REGULATION OF P190RHOGEF BY ACTIVATED RHO AND RAC GTPASES: AMPLIFICATION AND CROSSTALK

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DEDICATION

I would like to thank my Mentor, Dr. Paul Sternweis, for all the support, guidance, and opportunities he has given me throughout my academic journey. I thank the members of my Graduate Committee for their advice and supervision of my dissertation. I also like to thank all the members of the Sternweis Lab, both past and present, for their wonderful support. Lastly, I am exceedingly grateful for my family, because I would not have achieved this milestone without their constant prayers and words of encouragement.

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by

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The Rho family of monomeric GTPases regulates a wide range of cellular processes including cytoskeletal structure, motility, cell division, gene transcription, vesicular transport, and various enzymatic activities. Activation of Rho proteins largely depends on Rho Guanine nucleotide Exchange Factors (RhoGEFs), which catalyze the exchange of GTP for GDP on Rho. For classical RhoGEFs, the exchange activity resides in a Dbl homology (DH) domain, which is linked to a pleckstrin homology (PH) domain that subserves various functions. We have crystallized and solved structures of the PH domain from p190RhoGEF bound to either RhoA•GTP or Rac1•GTP. The interfaces between activated Rac1 or RhoA with the PH domain utilize the same hydrophobic surface on the PH domain. Similar to RhoA, activated

Rac1 interacts with the PH domain via its effector-binding surface albeit burying less surface area than the interaction of RhoA with the PH domain. Consequently, the PH domain of p190RhoGEF has a higher affinity for RhoA than Rac1. Both activated RhoA and Rac1 can stimulate exchange of nucleotide on RhoA by localization of the p190RhoGEF to the substrate, RhoA•GDP, *in vitro*; mutations of key hydrophobic residues in the PH domain abolish this stimulation. Among members of the homologous Lbc subfamily of RhoGEFs, p190RhoGEF exhibited the greatest capacity for regulation by Rac1. While interaction of RhoA with the PH domain provides a mechanism for direct positive feedback, the novel interaction of activated Rac1 with the PH domain of p190RhoGEF reveals a potential mechanism for cross-talk regulation between the Rho and the Rac signaling pathways.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Medina, F., Carter, A.M., Dada, O., Gutowski, S., Hadas, J., Chen, Z., and Sternweis, P.C. (2013). Activated RhoA Is a Positive Feedback Regulator of the Lbc Family of Rho Guanine Nucleotide Exchange Factor Proteins. Journal of Biological Chemistry *288*, 11325-11333.

Dada, O., Gutowski, S., Brautigam, C., Chen, Z., and Sternweis, P.C. Direct Regulation of p190RhoGEF by Activated Rho and Rac GTPases. (manuscript in revision)

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AKAP13 – a-kinase anchor protein 13 RhoGEF

AUC – analytical ultracentrifugation

CFP – cyan fluorescent protein

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

Dbl – diffuse B cell lymnphoma

Dbs – Dbl's big sister

DH – Dbl-homology

Dmut – dual-mutant

FAK – focal adhesion kinase

FRET – fluorescence resonance energy transfer

G protein – guanine nucleotide binding protein

GAP – GTPase activating protein

GDI – guanine nucleotide dissociation inhibitor

GEF – guanine nucleotide exchange factor

GPCR – G protein coupled receptor

GDP – guanosine 5' diphosphate

GTP – guanosine 5' triphosphate

GTPγS – guanosine 5' O-(2-thiophosphate)

GST – glutathione S-transferase

ITC – isothermal titration calorimetry

IPTG – isopropyl -β-D-thiogalactopyranoside

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

PIP_2 – L-α-phosphatidylinositol-4,5-bisphosphate

PMSF – phenylmethysulfonyl fluoride

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

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

PRG – PDZ-RhoGEF

PS – L-α-phosphatidylserine

RGNEF – mouse version of p190RhoGEF

RGS – regulator of G protein signaling Rho – Ras homology

ROCK – rho-associated coiled-coil-containing protein kinase

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TAME – N^{α}-p-tosyl-L-arginine methyl ester

TIAM1 - t-lymphoma invasion and metastasis-inducing protein 1

TLCK – N^{α}-p-tosyl-L-lysine chloromethyl ketone

TPCK – tosylphenylalanyl chloromethyl ketone

TEV – tobacco etch virus

WT – wild-type

YFP – yellow fluorescent protein

CHAPTER ONE

INTRODUCTION

Cellular Signaling

Signal transduction is fundamental to cellular functions including proliferation, differentiation, metabolism, and programmed cell death. Integrated response pathways allow cells to coordinate behavior while constantly sensing external inputs from their environment and monitoring intracellular function. Dysregulation of these signaling pathways prevents proper cell function and can lead to a variety of human diseases. Therefore, it is important to understand the mechanisms of the various cellular regulatory pathways and how they integrate to maintain cellular viability and response. One important class of signaling molecules that impact many signaling pathways are the small monomeric G-proteins, which include the Rho subclass (Lu et al., 2009; Schwartz, 2004).

Signaling Through Rho family of GTPases

Rho GTPases compose a major subfamily of the Ras superfamily of small monomeric GTPases. RhoA, Rac1, and Cdc42 are the most well characterized members due to their roles in the regulation of the actin cytoskeleton, cell migration, and cell division. Twenty-two genes encoding 22 distinct, yet homologous proteins in this subfamily have been identified in mammals (Ridley, 2006). These GTPases are organized into 8 sub families based on their

sequence homology. RhoA and its two other isoforms, RhoB and RhoC, were first identified in humans (Madaule and Axel, 1985) and share 88% identity among their amino acid sequences (Wheeler and Ridley, 2004). Rac1 is grouped in a subfamily that includes Rac2 and Rac3 isoforms and RhoG (Boureux et al., 2007). The Rac isoforms are 93% homologous in amino acid sequence, while only 72% homologous with RhoG (Heasman and Ridley, 2008). Cdc42 was first discovered as a cell-cycle mutant during experiments on the yeast, *Saccharomyces cerevisiae* (Etienne-Manneville, 2004). Later studies established the importance of Cdc42 in the development of the fruit fly, *Drosophila melanogaster* (Genova et al., 2000), and in the nematode, *Caenorhabditis elegans* (Gotta et al., 2001).

Rho proteins are molecular switches due their ability to adopt different conformational states in response to binding GDP or GTP (Vetter and Wittinghofer, 2001). In its active GTP-bound form, these GTPases bind and activate numerous downstream effector targets. Utilizing techniques such as affinity chromatography and yeast two-hybrid systems, scientists initially identified 30 effectors for Rho GTPases such as: serine, threonine, and tyrosine kinases, lipid kinases, lipases, and scaffold proteins (Bishop and Hall, 2000); subsequent studies added to this number (e.g., 50 effectors reported by (Jaffe and Hall, 2005) and continue to be elucidated. Thus, signaling through the Rho, Rac, and Cdc42 pathways regulates numerous cellular properties and activities including: establishment of polarity and morphology, proliferation, chemotaxis, invasion, phagocytosis, secretion of granules, neuronal development, and endosome trafficking (Etienne-Manneville and Hall, 2002; Heasman and Ridley, 2008). Therefore, effective regulation of Rho GTPases is essential to the coordination of many cellular functions.

Regulators of Rho GTPases

The major proteins that regulate these small GTPases are called Rho guanine nucleotide exchange factors (RhoGEFs) and GTPase-Activating Proteins (RhoGAPs) (**Figure 1.1**). RhoGEFs are compose of a family of 69 members and are characterized by tandem Dbl homology (DH) and pleckstrin homology (PH) domains (Rossman et al., 2005). The highly homologous DH domains, which contain the site for catalyzing nucleotide exchange on Rho GTPases, received its name from the first RhoGEF identified in a human diffuse B cell lymnphoma, Dbl RhoGEF (Eva and Aaronson, 1985). The Pleckstrin Homology (PH) domain is the 11th most abundant domain class in the human genome best known for frequently binding to polyphosphoinositides (Lemmon, 2007). It was originally identified as a stretch of 100–120 amino acids that appears twice in the platelet protein pleckstrin and is found in many molecules involved in cellular signaling, cytoskeletal organization, membrane trafficking and/or phospholipid modification (Lemmon and Ferguson, 2000; Tyers et al., 1988). RhoGEFs activate GTPases by facilitating the dissociation of GDP nucleotide from the protein leading to the subsequent association of GTP, due to its 10-fold higher concentration in the cellular environment compared to GDP (Cerione and Zheng, 1996). Activated GTPases can be turned off by a 70 member class of proteins called GTPase activating proteins (RhoGAPs). To overcome the slow intrinsic GTPase activity of small G-proteins, RhoGAPs enhance the hydrolysis of the gamma phosphate of GTP, leading to the inactivation of the GTPase (Tcherkezian and Lamarche-

Vane, 2007). In its inactivated form, GTPases can be regulated by a third class of proteins called guanine nucleotide dissociation inhibitors (RhoGDIs). These proteins, of which there are only 3 members, bind to the C-terminal lipid modifications on Rho GTPases, limiting their localization to the plasma membrane, thereby creating a cytosolic pool of inactive GTPases (Olofsson, 1999).

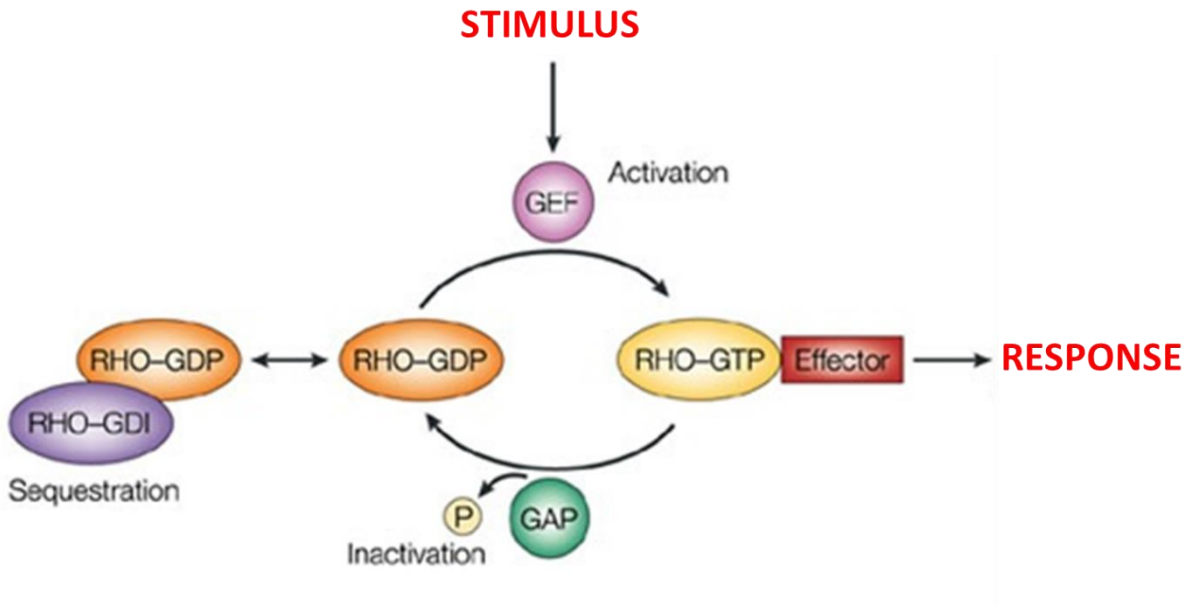

Figure 1.1. Regulators of Rho GTPases. RhoGEFs catalyze the exchange of GDP for GTP on the Rho GTPase. In this active state, the GTPase binds to effectors leading to downstream response. RhoGAPs enhances the hydrolysis of GTP to GDP which inactivates the Rho GTPase. RhoGDIs binds to inactivated Rho GTPases inhibiting their localization to the plasma membrane. *Figure modified from Sahai, E., and Marshall, C.J. (2002).*

Regulation of RhoGEFs

Guanine nucleotide exchange factors (GEF) undergo numerous forms of regulation that include direct association with proteins, lipids or second messengers that modulate their activity as well as posttranslational modifications that alter function. This regulation is accomplished through various mechanisms which include: translocation of the GEF to substrate, release of an autoinhibitory domain, or promotion of allosteric changes in the catalytic domain of the GEF (Bos et al., 2007). One example of regulation by the mechanism of translocation occurs with RalGDS, a GEF for Ral. RalGDS has a Ras-association (RA) domain that binds Ras-GTP; this interaction facilitates translocation of the GEF to the plasma membrane where it can activate its substrate, membrane associated Ral-GDP (Urano et al., 1996). An example of regulation by release of an autoinhibitory domain occurs in the Vav family of RhoGEFs. Phosphorylation of a key tyrosine upstream to the DH-PH catalytic domain of Vav by Src family kinases facilitates removal of the inhibitory N-terminal region from the Vav active sight thus increasing the activity of the GEF (Aghazadeh et al., 2000).

Well-defined domains can play important roles in the regulation of Dbl family RhoGEFs. RH-RhoGEFs, a subfamily of the Lbc family of RhoGEFs, is characterized by its N-terminal regulator of G protein signaling homology (RH) domain. Interaction of heterotrimeric G_{12} and G_{13} proteins with the RH domains of p115RhoGEF (p115) and leukemia-associated RhoGEF (LARG) directly stimulates the nucleotide exchange activity of the RhoGEFs on their substrate, RhoA (Hart et al., 1998; Sternweis et al., 2007; Suzuki et al., 2009). Another primary function of RH domains is localization of the RH-RhoGEFs to the

plasma membrane via this binding to activated G proteins (Bhattacharyya et al., 2009). Thus, hormones acting on G protein coupled receptors can cause stimulation of RhoA by simply utilizing this interaction to localize RhoGEFs to their membrane-associated substrate (Carter et al., 2014).

A role for the PH domain of RH-RhoGEFs was suggested by the novel observation of the interaction between the PH domain of PDZRhoGEF (PRG) and its activated substrate, RhoA (Chen et al., 2010); the inability of this binding interaction to affect basal stimulation of RhoA in solution suggested an alternative mechanism. This was subsequently demonstrated when activated RhoA was shown to stimulate the activity of PRG by localizing the RhoGEF to the surface of membrane vesicles containing membrane bound substrate (Medina et al., 2013). Mutating the essential residues on the PH domain necessary for binding activated RhoA attenuated this stimulation. Mutation of this binding site also attenuated the ability of PRG to activate RhoA by overexpression in cells. These observations, both *in-vitro* and *in-vivo*, were expanded to the other 6 members of the Lbc family of RhoGEFs thus defining a general mechanism of positive feedback regulation for these RhoGEFs (Medina et al., 2013). Whether other members of the Rho subfamily of Ras GTPases can bind and regulate Lbc RhoGEFs is examined in this dissertation.

Another form of regulation mediated through the PH domain involves its binding to phosphoinositides (PIs). Interaction with PIs is known to stimulate the intrinsic exchange activity of Dbl family GEFs. One such GEF, Phosphatidylinositol 3,4,5-triphosphate-dependent Rac exchanger 1 (P-Rex1) binds to PtdIns(3,4,5)P_3 with its PH domain thereby activating its exchange activity (Hill et al., 2005; Welch et al., 2002). Vav1 is also activated

by binding to PtdIns(3,4,5)P_3 with its PH domain, although to a lesser degree than P-Rex1 (Das et al., 2000; Han et al., 1998). In both cases, other factors such as: P-Rex1's binding to Gβγ subunits or phosphorylation of Vav1 mediated by its association with PtdIns(3,4,5)P_3 can stimulate their intrinsic GEF activities; therefore a combination of these regulatory mechanisms are necessary to achieve full GEF activity *in vivo* (Viaud et al., 2012).

p190RhoGEF – Discovery and Characterization

P190RhoGEF, also known as RGNEF (mouse) and designated as ARHGEF28, is an ubiquitously expressed regulator of RhoA (Gebbink et al., 1997). This protein contains the classic tandem DHPH domain motif common to the Dbl family of RhoGEFs, as well as a leucine-rich domain, a zinc-finger region, and a coil-coil motif (**Figure 1.2**) (van Horck et al., 2001).

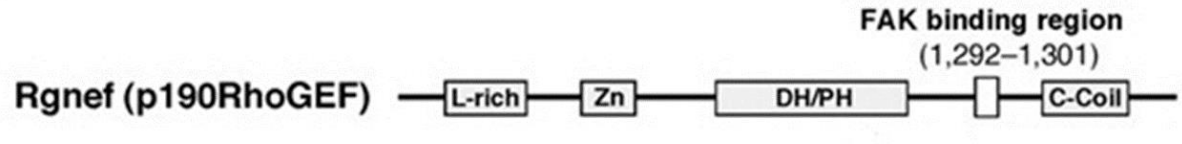

Figure 1.2. The Domains of p190 RhoGEF. *Figure modified from Yu, H. G., et al. (2011).*

P190RhoGEF specifically activates Rho isoforms (RhoA, RhoB, and RhoC), but not Rac1 or Cdc42 (Jaiswal et al., 2011; van Horck et al., 2001). Numerous binding partners for this RhoGEF have been identified including: a direct association of the C-terminal region of

focal adhesion kinase (Zhai et al., 2003) and 14-3-3 proteins (Zhai et al., 2001) with sites in the coil-coil region of p190RhoGEF, and an interaction of the scaffold protein, JIP-1, with a site C-terminal to the coil-coil region (Meyer et al., 1999). The C-terminal region of p190RhoGEF can also associate with microtubules (van Horck et al., 2001) and the proximal untranslated region of mRNA for light neurofilament subunit (Cañete-Soler et al., 2001). These binding interactions have suggested the importance of p190RhoGEF in focal adhesion formation, cancer cell motility, and neurodegeneration (Lim et al., 2008; Lin et al., 2005; Yu et al., 2011).

p190RhoGEF – Role in Cancer and Neurodegenerative Diseases

Colorectal cancer is the second most lethal neoplasia worldwide, yet there is still a lot to learn about the roles of Rho GTPases in its development and progression (Jemal et al., 2006; Leve and Morgado-Díaz, 2012). One important discovery is the upregulation of both Tiam1 and p190RhoGEF (RGNEF) in colorectal cancer (Malliri et al., 2006; Yu et al., 2011). Both mRNA for RGNEF and the protein itself are increased in the tumor sections from patients at later stages of colon cancer progression (Yu et al., 2011). RGNEF is not only important for tumor cell motility, but also size and invasiveness; overexpression of the C-terminal domain of RGNEF generated smaller, less invasive tumors in an orthotropic mouse model (Yu et al., 2011). The invasive phenotype of tumors is due to invadopodia, cellular structures that degrade nearby extracellular matrix; RhoA and RhoC are critical for the creation of these structures (Narumiya et al., 2009; Weaver, 2006). In one study, the

localization of RGNEF and subsequent activation of RhoC in invadopodia is proposed to facilitate formation of these structures in breast carcinoma cells (Bravo-Cordero et al., 2011).

Various Dbl-family RhoGEFs have been shown to play a role in the pathology of neurodegenerative diseases; including recent research linking RGNEF and amyotrophic lateral sclerosis. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder in patients suffering from an increasing loss of motor neurons resulting to their eventual death usually within 3 years of diagnosis (Hardiman et al., 2011). One characteristic of this disease is the absence of mRNA for NEFL (neurofilament light protein) in the spinal motor neurons (Bergeron et al., 1994); this leads to the formation of neurofilament (NF) inclusions (Kondo et al., 1986), the pathological hallmark of ALS. Altering the expression of NF in motor neurons in both an in-vivo and in-vitro contexts can cause motor neuron degeneration. Therefore, the regulation of NF RNA metabolism plays a role in motor neuron death and ALS pathogenesis (reviewed in Droppelmann et al., 2014). Human RGNEF, just like its mouse counterpart, binds NEFL mRNA, yet this interaction destabilizes NEFL mRNA and decreases protein expression (Droppelmann et al., 2013; Volkening et al., 2010). The presence of hRGNEF in neuronal cytoplasmic inclusions (NCIs) detected in the motor neurons of ALS patients also implies the importance of this protein in the disease (Droppelmann et al., 2013; Keller et al., 2012a).

Goals of Thesis Research Project

The goals of this research project were to further characterize the functional properties of RGNEF/p190RhoGEF by examining the uniqueness of the binding interaction between p190RhoGEF and GTPases, defining the mechanisms of potential regulation that they represent, and exploring their biological relevance.

CHAPTER TWO

METHODOLOGY

Plasmids and Constructs for Expression of Proteins

The PH domains of p190 (E1049-K1194), PRG (T927-P1085), LARG (A981-K1135), GEFH1 (E437-P574), p114 (Y302-P444), p115 (M609-A766), and AKAP13 (Y2195-N2333) RhoGEFs were amplified by PCR from their respective WT DH/PH pGEX-KG-TEV plasmids (Medina et al., 2013) with oligomers that incorporated a C-terminal His6-tag. These inserts were cloned into the pGEX-KG-TEV vector. PH domains containing double mutations in their binding interfaces were amplified by PCR from pGEX-KG-TEV plasmids containing p190 DH/PH (F1154A, I1156A), PRG DH/PH (F1044A, I1046E), LARG DH/PH (F1098A, I1100E), GEFH1 DH/PH (F539A, I541E), p114 DH/PH (F407A, I409E), p115 (Y726A, L728E), and AKAP-Lbc DH/PH (F2299A, I2301E) (Medina et al., 2013) with oligomers that incorporated a C-terminal His6-tag. The inserts were cloned into the pGEX-KG-TEV vector. The WT DH/PH pGEX-KG-TEV plasmids: p190 (D822-K1194), PRG (D712-P1085), LARG (D761-K1135), AKAP-Lbc (M1919-N2333), and p114 (E72-P444) were generated as previously described (Medina et al., 2013). The coding regions of WT FL-RhoA, WT FL-RhoB, WT FL-RhoC, WT FL-Rac1, WT FL-Rac2, and WT FL-Rac3 (obtained from cDNA Resource Center) were subcloned into pGEX-KG-TEV. WT RhoAΔC (residues 1-181) pGEX-KG-TEV was generated as previously described (Chen

et al., 2010). The coding region of WT Rac1ΔC (residues 1-182) were inserted into pGEX-KG-TEV vector. WT FL-RhoA.His_6 and WT FL-Rac1.His_6 pGEX-TEV plasmids was generated as previously described (Medina et al., 2013). A fluorescent PRG-PH domain was generated by inserting the Cerulean fluorescent protein (CFP) in between G1014 and S1015 of the PRG PH coding region. Fluorescent p190-PH was created by fusing CFP to the N-terminus of the p190 PH domain with a Gly-Ser linker. Both were cloned into the pGEX-KG-TEV vector. Construct of full length human RhoA with YFP fused to the N-terminus was cloned into pGEX-KG-TEV as described previously (Medina et al., 2013).

Protein Expression and Purification

All proteins were expressed in *E. coli* strain BL21(DE3) cells grown in LB medium at 22 °C overnight with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG); cells were collected by centrifugation and frozen for future use. Frozen cells from 1 L of medium were thawed and suspended with 50 mL of lysis buffer (50 mM NaHEPES, pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10 μM GDP) and protease inhibitors (23 μg/ml phenylmethylsulfonyl fluoride, 22 μg/ml N^{α}-*p*-tosyl-*L*-lysine chloromethyl ketone, 22 μg/ml tosylphenylalanyl chloromethyl ketone, 22 μg/ml pepstatin A, 1 μg/ml N^{α}-*p*-tosyl-*L*-arginine methyl ester). Lysozyme (2 mg/ml), DNase I (50 ug/ml) and $MgCl_2$ (5 mM) were added and cells were lysed for 60 min at 4°C, followed by centrifugation at 35,000 rpm to remove particulate material. GST-tagged fusion proteins were extracted from the soluble fraction by affinity chromatography with 2 mL of Glutathione Sepharose 4B (GE Healthcare). Resins

with GST-fusion proteins bound were suspended with lysis buffer and incubated overnight at 4 °C in the presence of 0.5 mg TEV protease to release and elute the protein from the immobilized GST-tag. GST-fusion proteins not undergoing TEV treatment were released from the resin by eluting with lysis buffer containing 15 mM glutathione. His_6-tagged proteins released from the resin were further purified by affinity chromatography with Ni-NTA agarose resin (Qiagen). Enriched proteins were further purified with a Mono Q anion exchange column (GE Healthcare) that had been pre-equilibrated with Buffer A (25 mM NaHEPES, pH 8.0, 5 mM β-mercaptoethanol, 2 mM $MgCl_2$). Elution was accomplished with a linear gradient of 0 to 0.5 M NaCl in Buffer A. Solutions for purification with MonoQ were supplemented with GDP (10 μM) when purifying GTPases.

Preparation of RhoA with bound mantGDP

His_6-RhoA was loaded with *N*-methylanthraniloyl-GDP (mant-GDP; Invitrogen) by incubation of 200 μM RhoA with 1mM mant-GDP) in Buffer C (20 mM NaHEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, 200 mM NaCl) plus 1 mM $MgCl_2$ for 20 h at 25 °C. The RhoA with bound mant-GDP was exchanged into Buffer A plus 5 mM $MgCl_2$ by dilution and concentration with an Amicon Ultra filtration device to remove excess nucleotide. A second incubation of 200 μM RhoA with 1 mM mant-GDP in Buffer A plus 5 mM $MgCl_2$ for 20 h at 25 °C was performed to ensure complete loading. RhoA loaded with mant-GDP was separated from free nucleotide by gel filtration through tandem Superdex 200 and Superdex

75 10/100 GL columns with 20mM NaHEPES, pH 8.0, 5 mM β-mercaptoethanol, 200 mM NaCl, and 5 mM $MgCl_2$.

Activation of GTPases

Purified RhoA, Rac1, and Cdc42 GTPases were exchanged into binding buffer (25 mM NaHEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, and 10 μM GDP) and concentrated to 200-500 μM protein. The concentrated proteins were adjusted to 0.5 mM $MgSO_4$ and 1 mM GTPγS, and incubated at room temperature for 24 hours. Proteins loaded with GTPγS was separated from free nucleotide by gel filtration through tandem Superdex 200 and Superdex 75 10/100 GL columns with 20mM NaHEPES, pH 8.0, 5 mM β-mercaptoethanol, 200 mM NaCl, and 5 mM $MgCl_2$.

Pulldown Assays

Immobilized GST-tagged Rho and Rac proteins were used to compare their relative ability to bind purified His_6-tagged PH domains. The GST-GTPases, either basal (GDP) or pre-activated with GTPγS, were mixed with 10 μl of glutathione-Sepharose 4B resin in 100 μl of Buffer A (50 mM NaHEPES, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 0.3% (v/v) Triton X-100, and 0.01% (w/v) BSA) and incubated for 30 min at 4 °C. The resin was washed with Buffer A, and His_6-p190 PH domain was added to the immobilized GST-Rho or Rac in Buffer A (100 μl) containing either 10 μM GDP or 10 μM

GTPγS for basal or pre-activated Rho or Rac, respectively. The mixtures were incubated for an addition 30 min at 4 °C and the resin rapidly separated by spinning in a microcentrifuge. Supernatants containing free PH domains were removed and the resins rapidly washed four times with 600 μl of cold Buffer A. PH domains bound to the resins were released by incubating in 30 μl of Buffer A containing 15 mM glutathione for 5 min, subjected to SDS-PAGE, and visualized by silver staining or immunoblot analysis using an anti-His_6 monoclonal antibody (R&D Systems).

Creation of Phospholipid Vesicles

Unilamellar phospholipid Vesicles were prepared by extrusion through an Avanti Mini-Extruder using a 100-nm polycarbonate membrane (Avanti Polar Lipids). All lipids were obtained from Avanti. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (poPE), 1-palmitoyl 2-oleoyl-*sn*-glycero-3-phosphocholine (poPC), and 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (18:1 DGS-NTA(Ni)) were mixed in solvent at a molar ratio of 4.75:5:0.25, respectively. The mixture was dried under a stream of N_2 gas for 30 min and placed under vacuum overnight. The lipids were then suspended to a total concentration of 10 mM in 1ml of buffer (20mM NaHEPES, pH 7.5, 200mM NaCl, 2mM $MgCl_2$, 5 mM β-mercaptoethanol). After five freeze thaw cycles using an ethanol/dry ice bath, the mixture was passed through the extruder for 11 passages to form vesicles with a diameter of ~100 nm.

Vesicle Binding Assay – Size Exclusion Column

Unilamellar phospholipid vesicles doped with nickel chelating lipid were made by extrusion as described above with the following modifications: poPE, poPC, L-α-phosphatidylserine (PS), L-α-phosphatidylinositol-4,5-bisphosphate (PIP_2), and DGS-NTA(Ni) were mixed in solvent at a molar ratio of 4.4:4.4:1:0.30:0.25, respectively. WT p190-PH (2 μM), WT Dbs-PH (2 μM), and His_6-RhoA or His_6-Rac1 (12.5 μM) proteins as indicated were mixed with vesicles (0.5 mM lipid, ~ 5 nM vesicles) in assay buffer (20 mM NaHEPES, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol) for 15 min at 25 °C, then 5 min on ice. Columns containing 7 mL of Aca34 polyacrylamide-agarose resin (Pall Life Sciences) were equilibrated with assay buffer at 4 °C. The various protein-vesicle mixtures (200 μL) were added to the columns followed by an additional 200 μL of assay buffer after the initial volume had entered into the resin. Twenty elution fractions (400 μL) were collected in eppendorf tubes, aliquots subjected to SDS-PAGE, and visualized by silver staining.

Nucleotide Exchange Assays in Solution

GTPases (RhoA, Rac1, or Cdc42 at 1 μM) were incubated in a 200 μl cuvette with 5 μM mant-GTP in reaction buffer (25 mM NaHEPES, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol, and 5 mM $MgCl_2$). The exchange reaction was started by the addition of 1 μM

p190 DH·PH protein and the change in fluorescence was monitored with a Fluorolog-3 spectrofluorometer at 25 °C at λex = 356 nm, λem = 445 nm, and slits = 1/1 nm.

Nucleotide Exchange Assays with Phospholipid Vesicles

His_6-RhoA•mant-GDP (1 μM) and His_6-RhoA or His_6-Rac1 proteins as indicated were mixed with vesicles (0.5 mM lipid, ~ 5 nM vesicles) in assay buffer (20 mM NaHEPES, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 0.01% (w/v) bovine serum albumin) for 1 min at 25 °C to allow association of the proteins with the DGS-NTA-Ni on the vesicle surface. All reactions were started with the addition of RhoGEF proteins and the change in fluorescence was monitored with a Fluorolog-3 spectrofluorometer at 25 °C at λex = 356 nm, λem = 445 nm, and slits = 1/1 nm.

FRET Competition Assay (Intermolecular FRET Assay)

Intermolecular FRET was used to measure association of 1 μM YFP-RhoA•GTPγS with 1 μM CFP-PRG-PH or 2 μM YFP-RhoA•GTPγS and 2 μM CFP-p190-PH. Competition was measured by titration of non-fluorescent activated GTPases or non-fluorescent PH domains. Assays were done in 20 mM NaHEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 5 mM $MgCl_2$ in a volume of 140 μl utilizing a Fluorolog-3 fluorometer at 25 degrees at λ_{ex} = 433 nm, λ_{em} scanned from 450 to 560 nm, slits = 1/1 nm. Changes in FRET were reported as the ratio of fluorescence at 525 nm over 475 nm.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was performed utilizing a MicroCal iTC200 (Malvern Instruments). All protein samples were exchanged into ITC buffer (25 mM TrisCl, pH 8, 100 mM NaCl, 1 mM $MgCl_2$) by size-exclusion chromatography prior to calorimetric characterization. Contents of the sample cell were stirred continuously at 750 rpm during the experiment. A typical titration of a PH domain with activated RhoA involved 19 injections at 2-min intervals of 2 μl PH (200 or 500 μM) into a sample cell containing ca. 0.2 ml of activated RhoA (20 or 50 μM) at 20 °C. The data were then analyzed by integrating the heat effects of injection normalized to the amount of injected proteins, then performing curve fitting based on a 1:1 binding model (A+B <-> AB Heteroassocation) using NITPIC and SEDPHAT software, respectively (Keller et al., 2012b; Zhao et al., 2015). All ITC figures were rendered using GUSSI (Brautigam, 2015).

Analytical Ultracentrifugation

Analytical ultracentrifugation (AUC) was utilized to study the interaction of activated RhoA (RhoA•GTPγS) and the PH domain of p190 in a titration series, with RhoA•GTPγS held constant at 4 μM, and the concentration of p190 PH varied between 0.4 and 40 μM. For this experiment, five titration points were examined. To conduct this study, 400 μL of buffer (25mM NaHEPES, pH 8, 200mM NaCl, 5mM $MgCl_2$) and the RhoA•GTPγS/p190-PH

mixtures (which had been equilibrated overnight at 4 °C with the same buffer) were pipetted into the reference and sample sectors, respectively, of a 1.2-cm Epon charcoal-filled dual-sectored centerpiece that had been sandwiched between two sapphire windows. The centrifugation cells were placed in an An50-Ti rotor, which was incubated at 20 °C under vacuum for several hours before centrifugation was started. A Beckman Optima XL-I (Beckman-Coulter, Fullerton, CA) centrifuge was used to perform the experiment using a rotor speed of 50,000 rpm, and the data were collected using the on-board spectrophotometer tuned to a wavelength of 280 nm. The *c*(*s*) analyses were carried out using SEDFIT (Schuck, 2000). These analyses were compiled and assembled into a weighted-average sedimentation coefficient isotherm (Dam and Schuck, 2005; Schuck, 2003) using GUSSI (Brautigam, 2015). SEDPHAT (Dam and Schuck, 2005) was used to analyze the isotherm; during this analysis, the sedimentation coefficient of the RhoA•GTPγS:p190 PH complex was fixed at the estimated value of 2.9 S. This latter value agrees roughly with a calculated sedimentation coefficient of 2.8 S for the complex based on modeling (García De La Torre et al., 2000). All *c*(*s*) distribution and isotherm figures were rendered using GUSSI (Brautigam, 2015).

CHAPTER THREE

P190RHOGEF INTERACTS WITH ACTIVATED GTPASES

INTRODUCTION

A series of preliminary dot blot assays utilized the isolated DH-PH domains from various RhoGEF proteins to test whether these proteins are capable of binding to either the active form of RhoA, Rac1, or Cdc42 GTPases. Prior published results demonstrated an interaction of several RhoGEFs with activated RhoA (Medina et al., 2013). One unexpected result was the ability of p190RhoGEF, a member of the Lbc RhoGEF family, to bind the activated state of both RhoA and Rac1 GTPases (Jana Hadas, personal communication).

These results coincided with the work of a former colleague, Frank Medina, who identified a novel interaction between PRG RhoGEF and the activated state of RhoA (Chen et. al, 2010). Using pulldown assays, PRG RhoGEF was shown to have a strong binding with nucleotide free RhoA, as expected from its activity as a nucleotide exchange factor for RhoA. Interestingly, in the same assay, PRG bound to active RhoA better than the nucleotide free form of the GTPase. Chen and Medina went on to pinpoint the binding interaction between active RhoA and PRG to the PH-Domain of the RhoGEF. While the PH domains within the Dbl family of RhoGEFs are known for frequently binding to polyphosphoinositides, their action by associating with other proteins had been largely uncharacterized.

RESULTS

WT p190 PH-domain binds to RhoA and Rac1 GTPases

Due to the recent observation that p190RhoGEF, a Dbl family member, binds to active forms of RhoA through its PH domain (Medina et al., 2013), one of the major questions I set out to address with my thesis research was whether the binding of activated Rac1 to p190RhoGEF also utilized the PH domain and whether this binding is unique to the this RhoGEF. I first needed to verify the interactions implicated for p190RhoGEF by the dot blot assays.

I cloned and purified the PH-domain of p190RhoGEF with a c-terminal His_6 tag to use in pull-down assays with immobilized GST-RhoA or GST-Rac1 (from bacterial expression) either in the inactive state (excess GDP) or preloaded with GTPγS (*GTP*). PH domains bound to the immobilized GTPases after washing were subjected to SDS-PAGE and visualized by immunoblotting using an anti-His_6 monoclonal antibody. Both GTPases were able to pulldown the PH domain of p190, albeit greater amounts of the PH domain were associated with the active form of RhoA compared to Rac1 (**Figure 3.1**). These results confirm that p190RhoGEF is capable of binding directly and selectively to the activated states of both RhoA and Rac1 through its PH-domain.

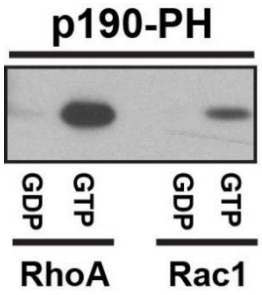

Figure 3.1 The PH domain from p190 binds activated RhoA and Rac1. Purified His_6-p190 PH domains (12 pmol) were incubated with immobilized basal or active forms of GST-RhoA (80 pmol) or GST-Rac1 (80 pmol). PH domains that bound to the resin were eluted, separated by SDS-PAGE and visualized by immunoblotting.

PH-domains from various Lbc family RhoGEFs Bind to RhoA and Rac1 GTPases Differently

As initially reported, PRG associates with activated RhoA through its PH-domain, while p115RhoGEF, highly homologous with PRG, did not show detectable association with the activated GTPase (Chen et. al, 2010). Due to the fact that the PH-domains from the seven members of the Lbc family RhoGEFs are highly homologous in the region important for binding RhoA, I decided to test their ability to associate with activated RhoA and Rac1 GTPases. The goal of these experiments was to verify that other domains could bind to activated RhoA as suggested by dot blots and to test whether the interaction of Rac1 is a characteristic specific to p190RhoGEF.

To investigate the ability of the Lbc family RhoGEFs to bind activated RhoA, I cloned and purified the PH-domains of PRG (T927-P1085), LARG (A981-K1135), AKAP13 (Y2195-N2333), GEFH1 (E437-P574), p114 (Y302-P444), p115 (M609-A766), and p190 (E1049-K1194) RhoGEFs as described in the methods of Chapter 2. Purified GEF PH.H6 domains were incubated with immobilized basal GST-RhoA with excess GDP (*GDP*), or pre-activated GST-RhoA with excess GTPγS *(GTPγS)*. PH domains bound to the resin after washing were subjected to SDS-PAGE and visualized by immunoblotting using an anti-His_6 monoclonal antibody. The PH-domains of PRG, LARG, AKAP13, p114, and p190 associated with the pre-activated RhoA GTPase. Yet, in the same experiment, the PH-domains of GEFH1 and p115 did not show binding to activated RhoA (**Figure 3.2**, **published as Figure 2 in Medina et al., 2013**).

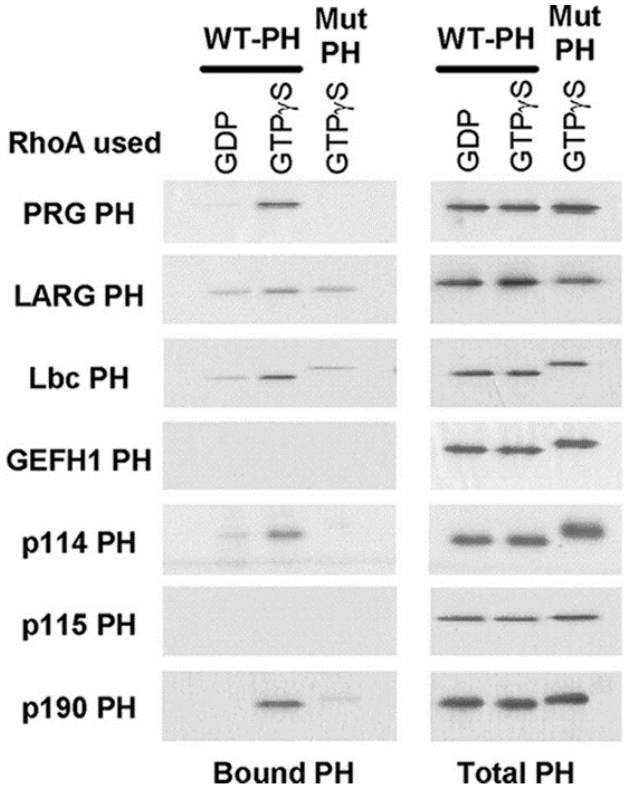

Figure 3.2 Interaction of PH domains from Lbc RhoGEFs with activated RhoA. Purified His_6-PH domains (40 pmol) were incubated with immobilized basal or active forms of GST-RhoA (80 pmol). PH domains that bound to the resin were eluted, separated by SDS-PAGE and visualized by immunoblotting. Mutant PH domains contained the mutations indicated in Chapter 3's text. The reason for slower migration of some of the mutated PH domains is unknown. *Figure modified from (Medina et al., 2013).*

To investigate the ability of the Lbc family RhoGEFs to bind activated Rac1, I utilized the purified PH-domains of PRG, LARG, GEFH1, and p190. I chose to test these particular proteins because they represented members from 2 subsets of the Lbc family of RhoGEFs: the RGS-RhoGEFs (PRG, LARG) and non-RGS RhoGEFs (GEFH1, p190). GST-Rac1 was cloned and expressed as described in chapter 2. Purified GEF PH.H6 domains were incubated with immobilized pre-activated GST-Rac1 with excess GDP *(GDP)*, or excess GTPγS *(GTP)* in a similar fashion to the pulldown assay described above. Out of the four PH-domains tested, only the PH-domain from p190 showed any binding interaction to activated Rac1 under these conditions **(Figure 3.3)**.

These results demonstrate that the PH-domains from various Lbc family RhoGEFs can associate with RhoA, but only p190RhoGEF displayed binding to Rac1.

WT p190 PH-domain binds to Isoforms of Rho (A,B,C) and Rac (1,2,3) GTPases

A second question is whether there is specificity in the association of the p190 domain for isoforms of Rho and Rac proteins. There are three closely related isoforms of Rho GTPase (RhoA, RhoB, and RhoC) (Madaule, P., and Axel, R., 1985); RhoA shares over 85% amino acid identity with RhoB and RhoC (Arthur et al, 2002). Any selectivity in binding among the isoforms would suggest selective regulation of the GEF by isoform specific pathways. Any differences would lead to future studies identifying residues on the GTPase and PH domain important for selectivity and attempts to identify physiological relevance.

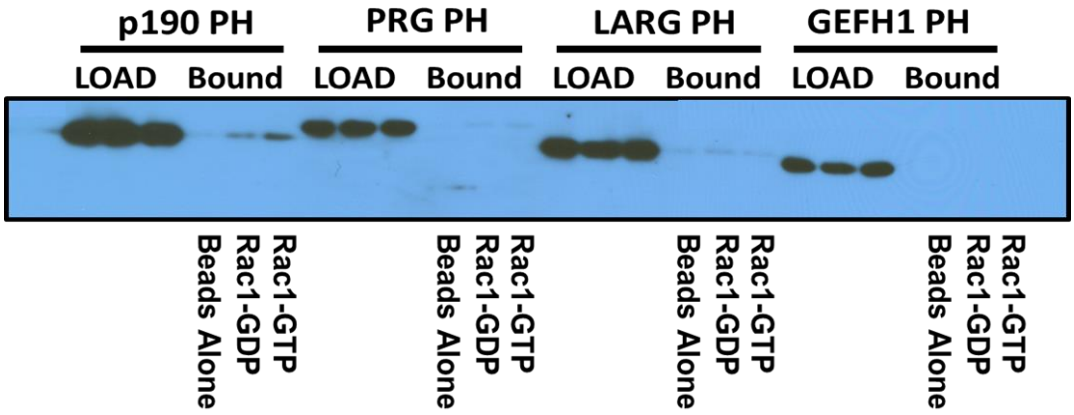

Figure 3.3 Interaction of PH domains from Lbc RhoGEFs with activated Rac1. Purified His_6-PH domains (12 pmol) were incubated with immobilized basal or active forms of GST-Rac1 (80 pmol) or resin. PH domains that bound to the resin were eluted, separated by SDS-PAGE and visualized by immunoblotting.

To test the hypothesis that the PH domain of p190RhoGEF could selectively bind to certain isoforms of Rho and Rac GTPases, I cloned and purified the GST-tagged GTPases (RhoA, RhoB, RhoC, Rac1, Rac2, and Rac3) as described in chapter 2. When tested in subsequent pulldown assays, I observed that the PH domain of p190 was capable of binding to all the tested isoforms of activated Rho (RhoA, RhoB, and RhoC) and Rac (Rac1, Rac2, and Rac3); as with RhoA and Rac1, the p190 PH domain associated less with all activated Racs compared to activated RhoA (**Figure 3.4 and Figure 3.5**).

Crystal Structure of WT p190 PH-domain bound to RhoA or Rac1 GTPases

A collaboration with Dr. Zhe Chen led to the successful determination of the crystal structures of the p190RhoGEF PH domain in complex with activated RhoA or Rac1 (**Figure 3.6**). The structures demonstrate that the binding between these two proteins involved the hydrophobic beta-sheets on the PH-domain and the switch regions on the GTPase; thus, both RhoA and Rac1 use a similar binding motif for interaction with the PH domain of p190. Interestingly, we discovered that this PH:GTPase binding interface is essentially the same as that defined by the crystal structure of RhoA bound to the PH-domain of PRG (Chen et al., 2010). .

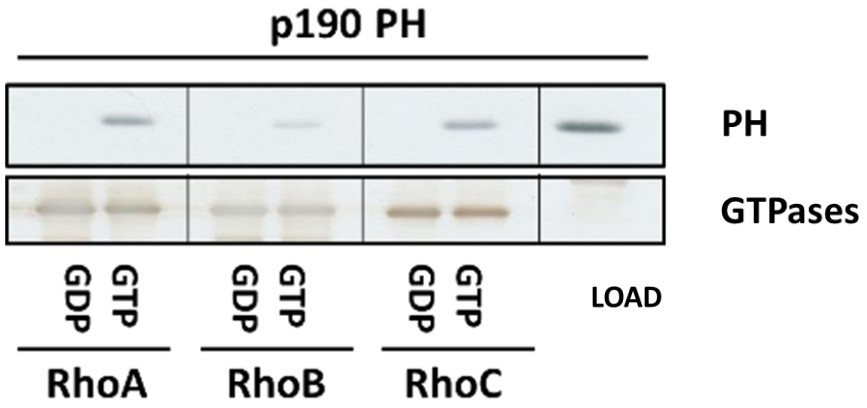

Figure 3.4 The PH domain of p190 binds activated Rho Isoforms. Purified His_6-p190 PH domains (12 pmol) were incubated with immobilized basal or active forms of GST-RhoA, RhoB, and RhoC (80 pmol). PH domains that bound to the resin were eluted, separated by SDS-PAGE and visualized by silver staining. *Upper bands*, p190 PH domain. *Lower bands*, GTPases.

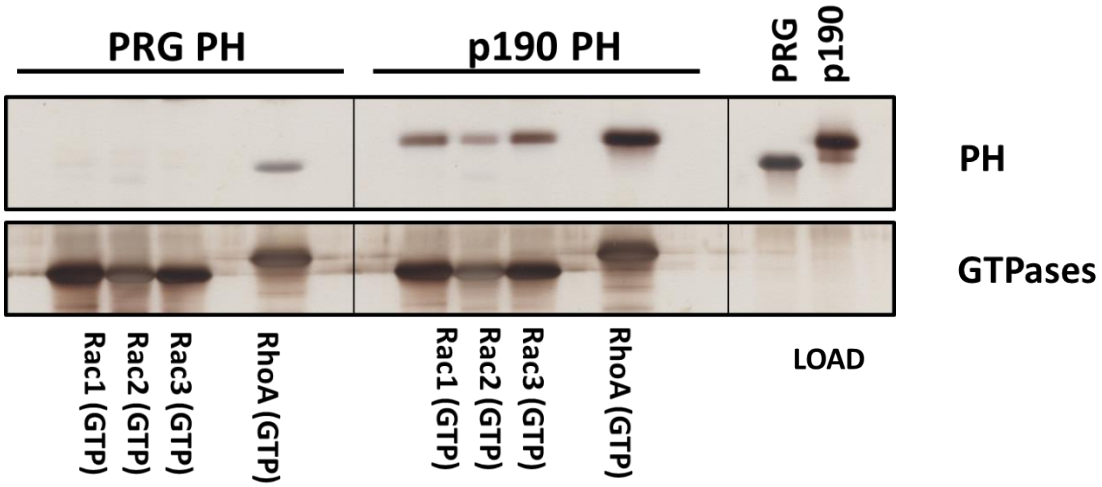

Figure 3.5 The PH domain of p190 binds activated Rac Isoforms. Purified His_6-p190 and His_6-PRG PH domains (12 pmol) were incubated with immobilized active form of GST-Rac1, Rac2, and Rac3 (80 pmol). PH domains that bound to the resin were eluted, separated by SDS-PAGE and visualized by silver staining. *Upper bands,* PRG or p190 PH domains. *Lower bands,* GTPases.

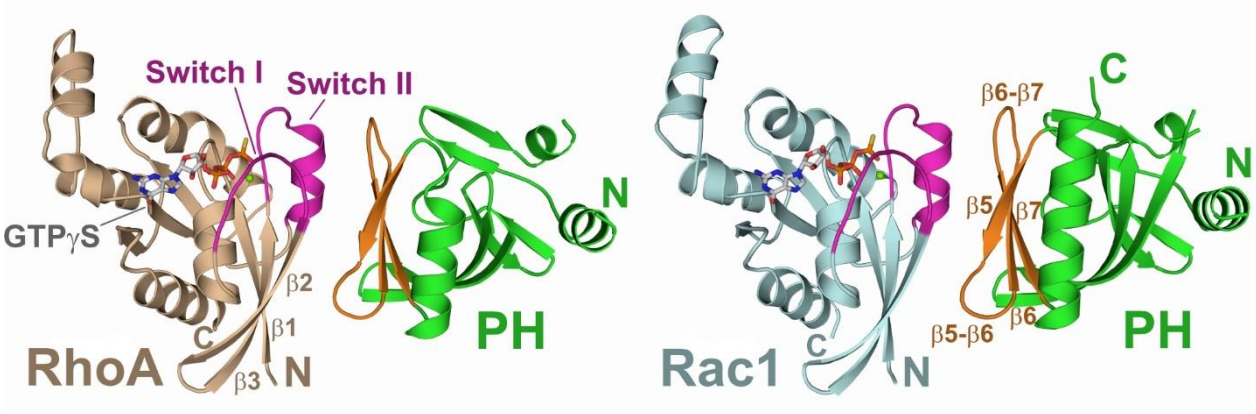

Figure 3.6. Structures of the p190 PH domain in complexes with activated GTPases. Ribbon diagrams depicting tertiary structures of p190-PH in a complex with RhoA•GTPγS (left) or Rac1•GTPγS (right). P190-PH is colored green, with the C-terminal layer of β-strands colored orange. RhoA is colored wheat, with switch regions colored purple. Rac1 is colored cyan, with switch regions colored magenta. GTPγS and magnesium ion are depicted as ball-and-stick models and colored as follows. Oxygen, nitrogen, carbon and phosphorous atoms are colored red, blue, grey, and yellow, respectively. Magnesium is colored green. *Figure modified from Dr. Zhe Chen.*

Affinity Measurements of Interactions Between the PH-domains of Lbc family RhoGEFs (p190, PRG, AKAP13, and GEFH1) and RhoA or Rac1 GTPases

Data from the various pulldown assays described above demonstrate that p190RhoGEF can interact with both RhoA and Rac1 GTPases. I was next interested in determining the binding affinity between the PH domain of p190RhoGEF and the RhoA and Rac1 GTPases. Small-volume isothermal titration calorimetry (SV-ITC) and analytical ultracentrifugation (AUC) were used to try to quantify the binding affinity of p190RhoGEF to activated RhoA and Rac1. I conducted these experiments under the guidance and support of Dr. Chad Brautigam and Dr. Thomas Scheuermann at the Macromolecular Biophysics Resource core facility at UT Southwestern. ITC used titration of pre-activated RhoA or Rac1 into the sample cell of a MicroCal ITC200 containing the PH domain of p190. Analysis of heat evolved by binding at each step indicated that the binding affinity between p190 PH and RhoA was about 2 μM (1.7 μM – 2.6 μM, 68.3% confidence intervals) (**Figure 3.7**). This result is supported by a second affinity measurement obtained by AUC, wherein the same protein pair predicted a binding affinity of 2.9 μM (2.3 μM – 3.7 μM, 68.3% confidence intervals) (**Figure 3.8**). Interestingly, when I measured the binding affinities from the PH domains of other Lbc family RhoGEFs with RhoA, I obtained affinity measurements from as low as 1.2 μM (PRG) up to 13 μM (p114) (**Figure 3.9 – 3.11**). Unfortunately, all attempts to directly measure the binding affinity between p190 PH and Rac1 by ITC or AUC were either unsuccessful or inconclusive. To overcome this unexpected setback, a colleague of mine, Stephen Gutowski, utilized a FRET competition assay to determine the relative affinity of

p190's PH domain to activated Rac1. Briefly, the loss of intermolecular FRET between CFP-p190-PH and activated YFP-GTPases due to the titration of non-fluorescent activated GTPases was measured by fluorometry. The data from his assays suggests that the affinity for activated Rac1 to the PH-domain of p190 is 10-fold lower than affinity for activated RhoA tested in the same assay (**Figure 3.12**). All experimental studies of the binding affinity between the PH domain of p190RhoGEF with RhoA and Rac1 GTPases are summarized in (**Table 3.1**).

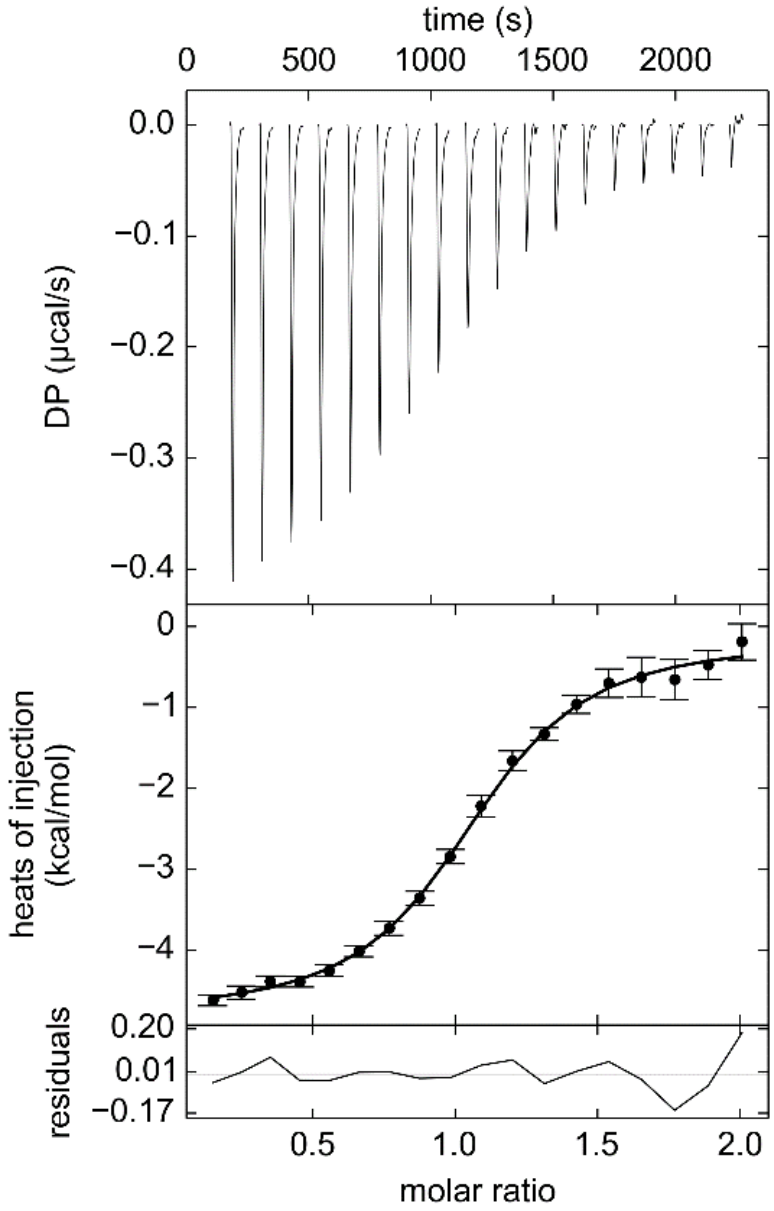

Figure 3.7. ITC - p190 PH and RhoA-GTPγS. Measurement of binding affinity between the p190 PH domain and RhoA•GTPγS by isothermal titration calorimetry. Kd = 2 μM (1.7 μM – 2.6 μM, 68.3% confidence interval). Error bars represents the uncertainty of each individual integrated isotherm peak assigned by the program, NITPIC.

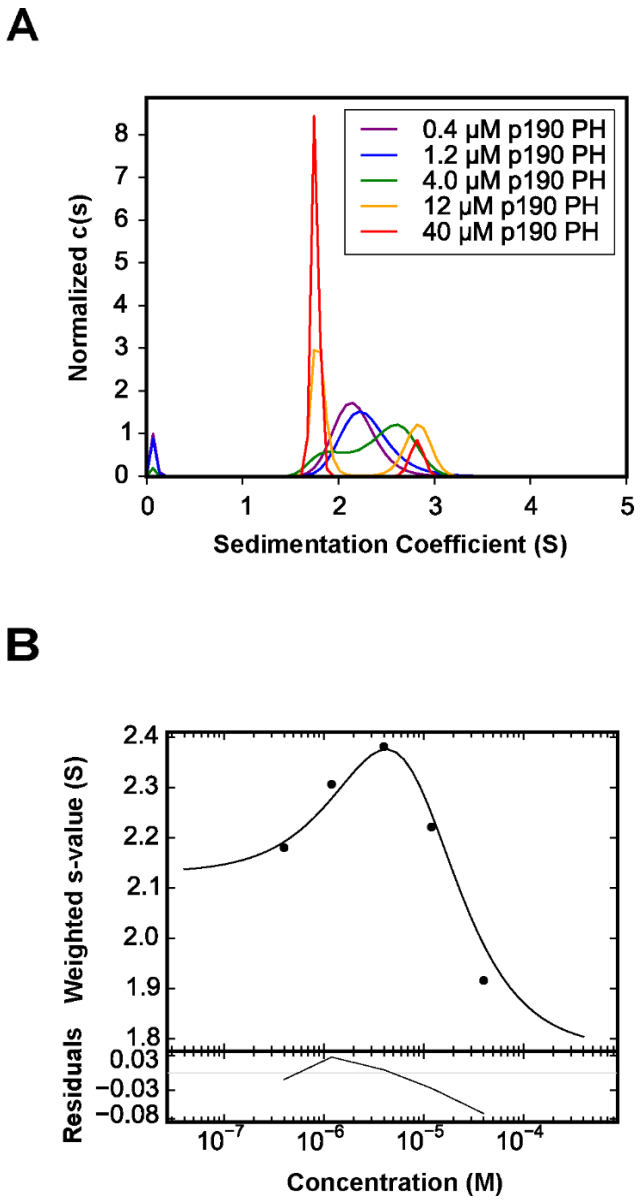

Figure 3.8. AUC – p190 PH and RhoA-GTPγS. Measurement of p190-PH and RhoA•GTPγS interactions by sedimentation velocity analytical ultracentrifugation (SV-AUC). **A.** Sedimentation coefficient distribution for p190-PH and RhoA•GTPγS. The concentrations of p190-PH titrated into 4 μM RhoA•GTPγS were 0.4 μM (purple), 1.2 μM (blue), 4 μM (green), 12 μM (orange), and 40 μM (red). The *c*(*s*) distributions were normalized by the total signal of the respective experiments. **B.** Isotherm of weighted-average sedimentation coefficients. This was obtained by integration of the *c*(*s*) distributions shown in panel A. Analysis of the isotherm resulted in a binding constant (*K*D) of 2.9 μM (2.3 μM – 3.7 μM, 68.3% confidence interval).

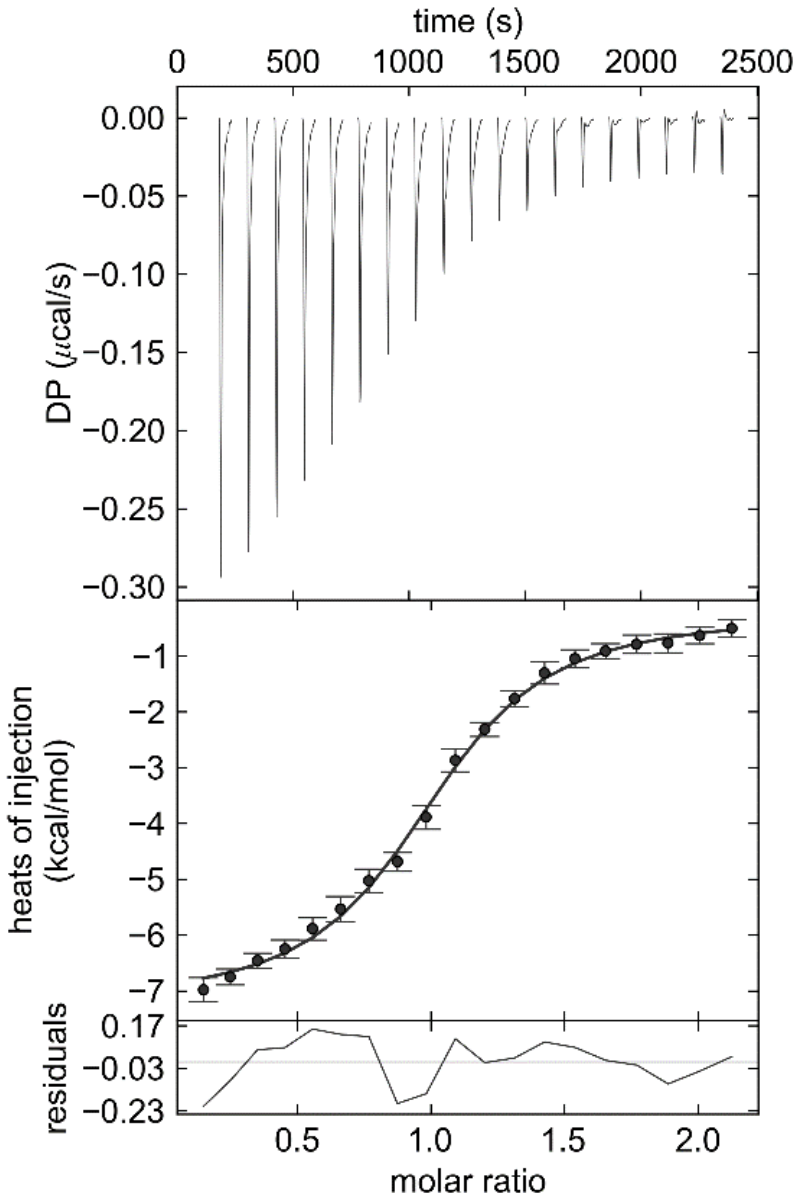

Figure 3.9. ITC – PRG PH and RhoA-GTPγS. Measurement of binding affinity between the PRG PH domain and RhoA•GTPγS by isothermal titration calorimetry. Kd = 1.2 μM (0.8 μM – 1.4 μM, 68.3% confidence interval). Error bars represents the uncertainty of each individual integrated isotherm peak assigned by the program, NITPIC.

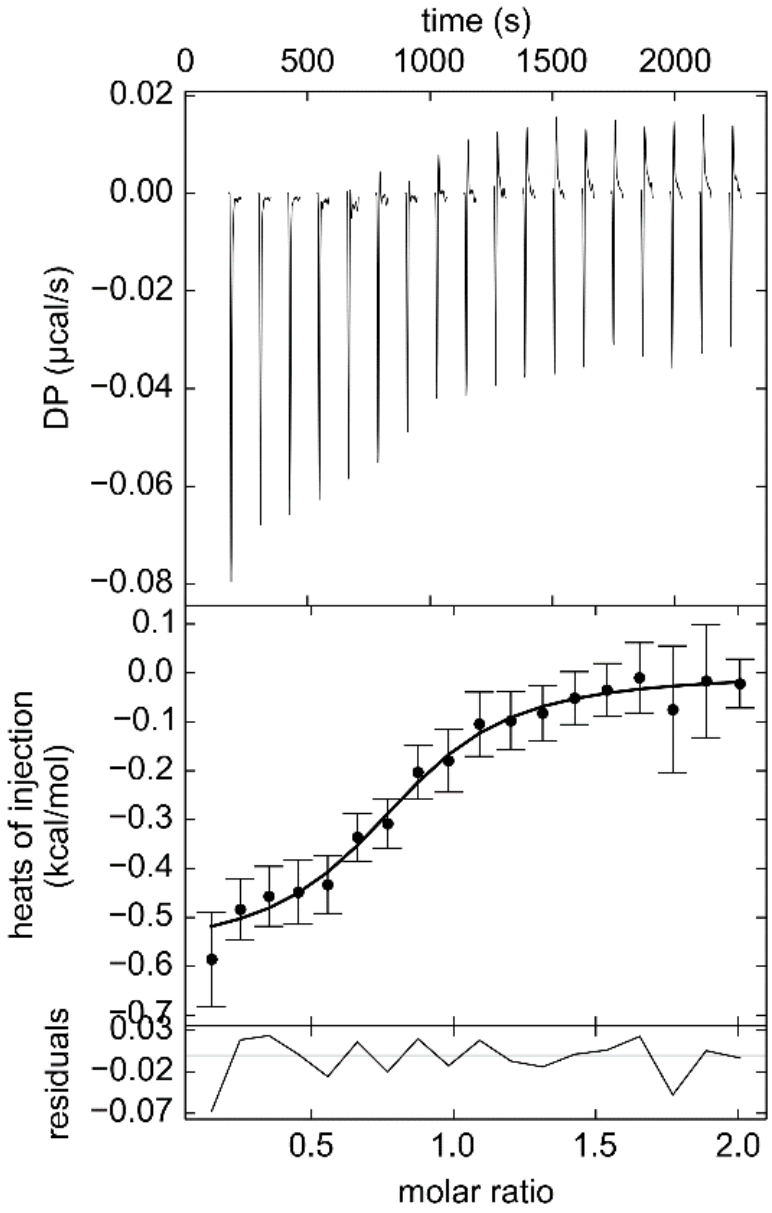

Figure 3.10. ITC – AKAP13 PH and RhoA-GTPγS. Measurement of binding affinity between the AKAP13 PH domain and RhoA•GTPγS by isothermal titration calorimetry. Kd = 3.1 µM (1.5 µM – 7 µM, 68.3% confidence interval). Error bars represents the uncertainty of each individual integrated isotherm peak assigned by the program, NITPIC.

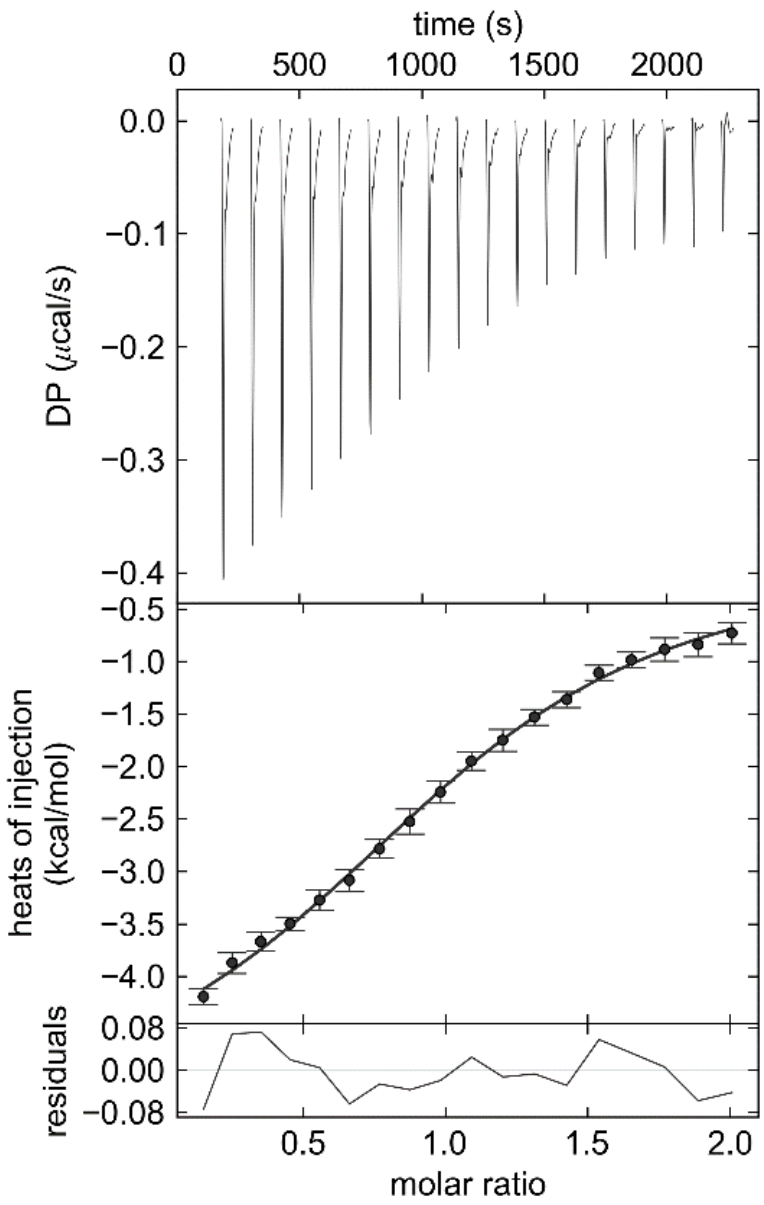

Figure 3.11. ITC – p114 PH and RhoA-GTPγS. Measurement of binding affinity between the p114 PH domain and RhoA•GTPγS by isothermal titration calorimetry. Kd = 13 μM (9.4 μM – 18.7 μM, 68.3% confidence interval). Error bars represents the uncertainty of each individual integrated isotherm peak assigned by the program, NITPIC.

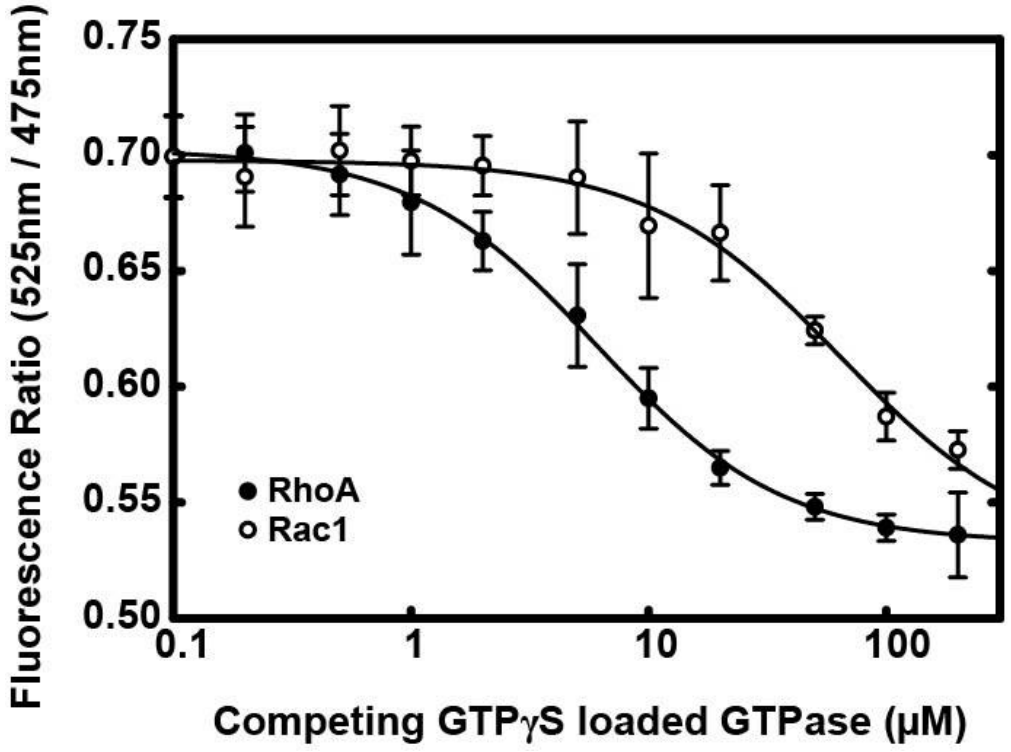

Figure 3.12 FRET Competition Assay – p190 PH, RhoA-GTPγS, and Rac1-GTPγS. *Stephen Gutowski performed this experiment.* The ability of non-tagged RhoA•GTPγS and Rac1•GTPγS to bind to the p190 PH domain was measured by competition of FRET produced by binding of 2 uM YFP- RhoA•GTPγS to 2 μM CFP-p190-PH. Error bars are the standard deviation of 3 independent experiments. Relative affinity for RhoA = 6.1 μM (*IC50*), Relative for Rac1 = 64 μM (*IC50*)

Experiment	Proteins Tested	Measured Affinity (Kd)
SV-ITC	P190 PH and RhoA GTPγS	2 μM (1.7 μM – 2.6 μM)
SV-ITC	PRG PH and RhoA GTPγS	1.2 μM (0.8 μM – 1.4 μM)
SV-ITC	AKAP13 PH and RhoA GTPγS	3.1 μM (1.5 μM – 7 μM)
SV-ITC	P114 PH and RhoA GTPγS	13 μM (9.4 μM – 18.7 μM)
SV-ITC	P190 PH and Rac1 GTPγS	---
AUC	P190 PH and RhoA GTPγS	2.9 μM (2.3 μM – 3.7 μM)
AUC	P190 PH and Rac1 GTPγS	---
FRET Competition	P190 PH and RhoA GTPγS	6.1 μM *(IC50)*
FRET Competition	P190 PH and Rac1 GTPγS	64 μM *(IC50)*

Table 3.1 Summary of all PH-domain and Activated GTPases Affinity Measurements. SV-ITC and AUC data were obtained by myself. The FRET Competition data were obtained by Stephen Gutowski.

DISCUSSION

Preliminary experiments identified p190RhoGEF as having the unique ability to bind both RhoA and Rac1. The goal of the studies reported in this chapter was to obtain a comprehensive picture of the interaction space between these GTPases and the related family of RhoGEFs. With the use of pulldown assays, I was able to confirm that the PH-domain of p190 is capable of binding to the active form of RhoA and Rac1. The ability for the PH-domain to interact with activated RhoA is a feature broadly shared among many members of the Lbc family RhoGEFs including: PRG, LARG, AKAP13, p114, and p190. I have also

shown that PH-domains of select members of the Lbc family of RhoGEFs (PRG, LARG, GEFH1) did not show detectable binding to activated Rac1 under conditions which detected binding of the GTPase to p190RhoGEF; this supports the assignment of novel binding characteristics of the p190 protein. The significance of this finding encourages future studies into the potential role these interactions play in the crosstalk regulation between the Rac and Rho pathways (Guilluy et al., 2011).

To get a better understanding of the binding between p190 and its two GTPase partners, my colleague Dr. Zhe Chen successfully generated crystal structures of the isolated PH-domain of p190RhoGEF in complex with activated RhoA or Rac1. The first structure, p190 PH bound to RhoA, is a second example involving a complex between an Lbc RhoGEF PH domain and its activated substrate, RhoA•GTPγS. The second structure is unique due to it being the first example of the same RhoGEF in complex with a second GTPase, Rac1•GTPγS. These structures reveal that both RhoA and Rac1 share a similar binding motif with the PH-domain of p190. The information gleaned from these two structures with help guide future mutagenesis efforts in identifying the important residues found in the binding interface between PH-domain of p190RhoGEF and RhoA and Rac1 GTPases.

My efforts to quantify the binding affinities of the PH-domain of p190RhoGEF to both activated RhoA and Rac1 were partially successful. I succeeded in measuring the affinity of p190-PH with activated RhoA by two different type of biophysical techniques; ITC indicated a Kd of 2 μM while AUC indicated a similar Kd of 3 μM. Unfortunately, all efforts to directly measure the binding affinity of p190-PH with activated Rac1 were unsuccessful. An alternative competition approach determined that p190-PH has a 10-fold

higher affinity for RhoA than Rac1, therefore suggesting the affinity of p190-PH with activated Rac1 is around 20 μM. Experiments in Chapter 4 will attempt to determine the reasons for this difference in binding affinity; Chapter 5 will look at potential effects of these differences on function.

CHAPTER FOUR

MUTAGENESIS STUDIES – AMINO ACID RESIDUES IMPORTANT FOR THE PH:GTPASE BINDING INTERFACE

INTRODUCTION

A crystal structure of the binding interaction between PRG PH domain and RhoA•GTPγS showed that binding occurred between the switch regions of RhoA and a conserved hydrophobic patch in the PH domain (Chen et al., 2010). Also we have previously shown that mutating key residues in the PH domains of various Lbc RhoGEFs can abolish binding to activated RhoA (Medina et al., 2013). Since p190RhoGEF's PH-domain is capable of binding to both RhoA and Rac1, it would be of interest to examine the amino acids residues found in the PH:GTPase binding interface and elucidate any roles they play in the association of p190-PH and GTPases.

RESULTS

Introducing Mutations to the WT p190 PH-domain Inhibits Binding to RhoA and Rac1 GTPases

Since the PH domain of p190RhoGEF binds to both RhoA and Rac1, the previous defined double mutation (F1154A and I1156A) that was shown to disrupt binding to RhoA (**Figure 3.2**) was tested for its ability to disrupt binding to Rac1. In a pulldown assay similar to Figure 3.1, the mutations in the PH domain of p190 attenuated binding to activated Rac1 as well as activated RhoA (**Figure 4.1**).

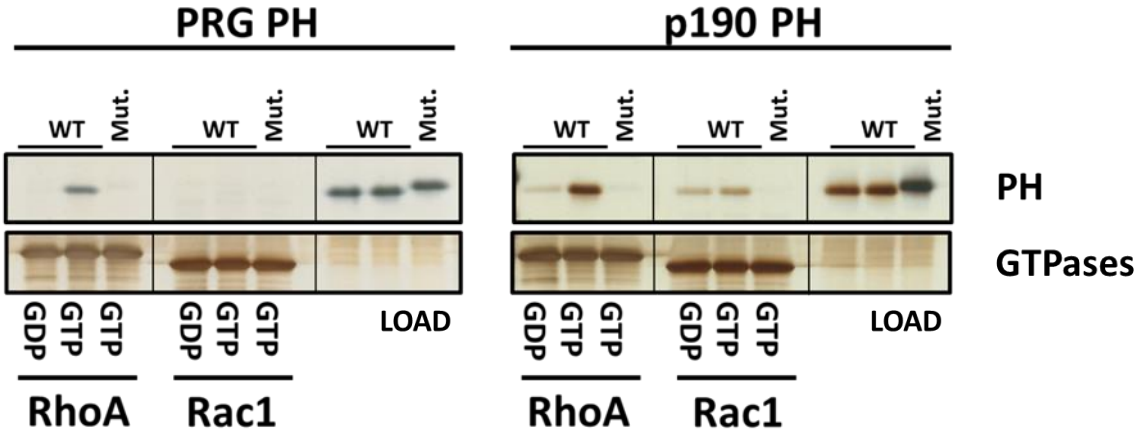

Figure 4.1. Mutations on p190 PH-domain reduces its ability to bind to the active form of RhoA and Rac1. Purified p190 PH domains (WT or F1154A/I1156A mutant) (12 pmol) were incubated with immobilized GST-RhoA or GST-Rac1 (80 pmol) with excess GDP or pre-activated with GTPγS. PH domains bound to the resin was subjected to SDS-PAGE and visualized by silver staining. *Upper bands*, PRG or p190 PH domains. *Lower bands*, GTPases.

Introducing Mutations to RhoA Inhibits Binding to WT PRG PH-domains

With verification of the interface between the GTPases and the p190 PH via mutants (F1154A, I1156A) in the PH domain, a new focus was to identify amino acids on either

GTPase that are important for interacting with the PH domain. In the long run, this could define the structural reasons for differences in affinity between the GTPases. One of my earliest efforts to examine this was done to support the thesis project of my colleague, Frank Medina. The goal was to identify mutations on RhoA that would inhibit its binding to the PH-domain of PRG thereby further verifying the functional interface for binding (Chen et at., 2010). An initial residue targeted was Val43 because Medina had shown that RhoC had a much lower apparent affinity for the PRG PH domain (Medina 2012 thesis) and the only obvious difference in interfacial residues was an isoleucine at position 43 in RhoC rather than a valine. Mutation of the Val to Ile in RhoA generated a protein that was a normal substrate for the PRG RhoGEF relative to WT RhoA (**Figure 4.2**) but with a markedly reduced affinity of the activated GTPase for the PH domain (**Figure 4.3**). A secondary amino acid on RhoA targeted for disruption of binding to the PH domain was Alanine-56. I mutated Ala-56 with amino acids that were either negatively charged (A56E), hydrophobic (A56L), or positively charged (A56R) in the context of the RhoA V43I protein. Only the RhoA V43I/A56L mutant was still a substrate for PRG RhoGEF in a similar fashion to WT RhoA (**Figure 4.4**). When tested for binding to the PH domain of PRG, activated RhoA V43I/A56L showed no ability to interact in a pull-down assay (**Figure 4.3**). Thus, the double mutation clearly attenuates the interaction of RhoA with the PH domain while retaining function as a substrate for the PRG.

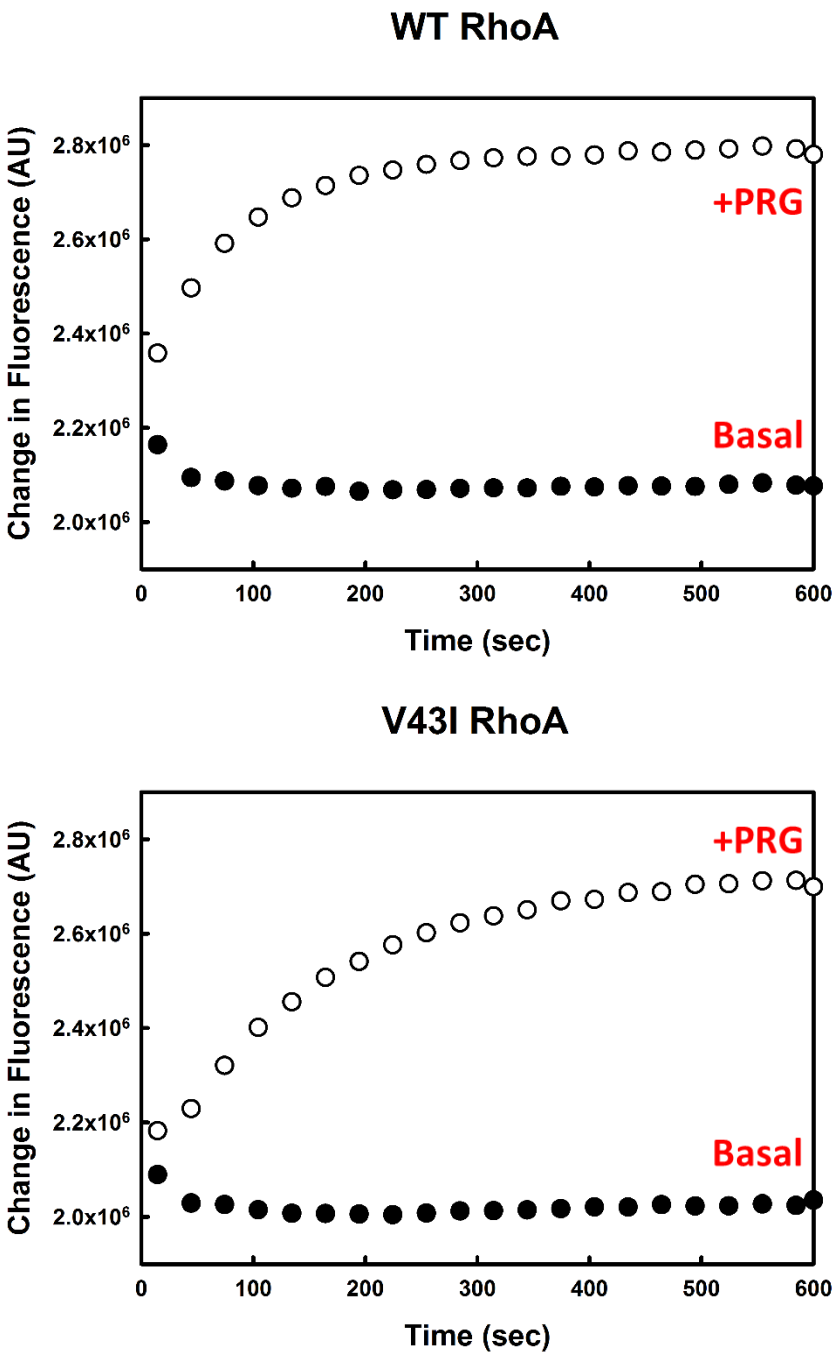

Figure 4.2. Ability of PRG RhoGEF to catalyze exchange of RhoA V43I mutant. Nucleotide exchange assays were performed with 50 nM PRG DH·PH domains (non-His tagged), 2 μM WT RhoA, 2 μM RhoA V43I mutant, and 9.3 μM mant-GTP. Rates of nucleotide exchange reaction were monitored by the increase in mant-GTP fluorescence intensity as a result of its association with GTPases.

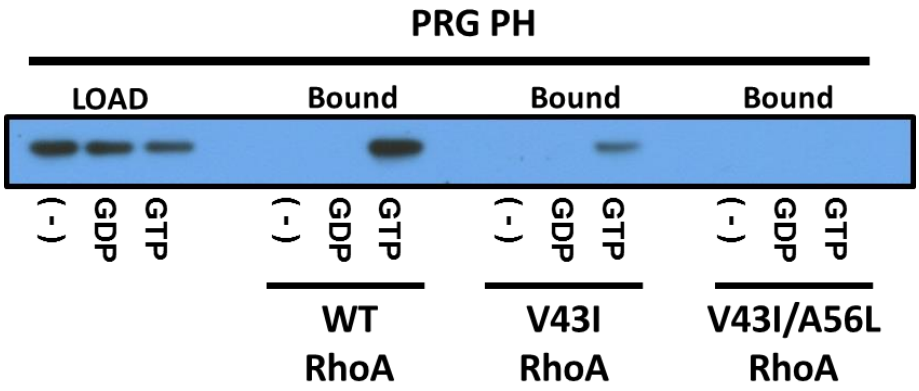

Figure 4.3. Mutating the V43 and A56 amino acids on RhoA alters its binding to the PH-domain of PRG RhoGEF. Purified PRG PH domains (12 pmol) were incubated with immobilized basal or pre-activated GST-RhoA (WT or mutants) (80 pmol) with excess GTPγS. PH domains bound to the resin was subjected to SDS-PAGE and visualized by immunoblotting.

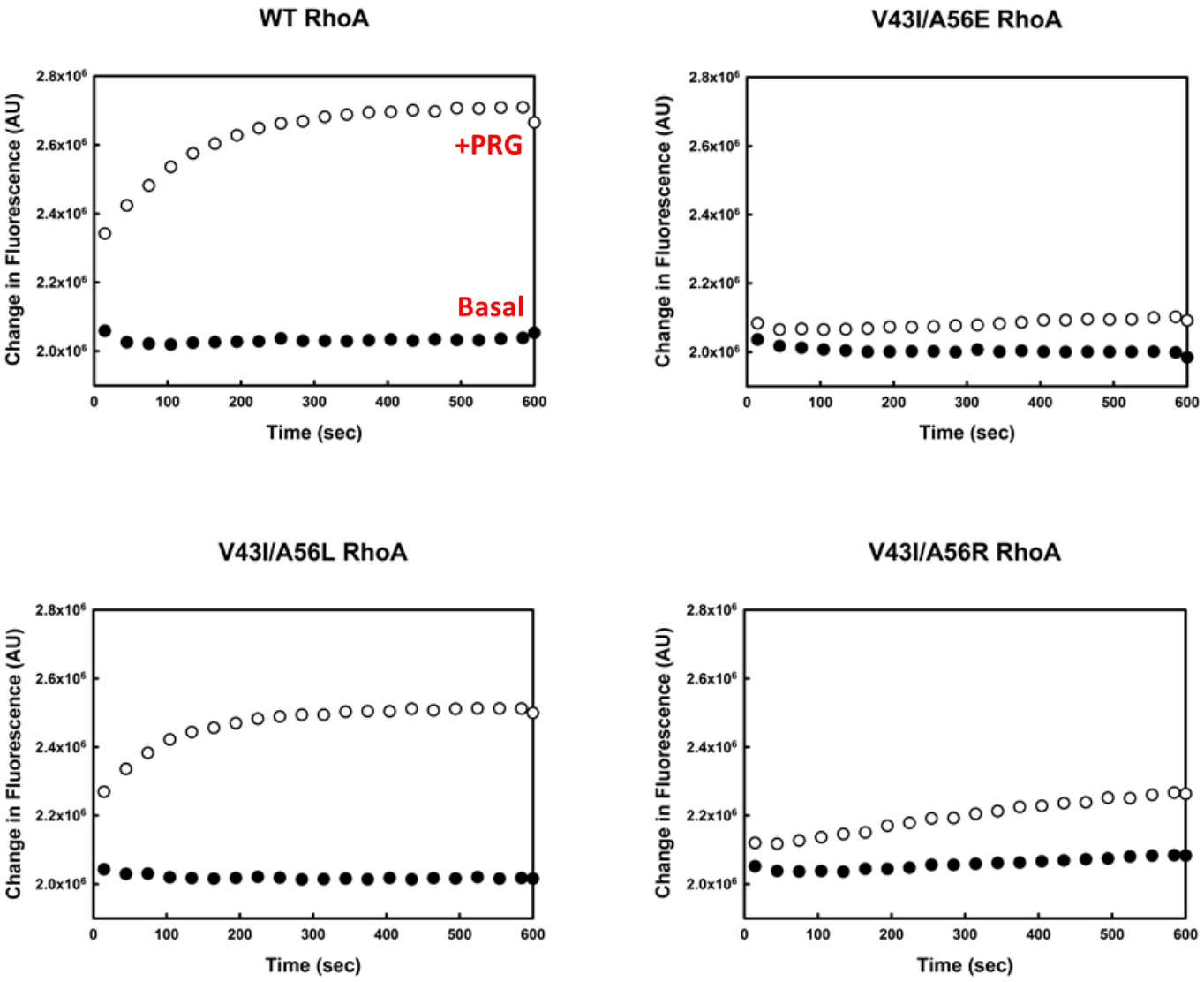

Figure 4.4. Ability of PRG RhoGEF to catalyze exchange of RhoA mutants (V43I/A56E, V43I/A56L, and V43I/A56R). Nucleotide exchange assays were performed with 50 nM PRG DH·PH domains (non-His tagged), 2 μM WT RhoA, 2 μM RhoA mutants (V43I/A56E, V43I/A56L, and V43I/A56R), and 9.3 μM mant-GTP. Rates of nucleotide exchange reaction were monitored by the increase in mant-GTP fluorescence intensity as a result of its association with GTPases.

Introducing Mutations to RhoA Alters its Binding to WT p190 PH domains

Since mutating specific residues on Rho GTPases can reduce its binding interaction with the PH-domain of a RhoGEF, this technique might be useful to create proteins that interact more tightly with the same PH-domain. To test this hypothesis, I utilized the different affinity properties of the isoforms of Rho. As noted above, PRG-PH binds more tightly to RhoA than RhoC (Medina 2012 thesis). In Figure 4.3, I showed that the RhoA V43I mutant bound weaker to the PH-domain of PRG. This loss of interaction was also seen with PH domain of p190RhoGEF (**Figure 4.5**). Interesting, the mirror mutation in RhoC, RhoC I43V, produced a mutant GTPase that increased its binding to p190-PH compared to WT RhoC (**Figure 4.5**).

Introducing "Rac1-like" Mutations on RhoA Inhibits Binding to p190-PH and PRG-PH

Since complementary mutagenesis of Rho isoforms can alter binding of the GTPases to the PH domain of p190RhoGEF, I employed this technique to identify key amino acids on RhoA and Rac1 that might explain their differences in binding affinity to p190. The crystal structures (**Figure 3.6**) from my colleague, Dr. Zhe Chen, provided us with targets to mutate to help answer this question. There are many residues that interact with the PH domain of p190 that are identical between RhoA and Rac1. They include Val-38 and Phe-39 from switch I of RhoA, two leucine residues (Leu-69 and Leu-72) from switch II of RhoA, as well

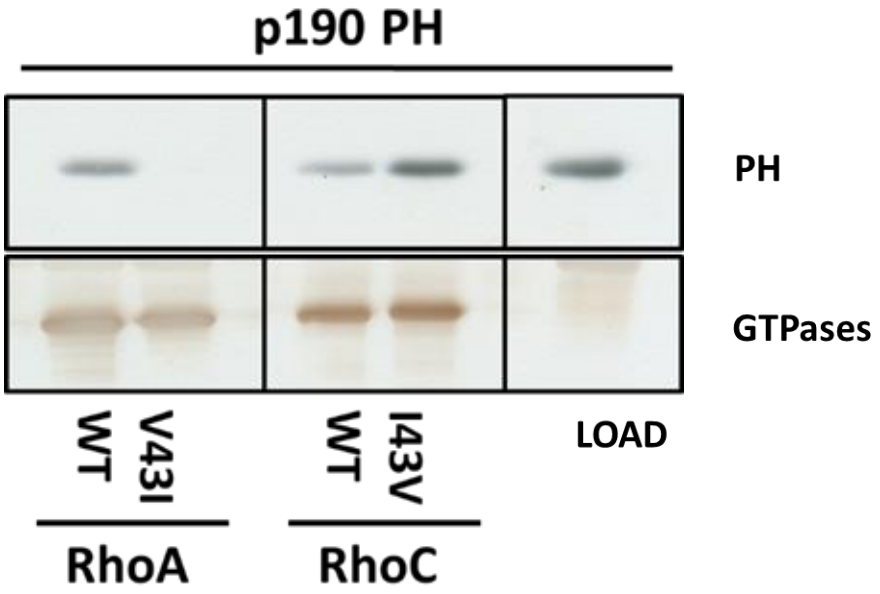

Figure 4.5. Mutating the V43 amino acid on RhoA or RhoC alters its binding to the PH-domain of p190 RhoGEF. Purified p190 PH domains (40 pmol) were incubated with immobilized pre-activated GST-RhoA or RhoC (WT or mutants) (80 pmol) with excess GTPγS. PH domains bound to the resin was subjected to SDS-PAGE and visualized by silver staining. *Upper bands,* p190 PH domain. *Lower bands,* GTPases.

as a tryptophan residue (Trp-58) from the β3 strand of RhoA preceding switch II. The corresponding set of residues in Rac1, including Val-36 and Phe-37 from switch I, two leucine residues (Leu-67 and Leu-70) from switch II, and Trp-56 from β3 (**Figure 4.6**). However, glutatmate-40 in RhoA, whose side chain forms two salt bridges with the extending side chain of Arg-1144 from p190-PH, is replaced by an aspartic acid in Rac1, which alters this interaction, presumably due to its reduced length. I cloned and purified a RhoA E40D mutant to test whether the loss of the salt bridges (mimicking Rac1) would weaken its binding affinity to p190-PH. My colleague, Stephen Gutowski, utilized a FRET Competition assay, as in Figure 3.12, to test this hypothesis. Surprisingly, mutating the glutamate residue to aspartic acid did not reduce RhoA's binding affinity to p190 compared to WT RhoA (**Figure 4.7**). To make RhoA more like Rac1, the amino acids E40, V43, and A56 of RhoA were altered to the corresponding amino acids (D, S and G) of Rac1, respectively. All three mutations combined resulted in only a 2-fold reduction in affinity of RhoA to p190 PH (**Figure 4.8**, **quantified in Figure 4.9**).

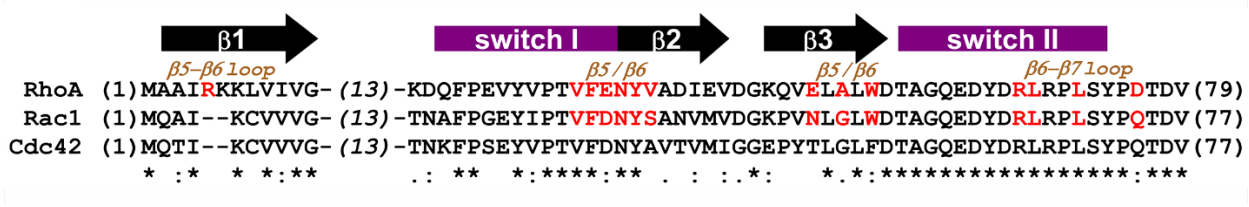

Figure 4.6. Residues involved in the p190-PH:GTPase binding interface. Partial sequence alignment of Rho family small GTPases. The two flexible switch elements are indicated by purple blocks on top. Residues in RhoA or Rac1 that are involved in contacts with the p190 PH domain are colored red. Corresponding secondary elements in p190-PH contacting these residues are labeled in italic and colored brown on top of the residues. *Figure modified from Dr. Zhe Chen.*

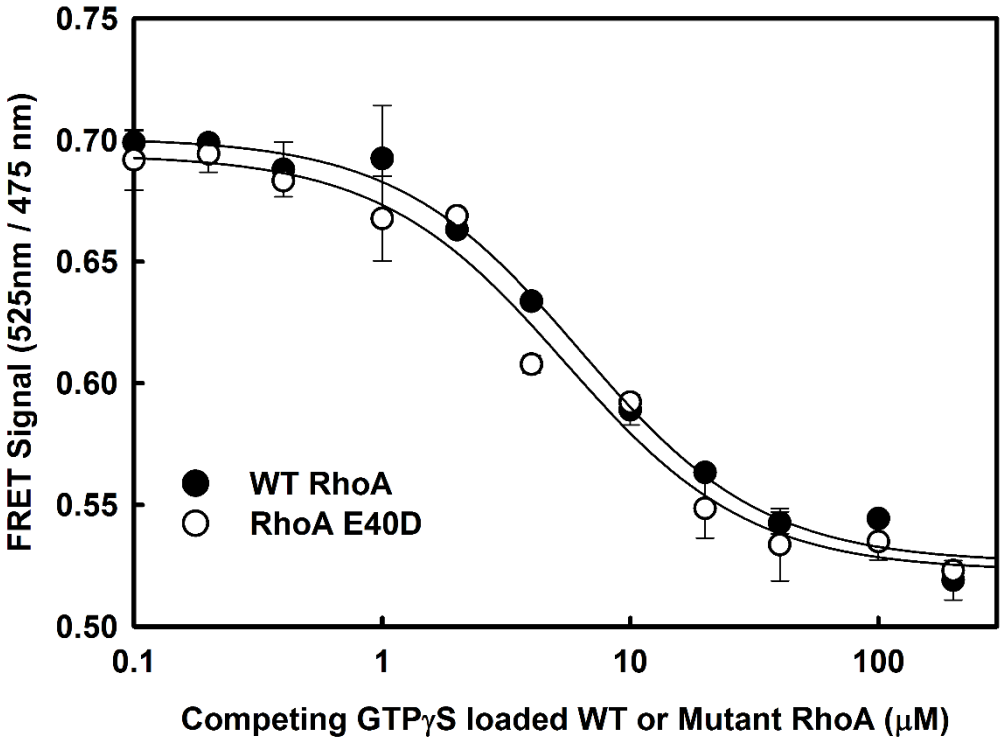

Figure 4.7. Single mutation on RhoA does not reduce its ability to bind to the p190 PH domain. *Stephen Gutowski performed this experiment.* The ability of non-tagged wild-type RhoA•GTPγS or mutant (E40D) RhoA•GTPγS to bind to the p190-PH-domain was measured by competition of FRET produced by binding of 2 μM YFP-RhoA•GTPγS to 2 μM CFP-p190-PH.

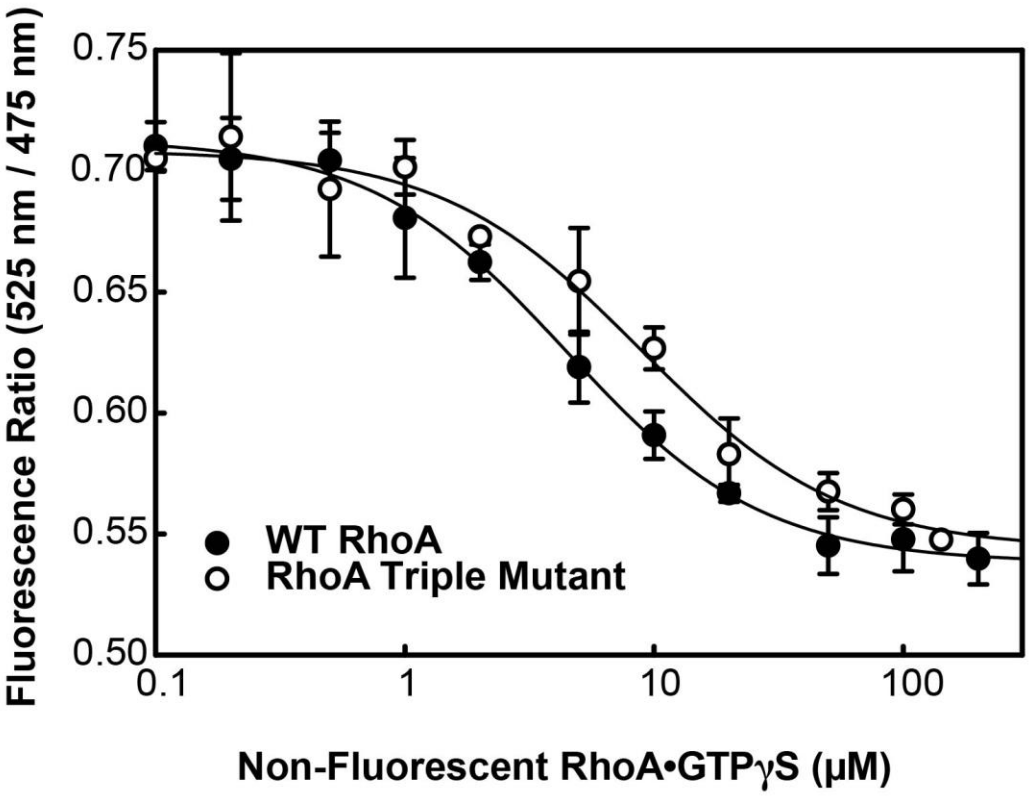

Figure 4.8. Mutations on RhoA reduce its ability to bind to the p190 PH domain. *Stephen Gutowski performed this experiment.* The ability of non-tagged wild-type RhoA•GTPγS or mutant (E40D/V43S/A56G) RhoA•GTPγS to bind to the p190 PH domain was measured by competition of FRET produced by binding of 2 μM YFP-RhoA•GTPγS to 2 μM CFP-p190-PH. Error bars in panel are the standard deviation of 3 independent experiments.

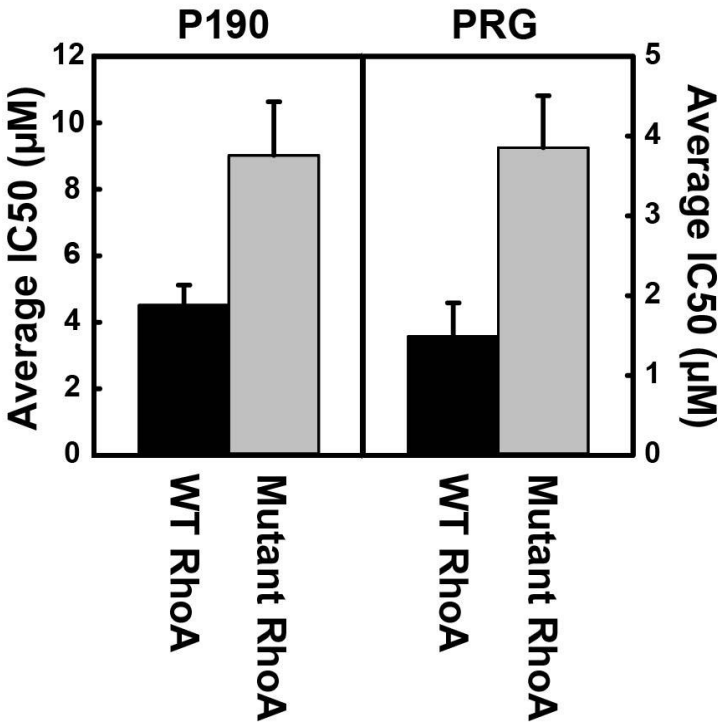

Figure 4.9. Mutations on RhoA reduce its ability to bind to the p190 and PRG PH domain. The relative ability of non-tagged wild-type RhoA•GTPγS or mutant (E40D/V43S/A56G) RhoA•GTPγS to bind to the p190-PH-domain or PRG-PH-domain was measured by competition of FRET. IC50 values were averaged from 3 separate titrations of each Rho; error bars are std dev.

Introducing "RhoA-like" Mutations on Rac1 Does Not Improve its Binding to p190-PH or PRG-PH

To identify residues on Rac1 that contribute to its weaker binding affinity to p190-PH compared to RhoA, I utilized a gain of function mutagenesis approach to generate Rac1 mutants that mimic RhoA. The first Rac1 residues I targeted were Ser-41 and Gly-54, residues that interact with p190-PH (**Figure 4.6**), but are found in a secondary periphery binding interface. I cloned and purified Rac1 mutants (S41V, G54A, and S41V/G54A) whose amino acids were to switched to the corresponding amino acids in RhoA. The introduced mutations did not increase Rac1 binding to p190-PH when tested in pulldown assays (**Figure 4.10**), isothermal titration calorimetry (**Figure 4.11**), or binding to PRG-PH by FRET competition assay (**Figure 4.12**). As mentioned above, Rac1 makes fewer salt bridge connections to the Arg-1140 residue on the p190 PH domain due having aspartic acid at position 38, while the equivalent RhoA residue is a glutamic acid. Therefore I cloned and purified a Rac1 D38E mutant and tested for change in binding affinity to p190-PH by isothermal titration calorimetry. Introducing this mutation to Rac1 did not improve the ability to measure any clear affinity to p190 (**Figure 4.13**).

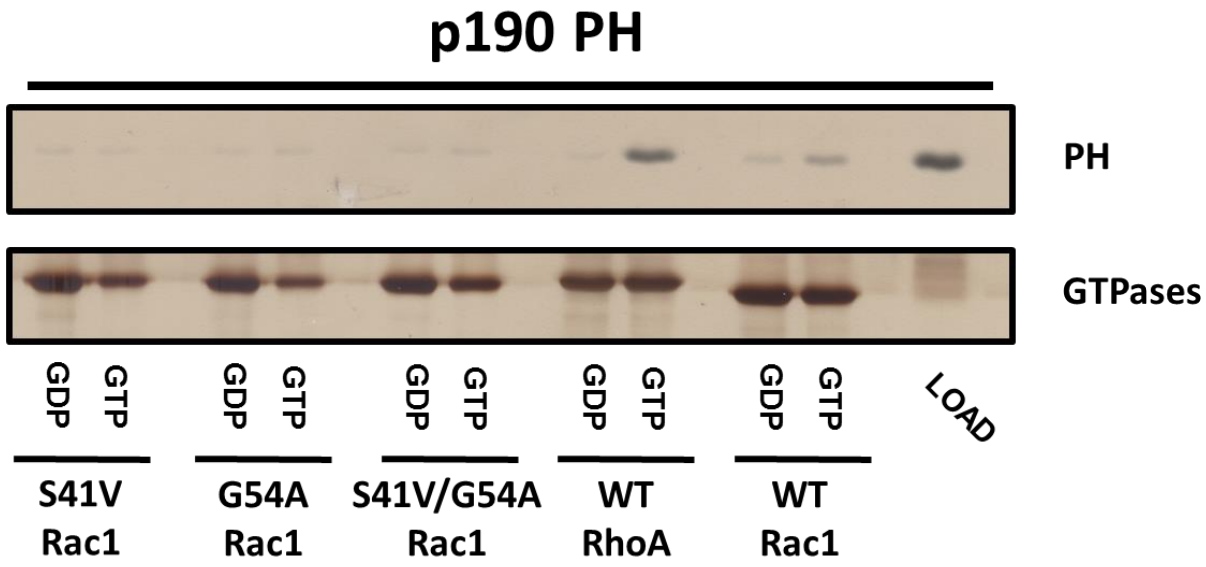

Figure 4.10. Mutating the S41 and G54 amino acids on Rac1 does not improve its binding to the PH-domain of p190 RhoGEF. Purified p190 PH domains (12 pmol) were incubated with immobilized pre-activated mutant GST-Rac1 (S41V, G54A, S41V/G54A) (80 pmol), wild-type GST-RhoA (80 pmol), and wild-type GST-Rac1 (80 pmol) with excess GTPγS. PH domains bound to the resin was subjected to SDS-PAGE and visualized by silver staining. *Upper bands,* p190 PH domain. *Lower bands,* GTPases.

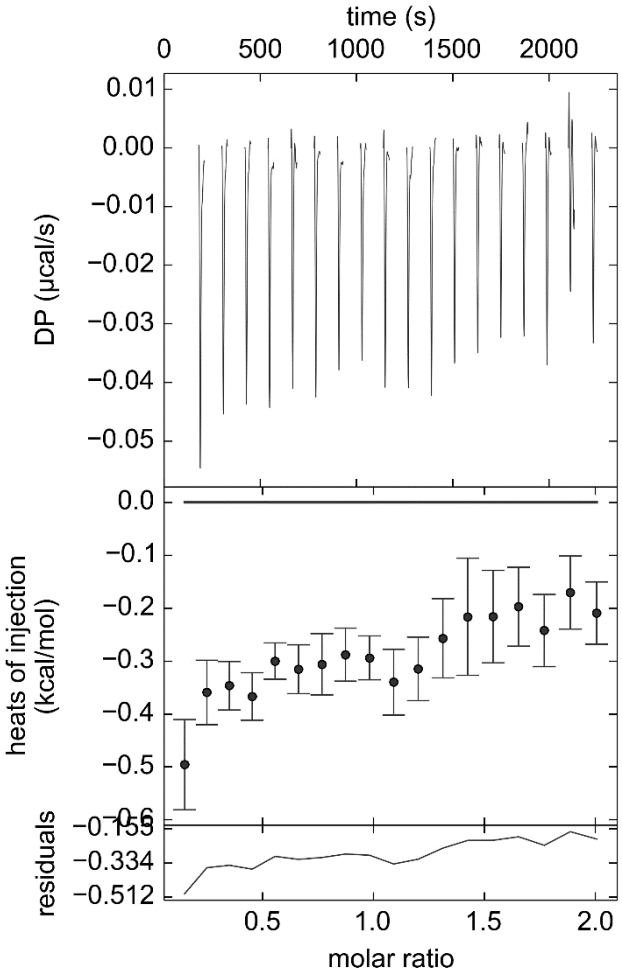

Figure 4.11. ITC - p190 PH and Rac1 S41V/G54A-GTPγS. Measurement of binding affinity between the p190 PH domain and mutant (S41V/G54A) Rac1•GTPγS by isothermal titration calorimetry. Error bars represents the uncertainty of each individual integrated isotherm peak assigned by the program, NITPIC.

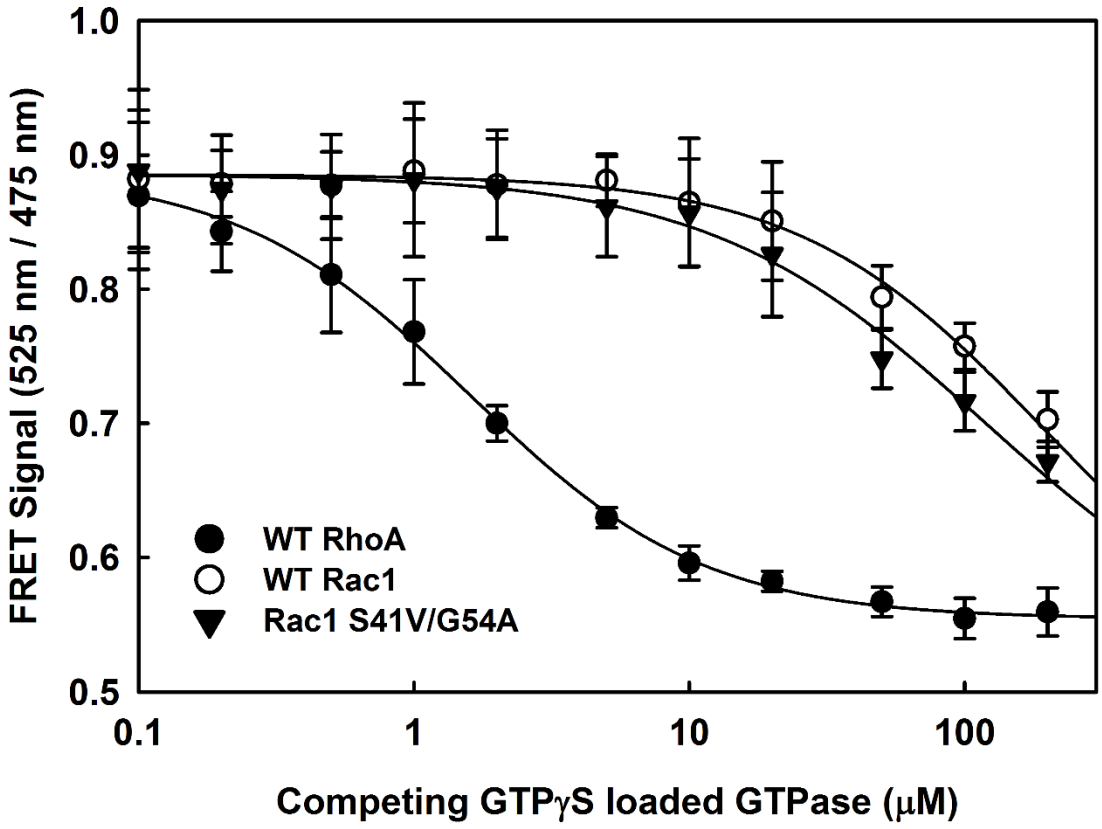

Figure 4.12. Double mutation on Rac1 does not improve its ability to bind to the PRG PH domain. *Stephen Gutowski performed this experiment.* The ability of non-tagged wild-type RhoA•GTPγS, wild-type Rac1•GTPγS, or mutant (S41V/G54A) Rac1•GTPγS to bind to the PRG-PH-domain was measured by competition of FRET produced by binding of 1 μM YFP-RhoA•GTPγS to 1 μM CFP-PRG-PH.

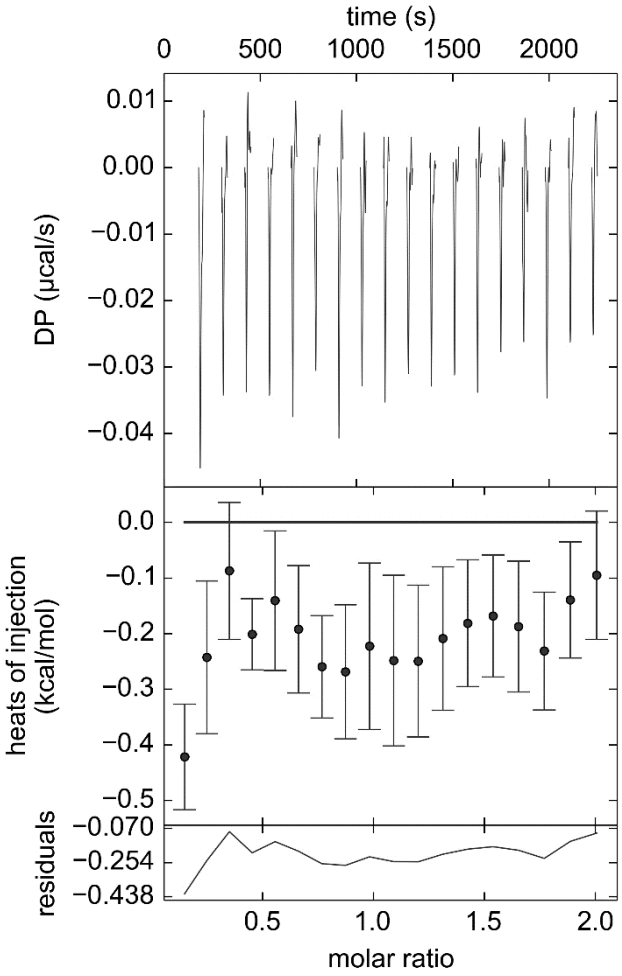

Figure 4.13. ITC - p190 PH and Rac1 D38E-GTPγS. Measurement of binding affinity between the p190 PH domain and mutant (D38E) Rac1•GTPγS by isothermal titration calorimetry. Error bars represents the uncertainty of each individual integrated isotherm peak assigned by the program, NITPIC.

DISCUSSION

p190RhoGEF is capable of binding to the active forms of RhoA and Rac1 GTPases through its PH domain, with our crystal structures demonstrating they share a common binding motif. My mutagenesis efforts targeting key amino acid residues on the PH-domain of p190 abolished the binding of the PH-domain to both RhoA and Rac1 in my protein binding assays; this confirms the shared common binding interface between the PH-domain and both GTPases. The result also demonstrates that p190's PH domain can only be occupied by either RhoA•GTPγS or Rac1•GTPγS, but not both proteins at the same time. Cells generate distinct zones of activated GTPases to regulate various cellular functions (Etienne-Manneville and Hall, 2002), therefore p190's interaction with either RhoA or Rac1 might be determined by which GTPase is present in its local environment. Yet the lack of identifying a p190-PH mutant that selectively binds either RhoA or Rac1 would make it challenging to test this hypothesis in future in-vivo experiments.

Mutating specific residues on GTPases can alter their binding affinity to the PH domain of RhoGEFs. In the case with RhoA, introducing single point mutations either abolished or improved binding of the GTPase to the PH domains of PRG and p190RhoGEF. Crystal structures of PRG RhoGEF (Chen et al., 2010) and p190RhoGEFs have identified numerous residues on RhoA that interact with the PH domain. One such residue was Val-43, mutating it to isoleucine reduced binding to PH domains of both RhoGEFs, although it had a stronger affect on p190 compared to PRG. This could be due to the higher affinity PRG has

for RhoA compared to p190, in fact multiple mutations were necessary to achieve a complete loss of binding, as seen in the RhoA V43I/A56L pulldown assay. Surprisingly, mutating residue 43 on RhoC to mimic RhoA (RhoC I43V) was sufficient to increase its binding affinity to p190-PH. While RhoA and RhoC share many amino acids that contact the PH domain (Medina 2012 thesis), mutating key residues that interact with the core binding region of the PH domain could explain observed differences in affinities.

While mutation of single residues can explain differences in binding affinity between Rho isoforms, mutagenesis efforts to explain Rac1's 10-fold weaker binding affinity for p190-PH compared to RhoA were more challenging. Based on our crystal structures, one of the targets focused on differences in interaction by RhoA and Rac1with the PH domain residue Arg-1140, located in the conserved core binding interface. The Glu-40 residue on RhoA makes multiple salt bridge connections to Arg-1140, while its equivalent Asp-38 residue on Rac1 does not. Yet changing this residue in RhoA, RhoA E40D, does not generate a loss of function mutant based on retention of binding affinity towards p190-PH in our FRET competition assay. Only the introduction of two other "Rac1-like" mutations on RhoA (RhoA E40D/V43S/A56G) gave a modest 2-fold reduction in binding affinity. All efforts to introduce gain of function mutations in Rac1 to improve its binding affinity to p190-PH were not successful. It possible that only a more extensive mutagenesis effort on Rac1, examining every residue target revealed by our crystal structures, would identify crucial residues capable of increasing binding affinity. Alternatively, any observed affinity differences between RhoA and Rac1 proteins are influenced by factors such as: differences in electrostatic potentials on the GTPase's binding surface (Chen et al., 2010), changes of

position of p190-PH as it interacts with different GTPases, and differences in the surface areas of the PH:GTPases binding interface (manuscript in revision), things that cannot be resolve by simple residue mutagenesis. All of my mutagenesis efforts to alter the binding affinity of both RhoA and Rac1 GTPases to the PH domain of RhoGEFs are summarized in (**Table 4.1**).

Mutant GTPase	Rational	Result(s)
RhoA V43I **RhoA V43I/A56L**	Mutating key amino acids on RhoA identified to be important for binding to PH-domain based on published crystal structure (Chen et al., 2010).	Decreased or loss of binding to PRG-PH in pulldown assay.
RhoC I43V	Mutation on RhoC makes it more like RhoA, based on XPLN GEF paper (Arthur et al., 2002).	Increased binding to p190-PH in pulldown assay.
RhoA E40D	Mutation on RhoA makes it more like Rac1, because Rac1 D38 has fewer salt bridge connections to Arg-1140 on p190 PH domain.	No difference in relative IC50 with mutant RhoA vs WT RhoA in FRET Competition Assay.
RhoA E40D/V43S/A56G	Mutating multiple key amino acids on RhoA to make it more like Rac1.	Two-fold decrease in relative IC50 with triple mutant RhoA vs WT RhoA in FRET Competition Assay.
Rac1 S41V **Rac1 G54A** **Rac1 S41V/G54A**	Mutating the secondary periphery binding site on Rac1 to be like RhoA.	Saw no improvement in binding to WT p190 PH in pulldown assay vs WT Rac1. Tested in ITC with WT p190 PH, data suggest very weak binding, similar Rac1 D38E result. No significant difference in relative IC50 with double mutant Rac1 vs WT Rac1 in FRET Competition Assay.
Rac1 D38E	Mutation on Rac1 makes it more like RhoA, because RhoA E40 makes four salt bridge connections to Arg-1140 on the p190 PH domain.	Tested in ITC with WT p190 PH, data suggest very weak binding.

Table 4.1 Summary of all RhoA and Rac1 Mutagenesis Studies.

CHAPTER FIVE

IDENTIFYING MECHANISMS OF P190RHOGEF REGULATION BY ACTIVATED RHOA AND RAC1

INTRODUCTION

A simple explanation for the binding of activated GTPases to RhoGEFs would be allosteric feedback regulation of the GEF's activity. Activated Ras does produce direct feedback regulation of its GEF, Son of Sevenless (SOS). Ras-GTP binds to SOS at a distinct site separate from that mediating nucleotide exchange activity. The interaction with Ras-GTP increases the exchange activity of the GEF on Ras by displacing an autoinhibitory region in the RasGEF domain (Margarit et al., 2003). Therefore, one potential regulatory mechanism invoked by the interaction of activated Rac1 and RhoA with p190RhoGEF could be to directly influence the intrinsic nucleotide exchange activity of RhoGEF. Translocation is believed to be another mechanism for regulation of RhoGEFs by bringing the protein into close proximity with its substrate GTPases. This would involve localization of the RhoGEF to the plasma membrane, the presumed location of its geranylgeranylated substrate, free RhoA (Bhattacharyya et al., 2009; Medina et al., 2013). A former colleague, Dr. Angela Carter, has demonstrated that chemically induced localization of an RGS-RhoGEF to the plasma membrane can lead to activation of RhoA on a timescale and magnitude similar to ligand-induced stimulation (Carter et al., 2014). Therefore, another

focus of this chapter will be to examine whether recruitment of p190RhoGEF to the plasma membrane is a viable regulatory mechanism for RhoA activation.

RESULTS

Specificity of p190RhoGEF's Exchange Activity on Various GTPase Substrates (RhoA, Rac1, and Cdc42)

p190RhoGEF has been characterized in the literature as a specific exchange factor for RhoA, but not Rac1 or Cdc42 (van Horck et al., 2001). I set out to verify this finding by making the DH/PH domains of p190 and examining its activity by measuring the association of mant-GTP to various GTPases. The isolated DH/PH domain of p190 was capable of facilitating the exchange of the fluorescently labeled nucleotide unto RhoA GTPase, thus confirming the observations as described in the literature. Interestingly, compared to the isolated DH/PH domain of PRG (50 nM), an Lbc family RhoGEF extensively studied by my colleague Frank Medina, the nucleotide exchange capability of p190 DH/PH (1 μM) was much less effective. P190 DH/PH in these experiments was unable to facilitate exchange on either Cdc42 or Rac1 GTPases (**Figure 5.1**). The ability of a Cdc42-selective GEF (50 nM Dbs) and Rac1-specific GEF (1 μM Tiam1) provides controls to demonstrate the viability of the cdc42 and Rac1.

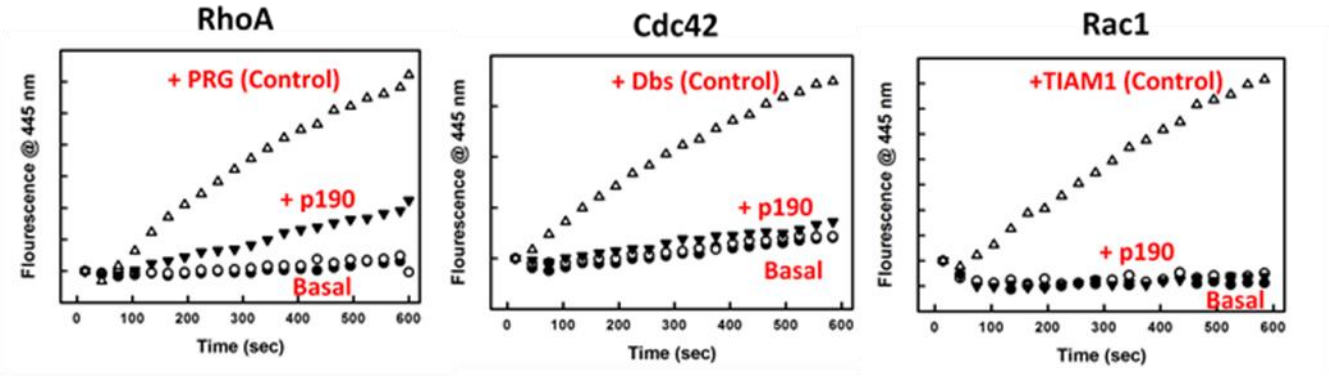

Figure 5.1. p190 RhoGEF is specific for RhoA. Nucleotide exchange assays were performed with 1μM p190 DHPH domains (non-His tagged) and 1μM GTPases (RhoA, Cdc42, Rac1). Rates of nucleotide exchange reaction were monitored by the increase in mant-GTP fluorescence intensity as a result of its association with GTPases. The DHPH domains of PRG (50 nM), Dbs (50 nM), and TIAM1 (1μM) were used as positive controls for RhoA, Cdc42, and Rac1 respectively.

Activated RhoA and Rac1 Facilitates Stimulation of RhoA by p190RhoGEF in a Membrane-delimited System

Binding of some PH domains to phospholipids has been proposed as a mechanism to localize Dbl family RhoGEFs to the plasma membrane. However, the PH domains of Dbl family members bind with low affinity to phospholipids, implying that these interactions are insufficient for membrane localization (Rossman et al., 2005). This begs the question, are there other binding partners on the membrane that are necessary for localization of RhoGEFs to the plasma membrane? Recent papers from former colleagues in my lab have shown that receptors, certain G-protein subunits, and activated GTPases can serve as important binding partners for the localization of RhoGEFs to the plasma membrane (Carter et al., 2014. Medina et al., 2013; Pascoe et al., 2015). In the case of active GTPases, my colleague, Frank Medina, devised an *in-vitro* phospholipid vesicle assay in which only activated RhoA bound to the vesicle surface stimulated nucleotide exchange by the PRG RhoGEF on RhoA bound to the vesicle surface; soluble activated RhoA provided no stimulation (Medina et al., 2013).

Therefore, I hypothesized that I would observe a similar effect with both activated RhoA and Rac1 towards p190RhoGEF when I used substrate localized to phospholipid vesicles. I utilized the assay as described (Medina et al., 2013) to measure exchange of mant-GDP from RhoA by the DH-PH domain of p190 in the absence or presence of activated Rac1 and RhoA sequestered to the phospholipid membrane. The addition of pre-activated RhoA.H6 to the phospholipid vesicles led to an increase in p190's exchange activity on

RhoA-mantGDP compared to p190 GEF alone (**Figure 5.2**). A similar effect was also observed when pre-activated Rac1.H6 was added to phospholipid vesicles, albeit not as dramatic as with RhoA.H6 (**Figure 5.2**).

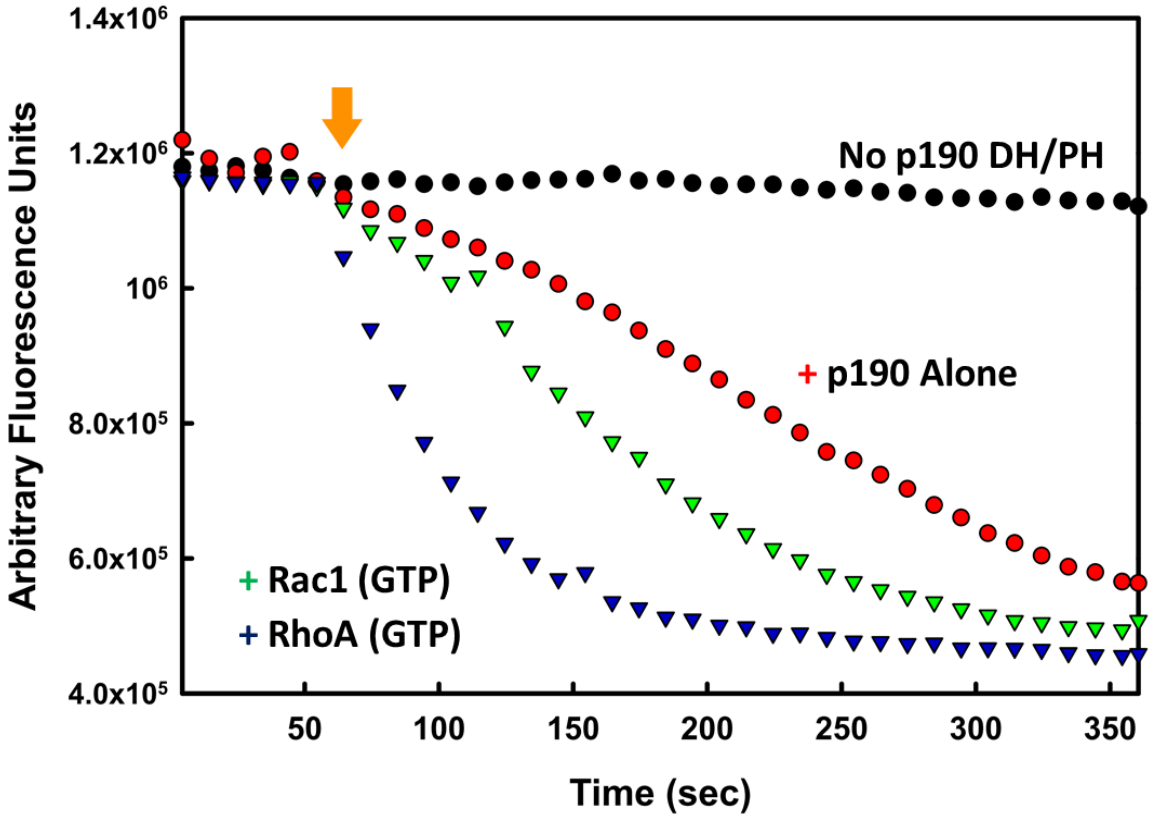

Figure 5.2. Binding of p190 RhoGEF to activated RhoA and Rac1 on phospholipid vesicles enhances nucleotide exchange activity. Nucleotide exchange assays were performed with nickel-containing phospholipid vesicles, p190 DHPH domains (non-His tagged), His-tagged mant-GTP RhoA, His-tagged pre-activated RhoA, and His-tagged pre-activated Rac1. Rates of nucleotide exchange reaction were monitored by the decrease in mant-GTP fluorescence intensity as a result of its dissociation from RhoA.

The difference in efficacy between membrane localized RhoA and Rac1 is compared by titration of the pre-activated GTPases over a range of 16-4000 nM. Both pre-activated GTPases stimulated p190's GEF activity on RhoA-mantGDP substrate in a dose dependent manner (**Figure 5.3 and 5.4**).

I next determined if the active state of the GTPases was necessary for its ability to enhance the nucleotide exchange activity of p190RhoGEF on membrane associated substrate. To test this, I again performed nucleotide exchange assays, but this time I added equal concentrations of either the pre-activated or basal RhoA.H6 and Rac1.H6 to the cuvette. In these experiments, I observed no obvious difference in the exchange rate of p190RhoGEF in the presence of the inactive form of Rac1 bound to the vesicles compared to GEF alone. A robust increase in exchange rate was observed when the active form of Rac1, sequestered to the vesicle, was introduced into the assay (**Figure 5.5**). Similar results were seen with RhoA; only the active form of RhoA stimulated p190's nucleotide exchange activity on its substrate (**Figure 5.6)**.

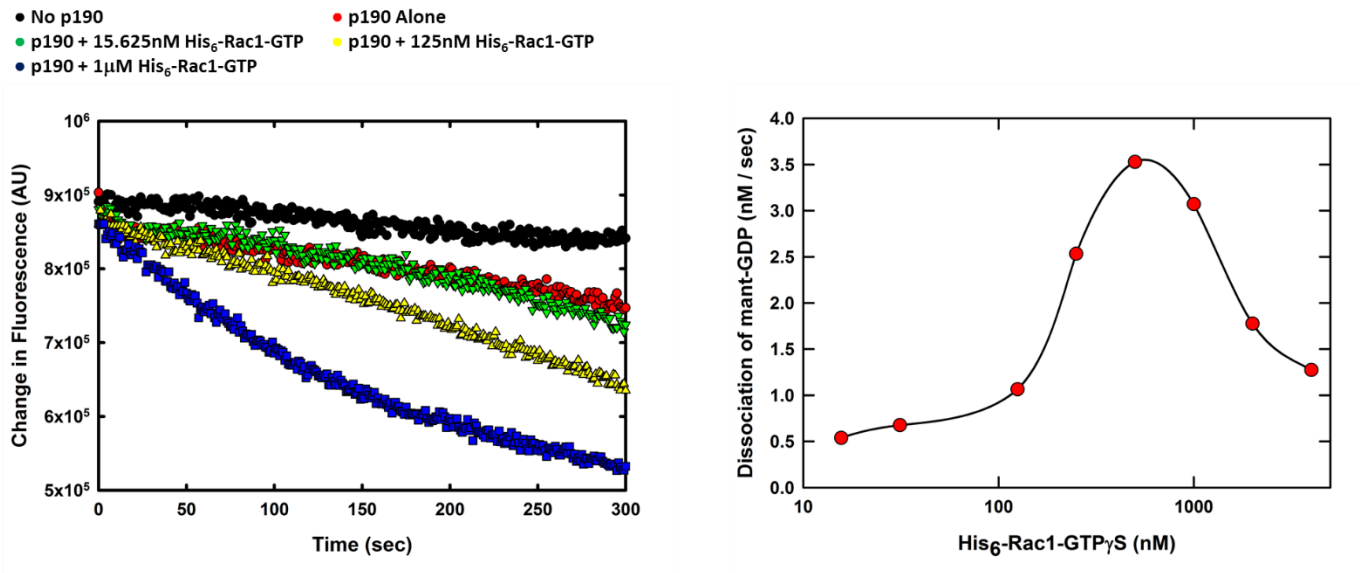

Figure 5.3. The active form of Rac1 can enhance the GEF activity of p190 RhoGEF in a dose-dependent manner. Nucleotide exchange assays were performed with nickel-containing phospholipid vesicles, 250 nM WT p190 DHPH domains (non-His tagged), 1 μM His-tagged RhoA(mant-GDP), 16 nM – 4 μM His-tagged pre-activated Rac1. Nucleotide exchange was monitored by the decrease of fluorescence in mant-GDP as it dissociated from RhoA. *Left panel*) Select concentrations - fluorometer data. *Right Panel*) Initial rates for dissociation of mant-GDP plotted against the concentration of His-tagged pre-activated Rac1 bound to vesicle. Log10 scale.

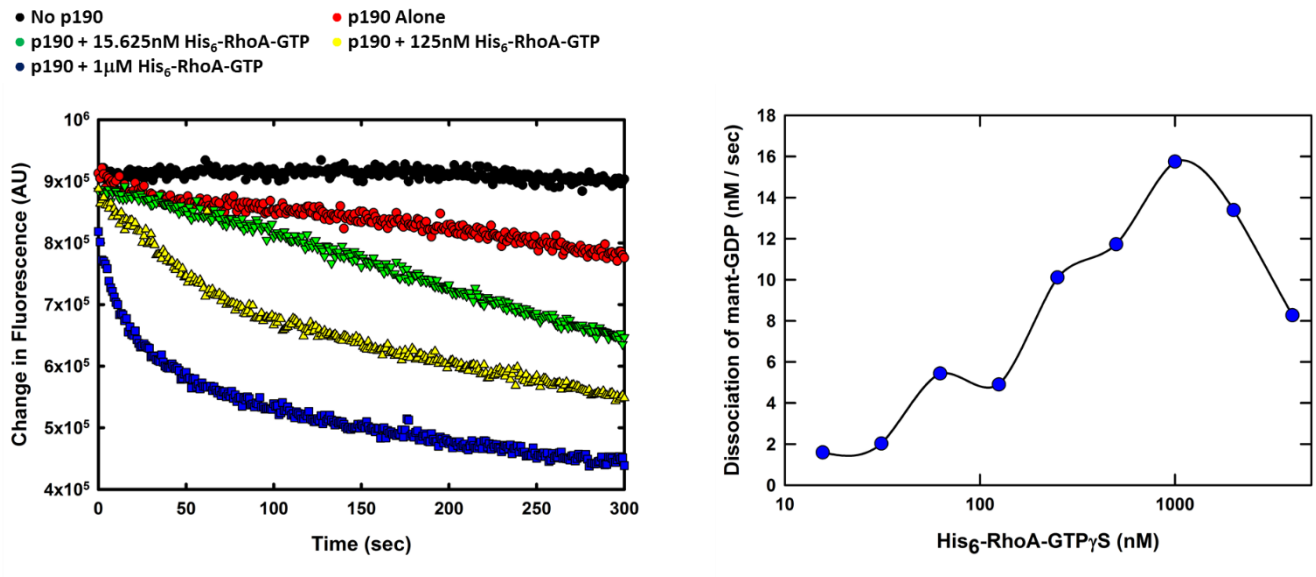

Figure 5.4. The active form of RhoA can enhance the GEF activity of p190 RhoGEF in a dose-dependent manner. Nucleotide exchange assays were performed with nickel-containing phospholipid vesicles, 250 nM WT p190 DHPH domains (non-His tagged), 1 μM His-tagged RhoA(mant-GDP), 16 nM – 4 μM His-tagged pre-activated RhoA. Nucleotide exchange was monitored by the decrease of fluorescence in mant-GDP as it dissociated from RhoA. *Left panel*) Select concentrations - fluorometer data. *Right Panel*) Initial rates for dissociation of mant-GDP plotted against the concentration of His-tagged pre-activated RhoA bound to vesicle. Log10 scale.

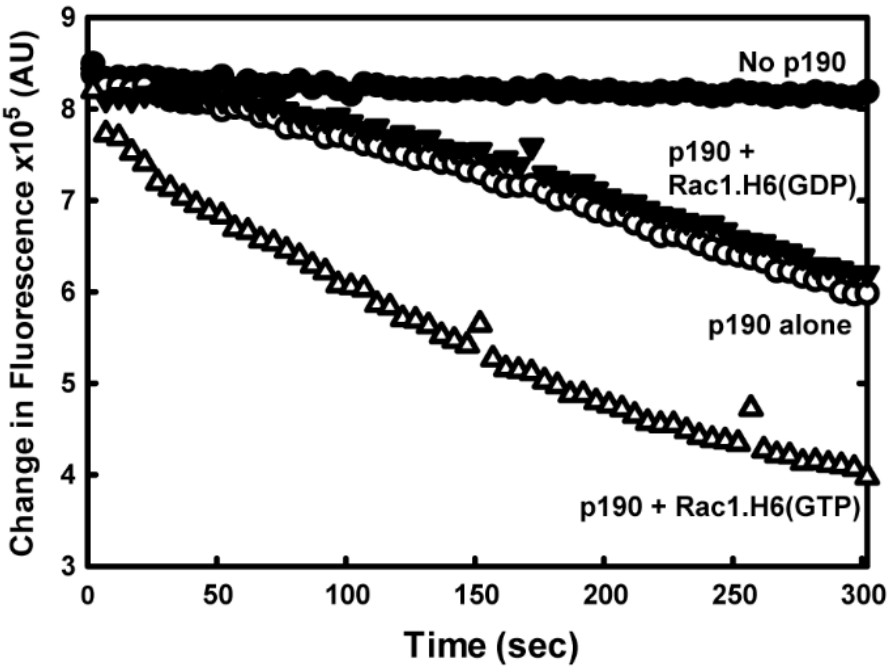

Figure 5.5. The active form of Rac1 can enhance the nucleotide exchange activity of p190 RhoGEF on RhoA associated with phospholipid vesicles. Nucleotide exchange assays were performed with nickel-containing phospholipid vesicles, 250 nM WT p190 DHPH domains (non-His tagged), 1 μM His-tagged RhoA(mant-GDP), 100 nM His-tagged pre-activated Rac1 or 100 nM His-tagged non-active Rac1. Nucleotide exchange was monitored by the decrease of fluorescence in mant-GDP as it dissociated from RhoA.

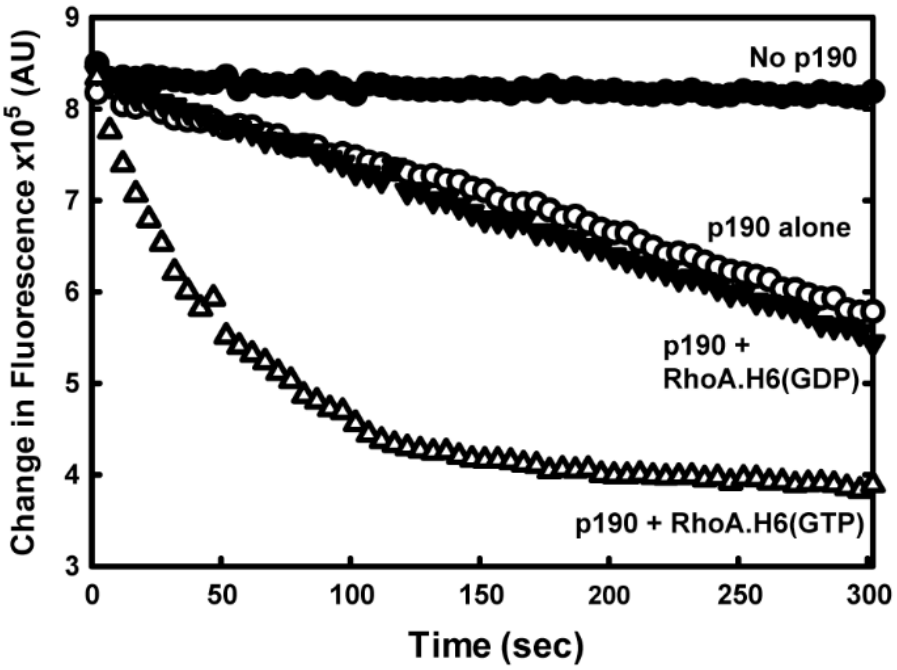

Figure 5.6. The active form of RhoA can enhance the nucleotide exchange activity of p190 RhoGEF on RhoA associated with phospholipid vesicles. Nucleotide exchange assays were performed with nickel-containing phospholipid vesicles, 250 nM WT p190 DHPH domains (non-His tagged), 1 μM His-tagged RhoA(mant-GDP), 100 nM His-tagged pre-activated RhoA, or 100 nM His-tagged non-active RhoA. Nucleotide exchange was monitored by the decrease of fluorescence in mant-GDP as it dissociated from RhoA.

Mutating the PH-domain of p190RhoGEF Attenuates GEF Activity on RhoA Facilitated by Activated GTPases in a Membrane-delimited System

Pulldown data have shown that mutations of key residues in the PH-domain of p190RhoGEF attenuated its binding to pre-activated RhoA and Rac1 (**Figure 4.2**). For these set of phospholipid vesicle experiments I used binding of mant-GDP to assess rates of nucleotide exchange on RhoA. Therefore, the increase in mant-GDP fluorescence intensity as a result of its association with RhoA was monitored. Mutating the same key residues in the DH-PH domain of p190 inhibited any increased GEF activity on RhoA in the presence of activated Rac1 localized to the vesicle membrane compared to the WT p190 DH-PH domains (**Figure 5.7**). Similar results were seen when activated RhoA was localized to the vesicle membrane, that is, GEF stimulation was lost with the mutant p190 DH-PH GEF compared to WT p190 DH-PH GEF (**Figure 5.8**).

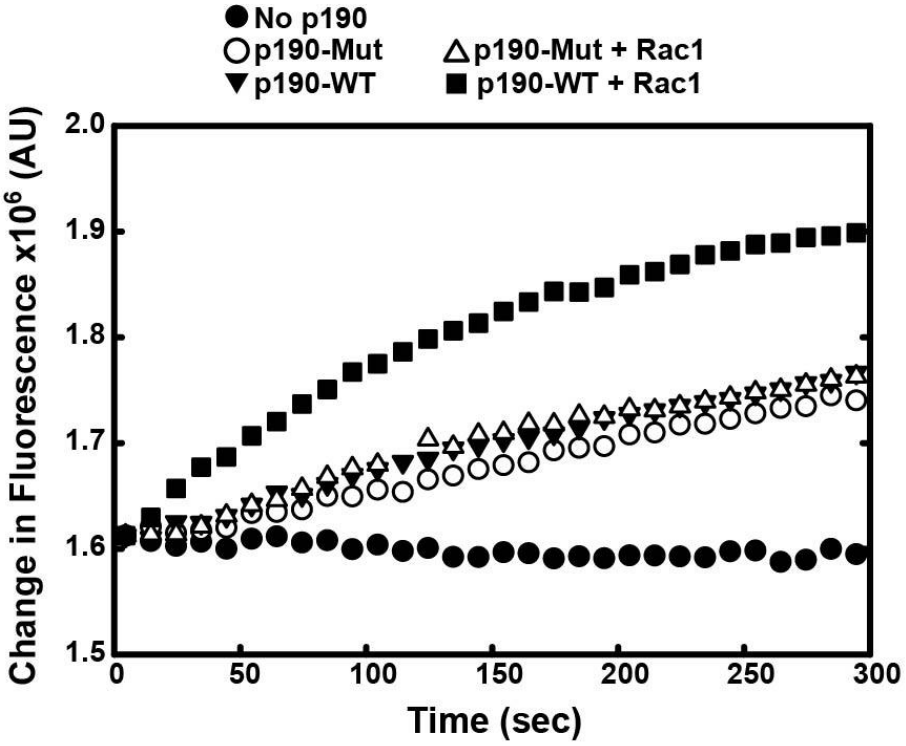

Figure 5.7. Mutations on the p190 PH domain reduce its ability to bind to the active forms of Rac1 and attenuate facilitation of p190RhoGEF activity on vesicle-delimited substrate. Nucleotide exchange assays were performed with nickel-containing phospholipid vesicles, 250 nM WT or Mutant p190 DHPH domains (non-His tagged), 1 μM His-tagged RhoA•GDP, and 100 nM His-tagged pre-activated Rac1. Rates of nucleotide exchange reaction were monitored by the increase in mant-GDP fluorescence intensity as a result of its association with RhoA•GDP.

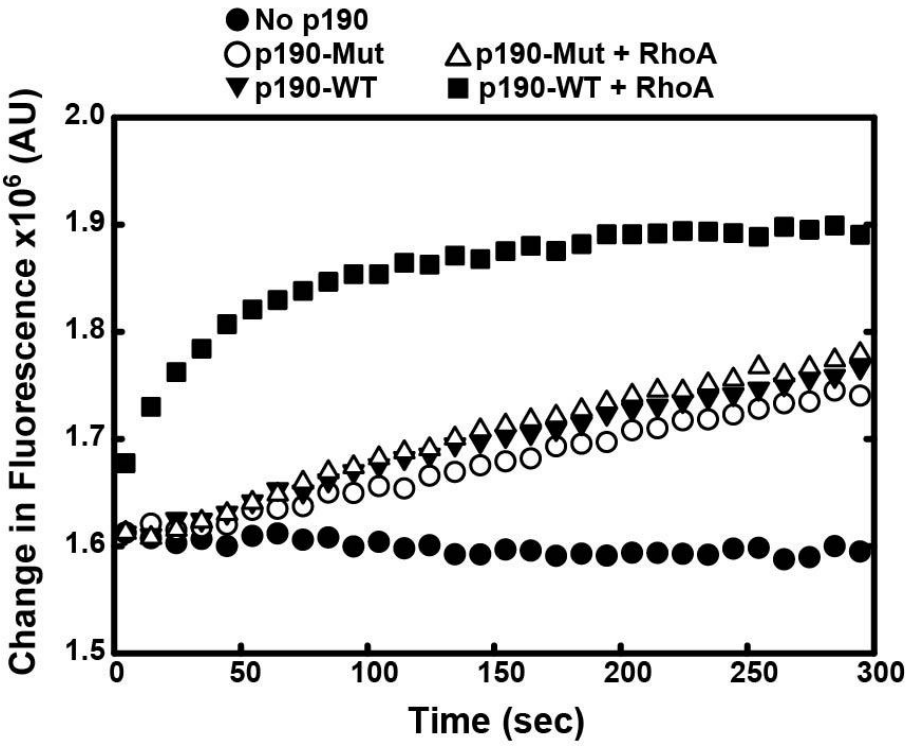

Figure 5.8. Mutations on the p190 PH domain reduce its ability to bind to the active forms of RhoA and attenuate facilitation of p190RhoGEF activity on vesicle-delimited substrate. Nucleotide exchange assays were performed with nickel-containing phospholipid vesicles, 250 nM WT or Mutant p190 DHPH domains (non-His tagged), 1 μM His-tagged RhoA•GDP, and 100 nM His-tagged pre-activated RhoA. Rates of nucleotide exchange reaction were monitored by the increase in mant-GDP fluorescence intensity as a result of its association with RhoA•GDP.

Comparison of the Stimulation of Various Lbc family RhoGEF's by Activated Rac1

The ability of Rac1 to facilitate activity of p190RhoGEF on membrane substrates in spite of its relatively low affinity and recognition of the same binding site as activated RhoA raised the question of whether this interaction is unique to p190RhoGEF, or also a property of other Lbc RhoGEFs. A preliminary pulldown assay, which examined the binding of activated Rac1 to the PH domains of various Lbc RhoGEFs (PRG, LARG, and GEFH1), did not reveal additional interactions (**Figure 3.3**). To further test for potential interactions, I used the DHPH domains of various Lbc family RhoGEFs in the more sensitive activity assay using substrate vesicles and localization of the GEFs. Nucleotide exchange activity of the indicated GEFs was assessed by the dissociation of mant-GDP from membrane association RhoA. In these assays, addition of activated Rac1.6His to the lipid membranes, caused a slight increase in the initial exchange rates of PRG (1.3 fold) and LARG (1.7 fold) and a more modest stimulation of AKAP-Lbc (2.4 fold) and p114RhoGEF (2.5 fold) compared to the respective RhoGEFs alone (**Figure 5.9**). However, these stimulations were small compared to the more efficacious action of Rac1 on p190RhoGEF (7.9 fold) in the same assays (**Figure 5.9**).

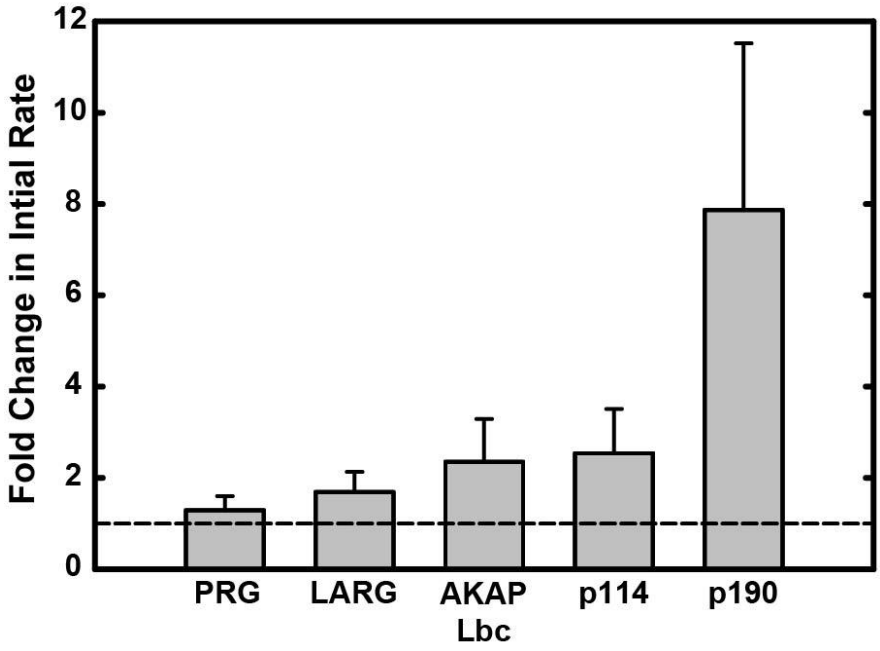

Figure 5.9. Stimulation of the initial rates of nucleotide exchange by various Lbc family RhoGEFs by pre-activated Rac1 on phospholipid vesicles. Nucleotide exchange assays were performed in a similar fashion as in Figure 5.5. Assays were initiated with the DHPH domains of PRG (25nM), LARG (25nM), AKAP-Lbc (100nM), p114RhoGEF (500nM), and p190RhoGEF (250nM). The initial exchange rates stimulated by addition of the RhoGEFs either in the absence or presence of 1 μM His_6-Rac1•GTPγS were determined; the fold increases in stimulation by activated Rac1 over the RhoGEF alone are shown. Data from three separate experiments were normalized by total signal and averaged; error bars are the standard deviation for each condition.

P190RhoGEF Binding to PIP_2 Does Not Facilitate its Localization to Lipid Membranes

The PH domain of p190RhoGEF binds to multiple phosphoinositides lipids when examined by dot blot assay (Miller et al., 2013). Binding of the PH domain to lipid second messengers PIP_2 and PIP_3 facilitates recruitment of various kinases and GEFs to the plasma membrane (Lemmon, 2008). Immunofluorescence data suggests that disrupting the lipid binding capabilities of p190 PH domain will prevent FAK from localizing to early adhesions and reduce the amount of adhesion complexes; this suggests that early in the cell's process of adhesion to fibronectin, p190RhoGEF acts as a scaffold through its association to PIP_2 at the plasma membrane (Miller et al., 2013). To test whether binding to PIP_2 is sufficient to localize p190RhoGEF to the plasma membrane, I utilized a vesicle binding assay that depends on separation by gel filtration (**Figure 5.10**). The DHPH of p190RhoGEF only shifted to earlier elution fractions containing PIP_2 phospholipid vesicles when the vesicles also contained immobilized activated RhoA or Rac1; in contrast, the Dbs PH domain that is known to bind to PIP_2 shows significant association with the vesicles.

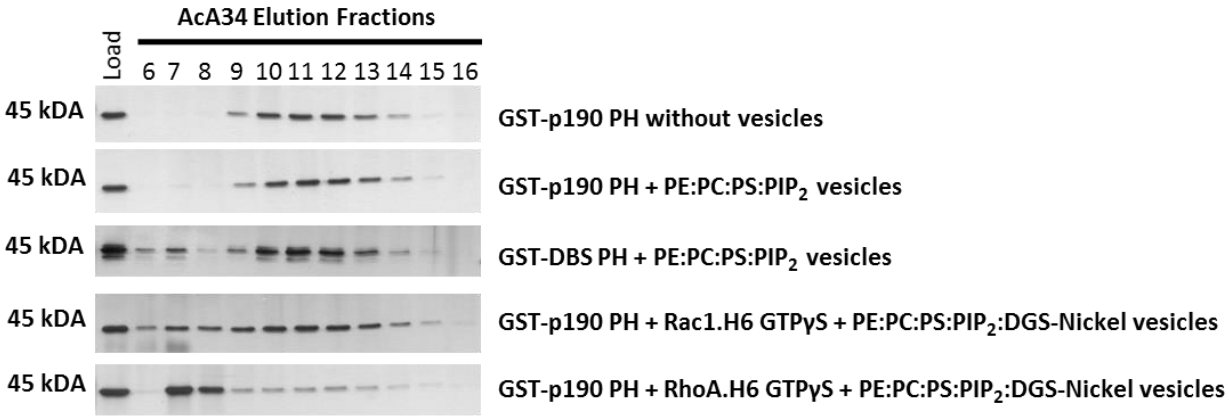

Fig 5.10. p190 PH domain binds to activated RhoA and Rac1, but not PIP_2, on phospholipid vesicles. Vesicle binding assays were performed with nickel-containing phospholipid vesicles, WT p190-PH (2 µM), WT Dbs-PH (2 µM), and His_6-RhoA or His_6-Rac1 (12.5 µM) proteins. Elution fractions collected off AcA34 polyacrylamide-agarose resin columns were subjected to SDS-PAGE, and visualized by silver staining.

DISCUSSION

The goal of this chapter was to elucidate the mechanism of p190RhoGEF regulation via its interactions with activated RhoA and Rac1. I began by testing the specificity of my purified p190 DH/PH protein to stimulate nucleotide exchange on the RhoA, Rac1, and Cdc42 GTPases. Confirming that p190 is a RhoA specific GEF is crucial to interpreting any results in which various combinations of either Rho, Rac, or Cdc42 were present in the same experiment. My results confirm p190's preferential exchange of RhoA and poor GEF activity as previously described in the literature (van Horck et al., 2001; Jaiswal et al., 2011). The variation in GEF nucleotide exchange efficiency by different Dbl family RhoGEFs is unknown (Jaiswal et al., 2011).

I next investigated whether this protein can undergo regulation of biological activity due to localization of the RhoGEF to the plasma membrane and its substrate, by reconstituting the signaling system in phospholipid vesicles. The addition of pre-activated RhoA and Rac1 bound to the vesicles enhanced the exchange activity of p190RhoGEF on vesicle associated RhoA and mutations in the PH-domain of p190RhoGEF abolished this effect *in vitro*. While both pre-activated RhoA and Rac1 helped anchor the GEF to the phospholipid membrane, a greater improvement in the initial exchange rate was seen with RhoA compared to Rac1. RhoA is a more potent stimulator of p190's GEF activity due its higher affinity (2 μM) for PH domain of p190 than Rac1 (20 μM), therefore a higher concentration of activated Rac1 in the environment is needed to get an initial stimulation of GEF activity similar to activated to RhoA. In the 6 minute timescale of these experiments, it

appears the efficacy of Rac1-mediated GEF stimulation is very similar to RhoA-mediated stimulation, because the fluorescence of each stimulation is plateauing towards a similar point. Therefore, in my vesicle assays, binding to membrane-localized RhoA initially sequesters more p190RhoGEF to the lipid membrane, making it a more efficient GEF towards its substrate, compared to membrane-localized Rac1.

The dependence of stimulations on localization support previous observations seen with other members of the Lbc RhoGEF family. In the case with RhoA, we believe that the binding of activated substrate to the PH domain of its RhoGEF is a mechanism of positive feedback regulation that allows the GEF to be sequestered within the vicinity of it substrate (Chen et al., 2010, Medina et al., 2013). Yet for Rac1, the sequestration of the p190 protein to the vesicle membrane by pre-activated Rac1 is a novel observation. This suggests a novel potential mechanism in which a non-substrate GTPase, in this case Rac1, is sufficient to localize an Lbc RhoGEF to the plasma membrane. This method of translocation has been observed for other GEFs. One example is RalGDS, a GEF for Ral. This GEF contains a Ras-association (RA) domain that allows it to bind Ras-GTP, which facilitates the translocation of RalGDS to the plasma membrane where it activates its substrate, Ral (Urano et al., 1996). In the case of Dbs, a GEF specific for RhoA and Cdc42, the authors demonstrated that activation of Rac1 correlates with Dbs mediated RhoA activation *in-vivo* (Cheng et al. 2004). They further show that the PH domain of Dbs is the site where Rac1•GTP binding occurs and the authors speculate that this interaction with Rac1•GTP at the plasma membrane is the mechanism for RhoA activation. Preliminary assays in which the isolated DHPH domain of Dbs interacts with membrane sequestered pre-activated Rac1 to stimulate nucleotide

exchange on membrane sequestered RhoA, supports their hypothesis (Stephen Gutowski, personal communication). Future experiments are being devised to explore whether p190RhoGEF can function in a similar fashion like Dbs in cells, facilitating the activation of RhoA activation in response to Rac1 activation. Phosphoinositides, such as PIP_2 and PIP_3, are capable of recruiting GEFs to the plasma membrane (Lemmon, 2008). It is hypothesized that this is the mechanism important for the p190RhoGEF mediated adhesion maturation downstream of integrin signaling (Miller et al., 2013). My vesicle binding results suggest that PIP_2 alone is not sufficient to localize p190 to the membrane, yet PIP_2 could be improving p190's localization to the membrane in the presence of activated GTPases. Performing vesicle exchange assays, in a similar fashion as Figure 5.2, would be a better way to elucidate the effect of PIs on p190's exchange activity at the plasma membrane when activated Rac1 or RhoA is added.

My assays of protein binding suggested that activated Rac1 is only capable of binding to p190RhoGEF. Yet when I examined 5 different Lbc family RhoGEFs (PRG, LARG, AKAP13, p114, p190), I was surprised to observe modest but significant stimulation of their activities by Rac1 in the localization dependent vesicle assays. It possible that the conditions I used in my pulldown assays were not optimal to detect any weak Rac-GTP interactions with the other GEFs. In spite of that, p190RhoGEF demonstrated the greatest improvement in GEF activity, compared to the other Lbc-RhoGEFs. It is interesting to note that the RGS-containing RhoGEFs (PRG and LARG) saw the smallest stimulation compared to the non-RGS RhoGEFs (AKAP-Lbc, p114, and p190). If translocation is the regulatory mechanism used by these RhoGEFs in-vivo, maybe RhoGEFs that lack other well defined binding

domains such as PDZ and RGS domains, have PH domains adapted to be sensitive to binding other activated GTPases that might be present in the plasma membrane. The RGS domains on PRG and LARG might be sufficient in bringing the GEF to the membrane in response to upstream cellular signals, while p190RhoGEF, whose lack of an RGS domain argues for its need to utilize other domains to sense and respond to upstream cellular signals.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

In summary, I characterized p190RhoGEF and its unique ability to interact with two members of the Rho family of GTPases, RhoA and Rac1 **(Figure 6.1**). My collaborator, Dr. Zhe Chen, crystallized and solved structures of the PH domain from p190-RhoGEF bound to RhoA•GTP, or Rac1•GTP. The interface between activated Rac1 and the PH domain is analogous to the one between activated RhoA and PH, utilizing the same hydrophobic surface on the PH domain. Similar to RhoA, activated Rac1 interacts with the PH domain via its effector-binding surface. The PH domain of p190RhoGEF has a higher affinity for RhoA than Rac1 GTPase. Mutating key residues on either GTPase will alter it affinity for p190's PH domain. Both activated RhoA and Rac1 can stimulate the RhoGEF activity of p190-RhoGEF *in vitro*, and mutations of key hydrophobic residues in the PH domain abolish this activation. P190RhoGEF exhibited the greatest stimulation of initial GEF activity by activated Rac1 in comparison to various Lbc family RhoGEFs. Therefore, with the data from my thesis project, I propose two models of p190RhoGEF regulation by GTPases: interaction of RhoA with the PH domain provides a mechanism for direct positive feedback (**Figure 6.2**), while the novel interaction of activated Rac1 GTPase reveals a potential mechanism for cross-talk regulation between the Rac and Rho signaling pathways (**Figure 6.3**).

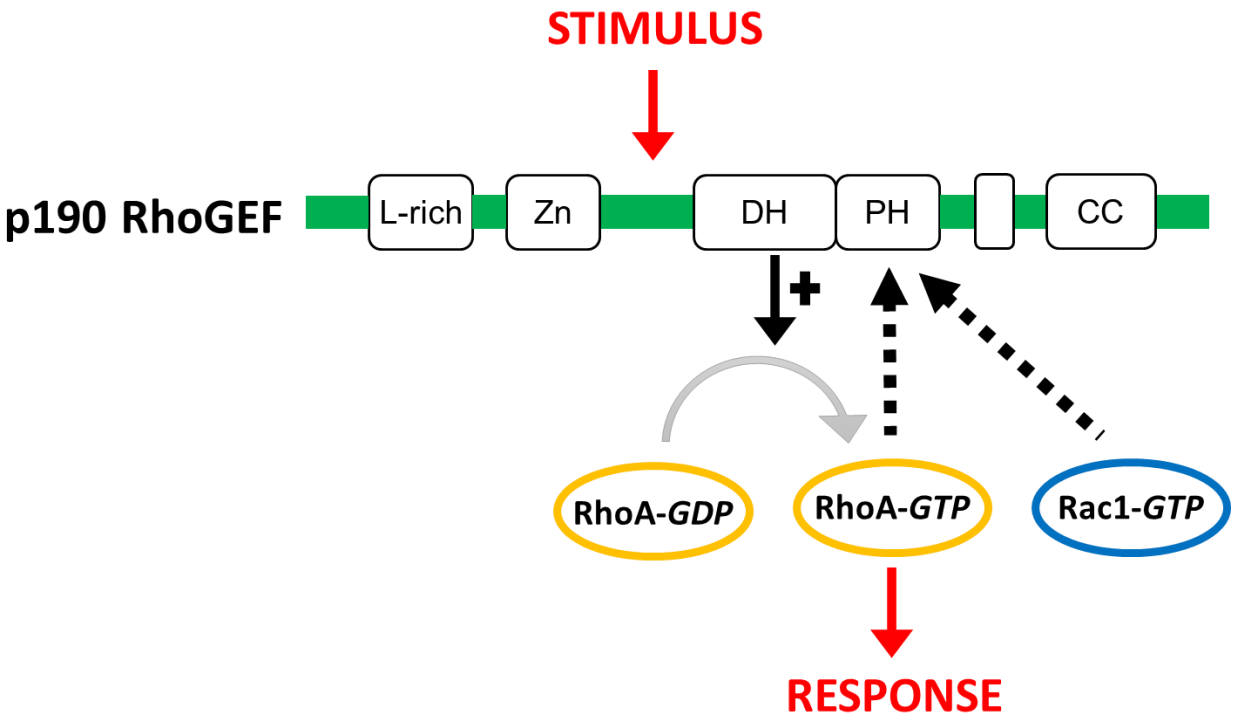

Figure 6.1. Regulation of p190RhoGEF by small GTPases. The active forms of RhoA and Rac1 interact with the PH domain of p190RhoGEF, thereby facilitating new mechanisms of RhoGEF regulation downstream of an activating stimulus.

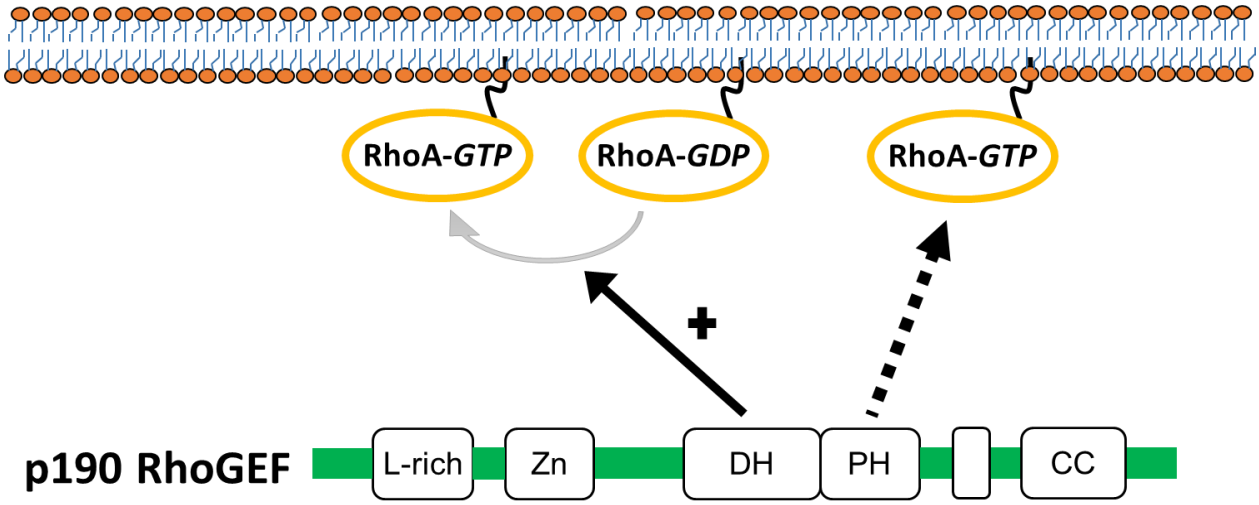

Figure 6.2. Model – Positive Feed Forward Mechanism. Binding to RhoA•GTP facilitates localization of p190RhoGEF to the plasma membrane where subsequent activation to RhoA•GTP provides binding sites for more p190RhoGEFs to associate and stimulate more GTPase activation.

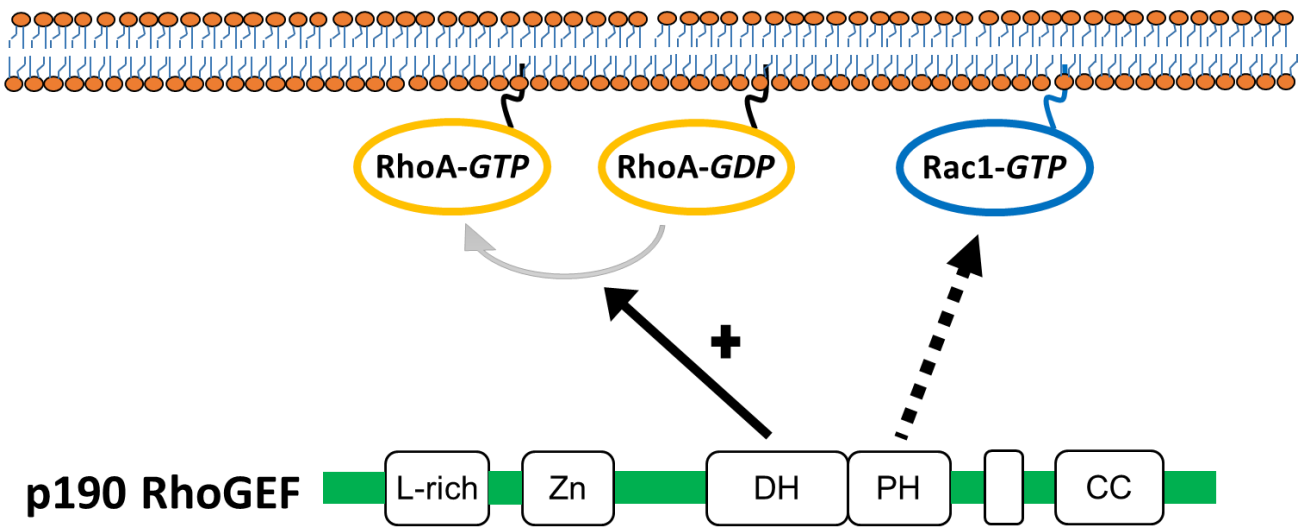

Figure 6.3. Model – "Trigger" Mechanism for RhoA Activation. Binding to Rac1•GTP (weak affinity) facilitates localization of p190RhoGEF to its substrate RhoA•GDP, where subsequent activation to RhoA•GTP provides higher affinity binding sites for other p190RhoGEFs to associated and activate more substrate.

How would p190RhoGEF play a role in crosstalk signaling? This could be through a mechanism in which Rac1 can facilitate the activation of RhoA by localization of p190RhoGEF to the plasma membrane. One study that supports this hypothesis found that the co-expression of activated Rac1 and Dbs in NIH3T3 cells correlates with subsequent RhoA activation. The authors state this phenomenon is mediated through Rac1 binding to the PH-domain of Dbs (Cheng et al., 2004). It would be interesting to explore whether p190RhoGEF can act in a similar manner in cells, facilitating the stimulation of RhoA activation in response to the activation of Rac1. To address this question, experiments are currently being performed in the lab, utilizing a heterodimerization system, to chemically induce localization of a constitutively active Rac1 mutant to the plasma membrane of cells co-expressing either wild-type p190RhoGEF or p190RhoGEF Y1003A (GEF-inactivating mutation) and measuring RhoA activation by a luciferase reporter assay. Concurrent experiments will utilize fluorescence microscopy to observe the translocation of FP-tagged p190RhoGEF to the plasma membrane of cells after treatment to induce membrane localization of the constitutively active Rac1 mutant.

I can only speculate about the physiological role the interaction of Rac1•GTP with p190RhoGEF has in-vivo. Many RhoGEFs play roles in various aspects of cellular motility, such as adhesion, contractility, and tail retraction (Chikumi et al., 2002, Dubash et al., 2007, Francis et al., 2006, Iwanicki et al., 2008, Lim et al., 2008, Miller et al, 2012, Zhai et al., 2003). In the case of p190RhoGEF, interaction with focal adhesion kinase (FAK) is crucial for the localization of the GEF to focal adhesions where it can facilitate

adhesion maturation (Zhai et al., 2003). Yet a more recent model suggests downstream of integrin signaling, p190RhoGEF associates with lipids at the plasma membrane acting as a scaffold for FAK localization to nascent focal adhesions (Miller et al., 2013). Downstream of integrin signaling the Rac and Rho signaling pathways play important roles in the regulation of focal adhesions; an initial activation of Rac1 promotes nascent adhesion formation, while the subsequent activation of RhoA promotes adhesion maturation (Guo et al., 2006, Guilluy et al., 2011). Perhaps p190RhoGEF's unique ability to interact with active form of Rac1, in combination with FAK and membrane lipid binding, is needed for sequestering the GEF in the vicinity of nascent adhesions and stimulating the subsequent focal adhesion maturation. One could test this hypothesis in the context of RGNEF -/- knockout fibroblasts which demonstrate reduced focal adhesion numbers after re-plating on fibronectin (Miller et al., 2012). The goal would be to first demonstrate that expression of FL-p190RhoGEF PH Mutant (mutation tested in Chapter 4) in CRISPR RGNEF knockout cells cannot rescue focal adhesion numbers compared to cells expressing WT FL-190RhoGEF. Next would be to examine the ability of a chimera FL-p190RhoGEF (a construct with the p190 PH domain replaced with the PH domain from PRG) to rescue focal adhesion numbers in RGNEF knockout cells. The rational behind a chimera p190 is due to PRG's lack of interaction with activated Rac1 (**Figure 3.3 and Chen et al., 2010**), its less robust stimulation by Rac1 compared to p190 (**Figure 5.9**), and the p190 PH mutation does not selectively affect binding to either Rac1 or RhoA (**Figure 4.1**). Successful completion of these experiments would establish the physiological importance of Rac1 interactions with p190 RhoGEF.

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