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Functional Analysis of Cdc42 Residues Required for Guanine Nucleotide Exchange*

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Guanine nucleotide exchange factors (GEFs) directly engage small GTPases to facilitate the exchange of bound GDP for GTP, leading to GTPase activation. Several recent crystal structures of GEFs in complex with Rho family GTPases highlight the conserved interactions and conformational alterations necessary for catalyzing exchange. In the present study, functional roles were defined for specific residues within Cdc42 implicated by the crystal structures as important for physiological exchange of guanine nucleotides within Rho GTPases. In particular, this study highlights the paramount importance of the phosphate-binding loop and interactions with the magnesium co-factor as critical for proper regulation of RhoGEF-catalyzed exchange. Other conformational alterations of the GTPases affecting interactions with the sugar and base of guanine nucleotides are also important but are secondary. Of particular note, substitution of alanine for cysteine at position 18 of Cdc42 leads to a fast cycling phenotype for Cdc42 with heightened affinity for RhoGEFs and produces a dominant negative form of Cdc42 capable of inhibiting RhoGEFs both *in vitro* and *in vivo*.

Similar to other members of the Ras superfamily, Rho family GTPases are biologically active when bound to GTP and are deactivated upon hydrolysis of GTP to GDP, a reaction typically accelerated by Rho GTPase-activating proteins (GAPs).[1] Guanine nucleotide exchange factors (GEFs) rapidly convert GTPases to their biologically active states by catalyzing the exchange of GDP for GTP. Currently, Dbl (diffuse B-cell lymphoma) family members constitute the largest group of GEFs for Rho GTPases (1–3) and are easily recognized by the invariant placement of a pleckstrin homology (PH) domain immediately carboxyl-terminal to a Dbl homology (DH) domain. Isolated DH domains typically possess significant exchange potential that can be enhanced by the adjacent PH domain (4, 5). The invariant linkage of DH and PH domains hints at a conserved function that is currently poorly understood.

As proposed for other GEFs, the reaction scheme for Dbl-stimulated exchange includes the formation of a low affinity GEF·GTPase·GDP·Mg^{2+} quaternary complex that rapidly converts to a high affinity GEF·GTPase binary complex concomitant with expulsion of GDP and Mg^{2+} (6). The reaction proceeds with the binding of GTP·Mg^{2+} to form an unstable quaternary complex of GEF·GTPase·GTP·Mg^{2+}, followed by dissociation of the GEF from the GTP-bound GTPase.

We have recently determined crystal structures of several unique DH/PH fragments in complex with their cognate Rho family GTPases (5, 7, 8), and the structures indicate a conserved mechanism of exchange. For example, Rho GTPases possess two "switch" regions that are conformationally sensitive to the state of bound nucleotide, and these switch regions occupy similar conformations in all current structures of Rho GTPases bound to GEFs. Certain conformational features of DH domain-bound Rho GTPases are also present in other GEF·GTPase structures, such as Sos1 (Cdc25 domain)·Ras (9), RCC1·Ran (10), and SopE·Cdc42 (11), and are indicative of conserved aspects of the catalyzed exchange mechanism utilized by various GEF families.

In order to more thoroughly understand the relative contributions of key structural features in facilitating guanine nucleotide exchange by RhoGEFs, Cdc42 and the Dbl family member Dbs (for Dbl's big sister) were mutated at critical sites, and the functional consequences were determined. This analysis indicates that mutations that affect Mg^{2+} binding are critical for efficient GEF-catalyzed exchange. Specifically, the methyl group of Ala^{59} within Cdc42 or its equivalent in Rac1 and RhoA normally impinges upon the Mg^{2+} binding site within the GEF·GTPase complexes. Consequently, substitution of alanine 59 to glycine in Cdc42 severely cripples GEF binding and exchange. Similarly, interactions that support the steric overlap of Ala^{59} and the Mg^{2+}-binding site also appear critical for nucleotide exchange. Mutations that affect interactions with the sugar or base of guanine nucleotides also have significant effects on exchange, but these effects are secondary to disruption of Mg^{2+} binding. One mutation (C18A) in Cdc42, designed to disrupt a hydrogen bond with the α-phosphate of guanine nucleotides, is particularly notable, since it confers a dominant negative phenotype upon Cdc42. Cdc42(C18A) is impaired in nucleotide binding and consequently binds Dbs with higher affinity than wild-type Cdc42. *In vivo*, Cdc42(C18A) exhibits a dominant-negative phenotype, presumably by sequestering exchange factors in nucleotide-depleted complexes analogous to

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[1] The abbreviations used are: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; WT, wild type; RU, response units; GST, glutathione *S*-transferase; PBD, p21 binding domain; mant, *N*-methylanthraniloyl.

other, better characterized variants of Rho GTPases (*i.e.* Cdc42(T17N)).

EXPERIMENTAL PROCEDURES

Guanine Nucleotide Exchange Assays—A carboxyl-terminal His_6-tagged, wild type DH/PH fragment (residues 623–967) expression construct was derived from a cDNA library of mouse brain as described previously (5). Mutations were introduced into wild type Dbs DH/PH domain and Cdc42 using the QuikChange site-directed mutagenesis kit (Stratagene) as per the manufacturer's instructions. cDNA sequences of all protein expression constructs were verified by automated sequencing. Dbs and Cdc42 proteins were expressed and purified as described (5).

Fluorescence spectroscopic analysis of *N*-methylanthraniloyl (mant)-GTP incorporation into bacterially purified Cdc42 and RhoA was carried out using a PerkinElmer Life Sciences LS 50B spectrometer at 25 °C. Exchange assays containing 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μM mant-GTP (Biomol) and a 1 or 2 μM concentration (as indicated) of either Cdc42 or RhoA protein, were prepared and allowed to equilibrate with continuous stirring. After equilibration, Dbs DH/PH domain was added to 200 nM, and the rates of GTP loading (k_{obs}) for wild type and mutant Cdc42 proteins were determined by monitoring the decrease in tryptophan fluorescence (λ_{ex} = 295 nm, λ_{em} = 335 nm) in response to binding mant-GTP (12–14). Because of their high intrinsic exchange rate, Cdc42(C18A) and Cdc42(V33A) were added to equilibrated exchange reactions containing 200 nM Dbs.

To test the inhibition of Dbs-catalyzed exchange by Cdc42(C18A), 400 nM Cdc42(WT) preloaded with mant-GDP was stimulated by 200 nM Dbs DH/PH in the presence of 2 μM Cdc42(WT) or Cdc42(C18A), each bound to GDP. Reaction conditions were similar to those described above except that 20 μM GDP was used in place of 100 μM GTP and exchange was followed by measuring the decrease in fluorescence resulting from mant-GDP release from Cdc42 (λ_{ex} = 360 nm, λ_{em} = 440 nm).

The rates (k_{obs}) of guanine nucleotide exchange were determined by fitting the data as single exponential decays utilizing GraphPad Prizm software. Data were normalized to wild type curves to yield the percentage of GDP released. All experiments were performed at least in duplicate.

Surface Plasmon Resonance—Cdc42 binding to Dbs was monitored using surface plasmon resonance with a BIAcore 2000 instrument at 25 °C. His-tagged Dbs DH/PH domain was immobilized to a nickel surface on a nitrilotriacetic acid sensor chip (BIAcore) as described by the manufacturer. Cdc42 solutions (ranging in concentration from 156 nM to 2.5 μM) were injected over the stable Dbs surface with or without 50 μM EDTA (used to chelate Mg^{2+} and remove nucleotide) at 25 μl/min for 25 s and allowed to dissociate for 60 s in phosphate-buffered saline. Raw data were normalized to the signal achieved due to binding a surface lacking Dbs. Normalized sensorgrams were aligned, and the steady state binding signal from each curve was fit to a single binding isotherm. The resulting dissociation constants are the mean of several sets of analyte concentrations.

Molecular Constructs—The pAX142 mammalian expression vector, pAX142-Cdc42(WT), and pAX-Cdc42(17N) have been described (15, 16). pAX142-Cdc42(C18A) was generated by PCR-based site-directed mutagenesis and verified by automated sequencing. pAX142-Dbl-HA1 contains a cDNA that encodes a transforming derivative of Dbl fused to an HA epitope tag (16). GST-PBD contains the Cdc42 binding domains from the Cdc42/Rac1 effector protein Pak3 (17). The NF-κB-*luc* and reporter construct utilized in the transcriptional assays have been described previously (18). pCMVnlac encodes the sequences for the β-galactosidase gene under the control of the cytomegalovirus promoter (provided by J. Samulski).

Cell Culture, Transfection, and Transient Reporter Gene Assays—COS-7 cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum. Cells were transfected by DEAE-dextran (COS-7) as described previously (17). Cells were allowed to recover for 30 h and subsequently starved in Dulbecco's modified Eagle's medium supplemented with 0.5% serum for 14 h before lysate preparation. Analysis of luciferase expression with enhanced chemiluminescence reagents and a Monolight 3010 luminometer (Analytical Luminescence, San Diego, CA) was described previously (19, 20). β-Galactosidase activity was determined using Lumi-Gal substrate (Lumigen, Southfield, MI) according to the manufacturer's instructions. All assays were performed in triplicate.

Cdc42 Activation Assays in Vivo—Affinity purification of GTP-Cdc42 was performed as described previously (21). Briefly, the p21 binding domain of Pak3 was expressed as a GST fusion (GST-PBD) in BL21(DE3) cells, immobilized to glutathione-coupled Sepharose 4B beads (Amersham Biosciences) (17), and used to precipitate activated GTP-bound Cdc42 from COS-7 cell lysates. Cells were washed in cold phosphate-buffered saline prior to lysis in 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 1 μg/ml phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation at 10,000 $\times$ *g* for 10 min at 4 °C and normalized for endogenous Cdc42 levels detected by monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Affinity purifications were carried out at 4 °C for 1 h, washed three times in an excess of lysis buffer, and then analyzed by Western blot.

RESULTS

Functional Analysis of the Exchange Mechanism—The structures of Tiam1 (T-cell lymphoma invasion and metastasis 1), Dbs, and intersectin in complex with their cognate Rho GTPases (5, 7, 8) suggest that DH domains catalyze exchange by repositioning the GTPase switch regions to disorganize the nucleotide binding pocket and directly occlude the Mg^{2+} binding site. As we have previously described (7), the repositioning of Cys^{18}, Val^{33}, Ala^{59}, and Glu^{62} (Cdc42 numbering) within the complexes appears essential for efficient guanine nucleotide exchange (Fig. 1). In order to better understand the contribution of these four residues to the exchange reaction, we have assessed the mechanistic effects of substitutions at these positions within Cdc42 using a combination of fluorescence spectroscopy to measure guanine nucleotide exchange rates and surface plasmon resonance to measure affinities of complex formation.

Cys^{18} and Val^{33} apparently undergo a concerted rearrangement within the nucleotide binding pocket upon complex formation, resulting in reorientation of the cysteine side chain relative to the nucleotide-bound structures of Rho GTPases. In this new rotamer conformation, Cys^{18} can no longer hydrogen-bond to a nucleotide α-phosphate (Fig. 1*B*), and loss of this hydrogen bond should reduce the affinity for nucleotide and promote exchange in the absence of GEF. Consequently, and as expected, substitution of Cys^{18} to alanine increases the spontaneous loading of mant-GTP onto Cdc42 (~0.0044/s *versus* 0.0003/s for Cdc42(WT)), partially mimicking the action of Dbs (Fig. 2*A*, Table I). The further stimulation of Cdc42(C18A) by Dbs (~0.0461/s) undoubtedly arises from GEF-induced alterations to portions of the active site outside the phosphate-binding loop.

Within the GEF·GTPase structures (5, 7, 8), the side chains of residues analogous to Val^{33} within Cdc42 are moved into the site normally occupied by Cys^{18}, promoting an alternate rotamer conformation of the cysteine residue that can no longer hydrogen-bond to the α-phosphate of guanine nucleotides (Fig. 1*B*). Therefore, substitution of Val^{33} to alanine within Cdc42 was designed to decouple structural alterations propagating from switch 1 to Cys^{18} within the phosphate binding loop. Since the rearrangement of Cys^{18} is predicted to be critically important to the GEF-catalyzed exchange reaction, mutation of Val^{33} was anticipated to hinder Dbs-catalyzed guanine nucleotide exchange. Experimentally, Cdc42(V33A) bound to Dbs with an affinity similar to Cdc42(WT) (Fig. 2, *G* and *J*, Table I). In addition, the Dbs-catalyzed rate of mant-GTP loading was only mildly impaired on Cdc42(V33A) (Fig. 2*B*, Table I). However, this observation is complicated by an ~5-fold increase in the intrinsic exchange rate within Cdc42(V33A) *versus* wild type (~0.0015/s *versus* ~0.0003/s, respectively), reducing the overall efficiency of Dbs-catalyzed exchange on Cdc42(V33A) (10-fold *versus* 72-fold stimulation for wild type) (Table I). These results are most easily explained by postulating that Val^{33} is needed not only to transmit structural alterations within switch 1 to Cys^{18} but also to maintain the overall integrity of the nucleo-

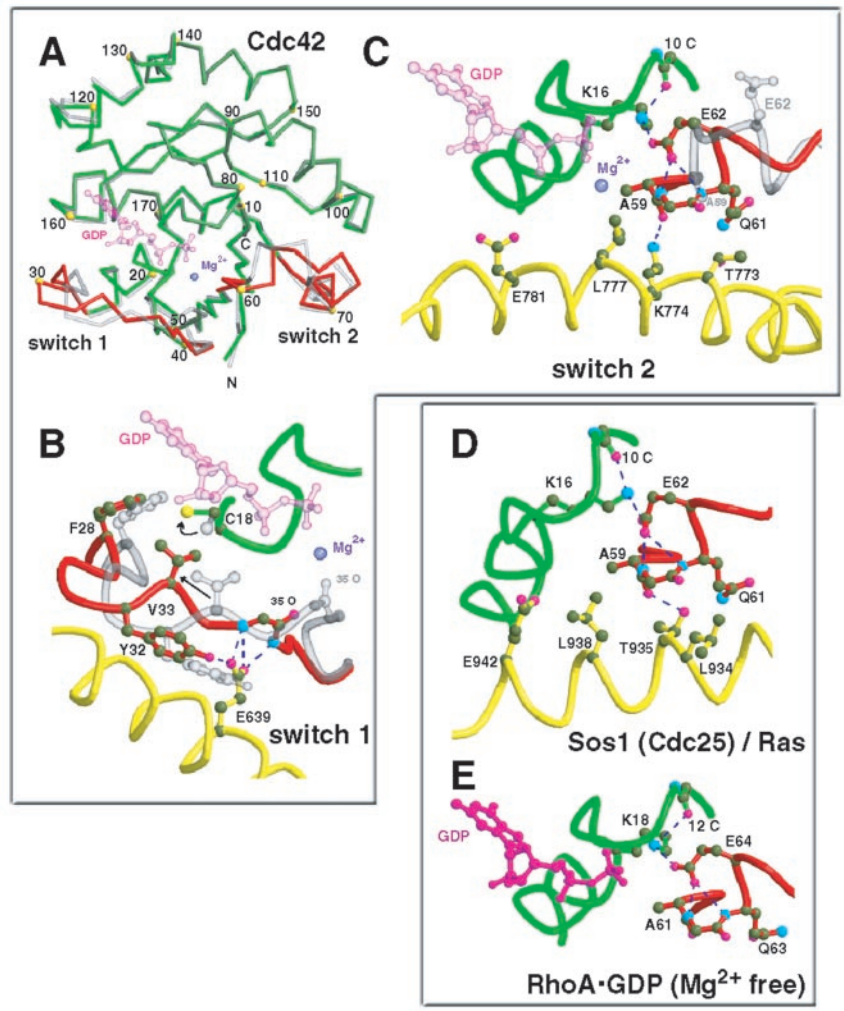

FIG. 1. **Remodeling of Cdc42 switches.** *A*, switches (*red*) of Cdc42 are remodeled upon binding Dbs. Dbs-bound Cdc42 (5) (*green*; every 10th residue *numbered*) is superimposed on Cdc42 (*transparent gray*) bound to GDP (coordinates provided by N. Nassar) (*transparent magenta*) and Mg^{2+} (*transparent blue*). Details of rearrangements for switches 1 (*B*) and 2 (*C*) are highlighted with *arrows*, indicating movements within Cdc42 upon binding Dbs (*yellow*), and *dashed lines*, indicating hydrogen bonds. Similar conformations of switch 2 are recapitulated in Sos1(Cdc25)·Ras (9) (*D*) and magnesium-depleted RhoA·GDP (30) (*E*). The *color scheme* is consistent with the earlier *panels*.

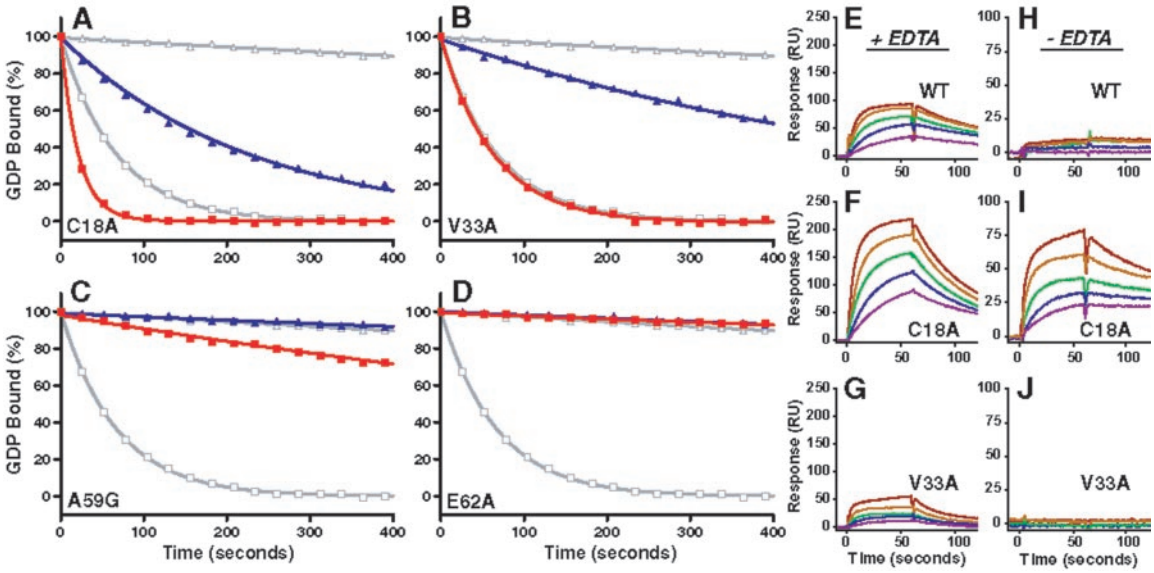

FIG. 2. **Kinetic analysis of Cdc42 guanine nucleotide exchange mechanism mutants.** Cdc42 mutants containing substitutions C18A (*A*), V33A (*B*), A59G (*C*), and E62A (*D*) were tested for the ability to be activated by Dbs. For each mutant, the intrinsic (*blue*, *closed triangles*) and Dbs-catalyzed (*red*, *closed squares*) guanine nucleotide exchange rates are shown relative to the equivalent exchange reactions (*gray*) for Cdc42(WT) (intrinsic (*open triangles*) and Dbs-catalyzed (*open squares*)). Sensorgrams of Cdc42(WT) (*E* and *H*), Cdc42(C18A) (*F* and *I*), and Cdc42(V33A) (*G* and *J*) showing binding to a Dbs DH/PH domain surface were measured in the presence (*E–G*) and absence (*H–J*) of 50 μM EDTA by surface plasmon resonance as described under "Experimental Procedures." Concentrations of Cdc42 proteins used are 0.156 μM (*magenta*), 0.313 μM (*purple*), 0.625 μM (*green*), 1.25 μM (*gold*), and 2.5 μM (*red*). *RU*, response units.

TABLE I

Rate and binding constants for Dbs-catalyzed guanine nucleotide exchange of wild type and mutant Cdc42 proteins

Rates (k_{obs}) of guanine nucleotide exchange for the various Cdc42 proteins were determined by fitting the data from Fig. 2 to a single exponential decay function. -Fold stimulation is the corresponding ratio of rates for the Dbs-catalyzed *versus* intrinsic exchange reactions. k_{obs} values are the mean of at least two experiments with S.D. values. K_d values are estimated from the SPR data in Fig. 2. NB, no binding detected.

2 μM Cdc42	Intrinsic rate (k_{obs})	Rate with 0.2 μM Dbs (k_{obs})	Stimulation	K_d (+EDTA)	K_d (−EDTA)
	$s^{-1} \times 10^{-3}$		*-fold*	*nM*	*nM*
WT	0.26 ± 0.01	18.74 ± 1.46	72 ± 5	425	NB
C18A	4.44 ± 0.08	46.07 ± 1.80	10 ± 0	178	457
V33A	1.49 ± 0.04	15.52 ± 0.18	10 ± 0	714	NB
A59G	0.19 ± 0.01	0.79 ± 0.04	4 ± 0	NB	NB
E62A	0.18 ± 0.00	0.18 ± 0.02	1 ± 0	NB	NB

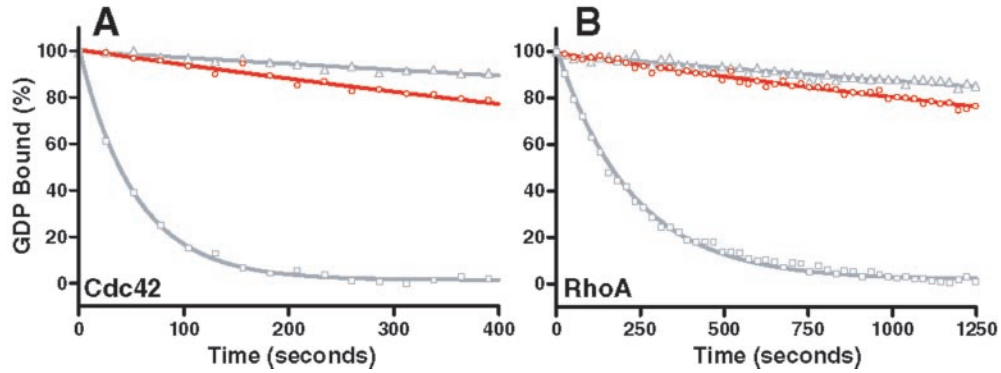

FIG. 3. **Dbs(E639A) exchange of Cdc42 and RhoA.** Dbs(E639A) (*red lines*, *open circles*) was tested for its ability to catalyze guanine nucleotide exchange of 1 μM Cdc42 (*A*) or RhoA (*B*). Also shown are the intrinsic (*gray lines*, *open triangles*) and wild-type Dbs-catalyzed (*gray lines*, *open squares*) exchange of Cdc42 and RhoA.

TABLE II

Rate constants of guanine nucleotide exchange reactions catalyzed by wild type and mutant Dbs proteins on Cdc42 and RhoA

Rates (k_{obs}) of guanine nucleotide exchange for wild type Cdc42 (left) or RhoA (right) stimulated by various Dbs proteins were determined by fitting the data from Fig. 3 as single exponential decays. The -fold stimulation for each Dbs protein reflects the ratio of k_{obs} measured for the GEF-containing reaction to the unstimulated reaction containing no GEF (none).

Dbs	Cdc42		RhoA	
	k_{obs}	Stimulation	k_{obs}	Stimulation
	$s^{-1} \times 10^{-3}$	*-fold*	$s^{-1} \times 10^{-3}$	*-fold*
None	0.28 ± 0.00		0.12 ± 0.00	
DH/PH (WT)	19.10 ± 0.01	68.9	4.49 ± 0.06	37.4
E639A	0.67 ± 0.00	2.4	0.21 ± 0.00	1.8

tide binding pocket irrespective of Dbs engagement.

Within conserved region 1 of the Dbs DH domain, the side chain of Glu^{639} is clearly critical for stabilizing switch 1 (Fig. 1*B*) and hydrogen-bonds with the hydroxyl of Tyr^{32} and the backbone nitrogens of Thr^{35} and Val^{36}. The equivalent of Glu^{639} is highly conserved among DH domains. Consistent with the necessity for Dbs to rearrange switch 1 of Cdc42 to effect exchange, mutation of the conserved glutamate (E639A) decreases the rates of Dbs-catalyzed exchange upon Cdc42 and RhoA 29- and 21-fold from wild-type rates, respectively (Fig. 3 and Table II). Residues analogous to Glu^{639} have been previously assessed for their role in nucleotide exchange for other Dbl family members (4, 22), and substitutions at these sites are consistently detrimental to catalyzed exchange, further indicating a conserved mechanism utilized among Dbl family proteins.

Ala^{59} of Cdc42 is repositioned upon complex formation with Dbs to occlude the Mg^{2+} binding site (Fig. 1*C*). Glu^{62} of Cdc42 supports this rearrangement as well as helping to preserve the integrity of the P-loop through interaction with Lys^{16}. Repositioning Ala^{59} (Ala^{59} in Rac1, Ala^{61} in RhoA) and Glu^{62} (Glu^{62} in Rac1, Glu^{64} in RhoA) occurs identically in the structures of Tiam1·Rac1, Dbs·Cdc42, and Dbs·RhoA, arguing for the importance of these altered conformations during nucleotide exchange of Rho GTPases. Consistent with this structural information, both the A59G and E62A mutations in Cdc42 are extremely deleterious to guanine nucleotide exchange (Fig. 2, *C* and *D*). Both Cdc42(A59G) and Cdc42(E62A) are essentially unresponsive to Dbs, and despite the fact that Glu^{62} undergoes no change in solvent exposure and the Ala^{59} methyl carbon loses only ~10 $Å^2$ upon complex formation, neither mutant displays measurable binding to Dbs as indicated by surface plasmon resonance (Table I). Furthermore, it is interesting to note that both A59G and E62A decrease the intrinsic rates of exchange for Cdc42, arguing that disruption of Mg^{2+} binding is important for spontaneous exchange within the GTPase.

C18A as a Dominant Negative Mutation—When a mutation in a GTPase decreases affinity for bound nucleotide, the GTPase may behave as a so-called "dominant negative" in the presence of its exchange factor, displaying increased affinity toward the GEF because it is less easily displaced through nucleotide binding. Accordingly, Cdc42(C18A) binds with increased affinity to Dbs both in the presence and absence of nucleotide (Fig. 2, *F* and *I*; Table I). Consistent with this behavior, Cdc42(C18A) effectively inhibits Dbs-catalyzed exchange of wild-type Cdc42 *in vitro* (Fig. 4). Similarly, Cdc42(C18A) inhibits the ability of Dbl, a close homologue of Dbs, to elicit transcription by NF-κB normally associated with Cdc42 activation *in vivo* (Fig. 5). Transcriptional inhibition by Cdc42(C18A) and the classical dominant-negative Cdc42(T17N) are roughly equal, indicating that Cdc42(C18A) is a potent dominant negative of Cdc42 activity.

DISCUSSION

In general, Dbl family proteins catalyze exchange without directly impinging upon the binding sites for either guanine nucleotides or the magnesium co-factor. Instead, RhoGEFs manipulate GTPase residues to promote ejection of GDP and

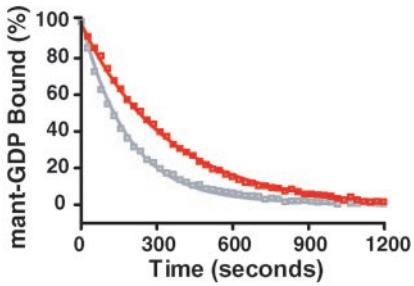

FIG. 4. **Cdc42 (C18A) inhibits Dbs exchange of wild type GTPase.** Inhibition of the Dbs-stimulated exchange of mant-GDP-loaded Cdc42(WT) by Cdc42(C18A). Reactions contained 200 nM Dbs DH/PH, 400 nM mant-GDP-loaded Cdc42(WT), 20 μM GDP, and a 2 μM concentration of either Cdc42(WT) (*gray*) or Cdc42(C18A) (*red*). Measured rates (k_{obs}) for each reaction were 0.0055 s^{-1} for Cdc42 and 0.0029 s^{-1} for Cdc42(C18A).

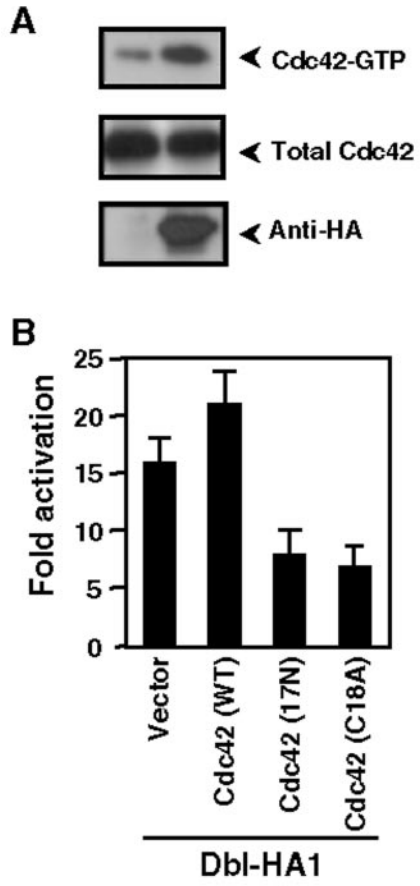

FIG. 5. **The Cdc42(C18A) mutant functions as a dominant inhibitor.** *A*, Dbl-HA1 activates Cdc42 in COS-7 cells. COS-7 cells were transiently transfected with 3 μg of pAX142 (− GEF) or pAX142-Dbl-HA1 (+ GEF), along with 3 μg of Cdc42(WT). Lysates were collected at 48 h and examined by Western blots for overall expression of Cdc42 and Dbl-HA1 as indicated as well as activated Cdc42 (GTP-bound) isolated by affinity purification with GST-PBD. *B*, Cdc42(C18A) blocks activation of an NF-κB-responsive transcriptional reporter by Dbl-HA1. COS-7 cells were co-transfected with 3 μg of pAX142 (vector), pAX142-Cdc42(WT), pAX142-Cdc42(17N), or pAX142-Cdc42(C18A), along with 3 μg of pAX142-Dbl(HA1), 2.5 μg of NF-κB-*luc*, and 500 ng of pCMVnlac as an internal control for transfection efficiency and/or growth inhibition. Luciferase and β-galactosidase levels were measured and expressed as -fold activation relative to the level of activation seen with empty vector control. Luciferase activity was then standardized relative to β-galactosidase activity. Data shown are representative of three independent experiments performed on triplicate plates. *Error bars* indicate S.D. values.

Mg^{2+}. The molecular rearrangements originally seen in Rac1 bound to Tiam1 (7) and recapitulated in the structures of Dbs·Cdc42 (5), Dbs·RhoA, and intersectin·Cdc42 (8) strongly support the assumption of a conserved exchange mechanism for all Dbl family proteins.

Specifically, repositioning of switch 1 of Cdc42 moves Phe^{28} away from the nucleotide base, displaces Thr^{35} so that it no longer interacts favorably with the Mg^{2+} ion, and, via Val^{33}, alters the side chain conformer of Cys^{18} so that it can no longer hydrogen-bond with the α-phosphate of GDP (Fig. 1*B*). The importance of Phe^{28} was highlighted in a previous study (23), where substitution of this residue to leucine in Cdc42 results in a "fast cycling" mutant with spontaneous exchange on par with Dbl-catalyzed exchange. Less well appreciated is the role of Cys^{18} of Cdc42 in binding nucleotides. However, the results presented here clearly demonstrate a critical role for Cys^{18} of Cdc42 in binding nucleotides and participating in a conserved exchange reaction catalyzed by RhoGEFs. With the exception of the three Rnd proteins (Rho6/Rnd1, Rho7/Rnd2, and Rho8/RhoE/Rnd3) that feature an alanine at position 18, all other Rho family members possess either a cysteine or the isosteric serine at this position. When Cys^{18} is substituted to alanine, Cdc42 loses the ability to bind nucleotide with high affinity, resulting in rapid exchange. Similarly, alanine at this position in the Rnd proteins may underlie their relatively high rates of spontaneous nucleotide exchange (24).

Like the Rnd proteins, Ras possesses an alanine at position 18 that cannot hydrogen-bond to the nucleotide α-phosphate. However, Ras, rather than binding weakly to nucleotides like the Rnd proteins, possesses other subtle differences in its active site and binds nucleotides with higher affinity than the Rho proteins (25, 26). Nevertheless, Powers *et al.* (27) have shown that the A25P mutation in yeast RAS (equivalent to A18P in human H-Ras) conferred a dominant-interfering phenotype. Examination of the nucleotide-bound H-Ras crystal structure (protein data bank code 121P) reveals that A18P would disrupt protein-nucleotide interactions primarily by introducing steric conflict with a bound nucleotide, unlike C18A in Cdc42, which introduces a smaller side chain and directly abolishes a positive protein-nucleotide interaction.

Furthermore, like Cdc42(C18A), Ras(D119N) has decreased affinity for nucleotides, resulting in increased affinity of Ras for its exchange factor, Sos1 (son of sevenless) (23, 28), with associated pleiotropic effects *in vivo*. For instance, at low expression levels, Ras(D119N) produces a dominant negative phenotype presumably by sequestering Sos1. However, at expression levels much higher than endogenous GEFs, Ras(D119N) is largely unbound by GEFs and overly active, since the mutation does not compromise effector binding and GTP is rapidly loading. Cdc42(C18A) may behave similarly, and more extensive analyses are necessary to fully understand the consequences of Cdc42(C18A) expression *in vivo*.

Finally, a naturally occurring mutation within Ras, A18T, occurs at the analogous position to Cys^{18} in Cdc42 and is associated with excellent prognosis for patients with malignant melanoma (29). Like Cdc42(C18A), Ras(A18T) also increases spontaneous nucleotide exchange rates, and we suggest that Ras(A18T) may inhibit Ras functions and associated tumor progression by sequestering GEFs.

The importance of stabilizing the conserved conformational alterations of switch 1 by RhoGEFs is further emphasized by the role of Glu^{639} of Dbs (Fig. 1*B*). This residue makes three conserved hydrogen bonds to switch 1 of Cdc42 that are crucial for reconfiguring switch 1 to promote nucleotide ejection. Not surprisingly, when Glu^{639} is substituted with alanine (E639A), Dbs only weakly catalyzes the exchange of nucleotides within Cdc42 or RhoA.

Dbl family proteins stabilize nearly identical conformations of switch 2 in Rho GTPases, and these conformations are similarly recapitulated in the structures of Ras (Fig. 1*D*) or Ran in complex with their exchange factors (9, 10) as well as the structure of RhoA bound to GDP but without magnesium (Fig. 1*E*) (30). This latter structure is particularly intriguing, since it may represent a stable intermediate along the reaction coordinate for exchange that is subsequently bound by GEFs (30). In light of this idea, the failure of Cdc42(E62A) to bind Dbs might be explained best as a recognition problem; *i.e.* Cdc42(E62A) is either never adopting the correct conformation of switch 2 conducive to Dbs-catalyzed exchange, or the sampling of this conformation is too short lived for productive engagement of Dbs. In favor of the idea that certain dynamic states of the switch regions promote binding in native GTPases, Spoerner *et al.* (31) have shown that mutation of Thr^{35} to serine in Ras dramatically alters the dynamic equilibrium of the effector loop to favor conformations incompetent for effector binding.

Although the side chain of Ala^{59} of Cdc42 does make minimal contact with Dbs, similar to the other RhoGEF·GTPase structures, the primary interaction is through its carbonyl oxygen, which interacts with a conserved positively charged side chain within the DH domain (Lys^{774} in Dbs; Lys^{1195} in Tiam1; Arg^{1384} via a water molecule in intersectin) (Fig. 1*C*). In theory, this interaction should be preserved in the A59G mutation. Nevertheless, Cdc42(A59G) has a greatly reduced affinity for Dbs under the surface plasmon resonance assay conditions. This reduction in binding may arise from the introduction of conformational freedom within switch 2, (similar to that postulated for Cdc42(E62A)), or the Ala^{59} side chain may be instrumental in the transient removal of Mg^{2+} in the absence of GEF. Since both the A59G and E62A substitutions significantly reduced the intrinsic rate of exchange of Cdc42, this latter explanation is an attractive possibility for either mutant.

Generally, mutations similar to E62A of Cdc42 in other GTPases (Ran(E70A), Ras(E62H)) also severely impair GEF-catalyzed nucleotide exchange (10, 32), suggesting that identical aspects of GEF-catalyzed exchange are conserved among different GTPase families. However, on a cautionary note, Ala^{59} of Ras bound to Sos (9) occupies the same position as Ala^{59} of Cdc42 bound to Dbs (Fig. 1, *C* and *D*), yet A59G in Ras has no effect on the rate of GDP release catalyzed by Sos (33). Therefore, the GEF-catalyzed exchange reactions for Ras and Rho GTPases do not appear identical despite significant structural similarities.

Recently, the structure of the bacterial RhoGEF, SopE, has been determined in complex with nucleotide- and Mg^{2+}-free Cdc42 (11). Interestingly, although SopE is specific for Cdc42 and Rac1 (34), the overall architecture of SopE is unrelated to DH domains. However, superpositioning of SopE-bound Cdc42 with Dbs-bound Cdc42 reveals that switches 1 and 2 of the GTPase are in nearly identical conformations when complexed to either GEF. In addition, key GEF/GTPase interactions with the switch regions have been preserved, with SopE featuring Asp^{124} stabilizing switch 1 (equivalent to Glu^{639} in Dbs) and Gln^{109} (equivalent to Asn^{810} in Dbs) involved in binding switch 2 (5, 11). Within SopE·Cdc42, Cys^{18}, Val^{33}, Ala^{59}, and Glu^{62} are reoriented to nearly identical positions relative to Dbs·Cdc42, and these residues are presumably similarly involved in nucleotide exchange. Overall, these structural similarities suggest that SopE catalyzes guanine nucleotide exchange of Rho GTPases similar to Dbl family proteins.

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