A Novel Regulator of p21-activated Kinases*

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Proteins of the p21-activated kinase (Pak) family have been implicated in the regulation of gene expression, cytoskeletal architecture, and apoptosis. Although the ability of Cdc42 and Rac GTPases to activate Pak is well established, relatively little is known about Pak regulation or the identity of Pak cellular targets. Here we report the identification of two closely related Pak-binding proteins, possibly arising from alternative splicing, designated p50 and p85(Cool-1) (cloned out of library). Both isoforms of Cool contain a Src homology 3 domain that directly mediates interaction with Pak3 and tandem Db1 homology and pleckstrin homology domains. Despite the presence of the Db1 homology-pleckstrin homology motif, a characteristic of Rho family activators, activation of Cdc42 or Rac by Cool is not detectable. Instead binding of p85(Cool-1), but not p50(Cool-1), to Pak3 represses its activation by upstream activators such as the Db1 oncoprotein, indicating a novel mechanism of regulation of Pak signaling.

The Rho family GTPases Rac1 and Cdc42 mediate diverse biological events including changes in the cytoskeletal architecture (1–3), stimulation of DNA synthesis (4), cellular transformation (5–8), and signaling to the nucleus (9–14). Many of the signaling pathways leading to the execution of these events involve the p21-activated kinases, Pak1–3;1 which are direct effectors of Cdc42 or Rac by Cool is not detectable. Instead binding of p85(Cool-1), but not p50(Cool-1), to Pak3 represses its activation by upstream activators such as the Db1 oncoprotein, indicating a novel mechanism of regulation of Pak signaling.

The apparent multiplicity of Pak-mediated signaling pathways suggests that Pak activity must be tightly regulated. This has been made all the more clear from the observations that Pak1 function is required for cellular transformation by Ras (8) and that Pak2 activation is involved in Fas-mediated apoptosis (21, 22). Here we describe the identification of two isoforms of a novel Pak-binding protein, probably resulting from alternative splicing of the same sequence, one of which is able to suppress Pak activation by upstream regulators.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—To identify proteins that interact with Pak3, the yeast strain L40 was co-transformed with Pak3 fused to the LexA DNA-binding domain, which regulates expression of both his3 and lacZ, and a HeLa cDNA library was fused with the Gal4 activation domain (23). Clones positive for β-galactosidase activity were rescued, and several clones that provided baindependent his3 and lacZ gene activation were sequenced. A 1-kilobase (EcoRI-XhoI) fragment of clone Y107 was used to screen an oligo(T)-primed, size fractionated (4–9 kilobases) U937 library (a gift from J. Burrows, Massachusetts Institute of Technology, Boston, MA) and a HeLa cDNA library (Stratagene). The U937 library yielded a full-length clone (A6) that was predicted to encode a protein of 436 amino acids, p50(Cool-1). Several partial clones were recovered from the HeLa library that appeared to be represent alternative spliced forms of Y107. One of these (clone 12a) was identical to a recently cloned cDNA, p85(Cool-1), but lacked the 3′ end encoding its C-terminal 31 amino acids. The p85(Cool-1) cDNA was generated by fusing this 3′ end, derived from the 3′ untranslated region of the Y907 clone A6, to clone 12a (p85(Cool-1) is therefore identical to p85(Cool-1)).

Plasmid Construction—The coding sequence of Pak3 was excised as a 1700-bp BamHI fragment from plasmid pJ3HmPak3 (12) and subcloned into pLexA (23). A BamHI site (GGF frame) was engineered in Cool-1 in front of the initiation methionine, and the BamHI-XhoI (1–730 bp) and XhoI-BglII (670 bp) fragments, which include the stop codon, were ligated into the BamHI site of plasmid BsgI to generate plasmid pBAS6Cool. The BamHI-EcoRI fragment from pBSA6Cool, encompassing the entire coding sequence of p85(Cool-1), was subcloned into Muc-tagged eukaryotic expression plasmid CMV6M to express p50(Cool-1) and pCMV6M(p85(Cool-1)-W43K) was constructed by three fragment ligation containing the 420-bp BamHI-XhoI polymerase chain reaction product, the 971-bp XhoI-EcoRI fragment from pBSA6Cool, and the 580-bp BamHI-EcoRI fragment from the CMV6M vector. Plasmid CMV6Mps85Cool (to express Muc-tagged p85(Cool-1)) was generated by ligating a BamHI-BsgI 1610-bp fragment (from clone 12a) and a polymerase chain reaction generated 340-bp BglI-EcoRI fragment (from the 3′ untranslated region of U937 clone A6) into the BamHI-EcoRI site of the CMV6M vector. Plasmid pCMV6Db1 was generated by ligating a BamHI fragment encoding oncogenic Db1 from plasmid pc11dB1 (a gift from Dr. Sandra Eva, Giannina Galini Institute, Geneva, Italy) into the BamHI site of pCMV6. pCMV6HA-Cdc42 (HA-tagged Cdc42), pGEX-PBD (GST-PBD), and J3HmPak3 (HA-Pak3) have been previously described (12).

Kinase Assays, Affinity Precipitation, and Immunoprecipitations—Kinase reactions were initiated by the addition of 2× kinase buffer (40 mM Hepes, pH 7.4, 20 mM MgCl2, and 4 mM MnCl2) and 2 μg [γ-32P]ATP (3000 Ci/mmol) for 3.5 min at room temperature. Reactions were stopped by the addition of 2× SDS sample buffer containing 20 mM EDTA. Affinity precipitation with GST-PBD was as described (24) except that COS cells were lysed in 25 mM Hepes, pH 7.4, 150 mM NaCl,

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1% Nonidet P-40, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates containing equal amounts of Pak3 were immunoprecipitated with anti-Pak3 primary antibody (a gift from Dr. S. Pelech, Kinetek Biotechnology Corporation, Vancouver, Canada).

Anti-Cool-1 antibody was prepared using His-tagged full-length p50Cool-1, treated with thrombin to cleave the His-tag and further resolved on a Mono Q-Sepharose column. Rabbit serum was collected 12 weeks after three injections with p50Cool-1.

RESULTS AND DISCUSSION

To identify potential Pak-binding partners we used the yeast two-hybrid screen. One positive clone was used to screen cDNA libraries and data bases (see "Experimental Procedures") to yield two Pak3-binding proteins, p50 and p85Cool-1. They share an N-terminal SH3 domain (amino acids 7–65), followed by a Dbl homology (DH) domain (amino acids 100–279) and an adjacent pleckstrin homology (PH) domain (amino acids 295–400) (Fig. 1A). Because p50 and p85Cool-1 are identical at the nucleotide level over amino acids 1–418, it is likely that they arise from alternative splicing of the same message. p85Cool-1 is identical to two recently cloned proteins, p85SPR (25) and β-PIX (26). The DH domain of the Cool proteins shows the highest sequence identity to the DH domains of Dbl (33%), Sdc1 (30%), Dbs (29%), Tiam-1 (29%), Still Life2 (29%), Cdc24 (26%), and Vav2 (24%). In addition to the genetic screen, we used recombinant GST-Pak3 (amino acids 148–239) to purify Pak-binding proteins from Src(Y527F)-transformed NIH 3T3 cells. Sequence obtained from an ~85-kDa Pak-binding protein was identical to portions of p50 and p85Cool-1 (data not shown). We also identified a closely related cDNA from the data bases, and we have found that its product also interacts with Pak3. This protein, Cool-2, is identical to the recently described α-PIX (26).

To determine whether the Cool proteins bind to Pak3 in mammalian cells, we transiently co-expressed Myc-tagged p50Cool-1 and HA-tagged Pak3 in COS cells and assayed for complex formation by immunoprecipitation and Western blot analysis (Fig. 1B). HA-Pak3 was detected in anti-Myc immunoprecipitants (Fig. 1B, lane 8, upper panel) and Myc-p50Cool-1 was detected in anti-HA immunoprecipitants (Fig. 1B, lane 13, lower panel). Mutation of a conserved tryptophan residue within the SH3 domain (W43K) eliminated the ability of p50Cool-1 to associate with Pak3 (Fig. 1B, lane 10, upper panel, and lane 14, lower panel), indicating that the SH3 domain of p50Cool-1 binds Pak3. The Src SH3-binding protein, Sam68, did not co-immunoprecipitate with Myc-p50Cool-1 (Fig. 1B, lane 9, upper panel), although it bound to the SH3 domain of Cool-1 in vitro, and SH3 domains from the Dbl family proteins Dbs and Vav did not bind Pak3 (data not shown), showing specificity of the Cool-1/Pak3 association in vivo. Pak3 contains four conventional (PXXP motif) SH3-binding sites (P1–P4); Pak3 containing Pro to Ala mutations in the P1-P4 sites, alone or in combination, were used to establish that these sites do not mediate Pak3-Cool-1 interactions (data not shown). While this manuscript was in preparation, Manser et al. reported binding of α-PIX and β-PIX (which correspond to Cool-2 and p85Cool-1) to residues 182–203 of Pak1, confirming the atypical (non-PXPPP motif) nature of Cool binding to Pak3 (26). Using a rabbit antiserum raised against full-length p50Cool-1, we detected two predominant proteins (from NIH 3T3 fibroblast lysates) that migrated at ~85 and ~78 kDa on a SDS-polyacrylamide gel (Fig. 1C, right panel, lane 1). Both of these proteins were detected in anti-Cool-1 immunoprecipitates (Fig. 1C, right panel, lane 2), as well as in anti-Pak3 immunoprecipitates (Fig. 1C, right panel, lane 3) and as well as in anti-Pak3 immunoprecipitates (Fig. 1C, right panel, lane 1), demonstrating an interaction between endogenous Cool proteins and Pak3 in NIH 3T3 cells. The less reactive band at ~50 kDa recognized by the anti-Cool-1 antibody (Fig. 1C, right panel, lane 1) may represent p50Cool-1; however, we were unable to determine whether p50Cool-1 was present in the immunoprecipitates because of the overlapping signal for IgG.

The presence of a DH-PIX tandem motif in Cool-1, a hallmark of Dbl family exchange factors (27), initially led us to consider the possibility that Cool-1 might activate Cdc42 or Rac. However, we were unable to detect stimulation of [3H]GDP dissociation from Cdc42 or Rac by recombinant p50Cool-1 purified from Escherichia coli or insect cells under conditions where the Dbl oncoprotein strongly stimulated GDP dissociation from Cdc42 or RhoA (data not shown).

We then considered the possibility that Cool-1 exchange activity may require cellular co-factors or post-translational mod-

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a S. Bagrodia, M. Hart, and R. Cerione, unpublished observations.

b S. J. Taylor, S. Bagrodia, and R. Cerione, unpublished observations.
Cdc42(Q61L)2. We next tested whether p50/p85 Cool-1 could di-
tial activators of Rac including Src(Y527F), Ras(G12V), and
Cool-1-mediated Rac1 activation in cells co-expressing poten-
of Rac1 but not Cdc42 (data not shown). We have not detected
p50Cool-1 completely blocked Cdc42(Q61L)-stimulated auto-
3
phosphorylation of Pak3 and strongly inhibited the phospho-

FIG. 2. Cool-1 does not detectably activate Cdc42 or Rac1 in vivo. COS cells were transfected with HA-Rac1 (top) or HA-Cdc42
(bottom) alone (lanes 1 and 9) or co-transfected with Myc-p50Cool-1 (lanes 2–7 and lanes 10–15) or with Dbl (lanes 8 and 16) for 48 h and then
serum-starved for another 4 h and treated with 100 ng/ml epidermal
growth factor (EGF) plus 25 ng/ml insulin-like growth factor-1 (IGF-1),
40 ng/ml platelet-derived growth factor (PDGF), 20% fetal calf serum,
50 ng/ml interleukin 1 (IL-1) for 10 min, or 200 μM methylene metha-
sulphonate (MMS) for 1 h as indicated. Cells were lysed and affinity-
precipitated with immobilized GST-PBD (GST-PBD AP, lanes 9–16),
and bound proteins were Western blotted and probed with anti-HA.
Lanes 1–8 represent 5% of the whole cell lysate (WCL) used in the
binding reaction.

FIG. 3. Cool-1 inhibits Dbl- and Cdc42-stimulated Pak3 activity.
A, COS cells were transiently co-transfected with Pak3 (0.5 μg),
Myc-p50Cool-1 (1 μg), Myc-p85Cool-1 (1 μg), Myc-p50W43KCool-1 (1 μg),
and Dbl (1 μg), and Pak3 kinase activity was measured in Pak3 immu-
oprercipitates using myelin basic protein (MBP) as a substrate. The top
part (bottom panel) of the blot was probed with anti-Pak3, and the
bottom part (top panel) of the blot was autoradiographed. Based on
Western blot analysis the amount of Dbl was similar in lanes 2–5,
the amounts of p50Cool-1 and p50(W43K)Cool-1 were also similar in lanes 3
and 5, and p85Cool-1 was present in a slightly larger amount. B, COS
cells were transiently transfected with 1 μg each of Myc-Pak3, Myc-
p50Cool-1, and Myc-p50W43KCool-1. Cells were lysed and affinity-pre-
cipitated with immobilized GST-Cdc42 (lanes 5, 7, 9, and 11) or with
constitutively active GST-Cdc42(Q61L) (lanes 6, 8, 10, and 12). Bound
proteins were Western blotted and probed with anti-Myc. Lanes 1–4
represent 10% of the whole cell lysate used in the binding reaction. C,
COS cells were transiently co-transfected with empty vector (lane 1) or
plasmids encoding HA-Pak3 (lanes 2–6, 0.5 μg of DNA), Myc-p50 Cool-1
(lanes 1, 4, and 6, 0.5 μg of DNA, and lane 5, 1.5 μg of DNA), Myc-p85
Cool-1 (lanes 3, 4, and 5, 0.5 μg of DNA, and lane 6, 1.5 μg of DNA).
Cells were lysed and affinity-precipitated (AP) with immobilized GST-
Cdc42L61 (-12 μg, lanes 7–12). Bound proteins were Western blotted,
and the blot was probed with anti-HA and anti-Myc. Lanes 7–12 of the
blot were reprobed with anti-Cool-1 to detect p50 Cool-1. Lanes 1–6
represent 10% of the whole cell lysate (10% WCL) used in the binding
reaction. Numbers above the lanes denote the ratio of p50 to p85
Myc-tagged Cool-1 cDNA that was transfected.

ifications as proposed for Vav, Sos, and Tiam-1 (28–31). To
measure Rac1 or Cdc42 activation in vivo, we used a modification
of a recently described assay for activated, GTP-bound Ras
(24). The PBD of Pak3 was expressed as a GST fusion protein
and immobilized by binding to glutathione-Sepharose beads.
The immobilized GST-PBD was used to precipitate activated
Cdc42 or Rac1 from transfected COS cell lysates (Fig. 2). In
untreated control cells, relatively low levels (<5%) of HA-Rac1
and Rac1 by Dbl is consistent with results from micro-

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Cdc42 to Pak3 by expressing Myc-tagged Pak3 and/or p50Cool-1 in COS cells and assaying the binding of Pak3 to GST-Cdc42 by affinity precipitation and anti-Myc-immunoblotting (Fig. 3B). Pak3 was precipitated by immobilized GST-Cdc42(Q61L) (GTPase-defective GTP-bound, lane 6) but not by GST-Cdc42 (GDP-bound, lane 5) and displayed a gel mobility shift due to Cdc42(Q61L)-stimulated autophosphorylation (compare lanes 1 and 6). (Pak3 autophosphorylation is sustained by the presence of Mg\(^{2+}\) during affinity precipitation.) Although p50Cool-1 expression did not affect Pak3 recovery in GST-Cdc42(Q61L) precipitates (compare lanes 1 and 6 with lanes 3 and 10), the precipitated Pak3 did not display the gel shift observed in the absence of p50Cool-1 or when co-expressed with p50Cool-1(W43K) (compare lanes 6, 10, and 12). Therefore, p50Cool-1 binding to Pak3 did not inhibit Cdc42 binding but did inhibit Cdc42-stimulated Pak3 autophosphorylation. Relative to Myc-Pak3, very little Myc-p50Cool-1 is detected in these precipitates (lane 10), which may be due to dissociation of p50Cool-1 from Pak3 during the precipitation and washing procedures but may also reflect the relatively poor ability of anti-Myc to detect Myc-tagged p50Cool-1 co-precipitated with activated Cdc42 and Pak3 (see below).

Unlike p50Cool-1, expression of p85Cool-1 did not inhibit Cdc42(Q61L)-stimulated Pak3 autophosphorylation (Fig. 3C, compare lanes 8 and 9), which is consistent with the results shown in Fig. 3A. As expected from the presence of identical SH3 domains, p50 and p85Cool-1 competed for binding to Pak3 in vitro (lanes 9–12). In these experiments, p50Cool-1 co-precipitating with Pak3 was not detected with anti-Myc (upper panel, lanes 8 and 10–12) but was readily detectable with an anti-Cool-1 antibody (lower panel, lanes 8 and 10–12). The fact that an ~3-fold excess of either p50Cool-1 or p85Cool-1 was able to significantly inhibit the binding of the other to Pak3 suggests that they have similar affinities for Pak3.

Our results suggest that p50Cool-1, by competing with endogenous p85Cool-1 or other Cool proteins for binding to Pak3, might sequester Pak3 away from its site(s) of activation. On the other hand p85Cool-1, which has a permissive effect on the stimulation of Pak activation by Dbl or other Rho family exchange factors, could play an important role in recruiting Pak to its sites of activation, possibly via its C-terminal region, which is not present in p50Cool-1. In support of this model, our preliminary data indicate that p50Cool-1 has a diffuse cytoplasmic localization, whereas p85Cool-1 is concentrated at focal adhesions (25). Moreover, HeLa cell p85Cool-1 (β-PIX) has recently been shown to localize to focal complexes and appears to mediate Pak1 recruitment to these sites by activated Cdc42 (26). However, this would not account for the ability of p50Cool-1 to inhibit Cdc42-stimulated Pak3 activity in vitro. Because both p50Cool-1 and p85Cool-1 appear to bind to a common site on Pak3, the specific inhibition of Pak3 activity by p50Cool-1 may be due to the differences in the C-terminal regions of p50 and p85Cool-1.

Protein kinases are often subject to multiple levels of regulation. The involvement of Pak1 in cellular signaling pathways leading to changes in gene expression or cytoskeletal architecture and their participation in both Ras-mediated transformation (8) and Fas-mediated apoptosis (21, 22) mandates that their activities be tightly controlled. The ability of p50Cool-1 to suppress and p85Cool-1 to permit Pak activity indicates that signaling through Pak-dependent pathways may be regulated by cell type, cell cycle, or developmental-specific expression patterns. It will be important to establish the role of DH and PH domains in Cool function and how differential expression of Cool impacts upon Pak signaling to different effector pathways.

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