Oncogenic Dbl, Cdc42, and p21-Activated Kinase Form a Ternary Signaling Intermediate through the Minimum Interactive Domains†

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ABSTRACT: Activation of many Rho family GTPase pathways involves the signaling module consisting of the Dbl-like guanine nucleotide exchange factors (GEFs), the Rho GTPases, and the Rho GTPase specific effectors. The current biochemical model postulates that the GEF-stimulated GDP/GTP exchange of Rho GTPases leads to the active Rho-GTP species, and subsequently the active Rho GTPases interact with and activate the effectors. Here we report an unexpected finding that the Dbl oncoprotein, Cdc42 GTPase, and PAK1 can form a complex through their minimum functional motifs, i.e., the Dbl-homolgy (DH) and Pleckstrin-homology domains of Dbl, Cdc42, and the PBD domain of PAK1. The Dbl−Cdc42−PAK1 complex is sensitive to the nucleotide-binding state of Cdc42 since either dominant negative or constitutively active Cdc42 readily disrupts the ternary binding interaction. The complex formation depends on the interactions between the DH domain of Dbl and Cdc42 and between Cdc42 and the PBD domain of PAK1 and can be reconstituted in vitro by using the purified components. Furthermore, the Dbl−Cdc42−PAK1 ternary complex is active in generating signaling output through the activated PAK1 kinase in the complex. The GEF−Rho−effector ternary intermediate is also found in other Dbl-like GEF, Rho GTPase, and effector interactions. Finally, PAK1, through the PDB domain, is able to accelerate the GEF-induced GTP loading onto Cdc42. These results suggest that signal transduction through Cdc42 and possibly other Rho family GTPases could involve tightly coupled guanine nucleotide exchange and effector activation mechanisms and that Rho GTPase effector may have a feedback regulatory role in the Rho GTPase activation.

The Rho family GTP-binding proteins, including RhoA, Rac1, and Cdc42, regulate cell cytoskeleton organization, transcription, membrane trafficking, DNA synthesis, and survival (*1*−*4*). Like many other Ras superfamily members, the Rho GTPases cycle between the inactive, GDP-bound state and the active, GTP-bound state (*5*). Stimulation of a variety of cell surface receptors can result in the activation of the Dbl family guanine nucleotide exchange factors (GEFs)[1] (*6*, *7*), the major class of Rho GTPase activators, and/or the suppression of two groups of negative regulators, the Rho GTPase-activating proteins (*8*) and Rho GDP-dissociation inhibitors (*9*), leading to the activation of individual Rho GTPase in a spatiotemperally controlled manner.

As the major class of positive regulators of Rho GTPases, the Dbl family of GEFs includes more than 60 members and shares sequence homology of ∼200 amino acid residues termed the Dbl homology (DH) domain that is almost invariably followed by another signaling motif, the Pleckstrin-homology (PH) domain (*6*, *7*). The DH domain is responsible for the Rho GTPase binding interaction and the GEF catalytic activity while the PH domain is involved in intracellular membrane targeting and/or direct GEF catalysis of Rho GTPases. Previous studies have also shown that in many Dbl family GEFs the DH and PH domains constitute the minimum structural module that is necessary and sufficient for the GEF activity in vitro and in vivo. The substrate specificity of individual Dbl family GEFs differs in many cases with some GEFs, such as Lbc, Cdc24, and Tiam1, appearing to be specific for one Rho GTPase (i.e., Rho, Cdc42, and Rac, respectively) whereas others display a broader range of substrate specificity (e.g., Dbl toward Rho, Rac, and Cdc42) (*6*, *7*). The mechanism of substrate discrimination has been attributed to the unique structural elements in the DH domain (*10*−*12*).

Upon activation, Rho GTPases can transmit divergent intracellular signals by direct binding to multiple effector proteins to elicit cellular responses (*13*). On the basis of the ability to selectively bind to the GTP-bound form of Rho GTPases, many Rho GTPase-interactive proteins have been identified to act as effectors in transducing upstream-specific signals to defined cellular activities. For example, WASP and N-WASP are important for Cdc42-mediated actin polymerization activity, PAK1 and PAK2 are involved in Cdc42- or Rac-regulated cell migration, and Rho-kinase and mDia serve to induce the active RhoA-mediated actin stress fiber formation and microtubule assembly. Previous structural

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[1] Abbreviations: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; mantGTP, *N*-methylanthraniloyl-GTP; PBD, p21 binding domain.

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mapping (*14*−*16*) and/or X-ray crystallography studies (*17*−*21*) have made it clear that the Rho GTPase−effector interactions are mediated by specific p21-binding domains (PBDs) found in the effectors, and the switch I and/or switch II regions of Rho GTPases that are most sensitive to the GTP-induced conformational change are invariably involved in engaging the effector PBDs.

The established biochemical model of the Rho GTPase signaling module, therefore, depicts that the activation step of Rho GTPases by GEFs precedes the effector binding and activation. It is also widely accepted that once activated the Rho GTPases would become GEF-independent in effector coupling and in inducing downstream cellular responses. Indeed, much of the available evidence of the cellular functions of Rho GTPases has been derived by using the constitutively active or dominant negative mutant approach (*1*, *22*), which precludes the detection of possible interplay among the GEFs, Rho GTPases, and the effectors should the interactions involve a conformation-sensitive, GDP/GTP exchange dynamic intermediate.

In this paper we report an unexpected finding that the DH/PH structural module of Dbl, the Rho GTPase Cdc42, and the PBD domain of PAK1 can form a ternary complex. We show that the ternary complex formation is dependent on the conformational state of Cdc42 and on the effective interactions between the DH domain of Dbl and Cdc42 and between Cdc42 and the PBD domain of PAK1. We also show that the ternary Dbl−Cdc42−PAK1 complex produces active signaling output from the activated PAK1 kinase. The complex formation ability could be generalized to other GEF−Rho GTPase−effector interactions. Finally, we observed a positive feedback regulation of the GEF-stimulated activation of Cdc42 by the PAK1 effector in a purified system and in cells. Our results thus suggest that signaling through Cdc42 and possibly other Rho GTPases involves tightly coupled guanine nucleotide exchange and effector activation mechanisms and that Rho GTPase effectors such as PAK1 may have a positive feedback regulatory role in Rho GTPase activation.

MATERIALS AND METHODS

cDNA Constructs. Constructs of $(HA)_3$-tagged Dbl(DH/PH) and its mutants, Cdc42 and its mutants, PAK1, proto-Dbl, Cdc24(DH/PH), Lbc(DH/PH), Ost(DH/PH), and TrioN-(DH/PH) were derived by subcloning the *Bam*HI−*Eco*RI fragments of the encoding cDNA sequences into the pKH3 vector (*23*, *24*). For myc-tagged expression, Cdc42 and its mutants were subcloned into the pCMV6 vector as described previously (*25*), and the myc-tagged PAK1 and its mutant H83L/H86L were kind gifts from Dr. J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA).

Transient Transfections. Cos-7 cells were plated in a 100 mm dish at a density of 1.5×10^6 in DMEM supplemented with 10% calf serum in a 5% CO_2 incubator at 37 °C (*26*). Upon reaching 90% confluency, cells were transfected with different cDNA plasmids by using LipofectAMINE reagents (Life Technologies) in the serum-free DMEM following the manufacturer's instructions. After a 5 h incubation, the cells were replaced in the growth medium and cultured for another 48 h before further analysis.

Expression and Purification of Recombinant Proteins. Expression and purification of GST, GST-Cdc42, GST-N17Cdc42, GST-L61Cdc42, GST-Dbl(DH/PH), GST-PAK1-(PBD), GST-PAK1(PBD-H83L/H86L), GST-PAK2(PBD), GST-Rhotekin(PBD), and GST-WASP(PBD) fusion proteins from the pGEX-KG and $(His)_6$-Cdc42, $(His)_6$-A38Cdc42, $(His)_6$-Cdc42C-7, $(His)_6$-N17Cdc42, $(His)_6$-L61Cdc42, and $(His)_6$-PAK1(PBD) fusion proteins from pET-15b vector transformed *Escherichia coli* were carried out as described previously (*24*−*27*). Production and purification of the baculovirus-mediated expression of $(His)_6$-Dbl(DH/PH), GST-Dbl(ND673-674AA), and GST-Dbl(H556A) from Sf9 insect cells were performed as described (*23*). The GST or $(His)_6$ tags of the fusions were removed by thrombin digestion when necessary. The concentration of proteins was estimated by Coomassie blue-stained SDS−PAGE or the Bradford assay with bovine serum albumin as a standard.

In Vitro Complex Formation Assay. Cdc42 was incubated with 1 mM GDP, GTP, or GTPγS at room temperature for 30 min in 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 0.5% Triton X-100, 0.5 mM dithiothreitol, 1 mg/mL BSA, and 1 mM EDTA before 5 mM $MgCl_2$ was added to stop the loading. Purified Dbl protein was added into this mixture, and the samples were agitated at 4 °C for 2 h. The glutathionine−agarose or Ni NTA−agarose immobilized fusion proteins were added and incubated for another 30 min. The coprecipitates were washed three times with the incubation buffer and analyzed by assay by the respective Western blotting (*23*).

In Vitro Guanine Nucleotide Exchange Assay. The time courses for [^{3}H]GDP dissociation from Cdc42 in the presence or absence of $(His)_6$-PAK1(PBD) and/or $(His)_6$-Dbl(DH/PH) were determined as previously described using the nitrocellulose filtration method (*23*). Briefly, Cdc42 loaded with [^{3}H]GDP was incubated with buffer containing 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 5 mM $MgCl_2$, 0.2 mM GTP, and 1 mM dithiothreitol supplemented with buffer, $(His)_6$-Dbl(DH/PH), or $(His)_6$-PAK1(PBD) together with $(His)_6$-Dbl. The reaction was stopped by diluting the reaction mixture into 10 mL of ice-cold stop buffer, and the protein-bound nucleotide was trapped by filtration through nitrocellulose filters.

Assays monitoring the loading of GTPγS to Cdc42 were performed similarly as described above for the GDP dissociation assay, except [^{35}S]GTPγS was used in place of GTP and no [^{3}H]GDP was preloaded onto Cdc42. Briefly, GDP-bound Cdc42 was incubated in a buffer containing 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, and 10 μM [^{35}S]GTPγS in the presence or absence of purified $(His)_6$-Dbl or Dbl with PAK1(PBD) at room temperature. The GTPγS binding was determined at various time points by diluting the reaction mixture (20 μL aliquots) into 10 mL of ice-cold buffer, and the protein-bound radioactive nucleotide was quantified by filter binding (*28*).

Fluorescence measurement of mantGTP binding was carried out by using an S 50B luminescent spectrometer (PerkinElmer Life Sciences) in an exchange buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 50 μM *N*-methylanthraniloyl-GTP (mantGTP), and 0.2 μM Cdc42 at 25 °C. Purified PAK1(PBD) was included where indicated. Dbl(DH/PH) (200 nM) was added to the mixture to initiate the reaction as described before (*28*). The mantGTP fluorescence changes during the exchange reactions were

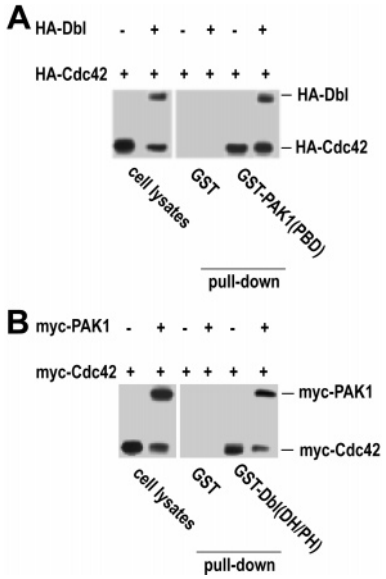

FIGURE 1: Dbl, Cdc42, and PAK1 can form a stable complex in cell lysates. HA-tagged onco-Dbl and Cdc42 (A) or myc-tagged PAK1 and Cdc42 (B) were coexpressed in Cos-7 cells. Cell lysates were subjected to the GST, GST-PAK1(PBD) (A), or GST-Dbl-(DH/PH) (B) fusion protein pull-down assay. Proteins bound to the GST fusions were resolved on SDS−PAGE and blotted with anti-HA or anti-myc antibody. Two percent of the total cell lysates was also analyzed in parallel.

monitored with an excitation wavelength at 360 nm and the emission wavelength at 440 nm.

PAK Kinase Activity Assay. Myc-PAK1 was transiently expressed in Cos-7 cells and was purified from the cell lysates by anti-myc immunoprecipitation. The immunoprecipitated proteins were washed and eluted by a myc peptide. The PAK1 activity was assayed at 30 °C for 30 min using 1 μg of myelin basic protein (MBP) as a substrate in a kinase assay buffer containing 5 μM [^{32}P]ATP. The phosphorylation was visualized by autoradiography after the reaction mixtures were separated by SDS−PAGE.

Effector Domain Pull-Down Assay. Cells transiently expressing comparable levels of various proteins were lysed at 4 °C in a buffer containing 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.2% sodium deoxycholate, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin. The cell lysates were then mixed with purified glutathionine−agarose immoblized fusion proteins (10 μg each) at 4 °C for 45 min. After incubation, the beads were washed twice in the lysis buffer, and the bound proteins were separated on SDS−PAGE, transferred onto the PVDF membrane, and immunoblotted with respective monoclonal antibodies (*22*).

RESULTS

The DH/PH Domains of Dbl and Cdc42 and the PBD Domain of PAK1 Can Form a Complex in the Cell Lysates. To examine the potential molecular interplay between the components of the GEF, Rho GTPase, and effector signaling module, we used the well-characterized onco-Dbl, Cdc42, and PAK1 trio as a model system in a transient cotransfection experiment followed by the GST fusion pull-down assay of the cell lysates. As shown in Figure 1A, GST-PAK1(PBD) readily formed a complex with Cdc42 in the cell lysates while GST alone did not pull down a detectable amount of Cdc42. Unexpectedly, GST-PAK1(PBD) also pulled down the onco-Dbl protein together with Cdc42. Similarly, GST-Dbl(DH/PH) was able to form a complex with Cdc42 or with Cdc42 and PAK1 when they were coexpressed in the cell lysates (Figure 1B). When the cells were cultured in 10% calf serum, there appears to be little advantage by Dbl in further stimulating the Cdc42 activity as shown in the PAK1(PBD) pull downs. The fact that GST-PAK1(PBD) could complex with cellular Cdc42 can be rationalized by the presence of the Cdc42−GTP species in the lysates whereas that GST-Dbl could complex with Cdc42 is likely due to the presence of Cdc42−GDP. The observations that GST-PAK1(PBD) could pull down both Cdc42 and onco-Dbl and that GST-Dbl(DH/PH) could complex with both Cdc42 and PAK1 suggest that the minimum functional domains of the trio, i.e., Dbl(DH/PH), Cdc42, and PAK1(PBD), are capable of forming an onco-Dbl−Cdc42−PAK1 complex in the cell lysates. However, the results do not exclude the possibility that Dbl(DH/PH) may bind to PAK1(PBD) and Cdc42 in separate interactions or that other cellular proteins are also involved in the complex formation.

Dependence of the Dbl−Cdc42−PAK1 Complex Formation on the Conformation State of Cdc42 and on the Dbl−Cdc42 and the Cdc42−PAK1 Interactions. To examine if the conformational state of Cdc42 could affect the complex formation, we coexpressed the GDP-bound dominant negative mutant N17Cdc42, the GTP-bound constitutively active mutant L61Cdc42 or V12Cdc42, the fast cycling mutant L28Cdc42, or wild-type Cdc42, with full-length PAK1 in Cos-7 cells and carried out the GST or GST-Dbl(DH/PH) pull-down assays. As shown in Figure 2A, while the immobilized GST-Dbl(DH/PH) could form a complex with wild-type Cdc42, L28Cdc42 or V12Cdc42, and PAK1, it also brought down N17Cdc42 and PAK1. However, GST-Dbl-(DH/PH) failed to precipitate the coexpressed L61Cdc42 and PAK1, similar to immobilized GST by itself. Since the cell lysates contained endogenous Cdc42 (and Rac1) and N17Cdc42 binds to PAK1 poorly, it is possible that the excess amount of GST-Dbl(DH/PH) might have brought down PAK1 through the endogenous Cdc42 when N17Cdc42 and PAK1 were coexpressed. Indeed, endogenous Cdc42 was detected in the GST-Dbl(DH/PH) coprecipitates by an anti-Cdc42 blot (data not shown; see Figure 5). On the other hand, the overexpressed L61Cdc42 would preferentially sequester the coexpressed PAK1 to exclude the formation of the Dbl−Cdc42−PAK1 complex mediated by the endogenous Cdc42. On the other hand, although L28Cdc42 or V12Cdc42 could exist in a higher proportion in the GTP-bound state than wild-type Cdc42, they appeared to behave differently from L61Cdc42 in complex formation with GST-Dbl(DH/PH) and PAK1 (Figure 2A). This may present another interesting distinction between the commonly used, active Cdc42 mutants (i.e., V12Cdc42, L61Cdc42, and L28Cdc42) in addition to previously suggested differences in RhoGDI interaction (*29*). These results suggest that the formation of the Dbl−Cdc42−PAK1 complex is sensitive to the conformational state of Cdc42.

Next, we tested if the Dbl−Cdc42−PAK1 complex formation is mediated by Cdc42 and whether it requires the pairwise interaction between Cdc42 and PAK1 and/or between Cdc42 and Dbl. Previously, it was shown that

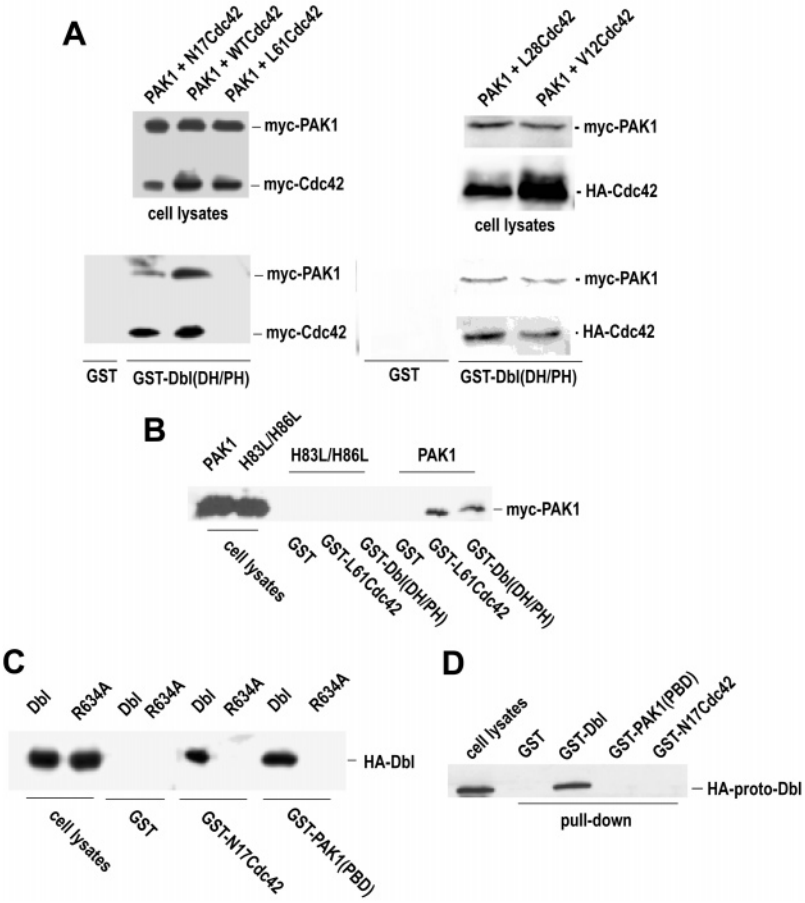

FIGURE 2: The Dbl or PAK1 mutants that disrupt the Dbl−Cdc42 or the Cdc42−PAK1 interaction are unable to form the Dbl−Cdc42−PAK1 complex. (A) Myc-tagged PAK1 was transiently coexpressed with myc-tagged WTCdc42, N17Cdc42, L61Cdc42, or HA-tagged L28Cdc42 or V12Cdc42 in Cos-7 cells. Cell lysates were subjected to the GST or GST-Dbl(DH/PH) pull-down assay and were probed by anti-myc or anti-HA Western blot. (B) Myc-PAK1 or myc-PAK1(H83L/H86L) mutant was expressed in Cos-7 cells, and cell lysates were subjected to GST, GST-L61Cdc42, or GST-Dbl pull down. The myc-PAK1 or myc-PAK1(H83L/H86L) in the lysates or pull downs were detected by anti-myc Western blot. (C) HA-tagged Dbl or Dbl(R634A) mutant was expressed in Cos-7 cells, and the lysates were subjected to GST, GST-N17Cdc42, or GST-Dbl pull-down assay followed by anti-HA blot. (D) HA-proto-Dbl was transiently expressed in Cos-7 cells. The cell lysates were pulled down by immobilized GST, GST-Dbl, GST-N17Cdc42, or GST-PAK1(PBD) and probed by anti-HA blotting.

residues His83 and His86 of PAK1(PBD) are involved in interaction with Asp38 and Tyr40 of Cdc42, and mutations of these two residues to leucine would cause a loss of Cdc42 binding activity (*25*). In addition, the DH domain mutant of Dbl, Dbl(R634A), is defective in substrate binding but retains the DH oligomerization activity (*23*, *24*). When the wild-type myc-PAK1 or myc-PAK1(H83L/H86L) was expressed in Cos-7 cells and subjected to GST, GST-Dbl(DH/PH), or GST-L61Cdc42 pull-down assay, wild-type myc-PAK1 readily coprecipitated with GST-L61Cdc42 or GST-Dbl(DH/PH) but myc-PAK1(H83L/H86L) failed to form a complex with either probe (Figure 2B). Similarly, while wild-type onco-Dbl in the cell lysates could coprecipitate with the immobilized GST-N17Cdc42 or GST-PAK1(PBD), the R634A mutant of onco-Dbl failed to associate with either GST-N17Cdc42 or GST-PAK1(PBD) (Figure 2C). Furthermore, proto-Dbl that exists in an autoinhibitory conformation precluding its direct binding interaction with Rho GTPase (*26*) was unable to complex with N17Cdc42 or PAK1(PBD) even though it remained active in forming an oligomer with Dbl(DH/PH) (Figure 2D). These results demonstrate that the interactions between Dbl(DH/PH) and Cdc42 and between Cdc42 and PAK1(PBD) are essential for the Dbl−Cdc42−PAK1 complex formation. It can also be inferred that Cdc42 acts as the key mediator in the complex.

Dbl, Cdc42, and PAK1 Can Form a Ternary Complex in a Purified, Reconstituted System. The above complex formation experiments were carried out in cell lysates that contained numerous cellular factors in addition to the transfected components. To determine whether the observed Dbl−Cdc42−PAK1 complex formation is through direct protein−protein interaction among the three components, we next attempted to reconstitute the complex binding interaction by using the purified Dbl(DH/PH), Cdc42, and PAK1(PBD) proteins. For this purpose, $(His)_6$-tagged wild-type Cdc42, N17Cdc42, L61Cdc42, and Cdc42C-7, a mutant Cdc42 bearing the C-terminal seven-residue truncation, as well as $(His)_6$-PAK1(PBD), GST-PAK1(PBD), and GST-PAK1-(PBD-H83L/H86L), were expressed in *E. coli*. The recombinant wild-type $(His)_6$-Dbl(DH/PH), GST-Dbl(ND673-674AA), and GST-Dbl(H556A) were expressed in Sf9 insect cells by the baculovirus-mediated induction. All proteins were purified to homogeneity by using the Ni^{2+}−agarose or glutathione−agarose affinity chromatography.

First, $(His)_6$-Dbl(DH/PH) was incubated with $(His)_6$-Cdc42, N17Cdc42, or L61Cdc42 in the presence or absence

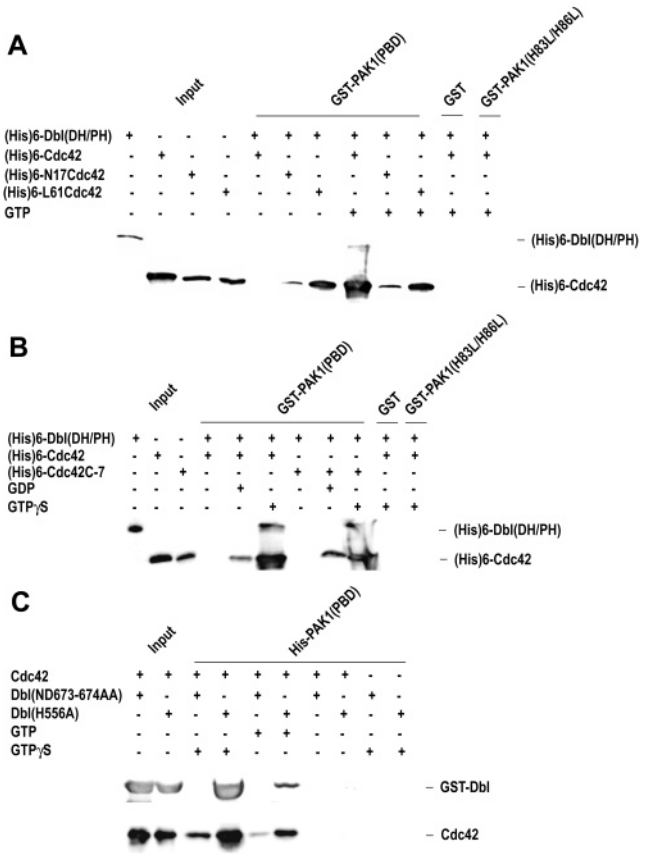

FIGURE 3: Reconstitution of the Dbl−Cdc42−PAK1 ternary complex in vitro by using the purified protein components. (A) The affinity-purified $(His)_6$-tagged Cdc42, N17Cdc42, or L61Cdc42 was incubated with $(His)_6$-Dbl in the presence or absence of 0.2 mM GTP. The immobilized GST, GST-PAK1(PBD), or GST-PAK1(PBD-H83L/H86L) was added to the solution, and the beads associated $(His)_6$-tagged proteins were probed by anti-His Western blotting. (B) $(His)_6$-Cdc42 or $(His)_6$-Cdc42C-7 was incubated with $(His)_6$-Dbl(DH/PH) in the presence or absence of 0.2 mM GDP or GTPγS. The mixtures were subjected to the GST, GST-PAK1-(PBD), or GST-PAK1(PBD-H83L/H86L) pull-down assay followed by anti-His Western blot analysis. (C) Purified Cdc42 alone or Cdc42 in combination with GST-Dbl(H556A) or Dbl(ND673−674AA) mutants was incubated with 0.2 mM GTP or GTPγS for 10 min. The mixtures were subjected to the pull-down assay by the bead-immobilized $(His)_6$-PAK1(PBD) protein. Protein inputs and pull downs were analyzed by anti-GST or anti-Cdc42 Western blot.

of 200 μM GTP, the various solution mixtures were subjected to immobilized GST, GST-PAK1(PBD), or GST-PAK1-(PBD-H83L/H86L) pull down, and the coprecipitates were analyzed by anti-His Western blotting. As shown in Figure 3A, Dbl(DH/PH) was able to coprecipitate with GST-PAK1-(PBD) in the presence of wild-type Cdc42 and GTP. In contrast, neither GST nor GST-PAK1(PBD-H83L/H86L) could form a complex with Cdc42 or Dbl. On the other hand, GST-PAK1(PBD) did not interact with Dbl(DH/PH) by itself, nor could it coprecipitate Dbl in the absence of wild-type Cdc42 or GTP or when Cdc42 was in the N17 or L61 mutant form. These results indicate that Dbl, Cdc42, and PAK1 could form a Dbl−Cdc42−PAK1 ternary complex through their minimum functional domains. However, unlike the pairwise interaction which can occur in almost stoichiometric ratio between Cdc42−GTP and PAK1 or between Dbl and Cdc42−GDP, only a small percentage (~1%) of the stoichiometrically mixed Dbl, Cdc42, and PAK1 could be detected in the coprecipitates, suggesting a transient or relatively weak binding interaction of the ternary complex formation. Further, the results suggest that the conformation state of Cdc42 and the presence of free guanine nucleotide are important for the ternary complex formation.

Second, because Cdc42 could form homodimers mediated by its C-terminal polybasic residues (*30*), to rule in or rule out the possibility that the observed ternary complex formation is due to the selective interaction of Dbl and PAK1 with distinct, dimerized Cdc42 molecules, we utilized the Cdc42C-7 mutant that has been shown to be defective in homodimerization (*30*) in the complex formation assay. Figure 3B shows that GST-PAK1(PBD) was capable of forming a ternary complex with Dbl in the presence of Cdc42C-7 like wild-type Cdc42, which was dependent upon the presence of free GTPγS. The Cdc42-binding defect PAK1(PBD-H83L/H86L) mutant was ineffective in pulling down either Dbl or Cdc42, similar to the GST alone with or without the addition of GTPγS. Interestingly, the presence of free GDP was disruptive to the ternary complex formation. Thus, the Dbl−Cdc42−PAK1 ternary complex formation is independent from the Cdc42 dimerization ability and is associated with the GTP loading to Cdc42.

Third, to further confirm the importance of the nucleotide exchange ability of Cdc42 in the complex formation, we used the Dbl DH domain mutant Dbl(ND673-674AA), which is able to bind to Cdc42 like wild-type Dbl but is catalytically deficient in GEF activity (*23*), and the DH domain mutant Dbl(H556A) that is defective in DH domain mediated oligomerization but remains capable of stimulating GDP/GTP exchange (*24*), in a $(His)_6$-PAK1(PBD) pull-down assay. We incubated a similar amount of Cdc42 with either Dbl(H556A) or Dbl(ND673-674AA) in the absence or presence of GTP or GTPγS. The $(His)_6$-PAK1(PBD) was able to coprecipitate with Dbl(H556A) but not Dbl(ND673-674AA), together with Cdc42 (Figure 3C). Again, the complex formation was contingent upon the presence of free GTP or GTPγS (Figure 3C). These results show conclusively that the catalytic GEF activity of Dbl, in addition to the Cdc42-binding activity, is required for the ternary complex formation.

The Dbl−Cdc42−PAK1 Complex Generates Active Signaling Output. At the basal state PAK1 exists in an autoinhibited conformation that can be activated by Cdc42 binding (*18*). To assess the effect of the complex formation on PAK1 activation, we have examined the PAK1 activity that is associated with the ternary complex. Purified myc-PAK1 was incubated in the complex formation buffer containing 200 μM GTP in the presence or absence of wild-type Cdc42 or L61Cdc42, and the mixtures were further subjected to the GST-Dbl(DH/PH) pull-down assay. The resulting coprecipitates were divided into two parts and were analyzed by anti-myc Western blotting and PAK1 kinase assay, respectively. Figure 4A shows that GST-Dbl(DH/PH) could form a complex with myc-PAK1 in the presence of wild-type Cdc42 but not in the presence of L61Cdc42 or in the absence of wild-type Cdc42. The free myc-PAK1 was mostly inactive but could be activated by the addition of L61Cdc42 (Figure 4B). The GST-Dbl(DH/PH) coprecipitated PAK1 was active in kinase activity when assayed by using MBP as a substrate (Figure 4B) whereas the GST-Dbl(DH/PH) pull downs in the absence of Cdc42 or in the presence

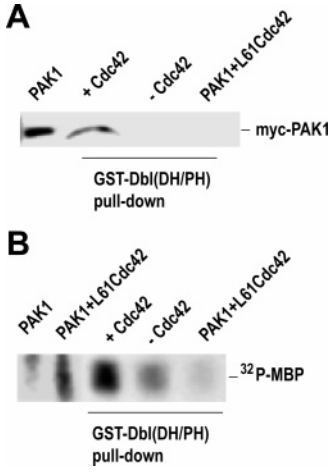

FIGURE 4: The Dbl−Cdc42−PAK1 ternary complex generates active PAK1 signal output. The myc-PAK1 was expressed in Cos-7 cells by transient expression. The protein was immunoprecipitated by using monoclonal anti-myc antibody and was eluted with a myc-antigen peptide. The purified myc-PAK1 was incubated with WTCdc42 or L61Cdc42 in the presence of 0.2 mM GTP, and the mixture or myc-PAK1 alone was further subjected to a pull-down assay by GST-Dbl fusion. The GST-Dbl-bound proteins were examined for the presence of myc-PAK1 by anti-myc Western blot (A) and for the PAK1 kinase activity in a kinase assay using MBP as a substrate (B).

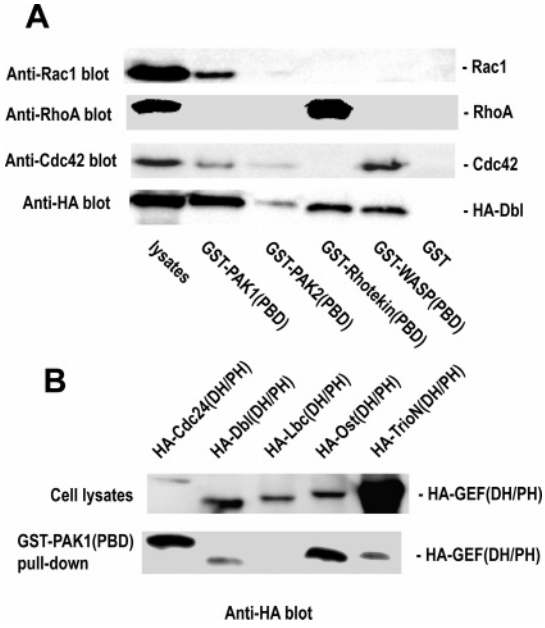

FIGURE 5: GEF−Rho GTPase−effector ternary complex formation in other GEF, Rho GTPase, and effector domain combinations. (A) HA-Dbl was transiently expressed in Cos-7 cells, and the cell lysates were subjected to the pull-down assay by immobilized GST, GST-PAK1(PBD), GST-PAK2(PBD), GST-Rhoteckin(PBD), and GST-WASP(PBD), respectively. Lysates and the pull downs were probed with anti-HA, anti-Rac1, anti-RhoA, or anti-Cdc42 monoclonal antibodies. (B) HA-tagged DH/PH domains of Cdc24, Dbl, Lbc, Ost, and TrioN were transiently transfected in Cos-7 cells, and the cell lysates were further probed by immobilized GST-PAK1(PBD) or GST-PAK1(PBD-H83L/H86L). The coprecipitates were analyzed by immunoblotting with the anti-HA antibody.

of L61Cdc42 showed little kinase activity. These data provide evidence that the Dbl−Cdc42−PAK1 complex is able to generate signaling output by the activated PAK1 kinase.

GEF−Rho GTPase−Effector Ternary Complex Formation in Other Rho GTPase Cases. Next we examined whether the observed Dbl−Cdc42−PAK1 complex formation is unique to this signaling module or could potentially apply to other Rho GTPase interactions with GEFs and effector domains. To this end, we generated a number of additional GST-effector domain fusion proteins, i.e., GST-PAK2(PBD), GST-Rhotekin(PBD), and GST-WASP(PBD), and carried out the pull-down assays in Cos-7 cell lysates expressing HA-Dbl(DH/PH). As shown in Figure 5A, GST-PAK2(PBD) could precipitate Dbl(DH/PH) from the lysates together with endogenous Cdc42 and Rac1 like GST-PAK1(PBD), albeit with a weaker affinity. GST-Rhotekin(PBD), a Rho-specific effector domain, was able to complex with Dbl(DH/PH) together with RhoA, while GST-WASP(PBD), a Cdc42-specific effector domain, could coprecipitate Dbl(DH/PH) and Cdc42. On the other hand, GST-PAK1(PBD) was able to coprecipitate the Cdc42 and/or Rac1 GEFs Cdc24, Dbl, Ost, and TrioN from the cell lysates expressing the respective GEFs but was ineffective in pulling down the Rho-specific GEF Lbc from Lbc-expressing lysates (Figure 5B). In comparison, GST-PAK1(PBD-H83L/H86L) was unable to complex with any of the GEFs tested or with endogenous Cdc42−Rac1 (data not shown). Taken together, these results suggest that the ability to form the GEF−Rho GTPase−effector complex may be a conserved feature of the Rho GTPase signaling mechanism, and the specificity of the ternary complex is consistent with the specificity of Rho GTPase interaction with respective GEFs and effectors.

PAK1 Can Potentiate the GTP-Loading Activity of GEF toward Cdc42. In addition to the effect on effector activation, we sought for other possible regulatory role of the GEF−Rho GTPase−effector interplay. We purified the $(His)_6$-tagged PAK1(PBD), Cdc42, and Dbl(DH/PH) by affinity chromatography (Figure 6A) and measured the GDP/GTP exchange rate of Cdc42 with various combinations of the three components. In the GDP dissociation assay, Dbl(DH/PH) could potently stimulate [^{3}H]GDP release from Cdc42 which was not significantly affected by the presence of excess PAK1(PBD) (Figure 6B). However, we repeatedly observed that PAK1(PBD) was able to further enhance the Dbl(DH/PH)-stimulated GTP loading onto Cdc42 in the [^{35}S]-GTPγS and mantGTP-loading assays (Figure 6C,D). The different ending fluorescence intensities in the Cdc42 + Dbl-(DH/PH) and the Cdc42 + Dbl(DH/PH) + PAK1(PBD) samples (Figure 6C, left panel) may represent the differences of Cdc42−mantGTP vs Cdc42−mantGTP bound to PAK1-(PBD) at the completion of the GEF reactions. As a control, the PAK1(PBD) binding defective mutant of Cdc42, Cdc42D38A (*27*), did not show any effect of PAK1(PBD) in the Dbl(DH/PH) stimulated GTP loading (Figure 6D), suggesting that Cdc42−effector domain interaction is required for the observed PAK1(PBD) potentiation of GTP binding. These in vitro data predicted that the presence of excess PAK1 could increase the GTP loading to Cdc42. To further test this possibility in cells, we coexpressed myc-PAK1 with HA-tagged N17Cdc42, L61Cdc42, or Cdc42 in Cos-7 cells and examined the total and endogenous Cdc42−GTP contents by the GST-WASP(PBD) pull-down assay. The expression of myc-PAK1, HA-Cdc42, or the Cdc42 mutants, as well as endogenous Cdc42, was probed with anti-myc or anti-Cdc42 immunoblotting, and the Cdc42−GTP species in the cell samples were compared. Figure 6E shows

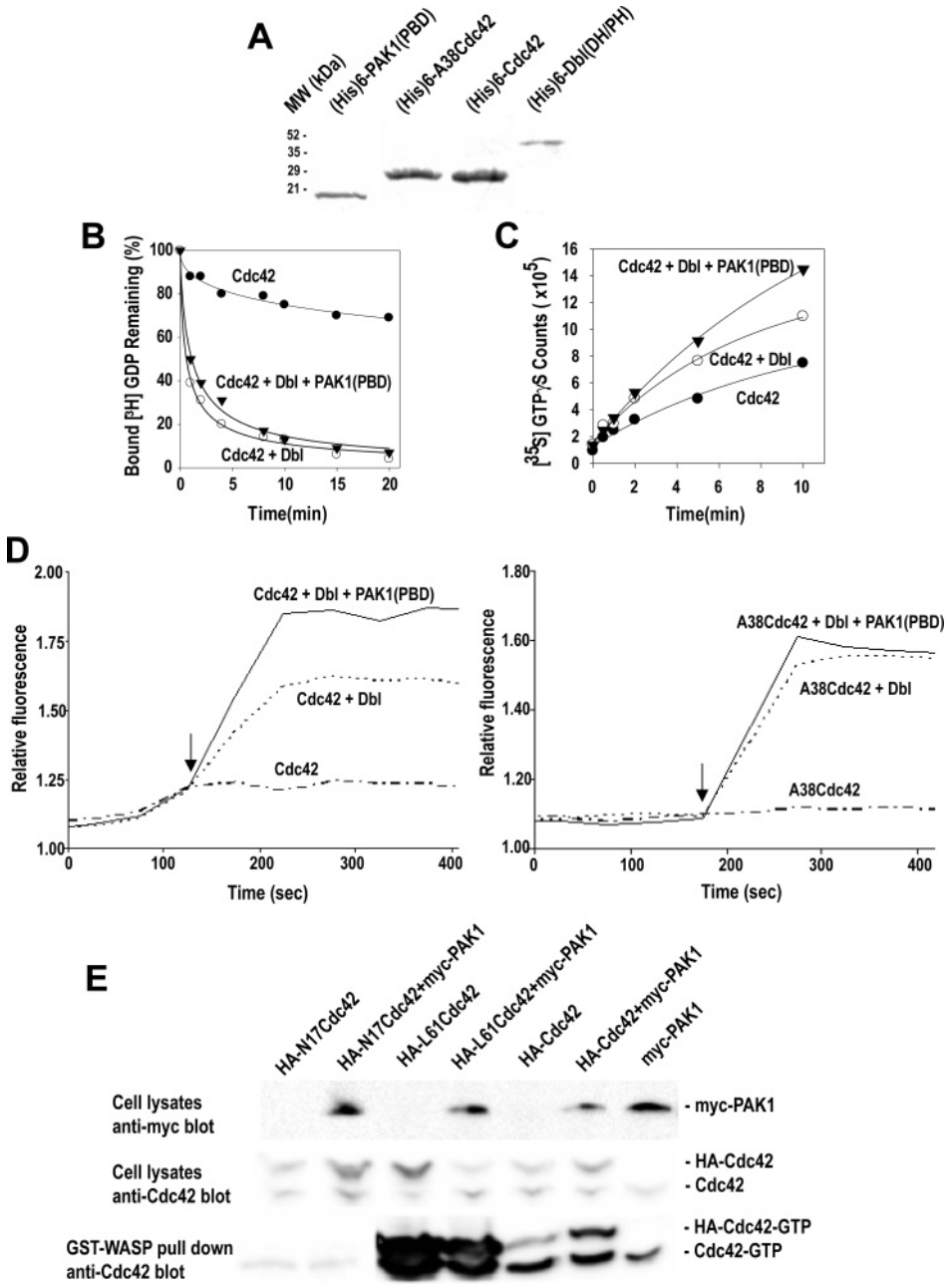

FIGURE 6: The GTP-loading activity of GEF is upregulated by PAK1. (A) Coomassie blue-stained SDS−PAGE of purified $(His)_6$ fusions of PAK1(PBD), Cdc42, A38Cdc42, and Dbl(DH/PH) used in the in vitro assays. (B, C) The ability of Dbl to stimulate [^{3}H]GDP dissociation from Cdc42 (B) or [^{35}S]GTPγS loading to Cdc42 (C) at various times in the presence of buffer (solid circles), 10 pmol of Dbl (open circles), or 10 pmol of Dbl + 50 pmol of PAK1(PBD) (solid triangles) was examined by the GDP dissociation or GTP-loading assay. (D) Time courses of the mantGTP loading reaction for Cdc42 or A38Cdc42 in the presence or absence of PAK1(PBD) under Dbl(DH/PH) catalysis. Wild-type or mutant Cdc42 was incubated in the reaction buffer containing 50 μM mantGTP, and Dbl(DH/PH) was added (arrows) to initiate the reaction. (E) HA-tagged N17Cdc42, WTCdc42, or L61Cdc42 was coexpressed with myc-PAK1 in Cos-7 cells. 48 h posttransfection, the cells were lysed and subjected to a pull-down assay by using immobilized GST-WASP. An aliquot of whole cell lysates and the WASP-associated Cdc42-GTP were separated by SDS−PAGE and immunoblotted with anti-myc or anti-Cdc42 antibody.

that whereas N17Cdc42 expression effectively suppressed endogenous Cdc42 activity, myc-PAK1 could significantly increase the total Cdc42−GTP level in cells, particularly the HA-Cdc42−GTP species. Further, L61Cdc42 overexpression led not only to a significant increase of total Cdc42−GTP but also to an increase of endogenous Cdc42−GTP content, suggesting a possible feedback regulation of Cdc42 activity. These data are consistent with the in vitro results suggesting that PAK1 could potentiate the GTP-loading activity of GEF to positively influence the Cdc42 activation process.

DISCUSSION

The widely accepted biochemical model of the Rho GTPase regulation and signaling mechanism postulates that the GEF preferentially recognizes the GDP-bound or nucleotide-free conformation of Rho GTPase to catalyze the exchange of bound GDP for GTP, which is in excess in the cell cytosol (Figure 7A; refs *6*, *7*, and *31*). Upon completion of GTP loading, the GEF dissociates from Rho−GTP and the active Rho−GTP subsequently binds to and activates the

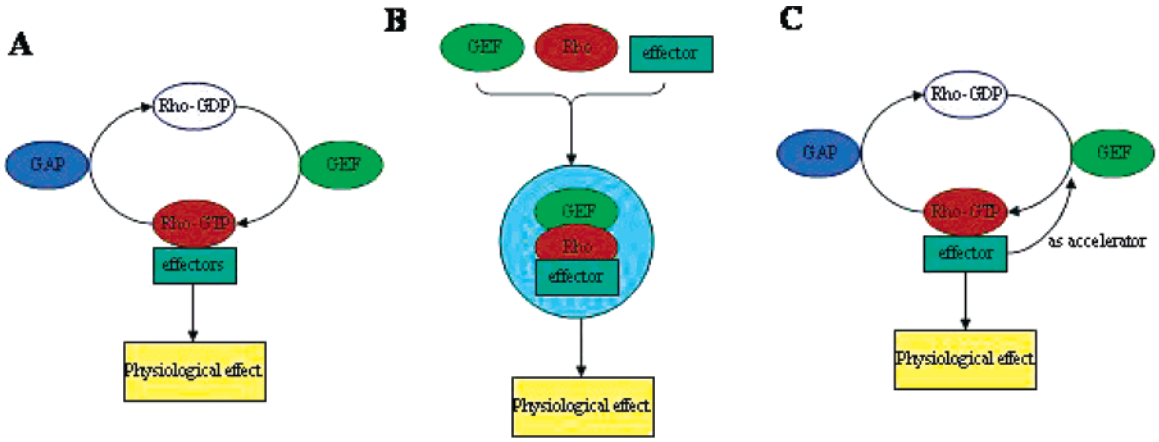

FIGURE 7: A model for the GEF−Rho GTPase−effector ternary interplay. **(**A) The current biochemical model postulates that the GEF-stimulated GDP/GTP exchange of Rho GTPase and the subsequent effector activation by Rho GTPase occur sequentially. (B) Our results are consistent with the existence of an alternative mechanism in which GEF, Rho GTPase, and effector can transiently form a ternary intermediate to directly generate the signaling output. (C) The Rho GTPase effectors may also positively regulate the GEF reaction by a feedback mechanism in the ternary complex to further enhance GTP loading to Rho GTPases.

effector. The Rho GTPase returns to the inactive, GDP-bound conformation through its intrinsic GTP hydrolysis activity that is facilitated by the GTPase-activating proteins and releases the effector when it becomes GDP bound (Figure 7A; refs *8* and *13*).

Although this GTPase cycle/effector coupling model is consistent with the available in vitro biochemical data gathered by studying pairwise interactions of the relevant components, a number of observations of Rho GTPase signaling in cells suggest that a highly coordinated and tightly coupled signal transduction mechanism may be at work to allow efficient signaling through the Rho GTPases. First, Rho GTPase activation and effector signaling in response to extracellular stimuli have fast kinetics and can occur within seconds in cells. For example, neutrophils can elicit morphological and motility/polarization changes mediated by Rac and Cdc42 and their respective effectors in response to the fMLP challenge, and these cellular changes can be detected in 5 s after fMLP stimulation (*32*). Such a fast rate of the signal transduction implies tightly coupled intermolecular interactions of signaling complexes along the Rho GTPase signaling axis. Second, a growing body of evidence indicates that the Rho GEFs, Rho GTPases, and the effectors can form a signaling complex through direct or indirect scaffolding interactions, therefore not only promoting the GEF to effector signaling efficiency but also contributing to the signaling specificity that is mediated by the identity of the components of the complex. For example, the Rac-specific GEFs, Tiam1 and Ras-GRF1, were reported to contribute to the signaling specificity of Rac via association with the scaffolding protein IB2/JIP2 that promotes Rac activation of a MAP kinase cascade (*33*, *34*), and the SH2 and SH3 regions of Vav were shown to ensure the proper phosphorylation of Vav and the subsequent engagement of downstream effectors (*35*). Interestingly, the Cdc42 effector WASP can recruit a Cdc42-specific GEF, intersectin, to a signaling complex through a SH3 domain mediated interaction (*36*), while the Cdc42 exchange factor−effector pair βPIX and PAK1 appears to exist in a tight complex in cells mediated by the SH3 domain binding (*37*). Thus, it is likely that in the physically associated GEF−Rho GTPase−effector complexes a more complicated molecular interplay could take place among the involved components to add sophistication to the mechanisms of effector activation and Rho GTPase regulation.

In the present study we have made an unexpected discovery that Cdc42 could form a complex with the Dbl GEF and the PAK1 effector through the minimum interactive domains. We first found that the immobilized GST-PAK1-(PBD) and GST-Dbl(DH/PH) could pull down Cdc42 and onco-Dbl or Cdc42 and PAK1 in the cell lysates (Figure 1). We then showed that the pairwise interactions between Dbl and Cdc42 and between Cdc42 and PAK1 were critical to the Dbl−Cdc42−PAK1 complex formation and that the conformational state of Cdc42 was also important for the complex formation (Figures 2 and 3). More convincingly, we demonstrated by using the purified Dbl(DH/PH), Cdc42, and PAK1(PBD) or their mutants that the Dbl−Cdc42−PAK1 ternary complex could exist in the presence of only these three components, and Cdc42 appeared to be the mediator of the Dbl−PAK1 association (Figure 3). Although only an estimated ~1% PAK1 could be found associated with the Dbl−Cdc42 complex when equal molar amounts of the three components were present, the complex-associated PAK1 was activated (Figure 4), indicating that the Dbl−Cdc42−PAK1 ternary complex can generate active signaling output. The observations that other GEFs, Rho GTPases, and effector domains could also form a similar complex (Figure 5) support a modified model of the signaling module in which the GEF may have a more active and direct role in effector activation by engaging Rho GTPase and the effector and the transient formation of the GEF−Rho−effector complex could act more efficiently to elicit cellular responses (Figure 7B).

How the DH/PH domains of GEF, Rho GTPase, and the effector PBD domain can come together to form the ternary complex requires further exploration. From the structural complexes of the pairwise interactions between the DH/PH of GEFs and Rho GTPases and between Rho GTPases and effector PBDs, it is not clear how Dbl(DH/PH) and PAK1-(PBD) could bind to Cdc42 simultaneously. In general, Rho GTPases utilize the switch I and II regions and the sequences between these GDP/GTP sensitive areas to make contact with the DH domain as seen in the Rac1-Tiam1, Cdc42-Dbs and RhoA-Dbs crystal structures (*10*−*12*). The sites of Rho

GTPases involved in interaction with effector PBDs vary from one PDB or Rho GTPase to another but typically include large contact areas consisting of multiple distinct structural patches on the GTPase surface in addition to specific residues of the switch I (*17*−*21*). The fact that wild-type Cdc42 but not the dominant negative or constitutively active Cdc42 mutant could sustain the complex formation, which would not be predicted from the conventional pairwise GEF−Cdc42−GDP or Cdc42−GTP−PAK1 binding interaction, suggests that a conformationally dynamic Cdc42 is required. Indeed, when GDP alone was present in the Dbl, Cdc42, and PAK1 mixture, the ternary complex failed to assemble (Figure 3B). The presence of free GTP or GTPγS, on the other hand, that allowed the GDP/GTP exchange on Cdc42, was able to trigger the complex formation (Figure 3B,C). A careful examination using a previously characterized DH domain mutant of Dbl that is defective in GEF catalytic activity but retains Cdc42 binding ability further helps to establish that the dynamic GDP/GTP exchange, involving the catalytic GEF activity of Dbl, is required for the ternary complex formation. It therefore appears that a pool of Cdc42 undergoing GDP/GTP binding transition under the Dbl catalysis remains associated with Dbl and that this pool of Cdc42 could transiently associate with PAK1(PBD), resulting in PAK1 activation. One possible mechanism that the DH domain and PDB could engage in the same Rho GTPase molecule simultaneously may be attributed to the flexibility of the switch regions of Rho GTPase, particularly in the course of GDP/GTP exchange, that might be induced to provide cryptic sites, to combine with the separate GEF and PBD recognition sites for the binding of both. This interpretation is supported by our recent finding that the GEF-catalyzed GDP/GTP exchange of Rho GTPases may undergo a dynamic one G-protein−two nucleotide (i.e., GDP−Rho−GTP) reaction intermediate, which would result in direct displacement of bound GDP by incoming GTP, instead of the commonly assumed nucleotide-free intermediate (Zhang and Zheng, unpublished results). It is difficult at this time to estimate quantitatively the percentage of Cdc42 that could undergo a GEF−Cdc42−effector intermediate compared to the Cdc42 species that become activated by the GEF reaction without the involvement of the effectors, since the ternary complex formation may be highly dynamic or with a relatively weak affinity and depends on the concentration and the molar ratios of the available GEFs, Cdc42, and effectors in specific intracellular locations. It would be ideal if a crystal structure of the ternary complex could be derived in the future to provide the detailed mechanistic insights, particularly on the possible allosteric conformational changes adopted by the Rho GTPase to accommodate two interacting molecules. Given the difficulty in producing a sufficient amount of the complex for the structural analysis, however, the attempt to obtain a structural model of the complex constitutes a major challenge.

In addition to generating transient signal output, the ternary complex may have other functional significance. Previously, it was shown that the small GTPase ARF could interact with its effector to facilitate the conversion of ARF−GTP from a low-affinity state for GTP to a high-affinity state in the GEF reaction, which in turn promotes the binding of ARF to the effector (*38*). A crystallographic analysis has revealed a direct feedback regulatory mechanism of the Sos−Ras interaction by which Ras−GTP could form a ternary complex with the Cdc25 domain and extended regions of Sos to further activate Sos−GEF activity toward Ras−GDP (*39*). DerMardirossian et al. (*40*) recently reported that PAK1 might serve to phosphorylate RhoGDI at Ser101/174 residues to promote RhoGDI dissociation from Rac1, suggesting a possible Rac-induced positive feed-forward regulatory mechanism. We found that the PAK1(PBD) could facilitate GEF-stimulated loading of GTP to Cdc42 (Figure 6), raising another interesting possibility that the effector may have a positive feedback regulatory role in the GEF reaction (Figure 7C). It remains to be seen if such a mechanism might be at work for other Rho GTPases. It is conceivable that there are a number of advantages for such feed-back or feed-forward mechanisms in cell regulation, one of which is that a modest increase of localized GEF or Rho GTPase activity in cells would result in amplified effector signaling while another is that it would allow rapid signal transmission within a short signaling circuit.

In summary, we show in the present study that Dbl, Cdc42, and PAK1 can form an active ternary complex through their minimum interactive domains, adding to the mechanistic perspective of Rho GTPase interaction with GEFs and effectors. Although the complex may not exist in abundance or in high affinity, transient formation of such a complex could provide a mechanism for increasing the signaling efficiency and specificity through the Rho GTPase signaling module. Further, the interesting discovery that Rho GTPase effectors such as PAK1 may positively influence the GEF activation of Rho GTPase raises new possibilities of the Rho GTPase signal amplification and adds to the increasingly complex Rho GTPase regulatory circuits.

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