Novel regulatory mechanisms for the Dbl family guanine nucleotide exchange factor Cool-2/ α -Pix

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The Cool-2 (cloned-out of library-2) protein (identical to α -Pix for Pak-interactive exchange factor) has been implicated in various biological responses including chemoattractant signaling and in certain forms of mental retardation. We show that when Cool-2 exists as a dimer, it functions as a Rac-specific guanine nucleotide exchange factor (GEF). Dimerization of Cool-2 enables its Dbl (diffuse B-cell lymphoma) and pleckstrin homology domains to work together (*in trans*) to bind specifically to Rac-GDP. Dissociation of dimeric Cool-2 into its monomeric form allows it to act as a GEF for Cdc42 as well as for Rac. The binding of either PAK (p21-activated kinase) or Cbl (Casitas B-lymphoma) to the SH3 domain of monomeric Cool-2 is necessary for the functional interactions between GDP-bound Cdc42 or Rac and the Cool-2 monomer. The $\beta\gamma$ subunit complex of large GTP-binding proteins, by interacting with PAK, stimulates the dissociation of the Cool-2 dimer and activates its GEF activity for Cdc42. Overall, these findings highlight novel mechanisms by which extracellular signals can direct the specific activation of Rac versus Cdc42 by Cool-2/ α -Pix.

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Introduction

Members of the Rho family of GTP-binding proteins function as molecular switches in biological response pathways that result in changes in the actin cytoskeletal architecture, the stimulation of cell cycle progression and gene transcription, and the regulation of intracellular trafficking activities (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998; Bar-Sagi and Hall, 2000; Erickson and Cerione, 2001). Three classes of proteins, GTPase-activating proteins (GAPs), guanine nucleotide dissociation inhibitors (GDIs), and guanine nucleotide exchange factors (GEFs), regulate the cycling of these GTP-binding proteins between their GDP-bound and GTP-bound states (Boguski and McCormick, 1993). Rho family proteins, such as Cdc42 and Rac, are activated by a number of up-

stream stimuli, as mediated by members of the Dbl (diffuse B-cell lymphoma) family of GEFs (Whitehead *et al.*, 1997; Hoffman and Cerione, 2002). One subgroup of the Dbl family, the Cool/Pix proteins (Cool for cloned-out of library-2 and Pix for Pak-interactive exchange factor) (Oh *et al.*, 1997; Bagrodia *et al.*, 1998; Manser *et al.*, 1998), is particularly interesting, as its members have been shown to exhibit a variety of functional activities and to elicit a diversity of cellular responses. We have characterized three members of the family: p50Cool-1, a longer splice variant form called p85Cool-1 (identical to β -Pix; Manser *et al.*, 1998), and a distinct gene product called p90Cool-2 (identical to α -Pix but from here on called Cool-2).

In addition to containing Dbl homology (DH) and pleckstrin homology (PH) domains, Cool family members have an SH3 domain, which they use to bind to PAK (p21-activated kinase) (Bagrodia *et al.*, 1998; Manser *et al.*, 1998) and promote signaling activities *in vivo* (Ku *et al.*, 2001; Lee *et al.*, 2001). Recently, it was demonstrated that the Cbl (Casitas B-lymphoma) proteins were also able to bind to the Cool proteins through their SH3 domain and compete with PAK–Cool interactions (Flanders *et al.*, 2003; Wu *et al.*, 2003). Within the middle portions of the p85Cool-1 and Cool-2 proteins are a proline-rich region and a Cat (Cool-associated tyrosine phosphosubstrate)/Git (G protein-coupled receptor kinase interactor) binding domain (Premont *et al.*, 1998; Bagrodia *et al.*, 1999). The Cat/Git proteins, also known as Pkl (paxillin-kinase-linker) (Turner *et al.*, 1999) and p95-APP1 (ArfGAP-putative, Pix-interacting, paxillin-interacting protein 1) (Di Cesare *et al.*, 2000), are members of the ArfGAP family and regulate the dynamics of focal adhesion complexes (Turner *et al.*, 2001). The Cool and Cat/Git proteins are often present in complexes containing paxillin and PAK that may mediate the integration of Arf and Rho family signaling activities at the cytoskeleton (Bagrodia and Cerione, 1999; Turner *et al.*, 1999, 2001). Recently, it was also reported that a complex between p85Cool-1 and p95-APP1 was required for Rac1B-mediated neurite outgrowth (Albertinazzi *et al.*, 2003).

The Cool-2 protein, unlike the Cool-1 proteins, contains a calponin homology (CH) domain that extends from its amino-terminus, and the disruption of this domain has been linked to certain (X-linked) forms of mental retardation (Kutsche *et al.*, 2000; Ramakers, 2002). Both Cool-2 and p85Cool-1 also have a leucine zipper near their carboxyl-terminal ends that enables dimerization to occur. In the case of p85Cool-1, dimerization was suggested to promote the formation of microvillus-like structures and membrane ruffles (Kim *et al.*, 2001; Koh *et al.*, 2001).

The Cool proteins were initially assumed to function as Rho family GEFs, given that they contained conserved DH and PH domains. However, thus far, only one member of the family, Cool-2, has exhibited significant GEF activity in standard biochemical assays (Feng *et al.*, 2002), whereas p85Cool-1 has been shown to bind preferentially to activated (GTP-bound) Cdc42 and to act as a target/scaffold protein in mediating the regulation of Cbl's E3 ubiquitin ligase activity (Wu *et al.*, 2003). Cool-2 strongly stimulates PAK activity

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(Bagrodia *et al*, 1999; Daniels *et al*, 1999; Feng *et al*, 2002), and it has been assumed that this results from Cool-2 acting as a GEF for Cdc42 or Rac. It was also reported that Cool-2 played a key role in chemoattractant signaling through its ability to activate Cdc42 (Li *et al*, 2003).

In this study, we have focused on the Cool-2 protein, given its involvement in a variety of biological responses, with the aim of better understanding how it functions as a GEF. We show for the first time that two novel regulatory mechanisms control its GEF activity. Under conditions where Cool-2 exists as a dimer *in vivo*, it functions as a Rac-specific GEF. We found that the carboxyl-terminal portion of the PH domain of Cool-2 works together with the DH domain through dimerization (i.e. *in trans*) to mediate a highly specific interaction with GDP-bound Rac. However, when Cool-2 is a monomer, it exhibits GEF activity toward Cdc42, as well as Rac, but only upon the binding of PAK or Cbl to its SH3 domain. Thus, differential regulation of the monomer-dimer equilibrium of Cool-2 by specific extracellular signals can dictate whether Rac- and/or Cdc42-coupled signaling pathways are activated.

Results

Cool-2 functions as a GEF for Rac-

The Cool-2 protein contains tandem DH and PH domains, which is characteristic of Rho family GEFs (Figure 1A). We

(Bagrodia *et al*, 1999) and others (Daniels *et al*, 1999) have previously shown that the SH3 domain of Cool-2 specifically interacts with a stretch of proline residues on PAK, resulting in the stimulation of PAK activity. Likewise, we found that a Cool-2 construct that contained just the SH3 and DH domains as well as most of the PH domain (SH3.PH(541).Cool-2 in Figure 1B) was able to activate PAK (Feng *et al*, 2002). We initially assumed that the Cool-2-mediated activation of PAK was the direct result of Cool-2 acting as a GEF for Cdc42 and/or Rac, as the GTP-bound forms of these proteins stimulate PAK activity (Manser *et al*, 1994; Bagrodia *et al*, 1995). However, as shown in Figure 2A, when Myc-tagged Cool-2 was expressed in COS-7 cells, then immunoprecipitated with anti-Myc and added to an assay incubation containing [3 H]GDP-bound Cdc42, no detectable Cdc42-GEF activity was measured for Cool-2 as indicated by the fact that little dissociation of [3 H]GDP from Cdc42 (i.e. the rate-limiting step for guanine nucleotide exchange) occurred over 20–25 min. This was similar to what was observed when Cdc42 was mixed with control lysates lacking Myc-Cool-2 (not shown) and in contrast to what occurred when EDTA was used to fully stimulate GDP dissociation by chelating Mg^{2+} . On the other hand, when the same type of assay was performed with Rac1, Cool-2 stimulated the dissociation of [3 H]GDP from Rac1, indicating that full-length Cool-2 acts as a specific Rac-GEF. Likewise, in binding experiments, we found

Figure 1 Domain structure of Cool-2 and different Cool-2 constructs. **(A)** Cool-2 contains CH, SH3, DH and PH domains, as well as a proline-rich region (PXXP), a Cat/Git binding domain (CBD), and a leucine zipper (LZ) region. The RSID (for Rac-specific interaction domain) is located at the C-terminal end of the PH domain. **(B)** Schematic representation of different truncated Cool-2 constructs and Cool-2 mutants.

Figure 2 The abilities of wild-type Cool-2 to bind and exhibit GEF activity toward Cdc42 and Rac. **(A)** COS-7 cells were transiently transfected with a plasmid expressing Myc-Cool-2 and the Cool-2 proteins were immunoprecipitated using an anti-Myc antibody. Recombinant (*E. coli*) GST-Cdc42 or GST-Rac was preloaded with [3 H]GDP. The exchange of bound [3 H]GDP for GTP γ S, as monitored by the dissociation of [3 H]GDP from Cdc42 or Rac, was assayed in the presence of immunoprecipitated Myc-Cool-2 or in the presence of lysates from cells not expressing Myc-Cool-2. The data shown are representative of three experiments. **(B)** The GST-Rac(T17N) and GST-Cdc42(T17N) fusion proteins were prepared as described in Materials and methods. Lanes 1–3 show the relative expression of the different Myc-Cool constructs based on Western blots of whole-cell lysates (WCLs) with anti-Myc antibody. Lanes 4–10 show the results of GST pull-down experiments, in which ~ 5 μ g of GST fusion proteins, immobilized on glutathione-agarose beads, was incubated with COS-7 cell lysates expressing Myc-Cool-2, Myc-p85Cool-1, or Myc-p50Cool-1 as described in Materials and methods. The co-precipitated Myc-tagged Cool proteins were detected by Western blotting with anti-Myc antibody.

that Cool-2 formed a stable complex with Rac but not with Cdc42. Figure 2B (lanes 5–7) shows that the nucleotide-depleted Rac construct, GST-Rac(T17N), was able to precipitate Myc-tagged Cool-2 from cells, as well as Myc-p50Cool-1 and to a lesser extent Myc-p85Cool-1 (the relative expression of these different Myc-Cool proteins is shown in lanes 1–3), whereas the corresponding GST-Cdc42(T17N) protein did not show a detectable interaction with either the Cool-1 or Cool-2 proteins (Figure 2B, lanes 8–10).

DH and PH domains of Cool-2 work together to exhibit GEF activity toward Rac-

Structural and mutagenesis studies have revealed a conserved mechanism by which Dbl family proteins catalyze nucleotide exchange on Rho-related GTP-binding proteins. For the case of Dbl, two conserved leucine residues within the

DH domain are essential for activity (Hart *et al*, 1994). The corresponding leucine residues are present within the DH domain of Cool-2 as well. A good deal is also known regarding how certain members of the Dbl family are able to exhibit specificity for their GTP-binding protein targets (Worthylake *et al*, 2000; Gao *et al*, 2001; Hussain *et al*, 2001; Hoffman and Cerione, 2002; Rossman *et al*, 2002; Snyder *et al*, 2002). For example, the Intersectin proteins are highly specific GEFs for Cdc42 whereas Tiam1 is highly selective for Rac (Worthylake *et al*, 2000; Hussain *et al*, 2001; Hoffman and Cerione, 2002; Snyder *et al*, 2002). Because full-length Cool-2 acted as a Rac-specific GEF (Figure 2A), we compared its sequence with that of Tiam1. Isoleucine 1187 within the DH domain of Tiam1 has been suggested to play a critical role in determining specificity for Rac (Snyder *et al*, 2002). The corresponding residue in Cool-2 appeared to be Ile 544, because like Ile 1187

in Tiam1, Ile 544 was immediately downstream from a leucine residue and 10 residues downstream from a glutamine residue (these residues are in bold in the sequence shown in Figure 1A). However, Ile 544 is located within the carboxyl-terminal end of the PH domain rather than within the DH domain. To test the functional importance of this region for the Rac-GEF activity of Cool-2, we changed Leu 543 and Ile 544 to arginine and valine, respectively (designated LIm.Cool-2 in Figure 1B). The Myc-tagged LIm.Cool-2 construct was transiently expressed in COS-7 cells and then assayed for its ability to stimulate the guanine nucleotide exchange activity of Rac1.

Figure 3A, left panel, compares the relative expression of Myc-LIm.Cool-2 in COS-7 cells with wild-type Myc-Cool-2 and other Cool-2 mutants that will be described below, and the right panel compares the relative amounts of these same Cool-2 constructs that were immunoprecipitated with anti-Myc and used in the GEF assays. As shown in Figure 3B, LIm.Cool-2 was ineffective at stimulating the dissociation of [3 H]GDP from Rac1. It was also incapable of stimulating PAK3 activity in cells (compare lane 1 (control) with lane 5 in the bottom panel of Figure 3C; the top panel shows the relative amounts of HA-tagged PAK3 expressed under the different experimental conditions).

We also generated a double mutant where the highly conserved leucine residues 383 and 384 within the DH domain of Cool-2 were changed to arginine and serine, respectively (designated DHm.Cool-2 in Figure 1B). We found that DHm.Cool-2 exhibited a relatively weak GEF activity (Figure 3B) and no detectable stimulation of PAK3 (lane 6 in the bottom panel of Figure 3C), consistent with the findings of Daniels *et al* (1999) when assaying PAK activation by a truncated version of Cool-2. Thus taken together, these results indicated that both the DH domain of Cool-2 and a newly identified region within the carboxyl-terminal end of the PH domain, which we designated as RSID (for Rac-specific interaction domain; see Figure 1A), were necessary for full Rac-GEF activity.

As a complimentary approach, we examined the ability of these and other Cool-2 mutants to bind to Rac. The LIm.Cool-2 and DHm.Cool-2 constructs were transiently expressed in COS-7 cells, as were full-length wild-type Cool-2, SH3.PH(541).Cool-2, and PH.LZ.Cool-2, which is comprised

of the PH domain and everything downstream (Figure 1B). Lysates were prepared from the different transfectants and then incubated with GST-Rac(T17N). Compared to wild-type Cool-2, both LIm.Cool-2 and DHm.Cool-2 showed weaker binding to GST-Rac(T17N) (Figure 3D, compare lane 2 with lanes 1 and 3, respectively, in the top panel; the bottom panel shows the GST-Rac(T17N) used in the different pull-down experiments). Neither SH3.PH(541).Cool-2 (lanes 4 and 6, top panel), which ends at residue 541 and thereby lacks

Figure 3 The DH and PH domains of Cool-2 work together to exhibit GEF activity toward Rac. (A) The left panel shows the relative expression in whole-cell lysates (WCLs) of different Myc-Cool-2 constructs used in the experiments shown in (B–D). The different Myc-Cool-2 constructs were expressed, alone (B, D) or together with HA-PAK3 (C) in COS-7 cells. The right panel shows the immunoprecipitation of the different Myc-Cool-2 proteins used in the GEF assays in (B) or in other experiments. (B) The dissociation of [3 H]GDP from Rac1 catalyzed by different Cool-2 constructs was assayed as described in the legend to Figure 2A and the data shown are representative of three experiments. (C) Assays of PAK activity (shown in the bottom panel) were performed for the different conditions indicated, using myelin basic protein (MBP) as a phosphosubstrate (see Materials and methods). The top panel compares the relative amounts of PAK3 expressed in the different cell lysates. (D) The top panel shows the binding of the different Cool-2 constructs to GST-Rac(T17N), performed as described in the legend to Figure 2B, and the bottom panel shows the relative amounts of GST-Rac(T17N) precipitated in the different experiments.

the complete RSID necessary for Rac-GEF activity, nor PH.LZ.Cool-2 (lane 5, top panel), which is missing the DH domain but includes the RSID, showed detectable binding to Rac. Thus, these results further indicate that the DH domain and residues within the carboxyl-terminal portion of the PH domain of Cool-2 are necessary for specific binding to Rac.

We have also examined whether a Cool-2 mutant that was unable to bind to PAK was able to stimulate PAK activity and/or exhibit GEF activity toward Rac1. A PAK-binding defective Cool-2 mutant was prepared by changing Trp 194 and Trp 195 to lysine residues. This Cool-2 mutant, designated as WWm.Cool-2 in Figure 1B, still exhibited GEF activity toward Rac1 (Figure 3B) but was incapable of stimulating PAK activity (Figure 3C, bottom panel, lane 7). Thus, while the binding of PAK to full-length Cool-2 was not essential for its Rac-GEF activity, this binding interaction was required for Cool-2-activated Rac molecules to stimulate PAK activity. This suggested that Cool-2 may not act catalytically to activate multiple Rac molecules and that once activated Rac is unable to dissociate from Cool-2 and engage PAK molecules that are not part of a Cool-2-PAK complex. Because Rac (or Cdc42) is typically in excess relative to the Cool-2 proteins in our assays, the inability of Cool-2 to act catalytically as a GEF may also explain as to why we often see less than complete dissociation of [3 H]GDP from the total pool of GTP-binding proteins.

Dimerization of Cool-2 is necessary for its specific interaction with Rac1-

Based on the results described in the preceding section, we assumed that any Cool-2 construct that contained both the DH domain and the RSID would function as a Rac-specific GEF. We therefore examined the abilities of some additional Cool-2 constructs to act as Rac-GEFs, including one that contained both the DH domain and RSID as well as a portion of the proline-rich region (SH3.PX.Cool-2) (Figure 4A), and another that included the full proline-rich domain (SH3.PXXP.Cool-2). Surprisingly, neither of these truncated Cool-2 molecules alone exhibited GEF activity (data not shown).

This led us to consider other possibilities regarding how the DH domain and RSID might work together to activate Rac1 specifically. One interesting possibility stemmed from the findings that p85Cool-1 dimerizes through its carboxyl-terminal leucine zipper (Kim *et al*, 2001). Cool-2 also contains a leucine zipper and so we wondered whether the dimerization of Cool-2 might allow cooperation (*in trans*) between its DH domain and RSID. Such a mechanism would be consistent with our findings that truncated Cool-2 constructs lacking the leucine zipper did not exhibit Rac-GEF activity.

To test this idea, we examined two Cool-2 constructs. One, designated CH.PXXP.Cool-2 (Figure 4A), begins at the amino-terminal CH domain and terminates just downstream from the proline-rich region and thus lacks both the Cat/Git-binding domain (CBD) and the leucine zipper. A second construct, SH3.CBD.Cool-2 (Figure 4A), starts at the SH3 domain and terminates just after the Cat/Git-binding domain and also lacks the leucine zipper. Both SH3.CBD.Cool-2 and CH.PXXP.Cool-2 stimulated PAK activity (compare lanes 1 and 4 in the bottom panel of Figure 3C, and lanes 1 and 3 in the bottom panel of Figure 4B, respectively), but SH3.CBD.Cool-2 was unable to bind to GST-Rac(T17N)

(Figure 3D, lane 7, top panel), and neither of these Cool-2 constructs alone stimulated [3 H]GDP dissociation from Rac (Figure 4C). Thus, Cool-2 constructs that lack the leucine zipper region do not exhibit the same capability as full-length Cool-2 to function as Rac-GEFs. However, as will be discussed in more detail in the next section, when these truncated Cool-2 constructs were coexpressed with PAK3, they were then able to function as Rac-GEFs and stimulate [3 H]GDP dissociation (Figure 4C).

To further demonstrate the importance of the leucine zipper for the Rac-specific GEF activity of full-length Cool-2, we generated a Cool-2 construct with specific mutations within this region. Leucine residues 711, 718, and 725 within the leucine zipper of Cool-2 conform to the 'LxxxxxL' motif. We therefore prepared a Cool-2 mutant in which leucine residues 711 and 718 were changed to arginine and serine, respectively (designated as LZm.Cool-2; Figure 4A). Myc-tagged LZm.Cool-2 or wild-type Cool-2 was then transiently expressed in COS-7 cells, either alone or together with HA-tagged p85Cool-1. As shown in Figure 5A (top panel, lanes 4 and 5), when these Cool-2 constructs were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), a single band with the expected mobility of the monomeric protein was always detected for Myc-LZm.Cool-2, whereas two bands were detected for full-length Cool-2 (lanes 1–3), with the slower mobility band reflecting dimer formation that was not fully disrupted even after treatment with SDS. We also found that HA-p85Cool-1 could be co-immunoprecipitated with Myc-Cool-2 (Figure 5B, bottom panel, lane 2), showing that heterodimerization between Cool-1 and Cool-2 can occur. However, HA-p85Cool-1 could not be co-immunoprecipitated with Myc-LZm.Cool-2 (Figure 5B, bottom panel, lane 1). Thus, the double mutation within the leucine zipper region of Cool-2 eliminated its ability to undergo dimerization. When we examined the ability of Myc-LZm.Cool-2, following its immunoprecipitation, to stimulate [3 H]GDP dissociation from Rac, we found that it was ineffective (Figure 5C). This is consistent with the idea that dimerization is necessary for the proper juxtaposition of the DH domain and RSID for the Rac-specific GEF activity exhibited by the full-length Cool-2 protein.

SH3 domain interactions enable monomeric Cool-2 to exhibit GEF activity toward Rac and Cdc42-

Given the importance of the leucine zipper for the Rac-specific GEF activity of Cool-2, how was it that the SH3.PH(541).Cool-2 construct, lacking the leucine zipper, was still able to activate PAK when expressed in cells (Figure 3C, bottom panel, lane 2; see also, Feng *et al*, 2002)? We wondered whether the binding of PAK to dimerization-defective Cool-2 constructs might stimulate their GEF activity and thereby account for their ability to activate PAK. Indeed, the coexpression of PAK3 with truncated Cool-2 constructs lacking the leucine zipper (SH3.CBD.Cool-2 and CH.PXXP.Cool-2) enabled these Cool-2 proteins to stimulate the dissociation of [3 H]GDP from Rac (Figure 4C). Likewise, coexpressing the shorter Myc-tagged SH3.PH(541).Cool-2 construct together with HA-tagged PAK3 (Figure 6A, panel a) gave rise to Rac-GEF activity (Figure 6B). Moreover, the coexpression of PAK3 together with SH3.PH(541).Cool-2 also enabled it to act as a GEF for Cdc42 (Figure 6B).

To confirm that it was the direct interaction between Cool-2 and PAK that was responsible for the GEF activity exhibited by SH3.PH(541).Cool-2, we mutated the conserved tryptophan residues 194 and 195 in the SH3 domain of Cool-2 to lysines (designated SH3.PH(541).Cool-2.WWm), as these residues are essential for binding to PAK (Bagrodia *et al*, 1998). When SH3.PH(541).Cool-2.WWm was coexpressed with PAK3 (Figure 6A, panel a), it was unable to stimulate the guanine nucleotide exchange activity of Cdc42 (Figure 6C). The same was true for Rac (data not shown).

We next asked whether the kinase activity of PAK was essential for Cool-2 to functionally couple to Cdc42 or Rac.

However, this was not the case, as the expression of a PAK3 construct that lacked the kinase domain (designated as PAK.KD in Figure 6A, panel b) was sufficient to enable SH3.PH(541).Cool-2 to stimulate [3 H]GDP dissociation from Cdc42 (Figure 6C). Moreover, when the adaptor protein Cbl-b, a member of the Cbl family that is enriched in breast cancer cells and also binds to the SH3 domain of the Cool proteins (Flanders *et al*, 2003), was coexpressed with SH3.PH(541).Cool-2 (Figure 6A, panel c), this enabled SH3.PH(541).Cool-2 to act as a GEF for Cdc42 (Figure 6C), as well as for Rac (Figure 6D). The effects of Cbl-b (as well as PAK3) were specific for the SH3.PH(541).Cool-2 construct, as

Figure 4 Dimerization of Cool-2 is necessary for its specific interaction with Rac1. (A) Schematic representation of different truncated Cool-2 constructs and Cool-2 mutants. (B) Top panel: COS-7 cells were transfected with a plasmid expressing HA-PAK3 together with plasmids expressing Myc-CH.PXXP.Cool-2 (lane 1), Myc-Cool-2 (lane 2), or control vector (lane 3). The middle panel compares the relative expression of HA-PAK3 for the different experimental conditions, and the bottom panel shows the results of PAK assays using MBP as a phosphosubstrate. (C) COS-7 cells were transfected with plasmids expressing Myc-CH.PXXP.Cool-2 or SH3.CBD.Cool-2, alone or together with a plasmid expressing HA-PAK3. The assays of [3 H]GDP dissociation from Rac were performed as described in the legend to Figure 2A and the data shown are representative of three experiments.

Figure 5 Dimerization of Cool-2 is necessary for its GEF activity toward Rac1. (A) SDS-PAGE profiles for Myc-Cool-2 or Myc-LZm.Cool-2, transiently expressed in COS-7 cells alone or together with HA-p85Cool-1. The higher bands in the top panel represent the Cool-2 dimer, whereas the lower bands represent the Cool-2 monomer. (B) Co-immunoprecipitation assays in which cell lysates coexpressing Myc-LZm.Cool-2 and HA-p85Cool-1, or Myc-Cool-2 and HA-p85Cool-1, were incubated with anti-Myc primary antibody. This experiment was carried out as described in Materials and methods. (C) The top panel compares the relative expression of Myc-Cool-2 and Myc-LZm.Cool-2 in whole-cell lysates (WCLs) (lanes 1 and 2) and the corresponding immunoprecipitation of the Cool-2 proteins (lanes 3 and 4). The bottom panel shows assays of [3 H]GDP dissociation from Rac1, performed as described in the legend to Figure 2A.

coexpression of Cbl-b with full-length Cool-2 (Figure 6A, panel d) did not alter its ability to act as a GEF for Rac (Figure 6D).

Apparently, the binding of PAK or Cbl to the SH3 domain of monomeric Cool-2 causes it to interact more effectively with Cdc42 or Rac. This is indicated from the binding experiments

presented in Figure 7B. In these experiments, GST-Cdc42(T17N) was incubated with the lysates from cells expressing Myc-SH3.PH(541).Cool-2 together with either HA-Cbl-b or HA-PAK3 (Figure 7A is the Western blot showing the expression of these proteins), and then precipitated with glutathione-agarose beads. The precipitates were blotted

Figure 6 Assays of [3 H]GDP dissociation from Rac or Cdc42 catalyzed by different Cool-2 constructs together with kinase-defective PAK3 or with Cbl-b. **(A)** Western blots for COS-7 cell lysates expressing Myc-SH3.PH(541).Cool-2 and HA-PAK3 (panel a), Myc-SH3.PH(541).Cool-2 and HA-PAK.KD (panel b), Myc-SH3.PH(541).Cool-2.WWm and HA-PAK3 (panel a), Myc-SH3.PH(541).Cool-2 and HA-Cbl-b (panel c), or Myc-Cool-2 and HA-Cbl-b (panel d). Assays of [3 H]GDP dissociation from Rac **(B, D)** or Cdc42 **(B, C)** in the presence of the indicated Cool-2, PAK3, and Cbl-b constructs. The assays were performed as described in the legend to Figure 2A and the data shown are representative of three experiments.

with anti-Myc to detect Cool-2 that co-precipitated with GST-Cdc42(T17N). The data presented in Figure 7B show that GST-Cdc42(T17N) was not capable of binding to SH3.PH(541).Cool-2 alone (i.e. see lane 1 in the top panel; the bottom panel shows the relative amounts of GST-Cdc42 used in the different experiments). However, an interaction was detected when SH3.PH(541).Cool-2 was coexpressed with either Cbl-b or PAK3 (lanes 2 and 3, respectively, in the top panel). The apparent differences in the effectiveness of the Cdc42–Cool-2 interaction observed in the presence of Cbl-b versus PAK3 is likely attributed to differences in the relative expression of these proteins (see Figure 7A).

These findings, when taken together with those described in the preceding section, suggest that Cool-2 constructs that are incapable of forming dimers can exhibit GEF activity toward Cdc42 (or Rac), provided that their SH3 domain is engaged by PAK or Cbl. This is further supported by the data shown in Figure 7C. Full-length Myc-Cool-2, following its immunoprecipitation from cells, was incapable of stimulating the nucleotide exchange activity of Cdc42, even when coex-

pressed with Cbl-b (Figure 7C). However, the dimerization-defective LZm.Cool-2 protein, when expressed together with Cbl-b, was able to exhibit Cdc42-GEF activity. The same was true when LZm.Cool-2 was coexpressed with PAK3 (data not shown).

Recently, it was suggested that the Cool-2/ α -Pix protein functioned in a chemoattractant signaling pathway to promote the activation of Cdc42 (Li *et al.*, 2003). This activation event required both the binding of PAK to Cool-2 and the binding of the G protein $\beta\gamma$ subunit complex to PAK. Given what we know about the necessity for Cool-2 to exist as a monomer to activate Cdc42, we suspected that the G protein $\beta\gamma$ complex, upon binding to PAK, might promote the monomeric state of Cool-2. Indeed, the addition of the retinal G protein $\beta_1\gamma_1$ subunit complex, together with histidine (His)-tagged, purified Cool-2 protein, to cell lysates expressing Myc-Cool-2 and HA-PAK3 completely blocked the ability of His-Cool-2 to form a dimer with Myc-Cool-2, as read out by their co-immunoprecipitation with anti-Myc antibody (compare lanes 2 and 3 in the top panel of Figure 8A). As expected,

Figure 7 SH3 domain-mediated association enhances the interaction of Cdc42 with monomeric Cool-2 proteins. **(A)** Western blot of whole-cell lysates (WCLs) of COS-7 cells expressing Myc-SH3.PH(541).Cool-2 alone (lane 1) or together with HA-Cbl-b (lane 2) or HA-PAK3 (lane 3). **(B)** GST pull-down assays were performed by incubating GST-Cdc42(T17N) with lysates from COS-7 cells expressing Myc-SH3.PH(541).Cool-2, alone (lane 1) or together with HA-Cbl-b (lane 2) or HA-PAK3 (lane 3) as described in Materials and methods. The co-precipitated Myc-SH3.PH(541).Cool-2 was detected by Western blotting with anti-Myc antibody (top panel); the relative amounts of GST-Cdc42(T17N) precipitated in the different experiments are shown in the bottom panel. **(C)** Full-length Myc-Cool-2 or Myc-LZm.Cool-2 was coexpressed with HA-Cbl-b in COS-7 cells (top panel, lanes 1 and 2), and then the Myc-Cool-2 proteins were immunoprecipitated (top panel, lanes 3 and 4) and [3 H]GDP dissociation from Cdc42 was assayed (bottom panel) as described in the legend to Figure 2A.

Myc-LZm.Cool-2 was unable to dimerize with His-Cool-2, even in the absence of added $\beta_1\gamma_1$ complex (see lane 1 in the top panel of Figure 8A).

Neither the addition of insect cell recombinant PAK3 nor retinal $\beta_1\gamma_1$ alone (Figure 8C shows the SDS-PAGE profiles for these purified proteins) was able to inhibit the ability of the purified His-Cool-2 protein to form a dimer with the transi-

ently expressed Myc-Cool-2 (compare lanes 3 and 4, respectively, in the top panel of Figure 8B with lane 1). However, when $\beta_1\gamma_1$ was added together with PAK3, a time-dependent inhibition of Cool-2 dimerization was observed (see lanes 6–8 in the top panel of Figure 8B), culminating in a complete block of the co-immunoprecipitation of His-Cool-2 with Myc-Cool-2 (lane 2 in the top panel).

We then set out to reconstitute the Cool-2-promoted activation of Cdc42 using purified proteins (Figure 8C). When the insect cell recombinant Cool-2 was incubated with PAK3 alone, there was no detectable stimulation of [3 H]GDP dissociation from Cdc42 (Figure 8D), consistent with the idea that PAK3 is not sufficient to promote the dissociation of dimeric Cool-2 into monomers. However, when Cool-2 was incubated with PAK3 together with the $\beta\gamma$ complex (i.e.

conditions that lead to the formation of Cool-2 monomers), we were able to reconstitute the Cdc42-GEF activity of Cool-2 successfully.

Discussion

The Cool/Pix proteins were originally identified through their abilities to bind to PAK (Bagrodia *et al*, 1998; Manser *et al*, 1998). Each of the members of the family share a common arrangement of SH3, DH, and PH domains, with the presence of tandem DH and PH domains leading to the expectation that these proteins would serve as upstream activators (GEFs) specifically complexed to a downstream target for Cdc42 or Rac. However, initially it was difficult to document that some of the members of the Cool/Pix family were indeed capable of GEF activity. For example, the expression of p50Cool-1 in cells resulted in an inhibition of Cdc42-stimulated PAK activity, whereas p85Cool-1 neither inhibited nor stimulated PAK activity (Bagrodia *et al*, 1998). On the other hand, Cool-2 strongly stimulated PAK activity. It was subsequently demonstrated that a truncated Cool-2 construct containing the SH3, DH, and PH domains, when assayed together with PAK3, was indeed capable of GEF activity as documented by its ability to stimulate the release of [3 H]GDP from Cdc42 (Feng *et al*, 2002). In addition, it was determined that an 18-amino-acid sequence, designated as T1, acted to repress GEF activity in the Cool-1 proteins, such that its removal yielded Cool-1 constructs capable of stimulating GDP dissociation from Cdc42, whereas addition of the T1 region to Cool-2 constructs inhibited their GEF function.

Given these findings, the questions have shifted toward understanding the underlying mechanisms by which the Cool proteins might function as GEFs. There have been reports suggesting that p85Cool-1 gives rise to cellular responses that are consistent with the activation of Rac or Cdc42 (Kim *et al*, 2001; Koh *et al*, 2001). Presumably, some form of signaling regulation is responsible for reversing the negative regulatory activity of the T1 region, thus converting p85Cool-1 into a functioning GEF. We expected that the mechanisms underlying the GEF activity for Cool-2 would be more straightforward, given its readily detectable activation of PAK in cells. However, surprisingly, we have found that Cool-2 is

Figure 8 G $\beta\gamma$ helps to activate the Cdc42-GEF activity of Cool-2. (A) The ability of Myc-Cool-2 versus Myc-LZm.Cool-2 to dimerize was examined in the presence of PAK3, and in the presence and absence of bovine retinal $\beta_1\gamma_1$, by assaying the ability of insect cell-expressed His-Cool-2 to be co-immunoprecipitated with Myc-Cool-2 that was transiently expressed in COS-7 cells (see Materials and methods). (B) The time-dependent dissociation of the dimers formed between His-Cool-2 and Myc-Cool-2, upon the addition of retinal $\beta_1\gamma_1$ and insect cell-expressed His-PAK3, was assayed as described in Materials and methods. 'Incubation time' refers to the time periods that $\beta_1\gamma_1$ and/or PAK3 were incubated with lysates expressing Myc-Cool-2 and containing His-Cool-2. (C) Coomassie blue staining of SDS-PAGE of purified protein preparations used in these experiments. Histidine-tagged Cool-2 and PAK3 were expressed and purified from insect cells (see Materials and methods), and GST-Cdc42 was expressed and purified from *E. coli*, with the GST tag being removed prior to the GEF assays. The retinal $\beta\gamma$ subunit complex was isolated as previously described (Phillips *et al*, 1989). (D) *E. coli*-expressed Cdc42 (1 μ g) was incubated with retinal $\beta\gamma$ (5 μ g) alone or with His-Cool-2 (4 μ g) and His-PAK3 (2 μ g) in the presence and absence of $\beta\gamma$, and then the dissociation of [3 H]GDP from Cdc42 was assayed as described in the legend to Figure 2A.

susceptible to a novel and complex set of regulatory mechanisms. Full-length Cool-2 acts as a specific GEF for Rac, with the dimerization of Cool-2 being an essential requirement for this specificity. Previous studies had shown p85Cool-1 dimerizes and that dimerization might be important for its cellular functions (Koh *et al*, 2001). Here we demonstrate that full-length Cool-2 can form homodimers as well as heterodimers with p85Cool-1, and that the dimerization of Cool-2 is mediated via a leucine zipper located near the carboxyl-terminal end of the protein. Moreover, the dimerization of Cool-2 enables it to specifically recognize GDP-bound Rac through the combined actions of two regions, namely the DH domain from one of the partner monomers and a region near the carboxyl-terminal end of the PH domain (designated the Rac-specific interaction domain or RSID) from the other monomer partner (Figure 9A).

Perhaps even more interesting from a regulatory standpoint is the fact that a second type of GEF activity can be manifested, but only when Cool-2 is in its monomeric state and bound through its SH3 domain to either PAK or Cbl.

Under these conditions, Cool-2 is able to stimulate the nucleotide exchange activity of either Cdc42 or Rac. Perhaps it is this functional interplay between PAK and Cool-2 that accounts for the findings that mutations in both PAK3 and Cool-2 give rise to certain forms of X-linked mental retardation (Ramakers, 2002). It is still unclear exactly how PAK or Cbl activates the GEF activity of monomeric Cool-2 proteins. One obvious possibility would be that the SH3 domain of monomeric Cool-2 folds over and blocks access to the DH domain. The binding of PAK or Cbl to the SH3 domain of Cool-2 might then prevent this intramolecular interaction from occurring and allow access to the DH domain. However, Cool-2 constructs that lack the SH3 domain and contain just the DH and PH domains would then be expected to show GEF activity, but this is not the case (data not shown). Rather, it seems more likely that the binding of PAK or Cbl to the SH3 domain of Cool-2 induces a conformational transition that changes the architecture of the DH domain, allowing it to couple to either Cdc42 or Rac functionally.

Figure 9 Dual regulatory mechanisms for the GEF activity of Cool-2. (A) Model depicting how the DH domain, RSID region, and dimerization work together to enhance the interaction between Rac and Cool-2. Cool-2 forms a dimer through its leucine zipper region. The DH domain from one Cool-2 molecule and the RSID region of the other Cool-2 molecule work together to bind Rac. (B) Monomeric Cool-2 shows GEF activity toward Cdc42 or Rac. The interaction of a G protein $\beta\gamma$ subunit complex with PAK and the binding of the PAK– $\beta\gamma$ complex to Cool-2 promote the dissociation of the Cool-2 dimer. The binding of PAK to the SH3 domain of monomeric Cool-2 induces a conformational change within the DH domain that enables GDP-bound Cdc42 or Rac to bind and undergo nucleotide exchange. The binding of Cbl-b to the SH3 domain of monomeric Cool-2 also activates its GEF activity (not shown), although Cbl-b alone is not sufficient to dissociate the Cool-2 dimer.

Thus, there may be receptor-coupled signaling systems that help to maintain Cool-2 in the dimeric state so that it acts specifically as a Rac-GEF and other types of signals that stimulate the dissociation of the Cool-2 dimer so that it can activate Cdc42. For example, it was recently reported that Cool-2 was acting downstream from G protein-coupled chemoattractant receptors in mouse neutrophils to specifically activate Cdc42, leading to F-actin formation and maintenance of cell polarity, two events crucial for the directional movement of these cells (Li *et al*, 2003). In this system, the activation of Cdc42 required the binding of PAK to Cool-2, as well as the interaction of PAK with the $G\beta\gamma$ complex that was released from the G_i protein when activated by the chemoattractant receptor. PAK alone was not sufficient to activate the Cdc42-GEF activity of Cool-2. These findings can be nicely explained based on what we now know about the mechanisms regulating Cool-2 function (Figure 9B). Specifically, we have shown that $G\beta\gamma$ complexes, by binding to PAK, are able to stimulate the dissociation of dimeric Cool-2 molecules to their monomeric state, resulting in the stimulated exchange of GDP for GTP on Cdc42. While PAK alone is not able to stimulate the dissociation of the dimer, the binding of PAK to the SH3 domain of monomeric Cool-2 is required for its GEF activity. Therefore, receptors that generate free $G\beta\gamma$ subunits can work together with PAK through Cool-2 to activate Cdc42, as apparently is the case in the chemoattractant-coupled signaling pathway. Still, some interesting questions remain. While chemoattractants activate Rac in human neutrophils (Benard *et al*, 1999), it is not clear that Cool-2 is essential for chemoattractant-dependent Rac activation (Li *et al*, 2003). Does this mean that in some cells Cdc42 (rather than Rac) preferentially couples to monomeric Cool-2? Exactly how is the binding of $G\beta\gamma$ to PAK translated into an effect on the monomer-dimer equilibrium of Cool-2? Are there other mechanisms by which this equilibrium can be perturbed, perhaps by the binding of proteins to Cbl, or through direct interactions with Cool-2 (e.g. via the Cat/Git/Pkl proteins)? How does PAK or Cbl alter the conformation of monomeric Cool-2 such that it becomes a functional GEF? Future studies will be directed toward addressing these issues.

Materials and methods

Plasmid construction

Plasmids pJ3HmPAK3 and pcDNA3-Myc-Cool-2 were prepared as described previously (Bagrodia *et al*, 1995, 1998; Flanders *et al*, 2003). The HA-tagged Cbl-b construct was a kind gift of Dr Stan Lipkowitz (National Cancer Institute, MD). The different Cool-2 and PAK3 constructs were prepared by amplifying the appropriate bases of the open reading frame of pJ3HmPAK3 and pcDNA3-Myc-Cool-2 and subcloning the PCR products into pcDNA3. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Cell culture, transfections, and co-immunoprecipitation assays

COS-7 cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (Invitrogen). Plasmids pJ3HmPAK3 (0.3–0.5 μ g/100 mm dish) and pcDNA3-Myc-Cool-2, as well as pcDNA3 encoding different Cool mutants (1.5–5.0 μ g/100 mm dish), were cotransfected into COS-7 cells using LipofectA-MINE (Invitrogen). Cells were lysed after 24–48 h in lysis buffer (20 mM Hepes, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin) at 4°C for 20 min. Lysates were prepared by

centrifugation at 12 000 g for 10 min at 4°C. Cell lysates coexpressing Myc- and HA-tagged proteins were incubated with anti-Myc primary antibody for 1.5 h on ice followed by mixing with protein A-Sepharose beads (Invitrogen) for 1 h. The beads were washed three times with lysis buffer and then resuspended in 2 $\times$ SDS-PAGE sample buffer. Proteins were eluted by boiling for 5 min, resolved on a 10 or 12% SDS-polyacrylamide gel and then transferred to an Immobilon-P membrane (Millipore). Proteins were probed with mouse monoclonal anti-Myc and anti-HA antibodies. Primary antibodies were detected with horseradish peroxidase-coupled sheep anti-mouse antibody by ECL (Amersham Biosciences).

Binding assays

The GST-Rac(T17N) and GST-Cdc42(T17N) fusion proteins were each expressed in *Escherichia coli* and purified by glutathione-agarose affinity chromatography. Cell lysates (~500 μ l) from COS-7 cells transiently expressing Myc- or HA-tagged proteins were combined with 50 μ l of a suspension of GST fusion proteins (~5 μ g) bound to glutathione-Sepharose beads and incubated at 4°C for 2–3 h. The beads were precipitated by microcentrifugation, washed three times with lysis buffer, and resuspended in 2 $\times$ SDS-PAGE sample buffer and Western blotting was performed as described in the preceding section.

Effects of $G\beta\gamma$ on Cool-2 dimerization

In one set of experiments, 1 ml of cell lysates coexpressing Myc-Cool-2 and HA-PAK3 was divided into two aliquots of 400 and 600 μ l. The first aliquot was incubated with 1.5 μ g of insect cell His-Cool-2 for 1 h on ice, while the other was mixed with 1.5 μ g of His-Cool-2 and 2.5 μ g of retinal $G\beta_1\gamma_1$ (prepared as described by Phillips *et al*, 1989) for 1 h on ice. Both incubations were then incubated with anti-Myc primary antibody for 1 h by mixing with protein A-Sepharose beads (Invitrogen) prior to immunoprecipitation and Western blotting.

In a second set of experiments, 5.4 ml of cell lysates expressing Myc-Cool-2 was incubated with 18 μ g of His-Cool-2 and 9 μ l of anti-Myc antibody for 1.5 h on ice. This mixture was then divided into aliquots (600 μ l) to which was added either 2 μ g of insect cell His-PAK3 alone or 2 μ g of either retinal $G\beta_1\gamma_1$ or heat-denatured $G\beta_1\gamma_1$ alone or in combination with 2 μ g of His-PAK3, for 60 min. The Myc-Cool-2 was immunoprecipitated from these incubations using protein G-Sepharose beads (Invitrogen), prior to Western blotting. When examining the time-dependent dissociation of the dimers formed between His-Cool-2 and Myc-Cool-2, the initial aliquots were incubated with protein G-Sepharose beads for 45 min, and then $\beta_1\gamma_1$ and PAK3 (2 μ g each) were added and the incubations were terminated at different times by the addition of 5 $\times$ SDS loading buffer.

Purification of PAK3 and Cool-2 from insect cells

Viruses expressing His-PAK3 and His-Cool-2 were produced using the Bac-to-Bac Baculovirus Expression Kit (Invitrogen). Insect (Sf21) cells were infected with virus at an MOI of 5 for 72 h. Cells were then lysed in buffer A (20 mM Tris-HCl, pH 8.0, 20 mM imidazole, 0.2% CHAPS, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin). The lysate was centrifuged at 14 000 rcf for 10 min and the supernatant was incubated for 30 min at 4°C with NTA agarose beads conjugated to nickel. The beads were washed three times with buffer A and then eluted in 10 ml of buffer B (20 mM Tris-HCl, pH 8.0, 200 mM imidazole, and 0.2% CHAPS).

PAK assays

COS-7 cells transiently expressing Myc-Cool-2 proteins and HA-PAK3 were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (Invitrogen) for 24–36 h, and then starved for 18–24 h. Cells were lysed in buffer A (40 mM Tris-HCl, pH 7.4, 1% Triton, 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin) and washed by centrifugation at 12 000 g for 10 min at 4°C. The lysates were incubated with anti-PAK3 primary antibody (Bagrodia *et al*, 1998) or anti-HA antibody for 2 h followed by mixing with protein A-Sepharose beads (Invitrogen) for 45 min. The beads were washed three times with buffer A, twice with 2 $\times$ phosphorylation buffer (10 mM MgCl₂ and 40 mM Tris-HCl, pH 7.4), and mixed with 1.0 μ g/sample of the substrate myelin basic protein (Sigma). PAK assays were initiated by adding 25 μ M ATP and 10 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) to the immunoprecipitation mixture and were

maintained at 25°C for 15 min. The reactions were stopped by adding 2 $\times$ SDS-PAGE sample buffer containing 20 mM EDTA. The phosphoproteins were resolved by SDS-PAGE (10 or 12% gel) and visualized by PhosphorImager analysis (Amersham Biosciences) prior to immunoblotting.

³H]GDP dissociation assays

Lysates prepared from COS-7 cells expressing different Myc-tagged Cool-2 constructs were incubated with anti-Myc antibody for 1.5 h followed by mixing with protein A-Sepharose beads for 45 min. The

beads were washed twice with lysis buffer and then with HMN buffer (10 mM Hepes, 5 mM MgCl₂, and 100 mM NaCl, pH 7.5). The immunoprecipitated Cool-2 proteins were incubated with 1 μ g of *E. coli*-expressed GST-Cdc42 or GST-Rac1, preloaded with [³H]GDP, in 140 μ l of reaction buffer that contained 500 μ M GTP γ S (to allow for [³H]GDP-GTP γ S exchange) at room temperature, and then 23- μ l aliquots were diluted into 1.5 ml of ice-cold termination buffer (20 mM Tris-HCl, 10 mM MgCl₂, and 100 mM NaCl, pH 7.4) at various time points. The percent [³H]GDP remaining on the filters was detected by scintillation counting.

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