eluted stepwise with 0.8 column volumes of buffer Q and NaCl gradient from 0.1 to 0.4 M. The p62<sup>PAK</sup>-containing fractions (eluted with 0.1 and 0.2 M NaCl) were pooled, diluted with an equal volume of buffer Q, and loaded onto a 30-ml Q-Sepharose column in Q buffer. The p62<sup>PAK</sup>-containing fractions (eluted with Q buffer containing 0.2 and 0.3 M NaCl) were pooled and adjusted to pH 7.5 with 1 ml Tris and loaded onto an 15-ml zinc-chelating Sepharose precharged with zinc (according to manufacturer’s protocol). The column was washed with 2 buffer (100 mM NaCl, 50 mM MES-NaOH, 1 mM EDTA) containing 0.5 mM phenylmethylsulfonyl fluoride and 1 μg/ml each of leupeptin, pepstatin, and apronitin. The fraction containing the peak of activity was pooled and dialyzed against 50 mM MES-NaOH, 1 mM EDTA, 1 mM DTT, and 1 μg/ml each of leupeptin, pepstatin, and apronitin. The dialyzed fraction was subjected to 15% polyacrylamide gel electrophoresis in the presence of 0.1% SDS. The gels were dried and autoradiographed. The peptide compositions of thrombin-activated γ-PAK dialyzed fraction were determined using a Mat Tek Tricolor peptide sequencer.
0.05% Triton X-100, pH 7.5. The p62\(^{AX}\) was eluted with Z buffer, pH 6.0, and with added 0.1 mM GTP-γ-S was loaded onto a 1-ml glutathione-Sepharose column containing GTP\(\gamma\)-S-glutathione 5'-transferase (GST)/Cdc42 (17). The column was washed with 3 volumes of Z buffer, pH 6.0; proteins were eluted with 3 volumes of Z buffer, pH 8.8, and lastly with 2 volumes of 10 mM glutathione. The flow-through was reloaded onto another GST-Sepharose column to recover remaining 0.5 ml of the column containing fraction was resolved on 9% SDS-polyacrylamide gel electrophoresis, and the p62 bands corresponding to ~20 kD of protein were excised. Gel pieces were subjected to total tryptic digestion, and peptides were recovered by washing with 50% acetonitrile. Peptides were separated by reverse-phase chromatography on a C18 column under standard conditions.

Cloning of p62\(^{AX}\) cDNA—PCR was performed by using degenerate oligonucleotides corresponding to peptides IVSIGDGP (5'-GATCAGAATGCTATCATGCTGATAACGGG-3') and WMAPEV (5'-GACTTCTGCITGGNGCCATCCT-A-3') shown in Fig. 3B. 30 cycles (1 min at 94°C, 2 min at 50°C, and 3 min at 72°C) were used to amplify rat testis cDNA. An amplified DNA fragment of ~600 base pairs (probe A in Fig. 3A) was subcloned and sequenced to confirm its identity. This was used to screen 1 × 10\(^9\) clones from a rat brain cDNA library (Stratagene), which yielded three independent λ phage clones with overlapping inserts containing the PCR cDNA sequence (insert size, ~1.5, 2.5, and 4 kb). The largest insert (clone A1 in Fig. 3A) was found to be truncated just before the expected p21 binding region present in the p62\(^{AX}\) sequence (137 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 0.4 mM NaH\(_2\)PO\(_4\), 5.6 mM glucose, 5 mM HEPES, pH 7.3, and 1 unit/ml apramycin) at a concentration of about 5 × 10\(^8\) cells/ml. To activate platelets, bovine thrombin (Sigma) was added at 1 unit/ml platelets, and the mixture was incubated at 37°C for 5 min or at times indicated. The cells were pelleted at 14,000 × g on the microfuge for 1 min and stored at ~70°C.

Immunoprecipitations—Thrombin-activated platelets were solubilized in IP buffer (50 mM HEPES, pH 7.3, 150 mM NaCl, 0.5% Triton X-100) buffer. Proteins were eluted with 2 × SDS-sample buffer, boiled for 5 min, and subjected to gel electrophoresis and Western blot analysis. p21 Binding, GAP Overlay, and In Vitro Kinase Assays—These assays were performed essentially as described previously (12, 17).

Western Blot Analysis—Following transfer of proteins to PVDF or nitrocellulose membranes, these filters were blocked overnight at 4°C in PBS containing 5% skimmed milk. All subsequent steps were at room temperature. Filters were washed (3 × 5 min) with PBS containing 0.1% Tween-20 (BDH) and incubated with primary antibody (at 1/1000 dilution) in 1% skimmed milk for 2 h. After washing (5 × 5 min) in PBS containing 0.1% Tween-20, the filters were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. The filters were then washed, and enhanced chemiluminescence signals were detected by placing to film (Amersham) at 10–60 s, depending on exposure.

Other Assays—Protein concentrations were measured using the Bradford assay with protein dye reagent (Bio-Rad) according to manufacturer's protocol.

**RESULTS**

**Localization of the Ubiquitous p62\(^{AX}\) Binding Protein**—The p68\(^{AX}\)/p42\(^{AX}\) binding isoforms of pAK (α- and β-PAK, respectively) (17, 20) are preferentially found in the brain while the 62-kDa binding protein is ubiquitous (17). This p62 could be partially purified from rat testes (containing the larger binding proteins) by chromatography on S-, Q-, and zinc chelating-Sepharose columns employing γ-3P-GTP-Cdc42 overlay for detection (Fig. 1A). Affinity chromatography with GTP\(\gamma\)-S-Cdc42 (Fig. 1B) then yielded two Cdc42-associated bands, the lower one being a proteolytic product of the p62 (17). Although the p62 did not bind strongly, appearing in all fractions of the GST/Cdc42 affinity column, microgram quantities were obtained from the p62 from a Coomassie-stained gel (Fig. 1B, lanes 23). The 68-kDa protein is residual albumin (added to the nucleotide exchange buffer in the GST-Cdc42 column). Gel-purified p62 and p60 proteolytic bands on tryptic digestion and reverse-phase chromatography yielded identical fingerprints (data not shown). The sequence of five peptides derived from p62 is shown in Fig. 3B. Only one (STMVGTPTWYMAPE) was identical to the predicted α-PAK sequence with all others containing at least one amino acid substitution. The p62 thus represented a distinct isoform, which we designated as α-PAK.

**Activation of p62\(^{AX}\) in Vitro**—As with p65\(^{AX}\), purified p62\(^{AX}\) was activated by Rac and Cdc42 (Fig. 2A). Cdc42 was more effective than Rac as determined by the increased auto-phosphorylation and myelin basic protein (MBP) phosphorylation. Both p62\(^{AX}\) and p62 were phosphorylated on serine and threonine residues. GTP\(\gamma\)-S-Cdc42 induced a larger mobility shift than GTP\(\gamma\)-S-Rac1, as monitored by Cdc42 overlays of the in vitro activated kinase (Fig. 2C, lanes 3-5). Phosphorylated p62\(^{AX}\) (p62) still bound Cdc42.

**Isolation and Sequencing of γ-PAK cDNAs—Degenerate oligonucleotides corresponding to the sequences IVSIGDGP and WMAPEV (Fig. 3B) were used as primers for PCR of rat testis cDNA. The expected 600-bp PCR product encoded one PAK-related sequence including NVLLMGEVS (Fig. 3B). This PCR product (probe A, Fig. 3A) was used to screen a rat brain cDNA library, which yielded three clones with overlapping inserts at the C-terminal end (Fig. 3A). The longest

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**Expression and Purification of γ-PAK**—The full-length γ-PAK and γ-PAK-1–210 in pGEX-2TK were expressed and purified from E. coli as GST fusion proteins.

**Other Assays**—Protein concentrations were measured using the Bradford assay with protein dye reagent (Bio-Rad) according to manufacturer's protocol.

**RESULTS**

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were eluted with 0.1, 0.2, 0.3, and 0.4 M NaCl (lanes 1-4, respectively). The p62-enriched fractions (*) were pooled and loaded onto a Cdc42 column. The flow-through (lane 1) was collected, and proteins were eluted with 0.1, 0.2, 0.3, and 0.4 M NaCl (lanes 2, 3, 4, and 5, respectively). The p62-enriched fractions (lanes 6-10) were collected (lanes 21-23) and loaded onto a zinc-chelating column. The flow-through was collected (lanes 24), and the pool containing fraction (lane 17) was next loaded onto a GTP-Sepharose column. The flow-through (lane 6) was collected, and proteins were eluted at pH 7.5, 6.0, and 5.0 (lanes 13-15). The p62-containing fraction (lane 16) was next loaded onto a GTP-S-Cdc42 affinity column (B). The flow-through (lane 17), pH 6.0 wash (lanes 18-20), and pH 8.8 eluted fractions (lanes 21-23) were collected. The GST/Cdc42 protein was eluted with glutathione (lane 24). The protein fractions were electrophoresed on 9% SDS gels, transferred onto nitrocellulose filters, and subjected to [γ-32P]GTP-Cdc42 binding assay (17). A Coomassie-stained gel corresponding to lanes 23 and 24 shows the purified p62 and p60 proteins and the GST/Cdc42 protein eluted from the affinity column.

Clone of 4 kb appeared to be truncated at its 5' end corresponding to a position before the p21 binding domain. Further screening with upstream sequences from the 4-kb clone (probe B, Fig. 3a) yielded clones containing upstream sequences that included the p21 binding domain. The cDNA of clone B4 contained 1756 nucleotides with a 1575-nucleotide open reading frame encoding a predicted 58-kDa protein of 524 amino acids (Fig. 3a). The putative translation start site began at nucleotide 49 (Fig. 3b), with upstream flanking sequences conforming to a translation initiation consensus sequence (24). The first five amino acids of γ-PAK showed homology to the predicted N-terminal sequence of α-PAK (Fig. 4).

Predicted Amino Acid Sequence of γ-PAK—The γ-PAK open reading frame encoded all five peptide sequences derived from purified p62 -PAK (underlined in Fig. 3b). γ-PAK showed 76% identity with α-PAK, with particular divergence in the linker region between the p21 binding domain (BD) and the kinase domain (Fig. 3a). The latter two shared >90% identity. Two features in the linker region are conserved, polyacidic (8 consecutive residues) and proline-rich sequences. γ-PAK showed 94% identity to human hPAK65 (25), suggesting it to be the rat homologue. However, the N-terminal hPAK65 sequence appeared truncated relative to the rat α- and γ-isozymes. A database search revealed hPAK65 5'-non-coding and 38 bp of putative coding sequence to be identical to human placental lactogen (somatomammotropin) cDNA (26). Thus, it is likely that the N-terminal 12 amino acids of hPAK65, which do not match the rat PAKs, are not present in the bona fide hPAK65 protein.

Expression of γ-PAK—γ-PAK mRNA was detected in all rat tissues examined, primarily as a 4.6-kb transcript (Fig. 5a). Two weaker ubiquitously transcribed bands of 7 and 4 kb may represent alternate spliced mRNAs or cross-hybridizing transcripts of other PAK isoforms. The mRNA expression pattern resembles that of the ubiquitous p62 -PAK binding protein (Fig. 5b), which is also similar in size to the in vitro translation product of clone B4 (Fig. 5b). We were unable to generate a full-length recombinant kinase as a GST-fusion protein. However, we were able to express truncated GST/γ-PAK1-210 as a 50-kDa fusion protein, which exhibited robust Cdc42 binding in overlay assays (Fig. 6a). GST-γ-PAK1-210 and GST/α-PAK1-250 bound [γ-32P]GTP-Cdc42 in a similar fashion, within the range of 0-500 ng of protein, consistent with the high conservation within the p21 binding domain. Both GST/α-PAK1-250 and GST-γ-PAK1-210 also inhibited intrinsic GTPase activity of Cdc42 similarly (Fig. 6b), doubling the half-life of bound GTP.
Fig. 3. **Isolation, nucleotide, and predicted amino acid sequence of γ-PAK.** A, schematic diagram showing the cloning strategy for γ-PAK cDNA. PCR was performed by using degenerate primers corresponding to peptides IVSIGDP and WMAPEV shown in Fig. 3B to amplify rat testis cDNA. An amplified 600-bp PCR product (probe A) was sequenced and used to screen a rat brain cDNA library. Three phage clones with insert sizes of 4, 1.5, and 2.5 kb (clones A1, A2, and A3, respectively) contained overlapping N-terminal sequences. The longest clone (A1) was truncated at the 5′-end, and an upstream probe (probe B) of 400 bp from clone A1 was used to rescreen the same library. Four clones of sizes 4, 0.9, 3, and 2 kb (clones B1, B2, B3, and B4, respectively) were obtained. Clone B1 was identical to clone A1, and clones B2 and B3 contained overlapping N-terminal sequences. The 2-kb clone (B4) contained the entire γ-PAK cDNA sequence including the translational start site. The restriction map for clone B4 is also shown. Bottom, a schematic diagram of the B4 open reading frame and the relative positions of the p21 binding and kinase domains, and untranslated regions. B, the cDNA and predicted amino acid sequences of γ-PAK. The five peptide sequences derived from purified p62PAK are underlined.
from 12 to 24 min at 20 °C. Full-length GST-α-PAK and GST-γ-PAK had the same effect on intrinsic hydrolysis, suggesting the kinase domain does not influence the p21 interaction under these conditions.

**Thrombin Activates p62PAK in Platelets**—Thrombin treatment of platelets activates several serine/threonine kinases, leading to processes including aggregation (21). Of the 12 rapidly activated serine/threonine kinases detected using a renaturable in situ kinase assay, the p170, p86, and p60 showed highest activity (23). The identities of these kinases, except p42–44 mitogen-activated protein kinase (27), are unknown.

Fig. 7 illustrates that addition of thrombin (1 unit/ml) rapidly increased the activities of three platelet kinases of 170, 86, and 62 kDa. The major Cdc42 binding protein of 62 kDa in platelet lysates had identical migration characteristics as the 62-kDa kinase (Fig. 7A). Comparison with brain and testes (left panel, Fig. 8A) showed platelets to have no novel Cdc42 interacting proteins. However, as we observed strong GAP activity in p85 and p50 in platelets even in the binding assays (Fig. 7B, seen as white bands), we subjected platelets and rat tissues to the GAP overlay technique (12). Brain cytosol contained GAPs (white bands) of ~150, 100, 85, 50, and 45 kDa as previously reported (Fig. 8A, lane 4, marked) with a faint band of 75 kDa (the white bands of 60–68 kDa result from [γ-32P]GTP-Cdc42 sequestration (binding) by the PAK isoforms rather than from GAP activity). Platelets contained Cdc42 GAPs of sizes 85, 75, 50, and 45 kDa (Fig. 8A, lane 6). To determine if these were due to proteins containing Rho GAP-like domains such as Abr or the p85a regulatory subunit of phosphatidylinositol 3-kinase, an immunoblot was assayed with appropriate antibodies. Abr, present in brain, was not detected in platelets; both tissues, however, apparently contained the p85 subunit (Fig. 8B).

Polyclonal antibodies against γ-PAK1–210, lacking the kinase domain, detected only the p62PAK and did not significantly cross-react with the α- and β- isoforms, as assessed by [γ-32P]GTP Cdc42 binding to immunoprecipitates from brain extract (Fig. 9C, Cdc42 overlay panel, cf. lanes 1 and 2). The platelet p62 was efficiently immunoprecipitated, exhibiting both Cdc42 binding and serine/threonine kinase activity in situ (Fig. 9, A and B, lanes 2). It was not tyrosine phosphorylated (data not shown). A serine/threonine kinase of about 86 kDa appeared to be communoprecipitated with p62PAK and which did not bind Cdc42 (Fig. 9, A and B, lanes 2). This p86 was not detected by the γ-PAK anti-serum (data not shown). Preincubation of the antibodies with γ-PAK1–210 fusion protein drastically decreased immunoprecipitation of both p62PAK and p86 (Fig. 9B, lanes 3). The p86 kinase was not detected in brain immunoprecipitates (Fig. 9C) (the brain p90 with weak kinase activity was not affected by the preincubation of the antibodies with γ-PAK fusion proteins, cf. lanes 2 and 3). Another renaturable immunoprecipitated brain protein kinase (p65) was detected (Fig. 9C, in situ kinase panel, lane 2). Although it did not bind Cdc42 (Fig. 9C, Cdc42 overlay panel, lane 2), its activity was diminished when the antibodies were preincubated with γ-PAK proteins (Fig. 9C, in situ kinase panel, lane 3). The identities of the p62PAK-associated protein kinases, p86 and p65, are under study.

**DISCUSSION**

In this study, we report the purification and subsequent cloning of a 62-kDa Cdc42/Rac binding protein, termed γ-PAK. γ-PAK is ubiquitously expressed in rat tissues, unlike α- and β-PAK, which are found in few tissues other than brain (20). Thus, γ-PAK reflects the widespread distribution of Rac and Cdc42 (28) and is prob-
ably a common target protein for signaling by these proteins. Purified native p62PAK activity toward MBP is increased on binding to GTP-Cdc42 and GTP-Rac in vitro. Unlike purified p65PAK, the p62PAK does not show decreased binding to Cdc42 upon activation. This is similar to results using recombinant hPAK65(25), except that we find PAK to be phosphorylated in both threonine and serine residues. hPAK65 and rat g-PAK appear to be homologues. The only non-conservative amino acid difference is at g-PAK G349, where hPAK65 contains an arginine; otherwise, this glycine is present in all mammalian and yeast PAK-like kinases that are known. Curiously, although the predicted hPAK65 contains 18 amino acid residues less than p62-PAK, its reported mobility represents a molecular mass of 65 kDa. This difference could be due to the presence of 12 amino acids derived from the placental lactogen mRNA sequence. It is likely that the reported hPAK65 cDNA does not exist as a bona fide mRNA in vivo, although a splicing event between two mRNAs is possible. The rat PAK N terminus shows blocks of homology to a-PAK and has been identified in two independent clones. The use of a probe derived from the conserved kinase domain of hPAK65 (25) will result in detection of mRNA of various PAK isoforms. The use of a probe sequence corresponding to the more variable linker domain allowed us to determine that the 4-kb mRNA is the dominant

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\[\text{FIG. 5. Analysis and distribution of g-PAK mRNA. A, Northern analysis of g-PAK mRNA. A Northern blot (Clonetech) containing ~2 \mu g of poly(A) RNA from various rat tissues was probed with a 800-bp BamHI-HpaII fragment of clone B4 (see Fig. 3A), labeled with [\alpha-32P]dCTP, and then reprobed with \beta-actin cDNA, similarly labeled. The tissue samples are as follows: brain (B), spleen (S), lung (Lu), liver (Li), skeletal muscle (SM), kidney (K), and testes (T). B, comigration of p62PAK and in vitro translated p62 product. Soluble proteins from tissues (150 \mu g) were assayed for [\gamma-32P]GTP-Cdc42 binding as described in Fig. 1. The tissue samples are as follows: brain (B), heart (H), kidney (K), liver (Li), lung (Lu), spleen (S), testis (Te), and thymus (Thy). An in vitro translation reaction with B4 cDNA was performed using a TNT-coupled reticulocyte lysate system according to manufacturer's protocol (Promega). The g-PAK translated products (5 and 10 \mu l of lysate, lanes 1 and 2) were separated on 9% SDS gel and exposed to Hyperfilm (Amersham).}\]

\[\text{FIG. 6. Comparison of p21 binding between a-PAK and g-PAK. A, a-PAK and g-PAK proteins bind GTP-Cdc42 with similar affinity. Various concentrations (0.05–1 \mu g) of the GST/g-PAK1–210 and GST/a-PAK1–250 fusion proteins were assayed for [\gamma-32P]GTP-Cdc42 binding as described in Fig. 1. The amount of [\gamma-32P]GTP-Cdc42 bound to the immobilized fusion proteins (a-PAK (f) or g-PAK (●)) was quantified with a phosphoimager (Molecular Dynamics), as shown in the graph. The results from duplicate experiments are indicated by empty or filled symbols. B, inhibition of Cdc42 GTPase activity by GST/g-PAK and GST/a-PAK. 1 \mu g of [\gamma-32P]GTP-Cdc42 was incubated at 20°C in the absence (●) or presence of 10-fold excess purified GST (○), GST/a-PAK1–250 ( ), full-length GST/a-PAK (●), or GST/g-PAK1–210 (○). At each time point, two 10-\mu l aliquots were absorbed onto nitrocellulose, washed, and assessed for bound radioactivity.}\]

\[\text{\gamma-PAK mRNA in rat tissues. Although the ~170-kDa Cdc42/Rac binding protein might be thought to arise by alternate splicing from the larger 7-kb mRNA, this family of binding proteins is not sequence related to PAK.\[2\]

\[\text{In platelets, the thrombin-activated p62 kinase (23) probably corresponds to p62PAK, suggesting the involvement of Rac and}\]

\[\text{\[2\] T. Leung and L. Lim, unpublished data.}\]
Cdc42 in platelet function. Thrombin also appears to require Rho proteins to mediate cell aggregation, which can be inhibited by C3 transferase, which ribosylates Rho (29). Rho has been reported to activate a downstream target, p85 phosphatidylinositol 3-kinase, in platelets (30). The role of Cdc42 and Rac is likely to involve the dramatic changes in cell shape that accompany platelet activation, since mammalian Cdc42 has been associated with cell spreading in monocytes (31), and microinjected Cdc42 (6, 7) and Rac (2) proteins can induce filopodia and membrane ruffling, respectively, in Swiss 3T3 cells. In yeast, the PAK-related kinase Ste20p is thought to lie at the top of the pheromone-induced mitogen-activated protein kinase cascade (18). This kinase has been implicated as a target for the Ste4/Ste18 βγ subunits and interacts with Cdc42 (32), suggesting that mammalian Rho p21s may regulate mitogen-activated kinase cascades.

Since Ras and Rac can co-operate in transformation (33) and a Ras target, phosphatidylinositol 3-kinase, has been suggested to mediate platelet-derived growth factor-induced membrane ruffling in fibroblasts (34), these p21s may signal both morphological and transcriptional changes in cells. A candidate Rho target is phosphatidylinositol 4-phosphate 5-kinase, which may mediate the effects of Rho on the cytoskeleton in the integrin signaling pathway (35). It is likely that Rho family

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**FIG. 7.** Thrombin stimulation of renaturable kinases in platelets. A, platelets were incubated with 1 unit/ml of thrombin at 37 °C for 0, 0.5, 1, and 3 min as indicated. The platelet lysates were separated by 9% SDS gels, transferred onto PVDF membranes, and assayed for kinase activity in situ. B, The p62 kinase comigrated with the major [γ-32P]GTP-Cdc42 binding activity in these extracts.

**FIG. 8.** Identification of Cdc42 GAPs and binding proteins in platelets. A, rat brain cytosol (lanes 1 and 4), purified testis p62PAK (lanes 2 and 5), and thrombin-activated platelet cytosol (lanes 3 and 6) were separated on 9% SDS gels, transferred onto PVDF membrane, and assayed for [γ-32P]GTP-Cdc42 binding (lanes 1–3) or for Cdc42-GAPs (lanes 4–6) by the overlay assay (12). B, rat brain cytosol (lane 1) and platelet cytosol (lane 2) were separated on 9% SDS gels, transferred onto nitrocellulose, and incubated with either anti-Abr antibody (13) or anti-p85 antibody (UBI).

**FIG. 9.** Immunoprecipitated p62 protein exhibits both Cdc42 binding activity and in situ kinase activity. A, and B, immunoprecipitation of p62PAK from platelets. Thrombin-activated platelet lysates (lanes T) were immunoprecipitated with either γ-PAK antibodies (raised to GST/γ-PAK1–210 fusion proteins) alone (lanes IP) or antibodies preincubated with GST/γ-PAK1–210 fusion proteins (lanes IP+C). The lysates or immunoprecipitates were separated on 9% SDS gels, transferred, and assayed for [γ-32P]GTP-Cdc42 binding activity or for kinase activity in situ. Strong signals in lane IP+C of the Cdc42 overlay correspond to the GST/γ-PAK1–210, which does not include the kinase domain. C, immunoprecipitation of p62PAK from brain supernatant. Rat brain supernatant (lanes T) was immunoprecipitated with γ-PAK antibodies alone (lane IP) or in the presence of GST/γ-PAK1–210 fusion proteins (lanes IP+C). The purified GST/γ-PAK1–210 protein was also shown as a reference (lanes C). The brain lysate and immunoprecipitates were separated on 9% SDS gels, Coomassie stained, or transferred onto PVDF membranes, and assayed for [γ-32P]Cdc42 binding activity or for kinase activity in situ.
p21s as with Ras p21s function in part by activating protein and lipid kinases. Although transforming tyrosine kinases, Ras and its target Raf kinases, are generally thought to act in cell proliferation and differentiation, it is noteworthy that proteins such as Ssr and mitogen-activated protein kinase are present in high concentrations in platelets (36, 27). Platelets undergo extensive cytoskeletal reorganization during activation (21); the rapidity of γ-PAK activation suggests a primary role for the kinase in these events. Additionally in mast cells, Rac and Rho have been reported to play a role in regulating secretion (37). The putative p62PAK-associated p86 kinase in platelets, which is also a rapidly activated serine/threonine kinase (23), may form a signaling complex perhaps mediated by the heterotrimeric G-proteins activated by thrombin. We have yet to determine whether this p86 kinase is responsible for the Cdc42 GAP activity observable in overlay assays. Further analysis of proteins associated with γ-PAK are likely to throw light on the role of PAK since some of these may well be downstream targets for the kinase.

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