Residues of the Rho Family GTPases Rho and Cdc42 That Specify Sensitivity to Dbl-like Guanine Nucleotide Exchange Factors*

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The Dbl-like guanine nucleotide exchange factor (GEF) Lbc oncprotein specifically activates the small GTP-binding protein Rho in mammalian fibroblasts to induce transformation and actin stress fiber formation, whereas another Dbl-related molecule, Cdc24, stimulates guanine nucleotide exchange of the Rho family GTPase Cdc42 to elicit effects on both gene induction and actin-based cytoskeleton change in Saccharomyces cerevisiae. To understand the mechanism of these functional interactions, we have taken a biochemical approach to probe the sites on Rho and Cdc42 that are involved in coupling to their respective GEFs, the Lbc and Cdc24 proteins. Point mutations in the switch II region of the small G-proteins, many of which would affect the interaction with GEF in the case of Ras, or a mutation in the switch I region that was identified as a contact site between Rab3A and Rab GEF had little effect on RhoA or Cdc42Hs with regard to the ability to interact with Lbc or Cdc24, suggesting that there exists a unique mechanism of regulation of the Rho family proteins by the Dbl GEFs. Analysis of a panel of chimeras (numbered by Cdc42Hs) is critical for binding of the RhoA mutation (D76Q) was found to be able to induce gene activation in fibroblast cells (4). Cdc42, on the other hand, was originally found in Saccharomyces cerevisiae playing roles in the regulation of polarized cell growth (5) and pheromone-stimulated gene induction (6), and its human homologue, Cdc42Hs, was shown to be involved in the actin-based filopodia formation (7, 8), the Jun N-terminal kinase-mediated transcriptional activation (9–11), and the stimulation of p70 S6 kinase in mammalian fibroblast cells (12). As in Ras, the cycling between the active GTP-bound and the inactive GDP-bound states of Rho family proteins is tightly regulated in cells (13). The guanine-nucleotide exchange factors (GEFs)1 accelerate the release of GDP from the small G-proteins, thereby facilitating GTP binding and the G-protein activations; the GTPase-activating proteins (GAPs) catalyze the conversion of the GTP-bound form of the G-proteins to the GDP-bound form by increasing their rates of GTP hydrolysis; and the GDP dissociation inhibitors may serve to stabilize the G-proteins at either state and to target them to specific intracellular locations.

A family of GEFs specific for Rho proteins has emerged over the past few years (14). Among the rapidly expanding Rho GEF family, members of which all contain a common motif designated as the Dbl homology domain (14), the Dbl oncprotein was the first to be shown to possess the ability to catalyze GDP/GTP exchange of a panel of Rho proteins, including RhoA and Cdc42Hs (15). Cdc24, the essential bud site assembly gene product in S. cerevisiae, acts as the physiological activator of Cdc42 during budding (16) and pheromone-induced mating (6). The Lbc oncprotein was found to induce foci and actin stress fiber formations in fibroblast cells through the Rho-mediated pathway (17), while Tiam-1 was shown to be an active GEF for Rac1 and Cdc42Hs and seems to influence the invasive capacity of T-lymphoma cells in a Rac-dependent manner (18). Recently, the FGD1 gene product, which is responsible for facioscapulohumeral dystrophy (Aarkog-Scott syndrome), was found to function as a Cdc42Hs-specific GEF (40). Other members of the Dbl family GEFs include the vav, ost, ect2, tim, lfc, and dbs oncogene products, the Bcr and Abl proteins, and the Ras activators Sos and (Ras-GRF) (14), yet their in vivo roles in the regulation of specific Rho proteins have to be determined.

The molecular mechanisms underlying the interaction between Rho family small GTP-binding proteins and Dbl-like GEFs remain unclear. It is thought that in analogy to the ligand-activated seven-transmembrane receptors, which in essence serve as GEFs for the heterotrimeric G-proteins (19), the GEFs for small GTP-binding proteins bind to the GDP-bound state of the proteins, and the GDP would then dissociate to yield nucleotide-free intermediates, which exhibit the highest affinities toward GEFs (15). Extensive biochemical and

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1 The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
yeast genetic studies to identify the sites on Ras involved in the interaction with its GEF Sos or Cdc25 have provided evidence that residues in the switch II region (residues 61–78 of Ras) are likely to be the major contributing sites for both GEF binding and GEF-stimulated catalysis (20–26). Mutagenesis studies of the Rab subfamily protein Rab3A of the Ras superfamily indicated that the residues encompassing the switch I domain (corresponding to residues 32–40 of Ras) are required for the responsiveness to GEF activity, and mutations in this region also significantly decreased the affinity of Rab3A for Rab3A GEF (27).

To determine whether the closely related subfamilies between the Ras superfamily members are reflected in similar modes of molecular interactions with their regulatory proteins, and in particular to begin to understand the mechanism of activation of Rho family proteins by Dbl-like GEFs, we have set out in this study to probe the sites of interactions between the Rho family proteins, Rho and Cdc42, and their respective GEFs, Lbc and Cdc24. The results presented here using a panel of chimeras made between RhoA and Cdc42Hs and by site-directed mutagenesis of RhoA and Cdc42Hs suggest that there exists a unique regulatory mechanism of Rho GTP-binding proteins to functionally couple to their GEFs, which requires the involvement of multiple regions of amino acids in their primary structure for GEF binding and subsequent catalysis of GDP release. The finding that different amino acid residues of RhoA and Cdc42Hs seem to be critical for interacting with their respective GEFs also raises the possibility that activation of each Rho family member by a specific GEF may engage in a distinct mechanism.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Site-directed mutants described in this work were generated by oligonucleotide-directed mutagenesis of RhoA and Cdc42Hs cDNAs cloned into M13p19 vector (Amersham Corp.) or by the polymerase chain reaction-based second extension amplification technique using the Pfu polymerase (Stratagene), with primers that contained the desired mutations. cDNAs of wild-type or mutant forms of RhoA and Cdc42Hs were subcloned into the BamHI and EcoRI sites of pGEX-KG vector to be expressed as glutathione S-transferase (GST) fusions (28). The sequences of mutagenized cDNA inserts were confirmed by manual or automated sequencing prior to protein expression. All point mutants used are described by single-letter amino acid denotations.

Construction of RhoA/Cdc42Hs Chimeras—RhoA/Cdc42Hs chimeric cDNAs were produced by using the polymerase chain reaction method using the Pfu polymerase, which generates blunt-ended DNA fragments in polymerase chain reactions. For example, to generate chimeras A (see Fig. 3), the product amplified from cDNA encoding Cdc42Hs with primers sandwiching the junction site (residue 21) and the 5′-end of the coding sequences containing a BamHI site was cloned together with the RhoA cDNA fragment, obtained with RhoA primers between the junction site and the 3′-end containing an EcoRI site, into the BamHI and EcoRI sites of pGEX-KG plasmid. The resulting chimeric insert in the vector was sequence-verified by automated fluorescence sequencing. All junctions of the chimeras were chosen at the most conserved regions between RhoA and Cdc42Hs (see Fig. 8). Amino acid residues in the chimeras are numbered according to the Cdc42Hs sequences, which match the corresponding residues of Ras before a 13-amino acid insert at residues 122–134, since RhoA contains an additional two amino acids at the amino terminus (see Fig. 8).

Expression of Recombinant Proteins—Expression and purification of GST fusion small GTP-binding proteins and Cdc42GAP from the pGEX vector-transformed E. coli were carried out as described previously (29). Production and purification of the Sf9 insect cell-expressed GST-Lbc, GST-Cdc42, and GST-Dbl were performed as described (15–17). The concentrations of proteins were estimated by Coomassie blue-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using bovine serum albumin as a standard, or by 32P]GTP binding for small GTP-binding proteins (27).

Guanine Nucleotide Exchange Assay—The [3H]GDP/GTP exchange of the RhoA/Cdc42Hs mutants and chimeras was measured at 24 °C as described (16). The exchange reactions were terminated at the indicated times by nitrocellulose filtration method, and the amounts of [3H]GDP remaining bound to the GTPases were normalized as the percentage of [3H]GDP bound at time 0. To assay the binding affinity of the G-protein mutants for GEF, a competitive binding protocol was employed (27). The known concentrations of mutant competitor were added into standard GEF assay mixtures containing 100 mM [3H]GDP, RhoA and ~0.1 μg of GST-Lbc, which is sufficient to cause 70% release of bound [3H]GDP from wild-type RhoA in 10 min.

GTPase Assay—The intrinsic and GAP-stimulated GTPase activities of RhoA/Cdc42Hs chimeras and mutants were measured as described (29) by the nitrocellulose filter-binding method.

Results

Lbc and Cdc24 Specifically Interact with Rho and Cdc42 GTPases, Respectively—The lbc oncogene product was the first mammalian member of the Dbl-like GEFs shown to function as a specific activator in vivo for Rho and to cause cellular transformation through the Rho signaling pathway (17). Fig. 1A shows that the insect cell-expressed GST-Lbc protein activates RhoA by stimulating the rate of [3H]GDP dissociation (compared with the sample containing GST alone), while in contrast, no detectable change in the time courses of GDP dissociation from Cdc42Hs was observed in the presence of GST-Lbc. Moreover, the Rho specificity of Lbc was also reflected in direct binding interactions, since as determined by the complex formation and competitive binding assays, Lbc does not bind to Cdc42Hs in vitro (Fig. 7).

In S. cerevisiae, both genetic and biochemical studies have clearly identified Cdc24 as an upstream component of Cdc42 signaling by serving as a Cdc24-specific GEF (16). Sharing 88% sequence identity with yeast Cdc42, the human homologue, Cdc42Hs, is able to complement cdc2 mutation (38) and, therefore, is able to act as an in vivo substrate for Cdc24. As shown in Fig. 1B, the purified GST-Cdc24 potently stimulates [3H]GDP/GTP exchange of GST-Cdc42Hs, to a similar extent as that of yeast Cdc42 (16). The effects of Cdc24 on Cdc24 appear to be specific, since neither recombiant Rho1 (16) nor RhoA (Fig. 1B) showed detectable responsiveness to Cdc24, and RhoA did not compete with Cdc42Hs for Cdc24 in a competitive binding assay (data not shown). From these results, we concluded that the specific interactions between RhoA and Lbc and between Cdc42Hs and Cdc24 provide representative cases to study the mechanism of activation of Rho GTPases by Dbl-like GEFs.

The Amino Acid Residues of RhoA and Cdc42Hs Required for Functional Interaction with GEF Are Distinct from That of Ras or Rab GTPases—A panel of mutants of RhoA and Cdc42Hs was constructed and expressed as active GEF fusion proteins (Fig. 2). These mutations were chosen for the purpose of comparison with that of Ras and Rab3A, two members of distinct subfamilies of the Ras superfamily that have been subjected to extensive mutational analysis with respect of their abilities to interact with GEFs. The close sequence relatedness among the Ras superfamily members (>27% sequence identity) (13) and...
the structural information of Ras (30), Arf (31), and Cdc42Hs (32) suggest that they share similar three-dimensional conformation and therefore might share a similar regulatory mechanism by GEF.

The switch I region mutants T35A and Y40K of the Rho proteins, which would impair effector interactions, demonstrated two extreme spectra of responsiveness to GEF stimulation; the T37A of RhoA and T35A of Cdc42Hs lost the responsiveness to Lbc or Cdc24 completely, whereas the Y42K of RhoA and the corresponding Y40K of Cdc42Hs had no detectable change in sensitivity to GEF when compared with wild-type proteins (Fig. 2). This contrasts with the effects on Rab3A and Ras with similar mutations; the former lost responsiveness to GEF in both of the corresponding mutations (27), and the

The later one suffered only ~3-fold reduction in GEF sensitivity with the T35A mutation (22). Extensive mutations in the switch II region of RhoA and Cdc42Hs, including the E64K, D67K, R70A, S73A, Y74A, D78K, and I80L mutants of RhoA and the Q61L, D63H, D65K, R66D, R68A, P69A, and F78L mutants of Cdc42Hs, did not change their sensitivity to GEF (Fig. 2), suggesting that these sites of RhoA and Cdc42Hs are not directly involved in interaction with GEF. In the case for Ras, both biochemical and genetic data have provided evidence that the switch II domain, including residues 61, 62, 63, 67, 69, and the distal switch II residues 73, 75, 76, and 78, forms direct interaction with GEF Cdc25, Ras-GRF, and/or Sos and that mutations in this region would abolish the responsiveness to Ras GEFs (20–26, 39). Based upon these findings, we conclude that the residues of RhoA and Cdc42Hs required to functionally
interact with their GEFs are distinct from that of Ras or Rab3A.

**Regions of Amino Acids in RhoA and Cdc42Hs That Specify Sensitivity to Lbc or Cdc24**—To assess the contributions of various regions of RhoA and Cdc42Hs in their interaction with Lbc and Cdc24, respectively, selected regions of RhoA or Cdc42Hs were substituted sequentially with the corresponding regions of Cdc42Hs or RhoA (Fig. 3). These RhoA/Cdc42Hs chimeras were expressed as stable GST fusion proteins and were readily purified from *Escherichia coli* by glutathione-agarose affinity chromatography (Fig. 4A) for functional assays of their intrinsic nucleotide-binding and GTPase activity, with respect to the activity of GEF or GAP stimulation. Substitution of residues 1–23 of RhoA with residues 1–21 of Cdc42Hs (chimera A) had no discernible effect on its ability to interact with Lbc in GDP/GTP exchange, while replacement of an additional 8 residues of RhoA (residues 24–31) with the corresponding portion of Cdc42Hs (chimera B) resulted in the loss of Lbc sensitivity (Figs. 3 and 4B). This indicates that the different sequences of the 8 amino acids between the GTPases include determinant(s) defining Lbc-specificity. Substitution of a stretch of amino acids of Cdc42Hs (residues 22–29) containing this region with that of RhoA (chimera C), however, was not sufficient to restore the responsiveness to Lbc (Figs. 3 and 4B), suggesting that an additional region(s) of RhoA is required. Construction of two additional chimeras between RhoA and Cdc42Hs further defined a second functional region important for the RhoA-Lbc interaction; replacement of residues 22–81 of Cdc42Hs by the corresponding sequences of RhoA (chimera D) resulted in the complete loss of Lbc responsiveness (Fig. 4B). Moreover, the intrinsic GDP dissociation rate was low and intrinsic nucleotide-binding properties of the chimeras were altered due to improper folding, all 10 chimeras tested above (chimeras A–J) were substantially less responsive to Lbc or Cdc24. In particular, chimera G substituted residues 1–29 of Cdc42Hs with the corresponding portion of RhoA and remained mostly responsive to Cdc24 (Fig. 4C). Thus, the important determinant(s) of Cdc42Hs involved in specific coupling with Cdc24 resides between amino acids 30 and 155, since chimera H, in which residues 30–155 of Cdc42Hs were replaced by the corresponding amino acids of RhoA (Fig. 3), was completely insensitive to Cdc24 (Fig. 4C). To determine whether more than one region of amino acids of Cdc42Hs in this central portion are required for interaction with Cdc24, as appeared to be the case for the RhoA-Lbc interaction, we constructed chimeras I and J, in which residues 121–191 and residues 82–120 and 156–191 of Cdc42Hs were substituted by the homologous sequences of RhoA, respectively (Fig. 3). Both failed to respond to Cdc24 stimulation (Fig. 4C), indicating that determinants in two regions of Cdc42Hs, residues 82–120 and residues 121–155, are contributing to the specific coupling between Cdc42Hs and Cdc24.

To rule out the possibility that the intrinsic biochemical properties of the chimeras were altered due to improper folding, all 10 chimeras tested above (chimeras A–J) were subjected to GEF and GAP assays using Dbl and Cdc24GAP as stimulants, respectively, which are capable of catalyzing the GDP dissociation or GTP hydrolysis of both RhoA and Cdc42Hs (15, 29). The chimeras, like wild-type RhoA and Cdc42Hs, retained responsiveness to both Dbl and Cdc24GAP (Fig. 3). Moreover, the intrinsic GAP dissociation rate was low and comparable with wild-type GTPases in every chimera tested (data not shown). Hence, the chimeras were synthesized properly, and the effects on the sensitivity to Lbc and Cdc24 observed reflected the requirements for specific determinants in each construct.

**Amino Acid Residues of Cdc42Hs and RhoA That Are Critical to GEF Sensitivity**—To determine the amino acid residues of RhoA and Cdc42Hs responsible for the specific interaction with their respective GEFs, we generated a number of site-directed mutants of RhoA and Cdc42Hs in the regions implicated by the chimeras to be required for interaction with Lbc or Cdc24. In the region of amino acids 24–31 of RhoA, two residues, Lys27 and Asp28, which are nonconserved in Cdc42Hs (Fig. 5), were mutated to those of Cdc42Hs, K27T, and D28N. The K27T mutant became insensitive to Lbc stimulation, while the neighboring mutation D28N behaved similarly to wild-type RhoA.
The few different amino acids in the region of residues 57–83 of RhoA were Trp 58, Asp 76, Ile 80, and Met 82, among which Asp 76 was the only one drastically different from the corresponding residue of Cdc42Hs (Gln 74) (Fig. 8). The point mutant D76Q of RhoA showed significantly reduced sensitivity to Lbc (Fig. 5A), whereas changes made at two other residues (I80F and M82V) did not affect the Lbc responsiveness (data not shown). It is therefore likely that the difference at residue 25 of Cdc42Hs had caused the loss of response in chimera B and that the distinct residue at position 74 of Cdc42Hs had resulted in the insensitivity of chimera E to Lbc stimulation (Fig. 3).

Based on the analysis of chimeras, the regions of amino acids of Cdc42Hs required for specific interaction with Cdc24 are apparently different from that of RhoA for interaction with Lbc (Fig. 3). We generated reverse mutants of Cdc42Hs that corresponded to the K27T and D28N mutations of RhoA, T25K, and N26D and found that neither behaved differently from wild-type Cdc42Hs in response to Cdc24 (Fig. 5B). This is consistent with the result obtained using chimera G (Fig. 3), which suggests that the amino end of Cdc42Hs (residues 1–29) does not contribute to Cdc24 specificity. A search in the region between residues 82 and 120 of Cdc42Hs for determinants required for interacting with Cdc24, as implicated by the results of chimera J (Fig. 3), revealed that the unique residue Gln 116 is a critical site, since mutant Q116K of Cdc42Hs completely lost responsiveness to Cdc24 (Fig. 5B). Additional mutagenesis scanning in this region of Cdc42Hs, including mutations of V85D, S88D, F90L, V93–94IP, and V98E, did not identify other sites critical for the interaction (data not shown), suggesting that Gln 116 is probably the only residue involved in this region. Notably, Gln 116 of Cdc42Hs (and Cdc42Sc) is the only exception at the NKXD motif of the Ras superfamily small GTP-binding proteins in which a highly conserved Lys is replaced by Gln, and changes in this motif have been shown to alter the guanine nucleotide binding properties of Ras and to affect the interaction with GEF SDC25 (30, 32). These results led us to conclude that the residues of Cdc42Hs that specify sensitivity to Cdc24 are distinct from those specifying sensitivity of RhoA to Lbc.

The observation that T35A mutation in the switch I domain
of both RhoA and Cdc42Hs lost GEF sensitivity (Fig. 2) led us to further examine the effect of mutations in this region of the GTPases. Double mutation of RhoA just adjacent to switch I, SE32-33 (SE33), where residues Glu32 and Val33 were mutated to the corresponding residues of Cdc42Hs, Ser30 and Glu31, showed no effect on Lbc coupling (Fig. 5A). Mutations of the highly conserved residues Tyr34 and Phe39 of RhoA (or Tyr32 and Phe37 of Cdc42Hs) to Lys and Glu, respectively, resulted in the loss of response to GEF stimulation (Fig. 5). To test whether the nonresponsive mutants in this region still behave as wild-type proteins in GDP-dissociation and to see if their insensitivity to Lbc or Cdc24 can be extended to the case for Dbl, the intrinsic and Dbl-stimulated rate of $[^{3}H]$GDP/GTP exchange of the Y32K, T35A, and F37E mutants of RhoA and Cdc42 (numbered by Cdc42) were compared with that of wild types. As shown in Fig. 6A, all three mutants of RhoA retained the wild-type intrinsic GDP/GTP exchange rate, and the T37A and F39E mutants remained as responsive to Dbl as wild type RhoA, while the Y34K mutant was insensitive to Dbl stimulation. Similar observations were made for the corresponding mutants of Cdc42 (data not shown). These results suggest that the Y32K, T35A, and F37E mutants (numbered by Cdc42) preserved the wild-type global conformation and that residue Tyr32 of the Rho proteins may provide a common site for Lbc, Cdc24, and Dbl interactions. Alternatively, these mutations, especially Y32K, have led to a perturbation of native structure that is not detectable by measuring the change of the basal exchange rate. Given that these mutations of RhoA and Cdc42Hs have been shown to be involved in effector or GAP binding (33, 34), these results further raised the possibility that residues at the switch I region of Rho proteins might provide a common interactive site for GEF, effector, and GAP.

To see whether the rest of the RhoA and Cdc42Hs point mutants that became insensitive to Lbc or Cdc24 still retained normal GDP dissociation and GTP hydrolysis properties, the intrinsic as well as the Dbl- or GAP-stimulated rates of $[^{3}H]$GDP release or $[^{32}P]$GTP hydrolysis of these mutants were examined. Fig. 6B shows that the K27T and D76Q mutants of RhoA had similar intrinsic rates of GDP-dissociation as wild-type RhoA and that they remained fully responsive to Dbl stimulation. The intrinsic GTPase activity as well as the GAP-catalyzed GTP hydrolysis of these mutants also appeared unaltered (Fig. 6C). A similar observation was made with the Q116K mutant of Cdc42Hs (data not shown). Thus, these mutants behave indistinguishably from wild-type proteins in these tests.

**Catalysis of GDP/GTP Exchange of RhoA by Lbc May Be Separable from Lbc Binding Interactions**—To determine the mechanism by which the mutations abolish sensitivity to GEF, a panel of mutants of RhoA that became insensitive to Lbc stimulation was tested for the ability to bind to Lbc by complex formation and by competitive inhibition. As shown in Fig. 7A, the glutathione-agarose-bound GST-T37A and GST-D76Q

![Graph A](image1.png)

**Fig. 6.** The intrinsic GDP/GTP exchange properties of RhoA mutants and the kinetics of their responsiveness to Dbl and Cdc42GAP. A, $[^{3}H]$GDP/GTP exchange rates of RhoA and the mutants Y34K, T37A, and F39E were measured in the presence of GST alone (closed symbols) or GST-Dbl (open symbols). The reaction conditions were same as in Fig. 1. RhoA with GST: ●, Y34K with GST; ○, T37A with GST; ▲, F39E with GST; ¶, RhoA with GST-Dbl; ▲, Y34K with GST-Dbl; ●, T37A with GST-Dbl; ○, F39E with GST-Dbl. B, time courses of $[^{3}H]$GDP/GTP exchange of wild-type RhoA and K27T and D76Q mutants in the presence (open symbols) or absence of GST-Dbl (closed symbols). ●, RhoA without GST-Dbl; ○, RhoA with GST-Dbl; ▲, K27T without GST-Dbl; ●, D76Q without GST-Dbl; ○, RhoA with GST-Dbl; ▲, K27T with GST-Dbl; ●, D76Q with GST-Dbl. C, intrinsic (closed symbols) and Cdc42GAP-stimulated (open symbols) $[^{32}P]$GTP hydrolysis of wild-type RhoA and K27T and D76Q mutants. ●, K27T without GAP; ○, RhoA without GAP; ▲, D76Q without GAP; ▲, K27T with GAP; ○, RhoA with GAP; ¶, D76Q with GAP.
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In in vivo situations (16, 17) provided the physiological relevance to study these couplings, particularly in the context of comparisons between the Rho-Lbc and Cdc42Hs-Cdc24 pairs themselves and of the interactions between Ras and Ras GEF.

By mutagenesis and chimera approaches we have made the following observations concerning the RhoA-Lbc and Cdc42Hs-Cdc24 interactions. (i) Mutations of RhoA and Cdc42Hs corresponding to the T35A mutant of Ras lost responsiveness to the respective GEFs completely, while the corresponding Y40K mutants of RhoA and Cdc42Hs showed no change in GEF-sensitivity; in addition, mutations in multiple sites of the switch II domain of RhoA and Cdc42Hs, including changes in many conserved residues between RhoA and Cdc42Hs, had no effect on GEF-stimulated GDP/GTP exchange. (ii) Substitutions of stretches of amino acids in two separate regions of RhoA (residues 24–31 and residues 57–83) and of Cdc42Hs (residues 82–120 and residues 121–155) with the homologous regions of Cdc42Hs and RhoA, respectively, resulted in functional chimeras (Dbl- and GAP-responsive) that are nonetheless Lbc- or Cdc24-insensitive. (iii) Mutations of the unique residues Lys27 and Asp76 of RhoA and Gln116 of Cdc42Hs to the corresponding amino acids of Cdc42Hs or RhoA led to the loss of Lbc or Cdc24 responsiveness. Moreover, mutations of the highly conserved residues Tyr32, Thr35, and Phe37 of RhoA and Cdc42Hs (numbered by the Cdc42Hs sequences) also resulted in the loss of response to GEF stimulation, while their intrinsic GDP/GTP exchange rates appear unaltered. (iv) The Lbc-insensitive mutants K27T and Y34K of RhoA suffered significantly reduced affinity toward Lbc compared with wild-type RhoA, while the T37A and D76Q mutants retained wild-type affinity. These observations are significant given that all chimeras and point mutants employed in this study retain the wild-type properties of GDP-release, and all but the Y32K mutants remain sensitive to Dbl and/or GAP; therefore, they are likely to have caused little or only local change in protein conformation by the modifications.

By modifying Ras, it has been shown that the switch I and switch II regions, as well as the network of interactions involved in the coordination of the Mg2⁺-nucleotide complex and the positioning of the guanine base, are crucial elements for Ras GEF SDC25 activity (20–26, 39). In particular, the T35A mutation in the switch I region, which changes the coordinates of Mg2⁺, suffered ~3-fold reduction in SDC25 sensitivity, while the switch II region mutants Q61L, E62H, and E63H had ~2-, 5-, and 5-fold reduction in SDC25 responsiveness, respectively (22). A number of studies have also provided in vivo evidence that changes in residues at 62, 63, 67, 69, 73/74, 75/77, 75, 76, and 78 in Ras disrupted the Ras GEF coupling (20–26, 39). In the case for Rab3A, extensive mutagenesis studies have assigned the switch I domain as a binding site for Rab3A GEF, including the Thr34 and Phe39 residues (corresponding to the Thr35 and Tyr40 of Ras), mutations of which severely impaired the affinity for Rab3A GEF (27). Our results of the RhoA and Cdc42Hs mutations in these regions (Fig. 8) indicate that mutants of the Ras family GTPases respond to their GEFs differently in sensitivity and/or affinity, compared with the similar mutants of Ras or Rab3A, although the switch I region, including the conserved residues Tyr32, Phe37, and the Mg2⁺-coordinating residue Thr35, still appears to be critical for the Lbc- or Cdc24-mediated activation of RhoA and Cdc42Hs. Thus, despite the close similarities between members of the Ras superfamily, a distinct activation mechanism of the Ras proteins involving different amino acids is at work to facilitate the GEF-induced guanine nucleotide exchange.

By analyzing the behavior of RhoA/Cdc42Hs chimeras, it is clear that the interaction of the Rho proteins with their GEFs...
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Fig. 8. Alignment of the amino acid sequences of RhoA, Cdc42Hs, and Ha-Ras. Numbering follows the Cdc42Hs sequences. Identical amino acids to the Rho sequences are represented as solid dots except those being mutated in this study. A gap in the alignment is denoted by a dash. The residues of RhoA and Cdc42Hs subjected to mutation in this study are underlined. The residues of RhoA implicated in coupling to Lbc are indicated by arrows, and the residues of Cdc42Hs found involved in interacting with Cdc24 are shown in boldface type. The secondary structures indicated below the aligned sequences are based on the x-ray crystallography data of Ha-Ras.

is complex. For example, substitution of residues 22–81 of Cdc42Hs with the homologous RhoA sequences (chimera D) was sufficient to confer Lbc responsiveness, yet replacement of residues 30–191 or residues 22–55 of Cdc42Hs with the RhoA sequences resulted in Lbc-insensitive chimeras (chimera B and E). Given that these effects were not the consequences of alteration of the biochemical properties of the proteins, we concluded that there exist at least two separate sites required for Lbc sensitivity in the regions spanning residues 24–31 and residues 57–83 of RhoA. Similarly, two sites in the primary structure of Cdc42Hs essential for the specific coupling with Cdc24 were identified, spanning the regions of amino acids 82–120 and amino acids 121–155, respectively. The existence of specific determinants at these regions was further demonstrated by mapping the distinct residues in the implicated regions that are required for the specificity, e.g. residues Lys27 and Asp76 of RhoA, and residue Gln116 of Cdc42Hs. In addition, the amino terminus of the switch I region, especially the conserved Tyr34, may serve as a common determinant required for binding of all Dbl-like GEFs (Fig. 8).

A possible explanation of how the D76Q mutation of RhoA was able to induce negative effects on the GEF reaction independently from a direct interaction may come from the examination of the GDP/GTP exchange mechanism. As in the case for Ras (22), the reaction sequence may constitute the alternation of nucleotide-free and nucleotide-bound states of Rho, a mechanism supported by the observations that the Dbl-like GEFs can form a stable complex with the nucleotide-depleted Rho protein and have very weak affinity for the GTP-bound Rho (15). It is therefore possible that the GEF activity is associated with a succession of specific conformational changes of Rho proteins that could influence the response to GEF even if the GEF interaction site is located in distal regions. According to the Ras three-dimensional structure (30), the charged residue Asp76 is situated at the distal switch II domain of the Ras structure, which is adjacent to the highly mobile L4 loop and thus may be involved in the catalytic aspect of the GEF action without directly contributing to the GEF binding event.

The roles of other regions of Cdc42Hs, e.g. the divergent region between amino acids 121 and 155 that is implicated by chimeras F and I (Fig. 3) in interaction with Cdc24, remain to be determined. However, it is clear that the residues of Cdc42Hs required for interacting with Cdc24 are distinct from those of RhoA for interacting with Lbc. For example, the unique Gln116 residue of Cdc42Hs (and Cdc42Sc), which deviates from the consensus Lys at the NXXD motif of the Ras superfamily responsible for interacting with the guanine base of bound guanine nucleotide (32) is found to be involved in the Cdc24-stimulated GDP/GTP exchange reaction, while the residue (Lys27) at the a1 region of RhoA that is required for RhoA-Lbc interaction does not seem to be important for the Cdc42-Cdc24 coupling (Fig. 5). It is therefore possible that distinct mechanisms of activation for Rho family proteins are employed in functional interaction with individual Dbl-like GEFs. This is further supported by the observation that the T35A and F37E mutants of RhoA and Cdc42Hs remain fully responsive to Dbl but are completely insensitive to Lbc or Cdc24 (Figs. 2, 5, and 6) and by our studies of Cdc42Hs-Dbl interaction with Cdc42Hs/Rac1 chimeras, the results of which indicate that yet another region in Cdc42Hs, the o3 helix, contains the determinant critical for the Dbl-catalyzed GDP/GTP exchange reac-
Rho and Cdc42 Residues That Specify Sensitivity to Dbl-like GEFs

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