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Ccpg1, a Novel Scaffold Protein That Regulates the Activity of the Rho Guanine Nucleotide Exchange Factor Dbs[▽]

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Dbs is a Rho-specific guanine nucleotide exchange factor (RhoGEF) with in vitro exchange activity specific for RhoA and Cdc42. Like many RhoGEF family members, the in vivo exchange activity of Dbs is restricted in a cell-specific manner. Here we report the characterization of a novel scaffold protein (designated cell cycle progression protein 1 [Ccpg1]) that interacts with Dbs and modulates its in vivo exchange specificity. When coexpressed in mammalian cells, Ccpg1 binds to the Dbl homology/pleckstrin homology domain tandem motif of Dbs and inhibits its exchange activity toward RhoA, but not Cdc42. Expression of Ccpg1 correlates with the ability of Dbs to activate endogenous RhoA in cultured cells, and suppression of endogenous Ccpg1 expression potentiates Dbs exchange activity toward RhoA. The isolated Dbs binding domain of Ccpg1 is not sufficient to suppress Dbs exchange activity on RhoA, thus suggesting a regulatory interaction. Ccpg1 mediates recruitment of endogenous Src kinase into Dbs-containing complexes and interacts with the Rho family member Cdc42. Collectively, our studies suggest that Ccpg1 represents a new class of regulatory scaffold protein that can function as both an assembly platform for Rho protein signaling complexes and a regulatory protein which can restrict the substrate utilization of a promiscuous RhoGEF family member.

Rho proteins are a subfamily of the Ras superfamily of small GTP binding proteins that control numerous cellular processes including actin organization, cell polarity, adhesion, motility, cell cycle progression, and vesicle transport (21). Due to the central role that Rho proteins play in the control of multiple cellular functions, the deregulation of their activities results in diverse aberrant phenotypes, including cancer. Thus, RhoA, Cdc42, and Rac1 are often overexpressed in breast, colon, and lung cancers, while RhoC is deregulated in pancreatic and inflammatory breast cancer (34).

Rho proteins function as molecular switches that cycle between an inactive GDP-bound form (GDP-Rho), and an active GTP-bound form (GTP-Rho). Once activated, Rho proteins are able to form productive interactions with a wide spectrum of effector molecules which mediate downstream responses (5). The levels of GTP-Rho in cells are tightly regulated by three families of proteins: Rho-specific guanine nucleotide exchange factors (RhoGEFs) that activate GTPases by stimulating the GTP-GDP exchange rate, Rho-specific GTPase-activating proteins (RhoGAPs) that downregulate Rho by stimulating the intrinsic rate of hydrolysis, and Rho-specific guanine nucleotide dissociation inhibitors that sequester Rho in inactive complexes (21).

The RhoGEFs are a large family of proteins that share a conserved Dbl homology/pleckstrin homology (DH/PH) domain tandem motif (33). The DH domain is unique to the RhoGEFs and generally contains all of the residues required for substrate recognition, binding, and exchange. This domain interacts directly with GTPases and preferentially binds substrates that are depleted of nucleotide and Mg^{2+} (37). The PH domains play a role in allosteric regulation of DH domain catalytic activity, membrane localization, and association with phosphoinositides (30, 32). The importance of RhoGEF-mediated regulation of Rho signaling is illustrated by the fact that many members of the RhoGEF family are potent oncogenes, and several have been shown to be rearranged in the context of human developmental disorders or cancer (41). The DH domains of RhoGEFs differ significantly in their substrate utilization. While some RhoGEFs are specific toward one GTPase, others are promiscuous and activate multiple Rho family members (35). Mechanisms controlling the specificity of RhoGEFs in vivo are poorly defined.

Dbs, and its Rat ortholog, Ost, are RhoA/Cdc42-specific RhoGEF family members that were independently identified in screens for cDNAs whose expression causes deregulated growth in NIH 3T3 fibroblasts (18, 40). Like many members of the RhoGEF family, Dbs/Ost has potent transforming activity, as measured by loss of contact inhibition, growth in low serum, anchorage-independent growth, and tumorigenicity in nude mice. Although Dbs activates both RhoA and Cdc42 in vitro, its in vivo exchange specificity seems to be cell type specific (9, 10). For example, Dbs can only activate RhoA when expressed in NIH 3T3 cells (10) and is a selective activator of Cdc42 in 293T cells (9).

Here we describe the characterization of a novel scaffold protein (designated Ccpg1, for cell cycle progression protein 1) that was identified as a binding partner for Dbs. Ccpg1 interacts with multiple RhoGEF family members, Cdc42, and the regulatory kinase, Src. Ccpg1 limits the ability of Dbs to utilize RhoA as a substrate, but not Cdc42. The isolated Dbs binding domain of Ccpg1 is not sufficient to

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suppress Dbs exchange and transforming activity, suggesting that Ccpg1-mediated inhibition of Dbs exchange and transformation requires an intact docking site for Dbs as well as regulatory sequences contained within the COOH terminus of Ccpg1. Thus, Ccpg1 has properties consistent with a structural platform that can assemble Rho signaling complexes and a regulatory protein that can limit the exchange activity of a promiscuous RhoGEF.

MATERIALS AND METHODS

Molecular constructs. The mammalian expression vector pAX142 has been described previously (39). pAX142-*dbs*-HA6 contains a cDNA encoding an oncogenic fragment of murine Dbs (residues 525 to 1097) fused to an NH_2-terminal hemagglutinin (HA) epitope tag (38). pAX142-*dbs*-HA1 encodes the full-length Dbs protein (residues 1 to 1149) fused to a COOH-terminal HA epitope tag (9). The pAX142-*dbs*-HA8 construct encodes residues 523 to 813 of Dbs, fused to an HA epitope tag. pAX142-*dbs*-HA9 encodes residues 791 to 967 of Dbs fused to an HA epitope tag (9). pAX142-*dbs*-HA10 was derived from the pAX142-*dbs*-HA8 plasmid using the Quickchange site-directed mutagenesis kit and encodes residues 623 to 813 of Dbs. The pAX142-*dbl*-HA1 construct encodes the DH/PH domains of Dbl fused to an HA epitope tag (38). pAX142-*bcr-(*415–876) encodes the DH/PH domains of Bcr fused to an HA epitope tag (22). pAX142-*vav*-HA2 encodes residues 66 to 845 of Vav1 (1). pUTSV1-*tiam1*-C1199 contains residues 393 to 1591 of Tiam1 fused to an HA epitope tag (25). pRKB-myc encodes the DH/PH domains of FGD1 (residues 375 to 710) fused to a myc epitope tag (29). pCDNA3-*cSrc*-HA encodes the full-length human Src kinase fused to a COOH-terminal HA epitope tag (26). pCDNA-Src527F contains a non-epitope-tagged constitutively active mutant of Src and was provided by Channing Der (University of North Carolina). The pAX142-*rhoA*, pAX142-*rhoA*(63L), and pAX142-*rhoA*(19N) constructs have been described previously (42). The pAX142-*cdc42*, pAX142-*cdc42*(12V), and pAX142-*cdc42*(17N) constructs have been described previously (38). Glutathione *S*-transferase (GST)–PDB and GST-C21 contain the Rho binding domains of Ccd42/Rac1 effector kinase PAK3 (3) and the RhoA effector protein Rhotekin (31), respectively. The $(SREm)_2$-*luc* and pAX142-β-galactosidase constructs used in the transcriptional assays have been described previously (38).

Molecular cloning of the Ccpg1 cDNA. The pAX142-*ccpg1* construct contains the full-length cDNA for the murine protein Ccpg1 fused to an NH_2-terminal FLAG epitope tag. The Ccpg1 cDNA was obtained by reverse transcription-PCR (RT-PCR)-based cloning using total RNA derived from NIH 3T3 mouse fibroblasts. The cDNA was synthesized using the SuperScript Choice system (Invitrogen) according to the manufacturer's instructions. Primers for PCR were designed based on the reported genomic sequence (Entrez Gene identification [ID] no. 72278, accession no. BC043049). The full-length Ccpg1 cDNA was cloned into the MluI site of the pAX142 vector and then fused to an NH_2-terminal FLAG epitope tag by PCR-based cloning. pAX142-*ccpg1*(1–307) encodes the 307 NH_2-terminal residues of Ccpg1 fused to an NH_2-terminal FLAG tag. The Ccpg1 deletion mutant constructs pAX142-*ccpg1*Δ311, pAX142-*ccpg1*Δ311, pAX142-*ccpg1*Δ457, and pAX142-*ccpg1*Δ638 were derived from the pAX142-*ccpg1* plasmid using PCR-based cloning. The pAX142-*ccpg1*ΔTransm construct encodes full-length Ccpg1 containing an internal deletion of the transmembrane domain (residues 219 to 241). cDNA sequences of all constructs were verified by automated sequencing.

Cell culture and transfection. 293T and COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose) supplemented with 10% fetal bovine serum (Benchmark; Gemini Biosciences) at 37°C with 10% CO_2. NIH 3T3 cells were maintained as described above in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (JRH). Cells were transfected with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Transformation assays. Primary focus formation assays were performed in NIH 3T3 cells as described previously (11). Briefly, NIH 3T3 cells were transfected using Lipofectamine 2000 reagent (Invitrogen). Focus formation was scored at 14 days by staining with 0.5% crystal violet.

Antibodies and immunoblotting. The following antibodies were used in the present study: mouse anti-Cdc42 (B-8; Santa Cruz), mouse anti-RhoA (26C4; Santa Cruz), rabbit anti-HA (Y-11; Santa Cruz), mouse anti-FLAG (M2; Sigma), anti-Myc (9E10; Santa Cruz), and rabbit anti-Src (Cell Signaling). Protein expression was detected by Western blot analysis using a secondary antibody conjugated to horseradish peroxidase (Calbiochem) and Luminol reagent (Santa Cruz). Quantitative Western blot analysis was performed using a secondary antibody conjugated to infrared fluorescent dyes (Alexa Fluor-680 from Molecular Probes or IRDye-800CW from Rockland). Membranes were imaged using a LI-COR Odyssey infrared imager, and the obtained integrated intensities of the relevant bands were used for quantitation.

Coimmunoprecipitations. Coimmunoprecipitations in COS7, 293T, and NIH 3T3 cells were performed as described previously (24). Proteins of interest were immunoprecipitated with anti-FLAG-M2 affinity gel by rotation at 4°C for 2 h. The immunoprecipitates were washed three times with cold lysis buffer and then resuspended in loading dye.

RhoA and Cdc42 activation assays. GST-PBD and GST-C21 were expressed as glutathione *S*-transferase fusions in *Escherichia coli* strain BL21(DE3) and were purified by immobilization on Sepharose 4B beads (Amersham) as described previously (28). 293T or COS7 cells were transfected using Lipofectamine 2000 reagent, incubated for 24 h, and serum starved in DMEM containing 0.5% serum for 16 h prior to harvesting the lysates. Total levels of endogenous Cdc42 and overexpressed HA-tagged RhoA were measured by quantitative Western blot analysis and normalized prior to affinity purification. Activated GTP-bound Cdc42 or RhoA was precipitated from the cell lysates using GST-PBD or GST-C21, respectively, according to the procedure described previously (10).

Transient-expression reporter gene assays. NIH 3T3 cells were cotransfected using Lipofectamine 2000 reagent (Invitrogen) with the reporter construct $(SREm)_2$-*luc*, β-galactosidase, and plasmids encoding proteins of interest. Cells were incubated for 24 h, and serum starved in DMEM containing 0.5% serum for 16 h. Luciferase activity was measured as described previously with enhanced chemiluminescence reagents using a Monolight 3010 luminometer (Analytical Luminescence) (22). β-Galactosidase activity was measured using Lumi-Gal substrate (Lumigen) according to the manufacturer's instructions. Luciferase activity was standardized relative to β-galactosidase activity. All experiments were performed three times on duplicate plates.

Immunostaining. Immunostaining of transiently transfected NIH 3T3 cells was performed as described previously (32). Briefly, NIH 3T3 cells were transiently transfected with 2 μg of plasmids encoding proteins of interest using the Lipofectamine 2000 reagent (Invitrogen). Ccpg1 expression was visualized using anti-FLAG antibody and red fluorescent Alexa Fluor 568-conjugated goat anti-mouse immunoglobulin G (Molecular Probes). Images were acquired using an Axiovert 200 M inverted fluorescence microscope (Zeiss) equipped with a ×100 oil-immersion objective, a CoolSNAP HQ camera (Photometrics, Pleasanton, CA) and Openlab acquisition software (Improvision, Sheffield, United Kingdom). Images were obtained by deconvolution from nine optical sections that were 0.2 μm apart. The maximum projection of the middle optical section of the deconvolved sections was exported into the TIF format and then processed using Adobe Photoshop 6.0 software.

Real-time RT-PCR assay of Ccpg1 expression. Total RNA was extracted using a QIAGEN RNeasy Mini kit according to the manufacturer's recommendations and quantified by spectrophotometer (A_{260}/A_{280}). Primer sets were designed using Primer3 software to recognize the mouse (forward, GAAGCGGCAACT GAAAAGAC; reverse, GAACAAACCCTTCTGGTGGA) or human (forward, GTCACACTTTTTCCCCTCCA; reverse, CTCAGTGGCCATAAAGCACA) forms of Ccpg1. Real-time RT-PCR was conducted using an iScript one-step RT-PCR kit with SYBR green (Bio-Rad) and a Rotor Gene-3000 thermocycler (Corbett research). 18S rRNA was used as the internal standard. A previously tested primer set was used to amplify 18S (rRNA) sequences (27). Expression levels of Ccpg1 mRNA in cultured cell lines were determined by the standard curve method using Rotor-Gene6 software.

siRNA inhibition of Ccpg1 expression. Predesigned small interfering RNA (siRNA) oligonucleotides targeted to human Ccpg1 mRNA (siRNA ID no. 140654, 15273, and 15177) and negative control siRNA (siRNA ID no. 4611) were purchased from Ambion. 293T cells were transfected with an siRNA cocktail containing equimolar amounts of Ccpg1 siRNA or scrambled control at a 150 nM concentration using SilentFect lipid reagent (Bio-Rad) according to the manufacturer's recommendation. Twenty-four hours later, cells were transfected with 3 μg of pAX142-*dbs*-HA6 plasmid or cognate vector using Lipofectamine 2000 reagent. Cells were incubated in growth medium for 24 h and serum starved in DMEM containing 0.5% serum for 16 h prior to harvesting the lysates. Levels of GTP-bound RhoA were measured by affinity precipitation assays as described above. Before cell lysis, approximately 2×10^5 cells were set aside for the analysis of Ccpg1 expression by RT-PCR.

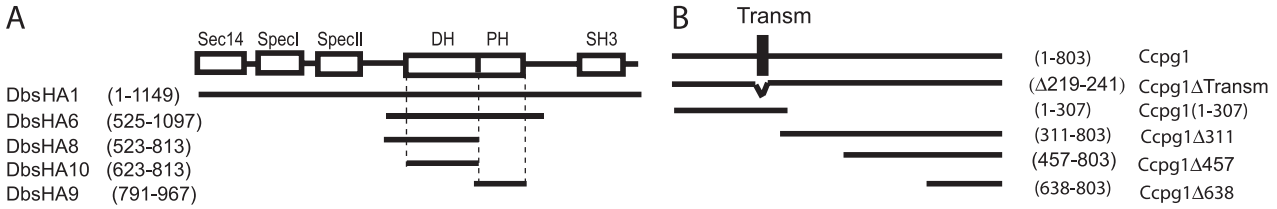

FIG. 1. Schematic representation of the domain structure of the Dbs (A) and Ccpg1 (B) proteins. Transm, transmembrane domain; Sec14, Sec14 homology domain; Spec I and Spec II, spectrin repeats I and II; SH3, Src homology domain. Numbering corresponds to proto-Dbs (A) or -Ccpg1 (B) amino acid sequences. Lines indicate the regions of the proteins included in the predicted translational products of the various truncation mutants.

RESULTS

Cell cycle progression 1 protein a novel binding partner for Dbs. The full-length Dbs protein contains several recognizable functional domains that include a regulatory NH_2-terminal Sec14 homology domain, spectrin repeats, a COOH-terminal SH3 domain, and a centrally located DH/PH domain module (Fig. 1A). In order to identify novel proteins involved in the regulation of Dbs, we analyzed the publicly available protein-protein interaction database (http://www.signaling-gateway.org/) of the Alliance for Cellular Signaling that contains data of high-throughput yeast two-hybrid screens. Ccpg1 was described in this database as a binding partner for murine Dbs.

Initially, we cloned the full-length mouse Ccpg1 cDNA by RT-PCR using total RNA derived from mouse NIH 3T3 fibroblasts. The Ccpg1 cDNA encodes an 803-amino-acid protein with a predicted molecular mass of approximately 88 kDa. An analysis of the Ccpg1 domain structure using the Modular Architecture Research Tool database revealed the presence of a transmembrane domain (residues 219 to 241) in the NH_2-terminal portion of the protein, but did not identify any known functional or protein-protein interaction motifs (Fig. 1B). Using the PHI-BLAST sequence analysis tool, we identified Ccpg1 homologs in rats, dogs, chickens, chimpanzees, and humans. The human ortholog of Ccpg1, also called CPR8 (for cell cycle progression restoration protein 8), was previously identified in a screen for human cDNAs that could overcome G_1 cell cycle arrest in *Saccharomyces cerevisiae* in response to the activation of the mating pheromone pathway (12). Although a cellular function for human CPR8 was not determined in this study, this data was consistent with a role in growth regulation. Since both Dbs and Ccpg1 have been implicated in growth regulatory pathways, we investigated the functional consequences of their interaction.

Dbs interacts with Ccpg1 in mammalian cells. First, we determined whether Dbs interacts with Ccpg1 in mammalian cells. For this analysis, we coexpressed full-length FLAG epitope-tagged Ccpg1 and full-length HA epitope-tagged Dbs in COS7 cells (Fig. 2A). For comparison, we also coexpressed FLAG-Ccpg1 with an HA epitope-tagged oncogenic derivative of Dbs (DbsHA6). FLAG-Ccpg1 was immunoprecipitated from the cell lysates with an anti-FLAG monoclonal antibody, and immunoprecipitates were analyzed for the presence of the Dbs protein by Western blot analysis using an anti-HA antibody. As shown in Fig. 2A, we could readily detect an interaction between Ccpg1 and both oncogenic and full-length Dbs in COS7 cells. Since DbsHA6 contains the isolated RhoGEF domain of Dbs, this data suggests that the docking site for Ccpg1 resides within the DH/PH domain tandem modules. Using an equivalent approach, we could also detect binding of DbsHA6 and Ccpg1 in 293T and NIH 3T3 cells (not shown).

Ccpg1 interacts with the DH and PH domains of Dbs. To further map the binding site of Ccpg1 within the Dbs DH/PH domain module, we subdivided this region into smaller fragments (Fig. 1A) and coexpressed them with full-length FLAG epitope-tagged Ccpg1 (Fig. 2B). FLAG-Ccpg1 was immunoprecipitated from cell lysates with an anti-FLAG monoclonal antibody, and immunoprecipitates were analyzed for the presence of the Dbs derivatives by Western blot analysis using an anti-HA antibody. As shown in Fig. 2B, we could detect binding of Ccpg1 with the Dbs-HA8 and Dbs-HA10 constructs, both of which encode the DH domain of Dbs, suggesting that residues 623 to 813 contain a binding site for Ccpg1. We could also detect a weak interaction between Ccpg1 and a Dbs fragment containing the isolated PH domain (DbsHA9), suggesting the existence of a second binding site.

Ccpg1 interacts with additional RhoGEF family members. Since we found that the DH domain contains a docking site for Ccpg1, we wondered whether Ccpg1 interacts with other RhoGEF family members. We assembled a panel of RhoGEFs with well-characterized in vitro exchange activities. Lsc is an

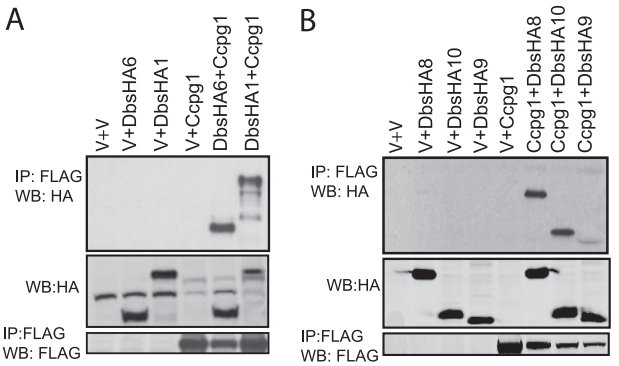

FIG. 2. Ccpg1 interacts with Dbs in mammalian cells. COS7 cells were transiently transfected with the indicated combinations of plasmids (V, cognate vector). Cell lysates were examined by Western blotting for expression of the Dbs fragments using an anti-HA antibody (WB: HA). Coimmunoprecipitations were then performed using an anti-FLAG affinity gel, and precipitates were examined by Western blotting with anti-HA antibody to detect the interactions. IP, antibody used for immunoprecipitations; WB, antibody used for Western blotting.

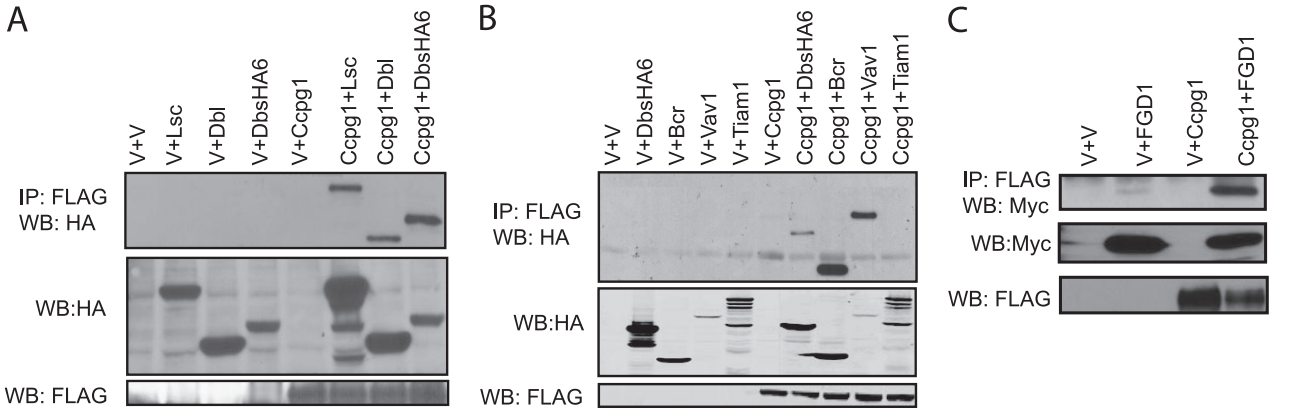

FIG. 3. Ccpg1 interacts with additional RhoGEF family members. COS7 cells were transiently transfected with the indicated combinations of plasmids (V, cognate vector). Cell lysates were examined by Western blotting for expression of the RhoGEFs using an anti-HA or anti-Myc antibody (WB: HA or WB: Myc, respectively). Coimmunoprecipitations were then performed using an anti-FLAG affinity gel, and precipitates were examined by Western blotting with an anti-HA (A and B) or anti-Myc (C) antibody to detect interactions. IP, antibody used for immunoprecipitations; WB, antibody used for Western blotting.

exchange factor that is specific for RhoA (14), while FGD1 and Tiam1 are specific for Cdc42 (44) and Rac1 (17), respectively. Dbl, Bcr, and Vav1 have broader exchange specificity. Dbl and Bcr are active toward RhoA and Cdc42 (16, 22), while Vav1 is active toward Rac1, RhoA, and Cdc42 (29). We coexpressed FLAG-Ccpg1 along with the DH/PH modules of HA-tagged Dbl, Lsc, Vav1, BCR, and Tiam1 (Fig. 3A and B), or myc epitope-tagged FGD1 (Fig. 3C) in COS7 cells. FLAG-Ccpg1 was immunoprecipitated from cell lysates with an anti-FLAG monoclonal antibody, and immunoprecipitates were analyzed for the presence of RhoGEFs by Western blot analysis using an anti-HA antibody or anti-myc antibody. As shown in Fig. 3, we could detect an interaction between Ccpg1 and either Dbl, Lsc, BCR, Vav, or FGD1, but not Tiam1. Thus, Ccpg1 has the capacity to interact with multiple members of the RhoGEF family.

Ccpg1 interacts with Cdc42. Since Ccpg1 interacts with the DH/PH domain modules of Dbs, which is a RhoA- and Cdc42-specific RhoGEF, we wondered if it can also interact with its substrates. In cells, Rho GTPases exist in two functionally distinct forms: an inactive GDP-bound form and an active GTP-bound form. Cdc42(12V) and RhoA(63L) are constitutively active mutants that accumulate in cells in the GTP-bound form, while Cdc42(17N) and RhoA(19N) are dominant inhibitory mutants that accumulate in the GDP-bound form. We coexpressed FLAG-Ccpg1 with either wild-type Cdc42 [Cdc42(WT)], Cdc42(12V), Cdc42(17N), wild-type RhoA [RhoA(WT)], RhoA(63L), or RhoA(19N) in COS7 cells and performed immunoprecipitations using an anti-FLAG antibody. Immunoprecipitates were analyzed for the presence of Cdc42 or RhoA using mouse anti-Cdc42 or anti-RhoA antibodies, respectively. As shown in Fig. 4A, we could readily detect an interaction between Ccpg1 and Cdc42(12V), but not Cdc42(WT) or Cdc42(17N). In contrast, we could not detect an association between Ccpg1 and any form of RhoA (Fig. 4B). This was not due to lack of sensitivity of the assay, since we could readily detect the interaction between DbsHA6 and Ccpg1, which was included as a positive control.

Ccpg1 restricts the exchange specificity of Dbs. Since the DH/PH modules of RhoGEFs contain all of the residues necessary for the exchange of GTP to GDP in the guanine nucleotide exchange reaction, it is reasonable to expect that proteins interacting with the DH/PH regions of GEFs could be involved in control of their activity or exchange specificity. Thus, we

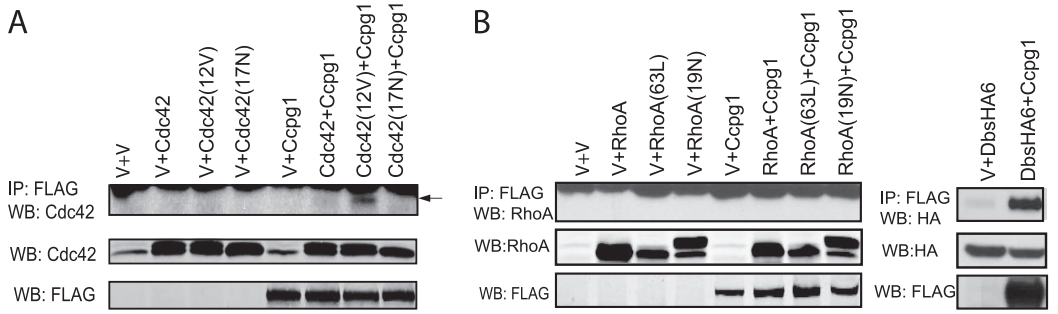

FIG. 4. Ccpg1 interacts with RhoGTPases. COS7 cells were transiently transfected with the indicated combinations of plasmids (V, cognate vector). Cell lysates were examined by Western blotting for expression of Cdc42 (A), nontagged RhoA (B), and DbsHA6 (B) using an anti-Cdc42, anti-RhoA, and anti-HA antibody, respectively. Coimmunoprecipitations were then performed using an anti-FLAG affinity gel, and precipitates were examined by Western blotting with an anti-Cdc42 (A), anti-RhoA (B), and anti-HA (B) antibody to detect the interactions. IP, antibody used for immunoprecipitations; WB, antibody used for Western blotting. An arrow indicates the position of the Cdc42-specific band.

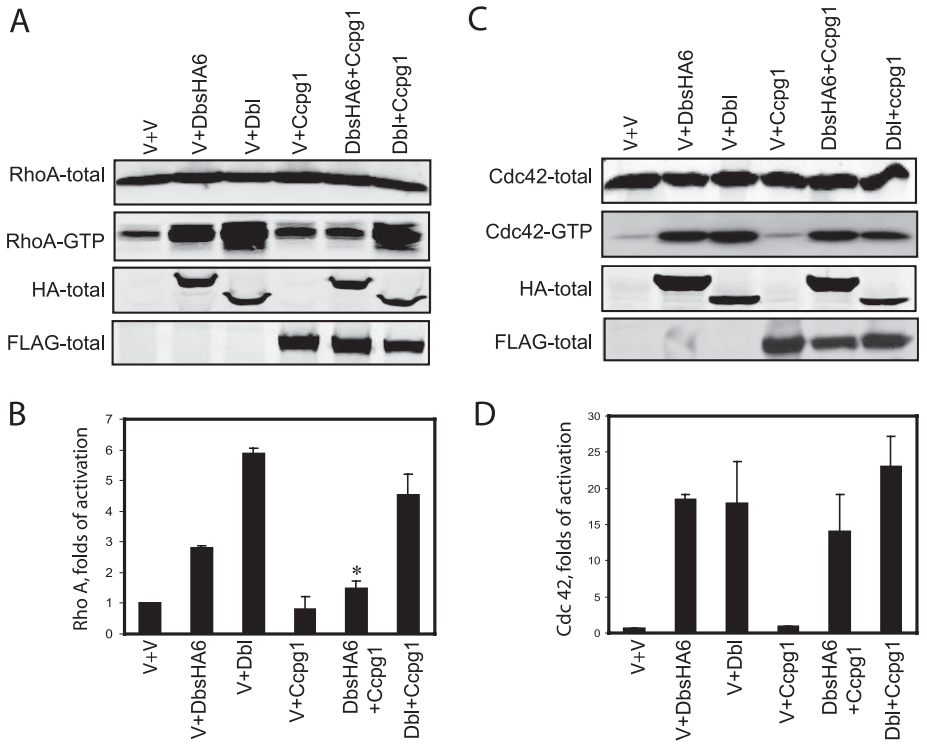

FIG. 5. Ccpg1 inhibits Dbs exchange activity toward RhoA but not Cdc42. COS7 cells (A) and 293T cells (C) were transiently transfected with the indicated combinations of plasmids (V, cognate vector). (A) HA-tagged wild-type RhoA protein was overexpressed under all conditions. Cell lysates were examined by quantitative Western blotting for expression of Dbs, Dbl (HA-total), and Ccpg1 (FLAG-total) using anti-HA and anti-FLAG antibodies, respectively. Total expression levels of RhoA (A [RhoA-total]) and Cdc42 (C [Cdc42-total]) were examined by quantitative Western blotting using an anti-HA or anti-Cdc42 antibody, respectively. Lysates were normalized for the expression of RhoA or Cdc42 and then subjected to affinity purification with GST-Rhotekin (A) or GST-PBD (C). GTP-bound RhoA (RhoA-GTP) or Cdc42 (Cdc42-GTP) was analyzed by quantitative Western blotting using an anti-HA or anti-Cdc42 antibody, respectively. (B and D) Relative levels of in vivo exchange activity of Dbs and Dbl proteins in the presence of the full-length Ccpg1 toward RhoA and Cdc42, respectively. Quantitations were performed using an Odyssey IR imager and expressed as integrated intensity units. The data presented were obtained by normalization for the expression levels of Dbs and Dbl proteins. The data shown are representative of three independent experiments and represent means and standard deviation of activation (fold). Significance of difference from the control was estimated by Student's *t* test for nonpaired values. An asterisk signifies $P < 0.01$. Bars not significantly different from the control values were left unmarked.

wondered whether the interaction of Ccpg1 with Dbs could affect its exchange activity. We coexpressed Ccpg1 and the Dbs protein in mammalian cells and tested its effect on the exchange activity of Dbs toward its target GTPases using affinity precipitation assays and quantitative Western blot analysis (Fig. 5).

Although RhoA and Cdc42 are known substrates for Dbs, the exchange activity of Dbs toward its target GTPases is restricted in a cell-type-dependent manner. Thus, Dbs can only activate endogenous RhoA in NIH 3T3 cells and endogenous Cdc42 in 293T cells, and it cannot utilize either substrate in COS7 cells (9, 10). Interestingly, Dbs can activate overexpressed RhoA in COS7 and 293T cells, suggesting that an inhibitory mechanism may be in place in these cell types. Initially, we tested the effect of Ccpg1 expression toward Dbs exchange activity on RhoA. We coexpressed FLAG-Ccpg1 along with DbsHA6 and HA epitope-tagged RhoA in COS7 cells. For comparison, we also included Dbl in this analysis since it is also a RhoA/Cdc42 exchange factor that interacts with Ccpg1 in this cell type. Consistent with our previous observations, cells expressing Dbs or Dbl alone displayed elevated levels of GTP-RhoA (Fig. 5A). Expression of Ccpg1 led to 40 to 60% inhibition of Dbs exchange activity toward RhoA (Fig. 5B). Similar data were obtained in NIH 3T3 cells (not shown). Surprisingly, the activity of the related GEF, Dbl, was not significantly affected by the expression of Ccpg1.

Next, we tested the effect of Ccpg1 expression on the exchange activity of Dbs toward Cdc42. For this analysis, FLAG-Ccpg1 was coexpressed with the DH/PH domains of Dbs or Dbl, in 293T cells, and the levels of endogenous Cdc42-GTP were measured. As shown in Fig. 5C and D, expression of Ccpg1 did not significantly reduce the exchange activity of Dbs or Dbl toward Cdc42. In fact, when we normalize against total Cdc42 and Dbl expression, we consistently see slightly elevated levels of Cdc42-GTP in the Dbl-expressing cell (Fig. 5D). We conclude that Ccpg1 can specifically inhibit Dbs exchange on RhoA, but not Cdc42. The effect of Ccpg1 expression on the exchange activity of other GEFs capable of interaction with this protein is under investigation.

Expression of Ccpg1 inhibits the transforming activity of Dbs, but not Dbl, in NIH 3T3 cells. Our previous studies showed that RhoA is a physiological target of Dbs-mediated transformation in NIH 3T3 cells. Since our in vivo GTP loading studies suggest that Ccpg1 expression inhibits Dbs ex-

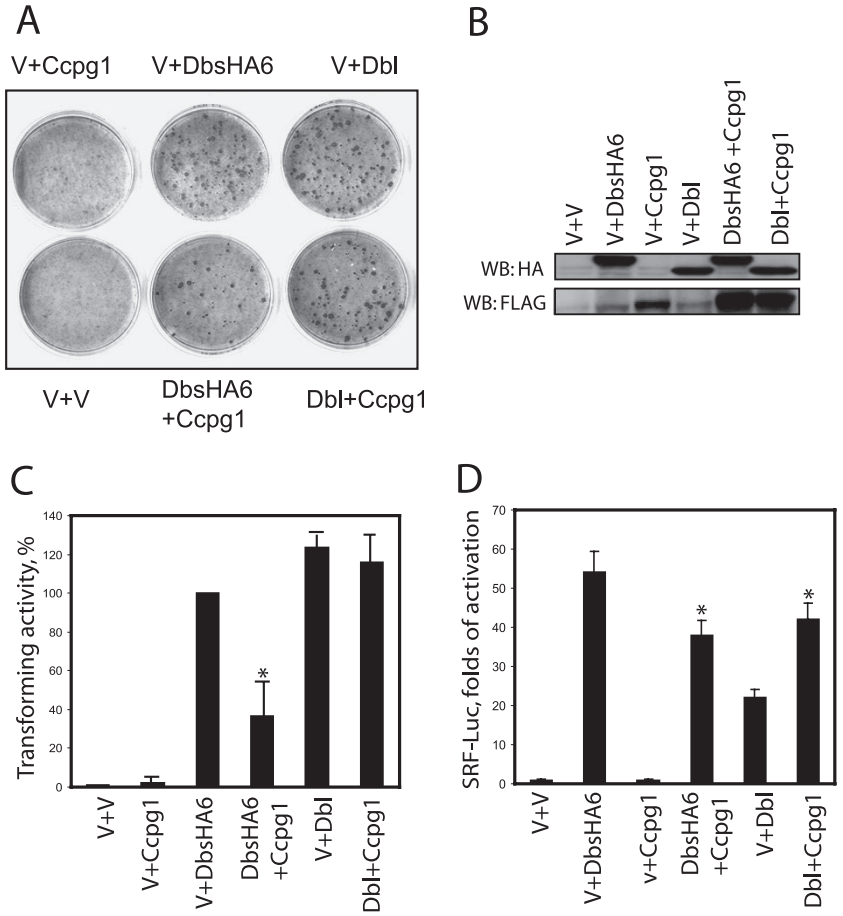

FIG. 6. Ccpg1 inhibits Dbs-mediated transformation and transcriptional activation. (A) Representative primary focus formation assay in NIH 3T3 cells. Transformation assays were performed as described in Materials and Methods (V, cognate vector). (B) Expression of the plasmids in NIH 3T3 cells was confirmed by Western blot analysis using anti-HA (WB: HA) or anti-FLAG (WB: FLAG) antibody. (C) Relative transforming activity of Dbs and Dbl oncogenes in the presence of Ccpg1. The relative transforming activity of the oncogenic Dbs was set as 100%. The data shown are representative of three independent experiments performed on duplicate plates and represent means and standard deviation of foci generated. (D) Transient-expression reporter gene assay in NIH 3T3 cells. NIH 3T3 cells were transiently transfected with the indicated combinations of plasmids along with the reporter construct $(SREm)_2$-*luc* and pAX142-β-galactosidase. Luciferase activity was normalized relative to β-galactosidase activity and then expressed as activation (fold) relative to the vector control. Data shown are representative of three independent experiments and represent means and standard deviation of activation (fold). Significance from the control was estimated by Student's *t* test for nonpaired values. An asterisk signifies $P < 0.01$. Bars not significantly different from the control values were left unmarked.

change activity toward RhoA, we wondered whether the expression of Ccpg1 also inhibits Dbs transforming activity. For this analysis, we coexpressed oncogenic derivatives of Dbs or Dbl, along with Ccpg1, in NIH 3T3 cells and performed a focus-formation assay (Fig. 6A and C). Consistent with the reduced RhoA activation, Dbs transforming activity was reduced up to 60% in the presence of Ccpg1. This reduction in transforming activity could not be attributed to differences in Dbs expression (Fig. 6B). Ccpg1 had no significant effect on the transforming activity of Dbl (Fig. 6A and C), which is consistent with the exchange data.

Expression of Ccpg1 inhibits transcriptional activation by Dbs. Our previous studies showed that Dbs can activate a serum response element reporter plasmid [$(SREm)_2$-*luc*] in NIH 3T3 cells in a RhoA-dependent manner (10). To further investigate the effect of Ccpg1 expression on Dbs signaling, we performed a transcription-coupled reporter assay using this construct (Fig. 6D). Consistent with the transformation and exchange assays, coexpression with Ccpg1 in NIH 3T3 cells inhibited activation of this reporter by Dbs. Interestingly, we observed a twofold increase in Dbl-mediated activation of the reporter in the presence of Ccpg1, which may reflect the elevated Cdc42 exchange activity that we observed in the affinity precipitation assays.

Ccpg1 localizes to the intracellular membranes where it colocalizes with a cytosolic pool of oncogenic Dbs. Next, we tested if Ccpg1 and Dbs colocalize when coexpressed in mammalian cells. In previous studies, we have observed that Dbs localizes to both the plasma membrane and the cytosol and that the cytosolic activity might be responsible for transformation (23). FLAG epitope-tagged Ccpg1 and HA-tagged DbsHA6 were coexpressed in NIH 3T3 cells, and their intracellular distribution was determined by indirect immunofluorescence using anti-FLAG or anti-HA antibodies, respectively. In accordance with our previous observations, DbsHA6 was found in both the cytosol, and the plasma membrane,

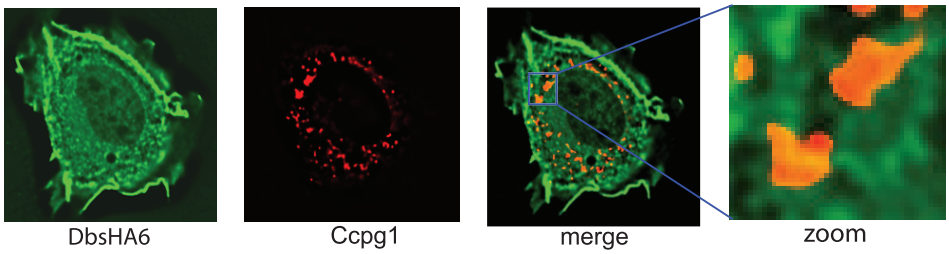

FIG. 7. Ccpg1 colocalizes with a cytosolic pool of Dbs. NIH 3T3 cells were transiently transfected with the indicated plasmids. Intracellular localization of Ccpg1 and DbsHA6 was visualized by indirect immunofluorescence using an anti-FLAG or anti-HA antibody, respectively. Images were obtained by deconvolution from nine optical sections that were 0.2 μm apart. The maximum projections of the middle optical section of the deconvolved sections are presented. Zoom indicates electronically enlarged area of intracellular membranes containing Ccpg1 and Dbs proteins.

where it was associated with membrane ruffles (Fig. 7). Sequence analysis of Ccpg1 predicts the existence of a transmembrane domain within the NH_2 terminus. Accordingly, we found that Ccpg1 localizes to punctate intracellular structures that are distributed throughout the cytosol, but with predominant staining in the perinuclear region. Within these structures, Ccpg1 colocalized with a cytosolic pool of Dbs (Fig. 7), which would be consistent with the inhibition of Dbs-mediated transformation that we observed in this cell type.

Knockdown of Ccpg1 expression by siRNA causes potentiation of Dbs exchange activity toward RhoA. To directly determine whether endogenous Ccpg1 can restrict Dbs exchange activity toward RhoA, we used siRNA to suppress Ccpg1 expression. First we compared expression levels of Ccpg1 in the cultured cell lines used in this study by real-time RT-PCR analysis. As shown in Fig. 8A, NIH 3T3 cells contain only residual levels of Ccpg1, while 293T cells have intermediate levels and COS7 cells express the largest amount of Ccpg1 mRNA. The presence of Ccpg1 correlates with the ability of Dbs to activate endogenous RhoA, with NIH 3T3 cells being permissive for such activation, while 293T and COS7 cells are restrictive (9, 10; this study). Next, 293T cells were transiently transfected with Ccpg1-specific siRNAs, incubated for 24 h to achieve down-regulation of Ccpg1 expression, and then transfected with plasmids encoding DbsHA6 or cognate vector. Levels of GTP-bound RhoA were then measured using affinity precipitation assays. As shown in Fig. 8B, we could achieve up to 65% inhibition of Ccpg1 expression in 293T cells by siRNA as measured by real-time RT-PCR. Under these conditions, Dbs was able to consistently activate endogenous RhoA in this cell type compared to vector or scrambled siRNA controls (Fig. 8C). These data link Ccpg1 and RhoA signaling in these cells and support an in vivo role for Ccpg1 as a regulatory protein involved in the regulation of the exchange specificity of a RhoGEF.

An interaction with Ccpg1 is not sufficient to limit Dbs transforming and exchange activity. To further understand the mechanism of Ccpg1-mediated regulation of Dbs transforming and catalytic activity, we mapped the binding site for Dbs within Ccpg1. Using secondary structure prediction algorithms, we developed a panel of FLAG epitope-tagged Ccpg1 deletion mutants (Fig. 1B). We then coexpressed DbsHA6 along with each panel member in COS7 cells and performed coimmunoprecipitation experiments using an anti-FLAG antibody. As shown in Fig. 9A, we could detect binding of Dbs to an NH_2-terminal fragment of Ccpg1 (residues 1 to 307). As shown in

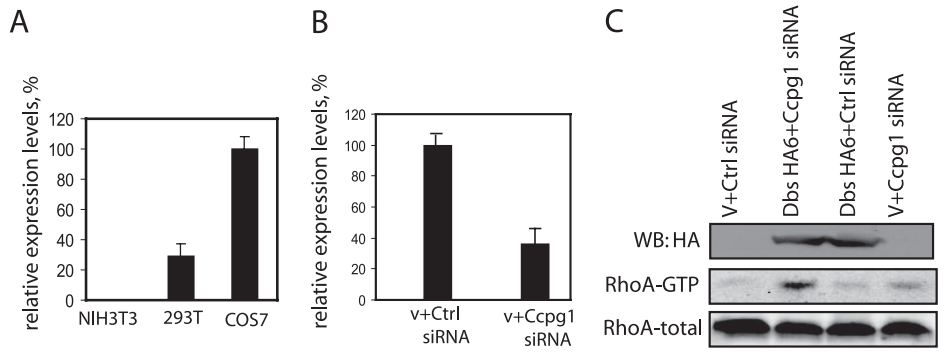

FIG. 8. Suppression of endogenous Ccpg1 expression potentiates Dbs exchange activity toward RhoA in 293T cells. (A) Relative expression levels of Ccpg1 mRNA in the indicated cell lines were measured by real-time RT-PCR. Expression levels of Ccpg1 mRNA in COS7 cells were set at 100%. (B) Inhibition of Ccpg1 expression in 293T cells by siRNA targeted to Ccpg1 (Ccpg1 siRNA) as measured by real-time RT-PCR. Expression levels of Ccpg1 in cells treated with the negative control siRNA (Ctrl siRNA) was set as 100%. For panels A and B, the data shown are an average of three independent experiments and represent means and standard deviation of the relative Ccpg1 expression levels. (C) 293T cells were transfected with Ccpg1 siRNA or control siRNA and incubated for 24 h. Then cells were transfected with plasmids encoding DbsHA6 or cognate vector, incubated for 24 h, and then serum starved for 18 h. siRNA-mediated inhibition of Ccpg1 expression was confirmed by real-time RT-PCR. Cell lysates were examined by Western blotting for expression of DbsHA6 (WB: HA) or total RhoA (RhoA-total) and then subjected to affinity purification with GST-Rhotekin as described in Materials and Methods. Levels of GTP-bound RhoA (RhoA-GTP) were analyzed by Western blotting using an anti-RhoA antibody.

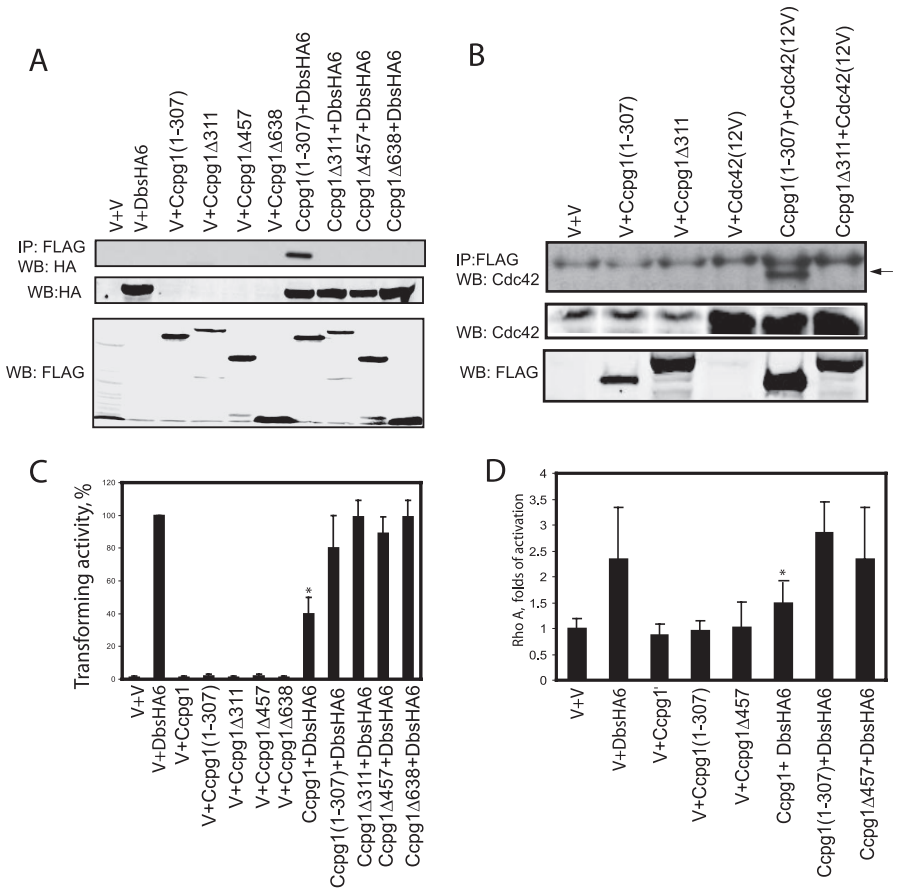

FIG. 9. Binding to Ccpg1 is not sufficient to limit Dbs transforming and exchange activity. (A and B) Coimmunoprecipitations were used to map the docking sites in Ccpg1 for Dbs (A) and Cdc42(12V) (B). COS7 cells were transiently transfected with the indicated combinations of plasmids. Cell lysates were examined by Western blotting for the expression of Dbs (A) or Cdc42(12V) (B) using an anti-HA antibody (WB: HA) or anti-Cdc42 antibody (WB: Cdc42). Coimmunoprecipitations were then performed using an anti-FLAG affinity gel, and precipitates were examined by Western blotting with an anti-HA (A) or anti-Cdc42 (B) antibody to detect the interactions. IP, antibody used for immunoprecipitations; WB, antibody used for Western blotting. An arrow indicates the position of the Cdc42-specific band. (C) Relative transforming activity of Dbs in the presence of the full-length Ccpg1 and the Ccpg1 deletion mutants as assayed by primary focus formation assay in NIH 3T3 cells. Transformation assays were performed as described in Materials and Methods and the legend to Fig. 6. (D) Relative levels of in vivo exchange activity of Dbs toward RhoA in the presence of the full-length Ccpg1 and Ccpg1 deletion mutants are shown. Affinity precipitation assays and quantitations were performed as described in Fig. 5. The data shown are representative of two independent experiments and represent means and standard deviation of activation (fold). Significance of difference from the control was estimated by Student's *t* test for nonpaired values. An asterisk signifies $P < 0.01$. Bars not significantly different from the control values were left unmarked.

Fig. 9B, we could also detect binding of Cdc42(12V) to the equivalent fragment.

Next, we tested the panel members for their ability to modulate Dbs transforming activity. As shown in Fig. 9C, the fragments of Ccpg1 that do not bind Dbs also do not inhibit Dbs transforming activity. However, the NH_2-terminal fragment of Ccpg1 that contains the Cdc42 and Dbs docking sites (residues 1 to 307) could not significantly inhibit Dbs-mediated transformation. This suggests that simply binding to Dbs is not sufficient for inhibition and that sequences which lie in the COOH terminus of Ccpg1 may actively limit Dbs biological activity. To confirm that the COOH terminus of Ccpg1 is inhibitory with respect to Dbs exchange activity, we performed in vivo exchange assays for Dbs in the presence of our panel of Ccpg1 deletion mutants (Fig. 9D). In agreement with the results of the transformation assay, we found that the Ccpg1 fragments that lack the Dbs binding site, or which lack the putative COOH-terminal regulatory domain, failed to block Dbs exchange activity toward exogenously expressed RhoA in COS7 cells. We conclude that Ccpg1-mediated inhibition of Dbs exchange and transformation requires an intact docking site for Dbs as well as the regulatory sequences contained within the COOH terminus of Ccpg1. Interestingly, removal of the transmembrane domain did not affect the ability of Ccpg1 to interact with Dbs and inhibit its transforming activity in NIH 3T3 cells (data not shown), suggesting that Ccpg1 does not simply inhibit Dbs by sequestering it onto intracellular membranes.

Src kinase interacts with COOH-terminal and NH_2-terminal fragments of Ccpg1. The COOH-terminal domain of Ccpg1 does not contain any known functional protein-protein interaction domains or catalytic domains. Since the COOH terminus is necessary for regulation of Dbs activity, we wondered if this domain could function as a docking site for regulatory molecules. Src family kinases play an important role in

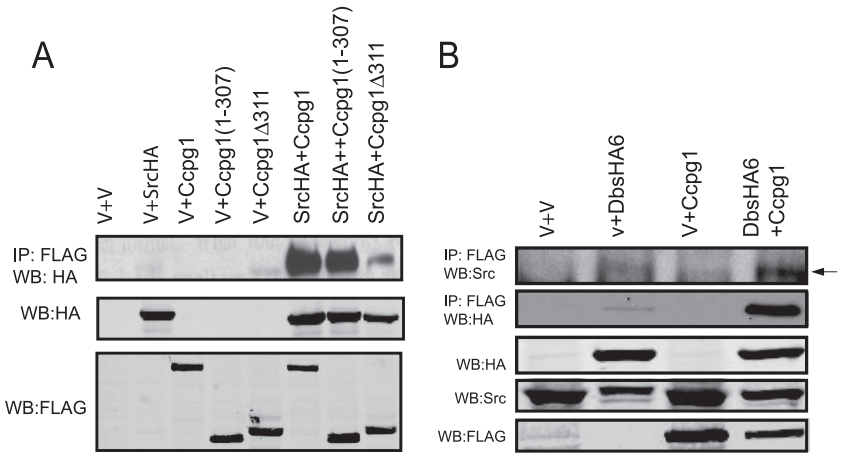

FIG. 10. Ccpg1 interacts with Src kinase and recruits it to Dbs-containing complexes. (A) COS7 cells were transfected with indicated plasmids. Coimmunoprecipitations were then performed using an anti-FLAG affinity gel, and precipitates were examined by Western blotting with an anti-HA (A) antibody to detect the interactions. (B) 293T cells were transfected with the indicated plasmids. An anti-FLAG affinity gel was used to precipitate Ccpg1/DbsHA6-containing complexes. Immunoprecipitates were then examined for the presence of HA-tagged DbsHA6 and endogenously expressed Src kinase using anti-HA or anti-Src antibodies, respectively. IP, antibody used for immunoprecipitations; WB, antibody used for Western blotting.

regulation of Rho-mediated signaling events (13). It was shown that Src kinase is required for the activation of Dbs downstream of the α1B-adrenergic receptor in 293T cells (43) and of Vav through phosphorylation of regulatory tyrosine residues (2). Initially, we tested if Src kinase could interact with Ccpg1 when coexpressed in mammalian cells. We coexpressed HA epitope-tagged full-length Src along with full-length and truncation mutants of Ccpg1 (Fig. 10A). Coimmunoprecipitations were performed using an anti-FLAG antibody, and immunoprecipitates were analyzed for the presence of Src by Western blot analysis using an anti-HA antibody. As shown in Fig. 10A, we could detect strong binding of Src kinase to full-length Ccpg1 and the NH_2-terminal domain of Ccpg1 containing the Dbs binding site. Interestingly, we also observed binding of Src to the COOH-terminal domain of Ccpg1, suggesting that the COOH terminus of Ccpg1 could also serve as a docking site for regulatory signaling molecules. In a parallel coimmunoprecipitation experiment, we were unable to detect a direct interaction between Dbs and Src (not shown), which is consistent with previous studies (43) and suggests that Ccpg1 may be required to support a functional interaction between these two proteins.

Ccpg1 recruits endogenously expressed Src kinase into Dbs-containing complexes. Next, we tested if Ccpg1 could couple natively expressed Src with Dbs. For this analysis, we coexpressed FLAG-tagged Ccpg1 along with HA-tagged DbsHA6 in 293T cells and immunoprecipitated the Ccpg1/DbsHA6 complex using an anti-FLAG antibody. These complexes were then analyzed for the presence of endogenously expressed Src kinase by Western blot analysis (Fig. 10B). Using this approach, we found that the Ccpg1/DbsHA6 complexes consistently contained detectable amounts of natively expressed Src kinase (Fig. 10B). However, we did not detect endogenously expressed Cdc42 in these complexes (not shown), suggesting that Ccpg1 may couple Dbs to another GTPase in this cell type. Alternatively, it is possible that the amounts of the endogenous GTP-bound Cdc42 associated with immunoprecipitated Ccpg1/DbsHA6 are below the detection limits of the anti-Cdc42 antibody used in our study.

DISCUSSION

One of the most intriguing questions in Rho protein biology is how the specificity of Rho-mediated signaling pathways is controlled. Emerging evidence suggests that such specificity can be regulated by RhoGEFs through their association with either scaffold proteins that direct the choice of downstream effectors (6, 7, 19, 20) or regulatory proteins which restrict their substrate usage (36). Thus, the interaction of Tiam1 with scaffolds like JNK-interacting protein 2, or spinophilin, was shown to cause specific activation of p38MAPK or p70 S6 kinase, respectively (6, 7). Similarly, the CNK scaffolding protein binds to Net1 and p115-RhoGEF and is required for the activation of the JNK-mitogen-activated protein kinase (MAPK) kinase cascade, but not other Rho-mediated pathways (19, 20). In contrast, the association of the Eph4A receptor with ephexin, a promiscuous exchange factor for RhoA, Rac1, and Cdc42, modulates its exchange activity leading to specific activation of RhoA (36).

Here we report the identification of a novel protein, Ccpg1, which combines the functional properties of a regulatory partner, and the binding properties of a scaffold protein, for Rho-mediated signaling events. Ccpg1 interacts with the DH/PH domain of the Cdc42/RhoA specific RhoGEF Dbs and restricts its exchange activity toward RhoA, but not Cdc42 in vivo (Fig. 5). Although Dbs is a potent activator of Cdc42 and RhoA in vitro, its exchange specificity in vivo is cell type specific. It can only activate endogenous RhoA when expressed in NIH 3T3 cells (10) and endogenous Cdc42 in 293T cells (9). Our data suggest that the interaction of Ccpg1 with Dbs might provide a molecular basis for the regulation of Dbs exchange specificity in vivo (Fig. 11). The expression levels of Ccpg1 in cultured cells correlate with the ability of Dbs to activate endogenous

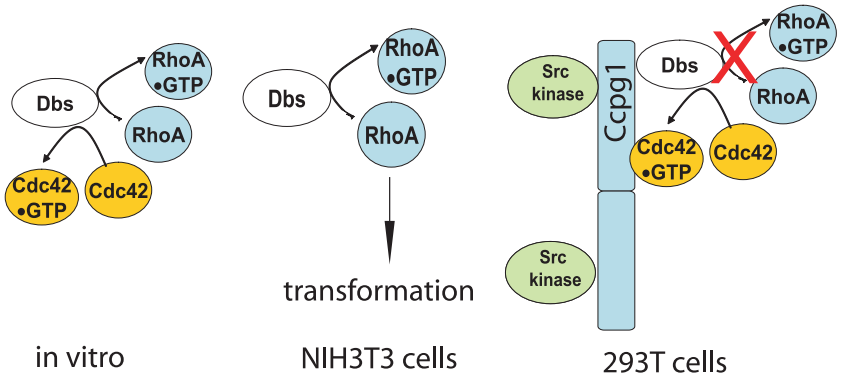

FIG. 11. A model for the regulation of Dbs transforming and exchange activity by Ccpg1. In vitro Dbs functions as a promiscuous exchange factor catalyzing exchange of GTP on Cdc42 and RhoA. When expressed in NIH 3T3 cells, which contain no detectable Ccpg1, Dbs is capable of activating endogenous RhoA and causing transformation. When Dbs is expressed in cells with high levels of Ccpg1 expression, such as 293T, the interaction with Ccpg1 leads to inhibition of Dbs exchange activity toward endogenous RhoA, but does not affect its ability to activate Cdc42. Whether or not Dbs activates endogenous Cdc42 in a Ccpg1-dependent manner is unclear. Interaction of the COOH-terminal region of Ccpg1 with regulatory kinases, such as Src, may be required to actively suppress Dbs exchange activity toward RhoA.

RhoA. Thus, NIH 3T3 cells do not express Ccpg1 and are permissive for the activation of RhoA and the corresponding transformed phenotype. In this model, expression of Ccpg1 in NIH 3T3 cells would cause inhibition of Dbs transforming activity due to inhibition of Dbs exchange activity toward RhoA. As predicted, 293T or COS7 cells, which express intermediate and high levels of Ccpg1 mRNA, respectively, do not support Dbs activity toward RhoA. Further support for the physiological role of Ccpg1 as a regulator of Dbs exchange activity toward RhoA was demonstrated by the ability of siRNA targeted to Ccpg1 to cause potentiation of Dbs exchange activity toward RhoA in 293T cells (Fig. 8C). Thus, our results suggest that Ccpg1 functions as a regulatory binding partner involved in the control of Dbs exchange specificity toward RhoA.

Although Ccpg1 can clearly restrict Dbs exchange activity toward RhoA, it is not yet clear that it does so in favor of Cdc42 activation. We did not find a correlation between expression levels of Ccpg1 and the ability of Dbs to activate endogenous Cdc42 in cultured cells. Thus, Dbs cannot activate Cdc42 in NIH 3T3 cells, where Ccpg1 is absent, and in COS7 cells, where Ccpg1 expression is high. Correspondingly, expression of Ccpg1 has no effect on the activity of Dbs toward Cdc42 in 293T cells (Fig. 5C and D). The currently available experimental data might simply suggest that Dbs exchange activity toward Cdc42 is independent of Ccpg1 binding. However, there is a possibility that the failure of Dbs to activate Cdc42 in NIH 3T3 and COS7 cells may reflect the absence of specific Ccpg1-associated regulatory factors in these cell types. Both possibilities are currently under investigation.

Although mammalian scaffold proteins such as hCNK are generally considered to be passive protein assembly platforms, recent data from yeast indicate that protein scaffolds might be actively involved in the regulation of signaling pathways. For example, the Ste5 scaffold of the yeast mating pheromone pathway allosterically activates autophosphorylation of the mitogen-activated kinase Fus3 (4). Once activated, Fus3 phosphorylates Ste5 and decreases the transcriptional output of the pathway. Evidence from the present study suggests that Ccpg1 may also play a more active role in RhoGEF regulation than simple recruitment. When we express an NH_2-terminal fragment of Ccpg1 that interacts with both Cdc42 and Dbs, it does not block Dbs exchange and transforming activity. This suggests that sequences within the COOH terminus of Ccpg1 are required to actively suppress the exchange activity of Dbs toward RhoA. In the tertiary structure of Ccpg1, the COOH terminus either may be directly involved in the inhibition of Dbs exchange activity toward RhoA or may serve as a binding site for regulatory proteins. For example, Src may recruit p190RhoGAP, which in turn may mediate down-regulation of RhoA activity in Dbs/Ccpg1/Src complexes (8).

Besides functioning as a regulatory binding partner for Dbs, Ccpg1 demonstrates properties characteristic of a scaffold protein. Thus, Ccpg1 interacts with multiple signaling molecules such as the regulatory kinase Src, the Rho family member Cdc42, and RhoGEFs. Interestingly, the docking for Ccpg1 appears to be conserved in other RhoGEF family members, including Dbl, Lsc, Bcr, FGD1, and Vav1. Although the physiological role of these interactions will have to be addressed in a separate study, it is clear from the present study that the interactions between Ccpg1 and related RhoGEFs are functionally distinct. Thus, whereas Ccpg1 inhibits Dbs activity for RhoA, it does not affect the activity of Dbl for the same substrate. This difference was confirmed by the inability of Ccpg1 to block Dbl transforming activity. Experiments are currently under way to determine whether this difference reflects the recruitment of distinct regulatory complexes or whether it reflects a difference in allosteric regulation of these RhoGEFs by Ccpg1.

Our finding that Ccpg1 interacts with Cdc42 in the GTP-bound form is similar to what has been observed for the hCNK scaffold protein (19). hCNK binds RhoA-GTP and a subset of the RhoGEF family members and links RhoA activation to Jnk-mediated signaling. Since Ccpg1 does not contain a consensus Cdc42/Rac1 interactive binding motif, nor does it share homology with the Rho binding domain of hCNK, the NH_2 terminus of Ccpg1 appears to contain a novel binding motif for Cdc42. Although it is formally possible that the interaction between Ccpg1 and Cdc42 occurs indirectly through Dbs, this seems unlikely since RhoGEFs typically do not form stable

associations with GTPases and Dbs does not interact with Cdc42-GTP in vitro or in vivo (9). The physiological role of Ccpg1 binding to Cdc42 remains to be determined. It is possible that after activation by Dbs, GTP-bound Cdc42 is recruited to Ccpg1, which then functions as an assembly platform for an as yet unknown Cdc42-mediated signaling cascade. Although Ccpg1 can interact with activated Cdc42 when both are overexpressed (Fig. 4A), we were unable to identify Cdc42 in immunocomplexes derived from 293T cells that contain Ccpg1, Dbs, and Src. It is possible that the Ccpg1 interaction with Cdc42 is transient and that levels of Ccpg1-associated Cdc42 are below the detection limit of the Cdc42 antibody used in this study. Since we currently lack information about Cdc42-mediated signaling pathways downstream of Dbs, further studies are required to assess the physiological consequences of the Ccpg1 interaction with Cdc42 and its role as a scaffold in such a signaling cascade.

Src has been shown to phosphorylate both Dbs and Vav, and in the latter case, phosphorylation is required to support exchange activity (2, 43). In the present study, we present evidence that Ccpg1 may be responsible for recruiting Src into complexes that contain Dbs. Although endogenous Src does not interact with either Dbs or Ccpg1 when they are expressed alone, we are readily able to detect recruitment of endogenous Src into the Ccpg1/Dbs-containing complexes. These observations suggest that interactions within the complex may be more stable than interactions between the individual components. They may also explain the previous finding that Src kinase cannot directly phosphorylate Dbs in an in vitro kinase assay using purified proteins (43). Since Dbs can only associate with Src in the presence of Ccpg1, Dbs may not serve as a substrate for Src in the absence of Ccpg1 in the in vitro kinase reaction.

Ccpg1 was first identified in an expression screen for human cDNAs that can rescue the Far1$^-$ phenotype in yeast (12). In *S. cerevisiae*, the mating pheromone signaling pathway is required to initiate gametic differentiation. This response to extracellular peptide hormones is associated with three broad, genetically separable, cellular responses: transcriptional activation, polarized changes in cell morphology, and a G_1 cell cycle arrest. Cell cycle arrest is primarily associated with inhibition of the Cln/Cdc28 complex through activation of the CDK inhibitor, Far1. Thus, whereas the transcriptional branch of the mating response pathway is normal in Far1 mutants, the mutants do not undergo G_1 arrest in response to mating factors. Although the molecular mechanism through which Ccpg1 can suppress the Far1 phenotype is unknown, a high level of Cln3 was noted in yeast cells that express Ccpg1. Moreover, Ccpg1 appears to regulate Cln3 posttranscriptionally since *CLN3* mRNA levels are not increased in these cells. Although there is no homolog of Ccpg1 in yeast, the striking cell cycle phenotype associated with its expression suggests that it has signaling capacity and thus may have a functional ortholog. For example, Bem1p is a yeast scaffold protein that interacts with both Cdc42p and the yeast RhoGEF family member Cdc24p (15). Interestingly, Bem1p contains a Cdk-phosphorylation consensus site at which it is both phosphorylated and activated in a Cln3-dependent manner. Thus, it is possible that Ccpg1 can function as a Bem1p ortholog in yeast that can override the pheromone-mediated G_1 arrest by actively recruiting and stabilizing Cln3.

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