MECHANISMS FOR REGULATION OF RHOA BY HORMONES

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DEDICATION

To my daughter, for her understanding and lack of complaint these last few months that I have focused so heavily on my work. I love you the most of all!

I would like to thank the members of my Graduate Committee, especially (especially, especially, especially) my mentor Paul Sternweis, for their continued guidance and patience. I thank all the members of the lab for being so willing to help with anything and everything and, of course, providing constant entertainment. I have to thank my parents, family, and friends for their ongoing encouragement and support, without which I would have given up long ago.

MECHANISMS FOR REGULATION OF RHOA BY HORMONES

by

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DISSERTATION / THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2012

MECHANISMS FOR REGULATION OF RHOA BY HORMONES

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The University of Texas Southwestern Medical Center at Dallas, 2012

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The family of RGS-RhoGEFs, which consists of p115RhoGEF, LARG, and PDZ-RhoGEF (PRG), are specific guanine nucleotide exchange factors (GEF) for the monomeric GTPase, RhoA. Like most RhoGEFs, these proteins contain tandem DH·PH domains that mediate their nucleotide exchange activity. Members of this family also contain a regulator of G-protein signaling (RGS) domain that interacts directly with the α subunit of G_{12} heterotrimeric GTPases and enhances the rate of intrinsic GTP hydrolysis by these proteins. While activated $G\alpha_{13}$ modestly stimulates the exchange activity of p115RhoGEF and LARG, it does not stimulate the intrinsic activity of PRG. All three RGS-RhoGEFs localize to the plasma

membrane upon expression of activated $G\alpha_{13}$ subunits indicating that regulation of the cellular localization of these RhoGEFs may be a fundamental mechanism for controlling their activity.

These studies examine translocation and formation of signaling complexes as mechanisms for regulating the RGS-RhoGEFs by hormones. A small molecule regulated heterodimerization system was used to rapidly and directly control the localization of the RGS-RhoGEFs. Acute localization of the RGS-RhoGEFs to the plasma membrane activates RhoA within minutes and to levels that are comparable to activation of RhoA by stimulation of EDG receptors with hormone. The activity of membrane localized RhoGEFs is not dependent on activation of $G\alpha_{13}$. These data demonstrate that simple translocation of the RhoGEFs can drive activation of the GTPases via the intrinsic exchange activity of the GEFs.

The conserved RGS domain was identified as a module capable of localizing the RhoGEFs by association with activated $G\alpha_{13}$ in response to hormone stimulation. Evidence is also provided that PDZ domains of PRG interact with selected EDG receptors and PH domains of various Lbc-RhoGEFs interact with activated RhoA. These interactions provide multiple anchoring points that may act cooperatively to secure the RhoGEFs to the plasma membrane in proximity to their substrate RhoA. Formation of such signaling complexes between receptors, G proteins, RhoGEFs, and RhoA may be further aided by constitutive oligomerization of EDG receptors. Formation of high order stable signaling complexes may be required to hold RhoGEFs at the plasma membrane for the timeframe necessary to modulate reorganization of actin cytoskeletal structures.

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PRIOR PUBLICATIONS

Sternweis, P. C., A. M. Carter, et al. (2007). "Regulation of Rho guanine nucleotide exchange factors by G proteins." Adv Protein Chem **74**: 189-228.

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LIST OF DEFINITIONS

BRET – bioluminescence resonance energy transfer

Cdc42 – cell division cycle 42

CFP – cyan fluorescent protein

Dbl – diffuse B cell lymphoma

Dbs – Dbl's big sister

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

DH – Dbl-homology

DMEM – Dulbeccos modified Eagle medium

EDG – endothelial differentiation gene

EDTA – ethylenediaminetetraacetic acid

EGF – epidermal growth factor

FAK – focal adhesion kinase

FBS – fetal bovine serum

FKBP – FK506 binding protein

FLuc – Firefly luciferase

FRB – FKBP12 rapamycin binding

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

GTP – guanosine 5’ triphosphate

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

GST – glutathione S-transferase

HBSS – Hank’s Balanced Salt Solution

HEK293 cells – human embryonic kidney 293 cell line

HEPES – N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]

IPTG – isopropyl β-D-thiogalactopyranoside

LARG – leukemia-associated RhoGEF

LB – Luria broth

Lbc– lymphoid blast crisis oncogene

LPA – lysophosphatidic acid

MDCK cells – Madin-Darby canine kidney epithelial cell line

MLC2 – myosin light chain 2

Myr – myristoylation

NTA – nitrilotriacetic acid

PBD – Cdc42/Rac binding domain of p21-activated kinase 1

PC3 cells – human prostate cancer cell line

PDZ – post synaptic density protein 95/Dlg-a/ZO-1

PH – pleckstrin homology

P*i* – inorganic phosphate

PLC – phospholipase C

PMSF – phenylmethysulfonyl fluoride

PRG – PDZ-RhoGEF

PVDF – polyvinylidene difluoride

Rapalog – rapamycin analog AP21967

RBD – Rhotekin binding domain

RGS – regulator of G protein signaling

Rho – Ras homology

RLuc – Renilla luciferase

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

RPMI – Roswell Park Memorial Institute

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

S1P – sphingosine-1-phosphate

SRE.L – serum response element like

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

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

TPCK – tosylphenylalanyl chloromethyl ketone

YFP – yellow fluorescent protein

WT – wild-type

CHAPTER ONE
Introduction

CELLULAR SIGNALING THROUGH G PROTEINS

Cells need to detect changes in their immediate environment and initiate proper responses to those changes in order to maintain cellular and organismal homeostasis, function, and survival. In eukaryotic cells, this is often accomplished through membrane spanning receptors that have access to both the extracellular and intracellular environments. Interactions with specific extracellular molecules can induce structural changes within membrane receptors that alter the manner in which these proteins interact with various intracellular binding partners, effectively transferring information about the outside environment to machinery inside the cell. There are multiple types of surface receptors that cells use to monitor the extracellular milieu. One large class of receptors, which are currently major targets for drug development, are multi-membrane spanning G protein-coupled receptors (GPCRs).

G Protein-Coupled Receptors

GPCRs are a large family of receptors that are characterized structurally by seven closely packed transmembrane helices. These receptors mediate downstream signaling through interaction with heterotrimeric G proteins, a class of signal transducing intracellular GTPases. There are three sub-classes of GPCRs, classes A-C, with the majority of receptors falling into the rhodopsin-like class A (Palczewski 2006). Class A receptors that can couple to the G_{12} family of heterotrimeric GTPases includes the serotonin, angiotensin, cholecystokinin, endothelin,

galanin, lysophosphatidic acid (LPA), acetylcholine, sphingosine-1-phosphate (S1P), tachykinin, thromboxane A2, and protease-activated receptors (Riobo and Manning 2005). Regulation of these receptors is complex and occurs on multiple levels including transcription, translation, post-translational modifications, membrane trafficking, and of course, ligand binding (Rosen et al 2009). The studies reported here will focus on signaling through the LPA and S1P receptors.

Multiple GPCRs have been found to exist as oligomers in the plasma membrane. The first GPCRs to be identified as obligate dimers for proper localization, ligand binding, and G protein activation were class C GPCRs (Pin et al 2005). Recently, evidence has accumulated that dimerization of class A GPCRs has a functional impact as well, including alterations in ligand affinity, G protein selectivity, and efficiency of G protein coupling (Rozenfeld and Devi 2011). G_{12} coupled class A receptors for S1P and LPA have been reported to form homo- and hetero-oligomers based on analyses using co-immunoprecipitation and two-hybrid interactions (Rosen et al 2009, Van Brocklyn et al 2002, Zaslavsky et al 2006); however, evidence for a functional impact of their dimerization is still lacking. Additionally, the methods used offer no information on cellular localization and may be compromised by non-specific receptor interactions.

Heterotrimeric GTPases

GPCRs couple to heterotrimeric GTPases which are composed of Gα, Gβ, and Gγ subunits. The Gβγ subunits are constitutive heterodimers and act as GDP dissociation inhibitors for the Gα subunits, the member that binds guanine nucleotide. These proteins are defined by activation states that are linked to a cycle of GDP/GTP nucleotide binding as shown in Figure 1. In the

inactive state, Gα subunits are bound to GDP and have high affinity for Gβγ dimers. Upon ligand binding, receptors bound to G proteins change conformation and promote release of GDP from Gα. This promotes efficient binding of GTP, which exceeds the concentration of GDP in cells. Binding of GTP to Gα initiates conformational changes that activate the G proteins leading to decreased affinity of Gα for Gβγ and receptors and generating increased affinity for the downstream effectors of both Gα and Gβγ (Sprang 1997). Gα subunits have intrinsic GTPase activity that hydrolyzes bound GTP to GDP, a mechanism for deactivating the proteins. However, the intrinsic activity of most GTPases is low and GTPase activating proteins (GAPs) known as regulators of G protein signaling (RGS) proteins are one set of regulators that operate by enhancing GTP hydrolysis rates and speeding G protein inactivation (Ross and Wilkie 2000).

There are four major classes of heterotrimeric G proteins based on their α subunit; G_s, G_i, G_q, and G_{12} (Sprang 1997). The α subunits of G_q and G_{12}, as well as Gβγ subunits, are involved in activation of the monomeric GTPase RhoA downstream of GPCR signaling (Aittaleb et al 2010). The studies contained in this document will focus on the regulation of RhoA through the α subunit of the G_{12} class of heterotrimeric G proteins. This class includes only two distinct Gα subunits, $G\alpha_{12}$ and $G\alpha_{13}$ (Sprang 1997).

Monomeric Rho GTPases

One set of downstream targets for G_{12} proteins is the RhoA subfamily of monomeric GTPases (Kurose 2003). RhoA, along with Cdc42 and Rac subfamilies, is a member of the Rho family of small monomeric Ras-like GTPases. Rho GTPases are best known for their

Figure 1: Heterotrimeric GTPase Cycle

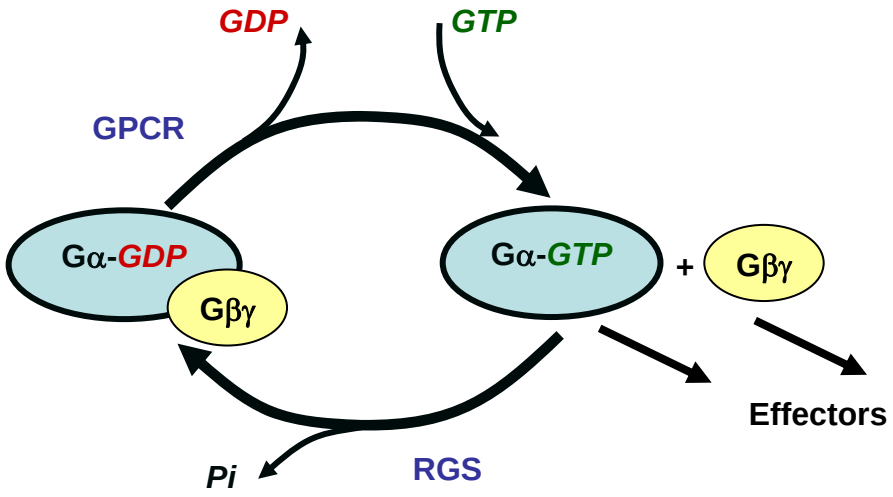


Figure 1. Heterotrimeric GTPase Cycle. G proteins are inactive when the α subunits are bound to GDP and associated tightly with Gβγ. Activated GPCRs promote dissociation of GDP from Gα and subsequent association of GTP leading to G protein activation and interaction with downstream effectors. Hydrolysis of GTP by the α subunit is promoted by interaction with RGS proteins and leads to inactivation of Gα and reassociation with Gβγ.

involvement in regulation of the actin cytoskeleton (Schmidt and Hall 2002). RhoA proteins activate polymerization of linear actin filaments through activation of the formins mDia1, mDia2, and DAAM1 (Goode and Eck 2007). The mechanism for activation of formins by RhoA is not completely understood, however, one step of activation is the relief of autoinhibition by binding of RhoA to the diaphanous inhibitory domain (Faix and Grosse 2006). In addition to targeting formins, RhoA also regulates the actin cytoskeleton through activation of Rho-associated coiled-coil-containing protein kinase (ROCK). One target of ROCK is myosin light chain II (MLC2). Phosphorylation of MLC2 promotes binding of myosin to actin, which increases actin bundling and provides the cellular machinery for contractility (Bishop and Hall 2000). In contrast to RhoA, Cdc42 and Rac proteins activate polymerization of branched actin

filaments through activation of WAVE/WASP proteins and the Arp2/3 complex (Goode and Eck 2007).

When overexpressed in cells, Rho proteins initiate contrasting cellular changes. RhoA proteins cause the broad formation of actin stress fibers throughout the cell, cdc42 proteins lead to the formation of multiple filopodia structures, and Rac proteins initiate the formation of lamellapodia (Hall 1998). However, recent evidence indicates that action of these GTPases is likely coordinated during normal cellular signaling events and not mutually exclusive (Machacek et al 2009).

The activity of Rho proteins is also determined by cycling between active-GTP and inactive-GDP bound states. Regulators of these proteins include guanine nucleotide exchange factors (GEFs), which promote binding of GTP and activation of Rho, and GAPs, which enhance hydrolysis of GTP and inactivation of Rho (Van Aelst and D'Souza-Schorey 1997). Rho proteins are prenylated on their C-termini. This modification is required for their interaction with a third regulator, RhoGDI. RhoGDI prevents dissociation of GDP from the GTPases and holds the inactive Rho proteins in the cytosol (Hori et al 1991). When not bound to RhoGDI, the lipid modification aids in localization of Rho proteins to plasma membrane. Importantly, to date, only Rho proteins free of RhoGDI have been found to productively interact with exchange factors, and thus, become activated (DerMardirossian and Bokoch 2005).

CHAPTER TWO
Current Literature Review

REGULATION OF RHOA THROUGH RGS-RHOGEFS

Over 60 RhoGEFs have been identified in mammalian genomes that are capable of activating Rho proteins (Rossman et al 2005). The RGS-RhoGEFs are a subfamily of GEFs that are classified by the presence of an RGS domain and are specific for RhoA proteins. This family consists of three members: p115RhoGEF, Leukemia Associated RhoGEF (LARG), and PDZ-RhoGEF (PRG). In addition to the RGS domain, all three of the RGS-RhoGEFs contain classical tandem Dbl and pleckstrin homology (DH·PH) domains, a characteristic of most RhoGEFs that are responsible for their exchange activity toward Rho GTPases. The DH·PH domains are followed by a C-terminal coiled-coil region that allows oligomerization of the RGS-RhoGEFs. In addition to these conserved regions, LARG and PRG both contain a PDZ domain at their N-termini that is involved in protein-protein interactions (Sternweis et al 2007).

Figure 2: RGS-RhoGEFs

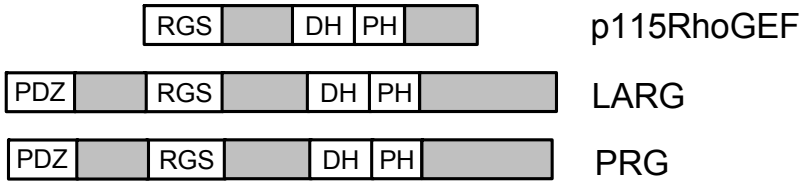


Figure 2: Diagram of the three RGS-RhoGEF family members; p115RhoGEF, LARG, and PRG. RGS and DH·PH domains are common to all family members. Both LARG and PRG contain N-terminal PDZ domains.

Physiology

The expression and physiologic function of the RGS-RhoGEFs varies among different tissues and cell types. p115RhoGEF is most highly expressed in hematopoetic cell lineages where it is involved in B cell homeostasis and migration, negative selection of T cells, and pseudopod formation and directional migration in neutrophils (Francis et al 2006, Girkontaite et al 2001, Harenberg et al 2005, Whitehead et al 1996). LARG is ubiquitously expressed and plays an important role in the development of multiple tissue types (Becknell et al 2003, Kourlas et al 2000, Kuner et al 2002, Medlin et al 2010). PRG is highly expressed in neuronal tissues where it is important for proper growth cone morphology and neurite retraction (Kuner et al 2002, Perrot et al 2002, Swiercz et al 2002, Togashi et al 2000). All three RGS-RhoGEFs have significant transforming potential when expressed to high levels and modulate migration of various cancer cell lines (Bourguignon et al 2006, Chikumi et al 2004, Guo and Zheng 2004, Hart et al 1996, Jiang et al 2010, Kelly et al 2006, Kitzing et al 2007). Additionally, human population studies have identified a single-nucleotide polymorphism in PRG that increases the risk of lung cancer in specific ethnic groups (Gu et al 2006).

Regulation

Regulation of the RGS-RhoGEFs is a highly complex process. The first level of control is through regulated expression and degradation of the proteins. The second level of control is through regulating the enzymatic activity of expressed RGS-RhoGEFs. There are two potential

mechanisms through which this could be accomplished: 1) modulation of intrinsic catalytic activity, or 2) modulation of exposure to substrate.

Expression and Degradation

Transcriptional regulation of RGS-RhoGEFs is apparent by the restricted expression patterns of both p115RhoGEF and PRG (Kuner et al 2002, Whitehead et al 1996). Additionally, the levels of LARG present in vascular smooth muscle cells is increased upon stimulation with the agonist angiotension II (Ying et al 2006), and levels of PRG in hippocampal neurons are decreased in response to neurotrophins (Lin et al 2011).

Regulation of Intrinsic Catalytic Activity by $G\alpha_{12/13}$

The RGS-RhoGEFs are unique in that they were the first identified bi-directional link between heterotrimeric and monomeric GTPases. The RGS domains of these three exchange factors interact specifically with the α subunits of the heterotrimeric G_{12} proteins (Kozasa et al 1998, Suzuki et al 2003, Wells et al 2002) and the exchange activity of p115RhoGEF and LARG can be stimulated *in vitro* by activated $G\alpha_{13}$ (Hart et al 1998, Suzuki et al 2003). While binding of $G\alpha_{13}$ to the RGS domain is required for stimulation of exchange activity in p115RhoGEF and LARG, the mechanism for activation was unclear until a third interaction site between $G\alpha_{13}$ and the DH domain was identified. Disruption of this interaction site completely abolished $G\alpha_{13}$ stimulation of the catalytic activity of p115RhoGEF (Chen et al 2012).

In addition to p115RhoGEF, which is directly stimulated by binding of activated $G\alpha_{13}$ to the DH domain, p63RhoGEF and Trio exhibit increased exchange activity toward RhoA in response

to stimulation with activated $G\alpha_q$. Unlike $G\alpha_{13}$, $G\alpha_q$ binds to the C-terminal extension of the PH domain of these GEFs, which relieves autoinhibition of the DH domain (Lutz et al 2007, Rojas et al 2007). To date, these are the only Gα subunits that are known to directly interact with exchange factors for monomeric GTPases.

The RGS domain of RGS-RhoGEFs, along with an acidic stretch of amino acids at the N-terminus of the domain, acts as a GAP toward $G\alpha_{12/13}$ *in vitro*, deactivating the proteins (Chen et al 2008, Kozasa et al 1998, Suzuki et al 2003). The physiological impact of this deactivation is not yet known. One possible outcome is attenuation of the incoming hormonal signal. In contrast, deactivation of $G\alpha_q$ by phospholipase C appears to enhance hormonal signaling by facilitating multiple cycles of activation (Biddlecome et al 1996).

PRG differs from the first two family members in that its RGS domain binds to the α subunits of G_{12} and G_{13}, but binding of activated $G\alpha_{13}$ to these sites does not affect the catalytic activity of the PRG (Wells et al 2002). Furthermore, the RGS domain of PRG lacks key residues in the acidic N-terminal extension that functions as a GAP (Chen et al 2008). The fact that exchange activity of PRG is not modulated *in vitro* by the presence of activated $G\alpha_{13}$ combined with the modest 2-4 fold activation of p115RhoGEF and LARG by $G\alpha_{13}$, indicates that additional mechanisms probably exist to regulate the activity of the RGS-RhoGEFs *in vivo*.

Phosphorylation

All three RGS-RhoGEFs are post-translationally phosphorylated. Thrombin initiates phosphorylation of p115RhoGEF and downstream activation of RhoA in human umbilical vein endothelial cells (Holinstat et al 2003). Activation of RhoA is also stimulated by TNF-α induced

phosphorylation of p115RhoGEF in endothelial bEnd.3 cells (Peng et al 2011). Focal adhesion kinase (FAK) phosphorylates LARG in response to stimulation of HEK293 cells with thrombin, which promotes long term elevation of active RhoA (Chikumi et al 2002). Fyn kinase phosphorylates LARG when human umbilical vein endothelial cells are exposed to tensional force (Guilluy et al 2011). Overexpression of Tec kinase leads to tyrosine phosphorylation of LARG which permits stimulation of LARG by activated $G\alpha_{12}$ (Suzuki et al 2003). Similarly, levels of active RhoA in HEK293 cells are increased upon tyrosine phosphorylation of PRG by FAK in response in thrombin stimulation (Chikumi et al 2002). Conversely, phosphorylation of PRG by p38 mitogen-activated protein kinase leads to ubiquitination and degradation (Lin et al 2011).

Phosphorylation of the RGS-RhoGEFs that results in increased cellular levels of active RhoA could be operating by either of the mechanisms stated above. The modifications could promote allosteric changes in the RhoGEFs that enhance their intrinsic catalytic activity or alternatively, promote allosteric changes that enhance their interaction with membrane associated proteins, promoting their co-localization with substrate.

Oligomerization

The C-termini of the RGS-RhoGEFs contains a coiled-coiled region that causes oligomerization of the proteins. PRG and LARG form both homo- and hetero-oligomers. Conversely, p115RhoGEF forms only homo-oligomers (Chikumi et al 2004). Disruption of oligomerization has no effect on the exchange activity of p115RhoGEF and the role that

oligomerization of the RGS-RhoGEFs plays in regulating their activities *in vivo* is unknown (Eisenhaure et al 2003).

The C-termini of the RGS-RhoGEFs, however, do have a role in regulation of activity of RGS-RhoGEFs that is independent of oligomerization. Removal of the C-terminus, while having little effect on the intrinsic exchange activity of purified RGS-RhoGEFs, promotes increased activation of RhoA in cells (Chikumi et al 2004, Eisenhaure et al 2003, Wells et al 2001). The mechanism for this increase in activity is not known, however, one hypothesis is that the C-terminus interacts with an unknown binding partner that sequesters the RhoGEFs away from the plasma membrane and prevents their interaction with the substrate RhoA.

Membrane Localization

Overexpression of constitutively activated $G\alpha_{12}$ in MDCK cells induces relocalization of GFP tagged p115RhoGEF, LARG, and PDZ-RhoGEF from the cytosol to the plasma membrane (Meyer et al 2008). Additionally, HEK293 cells stably expressing thromboxane A_2 receptors exhibit relocalization of endogenous p115RhoGEF to the plasma membrane following stimulation with the agonist U44619 (Bhattacharyya and Wedegaertner 2003).

The ability of plasma membrane localization of RhoGEFs to drive activation of RhoA is suggested by two separate studies. Expression of p115RhoGEF, which is constitutively targeted to the plasma membrane, drives cell rounding in PC12 cells (Bhattacharyya et al 2009). Additionally, overexpression of LARG containing mutations in the PH domain that cause cytosolic localization of the protein, elicits a lower increase in basal levels of active RhoA than overexpression of wild-type LARG in HEK293T cells. Furthermore, addition of tandem PH

domains from PLCγ, that are known to interact with phospholipids, to the mutant LARG proteins restores their ability to activate RhoA upon overexpression, presumably due to constitutive association with the plasma membrane (Aittaleb et al 2009). These studies are based on long-term overexpression of RhoGEFs and it remains unclear whether rapid membrane localization downstream of hormone stimulation can acutely activate RhoA.

There are multiple mechanisms through which membrane localization of endogenous RGS-RhoGEFs could be regulated. $G\alpha_{13}$ is localized to the plasma membrane: therefore binding of $G\alpha_{13}$-GTP to the RGS domain has the potential to translocate the RGS-RhoGEFs to the membrane and place them in close proximity with their substrate, free RhoA. The N-terminal PDZ domains of PRG and LARG bind to LPA receptors as well as single membrane spanning Plexins (Hirotani et al 2002, Swiercz et al 2002). Overexpression of isolated PDZ domains blocks LPA stimulation of RhoA, suggesting that binding between the RhoGEFs and the C-terminus of the receptor is important for receptor signaling (Yamada et al 2005). The PH domain from PRG was recently found to bind to the activated form of RhoA *in vitro*. Importantly, this interaction had no affect on the intrinsic catalytic activity of PRG (Chen et al 2010).

Collectively, these data suggest that plasma membrane localization is required for activity. However, it is not known if localization to the plasma membrane can facilitate RGS-RhoGEF activity on the time-scale required for hormone signaling. Constitutive localization of overexpressed RGS-RhoGEFs to the plasma membrane could simply result in long-term elevation in levels of activated RhoA that is not relevant to receptor signaling.

This Study

Regulation of the RGS-RhoGEFs is complex and occurs on multiple levels, including modulation of expression through transcriptional and translational regulators as well as regulators of protein degradation. There are two mechanisms through which the RGS-RhoGEFs may be regulated acutely following hormone stimulation of GPCRs. One is through modulation of intrinsic catalytic activity of the RhoGEFs. A second is modulation of the exposure of the RhoGEFs to their substrate RhoA. Either mode of regulation would provide a mechanism for acute activation of the RGS-RhoGEFs downstream of hormonal stimulation of G-protein coupled receptors (GPCRs). In fact, both mechanisms may work simultaneously. While activation of the catalytic activity of p115RhoGEF and LARG has been extensively documented, studies evaluating the role of translocation of the RGS-RhoGEFs in agonist promoted activation of RhoA are lacking.

The studies reported here focus on delineating the contribution of signaling complex formation and regulated membrane localization of RGS-RhoGEFS in acute hormone activation of RhoA through GPCRs. These studies show that acute localization is indeed sufficient to initiate activation of RhoA to extents that are comparable in both magnitude and kinetics to levels observed through hormone induced receptor signaling. It is further demonstrated that activated $G\alpha_{13}$ is not required for the activation of RhoA through translocation of the RGS-RhoGEFs, validating that the proteins have sufficient catalytic activity in the unstimulated state. The RGS region is clearly identified as a domain that responds to hormone treatment and participates in regulated translocation of the RGS-RhoGEFs. Evidence is provided that PDZ

domains interact with S1P receptors and that PH domains interact with activated RhoA in cells, both of which potentially assist in RhoGEF membrane localization. Finally, EDG receptors were verified to exist as oligomers at the plasma membrane, which has implications for efficient interactions with downstream signaling partners and assembly of RGS-RhoGEFs at the plasma membrane.

CHAPTER THREE

METHODS AND MATERIALS

Plasmids

The ARGENT heterodimerization kit containing pC_4-R_HE (encoding FRB) and pC_4M-F2E (encoding tandem FKBPs) was obtained from ARIAD Pharmaceuticals Inc. and is now available commercially through Clontech. All constructs were inserted into either pC_4-R_HE or Myc2pCMV5 for mammalian expression and pGEX-KG-TEV (Chen et al 2010) or pTrc D (Wells et al 2001) for bacterial expression. Constructs encode full-length proteins unless otherwise specified. Truncated constructs of human p115RhoGEF include: p115ΔRGS encoding amino acids (aa) 234-912, p115-RGS-DH·PH encoding aa 1-760, p115-DH·PH encoding aa 248-760, p115-PH encoding aa 609-765, and p115-RGS encoding aa 1-252. Truncated constructs of human PRG (long isoform) include: PRG-RGS-DH·PH encoding aa 330-1125, PRG-RGS encoding aa 330-532, PRG-PH encoding aa 967-1125, and PRG-PDZ encoding aa 2-127. Additional PH domain constructs from other RhoGEFs include human LARG-PH encoding aa 981-1137, human AKAP Lbc-PH encoding aa 440-584, mouse p114-PH encoding aa 302-444, human GEH1-PH encoding aa 206-574. Human Trio-N (aa 1226-1535 of Trio RhoGEF) was provided by Dr. John Sondek and cloned into the above stated vectors, both alone and 3' to the RGS fragments listed above. All chimeric constructs were designed with a 16 aa linker (GSGTGSGIDGTGSGTG) separating each protein. Human pEF-myc-Rac1 was provided by Dr. Helen Yin for mammalian expression. A 6His-tag was added to the C-terminus of full length human RhoA and a 9His-tag was added to Rac1 in place of the C-terminal CAAX-

box in the pGEX-KG-TEV vector for purification of GTPases for use in vesicle based nucleotide exchange assays. Vector coding for *C. botulinum* C3 transferase (Alberts et al 1998) and the pRL-TK vector coding for *Renilla reniformis* luciferase under the thymidine kinase promoter was provided by Dr. Melanie Cobb. Vector coding for firefly luciferase under the SRE.L promoter was previously constructed as described (Wells et al 2001). pGEX-2T-PBD and pGEX-2T-RBD were provided by Dr. M. A. Schwartz. Rat PRG-PDZ encoding aa 1-144 was used to produce purified GST-PRG-PDZ. Vector encoding GST-PSD95PDZ3 was provided by Dr. Rama Ranganathan. Human EDG receptor cDNA was obtained from cDNA.org and previously cloned into pcDNA3.1 and pFastBac for mammalian and SF9 expression, respectively. YFP, CFP, and RLuc coding regions were cloned onto the 3' end of receptors.

Protein Expression and Purification

$G\alpha_{13}$ with an N-terminal 6His-tag was expressed and purified from insect cells as described (Chen et al 2008). RhoAΔC (aa 1-181) was provided by Dr. James Chen. All other purified proteins were expressed in *E. Coli* strain BL21 (DE3) and cultured in LB medium overnight at 22^{o}C in the presence of 100 μM isopropyl-β-D-thiogalactopyranoside. Jana Hadas and Stephen Gutowski assisted with protein purifications.

p115-RGS-DH·PH, p115-DH·PH, PRG-RGS-DH·PH, Trio-N, p115-RGS-Trio-N, PRG-RGS-Trio-N, RhoA-6His, Rac1-9His

Cells expressing the desired proteins were lysed at 4°C in 20 mM NaHEPES pH 8.0, 5 mM β-mercaptoethanol, and protease inhibitors (1 μg/ml pepstatin A, 21 μg/ml phenylmethylsulfonyl fluoride, 21 μg/ml N^{α}-p-tosyl-*L*-lysine chloromethyl ketone, 21 μg/ml tosylphenylalanyl chloromethyl ketone, and 21 μg/ml N^{α}-p-tosyl-*L*-arginine methyl ester) by the addition of 1 mg/ml lysozyme for 1 hour and three cycles of rapid freeze-thaw. Following lysis, 5 mM $MgCl_2$, 100 mM NaCl, and 10 μg/ml DNAse were added for 1 hour and then cell debris removed by centrifugation at 35,000 *g* for 30 minutes. Ni-NTA agarose resin (Qiagen) was used to affinity purify HIS-tagged proteins. Proteins were cleaved from resin using TEV protease and further purified by Mono Q anion exchange column and by size exclusion chromatography with tandem Superdex 200/70 gel filtration columns (Amersham Pharmacia Biotech).

GST-PDZ, GST-PBD, GST-PH, GST-RBD

Cells were lysed at 4°C in 50 mM Tris pH 8.0, 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl, 0.2% Triton X-100 and protease inhibitors (1 μg/ml pepstatin A, 21 μg/ml phenylmethylsulfonyl fluoride, 21 μg/ml N^{α}-p-tosyl-*L*-lysine chloromethyl ketone, 21 μg/ml tosylphenylalanyl chloromethyl ketone, and 21 μg/ml N^{α}-p-tosyl-*L*-arginine methyl ester) by the addition of 1 mg/ml lysozyme for 30 minutes. Following lysis, 5 mM $MgCl_2$ and 10 μg/ml DNAse were added for 30 minutes and 4 cycles of sonication for 30 seconds performed before removal of debris by centrifugation at 35,000 *g* for 30 minutes. Glutathione-sepharose 4B resin (Amersham Pharmacia Biotech) was used to affinity purify GST-tagged proteins. Proteins were eluted with 15 mM glutathione then concentrated and buffer exchanged using amicon ultra centrifugal filter units.

Tissue Culture

HeLa cells used were the Tet-On line from Clontech. HeLa cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 0.1 mg/ml G418 and 10% Fetal Bovine Serum (FBS, Benchmark). PC3 cells were cultured in RPMI-1640 (Invitrogen) containing 10% FBS. All cells were maintained in a humidified incubator at 37 ^{o}C and 5% CO_2. Tet-On HeLa cells stably expressing HA-tagged-EDG receptors were generated by Stephen Gutowski following the protocol provided by Clontech and maintained in DMEM containing 0.1 mg/ml G418, 0.1 mg/ml hygromycin, and 10% FBS.

Microscopy

HeLa cells were seeded onto 6-well tissue culture (TC) dishes and transfected 2-4 hours later. After 20 hours, cells were trypsinized and replated onto glass bottom dishes. After 2 hours, cells were placed in optimem medium (Invitrogen) and incubated overnight. Cells were then placed in Hank's Balanced Salt Solution (HBSS) and visualized on a heated stage with a Ziess Axiovert 200M microscope and a Ziess 63 x 1.4 numerical aperture oil immersion objective. Image collection and analysis were performed using SLIDEBOOK 4.0 (Intelligent Imaging Innovations). Images were collected at CFPexc = 430 nm, CFPems = 470 nm, YFPexc = 500 nm, YFPems = 535 nm, FRETexc = 430 nm, FRETems = 535 nm, and 4 x 4 pixel binning. Corrected FRET (cFRET) was calculated by the following equation: [cFRET] = [FRET] – 0.56 × [CFP] – 0.035 × FRETexp/YFPexp × [YFP]; where FRET, YFP, and CFP are channel

intensities following background subtraction and FRETexp and YFPexp are exposure times for FRET and YFP, respectively .

Cell Fractionation

HeLa cells were seeded onto 100 mm TC plates and transfected 20 hours later. After 24 hours, cells were harvested in hypotonic lysis buffer (20 mM NaHEPES pH 7.5, 1 mM EDTA, 1 mM Dithiothreitol 10 mM NaCl, 1.5 mM $MgCl_2$, and protease inhibitors) and lysed by passage (10 times) through a 25G needle. Nuclei and unbroken cells were removed by centrifugation at 500 *g* for 5 minutes and then membranes separated from cytosol by centrifugation at 100,000 *g* for 15 minutes. Cytosolic fractions were diluted in 4X Laemmli Buffer and pellets were resuspended in 1X Laemmli Buffer. Samples were separated by SDS-PAGE, transferred overnight to PDVF membrane, and western blotted using anti-p115RGS and anti-PRGRGS antibodies, U2760 (Wells et al 2001) and 1186, respectively. Antiserum 1186 was prepared by Capralogics Inc., against the purified RGS domain (aa 286-539) of the PRG rat homolog, E48.

SRE.L Transcriptional Reporter Assay

HeLa cells or PC3 were seeded onto 48-well TC plates, grown for 20 hr and transfected with SRE.L, pRL-TK, and various combinations of indicated plasmids using Fugene HD transfection reagent (Promega). Four hours after transfection, cells were placed in optimem medium (Invitrogen) for 20 hour without serum. Cells were then stimulated with indicated

ligands and incubated for 5 hours. Cells were harvested and analyzed using the SRE.L transcriptional reporter assay kit from Promega and a Promega Glomax 20/20 luminometer. Luciferase activity is calculated as FLuc/RLuc luminescent signals. Fold activation is normalized to FLuc/RLuc for control (0.1 mg/ml BSA) stimulated cells.

Cellular Assay for Active Rho GTPases

HeLa cells were cultured on 100 mm TC plates. Twenty hours after seeding, cells were transfected with indicated plasmids for 4 hr and then cultured in optimem medium for 20 hour without serum. After serum starvation, cells were stimulated at 37 ^{o}C as indicated, placed on ice, rinsed once with 10 ml of ice cold PBS, and then lysed with 600 μl lysis buffer (50mM Tris pH 7.6, 10 mM $MgCl_2$, 500 mM NaCl, 2% IGEPAL CA-630, and protease inhibitors). Lysates were spun at 16,000 *g* for 2 minutes; small aliquots of the supernatant were removed for protein assays and the remaining supernatant was rapidly frozen in liquid N_2 and stored at -80^{o}C. Protein assays were performed using Precision Red Advanced Protein Assay Reagent #2 (Cytoskeleton) and used to adjust samples to equivalent protein amounts for analysis. Thawed soluble lysates were mixed with affinity beads, rotated for 30 min at 4 ^{o}C, and washed with 500 μl wash buffer (25mM Tris, pH 7.6, 30 mM $MgCl_2$, 40 mM NaCl) prior to elution.

Assay for activated RhoA

Agarose beads with bound GST-RBD (Rho binding domain of rhotekin) were obtained from Cytoskeleton and 15 μl of beads (50 μg GST-RBD) were used to isolate activated RhoA

from 450 μg lysate protein. Bound proteins were eluted with 1.5X Laemmli Buffer; eluates were separated by SDS-PAGE and transferred overnight to PDVF membranes for western blotting with anti-RhoA (Cytoskeleton).

Assay for activated Rac1

Purified GST-PBD (Cdc42/Rac binding domain of p21 activated kinase 1 (Benard and Bokoch 2002)) was incubated with glutathione agarose beads for 15 minutes and rinsed twice with lysis buffer; 40 μl of suspended beads containing 1 μg/μl GST-PBD were used to isolate activated myc-Rac1 from 450 μg of lysate protein. Beads were eluted with 2X Laemmli Buffer; eluates were separated by SDS-PAGE and transferred overnight to PDVF membranes for western blotting with anti-myc antibodies (Santa Cruz sc-40).

Preparation of Unilamellar Phospholipid Vesicles

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) (DGS-NTA(Ni)) were mixed in a mole ratio of 4.75:5:0.25, respectively, dried with a steady stream of N_2 gas for 30 minutes, and placed under a vacuum overnight. Lipids were then resuspended to 10 mM in 1 ml of buffer (20 mM NaHEPES pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol), subjected to 5 freeze-thaw cycles using an ethanol/dry ice

bath, then passed through an Avanti Mini-Extruder 21 times using a 100 nm polycarbonate membrane.

Nucleotide Exchange Assay

Binding of *N*-methylanthraniloyl-GDP or -GTP (mant-GDP or mant-GTP, Invitrogen) was performed on a Fluorolog-3 spectrofluorometer at 25 ^{o}C, excitation at 356 nm, emission at 445 nm, and slit widths of 1 nm. Vesicles (0.5 mM phospholipid) containing DGS-NTA(Ni) were mixed with 2 μM RhoA-6His or 2 μM Rac1-9His in reaction buffer (20 mM NaHEPES, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol). Mant-nucleotides (5 μM) were added and reactions started with the addition of RhoGEFs as indicated. When included, 6His-Gα_{13}, was added to vesicles with the other GTPases. Initial rates were estimated by linear regression and fold activation normalized to initial rate of exchange for Rho GTPases in the absence of RhoGEFs.

Pull-down assay for EDG receptor binding to GST-PDZ

SF9 cells, infected for 48 hours with virus encoding EDG receptors (Jana Hadas), were lysed in buffer 1 (50 mM NaHEPES, 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl, and protease inhibitors) by nitrogen cavitation. Nuclei and unlysed cells were removed by centrifugation at 400 *g* for 5 minutes. Membrane fractions were separated by centrifugation at 100,000 *g* for 40 minutes, supernant removed, and pellets resuspended in buffer 1. 0.5-2 mg

total protein was incubated in buffer 2 (buffer 1 plus 1% Triton X-100) at 4^{o}C for 1 hour. Insoluble fractions were removed by centrifugation at 100,000 *g* for 20 minutes. Supernant extracts were removed and incubated with GST-PDZ domains bound to sepharose beads at 4^{o}C for 30 minutes. Bound proteins were eluted with 1.5X Laemmli Buffer; eluates were separated by SDS-PAGE and transferred overnight to nitrocellulose membranes for western blotting with anti-HA (Sigma).

GAP assay

Loading of RhoA with GTP[γ–^{32}P]

RhoAΔC was buffer exchanged using an amicon ultra centrifugal filter unit into low $MgCl_2$ Buffer (25 mM NaHEPES, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM $MgCl_2$, 50 mM NaCl, 10 μM GDP). Loading reactions were performed in low $MgCl_2$ buffer using 100 μM RhoAΔC and 1 mM GTP (plus trace amounts of GTP[γ–^{32}P]), stopped by addition of 5 mM $MgCl_2$, and separated over G25 sepharose gel filtration column.

Basal hydrolysis by RhoA

0.5 μM RhoAΔC loaded with GTP[γ–^{32}P] was incubated in reaction buffer (20 mM NaHEPES, 1 mM dithiothreitol, 100 mM NaCl, 10 mM $MgCl_2$) at 25^{o}C; alone and in the presence of 5 mM GST, GST-RBD, or GST-PBD. Aliquots (20 μl) were removed at 0, 1, 5, 10, 20, 30, and 60 minutes and immediately added to 750 μl 5% activated charcoal (in 50 mM

NaH_2PO_4), centrifuged for 5 minutes at 3,000 *g*, and 500 μl of supernatant counted by liquid scintillation spectrometry.

Lysate induced hydrolysis

HeLa cells were scraped from a 100mm TC dish in 20 mM NaHEPES, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors. Cells were lysed by passage (15 times) through a 25G needle, lysates centrifuged at 100,000 g to remove debris, and supernatant recovered. Experiments were performed as described as above except temperatures were lowered to 4^{o}C and 100 μg total protein (supernatant) was added for each experiment.

BRET assay

HeLa cells were seeded onto 48-well TC plates, allowed to adhere for 4 hours and then transfected with YFP- and RLuc-tagged EDG receptors as indicated in figures. Twenty hours after transfection, cells were transferred onto solid white 96-well TC plates and incubated in serum free DMEM overnight. Cells were then washed with HBSS and incubated with 2 μM coelenterazine-*h* immediately prior to data collection using a POLARstar Optima plate reader from BMG LabTech. Emissions at 435 nm and 535 nm were simultaneously collected for 2 minutes prior to addition of agonist and for 4 minutes following agonist addition. The YFP channel emission (535 nm) was divided by the RLuc channel emission (435 nm) to obtain the BRET ratio.

CHAPTER FOUR
Results

TRANSLOCATION OF RHOGEFS TO THE PLASMA MEMBRANE ACTIVATES RHO GTPASES

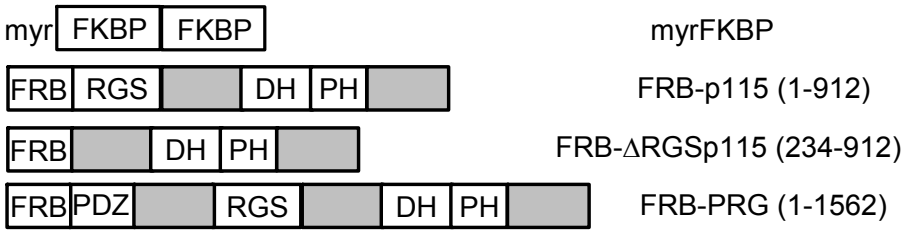
The RGS-RhoGEFs clearly translocate from the cytosol to the plasma membrane when exposed to activated $G\alpha_{12/13}$. This chapter provides evidence that translocation of RhoGEFs is a regulatory mechanism that controls activation of the RhoA signaling pathway. The ability of acute localization of RGS-RhoGEFs to stimulate activity of RhoA, as well as the ability of the RGS domain to detect hormone stimulation of cells and activate RhoGEFs, is examined.

Regulated Heterodimerization System Controls Localization of FRB Tagged RGS-RhoGEFs to the Plasma Membrane

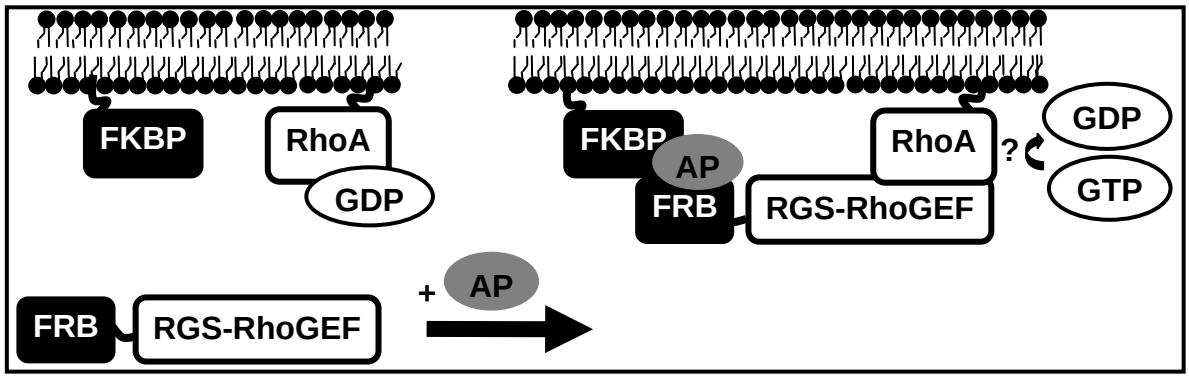
The ARGENT regulated heterodimerization system uses the rapamycin analog AP21967 (Rapalog) to acutely control dimerization of proteins of interest. The system consists of two binding partners, an FK506 binding protein (FKBP) that is constitutively localized to the plasma membrane by a myristoylation (myr) sequence at its N-terminus, and the cytosolic 93 amino acid region of the PI3K homolog FRAP (FRB) (Spencer et al 1993). Interaction of the two binding partners can be induced by addition of Rapalog, leading to effective translocation of FRB from the cytosol to the plasma membrane. Various FRB-RGS-RhoGEF fusion constructs were made to allow plasma membrane localization of exogenously expressed RGS-RhoGEFs to be regulated by the presence or absence of Rapalog (Fig. 4.1*A*-*B*).

Figure 4.1: System for regulated translocation of FRB-RGS-RhoGEFs

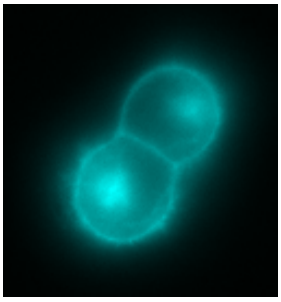
A.



B.



C.



D.

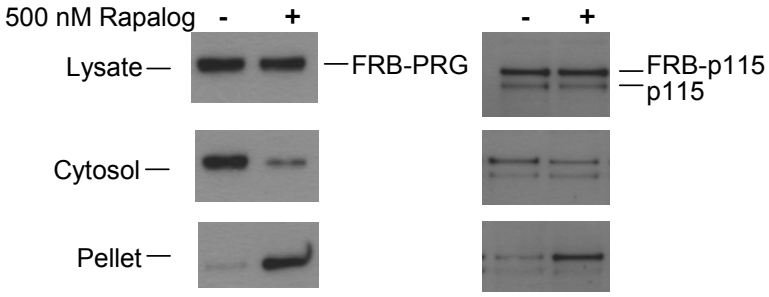


Figure 4.1. System for regulated translocation of FRB-RGS-RhoGEFs. (A) Diagram of constructs. (B) Diagram of translocation system. myrFKBP contains a myristoylation sequence at the N-terminus causing the domain to be localized to the plasma membrane. FRB-RGS-RhoGEFs are localized to the cytosol and only translocate to the membrane when binding to myrFKBP is induced with the rapalog AP. (C) myrFKBP localizes to the plasma membrane. HeLa cells transfected with myrFKBP-CFP and imaged by fluorescence microscopy. (D) Rapalog induces translocation of RGS-RhoGEFs from cytosolic to membrane fractions. HeLa cells were transfected with myrFKBP plasmid and plasmid encoding either FRB-p115 or FRB-

PRG. After 24 hours cells were stimulated with 500 nM Rapalog and hypotonically lysed. Lysates were fractionated by centrifugation and analyzed by western blotting.

Plasma membrane localization of myrFKBP was verified by fluorescence microscopy utilizing a CFP tagged myrFKBP (Fig. 4.1*C*). Localization of FRB-PRG and FRB-p115 in the presence and absence of Rapalog was examined using crude fractionation of HeLa cells. Both proteins localize to the cytosolic fraction in the absence of Rapalog. Levels of FRB-PRG and FRB-p115 in the cytosolic fraction decrease upon addition of Rapalog and concordantly, levels in the membrane fraction increase. This verifies that addition of Rapalog induces the proteins to translocate from the cytosol to the plasma membrane. In contrast, endogenous p115 does not redistribute to the membrane fraction upon treatment of cells with Rapalog (Fig. 4.1*D*).

Translocation of RGS-RhoGEFs is Sufficient to Activate RhoA

The small molecule regulated heterodimerization system was used to investigate whether induced membrane localization of FRB-PRG was capable of activating RhoA. In HeLa cells, using an SRE.L transcriptional reporter assay for detection of active RhoA, expression of increasing amounts of FRB-PRG raised basal activation of RhoA, as expected for overexpression of exogenous RhoGEF (Fig. 4.2*A*). Importantly, addition of Rapalog induced ~3 fold increase in reporter activity at lower concentrations of FRB-PRG expression. These results indicate that localization of PRG to the plasma membrane is sufficient to stimulate the signaling pathway. Transcription of the reporter plasmid was abolished by the expression of C3 transferase, verifying that activation of RhoA was required for regulation of the reporter (Fig. 4.2*B*).

Figure 4.2: Translocation of RGS-RhoGEFs Activates RhoA

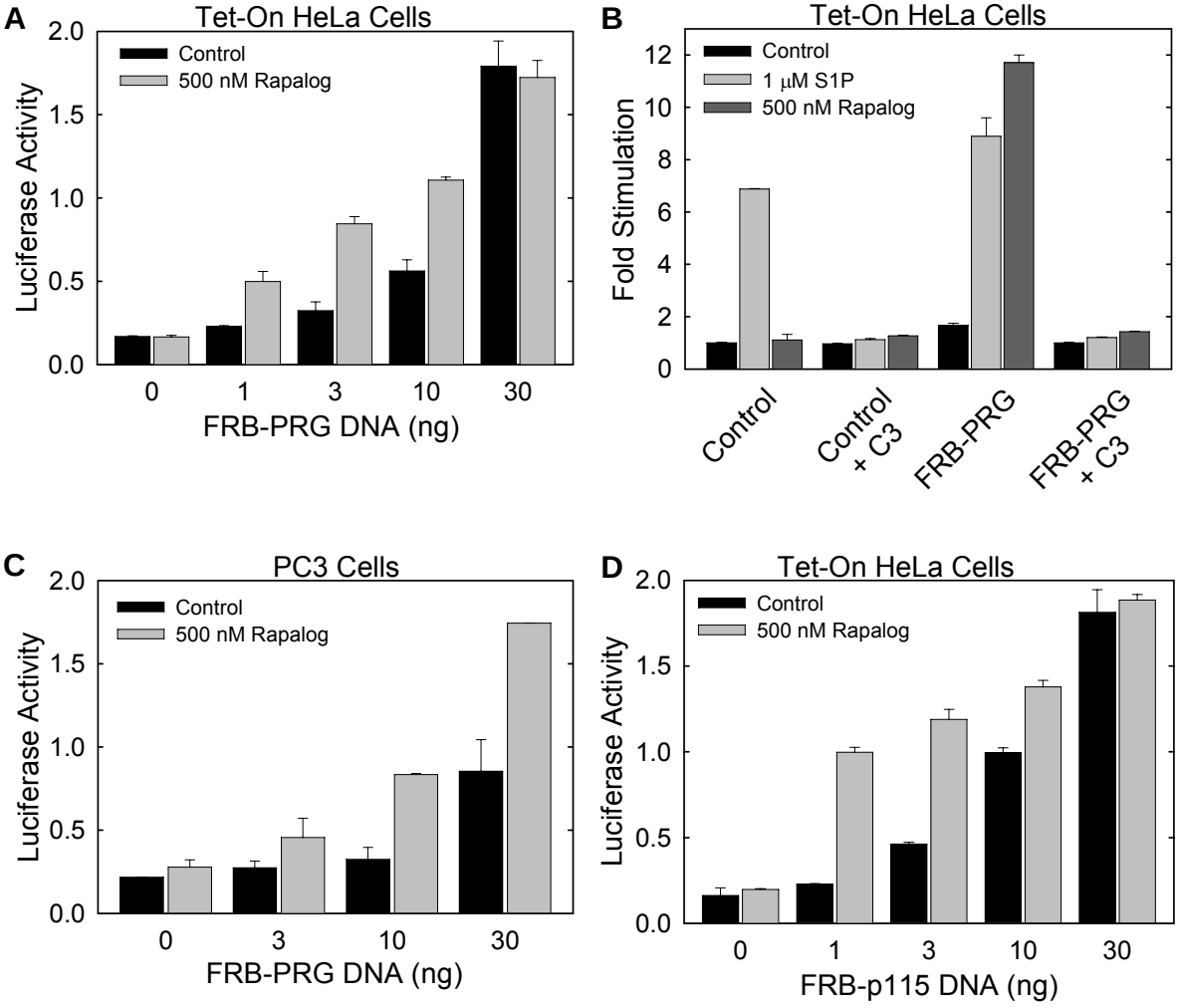


Figure 4.2. Translocation of RGS-RhoGEFs Activates RhoA. (A) Membrane localization of FRB-PRG activates RhoA responsive transcriptional reporter. HeLa cells were transfected with plasmids encoding the SRE.L reporter, myrFKBP, and FRB-PRG. Cells were serum starved for 20 hours and then stimulated with control (0.1 mg/ml BSA) or 500 nM Rapalog for 5 hours. Cells were then lysed and expression of RLuc and FLuc determined as stated in methods. (B) Activation of transcriptional reporter is dependent on RhoA activity. HeLa cells were transfected with plasmids encoding the SRE.L reporter, myrFKBP, and FRB-PRG either alone or in combination with C3 transferase. Cells were serum starved for 20 hours and then stimulated with control (0.1 mg/ml BSA), 1 μM S1P, or 500 nM Rapalog for 5 hours. Cells were then lysed and expression of RLuc and FLuc determined as stated in methods. (C) Activation of RhoA by membrane localization of FRB-PRG is not cell line specific. PC3 cells were transfected with plasmids encoding SRE.L reporter, myrFKBP, and FRB-PRG. Cells were then stimulated, processed, and analyzed as stated in *A*. (D) Membrane localization of FRB-p115 activates

RhoA. HeLa cells were transfected with plasmids encoding SRE.L reporter, myrFKBP, and FRB-p115. Cells were then stimulated, processed, and analyzed as stated in *A*.

Induced translocation of FRB-PRG was also shown to be an effective activator of RhoA in PC3 cells, demonstrating that this is not a unique phenomenon of HeLa cells (Fig. 4.2*C*).

The intrinsic exchange activity of p115RhoGEF can be stimulated *in vitro* by the addition of activated $G\alpha_{13}$, but only modestly (Hart et al 1998, Suzuki et al 2003). Localization to the plasma membrane could be an additional mechanism contributing to activation of this RhoGEF *in vivo*. Similar to FRB-PRG, induced translocation of FRB-p115 to the plasma membrane was sufficient to stimulate the SRE.L reporter of RhoA activity ~5 fold (Fig. 4.2*D*). Again, constitutive activation of RhoA was observed with increases expression of FRB-p115, even in the absence of Rapalog; this underscores the necessity for tight regulation of endogenous RhoGEFs.

$G\alpha_{12/13}$ Is Not Required for Activity of Translocated RGS-RhoGEFs

Although $G\alpha_{13}$ does not activate PRG *in vitro*, overexpression of PRG in combination with $G\alpha_{12}$ and $G\alpha_{13}$ has been reported to synergistically activate RhoA in cells (Suzuki et al 2003). Therefore, we tested whether hormone activation of $G\alpha_{13}$ would increase the activity of FRB-PRG translocated to the plasma membrane by the addition of Rapalog. In HeLa cells, S1P stimulates G_{13} via receptors of the EDG family. Treatment of cells with 1 μM S1P stimulated the transcriptional reporter for RhoA ~5 fold. Simultaneous addition of both S1P and Rapalog produced an essentially additive response. This indicates that activated $G\alpha_{13}$ does not further

enhance the catalytic activity of translocated FRB-PRG (Fig. 4.3*A*). $G\alpha_{13}$ modestly stimulates the exchange activity of p115RhoGEF *in vitro*. However, simultaneous stimulation of cells expressing FRB-p115 with both S1P and Rapalog also resulted in additive synthesis of the reporter. To further investigate any contribution of $G\alpha_{13}$ to the observed activity of membrane localized FRB-p115, we expressed an FRB-p115 construct missing the RGS region, FRB-ΔRGSp115, a domain required for regulation by $G\alpha_{13}$. Again simultaneous addition of both S1P and Rapalog resulted in additive levels of SRE.L activation (Fig. 4.3*B*). Together, these results show that $G\alpha_{13}$ activated by hormone does not enhance the catalytic activities of the translocated FRB-RhoGEFs. While this may represent an inability to further stimulate the enzymes on the membrane, it is also possible that $G\alpha_{13}$ activated by hormone and the myrFKBP targeting domain used in these experiments inhabit separate regions of the plasma membrane or are sterically hindered from interacting with each other.

While the translocated RhoGEFs may be insensitive to G_{13} activated by hormone, it is still possible that basal levels of active G_{13} contribute to their activity. To test for such dependence, the FRB-RhoGEFs were translocated in the presence of the overexpressed RGS domain of p115RhoGEF, a potent inactivator of the $G\alpha_{12}$ and $G\alpha_{13}$ proteins (Kozasa et al 1998). Although stimulation of the SRE.L reporter by S1P was almost completely abolished by overexpression of the RGS domain, activation due to Rapalog induced translocation of both FRB-PRG and FRB-p115 was not affected (Fig. 4.3*C, D*). This clearly demonstrates that activated $G\alpha_{12/13}$ is not required for the RGS-RhoGEFs to maintain their intrinsic catalytic activity and supports the hypothesis that recruitment of the GEFs to the plasma membrane, a site of localized substrate, is a feasible mechanism for their regulation. By this mechanism, activated $G\alpha_{12}$ or $G\alpha_{13}$ could

Figure 4.3: Gα13 is not required for catalytic activity of translocated RGS-RhoGEFs

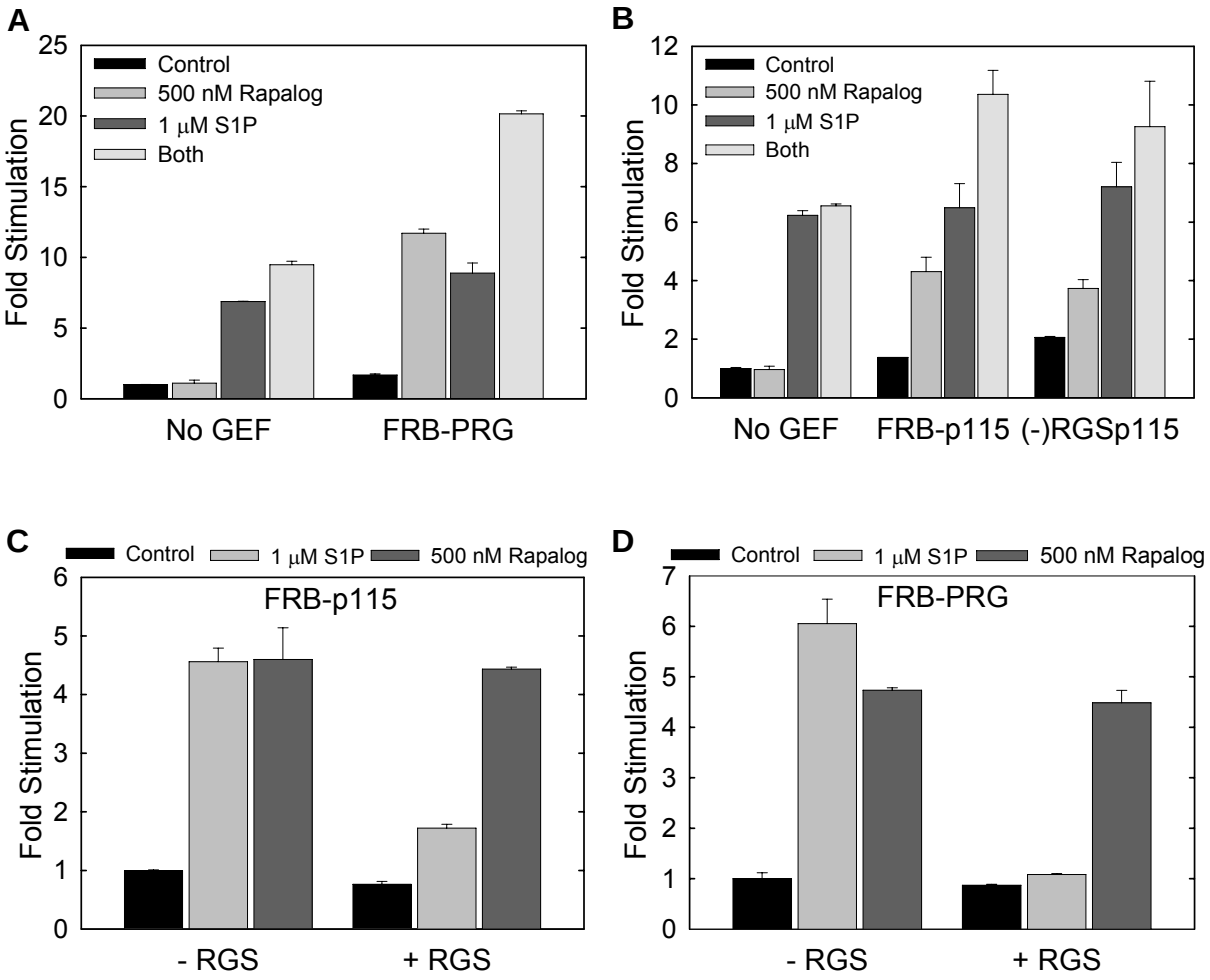


Figure 4.3. Gα13 is not required for catalytic activity of translocated RGS-RhoGEFs. (A) S1P stimulation of Gα13 does not enhance activity of translocated FRB-PRG. HeLa cells were transfected with myrFKBP, FRB-PRG, and SRE.L reporter plasmids. Cells were serum starved for 20 hours and then stimulated with 0.1 mg/ml BSA, 500nM Rapalog, 1μM S1P, or both Rapalog and S1P. Cells were then lysed and expression of RLuc and FLuc determined as stated in methods. (B) S1P stimulation of Gα13 does not enhance activity of translocated FRB-p115. HeLa cells were transfected with myrFKBP, FRB-p115 or FRB-ΔRGSp115, and SRE.L reporter plasmids. Cells were stimulated, processed, and analyzed as stated in *A*. (C, D) Blockage of Gα13 activity by p115-RGS does not effect activation of RhoA in response to translocation of FRB-RGS-RhoGEF. HeLa cells were transfected with myrFKBP, FRB-p115 or FRB-PRG, and SRE.L reporter plasmids either alone or in combination with the p115-RGS domain. Cells were stimulated, processed, and analyzed as stated in *A*.

activate RhoA by binding to the RGS domains of endogenous RGS-RhoGEFs and promoting their recruitment to the plasma membrane.

Magnitude and Kinetics of RhoA Activation.

Transcription of the RhoA responsive reporter occurs over hours whereas activation of RhoA by hormones occurs within minutes. In order to monitor acute formation of RhoA directly, the Rhotekin binding domain was used to rapidly isolate RhoA-GTP from cells. This allowed the ability of regulated translocation of the RGS-RhoGEFs to acutely activate RhoA to be assessed and compared with activation of RhoA observed upon treatment with hormones. Stimulation of HeLa cells with S1P produced a robust elevation in active RhoA within 1 minute, which began declining by 3 minutes after stimulation with hormone (Fig. 4.4*A, C*). Similarly, addition of Rapalog increased activation of RhoA within 1 minute, but levels of active RhoA continued to rise reaching a plateau around 3 minutes (Fig. 4.4*B, C*). This level of activation was sustained for at least 30 minutes (data not shown). A well-established mechanism for downregulation of G-protein coupled signaling pathways is desensitization and/or internalization of GPCRs (Gainetdinov et al 2004). This may account for the rapid decline in activation of RhoA by S1P. In contrast, translocation of the RhoGEF by Rapalog bypasses receptor and G protein activation and produces more prolonged stimulation by stably maintaining the RhoGEF at the plasma membrane. To directly compare the levels of activated RhoA produced in response to S1P and Rapalog, cells were stimulated within the same experiment for 3 minutes. Consistent with averaged data from multiple experiments (Fig 4.4*C*), the magnitude of activation of RhoA

Figure 4.4: The magnitude and kinetics of translocation induced activation is comparable to receptor mediated hormonal activation.

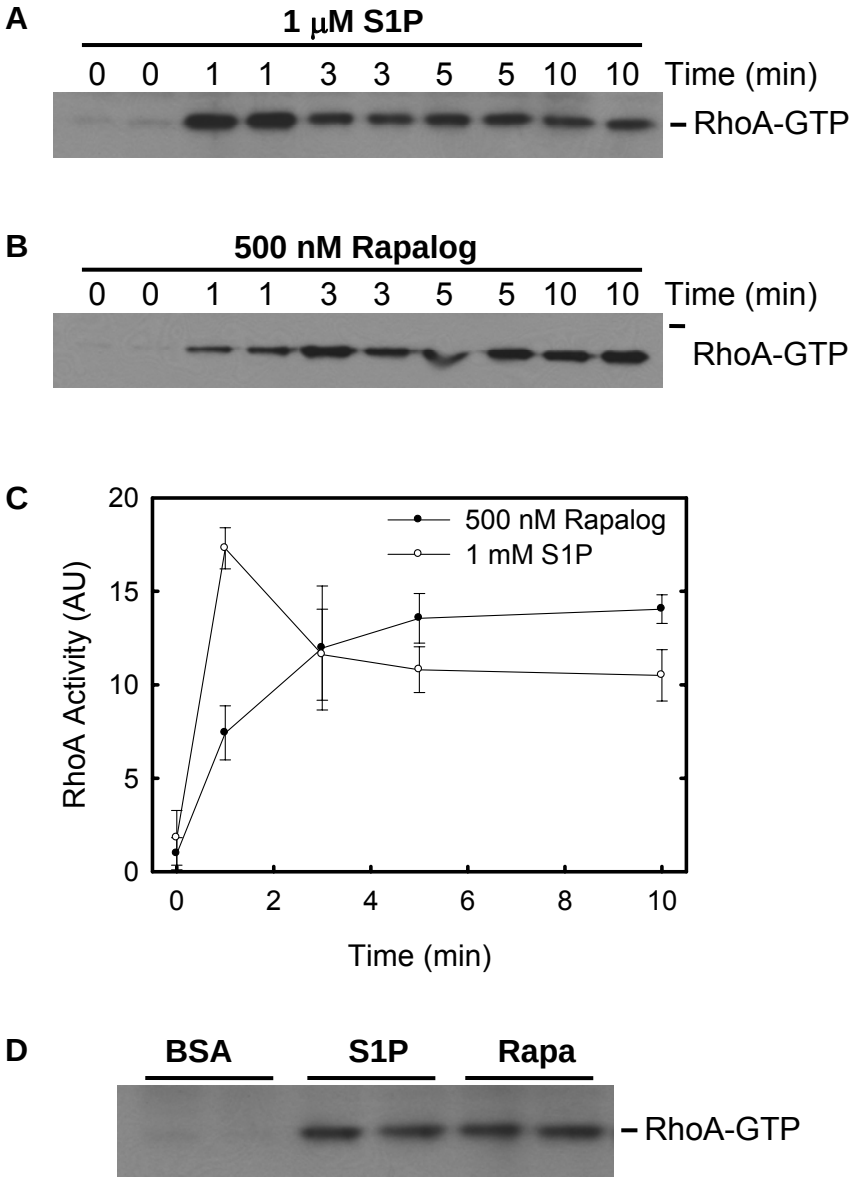


Figure 4.4. The magnitude and kinetics of translocation induced activation is comparable to receptor mediated hormonal activation. (A) HeLa cells were transfected with myrFKBP and FRB-p115 plasmids. Cells were stimulated with 1 μM S1P for the times shown and then lysed. GST-RBD bound to agarose beads was used to isolate active RhoA from the lysates and samples analyzed by western blot. (B) HeLa cells were transfected as in *A*, then stimulated with 500 nM Rapalog for the lengths of time shown and processed as in *A*. (C) Quantitation of results

represented in *A* and *B*. Western blot results from at least three experiments were quantitated using Image J. Band intensities for each point were normalized by the total sum of intensities within each experiment. Error bars reflect the standard deviation for each group of time points. (D) HeLa cells were transfected as in *A*, then stimulated with 0.1 mg/ml BSA, 1 μM S1P, or 500 nM Rapalog for 3 minutes and then processed as in *A*.

under these conditions was almost identical for hormone mediated and translocation induced activation (Fig. 4.4*D*). These data illustrate that activation of RhoA by both methods occurs within a comparable range.

The RGS domain of PRG can mediate hormonal activation of Rac by $G_{12/13}$

The RGS domains of RhoGEFs interact specifically with the active forms of $G\alpha_{12}$ and $G\alpha_{13}$; these G proteins are activated by GPCRs at the plasma membrane and may remain membrane associated following activation either through their interaction with receptor or through N-terminal acylation (Bhattacharyya and Wedegaertner 2000). Thus, a logical mechanism for recruitment of RGS-RhoGEFs to the plasma membrane in response to activation of GPCRs with hormones is interaction of their RGS domain with membrane-associated $G\alpha_{12}$ and $G\alpha_{13}$. To test if the RGS domain of PRG could act independently as a sensor of $G\alpha_{12}$ and $G\alpha_{13}$ activation, the domains were placed in front of the N-terminal set of DH·PH domains from the multifunctional Trio protein (Trio-N): the nucleotide exchange activity of this tandem DH·PH unit is specific for Rac (Debant et al 1996). Stimulation of HeLa cells with S1P normally activates RhoA, but not Rac1 (data not shown). Addition of the RGS domains to Trio-N should rewire this RacGEF to respond to the $G_{12/13}$ pathway and allow S1P to drive activation of Rac. For enhanced activity, HeLa cells that stably overexpress the EDG 5 receptor (EDGR5) were used. These cells also

stimulate RhoA, but not Rac1, in response to treatment with S1P (Fig. 4.5*B*). Figure 4.5*C* shows that expression of the PRG-RGS-Trio-N chimera in EDGR5 HeLa cells, but not expression of Trio-N alone, produced activation of Rac1 in response to stimulation with S1P. Levels of active Rac1 were higher in all cells transfected with Trio-N compared with PRG-RGS-Trio-N due to higher levels of expression of Trio-N alone (Figl 4.5*C*). The time course of activation of Rac1 by S1P through PRG-RGS-Trio-N is consistent with activation of endogenous RhoA by S1P (Fig. 4.5*D-F*). $G\alpha_{12/13}$ does not stimulate the intrinsic exchange activity of Trio-N; therefore, the observed activation of Rac1 in response to stimulation with S1P is a result of the effect of $G\alpha_{12/13}$ on the cellular localization of PRG-RGS-Trio-N. These data suggest that through binding to active $G\alpha_{12/13}$ in the plasma membrane, the RGS domain can respond to hormone stimulation, regulate the localization of RhoGEFs, and drive a signaling pathway.

Figure 4.5: The RGS domain of PRG can mediate hormonal activation of Rac1.

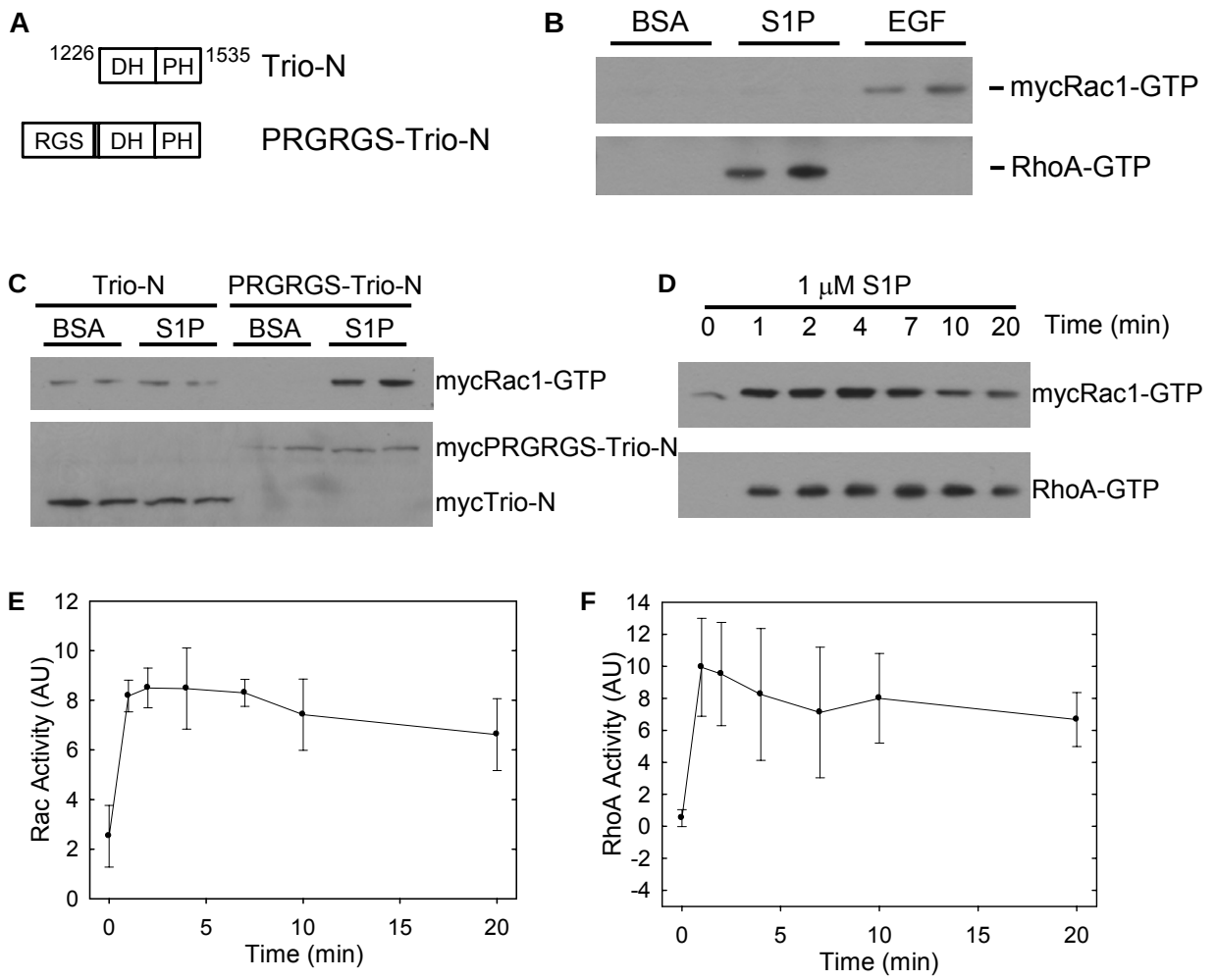


Figure 4.5. The RGS domain of PRG can mediate hormonal activation of Rac1. (A) Diagram of constructs. (B) S1P stimulates RhoA but not Rac1. EDGR5 HeLa cells were transfected with myc-Rac1 and stimulated with 0.1 mg/ml BSA, 1 μM S1P, or 50ng/ml EGF for 3 minutes and then lysed. GST-RBD and GST-PBD bound to agarose beads were used to isolate active RhoA and active myc-Rac1, respectively, and bound proteins were analyzed by western blot. (C) PRGRGS-Trio-N activates Rac1 in response to stimulation of cells with S1P. *Experiment performed by Stephen Gutowski*. Cells were transfected with myc- Rac1 and either Trio-N or PRGRGS-Trio-N plasmids, then stimulated with 0.1mg/ml BSA or 1 μM S1P for 3 minutes and lysed. *Top* - GST-PBD bound to agarose beads was used to isolate active Rac1 and bound proteins were analyzed by western blot. *Bottom* – Cell lysates were analyzed by western blot for expression of myc tagged exchange factors. (D) Timecourse of activation of Rac1 by PRGRGS-Trio-N in response to S1P. *Experiment performed by Stephen Gutowski*. Cells were transfected with myc- Rac1 and PRG-RGS-TrioN plasmids and stimulated with 1 μM S1P for the length of time shown. Cells were lysed and analyzed as in *A*. (E-F) Quantitation of results represented in

D. Western blot results from at least three experiments were quantitated using Image J. Band intensities for each point were normalized by the total sum of intensities within each experiment. Error bars reflect the standard deviation for each group of time points.

CHAPTER FIVE
Results

DOMAIN MODULES THAT FUNCTION IN TRANSLOCATION OF RGS-RHOGEFS

Translocation of RGS-RhoGEFs from the cytosol to the plasma membrane can clearly mediate activation of RhoA and suggests that formation of stable localized signaling complexes may play a key regulatory role in this pathway. The details of complex formation and how they may be regulated remain unclear. This chapter focuses on evidence that shows that multiple domains of the RGS-RhoGEFs interact with membrane-associated binding partners in a context dependent manner. Interactions of RGS domains with $G\alpha_{13}$, PDZ domains with receptor C-termini, and PH domains with active RhoA are examined.

RGS Domains Act as Recruiting Modules

The PRG-RGS domain placed in front of Trio-N facilitates activation of Rac1 in response to S1P, a traditional activator of RhoA (Fig 4.5*B*, *C*). Since the RGS domains interact specifically with the active forms of $G\alpha_{12}$ and $G\alpha_{13}$, it is logical that recruitment of RGS-RhoGEFS and PRG-RGS-Trio-N to the plasma membrane by hormone is through direct interaction of the RGS domains with membrane-associated $G\alpha_{12}$ and $G\alpha_{13}$. To test whether this simple mechanism of recruitment could acutely regulate the RGS-RhoGEFs, we used an *in vitro* phospholipid vesicle system. DGS-NTA(Ni) lipids, containing a nickel chelating head group, were included in vesicles to permit association of polyhistidine tagged proteins. This allowed localization of RhoA with a C-terminal 6-His tag and $G\alpha_{13}$ with an N-terminal 6-His tag to the surface of

vesicles, which then act as membrane delimited substrate and regulator, respectively. In this paradigm, the activity of RGS-RhoGEFs on sequestered substrate, monitored by binding of mant-GDP to RhoA, should be regulated by the presence or absence of vesicle associated $G\alpha_{13}$-AMF.

The presence of $G\alpha_{13}$-AMF on vesicles enhanced the initial rate of p115-RGS-DH·PH dramatically compared with the rate observed with inactive $G\alpha_{13}$ (Fig. 5.1*C*). This enhancement is not seen with p115-DH·PH, which is missing the RGS domain (Fig. 5.1*D*). Importantly, the activity of p115-DH·PH and p115-RGS-DH·PH toward RhoA free in solution is identical (Fig. 5.1*B*). The fact that no effect was observed with inactive $G\alpha_{13}$ suggests that the RGS domain of p115RhoGEF can act as a detector of $G\alpha_{13}$ activation and function to recruit p115RhoGEF to the plasma membrane where it comes into close proximity with RhoA substrate.

The intrinsic guanine nucleotide exchange activity of p115RhoGEF on RhoA can be stimulated 3-5 fold in solution by activated $G\alpha_{13}$ (Hart et al 1998). Recent studies have shown that this activation requires interaction of the helical domain of $G\alpha_{13}$ with the DH domain and specifically, a tryptophan at residue 507 within DH (Chen et al 2012). In Fig 5.1, the presence of $G\alpha_{13}$-AMF on the vesicle surface enhanced the activity of p115-RGS-DH·PH about 30 fold. This 30 fold enhancement could be the combination of stimulation of catalytic activity and localization of the proteins. To eliminate any contribution from enhancement of catalytic activity to the observed increases in rate, the activity of the W507E mutant of p115-RGS-DH·PH, which exhibits no change in catalytic activity in the presence of $G\alpha_{13}$ was measured (Chen et al 2012). Surprisingly, the initial rate of exchange activity of p115-RGS-DH·PH W507E in the presence and absence of activated $G\alpha_{13}$ is identical to the rate of wild-type

Figure 5.1: RGS domains function as recruiting modules.

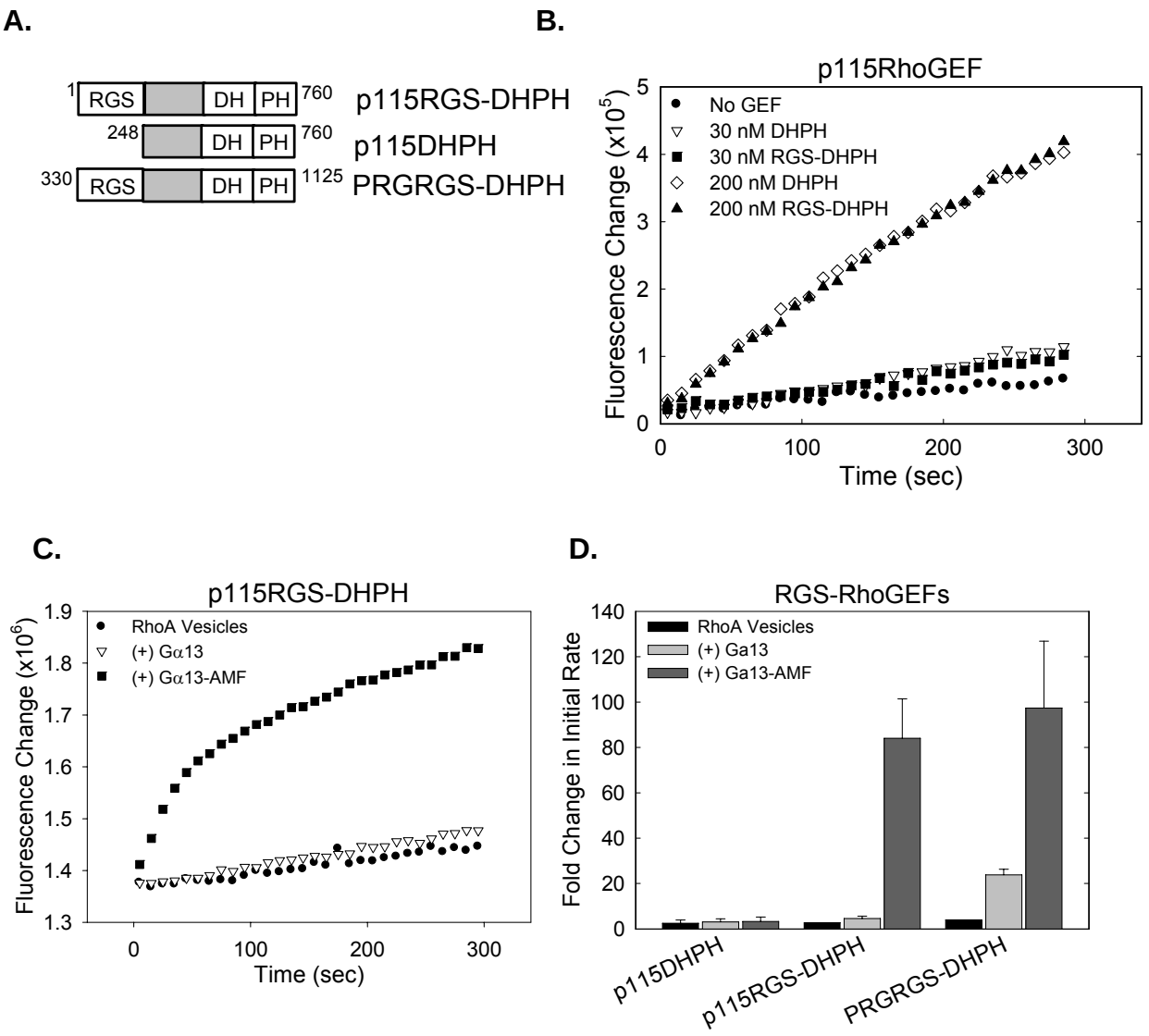


Figure 5.1. RGS domains function as recruiting modules. (A) Diagram of purified RhoGEFs used in exchange assays. (B) Purified p115-DH·PH and p115RGS-DHPH proteins have the same catalytic activity. RhoA-6His (2 μM) was incubated free in solution with 5 μM mant-GDP. Exchange reactions were started by addition of the indicated RhoGEF. (C-D) The RGS domain uses $G\alpha_{13}$ to localize RGS-RhoGEFs to vesicles to promote nucleotide exchange on membrane delimited RhoA. RhoA-6His (2 μM) bound to phospholipid vesicles was incubated with 5 μM mant-GDP either alone, or with 500 nM 6His-$G\alpha_{13}$, or 500 nM 6His-$G\alpha_{13}$-AMF. Exchange reactions were started by addition of 30 nM of the indicted RhoGEF. Time courses for p115RGS-DHPH are shown in *C* and initial rates compared as fold activation in panel *D*.

p115-RGS-DH·PH (Fig. 5.2). These results prove that activated $G\alpha_{13}$ can regulate RGS-RhoGEFs through simple enhancement of their co-localization with substrate alone. In this *in vitro* context, stimulation of intrinsic catalytic activity appears to offer no further benefit. It remains to be seen if activation of the intrinsic exchange activity of p115RhoGEF by $G\alpha_{13}$ may play an important role in the context of the cellular environment.

The RGS region forms a stable individual domain that appears to be capable of functioning as a detector of activated $G\alpha_{13}$ independent of the DH·PH domains of the RGS-RhoGEFs, as suggested in the previous chapter using PRG-RGS-Trio-N chimeras in HeLa cells (Fig 4.5). To test this hypothesis, the RGS domains from p115RhoGEF and PRG were placed in front of Trio-N, which functions as a RacGEF, and the activity of these chimeras on vesicle associated Rac1 were measured by binding of mant-GTP, both in the presence and absence of $G\alpha_{13}$-AMF. The activities of the chimeras toward Rac1 free in solution were similar to the activity of Trio-N alone (Fig. 5.3*B*). As observed with p115RhoGEF for RhoA, we found that the activity of both chimeras toward vesicle associated Rac1 was dramatically enhanced in the presence of $G\alpha_{13}$-AMF, whereas the activity of Trio-N alone was not (Fig. 5.3*C*). $G\alpha_{13}$-AMF does not stimulate the intrinsic catalytic activity of Trio-N; therefore, the enhancement of initial rate is solely contributed to the RGS domains localizing and concentrating the enzymes in proximity with their substrate. This demonstrates that binding of RGS domains to activated $G\alpha_{13}$ mediates rapid translocation of RhoGEFs to a lipid surface which facilitates efficient catalytic interaction with Rho GTPases.

Figure 5.2: Increased rates of exchange are due to localization and not stimulation of intrinsic catalytic activity.

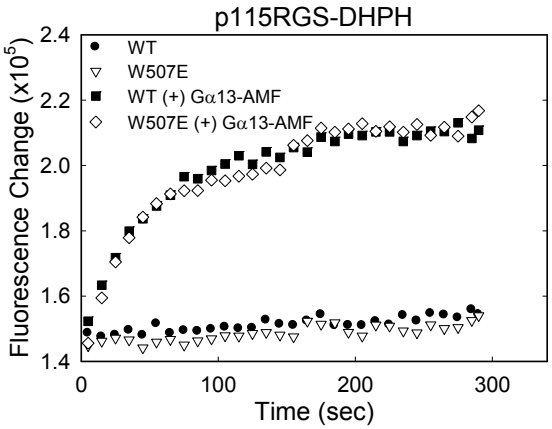


Figure 5.2. Increased rates of exchange upon recruitment of RhoGEFs to vesicles by $G\alpha_{13}$ are due to localization and not stimulation of intrinsic catalytic activity. RhoA-6His (2 μM) bound to vesicles was incubated with 5 μM mant-GDP; either alone or with 500 nM 6His-$G\alpha_{13}$-AMF. Exchange reactions were initiated by addition of 30 nM RhoGEF and change in fluorescence signal monitored over 5 minutes.

Figure 5.3: RGS domains mediate activation of Rac1 by $G\alpha_{13}$ in chimeric Trio-N proteins.

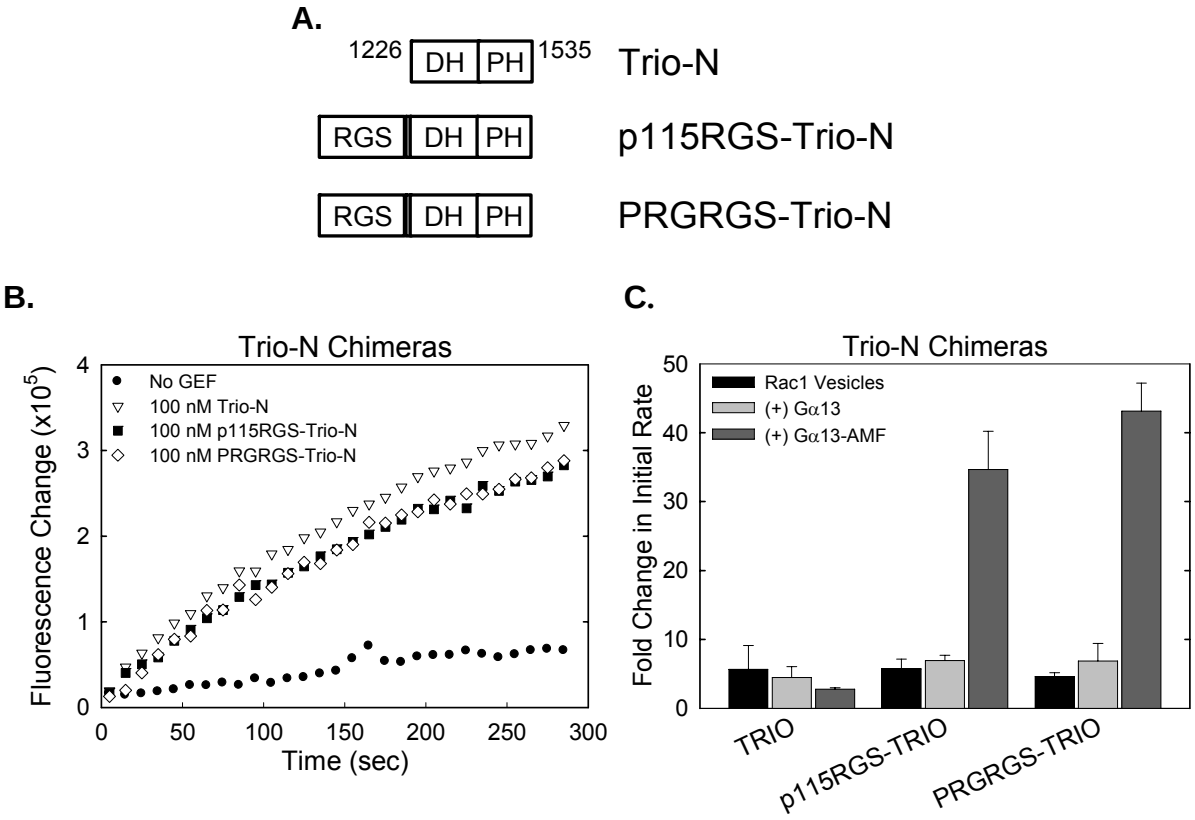


Figure 5.3. RGS domains mediate activation of Rac1 by $G\alpha_{13}$ in chimeric Trio-N proteins. (A) Diagram of purified RhoGEFs used in exchange assays. (B) Purified Trio-N and RGS-Trio-N chimeras have similar catalytic activity in solution. Rac1-9His (2 μM) was incubated free in solution with 5 μM mant-GTP. Exchange reactions were started by addition of the indicated RhoGEF. (C) RGS domains localize chimeric RacGEFs promoting nucleotide exchange on Rac1. Rac1-9His (2 μM) bound to vesicles was incubated with 5 μM mant-GTP either alone, or with 500 nM 6His-$G\alpha_{13}$, or with 500 nM 6His-$G\alpha_{13}$-AMF. Exchange reactions were started by addition of the RhoGEF and initial rates compared as fold activation.

PDZ Domains Bind to Cell Surface Receptors

The EDG receptor family includes LPA receptors (LPAR) 1-3 and S1P receptors (S1PR) 1-5. The C-termini of LPAR1, LPAR2, and S1PR2 contain a class I PDZ binding motif in which the last three amino acids of the proteins consist of a hydroxyl amino acid, followed by a variable residue, and ending in a hydrophobic amino acid (Fig. 5.4*A*) (Songyang et al 1997). PDZ domains and binding motifs are sites of protein-protein interactions that are commonly found in scaffolding proteins (Nourry et al 2003). Interestingly, LARG and PRG both contain PDZ domains at their N-termini that potentially interact with the EDG receptors. This has been shown for LPAR1 and LPAR2 by pull-down experiments of tagged receptors with purified PDZ domains from both GEFs (Yamada et al 2005). The same type of assay is shown in Figure 5.4*B* and expanded to include S1P receptors. For PRG-PDZ, binding occurred with LPAR1 and 2 (EDGR2 and 4), as expected, and was absent with LPAR3 (EDGR7), which lacks the C-terminal PDZ binding motif. Additionally, PRG-PDZ specifically interacted with S1PR2 (EDGR5) and not S1PR3 (EDGR3). No binding of the receptors to LARG-PDZ was observed (data not shown); however, functionality of the protein could not be verified with a positive control. Interestingly, S1PR2 not only contains a C-terminal PDZ binding motif but couples efficiently to $G\alpha_{12/13}$ as well; in contrast, S1PR3 does not contain a PDZ binding motif (Fig. 5.4*A*) and appears to couple to $G\alpha_{12/13}$ less efficiently (Sanchez and Hla 2004).

The functional importance of the interaction between the PDZ domain of PRG and S1P receptors was examined by overexpressing the isolated PDZ domain in HeLa cells and determining the effect on stimulation of RhoA by S1P. The PRG-PDZ domain reduces

Figure 5.4: The PRG-PDZ domain binds to EDG 5 receptors (S1PR2)

A. C-terminal 3 amino acids of EDGRs
(Class I Motif: S/T-X-Φ)

EDGR2 (LPAR1)	**S**-V-**V**
EDGR3 (S1PR3)	F-C-N
EDGR4 (LPAR2)	**S**-T-**L**
EDGR5 (S1PR2)	**T**-V-**V**
EDGR7 (LPAR3)	**S**-T-S

B.

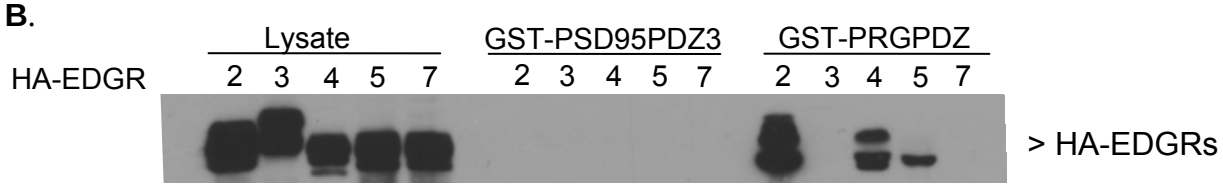


Figure 5.4. The PRG-PDZ domain binds to EDG 5 receptors (S1PR2). (A) Comparison of the C-terminal residues of EDG receptors; EDGRs 2, 4 and 5 contain a consensus sequence for the class I PDZ binding motif. (B) PRG-PDZ binds to EDGR5. Membrane extractions from SF9 cells expressing HA-tagged EDGR 2, 3, 4, 5, or 7 were incubated with purified GST-PRGPDZ and GST-PSD95PDZ3 proteins bound to glutathione agarose beads. Bound components were detected by western blot for the HA-tag.

stimulation by S1P of the transcriptional reporter for activation of RhoA in a concentration dependent manner (Fig. 5.5). This suggests that the PDZ domain can interact with S1P receptors *in vivo*, providing a mechanism for endogenous RhoGEFs to anchor themselves to membrane associated receptors and facilitate activation of RhoA. However, stimulation of RhoA by S1P in HeLa cells may or may not be entirely dependent on PDZ-containing RhoGEFs; an alternate explanation for inhibition of the system by expression of PRG-PDZ is steric interference of binding of the receptor to $G\alpha_{12/13}$ or other unknown signaling partners.

PH Domains of RhoGEFs Bind to Activated RhoA

The purified PH domain of PRG interacts specifically with RhoA-GTPγS and not RhoA-GDP *in vitro.* Importantly, binding of Rho-GTP to the PH domain does not preclude binding of the substrate, RhoA-GDP, to the DH domain and has no effect on the catalytic rate of exchange by PRG *in vitro* (Chen et al 2010). This suggests the PH domain may exhibit a feed-forward function as an additional anchoring point that would aid in the formation of a stable RhoA signaling complex.

The effect of overexpressing isolated PH domains from PRG in HeLa cells is shown in Figure 5.6*A*. PRG-PH domains inhibit stimulation of the transcriptional reporter of RhoA by S1P in a concentration dependent manner. Importantly, expression of a PRG-PH domain, PRG-PH F1044A, that contains a mutation to prevent interaction with activated RhoA, had no effect on the stimulation by S1P (Fig 5.6*B*). This shows that the PH domain from PRG can bind to active RhoA in cells and indicates the potential for anchoring of endogenous RhoGEFs to the

Figure 5.5: Expression of the PRG-PDZ domain blocks stimulation of RhoA by S1P.

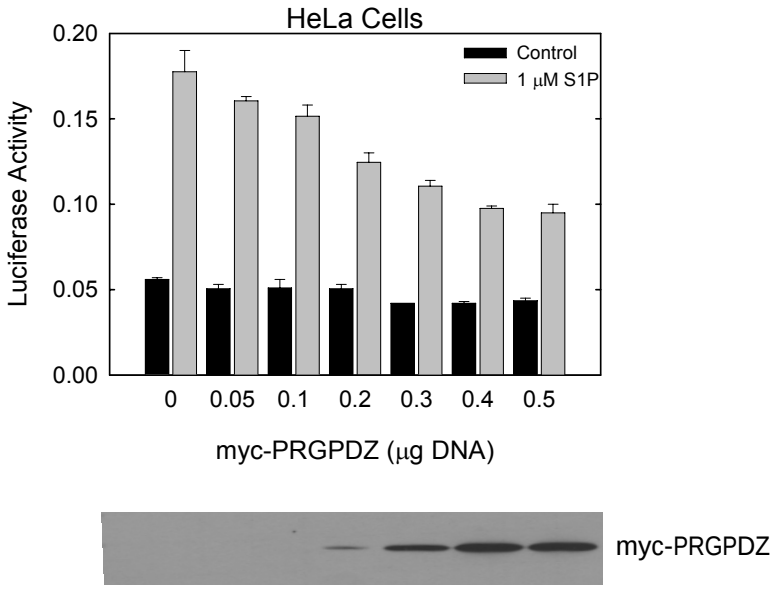


Figure 5.5. Expression of the PRG-PDZ domain blocks stimulation of RhoA by S1P. HeLa cells were transfected with SRE.L reporter plasmids and increasing amounts of myc-PRGPDZ plasmids. Cells were serum starved for 20 hours and then stimulated with control (0.1 mg/ml BSA) or 1 μM S1P for 5 hours. Cells were then lysed and expression of RLuc and FLuc determined as stated in methods.

plasma membrane as a mechanism for maintaining physiological activation of RhoA. However, in this experiment, binding of the PH domain alone to activated RhoA would also prevent RhoA's interaction with its downstream effectors, thus abrogating the effect of RhoA on transcription and the reporter plasmid used in these experiments.

PH domains are present in almost all RhoGEFs as a part of the canonical tandem DH·PH unit, with the DH domain supplying the essential site for the exchange activity toward Rho GTPases. The Lbc family of RhoGEFs contains the three RGS-RhoGEFs and four other homologous RhoGEFs from a second branch (Sternweis et al 2007). All of these proteins share specificity for RhoA, B and C as substrates and the PH domains of these Lbc-family RhoGEFs all contain a hydrophobic region within their PH domains homologous to the region of the PRG-PH domain that binds to RhoA-GTP (Aittaleb et al 2009). These domains were expressed in HeLa cells to examine the ability of PH domains from the broader Lbc family to block activation of RhoA in cells. As shown in Figure 5.6*B*, the PH domains from PRG, LARG, p115RhoGEF, GEFH1, and p114 all block activation of the reporter plasmid to varying degrees. Consistent with *in vitro* binding data (Chen et al 2010), the PRG-PH domain inhibits S1P signaling more potently than does the p115-PH domain. These data also suggest that the GEFH1-PH domain binds to active RhoA with higher relative affinity while the p114-PH domain associates more weakly.

Expression of the PH domain from AKAP-Lbc RhoGEF unexpectedly promoted activation of the transcriptional reporter plasmid used in these experiments. However, increased expression of the wild-type domain does block stimulation of the reporter via S1P whereas expression of a mutant PH domain, that lacks binding to activated RhoA (Olugbenga Dada, personal

communication), has no effect on stimulation via S1P (Fig 5.6*C*). Therefore, consistent with observations for PH domains from other Lbc family members, the AKAP-Lbc PH domain appears to bind to active RhoA in cells. In addition, expression of the domain has an unknown stimulatory effect on the reporter plasmid that is independent of the domain's ability to bind to activated RhoA. Together, these data suggest that binding between the PH domain of Lbc-RhoGEFs and active RhoA is a common theme.

A new tool for the detection of activated RhoA?

The Rho-binding domain (RBD) of rhotekin binds specifically to activated RhoA and is routinely used to trap active RhoA from cell lysates. The RBD is a tool well suited for this technique because it not only binds to active RhoA but also prevents hydrolysis of bound GTP by sterically hindering interaction of RhoA with endogenous GAP proteins in lysates (Ren and Schwartz 2000). This slowing of inactivation allows the time necessary to isolate active RhoA from cell lysates. However, RBD is relatively unstable which makes the development of new tools for the detection of activated RhoA desirable.

The functional impact of PRG-PH domain binding to activated RhoA is examined in Figure 5.7. PRG-PH domains have little impact on the basal rate of GTP hydrolysis by RhoA as measured by release of P^{32} from bound GTP[γ–^{32}P] (Fig. 5.7*A*). The addition of cell lysate greatly enhances the observed rate of hydrolysis. PRG-PH domains effectively inhibited this stimulation of hydrolysis by cell lysate to a similar extent as RBD (Fig. 5.7*B*). This highlights a functional impact of interaction of the PH domain with active RhoA and suggests that the PH domain is a good candidate for the development of improved tools for the detection of activated

Figure 5.6: PH domains block activation of a RhoA reporter by S1P.

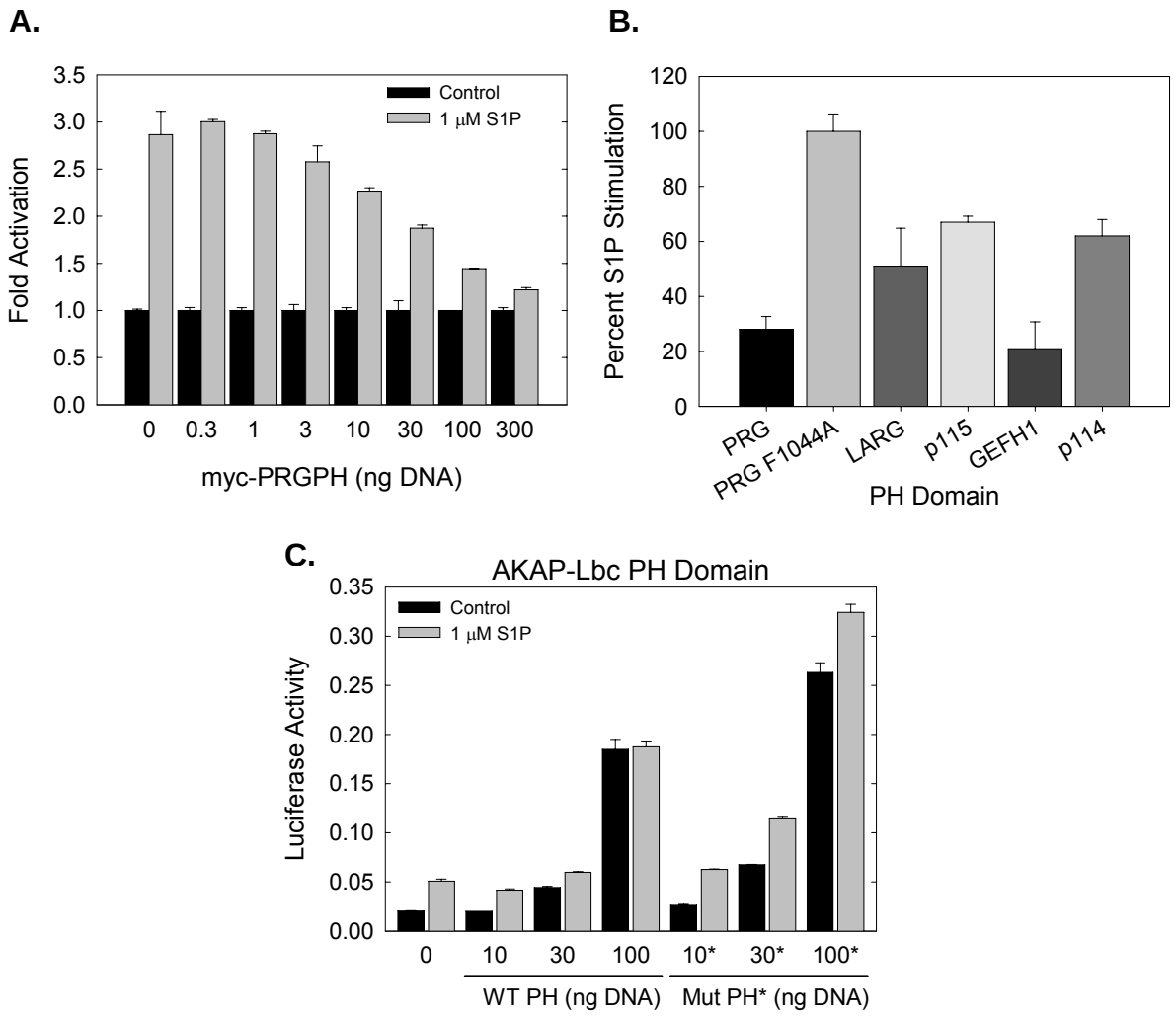


Figure 5.6. PH domains block activation of a RhoA reporter by S1P. (A) The PRG-PH domain inhibits S1P stimulation of the RhoA responsive transcriptional reporter in a concentration dependent manner. HeLa cells were transfected with SRE.L reporter plasmids and increasing amounts myc-PRG-PH plasmid. Cells were serum starved for 20 hours and then stimulated with control (0.1 mg/ml BSA) or 1 μM S1P for 5 hours. Cells were then lysed and expression of RLuc and FLuc determined as stated in methods. (B) PH domains from multiple Lbc-RhoGEF family members inhibit stimulation by S1P. Experiments were performed as in *A*, with increasing amounts of the specified myc-PH plasmids. Expression of the domains was compared by western blot using myc antisera and the percent of S1P stimulation (compared to the absence of PH domain expression) was quantitated at equivalent levels of expression. (C) The AKAP-Lbc PH domain inhibits stimulation of RhoA responsive transcriptional reporter by S1P. HeLa cells were transfected with SRE.L reporter plasmids and increasing amounts of myc-AKAP-Lbc PH or mutant PH plasmids. Experiments were performed and analyzed as in *A*.

RhoA. In fact, the PH domain of PRG does selectively extract the G14V activated mutant of RhoA from cells, compared to wild-type RhoA (Fig. 5.8). However, all current attempts to use the domain to isolate activated endogenous RhoA have failed (data not shown).

Figure 5.7: Functional binding of the PRG-PH domain to RhoA-GTP

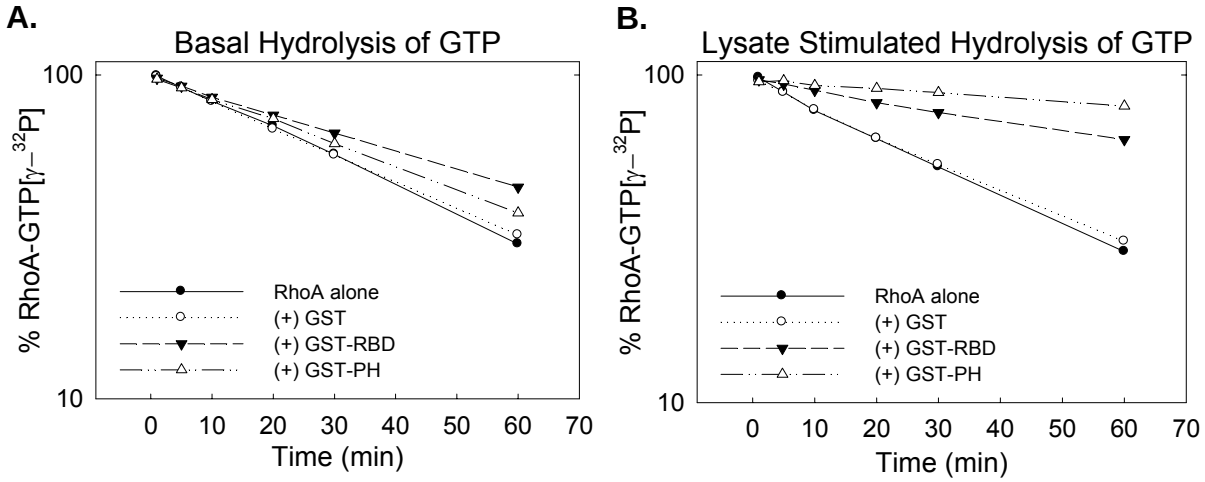


Figure 5.7. Functional binding of the PRG-PH domain to RhoA-GTP. (A) PRG-PH binding to RhoA-GTP has minimal effect on basal hydrolysis of GTP. RhoA was loaded with GTP[γ–^{32}P] and placed at room temperature for the amounts of time shown; either alone or with GST, GST-RBD, or GST-PH. (B) PRG-PH binding to RhoA-GTP inhibits lysate stimulated hydrolysis of GTP. RhoA was loaded with GTP[γ–^{32}P] and incubated at 4 ^{o}C in the presence of HeLa cell lysate for the amounts of time shown; either alone or with GST, GST-RBD, or GST-PH.

Figure 5.8: The PRG-PH domain extracts a constitutively activated form of RhoA from cell lysates.

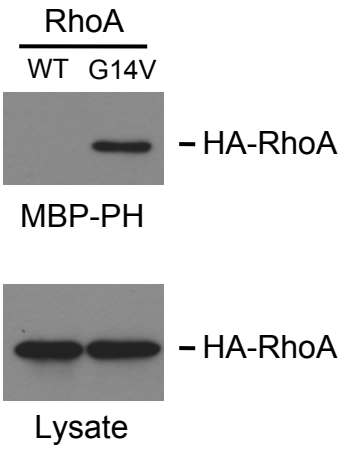


Figure 5.8. The PRG-PH domain selectively extracts a constitutively activated form of RhoA from cell lysates. HeLa cells were transfected with plasmids encoding HA-RhoA WT or HA-RhoA G14V (active mutant). After 4 hours, cells were placed in serum free optimem for overnight incubation before lysis. Purified MBP-PH bound to agarose beads was used to isolate RhoA from the lysates and then bead elutions were analyzed by western blot.

CHAPTER SIX
Results

OLIGOMERIZATION OF EDG RECEPTORS

The RGS-RhoGEFs exist in the cell as oligomers; p115RhoGEF forms homo-oligomers while LARG and PRG form homo- and hetero-oligomers (Chikumi et al 2004). In the case of LARG and PRG, the presence of PDZ domains at their N-termini would allow for multivalent interactions of oligomerized GEFs with oligomerized EDG receptors. This chapter focuses on evidence that EDG receptors are present at the plasma membrane as oligomers and, furthermore, that these oligomers exhibit functional interactions with tandem PDZ-PDZ domains.

EDG Receptors Form Oligomers in the Plasma Membrane

In addition to the binding of RhoGEFs to partners that are membrane localized, formation of higher order signaling complexes could be facilitated at the receptor level by assembly of receptors into homo- and hetero-oligomeric units. Various EDG receptors have been suggested to form oligomers through studies utilizing yeast-two hybrid analysis and co-immunoprecipitation experiments (Van Brocklyn et al 2002, Zaslavsky et al 2006). However, these assays provide no spatial information about oligomerization of the receptors and it is possible that the exogenously expressed receptors are not being processed properly by the cell, resulting in their co-localization simply through excessive concentration within the endoplasmic reticulum.

To gain information on the cellular localization of exogenously expressed receptors, chimeric EDG 2, 3, and 5 receptors tagged on their C-termini with YFP were analyzed in living cells. When expressed in HeLa cells, the YFP tagged receptors localize predominately to the plasma membrane, as determined by fluorescence microscopy (Fig 6.1*A*). Additionally, EDG 2 receptors tagged with both CFP and YFP were co-expressed and examined for potential resonance energy transfer. The corrected fluorescence resonance energy transfer (cFRET) signal shows that these membrane localized receptors are in sufficiently close proximity to exhibit productive energy transfer from the CFP of one receptor to the YFP on an adjacent partner (Fig 6.1*B*). These results demonstrate that exogenously expressed EDG receptors do localize to the plasma membrane of living cells and strongly suggest that EDG 2 receptors oligomerize at the plasma membrane and not solely within internal structures due to processing and/or mis-folding.

A larger group of EDG receptors were examined for homo- and hetero-oligomerization by bioluminescence resonance energy transfer (BRET) between receptors tagged with Renilla Luciferase (RLuc) and YFP. HA-tagged receptors were used in place of YFP-tagged receptors to gauge the expected signal in the complete absence of resonance energy transfer. EDG 3-RLuc receptors exhibited the highest degree of BRET when expressed with EDG 3-YFP receptors. BRET was lower between EDG 3 and both EDG 4 and 5 partners, however, the level of expression of the receptors was variable (data not shown), and therefore, no conclusion can be drawn about the preference of EDG 3 receptors to form homo- or hetero-oligomers. EDG 5-RLuc receptors, similarly to EDG 3-RLuc receptors, had the highest level of resonance energy transfer when expressed with EDG 5-YFP homo-oligomerization partner. In contrast, EDG 4-RLuc receptors exhibited a similar level of BRET regardless of the YFP containing receptor with

Figure 6.1: EDG receptors form oligomers at the plasma membrane.

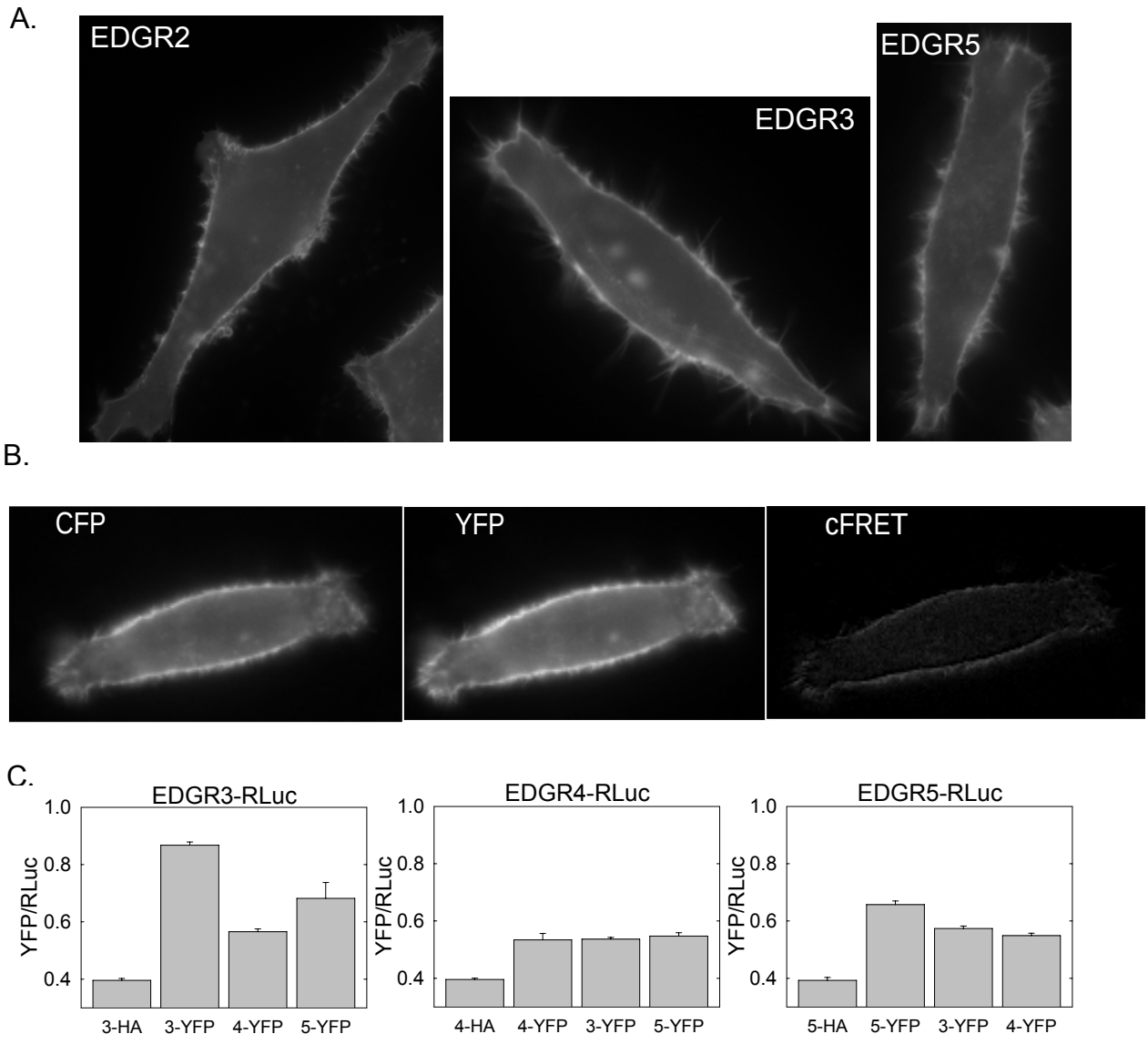


Figure 6.1. Oligomerization of EDG receptors (EDGRs). (A) EDGRs localize to the plasma membrane. HeLa cells were transfected with EDGRs tagged at their C-termini with YFP. Images were collected 48 hours post-transfection by excitation at 500 nm and emission measured at 535 nm. (B) EDG 2 (LPA 1) receptors interact at the plasma membrane. HeLa cells were co-transfected with EDG2Rs tagged at their C-termini with either YFP or CFP. Images were collected 48 hours post-transfection and analyzed at stated in the methods. (C) Multiple EDGRs form homo- and hetero-oligomers. HeLa cells were co-transfected with EDGRs tagged at their C-termini with either RLuc or YFP. 48 hours after transfection, cells were exposed to coelentrerazine-*h* and light emitted at 435 nm and 535nm was measured and compared as stated in the methods.

which it was paired (Fig 6.1*C*). Also, the BRET signal from all experiments using EDG 4-RLuc receptors was lower than that obtained with the other RLuc-tagged receptors. EDG 3 and 5 receptors appear oligomerize based on these data, however, more experiments are needed to confidently conclude that EDG 4 receptors oligomerize as well. It is possible that any receptor tagged with YFP, when paired with an RLuc-tagged partner, would exhibit a background BRET signal above that obtained with HA-tagged receptors.

None of the EDG receptors exhibited any change in BRET signal when stimulated with their cognate ligands. Importantly, the EDG receptors containing C-terminal RLuc and YFP tags could effectively activate RhoA to extents similar to WT receptors in response to agonist (data not shown), which validates their ability to functionally respond to hormones. Overall, the data suggest that any oligomeric state of these receptors may be constitutive and not regulated by ligand binding. However, receptors were only examined with C-terminal tags and the possibility of the location of the tag affects the ability of the receptors to alter their oligomeric state in response to ligand cannot be ruled out.

Tandem PDZ-PDZ Domains Interact More Effectively with Receptors than Single Domains

As illustrated in Fig 5.5, the PDZ domain of PRG inhibits stimulation of RhoA by S1P in HeLa cells, presumably by binding to the C-termini of receptors to alter function or sterically prevent association with their downstream signaling partners. If these receptors are present in the plasma membrane as functional oligomers, expression of tandem PDZ-PDZ domains that could interact with oligomeric receptors through bivalent interactions should inhibit the

stimulation of RhoA more effectively than single PDZ domains. Stimulation of the transcriptional reporter of RhoA activation is observed in HeLa cells upon treatment with S1P, but not LPA (data not shown). To avoid activation of RhoA that may be dependent on a mixed population of S1P receptors, PDZ-PDZ domains were expressed in HeLa cells stably expressing EDG 4 (LPA 2) receptors, and their effect on RhoA activation compared with expression of single PDZ domains. The tandem domains are more potent blockers of LPA stimulation of RhoA; this increased efficiency is consistent with more efficient interaction of tandem PDZ domains with dimeric or possibly higher order oligomers (Fig 6.2). In the future, use of mutant PDZ domains deficient in binding to class I motifs and receptors that lack the C-terminal binding sequence as negative controls would strengthen this conclusion.

Figure 6.2: Tandem PDZ-PDZ domains block receptor activity more effectively than single domains.

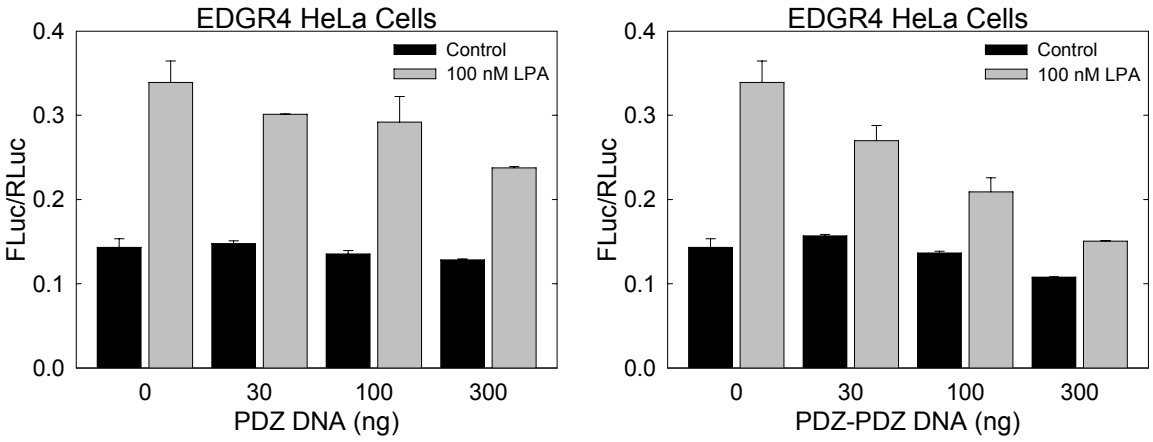


Figure 6.2. Tandem PDZ domains block receptor activity more effectively than single domains. HeLa cells were transfected with SRE.L reporter plasmids and increasing amounts of mycPRG-PDZ or mycPRG-PDZ-PDZ plasmids. Cells were serum starved for 20 hours and then stimulated with control (0.1 mg/ml BSA) or 1 μM S1P for 5 hours. Cells were then lysed and expression of RLuc and FLuc was determined as stated in methods.

CHAPTER SEVEN

DISCUSSION

RGS-RhoGEFs can potentially be regulated through multiple mechanisms including stimulation of their intrinsic exchange activity or their recruitment to a region of the cell with an enrichment of available substrate. Evidence exists to support both mechanisms. $G\alpha_{13}$ stimulates the exchange activity of p115RhoGEF and LARG *in vitro*, and all three RhoGEFs have been suggested to function synergistically with $G\alpha_{13}$ in cells (Hart et al 1998, Mao et al 1998, Suzuki et al 2003). But, all three RGS-RhoGEFs have been shown to localize to the plasma membrane upon addition of activated upstream regulators and expression of membrane localized LARG induces higher levels of active RhoA within cells than the cytosolic GEF (Aittaleb et al 2009). Since all three RGS-RhoGEFs exhibit significant exchange activity *in vitro* in the absence of $G\alpha_{13}$ (Sternweis et al 2007) and the population of RhoA that is free of RhoGDI and available for activation resides in the plasma membrane (DerMardirossian and Bokoch 2005), it was hypothesized that simple translocation of the RhoGEFs to the membrane and their substrate would suffice to drive the pathway.

The three aims of these studies were to: 1) examine the presence of EDG receptor-associated signaling complexes, 2) determine if translocation of RGS-RhoGEFs to the plasma membrane was sufficient to drive activation of the RhoA pathway, and 3) elucidate potential modules within the RGS-RhoGEFs that might facilitate their translocation and aid in the formation of signaling complexes. Formation of large signaling complexes at the plasma membrane could serve various functions within a cell. Pre-formed complexes, prior to signal initiation, would facilitate very rapid transduction of incoming extracellular information.

Context dependent complex formation would, by contrast, be useful as a regulatory node for pathways.

Translocation as a Regulatory Component

To examine the affect of acutely localizing the RGS-RhoGEFs, a regulated heterodimerization system that allows controlled translocation of the RGS-RhoGEFs to the plasma membrane in the absence of receptor mediated activation of $G\alpha_{13}$ was used. This system clearly illustrates that translocation is sufficient to activate RhoA. More importantly, translocation of RGS-RhoGEFs activates RhoA within minutes, consistent with the kinetics of RhoA activation observed upon stimulation of EDG receptors with hormone. The amounts of activated RhoA were similar whether stimulated by induced localization of RGS-RhoGEFs or addition of hormone. This data indicates that basal exchange rates of the RGS-RhoGEFs are sufficient for driving activation of the RhoA pathway and that localization of the proteins could account for hormone regulation.

Translocation to membranes may be a prominent mechanism for regulation across the family of 70 RhoGEFs. RhoGEF substrates, Rho, Rac, and cdc42 GTPases that are free of RhoGDI, all reside predominately within membranes (DerMardirossian and Bokoch 2005). This suggests that regulation of RhoGEF localization may be a general mechanism for controlling the activities of these GTPases. The observation that localization of RGS-Trio-N chimeras to membranes can stimulate activation of Rac1 supports this hypothesis. Examining the activity of a broad range of RhoGEFs utilizing the small molecule regulated heterodimerization system

would provide information about regulated localization as a common regulatory paradigm for RhoGEFs.

Although Rho GTPases reside within membranes, it is not likely that they are restricted to the plasma membrane. In fact, RhoB localizes predominately to endosomes (Ellis and Mellor 2000). It is likely that other Rho GTPases localize to various internal membrane structures as well. It would be interesting to determine if regulated localization of GEFs to specific organelles is a mechanism for controlling their activation of organelle-associated Rho GTPases. This should be feasible utilizing organelle specific targeting sequences in combination with the FKBP domain used in the regulated heterodimerization system employed here.

Role of $G\alpha_{12/13}$

The most highly characterized interaction between the RGS-RhoGEFs and a membrane localized protein is the binding of the RGS domains to $G\alpha_{12/13}$. The regulatory role of this binding interaction has been assumed to be stimulation of intrinsic activity of RGS-RhoGEFs. However, relocalization of the RGS-RhoGEFs to the site of $G\alpha_{13}$ activation (and RhoA concentration) is another effect of this binding interaction. Indeed, the chimeric RGS-Trio-N proteins used in the studies reported here, show that the RGS domain alone is sufficient to detect hormone activation of $G\alpha_{13}$ and responds by activating Rac1. This presumably reflects localization of Trio-N to the plasma membrane and its substrate. This is further supported by *in vitro* experiments examining activation of Rho GTPases upon localization of RhoGEFs to lipid vesicles via $G\alpha_{13}$. Interestingly, the fold increase in exchange rates achieved through

relocalization of p115RhoGEF, at least *in vitro*, far exceeds the fold increase in rates achieved through stimulation of intrinsic activity. Additionally, the W507E mutant of p115RhoGEF, which does not exhibit an increase in exchange rate upon exposure to activated $G\alpha_{13}$ (Chen et al 2012), activates RhoA on lipid vesicles identically to wild-type p115RhoGEF.

There are several possible interpretations of these results. First, the intrinsic exchange activity of the RhoGEFs may already be sufficient to maximally activate all available RhoA. This is supported by cellular experiments utilizing regulated translocation of FRB-RGS-RhoGEFs. Second, the lipid surface itself may induce conformational changes in the RGS-RhoGEFs that fully activate their intrinsic activity. It is not known how well effects observed with artificial lipid vesicles will translate to effects in natural cellular membranes. Third, activation of intrinsic activity may be relevant for achieving full activation downstream of hormone signaling in cells but not in the artificial systems used here. $G\alpha_{13}$-GDP·AlF_4^- ($G\alpha_{13}$-AMF) is a stable mimic of the transition state for GTP hydrolysis by $G\alpha_{13}$ and effectively activates the GTPase for *in vitro* experiments. However, $G\alpha_{13}$-GTP is the activated form produced in cells and the RGS domains of p115RhoGEF and LARG act as GAPs for $G\alpha_{13}$-GTP. Under these circumstances in which the levels of $G\alpha_{13}$-GTP may be rapidly depleted, stimulation of intrinsic activity of RhoGEFs may be critical.

Role of Conserved PH Domains

The association of activated RhoA with the PH domain of the RGS-RhoGEFs is another interaction that could aid in anchoring the RhoGEFs to the plasma membrane. The binding of

activated RhoA to the PH domain does not have any effect on the catalytic activity of any of the RhoGEFs examined thus far (Chen et al 2010) (Frank Medina and Olugbenga Dada, personal communications). Speculatively, the role of this third interaction site is to further stabilize the RhoGEFs at the site of substrate enrichment and provide a pathway for positive feedback regulation.

Binding between PH domains and activated RhoA is not limited to the RGS-RhoGEFs, but extends at least to their close relatives within the larger Lbc-RhoGEF family. Interestingly, PH domains are invariably found following all RhoGEF DH domains. The extreme evolutionary pressure that would generate this level of conservation is currently unknown. It is possible the domains bind to a wide and diverse range of activated GTPases that are membrane-associated. The conservation of this phenomenon among RhoGEFs could also indicate that cross-talk occurs between the various Rho GTPases themselves. The cdc42 and RhoA specific GEF, Dbs, has been reported to bind to activated Rac1 through its PH domain (Cheng et al 2004). This binding of the PH domain to activated Rac1 was suggested to lead to elevation in levels of active RhoA, although further evidence is needed.

Role of Oligomerized Receptors and PDZ Domains

The EDG receptors appear to exist as constitutive oligomers, although the functional impact of this oligomerization remains unknown. It is possible that oligomerization enhances receptor stability on the membrane surface, thus sustaining a relatively long-lived pool of ligand responsive proteins. It may also speed signal transduction by providing multiple interaction

points for signaling partners. Some EDG receptors bind not only G proteins, but oligomerized RGS-RhoGEFs as well. While steric hindrances may prevent coincident interaction of a single receptor with multiple downstream partners, oligomeric receptors could facilitate these interactions efficiently. Additionally, the RGS-RhoGEFs are also expressed as constitutive oligomers; therefore it is possible that multiple PDZ domains from oligomerized LARG and PRG would interact with several receptors simultaneously and that the presence of multiple interaction sites is necessary for stable binding of the RhoGEFs to receptors. This concept is supported by experiments demonstrating that tandem PDZ-PDZ domains block stimulation of RhoA by LPA more efficiently than single PDZ domains.

The ligand responsiveness of the interactions between PDZ domains and receptors is another point of interest. Activation of receptors induces conformational shifts that may reorient their C-termini and expose PDZ interaction sites. Modification of the Trio-N RacGEF to contain PDZ domains at the N-terminus would be useful in examining the ability of PDZ domains to recruit associated modules to the membrane upon receptor activation. Realistically, the C-termini of receptors may be constitutively exposed but not contribute to RhoGEF localization in the inactive state due to low binding affinity. If the affinity of PDZ domains for receptor C-termini is weak, tandem PDZ-PDZ domains attached to Trio-N might provide the affinity necessary to detect the interactions, either before (constitutively exposed), or after (allosterically regulated), stimulation with agonist. Again, this would provide evidence for potential functional interaction with dimerized RhoGEFs.

Final Perspectives

The most valuable information that could be provided about this system at this point would come from cellular reconstitution experiments in which endogenous RhoGEFs were knocked-down and replaced with various binding mutants. Mutant RhoGEFs have been produced that exhibit decreased binding between the PDZ domains and receptor C-termini, the RGS domains and $G\alpha_{13}$, and the PH domains and active RhoA (Chen et al 2010, Longhurst et al 2006). Additionally, expression of the W507E mutant of p115RhoGEF (Chen et al 2012) could provide extremely useful data about the necessity of $G\alpha_{13}$ stimulation of p115RhoGEF catalytic activity for adequate activation of RhoA. However, inability to re-couple exogenously expressed RhoGEFs to hormone signaling has precluded these experiments thus far.

It will be interesting to see how the regions of the RGS-RhoGEFs integrate to play a role in controlling their cellular localization and complex formation. It is likely that the PDZ, RGS, and PH domains all contribute to membrane anchoring, perhaps in a cooperative manner in the context of multiple binding sites as illustrated for PRG in Fig 7. Under this scenario, high affinity binding between the RGS domain and activated $G\alpha_{13}$ would act as an initial, diffusion controlled, detector of hormone signaling. Once the RGS domain pulls the RhoGEF to the plasma membrane, the higher effective concentrations would allow interaction of the lower affinity binding sites between the PDZ domain and receptors, providing effective secondary anchoring sites. Additionally, once a small amount of RhoA is activated, the PH domain could interact with active RhoA and aid in holding the RhoGEF at the membrane, exhibiting a feed-forward effect on signaling through processive turnover of substrate RhoA.

Why is this necessary? A simple explanation might be that cooperative binding between multiple sites may be required to hold the RhoGEFs at the plasma membrane for the timeframe necessary to generate productive and localized actin filaments downstream of RhoA activation. Recent work suggests that multivalency between binding partners promotes liquid-liquid demixing phase separations that are reflective of high affinity supracomplex formation and necessary for efficient actin structural reorganization (Li et al 2012). In the absence of coordinated binding, RhoGEFs only interact with membrane partners transiently, and basal Rho GTPase activities are kept low enough to avoid the level of actin polymerization required to alter cellular physiology. Additionally, at least a partial reinforcement mechanism for keeping endogenous RhoGEFs sequestered from the plasma membrane is their association with cytoskeletal elements or other cytoplasmic proteins (Banerjee and Wedegaertner 2004, Longhurst et al 2006). In a system where translocation is sufficient, this would limit signaling noise, which might be expected to be high. Under these conditions, where RhoGEF activators must compete with sequestering agents, a robust coordinated binding paradigm might be required to effectively stabilize the RhoGEFs at the plasma membrane in order to achieve the spatial and temporal regulation necessary to coordinate alterations in cytoskeletal structures.

Figure 7: Model for hormone activation of RhoA via RGS-RhoGEFS.

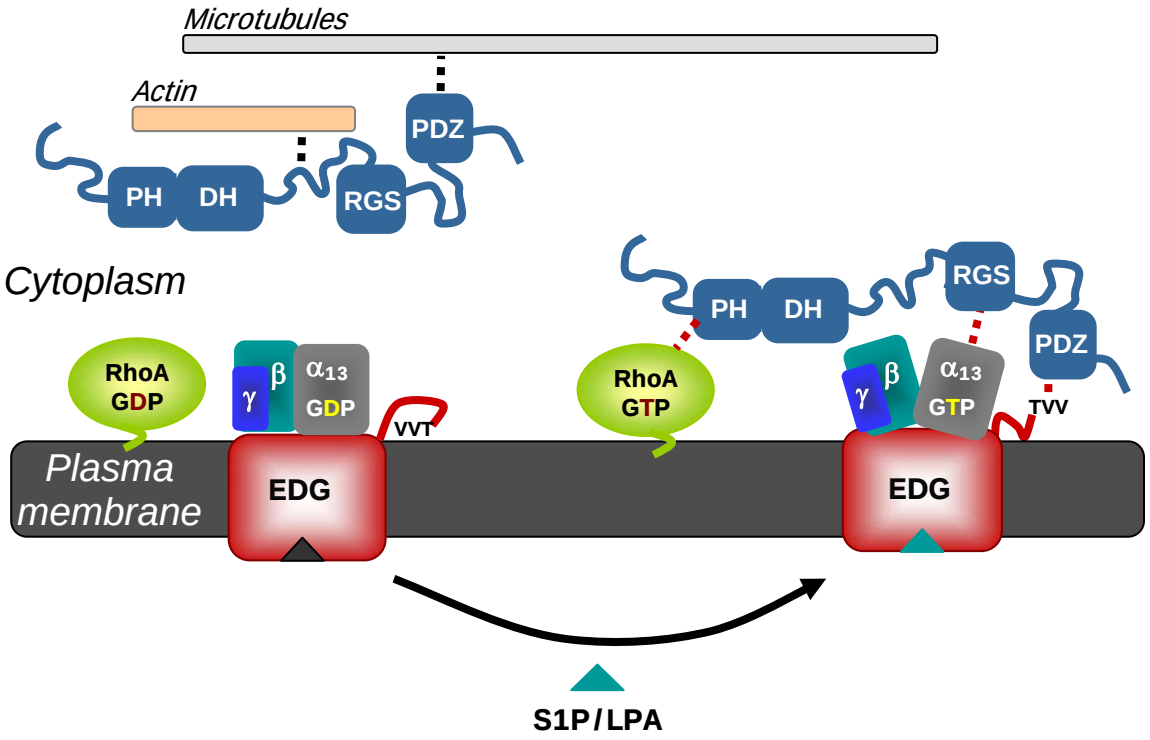


Figure 7. Model for hormone activation of RhoA via RGS-RhoGEFS. Under basal conditions, EDGRs and G proteins are inactive and PRG remains sequestered away from the membrane. Binding of hormone to receptors stabilizes their active conformation and initiates exchange of guanine nucleotide on $G\alpha_{12/13}$, activating the G proteins. The binding of activated $G\alpha_{12/13}$ to the RGS domain of PRG recruits the GEF to the membrane where it activates RhoA. This translocation is assisted by a second binding interaction between the PDZ domain of PRG and the C-terminus of the receptor. Once a portion of RhoA becomes activated, the PH domain of PRG can bind to activated RhoA providing a third interaction site to anchor the RhoGEF to the membrane. These multiple interaction sites likely exhibit cooperative binding, creating a stable signaling complex and securing the GEF at the plasma membrane in proximity with its substrate, RhoA.

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