Identification of a DOCK180-related Guanine Nucleotide Exchange Factor That Is Capable of Mediating a Positive Feedback Activation of Cdc42*  

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Cdc42, a member of the Rho subfamily of small GTPases, influences a wide range of activities including the establishment of cell polarity, migration, and the regulation of cell growth and differentiation. Here we describe the identification of an approximate 220-kDa protein that formed a stable complex with activated forms of Cdc42 and thereby showed characteristics of a downstream target/effectors for this GTPase. However, molecular cloning of the cDNA encoding this protein (p220) revealed that it was highly related to Zizimin-1 and identical in sequence to a gene product in a database designated DOCK11, which are members of the DOCK180 family of guanine nucleotide exchange factors (GEFs) for Cdc42 and Rac. Biochemical characterization shows that p220 is a specific GEF for Cdc42, with the GEF activity originating from its DHR2 (for DOCK homology region 2) domain. Nucleotide-depleted Cdc42 forms a stable complex with the DHR2 domain, whereas the binding of activated Cdc42 requires both the DHR2 domain and residues 66-126 within the amino-terminal portion of p220. Moreover, the full-length protein shows markedly higher GEF activity than the isolated DHR2 domain, whereas removal of the amino-terminal 126 amino acids necessary for binding-activated Cdc42 dramatically diminishes the activity. These and other results point to activated Cdc42 providing a positive feedback regulation of the GEF activity of p220. Thus, we refer to p220/DOCK11 as activated Cdc42-associated GEF, befitting its functional activity.

Cdc42 has been shown to regulate multiple cellular processes including actin cytoskeletal organization, membrane trafficking, cytokinesis, and cell polarity, as well as cell growth and differentiation (1–3). The activation of Cdc42 is triggered by a variety of stimuli including growth factors, cytokines, cell adhesion factors, and conditions leading to cell stress (4–10). More than a dozen downstream target/effectors for Cdc42 have been identified, including scaffold proteins like WASP and IQGAP, the y COP subunit of the COPI complex that coats trafficking vesicles, as well as different serine/threonine and tyrosine kinases including p21-activated kinase, mixed lineage kinase, and activated Cdc42-associated kinase (11–20). Thus, Cdc42 serves as a critical signaling switch converting an array of stimulatory inputs into a diversity of cellular responses and biological activities.

The activation of Cdc42 is directly catalyzed by guanine nucleotide exchange factors (GEFs).2 Dbl, which has potent transforming activity when truncated from its amino-terminal end, is the founding member of a family of GEFs for Cdc42 and related Rho GTPases (21). All of the members of this family are characterized by a tandem array of Dbl homology (DH) and Pleckstrin homology (PH) domains with the DH domains being primarily responsible for mediating GEF activity (22–24). In addition to these conserved sequences, most members of the Dbl family of GEFs contain other functional or protein-protein interaction domains that help to connect the activation of Cdc42 or other related Rho GTPases to a number of downstream signaling partners and cellular activities. For example, Intersectin-1L, a Cdc42-specific GEF, uses five SH3 and two EH domains, together with the tandem DH/PH domain, to couple the activation of Cdc42 to the endocytosis of cell surface receptors, presumably by organizing the F-actin structure along the surfaces of endocytic vesicles (25).

More recently, a new class of GEFs for Cdc42 and Rac that lack tandem DH and PH domains has been identified. Two examples are Zizimin-1 and DOCK180 (director of cytokinesis 180) (26, 27). Members of this family of GEFs have relatively high molecular mass (~200 kDa) and contain two conserved domains, designated CZH1 (for conserved Zizimin homology domain 1)/DHR1 (for DOCK homology region 1) and CZH2/DHR2. The CZH2/DHR2 domain represents the primary functional unit, because it catalyzes the nucleotide exchange activity of the GTPase substrates (26, 27). Although Dbl family GEFs are not found in plants, members of the Zizimin-1/DOCK180 family are conserved throughout yeast, plants, and animals (26, 27). Many members of the DOCK180 family, including DOCK180 itself, as well as DOCK2 and DOCK3, activate Rac to regulate cell adhesion, cell spreading, migration, and phagocytosis (28–30). However, the function of Zizimin-1, a specific GEF for Cdc42, is still not known.

In the classical model for the activation of small GTPases, the GEF interacts with the GDP-bound form of its substrate

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2 The abbreviations used are: GEF, guanine nucleotide exchange factor; ACG, activated Cdc42-associated GEF; DH, Dbl homology; PH, Pleckstrin homology; DHR, DOCK homology region; HA, hemagglutinin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; AMP-PNP, adenosine 5’-β,γ-iminotriphosphate; GTPγS, guanosine 5’-O-(thiotriphosphate); ACB, activated Cdc42 binding.
GTPase and catalyzes the dissociation of GDP. The relatively high cellular concentrations of GTP (relative to GDP) allow an exchange of the dissociated GDP for GTP, which then induces structural changes within the GTPase that enable it to interact with downstream signaling target/effector proteins. In many cases, the binding of GTP to the GTPase also prompts its dissociation from the GEF, thereby enabling the GEF to act catalytically in promoting GDP-GTP exchange on multiple GTPases. However, there have been some recent reports showing that GEFs can engage in stable complexes with the GTP-bound form of their substrate GTPase. One example is the Cool-2/α-Pix (cloned out of library 2/α p21-activated kinase interactive exchange factor) protein, which contains the tandem arrangement of DH and PH domains characteristic of Dbl family GEFs but was originally identified through its ability to bind to the Cdc42/Rac-target p21-activated kinase (31, 32). Cool-2/α-Pix forms a stable complex with activated (GTP-bound) Cdc42, which in turn strongly stimulates the GEF activity of Cool-2/α-Pix toward Rac. This has led to the suggestion that Cool-2/α-Pix is able to mediate a Cdc42-Rac signaling cascade (33).

In this report, we have identified a Cdc42-GEF that exhibits an interesting variation on the theme described above. This new GEF is a member of the Zizimin-1/DOCK180 family, and because it was originally isolated through its ability to bind activated forms of Cdc42, we refer to it here as activated Cdc42-associated GEF (ACG). ACG appears to represent a novel example of a GEF that responds to activated Cdc42 in a manner that may allow for positive feedback stimulation of GDP-GTP exchange on additional Cdc42 molecules.

MATERIALS AND METHODS

Chemicals, Plasmids, and Antibodies—The site-directed mutagenesis kits and Pfu DNA polymerase were purchased from Stratagene. Texas Red-conjugated anti-mouse IgG was obtained from Molecular Probes (Invitrogen). Monoclonal anti-HA antibody (HA.11) was from Covance, and the IMAGE cDNA clones were purchased from ATCC and Open Biosystems.

Preparation of GST Fusion Proteins and Their Use in Pull-down Assays—Escherichia coli containing plasmids encoding GST fusion proteins were cultured to a density of A600 of 1.0. Expression of the fusion proteins was induced by adding isopropyl β-D-thiogalactopyranoside (final concentration, 0.5 mM) to the culture medium for 3–5 h. The bacteria were precipitated by centrifugation at 6,000 × g for 10 min and the pellet was resuspended in a bacterial lysis buffer (40 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 μg/ml leupeptin, and 10 μg/ml aprotonin). The suspension was sonicated and centrifuged at 15,000 × g for 10 min. The supernatant was collected and incubated with glutathione-agarose beads (1:1 slurry in the bacterial lysis buffer) for 2 h at 4 °C with rotation. The beads were then washed (three times) with lysis buffer, and the immobilized GST fusion protein beads were resuspended in the same buffer.

For large scale pull-down assays, the GST fusion protein beads containing 40–80 μg of protein were incubated with 10 ml of mammalian cell lysates (10–20 mg of protein) at 4 °C for 2–3 h with rotation. The beads were washed (three times) with mammalian cell lysis buffer (40 mM Hepes, pH 7.4, 100 mM NaCl, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotonin) (8–10 ml each time) and resuspended with 2 × SDS-PAGE sample buffer. Following SDS-PAGE, the proteins that co-precipitated with the GST fusion proteins were visualized by staining with 0.5–1% Coomassie Blue. The bands of interest were excised and subjected to microsequencing by microcapillary reverse phase high pressure liquid chromatography nanoelectrospray tandem mass spectrometry on a Finnigan LC DECA quadrupole ion trap mass spectrometer at the Harvard Microchemistry Facility.

For small scale pull-down assays, the GST fusion protein beads containing 10–20 μg of protein were incubated with 0.5–1.0 ml of cell lysates (0.5–1.0 mg of protein) at 4 °C for 2–3 h with rotation. The beads were washed (three times) with lysis buffer and resuspended in 2 × SDS-PAGE sample buffer. The co-precipitated proteins were separated by SDS-PAGE and identified by immunoblotting.

Cell Culture, Immunoprecipitation, and Immunoblots—Mouse embryonic teratoma (P19) cells and COS7 cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and 5% CO2 at 37 °C. The transfection of plasmid DNA into COS7 cells was performed using the Lipofectamine transfection kit (Invitrogen). The cells were lysed after 48 h of transfection.

For the preparation of cell lysates, the cells were rinsed with PBS once and lysed with mammalian cell lysis buffer (0.5 ml/60-mm dish or 1 ml/100-mm dish) with rocking at 4 °C for 20 min. The lysates were collected and cleared by centrifugation at 14,000 rpm for 4 min and then immediately used.

For immunoprecipitation, the precleared cell lysates were incubated with primary antibody on ice for 30 min. Protein A beads were then added, and the mixture was incubated at 4 °C for 2 h with rotation. The beads were washed (three times) with mammalian cell lysis buffer, and the immunoprecipitated complexes were used either for GEF assays or directly dissolved in sample buffer for SDS-PAGE. Immunoblots were performed as suggested in the instructions accompanying the ECL immunoblot kits (Amersham Biosciences).

Immunofluorescence Staining—The cells were cultured on coverslips to 50–80% confluence. After the culture medium was removed, the cells were rinsed (two times) with PBS, fixed with 3.7% formaldehyde at 25 °C for 10 min, and permeabilized with 0.2% Triton X-100 in PBS at 25 °C for 10 min. Following washing with PBS, the cells were incubated with primary antibody at 37 °C for 30 min. The cells were then washed with PBS (three times) and incubated with secondary antibody that was conjugated with a fluorescent dye at 37 °C for 30 min. Finally, the cells were washed with PBS (three times for 10 min each time). The coverslips were mounted on glass slides, and immunofluorescence staining was monitored with a Leica confocal fluorescent microscope.

Guanine Nucleotide Exchange Assays—Nucleotide exchange activity was assayed as previously described (21). GST-Cdc42 or GST-Rac (typically 4 μg) was incubated with 10 μM [3H]GDP (12.6 Ci/mmol) in 25 μl of loading buffer (20 mM Tris-
HCl, pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM AMP-PNP), for 25 min at 25 °C. Immunoprecipitated ACG was added to the exchange assay mixture containing 20 mM Tris-HCl, pH 7.4, 80 mM NaCl, 5 mM MgCl₂, 1 mM AMP-PNP, 0.1 mM di-thiothreitol, 0.4 mg/ml bovine serum albumin, and 1 mM GTP·γ·S to a final volume of 40 μl and incubated at 25 °C for 15 min. Aliquots (10 μl) of the reaction mixture were removed and diluted into 0.5 ml of washing buffer (20 mM Tris-HCl, pH 7.4, 80 mM NaCl, 10 mM MgCl₂, and 10 mM β-mercaptoethanol) to stop the reaction. The samples were placed on BA85 filters and washed (three times) with washing buffer. The radioactivity on the filter, representing the amount of [3H]GDP bound to Cdc42 that was not exchanged with GTP·γ·S, was quantified by scintillation counting.

RESULTS
Identification of an ~220-kDa Binding Partner for Activated Cdc42—We have been using the mouse embryonic teratoma cell line P19 as a model system for examining the role of Cdc42 in cellular differentiation. In particular, we have used P19 cell lysates to perform pull-down experiments with GST-Cdc42 fusion proteins as a way of potentially identifying new Cdc42-regulatory proteins and target/effectors. Fig. 1A shows the results of an experiment where lysates were incubated with glutathione-conjugated agarose beads bound to the GTPase-defective GST-Cdc42(Q61L) mutant. The co-precipitated proteins were separated by SDS-PAGE and visualized by staining with Coomassie Blue (lane 2). A number of additional incubations were performed to control for nonspecific binding of bacterial proteins to GST-Cdc42 or for proteins from P19 cell lysates that might either associate directly with the GST moiety or with beads alone. These included incubations of GST-Cdc42(Q61L) with the lysis buffer (lane 1), GST with P19 cell lysates (lane 3), and glutathione-conjugated agarose beads with the cell lysates (lane 4). We found that a number of proteins appeared to selectively co-precipitate with GST-Cdc42(Q61L). The predominant protein of ~220 kDa, indicated by the black arrow, co-precipitated with GST-Cdc42(Q61L) in three different experiments. The 220-kDa Cdc42(Q61L)-binding protein (p220) is indicated by a black arrow. The results shown are representative of three experiments.

B, the amino acid sequence of ACG. The cDNA sequence of p220 (or ACG) has been deposited in GenBank™ (accession number AY692226). The PH domain is indicated by bold letters. The DHR1/CZH1 and the DHR2/CZH2 domains are boxed. The underlined letters are the peptide sequences obtained from microsequencing p220/ACG by the Harvard Microchemistry Facility. C, verification of the interaction between ACG and activated Cdc42. HA-tagged full-length ACG was expressed in COS7 cells. The glutathione-conjugated bead-bound GST, GST-Cdc42 (wild type), GST-Cdc42(Q61L), GST-Cdc42(Q61L,K183S,K184S), or Cdc42(Q61L,Y40C) were incubated with ACG-expressing COS7 cell lysates. The co-precipitated ACG was detected by immunoblotting with anti-HA antibody (top panel). The input from the lysates (10% of the total) is shown on the far-right side of the top panel. The relative amounts of the GST-Cdc42 fusion proteins used in the pull-down assays were visualized by staining with Coomassie Blue (bottom panel).

P19 cell lysates (Fig. 1A, lane 3), and glutathione-conjugated agarose beads with the cell lysates (Fig. 1A, lane 4). We found that a number of proteins appeared to selectively co-precipitate with GST-Cdc42(Q61L). The predominant pro-
tein was ~180 kDa (denoted by the white arrow in lane 2 of Fig. 1A) and was determined to be IQGAP1 (34, 35). Among the other most consistently observed bands that specifically bound to GST-Cdc42(Q61L) was one that corresponded to an ~220 kDa protein (see the black arrow in lane 2 of Fig. 1A). The p220 band was excised and subjected to microsequence analysis. Six peptide sequences were obtained and aligned against the NBCI GenBank data base using a Blast search (36). Four of the six peptides were identical to Zizimin-1, with the remaining two peptides showing ~90% identity. This indicated that p220 was a member of the Zizimin-1/DOCK180 family of GEFs. We further found that the peptide sequences XP_060056 and NP_653259, derived from computational analysis of the human genomic DNA sequence, completely matched the six peptide sequences of p220. Subsequently, Cote and Vuori (27) classified XP_060056 as a member of the DOCK180 superfamily and named it DOCK11. However, for the sake of simplicity, we refer to this protein as ACG, because this more accurately depicts its functional properties (see below).

To obtain the full-length cDNA encoding ACG, we used the derived genomic DNA sequences NM_144658 and XP_060056 to design primers to perform PCR, with a human brain cDNA library serving as a template. We also used Image clones 2329541, 2021991, 4671357, 5582516, and 5583671 to help assemble the cDNA encoding ACG. The full-length cDNA contains 6650 base pairs (GenBank accession number AY692226), corresponding to 2073 amino acid residues (Fig. 1B). The sequence for ACG was analyzed by a NCBI Blast search against GenBank and was found to contain three distinct domains. These are an amino-terminal PH domain (Fig. 1B, in bold), a DHR1/CZH1 domain

FIGURE 2. ACG is a Cdc42-specific GEF. A, HA-tagged DHR2 (amino acid residues 1539–2073) was expressed in COS7 cells. The cell lysates were incubated with glutathione-conjugated GST-Cdc42(Q61L), GST-Cdc42(T17N), GST-Cdc42 wild type (WT), GST-Rac(T17N), or GST-TC10(T31N) at 4 °C for 3 h with rotation. The co-precipitated DHR2 domain was detected by immunoblotting with anti-HA antibody (top panel). The relative amounts of the GST-Cdc42 fusion proteins used in the pull-down assays were visualized by staining with Coomassie Blue (bottom panel). The results shown are representative of two experiments. B, Cdc42-GEF assays with ACG and the DHR2 domain. The plasmids encoding HA-tagged ACG, the DHR2 domain, or pcDNA3 (vector control) were transfected into COS7 cells, and the HA-tagged proteins were immunoprecipitated by anti-HA antibody (top right panel). The immunoprecipitated complexes were used for the Cdc42-GEF assays, in which E. coli recombinant, His-tagged Cdc42 was loaded with [3H]GDP, and the dissociation of [3H]GDP for GTP was measured (see “Materials and Methods”). EDTA (10 mM) was used as a positive control for the assays. The results shown are representative of two experiments. C, assays were performed to determine whether ACG exhibits GEF activity toward Rac1. HA-tagged ACG was expressed in COS7 cells and then immunoprecipitated and assayed for Rac-GEF activity as described for B and under “Materials and Methods.” Insect cell recombinant Cool-2/α-Pix (2 μg), as well as EDTA (10 mM), was used as a positive control.
located approximately in the middle of the ACG molecule (boxed), and a DHR2/CZH2 domain found near the carboxyl-terminal end of the protein (boxed) (also see the schematic depiction of full-length ACG at the top of Fig. 3A). Given the presence of the DHR1 and DHR2 domains, ACG shows the typical earmarks of a member of the Zizimin-1/DOCK180 family of proteins.

To further confirm and characterize the interaction of ACG with activated forms of Cdc42, we overexpressed HA-tagged ACG in COS7 cells and examined its ability to be pulled down by GST-Cdc42 (wild type); GST-Cdc42(Q61L); GST-Cdc42(Q61L,K183S,K184S), which represents a GTPase-defective and γCOP binding-defective double mutant; and GST-Cdc42(Q61L,Y40C), which is GTPase-defective as well as defective for binding target/effectors like p21-activated kinase, mixed lineage kinase, and activated Cdc42-associated kinase. As shown in Fig. 1C, HA-tagged ACG was co-precipitated with Cdc42(Q61L), as well as with Cdc42(Q61L,K183S,K184S) (lanes 2 and 3), but not with wild-type Cdc42 nor with Cdc42(Q61L,Y40C) (lanes 1 and 4). Interestingly, the binding of Cdc42(Q61L,K183S,K184S) to ACG was much stronger than that of Cdc42(Q61L) (compare lanes 3 and 2), suggesting that ACG may compete with γCOP for Cdc42. Overall, these results confirmed that ACG forms stable complexes with activated forms of Cdc42.

**ACG Is a GEF for Cdc42**

DOCK180 has been shown to exhibit GEF activity toward Rac (37), whereas Zizimin-1 (also known as DOCK9) acts as a specific GEF for Cdc42 (26). In each of these cases, the GEF activity is mediated through a highly conserved region designated as the DHR2 or the CZH2 domain (26, 27). Thus, we were interested in seeing whether ACG exhibited GEF activity. We were unable to express either full-length ACG or its DHR2 domain in E. coli or to obtain sufficient levels in insect cells to allow biochemical characterization. However, we were able to express the DHR2 domain as an HA-tagged protein in COS7 cells and then assay its interactions with different GST-linked GTPases through pull-down assays. We found that unlike the case for full-length ACG, which binds preferentially to activated forms of Cdc42 (e.g. Cdc42(Q61L)), the recombinant HA-tagged DHR2 domain bound selectively to the nucleotide binding-defective Cdc42(T17N) mutant (Fig. 2A), as is typically the case for GEFs. The DHR2 domain of ACG did not bind to the nucleotide binding-defective mutant of Rac (Rac(T17N)) and only bound very weakly to the corresponding mutant for the related GTPase TC10 (TC10(T31N)). Thus, these data suggested that the DHR2 domain of ACG may exhibit specific GEF activity toward Cdc42.

To further examine this possibility, we transfected HA-tagged full-length ACG or the HA-tagged DHR2 domain in COS7 cells, immunoprecipitated these proteins with anti-HA antibody, and then assayed their GEF activity. We used anti-HA antibody that was immunoprecipitated from vector-transfected cell lysates as a negative control, and EDTA, which chelates Mg²⁺ and strongly stimulates GDP-GTP exchange on small Ras-related GTPases, as a positive control. As shown in
Active Cdc42-associated Guanine Nucleotide Exchange Factor

![Diagram](https://example.com/diagram.png)

**FIGURE 4.** Amino acids 66–125 of ACG are required for binding to activated forms of Cdc42. A, a schematic representation of full-length ACG and the amino-terminal truncation mutants that were assayed. B, HA-tagged ACG and its amino-terminal truncation mutants, ∆N65 and ∆N126, were expressed in COS7 cells. The glutathione-conjugated GST-Cdc42 (lane 3), GST-Cdc42(Q61L) (lane 2), or GST-Cdc42(T17N) (lane 1) were incubated with the cell lysates. The pull-down of ACG or its amino-terminal truncation mutants was detected by immunoblotting with anti-HA antibody. The inputs from the lysates (10% of the total) are shown on the far right side of the figure. The relative amounts of GST-Cdc42 fusion proteins used in the pull-down assays were visualized by staining with Coomassie Blue (bottom panel). The results shown are representative of two experiments. WT, wild type Cdc42.

Fig. 2B, full-length ACG was an effective GEF for Cdc42, such that its activity was comparable with that obtained upon treatment with excess EDTA, whereas the recombinant DHR2 domain caused only a minor increase in the rate of [3H]GDP dissociation from Cdc42, compared with the control condition (i.e. Cdc42 alone). These results were somewhat surprising, because often the isolated limit-functional domains of GEFs show much stronger activity than their full-length parental proteins, because of the need for regulatory events to activate the GEF activity of the latter. This suggested that full-length ACG can be effectively activated in cells. Fig. 2C further demonstrates that the GEF activity for ACG is specific for Cdc42. As previously reported, insect cell recombinant Cool-2/α-Pix shows Rac-GEF activity and stimulates the rate of [3H]GDP dissociation from Rac (38), whereas ACG is absolutely ineffective.

Characterization of the Interaction of ACG with Activated Forms of Cdc42—The preference shown by ACG in binding to activated forms of Cdc42 (Fig. 1) differs from what has been reported for full-length Zizimin-1, which selectively interacts with nucleotide-depleted forms of Cdc42 (26), as is the usual case for Cdc42-GEFs. This distinctive feature of ACG raised the possibility that activated forms of Cdc42 might exert regulatory effects on its Cdc42-GEF activity. To examine this possibility, we first needed to better define how activated Cdc42 binds to full-length ACG. A series of HA-tagged truncation mutants of ACG were prepared (Fig. 3A) and expressed in COS7 cells, and then their abilities to bind to either GST-Cdc42(Q61L), Cdc42(T17N), or wild-type GST-Cdc42 were determined through pull-down assays. Full-length ACG served as a positive control for binding to activated Cdc42(Q61L), whereas the isolated DHR2 domain was used as a positive control for binding to Cdc42(T17N).

Interestingly, when the amino-terminal 272 amino acid residues of ACG were removed, its binding preference switched from activated forms of Cdc42 (e.g. Cdc42(Q61L)) to the nucleotide-depleted Cdc42(T17N) (Fig. 3B, fourth panel from top). This suggested that the amino-terminal portion of ACG was not only necessary for binding to activated forms of Cdc42 but also interfered with and/or weakened the binding of nucleotide-depleted Cdc42 and thereby repressed GEF activity. Surprisingly, when the carboxyl-terminal DHR2 domain was deleted, binding to Cdc42(Q61L) was reduced (Fig. 3B, second panel from the top). Furthermore, a more substantial truncation that deleted residues 671–2073 resulted in the complete loss of binding to both activated and nucleotide-depleted forms of Cdc42 (Fig. 3B, third panel from the top). Taken together, these data suggest that the DHR2 domain is required for binding to activated as well as nucleotide-depleted forms of Cdc42. Apparently, the DHR2 domain represents the sole binding site for the latter, whereas the amino-terminal region of ACG together with DHR2 are necessary for optimal binding to activated Cdc42.

Because the truncation of the first 272 amino acid residues resulted in the loss of binding to Cdc42(Q61L) and an accompanying increase in the binding to Cdc42(T17N) (Fig. 3B), and because the PH domain of ACG is located within this region (Fig. 3A), we wondered whether the PH domain might play a role in regulating the binding of different forms of Cdc42. Thus, we generated two more amino-terminal truncation mutants of ACG, designated as ∆N65 and ∆N126 (Fig. 4A). Both of these truncation mutants still contained the PH domain. The HA-tagged ∆N65 and ∆N126 were transfected into COS7 cells, and their abilities to bind to different forms of Cdc42 were assessed by pull-down assays. To our surprise, ∆N126 showed little or no ability to bind to the Cdc42(Q61L) mutant but was able to form a complex with Cdc42(T17N) (Fig. 4B, third panel from top). On the other hand, the ∆N65 construct behaved similar to full-length ACG and bound preferentially to the dominant active Cdc42(Q61L) mutant (Fig. 4B, second panel from top). These data indicate that an essential region for binding-activated forms of Cdc42 is located within amino acid residues 66–126 of ACG and suggest that the PH domain is not directly responsible for this binding interaction. We have designated this region as the activated Cdc42 binding (or ACB) domain. We then asked the following question. Given that the interaction of activated forms of Cdc42 with full-length ACG requires both the ACB domain within the amino-terminal portion of the protein and the DHR2 domain, is it possible that these two domains are in close proximity to one another? The interaction of the ACB domain with the DHR2 domain might provide a binding site for activated Cdc42 that then enables it to further stimulate GEF
activity. We therefore co-expressed a Myc-tagged construct that included the ACB domain (Myc-N10–127) with HA-tagged DHR2 into COS7 cells and then examined whether these two domains could be co-immunoprecipitated from cell lysates. Fig. 5A shows that the Myc-N10–127 construct can be co-immunoprecipitated with HA-DHR2 from cells. However, when we directly compared the abilities of these two domains to interact in the presence of different forms of Cdc42, we consistently observed that activated Cdc42 mutants provided a marked advantage (Fig. 5B, top panel). These results further suggest that the ACB and DHR2 domains of ACG form a high affinity binding site for activated Cdc42.

**Activated Cdc42 Positively Regulates Signaling through ACG**—Given that we had identified the important determinants for the binding of activated forms of Cdc42 to ACG, we next wanted to examine the possible functional implications for this binding interaction. We were particularly intrigued by the possibility that the binding of activated forms of Cdc42 to ACG was important for enhancing its GEF activity, because this would potentially explain the marked differences in GEF activity exhibited by the full-length protein compared with the DHR2 domain. As a first step toward testing this idea, we assayed the GEF activities of the amino-terminal truncation mutants /H9004N65 and /H9004N126, because the former construct still binds to activated Cdc42, whereas the latter does not. Fig. 6 shows that the truncation mutant ΔN65 exhibited a strong GEF activity toward Cdc42 that was comparable with the activity of wild-type ACG. However, the truncation mutant ΔN126, which is ineffective in binding to activated Cdc42 but undergoes a detectable interaction with nucleotide-depleted Cdc42 (T17N), showed only very weak GEF activity toward Cdc42. These data suggest that for ACG to exhibit full GEF activity toward Cdc42, it needs to be capable of interacting with activated forms of the GTPase.

We took these studies one step further and examined the effects of the different ACG constructs on cell morphology. COS7 cells overexpressing wild-type ACG or the truncation mutant ΔN65 displayed multiple microspike structures (Fig. 7, panels a and b, respectively), which are a well established outcome of the activation of Cdc42 (4, 39). Cells overexpressing the DHR2 domain or the truncation mutant ΔN126 showed minimal changes in cell morphology. These results provide additional support for the notion that the activation of Cdc42 is necessary for the full GEF activity of ACG.
tion mutant ΔN126 showed little or no ability to elicit microspikes (Fig. 7, panels c and d). These findings corroborate the results of the nucleotide exchange assays shown in Fig. 6.

**DISCUSSION**

Two major families of GEFs for Rho GTPases have been identified and biochemically characterized. The first is the family of Db1-related proteins, in which case the founding member is the Db1 oncoprotein. Members of this family are characterized by a tandem arrangement of DH and PH domains, with the DH domain in many cases representing the minimal functional unit for catalyzing GDP-GTP exchange on its GTPase substrates. The second family is represented by DOCK180 and Zizimin-1. It was originally shown that the DOCK180 homologs CED-5 in *Caenorhabditis elegans* and Myoblast City in *Drosophila* were upstream activators of the Rac GTPase and involved in developmental processes that are important for the temporal and spatial control of the actin cytoskeleton (40, 41). In mammalian cells, DOCK180 (also known as DOCK-1) has been shown to activate Rac and to influence phagocytosis, cell adhesion, and migration processes (28–30, 37), whereas Zizimin-1 was reported to be a specific GEF for Cdc42 (26). Both Db1- and DOCK180-related GEFs have been conserved through evolution. A question of interest concerns whether these two families of GEFs share structural and functional properties as well as common regulatory cues. There are increasing examples of how members of the Db1 family are subject to sophisticated mechanisms of allosteric regulation (24). Here, we now show that the same appears to be true for a newly characterized member of the DOCK180 family that we refer to as ACG.

Our discovery of ACG arose from efforts to identify new potential regulatory proteins and target/effectors for activated Cdc42. Using different forms of GST-Cdc42 in pull-down
assays, we found that an ~220-kDa protein (p220) from P19 cell lysates bound preferentially to activated forms of the GTPase. This initially led us to believe that the protein was a downstream target/effecter for Cdc42. Upon molecular cloning and ultimately obtaining its primary amino acid sequence, we found unexpectedly that p220 was a member of the DOCK180/Zizimin-1 family. Thus, it seemed likely that this apparent Cdc42-target/effecter was also an upstream activator for Cdc42 or Rac, and hence we call it ACG. Although we showed that the isolated recombinant DHR2 domain from ACG was able to bind to nucleotide-depleted forms of Cdc42 as is typical for GEFs, it exhibited only a weak ability to stimulate GDP-GTP exchange activity. However, what was particularly surprising was that full-length ACG, when expressed in cells, showed relatively strong GEF activity toward Cdc42. This is highly unusual, at least for members of the DbI family of GEFs, where constructs containing just the DH and PH domains are typically more active than their corresponding full-length counterparts. Two examples for which we have significant experience are the Cdc42-GEF Intersectin-L and members of the Cool/Pix family of proteins (42, 43).

The ability of full-length ACG to activate Cdc42 led us to question whether the binding of activated Cdc42 to this GEF might elevate its activity. We first determined that activated forms of Cdc42 bind to a distinct region (designated the ACB) that lies between residues 66 and 126 within the amino-terminal portion of ACG, as well as to a second site located in the DHR2 domain. We then showed that deletion of the ACB, while leaving the vast majority of the ACG protein intact including its DHR1 and DHR2 domains, was sufficient to abrogate the enhanced GEF activity normally displayed by the full-length protein. Likewise, excising the amino-terminal determinants for binding-activated Cdc42 also prevented ACG from stimulating microspike formation in cells, a phenotype that arises from the activation of Cdc42 (4, 39). Thus, these findings now lead us to propose that ACG can mediate a positive feedback mechanism that regulates microspike formation in cells, a phenotype that arises from the activation of Cdc42 (4, 39). However, thus far, a limitation of these studies has been the lack of a reliable antibody for ACG so that we can rigorously correlate the reduction in the levels of this GEF with the loss of phenotype. Future studies will be directed toward establishing such a correlation as well as determining whether the same is true for P19 cells and other cell lines. We also will be interested in seeing whether other members of the DOCK180/Zizimin-1 family show the same capability for positive feedback regulation of GTPase-coupled signaling events. The region within the amino-terminal end of ACG that is essential for the binding of activated Cdc42 (i.e. the ACB domain) appears to be conserved in other members of the DOCK180/Zizimin-1 superfamily, such as Zizimin-1 (DOCK9), DOCK6, DOCK7, and DOCK10, although Zizimin-1 does not exhibit a detectable affinity for activated forms of Cdc42 (26). Thus, it also will be interesting to learn what accounts for the differences between ACG and Zizimin-1 and whether other mechanisms are responsible for regulating the Cdc42-GEF activity of Zizimin-1.

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REFERENCES

1. Johnson, D. I. (1999) Microbiol. Mol. Biol. Rev. 63, 54–105
2. Nobes, C. D., and Hall, A. (1995) Biochem. Soc. Trans. 23, 456–459
3. Cerione, R. A. (2004) Trends Cell Biol. 14, 127–132
4. Kozma, R., Ahmed, S., Best, A., and Lim, L. (1995) Mol. Cell. Biol. 15, 1942–1952
5. Shekarabi, M., and Kennedy, T. E. (2002) Mol. Cell Neurosci. 19, 1–17
6. Wong, K., Ren, X. R., Huang, Y. Z., Xie, Y., Liu, G., Saito, H., Tang, H., Wen, L., Brady-Kalnay, S. M., Mei, L., Wu, J. Y., Xiong, W. C., and Rao, Y. (2001) Cell 107, 209–221
7. Driggers, P. H., Segars, J. H., and Rubino, D. M. (2001) J. Biol. Chem. 276, 46792–46797
8. Ku, G. M., Yablonski, D., Manser, E., Lim, L., and Weiss, A. (2001) EMBO J. 20, 457–465
9. Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997) Nature 390, 632–636
10. Puls, A., Eliopoulos, A. G., Nobes, C. D., Bridges, T., Young, L. S., and Hall, A. (1999) J. Cell Biol. 112, 2983–2992
11. Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J., and Cerione, R. A. (1995) J. Biol. Chem. 270, 22731–22737
12. Teramoto, H., Coso, O. A., Miyata, H., Igishii, T., Miki, T., and Gutkind, J. S. (1996) J. Biol. Chem. 271, 27223–27228
13. Manser, E., Leung, T., Salihuddin, H., Tan, L., and Lim, L. (1993) Nature 363, 364–367
14. Yang, W., and Cerione, R. A. (1997) J. Biol. Chem. 272, 24819–24824
15. Symons, M., Derry, J. M., Karlak, B., Jiang, S., Lernahieu, V., McCormick, F., Francke, U., and Abo, A. (1996) Cell 84, 723–734
16. Erickson, J. W., Cerione, R. A., and Hart, M. J. (1997) J. Biol. Chem. 272, 24443–24447
17. Leung, T., Chen, X. Q., Tan, I., Manser, E., and Lim, L. (1998) Mol. Cell. Biol. 18, 130–140
18. Joberty, G., Petersen, C., Gao, L., and Macara, I. G. (2000) Nat. Cell Biol. 2, 531–539
19. Wu, W. J., Erickson, J. W., Lin, R., and Cerione, R. A. (2000) Nature 405, 800–804
20. Joberty, G., Perlunger, R. R., and Macara, I. G. (1999) Mol. Cell. Biol. 19, 531–539

Q. Lin, unpublished data.
Active Cdc42-associated Guanine Nucleotide Exchange Factor

6585–6597
21. Hart, M. J., Eva, A., Evans, T., Aaronson, S. A., and Cerione, R. A. (1991) Nature 354, 311–314
22. Schmidt, A., and Hall, A. (2002) Genes Dev. 16, 1587–1609
23. Cerione, R. A., and Zheng, Y. (1996) Curr. Opin. Cell Biol. 8, 216–222
24. Erickson, J. W., and Cerione, R. A. (2004) Biochemistry 43, 837–842
25. McGavin, M. K., Badour, K., Hardy, L. A., Kubiseski, T. J., Zhang, J., and Siminovitch K. A. (2001) J. Exp. Med. 194, 1777–1787
26. Meller, N., Irani-Tehrani, M., Kiosses, W. B., Del Pozo, M. A., and Schwartz, M. A. (2002) Nat. Cell Biol. 4, 639–647
27. Cote, J. F., and Vuori, K. (2002) J. Cell Sci. 115, 4901–4913
28. Wu, Y. C., and Horvitz, H. R. (1998) Nature 392, 501–504
29. Fukui, Y., Hashimoto, O., Sanui, T., Oono, T., Koga, H., Abe, M., Inayoshi, A., Noda, M., Oike, M., Shirai, T., and Sassuzki, T. (2001) Nature 412, 826–831
30. Namekata, K., Enokido, Y., Iwasawa, K., and Kimura, H. (2004) J. Biol. Chem. 279, 14331–14337
31. Bagrodia, S., Taylor, S. I., Jordon, K. A., Van Aelst, L., and Cerione, R. A. (1998) J. Biol. Chem. 273, 23633–23636
32. Manser, E., Looh, T. H., Koh, C. G., Zhao, Z. S., Chen, X. Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998) Mol. Cell 1, 183–192
33. Baird, D., Feng, Q., and Cerione, R. A. (2005) Curr. Biol. 15, 1–10
34. Hart, M. J., Callow, M. G., Souza, B., and Polakis, P. (1996) EMBO J. 15, 2997–3005
35. McCallum, S. J., Erickson, J. W., and Cerione, R. A. (1998) J. Biol. Chem. 273, 22537–22544
36. Altschul, S. F., Madden, T. M., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
37. Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998) Genes Dev. 12, 3331–3336
38. Feng, Q., Baird, D., and Cerione, R. A. (2004) EMBO J. 23, 3492–3504
39. Nobes, C. D., and Hall, A. (1995) Cell 81, 53–62
40. Reddien, P., and Horvitz, H. (2000) Nat. Cell Biol. 2, 131–136
41. Erickson, M., Galletta, B., and Abamyr, S. (1997) J. Cell Biol. 138, 589–603
42. Wang, J. B., Wu, W. J., and Cerione, R. A. (2005) J. Biol. Chem. 280, 22883–22891
43. Feng, Q., Albeck, J. G., Cerione, R. A., and Yang, W. (2002) J. Biol. Chem. 277, 5644–5650