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The Cool-2/α-Pix Protein Mediates a Cdc42-Rac Signaling Cascade

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Summary

Background: *C*loned-out *o*f *l*ibrary-2 (Cool-2)/*P*AK-*i*nteractive exchange factor (α-Pix) was identified through its ability to bind the Cdc42/Rac target *p*21-*a*ctivated *k*inase (PAK) and has been implicated in certain forms of X-linked mental retardation as well as in growth factor- and chemoattractant-coupled signaling pathways. We recently found that the dimeric form of Cool-2 is a specific guanine nucleotide exchange factor (GEF) for Rac, whereas monomeric Cool-2 is a GEF for Cdc42 as well as Rac. However, unlike many GEFs, Cool-2 binds to activated forms of Cdc42 and Rac. Thus, we have investigated the functional consequences of these interactions.
Results: We show that the binding of activated Cdc42 to the Cool-2 dimer markedly enhances its ability to associate with GDP bound Rac1, resulting in a significant activation of Rac-GEF activity. While the Rac-specific GEF activity of Cool-2 is mediated through the *D*bl *h*omology (DH) domain from one monomer and the Pleckstrin homology domain from the other, activated Cdc42 interacts with the DH domain, most likely opposite the DH domain binding site for GDP bound Rac. Activated Rac also binds to Cool-2; however, it strongly inhibits the GEF activity of dimeric Cool-2.
Conclusions: We provide evidence for novel mechanisms of allosteric regulation of the Rac-GEF activity of the Cool-2 dimer, involving stimulatory effects by Cdc42 and feedback inhibition by Rac. These findings demonstrate that by serving as a target for GTP bound Cdc42 and a GEF for Rac, Cool-2 mediates a GTPase cascade where the activation of Cdc42 is translated into the activation of Rac.

Introduction

Cdc42 and Rac are members of the Rho subfamily of Ras-related GTP binding proteins that have been implicated in a wide variety of cellular responses, including the regulation of the actin cytoskeletal architecture, cell shape and motility, intracellular trafficking, cell cycle progression, and malignant transformation [1–5]. Although both Cdc42 and Rac use a number of different targets/effectors to mediate these various cellular activities, among their best known targets are the p21-activated kinases (PAKs), a family of serine/threonine kinases [6]. Like the GTP binding proteins that serve as their upstream activators, the PAKs have been implicated in a wide range of cellular responses including actin cytoskeletal changes and nuclear events as mediated through their stimulation of different MAP kinase pathways. It has been assumed that the cellular activities of the PAKs need to be carefully regulated. This prompted the search for PAK binding partners that might serve such regulatory functions and led to the discovery of the Cool/Pix proteins [7, 8].

Members of the Cool/Pix family of proteins all contain an SH3 domain, followed by a tandem arrangement of Dbl homology (DH) and Pleckstrin homology (PH) domains [6]. This arrangement of DH and PH domains is characteristic of the Dbl family of guanine nucleotide exchange factors (GEFs) for Rho-related GTP binding proteins, and so it was assumed that the Cool/Pix proteins would also function as GEFs. Our laboratory has characterized three members of the Cool/Pix family, namely p50Cool-1, a longer splice variant called p85Cool-1 (identical to β-Pix), and a distinct gene product called Cool-2 (identical to α-Pix). However, based on these studies, it is clear that the Cool/Pix proteins do not all simply function as GEFs but rather exhibit a more complicated array of functional activities. In particular, p50Cool-1 appears to block the stimulation of PAK activity by Dbl and other GEFs [7], through an as yet undetermined mechanism. The p85Cool-1 protein does not interfere with PAK activity and is permissive for PAK activation, allowing activated Cdc42 or Rac molecules, generated by different Dbl-related proteins, to stimulate PAK [9]. Moreover, we recently made the rather unexpected finding that activated forms of Cdc42 bound to p85Cool-1, and this interaction played an important role in the regulation of EGF receptor ubiquitination, as catalyzed by the Cbl proteins [10].

Unlike the Cool-1 proteins, the expression of Cool-2 in cells leads to PAK activation [9, 11, 12], apparently arising from Cool-2's ability to function as a GEF [13]. Full-length Cool-2 normally exists as a dimer and under these conditions is a Rac-specific GEF [14]. Conditions that lead to the dissociation of the dimer to the monomer—for example, through the binding of a complex containing PAK and G protein βγ subunits to the SH3 domain of Cool-2—then allow Cool-2 to function either as a Rac- or Cdc42-GEF. Thus, there are various possible regulatory inputs, which, depending on how they influence the monomer-dimer equilibrium, dictate whether Cool-2 selectively promotes the activation of Rac or if it can also activate Cdc42. This most likely explains various findings that have implicated Cool-2 as being important in mediating the activation of one or the other of these GTP binding proteins in response to different extracellular stimuli [12, 15].

However, a rather puzzling aspect of Cool-2 function has been its ability, like that of p85Cool-1, to form a stable complex with activated forms of Cdc42 and Rac. Typically, GEFs only exhibit high-affinity binding to the nucleotide-depleted or GDP bound forms of their GTP binding protein substrates. Thus, we have set out to

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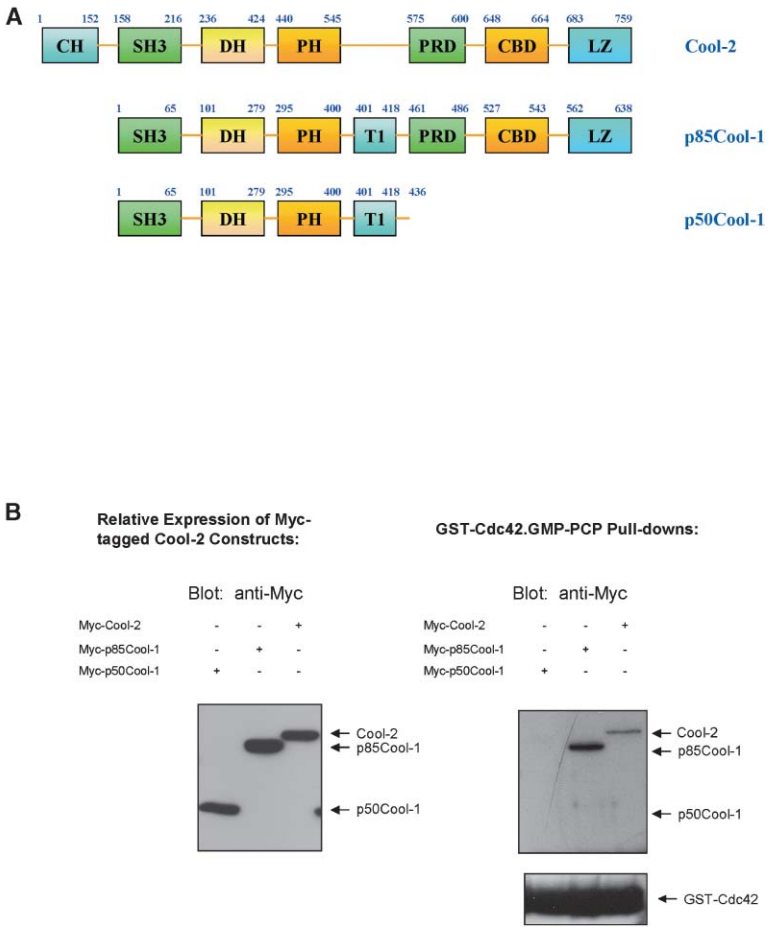


Figure 1. Domain Structures of Cool-2, p85Cool-1, and p50Cool-1 and the Relative Ability of the Cool/Pix Proteins to Bind to GMP-PCP Bound Cdc42

(A) Cool-2 contains calponin homology (CH), Src homology 3 (SH3), Dbl homology (DH), and Pleckstrin homology (PH) domains; a proline-rich region containing several PXXP motifs (PRD); a *C*ool-*a*ssociated *t*yrosine phosphosubstrate (Cat)/*G* protein-coupled receptor kinase *int*eractor (Git) binding (CBD) domain; and a leucine zipper (LZ) region. Cool-1 exists as two alternative splice variants. Although the Cool-1 proteins have a domain structure that is similar to that of Cool-2, their T1 regulatory domain is unique.

(B) COS-7 cell lysates expressing Myc-p50Cool-1, Myc-p85Cool-1, or Myc-Cool-2 (left panel) were incubated with GMP-PCP bound GST-Cdc42 immobilized on agarose beads as described in the Experimental Procedures. Washed beads were analyzed with an anti-Myc antibody to examine the relative binding capabilities of the different Cool proteins toward GST-Cdc42 (right panel, top). The lower panel on the right shows the relative amounts of GST-Cdc42-GMP-PCP precipitated in the different binding experiments.

understand the functional consequences of the interactions of Cool-2 with activated Cdc42 and Rac. In this report, we demonstrate that the binding of activated Cdc42 to the Cool-2 dimer has a marked stimulatory effect on its Rac-specific GEF activity. Activated versions of Cdc42 induce this allosteric regulation by binding to a site on the Cool-2 dimer that is distinct from and likely opposite to the site where GDP bound Rac sits. Interestingly, activated forms of Rac, which also bind to Cool-2, cause a striking inhibition of its Rac-specific GEF activity. Overall, these results suggest that Cool-2 is subject to novel allosteric regulatory mechanisms that enable it to play a pivotal role as a control switch in a GTPase cascade, converting the binding of activated Cdc42 into an activation of Rac. However, when sufficient levels of activated Rac are generated, a feedback inhibition then occurs that shuts off the GEF activity of Cool-2.

Results

Cool-2 Binds to the GTP Bound Form of Cdc42

Because the Cool/Pix proteins were identified based on their abilities to bind the Cdc42/Rac target PAK, and given that each member of the family contains tandem DH and PH domains (Figure 1A), it was initially assumed that they served as GEFs for either Cdc42 or Rac. Indeed, it has been demonstrated biochemically that Cool-2 can act as a GEF toward Rac and Cdc42 and that the specificity of its GEF activity is influenced by a monomer-dimer equilibrium [13, 14]. Dimeric Cool-2 exhibits a Rac-specific GEF activity, whereas monomeric Cool-2 can act as a GEF for either Rac or Cdc42. However, we have yet to find that either p50Cool-1 or p85Cool-1 exhibits classical GEF activity, as assayed by the stimulation of [^{3}H]GDP dissociation from Rac or Cdc42. The inability of the Cool-1 proteins to exhibit GEF activity was attributed to a sequence of 18 amino acids that lies just downstream from the PH domain, designated the T1 region (Figure 1A). Removal of the T1 region from p85Cool-1 restored GEF activity, whereas insertion of this region into the appropriate site on Cool-2 inhibited its GEF capability [13]. In fact, we recently reported that p85Cool-1, rather than acting as a GEF, can serve as a target for activated forms of Cdc42, and this has important consequences for the regulation of the Cbl-catalyzed ubiquitination of EGF receptors [10]. However, perhaps even more surprising was the finding that activated forms of Cdc42 were able to associate with dimeric Cool-2, given that it is a Rac-specific GEF and thus does not regulate the nucleotide exchange activity of Cdc42.

Figure 1B shows the results of GST pull-down assays, comparing the ability of GMP-PCP bound, *E. coli* recombinant GST-Cdc42 to associate with Myc-tagged forms of different members of the Cool/Pix family that were expressed in COS-7 cells. Activated, GMP-PCP bound GST-Cdc42 was capable of a stable interaction with Myc-tagged p85Cool-1, consistent with our earlier findings [10]. However, GMP-PCP bound Cdc42 also interacted with Myc-Cool-2, albeit to a slightly lesser extent than with Myc-p85Cool-1, but showed no detectable binding to Myc-p50Cool-1.

We next set out to examine how activated Cdc42 binds to Cool-2. A number of different Cool-2 constructs were prepared (Figure 2A) and assayed for their abilities to associate with GMP-PCP bound GST-Cdc42. As shown in Figure 2B (see the middle panels), all Cool-2 constructs containing an intact DH domain were capable of binding to activated Cdc42. These included the full-length Myc-Cool-2 protein, as well as an HA-tagged Cool-2 construct that started at the SH3 domain and ended after the proline-rich region (designated as SH3.PXXP.Cool-2 in Figure 2A) and a Myc-tagged construct that contained only the SH3 and DH domains (SH3-DH). However, a Cool-2 construct that contained just the SH3 domain (designated as SH3.Cool-2), or a construct that began just downstream of the DH domain and contained the remainder of the Cool-2 protein (designated as PH.LZ), was incapable of binding to activated Cdc42.

The DH domain of Cool-2 appears to be essential for the binding of activated Cdc42, because when two highly conserved leucine residues within this domain were mutated to arginine and serine, respectively (designated as DHm.Cool-2), the binding to GMP-PCP bound Cdc42 was eliminated (Figure 2B). It is worth noting that this differs from the case for GDP bound (as well as nucleotide-depleted) Rac, which is the target for the GEF activity of dimeric Cool-2. The DHm.Cool-2 protein still showed some ability to interact with GDP bound Rac, and it was only when mutations within the DH domain and the carboxy-terminal region of the PH domain (called the *R*ac-specific *i*nteraction *d*omain [RSID]) were combined that binding to Rac was fully inhibited [14]. In addition, we found that mutating leucine 543 and isoleucine 544 within the RSID to arginine and serine, respectively (designated as LIm.Cool-2), which significantly weakened the binding of GDP bound Rac [14], had no effect on the binding of activated Cdc42 (Figure 2B). Thus, while GDP bound Rac requires the DH domain and RSID on adjacent monomers within the Cool-2 dimer (i.e., in *trans*) to form a functional complex, GMP-PCP bound Cdc42 binds in a distinct manner, most likely at a site within the DH domain.

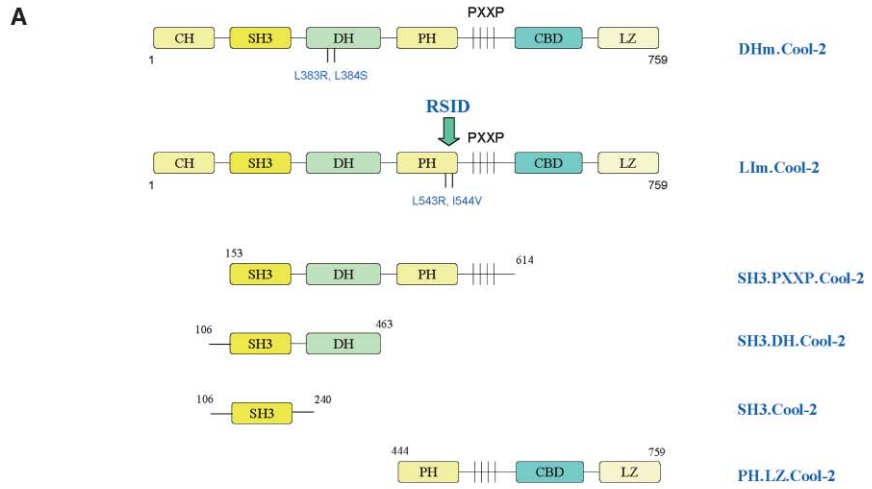
Activated Cdc42 Enhances the Interaction of GDP Bound Rac with Cool-2

We next wanted to determine the functional consequences of the binding of activated Cdc42 to Cool-2 and thus asked whether GMP-PCP bound Cdc42 might affect the interaction of Cool-2 with Rac. COS-7 cells were transiently transfected with the cDNAs encoding Myc-tagged Cool-2 and HA-tagged, nucleotide-depleted Rac(T17N), or with the cDNA encoding HA-Rac(T17N) alone. Purified, *E. coli* recombinant GST-Cdc42, preloaded with either GDP or GMP-PCP, was then incubated with lysates from the different COS-7 cell transfectants. As shown in Figure 3A (top panel), GST-Cdc42-GMP-PCP (lane 2), and to a much lesser extent GST-Cdc42-GDP (lane 1), were able to form a complex with the nucleotide-depleted Rac(T17N) protein, but only in cells that also expressed Cool-2. When Cool-2 was not coexpressed with Rac(T17N), GST-Cdc42-GMP-PCP showed no ability to associate with nucleotide-depleted Rac (Figure 3A, top panel, lane 3). Thus, these results argue for the existence of a stable ternary complex comprised of activated Cdc42, nucleotide-depleted Rac, and Cool-2. However, they also raised the question of whether the differences observed when using GMP-PCP bound Cdc42, versus GDP bound Cdc42, reflected a stronger binding affinity by activated Cdc42 for Cool-2, or an enhanced ability to impart a positive regulatory effect on the interactions between Rac and its GEF. Figure 3B (top panel) shows the results of experiments where GST-Cdc42-GMP-PCP and GST-Cdc42-GDP were incubated with insect (Sf21) cell lysates expressing histidine (His)-tagged Cool-2. It is clear that His-Cool-2 binds effectively to GMP-PCP bound Cdc42 under conditions where there is no detectable binding to GDP bound Cdc42, thus indicating that activated forms of Cdc42 exhibit a significantly stronger binding affinity for Cool-2.

We then examined the ability of Cdc42, Rac, and Cool-2 to form a ternary complex, using purified recombinant proteins. Full-length, wild-type Cool-2 was expressed and purified as a His-tagged protein from Sf21 cells, while Cdc42 and Rac were expressed and purified from *E. coli*. Figure 3C shows the SDS-PAGE profiles for these recombinant protein preparations. The binding of GST-Rac-GDP to His-tagged Cool-2, as assayed by the coprecipitation of these purified proteins with glutathione-agarose beads, was barely detectable under the conditions of this experiment (Figure 3D, top panel, lane 2). As expected, the relatively weak binding of His-Cool-2 to GST-Rac-GDP was completely eliminated when excess (wild-type) GDP bound Rac was included in the assay incubation (lane 8). However, when GMP-PCP bound Cdc42 was included in the assay, the binding of GST-Rac-GDP to His-tagged Cool-2 was markedly enhanced (lane 4). The stimulatory effects were specific for GMP-PCP bound Cdc42 and were not observed when the binding assays were performed in the presence of GDP bound Cdc42 (lane 5), nor when the binding incubations contained GMP-PCP bound Rac (Figure 3D, lane 6).

Activated Cdc42 Enhances the Rac-Specific GEF Activity of Cool-2

The initial demonstrations that full-length Cool-2 exhibited Rac-GEF activity were obtained by immunoprecipitating Myc-tagged Cool-2 from COS-7 cells and then assaying its ability to stimulate the exchange of [^{3}H]GDP for GTPγS, as readout by an accelerated rate of dissociation of the radiolabeled GDP from *E. coli*-expressed, purified Rac [14]. Figure 4A shows the results of a similar type of guanine nucleotide exchange assay, but using



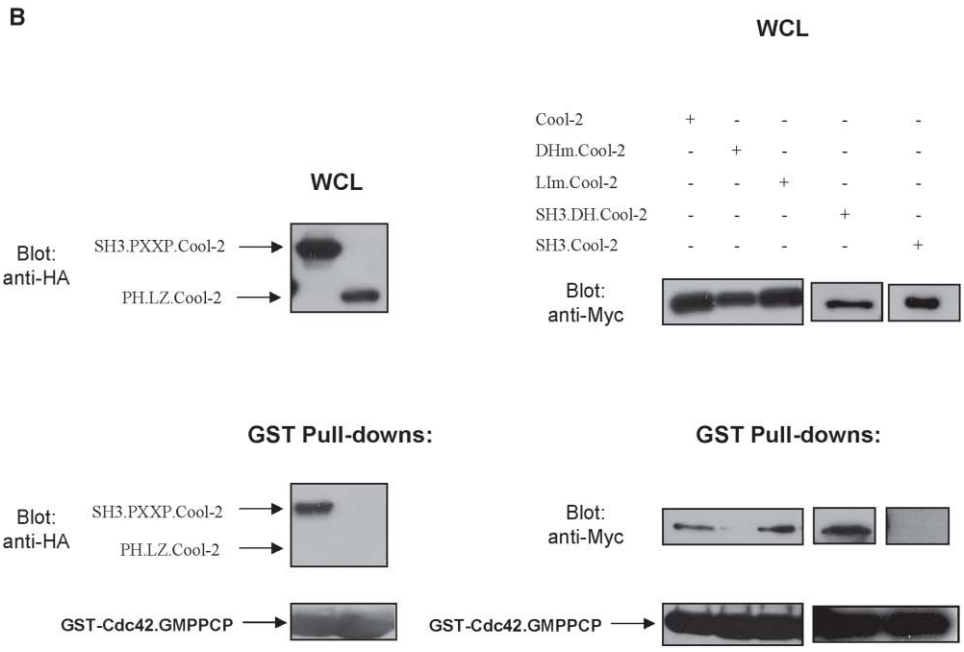


Figure 2. The Abilities of Different Cool-2 Constructs to Bind to GMP-PCP Bound GST-Cdc42

(A) Schematic representation of different truncated Cool-2 constructs and Cool-2 mutants. RSID, *R*ac-specific *i*nteraction *d*omain.

(B) Whole-cell lysates (WCL) from COS-7 cells expressing the indicated HA- or Myc-tagged Cool-2 constructs (shown in the upper panels) were incubated with GST-Cdc42 preloaded with GMP-PCP. The agarose beads containing GST-Cdc42 were washed and then subjected to Western blotting with anti-HA (middle panel, left) or anti-Myc antibody (middle panels, right) to examine the relative binding capabilities of the different Cool-2 constructs or mutants. The lower panels show the relative amounts of GST-Cdc42-GMP-PCP precipitated in the different binding experiments.

purified, insect cell-expressed His-tagged Cool-2. Whereas Rac alone shows a very slow rate of intrinsic [^{3}H]GDP-GTPγS exchange, the addition of His-Cool-2 to Rac stimulated the nucleotide exchange activity as indicated by the enhanced rate of dissociation of [^{3}H]GDP. However, when *E. coli*-expressed GMP-PCP bound Cdc42

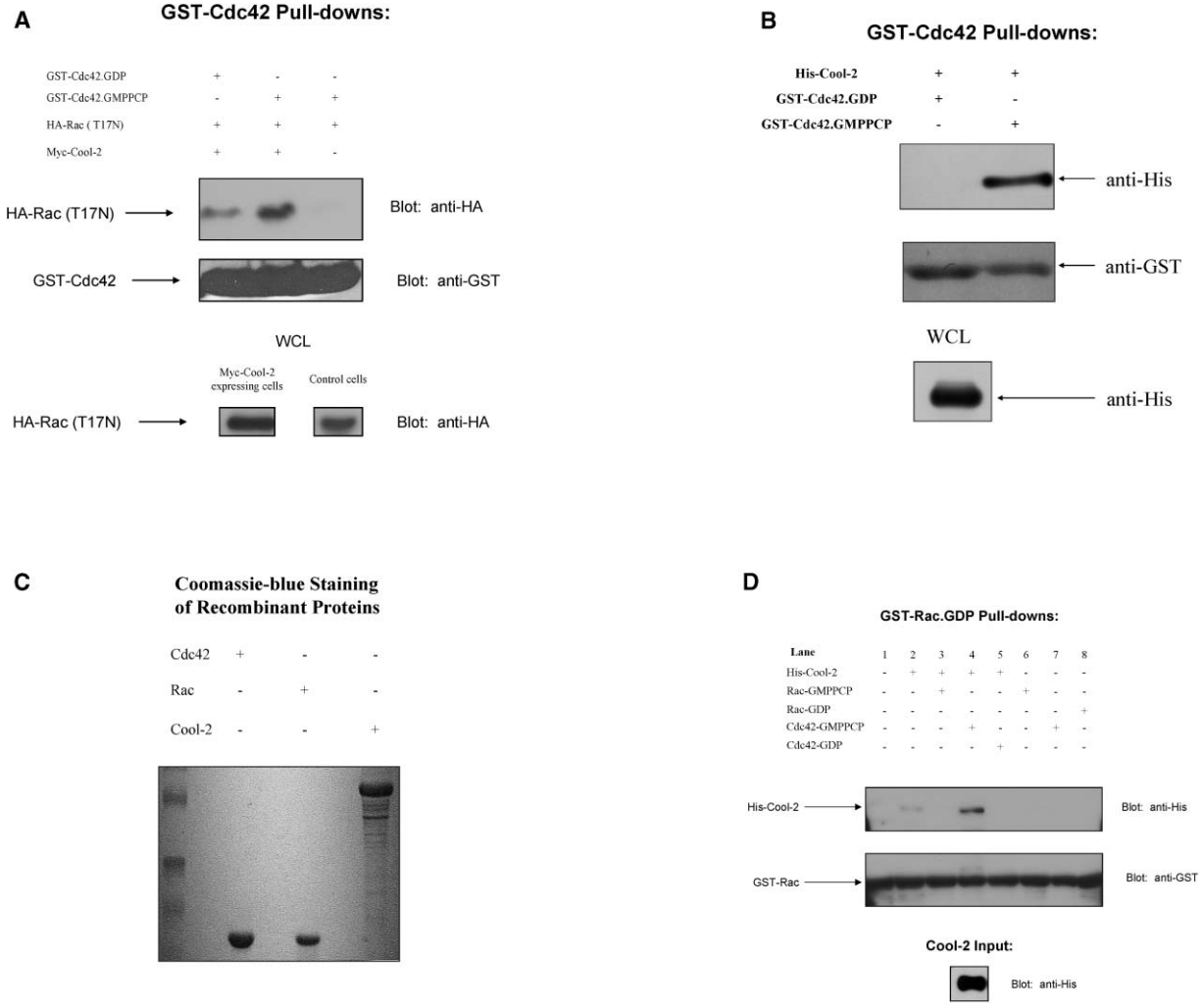


Figure 3. Activated, GMP-PCP Bound Cdc42 Enhances the Interactions between Cool-2 and Rac

(A) Whole-cell lysates (WCL) from COS-7 cells expressing Myc-Cool-2 and dominant-negative HA-Rac1(T17N) were incubated with immobilized GST-Cdc42 that had been preloaded with either GDP or GMP-PCP, while lysates from COS-7 cells expressing HA-Rac1(T17N) alone were incubated with GST-Cdc42 preloaded with GMP-PCP. Washed beads were analyzed with an anti-HA antibody to measure the extent that GMP-PCP bound GST-Cdc42 enhances the binding of HA-Rac1(T17N) to Myc-Cool-2 (upper panel). The middle panel shows the relative amounts of GST-Cdc42-GMP-PCP precipitated in the different binding experiments, and the lower panel shows the relative expression of HA-Rac1(T17N) in cells expressing Myc-Cool-2 or in control cells.

(B) Whole-cell lysates from insect (Sf21) cells expressing His-Cool-2 were incubated with immobilized GST-Cdc42 that was preloaded with GDP or GMP-PCP. The upper panel shows that His-Cool-2 binds to GMP-PCP bound GST-Cdc42 (as assessed by Western blotting with anti-His antibody) under conditions where there is no detectable binding to GDP bound GST-Cdc42. The middle panel shows the relative amounts of GST-Cdc42 precipitated in the different experiments, and the lower panel shows the expression of His-Cool-2.

(C) Coomassie blue-stained gel showing recombinant proteins used in the GST pull-down assays in Figure 3D: molecular weight standards (lane 1), Cdc42 (lane 2), Rac1 (lane 3), and His-Cool-2 (lane 4).

(D) GST-Rac1, bound to agarose beads, was incubated with 1 μg of each of the indicated recombinant proteins. The beads were washed and analyzed with an anti-His antibody to examine the relative binding of His-Cool-2 to GST-Rac1 under the indicated conditions (top panel). The middle panel shows the relative amounts of GST-Rac1 precipitated in the different binding experiments, and the lower panel shows the expression of His-Cool-2.

was added together with His-Cool-2, there was an even greater enhancement in the rate of nucleotide exchange. In fact, the rate of [^{3}H]GDP-GTPγS exchange on Rac that was obtained in the presence of Cdc42-GMP-PCP and His-Cool-2 approached the rate of EDTA-catalyzed nucleotide exchange, which represents the maximum rate possible, because EDTA effectively chelates Mg^{2+} and strongly stimulates GDP dissociation.

Given the ability of activated Cdc42 to stimulate the Rac-GEF activity of Cool-2, we were curious whether Cdc42 might also act as a positive feedback regulator, enabling dimeric Cool-2 to exhibit Cdc42-GEF activity, as well. However, we have not found this to be the case, as the addition of GMP-PCP bound Cdc42 to Cool-2 did not convert full-length Cool-2 into a Cdc42-GEF (Figure 4A). Figure 4B shows that GMP-PCP bound Cdc42 is also incapable of stimulating the GEF activity of monomeric Cool-2. In order to test this possibility,

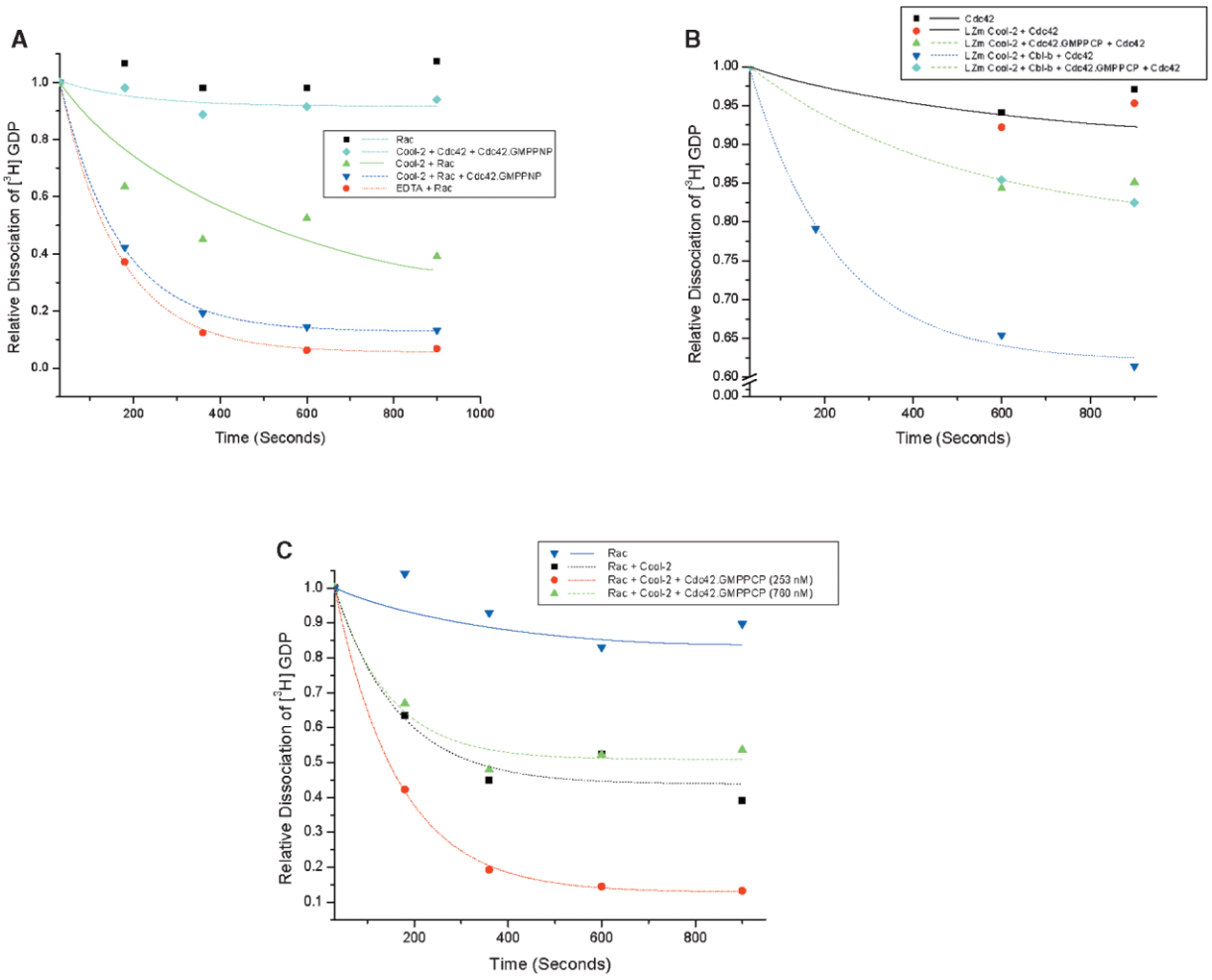


Figure 4. The GEF Activity of Cool-2 toward Rac Is Specifically Enhanced by GMP-PCP Bound Cdc42

(A) Relative dissociation of preloaded [^{3}H]GDP from Rac1 or Cdc42 in the presence of excess GTPγS and insect cell-expressed His-Cool-2. GMP-PCP bound Cdc42 (250 nM) enhances the GEF activity of His-Cool-2 (320 nM) toward Rac1 (250 nM) but not Cdc42 (250 nM). Rac1 alone is shown as a negative control, whereas Rac1 in the presence of excess EDTA represents a positive control.

(B) GMP-PCP bound Cdc42 inhibits the ability of His-Cool-2 monomers to stimulate [^{3}H]GDP release from Cdc42. COS-7 cells were transiently transfected with plasmids expressing dimerization-defective Myc-tagged LZm.Cool-2 and HA-tagged Cbl-b, or with Myc-LZm.Cool-2 alone. Proteins were immobilized on protein G beads, washed, and incubated with GMP-PCP bound Cdc42 (250 nM) as indicated. Recombinant (*E. coli*) Cdc42 (250 nM) was preloaded with [^{3}H]GDP. The exchange of bound [^{3}H]GDP for GTPγS, as monitored by the dissociation of the radio-labeled nucleotide from Cdc42, was assayed in the presence of immunoprecipitated Myc-Cool-2 (see Experimental Procedures; see also [14]).

(C) Time course for Cool-2-stimulated dissociation of [^{3}H]GDP from Rac in the presence and absence of Cdc42. This experiment was performed using His-Cool-2 (490 nM), Rac1 (250 nM), and Cdc42 (250 nM or 760 nM), as described in (A).

a dimerization-defective Myc-tagged Cool-2 construct that contains mutations within its leucine zipper region (i.e., leucine residues 711 and 718 were changed to arginine and serine, respectively; designated as LZm.Cool-2; see also [14]) was transiently expressed together with HA-tagged Cbl-b in COS-7 cells and then immunoprecipitated and assayed for its ability to stimulate [^{3}H]GDP dissociation from purified, recombinant Cdc42. The dimerization-defective Cool-2 construct was coexpressed with Cbl-b, because we had previously shown that Cbl-b binds effectively to the SH3 domain of Cool-2 and acts as a necessary cofactor for monomeric forms of Cool-2 to exhibit Cdc42-GEF activity [14]. However, we found that rather than stimulating the GEF activity of the LZm.Cool-2-Cbl-b complex, GMP-PCP bound Cdc42 blocked its GEF activity, presumably as an outcome of a competition with Rac-GDP for the sole DH domain. Consistent with this suggestion is our finding that maximal stimulation of the Rac-specific GEF activity of dimeric Cool-2, by GMP-PCP bound Cdc42, was achieved when the molar levels of activated Cdc42 were approximately half, or at most equivalent to, the molar levels of Cool-2 and Rac. As shown in Figure 4C, when the levels of GMP-PCP bound Cdc42 were increased so that they exceeded the molar levels of Cool-2 and Rac, Cdc42 was no longer able to stimulate the Rac-GEF activity of Cool-2. Presumably, at increasing levels of GMP-PCP bound Cdc42, both DH domains within the

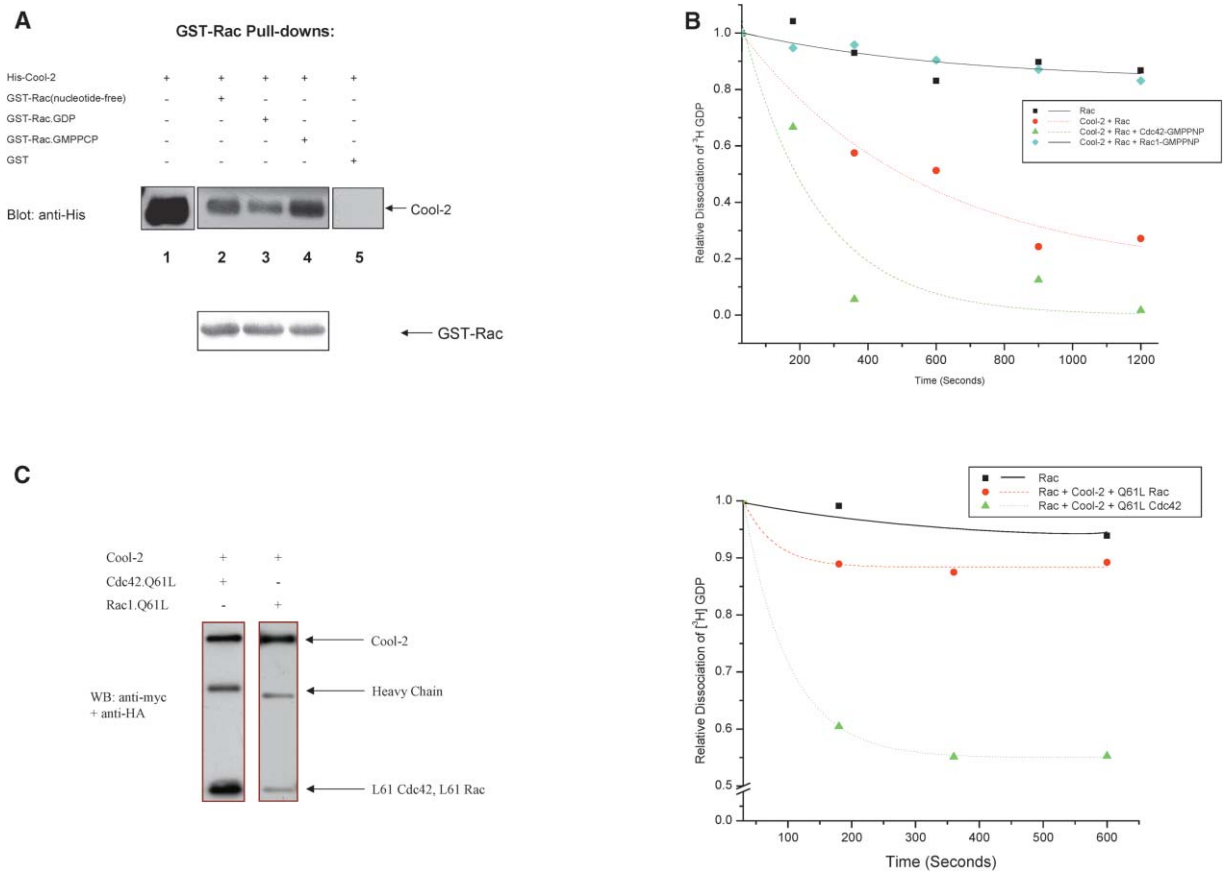


Figure 5. GMP-PCP Bound Rac Binds to Cool-2 and Inhibits Its Rac-Specific GEF Activity

(A) Lysates from insect (Sf21) cells expressing His-Cool-2 (lane 1) were incubated with nucleotide-depleted GST-Rac1 (lane 2), or with GST-Rac1 bound to either GDP (lane 3) or GMP-PCP (lane 4), or with GST alone (lane 5), as described in the Experimental Procedures. Washed beads were analyzed with an anti-His antibody to assay the relative binding of Cool-2 to the GST-Rac1 proteins (top panel). The bottom panel shows the relative amounts of GST-Rac1 precipitated in the different binding experiments.

(B) Cool-2-stimulated dissociation of [^{3}H]GDP from Rac1 is inhibited by GMP-PCP bound Rac1. The effects of GMP-PCP bound Cdc42 on the Rac-GEF activity of Cool-2 were examined as described in Figure 4A. The effects of GMP-PCP bound Rac1 (250 nM) were assayed with 320 nM His-Cool-2 and 300 nM Rac preloaded with [^{3}H]GDP.

(C) GTPase-defective Rac inhibits the Rac-GEF activity of Cool-2. COS-7 cells were transiently transfected with plasmids expressing Myc-tagged Cool-2 and either hemagglutinin (HA)-tagged Rac(Q61L) or HA-tagged Cdc42(Q61). The left panel shows the relative expression of Myc-Cool-2 and the HA-tagged GTP binding proteins. The right panel shows the results of assaying [^{3}H]GDP dissociation from *E. coli* Rac (250 nM), after immunoprecipitating Myc-Cool-2 from the Cdc42(Q61L)- and Rac(Q61L)-expressing cells.

Cool-2 dimer can be occupied by activated Cdc42 molecules, competitively blocking the binding of Rac-GDP and thereby inhibiting the Rac-specific GEF activity.

Activated Rac Inhibits the Rac-Specific GEF Activity of Dimeric Cool-2

Figure 5A shows the binding profile for the interactions of different nucleotide bound forms of *E. coli*-expressed, recombinant GST-Rac fusion proteins with full-length, insect cell-expressed His-tagged Cool-2. As expected, both nucleotide-depleted GST-Rac and GDP bound GST-Rac were able to associate with His-tagged Cool-2, when assayed in GST pull-down experiments (Figure 5A, top panel, lanes 2 and 3, respectively), whereas GST alone served as a negative control and did not precipitate His-Cool-2 (lane 5). However, like activated Cdc42, GMP-PCP bound GST-Rac was also able to form a stable complex with His-tagged Cool-2 (lane 4).

What was particularly interesting was that under the same conditions where activated Cdc42 stimulated the Rac-GEF activity of Cool-2, we found that GMP-PCP bound Rac caused a striking inhibition (Figure 5B). Moreover, when GTPase-defective Rac(Q61L) was coexpressed with Myc-Cool-2 in COS-7 cells, the Rac-GEF activity assayed for the immunoprecipitated Cool-2 was significantly diminished, such that the extent of [^{3}H]GDP dissociation from Rac under these conditions differed little from that measured for Rac alone (Figure 5C). However, when GTPase-defective Cdc42(Q61L) was coexpressed with Myc-Cool-2, the immunoprecipitated Cool-2 exhibited a strong stimulation of [^{3}H]GDP dissociation from Rac. Taken together, these findings have potentially important implications, as they would suggest that the GEF activity of Cool-2 is not only susceptible to stimulation by activated forms of Cdc42, but also to feedback inhibition by activated Rac. These results

may also explain why we have not found Cool-2 to act in a catalytic fashion in activating multiple Rac molecules. Under such conditions, the production of GTP bound Rac would shut down the GEF activity of Cool-2 and leave many of the GDP bound Rac molecules in the assay population unable to undergo Cool-2-stimulated nucleotide exchange.

Discussion

The Cool/Pix proteins have been implicated in a wide range of biological responses, including various receptor-coupled signaling pathways, and most recently, in the development of certain forms of X-linked mental retardation [12, 15–20]. Upon their discovery, these proteins were assumed to function as GEFs for Rho family members, in particular Rac and Cdc42, because they bind to the Cdc42/Rac target PAK and contain the tandem DH-PH domain arrangement that is characteristic of Dbl family members. Demonstrating their GEF activity has posed a number of challenges because of the complex regulatory mechanisms involved. However, we have recently established that for the case of the Cool-2 protein (also known as α-Pix), the GEF activity is tightly coupled to its monomer-dimer equilibrium [14]. When Cool-2 exists as a dimer, it functions as a Rac-specific GEF, whereas dissociation of the dimer to the monomeric state enables Cool-2 to act as a GEF for either Rac or Cdc42. It is interesting that interactions at the SH3 domain of Cool-2, either by PAK or Cbl, are necessary for monomeric Cool-2 to exhibit GEF activity. Neither the binding of PAK nor that of Cbl is sufficient to stimulate the dissociation of the Cool-2 dimer to the monomeric state, whereas the formation of a complex between PAK, and G$\beta\gamma$ subunits, which are generated as an outcome of G protein activation, stimulates the dissociation of Cool-2 dimers and activates Cdc42-GEF activity [14, 15]. It seems likely that other receptor-coupled signaling events, by influencing the monomer-dimer equilibrium of Cool-2, will then stimulate the selective activation of Rac versus Cdc42.

A particularly intriguing feature of the Cool/Pix proteins is their ability to bind activated forms of Cdc42 and Rac. In fact, we recently have shown that p85Cool-1, which preferentially binds activated Cdc42, acts as a Cdc42 target/scaffold protein by interfacing Cdc42 with c-Cbl [10]. The formation of a complex between activated Cdc42, p85Cool-1/β-Pix, and c-Cbl sequesters Cbl away from the EGF receptor, thus preventing Cbl-catalyzed ubiquitination. Under conditions where Cdc42 is constitutively active, the persistent sequestration of Cbl from the EGF receptor results in an accumulation of these receptors and cellular transformation [10].

Because activated Cdc42 is also able to form a stable complex with Cool-2, an important question seemed to be what the functional consequences of this interaction are. Here, we show that the binding of activated Cdc42 to Cool-2 causes a marked stimulation of its Rac-specific GEF activity. The stimulatory effects exhibited by Cdc42 are specific for dimeric Cool-2 molecules, as activated forms of Cdc42 do not stimulate the activity of monomeric Cool-2. Instead, activated Cdc42 inhibits the GEF activity of the Cool-2 monomer, presumably because GTP bound Cdc42 binds to the sole DH domain in a manner that is competitive with the binding of either GDP bound Cdc42 or Rac. Overall, these findings are consistent with the model presented in Figure 6. As depicted by the upper scheme in the figure, activated Cdc42 binds to the DH domain from one of the Cool-2 molecules making up the dimer. GDP bound Rac associates with the remaining available DH domain from the other Cool-2 molecule, as well as undergoes a *trans* interaction with the carboxy-terminal portion (i.e., the RSID) of the PH domain from the same Cool-2 molecule that binds Cdc42. Within this Cool-2 complex, activated Cdc42 induces a conformational change that strongly stimulates the GEF activity toward Rac. While we do not know the specific nature of this activating conformational change, it does not appear to influence the monomer-dimer equilibrium of Cool-2. If activated Cdc42 were stimulating the dissociation of the Cool-2 dimer into monomers, the GEF activity of Cool-2 would then become susceptible to a competitive inhibition by the active Cdc42 molecules. Rather, the allosteric activation elicited by GTP bound forms of Cdc42 may involve a positive regulatory influence on the orientation of the DH domain relative to the RSID used by the Cool-2 dimer to bind and specifically activate Rac.

Unlike Cdc42, the binding of activated Rac to Cool-2 strongly inhibits its Rac-GEF activity. We have found that this inhibition is essentially complete at levels of GMP-PCP bound Rac that are substoichiometric to the molar level of Cool-2. It may be that the binding of a single activated Rac molecule to the Cool-2 dimer is sufficient to sterically interfere with the binding of GDP bound Rac to the DH domain from one of the Cool-2 molecules making up the dimer and/or the RSID from the partner Cool-2 molecule. However, we also cannot rule out the possibility that the binding of activated Rac to the Cool-2 dimer confers an allosteric regulatory effect (opposite to that of Cdc42) that inhibits Rac-GEF activity. In either case, Rac would function as a feedback inhibitor of the GEF activity of dimeric Cool-2. As the levels of Rac-GTP increase due to Cool-2-stimulated GDP-GTP exchange, the GEF activity of the Cool-2 dimer will begin to shut down. Likewise, the production of GTP bound Rac or Cdc42 would be expected to lead to a feedback inhibition of either the Rac- or Cdc42-GEF activity exhibited by Cool-2 monomers, such as those generated by the binding of a PAK-G$\beta\gamma$ complex (see the lower scheme in Figure 6).

Our findings also suggest that under some circumstances, Cool-2 can mediate a GTPase cascade, by in effect translating the activation of Cdc42 into the activation of Rac. Other examples have been noted for GEFs being positively regulated by activated GTP binding proteins. For example, the Ras protein, upon activation by Son-of-sevenless (Sos), is able to bind to a distinct site on the Sos molecule and enhance its GEF activity [21]. It was proposed that this positive feedback regulation by GTP bound Ras might help to concentrate the active pool of Ras at specific membrane sites. Recently, a second example was reported where the binding of activated RhoG to Dock180/Elmo enhanced its GEF activity toward Rac [22]. However, to our knowledge, Cool-2 represents the first example of a Dbl family GEF that exhibits positive allosteric regulation, as im-

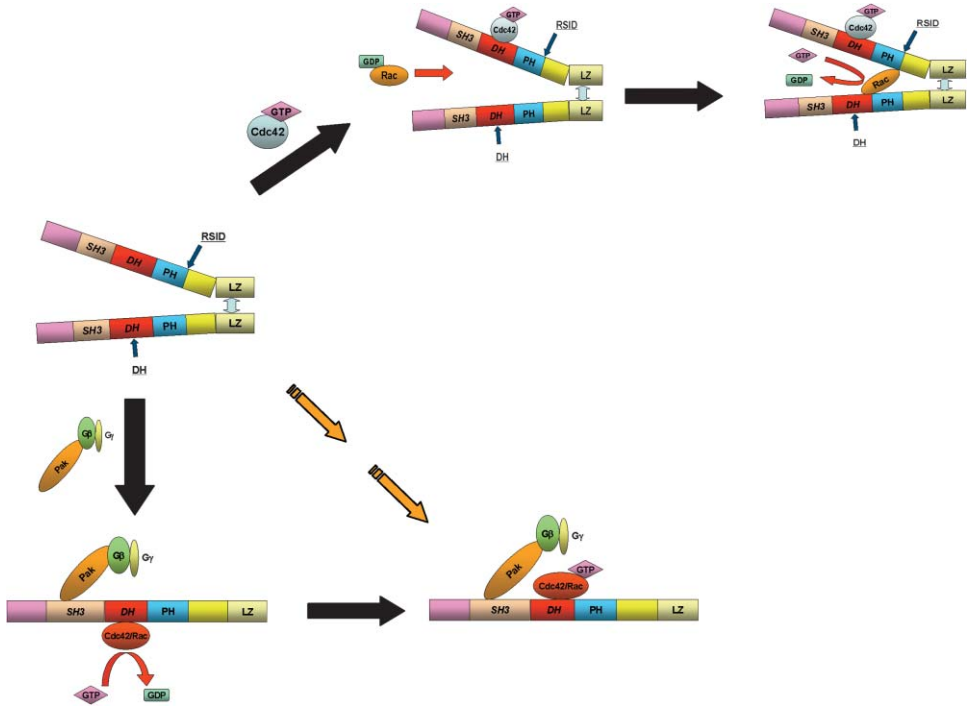


Figure 6. Model for the Activation and Stimulation of Cool-2 GEF Activity

In the absence of other binding partners, the Cool-2 dimer can act as a Rac-specific GEF. The upper scheme shows that, upon the binding of activated forms of Cdc42, an allosteric rearrangement occurs within the Cool-2 dimer that adjusts the binding interface in a more favorable arrangement for the binding of Rac, thereby allowing faster, more efficient exchange of GTP for GDP on Rac. Activated Rac is also capable of binding to the Cool-2 dimer (data not shown); however, this binding interaction inhibits further nucleotide exchange on Rac. Disruption of the Cool-2 dimer—for example, through the binding of a PAK3-Gβγ complex (lower scheme)—allows nucleotide exchange on Cdc42, as well as on Rac. Activated forms of Cdc42 or Rac are able to bind to monomeric Cool-2, competitively inhibiting further nucleotide exchange.

parted by one GTP binding protein (Cdc42) on the nucleotide exchange activity of another (Rac). Moreover, each of the other cases described above differs from that for Cool-2, because the activated GTP binding protein (Ras in the case of Sos, and RhoG in the case of Dock180/Elmo) binds to the same GEF molecule as the GDP bound substrate, at a site that is distinct from the GEF domain. However, in the case of Cool-2, activated Cdc42 binds to the DH domain, as does the substrate, GDP bound Rac. Consequently, the positive allosteric regulation imparted by Cdc42 can only occur in the Cool-2 dimer, but not in the monomer, where Cdc42 and Rac would compete for a single DH domain. Thus, dimeric Cool-2 serves a dual function as an upstream activator (Rac-GEF) and a downstream signaling target (i.e., for Cdc42). This dual function capability raises some interesting questions for future study. For example, are there any conditions where the binding of activated Cdc42 to p85Cool-1 can induce a GEF activity? What are the structural determinants that underlie the binding of activated forms of Cdc42 or Rac to the Cool-2 dimer, and how is it that these two GTP binding proteins appear to induce dramatically different regulatory effects on its GEF activity?

Experimental Procedures

Plasmid Construction

Plasmids pcDNA3-Myc-Cool-2, pcDNA3-Myc-p50Cool-1, and pcDNA3-Myc-p85Cool-1 were prepared as described previously [7, 9]. Rac1 was cloned into the BamHI-EcoRI site of a triple HA-tagged pKH_3 vector. The HA-tagged Cbl-b construct was a kind gift from Dr. Stan Lipkowitz (National Cancer Institute, MD). Different Cool-2 constructs were prepared by amplifying the appropriate bases of the open reading frame and subcloning the PCR product into a pcDNA3 or pKH_3 vector. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Each new plasmid was sequenced at the Cornell Biotechnology Resource Center.

Cell Culture and Transfections

COS-7 cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (Invitrogen). Plasmids pcDNA3-Myc-Cool-2, pcDNA3-Myc-p50Cool-1, and pcDNA3-Myc-p85Cool-1, as well as pcDNA3 or pKH3 encoding different Cool-2 mutants or constructs (2–4 μg/100 mm dish), were transfected into COS-7 cells using the LipofectAMINE transfection kit (Invitrogen). Cells were lysed after 24–48 hr in lysis buffer (20 mM HEPES [pH 7.4], 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) at 4°C for 20 min. If the lysates were used in GST pull-down experiments, 5 mM $MgCl_2$ was added to the lysis buffer. Lysates were prepared by centrifugation at 12,000 × g for 10 min at 4°C, and the supernatant was collected.

Protein Expression and Binding Assays

The GST-Rac1 and GST-Cdc42 fusion proteins were each expressed in *E. coli* BL21 (DE3) cells (Novagen) and purified by glutathione-agarose affinity chromatography in buffer A (20 mM HEPES, 5 mM $MgCl_2$, and 5 mM DTT). For the [^{3}H]GDP dissociation assays, the proteins were eluted from the glutathione-agarose beads by adding 10 mM glutathione to the buffer. The proteins were then liberated from their GST tag by overnight incubation with thrombin and 2 mM $CaCl_2$ at 4°C. Finally, the proteins were purified in buffer A on a G75 Sephadex column (Amersham BioSciences).

For the binding experiments, cell lysates from COS-7 cells transiently expressing Myc-tagged Cool-2, or lysates from insect cells expressing His-Cool-2, were combined with 5 μg of GST-Cdc42 or GST-Rac1 in a particular nucleotide bound state (e.g., preloaded with GMP-PCP) and immobilized on glutathione-agarose beads. The lysates were incubated with immobilized GST-Cdc42 (or GST-Rac) for 2 hr at 4°C. The beads were precipitated by microcentrifugation, washed three times with lysis buffer, and resuspended in 5× SDS-PAGE sample buffer. Proteins were eluted by boiling for 5 min and separated on a 10% or 12% SDS-polyacrylamide gel, transferred to an Immobilon-P membrane (Millipore), and probed with mouse monoclonal anti-Myc or anti-HA antibodies (Covance). Primary antibodies were detected with horseradish peroxidase-coupled sheep anti-mouse antibody by ECL (Amersham Biosciences).

Purification of His-Cool-2 from Insect (Sf21) Cells

Insect (Sf21) cells were maintained in Grace's Insect Media (Invitrogen) supplemented with 10% fetal bovine serum. A virus for full-length His-Cool-2 was produced using the Bac-to-Bac Baculovirus Expression Kit (Invitrogen). Sf21 cells were infected with the His-Cool-2 virus at a moi of 5 for 72 hr. Cells were lysed in buffer containing (20 mM Tris [pH 8.0], 20 mM imidazole, 0.2% CHAPS, and protease inhibitors). The lysate was centrifuged at 12,000 × g for 10 min, and the supernatant was incubated at 4°C for 30 min with NTA-agarose conjugated to nickel. The beads were washed three times with the lysis buffer and eluted in 20 mM Tris (pH 8.0), 200 mM imidazole, and 0.2% CHAPS. The protein was then concentrated to 1 μg/μl.

[^{3}H]GDP Dissociation Assay

Typically, His-Cool-2 purified from Sf21 cells was preincubated (at the concentrations indicated in the figure legends) with a predetermined amount of GMP-PCP bound Cdc42 or Rac1 for 30 min. This complex was then incubated with Cdc42 or Rac (typically 250 nM) preloaded with [^{3}H]GDP in 140 μl of reaction buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT) that contained 100 μM GTPγS (to displace the [^{3}H]GDP that is exchanged through the course of the experiment) at room temperature. At specific time points, 20 μl aliquots were diluted into 1 ml of ice-cold termination buffer (20 mM Tris, 100 mM $MgCl_2$, and 10 mM NaCl [pH 7.4]). The percent [^{3}H]GDP remaining on the filters was detected by scintillation counting.

If Cool-2 was prepared from COS-7 cells, lysates expressing different Myc-tagged Cool-2 constructs were incubated with anti-Myc antibody for 1.5 hr followed by mixing with protein G-Sepharose beads for 1 hr. The beads were washed twice with lysis buffer and then with HMN buffer (10 mM HEPES [pH 7.5], 5 mM $MgCl_2$, and 100 mM NaCl). The immunoprecipitated Cool-2 proteins were then incubated with Cdc42 or Rac as described above.

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