ACTIVATED RHOA POSITIVELY REGULATES EXCHANGE ACTIVITY OF PDZ-RHOGEF

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by

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DISSERTATION

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RhoA plays a key role in regulation of the actin cytoskeleton, cell migration and cell shape. Rho GTPases cycle between an inactive GDP-bound state and an active GTP-bound state. This cycle is mediated by guanine nucleotide exchange factors (GEFs) that increase the rate of dissociation of GDP by stabilizing the nucleotide-free state of the GTPases via their DH/PH domains; this facilitates binding of GTP and activation of the protein. The RGS subfamily of RhoGEFs (RGS-RhoGEFs) act as direct mediators of RhoA activation in response to stimulation of the heterotrimeric G12 and G13 proteins by hormone receptors. RhoGEFs usually bind most tightly to the nucleotide free form of RhoA, which represents the intermediate state for exchange of guanine nucleotides. Recently, our lab discovered that PDZ-RhoGEF (PRG), a member of the

RGS-RhoGEFs, bound tightly to both nucleotide-free and activated RhoA (RhoA·GTP). Using deletion analysis and pulldown assays, I was able to show that this interaction occurs between the PH domain of PRG and activated RhoA. James Chen was able to define the molecular determinants of this interaction by solving the crystal structure of the PRG-DH•PH-RhoA(GTPγS) complex. This structure revealed that the interface is comprised of the switch regions in RhoA and a conserved hydrophobic patch in the PH domain of PRG. Interestingly, activated RhoA does not regulate the exchange activity of PRG in solution. Here, I use reconstitution of the signaling pathway with phospholipid vesicles and recombinant proteins, to show that this interaction serves as a mechanism for spatially regulating PRG exchange activity, a feed-forward mechanism. We hypothesize that this feed-forward mechanism is also applicable *in vivo* and potentially may serve as a mechanism utilized by a larger group of RhoGEFs known as the Lbc subfamily of RhoGEFs.

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PRIOR PUBLICATIONS

Chen, Z., Medina, F., Liu, M.Y., Thomas, C., Sprang, S.R., and Sternweis, P.C. (2010). Activated RhoA binds to the pleckstrin homology (PH) domain of PDZ-RhoGEF, a potential site for autoregulation. The Journal of biological chemistry 285, 21070-21081.

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DGS-NTA(Ni) - 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)

DH - Dbl homology

Dmut – dual-mutant

GAP - GTPase-activating protein

GDI - guanine nucleotide dissociation inhibitor

GEF – guanine nucleotide exchange factor

GST - glutathione S-transferase

GTPγS - guanosine 5′-O-[γ-thio]triphosphate

LARG - leukemia-associated RhoGEF

Mant-GDP - 2′deoxy-3′O-N-methylanthraniloyl GDP

Mant-GTP - 2′deoxy-3′O-N-methylanthraniloyl GTP

PH - pleckstrin homology

poPC - 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

poPE - 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine

PRG - PDZ-RhoGEF

RGS - regulators of G protein signaling

ROCK - Rho-associated coiled-coil containing protein kinase

TEV - tobacco etch virus

WT – wild-type

Chapter 1

Introduction

The Rho Family of GTPases

Cells must be able to detect and respond to changes in their environment for survival and normal function. To fulfill this requirement, cells have developed signaling pathways operated by proteins to process external stimuli into a response. The Ras superfamily of monomeric GTP-binding proteins represent a large family of signaling molecules that play an important role in linking activation of cell surface receptors to the appropriate physiological response. The Rho (**R**as **ho**molog) family of GTPases make up one branch of the Ras superfamily and members are found in all eukaryotic organisms (Ridley, 2006). They are responsible for regulating numerous cellular functions including regulation of the actin cytoskeleton, cell migration, cell cycle progression, gene transcription, neurite growth and cell adhesion (Oleksy et al., 2006). In humans, 22 genes encoding at least 25 proteins have been described for the Rho family (Wennerberg and Der, 2004), and smaller numbers can be found in model organisms like *Saccharomyces cerevisiae* (5 members), *Caenorhabditis elegans* (10 predicted members) and *Drosophila melanogaster* (11 predicted members) (Jaffe and Hall, 2005). Based on primary sequence identity, structure, and cellular function, the Rho family can be divided into six subfamilies: RhoA-related, Rac-related, Cdc42-related, Rnd proteins, RhoBTB proteins and Miro proteins (Wennerberg and Der, 2004). The most well characterized members of these subfamilies are RhoA, Rac1 and Cdc42; these were the first members to be found to have an important role in the morphological responses of cells to extracellular stimuli (Wheeler and Ridley, 2004).

Structurally, Rho proteins are typically small (190-250 amino acids), usually consisting of only a GTPase domain and short extensions at both their N- and C-termini. Within their

GTPase domains, Rho family members share approximately 40-95% amino acid identity, which may explain why some effector proteins are able to interact with multiple Rho family members (Wennerberg and Der, 2004). In addition, Rho GTPases are usually modified post-translationally by prenylation (farnesylation or geranylgeranylation) at their C-termini, which enables their association with cell membranes (Ridley, 2006).

Like all GTPases, Rho proteins function as molecular switches cycling between two conformational states: a GTP-bound "active state" and a GDP-bound "inactive state" (Fig. 1.1). In the active state, Rho proteins bind to effector proteins with higher affinity and promote downstream signaling (Bishop and Hall, 2000). The transition from active to inactive states may then be governed by the intrinsically slow hydrolytic rate of GTP by the GTPase. Alternatively, it is most frequently assisted by the binding of GTPase-activating proteins (GAPs) that accelerate this intrinsic rate of hydrolysis (Sprang, 1997). Inactive Rho proteins can bind guanine nucleotide dissociation inhibitors (GDIs) that inhibit dissociation of bound GDP and protect the Rho proteins from misfolding and degradation (Garcia-Mata et al., 2011).

When the appropriate signals are received, activated guanine nucleotide exchange factors (GEFs) bind the free GTPases and stabilize their nucleotide-free conformation; this promotes dissociation of bound GDP and subsequent association of GTP (Sprang, 1997). Association of GTP causes a conformational change in two regions on Rho GTPases called switch I and switch II. Effector proteins respond to the conformational change in the switch regions by binding with increased affinity directly to the switch regions themselves (Vetter and Wittinghofer, 2001). Usually, the consequence of this interaction is the disruption of some type of intramolecular autoinhibitory interaction that results in exposure of a functional domain within the effector and initiation of a downstream response (Bishop and Hall, 2000). To date, over 50 effector proteins

have been identified for Rho, Rac, and Cdc42; these include several different types of kinases, lipases, oxidases and scaffold proteins (Jaffe and Hall, 2005). This multitude of effector targets illustrates the diverse variety of signaling pathways regulated by Rho, Rac and Cdc42 proteins. However, they are best known for their role in regulation of actin cytoskeleton (Raftopoulou and Hall, 2004).

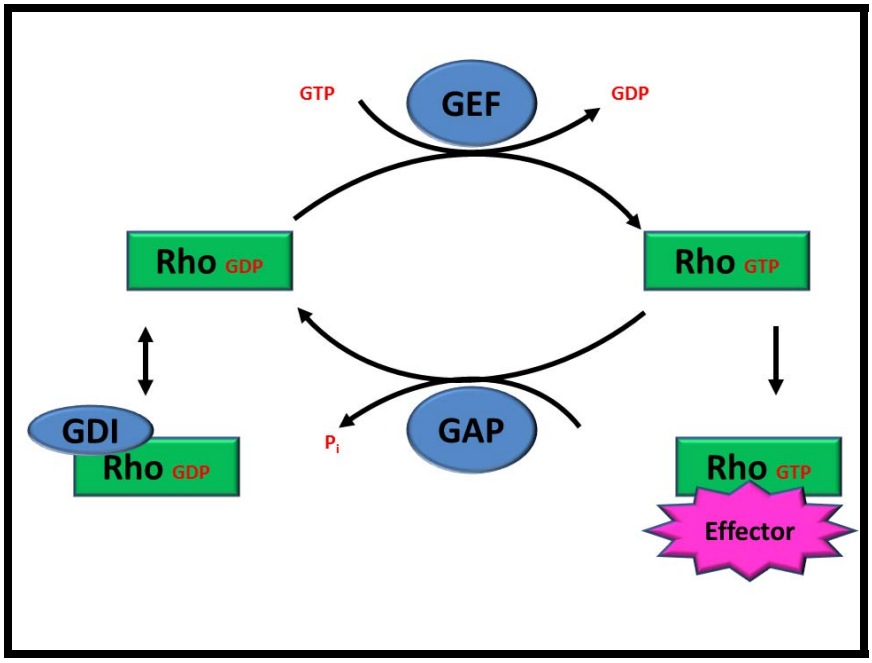


FIGURE 1.1 The GTPase Cycle. Rho GTPases cycle between a GTP-bound "active state" and a GDP-bound "inactive state." Guanine nucleotide exchange factors (GEFs) affect this equilibrium by binding RhoGTPases and stabilizing a nucleotide-free conformation, which promotes dissociation of GDP and association of GTP. In the active state, Rho proteins interact with effector proteins to promote downstream signaling. GTPase activating proteins (GAPs) bind to active Rho proteins and stimulate hydrolysis of GTP to GDP, which results in inactivation of the Rho protein. Inactive Rho proteins in cytosol are bound by guanine nucleotide dissociation inhibitors (GDIs) that prevent spontaneous dissociation of bound GDP and interaction with GEFs.

Rho-mediated regulation of the actin cytoskeleton

In eukaryotic cells, the actin cytoskeleton serves as the structural framework for cell shape, establishment of polarity, and provides the driving force in cell motility (Hall, 1998). One of the initial studies that was able to link Rho function to regulation of the actin cytoskeleton, was the observation that addition of an exoenzyme produced by the bacterium Clostridium botulinum to Vero cells caused a disappearance of actin filaments, but not microtubules (Chardin et al., 1989). The exoenzyme responsible for this phenotype, "C3 transferase," was at the time a newly described ADP-ribosyl transferase, which had just been shown to catalyze ADP-ribosylation on Rho (Aktories et al., 1989). Due to this unusual phenotype, the authors of this work proposed that ADP-ribosylation of Rho was responsible for this disruption in actin microfilaments, and that Rho may therefore be involved in cytoskeletal control (Chardin et al., 1989). This assumption was supported by subsequent work which observed that microinjection of various cells with Rho expressing cDNA and recombinant protein resulted in the organization of actin stress fibers (Paterson et al., 1990). Today, we know actin stress fibers as bundles of contractile actomyosin that are linked to the plasma membrane at integrin-rich focal adhesions and serve as mediators of cell contraction (Fig. 1.2) (Pellegrin and Mellor, 2007). Subsequently, experiments in Swiss 3T3 fibroblasts helped facilitate our understanding of the signaling networks involved in regulating these structural processes (Ridley and Hall, 1992). In the study, stimulation of serum-starved fibroblasts with growth factor resulted in assembly of new actin stress fibers accompanied by the formation of focal adhesions, and the appearance of membrane ruffles. Inhibition of endogenous Rho was then used to show that Rho is specifically required for assembly of actin stress fibers and formation of focal adhesions, however not for membrane

ruffles. This then affirmed that Rho is required in a signal transduction pathway induced by growth factors that results in the formation of focal adhesions and stress fibers (Ridley and Hall, 1992).

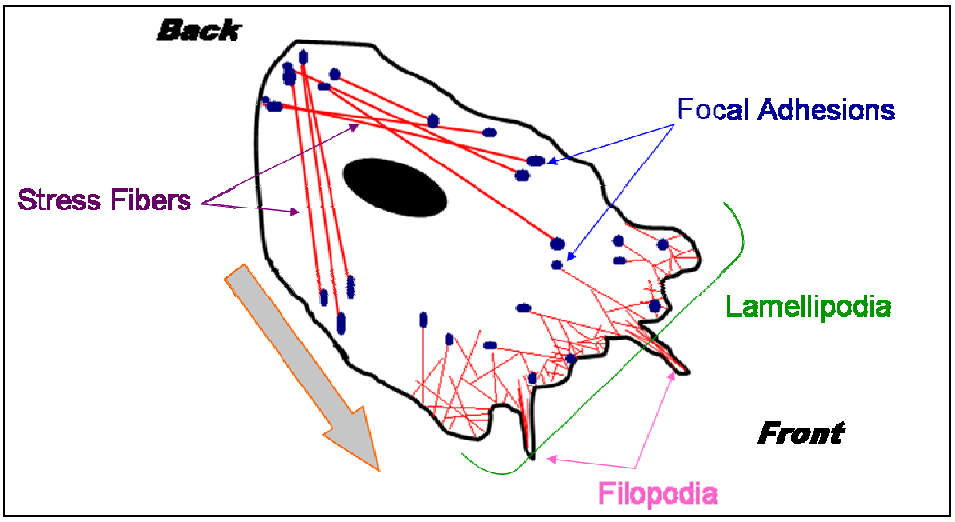


FIGURE 1.2 Rho Family Induced Actin-Based Structures in a Migrating Cell. Top view of migrating cell. Polymerized actin (red lines) is seen in the front and rear of the cell, linked to the plasma membrane at focal adhesions (blue dots). In cell migration, actin-based membrane protrusions at the front of the cell, induced by Cdc42 (filopodia) and Rac1 (lamellipodia), help to guide and provide the driving force required for the cell to move, respectively. RhoA-induced contractile actin:myosin filaments (stress fibers) in the cell body and at the rear, help the cell body to contract, and retract the rear-end (Jaffe and Hall, 2005; Raftopoulou and Hall, 2004; Ridley, 2006). Redrawn using (Raftopoulou and Hall, 2004) as distant source.

We now know of two major downstream effectors of RhoA involved in regulating stress fiber formation: the ROCK/ROK (a Rho-associated coiled-coil containing protein kinase) (Rho kinase/ROK) protein kinases (Ishizaki et al., 1996) and the diaphanous-related formin, mDia1(mammalian homolog of Drosophila diaphanous) (Narumiya et al., 2009; Pellegrin and Mellor, 2007; Watanabe et al., 1997).

ROCK-1 and ROCK-2 are serine/threonine kinases activated by RhoA. They are responsible for phosphorylating multiple targets in the stress fiber pathway, all of which promote myosin phosphorylation and increased contractility of actomyosin (Fig 1.3) (Pellegrin and

Mellor, 2007). For example, once activated, ROCK can directly phosphorylate myosin light chain 2 (MLC2); this phosphorylation leads to increased stress fiber contractility due to an increase in myosin ATPase activity (Katoh et al., 2001; Pellegrin and Mellor, 2007). In addition, ROCK can also promote myosin phosphorylation by interacting with and phosphorylating the myosin binding subunit (MBS) of myosin light chain phosphatase, which results in inactivation of phosphatase activity (Kawano et al., 1999). Lastly, activated ROCK proteins can also affect actin filaments by phosphorylating and activating LIM-kinase (LIMK), which can then phosphorylate and inactivate cofilin, an actin-depolymerizing and severing factor (Maekawa et al., 1999).

The activity of mDia1 is also required to generate the thick, parallel stress fibers characteristic of RhoA activation (Pellegrin and Mellor, 2007). mDia1 is the mammalian ortholog of *Drosophila* Diaphanous, and belongs to the family of Diaphanous-related formins (DRFs) (Raftopoulou and Hall, 2004; Ridley, 2006). Proteins from this family have an actin-nucleating region, referred to as an FH2 domain, which nucleates parallel, unbranched actin filaments (Ridley, 2006). Activated RhoA binds to mDia1 and relieves an auto-inhibitory interaction, which results in exposure of the FH2 domain that can bind to the barbed end of an actin filament and stimulate elongation of actin filaments (Fig. 1.3) (Jaffe and Hall, 2005; Zigmond, 2004). Thus, ROCK proteins and mDia1 play important cooperative roles in stimulating actin:myosin filament assembly, contractility, and stability (Raftopoulou and Hall, 2004).

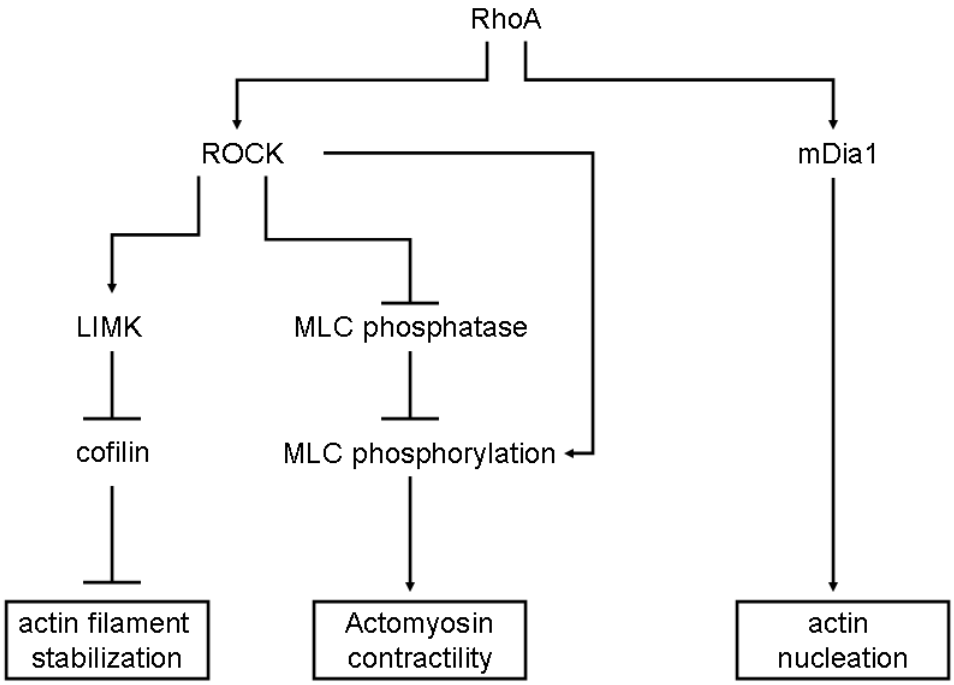


FIGURE 1.3 The RhoA Signaling Pathway. RhoA activated signaling pathways regulate the formation of actin stress fibers. RhoA has two major downstream effectors involved in regulating stress fiber formation: ROCK/ROK (a Rho-associated coiled-coil containing protein kinase) (Rho kinase/ROK) protein kinase and the diaphanous-related formin, mDia1. ROCK affects actin filament stabilization by phosphorylating and activating LIM kinase (LIMK), which then phosphorylates and inactivates cofilin, an actin-depolymerizing and severing factor. ROCK affects myosin light chain (MLC) phosphorylation by directly phosphorylating MLC, and also by phosphorylating the regulatory subunit of myosin light chain phosphatase, which inhibits its phosphatase activity. Increased phosphorylation of MLC increases actomyosin contractility. Finally, RhoA binds and activates mDia1 at the plasma membrane, which initiates the actin nucleation required for stress fiber formation (Pellegrin and Mellor, 2007). Redrawn using (Pellegrin and Mellor, 2007; Raftopoulou and Hall, 2004) as distant sources.

Regulation of Rho activity

The involvement of the Rho GTPase family in a diverse array of cellular functions and the high number of downstream targets (over 50 for Rho, Rac, and Cdc42) indicate a need for tight regulation of Rho family signaling (Jaffe and Hall, 2005; Sternweis et al., 2007). This regulation is mainly carried out by three types of proteins (GDIs, GAPs and GEFs) which, as mentioned previously (Fig. 1.1), control different aspects of the Rho GTPase cycle. Extracellular signals could differentially regulate any Rho family member by modifying any of these three proteins; however, in most cases, these signals act through GEFs (Raftopoulou and Hall, 2004).

The Dbl family of Rho guanine nucleotide exchange factors (RhoGEFs) consists of over 70 members and represents the largest family of direct activators of Rho proteins in humans (Rossman et al., 2005). Members of this family are characterized by the presence of tandem Dbl homology (DH) and pleckstrin homology (PH) domains. The DH domain serves as the main binding interface for GTPases and catalyzes nucleotide exchange on bound GTPases by stabilizing nucleotide-free GTPase intermediates (Rossman et al., 2005; Rossman and Sondek, 2005). Roles for DH-associated PH domains appear to vary among individual proteins, with functions ranging from targeting RhoGEFs to membranes via interaction with phosphoinositides, to assisting in nucleotide exchange (Aittaleb et al., 2009; Rossman et al., 2005). Aside from the canonical DH·PH unit, Dbl RhoGEFs are typically large and vary from one another in their additional protein domains. The RGS (regulator of G-protein signaling)-RhoGEF subfamily of proteins serve as one example of Dbl family proteins that utilize their additional domains to provide a link between activation of cell surface receptors coupled to the G12 family of heterotrimeric G proteins and regulation of RhoA-mediated signaling pathways (Sternweis et al.,

2007). The RGS-RhoGEF subfamily consists of three members, p115-RhoGEF, PDZ-RhoGEF (PRG), and leukemia-associated RhoGEF (LARG), all of which contain a RhoGEF regulator of G protein signaling (rgRGS) homology domain located N-terminal to the DH domain (Fig. 1.4) (Chen et al., 2010). RgRGS domains bind with high affinity to activated $G\alpha_{12/13}$ subunits and, in the case of p115 and LARG, stimulate the intrinsic GTPase activity of the $\alpha_{12/13}$ subunit (Kozasa et al., 1998; Suzuki et al., 2003). Interaction of activated $G\alpha_{13}$ with p115 and LARG also results in stimulation of RGS-RhoGEF nucleotide exchange activity towards RhoA (Hart et al., 1998; Suzuki et al., 2003). Finally, PRG and LARG are unique in that they possess PDZ domains at their far amino-termini that have been shown to mediate interactions with membrane bound proteins (Yamada et al., 2005).

A potential mechanism for activation of RhoA-mediated signaling by $G\alpha_{12/13}$-coupled receptors is straightforward for p115 and LARG; PRG, however, does not exhibit the same bidirectional relationship with α subunits. Although PRG interacts

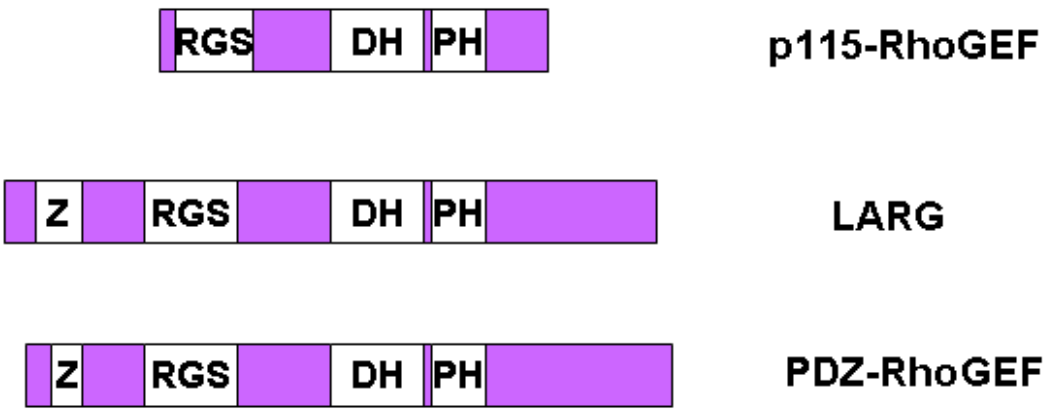


Figure 1.4 The RGS-RhoGEF Subfamily. A schematic diagram of the RGS-RhoGEF subfamily and their known structural elements. Alternate names include: p115-RhoGEF, Lsc (mouse)/Arhgef1; LARG, KIAA0382/Arhgef12; PDZ-RhoGEF, GTRAP48/KIAA0380/Arhgef11. Domains: RGS, regulator of G-protein signaling; DH, dbl homology; PH, pleckstrin homology; Z, PDZ (PSD-95/SAP90-Discs-larg-ZO-1).

with activated $G\alpha_{13}$, it neither functions as a GAP, nor is its exchange activity stimulated by the interaction. Thus, PRG distinguishes itself from its close homologs by employing a distinct mechanism for regulation of nucleotide exchange. Recently, a novel interaction between PRG and activated RhoA was suggested by pulldown experiments (Rumenapp et al., 1999). This was also observed by Mu-Ya Liu in the Sternweis group (personal communication) using purified proteins. To our knowledge, no further work has been done to investigate this observation. This thesis is devoted to characterizing this interaction and determining its functional relevance. A more thorough introduction to PRG and its unique properties will be given in chapter 3.

CHAPTER 2

Methodology

Jana Hadas assisted in cloning full-length and DH·PH constructs of PRG, wild-type as well as the point-mutants. James Chen cloned the PRG-PH domain construct. Steve Gutowski purified Rac1 and Cdc42 proteins.

Expression Constructs

The indicated coding regions of human PRG, LARG, p115-RhoGEF, AKAP-Lbc, p190-RhoGEF, GEF-H1 and the mouse form of p114-RhoGEF were subcloned into a modified pGEX-KG vector containing the protease recognition site for the tobacco etch virus (pGEX-KG-TEV) for proteolytic cleavage of the expressed domains from glutathione S-transferase (GST). Additional constructs were made in which a 6xHis tag was inserted at the C-termini of these sequences. The coding regions of human RhoA, RhoB, RhoC, Cdc42, Rac1, Rnd2, Rnd3 and RhoG were subcloned into the pGEX-KG or pGEX-KG-TEV vectors. Additional constructs were made for RhoA, Rac1 and Cdc42, in which a 6xHis tag (RhoA and Cdc42) or 9xHis tag (Rac1) was inserted at the C-termini for use in the phospholipid vesicles.

Expression and Purification of Proteins

Constructs of RhoGEF exchange factors were expressed in LB medium at 22°C overnight in *Escherichia coli* strain BL21(DE3) cells with 50 μM isopropyl-β-D-thiogalactopyranoside. Cells harvested from 1 liter of culture were resuspended with 30 ml of lysis buffer (50mM NaHEPES, pH 8.0, 200mM NaCl, 1mM dithiothreitol, 1mM EDTA, 1% (v/v) Triton X-100, and protease inhibitors). Cells were lysed by addition of 1mg/ml lysozyme and incubation with rotation for 30 minutes at 4°C. After lysis, 5mM $MgCl_2$ and 20 μg/ml DNAse I were added and the incubation continued with rotation for 60 minutes at 4 °C. GST-tagged fusion proteins were extracted from the soluble fraction of lysates by affinity chromatography with glutathione-Sepharose 4B (Amersham Biosciences). Protein-bound resin

was suspended with 50 mM NaHEPES, pH 8.0, 200 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, protease inhibitors and 1 mg TEV protease and incubated overnight at 4 °C to separate the desired protein from the GST tag. Soluble protein was then further purified by IMAC-Ni^{2+} affinity chromatography (Bio-Rad) or by size-exclusion chromatography using Superdex 200/75 tandem gel filtration columns (Amersham Biosciences).

GTPases were expressed in LB medium at 22 °C overnight in *Escherichia coli* strain BL21(DE3) cells with 50 μM isopropyl-β-D-thiogalactopyranoside. Harvested cells from 1 liter of culture were resuspended with 30 ml lysis buffer (50 mM NaHEPES, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10 μM GDP, and protease inhibitors). For Rnd proteins, GDP was replaced with 300 μM GTP. Cells were lysed by addition of 1 mg/ml lysozyme and rotation for 30 minutes at 4 °C. After lysis, 5 mM $MgCl_2$ and 20 μg/ml DNAse I were added and rotation continued for 60 minutes at 4 °C; alternatively, cells were sonicated. Lysates were cleared by centrifugation for 30 minutes at 35 K RPM and the GST-tagged fusion proteins were extracted from the soluble fraction by affinity chromatography with glutathione-Sepharose 4B (Amersham Biosciences). Protein-bound resin was eluted with 15 mM reduced glutathione or suspended with 50 mM NaHEPES, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10 μM GDP, 5 mM $MgCl_2$, protease inhibitors and 0.5 mg TEV protease for cleavage of the GTPases from the GST tag followed by incubation overnight at 4 °C to complete the cleavage. In the latter case, cleaved protein was further purified with a Mono Q anion exchange column (Amersham Biosciences) that had been pre-equilibrated with Buffer A (25 mM TrisCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$). Elution was accomplished with a linear gradient of 0-1M NaCl in Buffer A.

Activation of GTPases

GTPases were exchanged into 25 mM NaHEPES, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, and 10 μM GDP and concentrated to approximately 100 μM by centrifugation in a Amicon Ultra-4 centrifugal filter device (Millipore) (10K MWCO). The concentrated protein was then supplemented with 0.5 mM GTPγS and 0.5 mM $MgSO_4$ and incubated for 24 h at room temperature. Loading was assessed by quantitating percent loading in a parallel reaction using [^{35}S]-GTPγS.

Pulldown Assays

Immobilized GST-tagged RhoA (or other GTPase) was used to compare the relative ability of purified His_6-tagged RhoGEFs to bind RhoA in the presence of different guanine nucleotides. GST-RhoA (80 pmol) was mixed with 10 μl of glutathione-Sepharose 4B resin in 100 μl of incubation buffer (50 mM NaHEPES, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5 mM $MgCl_2$, and 0.3% (v/v) Triton X-100) and incubated for 30 min at 4 °C. The resin was washed with incubation buffer, and His_6-tagged RhoGEF proteins (12 pmol) were added to the immobilized GST-RhoA in incubation buffer (100 μl) containing no additional guanine nucleotide, 10 μM GDP, or 10 μM GTPγS. The mixtures were incubated on a rotating platform for 45–60 min at 4 °C, after which the Sepharose resin was pelleted using a microcentrifuge. Supernatants containing unbound RhoGEF were removed, and the resins were then washed twice with 500 μl of cold incubation buffer. RhoGEF bound to the resin was released by boiling in SDS sample buffer, and respective amounts bound were compared by

immunoblot analysis using anti-His_6 monoclonal antibody (R&D Systems). Each pulldown assay was repeated at least three times.

Nucleotide Exchange Assay (in Chapter 3 and 4)

RhoA was loaded with *N*-methylanthraniloyl-GDP (mant-GDP, Invitrogen) by incubating 30 μM RhoA with a 7-fold molar excess of mant-GDP in 50 mM NaHEPES, pH 8, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 0.5 mM $MgCl_2$ at room temperature for 24 h in the dark. Loaded RhoA was then purified by gel filtration columns to remove excess nucleotide. Fluorescence assays were performed on a Fluorolog-3 spectrofluorometer at room temperature (λ_{ex} = 356 nm, λ_{em} = 445 nm, slits = 1/1 nm). In each assay, 1 μM mant-GDP-loaded RhoA was incubated with 100 μM GDP in reaction buffer (25 mM NaHEPES, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol, and 5 mM $MgCl_2$) in a 200-μl cuvette. The exchange reaction was started by the addition of 100 nM PRG. Each measurement was repeated at least three times.

Preparation of (poPE:poPC) and (poPE:poPC:DGS-NTA(Ni) Phospholipid Vesicles

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine:1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine lipids (50 : 50) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine:1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine lipids:1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) lipids (47.5 : 50 : 2.5) in chloroform (Avanti Polar Lipids, Inc) were mixed in a glass tube, and then dried under a gentle stream of nitrogen gas for approximately 30 minutes. Tubes containing lipid films

were then put under house vacuum overnight at room temperature. Lipid films were then suspended in 20 mM NaHEPES, pH 7.5, 200 mM NaCl, 5 mM β-mercaptoethanol and 2 mM $MgCl_2$ until total lipid concentration was 10mM. Tubes containing suspensions were then covered and incubated for 15 minutes at room temperature with intermittent gentle vortexing to convert the lipid film into large multilamellar vesicles. The lipid preparations were then frozen in a dry ice/ethanol bath and then thawed in a 37°C water bath 4 cycles. Large multilamellar vesicles were then forced through a polycarbonate membrane with 0.1 μm pore size using a mini-extruder (Avanti Polar Lipids, Inc) to produce a uniform suspension of unilamellar vesicles (approximately 100 nm in diameter). Concentration of total lipid was measured by determination of phosphate reference assay.

CHAPTER 3

Activated RhoA Binds to the Pleckstrin Homology (PH) Domain of PDZ-RhoGEF

This chapter has been published in the Journal of Biological Chemistry (Chen et al., 2010). James Chen contributed to writing of this manuscript and generously allowed for use of his crystal structure in Figure 3.5A & B. Mu-ya Liu designed the original pulldown protocol which was modified by myself.

Introduction

Many approaches have been taken to characterize the role and function of the RGS-RhoGEFs since their initial discovery. One study (Rumenapp et al., 1999) focused on characterization of a newly identified potential RhoGEF termed KIAA0380 (today known as 'PDZ-RhoGEF' or PRG). In the study, activity assays done with purified recombinant proteins confirmed that PRG functions as a RhoA-specific GEF. Some members of the Dbl family, e.g. Ect-2, had previously been shown to bind RhoGTPases without catalyzing nucleotide exchange (Miki et al., 1993), suggesting a scaffolding role for recruitment or subcellular transportation. Therefore, the *in vitro* binding behavior of PRG to RhoA, Rac1 and Cdc42 was examined in all possible nucleotide-bound states (nucleotide-depleted, GDP-bound or GTPγS-bound) of the GTPases (Rumenapp et al., 1999). PRG bound to RhoA in both nucleotide-depleted and GTPγS-bound states (no binding to RhoA-GDP was detected) but failed to bind Rac1 or Cdc42, regardless of nucleotide-bound state. The authors acknowledge that binding of PRG to nucleotide-depleted RhoA agrees with the classical model by which DH domains are believed to facilitate nucleotide exchange, which is by stabilizing a nucleotide-free transition state of the Rho GTPase (Hart et al., 1994). However, binding to GTPγS-activated RhoA presented a more novel interaction. The authors speculated that, aside from its role as a GEF, perhaps this interaction indicated that PRG also acts as an effector of activated RhoA or serves as a transporter for the activated GTPase. Unfortunately, no further work was done to identify the physiological purpose for this interaction or to confirm if both conformations of RhoA (nucleotide-depleted and GTPγS-bound) bound to the same site on PRG.

About this same time, a former member of the Sternweis group, Mu-ya Liu, performed a similar set of pulldown experiments examining binding of GTRAP48 (rat homolog of PRG) to immobilized RhoA and the nucleotide dependence of this interaction. The results from these experiments were similar to those seen by Rumenapp and colleagues (Rumenapp et al., 1999); GTRAP48 bound to both nucleotide-depleted and GTPγS-bound RhoA. These results validated the published study and showed that this binding characteristic is an evolved trait shared by both human and rat homologs. The findings raise some fundamental questions. What is the binding site on PRG for activated RhoA? Does this binding affect the activity of the RhoGEF? What is the physiological role of this interaction?

In this chapter, I present work that identifies the PH domain of PRG as the binding site of activated RhoA. I begin by showing that I was able to successfully repeat the binding experiment done by Mu-ya Liu with PRG (human homolog of GTRAP48). I then recount how, using deletion analysis of PRG, I was able to identify that activated RhoA binds to the PH domain of PRG. Site-directed mutagenesis of the PRG active site showed that the active site does not contribute to this binding interaction. In addition, I present a crystal structure of a PRG-DH·PH-RhoA(GTPγS) complex, solved by my colleague James Chen, which reveals in greater detail that the binding interface involves the switch regions in RhoA and a conserved hydrophobic patch in the PH domain of PRG.

Results

PRG, unlike its close homolog p115-RhoGEF, binds to GTPγS-activated GST-RhoA

Investigation of the novel interaction between PRG and activated RhoA began by using PRG (the human homolog, PDZ-RhoGEF) to confirm the pulldown experiments initiated by Mu-ya Liu with GTRAP48. To accomplish this, a poly histidine-tagged full-length version of PRG was cloned into a modified pGEX-KG vector. Protein was expressed in a bacterial expression system (BL21 DE3) and purified via affinity chromatography as described in "Chapter 2". Previous work done by others has shown that Lsc (mouse form of p115-RhoGEF) behaves as a canonical GEF in that it only shows interaction with the nucleotide-depleted form of RhoA (Glaven et al., 1996). Therefore, its human homolog, p115-RhoGEF, was prepared (see chapter 2) as a control for the pulldown assays. Binding of both full-length PRG and p115-RhoGEF to immobilized GST-RhoA was then tested as shown in Fig. 3.1.

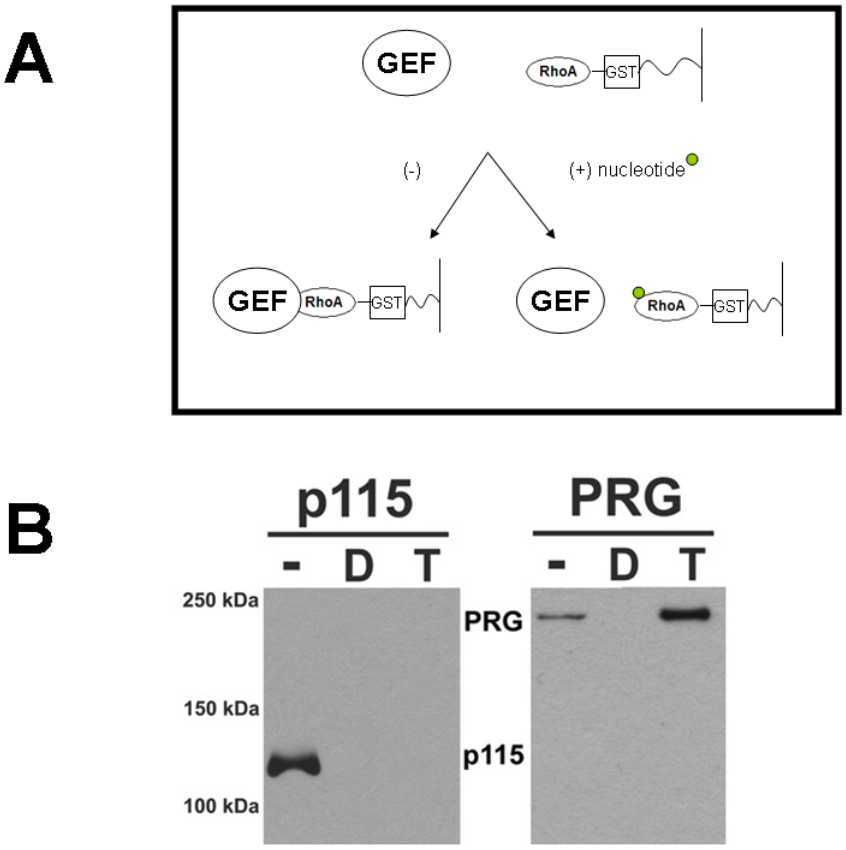


FIGURE 3.1 PRG binds to GTPγS-activated GST-RhoA. (A) Schematic diagram illustrating the pulldown experiment. Purified GST-tagged RhoA (80 pmol) was bound to glutathione Sepharose resin. Immobilized GST-RhoA was then incubated with purified p115-RhoGEF or PRG (12 pmol) with no additional guanine nucleotide (-), 10 μM GDP (D), or 10 μM GTPγS (T), as described in Chapter 2. RhoGEF that bound to immobilized GST-RhoA was released by boiling the resin in SDS sample buffer. **(B)** Western blot analysis of RhoGEF bound to immobilized GST-RhoA. Eluted proteins from GST resins were subjected to SDS-PAGE and visualized by immunoblotting using an anti-6His monoclonal antibody. Modified from (Chen et al., 2010).

The results from the pulldown assay (Fig. 3.1B) show that both p115-RhoGEF and PRG bound tightly to nucleotide-depleted RhoA, as expected for DH containing RhoGEFs which are known to bind and stabilize the nucleotide-free transition state of RhoA. Unlike p115-RhoGEF, however, PRG demonstrated tighter binding to RhoA in the presence of GTPγS (Fig. 3.1B). This binding presents a novel and unexplained phenomena. It should be noted that, although binding of PRG to GTPγS-activated RhoA was reported previously by Rumenapp *et al.,* 1999, PRG demonstrated preferential binding to nucleotide-free RhoA in their similar experiments.

The DH·PH domains of PRG are sufficient to bind GTPγS-activated GST-RhoA

PRG, like all RGS-RhoGEFs, is a large protein and contains multiple structural domains. Therefore, despite the fact that DH domains possess well characterized binding sites for RhoGTPases that facilitate exchange of guanine nucleotides (Rossman et al., 2005), it is possible that a second binding site may be responsible for binding activated RhoA. In an effort to address this possibility, deletion analysis of PRG was performed based on known domains and predicted secondary structural elements. Truncated fragments of PRG (Fig. 3.2A) were cloned, expressed in bacteria and purified via affinity chromatography as described in "Chapter 2." Following purification, protein fragments were then monitored for their ability to bind immobilized GST-RhoA using the pulldown assay (Fig. 3.2).

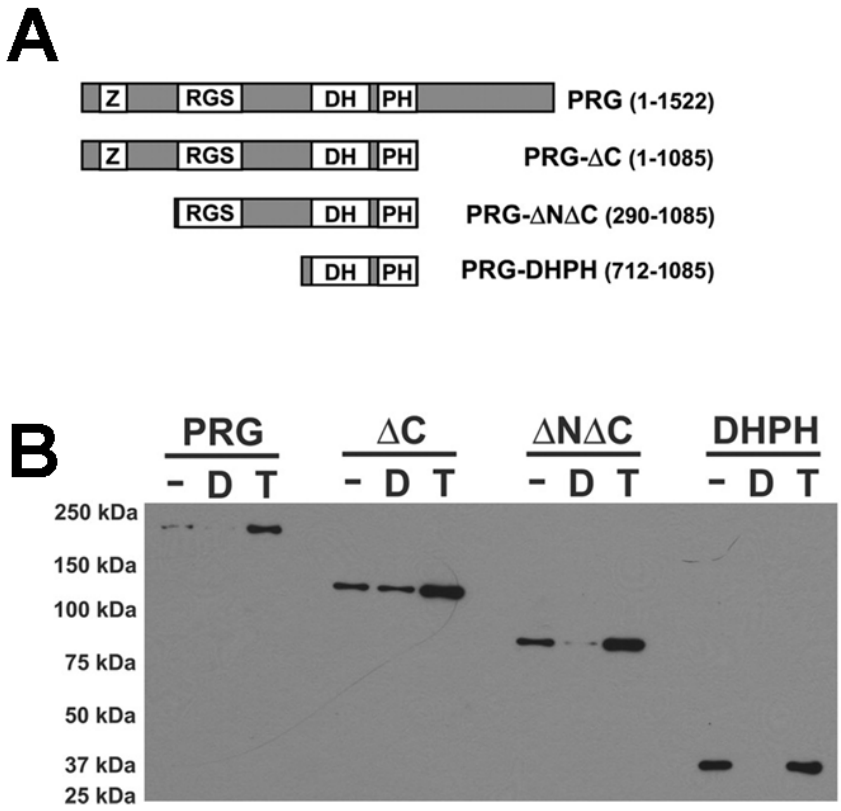


FIGURE 3.2 The DH·PH domains of PRG are sufficient to bind both inactive as well as activated GST-RhoA. (A) Diagram of PRG fragments used in this research; amino acids included in each fragment are listed to the right. PDZ domains are designated as 'Z.' **(B)** Purified full-length PRG, or truncations as described in **A**, were monitored for their ability to bind immobilized GST-RhoA in the absence or presence of various guanine nucleotides as described in Fig. 3.1A. Modified from (Chen et al., 2010).

The results from the pulldown assay (Fig. 3.2B) show that the PRG-DH·PH domains are sufficient for association with both inactive and activated forms of RhoA.

The active site of PRG does not contribute to binding GTPγS-activated RhoA

Although RhoGEF DH domains are traditionally credited as the domains responsible for catalyzing nucleotide exchange within Rho GTPases (Rossman et al., 2005), several studies now provide evidence that some DH-associated PH domains, including PRG, can help facilitate nucleotide exchange on GTPases (Derewenda et al., 2004; Liu et al., 1998). This suggests that for some RhoGEFs, tandem DH·PH domains serve as the core catalytic unit for facilitating nucleotide exchange. I have shown that tandem PRG-DH·PH domains are sufficient for binding activated RhoA (Fig. 3.2B). It is possible that the active site of PRG, which is responsible for binding GDP-bound RhoA and facilitating nucleotide exchange, is also the site responsible for binding GTPγS-activated RhoA. To test this possibility, an attempt was made to eliminate binding of PRG to GDP-loaded RhoA and nucleotide-free RhoA by mutation of the PRG active site. This was followed by assessment of the ability of these mutants to bind to GTPγS-activated RhoA (Figure 3.3). Mutation of the PRG active site was facilitated by analysis of the crystal structure of the PRG-DH·PH domains in complex with nucleotide-free RhoA (Derewenda et al., 2004). The authors defined an extensive interface between the DH domain of PRG and RhoA (Derewenda et al., 2004). Based on this interface, I chose to mutate three amino acid residues (R868, D873 and M879) on the surface of the DH domain that directly contact nucleotide-free RhoA. Amino acids predicted to interfere with and hamper binding to RhoA were individually substituted into these sites (R868G, D873W and M879Y) with the intention of creating a "DH-

dead" mutant incapable of binding RhoA in its active site. Each of these mutants could be expressed in bacteria to similar extents and purified like the wild-type domains. The ability of these mutants to stimulate nucleotide exchange was examined via *in vitro* activity assays. As evinced in these assays, each of these mutants displayed a diminished ability to stimulate nucleotide exchange compared to wild-type protein (Fig. 3.3A). This indicates that binding of RhoA to the PRG active site is perturbed by the inserted mutations. To confirm this deficiency, binding of these "DH-dead" mutants to RhoA and the nucleotide dependence of this interaction was examined by pulldown assay. The results from the pulldown assay reveal that binding of the mutants to RhoA in the absence of nucleotide has been abolished while there was little effect on their ability to bind RhoA in the presence of activated GTPγS (Fig. 3.3B). These results suggest that the active site of PRG does not participate in binding activated RhoA.

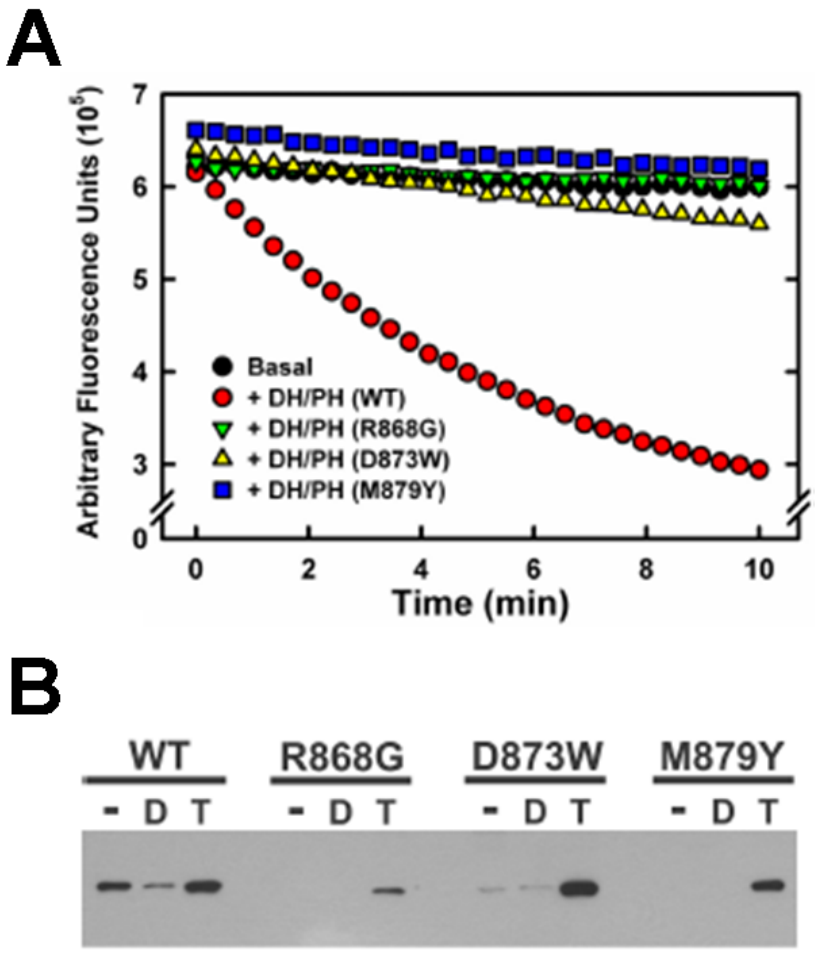


FIGURE 3.3 Mutation of the active site of PRG within its DH domain drastically inhibits exchange activity and ability to bind RhoA in the absence of nucleotide, but does not affect binding to activated RhoA. (A) Stimulation of dissociation of mantGDP from RhoA by wild-type or mutant PRG-DH·PH domains was monitored by the decrease in fluorescence of the dissociated mantGDP. For each reaction, 1 μM RhoA, pre-loaded with mantGDP, was mixed with 100 μM GDP at room temperature. The exchange reaction was started by addition of 100 nM PRG-DH·PH (wild-type, red solid circles; R868G, green triangles; D873W, yellow triangles; M879Y, blue squares) or buffer (solid black circles). Fluorescence (λ_{ex} = 356 nm, λ_{em} = 445nm) was then measured for 10 minutes. **(B)** Purified PRG-DH·PH domains, wild-type (WT) or mutants, were monitored for their ability to bind to immobilized GST-RhoA in the absence or presence of various guanine nucleotides as described in Fig. 3.1A. Modified from (Chen et al., 2010).

GTPγS-activated RhoA binds to the PH domain of PRG

In an effort to further refine the activated RhoA binding site on PRG, constructs of isolated PRG-DH and PRG-PH domains were designed (Fig. 3.4A). The expression and purification protocol for these isolated domains was identical to that of previous deletion constructs (*see* Chapter 2). Purified PRG-DH and -PH domains were examined for their ability to bind immobilized GST-RhoA and the nucleotide dependence of this interaction using a modified version of the previously described pulldown assay (Fig. 3.4B).

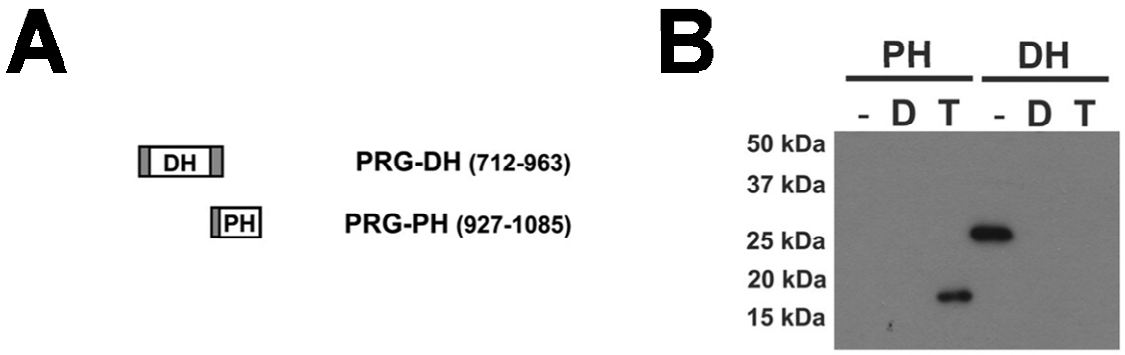
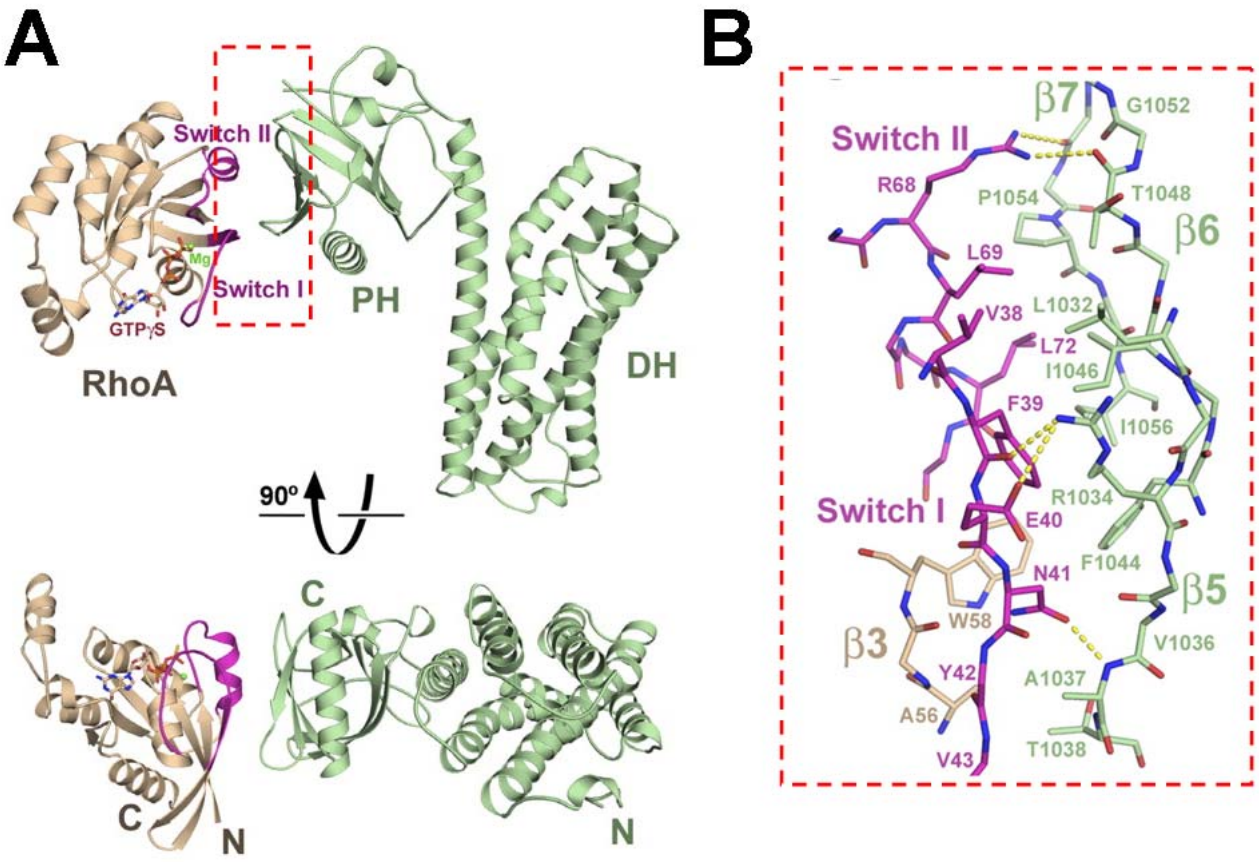


FIGURE 3.4 The PRG-PH domain is sufficient for binding of RhoA(GTPγS).
(A) Diagram of PRG fragments used in this research; amino acids included in each fragment are listed to the right. **(B)** Purified His-tagged PRG-DH and –PH domains were incubated with immobilized GST-RhoA and no additional nucleotide (-) or 10 μM GDP (D) or immobilized GST-RhoA pre-loaded with GTPγS (T). PH or DH domains bound to the resin were analyzed by immunoblotting as described in Fig. 3.1A. Figure modified from (Chen et al., 2010).

The results from the pulldown assay reveal that the PRG-DH domain demonstrated canonical GEF binding behavior binding strongly to RhoA in the nucleotide-free condition, but not to GTPγS-loaded RhoA. The PRG-PH domain, however, displayed the opposite effect, binding strongly to GTPγS-loaded RhoA, but not to nucleotide-free RhoA. This indicates that the PRG-PH domain is alone sufficient for binding of RhoA(GTPγS).

The molecular determinants of this interaction were identified by my colleague, James Chen, who was able to purify a complex of the PRG-DH·PH domains bound to RhoA(GTPγS), generate crystals, and solve a three-dimensional structure of the complex at 2.7 Å resolution (Fig. 3.5A) (Chen et al., 2010). This structure revealed that the interface is comprised of the switch regions in RhoA and a conserved hydrophobic patch in the PH domain of PRG. Upon closer inspection, this interface is populated with conserved hydrophobic residues from both the PH domain and RhoA (Fig. 3.5B).

To validate that this was a legitimate binding interface in solution, rather than an artifact of crystallization, numerous hydrophobic residues (A1037, F1044, I1046 and I1056) and one charged residue (R1034) on the interacting surface of the PH domain were chosen for site-directed mutagenesis. Amino acids that were likely to perturb association with RhoA(GTPγS) were then separately substituted into these sites. After purification of these mutant PRG-DH·PH domains, their ability to bind immobilized RhoA(GTPγS) was measured using the pulldown assay (Fig. 3.1A). This analysis shows that mutation of the selected residues on the PH domain significantly reduced the binding affinity of PRG-DH·PH proteins for GTPγS-activated RhoA (Fig. 3.5C). In contrast, binding of mutants to nucleotide-free RhoA was minimally affected. These results confirm that the binding interface is legitimate and the crystal structure is correct. Thus, binding of activated RhoA to PRG is mediated by the switch regions in RhoA and a hydrophobic patch in the PH domain of PRG.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Switch I
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GTPγS
RhoA
PH
DH
90°
C
N
C
N
B
β7
G1052
Switch II
R68
P1054
T1048
β6
L69
V38
L1032
L72
I1046
F39
I1056
Switch I
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A1037
A56
V43
T1038

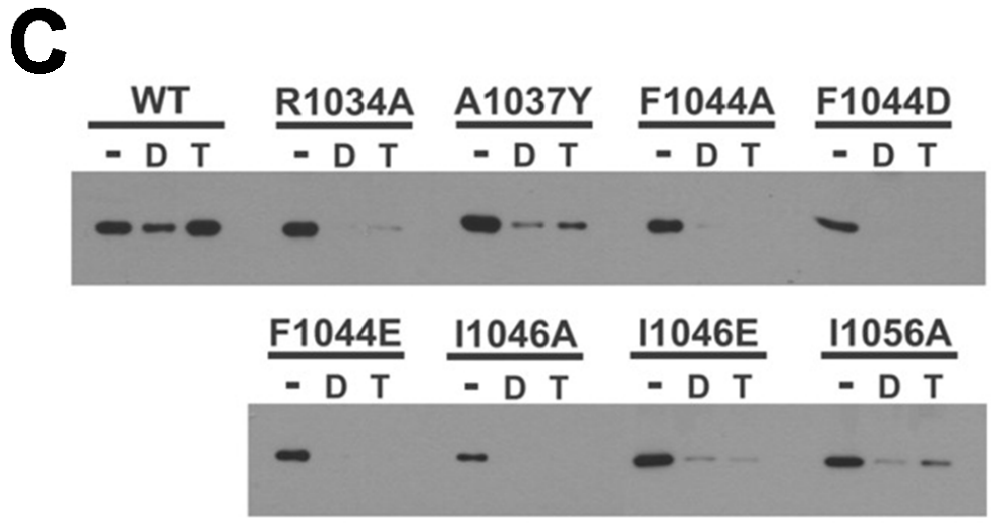

C
WT
R1034A
A1037Y
F1044A
F1044D
- D T
- D T
- D T
- D T
- D T
F1044E
I1046A
I1046E
I1056A
- D T
- D T
- D T
- D T

FIGURE 3.5 The PH-RhoA Interface. (A) Ribbon diagrams of tertiary structures of PRG-DH·PH in complex with RhoA·GTPγS. PRG-DH·PH is colored green. RhoA is colored wheat, with switch regions colored purple. GTPγS and magnesium ion are depicted as ball-and-stick models and colored as follows. Oxygen, nitrogen, carbon and phosphorus atoms are colored red, blue, wheat, and yellow, respectively. Magnesium is colored green. **(B)** Magnified view of contacts between switch regions of RhoA and β5-β6-β7 strands of the PH domain. Hydrogen bonds are drawn as dotted lines and colored yellow. Residues are labeled and color-coded as shown in A. **(C)** Purified PRG-DH·PH domains, wild-type (WT) or mutant, were monitored for their ability to bind immobilized GST-RhoA in the absence or presence of various guanine nucleotides as described in Fig. 3.1A. Figure modified from (Chen et al., 2010).

Discussion

In this chapter, I have shown that PRG (a member of the RGS-RhoGEFs) binds to both nucleotide-free and GTPγS-activated forms of RhoA. Binding to nucleotide-free RhoA is expected as exchange factors are known to stabilize nucleotide-depleted GTPases (Rossman et al., 2005). Binding to GTPγS-activated RhoA, however, presents a novel and unexplained phenomena. Furthermore, PRG appears to bind equally well, if not stronger to GTPγS-activated RhoA, than to nucleotide-free RhoA. This high binding affinity for both conformations of RhoA conflicts with previous work done by Rumenapp *et al.,* 1999. Their group was one of the first to identify that PRG can bind to both nucleotide-free and GTPγS-activated forms of RhoA, however, in their work binding appeared preferential to the nucleotide-free GTPase (Rumenapp et al., 1999). Possible reasons for this discrepancy include differences in experimental design, expression system employed and activity of recombinant proteins and the protocol for nucleotide loading of GTPases. Whatever the reason may be for this discrepancy, the results from both experiments agree that binding of PRG to activated RhoA is observable *in vitro* and may have a physiological role *in vivo*.

In addition to confirming that PRG is able to bind activated RhoA, I have shown by deletion analysis that the PH domain is sufficient for this interaction. To our knowledge this has never been shown before. Furthermore, using site-directed mutagenesis of the PRG active-site, I was able to produce recombinant mutant proteins with a significantly impaired ability to bind nucleotide-free RhoA and stimulate nucleotide exchange. Interestingly, binding of these DH-mutants to GTPγS-activated RhoA was unaffected by these mutations which suggests that the active site does not participate in binding activated RhoA.

Finally, my colleague James Chen was able to determine the three-dimensional structure of a PRG-DH·PH-RhoA(GTPγS) complex using X-ray crystallography. This structure revealed that the binding interface is comprised of the switch regions in RhoA and a conserved hydrophobic patch in the PH domain of PRG. By mutating specific residues on the PH domain of PRG, which contact RhoA(GTPγS) in the crystal structure, I was able to confirm that this is real binding interface in solution and not an artifact of crystallization.

CHAPTER 4

Binding of Activated RhoA to PDZ-RhoGEF Provides Positive Feedback Regulation of Exchange Activity

Figure 4.1 was published in the Journal of Biological Chemistry (Chen et al., 2010). Thanks to Steve Gutowski for all his assistance in the vesicle assays.

Introduction

Activated RhoA associates with its own regulator, PRG. This interaction involves the switch regions in RhoA and a conserved hydrophobic patch on the PH domain of PRG. This next study attempts to elucidate the physiological role of this interaction. As previously mentioned, tandem DH·PH domains of PRG serve as the core catalytic unit for facilitating nucleotide exchange. Thus, it is possible that binding of activated RhoA to the PH domain may directly affect this intrinsic activity in either a positive or negative manner. An example of this type of feedback mechanism between regulator and substrate was shown for the GTPase Ras and its exchange factor, Son of Sevenless (SOS) (Margarit et al., 2003). In this example, binding of Ras(GTP) to a second site on SOS, located distal to the active site, resulted in allosteric stabilization of the SOS active site and a significant increase in the catalytic efficiency of SOS (Margarit et al., 2003). Likewise, I hypothesized that binding of activated RhoA to the PH domain may serve as some form of feedback signal resulting in a change in the catalytic efficiency of PRG.

A second hypothesis proposed in my previous publication (Chen et al., 2010) is that binding of activated RhoA to the PRG-PH domain serves as a mechanism for localizing PRG to the plasma membrane. RhoA is prenylated *in vivo* and is presumed to be associated with the plasma membrane in cells (Ridley, 2006). Thus, localization of PRG to the plasma membrane via interaction with Rho(GTP) could serve as a mechanism for keeping PRG in close proximity with Rho(GDP) and prolong catalytic activity, a feed-forward mechanism. This hypothesis was supported by a study of LARG in which mutation of a conserved hydrophobic patch on the LARG PH domain minimally affected nucleotide exchange activity *in vitro,* but did impair the

ability of LARG to stimulate RhoA-dependent gene transcription and to induce actin stress fiber formation in cultured cells (Aittaleb et al., 2009). RhoA-dependent gene transcription in cells could be rescued by expression of fusion proteins of mutant LARG with exogenous membrane-targeting motifs; the authors suggested that the PH domain of Lbc subfamily members appears to be connected with membrane targeting. Lastly, the authors of this study were able to verify that the PH domain of LARG does not to bind phospholipids, suggesting that protein-protein interactions were the source of the membrane-targeting effect (Aittaleb et al., 2009).

I have investigated both of the proposed hypotheses as potential mechanisms for regulating PRG exchange activity and present the results of this investigation here.

Results

RhoA(GTPγS) does not affect nucleotide exchange activity of PRG for RhoA in solution

To examine if binding of activated RhoA to the PRG-PH domain directly influences guanine nucleotide exchange activity of PRG, I monitored nucleotide exchange activity of PRG *in vitro* in the absence or presence of saturating RhoA(GTPγS). In these assays, rates of nucleotide exchange were monitored by a decrease in the fluorescence intensity of mantGDP as a result of its dissociation from pre-loaded RhoA. The results from these assays (Fig. 4.1) indicate that the exchange activities of the PRG-DH·PH domains and of full-length PRG are not significantly affected by the presence of RhoA(GTPγS). This suggests that binding of activated RhoA to the PRG-PH domain does not serve as a feedback mechanism for regulating the intrinsic catalytic activity of PRG.

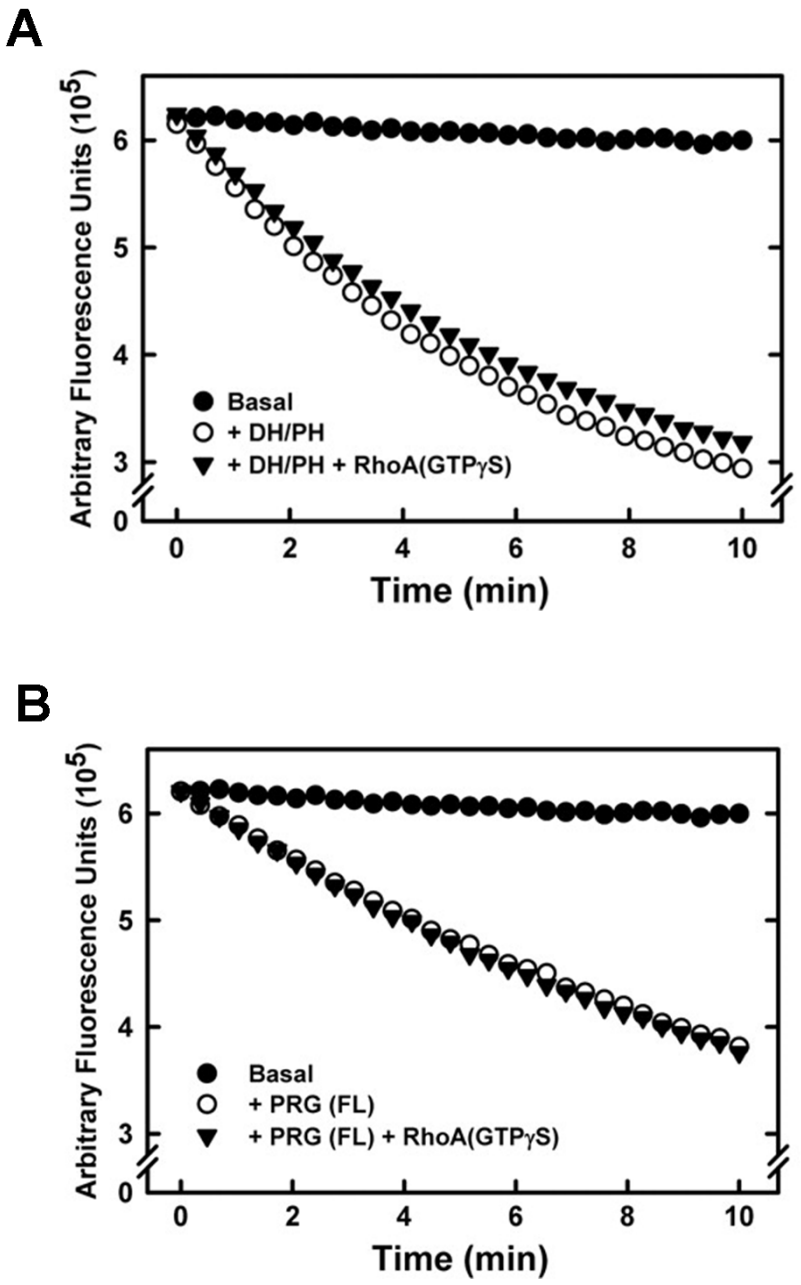


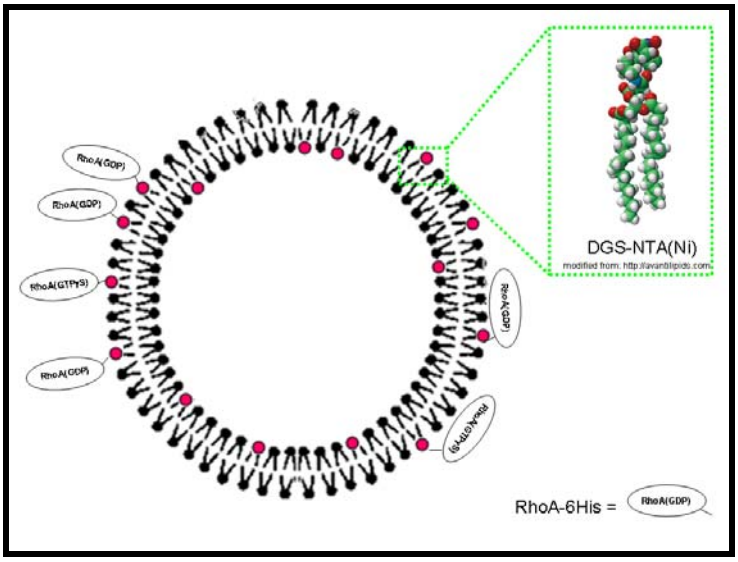
FIGURE 4.1 RhoA(GTPγS) does not affect nucleotide exchange activity of PRG for RhoA in solution. (A) Nucleotide exchange assays with PRG-DH·PH and RhoA. For each time course, 1 μM RhoA loaded with mantGDP was mixed with 100 μM GDP, and the exchange reaction was started at room temperature by the addition of buffer (*Basal, solid circles*) or 100 nM PRG-DH·PH alone (*open circles*) or 100 nM PRG-DH·PH with 1 μM RhoA(GTPγS) (*solid triangles*). The subsequent decrease in fluorescence (λ_{ex} = 356 nm, λ_{em} = 445 nm) was measured for 10 minutes. **(B)** The same nucleotide exchange assays as described in (A), but with full-length PRG and RhoA. Modified from (Chen et al., 2010)

Activated RhoA positively regulates exchange activity of PRG by localizing it to the membrane surface of phospholipid vesicles

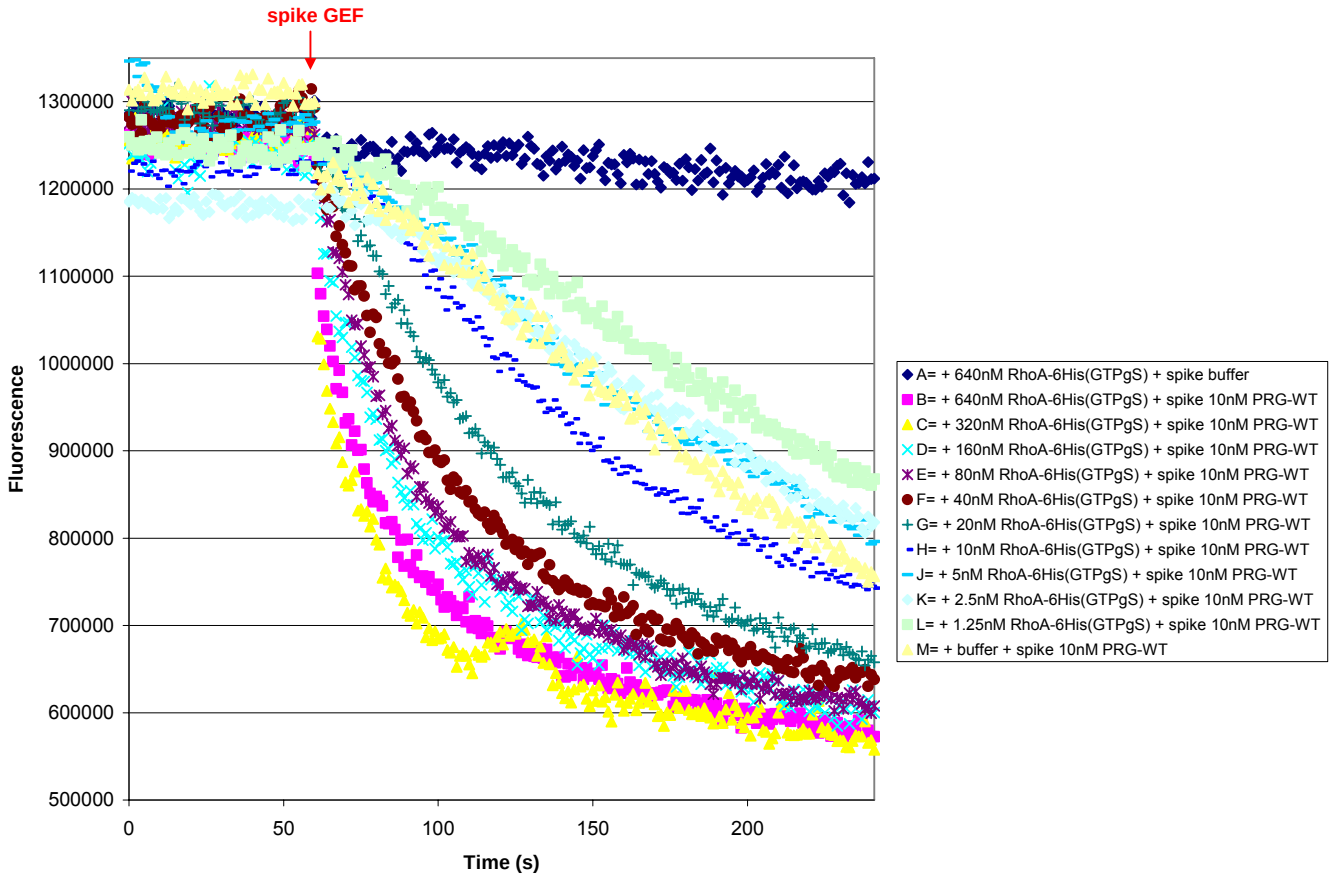
I used phospholipid vesicles and recombinant proteins to investigate RhoA(GTPγS)-mediated localization of PRG as a potential mechanism for regulation of PRG nucleotide exchange activity *in vitro* (Fig. 4.2A). The phospholipid vesicles used in this system were composed of poPE : poPC : DGS-NTA(Ni). DGS-NTA(Ni) is a commercially available phospholipid (Avanti, Inc.) modified with a nickel-chelating group covalently attached to the lipid molecule; this modification allows the lipid to bind recombinant proteins and peptides containing poly-histidine tags (Chikh et al., 2002). Thus, RhoA, which is normally associated with the membrane via C-terminal prenylation, can be bound to the surface of vesicles by a 6-His sequence in place of the hydrophobic modification.

The ability of membrane-bound RhoA(GTPγS) to increase the effective activity of PRG on membrane associated substrate is shown in Figure 4.2B. Under basal conditions, nucleotide exchange is very slow (condition A). Once PRG is added (condition M), there is a significant increase in the rate of exchange as monitored by the decrease in fluorescence. This signal remains unaffected by concentrations of RhoA(GTPγS) ≤ 5 nM. Once the concentration of activated RhoA reaches 10 nM there is a slight, yet noticeable increase in the exchange rate. This increase in exchange rate increases dramatically at 20 nM RhoA(GTPγS) and continues to increase dose-dependently until approximately 320 nM RhoA(GTPγS). These results indicate that RhoA(GTPγS) positively affects PRG nucleotide exchange activity. The dose-dependent

A


RhoA(GDP)
RhoA(GDP)
RhoA(GTPγS)
RhoA(GDP)
RhoA-6His(GDP)
RhoA(GTPγS)
DGS-NTA(Ni)
modified from: http://avantilipids.com
RhoA-6His =
RhoA(GDP)

B


spike GEF
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1200000
1100000
1000000
900000
800000
700000
600000
500000
Fluorescence
0
50
100
150
200
Time (s)
A= + 640nM RhoA-6His(GTPgS) + spike buffer
B= + 640nM RhoA-6His(GTPgS) + spike 10nM PRG-WT
C= + 320nM RhoA-6His(GTPgS) + spike 10nM PRG-WT
D= + 160nM RhoA-6His(GTPgS) + spike 10nM PRG-WT
E= + 80nM RhoA-6His(GTPgS) + spike 10nM PRG-WT
F= + 40nM RhoA-6His(GTPgS) + spike 10nM PRG-WT
G= + 20nM RhoA-6His(GTPgS) + spike 10nM PRG-WT
H= + 10nM RhoA-6His(GTPgS) + spike 10nM PRG-WT
J= + 5nM RhoA-6His(GTPgS) + spike 10nM PRG-WT
K= + 2.5nM RhoA-6His(GTPgS) + spike 10nM PRG-WT
L= + 1.25nM RhoA-6His(GTPgS) + spike 10nM PRG-WT
M= + buffer + spike 10nM PRG-WT

FIGURE 4.2 Titration of RhoA(GTPγS) in a reconstituted signaling system. (A) Illustration of reconstituted signaling system. A selected mixture of phospholipids (poPE : poPC : DGS-NTA(Ni)) at a molar ratio 47.5:50:2.5 was resuspended in a buffered saline solution to give large, multilamellar vesicles. The lipid suspension was then repeatedly passed through a polycarbonate filter with 0.1μm pores. Extrusion typically yields large, unilamellar vesicles (LUV) with a mean diameter of 120-140nm (http://avantilipids.com). These LUVs were mixed with controlled amounts of purified C-terminally tagged RhoA-6His for 1 minute at 25˚C. The result is LUVs bound with RhoA ready for experimentation. **(B)** Titration curve of RhoA(GTPγS) in the reconstituted signaling system. Dissociation of mant-GDP from pre-loaded RhoA was monitored by the decrease in fluorescence of dissociated mantGDP. For each reaction, 1 μM RhoA-6His pre-loaded with mant-GDP was mixed with 1 mM GDP, 5 nM large unilamellar vesicles (LUV) and increasing amounts of GTPγS-loaded RhoA or buffer at 25 ˚C for 1 minute. Fluorescence (λ_{ex} = 356 nm, λ_{em} = 445nm) was then measured for 1 minute. After this, the exchange reaction was started by addition of 10 nM PRG-DH·PH or buffer and fluorescence was measured for an additional 180 seconds.

response in exchange activity suggests that this mechanism for regulating exchange activity is sensitive to the concentration of RhoA(GTPγS).

To confirm that this positive effect on exchange activity was due to localization of PRG to the membrane of phospholipid vesicles via its interaction with membrane-bound RhoA(GTPγS) and not just the presence of phospholipid vesicles, I repeated the *in vitro* activity assay using untethered RhoA(GTPγS) (no His tag) (Fig 4.3A). To avoid confusion, unless it is written as “soluble”, all RhoA used in these experiments is hexa His-tagged and therefore bound to nickel-chelating phospholipid vesicles. As shown in figure 4.3A, when PRG is added to vesicles with membrane-bound RhoA(GTPγS), there is a significant increase in exchange activity, a feed-forward response. When the same amount of PRG is added to vesicles in the presence of non-tagged soluble RhoA(GTPγS), a much lower response in exchange is observed. This lower exchange rate is the same rate as that observed in the absence of RhoA(GTPγS).

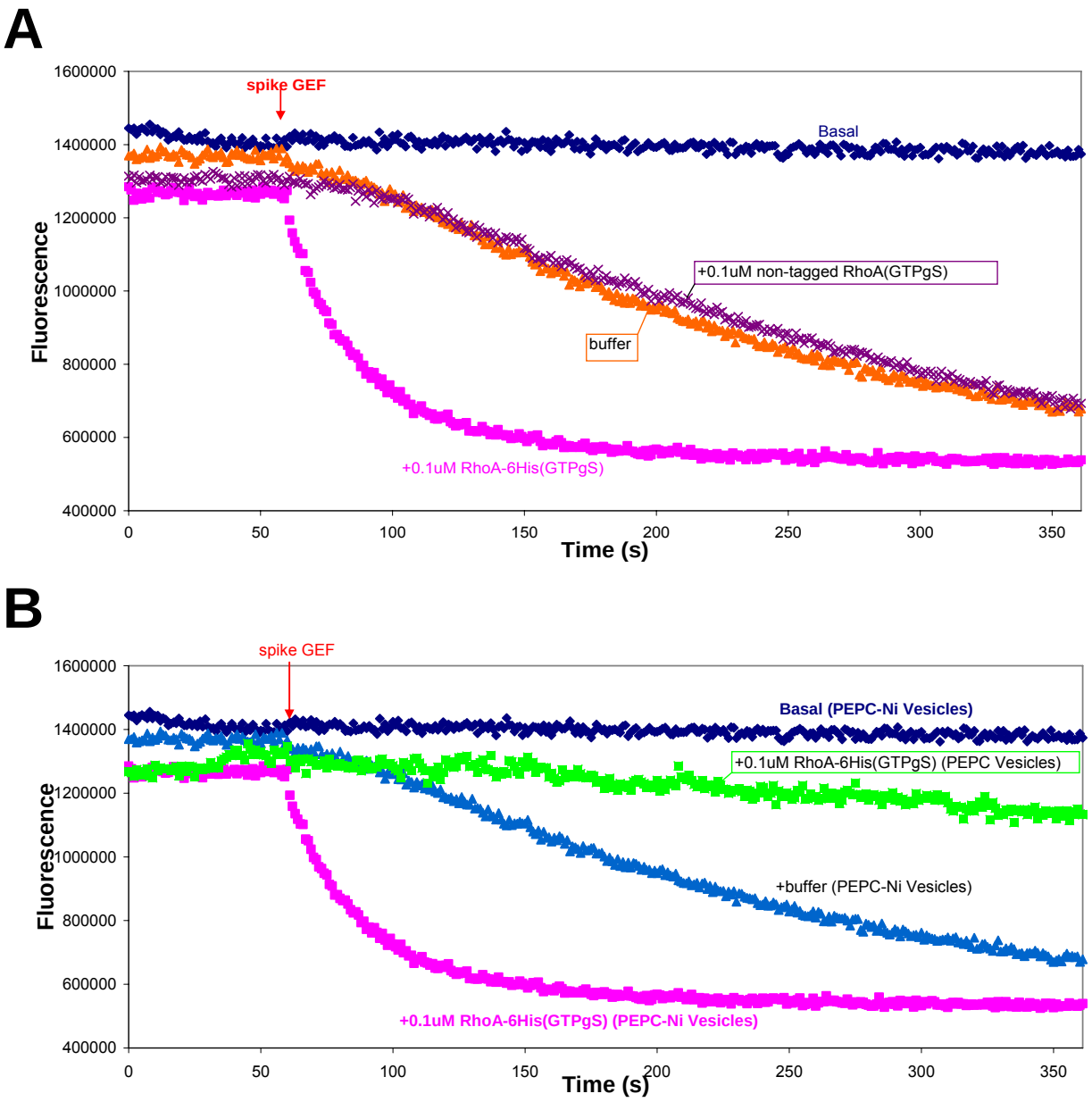


FIGURE 4.3 RhoA(GTPγS) positively regulates exchange activity of PRG by localizing it to the surface of phospholipid vesicles. (A) Soluble RhoA(GTPγS) is unable to regulate PRG. Dissociation of mantGDP from pre-loaded RhoA was monitored by the decrease in fluorescence of the dissociated mantGDP. For each reaction 1 μM RhoA-6His(mantGDP) was mixed with 1 mM GDP, 5 nM PEPC-Ni Vesicles and 0.1 μM RhoA-6His(GTPγS), buffer, or 0.1μM RhoA(GTPγS) and fluorescence monitored (λ_{ex} = 356 nm, λ_{em} = 445nm) at 25°C for 1minute. After this, the exchange reaction was started by addition of 10 nM PRG-DH·PH or buffer. Fluorescence was measured for 300 seconds. **(B)** Mant-GDP dissociation assay. For each reaction 1 μM RhoA-6His(mantGDP) was mixed with 1 mM GDP and 5 nM PEPC-Ni vesicles or 5 nM PEPC vesicles and 0.1 μM RhoA-6His(GTPγS) or buffer as indicated. Fluorescence was

monitored at 25°C for 1minute. After this, the exchange reaction was started by addition of 10 nM PRG-DH·PH or buffer and fluorescence measured for 300 seconds.

The positive effect of His-tagged RhoA(GTPγS) on nucleotide exchange is also lost when the reaction is done in the presence of phospholipid vesicles missing the nickel-chelating lipid, DGS-NTA(Ni) (Fig. 4.3B). Both of these results (figure 4.3) indicate that the positive effect of RhoA(GTPγS) on nucleotide exchange, the feed-forward response, is a result of RhoA(GTPγS) being stably bound to the membrane.

Our next goal was to identify if the positive effect of membrane-bound RhoA(GTPγS) on nucleotide exchange required high affinity binding between membrane-bound RhoA(GTPγS) and the PH domain of PRG. To investigate this, I compared the ability of wild-type PRG and a PRG mutant, which contained two point-mutations in the hydrophobic patch of the PH domain that abolishes binding to RhoA (see Table 5.1), to stimulate nucleotide exchange on RhoA bound to phospholipid vesicles in the presence of membrane-bound RhoA(GTPγS). The exchange activities of both PRG wild-type and PH-mutant proteins is the same with untethered RhoA in solution (data not shown). As shown in figure 4.4, there is very little nucleotide exchange in basal conditions. When wild-type PRG is added combination with RhoA-6His(GTPγS) there is a sharp increase in fluorescence indicating a high rate of nucleotide exchange (Fig. 4.4A). This exchange is completely dependent on the presence of RhoA(GTPγS), as indicated by the weak signal seen when

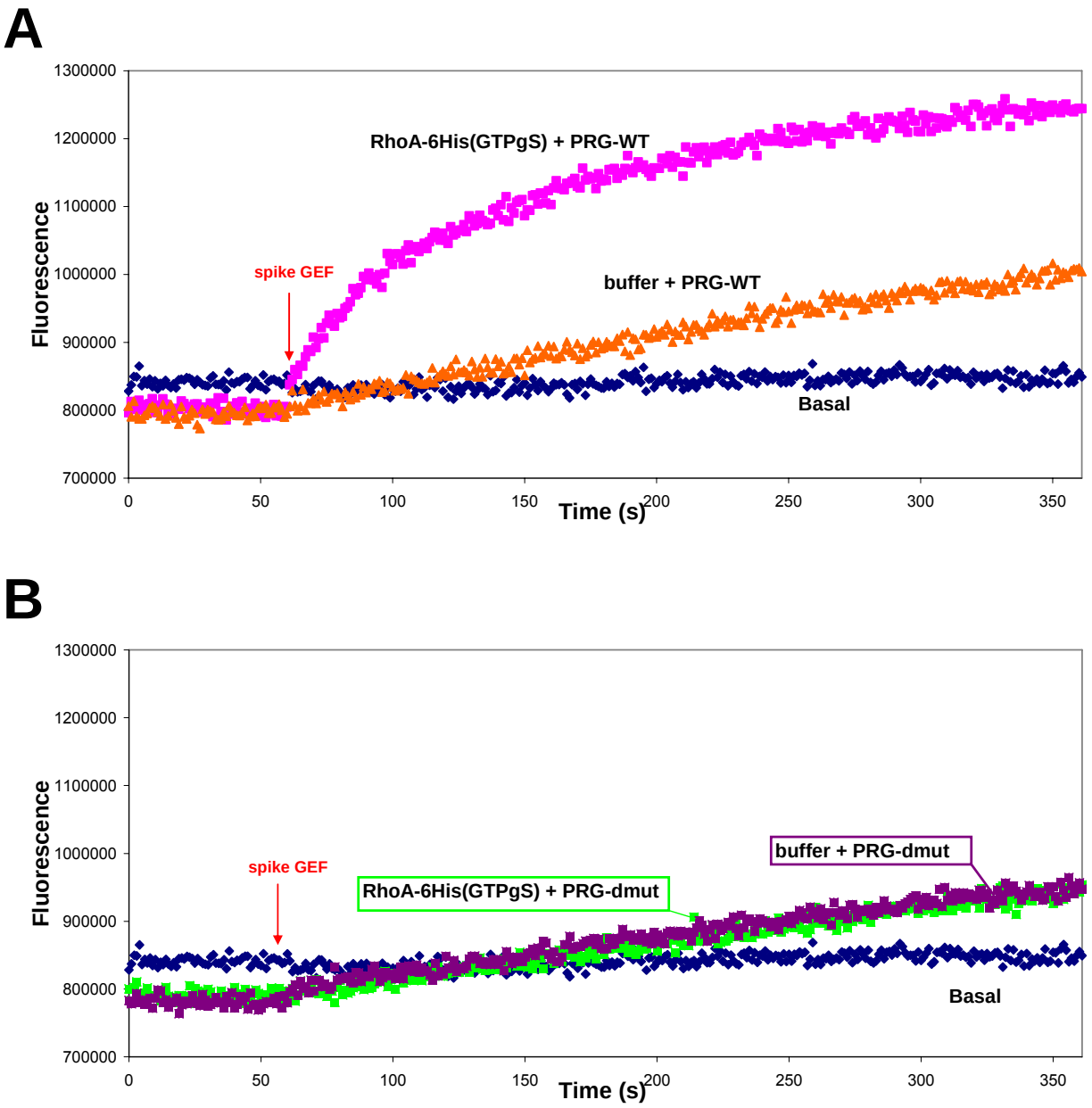


FIGURE 4.4 The feed-forward response requires high affinity binding between membrane-bound RhoA(GTPγS) and the PH domain of PRG. Association of mantGDP with RhoA was monitored by the increase in mantGDP fluorescence intensity as a result of its association with RhoA. For each reaction, 1μM RhoA-6His was incubated with 2.5 μM mantGDP, 5nM PEPC-Ni vesicles and 0.1 μM RhoA-6His(GTPγS) or buffer for 1 minute at 25°C. Fluorescence (λ_{ex} = 356 nm, λ_{em} = 445nm) was then measured for 1 minute. After this, the exchange reaction was started by addition of **(A)** 10nM PRG-DH·PH ("PRG-WT") or **(B)** 10nM PRG-DH·PH-Dual mutant ("PRG-dmut") (see Table 5.1). Fluorescence was then measured for 300 seconds.

RhoA(GTPγS) was omitted (Fig 4.4A). This same weak stimulation of exchange occurs when the PH-mutant of PRG is added to a new reaction, and occurs independently of membrane-bound RhoA(GTPγS) (Fig. 4.4B). This indicates that feed-forward response requires high affinity binding between membrane-bound RhoA(GTPγS) and the PH domain of PRG.

Finally, many of the residues in RhoA that contact the PRG-PH domain are conserved in other members of the Rho family (Figs. 3.5B and 5.1). This suggests that GTPases closely related to RhoA in amino acid identity or function may also be capable of facilitating the feed-forward response seen with activated RhoA. To investigate this possibility, I repeated the *in vitro* phospholipid vesicle assays using the closely-related GTPases Cdc42 and Rac1. As shown in figure 4.5, in the presence of membrane-bound RhoA(GTPγS), addition of PRG elicits a high increase in nucleotide exchange rate. However, no significant increase in exchange rate of the GEF is seen in the presence of membrane-bound Cdc42(GTPγS) or Rac1(GTPγS). This shows that the selectivity in binding observed in pulldown experiments (Fig.5.3A) is preserved in their ability to localize the GEF to substrate on the phospholipid vesicles. Thus, this interaction appears to be limited to a feed-forward response with PRG and does not subserve a mechanism for cross activation among the GTPase pathways.

Discussion

The goal of the work presented in this chapter was to identify a physiological role for binding of the PRG-PH domain to activated RhoA. It is possible that this binding reaction regulates some unknown function of PRG or perhaps has no function at all. However, because

activated RhoA binds to the PH domain, which participates in facilitating nucleotide exchange on RhoA, we believe that binding of activated RhoA to PRG plays a role in regulating exchange activity. I proposed two hypotheses how binding of activated RhoA might regulate exchange activity of PRG.

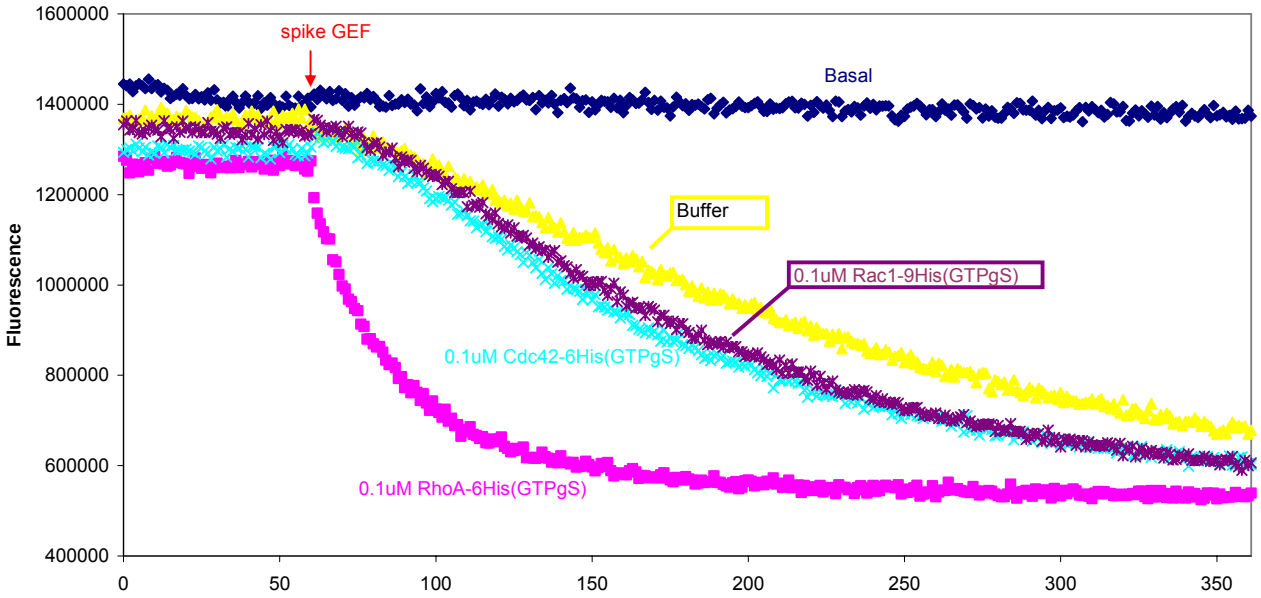


FIGURE 4.5 The PH domain of PRG demonstrates specificity in eliciting the feed-forward response. Dissociation of mant-GDP from pre-loaded RhoA was monitored by the decrease in fluorescence of the dissociated mant-GDP. For each reaction, 1 μM RhoA-6His(mant-GDP) was incubated with 1 mM GDP, 5 nM PEPC-Ni vesicles and 0.1μM RhoA-6His(GTPγS) or 0.1 μM Cdc42-6His(GTPγS) or 0.1 μM Rac1-9His(GTPγS) or buffer for 1 minute at 25°C. Fluorescence (λ_{ex} = 356 nm, λ_{em} = 445nm) was then measured for 1 minute. After this, the exchange reaction was started by addition of 10nM PRG-DH·PH or buffer, fluorescence was then measured for 300 seconds.

Both hypotheses are feedback regulation. One is direct, the other is indirect. The first hypothesis was that binding of activated RhoA to the PH domain may serve as some form of feedback signal resulting in a change in the intrinsic catalytic efficiency of PRG. We tested this hypothesis by monitoring the exchange activity of PRG in solution in the absence and presence of GTPγS-activated RhoA. In these experiments, both full-length PRG and its isolated DH·PH domains

demonstrated the same activity in the absence and presence of GTPγS-activated RhoA. This strongly suggests that binding of activated RhoA does not serve as a direct feedback signal on intrinsic catalysis. Our second hypothesis was that binding of activated RhoA to the PH domain serves as a mechanism for localizing PRG to the plasma membrane. Indeed, previous work on p115-RhoGEF and LARG has provided evidence that constitutive targeting of RGS-RhoGEFs to membranes represents a mechanism by which RGS-RhoGEFs can stimulate activation of RhoA (Aittaleb et al., 2009; Bhattacharyya et al., 2009). I used a reconstituted signaling system consisting of phospholipid vesicles and purified recombinant proteins to test whether activated RhoA could act as an acute feedback mechanism to enhance the ability of PRG to stimulate activation of RhoA. Testing this hypothesis *in vitro* allowed for easier control of protein concentrations and temporal measurements, as well as clear interpretation. Our initial experiment was to monitor the ability of soluble PRG to stimulate nucleotide exchange on RhoA bound to phospholipid vesicles in the presence of increasing amounts of membrane-bound RhoA(GTPγS). In this experiment, we observed a dose-dependent increase in exchange activity of PRG with increasing amounts of activated RhoA (Fig. 4.2B). We verified that this response in exchange activity was dependent on membrane localization of RhoA(GTPγS) (Fig. 4.3). Additionally, mutation of the conserved hydrophobic patch on the PRG-PH domain abolished this RhoA(GTPγS)-mediated response. We propose that PRG is targeted to the membrane via interaction of the PH domain with membrane-bound RhoA(GTPγS). This localization to the membrane increases the local concentration of GEF and substrate, RhoA(GDP), and supports processive activation of substrate while the GEF is held at the vesicle surface which results in an increase in exchange activity. Finally, we were able to show that the PH domain does demonstrate specificity for binding activated GTPase (Fig. 4.5). These results strongly suggest

that we have identified a novel mechanism for regulating PRG exchange activity. In this mechanism PRG exchange activity is facilitated by localization to the membrane via interaction with activated RhoA.

CHAPTER 5

Binding of Activated RhoA is a Shared Feature Among Members of the Lbc-RhoGEF Family

Figure Fig.5.3A was published in the Journal of Biological Chemistry (Chen et al., 2010).

Introduction

In humans, around one percent of the genome encodes proteins that either regulate or are regulated by Rho family GTPases (Jaffe and Hall, 2005). In this matrix of interacting proteins there are examples of both promiscuity and specificity. Thus far, I have presented evidence which shows that activated RhoA binds to the PH domain in PRG and this interaction positively regulates exchange activity of PRG in a reconstituted signaling system by maintaining positioning of PRG at the membrane. This regulatory mechanism may represent a more common phenomenon for regulating the exchange activity of RhoGEFs.

Binding of the PRG-PH domain to GTPases other than RhoA could potentially facilitate crosstalk between distinct GTPase signaling pathways or serve as a mechanism for localizing activation of RhoA to discreet sites within the cell. Interestingly, many of the residues in RhoA that contact the PRG-PH domain are conserved in other members of the Rho family (Figs. 3.5B and 5.1) (Chen et al., 2010). This suggests that the PRG-PH domain may be able to associate with additional Rho family members. I have investigated this hypothesis by pulldowns and present the results in this chapter. In addition to activated RhoA, PRG is able to interact with activated RhoB and RhoC isoforms, albeit to varying degrees.

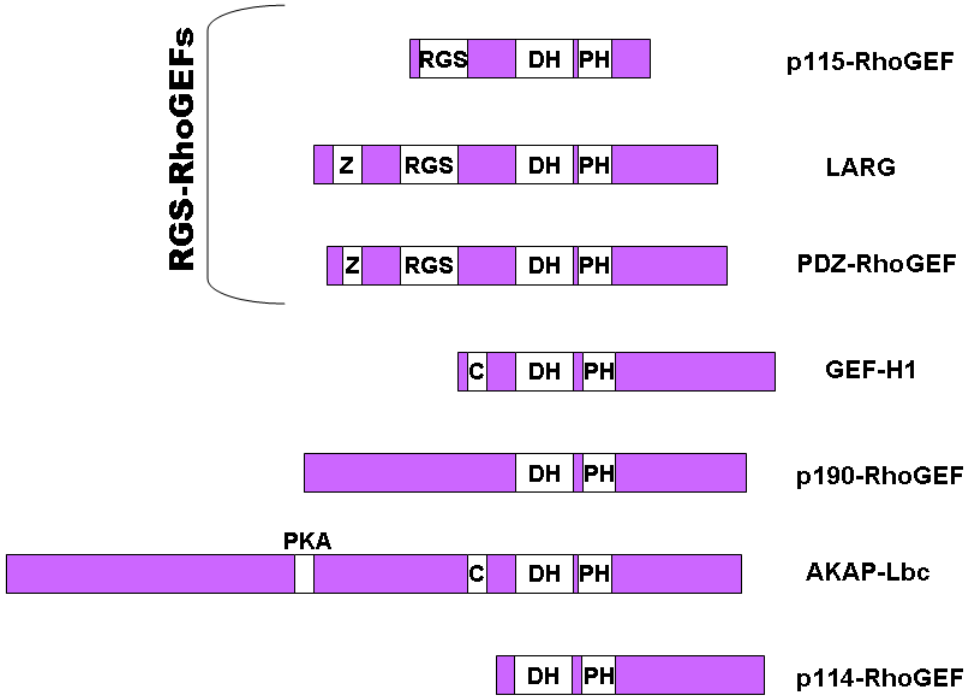
```
RhoA  1    -----------------MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENY
RhoC  1    -----------------MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENY
RhoB  1    -----------------MAAIRKKLVVVGDGACGKTCLLIVFSKDEFPEVYVPTVFENY
Cdc42 1    -------------------MQTIKCVVVGDGAVGKTCLLISYTTNKFPSEYVPTVFDNY
Rac1  1    -------------------MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNY
Rnd2  1    ---------------MEGQSGRCKIVVVGDAECGKTALLQVFAKDAYPGSYVPTVFENY
Rnd3  1    MKERRASQKLSSKSIMDPNQNVKCKIVVVGDSQCGKTALLHVFAKDCFPENYVPTVFENY
RhoG  1    -------------------MQSIKCVVVGDGAVGKTCLLICYTTNAFPKEYIPTVFDNY
                 43
                 ↓
RhoA       VADIEVDGKQVELALWDTAGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPE
RhoC       IADIEVDGKQVELALWDTAGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPE
RhoB       VADIEVDGKQVELALWDTAGQEDYDRLRPLSYPDTDVILMCFSVDSPDSLENIPEKWVPE
Cdc42      AVTVMIGGEPYTLGLFDTAGQEDYDRLRPLSYPQTDVFLVCFSVVSPSSFENVKEKWVPE
Rac1       SANVMVDGKPVNLGLWDTAGQEDYDRLRPLSYPQTDVFLICFSLVSPASFENVRAKWYPE
Rnd2       TASFEIDKRRIELNMWDTSGSSYYDNVRPLAYPDSDAVLICFDISRPETLDSVLKKWQGE
Rnd3       TASFEIDTQRIELSLWDTSGSPYYDNVRPLSYPDSDAVLICFDISRPETLDSVLKKWKGE
RhoG       SAQSAVDGRTVNLNLWDTAGQEEYDRLRTLSYPQTNVFVICFSIASPPSYENVRHKWHPE


RhoA       VKHFCPNVPIILVGNKKDLRNDEHTRRELAKMKQEPVKPEEGRDMANRIGAFGYMECSAK
RhoC       VKHFCPNVPIILVGNKKDLRQDEHTRRELAKMKQEPVRSEEGRDMANRISAFGYLECSAK
RhoB       VKHFCPNVPIILVANKKDLRSDEHVRTELARMKQEPVRTDDGRAMAVRIQAYDYLECSAK
Cdc42      ITHHCPKTPFLLVGTQIDLRDDPSTIEKLAKNKQKPITPETAEKLARDLKAVKYVECSAL
Rac1       VRHHCPNTPIILVGTKLDLRDDKDTIEKLKEKKLTPITYPQGLAMAKEIGAVKYLECSAL
Rnd2       TQEFCPNAKVVLVGCKLDMRTDLATLRELSKQRLIPVTHEQGTVLAKQVGAVSYVECSSR
Rnd3       IQEFCPNTKMLLVGCKSDLRTDVSTLVELSNHRQTPVSYDQGANMAKQIGAATYIECSAL
RhoG       VCHHCPDVPILLVGTKKDLRAQPDTLRRLKEQGQAPITPQQGQALAKQIHAVRYLECSAL


RhoA       TK-DGVREVFEMATRAALQARRGKKK-S------------------------------G
RhoC       TK-EGVREVFEMATRAGLQVRKNKRR-R------------------------------G
RhoB       TK-EGVREVFETATRAALQKRYGSQN--------------------------------G
Cdc42      TQ-RGLKNVFDEAILAALEPPETQPKRK-----------------------------C
Rac1       TQ-RGLKTVFDEAIRAVLCPPPVKKRKR-----------------------------K
Rnd2       SSERSVRDVFHVATVASLGRGHRQLRRTDSRRGMQRSAQLSGRPDRGN-EGEIHKDRAKS
Rnd3       QSENSVRDIFHVATLACVNKTNKNVKRNKSQRATKRISHMPSRPELSAVATDLRKDKAKS
RhoG       QQ-DGVKEVLAEAVRAVLNPTPIKR-GR-----------------------------S


RhoA       CLVL----
RhoC       CPIL----
RhoB       CINCCKVL
Cdc42      CIF-----
Rac1       CLLL----
Rnd2       CNLM----
Rnd3       CTVM----
RhoG       CILL----
```

FIGURE 5.1 Sequence Alignment of Select Rho Family GTPases. The entire protein sequence of the selected Rho family members was aligned using Clustal. Residues in RhoA involved in contacting the PRG-PH domain are colored *red*. Position 43 in RhoA is indicated by ↓**.** All sequences are human. GenBank identifiers are as follows: RhoA (20379114), RhoC (20379118), RhoB (20379116), Cdc42 (20379098), Rac1 (8574038), Rnd2 (2507301), Rnd3 (47606459), and RhoG (20379122).

The RGS-RhoGEFs belong to a larger subfamily of Lbc RhoGEFs which share closely related DH·PH domains (Fig. 5.2A) (Aittaleb et al., 2009). Interestingly, the hydrophobic patch on the PH domain of PRG that engages activated RhoA is highly conserved among all members in this subfamily (Aittaleb et al., 2009; Chen et al., 2010). In particular, a sequence alignment of the PH domains of Lbc RhoGEFs shows that many of the residues in PRG that contact activated RhoA are conserved within this subfamily (Fig. 5.2B). Previous work done by others on full-length p114-RhoGEF (Blomquist et al., 2000), p190-RhoGEF (van Horck et al., 2001) and GEF-H1 (Ren et al., 1998) has provided preliminary evidence that these RhoGEFs are able to bind activated RhoA. In addition to activated RhoA, GEF-H1 has also demonstrated the ability to bind GTPγS-activated Rac1 (Ren et al., 1998). To our knowledge, no further work has been done to characterize the interaction between these RhoGEFs and activated RhoA or to elucidate its physiological relevance.

I propose that binding of activated RhoA to PH domains may be a shared feature of the Lbc RhoGEFs. Although binding to activated RhoA had no direct effect on intrinsic exchange activity of PRG, it is still possible that binding of activated RhoA to other RhoGEFs may directly influence their exchange activity in a feedback type manner. I have investigated these possibilities by generating DH·PH constructs of the Lbc RhoGEFs, testing their ability to bind activated RhoA via pulldown, and measuring their exchange activity in the absence and presence of activated RhoA. The results of these experiments confirm that the majority of Lbc RhoGEFs are able to bind activated RhoA via pulldown. Furthermore, activated RhoA has no effect on the exchange activity of these constructs for RhoA(GDP) in solution.

A



B

```
PDZ-RhoGEF 964    ------------RKMIHEGPLTWRISKDKTLDLHVLLLEDLLVLLQKQDEKLLLKCHSK
LARG      1019    -------------KMIHEGPLVWKVNRDKTIDLYTLLLEDILVLLQKQDDRLVLRCHSK
p115       647    -------------KLVHEGPLTWRVTKDKAVEVHVLLLDDLLLLLQRQDERLLLKSHSR
AKAP-Lbc  2217    KSGQMFAKEDLKRKKLVRDGSVFLKNAAGRLKEVQAVLLTDILVFLQEKDQKYIFASL--
p114       374    ------------------------------------------------DQKYVFASV--
p190-RhoGEF1086   -------------TLLYDGLVYWKTATGRFKDILALLLTDVLLFLQEKDQKYIFAAV--
GEFH1      473    --------------LIHDGCLLWKTATGRFKDVLVLLMTDVLVFLQEKDQKYIFPTL--

PDZ-RhoGEF        TAVGSSDSKQTFSPVLKLNAVLIRSVATDKRAFFIICTSKLGPPQIYELVALTSSDKNTW
LARG              ILASTADSKHTFSPVIKLSTVLVRQVATDNKALFVISMSDNG-AQIYELVAQTVSEKTVW
p115              TLTPTPDGKTMLRPVLRLTSAMTREVATDHKAFYVLFTWDQE-AQIYELVAQTVSERKNW
AKAP-Lbc          ------DQ---KSTVISLKKLIVREVAHEEKGLFLISMGMTD-PEMVEVHASSKEERNSW
p114              ------DS---KPPVISLQKLIVREVANEEKAMFLISASMQG-PEMYEMYTSSKEDRNIW
p190-RhoGEF       ------DQ---KPSVISLQKLIAREVANEERGMFLISASSAG-PEMYEIHTNSKEERNNW
GEFH1             ------D----KPSVVSLQNLIVRDIANQEKGMFLISA--AP-PEMYEVHTASRDDRSTW

PDZ-RhoGEF        MELLEEAVR
LARG              QDLICRMAA
p115              CALITETAG
AKAP-Lbc          IQIIQDT--
p114              MAHIRRA--
p190-RhoGEF       MRRIQQAVE
GEFH1             IRVIQQSVR
```

FIGURE 5.2 The Lbc RhoGEF Family. (A) A schematic diagram of the Lbc RhoGEF family and their known structural elements. Alternate names include: p115-RhoGEF, Lsc (mouse)/Arhgef1; LARG, KIAA0382/Arhgef12; PDZ-RhoGEF, GTRAP48/KIAA0380/Arhgef11; p190-RhoGEF, Rip2/RGNEF; AKAP-Lbc, AKAP13; p114-RhoGEF, KIAA0521/Arhgef18. Domains: RGS, regulator of G-protein signaling; DH, dbl homology; PH, pleckstrin homology; Z, PDZ (PSD-95/SAP90-Discs-larg-ZO-1); C, C1 homology domain; PKA, binding site for protein kinase A. **(B)** A sequence alignment of the PH domains of Lbc RhoGEF family members was done with Clustal. Residues in PDZ-RhoGEF that contact activated RhoA are colored *red*. All sequences are human except p114 (mouse). GenBank identifiers are as follows: PDZ-RhoGEF (55662301), LARG (7662088), p115 (34395524), AKAP-13 (21493029), p114 (38614249), p190-RhoGEF (219520818), and GEFH1 (253735775).

Results

The PRG-PH domain binds RhoB and RhoC isoforms

Several Rho family members with high similarity to RhoA (RhoB, RhoC, Cdc42, Rac1, Rnd2, Rnd3 and RhoG) were expressed in bacteria as GST-fusion proteins and purified by affinity and conventional chromatography. With the exception of Rnd2 and Rnd3, the GTPases were pre-loaded with GTPγS. Rnd2 (Rho7) and Rnd3 (RhoE) are atypical Rho family members that lack amino acids required for GTPase activity and as such are constitutively bound to GTP (Riou et al., 2010). Binding to PRG was then assessed using a modified version of the pulldown assay (Fig. 3.1A). The results from the pulldown assays reveal that the PRG-DH domain binds nucleotide-free RhoA, RhoB and RhoC with similar affinity (Fig. 5.3A and B). This is not surprising as all three isoforms share significant amino acid identity (~85%) and are believed to interact with the same GEFs and effector proteins (Wennerberg and Der, 2004). In the same group of

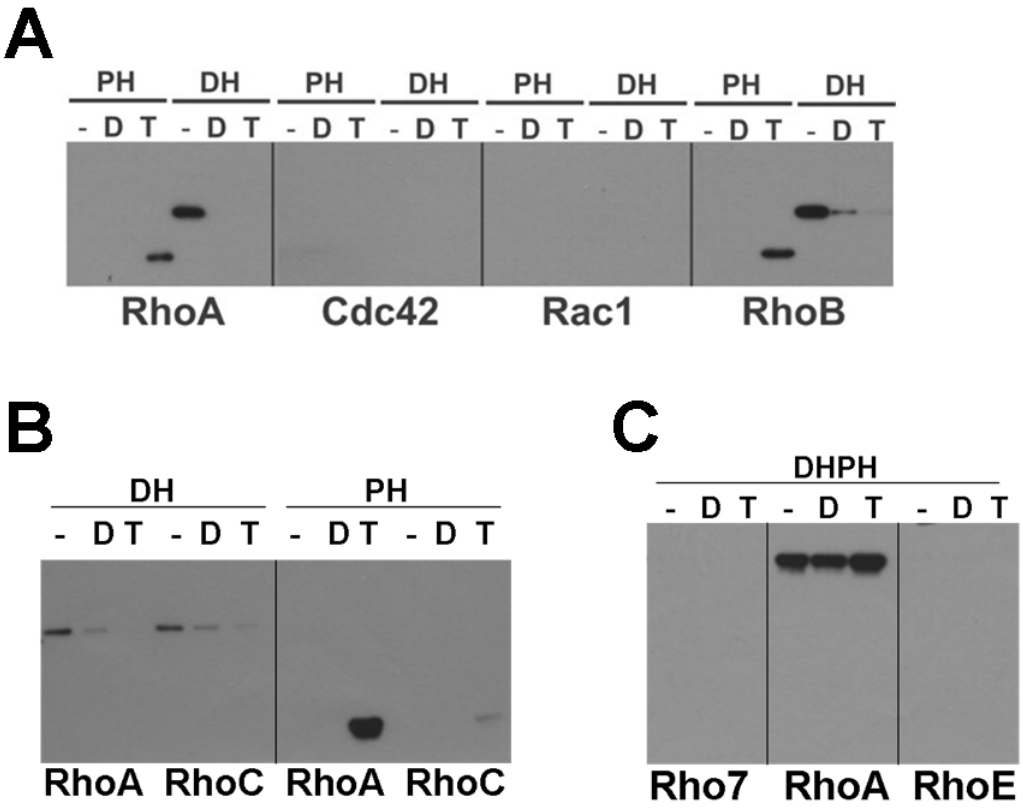


FIGURE 5.3 The PH Domain of PRG Binds RhoB and RhoC Isoforms. (A and B) Purified His-tagged PRG-PH and PRG-DH domains were incubated with immobilized GST-RhoA, GST-Cdc42, GST-Rac1, GST-RhoB, or GST-RhoC with no additional nucleotide *(-)* or 10μM GDP (*D*) or with immobilized GST-GTPases pre-loaded with GTPγS (*T*). Bound PH or DH domains were then analyzed by immunoblotting as described in Fig. 3.1A (Chen et al., 2010). **(C)** Purified His-tagged PRG-DH·PH was incubated with immobilized GTP-bound GST-Rho7 and GST-RhoE with no additional nucleotide *(-)* or 10μM GDP *(D)* or 10μM GTPγS *(T)*. PRG-DH·PH bound to the resin was analyzed by immunoblotting as described in Fig. 3.1A. Note: GST-RhoA was included in this assay as a control and treated as in (A).

experiments, the PRG-PH domain bound to RhoA, RhoB and RhoC in the GTPγS-activated state; however, the affinity for RhoC appeared to be much lower (Fig. 5.3A and B). No binding was observed with Cdc42, Rac1 (Fig.5.3A), Rho7, RhoE (Fig. 5.3C) or RhoG (data not shown) in any of the three conditions.

Additional Lbc RhoGEF family members bind activated RhoA

My next goal was to identify if the remaining Lbc RhoGEF family members are also able to bind activated RhoA through their PH domains. To address this possibility I cloned the DH·PH tandem domain constructs of each of the Lbc RhoGEF family members based on existing structural information in the literature and predicted secondary structural elements (Table 5.1).

RhoGEF	DH·PH	Mutations
p115-RhoGEF	P395-A766	(Y726A, L728E)
PRG	D712-P1085	(F1044A, I1046E)
LARG	D761-K1135	(F1098A, I1100E)
AKAP-Lbc	M1919-N2333	(F2299A, I2301E)
p114-RhoGEF	E72-P444	(F407A, I409E)
p190-RhoGEF	D822-K1194	(F1154A, I1156A)
GEF-H1	E206-P574	(F539A, I541E)

TABLE 5.1 DH·PH Domain Constructs of the Lbc RhoGEF Subfamily. The DH•PH constructs used in this work are indicated by their N- and C-terminal amino acids. Dual point-mutations created within the conserved hydrophobic patch are also indicated. Residue numbering is based on sequences in GenBank (see legend in Fig. 5.2 for GenBank identifiers).

The DH·PH domains were expressed in bacteria, and purified by affinity chromatography (see Chapter 2). Purified DH·PH domains were then examined for their ability to bind immobilized GST-RhoA by pulldown assay. The results from these assays show that the DH·PH domains of GEF-H1, p190-RhoGEF and LARG are able to bind both nucleotide-free and GTPγS-loaded states of RhoA (Fig. 5.4). Unfortunately, due to complications with non-specific binding and sensitivity of the pulldown assay, I have been unable to observe reliable binding results for AKAP-Lbc or p114-RhoGEF in any of the nucleotide-bound states of RhoA. The binding of the LARG-DH•PH domain construct to nucleotide-free RhoA and GTPγS-activated RhoA is reproducible and is further seen with the full-length version of the protein (data not shown). Because the smallest construct used in these experiments to monitor binding is a construct of the tandem DH•PH domains it is still possible that binding to activated RhoA occurs at a site distal to the conserved hydrophobic patch on the PH domain. Mutation of select amino acid residues in this hydrophobic region of PRG attenuated binding of PRG to activated RhoA (Fig. 3.5C). In an effort to confirm the binding site responsible for binding activated RhoA, homologous mutations were made in two highly conserved hydrophobic residues in the hydrophobic patch of the LARG-PH domain (Table 5.1). PRG was similarly mutated as a positive control. Mutant protein was then expressed, purified and tested for binding to activated RhoA as shown for the wild-type protein (Fig. 5.4B). The results from this experiment show that while binding of the "Dual-mutant" (Dmut) DH•PH domains to nucleotide-free RhoA is unaffected by the selected point mutations, binding to GTPγS-loaded RhoA is significantly impaired. This confirms that binding of LARG to activated RhoA occurs through binding of activated RhoA to the conserved hydrophobic patch on the PH domain of LARG. Similar experiments with the DH·PH domains of GEF-H1 and p190-RhoGEF were not completed to non-specific binding issues. However,

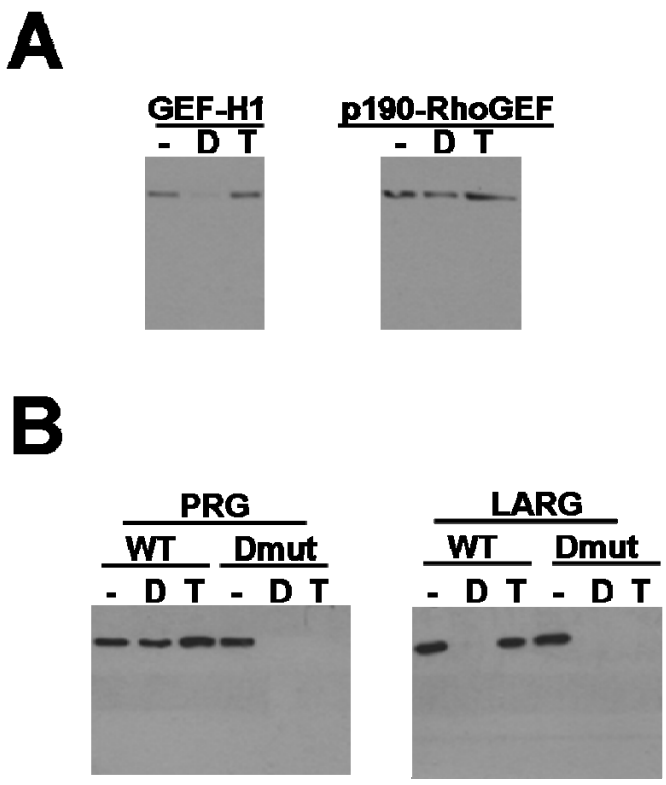


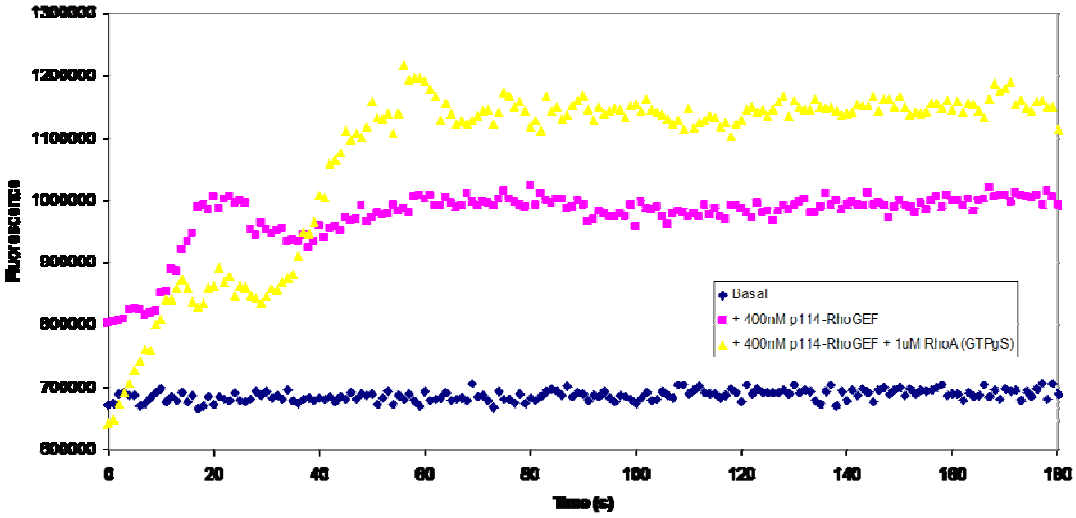
FIGURE 5.4 Additional Lbc RhoGEF Family Members Bind Activated RhoA. (A) Purified His-tagged DH·PH domain constructs of GEF-H1 and p190-RhoGEF were incubated with immobilized GST-RhoA with either no additional nucleotide *(-)* or 10μM GDP *(D)* or with immobilized GST-RhoA pre-loaded with GTPγS *(T)*. DH·PH domains bound to the resin were analyzed by immunoblotting as described in Fig. 3.1A. **(B)** Purified His-tagged DH·PH domain constructs of PRG and LARG, both wild-type *(WT)* and dual-mutant *(Dmut)*, were incubated with immobilized GST-RhoA with either no additional nucleotide *(-)* or 10μM GDP *(D)* or with immobilized GST-RhoA pre-loaded with GTPγS *(T)*. DH·PH domains bound to the resin were analyzed as in (A).

direct binding of activated RhoA to the isolated PH domains of these proteins was confirmed by my colleagues (Olugbenga Dada and Jana Hadas, personal communication).

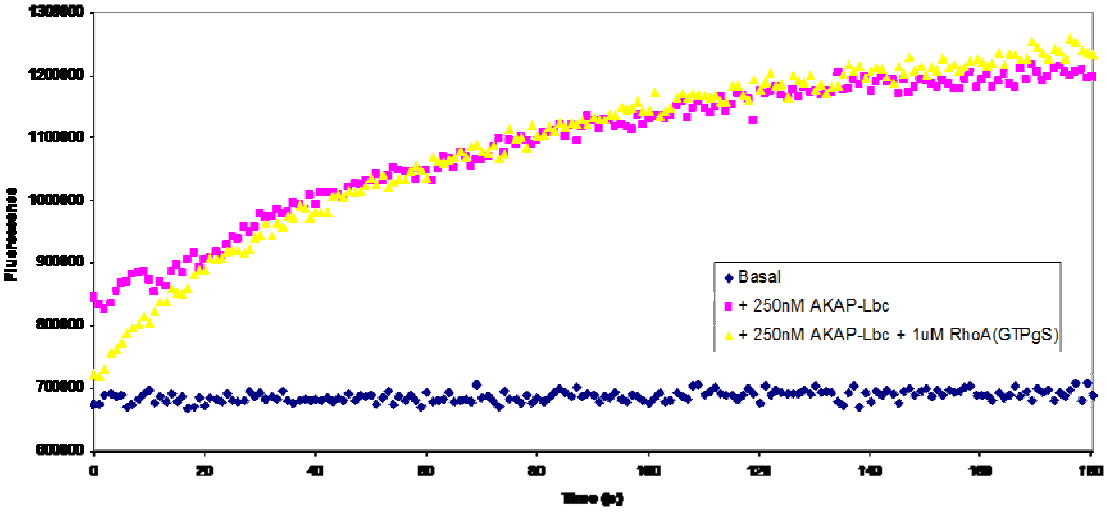
Activated RhoA does not affect intrinsic exchange activity of Lbc RhoGEF family members

Although binding to activated RhoA had no direct effect on intrinsic exchange activity of PRG, it is still possible that binding of Lbc RhoGEF family members to activated substrate may directly influence their exchange activity in a feedback type manner, as was observed for Son of Sevenless (SOS) and activated Ras (Margarit et al., 2003). To address this possibility, the nucleotide exchange activity of Lbc RhoGEF family members on Rho(GDP) was monitored in the absence and presence of GTPγS-activated RhoA in solution (Fig. 5.5). As evinced in these assays, RhoA(GTPγS) had either no or modest (<2 fold) effects on the exchange activity of the Lbc RhoGEF family members. This suggests that binding of activated RhoA does not significantly influence the intrinsic exchange activity of Lbc RhoGEF family members, although these results require further verification. Although not shown here, the same results were seen in assays performed by my colleague Olugbenga Dada on p190-RhoGEF (personal communication).

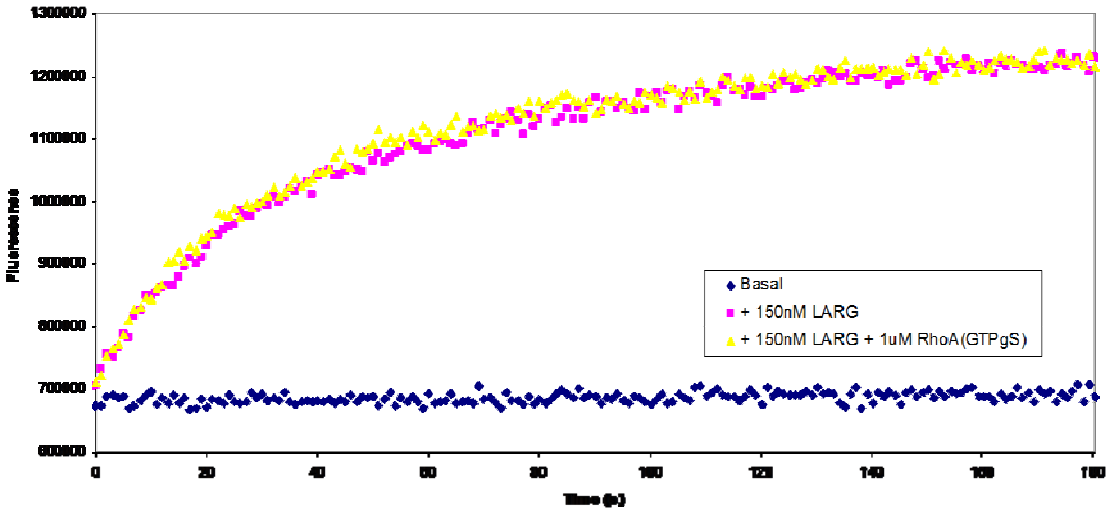
A


Basal
+ 400nM p114-RhoGEF
+ 400nM p114-RhoGEF + 1uM RhoA(GTPgS)

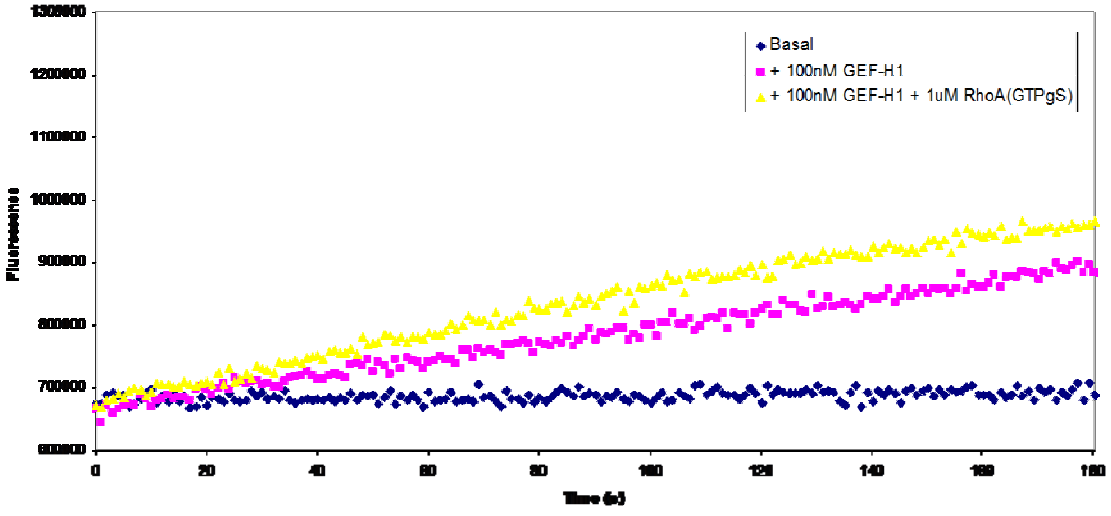
B


Basal
+ 250nM AKAP-Lbc
+ 250nM AKAP-Lbc + 1uM RhoA(GTPgS)

C


Basal
+ 150nM LARG
+ 150nM LARG + 1uM RhoA(GTPgS)

D


Basal
+ 100nM GEF-H1
+ 100nM GEF-H1 + 1uM RhoA(GTPgS)

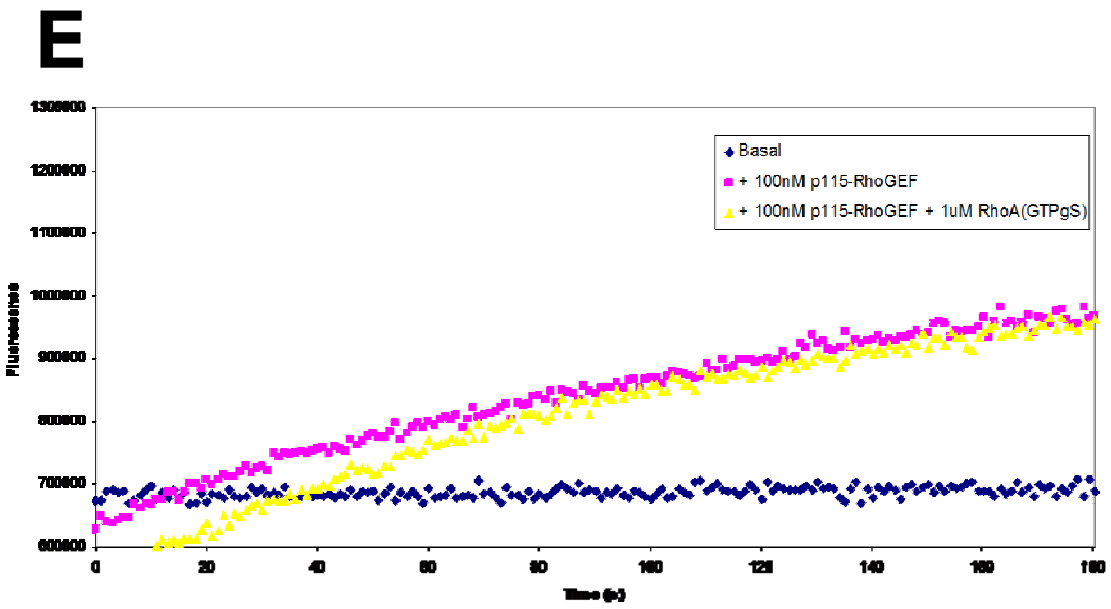


FIGURE 5.5 MantGDP Association Assays with Lbc RhoGEF Family Members and RhoA. In these assays, rates of nucleotide exchange are monitored by the increase in mantGDP fluorescence as a result of its association with RhoA. **(A-E)** Basal, 1μM RhoA was mixed with 2.5μM mantGDP and 1μM RhoA(GTPγS) for 1 minute at 25°C, and the exchange reaction was started by the addition of buffer *(Basal, blue diamonds)*. The subsequent increase in fluorescence (λ_{ex}= 356 nm, λ_{em}= 445 nm) was measured for 3 minutes. Plus Lbc RhoGEF, 1μM RhoA was mixed with 2.5μM mantGDP and buffer *(pink squares)* or 1μM RhoA(GTPγS) *(yellow triangles)* for 1 minute at 25°C, and the exchange reaction was started by the addition of purified DH·PH construct. Fluorescence was then measured for 3 minutes.

Discussion

Many of the residues in activated RhoA that contact the PH domain of PRG are conserved in the Rho family of GTPases (Figs. 5.1). This increases the likelihood that related GTPases will be able to associate with the PRG-PH domain. In Chapter 5, I have investigated this possibility by examining the ability of PRG to bind Rho family GTPases via pulldown. The results from these assays reveal that the PRG-DH domain binds to nucleotide-free RhoA, RhoB and RhoC with similar affinity. In contrast, the PRG-PH domain binds strongly to GTPγS-activated RhoA and RhoB isoforms and weakly to GTPγS-activated RhoC (Fig. 5.3). Binding of

RhoA, RhoB and RhoC isoforms to the same proteins or domains is expected considering that these isoforms are 85% identical at the amino acid level (Sloan et al., 2012). However, the rationale for the difference in affinity of the PH domain for activated RhoA and RhoB versus RhoC is unknown. One potential reason that could account for this discrepancy is a difference in a single amino acid residue. More specifically, each of the residues in RhoA that make contact with the PRG-PH domain in the PRG-DH·PH-RhoA·GTPγS complex are conserved in RhoB and only differ from RhoC in a single residue at position 43 (Fig. 5.1). Interestingly, in work done by others on the Dbl family exchange factor XPLN, investigators were able to demonstrate that XPLN selectively associates with and stimulates nucleotide exchange on RhoA and RhoB but not RhoC (Arthur et al., 2002). In this work they go on to show that the inability of XPLN to facilitate nucleotide exchange on RhoC is mediated by an isoleucine at position 43 in RhoC, a position occupied by a valine in RhoA and RhoB (Arthur et al., 2002).

I used site-directed mutagenesis to replace the isoleucine at position 43 in RhoC with a valine to test if this single amino acid change could affect the interaction of the PRG-PH domain with RhoC. My colleague Olugbenga Dada then compared binding of the PRG-PH domain to wild-type RhoC and the point-mutant (RhoC-I43V) by pulldown. Surprisingly, the PRG-PH domain appeared to bind to RhoC-I43V with much higher affinity compared to wild-type RhoC (personal communication). These results suggest that the PRG-PH domain is highly selective with regard to its binding partners and is capable of discriminating between closely-related isoforms of proteins.

In a related note, the hydrophobic patch on the PH domain of PRG that interacts with activated RhoA is highly conserved in the Lbc RhoGEF family, and many of the residues in PRG that make contact with activated RhoA in the PRG-DH·PH-RhoA(GTPγS) complex are also conserved among Lbc RhoGEF family members. Therefore, I investigated whether binding to activated RhoA may be a shared feature of the Lbc RhoGEF family. LARG was clearly capable of selective interaction with activated RhoA and attenuation by specific mutations strongly indicate that this binding occurred via the conserved hydrophobic patch on the PH domain of LARG (Fig. 5.4B). Due to non-specific binding, I was unable to reproduce the binding results observed with GEF-H1 and p190-RhoGEF, however, my colleague Olugbenga was recently able to generate isolated PH domain constructs of the Lbc RhoGEF family members, and using these constructs he was able to show by pulldown that LARG, AKAP-Lbc, p114-RhoGEFand p190-RhoGEF, all selectively bind to GTPγS-loaded RhoA (manuscript in preparation). Furthermore, by making specific point mutations ("dual-mutants") in the constructs he was able to confirm that binding occurred with the conserved hydrophobic patch on these constructs. In addition, my colleague, Jana Hadas, was able to incorporate the PH domain constructs cloned by Olugbenga into a dot blot assay which revealed that, excluding p115-RhoGEF, each of the Lbc RhoGEF family members are capable of interacting with GTPγS-activated RhoA through a conserved hydrophobic patch on their PH domains (manuscript in preparation). These results strongly suggest that binding to activated RhoA is a shared feature among the majority of Lbc RhoGEF family members.

Using activity assays I have confirmed that binding to activated RhoA does not significantly affect the intrinsic exchange activity of DH·PH tandem domain constructs of Lbc RhoGEF family members (Fig. 5.5). A point must be made, however, that these assays were

performed with purified DH·PH domain constructs and must be repeated with purified full-length versions of these proteins.

It is possible that, as seen with PRG in chapter 4, binding of Lbc RhoGEF family members to activated RhoA serves as a mechanism for localizing Lbc RhoGEF family members to the membrane. Testing activity of these proteins in a reconstituted vesicle system can be used to test this hypothesis. If these proteins are regulated by this type of feed-forward mechanism, then a new mechanism has been identified for regulation of an entire subfamily of RhoGEFs, the Lbc RhoGEFs.

CHAPTER 6

Conclusions and Recommendations

PRG is a RhoA-selective guanine nucleotide exchange factor that has been shown to bind to GTPγS-activated RhoA (Chen et al., 2010; Rumenapp et al., 1999). I have investigated the physiological significance of this interaction and present the results of my investigation in this dissertation. The main goals of this investigation were to identify how the binding interaction was taking place, identify the function of the interaction, and to examine the prevalence of this interaction among related-proteins. In chapter 3, I investigated how the binding reaction was taking place by deletion analysis and site-directed mutagenesis of PRG. Through these experiments I was able show that the PH domain of PRG is sufficient for binding activated RhoA. This was confirmed and examined at the molecular level by my colleague James Chen. In his crystal structure (Fig. 3.5), James was able to identify that binding of PRG to activated RhoA occurs between a conserved hydrophobic patch on the PH domain of PRG and the switch regions on RhoA.

In chapter 4, I attempted to identify the physiological function of this interaction. I proposed two mechanisms as hypotheses to test. The first hypothesis was that binding of activated RhoA to the PRG-PH domain directly regulates the intrinsic catalytic efficiency of PRG. I tested this possibility by examining the exchange activity of PRG on soluble RhoA in the absence and presence of GTPγS-loaded RhoA (Fig 4.1). In these experiments, GTPγS-loaded RhoA did not affect the exchange activity of PRG for RhoA(GDP) in solution, suggesting that direct regulation of catalytic activity is not a mechanism. My second hypothesis was that binding of activated RhoA to the PRG-PH domain serves as a mechanism for localizing PRG to the plasma membrane. Localization to the membrane could serve as an indirect mechanism for stimulating the enzyme by increasing local concentrations of GEF with its substrate, RhoA(GDP), resulting in an increase in turnover of RhoA. I tested this by reconstituting the

signaling system using phospholipid vesicles and recombinant proteins. In my experiments, PRG demonstrated a dose-dependent increase in exchange activity with increasing amounts of membrane-bound activated RhoA. This increase in exchange activity was dependent on membrane localization of activated RhoA and conservation of the hydrophobic patch on the PH domain of PRG. These results suggest that localization of PRG to the membrane via interaction with membrane-bound activated substrate is sufficient for stimulation of RhoA turnover through enhanced utilization of intrinsic catalytic activity.

Finally, in chapter 5, I examined if binding between regulator and activated substrate is a unique feature between PRG and activated RhoA, or if closely-related proteins also demonstrated this binding behavior. I first examined this by monitoring the ability of PRG to bind RhoA-related proteins by pulldown. The results from these experiments show that the PRG-PH domain binds to GTPγS-activated RhoA and RhoB with similar affinity, and binds significantly weaker to GTPγS-activated RhoC. Thus, the PRG-PH domain is able to selectively bind closely related isoforms of RhoA and does so with varying affinity.

I also tested other exchange factors that are closely-related to PRG for their ability to interact with activated RhoA. I chose to begin this search by examining the remaining members of the Lbc subfamily of RhoGEFs, of which PRG is a member. Members of this subfamily have the most closely-related DH·PH domains to PRG based on primary sequence and previous work has recognized that members of this family all share a common hydrophobic patch on the solvent exposed surface of their PH domains (Aittaleb et al., 2009). Therefore, I examined the ability of these proteins to bind GTPγS-activated RhoA via pulldown. The results from these assays indicate that, in addition to PRG, LARG also selectively bound to activated RhoA, and does so through a conserved hydrophobic patch on its PH domain. In preliminary experiments, I also

observed evidence that GEF-H1 and p190-RhoGEF exhibit this binding behavior. My colleague, Olugbenga Dada, recently generated isolated PH domains of the Lbc RhoGEF family members (both wild-type and "Dual-mutant" constructs), and could use these constructs to reliably show by pulldown that LARG, AKAP-Lbc, p114-RhoGEF and p190-RhoGEF, all selectively bound to GTPγS-loaded RhoA; mutations in the conserved hydrophobic patch on the PH domains verified this as the site of interaction (manuscript in preparation). These results collectively indicate that binding to activated RhoA is a shared feature among the majority of Lbc subfamily RhoGEFs. In addition to this, I have also verified that binding of activated RhoA does not significantly affect the intrinsic catalytic activity of these RhoGEFs (Fig. 5.5). Therefore, there is a strong possibility that, like PRG, binding of these additional Lbc RhoGEFs to activated RhoA serves as a mechanism for spatially regulating exchange activity. If this is the case, then we have discovered a new mechanism for regulating exchange activity of a small subfamily of RhoA-specific exchange factors, a feed-forward mechanism. This mechanism appears to be unique in that in that it can occur on its own, as we have seen with PRG in the reconstituted signaling system, or in conjunction with a distinct signaling pathway or localizing domain (i.e. the RGS or PDZ domain). Regardless, of the initial stimuli or signaling pathway, this mechanism could serve as a way to perpetuate the original signal or perhaps even enhance it. Binding of additional GTPases to the PH domains of Lbc RhoGEFs could also serve as a mechanism for facilitating crosstalk between distinct GTPase signaling pathways. In deed, the PH domain is the 11th most common domain in the human genome, and the majority of Dbl RhoGEFs contain a PH domain, therefore, it is highly possible that additional proteins also utilize the feed-forward mechanism to stimulate signaling. In addition, an important goal for future work is to identify the mechanism(s)

employed by the cell to regulate or terminate the feed-forward response. These exciting possibilities warrant further investigation of PH-containing RhoGEFs.

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