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Expression of Constitutively Active α-PAK Reveals Effects of the Kinase on Actin and Focal Complexes

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The family of p21-activated protein kinases (PAKs) appear to be present in all organisms that have Cdc42-like GTPases. In mammalian cells, PAKs have been implicated in the activation of mitogen-activated protein kinase cascades, but there are no reported effects of these kinases on the cytoskeleton. Recently we have shown that a *Drosophila* PAK is enriched in the leading edge of embryonic epithelial cells undergoing dorsal closure (N. Harden, J. Lee, H.-Y. Loh, Y.-M. Ong, I. Tan, T. Leung, E. Manser, and L. Lim, Mol. Cell. Biol. 16:1896–1908, 1996), where it colocalizes with structures resembling focal complexes. We show here by transfection that in epithelial HeLa cells α-PAK is recruited from the cytoplasm to distinct focal complexes by both Cdc42^{G12V} and Rac1^{G12V}, which themselves colocalize to these sites. By deletion analysis, the N terminus of PAK is shown to contain targeting sequences for focal adhesions which indicate that these complexes are the site of kinase function in vivo. Cdc42 and Rac1 cause α-PAK autophosphorylation and kinase activation. Mapping α-PAK autophosphorylation sites has allowed generation of a constitutively active kinase mutant. By fusing regions of Cdc42 to the C terminus of PAK, activated chimeras were also obtained. Plasmids encoding these different constitutively active α-PAKs caused loss of stress fibers when introduced into both HeLa cells and fibroblasts, which was similar to the effect of introducing Cdc42^{G12V} or Rac1^{G12V}. Significantly dramatic losses of focal adhesions were also observed. These combined effects resulted in retraction of the cell periphery after plasmid microinjection. These data support our previous suggestions of a role for PAK downstream of both Cdc42 and Rac1 and indicate that PAK functions include the dissolution of stress fibers and reorganization of focal complexes.

The p21-activated protein kinase (PAK) family of Cdc42/Rac1-activated kinases were first identified as GTPase inhibitory proteins in p21 overlays and the most abundant proteins specifically binding the GTP-bound forms of Cdc42 and Rac1 (34, 35). The brain-enriched α-PAK, which is directly activated by these GTPases and is closely related to *Saccharomyces cerevisiae* Ste20p (36), is a member of a family of ubiquitous mammalian kinases (5, 37, 39, 52). Evidence for the proposal that Ste20p was a yeast Cdc42p-dependent protein kinase (36) which potentially links cell polarization to the mating-pheromone-responsive mitogen-activated protein (MAP) kinase pathway has recently been obtained (50, 59). Significantly, the guanine nucleotide exchange factor for Cdc42p, *dbl*-related Cdc24p, interacts with and is potentially activated by the heterotrimeric G-protein β-subunit Ste4p (59). In mammalian cells, the heterotrimeric G-protein-coupled bradykinin receptor has also been shown to activate Cdc42 (28).

PAKs are distinguished by the presence of sequence conserved kinase and p21-binding and activation domains. Three mammalian PAK isoforms have been described to date: the brain-enriched rat 68-kDa α-PAK (36), corresponding to human PAK1 (hPAK1) (27); the rat 65-kDa β-PAK (37) isoform, which is essentially identical to mouse PAK3 (5); and the smaller ubiquitous 62-kDa γ-PAK (52), corresponding to hPAK2 and hPAK65 (27, 39). γ-PAK has also been described as a protease-activated kinase (24) and an H4/S6 kinase (4). A role for mammalian PAKs as mediators of Cdc42 and Rac1 signalling to JNK and p38 MAP kinases has been reported (6, 7, 58). Indeed, there may be a common role for a wider family of kinases with similarity to PAK, including the germinal center kinase (46), as upstream regulators of stress-activated MAP kinase pathways. PAK proteins may therefore participate in transcriptional activation pathways utilizing Cdc42 and Rac1 (10, 20, 40).

PAK is one of several effector targets for Cdc42 and Rac1. The motif binding to Cdc42 and Rac1 identified in the activated Cdc42-associated kinase (ACK), PAK, and Ste20p (36) has been used to identify other novel gene products which are potential effectors of Cdc42 or Rac1 (8). However, [γ-^{32}P]p21 overlays of tissue and cell extracts with Cdc42 and Rac1 show that signals from PAKs predominate, suggesting these to be major ubiquitous targets (36). A p62 neutrophil Cdc42 target has recently been shown to correspond to the Wiskott-Aldrich syndrome protein, which appears to regulate actin polymerization (51) by binding to GTP-Cdc42 and perhaps GTP-Rac1 (3).

Since Rac1 and Cdc42 have established roles in the morphological organization of cultured cells (28, 42, 47), we have sought to determine which aspects might be mediated by PAK family kinases. In *Schizosaccharomyces pombe*, disruption or overexpression of the *pak1* gene product has marked effects on cell shape, actin localization, and mating (38, 44). The *S. cerevisiae* PAK-related Cla4p kinase appears to be required for normal polarized growth and localization of the septin ring during cytokinesis (11). We have now mapped the autophosphorylation sites on recombinant α-PAK and substituted acidic residues with the aim of producing constitutively active α-PAK mutants. We also constructed two chimeras of α-PAK with Cdc42, one with the C-terminal localization sequence (PAK-

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CAAX) and the other containing Cdc42^{G12V} itself (PAK-CC) with elevated activity. The morphological effects of these different constitutively active kinases were analyzed by transient transfection or microinjection of the various cDNA constructs. All of the active constructs abolished intracellular stress fibers, which is similar to effects of Cdc42^{G12V} and Rac^{G12V} in epithelial cells. We show that α-PAK is recruited from a soluble pool to Cdc42- and Rac1-dependent focal complexes, perhaps in a manner analogous to that observed in vivo with the *Drosophila* PAK (DPAK) (17). Higher levels of membrane-targeted PAK-CAAX or cytoplasmic PAK-CC were also able to cause dissolution of focal adhesions (FAs), indicating that PAK may be recruited by Cdc42 and Rac1 to promote the turnover of these structures.

MATERIALS AND METHODS

Expression and purification of recombinant proteins. The cDNA sequence of α-PAK was engineered for cloning into bacterial and mammalian expression vectors as follows. At the 5′ end, a *Bam*HI-containing linker sequence was introduced by PCR (gga tcc ACA ATG→); the first uppercase A is subsequently referred to as nucleotide 1 of the α-PAK sequence and where the ATG encodes amino acid residue 1. Recombinant glutathione *S*-transferase (GST)/α-PAK^{L404S} was expressed from plasmid pGEX-2T containing the α-PAK^{L404S} cDNA sequence cloned as a *Bam*HI-*Eco*RI fragment (nucleotides 1 to 1785). The wild-type (α-PAK^{L404}) construct was repaired with a 144-bp fragment of cDNA derived from PCR with a correcting oligonucleotide between the *Hin*dIII (at nucleotide 1124) and *Nco*I (nucleotide 1268) sites. Wild-type and hyperactive α-PAK constructs were routinely transformed into *Escherichia coli* BL21 or JM109 grown at 30°C. Fusion proteins were purified on glutathione-Sepharose (Pharmacia-LKB) as described previously (34).

Phosphopeptide analysis. GST/α-PAK bound to glutathione-Sepharose beads was dephosphorylated by incubation with either 0.1 mg of potato acid phosphatase (Sigma) per ml in 50 mM morpholine ethanesulfonic acid (MES; pH 5.8)–0.5 mM $MgCl_2$–0.05% Triton X-100 with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg each of aprotinin and pepstatin (Sigma) per ml or brain cytosol extracted in 50 mM Tris (pH 8.0)–0.5 mM $MgCl_2$–0.05% Triton X-100 with 0.5 mM PMSF and 1 μg each of aprotinin and pepstatin per ml and subjected to G-25 chromatography in the same buffer to remove ATP. The treated recombinant GST/α-PAK or inactive GST/α-PAK^{L404S} was incubated with an equal amount of GTPγS-Cdc42 and 20 μM [γ-^{33}P]ATP or [γ-^{32}P]ATP for 10 min and then chased with 0.5 mM ATP for 20 min at 30°C in kinase buffer (50 mM HEPES [pH 7.3], 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 0.05% Triton X-100). After Coomassie blue staining, proteins bands were excised from sodium dodecyl sulfate (SDS)–9% polyacrylamide gels and subjected to in-gel tryptic digestion (49). Tryptic peptides derived from protein were separated on a reverse-phase C_{18} column. The ^{32}P-labeled phosphorylated peptides were located by scintillation counting; these fractions were lyophilized, and 50% of this material was subjected to Edman sequencing. The remainder was resuspended in 6 M HCl and heated to 110°C for 2 h. After lyophilization, the hydrolysate was dissolved in 10 μl of a 0.5-mg/ml mix of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma), and 5 μl was spotted onto phosphocellulose thin-layer chromatography plates (Kodak) and analyzed by electrophoresis (750 V) in 5% acetic acid–0.5% pyridine.

Mutagenesis of α-PAK. For the kinase domain mutants, oligonucleotide primers corresponding to the required mutated cDNA sequences were introduced into the α-PAK sequence by replacing the *Hin*dIII-*Nco*I sequence with a PCR-derived fragment (as described above). PCR was performed with Vent polymerase (New England Biolabs) using 5 ng of plasmid template with 15 cycles as follows: 94°C, 1 min; 50°C, 1 min; 72°C, 1 min. For mutations in the N-terminal regulatory sequence, a two-step PCR method was used. In the first round, cDNA was amplified between a mutated primer and another upstream of the 5′ cloning site (*Bam*HI). A second PCR was performed with a directly adjacent primer (3′ to the first) in the opposite direction, using an internal α-PAK primer 3′ to the internal *Hin*dIII site (α-PAK position 1124). Corresponding fragments were recovered after agarose gel electrophoresis and ligated to each other. A second round of PCR was then performed with only the 5′ and 3′ flanking primers to yield cDNA which was cloned into *Bam*HI and *Hin*dIII sites of the cDNA. The α-PAK sequence from *Bgl*II (nucleotide 755) to the 3′ *Eco*RI site was then replaced; the amplified *Bam*HI/*Bgl*II segment of the cDNA was completely sequenced.

Construction of mammalian cell expression vectors. The pXJ40 vector (56) containing the cytomegalovirus enhancer/promoter was modified with annealed oligonucleotides encoding the hemagglutinin (HA), AU1, or FLAG epitope. These sequences, corresponding to M**YPYDVPDYA**GS, M**DTYRYI**GS, or M**DYKDDDDK**GGS (epitopes in boldface), were inserted between the *Eco*RI and *Bam*HI sites at the 5′ of the polylinker, and translation initiation was enhanced by use of the Kozak consensus sequence ACC ATG following the *Eco*RI site. The *Bam*HI site (encoding GS) is in the same frame as for pGEX vectors; p21 and PAK constructs were moved from pGEX-4T-1 (cloned in the *Bam*HI/*Eco*RI site) to the pXJ40-derived vectors as *Bam*HI/*Xho*I fragments. The kinase-dead lysine (K^{298})-to-alanine mutation was created by using a mutant oligonucleotide which spanned the internal *Bal*I site (nucleotide 888) to amplify the α-PAK sequence between this and the *Hin*dIII site (nucleotide 1124): the 236-bp mutant *Bal*I/*Hin*dIII fragment was ligated into pXJHA-α-PAK cut with the same enzymes. An *Xho*I restriction site replacing the termination codon was introduced into the α-PAK sequence (yielding α-PAK^{Xho1}) by amplification of 512 bp of sequence from the internal *Hin*dIII site (nucleotide 1124) to the termination codon (nucleotide 1636) and ligating the insert into pXJHA-α-PAK cut with *Hin*dIII and *Xho*I. The cDNA sequence corresponding to last 15 amino acids of Cdc42 (41) was then spliced as an *Xho*I/*Not*I fragment to the pXJHA-α-PAK^{Xho1}, where the *Xho*I site encodes codons 174 and 175 (LE) of Cdc42. To create PAK-CC, a 5′ PCR primer containing an *Xho*I site preceding a G_6 sequence (i.e., encoding LEGGGGGG**MQTIK**→ [Cdc42 sequence in boldface]) and a 3′ primer incorporating a stop codon after Cdc42 residue 175 were used to the amplify Cdc42^{G12V} template. The *Xho*I/*Kpn*I-digested fragment was cloned into the C terminus of pXJHA-α-PAK^{Xho1}. Fidelity of all products was checked by sequencing the amplified region of the cDNA. Constructs for expression of α-PAK$^{1\text{-}250}$ and α-PAK$^{1\text{-}150}$ in HeLa cells were derived by cloning the *Bam*HI/*Bgl*II or *Bam*HI/*Xmn*I cDNA fragments into the pXJ-FLAG vector.

Cell culture and staining. COS-7 cells were maintained in Dulbecco modified Eagle medium with 1 g of glucose per liter and 10% fetal bovine serum (FBS) and were transfected in medium containing 1% serum by using Lipofectamine (Gibco-BRL) according to the manufacturer's protocol. Five micrograms of each pXJ plasmid and 50 μl of Lipofectamine were added per 90-mm-diameter dish; cells were maintained for 20 h before harvesting.

HeLa cells were maintained in minimal essential medium containing 10% FBS. Subconfluent cells plated on coverslips for 48 h were microinjected with the different constructs (50 ng/μl unless otherwise indicated) by using an Eppendorf micromanipulator. For transfection studies, cells were plated onto glass chamber slides (Nunc) and after 1 to 2 days treated with a complex containing 0.5 μg of each plasmid and 3 μl of Lipofectamine per 2- by 2-cm well in 1 ml of medium containing 5% FBS. After 16 h, cells were washed once with phosphate-buffered saline (PBS) (room temperature) and fixed with 3% paraformaldehyde (20 min at room temperature). Primary antibodies were incubated in PBS–0.5% Triton X-100 for 2 h at 30°C at the following dilutions: anti-vinculin monoclonal antibody (MAb) (hVIN-1; Sigma), 1:300; affinity-purified rabbit anti-α-PAK, 1:50; anti-HA or anti-FLAG MAb (IBI), 5 μg/ml; antipaxillin MAb (Transduction Laboratories), 1 μg/ml. Fluorescein isothiocyanate-conjugated second antibodies (1:100) and rhodamine-conjugated second antibodies (1:50; Boehringer Mannheim) were incubated for 1 h at 24°C. To visualize polymerized actin, cells were stained with rhodamine-conjugated phalloidin (1 μg/ml; Sigma) for 1 h at room temperature.

Western blots and immunoprecipitation. Proteins were transferred to polyvinylidene difluoride membranes (NEN) and probed with rabbit anti-α-PAK antibodies (1:500) or MAb 12CA5 (0.5 μg/ml) for 2 h at room temperature. Horseradish peroxidase-coupled second antibodies (1:4,000; DAKO) were incubated for 1 h. Bands were visualized with hyperfilm in the presence of luminol (Amersham) for 10 to 60 s, depending on signal intensity. For immunoprecipitation, cell extracts were diluted to 4 mg/ml in cell lysis buffer (25 mM HEPES [pH 7.3], 0.3M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 0.5% Triton X-100, 5% glycerol, 0.5 mM PMSF, 1 μg each of aprotinin and pepstatin per ml). Extracts (0.4 mg) were incubated with 2.5 μg of MAb 12CA5 for 2 h and then passed through 50 μl of protein A-Sepharose 4B columns (Sigma). After washing with 200 μl of cell lysis buffer and 2 ml of PBS–0.1% Triton X-100, the kinase reaction was carried out in buffer containing 50 mM HEPES (pH 7.3), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, and 0.05% Triton X-100 with 20 μM [γ-^{33}P]ATP.

RESULTS

Localization of autophosphorylated residues in α-PAK. Native brain PAK undergoes GTP-p21-stimulated autophosphorylation which is associated with a potent increase in kinase activity (36). Recombinant PAK proteins purified from *E. coli*, although already partly activated, displayed a similar response to the GTPases (37). However, we subsequently found that the recombinant α-PAK contained a point mutation (L404S) which resulted in lower basal activity (37) and enabled its selection since wild-type α-PAK is active and lethal in *E. coli*. We have now established that plasmids encoding wild-type α-PAK can be propagated at 30°C. The α-PAK^{L404S} kinase, which does not become significantly activated in the bacteria but is activated by the GTP forms of Cdc42 and Rac1, thus provides an invaluable tool to study PAK activation by using recombinant kinase; this is not possible with any of the three

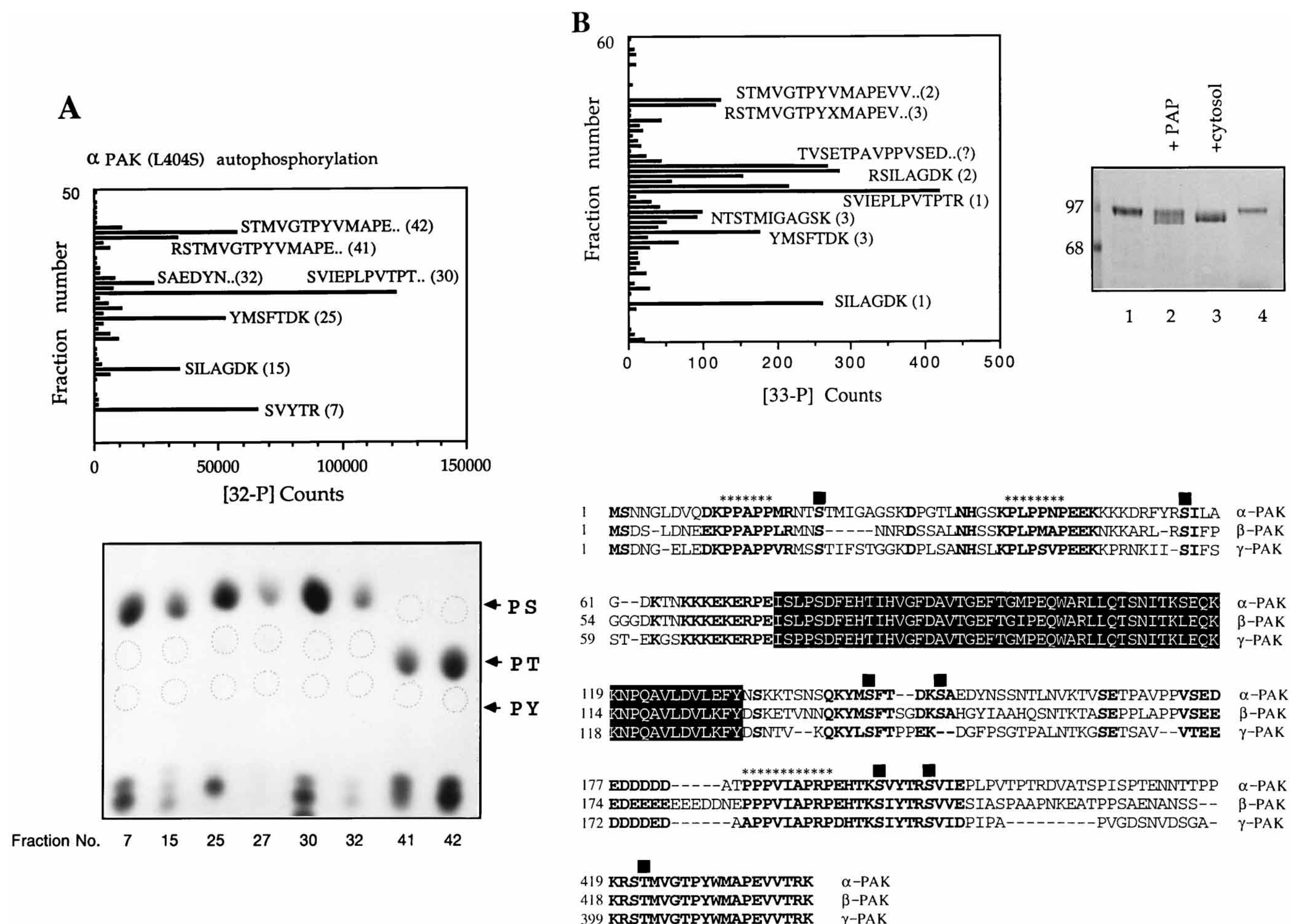


FIG. 1. Localization of α-PAK autophosphorylation sites. (A) Recombinant GST/α-PAK^{L404S} (40 μg in 100 μl) was labeled in the presence of 40 μg of GTPγS-Cdc42 and 20 μM [γ-^{32}P]ATP (50 μCi) for 10 min at 30°C and then chased for 20 min with 0.5 mM ATP. After electrophoresis on a 9% gel, GST/PAK bands were excised and digested with trypsin. Counts associated with the high-pressure liquid chromatography-purified tryptic peptide fractions are shown. Those peptides containing >2,000 cpm were taken for N-terminal sequencing; a portion was also subjected to complete hydrolysis and phosphoamino acid analysis (fractions numbers given). The positions of ninhydrin-stained phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) which were also run are indicated; the autoradiogram was obtained by exposing the thin-layer chromatography plate to film overnight. (B) Potato acid phosphatase (PAP; lane 2) incompletely dephosphorylated GST/α-PAK, as judged by mobility shift; however, mixed phosphatases from brain cytosol appeared efficient in this respect (lane 3). Recombinant wild-type GST/α-PAK was dephosphorylated on the column; after washing, the cytosol-treated kinase was eluted and activated with GTPγS-Cdc42 (lane 4) as for panel A except that [γ-^{33}P]ATP was used. Peptides with >100 cpm (without added scintillation fluid) were subjected to N-terminal Edman sequencing. At each cycle, half the material was taken for counting; the cycle number associated with release of the radiolabel is given in parentheses. The positions of the mapped phosphorylated residues are shown (black squares); these do not occur within the p21-binding domain (boxed), but S21 and S198 are close to potential SH3-binding sequences (asterisks).

wild-type mammalian PAKs, which are purified from *E. coli* essentially in their activated state.

Initial experiments to map the sites of autophosphorylation during Cdc42-mediated α-PAK activation were conducted with the attenuated α-PAK^{L404S} protein (Fig. 1A), which becomes activated more than 100-fold in the presence of GTPγS-Cdc42 (37). Autophosphorylation was carried out with 20 μM [γ-^{32}P]ATP for 10 min and then chased with 0.5 mM ATP to completion as assessed by the upward shift in mobility of hyperphosphorylated GST/α-PAK protein. Tryptic ^{32}P-labeled phosphorylated PAK peptides were then located from Cerenkov radiation (Fig. 1A). A proportion of labeled peptides was subjected to Edman sequencing, and the remainder was taken for phosphoamino acid analysis (Fig. 1A, lower panel). The only phosphothreonine-containing peptide was derived from sequence in the kinase domain (STMVGTPYWMAPEVVTR [Fig. 1B, lower panel]), in a segment often involved in kinase activation (for a review, see reference 25). Since the activation of recombinant α-PAK^{L404S} might not be identical to that of wild-type α-PAK, the latter was subjected to similar analysis. GST/α-PAK (which was already active) was first dephosphorylated in vitro (Fig. 1B, lane 3) and then rephosphorylated in the presence of GTPγS-Cdc42 and [γ-^{33}P]ATP (Fig. 1B, lane 4). In the case of the peptide identified as derived from cleavage after K162 (i.e., TVSETPA. . .), there was no significant release of label over 20 sequencing cycles. It is likely that the label in this peptide was at S198, given poor tryptic cleavage at K197. With the exception of residue S21, all phosphorylated residues in wild-type α-PAK corresponded to those mapped on the α-PAK^{L404S} protein, and most were within sequences that are well conserved among mammalian PAKs (Fig. 1B, lower panel).

Generation of constitutively active PAK mutants. A wide variety of protein kinases, including cyclic AMP-dependent kinase (cAPK), Ca/calmodulin-dependent kinase 1, ERK-2, and Src family tyrosine kinases, have been shown to require phosphorylation events within the kinase domain for activation (reviewed in reference 25). The approach of generating con-

stitutively activated kinase by replacement of phosphorylated residues with acidic ones has been successful in the cases of p34*cdc2* (16), MEK1 (23), and protein kinase C (43). The effect of such substitutions in PAK was tested by measuring the kinase activity of immunoprecipitated HA-PAK from transfected COS-7 cells. The mutant α-PAK^{T422E}, (see Fig. 3A), but not others with substitutions of phosphorylated serine residues (data not shown), showed activity above basal levels.

The HA-α-PAK recovered from COS-7 cells had low or undetectable kinase activity, but cotransfection with the active (G12V) form of AU1-tagged Cdc42 or Rac1 yielded enzyme with increased activity toward myelin basic protein (MBP) (Fig. 2A). It was possible to observe a partial mobility shift of the kinase in Western blots of total proteins from these cells (Fig. 2B); often during the in vitro kinase reaction, a larger fraction of the kinase became shifted (Fig. 2A). The active PAK recovered from COS-7 cells cotransfected with Cdc42^{G12V} could be further substantially activated in vitro (Fig. 3C). This result suggests that in vivo, PAK either does not become fully activated by Cdc42 or, more likely, is subjected to some form of intracellular down-regulation upon activation. The HA-tagged p21s and HA-PAK were found to be expressed at equivalent levels (Fig. 2B), showing that Cdc42 was not limiting. The strong stimulation of immunoprecipitated α-PAK kinase activity in vitro by GTPγS-Cdc42 (Fig. 2C) also resulted in the consistent phosphorylation of coprecipitating presumptive PAK substrates of 75 and 90 kDa. Phosphorylation of the pp75 was detected only when α-PAK was expressed as truncated PAK$^{61-544}$ because its mobility was similar to that of phosphorylated HA-α-PAK. The in vitro activation of immunoprecipitated PAK allowed us to assess the extent of their activation within transfected cells: by this criterion, α-PAK^{T422E} was only moderately (~5%) active.

We were able to generate a number of other constructs which exhibited higher activity (Fig. 3A and B). First, the amount of negative charge within the kinase activation loop was increased by mutating both S421 and T422 to aspartic acid and glutamic acid, respectively (PAK^{DE}). This construct showed higher activity than with the single substitution but could be further activated in vitro by addition of GTP-Cdc42 (Fig. 3C). Second, in an approach analogous to that used by Leevers et al. to produce constitutively active Raf (30), we fused the CAAX box derived from the last 15 amino acids of Cdc42 onto the C terminus of α-PAK. Immunoprecipitates of this protein also showed increased activity (Fig. 3B), which might relate to its colocalization to sites also enriched for its activator Cdc42. However, there was no apparent synergism when this CAAX box was fused to PAK^{DE}. Third, we constructed cDNAs in which the activator Cdc42^{G12V} was covalently fused to the kinase, a strategy that was successful in the case of the p85 and p110 subunits of phosphatidylinositol 3-kinase (22). Such an α-PAK/Cdc42^{G12V} chimera (designated PAK-CC), which was expressed at levels similar to those of the other α-PAK constructs (Fig. 3B), showed much more robust in vitro activity toward MBP than the other PAK constructs. This chimera lacked the last 13 amino acids of Cdc42, and is therefore not expected to interact with membranes, and contained a polyglycine linker between the two protein moieties (see Materials and Methods). A C-terminal fusion of the unrelated green fluorescent protein from Aequorea victoria to PAK, used as a control, did not result in PAK activation (data not shown). Compared with α-PAK, immunoprecipitates of PAK^{DE} did not show clear labeling of the pp90 in vitro (Fig. 3C), suggesting that less of this protein was present or it was already phosphorylated. These various PAK constructs were used to assess kinase function in relation to cell morphology.

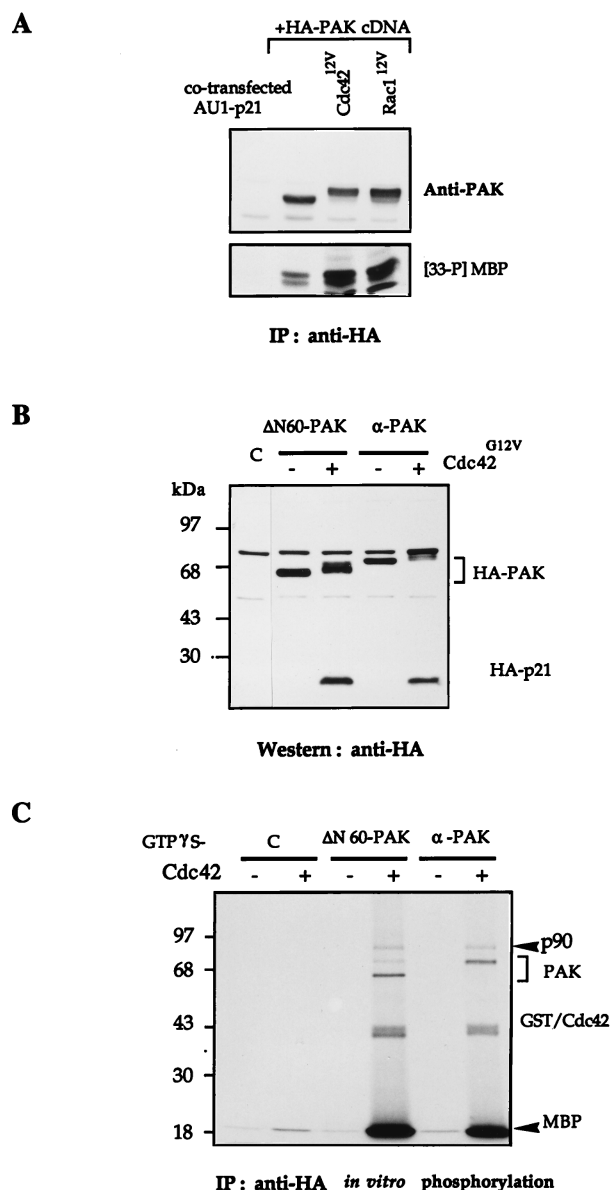


FIG. 2. In vivo and in vitro activation of immunoprecipitated HA-α-PAK. (A) COS-7 cells were transfected with pXJHA-α-PAK (lane 2) alone or with pXJAU1-Cdc42^{G12V} or pXJAU1-Rac1^{G12V}. HA-tagged protein was immunoprecipitated (IP) by MAb 12CA5 and incubated with 20 μM [γ-^{33}P]ATP and 0.1 mg of MBP per ml as substrates. After 15 min at 30°C proteins, were denatured by heating in SDS sample buffer and run on 9% gels for Western analysis or 12.5% gels for autoradiography. (B) Western analysis of COS-7 cell extracts after transfection with HA-tagged α-PAK or α-PAK$^{61-544}$ (ΔN60) with or without HA-tagged Cdc42^{G12V}. The tagged p21 and PAK were expressed at similar levels; in the presence of Cdc42^{G12V}, there was a partial shift in the mobility of PAK. The p75-immunoreactive band was also detected in control untransfected cells (lane C). (C) Similar activation of immunoprecipitated α-PAK and α-PAK$^{61-544}$ (Δ60) in vitro by GTPγS-Cdc42. The pp90 PAK substrate coprecipitated with both HA-α-PAK and HA-ΔN60-PAK. The pp75 substrate detected with HA-ΔN60-PAK was not immunologically related to PAK (data not shown). Phosphorylation of GST-Cdc42 fusion protein occurs at the PKA site derived from the pGEX-2TK.

Cdc42 and Rac1 promote association of PAK with focal complexes. When α-PAK was expressed following microinjection of the cDNA into a variety of cell lines, we observed dispersed cytoplasmic distribution of the kinase. We investigated the effects of p21s on α-PAK distribution by plasmid

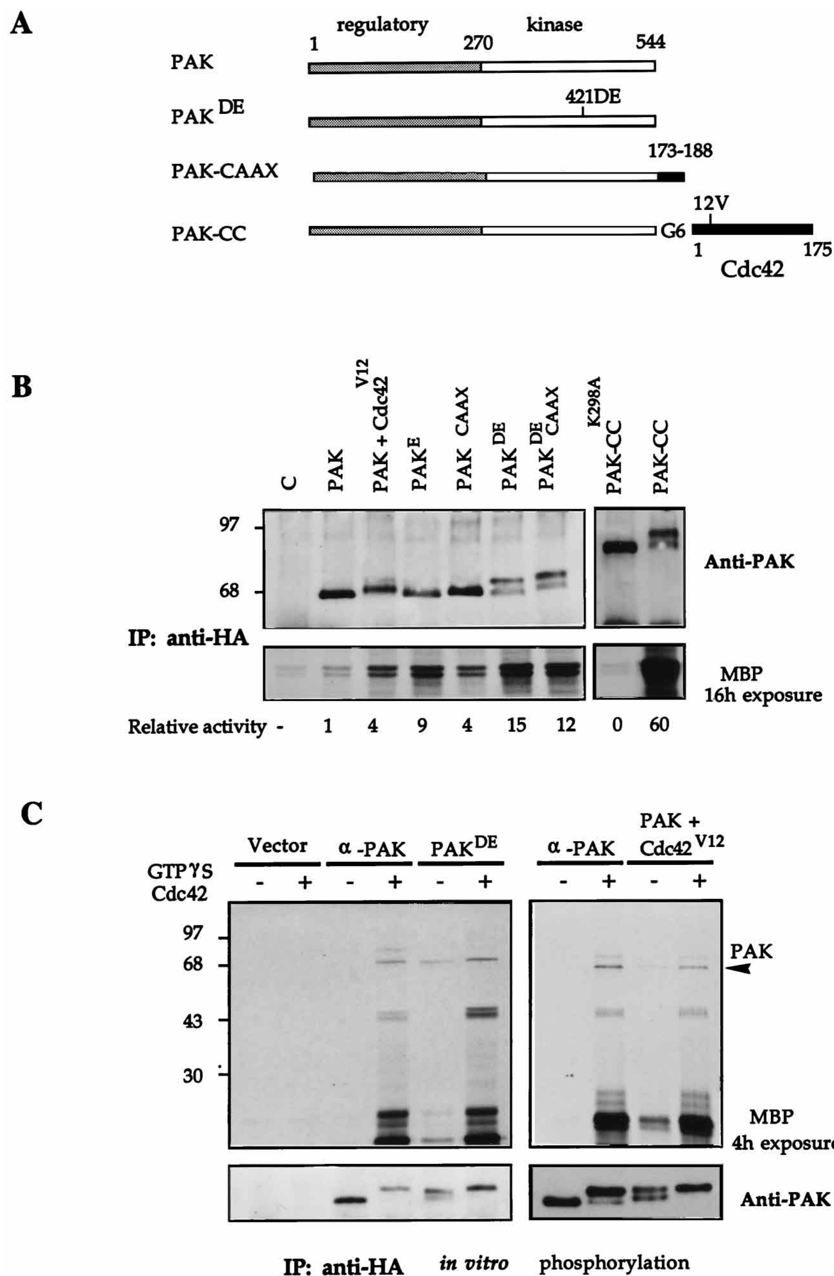


FIG. 3. Generation of constitutively activated PAK proteins. (A) Schematic structure of the three constitutively active kinase constructs used to analyze the effects of PAK in HeLa cells. (B) Activity of immunoprecipitated (IP) COS-7-expressed HA-tagged kinase assayed as described for Fig. 3. The constructs are abbreviated as follows: PAK^{T422E} as PAK^{E}, PAK-$Cdc42^{173-188}$ as PAK-CAAX, $PAK^{S421D,T422E}$ as PAK^{DE}, and PAK-G6-$Cdc42^{1-175(G12V)}$ as PAK-CC. The relative kinase activities indicated were typical for the constructs under consideration. (C) Phosphorylation of PAK, MBP, and p90 protein during in vitro activation of HA immunoprecipitates with GTPγS-Cdc42. Both immunoprecipitated α-PAK^{DE} and α-PAK cotransfected with AU1-$Cdc42^{G12V}$ exhibited partial mobility shifts without added GTPγS-Cdc42.

transfection into HeLa cells because these cells lack endogenous immunoreactive α-PAK. The epithelial HeLa cells show a reorganization of their actin cytoskeleton when an activated version of Cdc42, Rac1, or RhoA is expressed (14) which parallel those changes found in fibroblasts (28, 42, 47, 48). These include Cdc42-induced filopodia (and ruffle) formation and Rac1-induced ruffling (shown in Fig. 7 and 8). These initial experiments indicated that when cotransfected with an activated version of Cdc42 or Rac1, transfected α-PAK became concentrated in punctate structures which resembled focal complexes. Although RhoA activity appears responsible for maintenance of FAs in adherent cells, Cdc42 and Rac1 have also been shown to promote the formation of new, smaller focal complexes in serum-starved fibroblasts in the presence of C3 toxin (42). Overexpression of wild-type PAK in itself never altered the appearance of the FAs, perhaps because of tight regulation of its activity (see above).

HeLa cell FAs (as detected by staining of paxillin) were largely abolished on transfection of the dominant inhibitory $RhoA^{T19N}$, but this did not alter the distribution of cotransfected PAK, which remained cytoplasmic (Fig. 4a). The use of $RhoA^{T19N}$ allowed us to examine the relationship of PAK to

the novel focal complexes whose formation was induced with $Cdc42^{G12V}$ or $Rac1^{G12V}$ (Fig. 4b and c and reference 42) and facilitated by inhibitors of RhoA. The α-PAK cotransfected with $Cdc42^{G12V}$ or $Rac1^{G12V}$ in this case became localized predominantly at the cell periphery in these focal complexes. In contrast, $RhoA^{G14V}$ promoted the formation of the larger complexes (FAs) at the cell surface adjacent to the substratum, but PAK staining was not concentrated in the corresponding region (arrow in Fig. 4d). The $Cdc42^{G12V}$-expressing cells were more rounded and decorated with a fringe of centripetal focal complexes which appeared to underlie the filopodia and retraction fibers. The variability in PAK expression allowed us to observe that in cells showing low levels (Fig. 4b, arrow) PAK appeared to be exclusively located in these paxillin-containing centripetal structures. Higher levels of PAK were often associated with a decrease in the size of these complexes (Fig. 4b, arrowheads), with PAK but not paxillin also accumulating at cell-cell junctions (as did HA-$Cdc42^{G12V}$ [Fig. 5a]). The $Rac1^{G12V}$-expressing HeLa cells were typically very flattened where PAK exhibited peripheral staining at positions occupied by closely spaced bead-like focal complexes (Fig. 4c). In the absence of $RhoA^{T19N}$, $Rac1^{G12V}$-transfected cells displayed focal complexes (Fig. 5b) which were significantly different from either the punctate Rho type (Fig. 5c, right) or peripheral Rac1 type and therefore might represent hybrid RhoA-Rac1 complexes.

Since in their activated forms Cdc42 and Rac1 associate tightly with PAK, it seemed highly likely that each p21 would colocalize with PAK at focal complexes. Analysis of epitope-tagged HA-$Cdc42^{G12V}$ and HA-$Rac1^{G12V}$ (expressed at low levels) showed such a colocalization with transfected PAK. HA-$Cdc42^{G12V}$ was clearly enriched at the positions corresponding to peripheral focal complexes, as was PAK (Fig. 5a, arrows). HA-$Rac1^{G12V}$ was clearly associated with PAK at peripheral as well as internal complexes (Fig. 5b, arrows), probably representing Rac/Rho-type FAs which are seen in these cells not transfected with $RhoA^{T19N}$. HA-$Rac1^{G12V}$ also appeared to be enriched in membrane ruffles at the junctions between cells.

Unlike the case for wild-type PAK, transfection of the active chimeric PAK-CAAX (containing the Cdc42-derived C-terminal 15 amino acids) can lead to its association with FAs (Fig. 5c). This result suggests that the presence of this membrane anchor enabled PAK to associate with FAs (Fig. 5c) or alternatively to stabilize (active) PAK binding, which would otherwise be transient. However, this observation was restricted to lower levels of PAK-CAAX since higher level of expression caused a profound loss of paxillin-stained FAs (Fig. 5d). Often faint residual complexes were detected at the periphery, thus allowing the cells to remain attached. When cells were probed with antibodies against phosphotyrosine which predominantly detect FAs, cells overexpressing PAK-CAAX also lacked these structures (data not shown).

To localize the regions of PAK mediating focal complex localization, two constructs expressing the N-terminal noncatalytic region were made. Unlike the full-length kinase, PAK^{1-250} bound to HeLa FAs (by paxillin staining), even in the absence of cotransfected $Cdc42^{G12V}$ (Fig. 6A). It also localized to Cdc42-induced focal complexes (data not shown). PAK^{1-150} was retained within the cytoplasm, not being found either in FAs or in Cdc42-induced focal complexes. A kinase inactive mutant α-PAK^{K298A}, like PAK^{1-250}, could also associate with FAs in the absence of cotransfected p21 (Fig. 6B). The interactions of mutant or truncated PAK proteins with HeLa Rho-type FAs, unlike that with Cdc42- or Rac1-induced focal complexes, thus appeared to be independent of p21. This was tested by using a p21-binding mutant, α-PAK^{K298A}(H83/86L), having <2% of wild-type Cdc42-binding (60), which indeed exhibited similar focal staining. Deletion of PAK residues 1 to 60 (ΔN60-PAK^{K298A}) abolished this FA association. In all of these cases, cells expressing these constructs had apparently normal paxillin-stained FAs. Thus, α-PAK residues 1 to 60 and 150 to 250 appear to contain sequences that are responsible for targeting the kinase to FAs. The ability of the N-terminal noncatalytic region of PAK but not full-length wild-type kinase to associate with FAs suggests that targeting sequences are normally cryptic.

Phosphotyrosine cannot be detected in immunoprecipitated PAK (15), indicating this is not a means for PAK to associate with FAs. The observation that PAK interacted in vitro with SH3 domains of Nck and phospholipase Cγ (5) led to the recent demonstration that Nck can bind to PAK in vivo via its N-terminal proline-rich sequence that is conserved among mammalian, *Drosophila*, and *Caenorhabditis elegans* PAKs (15). Though Nck could potentially mediate PAK binding to phosphotyrosine-rich focal complexes, we have not been able to locate this adapter protein in these structures by using commercially available anti-Nck (data not shown).

Active PAK down-regulates stress fibers and FAs. Since Cdc42 and Rac1 promote different effects (i.e., formation of filopodia or lamellipodia) while both p21s are able to recruit PAK (Fig. 4), what is the role of PAK in these processes? Because transfection of PAK-CAAX and PAK-CC can cause a catastrophic loss of focal adhesions and stress fibers (Fig. 5d and data not shown), we investigated shorter-term effects by nuclear microinjection of plasmids encoding constitutively active PAKs. In HeLa cells, $Cdc42^{G12V}$ expression is associated with the appearance of filopodia and ruffles and loss of associated stress fiber (Fig. 7 and 8). The major effect of α-PAK^{DE} was the loss of strongly staining actin stress fibers from the interior of injected cells (Fig. 7). Such losses also occurred in cells transfected with either $Cdc42^{G12V}$ or $Rac1^{G12V}$ expressed at similar levels (compare levels of expressed HA tag in Fig. 7). The loss of internal stress fibers due to $Cdc42^{G12V}$ has been noted in fibroblasts (28) and in HeLa cells (14). As with this latter study, we found that following overnight transfection with plasmid encoding $Cdc42^{G12V}$, cells exhibited complete loss of stress fibers and the formation of actin-rich punctate podosomes (not shown).

Figure 8 shows phase-contrast time-lapse images of HeLa cells microinjected with the plasmid (50 ng/μl) encoding $Cdc42^{G12V}$ or PAK-CC. Consistently, morphological changes became evident 60 to 90 min after injection with the $Cdc42^{G12V}$ or PAK-CC plasmid. With $Cdc42^{G12V}$, there was appearance of filopodia and retraction fibers (arrows), production of ruffles, and a rounding of the cells. PAK-CC-injected cells consistently exhibited signs of retraction ~1 h after injection (arrow) followed by a complete collapse toward the cell body, leaving process-like structures radiating from positions previously occupied by the cell margin. A control plasmid, the α-PAK^{K298A} inactive chimeric construct (with no detectable activity [Fig. 3B]) or the $Cdc42^{G12V}$1-175 plasmid, did not elicit any of these responses up to 4 h after injection (data not shown). The former control indicates it is the kinase activity which is responsible for the morphological effects; the latter indicates that the Cdc42 portion of the protein was not responsible for these morphological changes, consistent with reports that C-terminally truncated Ras^{G12V} is biologically inactive (13). Thus, expression of constitutively active PAK-CC and $Cdc42^{G12V}$ driven from the same expression plasmids following their nuclear microinjection (Fig. 2 and 7) was able to elicit clear phenotypic changes in HeLa cells over similar time

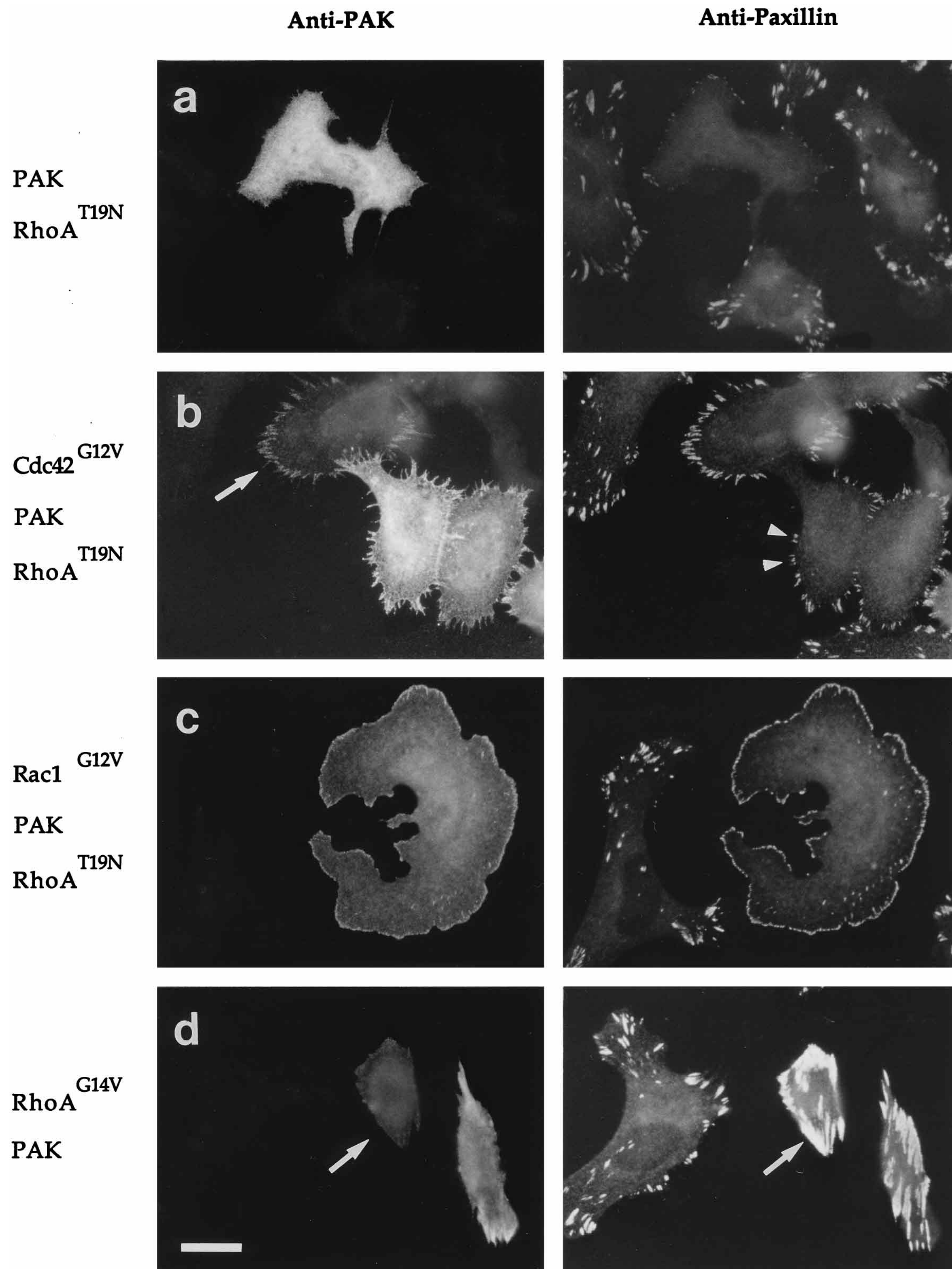


FIG. 4. Activated Cdc42 and Rac1 can recruit PAK to focal complexes. Fluorescence micrographs of HeLa cells showing the distribution of α-PAK, as detected by anti-PAK antibodies, and endogenous paxillin. Transfections of expression plasmids encoding α-PAK, HA-Cdc42^{G12V}, HA-Rac1^{G12V}, and FLAG-RhoA^{T19N} as indicated were carried out with Lipofectamine. Cells were fixed for 16 h after DNA addition in the presence of 5% serum. The α-PAK staining was concentrated into characteristic focal complexes in the presence of Cdc42^{G12V} or Rac1^{G12V} but not with RhoA^{G14V}. In parallel experiments, cells were instead stained for anti-PAK and anti-FLAG (RhoA^{T19N}) immunoreactivity, confirming that these proteins were always coexpressed in the same cells. Bar, 10 μm.

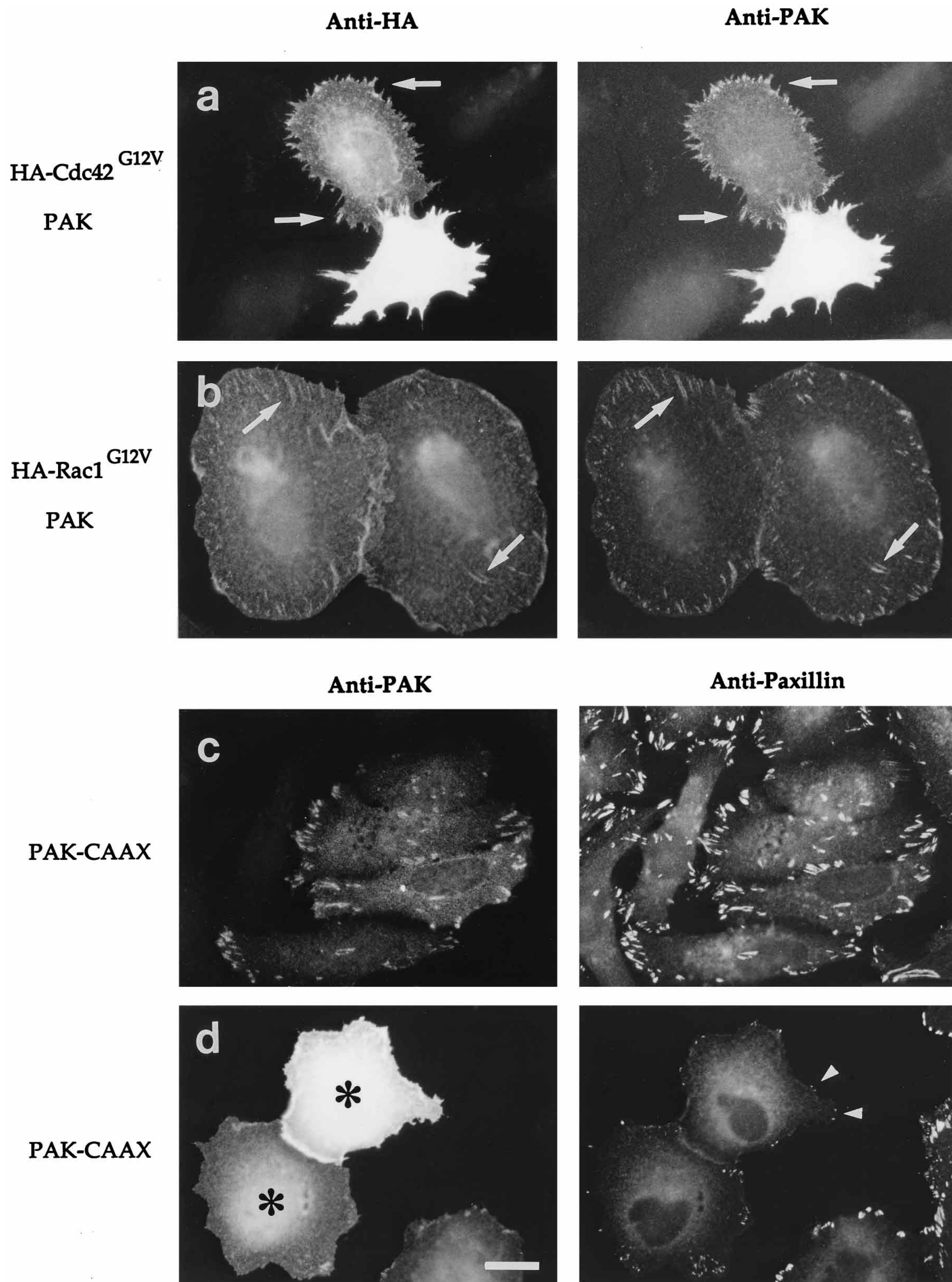
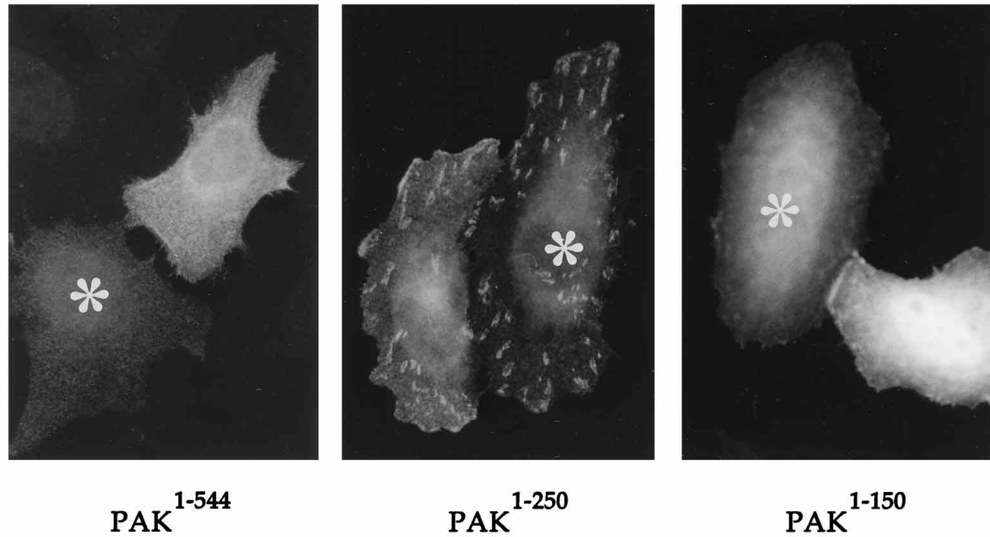


FIG. 5. Active Cdc42 or Rac1 colocalizes with PAK to focal complexes. HeLa cells were observed 16 h after addition of expression vectors encoding the proteins as indicated. Cells consistently expressed similar levels of both PAK and p21 (a). At low levels, these proteins clearly colocalized (arrows) to characteristic peripheral focal complex structures with $Cdc42^{G12V}$ or more interior located complexes with $Rac1^{G12V}$ (b). The PAK-CAAX protein could be found in typical Rho-type focal complexes in the absence of cotransfected p21 (c). At higher PAK-CAAX expression levels, cells exhibited few or no focal complexes (d), with typical residual structures such as those indicated (arrowheads). Bar, 10 μm.



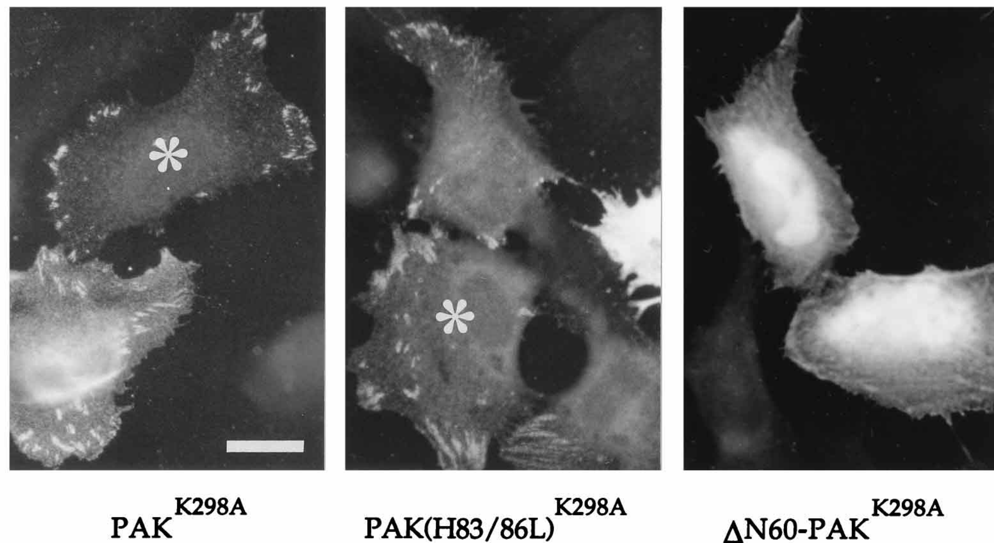


FIG. 6. The N terminus of α-PAK contains an FA targeting signal. (A) HeLa cells were transfected with α-PAK cDNAs encoding the amino acid residues indicated. While full-length PAK (PAK^{1-544}) was evenly distributed throughout the cytoplasm, PAK^{1-250} clearly localized to FAs but PAK^{1-150} protein did not show such punctate staining with anti-PAK antibodies. (B) FA staining was observed with kinase-inactive α-PAK^{K298A} and a p21-binding mutant, α-PAK^{K298A}(H83/86L), but not with ΔN60-PAK^{K298A} lacking the first 60 residues. Cells expressing low level of transfected PAK proteins are marked (asterisks). Paxillin staining was normal in all transfected cells. Bar, 10 μm.

courses. The contracted PAK-CC cells were not dead: such cells could be identified by PAK staining after an overnight period during which many recovered morphologically.

When PAK-CC-expressing HeLa cells were fixed and stained 1 h postinjection, at the time just prior to retraction, we observed complete loss of intracellular stress fibers and peripheral FAs (Fig. 9A). To determine whether this reflected abolition by PAK-CC of Rho-induced structures, cells were coinjected with plasmids encoding $RhoA^{G14V}$ and PAK-CC. These coinjected cells displayed an increase in stress fiber content (Fig. 9A, top right panel). These results show that PAK-CC disassembles Rho-dependent structures and that this effect can be counteracted by activated Rho. To confirm that the morphological effects of PAK occurred in other cell types, we analyzed Swiss 3T3 fibroblasts injected with a PAK-CC or PAK-CAAX expression plasmid. Both constructs (but not the wild-type kinase) gave rise to similar cell retraction. Figure 9B shows typical reorganization of stress fibers and loss of FAs when these fibroblasts were stained for actin and paxillin 1 h postinjection: by 2 h (Fig. 9c), injected cells were completely retracted. These data suggest that PAK acts through a pathway common to different cell types, causing disassembly of FAs and stress fibers (Fig. 10) and leading to retraction.

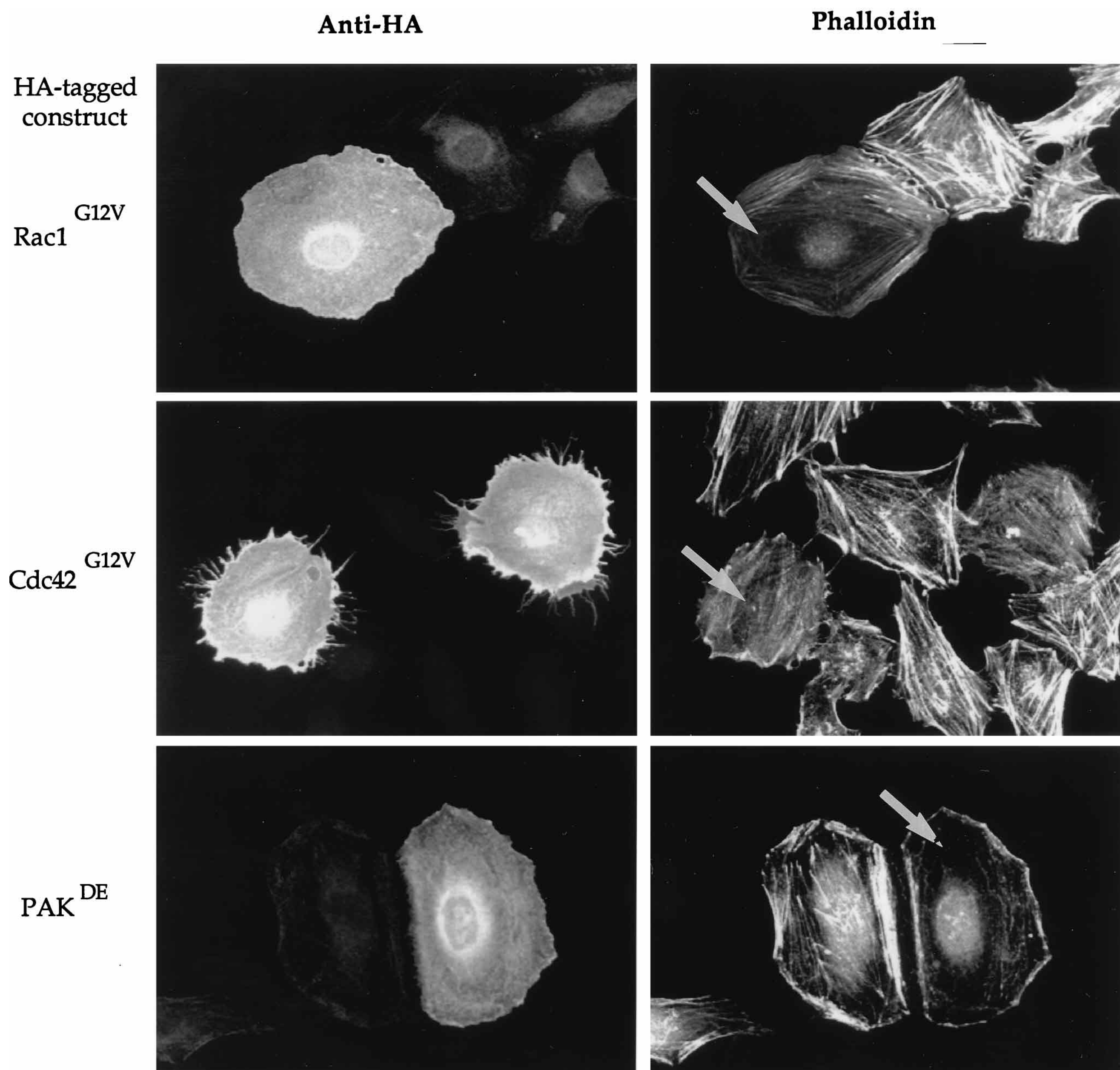


FIG. 7. Expression of active PAK^{DE}, Cdc42^{G12V}, and Rac1^{G12V} leads to loss of stress fibers. HeLa cells plated on glass coverslips in 10% serum were microinjected with pXJHA plasmids (50 ng/μl). Confocal images of cells expressing the HA-tagged proteins are shown, with Cdc42^{G12V} protein being enriched at the cell margin. Arrows indicate injected cells in which there was a clear loss of strongly staining stress fibers. In each case, these are representative of at least 20 injected cells. The cells were fixed for 3 h after injection.

DISCUSSION

The significance of PAK autophosphorylation. PAK family kinases have been implicated as acting at the summit of MAP kinases cascades in both yeast (50, 59) and mammalian (6, 7, 58) cells. Their own activation is initiated by the binding of GTP-Cdc42 or GTP-Rac1, which promotes autophosphorylation. In cotransfection studies described here (Fig. 2 and 3), we find that the activity of immunoprecipitated HA-tagged PAK is increased equally upon cotransfection with either Cdc42^{G12V} or Rac1^{G12V}. However, this p21-mediated activation in vivo is much less than achievable in vitro. Thus, the in vivo activated PAK is further substantially stimulated when incubated with GTP-Cdc42 or GTP-Rac1. Since PAK is colocalized with the p21s (Fig. 5) and therefore able to bind and be activated by either p21, our results suggest that PAK is subject to strong negative regulation in vivo. The large number of α-PAK autophosphorylation sites implies that regulation of the kinase can be quite complex. The site equivalent to T422 in Ste20p is required for this kinase to exert its biological activity within the mating pheromone response pathway (55), although Ste20p does not contain sites equivalent to those mapped in the α-PAK regulatory domain. Two autophosphorylation sites identified in a proteolytically active 40-kDa fragment of a purified placental PAK (4) appear to be equivalent to α-PAK

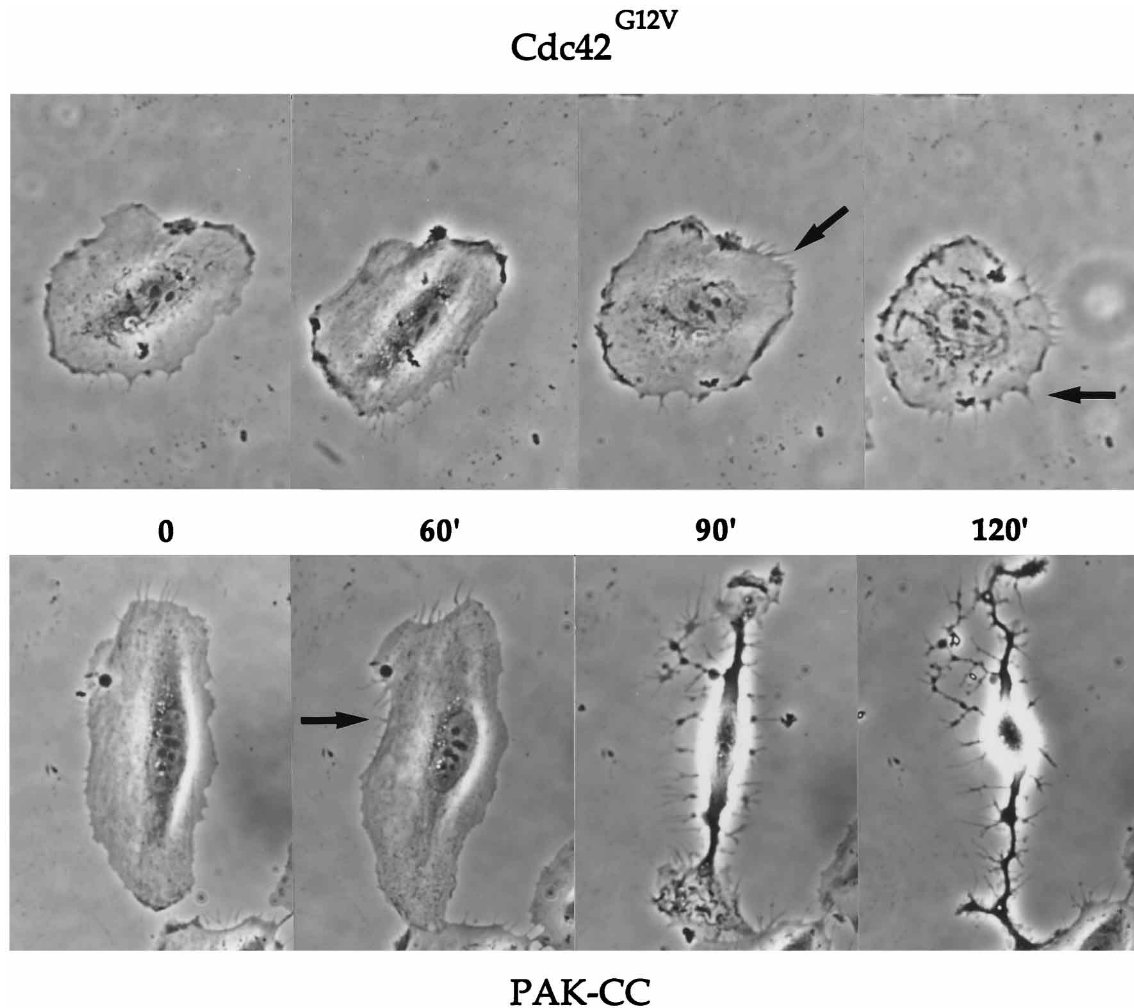


FIG. 8. Microinjection of the PAK-CC expressing cDNA construct causes rapid cell shape changes. Phase-contrast micrographs show HeLa cells following nuclear microinjection of plasmid (50 ng/μl) encoding Cdc42^{G12V} or PAK-CC. After injection cells, were maintained at 37°C in 10% serum and photographed at the indicated times (minutes). The phenotype with Cdc42^{G12V} included production of filopodia (arrow, panel 2) and retraction fibers (panel 3) in addition to membrane ruffles. PAK-CC injected cells exhibited retraction of peripheral membranes starting at 60 min (arrow).

S203 and T422, which suggests that common regulatory sites exist among mammalian PAKs. Since N-terminally truncated PAK is active (45) or is activated by proteolysis, Benner et al. have suggested that the N terminus of PAK contains a kinase inhibitory domain (4). The interaction of the inhibitory N terminus with the catalytic domain may generate a more compact structure for the inactive kinase (shown schematically in Fig. 10), which is reflected by its greater electrophoretic mobility compared with the activated and relatively unfolded kinase on SDS-polyacrylamide gels (Fig. 1B).

That COS-7 cell-expressed α-PAK^{T422E} is only partially activated is not unexpected, since equivalent substitutions in other proteins can generate nonfunctional (e.g., cAPK^{T197D} [1]) or at best partially active (e.g., ERK-2^{T183E} [57]) kinase. It is not clear why and how bacterially expressed mammalian PAKs are phosphorylated and active. One might predict that certain amino acid substitutions in the p21-binding domain of PAK cause kinase activation by mimicking conformational changes associated with binding of GTP-p21; indeed, the constitutively active β-PAK (mouse PAK-3) protein (6) and the active α-PAK (hPAK1^{L107F}) mutant selected in *S. cerevisiae* (7) both contain amino acid substitutions in the p21-binding region.

PAK as a focal complex protein. Recruitment of PAK to focal complexes by activated Cdc42 and Rac1 (when p21 and PAK are expressed at low levels as shown in Fig. 4) strongly suggests that both p21s utilize PAK in vivo and that PAK probably participates in the dynamics of these focal complexes. Analogous DRac-dependent focal complexes have been identified at the leading edge of epidermal cells whose morphological changes drive dorsal closure of the *Drosophila* embryo. These structures are enriched for DPAK, integrin, and phosphotyrosine (17). Here we show that the regulatory N-terminal half of α-PAK (which is significantly conserved in DPAK) can bind to FAs which have components in common with focal complexes. This PAK$^{1-250}$ region has no similarity to the FA targeting domain of the p125^{FAK} localized to residues 853 to 1012 in the kinase C terminus (18). Since transfected kinase-inactive α-PAK^{K298A} and truncated PAK$^{1-250}$ are targeted to FAs, kinase activity is not required for binding. The FA localization of transfected PAK^{K298A}(H83/86L) with defective p21 binding indicates that PAK interaction with Cdc42 or Rac1

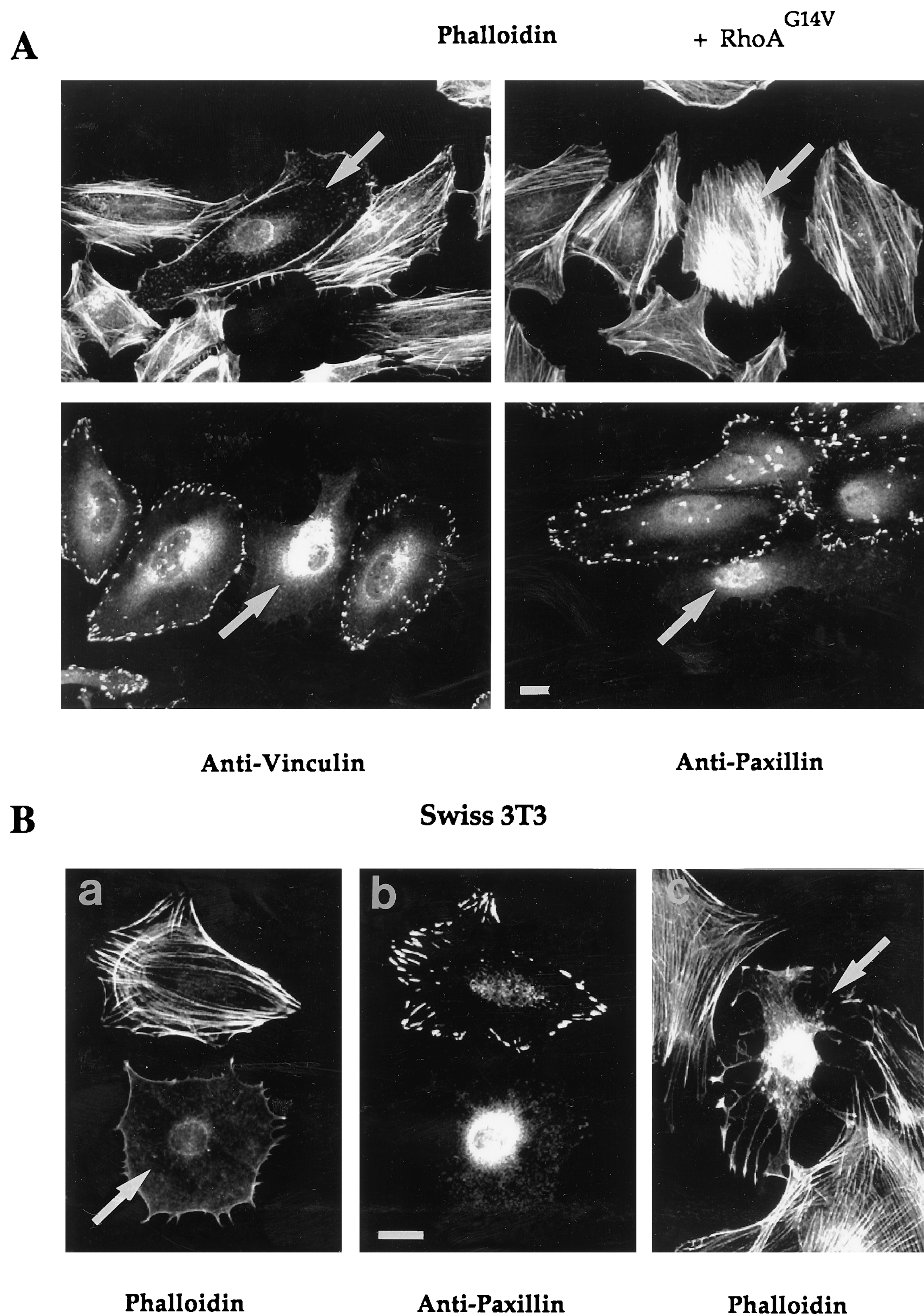


FIG. 9. PAK-CC expression causes loss of FAs and actin stress fibers. (A) HeLa cells microinjected with plasmids (arrows) encoding PAK-CC alone or in combination with RhoA^{G14V} were fixed after 1 h of injection and stained with the appropriate antibodies. (B) Swiss 3T3 cell were injected under the same conditions as used for panel A. After 1 h (panels a and b), cells were fixed and stained for paxillin or phalloidin as indicated. Panel c shows the typical morphology of cells 2 h postinjection. Confocal images were representative of at least 10 injected cells. Bar, 10 μm.

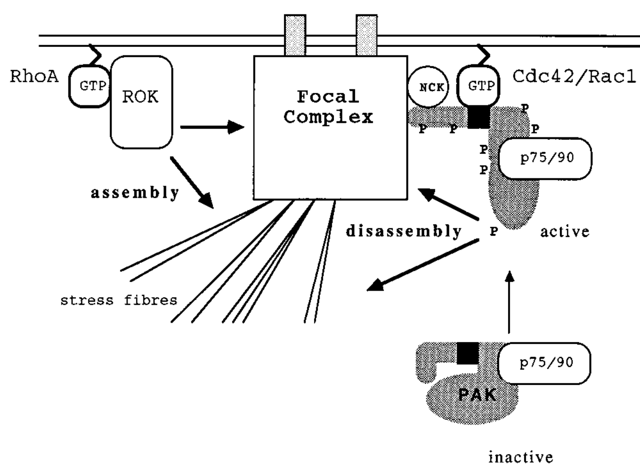


FIG. 10. A model for the role of PAK in focal complex dynamics. The model shows targeting of PAK by GTP-Cdc42 or GTP-Rac1 to a Rho-dependent FA complex, which is characterized by a larger size than Cdc42- and Rac1-dependent focal complexes and numerous associated stress fibers. In the case of the Rho-dependent FAs, their formation and that of associated stress fibers are driven by interaction of RhoA with the downstream kinase ROK (32); Cdc42 and Rac1 may utilize other proteins whose kinase domains are similar to that of ROK in the formation of their own complexes. All such complexes contain common components such as paxillin, vinculin, and talin. The interaction of PAK with Cdc42 and Rac1 leads to autophosphorylation at a number of sites and conformational changes that unmask the focal complex binding site(s). Activated PAK exerts a local effect to promote dissolution of these focal complexes and stress fibers. Activated kinase can phosphorylate putative p75 and p90 targets which might play a role in these functions. PAK may have a more limited action on Cdc42- and Rac1-dependent focal complexes because of concomitant down-regulation (see text).

may not be absolutely essential for FA localization of PAK. These observations are incorporated in our model of Cdc42 and Rac1 recruitment of PAK to focal complexes (Fig. 10). The association of PAK with its p21 partner disrupts intramolecular interactions between regulatory and catalytic domains of PAK, leading to PAK activation and unmasking of the focal complex binding site(s). In support of PAK intramolecular interactions, we have identified a kinase autoinhibitory domain in the N terminus of PAK (60). These interactions may be disrupted in the K298A mutant through structural alterations in the mutated kinase domain lacking the conserved lysine that coordinates ATP. Recombinant GST/α-PAK^{K298A} protein is recovered in a highly degraded state, indicative of an opening up of the protein structure which increases susceptibility to proteolysis.

PAK may be involved with phosphorylation of target proteins that include the p75 and p90 proteins (Fig. 10). The p90 substrate in COS-7 cells could be equivalent to the tyrosine-phosphorylated 90-kDa protein detected in PAK1 immunoprecipitates (15). The p90 therefore represents a potential link of PAK to phosphotyrosine-enriched components of the focal complexes, as is the adapter protein Nck, which contains both SH3 and SH2 domains and binds to the N terminus of PAK (15). Our observation that in addition to PAK, focal complexes contain a high concentration of activated forms of Cdc42 and Rac1 is consistent with a report of Cdc42 being translocated to the cytoskeleton by an integrin-mediated process in human platelets (12). Similarly, RhoA has been shown to be recruited by interaction of fibronectin-coated beads with fibroblasts to FA-like complexes (9). It is likely that other proteins involved with Rho-p21 signalling are present in focal complexes. Examples are the putative Rho guanine nucleotide exchange factor (GEF) Vav, which binds the FA protein zyxin (21), and a FAK-associated RhoA/Cdc42 GTPase-activating protein (GAP) (19).

PAK as a modulator of the cytoskeleton downstream of p21s. Activated Cdc42 and Rac1 promote formation of peripheral actin structures such as filopodia and lamellipodia, as well as new focal complexes associated with these structures. Our findings that PAK does not directly promote formation of peripheral actin structures concur with evidence that PAK is not required for chemoattractant (Rac-mediated) membrane ruffling in neutrophils, although PAK-1 and PAK-2 (α- and γ-PAK) do become activated (27). Instead, PAK serves to down-regulate focal adhesions and stress fibers as illustrated in Fig. 10. The effects of constitutively active PAK and the observations in Fig. 7 relating to stress fiber loss indicate an antagonism between Cdc42-Rac1 and RhoA. Although it is an attractive proposition that Cdc42 lies at the top of a simple linear signaling cascade i.e., Cdc42→Rac→Rho, which might be inferred from the work of Nobes and Hall (42), the prediction that Cdc42^{G12V} expression leads to an accumulation of stress fibers similar to that seen with RhoA^{G14V} is not borne out. Such effects have not been documented, but rather stress fiber loss occurs (Fig. 7 and reference 14). The dissolution of Rho-dependent stress fibers and FAs, promoted by PAK and which accompanies formation of Cdc42 or Rac1-induced structures, may be necessary for mechanostructural reasons related to cell movement and/or release of common components required for the newly formed structures.

Activated PAK is targeted to FA, by unmasking of FA-binding sites, where its kinase activity presumably is responsible for events leading to FA dissolution (and stress fiber disassembly). PAK is also targeted to newly formed Cdc42- and Rac1-dependent focal complexes, via the FA-binding domain unmasked on activation (regardless of whether activation is partial or complete). At present we cannot state whether Cdc42 and Rac1 present in these complexes supplement recruitment of PAK or whether it is PAK which brings the p21s to these complexes, upon binding and activation. The difference between PAK action on FAs (which are dissolved) and on the smaller focal complexes (which appear to be partially refractory to PAK) may relate to the in vivo down-regulation of PAK that we have observed. It is possible that certain phosphatases which down-regulate PAK and limit its disassembling activity are present in focal complexes but not FAs.

The autophosphorylation sites of PAK represent likely targets for such phosphatases. These sites, which are distributed in different PAK domains, appear to be involved in separate functions. Threonine 422 autophosphorylation, which is essential for activity toward exogenous substrates, is not required for p21-driven N-terminal serine autophosphorylation (data not shown). Thus, dephosphorylation of T422 would cause loss of catalytic activity but may not interfere with maintenance of the open PAK conformation (shown schematically in Fig. 10) required for FA binding. Further experiments to determine the functional relevance of the sites of in vivo PAK phosphorylation are clearly required. A strong negative regulation of PAK would explain why we and others have never observed any significant proportion of gel-shifted endogenous PAK in extracts derived from growth factor-treated cells, in contrast to other signalling molecules such as MAP kinase or S6 kinase. PAK cotransfected with Cdc42^{G12V}, or PAK-CAAX proteins, is not highly active (Fig. 3B and C), and only by substantial overexpression of PAK and p21 can significant hyperphosphorylated PAK be observed in vivo (Fig. 2 and 3). This cellular down-regulation may be a means of balancing the opposing activities of Cdc42 and Rac1 in promoting both the assembly and disassembly of focal complexes. Although PAK might be

targeted to its site of action by Cdc42 and Rac1, only a fraction of the kinase may be actively participating to facilitate focal complex turnover. This leaves the possibility that the nonkinase domain participates in other functions within the focal complex.

That PAK does act on focal complexes, albeit on a limited basis, is supported by our observations that in $Rac1^{G12V}$-transfected cells, increasing levels of cotransfected PAK and $Rac1^{G12V}$ correlate with decreases in the size of focal complexes (data not shown). This action can also explain why cells expressing activated Cdc42 or Rac1 do not massively accumulate focal complexes as seen with $RhoA^{G14V}$ (Fig. 4d).

In addition to PAK, other Rho-p21 effectors are potentially linked to cytoskeletal changes. Wiskott-Aldrich syndrome protein has been implicated in actin polymerization events downstream of Cdc42, perhaps through recruitment of profilin-like proteins (51), and POR1 may participate in Rac1-mediated ruffle formation (53), although neither of these proteins is widely expressed. Interestingly, the Rac GAP *n*-chimaerin can cooperate with Rac1 and Cdc42 to induce formation of both lamellipodia and filopodia (29), suggesting that the diverse family of GAPs for the Rho-p21 family are intimately linked to cytoskeletal events. Putative effectors of RhoA signalling include protein kinase N (2, 54), which is related to rhophilin (54), and the Rho-associated kinases (ROKs) (31, 32). We have recently established that ROKα acts downstream of RhoA to promote the formation of actin stress fibers and focal complexes in both HeLa and fibroblast cells (32). This p160 serine/threonine kinase may mediate its effects via phosphorylation of a subunit of myosin light-chain kinase phosphatase (26). Since Cdc42 and Rac1 also promote focal complex formation, it is particularly interesting that the ubiquitous p180 Cdc42/Rac1-binding proteins detected in p21 overlays (36) contain a kinase domain related to that of ROKs (33). In the model shown in Fig. 10, ROK is shown to be acting downstream of RhoA in promoting formation of FAs; perhaps the p180 kinases carry out an analogous function during Cdc42- and Rac1-driven focal complex formation.

Since both Cdc42 and Rac1 produce highly dynamic structures (i.e., filopodia or membrane ruffles), the turnover of focal complexes (and peripheral actin structures) is likely to be important. The highly elevated PAK levels in certain adult neurons (37) could reflect their requirement for cytoskeletal plasticity. Similarly, DPAK is enriched in the leading edge of the *Drosophila* embryo epidermis; these cells have to undergo extensive cell shape changes and directed movement to achieve dorsal closure (17), a process perhaps requiring additional PAK activity in these cells. Thus, PAK activation could be critical for the cycle of focal complex recruitment-disassembly to facilitate the dynamic remodelling of the cytoskeleton.

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