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). 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. 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 vitro, Cdc42(C18A) exhibits a dominant-negative phenotype, presumably by sequestering exchange factors in nucleotide-depleted complexes analogous to...
EXPERIMENTAL PROCEDURES

Guanine Nucleotide Exchange Assays—A carboxyl-terminal His-tagged, wild type DH/PH fragment (residues 823-967) 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-methylnitraniloi (malt)-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 MgCl2, 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 (kon) for wild type and mutant Cdc42 proteins were determined by monitoring the decrease in tryptophan fluorescence (λex = 285 nm, λem = 335 nm) in response to binding mant-GTP (12-14). Because of their high intrinsic exchange rate, Cdc42(C18A) and Cdc42(W33A) 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 (λex = 360 nm, λem = 440 nm).

The rates (kon) of guanine nucleotide exchange were determined by fitting the data as single exponential decays utilizing GraphPad Prism 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 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 Mg2+ 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 of a surface lacking Dbs. Normalized sensorgrams were aligned, and the resulting dissociation constants are the mean of several sets of anlyte 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-DH-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). pCMVlac 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 lysis preparation. Analysis of luciferase expression with enhanced chemiluminescence reagents and a Monolight 3010 lumino-meter (PerkinElmer Life Sciences, 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-Cl, pH 7.4, 2 mM MgCl2, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aproton, and 1 μg/ml phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation at 10,000 × 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 Mg2+ binding site. As we have previously described (7), the repositioning of Cys18, Val33, Ala50, and Glu62 (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.

Cys18 and Val33 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, Cys18 can no longer hydrogen-bond to a nucleotide α-phosphate (Fig. 1B), 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 Cys18 to alanine increases the spontaneous loading of mant-GTP onto Cdc42 (~0.004/s versus 0.0003/s for Cdc42(WT)), partially mimicking the action of Dbs (Fig. 2A, Table I). The further stimulation of Cdc42(C18A) by Dbs (~0.046/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 Val33 within Cdc42 are moved into the site normally occupied by Cys18, promoting an alternate rotamer conformation of the cysteine residue that can no longer hydrogen-bond to the α-phosphate of guanine nucleotides (Fig. 1B). Therefore, substitution of Val33 to alanine within Cdc42 was designed to decouple structural alterations propagating from switch 1 to Cys18 within the phosphate binding loop. Since the rearrangement of Cys18 is predicted to be critically important to the GEF-catalyzed exchange reaction, mutation of Val33 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. 2B, 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 Val33 is needed not only to transmit structural alterations within switch 1 to Cys18 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 (S) (green; every 10th residue numbered) is superimposed on Cdc42 (transparent gray) bound to GDP (coordinates provided by N. Nassar) (transparent magenta) and Mg²⁺ (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 F) and Cdc42(C18A) (G and H) 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), 0.312 µM (purple), 0.625 µM (green), 1.25 µM (gold), and 2.5 µM (red). RU, response units.
**Mechanism of Rho GTPase Activation**

**Table I**

| 2 μM Cdc42 | Intrinsic rate ($k_{obs}$) | Rate with 0.2 μM Dbs ($k_{obs}$) | Stimulation | $K_r$ (+EDTA) | $K_r$ (-EDTA) |
|------------|---------------------------|---------------------------------|-------------|---------------|---------------|
|            | $s^{-1} \times 10^{-3}$   | $s^{-1} \times 10^{-3}$          | $\text{fold}$ |               |               |
| 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                    | 4 ± 0       | 714           | NB            |
| A59G       | 0.19 ± 0.01               | 0.79 ± 0.04                     | NB          | 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**

| Dbs | Cdc42 | RhoA |
|-----|-------|------|
|     | $k_{obs}$ | Stimulation | $k_{obs}$ | Stimulation |
|     | $s^{-1} \times 10^{-3}$ | $\text{fold}$ | $s^{-1} \times 10^{-3}$ | $\text{fold}$ |
| None | 0.28 ± 0.00 | 0.12 ± 0.00 | 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 |

**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...
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 Phe28 away from the nucleotide base, displaces Thr35 so that it no longer interacts favorably with the Mg2+ ion, and, via Val33, alters the side chain conformer of Cys18 so that it can no longer hydrogen-bond with the α-phosphate of GDP (Fig. 1B). The importance of Phe28 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 Cys18 of Cdc42 in binding nucleotides. However, the results presented here clearly demonstrate a critical role for Cys18 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 Cys18 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 (gon gf evenless) (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 Cys18 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 Glu639 of Dbs (Fig. 1B). 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 Glu639 is substituted with alanine (E639A), Dbs only weakly catalyzes the exchange of nucleotides within Cdc42 or RhoA.
Db family proteins stabilize nearly identical conformations of switch 2 in Rho GTPases, and these conformations are similarly recapitulated in the structures of Ras (Fig. 1D) or Ran in complex with their exchange factors (9, 10) as well as the structure of RhoA bound to GDP but without magnesium (Fig. 1E) (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 Thr35 to serine in Ras switch regions promote binding in native GTPases, Spoerner et al. (31) have shown that mutation of Thr35 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 Ala59 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 (Fig. 1C). In theory, this interaction should be preserved in the A59G mutation. Nevertheless, Cdc42(A59G) has a greatly reduced affinity for Dbs under the surface plasma 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 Ala59 side chain may be instrumental in the transient removal of Mg2+ 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 nucleotide exchange (10, 32), suggesting that identical