The Molecular Basis of RhoA Specificity in the Guanine Nucleotide Exchange Factor PDZ-RhoGEF*

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The Dbl homology nucleotide exchange factors (GEFs) activate Rho family cytosolic GTPases in a variety of physiological and pathophysiological events. These signaling molecules typically act downstream of tyrosine kinase receptors and often facilitate nucleotide exchange on more than one member of the Rho GTPase superfamily. Three unique GEFs, i.e. p115, PDZ-RhoGEF, and LARG, are activated by the G-protein coupled receptor pathway. The interaction with the DH domain, it is not a selectivity determinant, and its interaction with PDZ-RhoGEF is unfavorable. The key selectivity determinants are dominated by polar contacts involving residues unique to RhoA. We find that selectivity for RhoA versus Cdc42 is defined by a small number of interactions.

The Rho family GTPases, comprised of 22 distinct, yet homologous proteins encoded in the human genome, act as molecular switches that regulate a plethora of cellular functions including regulation of actin cytoskeleton, cell cycle progression, gene transcription, neurite growth, cell adhesion, cell migration, and others (1–7). The most ubiquitous and the best studied of these are RhoA, Rac1, and Cdc42 (1, 3, 5). The upstream stimuli activating Rho-mediated processes typically involve both tyrosine kinase receptors and G-protein-coupled receptors. The signals are then transmitted either directly or through cascades of protein-protein interactions, to guanine nucleotide exchange factors (GEFs), i.e. large, multidomain cytosolic proteins that are capable of catalyzing the exchange of the GDP nucleotide on the GTPase for GTP and therefore activate the respective signaling pathway (8). The GEFs can either activate specific Rho-GTPases or several of them. A vast majority of Rho-specific GEFs physically act on the respective GTPase through two intimately linked domains, i.e. a 200-residue-long Dbl homology (DH) domain and an adjacent, 100-residue-long PH domain (9). Genomic analyses reveal around 70 such GEFs in the human genome, several of which are products of oncogenes linked to various forms of cancer (8). It is generally assumed that the isolated DH-PH tandem is constitutively active and that in their nascent, inactive form in the cytosol the GEFs inhibit that activity through supramodular interdomain interactions (10).

Because of its biological importance, the mechanism by which the DH-PH fragment exerts its function has been under considerable scrutiny. It has been shown early on, that the DH-domain specifically binds to and stabilizes nucleotide-free form of its cognate Rho-GTPase (11). This alone could tip the equilibrium toward a GTP-containing complex, because cells contain about 10 times as much GTP as GDP, and upon release from the complex with GEF, the GTPase, which binds both nucleotides with approximately the same affinity, is much more likely to bind the more abundant GTP. Two questions are particularly intriguing about the activation mechanism of the Rho-GTPase: 1) why are some GEFs specific for one GTPase while others activate indiscriminately two or more targets; and 2) what exactly is the role of the PH domain which invariably accompanies the DH domain at the C terminus.

In the past few years our understanding of the mechanistic aspects of DH-PH interactions with Rho-GTPases has been significantly expanded because of a number of crystallographic investigations which revealed atomic models of several complexes. These studies included the DH-PH tandem of Tiam1 in complex with Rac1 (12), Dbs in complexes with Cdc42 (13) and RhoA (14), and ITSN (intersectin) in complex with Cdc42 (14). The studies revealed that the DH domain is primarily responsible for the nucleotide exchange function and GTPase recognition and that it interacts with the respective GTPase in a conserved fashion. In general terms, there are three distinct surface patches on the GTPase, which come into contact with any DH domain. The first two involve residues within switch I and switch II of RhoA. These generic contacts pry the nucleotide binding site open and are responsible for the stabilization of the nucleotide free GTPase. However, these highly conserved interactions of both switch regions do not include structural determinants required for specific recognition of GTPase by a given member of Dbl family and so in the absence of additional...
interactions they could conceivably allow for the binding of any DH domain to any Rho-GTPase. The actual specificity-defining epitope on the GTPase includes the solvent-exposed residues from the β1, β2, and β3 strands. On the basis of the characterized crystal structures of complexes of GTPases with respective DH-PH tandems, it has been possible to formulate some general schemes by which selectivity is achieved (8, 14–17). However, these results do not explain all the aspects of GTPase selectivity by various highly specific GEFs. One such family, acting downstream of G-protein-coupled receptors, includes RhoA (residues 1–193), Cdc42 (residues 1–191), and Rac1 (residues 1–192) were individually cloned into a modified pET vector (28) in fusion with a His6 tag. The QuikChange™ site-directed mutagenesis kit from Stratagene, Inc. was used to generate all mutants described in this paper, according to manufacturer’s instructions.

Expression and Purification of Mutant Proteins—The wild type form and mutants of DH-PHPRG were expressed as GST fusion proteins in the BL21(DE3)-RIL E. coli cells. Transformed cells were grown at 37 °C in LB medium in the presence of 100 μg/ml ampicillin to an A600 of 0.8–1.0 and induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 21 °C overnight. Purification protocol was based on the procedure described for the wild type DH-PHPRG (29) except for the ion-exchange chromatography (HiTrap Q-Sepharose, Amersham Biosciences), which replaced size exclusion as the final purification step. Briefly, the N-terminal-tagged mutants were purified by glutathione-Sepharose 4B affinity chromatography, subsequently digested with recombinant tobacco etch virus (30), and passed over the second glutathione-Sepharose 4B column (Amersham Biosciences) to remove the GST moiety. Following the ion-exchange chromatography, the samples were estimated to be at least 95% pure as judged by SDS-PAGE. The protein concentration was determined using UV absorption at λ = 280 nm. RhoA, Cdc42, and Rac1 mutants were purified by nickel-nitriolotriacetic acid-agarose affinity chromatography according to the protocol described previously for wild type RhoA, Cdc42, and Rac1 GTPases (27). His6 tags were removed by recombinant tobacco etch virus protease. Prior to nucleotide exchange, assays purified GTPases were incubated with excess GDP (ICN Biomedicals, Inc.) in the presence of EDTA. After 1 h, excess MgCl2 was added, and the unbound nucleotide was removed using a HiPrep desalting column (Amersham Biosciences).

**GUANINE NUCLEOTIDE EXCHANGE ASSAY** —The rate of guanine nucleotide exchange was monitored by the increase in the N-methylanthraniloyl-GTP (Mant-GTP) fluorescence intensity as a result of its binding by the GTPase. Mant-GTP analog was synthesized according to the published protocol (31). GDP, GTP, N-methylsiaoic anhydride, and triethylamine were purchased from Sigma. The exchange reaction was monitored using a Jasco FP-750 spectrofluorimeter at 21 °C, in a 1-cm stirred quartz cuvette in a buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1 mM DTT, 5% glycerol. Briefly, the exchange reaction mixture containing 1 μM GDP-preloaded GTPase and 500 nM Mant-GTP was allowed to equilibrate, while stirred, and the relative fluorescence increase of the Mant-GTP (λex = 356 nm, λem = 445 nm, slits = 5 nm/5 nm) was monitored simultaneously. After ~200 s equilibration time, the DH/PH tandem, or the isolated DH domain, was added at 100 nM and the stimulated exchange reaction was continued for 600 s. The initial rates of guanine nucleotide exchange were determined by linear regression analysis of data corresponding to 10–100 s after DH/PH stimulation, depending on the mutant’s activity. Parallel, intrinsic exchange activity in the absence of the DH/PH tandem was measured for each mutant after equivalent equilibration time and used as control. Each nucleotide exchange assay was carried out three times, and the presented data constitute an average. The average variation among measured rate did not exceed 15%. Fold stimulation values were calculated as the ratio of the initial exchange rate of DH or DH-PH-stimulated reaction to the intrinsic rate of exchange for the wild type or mutant GTPase.

**RESULTS AND DISCUSSION** —The recombinant DH-PHPRG fragment (residues 712–1081) and the isolated DH domain were cloned into pDEST15 vector in fusion with glutathione-S-transferase (GST) as described in previous study (27). The clone used in this study contained an accidental mutation, N715T, which was a result of a cloning artifact. This mutation was propagated in all derivative clones obtained by mutagenesis. We determined that this mutation fractionally increases the catalytic activity of the DH-PH tandem variants but does not impact on the comparative results presented in this study. Human RhoA (residues 1–193), Cdc42 (residues 1–191), and Rac1 (residues 1–192) were individually cloned into a modified pET vector (28) in fusion with a His6 tag. The QuikChange™ site-directed mutagenesis kit from Stratagene, Inc. was used to generate all mutants described in this paper, according to manufacturin’s instructions.

**EXPERIMENTAL PROCEDURES** —Site-directed Mutagenesis of RhoA, Cdc42, Rac1, and DH-PH—The recombinant DH-PHPRG fragment (residues 712–1081) and the isolated DH domain were cloned into pDEST15 vector in fusion with glutathione-S-transferase (GST) as described in previous study (27). The clone used in this study contained an accidental mutation, N715T, which was a result of a cloning artifact. This mutation was propagated in all derivative clones obtained by mutagenesis. We determined that this mutation fractionally increases the catalytic activity of the DH-PH tandem variants but does not impact on the comparative results presented in this study. Human RhoA (residues 1–193), Cdc42 (residues 1–191), and Rac1 (residues 1–192) were individually cloned into a modified pET vector (28) in fusion with a His6 tag. The QuikChange™ site-directed mutagenesis kit from Stratagene, Inc. was used to generate all mutants described in this paper, according to manufacturer’s instructions.
Met966, which is H-bonded to Asn929 within the C-terminal nucleotide exchange enhancement (data not shown). However, two residues on PH to Ala have no significant impact on the those reported previously, i.e. 124-fold for RhoA and weak, but measurable 3-fold for Cdc42. No measurable activity was detected for Rac1 (Table 1).

We then asked if the PH domain plays a role in GTPase selectivity. The crystal structure of the DH-PHPRG-RhoA complex (27) shows that the contact between the PH domain and RhoA is limited to only two H-bonds: one involving Asn1068 within the switch II of RhoA and another between Ser1065 and the main chain amide of Glu997. Mutations of either of the two residues on PH to Ala have no significant impact on the nucleotide exchange enhancement (data not shown). However, there is also an indirect contact, involving the carbonyl of Met966, which is H-bonded to Asn929 within the C-terminal fragment of the α6 helix of the DH domain, which in turn is H-bonded to Arg68 within the switch II of RhoA. When Asn929 is mutated to Ala, the stimulation is significantly reduced (data not shown). This is most likely due to the stabilizing effect that the PH domain exerts on the long helix, which connects it with the DH domain (α6 helix of the DH domain) and to a much lesser extent on the direct interaction of the PH domain with the GTPase. Thus, we conclude that for all practical purposes the functionally important DH-PH/RhoA contacts defining the GTPase specificity exclusively involve the DH module.

We asked, therefore, if an isolated DH domain was capable of mediating nucleotide exchange on RhoA. We designed three DH-domain constructs, i.e. 712–943, 729–939, and 712–932. Of these, the last one was expressed at high levels in E. coli and had the ability to stimulate nucleotide exchange on RhoA ~48-fold but not at a level of the DH-PH tandem. The 729–939 variant of DH domain has marginal activity, as reported previously (27), but this appears to be primarily a result of poor stability (data not shown). Thus, although the PH domain does not seem to play a direct role in defining the GEF’s specificity, its presence is required for the expression of full activity and therefore we used the full DH-PHPRG tandem in all subsequent experiments.

### Table 1

| GTPase (intrinsic) | DH-PH PRG | Cdc42 wt | Rac1 wt |
|-------------------|-----------|---------|---------|
| wt                | 33.6      | 123     | 1       |
| 867Q              | 12.1      | 45      | 1.5     |
| R867H             | 14        | 52      | 1.5     |
| R868K             | 7.5       | 28      | 1.5     |
| R868G             | 1.4       | 5       | 0.5     |
| L869A             | 5.3       | 20      | 0.5     |
| L869M             | 17.9      | 66      | 0.5     |
| R872S             | 28        | 104     | 1.5     |
| R872D             | 14        | 52      | 1.5     |
| D873S             | 23.5      | 87      | 1.5     |
| D873N             | 5.4       | 20      | 0.5     |
| I876L             | 16.9      | 63      | 1.5     |
| I876M             | 7.1       | 26      | 0.5     |
| I876V             | 39.3      | 145     | 1.5     |
| I876P             | 60.9      | 225     | 0.5     |

| GTPase (intrinsic) | DH-PH PRG | Cdc42 wt | Rac1 wt |
|-------------------|-----------|---------|---------|
| wt                | 0.27      | 0.19    | 0.05    |
| 867Q              | 0.12      | 0.06    | 1       |
| R867H             | 0.15      | 0.07    | 1       |
| R868K             | 0.16      | 0.08    | 1.6     |
| R868G             | 0.17      | 0.09    | 1.5     |
| L869A             | 0.18      | 0.09    | 1.6     |
| L869M             | 0.19      | 0.1     | 0.5     |
| R872S             | 0.2       | 0.1     | 1.5     |
| R872D             | 0.2       | 0.1     | 1.5     |
| D873S             | 0.2       | 0.1     | 1.5     |
| D873N             | 0.2       | 0.1     | 1.5     |
| I876L             | 0.2       | 0.1     | 1.5     |
| I876M             | 0.2       | 0.1     | 1.5     |
| I876V             | 0.2       | 0.1     | 1.5     |

| GTPase (intrinsic) | DH-PH PRG | Cdc42 wt | Rac1 wt |
|-------------------|-----------|---------|---------|
| wt                | 0.27      | 0.19    | 0.05    |
| 867Q              | 0.12      | 0.06    | 1       |
| R867H             | 0.15      | 0.07    | 1       |
| R868K             | 0.16      | 0.08    | 1.6     |
| R868G             | 0.17      | 0.09    | 1.5     |
| L869A             | 0.18      | 0.09    | 1.6     |
| L869M             | 0.19      | 0.1     | 0.5     |
| R872S             | 0.2       | 0.1     | 1.5     |
| R872D             | 0.2       | 0.1     | 1.5     |
| D873S             | 0.2       | 0.1     | 1.5     |
| D873N             | 0.2       | 0.1     | 1.5     |
| I876L             | 0.2       | 0.1     | 1.5     |
| I876M             | 0.2       | 0.1     | 1.5     |
| I876V             | 0.2       | 0.1     | 1.5     |

FIGURE 1. A, a molecular surface representation of RhoA. The specificity patch including β1, β2, and β3 strands (yellow) as well as switch I and switch II regions (magenta) are highlighted and presented as ribbons. B, alignment of non-conserved residues within the specificity-defining regions of RhoA, Cdc42, and Rac1.

The crystal structure of the DH-PHPRG tandem in complex with RhoA (Fig. 2A) shows that the specificity-defining epitope on the target RhoA GTPase involves residues from the β1, β2, and β3 strands, i.e. Argβ5, Gluβ10, Valα3, Aspα5, Gluα4, Trpα8, and Aspα76 from switch 2 loop (Fig. 1A). Except for Trpα8, which is also found in the homologous position in Rac1 (Trpβ6), all these residues are unique to RhoA (Fig. 1B), and they interact primarily with the cluster of amino acids located on the α5 helix of the DH domain, specifically Argβ6, Argβ8, Leuα6, Argβ7, Aspβ73, and Ileα76 (Fig. 2, B and C). To better understand the functional aspects of this interface we prepared a series of single and multiple site mutants of both RhoA and DH-PHPRG. The functional properties of these mutants are shown in Tables 1 and 2.

The Trpα5, Valα3 “Cradle”—Residues Leuα6 and Ileα76 of the DH domain are part of a hydrophobic cradle, which binds the solvent-exposed hydrophobic side chains of Valα3 and Trpα8 on RhoA (Fig. 2B). In GEFs other than PDZ-RhoGEF, the amino acids corresponding to Leuα69 and Ileα767 have been shown to be critical for RhoA/Rac1 recognition and also for recognition of Cdc42 through interaction with Pheα6 in the latter GTPase (14, 15). A leucine, with its bulky side chain (equivalent to Ileα76) in DH-PHPRG, is preferred in all Cdc42-specific GEFs, which accommodate the smaller Pheα6 of Cdc42. In contrast, an isoleucine is found at this position in those GEFs that recognize the indole moiety of Trp in RhoA and Rac1. Thus, mutations Leu1376 → Ileα76 (Cdc42-specific GEF) and Leu1366 → Ileα76 (RhoA-specific GEF) allowed both GEFs to catalyze exchange in Rac1 (14, 15). Moreover, a Trpα8 → Phe substitution in Rac1 resulted in the complete loss of its sensitivity to Tiam1 (Rac-specific GEF) and allowed for recognition by ITSN (Cdc42-specific GEF) (16, 32). Based on these observations,
Trp$_{56}$ of Rac1 (equivalent to Trp$_{58}$ in RhoA) was proposed as a necessary and sufficient specificity determinant for recognition by Rac-specific GEFs (32). To better understand the role of Trp$_{58}$ for specific recognition of RhoA by DH-PH PRG we mutated this residue to Phe. Surprisingly, the Trp$_{58}$ Phe mutant of RhoA shows significantly enhanced sensitivity to DH-PH PRG with 198-fold stimulation in contrast to the 124-fold stimulation observed for wild type GTPase (Table 2 and Fig. 3A).

In addition to Trp$_{58}$, Val$_{43}$ is also an important component of the hydrophobic “knob” on RhoA. Its side chain is in van der Waals contacts with Leu$_{869}$ and Arg$_{868}$ of DH-PH PRG. In Cdc42 and Rac1, the equivalent position is occupied by Ala and Ser, respectively. When Val$_{43}$ is replaced in RhoA with a Ser, the exchange activity is reduced ~25-fold, the most critical impairment among all tested mutants of RhoA (Table 2 and Fig. 3A).

We then asked how changes in the Trp$_{58}$-Val$_{43}$ cradle of the DH domain affect its functionality. We noted that the position equivalent to Ile$_{876}$ in PDZ-RhoGEF and other Rho-specific GEFs is not restricted to an isoleucine and that other hydrophobic residues are found in this position, e.g. Pro in p115 and LARG, Leu in Lfc and Lbc, Met in Vav, and Val in Vav2. In contrast, Cdc42-specific GEFs prefer leucine and Rac1-specific GEFs isoleucine at the equivalent position. We made four single-site mutants at position 876, replacing the wild type Ile with the four above listed amino acids. The activity of these mutants correlates inversely with the size of the substituted side chain. Methionine, the largest of the four, reduces the activity 4-fold, while, in contrast, a Pro results in a nearly 2-fold increase in activation (Table 1 and Fig. 3B). This trend is consistent with the behavior of the Trp$_{58}$ Phe mutant of RhoA (Table 2 and Fig. 3A). Thus, either reducing the size of the residue in position 58 of RhoA, or increasing the size of the corresponding pocket on the DH domain, increases significantly the activity of DH-PH PRG. Clearly, Trp is not an optimal residue on the RhoA GTPase for activation by PDZ-RhoGEF. In accordance with this observation, a substitution of Trp for Phe$_{56}$ in Cdc42 completely abolishes the already weak activity of DH-PH PRG toward Cdc42 (data not shown). However, the Ile$_{876}$ Leu variant of DH-PH PRG is not sufficient to rescue the exchange rate on Cdc42 (Table 1).

The second residue, which is critical for the functionality of the cradle, is Leu$_{869}$. Its replacement with alanine in DH-PH PRG significantly reduces exchange activity on RhoA, from 124- to 20-fold (Table 1 and Fig. 3B). The majority of known RhoA-specific GEFs retain a Leu at the position equivalent to Leu$_{869}$, whereas Cdc42-specific GEF-ITSN has a slightly larger Met in this position, allowed by the presence of alanine in position 41 of Cdc42 (Val in RhoA). The Leu$_{869}$ Met mutation did not, however, increase the sensitivity of DH-PH PRG toward Cdc42 (Table 1).

The results of our experiments differ from those reported for the equivalent Leu$_{759}$ and Leu$_{766}$ in Dbs (15), which in vitro acts on both RhoA and, to lesser extent, Cdc42. Mutation of Leu$_{759}$ Dbs (equivalent to Leu$_{869}$ in PDZ-RhoGEF) to both Met and Ile, crippled GDP exchange on RhoA, from 124- to 20-fold (Table 1 and Fig. 3B). The majority of known RhoA-specific GEFs retain a Leu at the position equivalent to Leu$_{869}$, whereas Cdc42-specific GEF-ITSN has a slightly larger Met in this position, allowed by the presence of alanine in position 41 of Cdc42 (Val in RhoA). The Leu$_{869}$ Met mutation did not, however, increase the sensitivity of DH-PH PRG toward Cdc42 (Table 1).
exchange on RhoA but did not affect the Cdc42 exchange. Both mutations resulted in a gain of function, which conferred on Dbs the ability to catalyze nucleotide exchange on Rac1. This was particularly evident for the L766IDbs mutant, which showed 57-fold activation of Rac1. The Cdc42 F56W mutant is also a good substrate for Dbs (L766I), with 81-fold stimulation of nucleotide exchange. Interestingly, a double mutant of both Leu759 and Leu766 to Met abolishes exchange on Cdc42 while maintaining exchange on RhoA.

The Salt Bridge “Zipper”—Considering that Trp58 does not constitute a sufficiently cohesive interaction favoring RhoA, we set out to determine the specific role played by the cluster of charged residues, previously implicated as specificity determinants in the DH domain and involving residues Arg867, Arg868, Arg872, and Asp873 (Fig. 2C). We mutated Arg867, Arg868, Arg872, and Asp873 individually to amino acids that occur at these locations in other exchange factors with distinctly different specificities. All mutants show lower activity on RhoA, with mutants in position 868 showing the most dramatic effects. Even a conservative substitution to a Lys reduced nucleotide exchange stimulation from 124- to 28-fold, while mutation to Gly, a residue found at this position in intersectin, reduces it to mere 5-fold (Table 1 and Fig. 3A). A replacement of Asp45 with Asn in RhoA shows a half the rate enhancement of nucleotide exchange on RhoA (14), in which a Lys in this position interacts with Asp45 and Glu54 of RhoA. A Lys→Ala mutant of Dbs shows a half the rate enhancement of nucleotide exchange on RhoA (14), a significantly smaller difference than that caused by the removal of Arg868 on DH-PH<sup>PRG</sup>. Crystal structures of DH-PH<sup>LARG</sup> (26) and DH-PH<sup>PRG</sup> (27) in complexes with RhoA both show that the Arg in question is involved in a multiple H-bond network with both Glu54 and Asp45 of RhoA. The higher resolution structure of DH-PH<sup>PRG/PRG</sup> (27) allows for a more reliable visualization of specific hydrogen bonds, although both models indicate that Arg868 forms two strong H-bonds to Asp45 and interacts via a single bond with Glu54 (Fig. 2C). Accordingly, the replacement of Asp45 with Asn in RhoA has more dramatic consequences than replacement of Glu54. Moreover, a double mutant of RhoA (D45N/E54Q) allows for only 9-fold enhancement of nucleotide stimulation (Table 2 and Fig. 3A). Thus, considering how Asp45 and Glu54 are both important for recognition, it is not surprising that Arg868, which engages both these amino acids, is critically important. While lysine retains the electrostatic attraction between the proteins, it is unable to form the three H-bonds, which seem to constitute the key structural determinant for the recognition of RhoA. Interestingly, previously published mutational studies of Rac1 (32) show that Rac1 residues corresponding in RhoA to Val43, Asp45, and Glu54, i.e. Ser41, Asn43, and Asn52, do not play a role in recognition by either Trio, GEF-H1, or Tiam1. Thus, this epitope is associated with a new functionality for RhoA, rather than one with variable functionality, as is the case with Trp58.

The crystal structure of the DH-PH<sup>PRG</sup> in complex with RhoA also reveals yet another unique electrostatic interaction, involving Arg<sup>5</sup> of RhoA and Asp<sup>873</sup> of DH-PH<sup>PRG</sup> (Fig. 2C). No such contact is possible for either Cdc42 or Rac1, which have Thr and Ala, respectively, at the position of Arg5 in RhoA. To verify the importance of this salt bridge we mutated both Arg<sup>5</sup> of RhoA and Asp<sup>873</sup> of DH-PH<sup>PRG</sup>. The R5A RhoA mutant shows ~10-fold reduced response to DH-PH<sup>PRG</sup> as compared with the wild type (Table 2 and Fig. 3A). A replacement of Asp<sup>873</sup> with a neutral Asn results in a similar (~6-fold) reduction of the exchange activity of DH-PH<sup>PRG</sup> (Table 1 and Fig. 3C). Finally, two more salt bridges Asp<sup>867/PRG</sup>-Glu<sup>40/RhoA</sup> and Asp<sup>872/PRG</sup>-Asp<sup>76/RhoA</sup> exert moderate effects on the recognition of RhoA by PDZ-RhoGEF (Tables 1 and 2 and Fig. 3, A and C).
Conversion of Cdc42 and Rac1 into Substrates for DH-PHPRG—The critical changes in nucleotide exchange due to specific amino acid replacements suggest that susceptibility to PDZ-RhoGEF may be defined by the synergistic action of just a few residues on RhoA. To test whether such a limited surface epitope could effectively mimic RhoA, we engineered several mutants of Cdc42 and Rac1, in which positions 3, 41, 43, and 52, which correspond to Arg$^5$, Val$^{43}$, Asp$^{45}$, and Glu$^{54}$ in RhoA, are mutated to their RhoA counterparts (Table 3). Just a double substitution T3R/A41V in Cdc42 significantly elevated the susceptibility of GTPase to PDZ-RhoGEF, from 3- to 39-fold with additional increase of up to 64-fold for the quadruple variant T3R/A41V/T43D/T52E (Table 3 and Fig. 3D). Thus, only four mutations result in 20-fold higher sensitivity of Cdc42 to the catalytic activity of DH-PHPRG, converting GTPase to a substrate that is half as good kinetically as RhoA. In contrast, analogous variants of Rac1 behaved differently. The double mutant A3R/S41V has no impact on susceptibility to nucleotide exchange, and only moderate enhancement of sensitivity (up to 14-fold stimulation) was observed for the quadruple mutant A3R/S41V/N43D/N52E (Table 3 and Fig. 3D).

CONCLUSIONS

The specificity of Dbl homology nucleotide exchange factor determinates the interplay of numerous signaling and regulatory pathways in the cell. The GEFs of the p115 family show a unique pattern of acting downstream of Go$^{12/13}$-coupled receptors and activating selectively RhoA. The mechanisms by which these GEFs accomplish this selectivity have not been well studied to date. We show that in the case of PDZ-RhoGEF, this selectivity is due primarily to the synergistic functionality of several charged residues in the DH domain, namely Arg$^{67}$, Arg$^{68}$ and Arg$^{872}$, and Asp$^{873}$. These residues interact intimately with a unique cluster of amino acids on the solvent accessible face of RhoA, i.e. Arg$^{5}$, Asp$^{45}$, Ghu$^{54}$, and Asp$^{76}$. No more than four mutations can dramatically alter the susceptibility of a GTPase to the catalytic activity of GEF. For example, the T3R/A41V/T43D/T52E mutant of Cdc42 is half as good a substrate as RhoA for DH-PHPRG.

It has not been possible to reproduce these susceptibility changes in Rac1 using a similar set of mutations, and it is not easy to rationalize these results. One of the mechanisms by which Rac1 is strongly disfavored may include Trp$^{56}$. We show here that Trp$^{38}$ in RhoA (equivalent to Trp$^{56}$ in Rac1) is sterically unfavorable, and Trp$^{56}$ was not one of the mutated residues in the quadruple variant of Rac1. It is possible that the introduction of favorable interactions in Cdc42, including the salt bridge zipper, is sufficient to turn it into a suitable substrate for PDZ-RhoGEF, while in Rac1 Trp$^{56}$ continues to generate a repulsive force.

This work, along with previous studies, demonstrates that even single sporadic mutations in Dbl homology GEFs have the potential to significantly alter substrate specificity. In evolutionary terms, such mutations and associated gene duplication events can lead to major phenotype changes as new signaling pathways are created. Unfortunately, sporadic mutations may also lead to malignant transformation as a result of changes in signaling networks.

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