Substrate Specificity and Recognition Is Conferred by the Pleckstrin Homology Domain of the Dbl Family Guanine Nucleotide Exchange Factor P-Rex2*

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Dbp family guanine nucleotide exchange factors (GEFs) are characterized by the presence of a catalytic Dbl homology domain followed invariably by a lipid-binding pleckstrin homology (PH) domain. To date, substrate recognition and specificity of this family of GEFs has been reported to be mediated exclusively via the Dbl homology domain. Here we report the novel and unexpected finding that, in the Dbp family Rac-specific GEF P-Rex2, it is the PH domain that confers substrate specificity and recognition. Moreover, the β3β4 loop of the PH domain of P-Rex2 is the determinant for Rac1 recognition, as substitution of the β3β4 loop of the PH domain of Dbp (a RhoA- and Cdc42-specific GEF) with that of P-Rex2 confers Rac1-specific binding capability to the PH domain of Dbp. The contact interface between the PH domain of P-Rex2 and Rac1 involves the switch loop and helix 3 of Rac1. Moreover, substitution of helix 3 of Cdc42 with that of Rac1 now enables the PH domain of P-Rex2 to bind this Cdc42 chimera. Despite having the ability to recognize this chimeric Cdc42, P-Rex2 is unable to catalyze nucleotide exchange on Cdc42, suggesting that recognition of substrate and catalysis are two distinct events. Thus substrate recognition can now be added to the growing list of functions that are being attributed to the PH domain of Dbp family GEFs.

The Rho family of G proteins are members of the Ras superfamily (1, 2). A hallmark of G proteins is their ability to undergo conformational changes upon binding to either GTP or GDP. These G proteins therefore function as binary switches, as different partner proteins within the cell recognize the GTP-bound “on” state and the GDP-bound “off” state of the protein. There are twenty Rho family members identified to date, with RhoA, Rac1, and Cdc42 being the most well characterized members (3). They carry out a wide and diverse range of functions, from regulating the actin cytoskeletal network to the interactions, it cannot explain the specificity of all GEF-Rho subunit interactions (25). Dbs and Dbl are able to activate both Cdc42 and RhoA (23). Vav1 and Vav2 catalyze exchange on RhoA, Rac1, and Cdc42 (24). The structural basis for this recognition process is only beginning to be understood (25). The crystal structures of the Rac1-Tiam1, Cdc42-Dbs, Dbs-RhoA, and Cdc42-Intersectin complexes show that the CR1 and CR3 regions of the DH domain of the GEF make extensive contact with the β2β3 strands of the GTPases (25–28). These regions were therefore predicted to determine the specificity of these interactions. Based on these crystal structure complexes, Trp56 of Rac1 was identified as one of the critical residues for recognition by Rac-specific GEFs (27). Mutation of this residue to Phe, the corresponding residue in Cdc42, was sufficient to abolish binding by Tiam1. Moreover, this mutant Rac1 could now be recognized by Intersectin, a Cdc42-specific GEF. Although this region clearly determines the specificity of some of these interactions, it cannot explain the specificity of all GEF-GTPase interactions (25). Moreover, the contribution of the PH domain in these interactions has remained unexplored.

P-Rex1 was isolated from neutrophils based on its activity as a Rac-specific GEF (29). Its activity was conditional on the presence of phosphatidylinositol 3,4,5-trisphosphate or Gβγ subunits. P-Rex1 plays a crucial role in stimulating the neutrophil-oxidative burst (29). Our biochemical characterization of P-Rex2, a homologue of P-Rex1, shows that it also is a

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¶ The abbreviations used are: Dbl, diffuse B-cell lymphoma; GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; GST, glutathione S-transferase; Dbs, big sister of Db; GTPγS, guanosine 5′-3′-O-(thio)triphosphate.

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Rac-specific GEF. In this report, P-Rex2 is shown to recognize Rac1 in an unusual way. The specificity of this interaction is conferred by the PH domain of P-Rex2 and not by the DH domain. Specificity of substrate interaction has never before been attributed to the PH domain of a Db family GEF. This report demonstrates that, within the PH domain, the β3β4 loop is necessary and sufficient to confer this specificity. In addition, the PH domain of P-Rex2 appears to make contact with the switch loop and helix 3 of Rac1. However, although helix 3 of Rac1 is sufficient to confer binding to a chimeric GTPase, the helix by itself is not sufficient to allow P-Rex2 to catalyze nucleotide exchange. This suggests that substrate recognition and catalysis by the P-Rex2 GEF have distinct requirements.

EXPERIMENTAL PROCEDURES

Materials—The wild-type bacterial expression construct for RhoA was created by PCR amplification and cloned into the Smal/NotI sites of pGEX-4T-1 (Amersham Biosciences). Bacterial expression constructs for Rac1 and Cdc42 were kind gifts from Prof. Gary Bokoch (The Scripps Research Institute, La Jolla, CA). The DHPh domains of Db and the PH domain of Dbs were PCR-amplified from the human mammary cDNA library (Clontech) and cloned into the pcDNA3.1D V5-His-TOPO vector (Invitrogen). The DHPh domain (residues 22–231), the PH domain of Dbs were PCR-amplified from the human mammary cDNA library (Clontech) and cloned into the pcDNA3.1D V5-His-TOPO vector (Invitrogen). The DHPh domain (residues 241–370), and the DHPh domains of P-Rex2 (residues 22–370) were cloned into pcDNA3.1D V5-His-TOPO vector by PCR. The β3β4 loop deletion (amino acid residues 277–296), mutant of the PH domain of P-Rex2, and the Rac1W56F mutant were created using the QuikChange site-directed mutagenesis kit from Stratagene, according to the manufacturer’s protocol. The Dbs/P-Rex2 β3β4 loop chimeric PH domain was created by PCR as follows. The 5′ end of the chimera was created by PCR using oligos 5′-CATCTGTCGACTG-3′ (antisense) and 5′-GATCTGGTTCCGCGTGGATCCAAAATTATTTCAGC-3′ (sense). The 3′ end of the chimeric gene was amplified using oligos 5′-GGACAAGACAGCCCACATCAAGGCTACATGGTACATGATCTTTTTTCCCATGCTAAGACAGCCATGTCGACTG3′ (sense) and 5′-CGGCTCTGTGACTGGAGCATCGTACGACTGCAGTCGAGCT-3′ (antisense). The 3′ and 5′ ends of the gene obtained by PCR were purified and were merged together by PCR using oligos 5′-CACATCGTCTGATCGAGGTGAGGGCTCATTCAGC-3′ (sense) and 5′-GACCGTCGTCGACTGCTGCTGATGCAGGGCTCATTCAGC-3′ (antisense). The full-length PCR product was subsequently purified and cloned into the pcDNA3.1D V5-His-TOPO vector by TOPO cloning. The bacterial expression construct for the Cdc42Rac1 helix 3 chimera was created by PCR as follows. The 5′ end of the chimera was created by PCR using oligos 5′-GATCTGGTTCCGCGTGGATCCAAAATTATTTCAGC-3′ (antisense) and 5′-CACATTTCCTTCAACGATGCCAAATAGGATGAGGCCACAGGG-3′ (antisense). The 3′ end of the chimera was created by PCR using oligos 5′-GATCTGGTTCCGCGTGGATCCAAAATTATTTCAGC-3′ (antisense) and 5′-CATCTGTCGACTGCTGCTGATGCAGGGCTCATTCAGC-3′ (antisense). The 5′ and 3′ ends of the gene obtained by PCR were purified, mixed together, and the full-length chimeric gene was PCR-amplified by PCR using oligos 5′-GATCTGGTTCCGCGTGGATCCAAAATTATTTCAGC-3′ (antisense) and 5′-CATCTGTCGACTGCTGCTGATGCAGGGCTCATTCAGC-3′ (antisense). The PCR product was digested with BamHI and EcoRI and was ligated into the pGEX-2T vector (Amersham Biosciences) cut with the same enzymes.

Phosphorylation of Proteins—GST and GST-tagged Rac1, RhoA, and Cdc42 were expressed and purified from Escherichia coli as described elsewhere (30).

Complex Formation Assay—The complex formation assay was performed essentially as described previously (31). The DHPh domains of P-Rex2, Db, or Dbs were labeled with [35S]Met using the rabbit reticulocyte lysate-coupled transcription translation system (Promega) for 90 min at 30 °C in a total reaction volume of 10 μL, according to the manufacturer’s instructions. 2 μL of this reaction was then incubated with 5 μg of purified GST, GST-Rac1, GST-RhoA, or GST-Cdc42 in 500 μL of complex formation assay buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 2.0 mM EDTA, and 0.5% Triton X-100) with 25 μL of glutathione-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C. The beads were then boiled in SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE, transferred onto a nitrocellulose membrane, and detected by autoradiography. All experiments were repeated at least three times to ensure the reproducibility of the experiments. The data shown are representative of each of these data sets.

Rac1 was loaded with GDP or GTP·S, as described earlier (31). Briefly, 12.5 μM purified GST-Rac1 was incubated with 20 μM GDP or GTP·S in loading buffer (10 mM Hepes, pH 7.5, 100 mM NaCl, 7.5 mM EDTA) at 23 °C for 25 min. The complexes were stabilized using 20 mM MgCl2, 5 μg of the loaded protein was then used for the complex formation assay in a modified complex formation assay buffer with 20 mM MgCl2 instead of 2.0 mM EDTA.

Guanine Nucleotide Exchange Assay—The in vitro GEF assay was performed on a Mithras LB 940 (Berthold Technologies) fluorescence spectrophotometer by modifying a basic protocol described elsewhere (32). 2 μM purified GST-tagged Rho family G protein was incubated with 100 μM purified GST-tagged P-Rex2 and 400 μM N-methylanthraniloyl-GDP (Molecular Probes) in 50 μL of exchange buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 50 μg/μL bovine serum albumin, and 10% glycerol). Excitation and emission wavelengths were 355 nm and 460 nm, respectively.

RESULTS

The Rac1W56F Mutation Does Not Affect P-Rex2 Binding—Comparison of the crystal structure complexes of Tiam1 and Rac1 with other Rho GTPase-GEF complexes has identified the Trp56 residue of Rac1 as a key determinant of specificity for Rac-specific GEFs (27). This residue of Rac1 was shown to interact with a conserved Ile in the DH domain of Rac-specific GEFs. However, sequence alignment of the DH domain of P-Rex2 with that of other GEFs shows that this conserved Ile is not present in the DH domain of P-Rex2 or P-Rex1 (Fig. 1A).

Because mutation of the Trp56 residue of Rac1 to Phe is sufficient to abolish binding by the DHPh domain of the Rac1-specific GEF Tiam1, we decided to test whether this mutation does not affect the binding ability of the DHPh domain of P-Rex2. The DHPh domain of P-Rex2 labeled with [35S]Met using a rabbit reticulocyte lysate was tested for its ability to bind GST or GST-tagged forms of Rac1 WT or Rac1W56F mutant in a pull-down assay. The proteins were transferred onto a nitrocellulose membrane, and binding was detected by autoradiography. The total lysate shown represents the input of the experiment. Ponceau S staining of the membrane detected the protein levels. WT, wild-type.

### FIG. 1. Determinants of specificity of P-Rex2/Rac1 interaction differ from those of other Rac1-specific GEFs.

| GEF        | a5helix | Substrate  |
|------------|---------|-----------|
| hP-Rex2    | GKRKENRLVPLDQVPTFPQR1CKYP | Rac1      |
| hP-Rex1    | GKRKTDDLPSVQPTFPQR1CKYP  | Cdc42     |
| Tiam1      | NPPQCGSTRLELQKPTFPQR1CKYP | RhoA & Cdc42 & Rac1 |
| Trp        | RGLLI--AESIYSGKPVPRRQKTVQY |
| UNC-73     | -GLEINRMALSDQKPVPRRQKTVQY |
| TFSN       | MDPRCMGMPFLSPKPTFPQR1CKYP |
| Cdc42      | KWLQMDTIALDDQKPVPRRQKTVQY |
| Dbs        | QKLLKMLKLSLDQKPVPRRQKTVQY |
| Db         | QRLKGLRQLSLSDQKPVPRRQKTVQY |
| Vz         | KVQDQFKQLDLVNYVRKPVPRRQKTVQY |

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2 R. E. Joseph and F. A. Norris, manuscript submitted for publication.
PH Domain Confers Substrate Recognition and Specificity

**Fig. 2.** Substrate recognition and specificity is conferred by the PH domain of P-Rex2. A, the DH, PH, and DHPH domains of P-Rex2 were labeled with [35S]Met and tested for their ability to bind GST or GST-tagged forms of RhoA, Rac1, or Cdc42 in a pull-down assay as before. The DHPH domain of Dbl was used as a positive control. Binding was detected as before. B, dose response curve of P-Rex2 PH domain with Rac1. The PH domain of P-Rex2 was labeled with [35S]Met and tested for its ability to bind to varying amounts of GST-tagged Rac1. Ponceau S staining of the membrane shows the protein levels.

**Fig. 3.** The β3β4 loop of the PH domain of P-Rex2 is necessary for its interaction with Rac1. A, alignment of the β3β4 loop of the PH domain of P-Rex2 with that of other PH domains. The predicted secondary structure is based on the crystal structure of the Grp1 PH domain and is indicated by the two β-sheets. B, the wild-type (WT) PH domain of P-Rex2 and β3β4 deletion mutant of the PH domain of P-Rex2 were labeled with [35S]Met and tested for their ability to bind GST or GST-tagged Rac1WT in a pull-down assay as before. Binding was quantified by densitometry and normalized against that of the WT PH domain. Protein levels were assessed by Ponceau S staining.

**Fig. 4.** The β3β4 loop of the PH domain of P-Rex2 is sufficient for its interaction with Rac1. The PH domains of P-Rex2, Dbs, and the β3β4 chimeric PH domain of Dbs were labeled with [35S]Met and tested for their ability to bind GST or GST-tagged RhoA, Rac1, or Cdc42 in a pull-down assay as before. Binding was detected by transferring the proteins onto a nitrocellulose membrane followed by autoradiography. Ponceau S staining shows the protein levels.

Suggested that the mode of interaction of P-Rex2 with Rac1 was different from that of other Rac-specific GEFs.

**Substrate Recognition Occurs via the PH Domain of P-Rex2**—Substrate recognition by the Dbl family of GEFs has been thought to be mediated exclusively by the DH domain (25). The above result indicates that P-Rex2 might be an exception. To determine the domain of P-Rex2 that recognizes its Rac1 substrate, the DH, PH, and DHPH domains of P-Rex2 were expressed and labeled with [35S]Met using a reticulocyte lysate and tested for their ability to interact with GST-Rac1 in a pull-down assay. The DHPH domain of Dbl, which interacts with Rac1 as well as RhoA and Cdc42, was used as a positive control. As shown in Fig. 2A, surprisingly, the PH domain alone of P-Rex2 was able to interact with Rac1, whereas the DH domain alone of P-Rex2 was unable to do so. Moreover, the PH domain of P-Rex2 was able to discriminate between Rac1, RhoA, and Cdc42 and bind specifically to Rac1. Increasing amounts of GST-tagged Rac1 were able to associate with increasing amounts of the PH domain, indicating that the levels of GTPase being used in the pull-downs were not saturating (Fig. 2B). This is the first demonstration of the ability of a PH domain of a Dbl family GEF to recognize its G protein substrate.

The β3β4 Loop Region of the PH Domain of P-Rex2 Is Required for Rac1 Interaction—The crystal structure of the Dbl family GEF Dbs in complex with its substrate Cdc42 shows that, in addition to interaction of the DH domain with Cdc42, the PH domain of Dbs has an extended β3β4 loop, which makes contact with the switch loop 2 and helix 3b of Cdc42 (28). Alignment of the PH domain of P-Rex2 with other PH domains shows that the P-Rex2 PH domain also has an extended β3β4 loop (Fig. 3A). To determine whether this loop of P-Rex2 makes contact with Rac1, a β3β4 loop deletion mutant of P-Rex2 PH domain was tested for its ability to bind Rac1. As shown in Fig. 3B, the β3β4 loop deletion caused a >80% reduction in the ability of the P-Rex2 PH domain to bind Rac1. This suggests that the β3β4 loop of the PH domain of P-Rex2 is required for the interaction with Rac1.

The β3β4 Loop Region of the PH Domain of P-Rex2 Is the Determinant of Rac Interaction—Although deletion of the β3β4 loop of the PH domain of P-Rex2 caused a dramatic decrease in Rac1 binding, it was possible that this mutation affected the overall structure of the PH domain. The Dbl family GEF Dbs catalyzes exchange specifically on RhoA and Cdc42 and not on Rac1. Binding studies with the PH domain alone of Dbs showed that it did not interact with RhoA, Rac1, or Cdc42 (Fig. 4). To determine whether the β3β4 loop of the PH domain of P-Rex2 would be sufficient to confer Rac1 recognition, we created a chimeric PH domain in which the β3β4 loop region of the Dbs PH domain was replaced with that of P-Rex2. As shown in Fig. 4, this chimeric PH domain acquired the ability to specifically interact with Rac1 and not RhoA and Cdc42. This demonstrated that the β3β4 loop of the PH domain of P-Rex2 is sufficient to produce Rac1 interaction.

Nucleotide Binding of Rac Influences Interaction with the PH Domain of P-Rex2—The crystal structure of Dbs in complex with Cdc42 shows that, in addition to contacts between the DH domain and Cdc42, the PH domain makes contact with the GTP-sensitive switch loop 2 of Cdc42 (28). To test whether the PH domain of P-Rex2 can also sense the nucleotide status of Rac1, purified GST-tagged Rac1 was loaded with GDP or GTP-γS and tested along with nucleotide-free Rac1 for the ability to bind the PH domain. As shown in Fig. 5, the nucleotide-bound forms of Rac1 showed a dramatic reduction in the ability to bind the PH domain. This suggests that the PH domain of P-Rex2 makes nucleotide-sensitive contacts with Rac1, possibly in the switch 2 loop region, by analogy to what was seen in the Dbs/Cdc42 crystal structure contacts.

Helix 3 of Rac Is the Region That Determines the Rac1 Specificity of Binding of the PH Domain of P-Rex2—The crystal structure of Cdc42 with Dbs shows that the Dbs PH domain also makes contact with helix 3b of Cdc42 (28). This region is adjacent to the switch 2 loop of Cdc42. Importantly, the primary sequence of helix 3 differs between Rac1 and Cdc42 (Fig.
PH Domain Confers Substrate Recognition and Specificity

The PH domain of P-Rex2 interacts with the switch loops of Rac1. GST-tagged Rac1 was loaded with GTPyS or GDP and tested for their ability to interact with the PH domain of P-Rex2 as before. The PH domain of P-Rex2 showed diminished binding to the GDP-loaded form of Rac1 as compared with the nucleotide-free form of Rac1 (Rac1NF). Ponceau S staining of the nitrocellulose membrane shows protein levels.

**DISCUSSION**

Activation of Dbl family GEFs in vivo lead to the activation of specific G protein signaling pathways (6). This specificity hinges on the ability of the GEFs to discriminate among the different G proteins present within the cell. Determination of the determinants of specificity of interaction between Dbl family GEFs and their cognate Rho GTPases is therefore necessary to understand signaling specificity. The crystal structures of Tiam1, Dbs, and Intersectin with Rac1, Cdc42/RhoA, or Cdc42, respectively, have provided a major breakthrough for identifying critical residues that determine the specificity of these interactions (25–28). Attention has been focused on the catalytic DH domain in particular, as it was found to make major contact with the GTPase. Alignment of the primary sequence of the DH domain of Rac1-specific GEFs has isolated a conserved Ile residue that makes contact with Trp 

**FIG. 5.** The PH domain of P-Rex2 interacts with the switch loops of Rac1. GST-tagged Rac1 was loaded with GTPyS or GDP and tested for their ability to interact with the PH domain of P-Rex2 as before. The PH domain of P-Rex2 showed diminished binding to the GTPyS- or GDP-loaded form of Rac1 as compared with the nucleotide-free form of Rac1 (Rac1NF). Ponceau S staining of the nitrocellulose membrane shows protein levels.

**FIG. 6.** Helix 3 of Rac1 confers specificity to the interaction between Rac1 and the PH domain of P-Rex2. A, alignment of helix 3 of Rac1 with that of Cdc42. B, the PH domain of P-Rex2 was tested for its ability to interact with the PH domain of Cdc42 chimera as before. Ponceau S staining of the nitrocellulose membrane shows protein levels.

P-Rex2 Does Not Catalyze Exchange on Chimeric Cdc42 with Helix 3—The previous binding experiments demonstrated that the PH domain of P-Rex2 has a role in the recognition of Cdc42. The PH domain of P-Rex2 was tested for its ability to interact with the PH domain of Cdc42 chimera as before. Ponceau S staining of the nitrocellulose membrane shows protein levels.

**FIG. 7.** P-Rex2 does not catalyze exchange on the Cdc42 chimera. In vitro GEF activity of P-Rex2 was monitored by the increase in fluorescence associated with the binding of N-methylanthraniloyl-GDP by Rac1 or Cdc42 chimera. The blanks for Rac1 and Cdc42 chimera are indicated by filled triangles and open circles, respectively. The effect of the concentration of P-Rex2 along with 0.4 μM Gβγ subunits to Rac1 (filled squares) or the Cdc42 chimera (open triangles) is shown.
domain recognizes Rac1 by interacting with the switch loop and helix 3. Substitution of helix 3 of Cdc42 with that of Rac1 enabled the PH domain to interact with this chimeric protein. Moreover, it is likely that the PH domain of P-Rex2 interacts with switch loop 2 of Rac1 rather than switch loop 1 because of its proximity with helix 3б. Even though P-Rex2 could interact with the Cdc42 chimera, it was unable to catalyze exchange on it. This suggests that substrate recognition and catalysis are two distinct events. Although the PH domain may recognize the substrate, it is the contacts being made by the catalytic DH domain that determine enzymatic activity. Other Dbl family GEFs have been previously shown to interact with many GTPases; however, they could catalyze exchange on a limited subset of these GTPases. Dbl, for example, can bind to RhoA, Rac1, and Cdc42 but will catalyze exchange on only RhoA and Cdc42 (28, 37). Further studies will be required to delineate the contacts that are being made in these two distinct processes.

The structure of the DHPH domain of Dbl family GEFs shows that the orientation of the PH domain with respect to the DH domain can vary widely (28). The region in between the DH and PH domains varies greatly between different GEFs. However, it is this intervening region that determines the placement of the PH domain relative to the DH domain. Moreover, the NMR structure of Sos shows that this linker region is extremely flexible (13). This suggests that the orientation of the PH domain with the Cdc42 chimera, it was unable to catalyze exchange on it. This suggests that substrate recognition and catalysis are two distinct events. Although the PH domain may recognize the substrate, it is the contacts being made by the catalytic DH domain that determine enzymatic activity. Other Dbl family GEFs have been previously shown to interact with many GTPases; however, they could catalyze exchange on a limited subset of these GTPases. Dbl, for example, can bind to RhoA, Rac1, and Cdc42 but will catalyze exchange on only RhoA and Cdc42 (28, 37). Further studies will be required to delineate the contacts that are being made in these two distinct processes.

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Addendum—While our manuscript was under revision, two other laboratories reported the characterization of P-Rex2 (38, 39).

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