Structural Determinants of RhoA Binding and Nucleotide Exchange in Leukemia-associated Rho Guanine-Nucleotide Exchange Factor*

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Rho guanine-nucleotide exchange factors (RhoGEFs) activate Rho GTPases, and thereby regulate cytoskeletal structure, gene transcription, and cell migration. Leukemia-associated RhoGEF (LARG) belongs to a small subfamily of RhoGEFs that are RhoA-selective and directly activated by the Akt1 family of heterotrimeric G proteins. Herein we describe the atomic structures of the catalytic Dbl homology (DH) and pleckstrin homology (PH) domains of LARG alone and in complex with RhoA. These structures demonstrate that the DI/PH domains of LARG can undergo a dramatic conformational change upon binding RhoA, wherein both the DI and PH domains directly engage RhoA. Through mutational analysis we show that full nucleotide exchange activity requires a novel N-terminal extension on the DH domain that is predicted to exist in a broader family of RhoGEFs that includes p115-RhoGEF, Lbc, Lfc, Net1, and Xpln, and identify regions within the LARG PH domain that contribute to its ability to facilitate nucleotide exchange in vitro. In crystals of the DH/PH-RhoA complex, the active site of RhoA adopts two distinct GDP-excluding conformations among the four unique complexes in the asymmetric unit. Similar changes were previously observed in structures of nucleotide-free Ras and Ef-Tu. A potential protein-docking site on the LARG PH domain is also evident and appears to be conserved throughout the Lbc subfamily of RhoGEFs.

Rho GTPases are molecular switches that cycle between an active GTP-bound and an inactive GDP-bound state. In their activated form, Rho GTPases bind to effector proteins that regulate the actin cytoskeleton, gene expression, and cell cycle progression (1). Members of the three principal Rho GTPase subfamilies (Rho, Cdc42, and Rac) exert distinct morphological effects on cells and promote transcriptional activation through unique pathways. All are thought to play important roles in cellular transformation and metastasis (2, 3).

Rho GTPases are converted into their active, GTP-bound form by a large family of ~50 guanine-nucleotide exchange factors (RhoGEFs)† that have a catalytic domain homologous to that of the Dbl oncoprotein (the DH domain) (4). In most Dbl family RhoGEFs, the DH domain is positioned immediately N-terminal to a pleckstrin homology (PH) domain. Structures of DH/PH tandem domains from several RhoGEFs have been determined, either alone (Sos, Trio-N, and Dbs) (5–7) or in complex with their substrate GTPases (Tiam1-Rac1, Dbs-Cdc42, Dbs-RhoA, and intersectin-Cdc42) (8–10). The DH domain is an oblong helical bundle that facilitates nucleotide exchange by forming a stable complex with a nucleotide-free conformation of the Rho GTPase. The bulk of the DH domain-GTPase interface is formed between residues in the a1, a5, and a6 segments of the DH domain and the switch 1 and 2 elements of the GTPase. These contacts are highly conserved and define the basis for disruption of the nucleotide and magnesium binding sites of the GTPase. Based on the various DH/PH-GTPase crystal structures and biochemical studies (10), the a4-a5 loop region of the DH domain was shown to be important for determining specificity, because it interacts with subfamily-specific residues near the N terminus of the GTPase. Tiam1 is selective for Rac1, intersectin for Cdc42, and Dbs for both Cdc42 and RhoA. However, no structures have yet been reported for a RhoA-selective DH domain.

The core of the RhoGEF DH domain is a flattened, seven-stranded ␤-barrel capped with a characteristic C-terminal helix (␣C). The role of the PH domain is complex and varies among RhoGEF subfamilies. The PH domain can help localize RhoGEFs to membranes (6, 11–13), which is an important step for many cytosolic RhoGEFs, because Rho GTPases are geranylgeranylated at their C termini and therefore typically membrane-associated. The PH domain may also target the RhoGEF directly to the cytoskeleton where many downstream effectors of RhoA are found (14–16). In some cases, the PH domain also seems to play a role in regulating catalytic activity (15, 17–19). For example, residues within the PH domain of Dbs interact directly with the bound Rho GTPase (9, 10) and enhance in

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‡ The abbreviations used are: RhoGEF, Rho guanine-nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; LARG, leukemia-associated RhoGEF; PEG, polyethylene glycol; mant-GDP, N-methylanthraniloyl-GDP; r.m.s.d., root mean square deviation; RH, regulator of G protein signaling (RGS) homology; MIRAS, multiple isomorphous replacement with anomalous scattering.
vitro nucleotide exchange on Cdc42 and RhoA 11- and 24-fold, respectively (9, 20). Conversely, the PH domains of Sox and the C-terminal DH/PH domains of Trio appear to inhibit nucleotide exchange (16, 21).

The relative orientation of the PH domain with respect to the DH domain is similar in the structures of intersectin, Dbs, and Trio-N, suggesting a common functional role. However, only in the Dbs-RhoA and -Cdc42 complexes have direct contacts between the PH domain and the GTPase been observed. These contacts are important for PH domain-assisted nucleotide exchange in vitro and Dbs function in vivo (18). Intersectin does not form analogous contacts between its PH domain and Cdc42 and does not exhibit PH domain-assisted nucleotide exchange (22). The PH domain of Trio-N assists in nucleotide exchange (16), however a structure of the Trio-N DH/PH domains in complex with their substrate GTPase is not available.

Leukemia-associated RhoGEF (LARG) and its close homologs, p115-RhoGEF and PDZ-RhoGEF, are RhoA-selective RhoGEFs that are directly regulated by activated Ga12/13 proteins and thereby play a key role in oncogenic transformation induced by G protein-coupled receptors (23–25). All three RhoGEFs contain a regulator of G protein signaling (RGS) homology (RH) domain positioned ~200 residues N-terminal to their DH/PH domains and are therefore referred to as the RH-RhoGEFs (24). The RH domain binds to and serves as a GTPase-activating protein for activated Ga12/13 (26, 27). At the same time, the binding of Ga12/13 (27, 28) and possibly Go4 (29) stimulates nucleotide exchange on RhoA. The PH domains of LARG (30) and p115-RhoGEF (31) are required for full catalytic activity in vitro and enhance nucleotide exchange ~2- and 14- to 24-fold, respectively. Whereas the contributions of the LARG and other RhoGEF PH domains toward nucleotide exchange in vitro are relatively small (2- to 24-fold), it is anticipated that they have much more profound effects in vivo, as was shown for Dbs (18).

To better understand the structure and regulation of human LARG, we initiated crystallographic studies of its DH/PH domains (32). Herein we report atomic structures of the DH/PH domains of LARG and their complex with a soluble (unphosphorylated) form of human RhoA. These structures reveal novel interactions between the LARG DH and PH domains and RhoA. Using site-directed mutagenesis and fluorescence-based nucleotide-exchange assays, we show that these interactions are important for LARG-mediated nucleotide exchange in vitro.

Experimental Procedures

Cloning, Expression, and Protein Purification—The cloning, expression, and purification of the LARG DH/PH fragment and TEV protein were as previously described (32). DNA encoding the LARG DH domain (residues 765–986) was cloned into a modified pMAL expression vector (pMALc2H6,7,8,9) using BamHI and SalI restriction sites. The pMALc2H6,7,8,9 expression vector was generated by inserting oligonucleotides encoding a decahistidine (H10) tag followed by a TEV protease recognition site between Aval and EcoRI of the pMALc2X vector (New England Biolabs). Expression and purification of the LARG DH domain was as described previously for the DH/PH domains except that protein was expressed at 20 °C, the MBP-DH fusion protein was dialyzed against buffer containing 100 mM NaCl, and finally digested with 2% (w/v) TEV protease. Fractions containing the DH domain were pooled, concentrated to ~5 mg/ml, and stored at ~80 °C.

The DNA sequence encoding 1–193 of human RhoA was cloned from the pSEXG-RhoA vector (T. Kozasa, University of Illinois at Chicago, Medical Center) into pMALc2H6,7,8,9 using the EcoRI and SalI restriction sites. Protein expression from Rosetta (DE3) pLysS cells transformed with the pMALc2H6,7,8,9-RhoA vector was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 30 °C and harvested after 4–6 h. Lysis and purification was as described above for the LARG DH domain, except that cells were lysed in the presence of 50 μM GDP. Buffers for nickel-nitrirotriacetic acid columns also contained 10% glycerol, 10 mM MgCl2, and 5 μM GDP, and the gel filtration buffer contained 1 mM MgCl2 and 40 μM GDP. RhoA was concentrated to ~5 mg/ml and stored at ~80 °C. The coding region for residues 1–192 of human Rac1 and residues 1–191 of human Cdc42 were amplified from pCDNA1-rac1 and pCDNA-cdc42 (gifts from S. Hama [Washington University at St. Louis, St. Louis, MO] and A. Hall [University of California, San Francisco]) and cloned into EcoRI and SalI sites. These proteins were then purified as described above for RhoA.

Purification of the RhoA-DH/PH Complex—The DH/PH domains of LARG were mixed with a 2-fold molar excess of RhoA and diluted 10-fold with complex buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 10 mM EDTA, 2 mM dithiothreitol). After incubation for 10 min on ice, the complex was loaded onto an S200 16/60 size-exclusion column pre-equilibrated in complex buffer supplemented with 1 mM EDTA. Fractions containing the 1:1 RhoA-DH/PH complex were pooled, concentrated to about 8 mg/ml, and stored at ~80 °C until crystallization.

Crystallography and Data Collection—Crystallization of and data collection from the LARG DH/PH domains were described previously (32) (Table 1). DH/PH-RhoA complex crystals were formed by vapor diffusion using wells containing 50 mM sodium phosphate, pH 7.4, 11% PEG 8K, 0.6 M NaCl, and 5 mM EDTA. The crystals grew as long rods that can approach 1 mm in length and have 72% solvent content. Native data from the DH/PH-RhoA complex were collected from a single crystal at 90 K harvested in cryoprotectant solution (15% PEG 400, 50 mM sodium phosphate, pH 7.4, 20 mM HEPES, pH 8.0, 15% PEG 8K, 0.6 M NaCl, 5 mM MgCl2, and 40 mM MgCl2). After incubation for 10 min on ice, the complex was loaded onto an S200 16/60 size-exclusion column pre-equilibrated in complex buffer supplemented with 1 mM EDTA. Fractions containing the 1:1 RhoA-DH/PH complex were pooled, concentrated to about 8 mg/ml, and stored at ~80 °C until crystallization.

Structure Determinations—The structure of the LARG DH/PH domains was determined using a combination of MIRAS and molecular replacement. Xenon and NaBr derivatives were generated as described previously (32) and a homology model of the LARG DH domain, based on three-dimensional coordinates (33), was manually placed into the resulting MIRAS-phased electron density map. Phases from molecular replacement and MIRAS were then combined, and the DH domain was refined using CNS (34). Subsequently, a solvent-flattened electron density map allowed placement of a homology model of the LARG DH domain. The structure was refined using rounds of maximum-likelihood refinement by either CNS or REFMAC (35 alternating with model building in the program O (36). In the final cycles of refinement, two isotropic B-factors were used in conjunction with TLS refinement (37). Modeling of the DH/PH domains was ultimately assisted by the structure of the DH/PH-RhoA complex, which facilitated interpretation of poorly ordered regions of the structure, particularly the N-terminal extension of the DH domain, and the p1β2 and β6-βn loops of the PH domain.

The 2.2-Å crystal structure of the LARG DH/PH-RhoA complex was determined by molecular replacement using the LARG DH domain modeled in complex with nucleotide-free RhoA (10). The PH domains of the four complexes in the asymmetric unit were later fit by hand. The structure was refined and built as described for the LARG DH/PH domains, except that 4-fold NCS restraints were imposed on structurally equivalent regions of each DH/PH-RhoA complex using REPEN. As a consequence of the high degree of identity between subunits became apparent, these restraints were gradually loosened and/or eliminated. For all DH domains and RhoA subunits, main-chain and side-chain densities are well defined. The A and C chain PH domains are better ordered than the E and G PH domains and have even more ordered loops than the PH domain of the 2.1-Å uncomplexed structure. To verify the resulting model, σa-weighted phases (38) were generated from the coordinates and refined with twenty cycles of solvent flattening and averaging in the program DM (39). Multidomain 4-fold averaging was used for all parts of the structure except for the RhoA subunits, which were subjected to 3-fold averaging owing to the observed conformational change in the B chain, whose density was omitted from averaging. Correlation between the averaged DM map and the 2|Fobs|–|Fcalc| Fourier map generated by REFMAC was 95% for main-chain atoms and 92% for side-chain atoms. The omit map shown in Fig. 5a was generated in a similar fashion, except that the B chain of RhoA was left out of the initial model used to generate phases.

Two residues in each subunit of LARG, Ser-833 and Asp-1054, fall within the disallowed region of the Ramachandran plot. In other atomic structures of DH domains, residues equivalent to Ser2362 have the same stereochemistry (24). However, the stereochemistry of Asp-1054 is in contradiction to that of a type I β-turn in the PH domain, a position usually occupied by glycine. In the DH/PH structure, the residue Asn-765 of the DH domain and residues 999–1007 in the βN-αN loop and 1062–1074 in the β4 strand of the PH domain could not be modeled. In the “A” and “C” DH/PH chains of the LARG DH/PH-RhoA complex, Asp-765 at the N terminus and residues 1064–1074 in the β4 insertion of the PH domain do not have interpretable electron density and were not modeled in the
**DH/PH Domains of LARG and Their Complex with RhoA**

**RESULTS**

The Structure of the LARG DH/PH Domains—As in previously determined DH domain structures, the core of the LARG DH domain is comprised of six major helical segments (9), wherein segments 2, 3, and 5 are broken into several distinct α-helices (Fig. 1a).

The DH domain, however, has a novel N-terminal extension (residues 766–781) composed of two short helices, αN1 and αN2, that bury the side chain of Trp-769 against the α1 helix of the DH domain (Figs. 1a and 2a). This extension was included in the DH/PH fragment used for crystallization because of its high sequence homology among the Lbc subfamily of RhoGEFs (4), which includes the RH-RhoGEFs, Lbc, Lfc, Net1, Xpln, and intersectin (Fig. 2b). Otherwise, the LARG DH domain is quite similar to that of its closest homolog of known structure, intersectin (r.m.s.d. of 1.3 Å for 187 equivalent Ca atoms), whose structure was determined without the corresponding N-terminal extension (10). The most pronounced difference between the LARG and intersectin DH domains occurs within the region spanning the α2-α3 loop and the first helix of the third helical segment, which is on the opposite side of the DH domain from the GTPase binding site.

The PH domain of LARG has notable structural differences from other RhoGEF PH domains of known structure. The β3-β4 loop of LARG is an abrupt β-turn, whereas the analogous loops in the Tiam1-Rac1 and Dbs-Cdc42 complexes are extended (8, 9) (Fig. 1c). In the case of Dbs, the extended β3-β4 loop forms direct contacts with Cdc42 (9). Although Dbs has a continuous β4 strand, intersectin has a three-residue bulge (residues 1515–1517) and LARG has a disordered 17-residue insertion (residues 1060–1077) within the strand. The function of this insertion in LARG and other RH-RhoGEFs is not known, although it projects along what is anticipated to be the membrane binding surface of the PH domain (Fig. 1b) and therefore could be involved in membrane association.

**Embrace of RhoA by the LARG DH/PH Domains**—The LARG DH/PH-RhoA complex crystallized as a tetramer with pseudo-C4 non-crystallographic symmetry (Fig. 1b, inset). Although oligomers that associate with membranes often have cyclic symmetry, this tetramer is probably not physiologically significant, in part because the observed subunit contacts are not conserved in other RH-RhoGEFs. The DH/PH chains are labeled A, C, E, and G, and their respective RhoA subunits are B, D, F, and H. Thus the four DH/PH-RhoA complexes are referred to as being composed of the A:B, C:D, E:F, or G:H chains.

The most striking change in the LARG DH/PH domains upon binding RhoA is the roughly 30° rotation of the PH domain relative to the DH domain, such that both domains embrace RhoA (Figs. 1b and 3). This conformational change occurs by virtue of a bend at the end of the α6 helix of the DH domain that spans residues 976–983 (thus the “PH domain” of LARG is therefore defined as spanning residues 984–1138). The result-

### Table 1

| DH/PH | DH/PH-RhoA |
|-------|------------|
| Wavelength (Å) | 1.000 | 1.069 |
| Space group | C2 | C2 |
| Unit cell (Å, °) | a = 193.6 | a = 294.9 |
| | b = 45.9 | b = 95.0 |
| | c = 74.7 | c = 157.0 |
| | β = 107.5 | β = 94.0 |
| Resolution limit (Å) | 2.07 | 3.2 |
| Unique reflections (total) | 35,784 (814,907) | 64,268 (1,360,421) |
| Completeness (%) | 97.5 (93.7)* | 91.2 (80.4) |
| Rsym (%) | 4.6 (36.5) | 8.3 (57.4) |
| Average I/σ(I) | 21.0 (3.3) | 11.4 (1.4) |
| Resolution range for refinement (Å) | 24 to 2.07 | 15 to 3.22 |
| Total reflections used | 35,784 | 60,392 |
| Number of protein atoms | 2,980 | 17,001 |
| Number of water molecules | 91 | 0 |
| r.m.s.d. bond lengths (Å) | 0.02 | 0.02 |
| r.m.s.d. bond angles (°) | 1.7 | 1.8 |
| Rwork (%) | 23.3 | 24.8 |
| Rfree (%) | 27.6 | 29.1 |
| Average B-factor (Å²) | 36.5 | 30.4 |

*Values in parentheses refer to the highest resolution shell (DH/PH: 2.12–2.07 Å; DH/PH-RhoA: 3.31–3.2 Å).

**Nucleotide Exchange Assay—**GTPases were loaded with N-methylanthraniloyl-GDP (mGDP) by incubating 180 μM RhoA with a 10-fold molar excess of mGDP in loading buffer (20 mM HEPES, pH 8.0, 100 mM NaCl, 4 mM EDTA, 1 mM dithiothreitol) for 1.5 h on ice. Subsequently, the GTPase was exchanged into reaction buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol) via a G10 gel filtration column. The exchange reaction was then started by the addition of 100 nM LARG fragment, and Δ absorbance at 280 nm, slits 2/2 nm, in a 200-μl cuvette. The exchange reaction was then started by the addition of 100 mM DH/PH fragment, and Δ absorbance was measured at 280 nm, slits 2/2 nm, in a 200-μl cuvette.

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The orientation of the LARG PH domain with respect to the DH domain is strikingly similar to those of Dbs in complex with either Cdc42 or RhoA (9, 10), Dbs alone (7), and Trio-N (6). However, the α6 segment of LARG is 10 residues longer, and thus the PH domain in the LARG DH/PH-RhoA complex is translated about 13 Å further along the direction of α6 (Fig. 1c). The residues from RhoA that lose the most accessible surface area upon their interactions with the LARG PH domain are Glu-97 in the α3 helix, whose carboxylate interacts with the N terminus of the αC helix, and Arg-68 from switch 2, which packs against residues from αN and the β1 strand of the PH domain (Fig. 3b).

The PH domain of each LARG subunit has a slightly different orientation in the complex, varying as much as 10° when their DH domains are superimposed. Even so, each PH domain maintains similar contacts with RhoA (Fig. 3a). The observed rotation axis among the complexed PH domains is in a direction roughly orthogonal to that of the 30° collapse of the PH domain relative to the unbound DH/PH domains. The accessible surface area buried between the PH domain and RhoA is on...
average 160 Å², varying between 240 Å² in the C.D complex to 40 Å² in the E.F complex, wherein the PH domain appears nearly disengaged from RhoA.

The conformation of the individual DH and PH domains in their complex with RhoA is similar to their counterparts in the unbound DH/PH structure (r.m.s.d values of 0.85 and 0.84 Å, respectively). Upon binding RhoA, the largest conformational change in the DH domain occurs within the α1N/α2N extension and the α4 region. The entire α1N/α2N extension shifts 2–3 Å toward RhoA in the complex. The α2-α3 loop, which interacts extensively with the α1N/α2N extension, shifts similarly (Fig. 3a). The side chain of Glu-790 from the α1 helix assumes a bent conformation that enables it to form two backbone hydrogen bonds with the N terminus of α1N and to pack against the aromatic ring of RhoA-Tyr-34 (Fig. 2a). The largest change in the α4 region occurs in the α4-α5 loop, which directly engages RhoA (e.g. 2 Å for the Ca of Arg-923 away from RhoA relative to the DH/PH structure) (Fig. 3a). The internal conformation of the LARG PH domain is essentially unchanged upon binding RhoA, although its loops become better ordered. For example, the 18-residue βN-3 helix loop becomes ordered in the A and C chains of the complex (Fig. 1b), and contains an extra helix (αNb, residues 1005–1013). A similar phenomenon was noted for the Dbs PH domain upon comparing its GTPase-free and bound structures (7).

**The LARG DH-RhoA Interface**—As observed in other DH-GTPase complexes, a region of switch 2 of RhoA (residues 61–68) reorganizes in the LARG-RhoA complex such that the side chain of Ala-61 projects into the magnesium binding site and Glu-64 occludes the γ-phosphate binding site of RhoA. Residues 27–40 of RhoA, which includes switch 1, are also restructured such that the nucleotide binding site of RhoA becomes more solvent-exposed. Switch 1 appears to be better ordered in the LARG DH/PH-RhoA complex than in other DH/PH-GTPase complexes, perhaps by virtue of its additional contacts with the α1Nα2N2 extension.

The structure of LARG is the first of a RhoA-selective RhoGEF, and, as previously proposed (10), it appears to use the α4-α5 loop region to dictate substrate specificity (Fig. 4). Arg-923 in the α4-α5 loop of LARG forms salt bridges with both Asp-45 and Glu-54 of RhoA, which are substituted by shorter polar side chains in Rac1 and Cdc42 (Fig. 4a). Arg-923 is invariant throughout the Lbc subfamily of RhoGEFs except in intersectin, which is specific for Cdc42 and has a glycine at the equivalent position. Within the same region, Arg-5, Val-43, and Asp-76 of RhoA appear to form additional RhoA-specific con-
FIG. 3. Contacts between the LARG PH domain and RhoA. a, the four different LARG PH domains in the asymmetric unit of the DH/PH-RhoA crystals (Cα traces: the A chain is colored yellow, C chain is magenta, E chain is green, and G chain is red) adopt slightly different orientations with respect to the DH domain and the RhoA subunit (rendered as a blue transparent surface). However, their interface with RhoA is held relatively fixed compared with the β1-β2 loop region, where the rotational differences among the PH domains are exaggerated. The β1-β2 loop of the G chain is not shown, because it is disordered in the crystal structure, but it would occupy a position similar to the A chain. Shown in black is the Cα trace of the DH domain of the uncomplexed LARG DH/PH domains with its PH domain omitted for clarity. Conformational differences between the GTPase-bound and GTPase-free structures of the DH domain are evident in the αN/αN2 extension and the α2-α3 loop, which rotate as a unit to interact with switch 1 of RhoA, as well as in the α4-α5 loop, which makes specificity-determining contacts with the GTPase. b, interface between the PH domain and RhoA. The subunits are from the C:D DH/PH-RhoA complex, and the RhoA side chains shown are those that lose the most accessible surface area upon binding the LARG PH domain. RhoA-Glu-97 interacts with the N terminus of the LARG αC helix, where it can form hydrogen bonds with backbone amide groups and the side chain of Ser-1118. RhoA-Arg-68 contacts both Arg-986 and Glu-1023 of the PH domain and forms a hydrogen bond with the side chain of Asn-983. In some of the complexes, RhoA-Arg-68 also appears to form a salt bridge with LARG-Glu-982. Glu-1023 forms a salt bridge with Arg-986.
contacts with LARG (Fig. 4). The side chain of Trp-58 of RhoA is completely buried in the LARG interface and potentially forms a hydrogen bond with the carboxylate of LARG-Asp-928 (Fig. 4b), a residue conserved as aspartate or glutamate among Lbc subfamily RhoGEFs except for intersectin, which has a serine at the equivalent position.

Alternative Conformations of Nucleotide-free RhoA—Overall, the conformation of RhoA in the LARG DH/PH-RhoA complex is similar to that of the GTPase in other DH/PH-GTPase complexes. However, there are some interesting structural differences (Fig. 5). In all four complexes of the LARG DH/PH-RhoA structure, the backbone carbonyl of Gly-14 in the P-loop has flipped so that it occludes the binding site for the nucleotide (data not shown). Asn-117, positioned next to the P-loop, adopts a rotamer not observed in previously determined RhoA structures. Strong electron density is observed in the a-phosphate binding site of all four RhoA subunits and was modeled as inorganic phosphate due to its presence in the crystallization buffer (Fig. 5a).

In the A:B LARG-RhoA complex, crystal contacts have trapped RhoA in a conformation wherein the purine-binding site of the nucleotidase is also occluded (Fig. 5a). Residues 160–164 of RhoA, which contain the SAK motif, shift by up to 3.4 Å into the purine-binding pocket of RhoA. An analogous collapse into the purine-binding pocket of RhoA is also observed in the Ras-Sos complex (41). This change is accompanied by a shift of residues 31–35 in switch 1 of RhoA further away from the nucleotide-binding site, such that the Ca atom of Val-33 shifts by 1.6 Å.

Role of the αN1/αN2 Extension—The structure of the LARG DH domain revealed the presence of a novel N-terminal extension that directly contacts switch 1 of RhoA. We investigated the role of the αN1/αN2 extension in nucleotide exchange by using site-directed mutagenesis and a fluorescence resonance energy transfer-based assay that monitors the release of N-methylanthraniloyl-GDP (mant-GDP) from RhoA (see “Experimental Procedures” and Fig. 6). To test whether the αN1/αN2 extension could influence nucleotide exchange activity, we either deleted the extension (ΔN) or perturbed its hydrophobic core (W769A or W769D). To evaluate the role of Glu-790 from the α helix of the DH domain (Fig. 2e), which packs against RhoA-Tyr-34 and forms two hydrogen bonds with the backbone of the αN1/αN2 extension, we mutated the residue to glycine (E790G), its equivalent in intersectin. To test whether the extension-switch 1 interface contributes to the substrate specificity of LARG, RhoA-Lys-27 and -Val-33 were mutated to threonine and glutamate, respectively, their counterparts in both Rac1 and Cdc42. All mutant proteins expressed similarly to wild-type and could be purified to the same level of homogeneity (data not shown), suggesting that they are not misfolded or otherwise destabilized.

The LARG-ΔN, W769A and W769D mutants all reduced nucleotide exchange of RhoA to 15–20% of the activity of the wild-type DH/PH domains (Fig. 6a and Table I). The E790G mutant of LARG was similarly deficient at nucleotide exchange. Furthermore, neither wild-type, W769A, W769D, nor ΔN mutants of LARG could catalyze nucleotide exchange on Rac1 or Cdc42 in our assays (data not shown). Thus, the αN1/αN2 extension and Glu-790 are important for nucleotide exchange on RhoA, but apparently not for substrate specificity.

Surprisingly, neither the K277T nor the V33E mutations of RhoA exhibited a significant loss of nucleotide exchange relative to wild-type RhoA (Table II). Therefore, it appears that the observed contacts between αN1/αN2 extension and switch 1 are not important. However, the αN1/αN2 extension may still have an indirect effect on binding RhoA. Disruption or deletion of the αN1/αN2 extension could allow the side chain of Glu-790 of LARG to adopt a conformation that interferes with GTPase binding. To test this hypothesis, we constructed the LARG-E790GΔN double mutant, which could potentially rescue the
activity of the ΔN deletion. However, the E790G/ΔN mutation was just as deficient at catalyzing nucleotide exchange on RhoA as the other αN1/αN2 extension mutations (Fig. 6a and Table II). Therefore, although Glu-790 may be constrained in a favorable conformation by the αN1/αN2 extension mutations (Fig. 6a), its contact with RhoA-Tyr-34 also appear important for nucleotide exchange on RhoA.

The LARG PH Domain-RhoA Interface and Its Role in Nucleotide Exchange—Contacts between a RhoGEF PH domain and the GTPase substrate have only previously been observed in complexes of the Dbs DH/PH domains (9, 10). We therefore tested the contribution of the LARG PH domain toward nucleotide exchange using site-directed mutagenesis and the fluorescence resonance energy transfer-based nucleotide exchange assay described above. First, we compared the rates of nucleotide exchange on RhoA catalyzed by either the LARG DH/PH domains or the DH domain alone (Fig. 6b). Consistent with previous studies of LARG and the closely related p115-RhoGEF (30, 31), the DH domain of LARG catalyzed nucleotide exchange less efficiently than the DH/PH domains (Fig. 6b and Table II).

The two residues of RhoA that bury the most accessible surface area with the LARG PH domain are Glu-97 and Arg-68 (Fig. 3b). RhoA-Glu-97, which introduces electrostatic repulsion and/or steric collision with RhoA-Glu-97, reduced the exchange rate to the level of the DH domain alone (Fig. 6b and Table II). However, nucleotide exchange of RhoA-E97A was nearly identical to that of wild-type RhoA. It is possible that the RhoA-E97A mutation was not severe enough to abrogate beneficial packing between the PH domain and RhoA.

RhoA-Arg-68 interacts with the αN helix and the β1 strand of the PH domain (Fig. 3b). Because our LARG DH domain fragment (residues 765–1019) (30) were similarly deficient at nucleotide exchange when compared with the intact DH/PH domains, the residues within αN (residues 983–993) appear, at least by themselves, incapable of facilitating nucleotide exchange. We therefore targeted Glu-1023 of the PH domain for site-directed mutagenesis because of its sequence conservation among the RH-RhoGEFs, its contact with RhoA-Arg-68, and its involvement in a hydrogen bond network with the αN helix (Fig. 3b). The nucleotide exchange activity of the LARG-E1023A mutant was diminished to that of the DH domain alone (Fig. 6b and Table II). As expected, the E1023R mutation, which introduces electrostatic repulsion and steric collisions with both LARG-Arg-986 and RhoA-Arg-68, was even less active than E1023A.

We then assessed the ability of the wild-type DH/PH and DH domains of LARG to catalyze nucleotide exchange on RhoA-
For each time course, $1 \mu M$ RhoA loaded with mant-GDP was incubated with $100 \mu M$ GTP at $25^\circ C$, and the exchange reaction was started by the addition of 100 nM LARG fragments except in the case of GTPase alone (baseline). The subsequent decrease in fluorescence ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 430$ nm) was then measured for 300 s. Each curve shown is the average of 2–3 measurements. a, Perturbation of the $\alpha N1/\alpha N2$ extension of LARG dramatically reduces the rate of nucleotide exchange on RhoA versus the wild-type DH/PH domain (a, $\Delta N$ (×), E790G (●), and $\Delta N/E790G$ (●). The W769D and W769A mutations (not shown) have similar exchange rates as $\Delta N$ (Table II). b, mutation of Glu-1023 eliminates PH domain-assisted nucleotide exchange. The PH domain of LARG assists nucleotide exchange on RhoA, as seen by comparing the curves for DH/PH (a) versus that of the DH domain (residues 765–986, □ alone. The E1023A mutation (*), which is predicted to disrupt a network of hydrogen bonds with the $\alpha C$ helix near the RhoA interface (Fig. 3b), reduces the rate of nucleotide exchange to the level of the DH domain. The E1023R mutation introduces a side chain that creates steric overlap and charge repulsion, and, as expected, reduces the catalytic rate even further. The S1118D mutation (not shown), which was designed to disrupt the contact between RhoA-Glu-97 and the N terminus of the LARG $\alpha C$ helix (Fig. 3), also reduces the catalytic rate by 50%. These results suggest that at least one role of the LARG DH/PH domains is dimeric in solution (data not shown). However, there is no evidence from size exclusion chromatography that the DH/PH domains are dimeric in solution (data not shown). The residues that compose this solvent-exposed patch are highly conserved among the Lbc subfamily RhoGEFs, suggesting a functional role.

**Table II**

| LARG fragment | Rate of nucleotide exchange $k_{obs}$ $s^{-1} \times 10^{-3}$ |
|---------------|-------------------------------------------------------------|
| None          | 0.15 ± 0.007                                               |
| DH/PH-(765–1138) | 5.0 ± 0.25 (33)                                    |
| DH/PH-ΔN      | 1.0 ± 0.07 (6.8)                                          |
| DH/PH-W769A   | 0.75 ± 0.02 (5)                                           |
| DH/PH-W769D   | 0.68 ± 0.02 (4.5)                                          |
| DH/PH-E790G   | 1.3 ± 0.06 (8.6)                                          |
| DH/PH-E790G/ΔN| 1.2 ± 0.02 (8)                                            |
| DH/PH-S1118D  | 3.3 ± 0.7 (22)                                            |
| DH/PH-E1023A  | 3.6 ± 0.1 (24)                                            |
| DH/PH-E1023R  | 2.2 ± 0.04 (15)                                           |
| DH-(765–986)  | 3.7 ± 0.25 (25)                                           |

$^a$To calculate $k_{obs}$ each curve was fit to a single-order exponential decay with Prism version 4.0. Each data point is the average of at least three measurements.

$^b$ND, not determined.

$^c$Numbers in parentheses correspond to the fold rate enhancement over the basal rate in the absence of LARG, if measured.

R68A, a mutant that had no effect on nucleotide exchange rates catalyzed by Dbs, although mutation of the contacting Dbs PH domain residue (Tyr-889) diminished nucleotide exchange (9). Interestingly, the LARG DH/PH domains could catalyze nucleotide exchange about 4-fold more efficiently on RhoA-R68A than wild-type RhoA (Table II). The molecular basis for this effect could be due to the removal of electrostatic repulsion between the RhoA-Arg-68 and LARG-Arg-986 side chains (Fig. 3b). RhoA-R68A was an equally good substrate for our DH domain fragment of LARG (residues 766–986, Table II) as it was for the DH/PH domains. These results suggest that at least one role of the LARG PH domain could be to help compensate for unfavorable, yet apparently necessary, contacts between RhoA-Arg-68 and the $\alpha 6/\alpha N$ helix of LARG, perhaps by burying additional accessible surface area (e.g. between $\alpha C$ and RhoA-Glu-97).

A Potential Protein Docking Site on the LARG PH Domain—In the DH/PH crystals, the PH domain forms a 2-fold crystallographic dimer interface that buries 800 Å$^2$ of surface area. The interface consists of a solvent-exposed hydrophobic patch on the $\beta 5-\beta 7$ sheet of the PH domain, which includes the side chains of Leu-1086, Phe-1098, Ile-1100, Ala-1107, and Ile-1109 (Fig. 1b). Strikingly, the hydrophobic patch of all four PH domains in the asymmetric unit of the LARG-RhoA crystals form similar non-crystallographic dimer contacts. However, there is no evidence from size exclusion chromatography that the LARG DH/PH domains are dimeric in solution (data not shown). The residues that compose this solvent-exposed patch are highly conserved among the Lbc subfamily RhoGEFs, suggesting a functional role.

**DISCUSSION**

The structures of both the GTPase-bound and free states of a DH/PH domain have only previously been reported for Dbs (7, 9). Although there were no major conformational differences...
between the two states of the Dbs DH/PH domain, there is a
dramatic conformational change between the DH and PH dom-
ains of LARG upon binding RhoA (Fig. 1, a and b). Given the
longer α6 helix of the LARG DH domain, which extends the PH
domain further from the surface of the DH domain than in Dbs
(Fig. 1c), it is possible that the LARG PH domain simply has
greater conformational freedom when not in complex with
RhoA. However, it is striking that the relative orientation of
the DH and PH domains of LARG while in complex with RhoA
are essentially the same as those of Dbs and Trio-N, even
though they are distantly related within in the RhoGEF family
(Fig. 1c) (4). The distinct conformations exhibited by structures
of the Sos (5) and Tiam1 (42) DH/PH domains may therefore
represent exceptions, and not the rule.

A characteristic shared by both LARG and Dbs that may help
account for the structural similarity of their DH/PH domains is
the fact that residues from their PH domains interact directly
with the GTPase substrate. In Dbs RhoGEF, a triad of inter-
acting residues (Dbs-His-814, Dbs-Tyr-889, and RhoA-Asp-65)
appears most important for PH domain-assisted nucleotide
exchange in vitro (9). Upon superposition, there is no residue in
LARG equivalent to Dbs-Tyr-889, although the side chain of
LARG-Lys-979 occupies the same approximate position as Dbs-
His-814. LARG-Lys-979 does not, however, appear to make an
analogous contact with RhoA-Asp-65. These differences may
account for the smaller catalytic rate enhancement provided by
the LARG PH domain compared with that of Dbs. In LARG, we
have instead identified Ser-1118 (of c) and Glu-1023 (of β) as
residues in the PH domain whose mutation lead to reduced
nucleotide exchange. Because Glu-1023 of LARG only forms a
small part of the interface with RhoA (Fig. 3b), its more impor-
tant functional role could be to stabilize the α6/αN helix in a
conformation more competent to bind RhoA (Fig. 3b). An ana-
logous role was proposed for Tyr-889 in the Dbs PH domain (9).

Intersectin, another Lbc subfamily RhoGEF, contains a PH
domain that does not facilitate nucleotide exchange on its
GTPase substrate in vitro (22). Accordingly, its PH domain
does not contact either the DH domain or the GTPase substrate
in the intersectin-Cdc42 crystal structure (10). The residues of
the LARG PH domain that make specific contacts with RhoA
(Fig. 3b) are conserved in intersectin with the exception of
LARG-Glu-1023, which is substituted by serine. This change
may be enough to abrogate PH domain-assisted nucleotide
exchange in intersectin, just as the LARG E1023A and E1023R
mutations abolish it in LARG (Fig. 6b and Table II).

The LARG DH domain has a novel αN1/αN2 extension that
directly interacts with RhoA. Other Lbc subfamily RhoGEFs,
such as Lbc and Lfc, are predicted by sequence analysis to have
a similar extension (Fig. 2b). We have shown through deletion
or point mutation of this extension in LARG that catalytic
activity in vitro is diminished when the extension is removed or
its structure perturbed (Fig. 6a and Table II). Based on our
data, one possible role for the αN1/αN2 extension may be to fix
the conformation of Glu-790 in a manner that allows it to pack
optimally against the side chain of RhoA-Tyr-34, a switch 1
residue. Given its influence on the rate of nucleotide exchange
(Table II), and its conformational flexibility (Fig. 3a), the αN1/
αN2 extension could serve as a “switch” that can be perturbed
and/or reorganized by contacts with other domains of LARG
(e.g. the RH domain) or with other regulatory proteins (e.g.
Gαq). Perhaps not coincidentally, Tyr-174 of the RhoGEF
Vav, which is phosphorylated by Src family kinases to relieve
autoinhibition (43), is equivalent by sequence alignment to
Trp-769 of LARG (Fig. 2b). If the αN1/αN2 extension is in-
deed such a “switch,” then nature will have delegated regu-
lation of RhoGEF activity to the same small region N-
terminal to the DH domain in even as divergently related
enzymes as LARG and Vav.

As predicted by extensive analysis of the Dbs-Cdc42, Dbs-
RhoA, Tiam1-Rac1, and intersectin-Cdc42 complexes (10),
structural elements that dictate the specificity of LARG for
RhoA are primarily found in the α4-α5 loop region of the DH
domain (Fig. 4). Salt bridges between LARG-Arg-923 and the
N-terminal region of the GTPase (Fig. 4a) help select for RhoA,
but they are not sufficient to select against Cdc42, because Dbs
has Lys-758 in the equivalent position and can utilize both
Cdc42 and RhoA as substrates. Accommodation of RhoA-
Trp-58 (Phe-56 in Cdc42) will help LARG discriminate against
Cdc42, but not Rac1, which also has tryptophan at the equiva-
lent position (42). However, the α4-α5 loops of LARG, Tiam1,
and Dbs are not structurally equivalent, and multiple interac-
tions between these loops and the GTPase are likely respon-
sible for additional selectivity. For example, RhoA-Val-43, which
is buried by the α4-α5 loop of LARG (Fig. 4b), is substituted by
serine in Rac1 and alanine in Cdc42.

In all reported DH/PH-GTPase structures, the GTPase as-
sumes a conformation that is incompatible with GTP binding
due to a reorganization of switch 2 that disrupts its Mg2+-
and γ-phosphate binding sites. However, this conformation is
not necessarily GDP-exclusive. The Mg2+-free structure of RhoA
in complex with GDP (44) demonstrated that RhoA can bind GDP
even when switch 2 adopts the same conformation as when it does
when bound to a DH domain. Furthermore, LARG-RhoA crys-
tals can be grown in the presence of GDP, and strong difference
class density corresponding to GDP can be observed bound in
the RhoA active site. Therefore, the novel changes we observe in
the P loop and the purine-binding pocket of the LARG-RhoA
complex (Fig. 5) probably reflect the natural plasticity of the
GTPase active site when nucleotides are absent, rather than an
active exchange mechanism exerted by the LARG DH domain.
This idea is further supported by the fact that no part of the
LARG DH domain comes into obvious direct or indirect contact
with either the P loop or the NXXD and SAK motifs of the
GTPase. It remains to be shown whether the DH domain-
mediated exchange mechanism requires RhoA to assume dis-
tinct nucleotide-free, GDP-bound, and GDP-bound states while
bound to the DH domain, as has been proposed for Cdc25 on
Ras (45) or Sec-7 on Arf (46). Our preliminary data indicate
that at least the DH/PH-RhoA and DH/PH-RhoA-GDP states
occur for LARG, and that their structures will be quite similar.

Finally, the PH domain of LARG, and perhaps all Lbc sub-
family RhoGEFs, has an exposed hydrophobic patch (Fig. 1b)
that could interface with other domains of LARG or other
regulatory proteins. If the LARG PH domain docks with the
plasma membrane in the same general orientation as other
well characterized PH domains (e.g. PLC-δ1 (47)), this patch
would be able to interact laterally with other peripheral mem-
brane proteins/domains. Because the PH domain of Lbc
binds specifically to tubulin (14), and that of Lbc to actin (15), one
intriguing possibility is that this conserved hydrophobic patch
could be involved in targeting these RhoGEFs to the cytoskel-
eton. Other actin and microtubule binding domains likewise
feature a solvent-exposed hydrophobic patch that is thought to
be important for filament binding (48, 49). A better under-
standing of the roles of this putative protein-docking site and
the αN1/αN2 extension of LARG will be facilitated by deter-
imining atomic structures of larger fragments of LARG that
include its RH domain and by evaluating the effects of point
mutants that perturb these structural elements on the function
of full-length LARG in vitro.

2 G. Gao, personal communication.
