A Tyrosine-phosphorylated Protein That Binds to an Important Regulatory Region on the Cool Family of p21-activated Kinase-binding Proteins*

(Received for publication, February 5, 1999, and in revised form, May 7, 1999)

Shubha Bagrodia‡, Dennis Bailey‡, Zoe Lenard‡, Matt Hart§, Jun Lin Guan‡, Richard T. Premont†, Stephen J. Taylor‡, and Richard A. Cerione†**

From the ‡Department of Molecular Medicine, |Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853-6401, §Onyx Pharmaceuticals, Richmond, California 94806, and the ¶Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710

The p21-activated kinases (Pak) are major targets of the small GTPases Cdc42 and Rac. We, and others, recently identified a family of proteins termed Cool/Pix, which interact with Pak3. In cells, p50Cool-1 suppresses Pak activation by upstream activators; p85Cool-1 has a permissive effect on Pak activation, and we now show that the closely related Cool-2 stimulates Pak kinase activity. To understand the differential regulation of Pak by Cool proteins, we screened for Cool-interacting proteins by affinity purification and microsequencing. This has led to the identification of two closely related proteins called Cat (Cool-associated, tyrosine phosphorylated), which contain a zinc finger followed by three ankyrin repeats. Cat-1 is identical to the recently identified binding partner for the β-adrenergic receptor kinase (βARK or GRK-2), which was shown to have Arf-GAP activity. Cat-1 and Cat-2 both bind to the COOH-terminal region of p85Cool-1 and p85Cool-2 but do not bind to p50Cool-1. Cat-1 is tyrosine-phosphorylated in growing NIH 3T3 fibroblasts, and its tyrosine phosphorylation is increased following cell spreading on fibronectin, decreased in cells arrested in mitosis, and increased in the ensuing G1 phase. Cat proteins are tyrosine-phosphorylated when co-expressed in cells with the focal adhesion kinase Pak and Src. These findings suggest that in addition to playing a role in Cool/Pak interactions, Cat proteins may serve as points of convergence between G protein-coupled receptors, integrins, Arf GTPases, cell cycle regulators, and Cdc42/Rac/Pak signaling pathways.

The GTP-binding proteins Cdc42 and Rac initiate signaling pathways that impact on cell cycle progression and influence cell shape and the actin cytoskeleton (1–6). Among the best known targets of these GTP-binding proteins are the p21-activated serine/threonine kinases (Paks)1 (7, 8). At present, there are three closely related members of the family (Pak1–3) and a recently discovered but less closely related member named Pak4 (9). The mammalian Paks are homologous to the Saccharomyces cerevisiae Ste20 protein kinase, which plays an essential role in the pheromone/mating factor pathway by regulating a protein kinase cascade that leads to the nucleus and stimulates the mitogen-activated protein kinases FUS3/KSS1 (10). It was subsequently appreciated that both Cdc42 and Rac also initiate signaling cascades to the nucleus that involve activation of the stress responsive mitogen-activated protein kinases, c-Jun kinase, and p38 (11–14) and that in some situations, this signaling is mediated via Cdc42/Rac-stimulation of Pak activity (13, 15).

Pak has been implicated as an important mediator in triggering actin cytoskeletal and cell morphological changes (16–21). In some cases, it appears that Pak activation antagonizes RhoA-mediated signaling, resulting in dissolution of actin stress fibers and a decrease in the formation of focal complexes (17). It has also been reported that the overexpression of Pak leads to actin cytoskeletal rearrangements, which do not depend on its kinase activity (16), and that Pak is essential for neurite extension in rat adrenal PC12 cells (19). Recently it has been shown that Pak is required for cell transformation by Ras (22) and that phosphorylation of Raf-1 by Pak may play an important role in Ras-mediated transformation (23). Pak may therefore function as critical control proteins for a variety of Cdc42- and Rac-stimulated cellular responses making it likely that they are tightly regulated both in terms of their activity and cellular localization. Recent developments are consistent with an intricate regulation of the Paks, because a family of Pak-binding proteins named Cool (cloned out of library; Ref. 24), Pix (Pak-interacting exchange factor; Ref. 25), or p85SPR (SH3 domain containing, proline-rich; Ref. 26) has been identified. These proteins share tandem SH3, Dbl homology, and pleckstrin homology domains and associate with Paks via their SH3 domain. The presence of the Dbl homology domain implies a guanine nucleotide exchange factor (GEF) activity for the Cool proteins, which by directly activating Cdc42 and/or Rac would activate Pak. p85Cool-1 (identical to β-Pix and p85SPR) has been shown to have weak (25) or undetectable (24) exchange factor activity against Rac1 and no detectable activity against Cdc42 (24, 25). This suggests that either the exchange factor activities of Cool proteins are tightly regulated or that their mechanism of action does not involve exchange factor activity. This possibility is supported by the observation that p50Cool-1, a splice variant of p85Cool-1 that contains the presumptive exchange factor domain, inhibits rather than activates Pak (24). p85Cool-1 does not inhibit Pak activity but rather has a permissive effect on the stimulation of Pak by the GEF Dbl (24). The differences in the functional outcomes of the...
binding of p50Cool-1 and p85Cool-1 to an identical site on the Pak proteins suggest that the extended carboxyl-terminal region of p85Cool-1 might prevent it from competitively inhibiting Pak autophosphorylation and phosphorylation of substrates.

In the present work, we describe the identification and characterization of a family of proteins that bind to the carboxyl-terminal region of p85Cool-1 and do not bind to p50Cool-1. One of these is a 95-kDa protein that is tyrosine-phosphorylated in vivo and therefore has been designated Cat-1 (Cool-associated, tyrosine phosphorylated). A second highly related gene product, M, ~85 kDa, was cloned and named Cat-2. In addition to their specific binding to p85Cool-1, both Cat-1 and Cat-2 are also capable of binding to the carboxyl-terminal domain of Cool-2 (identical to α-Pix), a member of the Cool/Pix/p85SR family that unlike Cool-1 activates Pak3 when the two proteins are co-expressed in cells. The abilities of the Cat proteins to selectively bind to Cool proteins, which promote rather than repress Pak activity, as well as our finding that Cat is tyrosine phosphorylated in a cell adhesion and cell cycle-dependent manner, suggest that Cool-Cat complexes may play key roles in Pak regulation.

**EXPERIMENTAL PROCEDURES**

**Cloning of Cat-2 cDNA**—Based on peptide microsequencing results, oligonucleotides 5'-GATGCAGTCCTGGTGGCCAG-3' and 5'-TCGT-GTTGATGAGTACGCAGG-3' corresponding to DAVWLT and PYESSTR, respectively, encoded by the human KIAA0148 gene were used to amplify a 96-bp PCR product from a lambda ZapHeLa cDNA library (Stratagene). The PCR product was subcloned into PCR2.1 (Invitrogen) and sequenced using an automated sequencer (Applied Biosystems). A lambda Zap mouse NIH 3T3 cDNA phage library (Stratagene) was screened with the 96-bp PCR product labeled by randomly priming with [α-32P]dCTP (Prime-gene kit, Stratagene). Eight positive plaques were picked (25). The excised cDNA inserts were partially sequenced. Several clones contained sequences related to the KIAA0148 gene. The longest clone (~2100 bp) was sequenced and determined to lack the 5'-end. The 5'-end of Cat-2 containing the initiation ATG (~400 bp) was cloned by 5'-rapid amplification of cDNA ends (Life Technologies) from mouse brain poly(A) RNA (CLONTECH).

**Cloning of Cool-2—**Cool-2 was cloned from a human hippocampus lambda Zap cDNA library (Stratagene) using a probe containing nucleotides 658–1324 that were generated using primers based on the GenBank™ accession P23304 sequence.

**Plasmid Construction**—Full-length Cat-2 was cloned into the BamHI site of hemagglutinin (HA)- or Myc-tagged pcDNA3.1Aack-2 or a Myc-tagged pcDNA3 vector as an ~2480-bp insert. Full-length Cool-2 was cloned into the XbaI and PclI/Sous1 sites in the Myc-tagged vector JCM as an ~2480-bp insert. Cool-2 from the JCM vector was also subcloned into the XbaI/EcoRI sites of the Myc-tagged pcDNA3 vector. The COOH-terminal domain of Cool-1 spanning amino acids 369–649 was cloned by PCR and subcloned as a BamHI-EcoRI fragment into Myc-tagged pcDNA3 vector. The plasmid expressing COOH-terminal Flag-tagged rat Git-1 (2 protein-coupled receptor kinase interactor-1) has been described previously (27). Plasmids expressing p50Cool-1, p85Cool-1, p56(W43K) Cool-1, and Dbl have all been described (24). The Dbl homology domain of Cool-1 spanning amino acids 369–649 was amplified by PCR and cloned as a BamHI-EcoRI fragment into Myc-tagged pcDNA3 vector. A cDNA fragment encoding amino acids 148–238 of Pak3 was amplified by PCR and subcloned into the BamHI-SalI site of the pGEK-KG plasmid to express the Cool-binding domain (CBD). Amino acids 537–866 of Cool-2 were amplified by PCR and subcloned into the BamHI-EcoRI site of plasmid pGEK-KG. The triple HA-tagged Pak, the kinase-defective HA-Fak (K454R), and HA-Fak(Y397F) expression plasmids have been described previously (28).

**RESULTS**

**Cool-2 Activates Pak**—We have previously shown that a novel Pak3 binding partner, named p50Cool-1 (cloned out of library), inhibits Pak3 activity and its stimulation by upstream activators. A larger splice variant designated p85Cool-1, which is identical to β-Pix (25), competes with p50Cool-1 for binding to Pak3 but is not inhibitory and instead has a permissive effect on Pak3 activity (24). A related gene product, which we named Cool-2 and is identical to α-Pix (24, 25), also competes with p50Cool-1 for binding Pak3; however, it stimulates rather than inhibits Pak3 activity. These data are presented in Fig. 1. In this experiment, Pak3 was co-expressed with the different Cool proteins or with other members of the Dbl family, in COS cells. Pak3 was then precipitated, and its activity to phosphorylate exogenous substrates was assayed using myelin basic protein. In the absence of a stimulatory factor (e.g. activated Cdc42 or Rac), p50Cool-1 had no detectable effect on myelin basic protein phosphorylation, whereas p56(W43K) Cool-1 showed a modest (~2-fold) stimulation. However, like Dbl, Tiam-1 and Vav, which have been shown to act as guanine nucleotide exchange factors for Cdc42 or Rac (30–32) and thereby activate Cdc42/Rac-targets (e.g. Pak3), co-expression of Cool-2 with Pak3 also significantly stimulated Pak3 activity. These findings raise the interesting question of why Cool-2 is able to bind and stimulate Pak3 activity, or in the case of p50Cool-1 permissively support Pak3 activity, whereas p50Cool-1 acts as a competitive inhibitor of autophosphorylation and substrate phosphorylation. We reasoned that the extended carboxyl terminus present within p85Cool-1 and p85Cool-2, relative to p50Cool-1, may allow a protein-binding interaction that...
prevents these Cool molecules from blocking access to the protein kinase active site of Pak3. Thus, we set out to identify a binding partner that was capable of associating with p85Cool-1 and/or p85Cool-2 but not p50Cool-1.

A Tyrosine-phosphorylated 95-kDa Protein Co-precipitates with Cool and Pak3—Our first clue to the existence of a cellular protein that binds specifically to p85Cool-1 or Cool-2, but not p50Cool-1, came from biochemical studies aimed at identifying Pak3-associated proteins. As shown in Fig. 2A, we initially noticed a tyrosine-phosphorylated 95-kDa protein that co-immunoprecipitated with Pak3 from NIH 3T3 cell lysates (lane 6). A tyrosine-phosphorylated protein of identical mass was also detected when cell lysates were affinity precipitated with a GTPase-defective, constitutively active Cdc42 mutant, GST-Cdc42(Q61L) (Fig. 2A, lane 4) but not when lysates were incubated with GST-Cdc42(GDP) (lane 3). Thus, the 95-kDa tyrosine-phosphorylated protein (originally designated p95) appeared to associate with GST-Cdc42, perhaps via its association with Pak3.

To delineate the region on Pak3 that was responsible for its association with p95, different GST-Pak3 fusion proteins were prepared and used as affinity reagents. A GST fusion protein that contained amino acid residues 148–238 of Pak3 was able to affinity precipitate the tyrosine-phosphorylated p95 from NIH 3T3 cell lysates (Fig. 2A, lane 5). We refer to this region on Pak3 as the CBD, because we have found that it is sufficient for binding the Cool proteins. The fact that the CBD was essential for the association of p95 raised the possibility that p95 may bind indirectly to Pak3 via Cool. This was further suggested when we were able to co-immunoprecipitate p95 with Cool from NIH 3T3 cells using an anti-Cool antibody (Fig. 2B, lane 1). We were not able to detect endogenous Pak in the anti-Cool immunoprecipitates (possibly because there is less Pak than Cool or p95 in NIH 3T3 cells and/or because the Cool antibody interferes with the Pak3-binding site on Cool). Therefore, co-precipitation of p95 with Cool-1 could not be attributed to a p95-Pak interaction, but most likely reflected a direct interaction between p95 and Cool.

We next set out to establish that the tyrosine-phosphorylated p95 that co-immunoprecipitated with Cool was identical to the 95-kDa protein that co-immunoprecipitated with Pak-3, as well as affinity precipitated with GST-CBD. The serial depletion of tyrosine-phosphorylated p95 from Src(Y527F)-transformed NIH 3T3 cell lysates resulted in a decrease in the amount of tyrosine-phosphorylated p95 associated with GST-CBD (Fig. 2C, lanes 1–3) and in the amount of p95 associated with GST-Cool-2 (residues 537–666) (lane 4 where p95 was depleted from lysates versus lane 5 where it was not depleted). These data argue that the tyrosine-phosphorylated p95 that associated with Pak3 and GST-CBD was identical to the 95-kDa phosphoprotein that bound to GST-Cool-2 (residues 537–666). Because p95 was a Cool-associated and tyrosine-phosphorylated protein, we refer to it as Cat-1. It should be noted that the ability of Cat-1 to associate with activated Cdc42 (Fig. 2A, lane 4) is apparently through a quaternary Cdc42-Pak3-Cool-Cat complex.

Identification of Mouse p95 Cat-1 as Git-1 and Molecular Cloning of a Related Protein, Cat-2—Using GST-CBD as an affinity reagent, we set out to purify Cat-1 from Src(Y527F)-transformed cells, which can be grown to high density and appeared to contain relatively large amounts of the tyrosine-phosphorylated p95 Cat protein. Silver staining revealed two major bands of Mr ~86 and 95 kDa that were co-precipitated with GST-CBD (Fig. 3A, lanes 2 and 3). The protein band at ~86 kDa is p85Cool-1, as determined by microsequence analysis. Three peptides obtained from microsequencing of the 95-kDa protein were identical to a recently identified protein designated Git-1, which was discovered based on its ability to bind the β-adrenergic receptor kinase (GRK-2) (27). The same three peptides obtained from the 95-kDa protein also showed close matches with an ~50-kDa human protein of unknown function, KIAA0148 (peptide 1, 15/15 identity; peptide 2, 16/16 identity; peptide 3, 13/16 identity, 15/16 similarity). We also identified an additional related protein through molecular cloning, using oligonucleotides derived from the KIAA0148 sequence to screen an NIH 3T3 cDNA library. We isolated a full-length clone that encodes a protein of 708 amino acids that we termed Cat-2 (Fig. 3B). As shown below, both Cat-1 and Cat-2 are Cool-binding proteins. Cat-2 appears to be 81% identical and 87% similar to a paxillin-binding protein designated paxillin kinase linker (Pkl) (45), and Cat-2 is 69% identical and 78% similar to rat Git-1 and 86% identical and 89% similar to human KIAA0148.

In summary, we believe that Cat-1 is the mouse homolog of rat Git-1 and that Cat-2 is an alternatively spliced homolog of human Pkl and KIAA0148. Both Cat-1 and Cat-2 are widely distributed in a variety of tissues including heart, brain, and skeletal muscle (data not shown).

Blot sequence analysis showed that like Cat-1, the Cat-2 protein has a Gea type (33) C2C2H2 zinc finger motif (amino acids 11–44), which is part of a larger conserved domain (amino acids 3–77), and a triple ankyrin repeat region (amino acids 126–226) (Fig. 3C). Both Cat-1 and Cat-2 also show sequence similarity to ASAP1, a phospholipid-dependent Arf-GAP that associates with and is phosphorylated by Src (34), mammalian Arf-GAP1 (35), and yeast Gsc1 (36), as well as sharing homology with the tumor suppressor BRCA1-associated ring domain protein, BARD1 (accession number 495973), the phosphatidylinositol 1,4,5-trisphosphate-binding protein, centaurin-α (37), and several proteins of unknown function in the data base. So far, Arf-GAP, Git-1, and ASAP1 have been shown to have Arf-GAP activity (27, 34, 35). p95 Cat-1 and p95 Cat-2 also contain a number of potential SH3-binding sites (PXXP).

Both Cat-1 and Cat-2 bind to p85 Cool-1 in mammalian cells. This was shown by transiently co-expressing Flag-tagged Cat-1 or HA-Cat-2 with Myc-tagged Pak3, Myc-p50 Cool-1, Myc-p85 Cool-1, or Myc-p5(W43K Cool-1) (an SH3 domain point mutant that is defective in binding Pak3 (24)) and then assaying for complex formation by co-immunoprecipitation and Western blot analysis (Fig. 4). Flag-Cat-1 (Fig. 4A) and HA-Cat-2 (Fig. 4, A, lane 1).
Incubated with either anti-Cool polyclonal antibody (Wcl, whole cell lysates, A, whole cell lysates from NIH 3T3 fibroblasts were incubated with either anti-Cool polyclonal antibody (lane 1) or protein A-Sepharose alone (lane 2). Bound proteins were processed as in A. C, cell lysates from NIH 3T3 cells overexpressing Src (Y527F) were incubated with GST-CBD for 2 h and then precipitated (lane 1); the supernatant from lane 1 was reincubated with fresh GST-CBD for an additional 2 h and then precipitated (lane 2), and the supernatant from lane 2 was reincubated with fresh GST-CBD for 1 h and then precipitated (lane 3). GST-Cool-2 (residues 537–666) was incubated with either the depleted supernatant from lane 3 (lane 4) or with nondepleted lysate (lane 5). Bound proteins were detected by Western blotting with anti-phosphotyrosine antibody 4G10 (UBI).

B and C) were each detected in anti-Myc p85Cool1 and p85Cool2 immunoprecipitates (Fig. 4A, lanes 1 and 2; Fig. 4B, lane 3; and Fig. 4C, lanes 3 and 4) and did not associate with Pak3 (Fig. 4B, lane 1) or p50Cool (Fig. 4B, lane 2). The Cat/Cool interactions are not dependent on the SH3 domain of Cool, as p85Cool1 point-mutated in the SH3 domain still interacts with both Cat-1 (Fig. 4A, lane 5) and Cat-2 (Fig. 4B, lane 4). The interactions between the Cat and Cool molecules must involve the carboxyl-terminal domains of p85Cool1 or p85Cool2, because p50Cool, which does not bind the Cat proteins, is otherwise identical to p85Cool1. As shown in Fig. 4A, 4 and 4C, amino acid residues 369–649 of p85Cool1 are sufficient for binding Flag-tagged Cat-1 (lane 3) and HA-tagged Cat-2 (lane 5).

Cell Cycle and Cell Adhesion-dependent Regulation of Tyrosine Phosphorylation of p95—Given that the Paks and their upstream activators, Cdc42 and Rac, have been implicated in the regulation of cell cycle progression (1), we were interested in examining the tyrosine phosphorylation of Cat-1 during the cell cycle (Fig. 5). We expressed Flag-tagged Cat-1 in NIH 3T3 cells, arrested the transfected cells in mitosis by nocodazole treatment, and analyzed tyrosine phosphorylation of Cat-1 in anti-Flag immunoprecipitates by anti-phosphotyrosine immunoblotting (Fig. 5A). Consistent with our results with endogenous p95, we found that Cat-1 was tyrosine phosphorylated in unsynchronized NIH 3T3 cells (lane 2). However, phosphotyrosine was not detectable in Cat-1 immunoprecipitated from mitotic cells (lane 3) indicating that Cat-1 was tyrosine dephosphorylated when cells were in mitosis. When cells were released from mitotic arrest, Cat-1 was again phosphorylated on tyrosine (lane 4). These results were consistent with those obtained when examining the tyrosine phosphorylation of endogenous p95. Tyrosine-phosphorylated p95 was affinity precipitated by GST-CBD and co-immunoprecipitated with Cool from unsynchronized NIH 3T3 cell lysates (Fig. 5C, lanes 4 and 7). However, in cells arrested in mitosis (Fig. 5B, middle panel), tyrosine-phosphorylated p95 was not detected in either GST-CBD affinity precipitates (Fig. 5C, lane 5) or in anti-Cool immunoprecipitates (lane 8). When cells were released from mitosis, a rapid reappearance (within 1 h) of tyrosine-phosphorylated p95 in GST-CBD affinity precipitates and anti-Cool immunoprecipitates (lanes 6 and 9) was detected. The reappearance of tyrosine-phosphorylated p95 correlated with the readherence and spreading of the early G1 cells to the substratum (Fig. 5B, right panel). We therefore examined whether tyrosine phosphorylation of Cat-1 was dependent on cell adhesion. NIH 3T3 cells were trypsinized and replated on plates coated with fibronectin, polylysine, or bovine serum albumin, and tyrosine phosphorylation of endogenous p95 was analyzed in GST-CBD affinity precipitates (Fig. 6A). Tyrosine phosphorylation of p95 was detectable only after plating on fibronectin (lane 5). Tyrosine phosphorylation was induced within 15 min of plating on fibronectin and continued to increase when cells were plated for up to 1 h (Fig. 6B, lanes 6–8).

These results suggested that tyrosine phosphorylation of Cat-1 may occur via integrin-mediated signaling pathways, which led us to examine the possibility that Cat-1 and/or Cat-2 might be phosphorylated by tyrosine kinases that have been implicated in integrin signaling (Fig. 7). COS cells were co-transfected with Myc-tagged Cat-2 and wild-type HA-tagged Fak, kinase-defective HA-Fak(K454R), or HA-Fak mutated at an autophosphorylation site (Y397F), and then immunoprecipitated Cat-2 was analyzed by Western blotting with anti-phosphotyrosine (Fig. 7A). Tyrosine-phosphorylated Cat-2 was not detected in control cells (transfected with vector alone, lane 2);
FIG. 3. Purification and molecular cloning of Cat. A, cell lysates from Src (Y527F)-transformed cells (527) or lysis buffer alone (LB) were affinity precipitated with GST-CBD (lanes 1–3) or GST alone (lane 4). Bound proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by silver staining. The positions of p95 and molecular mass standards are indicated. B, schematic representation and amino acid sequence of mouse Cat-2. The coding sequence of Cat-2 contains a zinc finger region (residues 3–77) containing the motif CXXC16CXXC, where X is any amino acid (residues 11–44) and three ankyrin repeat regions (residues 126–226). Amino acid sequence alignment was done using the Jotun Hein cluster method. Peptides 1–3, obtained from microsequencing of p95, were identical to residues 152–166, 352–370, and 371–386 in Git-1, respectively. Mouse Cat-2 is 69 and 86% identical to the amino acid sequence of rat Git-1 and human KIAA0148, respectively.
Myc-tagged proteins (lanes 1–3, 5) or empty vector alone with a plasmid expressing FLAG-tagged Cat-1/Git-1. Anti-Myc (9E10) immunoprecipitates (IP) were probed with either anti-FLAG (M2) or anti-Myc antibody. Cool-1 (369–649) expresses the COOH-terminal 280 amino acids of p85Cool-1 (Fig. 7B). The fact that the Cat protein still showed detectable tyrosine phosphorylation in cells expressing kinase-defective Fak (lane 3) suggests that an additional kinase(s) is involved. One possibility is Src, because autophosphorylated Tyr-397 of Fak serves as a docking site for Src family tyrosine kinases and may mediate Src activation and recruitment to focal adhesions (42). We have examined the tyrosine phosphorylation of FLAG-tagged Cat-1 in Src-transformed NIH 3T3 cells (Fig. 7B) and found that tyrosine phosphorylation of Cat-1 was significantly increased compared with normal NIH 3T3 cells (lanes 2 and 4). Thus, the tyrosine phosphorylation of Cat that accompanies the exit of cells from mitosis into G1, and adherence to fibronectin is likely to involve both the Fak and Src tyrosine kinases. These results, taken together with the recent finding that Cat-2/Pk1 has paxillin binding capability (45), raise some interesting possibilities regarding a role for the Cat proteins in interfacing integrin signaling with Pak activation (43).

FIG. 4. Cat proteins associate with Cool in cells. A, COS cells were transiently transfected with plasmids expressing the indicated Myc-tagged proteins (lanes 1–3, 5) or empty vector alone with a plasmid expressing FLAG-tagged Cat-1/Git-1. Anti-Myc (9E10) immunoprecipitates (IP) were probed with either anti-FLAG (M2) or anti-Myc antibody. Cool-1 (369–649) expresses the COOH-terminal 280 amino acids of p85Cool-1. B, COS cells were transiently transfected with plasmids expressing the indicated Myc-tagged proteins along with HA-tagged Cat-2. Anti-Myc immunoprecipitates were Western blotted with anti-HA or anti-Myc. A portion of the whole cell lysate (WCL) was Western blotted with anti-HA to show expression of HA-Cat-2. C, COS cells were transiently transfected with plasmids expressing the indicated Myc-tagged proteins along with HA-Cat-2. Anti-Myc immunoprecipitates were Western blotted with anti-HA. A portion of the whole cell lysate was Western blotted with anti-Myc.

however, tyrosine phosphorylation of Cat-2 was readily detectable in cells expressing wild-type Fak (lane 3). A decrease in the levels of tyrosine phosphorylation of Cat-2 was observed in the presence of either kinase defective-Fak or Y397F-mutant Fak (lanes 4 and 5). The fact that the Cat protein still showed detectable tyrosine phosphorylation in cells expressing kinase-22398

Paks participate in a diversity of biological responses including regulation of actin cytoskeleton changes and signaling pathways that underly cellular stress responses and impact on cell growth (7, 8, 17–19, 21, 44). This implies that the Paks will be subject to a variety of regulatory mechanisms. At present, the best understood mechanism involves the binding of activated, GTP-bound forms of Cdc42 and Rac to an amino-terminal domain within Pak (p21-binding domain), which de-represses an intramolecular inhibitory interaction and increases access to the Pak active site. More recently, a new family of regulatory molecules have been identified, Cool/Pix, whose members bind to Pak and 3 at a proline-rich sequence downstream from the p21-binding domain (24, 25). The Pix proteins were proposed to recruit and activate Rac, which in turn could stimulate the bound Pak (25). However, recent studies with the Cool proteins (p85Cool-1 is identical to β-Pix, and Cool-2 is identical to α-Pix) suggest that more complex regulatory mechanisms may be in place. Although it was reported that the Pix proteins are capable of GEF activity (25), thus far we have not been able to convincingly show that p85Cool-1 or p85Cool-2 directly stimulates the nucleotide exchange activity of Rac or Cdc42 in vitro; we have also failed to detect a GEF activity for the Cool proteins in cellular assays of Cdc42/Rac activation under conditions where the well characterized GEFs for Cdc42 and/or Rac, Dbl, Tiam-1, and Vav all showed GEF activity (24, 25). These findings raise the possibility that p85Cool-1 and p85Cool-2 may act as cofactors to support the activation of Pak by GTP-bound Cdc42 or Rac, rather than stimulating Pak activity by directly activating these GTP-binding proteins. Moreover, p50Cool-1 inhibits rather than stimulates Pak activity, suggesting that the Cool proteins may also act as direct allosteric regulators of Pak.

A particularly interesting aspect of Cool function is that although p50Cool-1, p85Cool-1, and p85Cool-2 all appear to bind to an identical region on Pak such that they compete with each other for binding, the larger Cool proteins are not inhibitory, and in fact Cool-2 stimulates Pak kinase activity. Inhibition of Pak activity by p50Cool-1 appears to be competitive, possibly by blocking access of substrates, including Pak autophosphorylation sites. Because p85Cool-1 and p85Cool-2 which both have an extended COOH terminus downstream of the SH3/Dbl homology/pleckstrin homology domain, do not inhibit Pak, it is possible that these larger Cool proteins are conformationally distinct from p50Cool-1 when bound to Pak. The most likely possibility for these functional differences between the different Cool molecules is that a protein(s) binds to the extended carboxyl-terminal domains of p85Cool-1 and p85Cool-2 and main-

2 S. Bagrodia, D. Bailey, Z. Lenard, M. Hart, J. L. Guan, R. T. Premont, unpublished data.
tains these Cool molecules in an optimal orientation for supporting activation, rather than inhibition, of Pak activity.

We have now identified two proteins that bind to the carboxyl-terminal domains of p85Cool-1 and p85Cool-2 but do not bind to p50Cool-1 and thus are candidates for playing a role in Pak regulation. One of these proteins is a tyrosine-phosphorylated protein, Cat-1, which is identical to Git-1, a recently identified ARK-associated protein (27). Cat-2 is a homolog of a recently identified paxillin-binding protein (Pkl) (45). Both Cat-1 and Cat-2 contain a putative zinc finger domain that shares similarity with mammalian and yeast proteins shown to possess Arf-GAP activity and indeed Git-1 is an Arf-GAP (27). Thus, in addition to playing a regulatory role in Cool/Pak interactions, the Cats are likely to serve as points of convergence for a diversity of signaling molecules including integrins, heterotrimeric G protein-coupled receptors, Arf, and Cdc42/Rac.

A particularly interesting possibility is that the Cats serve as important control points for mediating integrin- and cell cycle-dependent regulation of Pak. We have found that the tyrosine phosphorylation of Cat-1 is decreased in mitotic cells but increased early in the following G1 phase and upon cell adhesion to a fibronectin matrix. The Cats may be involved in recruiting Pak-Cool complexes to cellular sites for integrin signaling such as focal adhesions. This would be consistent with the paxillin binding capability of Cat-2/Pkl. It has also recently been reported that Pak activity is stimulated following cell adhesion (43), so it is attractive to consider that this occurs via a Cat-Cool-Pak signaling complex in which Cat maintains Cool in an appropriate conformation for a stimulatory rather than inhibitory signal to Pak. We were able to show that Cat-1 and Cat-2 are tyrosine phosphorylated after co-expression with activated Src or Fak. Although we do not know whether either of these tyrosine kinases directly phosphorylates Cat-1 or Cat-2 in cells, it is tempting to speculate that the Cats may couple Fak/Src tyrosine kinase pathways to Pak serine/threonine kinase pathways. Interestingly another protein possessing an Arf-GAP zinc finger domain/ankyrin repeat motif, ASAP1, has recently been shown to be a direct target of activated Src (34).

Thus far, we have been unable to detect significant stimulation of Pak3 kinase activity following co-expression of Cat-1 or

Fig. 5. Cell cycle-dependent Cat-1 tyrosine phosphorylation. A, NIH 3T3 cells were transiently transfected with FLAG-tagged Cat-1/Git-1; cells were then trypsinized and replated. Unsyncronized (U), cells arrested in mitosis with nocodazole (M), or cells released from nocodazole block for 90 min (MR) were lysed, and FLAG-tagged Cat-1/Git-1 was immunoprecipitated with an anti-FLAG M2 monoclonal antibody or lysates from unsynchronized cells were incubated with protein G-Sepharose alone (lane 1). Immunoprecipitates (IP) were Western blotted with either anti-phosphotyrosine or with anti-FLAG antibody. B, the panels show unsynchronized NIH 3T3 cells, cells arrested in mitosis by nocodazole, and cells released from a nocodazole block for 1 h. Mitotic cells are round and loosely attached to the plate and respread within 1 h of release. C, NIH 3T3 cell lysates from unsynchronized (U), mitotic (M), or cells released from the nocodazole block (MR) were incubated with GST-CBD or anti-Cool polyclonal antibody as indicated. Bound proteins were Western blotted with either anti-phosphotyrosine antibody 4G10, (lanes 1–9) or anti-Cool antibody (lanes 4–9). Lanes 1–3 represent a fraction of the whole cell lysate (wcl) used in the binding reaction.

Fig. 6. Stimulation of p95 tyrosine phosphorylation after cell spreading on fibronectin. A, serum-starved NIH 3T3 cells were trypsinized and replated on plates coated with bovine serum albumin (B), fibronectin (F), or poly-L-lysine (P) for 1 h. Cells were lysed and affinity precipitated with GST-CBD. Whole cell lysates (wcl) and proteins affinity precipitated with GST-CBD were detected by Western blotting with antiphosphotyrosine. B, serum-starved NIH 3T3 were trypsinized and replated on fibronectin-coated plates for 15, 30, or 60 min or on bovine serum albumin coated plates for 60 min and processed as in A.
Fig. 7. Cat proteins are tyrosine phosphorylated by Fak and Src. COS cells were transiently transfected with Myc-tagged Cat-2 or co-transfected with HA-tagged wild type (WT), kinase-defective (KD), or the autophosphorylation site-mutated (Y397F) Fak as indicated. Anti-Myc immunoprecipitates (IP) were Western blotted with anti-Myc or anti-phosphotyrosine antibody. A portion of the whole cell lysate (wcl) was Western blotted with anti-HA to show Fak expression. B, NIH 3T3 or Src (Y527F)-transformed NIH 3T3 cells were transiently transfected with FLAG-Cat-1/Git-1. Anti-FLAG immunoprecipitates (IP) were Western blotted with anti-phosphotyrosine or anti-FLAG antibody (A6).

Cat-2 with Pak3. This may mean that Cat proteins are not limiting in the cell. However, it may also suggest that additional signals, possibly from integrin or cell cycle signaling pathways, are needed to promote Cat- and Cool-mediated Pak activation. Because Paks have been shown to be involved in terminal cellular events such as transformation and apoptosis, it is not surprising that Pak activity is tightly regulated and that its activation may require multiple inputs. The Cat proteins are likely to play important roles in integrating these signals to appropriately regulate Pak activity. Future efforts will be devoted to identifying dominant-negative Cat mutants that can be used to block Pak activation and thereby help delineate the different signaling pathways responsible for Pak regulation.

Acknowledgments—We thank Dr. Frits Michiels for the HA-Tiam-1 plasmid, Dr. Takahiro Nagase for his generous gift of the KIAA0148 cDNA clone HA3431, Drs. Robert J. Lefkowitz, Wannian Yang, and Jihe Zhao for helpful discussions, and Drs. Chris Turner and Michael Brown for sharing unpublished results. We also thank Cindy Westmiller for expert secretarial assistance.

REFERENCES
1. Olsen, M. F., Ashworth, A., and Hall, A. (1995) Science 269, 1270–1272
2. Ridley, A. J., and Hall, A. (1995) Cell 70, 389–399