Multiple Domain 'Nexus' Proteins in Receptor-Mediated Cell Signaling

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ABSTRACT

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(Under the direction of Dr. David P. Siderovski)

Signal transduction is the fundamental biological process of converting changes in extracellular information into changes in intracellular functions. It controls a wide range of cellular activities, from the release of neurotransmitters and hormones, to integrated cellular decisions of proliferation, differentiation, survival, or death. The vast majority of extracellular signaling molecules exert their cellular effects through activation of G protein-coupled receptors (GPCRs); however, the G-protein coupled paradigm is by no means the exclusive mechanism of membrane receptor signal transduction. Polypeptide ligands such as nerve growth factor act exclusively on receptor tyrosine kinase receptors (RTKs) to promote signaling. GPCRs and RTKs both form an interface between extracellular and intracellular physiology by converting hormonal signals into changes in intracellular metabolism and ultimately cell phenotype. Initially, it was thought that GPCRs and RTKs represented linear and distinct signaling pathways that converge on downstream targets to regulate cell division and gene transcription. However, activation of second messenger generating systems do not fully explain the range of effects of GPCR or RTK activation on biological processes such as differentiation and cell growth. Recent work has revealed that GPCR and RTK signaling pathways are not

mutually exclusive of one another; in fact, they often function as partners, forming complex signaling networks through scaffold/nexus proteins.

The work described herein examines the complexity of signal regulation by multifunctional nexus proteins. I showed that the activation of phospholipase C-ε by $G\alpha_{12/13}$-coupled receptors occurs through a mechanism involving the small GTPase Rho. I demonstrated the usefulness and complexities of 'regulators of G-protein signaling' (RGS proteins) for discerning the Gα selectivity of GPCR signaling. Finally, I found that RGS12, in addition to regulating Gα signaling, acts as a Ras/Raf/MEK scaffold in nerve growth factor-mediated differentiation. The work presented here expands our understanding of how multiple domain proteins facilitate convergence and cross-regulation of RTK, heterotrimeric G-protein, and Ras-superfamily signaling.

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Φ	Hydrophobic amino acid
2-MeSADP	2-methylthioadenosine diphosphate
3D	Three dimensional
7-AAD	7- aminoactinomycin D
Ala or A	Alanine
Arg or R	Arginine
ASM	Airway smooth muscle
Asn or N	Asparagine
Asp or D	Aspartic Acid
AT1aR	angiotensin type 1a receptor
ATP	Adenosine 5'-triphosphate
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
C-TAK1	Cdc25C-associated kinase 1
cDNA	Complementary DNA
CNS	Central nervous system
CRD	Cysteine-rich C1 domain
DH	Dbl homology
DHR	Discs-large homology repeat
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid

DRG	Dorsal root ganglion
Glu or E	Glutamic Acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
Elk-1	E-26-like protein 1
EPAC	Exchange protein activated by cAMP
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GABA	Gamma (γ)-aminobutyric acid
GAP	GTPase-accelerating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine 5'-diphosphate
GEF	Guanine nucleotide exchange factor
GIPC	GAIP-interacting protein, C terminus
Gly or G	Glycine
GoLoco	$G\alpha_{i/o}$-loco
GPCR	G protein-coupled receptor
GRK	G-protein receptor kinase
GTP	Guanosine 5'-triphosphate
HA	Hemagglutinin
His or H	Histidine
Ig-C2	Immunoglobulin-like C2 type

$Ins(1,4,5)P_3$	Inositol (1,4,5)trisphosphate
JIP	JNK-interacting protein
JNK	c-Jun N-terminal kinase
kDa	kilodaltons
KSR	Kinase suppressor of Ras
LARG	Leukemia-associated RhoGEF
Leu or L	Leucine
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MEK	MAP kinase/ERK kinase
MEKK	MEK kinase
MP1	MEK partner 1
NGF	Nerve growth factor
NSAIDs	Non-steroidal anti-inflammatory drugs
OSM	Osmosensing scaffold for MEKK3
p115-RGS	RGS domain of p115RhoGEF
$p75^{NTR}$	p75 neurotrophin receptor
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDL	Poly-D-lysine
PDZ	Postsynaptic density protein / *Drosophila melanogaster* discs-large protein / Zona occludens 1 protein homology

PH	Pleckstrin homology
Phe or F	Phenylalanine
PtdIns(3)P	Phosphatidylinositol 3-phosphate
PtdIns(5)P	Phosphatidylinositol 5-phosphate
PLC	Phospholipase C
Pro or P	Proline
PTB	Phosphotyrosine binding
PtdIns(4,5)P_2	Phosphatidylinositol (4,5)bisphosphate
RA	Ras-associating/-association
RBD	Ras-binding domain
RGS	Regulator of G-protein signaling
RNA	Ribonucleic acid
RSK	90 kDa ribosomal S6 kinase
RTK	Receptor tyrosine kinase
RU	Resonance units
S1P	Sphingosine 1-phosphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sef	Similar expression to *fgf* genes
Ser or S	Serine
SH2	Src homology 2
SH3	Src homology 3
siRNA	Small interfering RNA
Sos	Son of sevenless

SPR	Surface plasmon resonance
Ste	Sterile
Thr or T	Threonine
TrkA	Tropomyosin-related kinase A
Tyr or Y	Tyrosine
UDP	Uridine 5’-diphosphate
UTP	Uridine 5’-triphosphate
Val or V	Valine
ZO-1	Zonula occludens 1

CHAPTER 1

INTRODUCTION

1.1 The Importance of Cellular Signaling

The ability of organisms to sense and react to their environment is critical to their survival. Individual cells within an organism must be able to detect the presence of extracellular molecules and to initiate a variety of intracellular responses. To enable organisms to do this, a complex range of signaling pathways have evolved that are carefully orchestrated and controlled, the primary components and principles of which are similar across a diverse range of organisms.

One mechanism of cell communication is via the release and detection of signaling molecules such as cytokines, growth factors, neurotransmitters, and hormones. These extracellular signals are responsible for a variety of cellular responses including migration, secretion, apoptosis, proliferation, and differentiation. To respond to such a signal in the extracellular environment, the cell must have the capacity to bind to a signaling molecule. In the majority of cases, hormones bind to receptors on the cell surface to stimulate specific signal transduction pathways in the cell.

1.2 Signal Transduction at the Receptor

Signal transduction is the fundamental biological process of converting changes in extracellular information into changes in intracellular functions. It controls a wide range of cellular activities, from the release of neurotransmitters and

hormones, modulation of transmembrane ion flux and activation or repression of gene expression, to integrated cellular decisions of proliferation, differentiation, survival or death. The vast majority of extracellular signaling molecules exert their cellular effects through activation of G protein-coupled receptors (GPCRs). GPCRs form an interface between extracellular and intracellular physiology by converting hormonal signals into changes in intracellular metabolism and ultimately cell phenotype [1, 2].

1.2.1 Heterotrimeric G-protein signal transduction

The standard model of GPCR signal transduction has long been restricted to a three-component system: receptor, heterotrimeric G-protein, and effector [3] (Figure 1.1). The receptor, a cell-surface protein that spans the membrane seven times, is coupled to a membrane-associated heterotrimeric complex that comprises a GTP-hydrolyzing Gα subunit and a Gβγ dimeric partner. Agonist-induced conformational changes enhance the guanine nucleotide-exchange factor (GEF) activity of the receptor, leading to the release of GDP (and subsequent binding of GTP) by the Gα subunit. On binding GTP, conformational changes within the three 'switch' regions of Gα allow the release of the Gβγ dimer. Gα·GTP and Gβγ subunits regulate the activity of target effector proteins such as adenylyl cyclases, phospholipase C (PLC) isoforms, ion channels, and cyclic nucleotide phosphodiesterases, which regulate multiple downstream signaling cascades that integrate key biological processes such as development, visual excitation, cardiac contractility, and glucose metabolism [4]. The intrinsic GTP hydrolysis (GTPase) activity of Gα resets the cycle by forming Gα·GDP which has low affinity for effectors

but high affinity for Gβγ. Reassociation of Gα·GDP with Gβγ reforms the inactive, GDP-bound heterotrimer (Gαβγ) which completes the cycle. Perturbations in G-protein signaling characterize many human ailments [4]. Accordingly, the therapeutic effects of many current drugs are due to their capacity to bind target receptors and regulate G-protein signaling.

1.2.2 Receptor Tyrosine Kinase Signal Transduction

Many extracellular signals act through GPCRs to activate second messenger pathways; however, the G-protein coupled paradigm is by no means the exclusive mechanism of membrane receptor signal transduction. Polypeptide ligands such as nerve growth factor (NGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and insulin act exclusively on receptor tyrosine kinase receptors (RTKs) [5-8].

All RTKs, with the exception of the insulin receptor family, are monomeric in the absence of ligand (Figure 1.2). They possess an extracellular ligand binding domain that is connected to a cytoplasmic region by a single transmembrane helix. The cytoplasmic region contains a tyrosine kinase domain which possesses catalytic activity and regulatory sequences. Ligand binding to the extracellular region of RTKs leads to dimerization of monomeric receptors, resulting in autophosphorylation of specific tyrosine residues in the intracellular portion [9]. Generally, tyrosine autophosphorylation either stimulates the intrinsic kinase activity of the receptor, or generates recruitment sites for downstream signaling adaptors or enzymes containing phosphotyrosine-recognition domains, such as the phosphotyrosine binding (PTB) domain or the Src homology 2 (SH2) domain [10].

Activation of signaling molecules by RTKs generally requires not only stimulation of receptor catalytic activity but also localization of these molecules to the activated receptor. For instance, autophosphorylation of Tyr766 in FGF receptor-1 (FGFR-1) provides a high affinity binding site for the SH2 domain of PLC-γ [11]. The association of the SH2 domain with phosphorylated Tyr766 is critical for the activation of PLC-γ by FGFR-1, as a point mutant in Tyr766 prevents binding, phosphorylation, and stimulation of the PLC [11].

1.2.3 Integration of GPCR and RTK Signal Transduction Pathways

Initially, it was thought that GPCRs and RTKs represented linear and distinct signaling pathways that converge on downstream targets such as MAPKs to regulate cell division and gene transcription (Figures 1.1 and 1.2). However, activation of second messenger generating systems do not fully explain the full range of effects of GPCR or RTK activation on biological processes such as differentiation and cell growth. Recent work has revealed that these two signaling pathways are not mutually exclusive of one another; in fact, they often function as partners, forming complex signaling networks. The process by which GPCRs and RTKs share information and modulate each other is termed “cross-talk”, and can occur between GPCRs and RTKs at several levels.

GPCRs initiate cross-talk in many ways, one of which is a phenomenon termed “transactivation”. Transactivation occurs when activation of GPCRs results in tyrosine phosphorylation and subsequent activation of RTKs [12, 13]. Activation of RTKs leads to the recruitment of scaffold proteins such as Shc, Grb2, and Crk, via their SH2 domains. In turn, these proteins, via SH2 or SH3 domains, bind to

additional adaptor proteins and signaling molecules. Several GPCR agonists, including angiotensin II, lysophosphatidic acid, bradykinin, and endothelin, transactivate RTKs such as the platelet-derived growth factor receptor (PDGFR) and the epidermal growth factor receptor (EGFR) (reviewed in [14]).

Different concepts have emerged to explain mechanisms of transactivation. Molecules such as PKC, Src, and reactive oxygen species mediate RTK transactivation (reviewed in [14]). Activation of GPCRs stimulates extracellular proteases to cleave a RTK ligand molecule to its active, RTK-binding form, which in turn activates the RTK [15]. Adaptor proteins such as Gab1 and IRS-1, which serve as docking sites for multiprotein complexes at the RTK, also are implicated as mediators of GPCR-induced RTK transactivation. Additionally, GPCR activation stimulates the activity of protein tyrosine phosphatases that inactivate RTKs.

In some cases, the G-protein involvement of RTK activation is proximal to, and downstream of, the RTK. For instance, it has been reported that activated RTKs can induce the activation of G-proteins by promoting the dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ dimer leading to downstream signaling [16]. Alternatively, stimulation of a RTK can lead to a direct association between GPCRs and RTKs through scaffold proteins, such as β-arrestins, regulator of G protein signaling (RGS) proteins, and G-protein receptor kinases (GRKs) to activate downstream effectors [17-21]. Recently, phosphorylation of GPCRs by RTKs was identified as an additional mode of cross-talk. For example, insulin-mediated phosphorylation of the β_2-adrenergic receptor creates a platform that binds SH2- and SH3-domain-

containing proteins such as Grb2 [22]. These data indicate the involvement of GPCRs both upstream and downstream of RTK signal transduction.

As mentioned above, one level of cross-talk between GPCRs and RTKs is through RGS proteins. RGS proteins normally function as GTPase-accelerating proteins (GAPs) for Gα subunits thereby terminating GPCR-mediated signaling [23]. However, there are a few examples where RGS proteins appear to be involved in regulating signal transmission from RTKs and small GTPases. For instance, GIPC ("GAIP-interacting protein, C terminus") specifically binds to a C-terminal PDZ-binding sequence that is unique to RGS19 (GAIP), and both proteins associate at distinct sites on the high-affinity neurotrophin receptor, TrkA. TrkA and GIPC colocalize on putative retrograde transporter vesicles in neuronal cell bodies, while RGS19 is found on clathrin-coated vesicles near the plasma membrane [17]. Druey proposed that GIPC might function to physically join the TrkA receptor on one type of transport carrier to a GPCR regulated by RGS19 located on a distinct intracellular vesicle [24].

Cho *et al.* provided evidence suggesting that G-proteins are involved in PDGFR signal transmission [25]; specifically, they demonstrated that overexpression of recombinant RGS5 reduces PDGF-stimulated MAPK activation in human aortic smooth muscle cells. Regulation of the RGS protein itself by RTKs may also play a role in cross-talk. EGF promotes phosphorylation of RGS16 on conserved tyrosine residues (Y168 and Y177) in the RGS-box [26], and it has been suggested that this phosphorylation increases the stability and GAP activity of RGS16. It is possible that the EGFR inhibits Gα_i-dependent Ras-MAPK activation in a feedback loop by

enhancing RGS16 activity through tyrosine phosphorylation. RGS12, a multidomain-containing RGS protein, also regulates RTK signaling. Specifically, we have shown that RGS12 inhibits signal transmission from the PDGFβR in guinea-pig airway smooth muscle (ASM) cells [18]. RGS12 appears to reduce PDGF-dependent activation of MAPK in ASM cells not by its GAP activity, but by removing the PDGFβR from the cell surface. Interestingly, the PDZ and PTB domains in the N-terminus of RGS12 appear to be functionally important for this regulation; the N-terminus in isolation is sufficient for inhibiting receptor activity and localizing the receptor to cytoplasmic vesicles, and RGS14, which has a similar architecture as RGS12, but lacks the PDZ and PTB domains, exhibits a different subcellular localization compared with RGS12 [18].

Scaffolds for the MAPK pathway also facilitate signal integration between RTK and GPCR signaling. For example, MEK partner 1 (MP1) serves to integrate Rho GTPase activation and MAPK signaling [27]. β-arrestins couple GPCR signaling with the MAPK cascade [28]. As will be described below, and in Chapters 2-4, RGS12 and PLC-ε are both multidomain containing proteins that function as scaffolds/nexus proteins that facilitate signal integration of small and large GTPases.

1.3 Phospholipase C and Signal Transduction

Stimulation of phosphoinositide-hydrolyzing PLC isozymes by extracellular stimuli such as neurotransmitters, hormones, chemokines, inflammatory mediators, and odorants is one of the major signal transduction pathways used by cell surface receptors to mediate downstream signaling events [29]. At least six classes of PLC isozymes underlie these signals: PLC-β, PLC-γ, PLC-δ, PLC-ε, PLC-ζ, and PLC-η

(Figure 1.3) [30-32]. Until recently, PLC-β was the isozyme most commonly found to be activated by GPCRs and heterotrimeric G-proteins. GPCRs activate PLC-β enzymes either via release of α-subunits of the G_q family of G-proteins [33-36] or by Gβγ dimers from activated G_i family members [37-39]. In contrast, PLC-γ, PLC-δ, and PLC-ζ isoforms differ largely in their regulatory mechanisms. PLC-γ enzymes are regulated by receptor and non-receptor tyrosine kinases [40-42]. PLC-δ isoforms may be regulated by Ca^{2+} [43] and/or the high-molecular-weight G-protein (G_h) [31, 44]; however, the mechanisms by which PLC-δ enzymes couple to and are regulated by membrane receptors is less clear [31]. PLC-ζ is reportedly responsible for sperm-mediated Ca^{2+} oscillations that occur during fertilization [32]. PLC-η, the most recently identified PLC, appears to be regulated by Gβγ dimers [45-47].

1.3.1 Phospholipase C-ε

A novel class of PLC was first revealed with the identification of the protein PLC210 in a screen for *C. elegans* Ras (LET-60) effectors [48]. Cloning of the full coding sequence of PLC210, the prototypical member of the PLC-ε family, identified functional domains not previously described in other PLCs. PLCs generally contain a PH domain, an EF-hand domain, X and Y catalytic domains, and a C2 domain (notably PLC-ζ lacks a PH domain) (Figure 1.3). However, PLC210 and mammalian PLC-ε uniquely possess an N-terminal CDC25-homology domain and two C-terminal Ras-association (RA) domains [48-51]. It is now known that upstream regulators of PLC-ε include Ras subfamily [49, 51] and Rho subfamily [52] GTPases, as well as subunits of the heterotrimeric G-protein family [50, 53]. Activation of PLC-ε by

GPCRs coupled to Gα subunits of the $G_{i/o}$, $G_{12/13}$, and G_s families has also been demonstrated, revealing that PLC-ε is yet another PLC isozyme regulated by GPCRs [54-56]. In addition to generating the second messengers Ins(1,4,5)P_3 and diacylglycerol, PLC-ε has also been shown to trigger other downstream signals independent of its phosphoinositide-hydrolyzing activity. PLC-ε, via the CDC25-homology domain at its amino terminus, functions as a GEF for Ras-family GTPases [50, 51, 57, 58]. In light of these findings, PLC-ε appears to be a candidate scaffold protein to integrate and mediate crosstalk between monomeric and heterotrimeric G-proteins [59].

PLC-ε contains tandem Ras-association domains (RA1 and RA2) (Figure 1.3); thus, the observation that various monomeric G-proteins activate PLC-ε was not surprising. However, further examination of small GTPase activation of PLC-ε has revealed that both RA-dependent as well as RA-independent interactions can occur. Specifically, the Ras family G-proteins H-Ras, R-Ras, TC21, Rap1A, Rap2A, and Rap2B stimulate PLC-ε in a RA2-dependent manner, whereas Ral, Rho, and Rac activation of PLC-ε appears to be primarily RA-independent [49, 52, 55, 60]. The mechanism by which Ral and Rac activate PLC-ε is unknown; however, the interaction and mode of activation of PLC-ε by Rho has been elucidated [49, 52, 55, 61]. Wing and colleagues [52] identified a unique 65 amino-acid insert within the catalytic core of PLC-ε, not present in other PLC isozymes, as the region within PLC-ε that imparts responsiveness to Rho. Interestingly, this region also appears to be essential for $G\alpha_{12/13}$ activation of PLC-ε. Thus, it is possible that $G\alpha_{12/13}$ activation

of a Rho-GEF such as p115RhoGEF or LARG leads to activation of Rho and subsequently of PLC-ε. Heterotrimeric G-protein activation of PLC-ε by $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_s$, and $G\beta\gamma$ has been demonstrated upon cellular co-transfection [53]; however, whether heterotrimeric G protein-mediated activation requires direct interaction of these subunits with PLC-ε is unclear. Demonstration that PLC-ε activation occurs via monomeric GTPases known to be downstream of heterotrimeric G-proteins suggests that heterotrimeric G protein-promoted PLC-ε stimulation is more likely indirect, and more closely resembles that of the novel PLC-β interactions described below.

1.3.2 Novel Phospholipase C Interactions

Until recently, regulation of PLC-β isozymes by GPCRs was thought to primarily occur via direct interactions with either Gα subunits of the G_q family or Gβγ subunits [31]. However, the assumption that PLC-β signaling is solely regulated by heterotrimeric G-proteins was dramatically altered with the observation by Illenberger and colleagues that members of the Rho subfamily of small GTPases, specifically Rac1 and Rac2, activate PLC-β isozymes [62, 63]. This finding raises the question of how integrated regulation of these isozymes by small GTPases and heterotrimeric G-proteins occurs, and within what signaling cascades does this phenomenon elicit specific cellular responses. In addition, these findings highlight the possibility that heterotrimeric G-protein activation of PLC-β isozymes might be synergistic via direct and indirect mechanisms involving Gβγ. For instance, Gβγ subunits activate Rac directly via the Rac-GEF P-Rex1 [64]. Thus, it may be that in certain signaling cascades, Gβγ subunits from heterotrimeric G-proteins might

stimulate PLC-β directly *and* activate a Rac-GEF such as P-Rex1 to increase Rac-GTP levels, thus activating PLC-β indirectly. Although PLC-β activation via this type of mechanism has not been demonstrated, activation of PLC-ε by $G\alpha_s$-coupled receptors via an analogous pathway has been described, as detailed below.

Schmidt and colleagues [56, 65, 66] made the observation that $G\alpha_s$-coupled receptors are capable of activating PLC-ε, and that this activation is dependent upon both heterotrimeric and monomeric G-proteins. Specifically, β_2-adrenergic-, M_3-muscarinic-, and prostaglandin E_1 receptor-mediated activation of PLC-ε was reported [56, 65], with the mechanism of activation hypothesized as follows. $G\alpha_s$-coupled receptors stimulate adenylyl cyclase, which results in increased cyclic AMP levels and thus activation of the Rap-GEF EPAC (exchange protein activated by cAMP) [67, 68]. Once activated, EPAC is thought to catalyze GTP-loading on Rap2B, leading to activation of PLC-ε. In addition to providing a potential mechanism by which GPCRs activate a PLC isozyme via integration of heterotrimeric and monomeric G-protein signaling, the findings of Schmidt and colleagues also provide evidence for a positive interaction between cAMP-promoted and PLC signaling pathways.

In addition to GPCR-mediated stimulation of PLC-ε, RTK-mediated regulation has been observed. RTKs such as those for EGF and PDGF have been shown to activate PLC-γ enzymes by recruitment of the enzyme to the autophosphorylated receptor and subsequent tyrosine phosphorylation [31]. In contrast, the mechanism of PLC-ε activation by RTKs appears to involve small GTPases. Specifically, Ras and Rap GTPases have been reported to participate in the activation of PLC-ε in a

number of cell types [51, 55, 57, 58]. The mechanism of activation of PLC-ε by these GTPases appears to involve the RA2 domain, as mutations in RA2 either reduce or completely inhibit activation of the enzyme by the EGF receptor [55].

The direct contribution of PLC-γ to the activation of PLC-ε has also been examined. Song *et al.* found that a PDGF receptor mutant, deficient with respect to PLC-γ activation, still activates PLC-ε, via H-Ras and Rap1A as intermediaries [58]. Recently, however, Stope *et al.* reported that the mechanism of PLC-ε stimulation by the EGF receptor in HEK-293 cells involves not only small GTPase activation, but also PLC-γ mediated activation [69]. Specifically, the EGF receptor was identified as a "platform" that assembles and activates two direct effectors, PLC-γ1 and the nonreceptor tyrosine kinase c-Src. Upon activation, PLC-γ1 and c-Src recruit and activates the Ca^{2+}/diacylglycerol-regulated guanine nucleotide exchange factor for Ras-like GTPases, RasGRP3, via second messenger formation and tyrosine phosphorylation, respectively. Once active, RasGRP3 catalyzes nucleotide exchange on Rap2B, inducing activation of this small GTPase. Active Rap2B then binds to PLC-ε and translocates the lipase to the plasma membrane where it can efficiently propagate signaling.

1.3.3 Physiological Function of PLC-ε

The molecular mechanisms of PLC-ε regulation have been intensively studied; however, little is known about the function of PLC-ε in physiological processes. Studies indicate that the regional and temporal expression profile of each PLC isoform may account for its physiological function [31]. For example, PLC-β1 is highly expressed in the hippocampus and cerebral cortex [70], and PLC-β1 knockout

mice exhibit minor developmental abnormalities in the hippocampus and develop epilepsy [71]. To begin to understand the physiological function of PLC-ε, Kataoka and colleagues examined the spatial and temporal expression patterns of PLC-ε in the central nervous system of mouse embryos and adults [72]. The induction of PLC-ε expression appears to be associated specifically with the commitment of neuronal precursor cells to the neuronal lineage, and seems to persist after terminal differentiation into neurons [72]. In contrast to PLC-β1 which exhibits region-specific expression [70], PLC-ε expression is observed in almost all regions containing mature neurons [72]. These results suggest that PLC-ε may be involved in a more general aspect of neuronal differentiation and neuronal function than a region-specific isoform such as PLC-β1, which is critical for very selective neuronal functions such as those associated with the hippocampus. It is possible that PLC-ε, via Ras and/or Rap regulation, may have a general role in fibroblast growth factor and neurotrophic factor signaling, both of which have been implicated in neuronal development.

Recently, the physiological function of PLC-ε in the nematode *C. elegans* was addressed. *C. elegans* ovulation and fertility are regulated by an Ins(1,4,5)P_3 signaling pathway activated by the receptor tyrosine kinase LET-23 [73, 74]. PI-PLCs generate Ins(1,4,5)P_3 by catalyzing the hydrolysis of PtdIns(4,5)P_2 into Ins(1,4,5)P_3; thus, it is possible that an enzyme involved in generation of Ins(1,4,5)P_3 would also play an important regulatory role in fertility and ovulation. Kataoka and colleagues used deletion mutants of the PLC-ε gene in *C. elegans*, *plc-1*, to investigate the role of the gene in ovulation. Two deletion alleles were generated

that removed regions important for the catalytic activity of PLC-ε, and both exhibited reduced fertility as a result of ovulation defects [75]. These results provide the first genetic analysis of the physiological function of PLC-ε in an intact organism, and suggest that PLC-ε is required for proper ovulation in *C. elegans*, consistent with previous studies that show the requirement of precise control of Ins(1,4,5)P_3 signaling levels for normal ovulation [73].

Studies with PLC-ε(-/-) mice have added further complexity to our understanding of the potential role(s) PLC-ε plays in physiological processes. The role of PLC-ε in de novo skin chemical carcinogenesis was recently examined in mice containing genetically inactivated PLC-ε. PLC-ε(-/-) mice exhibited delayed onset and markedly reduced incidence of skin squamous tumors in response to chemical carcinogens [76]. Additionally, the papillomas formed in PLC-ε(-/-) mice failed to undergo malignant progression, and the skin failed to exhibit basal layer cell proliferation and epidermal hyperplasia. Interestingly, activated H-Ras, which itself activates PLC-ε, was detected in all tumors irrespective of the PLC-ε background. These results suggest that PLC-ε plays a critical role in tumor development downstream of Ras signaling, and may be a molecular target for the development of anticancer drugs.

A second study in mice examined the role of PLC-ε in cardiac function and disease after upregulated PLC-ε protein and RNA expression were detected in human hearts during heart failure and in mice models of cardiac hypertrophy [77]. PLC-ε(-/-) mice had decreased cardiac function, decreased contractile response to acute isoproterenol administration, and were more susceptible to development of

hypertrophy under chronic stress. Additionally, cardiac myocytes isolated from PLC-ε(-/-) mice showed decreased responsiveness to β-adrenergic receptor stimulation. These data suggest that PLC-ε is required for maximum efficacy of the β-adrenergic receptor system in cardiac myocytes, and that loss of PLC-ε signaling appears to sensitize the heart to development of hypertrophy in response to chronic cardiac stress [77]. As previously mentioned, a signaling pathway from the β_2-adrenergic receptor, through $G\alpha_s$, cAMP, EPAC, and Rap2B to PLC-ε has been proposed based on studies in cells transfected with PLC-ε. Thus, it remains to be determined whether the *in vivo* PLC-ε regulatory signaling network in the heart intersects with β-adrenergic receptor signaling pathways and/or other pathways. The studies performed in *C. elegans* and in mice highlight the complexity of the role PLC-ε plays in physiological processes. Future studies examining the cellular function and regulation of PLC-ε both *in vitro* and *in vivo* will help to merge the gap between molecular and functional analyses of PLC-ε regulation, and thus provide evidence in support of PLC-ε as a critical player in mammalian physiology. In Chapter 2, I describe examination of GPCR-mediated regulation of inositol lipid signaling through a mechanism involving $G\alpha_{12/13}$, Rho, and PLC-ε. The results of these studies are consistent with the idea that $G\alpha_{12/13}$- and Rho-dependent activation of PLC-ε occurs downstream of both LPA- and thrombin-activated receptors, and that the regulation of PLC-ε by $G\alpha_{12/13}$ occurs at least in part through activation of Rho.

1.4 RGS Proteins as Negative Regulators of GPCR Signaling

In the aforementioned standard model of GPCR signaling, the duration of G-protein signaling through effectors is thought to be controlled by the lifetime of the

Gα subunit in its GTP-bound form. It was originally thought that the duration of signaling could be modulated by only two factors: the intrinsic GTP hydrolysis rate of Gα and acceleration of that rate by some Gα effectors that exhibit "GTPase-accelerating" or "GAP" activity (*e.g.*, phospholipase C-β), [78]. In 1996, a new superfamily of Gα GAPs was discovered: the RGS ("regulator of G-protein signaling") proteins [79-83]. Each RGS protein contains a hallmark ~120 amino-acid RGS domain (or "RGS-box") which contacts Gα subunits, and dramatically accelerates GTPase activity [84-86]. Crystal structures of RGS4 bound to $G\alpha_{i1}$·GDP·AlF_4^- [87] and RGS9 bound to Gα-transducin·GDP·AlF_4^- [88] revealed the precise molecular mechanism of GTPase acceleration: the RGS domain contacts Gα at its conformationally-flexible switch regions to stabilize the transition state of GTP hydrolysis (as mimicked by the planar ion aluminium tetrafluoride [AlF_4^-]). The discovery of RGS proteins and their GAP activity towards Gα subunits resolved apparent timing paradoxes between observed rapid physiological responses mediated *in vivo* by GPCRs and the slow hydrolysis activity of the cognate G-proteins seen *in vitro* (*e.g.*, retinal photoreception; ref. [89]). Many RGS proteins are now known to catalyze rapid GTP hydrolysis by isolated Gα subunits *in vitro* and to attenuate and/or modulate GPCR-initiated signaling *in vivo* (reviewed in [90-92]); accordingly, RGS proteins are considered key desensitizers of heterotrimeric G-protein signaling pathways and excellent drug discovery targets [23].

1.4.1 RGS Protein Selectivity/Determinants

RGS proteins specific for $G_{i/o}$, G_q, $G_{12/13}$, and G_s Gα subunits have been described [23]. The RGS domain is critical for the GAP activity of each RGS protein

on the Gα subunit; however, the mechanism by which each protein inhibits signaling differs, and can depend on sequence beyond the RGS-box. In fact, regions outside of the RGS domain can also provide selectivity at the level of the receptor itself.

The GPCR signaling regulator, G-protein receptor kinase 2 (GRK2), contains a conserved N-terminal RGS-box that does not stimulate GTPase activity, but rather sequesters Gα subunits of the G_q family [93-95]. In contrast to RGS proteins that act on both $G\alpha_q$ and $G\alpha_i$ subunits (*e.g.*, RGS2 and RGS4), the RGS-box of GRK2 is selective for Gα subunits of the G_q family, and thus, is a potent effector antagonist for G_q-coupled GPCRs. However, GRK2 has a multidomain architecture (Figure 1.4), and therefore regulates more than GPCR signaling through $G\alpha_q$. The C-terminal fragment of GRK2 competitively binds Gβγ via its pleckstrin-homology (PH) domain [96], and the kinase domain phosphorylates and desensitizes GPCRs [97].

RGS2, a founding member of the RGS protein family, accelerates the GTPase activity of Gα subunits of the G_q family *in vitro* [98, 99]. However, in receptor reconstitution and cellular assays, RGS2 acts as a negative regulator of both $G_{i/o}$- and G_q-coupled receptor signaling [99]. These findings suggest that assay conditions alter the G protein specificity of RGS2 from $G\alpha_q$ to both $G\alpha_q$ and $G\alpha_{i/o}$.

RGS4 stimulates the GTPase activity of Gα subunits of the $G_{i/o}$ [84, 100] and G_q family [99, 101] via its RGS-box. Similar to RGS2, RGS4 contains little sequence beyond the RGS-box. However, RGS4 has been demonstrated to exert receptor-selective inhibitory activity via its amphipathic alpha-helical N-terminus [102]. In addition, the N-terminus also has been shown to confer high potency inhibition of G_q-mediated receptor signaling *in vivo* [102].

The guanine nucleotide exchange factor (GEF) for Rho, p115 RhoGEF, contains an N-terminal RGS-box and acts as a GAP for $G\alpha_{12}$ and $G\alpha_{13}$ [103]. The GAP activity of p115 RhoGEF requires sequences flanking the RGS-box [104]. Specifically, without the N-terminus of the protein, the RGS-box is nonfunctional. Similar to GRK2, p115 RhoGEF also contains domains beyond the RGS-box. p115 RhoGEF contains a tandem Dbl-homology domain (DH/RhoGEF) and PH domain C-terminal cassette (Figure 1.4) that exhibits RhoA-specific GEF activity [105]. Thus, p115 RhoGEF, similar to GRK2 and other multidomain RGS proteins, regulates more than just the termination of $G\alpha$ signaling. In Chapter 3, I describe the utilization of RGS-box proteins to evaluate G-protein selectivity in receptor-promoted signaling. My results suggest that RGS proteins can be used to delineate $G\alpha$ selectivity of GPCRs. However, the findings also highlight the importance of using additional RGS proteins or other inhibitors of $G\alpha$ signaling when employing RGS-box constructs to delineate signaling pathways.

RGS-box-independent regulation of GPCR signaling has also been demonstrated. The N-terminus of RGS2 inhibits both GTPase-deficient, constitutively active mutant of $G\alpha_s$(Q227L)- and β_2-adrenergic receptor-stimulated cAMP accumulation in HEK 293 cells expressing type V adenylyl cyclase [106, 107]. Similarly, RGS16, a GAP for $G\alpha_{i/o}$ and $G\alpha_q$, binds to $G\alpha_{13}$ and inhibits $G\alpha_{13}$-mediated signal transduction via its amino terminus [108]. RGS3 employs a mechanism independent of its $G\alpha$ GAP activity to impair $G\beta\gamma$ signaling. Specifically, RGS3 lacking a functional RGS-box potently inhibits $G\beta\gamma$-mediated inositol phosphate production and activation of Akt and MAPK by binding $G\beta\gamma$ subunits,

effectively interfering with their availability for effector activation [109]. RGS12, a multidomain RGS protein, inhibits both $G_{12/13}$- and $G_{q/11}$-coupled receptor signaling when overexpressed in cells [110]. *In vitro*, the RGS-box of RGS12 acts as a GAP for $G\alpha_{i/o}$ subunits, but not $G\alpha_q$ nor $G\alpha_s$ [111]; thus, the mechanism of this inhibition remains to be determined, but most likely does not occur via the RGS-box.

1.5 Multiple Domain-Containing RGS Proteins

Since the discovery of the first RGS proteins in 1996, it has become apparent that the signature RGS-box is a modular protein domain that is found in many contexts (Figure 1.4). Nine subfamilies of RGS proteins have been characterized to date which differ considerably in their overall size, amino-acid identities, and tissue distribution [23]. The identification of multidomain RGS proteins has led to a new appreciation of these molecules as being more than just GAPs for $G\alpha$ subunits [90, 92].

1.5.1 The D- or R12-Subfamily of RGS Proteins

The D- or R12-subfamily of RGS proteins contains only three members, namely RGS10, RGS12, and RGS14 [112], and all three proteins act as GAPs for $G\alpha_i$-family $G\alpha$ subunits *in vitro* [85, 111, 113, 114]. RGS10 belongs to the R12-subfamily given its RGS-box sequence similarity; however, at 173 amino-acids, it is the smallest member and contains only a very short N-terminal tail and an RGS-box. In fact, RGS10 is more structurally similar to members of the R4-subfamily of RGS proteins, such as RGS2, that lack additional regulatory domains. In contrast, RGS12 and RGS14 are much larger proteins [115], and contain numerous functional domains beyond the hallmark RGS-box, as described below.

1.5.2 RGS12

RGS12 is an example of an RGS protein with numerous signaling regulatory elements. RGS12 and a mammalian paralogue of RGS12, RGS14, were originally cloned from the rat C6 glioblastoma cell line using degenerate PCR primers directed to sequences conserved within all RGS domains [115, 116]. RGS12 and RGS14 both contain an RGS-box, tandem RBDs (Ras-binding domains), and a GoLoco ($G\alpha_{i/o}$-Loco interaction) motif (Figure 1.4). However, RGS12 also has an N-terminal extension containing two additional protein-protein interaction domains: a PDZ (Postsynaptic density-95 / Discs-large / Zona occludens 1 protein homology) domain and a phosphotyrosine-binding (PTB) domain. This N-terminal extension is highly evolutionarily conserved among mammalian, avian, and model organism genomes. For example, the longest isoform of the Drosophila protein Loco ("isoform D") shares the complete PDZ/PTB/RGS/RBDs/GoLoco architecture with human, rodent, and chicken RGS12 [117]. The domain architecture of RGS12 suggests multiple functions (see below) and work done in our lab and others supports this hypothesis (Figure 1.5) [23].

1.5.3 PDZ Domain

PDZ domains are ~90 amino-acid modular protein interaction domains that mediate specific protein-protein interactions that underlie the assembly of large protein complexes involved in signaling or subcellular transport. These domains were originally termed DHR (for Discs-large homology repeat) [118] or GLGF repeats (after a signature Gly-Leu-Gly-Phe sequence found in the domain) [119]. PDZ domains are now primarily known by an acronym of the first three PDZ-

containing proteins identified: the postsynaptic protein PSD-95/SAP90, the *Drosophila* septate junction protein Discs-large, and the tight junction protein zonula occludens 1 (ZO-1) [120]. Since their initial discovery, PDZ and PDZ-like domains have been recognized in numerous proteins from organisms as diverse as mammals, bacteria, yeast, plants, *Caenorhabditis elegans*, and *Drosophila* [121]. In fact, they are among the most abundant protein domains represented in multicellular eukaryotic genomes.

A canonical PDZ domain comprises six β-strands (A to F) and two α-helices (A and B), and carboxyl peptide ligands bind to an extended groove formed by the βB strand and the αB helix of the domains by augmenting the βB strand in an antiparallel fashion [122]. The binding specificity of each PDZ domain is ultimately determined by the interaction of the first residue of helix αB (αB1) and the side chain of the -2 residue of the C-terminal ligand [123]. In class I PDZ interactions, such as those of PSD-95 and NHERF, a serine or threonine residue occupies the -2 position (-*X*-S/T-*X*-Φ; where Φ is a hydrophobic amino-acid) [124, 125]. The hydroxyl group of serine or threonine forms a strong hydrogen bond with the N-3 nitrogen of a highly conserved histidine residue at position αB1 in class I PDZ domains [122]. In comparison, class II PDZ interactions, such as those of PICK1 and CASK, are characterized by hydrophobic residues at both the -2 position of the peptide ligand (-*X*-Φ-*X*-Φ) and the αB1 position of the PDZ domain [123]. A third class of PDZ domains, such as nNos and Mint1/X11, prefers negatively charged amino-acids at the -2 position (-*X*-D/E-*X*-Φ) [126, 127]. Class III specificity is determined by the

coordination of the hydroxyl group of a tyrosine residue at position αB1 in the PDZ domain with the side chain carboxylate of the -2 residue [126, 128].

The RGS12 PDZ domain has a class I binding specificity (*i.e.*, C-terminal S/T-*X*-L/V motifs) and interacts with the C-terminus of the interleukin-8 receptor CXCR2 *in vitro* [111], and *in vivo* (unpublished results). RGS12 prefers the motif T-*X*-L versus S-*X*-L. In addition to CXCR2, RGS12 can bind to the C-termini of MEK2 (RTAV), p90RSK1 (STTL), and PDGFRβ (DSFL) (Chapter 4, unpublished results, and [18], respectively). However, RGS12 does not bind indiscriminantly to C-terminal S/T-x-L/V sequences. For example, the PDZ domain of RGS12 does not bind to the β_2-adrenergic receptor (DSPL), the metabotropic glutamate 1α receptor (SSTL), nor the neuropeptide Y receptor 2 (ATNV) *in vitro* [111]. Additionally, RGS12 does not bind corticotrophin releasing hormone receptors 1 and 2 (STAV and TTAV, respectively) *in vivo* (unpublished results), suggesting that other factors play a role in RGS12 PDZ selectivity beyond the conserved S/T-x-L/V motif.

In collaboration with the laboratory of Dr. Nigel Pyne (Strathclyde Institute, Glasgow), we recently observed that RGS12 forms a complex with the sphingosine 1-phosphate ($S1P_1$) receptor and the platelet-derived growth factor-β (PDGFβ) receptor in guinea-pig airway smooth muscle cells, and thereby reduces the ability of these receptors to activate MAPK [18]. Specifically, we found that overexpression and subsequent association of RGS12 with the complex induces agonist-independent internalization of the PDGFβ and $S1P_1$ receptors into endocytic vesicles. Significantly, RGS12-directed internalization of the receptor complex demonstrates a potential novel role for RGS12 in promoting down-regulation or

internalization of receptor complexes from the cell surface. Although the mechanism by which RGS12 mediates PDGFβ receptor-$S1P_1$ receptor complex internalization is unknown, the N-terminal PDZ and PTB domains of RGS12 are responsible for the binding, and it is likely that this region is also responsible for the highly specific localization of the complex to endosomes. Similar to full-length RGS12, expression of the N-terminal PDZ-PTB domain tandem in isolation induced the internalization of the complex into endocytic vesicles [18] suggesting that this N-terminal domain cassette of RGS12 is responsible for the localization of the complex.

1.5.4 PTB Domain

PTB domains are modular 100-170 amino-acid domains that participate in protein-protein interactions [129, 130]. PTB domains are present in a number of signaling scaffold proteins, including Shc [131], Dab-1 [132], X11 [133], JIP-1 [134], and Numb [135]. Originally characterized as binding to the consensus amino-acid sequence Asn-Pro-X-pTyr (NPXpY), PTB domains are now known to have diverse binding capabilities. Some PTB domains bind peptides without the consensus motif or even independently of tyrosine phosphorylation [135]. Typical substrates for PTB domain-containing proteins include growth factor receptors, integrin cytoplasmic tails, GTPase regulatory enzymes, and components of endocytosis machinery; thus, PTB domains are often localized either to membranes or to juxtamembrane regions.

An additional feature of many PTB domains is their ability to bind directly to liposome-associated or free phospholipid head groups [131, 132, 136]. Phosphoinositide binding by Dab-1 and Shc PTB domains has been proposed to mediate membrane localization of these proteins. The site for phospholipid binding

in these proteins exists in a pocket distinct from that of the peptide binding pocket and involves interactions with basic residues. The Numb PTB also binds various phospholipids, including phosphatidylinositol (4,5)bisphosphate [PtdIns(4,5)P_2], a phospholipid involved in actin remodeling [136]. Although the residues in Numb involved in phospholipid binding have not been determined, three-dimensional (3D) modeling of the PTB domain reveals that the protein possesses highly favorable basic regions to accommodate acidic phospholipid head groups [129]. Similar to the Numb PTB domain, 3D electrostatic contouring of a model of the RGS12 PTB domain reveals a conserved pocket that is likely to bind phospholipids [129]. Mapping of the RGS12 PTB model for surface electrostatic potential indicates that this conserved pocket contains a number of positively-charged residues (i.e, R255, R260, R262, and R308) and appears to form a “crown” for coordination of acidic phospholipids. Preliminary data suggest that RGS12 preferentially binds to PtdIns(3)P and PtdIns(5)P (unpublished results); however, the structural determinants of this interaction remain to be determined.

Schiff and colleagues found that RGS12 is specifically involved in establishing the rate of desensitization of $GABA_B$ receptor-mediated, voltage-independent inhibition of presynaptic $Ca_v2.2$ (N-type) calcium channels in embryonic chick dorsal root ganglion (DRG) neurons [137]. For example, microinjection of DRG neuron cell bodies with recombinant full-length RGS12, but not other RGS proteins such as GAIP or RGS14, dramatically accelerates the time to termination of GABA-mediated $Ca_v2.2$ channel inhibition. Conversely, microinjection of antibodies against RGS12,

but not other RGS proteins, dramatically prolongs the effect of calcium channel modulation by this major inhibitory neurotransmitter, γ-aminobutyric acid (GABA).

The PTB domain facilitates the recruitment of RGS12, in a transient, phosphotyrosine-dependent manner, to the SNARE-binding or "synprint" region in loop II-III of the $Ca_v2.2$ channel [137, 138], and thereby determines the desensitization rate of $GABA_B$-receptor mediated inhibition of calcium current in DRG neurons [137]. Studies examining the interaction of RGS12 with the synprint region of the $Ca_v2.2$ channel also revealed that the binding motif in the channel is not a canonical PTB binding motif [138]. As described above, most PTB domains recognize the consensus NPXY motif, either in a phosphotyrosine-dependent or independent manner [139]. However, variations of this motif have been identified, suggesting a wider binding specificity for PTB domains than once thought. RGS12 binds to the channel in a phosphotyrosine-dependent manner and to a region that contains the residues EALY [138]. In addition to these residues, high affinity binding to the channel is also dependent upon residues C-terminal to the phosphotyrosine, suggesting that this interaction may be a new mode of peptide recognition for PTB domains.

1.5.5 RGS Domain

The majority of RGS-box-containing proteins negatively regulate GPCR signaling by stimulating the intrinsic GTPase activity of $G_{i/o}$, G_q, $G_{12/13}$, and G_s Gα subunits [23]. The RGS domain of RGS12 is no exception. RGS12 is capable of stimulating the intrinsic GTP hydrolysis rate of both $G\alpha_i$ and $G\alpha_o$ subunits *in vitro*, but not of $G\alpha_q$ nor $G\alpha_s$ [111]. RGS12 does not appear to require regions beyond the

RGS domain for this activity, as the isolated RGS-box is capable of increasing the GTPase activities of $G\alpha_{i/o}$ subunits [111].

$GABA_B$ receptors couple to $G\alpha_o$ to inhibit $Ca_v2.2$ channels in embryonic chick DRG neurons [140]. As described above, RGS12 is involved in establishing the rate of desensitization of $GABA_B$ receptor-mediated inhibition of $Ca_v2.2$ calcium channels [137]. RGS12 may act to terminate the signal by two mechanisms: RGS12 may directly modulate channel activity; alternatively, the channel may be used as a 'scaffold' to recruit RGS12 to the vicinity of $G\alpha_o$ and functional effects may arise from RGS12 enhancing the GTPase activity of $G\alpha$. Whether either or both of these mechanisms are in operation remains to be determined.

1.5.6 Tandem Ras-Binding Domains

GTPases are proteins that bind and hydrolyze GTP. Ras is a GTPase that is involved in many signal transduction processes such as control of growth, apoptosis, and differentiation [141, 142]. Ras, and other GTPases, act as molecular switches by cycling between an 'on' and 'off' state [143]. In the 'off' state, the GTPase is tightly bound to GDP, until GEFs promote GDP-dissociation, and subsequent loading of GTP. Once bound to GTP, Ras interacts with Ras effectors, proteins that preferentially bind to the activated GTP-bound form of Ras.

The first *bona fide* effector of Ras to be identified was c-Raf-1 [144, 145]. c-Raf-1 contains a 81 amino-acid Ras binding domain (RBD) that is sufficient to bind to Ras and Rap isoforms in a GTP-dependent manner. Structural studies revealed that the RBD of c-Raf-1 has an ubiquitin fold that interacts with Ras and Rap by forming an inter-protein β-sheet, which involves the outer strands of both proteins

[146-148]. Intriguingly, the interface of the complex is determined to a large extent by complementary charge interactions, *i.e.* the surfaces of Ras and c-Raf-1 RBD are negatively and positively charged, respectively [147]. Approximately 20 RBDs have been identified in human proteins [149, 150]; however, whether all of them are true Ras effectors remains to be determined [141]. Isolated RBDs have been described in several proteins including Tiam-1 and c-Raf-1; in comparison, a tandem repeat of two RBDs has only been described in RGS12 and RGS14 [149, 150].

Domains homologous to the RBD in c-Raf-1 were identified in tandem in both RGS12 and RGS14 [151]. A multiple sequence alignment of the RBDs revealed that RBD1 of both RGS12 and RGS14 contains many residues that are conserved in human c-Raf-1. For example, the critical arginine (R89) that lies at the center of the Rap1A/c-Raf-1 binding interface is conserved in RGS14 [148]. Interestingly, this arginine residue is substituted with a positively charged histidine residue in RGS12. These results suggest that the RBDs identified in RGS14 and RGS12 likely bind Ras-like GTPases. The RBDs of RGS14 interact with both Rap and Ras GTPases *in vitro* [152-154], and the RBDs of RGS12 interact with activated H-Ras *in vivo* (Chapter 4). Moreover, it has been suggested that RBD1 of RGS14 mediates the binding to Ras and Rap, while RBD2 does not appear to interact with GTPases [152-154]. In the case of the cell junction and Ras-interacting protein AF6, which contains tandem Ras-association domains, the first domain binds Ras with high affinity whereas the second domain binds with low affinity [141]. Thus, it is possible that the first RBD of RGS12 and RGS14 is a high-affinity binding domain, whereas the second is low-affinity, although this hypothesis remains to be tested.

1.5.7 GoLoco Motif

Following the cloning of RGS12 and RGS14, a novel 19 amino-acid region within both proteins was identified that is also present in other Gα-interacting proteins [151, 155]. As the existence of the GoLoco motif was initially inferred from the detection of a second $G\alpha_i$-binding site within Loco, the *Drosophila* RGS12 homologue [117], it was termed $G\alpha_{i/o}$-Loco, or GoLoco motif [155]. GoLoco motifs have since been identified in several distinct classes of proteins encoded in metazoan genomes, including the *Drosophila* proteins Loco and Pins, the mammalian proteins RGS12, RGS14, Pcp-2, Rap1GAP, G18, LGN, and AGS3, and the *Caenorhabditis elegans* proteins GPR-1 and GPR-2 [156]. To date, all characterized GoLoco motifs have been found to interact with $G\alpha_{i/o}$ subunits and thereby prevent their spontaneous release of GDP: an activity referred to as guanine nucleotide dissociation inhibitor (GDI) activity [156].

Functional characterization of the GoLoco motif regions of RGS12 and RGS14 revealed that both proteins interact exclusively with $G\alpha_i$ subunits (bar $G\alpha_{i2}$ [157, 158]) in their GDP-bound forms [159]. Both regions exhibited GDI activity in GTPγS binding assays, inhibiting the rate of exchange of GDP for GTP by $G\alpha_{i1}$, and stabilized $G\alpha_{i1}$ in its GDP-bound form, inhibiting the increase in intrinsic tryptophan fluorescence stimulated by AlF_4^- [159]. These results indicate that both RGS12 and RGS14 harbor two distinctly different Gα interaction sites, suggesting that both proteins participate in a complex coordination of G-protein signaling beyond simple Gα GAP activity.

The GoLoco motif binds to $G\alpha_i$·GDP subunits and prevents concomitant $G\beta\gamma$ association [157, 160]; thus, it is possible that the GoLoco motif plays a role in modulating GPCR signaling pathways. To address this possibility, Oxford and colleagues examined whether a peptide comprising the GoLoco motif of RGS12 could selectively uncouple $G\alpha_i$-coupled GPCR signaling by the $G\alpha_i$-coupled D_2-dopamine receptor. Intracellular microinjection of the RGS12 GoLoco peptide into mouse pituitary gland-derived AtT-20 cells antagonized D_2-dopamine receptor coupling to the activation of Kir3.1/3.2 potassium current, but did not affect $G\alpha_o$-coupled somatostatin-induced current [157]. These results support the possibility that $G\alpha_i$-coupled GPCR signaling pathways may be the direct targets of GoLoco protein modulation.

In addition to regulating $G\alpha$ signaling, the GoLoco motif may play a role in the localization of RGS12 in cells. RGS12 appears to be subcellularly localized in a punctate pattern when overexpressed in primary airway smooth muscle cells, as well as in PC12 cells, HEK 293T cells, and primary DRG neurons (Chapter 4, [18], and unpublished results). Although the exact mechanism by which RGS12 localizes to endosomes is unknown, it is possible that the $G\alpha_i$-binding activity of the C-terminal GoLoco motif plays a role; we have previously reported a loss-of-function mutation in the GoLoco motif that mislocalizes RGS12 to the nucleus [18]. These findings, as well as those described herein, suggest that the multiple functional domains found within RGS12 may cooperate to define the spatial and temporal nature of a RGS12-coordinated signaling output initiated from RTKs and both monomeric and heterotrimeric G-protein subunits.

1.5.8 *Drosophila loco*

The *Drosophila* gene *loco* was originally identified in a screen for glial cell-specific genes, and encodes an RGS domain-containing protein with the same multiple domain architecture as RGS12 and RGS14 [117]. It is now known that mammalian RGS12 and RGS14 are the orthologues of *Drosophila* Loco [156]. Loco is essential for multiple processes in *Drosophila* development including dorsal/ventral axis formation, neuroblast asymmetric cell division, and nurse cell dumping [161, 162]. The *loco* gene is expressed in lateral glial cells in the developing embryonic central nervous system and is required for correct glial cell differentiation [117]. Normal glial-glial cell contacts are absent in *loco*-deficient flies, which results in a loss of the blood-brain barrier, and subsequent gross locomotor defects in surviving mutants, hence the moniker “locomotion defects”. Relatively little is known about the specific molecular mechanisms underlying glial cell development, although the specific interaction between Loco and $G\alpha_i$ suggests a function of G-protein signaling in this process [117]. Recently, Loco, the GPCR Moody, and the $G\alpha$ subunits $G\alpha_i$ and $G\alpha_o$ have been found expressed in surface glia, and genetic studies suggest that these four proteins act as part of a common signaling pathway critical for blood-brain barrier formation [163, 164].

As will be described in Chapter 4, our data on RGS12 as a MAPK scaffold is consistent with *Drosophila* studies. For example, we found that RGS12 preferentially binds to activated H-Ras, and this is consistent with yeast two-hybrid data demonstrating that Loco interacts with activated *Drosophila* Ras1 [165]. Additionally, the localization of Loco in surface glia is punctate throughout the cytoplasm [164],

paralleling our own observations in primary airway smooth muscle cells, PC12 cells, HEK 293T cells, and primary DRG neurons ([18], Chapter 4, and unpublished results). These results, and findings described in Chapter 4, suggest that RGS12 might constitute an evolutionarily-conserved molecular link that integrates RTK signaling with GPCR signaling in both vertebrates and non-vertebrates.

1.6 Mitogen-Activated Protein Kinases

Mitogen-activated protein kinase (MAPK) cascades are evolutionarily conserved in a variety of signaling pathways that regulate various cellular processes, including apoptosis, gene transcription, differentiation, and growth [166-169] (Figure 1.6). These cascades culminate in activation of a member of one of three major kinase classes: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), or the p38 kinases. All MAPK cascades display a three-tier architecture in which the last member of the cascade, “MAPK” (an ERK, JNK, or p38 kinase), is activated by phosphorylation at tyrosine and threonine residues by the second member of the cascade: “MAP2K”. MAP2K is activated by threonine and serine phosphorylation by the first member of the cascade: “MAP3K”. MAP3K is activated by a variety of mechanisms depending on the system; with the Raf family of MAP3Ks, this activation is primarily via Ras-family GTPases [170]. Thus, the ERK version of the MAPK signaling cascade proceeds via Ras·GTP-mediated activation of a member of the Raf family (MAP3K-tier), which in turn activates MEK1/2 (MAP2K-tier), and leads to ERK1/2 activation (MAPK-tier) [166, 171].

1.6.1 MAPK Cascades and the Importance of Scaffold Proteins

The activation of a MAPK signaling cascade is highly ordered and involves tight regulation of all modular components of the cascade. As the ERK MAPK cascade is activated in a seemingly ubiquitous fashion in response to a wide variety of extracellular stimuli, mechanisms must exist to link specific inputs with appropriate biological outcomes [172-174]. Scaffold proteins bind numerous members of a particular signaling cascade to form a multimolecular complex and, as such, serve as organizing centers to enhance functional interactions between components of MAPK pathways. Several MAPK scaffold proteins have been identified in both yeast and mammals, with the *S. cerevisiae* scaffold protein Ste5 serving as the archetype [167, 175]. Ste5 is the product of a "sterile" gene, so-named given that loss-of-function ste5 mutants fail to progress through pheromone-induced mating [176, 177]. Ste2 and Ste3 are pheromone-responsive GPCRs that activate the Gα subunit Gpa1 and the G$\beta\gamma$ subunit Ste4/Ste18. The Ste5 scaffold protein tethers the protein kinases Ste11 (MAP3K), Ste7 (MAP2K), and Fus3 (MAPK) simultaneously through separable binding sites [178], and recruits these kinases to the plasma membrane—the site at which other proteins (*i.e.*, Ste4/Ste11 and the PAK-like kinase Ste20) participate in pheromone-dependent activation of the MAPK cascade [179]. Ste5-mediated compartmentalization of enzymatic components of the mating pathway not only increases signaling efficiency, but also generates specificity by insulating the pheromone pathway from parallel pathways that also use these universal signaling components [175]. For example, both Ste11 and Ste7 also participate in nitrogen sensing and pseudohyphal growth initiation; however, formation of a pre-existing

complex by Ste5 specifically connects Ste11 and Ste7 to Fus3, thereby facilitating activation of this but not other MAPK-tier kinases [175] (cf. [180]).

1.6.2 Mammalian MAPK Cascade Scaffolds

Proteins with scaffolding functions have now been identified in mammalian MAPK cascades (Table 1.1) [181]. For example, MEK partner 1 (MP1) specifically binds MEK1 and ERK1 and facilitates ERK1 activation [182], whereas JNK-interacting protein (JIP)-1 binds MLK3 and DLK (MAP3Ks), MKK7 (MAP2K), and c-Jun kinase (JNK, a MAPK) to regulate the specific biological activities of the JNK pathway [183]. Osmosensing scaffold for MEKK3 (OSM) binds Rac, MEKK3, and MKK3 to coordinate p38 MAPK activation in the cellular response to hyperosmotic shock [139]. These scaffold proteins can bind to MAPK components constitutively; however, there are examples of scaffold associations that are agonist-dependent [184]. For instance, the ERK MAPK scaffold “kinase suppressor of Ras” (KSR-1) associates constitutively with MEK, 14-3-3, Hsp90, and Cdc37 [185-190], but only binds MAPK and Raf kinases in a Ras-dependent manner [185, 187, 189, 191]. Scaffolds are also found in signaling pathways outside the MAPK cascade (*e.g.*, GPCR signaling) [192-194], suggesting that their function and requirement are widespread.

β-arrestins have a well-established role in the desensitization and termination of GPCR signaling; however, a growing body of evidence indicates that they also function as MAPK scaffolds. Traditionally, ligand binding and subsequent phosphorylation of GPCRs by GRKs promotes translocation of β-arrestins from the cytoplasm to the receptor, where they interact directly with the receptor. Upon

binding, β-arrestins uncouple the receptor from G-proteins and target it for endocytosis. Additionally, β-arrestins mediate interaction of the GPCR with a variety of signaling proteins, including components of the MAPK cascade [28, 195]. Complexes have been identified that contain a GPCR, β-arrestin, Raf, MEK, and ERK. For example, activation of the angiotensin type 1a receptor (AT1aR) leads to the assembly of a complex containing the receptor, β-arrestin-2, c-Raf-1, MEK1, and ERK2 in HEK 293 and COS-7 cells [28]. Significantly, assembly of this complex is promoted by binding of c-Raf-1 to β-arrestin, and contributes to AT1aR-mediated activation of ERK; *e.g.*, overexpression of β-arrestin increases angiotensin-mediated ERK activation [196]. These results suggest that β-arrestin can function as a scaffold protein that promotes the stable association of MAPK signaling proteins with GPCRs, and this association appears to facilitate efficient and proper signaling to ERK.

The stoichiometry of components participating in a scaffold complex is critical for optimal signal processing. Depending on the relative concentration of the scaffold, output from a MAPK signaling cascade can either be disrupted or favored. This phenomenon, the “pro-zone” effect [197] or “combinatorial inhibition” [198], arises because at low scaffold concentrations the kinases are in excess and formation of a functional complex containing each kinase is likely to occur; in contrast, when scaffold is in excess, non-functional complexes containing less than the full complement of components becomes more prevalent [198]. Inhibition or promotion of MAPK signaling has been observed with the scaffold proteins KSR-1 and MP1. KSR-1 was found to enhance MAPK signaling at low levels by cooperating

with activated Ras, whereas, at high levels, KSR-1 antagonized MAPK activation [185]. Similarly, organization of a productive MP1/MEK1/ERK1 complex can be blocked or promoted depending on the concentration of the scaffold [182]. These findings emphasize that the relative stoichiometry of scaffold to MAPK cascade component is crucial for accurate and efficient signal transduction [198, 199], and highlight the caution that one needs to implement when executing and analyzing scaffold protein experiments, *e.g.*, JIP-1 [183, 200].

MAPK scaffolds not only create functional MAPK modules of unique composition and regulate their activation by specific upstream stimuli, they also provide both spatial and temporal control of MAPK signaling; thus, the localization of a scaffold complex is critical for appropriate and optimal signal processing. The MAPK scaffold KSR, for example, is inactive in the cytosol. However, in response to EGF stimulation of the EGFR, KSR translocates to the plasma membrane to facilitate Ras activation of the Raf/MEK/ERK cascade [201]. The scaffold function of MP1 is dependent upon binding to p14, an adaptor protein found on the cytoplasmic side of late endosomes [202]. Mislocalization of the MP1/p14 complex to the plasma membrane fails to promote Ras activation of ERK. Thus, MP1 is not required for EGFR activation of ERK at the plasma membrane, but for prolonged activation of ERK on late endosomes.

β-arrestin, as described above, facilitates c-Raf-1 activation of MEK1 and ERK2 [28]. Interestingly, β-arrestin-associated ERK remains sequestered in the cytosol, and in this way, directs substrate selectivity [195, 196]. Sef ("similar expression to *fgf* genes") is a Golgi-associated MEK/ERK scaffold that binds

activated MEK and promotes its phosphorylation of ERK [203]. Upon complex formation, Sef prevents activated ERK translocation to the nucleus, consequently precluding ERK phosphorylation of the nuclear transcription factor E-26-like protein 1 (Elk-1). Instead, Sef-associated ERK promotes phosphorylation of the cytosolic 90 kDa ribosomal protein S6 kinase 2 (p90RSK2). These results demonstrate that scaffold proteins can spatially regulate MAPK signaling by specifying substrate selection.

1.6.3 Nerve Growth Factor and TrkA

Nerve growth factor (NGF), the prototypical neurotrophin, was discovered 50 years ago by Cohen, Levi-Montalcini, and Hamburger as a factor required for axonal growth from explants [204]. NGF can be isolated in two forms, 7S or 2.5S, with S designating the sedimentation coefficient. The 7S form of NGF is a high-molecular-weight complex containing two copies of each of three types of polypeptide chains (α, β, γ). The 2.5S NGF, conversely, consists of the β-subunit only and is abundant in mouse submandibular glands [205]. NGF mediates its effects through binding to two different receptors—the tropomyosin-related kinase A (TrkA) receptor and the p75 neurotrophin receptor ($p75^{NTR}$). The $p75^{NTR}$ belongs to the tumor necrosis factor receptor superfamily, and was the first identified receptor for NGF [206, 207]. TrkA was originally discovered as a rearrangement of non-muscle tropomyosin and a tyrosine kinase, and subsequently was identified as a receptor for NGF [208-211].

TrkA is a prototypical RTK that contains an extracellular domain composed of two distinct subsets of cell adhesion-related motifs: at the amino-terminal end, arrays of three leucine-rich motifs flanked by two cysteine clusters. Next to the second

cysteine cluster, two immunoglobulin-like C2 type domains (Ig-C2) stretch down to a single transmembrane domain. Facing the inner side of the membrane, Trk receptors display a cytoplasmic region with an intracellular tyrosine kinase domain followed by a short carboxy-terminal extension [212, 213]. Binding of NGF to TrkA occurs mainly through the Ig-C2 domains, although the leucine-rich motifs and the cysteine clusters may be involved [213]. The affinity of NGF for TrkA is modulated by the presence of $p75^{NTR}$, which enhances specificity for NGF versus neurotrophin-3 [214]. In fact, formation of high-affinity binding sites for NGF requires the presence of an appropriate ratio of $p75^{NTR}$ and TrkA receptors [215].

The signaling pathways activated by NGF regulate many neuronal functions including differentiation, cell survival, dendritic arborization, axonal growth, plasticity, synapse formation, and axonal guidance [213]. Upon binding NGF, TrkA receptors dimerize and autophosphorylate on multiple tyrosine residues in *trans*, which leads to recruitment of different adaptors and enzymes, and activation of multiple coordinated signaling pathways. Two tyrosine residues on TrkA, Y490 and Y785, are phosphorylated in response to NGF and serve as major docking sites for binding of adaptor proteins and enzymes. Specifically, Y490 and Y785 primarily recruit Shc and PLC-γ, respectively [216]. Phosphorylated tyrosine residues located in the catalytic loop of the tyrosine kinase domain (Y679, Y683, Y684) or in the carboxyl-terminal region (Y794), can also recruit signaling molecules such as SH2B, APS, and Grb2 [217, 218].

1.6.4 PC12 Cells

The adrenal glands are adjacent to the kidneys, and are composed of two distinct layers, the adrenal cortex and the adrenal medulla. The adrenal medulla, which comprises the central 10-20% of the adrenal gland, is derived from neuroectodermal cells of the sympathetic ganglia, and secretes the catecholamines epinephrine and norepinephrine in response to sympathetic neural stimulation to the medullae [219]. Cells of the adrenal medulla are known as pheochromocytes or chromaffin cells [220]. There have been a variety of continuous cell lines derived from the adrenal medulla, with the archetypal being the rat PC12 cell line, which was first established from a transplantable rat adrenal pheochromocytoma [221].

PC12 cells are a useful system for studying how hormones, growth factors, and neurotrophins initiate multiple signaling pathways that converge on specific cellular targets to execute complex processes, such as survival, proliferation, and differentiation. Specifically, PC12 cells provide an example of how one signaling cascade, namely the Raf/MEK/ERK pathway, can promote distinct biological outcomes (Figure 1.7). For example, EGF and NGF use the Raf/MEK/ERK pathway to elicit proliferation and differentiation of PC12 cells, respectively. Both acidic and basic FGF can reproduce the entire spectrum of PC12 cell responses previously shown to be elicited by NGF [222]. The duration of ERK signaling by EGF *versus* NGF may explain these very distinct responses. EGF, acting through the EGF receptor, induces a transient activation of ERK which results in proliferation [223, 224]. In contrast, NGF acting through the TrkA receptor, induces both transient and prolonged phosphorylation of ERK, with prolonged activation required for

differentiation [225, 226]. It is possible that the distinct biological consequences of transient versus prolonged activation of ERK reflect temporal differences in nuclear accumulation of ERK, and subsequent activation of transcription factors and changes in gene expression [224]. Alternatively, scaffold or adaptor proteins within the cell may promote differential ERK substrate selection by localizing ERK to areas other than the nucleus, such as endosomes or the cytoplasm.

Promotion of differentiation by NGF appears to progress through a cascade involving, but not limited to, the TrkA receptor, the small GTPases Ras·GTP and Rap1·GTP, c-Raf-1 and B-Raf, MEK1/2, and ERK1/2 [227]. Thus, in addition to the time course of ERK activation in PC12 cells, the combination of signaling components activated by NGF *versus* EGF may result in different outcomes. In fact, scaffold proteins exist that may promote formation of stable complexes that compartmentalize and regulate different responses. For example, both the scaffold proteins KSR and DYRK1A promote neurite outgrowth in PC12 cells by binding to and coordinating MEK/ERK and Ras/B-Raf/MEK1, respectively [187, 228]. In Chapter 4, I describe several lines of experimentation that support the hypothesis that RGS12 is a novel MAPK scaffold that facilitates the assembly and regulation of the Ras-Raf-MEK cascade in PC12 cells. RGS12 appears to selectively regulate NGF- but neither EGF- nor bFGF-mediated signaling in PC12 cells, and this may be due, at least in part, to a direct interaction with the TrkA receptor.

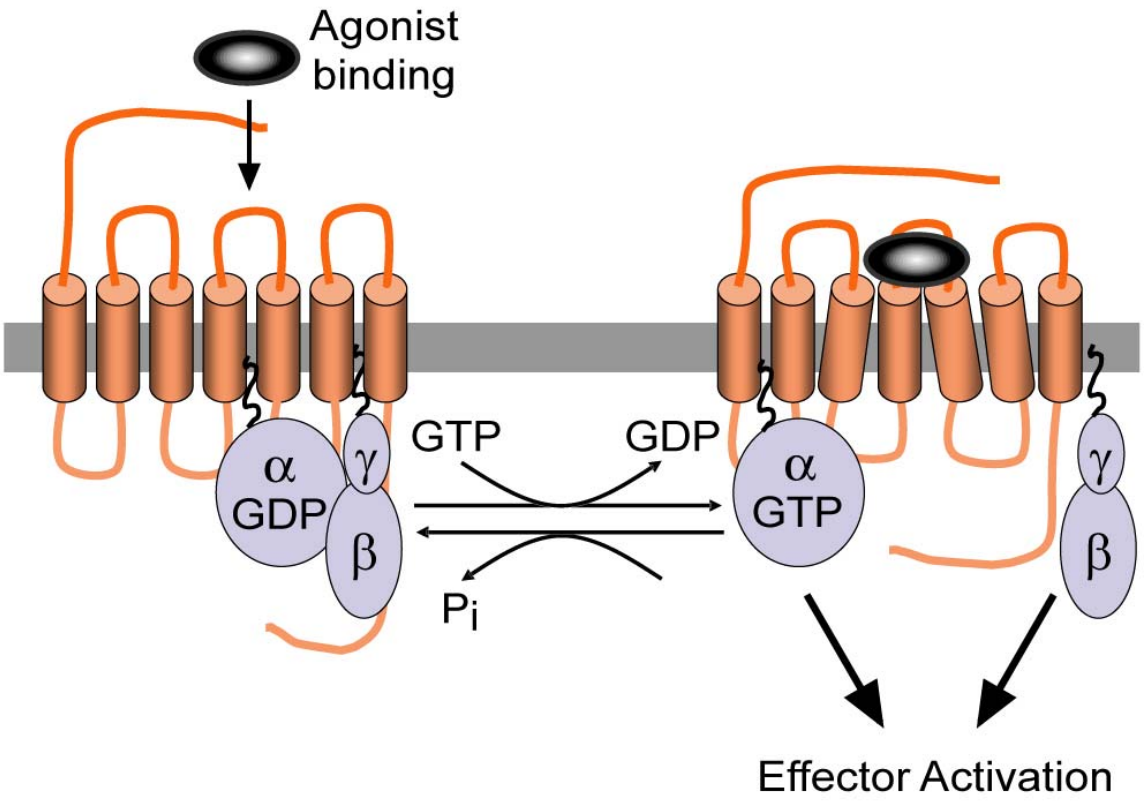

Figure 1.1. Standard model of the GDP/GTP cycle governing activation of heterotrimeric G protein-coupled receptor (GPCR) signaling pathways. In the absence of ligand, the Gα subunit is GDP-bound and closely associated with the Gβγ heterodimer. This Gα·GDP/Gβγ heterotrimer interacts with the cytosolic loops of a seven-transmembrane-domain G protein-coupled receptor (GPCR). Gβγ facilitates the coupling of Gα to receptor and also acts as a guanine nucleotide dissociation inhibitor for Gα·GDP, slowing the spontaneous exchange of GDP for GTP. Agonist-bound GPCRs act as guanine nucleotide exchange factors by inducing a conformational change in the Gα subunit, allowing it to exchange GTP for GDP. Gβγ dissociates from Gα·GTP, and both Gα·GTP and Gβγ are competent to signal to their respective effectors. The cycle returns to the basal state when Gα hydrolyzes the gamma-phosphate moiety of GTP.

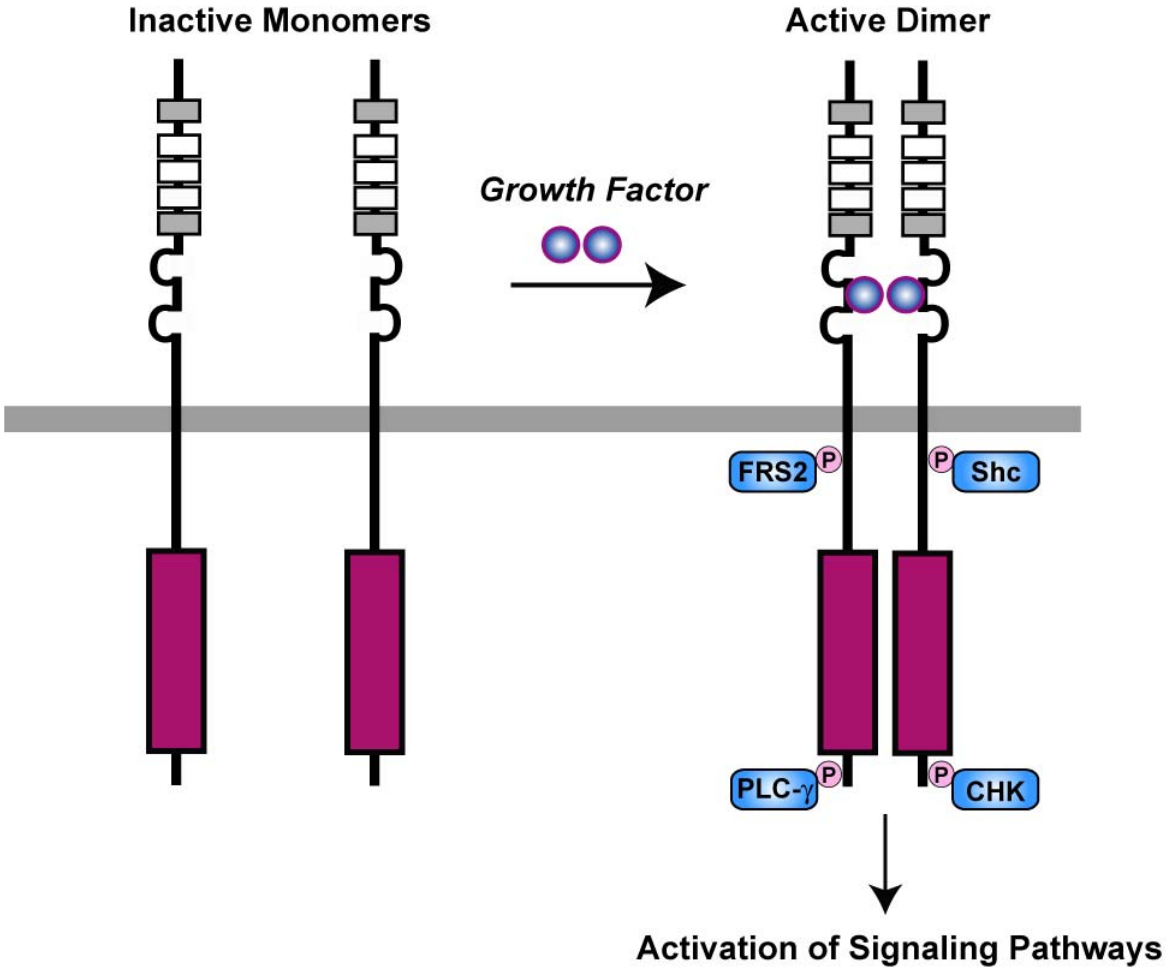

Figure 1.2. Receptor tyrosine kinase activation (adapted from [213]). In the absence of ligand, all RTKs (except for the insulin receptor) are monomeric. Ligand binding to the extracellular region leads to dimerization of monomeric receptors, resulting in autophosphorylation of specific tyrosine residues in the intracellular portion. Generally, tyrosine autophosphorylation either stimulates the intrinsic kinase activity of the receptor, or generates recruitment sites for downstream signaling adaptors such as FRS2, Shc, PLC-γ, or CHK. These interactions trigger the activation of various signaling pathways, which result in cellular processes such as survival, cell proliferation, and differentiation.

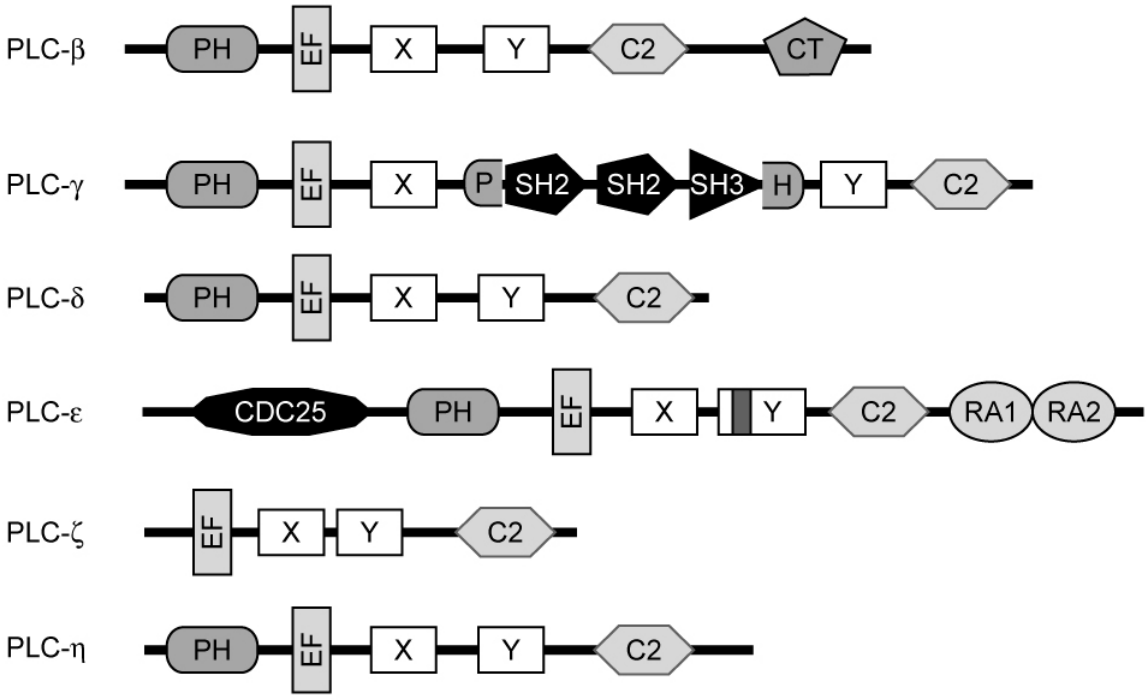

Figure 1.3. Domain architecture of mammalian phospholipase C (PLC) family members. The six classes of PLC isoforms are distinguished by differing structural domains. The hallmarks of all PLC family members, except for PLC-ζ, are an N-terminal PH domain, and EF, X, Y and C2 motifs forming the catalytic core for phosphoinositide hydrolysis. Domain identification: PH: pleckstrin homology domain; EF: EF-hand domain; X and Y: XY box/catalytic TIM barrel; C2: Ca^{2+}/lipid-binding domain; CT: C-terminal domain; SH2: Src-homology-2 domain; SH3: Src-homology-3 domain; CDC25: cell division cycle protein 25-like domain; RA: Ras-associating domain; shaded area in PLC-ε Y box: unique 60-70 amino acid insert in the Y domain required for Rho activation of PLC activity.

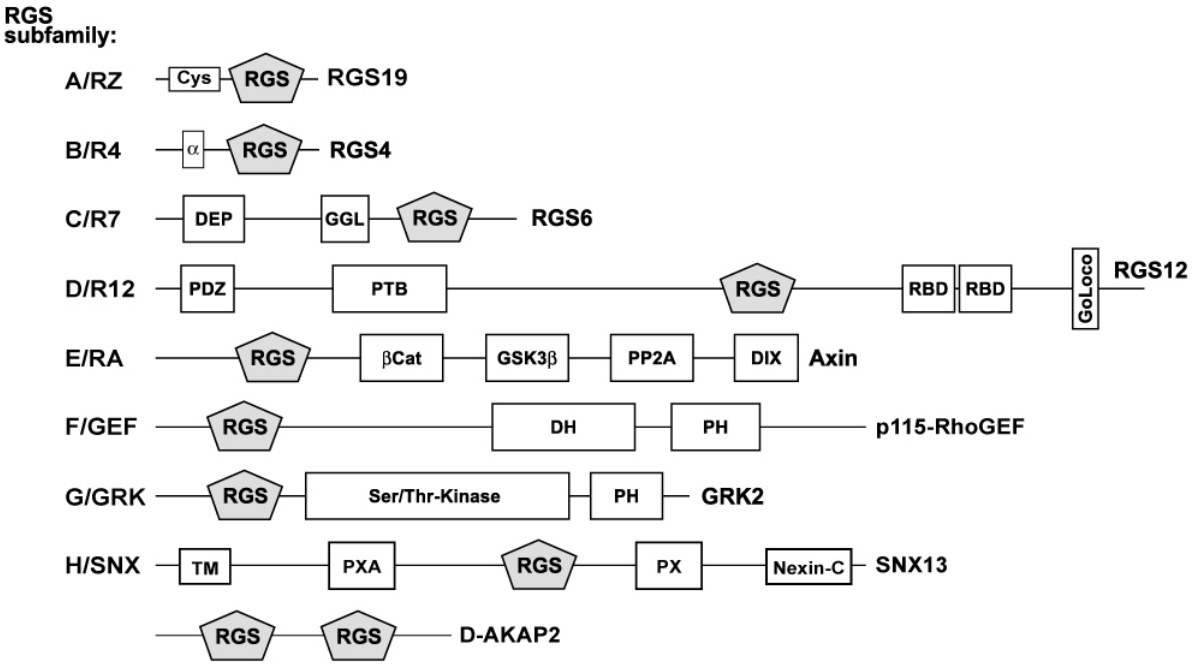

Figure 1.4. Schematic of the varied multidomain architectures of RGS family proteins. RGS subfamily nomenclature follows that first established by Wilkie and Ross [265]. Abbreviations used are: Cys: cysteine-rich region; RGS: regulator of G-protein signaling domain; α: amphipathic helix; DEP: Dishevelled/EGL-10/Pleckstrin homology domain; GGL: Gγ-like domain; PDZ: PSD-95/Dlg/ZO-1 homology domain; PTB: phosphotyrosine-binding domain; RBD: Ras-binding domain; GoLoco: $G\alpha_{i/o}$-Loco interacting motif; βCat: β-catenin binding domain; GSK3β: glycogen synthase kinase-3β binding domain; PP2A: phosphatase PP2A binding domain; DIX: domain present in Dishevelled and Axin; DH: Dbl-homology domain; PH: Pleckstrin homology domain; Ser/Thr-kinase: serine-threonine kinase domain; TM: transmembrane domain; PXA: phosphatidylinositol-associated domain; PX: phosphatidylinositol-binding domain; Nexin-C: Sorting nexin C-terminal domain.

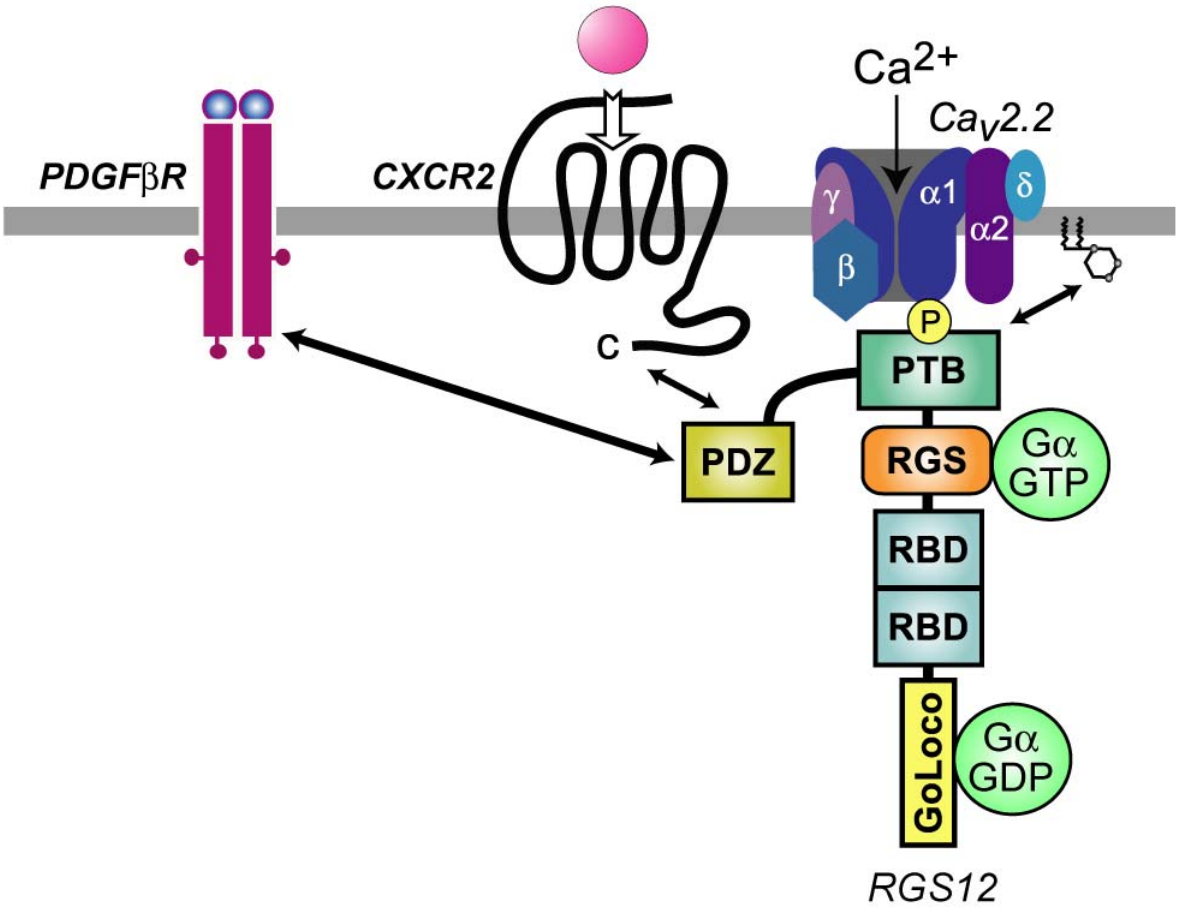

Figure 1.5. The multidomain RGS protein RGS12 and its known interactors. The PDZ domain binds the C-termini of GPCRs including that of the IL-8 receptor CXCR2. The PDZ domain of RGS12 has also been shown to interact with the PDGFβR, and the PDZ and PTB domain N-terminus regulates PDGFβR localization and signaling to ERK. The PTB domain binds to the synprint ("synaptic protein interaction") region of the Cav2.2 channel; this interaction is dependent on neurotransmitter-mediated phosphorylation of the channel by Src tyrosine kinase. The PTB domain also preferentially binds to monophosphorylated phosphatidylinositides such as PI(3)P. The RGS-box acts as a GAP for $G\alpha_i$-family $G\alpha$ subunits. The GoLoco domain prevents guanine nucleotide exchange by binding GDP-bound $G\alpha_i$.

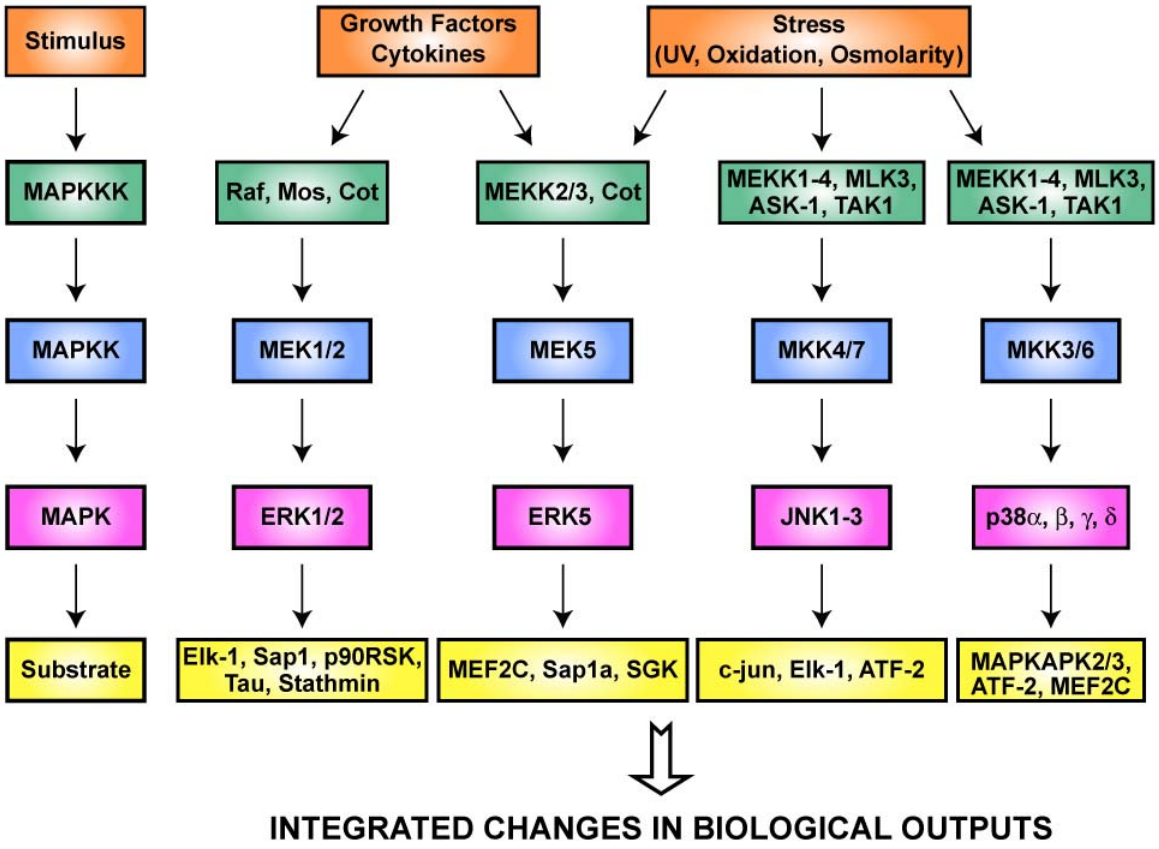

Figure 1.6. Mammalian MAPK signaling pathways (adapted from [169]). The mammalian MAPK family consists of four major subgroups: ERK1/2, ERK5, JNK, and p38 MAPKs, which are activated by phosphorylation at tyrosine and threonine residues by their specific MAPKKs. MAPKKs are activated by phosphorylation at threonine and serine residues by their specific MAPKKKs. MAPKKKs are activated by a variety of mechanisms in response to stimulation by growth factors, cytokines and stresses. Activated MAPKs phosphorylate various protein substrates including transcription factors (e.g., Elk-1 and c-jun), kinases (e.g., p90RSK and SGK), and cytoskeletal proteins (e.g., Tau and Stathmin), which is critical for appropriate cellular responses to extracellular stimuli.

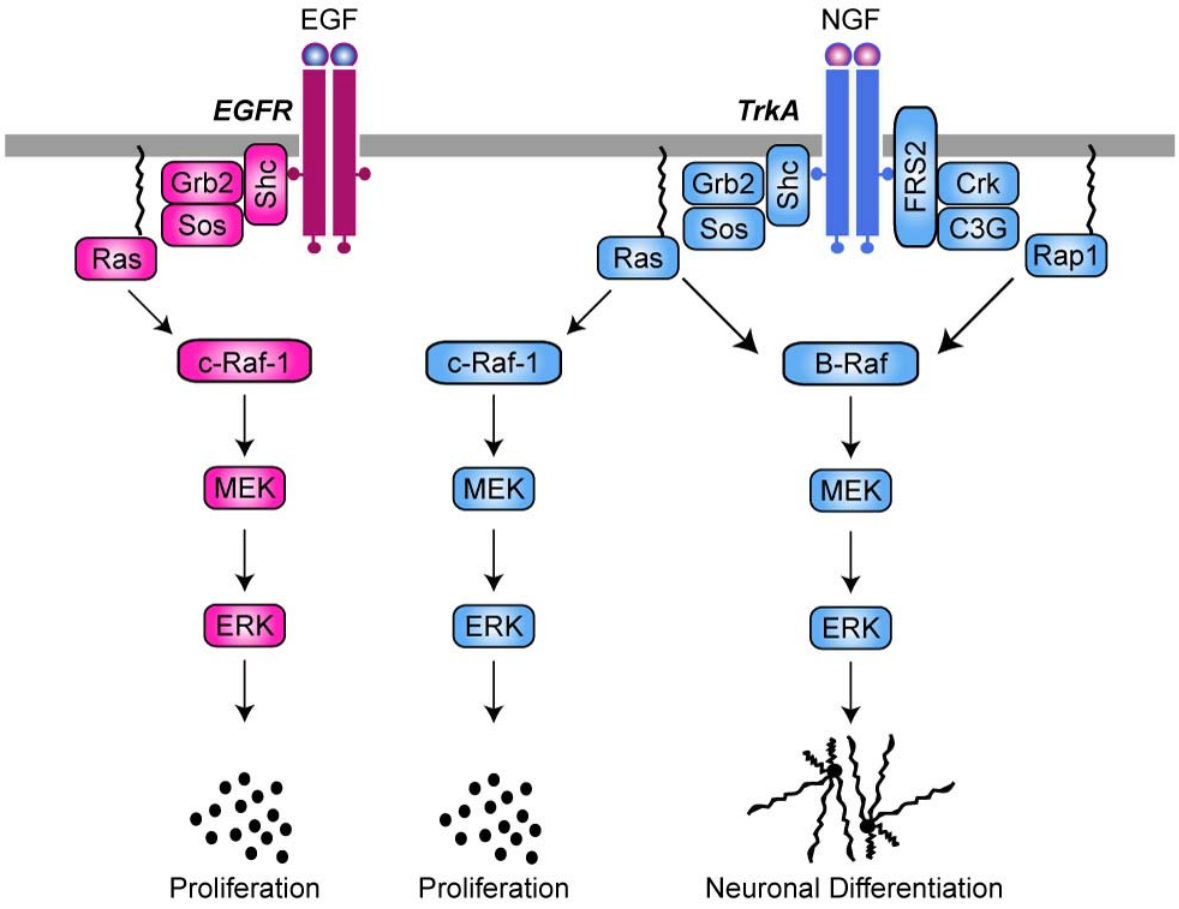

Figure 1.7. PC12 signaling pathways involving the MAPK cascade (adapted from [306]). Different outcomes are activated by EGF and NGF, but both growth factors employ similar signal transduction machinery. EGFR activation recruits Shc, which acts as a phophorylation-dependent scaffold for the adaptor Grb2 and its binding parner Sos. Sos, a Ras-GEF, activates Ras, which in turn activates c-Raf-1, MEK, and ERK transiently, resulting in cell proliferation. The NGF receptor TrkA also activates ERK transiently through a similar mechanism. In addition, sustained activation of ERK stemming from NGF treatment appears to involve activation of both Ras and Rap1, and B-Raf. The small GTPase Rap1 is recruited to the TrkA receptor by the phosphorylation-dependent scaffold FRS2, which subsequently recruites Crk/C3G. C3G, a Rap-GEF, activates Rap1, which activates B-Raf, MEK, and ERK. Prolonged ERK activation by Ras and Rap1 results in neuronal differentiation.

TABLE 1.1. Selected mammalian scaffold proteins (adapted from [180])

Name	Protein Interactions	Scaffold Function
MP1	MEK1, ERK1, p14	Activation of ERK at late endosomes
JIP-1	Akt, ApoER2, APP, JNK, KLC, Megalin, MKK7, MLK, MPK7, p190RhoGEF, Ras-GRF, Tiam1	JNK activation Kinesin cargo
OSM	Actin, Rac, MEKK3, MKK3	Activation of p38
KSR	Raf, MEK1/2, ERK1/2, 14-3-3, C-TAK1, Gβγ	Ras activation of ERK
β-arrestin-1,2	c-Raf-1, MEK1, ERK2	GPCR activation of ERK
β-arrestin-2	ASK1, MKK4, JNK3	GPCR activation of JNK3
DYRK1A	Ras, B-Raf, MEK1	Ras activation of ERK

CHAPTER 2

$G\alpha_{12/13}$- AND RHO-DEPENDENT ACTIVATION OF PHOSPHOLIPASE C-ε BY LYSOPHOSPHATIDIC ACID AND THROMBIN RECEPTORS

2.1 Abstract

Since phospholipase C epsilon (PLC-ε) is activated by $G\alpha_{12/13}$ and Rho family GTPases, we investigated whether these G-proteins contribute to the increased inositol lipid hydrolysis observed in COS-7 cells following activation of certain G protein-coupled receptors. Stimulation of inositol lipid hydrolysis by endogenous lysophosphatidic acid (LPA) or thrombin receptors was markedly enhanced by the expression of PLC-ε. Expression of the LPA_1 or PAR1 receptor increased inositol phosphate production in response to LPA or SFLLRN, respectively, and these agonist-stimulated responses were markedly enhanced by co-expression of PLC-ε. Both LPA_1 and PAR1 receptor-mediated activation of PLC-ε were inhibited by co-expression of the RGS domain of p115RhoGEF, a GTPase-activating protein for $G\alpha_{12/13}$, but not by expression of the RGS domain of GRK2, which inhibits $G\alpha_q$ signaling. In contrast, activation of the Gq-coupled M1 muscarinic or $P2Y_2$ purinergic receptor was neither enhanced by co-expression with PLC-ε nor inhibited by the RGS domain of p115RhoGEF, but was blocked by expression of the RGS domain of GRK2. Expression of the Rho inhibitor C3 botulinum toxin did not affect LPA- or SFLLRN-stimulated inositol lipid hydrolysis in the absence of PLC-ε, but completely prevented the PLC-ε-dependent increase in inositol phosphate accumulation.

Similarly, C3 toxin blocked the PLC-ε-dependent stimulatory effects of the LPA_1, LPA_2, LPA_3 or PAR1 receptor, but had no effect on the agonist-promoted inositol phosphate response of the M1 or $P2Y_2$ receptor. Moreover, PLC-ε-dependent stimulation of inositol phosphate accumulation by activation of the EGF receptor, which involves Ras- but not Rho-mediated activation of the phospholipase was unaffected by C3 toxin. These studies illustrate that specific LPA and thrombin receptors promote inositol lipid signaling via activation of $G\alpha_{12/13}$ and Rho.

2.2 Introduction

Many extracellular hormones, neurotransmitters, and growth factors exert their physiological effects by mechanisms that in part involve phospholipase C-catalyzed breakdown of phosphatidylinositol (4,5)P_2 into the Ca^{2+}-mobilizing second messenger inositol (1,4,5)P_3 and the protein kinase C-activating second messenger diacylglycerol [31, 229]. For example, extracellular stimuli that activate members of the large family of seven transmembrane-spanning heterotrimeric G protein-coupled receptors (GPCRs) activate PLC-β isozymes by release of α-subunits of the Gq family of G proteins [33-35] or by release of Gβγ dimers from activated Gi [37-39]. In contrast, PLC-γ enzymes are activated by tyrosine phosphorylation following activation of receptor and nonreceptor tyrosine kinases [40, 42].

PLC-ε, which possesses Ras-associating (RA) domains at its carboxy terminus, was initially identified in *C. elegans* as a Ras-binding protein [48]. Mammalian PLC-ε is activated by coexpression with Ras [49, 51] as well as by activators of GEFs that in turn promote formation of active Rap or Ras [56, 65, 230]. For example, Gs-coupled GPCRs promote PLC-ε-dependent inositol lipid signaling

through activation of the cyclic AMP-activated GEF, EPAC, which in turn activates Rap1A [56]. Initial studies of mammalian PLC-ε revealed activation by $G\alpha_{12}$ and $G\alpha_{13}$ but not by $G\alpha_q$ [50, 53], and $G\beta\gamma$ also has been shown to activate this PLC isozyme [53].

Coexpression of Rho family GTPases with PLC-ε results in marked stimulation of inositol lipid hydrolysis [52]. PLC-ε mutants that lack functional RA domains retain activation by Rho, indicating that Rho family GTPases regulate this PLC isozyme by a mechanism distinct from that utilized by Ras and Rap. Observation of GTP-dependent activation of purified PLC-ε by purified RhoA illustrates that the stimulatory action of Rho in inositol lipid signaling is direct [61]. GEFs for Rho are downstream effectors of $G\alpha_{12/13}$ [231-235]. Thus, observation of Rho-dependent activation of PLC-ε suggests that GPCRs that activate $G\alpha_{12/13}$ promote inositol lipid signaling through activation of Rho.

With the goal of establishing whether receptor-mediated regulation of inositol lipid signaling occurs through a mechanism involving $G\alpha_{12/13}$, Rho, and PLC-ε, we have studied regulation of PLC-ε-promoted inositol lipid hydrolysis by endogenous and recombinant GPCRs expressed in COS-7 cells. The results of these studies are consistent with the idea that $G\alpha_{12/13}$- and Rho-dependent activation of PLC-ε occurs downstream of both LPA- and thrombin-activated receptors, and that the regulation of PLC-ε by $G\alpha_{12/13}$ occurs at least in part through activation of Rho.

2.3 Materials and Methods

Materials. Expression vectors (in pcDNA3.1) for the human M1 muscarinic cholinergic, LPA_1, LPA_2, and LPA_3 receptors were purchased from the UMR cDNA

Resource Center (Rolla, MO). An expression vector encoding the human $P2Y_2$ receptor was described previously [236]. The plasmid encoding wildtype EGF receptor is described in [237]. A pCMV-Script vector encoding FLAG-tagged rat PLC-ε was generously provided by Grant Kelley, SUNY Syracuse. An expression vector for C3 botulinum toxin was obtained from Channing Der, University of North Carolina. cDNA encoding the first 240 amino-acids of human p115RhoGEF was subcloned in-frame with an N-terminal tandem hemagglutinin (HA)-epitope tag into a modified pcDNA3.1 vector [238]. cDNA encoding amino-acids 45-178 of bovine GRK2 (designated GRK2-RGS) in frame with an N-terminal HA epitope tag in pcDNA3 was kindly provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). LPA (1-oleoyl-sn-glycerol 3-phosphate sodium salt) was purchased from Sigma-Aldrich (St. Louis, MO), and dissolved in water containing 1.0% fatty acid-free BSA. The PAR1 receptor agonist peptide, SFLLRN, was synthesized as the carboxyl amide and purified by reverse phase high pressure liquid chromatography (UNC Peptide Facility, Chapel Hill, NC). UTP, carbachol, and epidermal growth factor (EGF) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were from sources previously noted [52, 53, 61].

Cell Culture and Transfection of COS-7 Cells. COS-7 cells were plated in 12-well or 96-well culture dishes and maintained in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 10% CO_2/90% air atmosphere. The indicated DNA expression vectors were transfected into COS-7 cells using Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN) at a ratio of 3:1 (Fugene:DNA) following the

manufacturer's protocol. Empty vector DNA was used as necessary to maintain a constant total amount of DNA per well.

Measurement of [^{3}H]Inositol Phosphates. Approximately 24 h after transfection, the medium was replaced with inositol- and serum-free DMEM containing 1 μCi/well [*myo*-^{3}H]inositol (American Radiolabeled Chemicals, St. Louis, MO). Phospholipase C activity was quantified twelve h after labeling by incubation in inositol-free DMEM containing 10 mM LiCl either in the absence of a receptor agonist or in the presence of 10 μM LPA, 50 μM SFFLRN, 100 μM carbachol, 100 μM UTP, or 100 ng/mL EGF. The reaction was stopped after 30-60 min by aspiration of the medium and addition of ice-cold 50 mM formic acid. Following neutralization with 150 mM ammonium hydroxide, the accumulation of [^{3}H]inositol phosphates was quantified by Dowex chromatography as previously described [53].

Western Blotting. COS-7 cells were seeded at 60,000 cells per well in a 12 well plate and transfected 24 hours later with pcDNA3, myc-PLC-ε, or myc-PLC-ε with 0.3 ng, 3 ng, or 30 ng C3 toxin using FuGENE 6 transfection reagent as described previously. Forty-eight hours post-transfection the cells were lysed on ice in 200 μL lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 1% Triton X-100, containing protease inhibitors). COS-7 cell lysates were sonicated in an ice water bath for 5 min and then centrifuged at 13,000 x *g* for 20 min at 4 °C. The supernatant was removed, mixed 1:1 with 5x Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE and transfer to nitrocellulose. Western blotting was performed using anti-myc clone 9E10 (Roche Applied Science, Indianapolis, IN) and anti-α-tubulin clone B-5-1-2 (Sigma-Aldrich, St. Louis, MO) primary antibodies, a

secondary anti-mouse IgG antibody conjugated to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ), and enhanced chemiluminescence (Denville Scientific Inc., Metuchen, NJ).

2.4 Results

PLC-ε-dependent promotion of inositol lipid signaling by endogenous LPA and thrombin receptors.

We recently reported that Rho GTPases directly activate PLC-ε [52, 61]. To begin to address potential GPCR-mediated regulation of this PLC isozyme through a Rho-dependent signaling pathway, we screened COS-7 cells for the functional presence of GPCRs that exhibit PLC-ε-dependent activation of inositol lipid signaling. Incubation of cells with histamine, prostaglandin E_2, carbachol, adenosine, norepinephrine, somatostatin, or a combination of P2Y receptor agonists (UTP, 2MeSADP, ATP, UDP, and UDP-glucose) all failed to elevate inositol phosphates in a PLC-ε-dependent manner (data not shown). In contrast, as was recently reported by Kelley *et al*. [55], incubation of PLC-ε-expressing cells with LPA (10 μM) or with the agonist peptide SFLLRN (50 μM) resulted in an increase in inositol phosphate levels compared to the very low responses observed to LPA or SFLLRN in control cells (Figure 2.1).

PLC-ε-dependent stimulation of inositol phosphate accumulation by molecularly defined LPA and thrombin receptors.

Since LPA is the cognate agonist for at least three different GPCRs, we individually expressed the LPA_1, LPA_2, or LPA_3 receptors in COS-7 cells with the goal of determining the extent to which these signaling proteins exhibit PLC-ε-

dependence in their action. LPA-stimulated inositol phosphate accumulation was reproducibly increased in LPA_1 receptor-expressing cells compared to empty vector-transfected cells, and was enhanced by ~10 fold in LPA_2 or LPA_3 receptor-expressing cells (Figure 2.2). Coexpression of the LPA_1 receptor with PLC-ε markedly enhanced the inositol phosphate response to LPA. Coexpression with PLC-ε also increased the LPA-promoted inositol lipid signaling response in LPA_2 or LPA_3 receptor-expressing cells, although the enhancement observed with PLC-ε was both variable and of much smaller magnitude, *i.e.* from no effect to approximately 2-fold increases in response, than that observed with the LPA_1 receptor (Figure 2.2). To determine whether the lack of effect of the LPA_2 and LPA_3 receptors on PLC-ε activation was due to a depletion of phosphatidylinositol (4,5)P_2, we examined the ability of other $G\alpha_q$-coupled receptors to promote inositol lipid signaling. $G\alpha_q$-coupled receptors such as $P2Y_4$ and $P2Y_{11}$ produced much larger responses than the LPA_2 and LPA_3 receptors (data not shown), suggesting that the system is not saturated. Additionally, selective inhibition of the large $G\alpha_q$-stimulated response of the LPA_2 and LPA_3 receptors by the RGS domain of GRK2 [93, 238] did not uncover a PLC-ε-dependent response by these receptors (data not shown). Therefore, the LPA_1 receptor promotes signaling responses in COS-7 cells that are markedly dependent on the presence of PLC-ε, whereas the inositol lipid signaling response in LPA_2 or LPA_3 receptor-expressing cells is less affected by expression of PLC-ε.

Given the effects of PLC-ε on agonist-promoted responses of the molecularly defined LPA receptors, we also tested the activities of the PAR1 receptor, another

GPCR known to couple to $G\alpha_{12/13}$- and Rho-regulated pathways, and two receptors, the M1 muscarinic cholinergic receptor and the nucleotide-activated $P2Y_2$ receptor, known to primarily activate Gq-regulated signaling pathways. Expression of the PAR1 receptor in the presence of PLC-ε resulted in a marked increase in SFLLRN-promoted inositol phosphate accumulation (Figure 2.3A). In contrast, expression of the M1 muscarinic receptor (Figure 2.3B) or $P2Y_2$ receptor (Figure 2.3C) resulted in a marked increase in agonist-promoted inositol lipid response that was not further augmented by coexpression with PLC-ε. The large increase in basal [^{3}H]inositol phosphate accumulation illustrated in Figure 2.3C is due to basal release of ATP and UTP from COS-7 cells, which in turn activates the expressed $P2Y_2$ receptor [239-241].

RGS protein-selective inhibition of PLC-ε-dependent signaling by the LPA_1 and PAR1 receptors.

Activation of both LPA [242, 243] and thrombin [243, 244] receptors has been reported previously to result in activation of $G\alpha_{12/13}$- and Rho-promoted signaling pathways. To address the potential roles of $G\alpha_{12/13}$ as well as $G\alpha_q$ in the responses to these GPCRs, we applied the RGS domain of p115RhoGEF (p115-RGS), which acts as a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$ [103, 238], and GRK2-RGS, which is known to bind selectively to $G\alpha_q$ and inhibit $G\alpha_q$ signaling [93, 238]. Co-expression of p115-RGS with the LPA_1 receptor or PAR1 receptor had no effect on agonist-stimulated inositol phosphate accumulation in the absence of PLC-ε (Figures 2.4A and 2.4B). However, the elevated agonist-promoted inositol phosphate response observed in cells coexpressing these receptors with PLC-ε was essentially

completely inhibited by co-expression with p115-RGS. In contrast, p115-RGS had no effect on agonist-stimulated inositol phosphate responses promoted by the M1 muscarinic receptor (Figure 2.4C) or $P2Y_2$ receptor (data not shown) in the absence or presence of PLC-ε. Conversely, expression of $G\alpha_q$-binding GRK2-RGS significantly inhibited M1 (Figure 2.4) and $P2Y_2$ (data not shown) receptor-promoted signaling but had no effect on the inositol lipid signaling response to the LPA_1 (Figure 2.4A) or PAR1 (Figure 2.4B) receptor either in the absence or presence of expression of PLC-ε.

C3 toxin inhibits $G\alpha_{12/13}$-, LPA-, and thrombin-promoted activation of PLC-ε.

The data presented thus far implicate $G\alpha_{12/13}$ in the mechanism of activation of PLC-ε by LPA and thrombin receptors. In contrast, the M1 muscarinic and $P2Y_2$ receptors apparently regulate inositol lipid signaling by mechanisms that involve neither $G\alpha_{12/13}$ nor PLC-ε. To address the potential role of Rho in GPCR-promoted activation of PLC-ε, we utilized C3 toxin to inactivate Rho. Expression of GTPase-deficient mutants of $G\alpha_{12}$ ($G\alpha_{12}$Q229L) or $G\alpha_{13}$ ($G\alpha_{13}$Q226L) had no effect on accumulation of inositol phosphates in control COS-7 cells (Figure 2.5A). However, co-expression of these $G\alpha_{12}$ or $G\alpha_{13}$ mutants with PLC-ε resulted in a marked increase in [^{3}H]inositol phosphate accumulation compared to [^{3}H]inositol phosphate levels in cells transfected with PLC-ε alone. The capacity of $G\alpha_{12}$ and $G\alpha_{13}$ to activate PLC-ε was lost with transfection of increasing amounts of an expression vector for C3 botulinum toxin (Figure 2.5B). Loss of responsiveness was not due to a nonspecific effect on inositol lipid signaling since the capacity of GTPase-deficient $G\alpha_q$ ($G\alpha_q$Q209L) to promote phosphoinositide hydrolysis was largely retained. To

determine whether the reduction of PLC-ε activity was due to a decrease in the expression level of PLC-ε, we immunoblotted cell lysates expressing PLC-ε alone, or PLC-ε in the presence of increasing amounts of C3 toxin. The expression of PLC-ε in the presence of 0.3 ng, 3 ng, or 30 ng C3 toxin was not significantly altered (Figure 2.5C), suggesting that the lack of activity of PLC-ε in the presence of C3 toxin is not due to the inhibition of PLC-ε expression. Thus, Rho is downstream of $G\alpha_{12}$ and $G\alpha_{13}$ in the PLC-ε-dependent signaling response measured under these conditions.

The potential contribution of Rho to the PLC-ε-dependent stimulation of inositol lipid signaling by the endogenous LPA and thrombin receptors of COS-7 cells also was examined by measuring inositol phosphate accumulation following expression of C3 toxin. Whereas the capacity of LPA (Figure 2.6A) or SFLLRN (Figure 2.6B) to stimulate phosphoinositide hydrolysis in the absence of PLC-ε was not affected by transient expression of C3 toxin, PLC-ε-dependent elevation of inositol phosphates in response to both agonists was entirely lost in C3 toxin-expressing cells in a concentration dependent manner (Figures 2.6A and 2.6B). These results suggest that endogenous LPA and thrombin receptors of COS-7 cells activate a Rho GTPase(s), which in turn activates PLC-ε.

C3 toxin-mediated inhibition of PLC-ε-dependent inositol lipid signaling by molecularly defined LPA and thrombin receptors.

To determine whether Rho also is involved in PLC-ε-dependent inositol lipid signaling by molecularly defined LPA receptors and the PAR1 receptor, C3 toxin was coexpressed with each of these receptors in the absence or presence of PLC-ε.

The increased response to LPA conferred by LPA_1 receptor expression was not affected by C3 toxin (data not shown). In contrast, the large PLC-ε-dependent response to LPA observed in LPA_1 receptor-expressing cells was completely inhibited in a concentration dependent manner by coexpression of C3 toxin (Figure 2.7A). C3 toxin also had no effect on the LPA-promoted inositol phosphate response in COS-7 cells expressing either the LPA_2 or LPA_3 receptor alone (data not shown), but blocked completely and in a concentration dependent manner the PLC-ε-dependent effects of LPA mediated through these two receptors (Figure 2.7B and 2.7C). Expression of C3 toxin also resulted in loss of PLC-ε-dependent but not PLC-ε-independent inositol lipid signaling of the PAR1 receptor (Figure 2.8A). In contrast, agonist-stimulated inositol phosphate accumulation promoted by the M1 muscarinic cholinergic receptor (Figure 2.8B) or the $P2Y_2$ purinergic receptor (Figure 2.8C) was neither enhanced by expression of PLC-ε nor inhibited by coexpression of C3 toxin.

Although expression of C3 toxin did not affect expression of PLC-ε (Figure 2.5C and data not shown), the elevated levels of basal [^{3}H]inositol phosphate accumulation after PLC-ε expression were suppressed by coexpression of C3 toxin, *e.g.* see Figures 2.7 and 2.8. PLC-ε-dependent elevation of [^{3}H]inositol phosphate accumulation also was inhibited by p115-RGS (Figure 2.4A and data not shown), suggesting that a $G\alpha_{12/13}$/Rho-dependent pathway activates expressed PLC-ε in the absence of added receptor agonists. An analogous effect is observed with overexpression of Gq-coupled P2Y receptors (Figure 2.8C) in the absence of application of exogenous agonist due to autocrine release of cognate adenine and uridine nucleotide agonists [239, 241, 245]. Nonetheless, these results do not rule

out the possibility that C3 toxin nonspecifically inhibits activation of PLC-ε by all activators. Thus, we also examined EGF receptor-mediated activation of PLC-ε, which occurs via a $G\alpha_{12/13}$/Rho-independent mechanism involving binding of Ras subfamily GTPases to the carboxyl terminal RA domains of the enzyme [49, 51, 55, 69]. As illustrated in Figure 2.9B, PLC-ε-dependent effects of EGF on [^{3}H]inositol phosphate accumulation were observed in COS-7 cells coexpressing the EGF receptor. The effect of C3 toxin on EGF-promoted activation of PLC-ε was examined in a series of experiments in which PLC-ε-dependent accumulation of [^{3}H]inositol phosphates was quantified in the presence of EGF versus LPA in EGF receptor-expressing cells versus LPA_1 receptor-expressing cells, respectively. Whereas expression of increasing amounts of C3 toxin inhibited LPA_1 receptor-promoted [^{3}H]inositol phosphate accumulation (Figures 2.7A and 2.9A), little if any effect of C3 toxin on PLC-ε dependent stimulatory effects of the EGF receptor were observed (Figure 2.9B).

2.5 Discussion

PLC isozymes contain a highly conserved catalytic core, as well as additional domains that render these isozymes susceptible to different modes of regulation [31]. PLC-ε is activated by Ras and Rho GTPases as well as by subunits of heterotrimeric G proteins including $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_s$, and $G\beta\gamma$ [49-53, 56]. Activation by Ras subfamily GTPases occurs as a consequence of direct interaction of these G proteins with RA domains in the carboxy terminus of PLC-ε [49, 51]. We illustrated recently that Rho binds to undefined sequence in the catalytic core of PLC-ε activating the isozyme through a mechanism that does not require the RA domains

[52, 61]. In contrast, the mechanism(s) whereby PLC-ε is activated by subunits of heterotrimeric G proteins remains largely unclear and may not be direct. Data reported in the current study indicate that activation of PLC-ε by $G\alpha_{12}$ and $G\alpha_{13}$ after expression in COS-7 cells is dependent on functional Rho. Moreover, these results are consistent with the conclusion that activation of PLC-ε by receptors for LPA and thrombin is dependent on activation of both $G\alpha_{12/13}$ and Rho.

The downstream signaling responses promoted by GPCRs through $G\alpha_{12/13}$-dependent mechanisms have been less clearly defined than those dependent on activation of Gα-subunits of the Gs, Gi, and Gq families. Nonetheless, marked morphological and cell proliferative changes are consistently observed with introduction of GTPase-deficient mutants of $G\alpha_{12}$ or $G\alpha_{13}$ in various cell types [246, 247], and a variety of effectors are stimulated downstream of activation of $G\alpha_{12/13}$ [243, 246]. Some of the cellular responses promoted by $G\alpha_{12/13}$ are mimicked by activated Rho, and activation of Rho occurs in many if not all cells in which $G\alpha_{12}$ or $G\alpha_{13}$ is activated [247]. A large family of RhoGEFs [231-235], *e.g.* p115RhoGEF, leukemia-associated RhoGEF (LARG), PDZ-RhoGEF, and Lbc-RhoGEF, are among the best studied of the effector proteins directly regulated by $G\alpha_{12/13}$. Moreover, the observation that certain $G\alpha_{12/13}$-coupled GPCRs produce cellular effects that involve Rho [247] or RhoGEFs [248] suggests that the putative Rho-activated PLC-ε signaling pathway implied from our previous studies is logically extended to GPCRs that activate Rho GTPases through activation of $G\alpha_{12/13}$ [246, 247, 249, 250].

The recent observation by Kelley and coworkers [55] of activation of PLC-ε by natively expressed LPA and PAR receptors of COS-7 cells was confirmed by the results reported here. Whereas the inositol lipid signaling response of these GPCRs was not affected by C3 toxin in the absence of PLC-ε expression, the complete inhibition of PLC-ε-dependent signaling from these receptors by C3 toxin is consistent with the conclusion that LPA and thrombin receptor-promoted activation of PLC-ε occurs through activation of Rho. Previous studies of the three subtypes of LPA receptors have suggested that these GPCRs couple to multiple G proteins [242, 243]. Expression of the LPA_1, LPA_2, or LPA_3 receptors all resulted in an enhanced inositol phosphate response to LPA in COS-7 cells. The large increase in LPA-stimulated response observed when the LPA_1 receptor was coexpressed with PLC-ε and the inhibition of this augmented response by p115-RGS or C3 toxin are consistent with the known coupling of this GPCR to $G\alpha_{12/13}$ [242, 243]. Moreover, these results are consistent with the conclusion that the LPA_1 receptor activates PLC-ε through $G\alpha_{12/13}$-promoted activation of Rho. Observation of a large PLC-ε-dependent signaling response with expression of the PAR1 receptor also was consistent with the known coupling of this receptor to $G\alpha_{12/13}$ and Rho signaling pathways [243, 244].

Over fifty RhoGEFs exist [251], and therefore, Rho is activated by many different signaling pathways in addition to those involving $G\alpha_{12/13}$. Indeed, $G\alpha_q$ also promotes activation of Rho through mechanisms that apparently are independent of inositol lipid hydrolysis [247, 252], and $G\alpha_q$-activated RhoGEFs have been proposed to exist [231, 252, 253]. Thus, GPCRs potentially regulate PLC-ε through $G\alpha_q$-

dependent signaling pathways although this apparently does not occur in COS-7 cells with the M1 muscarinic or $P2Y_2$ purinergic receptors. That is, whereas large increases in agonist-stimulated inositol lipid hydrolysis were observed after expression of the Gq-coupled M1 muscarinic or $P2Y_2$ receptors, no activation of PLC-ε by these two receptors was observed. Perhaps Gq-regulated RhoGEFs are not expressed in COS-7 cells or lack cellular localization with the LPA and thrombin receptors and PLC-ε.

The RhoGEF responsible for LPA and thrombin receptor-promoted activation of PLC-ε in COS-7 remains to be identified. Indeed the work of Wang *et al.* [248] in PC-3 prostate cancer cells indicates that the LPA and thrombin receptors may activate Rho through distinct RhoGEFs. Whereas the PAR1 receptor utilized the $G\alpha_{12/13}$-activated RhoGEF LARG, LPA receptor-promoted signaling involved another $G\alpha_{12/13}$-activated RhoGEF, PDZRhoGEF. Interestingly, Yamada *et al*. [254] recently reported that the carboxy termini of the LPA_1 and LPA_2 receptors, but not the LPA_3 receptor, interact with the PDZ domain of PDZ-RhoGEF, and that mutation of the carboxy terminus of the LPA_1 and LPA_2 receptors results in loss of capacity of LPA to promote activation of Rho.

The most parsimonious interpretation of the current data is that LPA and thrombin receptors natively expressed in COS-7 cells as well as recombinant LPA_1 and PAR1 receptors overexpressed in these cells all potently activate PLC-ε as a downstream consequence of activation of $G\alpha_{12/13}$ and Rho. LPA and thrombin receptors also are known to activate Gq and Gi, and therefore, also regulate inositol lipid signaling through activation of PLC-β isozymes. The relative contribution of

these different inositol lipid signaling pathways almost certainly will vary widely across cell types, and it will be important to establish the relative contribution of Rho-dependent activation of PLC-ε in the physiological responses to LPA and thrombin.

Independent binding of activated Rho and Ras subfamily GTPases to PLC-ε implies complex physiological regulation of this inositol lipid hydrolyzing isozyme from multiple cell surface receptors. Activation of PLC-ε by EGF receptors was shown to be dependent on intact RA domains and apparently involves GEF-promoted activation of Ras or Rap [55]. Activation of Rap1A and consequential binding of GTP-bound Rap1A to the carboxy-terminal RA domains also accounts for activation of PLC-ε by $G\alpha_s$-coupled GPCRs, which activate adenylyl cyclase, elevate cyclic AMP levels, and therefore, activate a cyclic AMP-regulated GEF for Rap1A [56, 65, 230]. $G\alpha_{12}$, $G\alpha_{13}$, and/or other $G\alpha$ subunits may yet prove to be direct activators of PLC-ε. However, the data presented here indicate that Rho accounts for much if not all of the activation of this isozyme by $G\alpha_{12}$ and $G\alpha_{13}$, and the direct regulation of PLC-ε illustrated to date involves binding of Rho family GTPases in the catalytic core of the isozyme and binding of Ras family GTPases in the RA domains of the carboxy terminus.

The physiological roles played by PLC-ε have yet to be defined. However, mice lacking functional PLC-ε exhibit defects in heart semilunar valve development [255] and marked reduction of the incidence of skin tumors in a chemical carcinogen-induced model [76]. Furthermore, PLC-ε is specifically induced in the developing mouse brain [72], suggesting a role for this phospholipase in neuronal differentiation. Rho-mediated pathways downstream of receptors for LPA, thrombin,

and other extracellular signaling molecules subserve important roles in neuronal growth and differentiation [256]. The current work suggests that it will be important to establish the contribution of Rho-regulated PLC-ε in various aspects of neuronal development and function.

In summary, the current work together with previous studies illustrating that Rho directly activates PLC-ε, are consistent with the idea that PLC-ε is a downstream effector of GPCRs that activate $G\alpha_{12/13}$- and consequentially activate Rho. LPA, thrombin, and other receptors that activate $G\alpha_{12/13}$ also predictably activate Gq. The relative contribution of PLC-ε versus PLC-β isozymes in the physiological action of these $G\alpha_{12/13}$-activating GPCRs will be important to establish. Similarly, RhoA, RhoB, and RhoC robustly activate PLC-ε and it will be important to understand the extent this PLC isozyme is involved in the wide range of cellular processes known to be regulated by these GTPases.

2.6 Acknowledgments

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2.7 Footnotes

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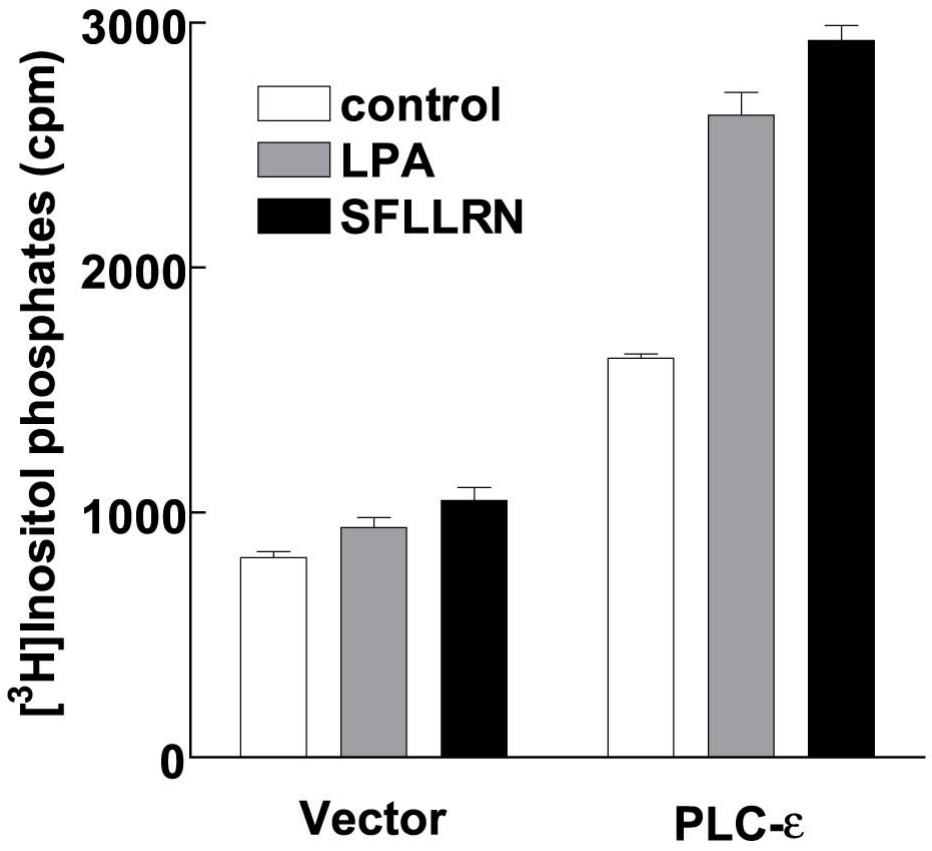

Figure 2.1. PLC-ε-dependent stimulation of inositol phosphate accumulation by endogenous LPA and thrombin receptors in COS-7 cells. [^{3}H]Inositol phosphate accumulation was measured as described in Methods in COS-7 cells transfected with empty vector or with an expression vector for PLC-ε. Incubations were in the presence of vehicle, 10 μM LPA, or 50 μM SFLLRN.

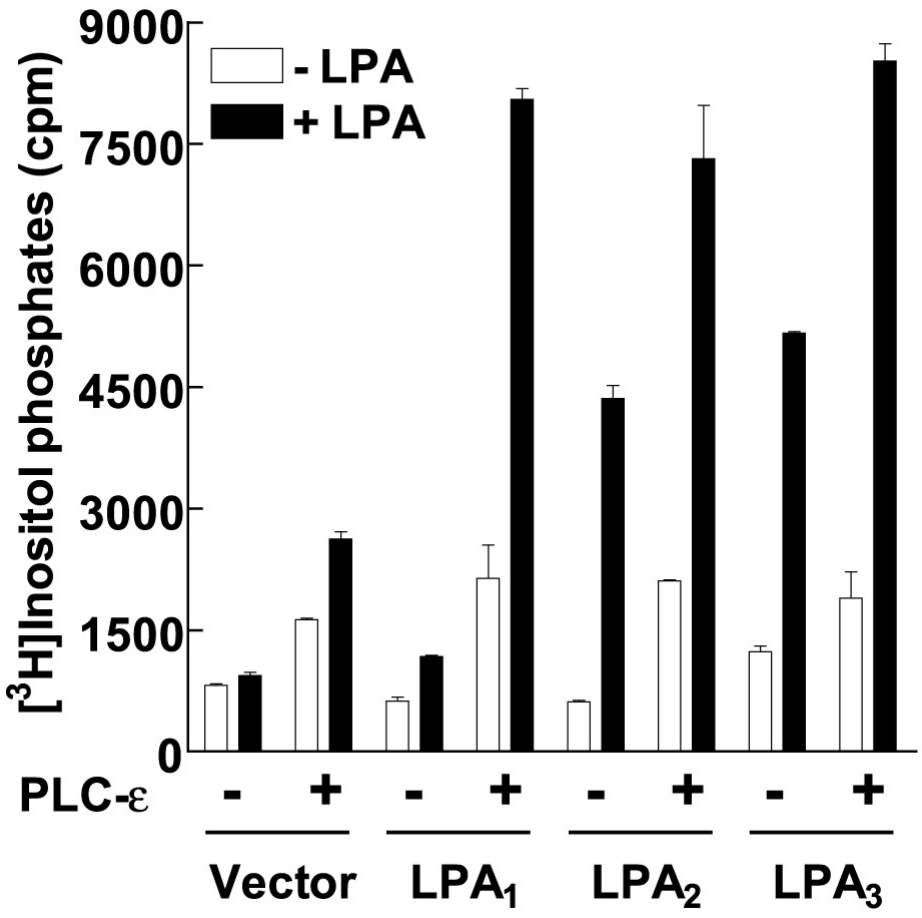

Figure 2.2. PLC-ε-dependence of LPA-stimulated [^{3}H]inositol phosphate accumulation in cells expressing empty vector, LPA_1, LPA_2, or LPA_3 receptor. COS-7 cells were transfected with an expression vector for the LPA_1, LPA_2, or LPA_3 receptor in the absence or presence of an expression vector for PLC-ε. [^{3}H]Inositol phosphate accumulation was quantified in the absence or presence of 10 μM LPA as described in Methods.

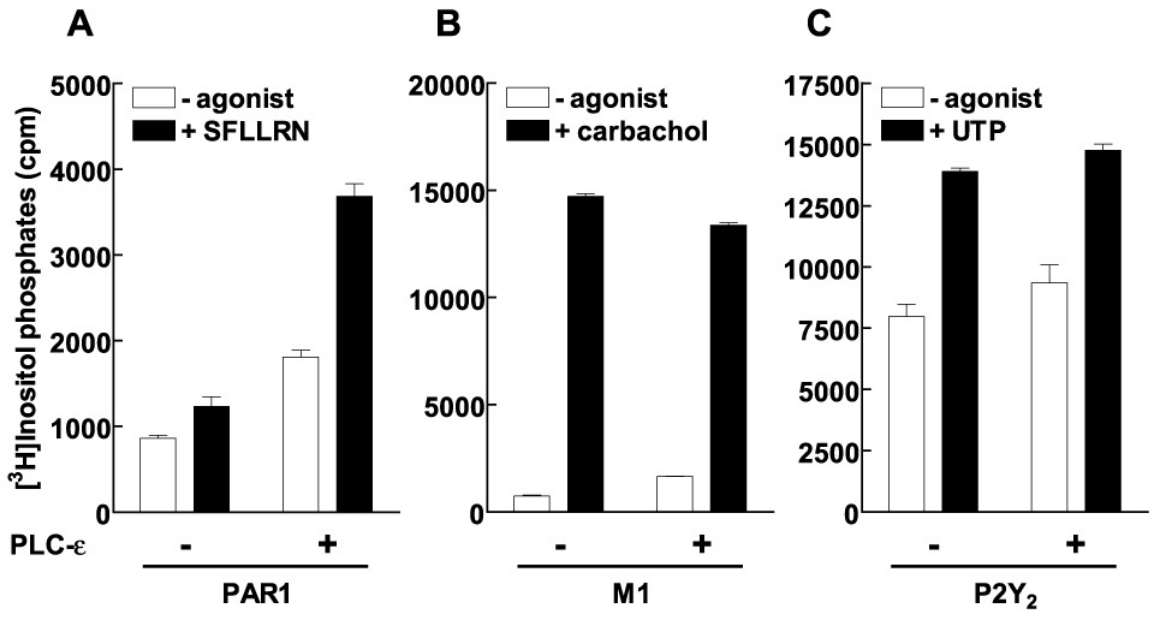

Figure 2.3. Differential effects of PLC-ε on agonist-stimulated [^{3}H]inositol phosphate accumulation in cells expressing the PAR1 receptor versus the M1 muscarinic cholinergic or P2Y$_2$ receptor. COS-7 cells were transfected with an expression vector for the PAR1 (**A**), M1 muscarinic cholinergic (**B**), or P2Y$_2$ (**C**) receptor in the absence or presence of an expression vector for PLC-ε. [^{3}H]Inositol phosphate accumulation was quantified in the PAR1, M1, or P2Y$_2$ receptor expressing cells in the absence or presence of 50 μM SFLLRN, 100 μM carbachol, or 100 μM UTP, respectively.

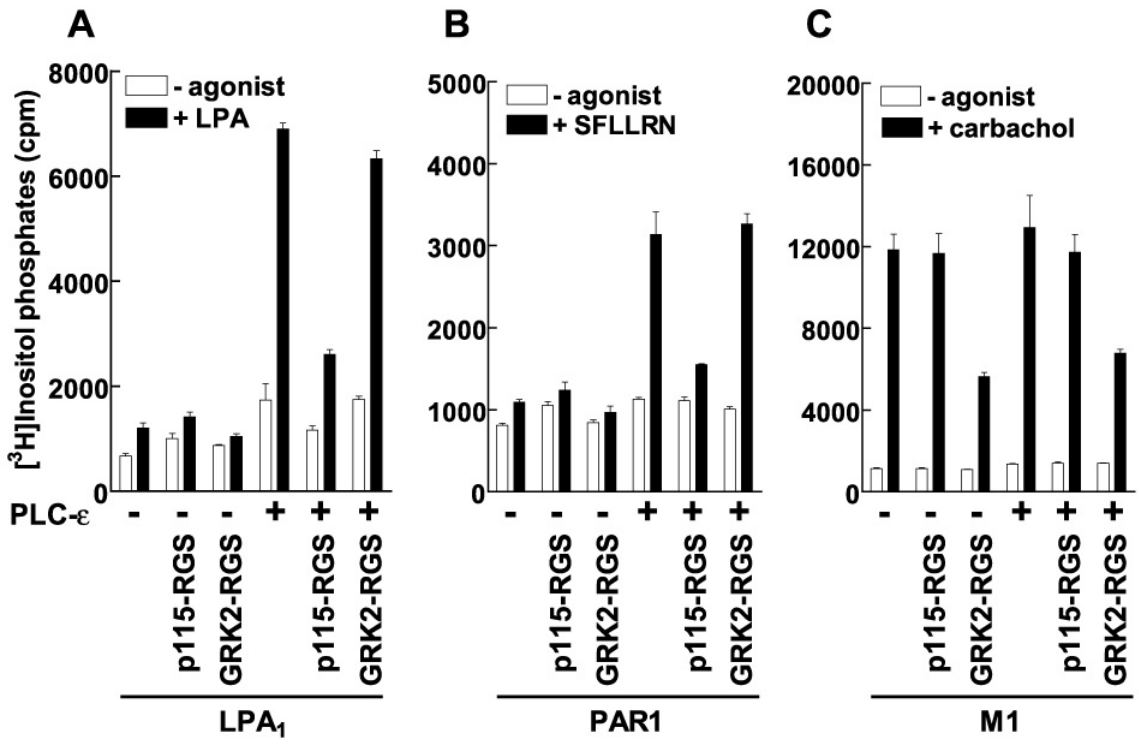

Figure 2.4. Differential effects of RGS proteins on LPA_1 receptor- and PAR1 receptor- versus M1 muscarinic receptor-promoted [^{3}H]inositol phosphate accumulation in PLC-ε-expressing cells. COS-7 cells were transfected with the LPA_1 receptor, the PAR1 receptor, or the M1 muscarinic cholinergic receptor in the absence or presence of PLC-ε and with either p115-RGS or GRK2-RGS. [^{3}H]Inositol phosphate accumulation was quantified in the LPA_1, PAR1, or M1 receptor-expressing cells in the absence or presence of 10 μM LPA, 50 μM SFLLRN, or 100 μM carbachol, respectively as described in Methods.

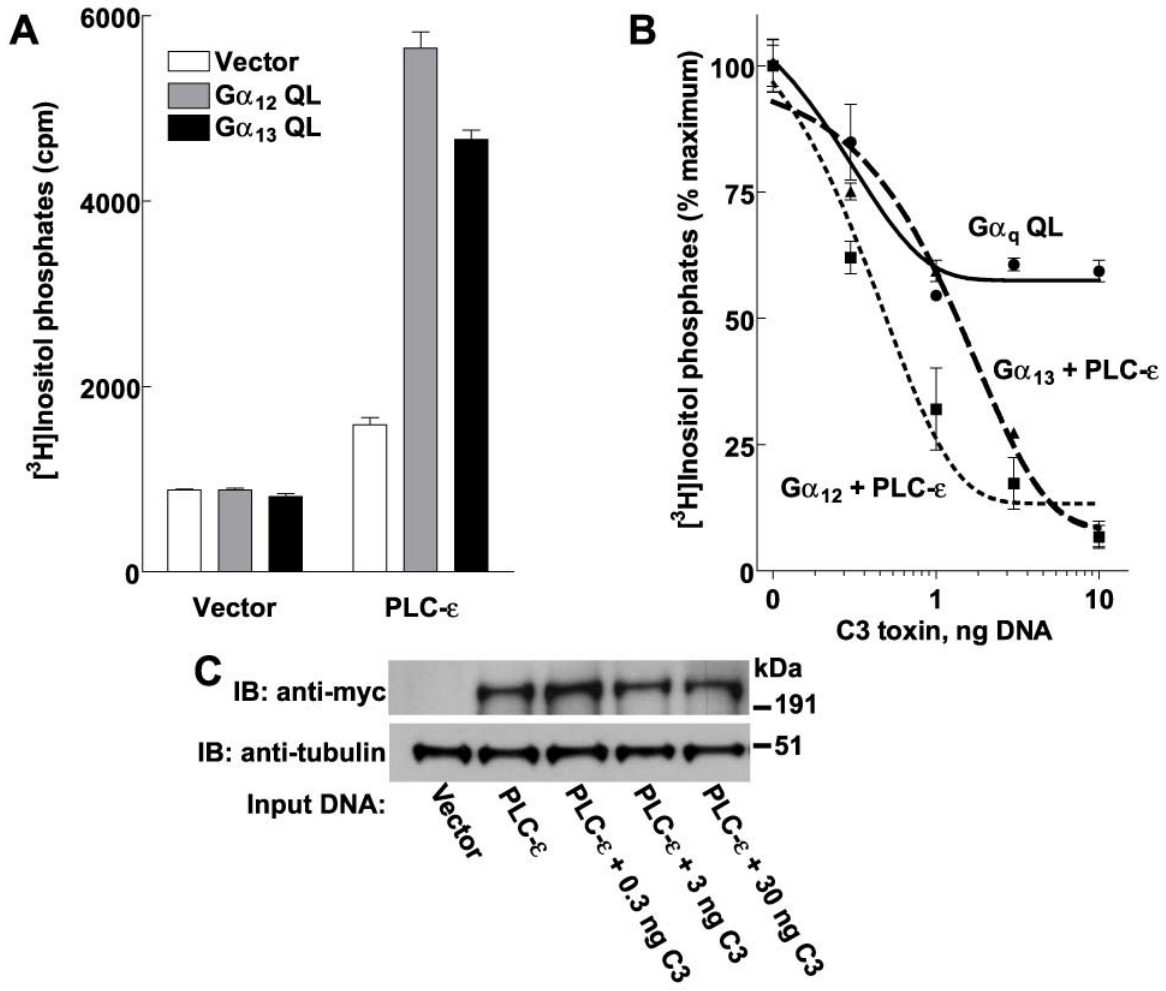

Figure 2.5. C3 toxin-mediated inhibition of the activation of PLC-ε by $G\alpha_{12}$ and $G\alpha_{13}$. **A**, COS-7 cells were transfected with empty vector, GTPase-deficient $G\alpha_{12}$ ($G\alpha_{12}$ Q226L), or GTPase-deficient $G\alpha_{13}$ ($G\alpha_{13}$ Q229L), in the absence or presence of transfection of an expression vector for PLC-ε. **B**, COS-7 cells were transfected with PLC-ε + $G\alpha_{12}$, PLC-ε + $G\alpha_{13}$, or $G\alpha_q$(Q209L) and with the indicated amounts of an expression vector for C3 toxin. [^{3}H]Inositol phosphate accumulation was measured as described in Methods. Data is expressed as percentage of maximum, with the average maximum for each as follows: PLC-ε + $G\alpha_{12}$, 6693 cpm; PLC-ε + $G\alpha_{13}$, 12,447 cpm; $G\alpha_q$(Q209L), 7947 cpm. **C**, COS-7 cells were transfected with empty vector, myc-PLC-ε, or myc-PLC-ε in the presence of 0.3 ng C3 toxin, 3 ng C3 toxin, or 30 ng C3 toxin. Forty-eight hours post-transfection the cells were lysed and mixed 1:1 with 5x Laemmli sample buffer. The lysates were subjected to SDS-PAGE and immunoblotted for the presence of myc-PLC-ε and α-tubulin as indicated.

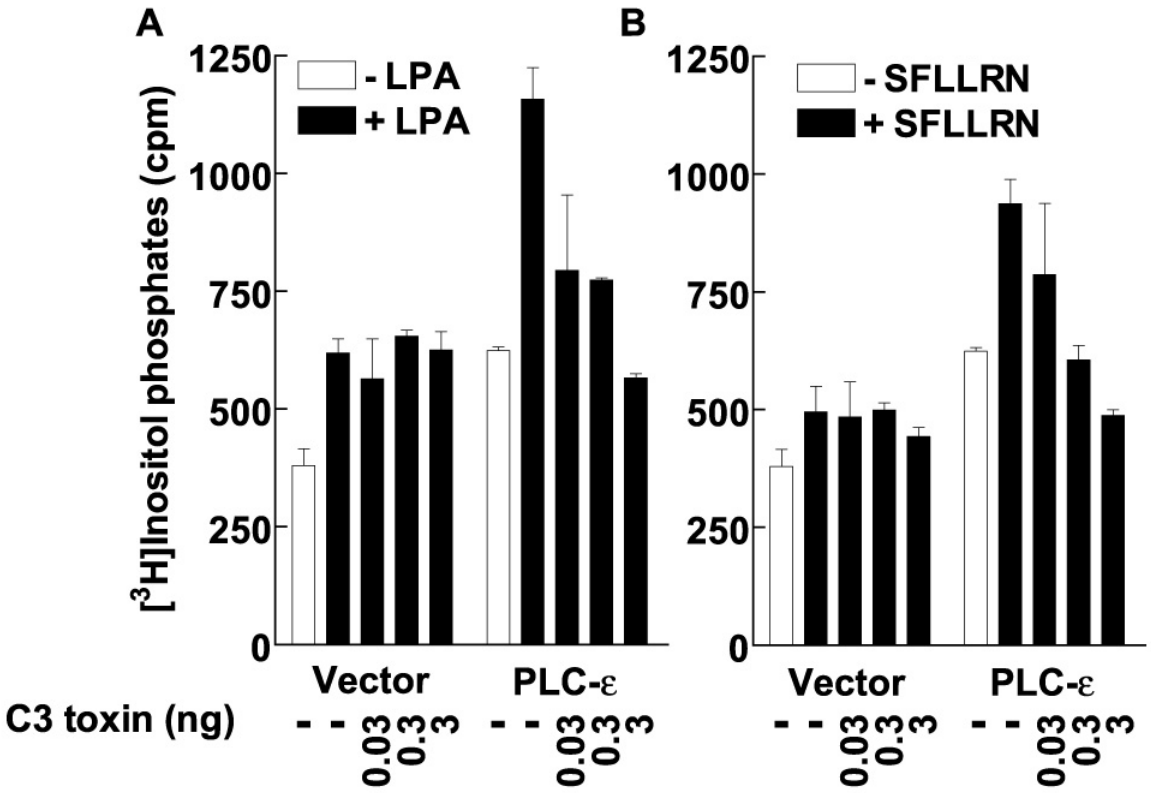

Figure 2.6. Effect of C3 toxin on PLC-ε-dependent signaling of endogenous LPA and thrombin receptors. COS-7 cells were cotransfected with the indicated amounts of an expression vector for C3 toxin and either empty vector or an expression vector for PLC-ε. [^{3}H]Inositol phosphate accumulation was measured in the absence or presence of 10 μM LPA or 50 μM SFLLRN as described in Methods.

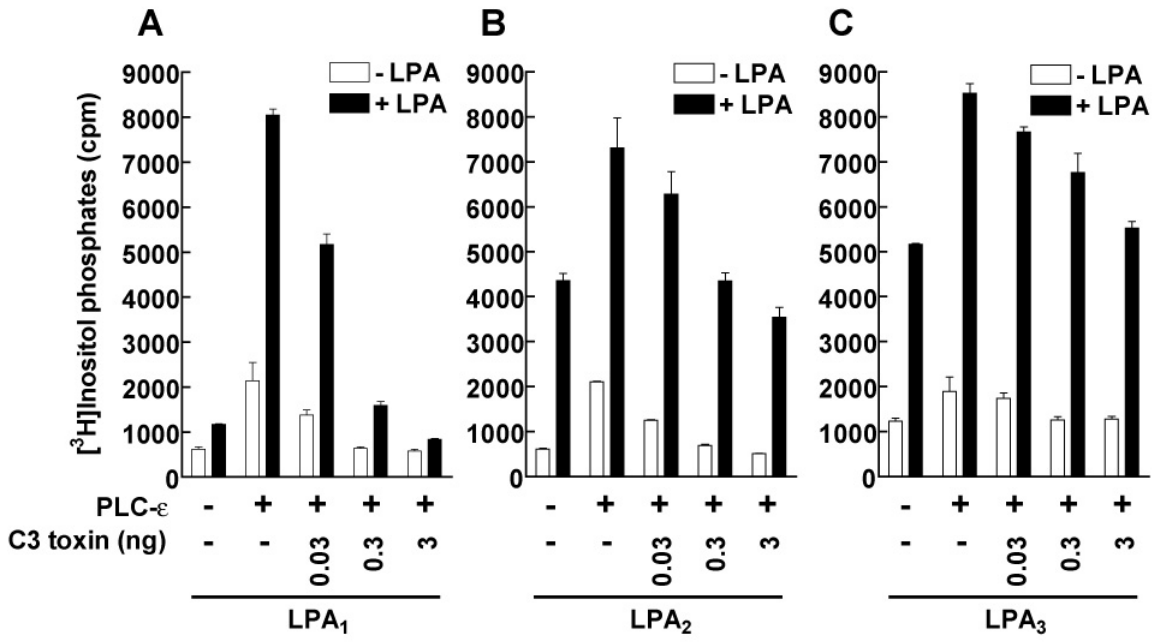

Figure 2.7. C3 toxin-mediated inhibition of LPA-stimulated [^{3}H]inositol phosphate accumulation in COS-7 cells expressing PLC-ε and the LPA_1, LPA_2, or LPA_3 receptor. COS-7 cells were transfected with the indicated amounts of C3 toxin DNA and expression vectors for PLC-ε and the LPA_1, LPA_2, or LPA_3 receptor. [^{3}H]Inositol phosphate accumulation was quantified in the absence or presence of 10 μM LPA as described in Methods.

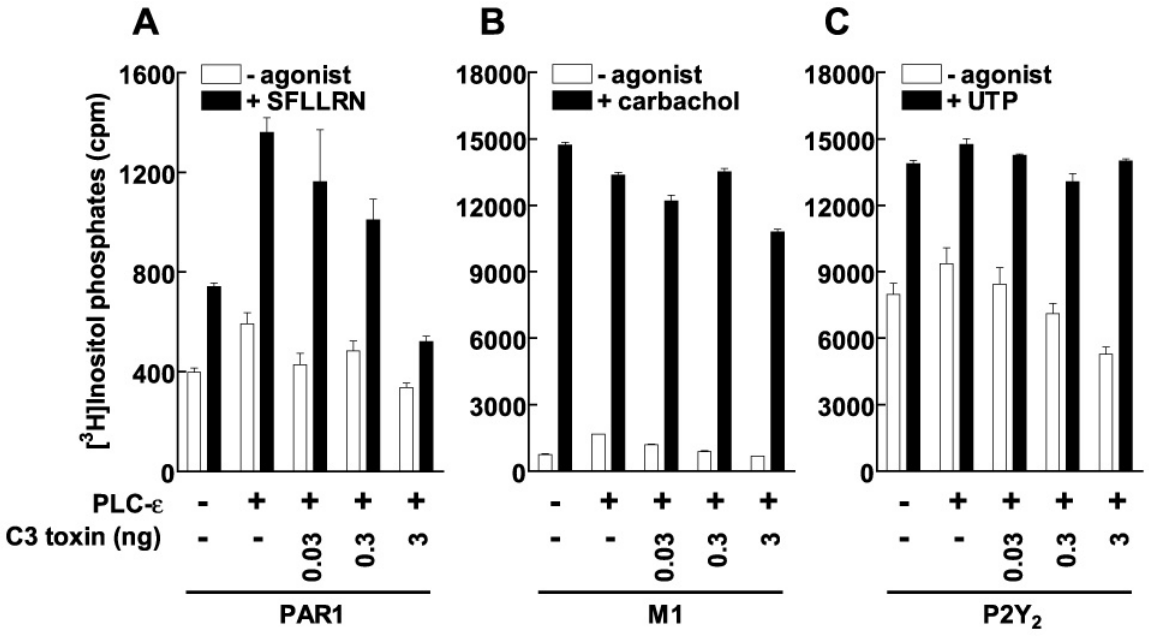

Figure 2.8. Differential effect of C3 toxin on PAR1 receptor- versus M1 muscarinic cholinergic and $P2Y_2$ receptor-promoted [^{3}H]inositol phosphate accumulation in PLC-ε expressing cells. COS-7 cells were transfected with the indicated amounts of C3 toxin DNA and expression vectors for PLC-ε and the **(A)** PAR1, **(B)** M1 muscarinic cholinergic, or **(C)** $P2Y_2$ purinergic receptor. [^{3}H]Inositol phosphate accumulation was quantified in the PAR1, M1, or $P2Y_2$ receptor-expressing cells in the absence or presence of 50 μM SFLLRN, 100 μM carbachol, or 100 μM UTP, respectively.

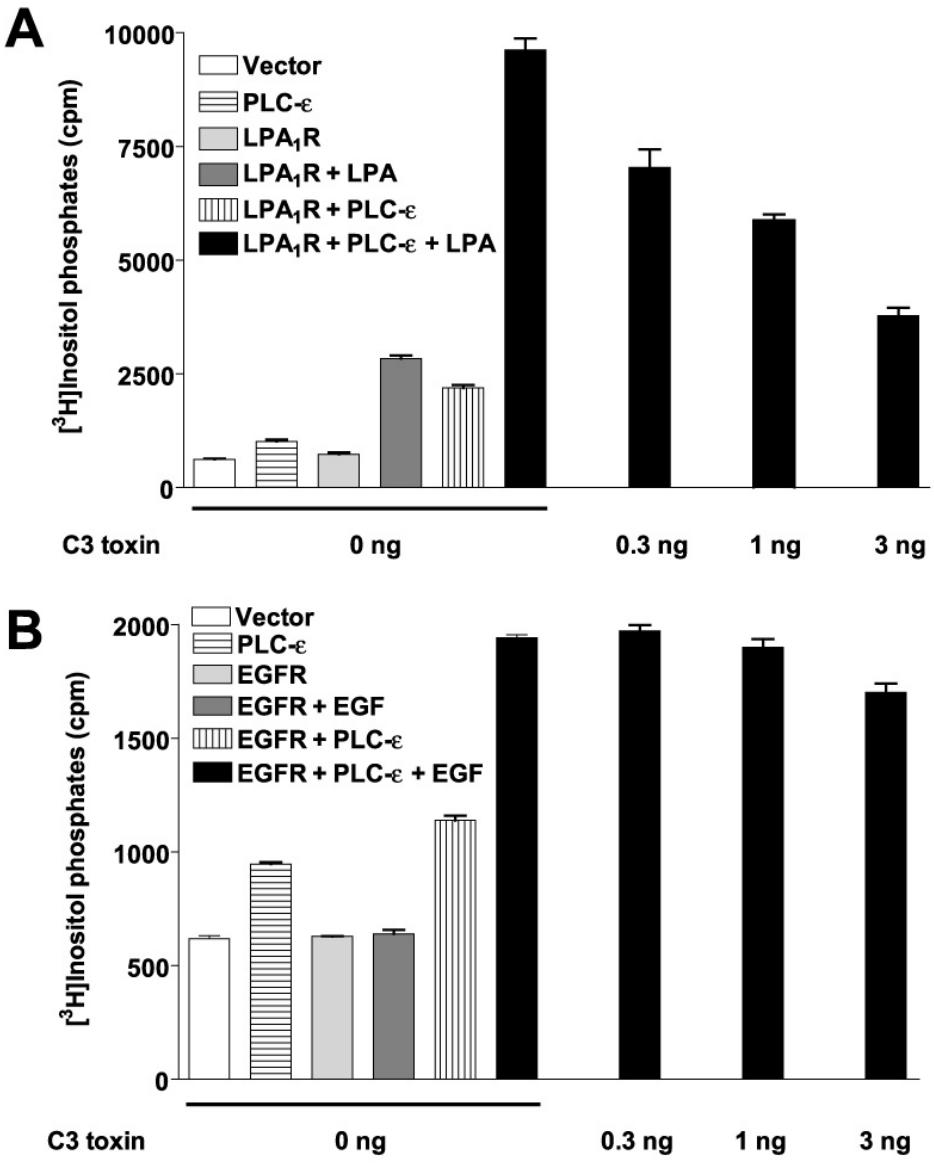

Figure 2.9. Lack of effect of C3 toxin on EGF receptor-promoted activation of PLC-ε. COS-7 cells were transfected with the indicated amounts of C3 toxin DNA and expression vectors for PLC-ε and the **(A)** LPA_1 or **(B)** EGF receptor. [^{3}H]Inositol phosphate accumulation was quantified in the LPA_1 or EGF receptor-expressing cells in the absence or presence of 10 μM LPA or 100 ng/mL EGF, respectively.

CHAPTER 3

APPLICATION OF RGS-BOX PROTEINS TO EVALUATE G PROTEIN SELECTIVITY IN RECEPTOR-PROMOTED SIGNALING

3.1 Abstract

Regulator of G protein signaling (RGS) domains bind directly to GTP-bound Gα subunits and accelerate their intrinsic GTPase activity by up to several thousand-fold. Selectivity of RGS proteins for individual Gα subunits has been illustrated. Thus, expression of RGS proteins can be used to inhibit signaling pathways activated by specific G protein-coupled receptors (GPCRs). In this chapter, we describe the use of specific RGS domain constructs to discriminate between $G_{i/o}$, G_q, and $G_{12/13}$-mediated activation of phospholipase C (PLC) isozymes in COS-7 cells. Overexpression of the N-terminus of GRK2 (amino-acids 45-178) or p115 RhoGEF (amino-acids 1-240) elicited selective inhibition of $G\alpha_q$- or $G\alpha_{12/13}$-mediated signaling to PLC activation, respectively. In contrast, RGS2 overexpression was found to inhibit PLC activation by both $G_{i/o}$- and G_q-coupled GPCRs. RGS4 exhibited dramatic receptor selectivity in its inhibitory actions; of the $G_{i/o}$- and G_q-coupled GPCRs tested (LPA_1, LPA_2, $P2Y_1$, $S1P_3$), only the G_q-coupled lysophosphatidic acid receptor LPA_2 was found to be inhibited by RGS4 overexpression.

3.2 Introduction

Many extracellular stimuli, such as neurotransmitters, hormones, chemokines, inflammatory mediators, and odorants exert their effects by activating

phosphoinositide-hydrolyzing PLC isozymes [29]. Five classes of PLC isozymes underlie these signals: PLC-β, PLC-γ, PLC-δ, PLC-ε, and PLC-ζ [31, 32]. The G protein-coupled receptor (GPCR) superfamily activates PLC-β enzymes through activation of α subunits of the G_q family of G protein heterotrimers or by Gβγ dimers released from activated G_i and potentially from other heterotrimeric G proteins [31]. PLC-ε is regulated by Ras and Rho GTPases, as well as by $G\alpha_{12/13}$ and Gβγ subunits of heterotrimeric G proteins [59] and therefore also is activated by GPCRs [54, 55].

Identification of the specific G proteins involved in GPCR-promoted signaling pathways is a critical step in understanding the mechanism by which receptors mediate particular downstream signaling events. Tools such as pertussis toxin, Gβγ "sinks" such as Gα-transducin or the C-terminal fragment of the G protein-coupled receptor kinase 2 (GRK2), antisense oligonucleotides, RNA interference, or GoLoco motif peptides can be applied as reagents to delineate the G protein subunits involved in GPCR-mediated activation of PLC. However, such reagents are limited to specific G proteins (*e.g.* pertussis toxin and GoLoco motif peptides only uncouple GPCRs from heterotrimers of the $G_{i/o}$ family), may result in more widespread, non-specific effects, or are expensive and time-consuming (*e.g.* antisense RNAi).

A potentially important new means for modification of GPCR signaling involves application of RGS proteins. RGS proteins contain a conserved ~120 amino-acid "RGS-box" that accelerates Gα-mediated GTP hydrolysis, and therefore, inhibits GPCR-promoted signaling [23]. Although several investigators have applied RGS proteins to inhibit G protein-dependent signaling, *e.g.* a decrease in D_2

dopamine receptor stimulated signaling occurred in the striatum after ectopic expression of RGS9 [257], utilization of RGS proteins to control GPCR function has not been fully elucidated.

Greater than 30 RGS domain-containing proteins have been identified to date, and many of these are negative regulators of G protein signaling. Given that certain RGS proteins exhibit selectivity for specific $G_{i/o}$, G_q, $G_{12/13}$, and G_s $G\alpha$ subunits [23], these RGS proteins potentially can be utilized to delineate the G proteins involved in signaling pathways activated by GPCRs. In this chapter, we describe the use of RGS proteins as antagonists of GPCR signaling, and examine their effectiveness in delineating the G protein coupling profile of a number of GPCRs using the inositol lipid signaling pathway as an example of their application.

3.3 Methodology

PLC isozymes catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to 1,2-diacylglycerol and inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] in response to activation of cell surface receptors. The production of Ins(1,4,5)P_3 is measured using a phosphoinositide hydrolysis assay that quantifies the amount of inositol phosphates produced as a result of PLC activation. Both PLC-β and PLC-ε are stimulated by GPCRs through activation of G_q, $G_{i/o}$, and $G_{12/13}$ heterotrimers. Conversely, the activity of RGS proteins as negative regulators of GPCR signaling can be evaluated using inositol phosphate accumulation as a biochemical assay of receptor activation. The methodology described in this chapter uses COS-7 cells co-transfected with GPCRs, RGS proteins, and in some cases

PLCs, and the phosphoinositide hydrolysis assay as a read-out, to examine the specificity and capacity of specific RGS proteins to inhibit GPCR activation of PLC.

3.4 Materials and Methods

1. RGS Constructs

A plethora of RGS proteins act on Gα subunits of the $G_{i/o}$, G_q, $G_{12/13}$, and G_s families. We have used the following RGS proteins to discriminate between $G_{i/o}$, G_q, and $G_{12/13}$-promoted signaling: the RGS-box of GRK2 (GRK2RGS), RGS2, RGS4, and the RGS-box of p115 RhoGEF (p115RGS). RGS2 and RGS4 are essentially limited to a core RGS domain structure with short N- and C-terminal polypeptide extensions, whereas GRK2 and p115 RhoGEF have an easily-delineated multidomain architecture (Figure 3.1). cDNA encoding full-length RGS4 with a triple hemagglutinin (3X HA)-epitope tag was obtained from the Guthrie Research Institute (Sayre, PA; www.cdna.org), cDNA encoding full-length RGS2 was subcloned in-frame with an N-terminal histidine (His_{10})-epitope tag into pcDNA3.1(-) (Invitrogen Corp., Carlsbad, CA) [258], cDNA encoding amino-acids 45-178 of GRK2 in-frame with an N-terminal HA-epitope tag in pcDNA3 was kindly provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA), and cDNA encoding the first 240 amino-acids of human p115 RhoGEF was subcloned in-frame with an N-terminal tandem HA-epitope tag into a modified pcDNA3.1 vector [111].

2. Other Constructs

cDNAs comprising the coding sequence of the human LPA_2 and $S1P_3$ receptor were inserted into the pCR3.1 vector. cDNA comprising the coding sequence of the mouse LPA_1 receptor was inserted into the pcDNA3 vector. The

LPA_1, LPA_2, and $S1P_3$ receptors were generously provided by Dr. Kevin R. Lynch (University of Virginia, Charlottesville, VA). The human purinergic $P2Y_1$ receptor, bearing an N-terminal HA-epitope tag, was cloned as previously described [259]. Rat PLC-ε in pCMV-script [49] was a kind gift of Dr. Grant Kelley (SUNY Syracuse, NY). The cDNA of the constitutively active mutant of mouse $G\alpha_{12}$ ($G\alpha_{12}$ Q229L ($G\alpha_{12}$QL)) was kindly provided by Dr. Channing Der (University of North Carolina, Chapel Hill, NC). The constitutively active mutants of human $G\alpha_q$ ($G\alpha_q$ Q209L ($G\alpha_q$QL)) and human RhoB (RhoB G14V (RhoBGV)) were obtained from Guthrie Research Institute (Sayre, PA; www.cdna.org). The carboxyl-terminal domain of GRK2 (GRK2ct) was a kind gift of Dr. Robert J. Lefkowitz (Duke University, Durham, NC).

3. Cell Culture and Transfection of COS-7 Cells

COS-7 cells are maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 200 U penicillin, and 0.2 mg/mL streptomycin (all obtained from Gibco, Invitrogen Corp, Carlsbad, CA) at 37 °C in a 5% CO_2/95% air humidified atmosphere. To plate COS-7 cells, remove medium from cells, wash cells once with 5 mL phosphate buffered saline (PBS), remove PBS, and add 4 mL 0.25% trypsin/EDTA (all obtained from Gibco, Invitrogen Corp, Carlsbad, CA). Following a 5 minute (min) incubation at 37 °C, suspend the cells in 10 mL of medium, remove 10 μL, and determine cell number with a hemocytometer. A confluent 162 cm^2 flask of COS-7 cells will yield approximately 10 mL of cells at a concentration of 1 x 10^6 cells/mL. Bring cells to a density of 60,000-75,000 cells/mL with medium, plate 1 mL of cells/well in 12-well culture dishes (CoStar 3512), and incubate overnight (approximately 8 to 12 hours). Following

overnight incubation, prepare the transfection reagent in serum-free DMEM. Specifically, dilute 2 μL of FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) directly into 41 μL serum-free DMEM. Make sure not to touch the side of the tube with the FuGENE 6 reagent, since this will significantly reduce transfection efficiency. Once diluted, flick the tube several times to mix (do not vortex), and incubate at room temperature (RT) for 5 min. While waiting, dilute all DNA plasmids to 100 ng/μL with sterile water. To each tube containing the 43 μL FuGENE 6/serum-free DMEM mix, add 700 ng total of the necessary DNA expression plasmids. For most experiments described in this chapter, 50 ng of GPCR, 100 ng of PLC-ε, and 550 ng of RGS protein DNA plasmids were added to the 43 μL mix (0.5 μL, 1 μL, and 5.5 μL of 100 ng/μL dilutions, respectively). When one of the above plasmids was not required, the total amount of DNA was brought up to 700 ng with empty pcDNA3 vector. Once the DNA is added to the FuGENE 6/serum-free mix, flick the tube several times to mix (do not vortex), and incubate at RT for 15-45 min to allow the DNA to complex with the FuGENE 6 transfection reagent. Add 50 μL of complexes to each well and return the culture dishes to the 37 °C, 5% CO_2/95% air atmosphere.

4. Phosphoinositide Hydrolysis Assay

Dilute 1 μCi (1 μL) of [*myo*-^{3}H]inositol (American Radiolabeled Chemicals, catalogue# ART 116A-myo-inositol, [2-3H(N)]) into 400 μL serum-free inositol-free DMEM (ICN Biomedicals, Inc., Aurora, Ohio, catalogue# 1642954), and mix by inverting the tube several times. Approximately 12-24 h after transfection, remove the medium from each well and replace with 400 μL of medium consisting of serum-

free inositol-free DMEM containing [*myo*-^{3}H]inositol (1 μCi/well). Return the dishes to the tissue culture incubator and incubate for 12-18 h at 37 °C. Phospholipase C activity is quantified 12-18 h after labeling by treating cells for 30-45 min in inositol-free DMEM containing 10 mM LiCl (to inhibit inositol phosphatases). Hormone-stimulated PLC activity was determined in the experiments illustrated below by incubating cells in the absence or presence of lysophosphatidic acid (LPA; 1-Oleoyl-sn-glycerol 3-phosphate sodium salt dissolved in water containing 1.0% fatty acid-free BSA), S1P (sphingosine-1-phosphate dissolved in water containing 1.0% fatty acid-free BSA), or the purinergic receptor agonist 2-MeSADP (2-methylthioadenosine diphosphate) (LPA and S1P, Sigma, St. Louis, MO; 2-MeSADP, Research Biochemicals Inc., Natick, MA). The reaction is terminated after 30-45 min by aspirating the medium and adding 750 μL of ice-cold 50 mM formic acid (Fisher Scientific, formic acid 88%, catalogue# A118P) for a minimum of 15 min at 4 °C. Neutralize cell extracts with 250 μL of 150 mM NH_4OH (Fisher Scientific, ammonium hydroxide, catalogue# A669), and add 1 mL neutralized sample to 1 mL packed volume columns of AG 1-X8 anion-exchange gel resin (Bio-Rad, catalogue# 140-1454, formate form, 200-400 mesh size). Poly-Prep chromatography columns (Bio-Rad, catalogue# 731-1550) are utilized since they contain a filter for stacking the resin, and also have a large volume capacity to accommodate washes up to 10 mL. Prior to addition of cell extracts, the columns should be prepared by washing with 5 mL of 2 M ammonium formate (Fisher Scientific, ammonium formate, catalogue# A666)/0.1M formic acid followed by two 10 mL washes of deionized water. After loading the cell extracts, wash the columns with 10 mL deionized water

followed by 10 mL of 50 mM ammonium formate, and elute the [^{3}H]inositol phosphates into scintillation vials by adding 5 mL 1.2 M ammonium formate/0.1 M formic acid to the column. Add 10 mL scintillation fluid (Fisher Scientific, Scintisafe Gel Cocktail, catalogue# SX24-5) to each vial and shake vigorously until the mixture becomes clear. If the mixture remains a "milky" color, add more scintillation fluid and repeat the shaking step. Quantify radioactivity by scintillation counting [260].

Note: A convenient indicator of proper neutralization of the cell extracts, and an easy way to keep track of which columns have already received sample, is as follows. Add 1-2 mL of inositol-free DMEM to the packed gel resin, which does not alter the binding capacity or specificity of the gel resin for the [^{3}H]inositol phosphates. The gel resin will appear purple-pink in color, and a pale purple color should remain after washing the columns twice with deionized water. Addition of 5 mL of 2 M ammonium formate/0.1M formic acid changes this color from purple to yellow (presumably due to phenol red in the medium, a pH indicator dye). However, following two to three washes with 10 mL of deionized water, the columns return to a pale purple color. Upon addition of neutralized sample, each column will turn yellow providing indication of proper neutralization of sample, and indication of the addition of the samples to each column.

3.5 Background

In choosing an RGS protein to use in interrogating GPCR coupling specificity, a number of properties of these proteins should be considered including the specificity of the RGS-boxes for each activated Gα subunit, the mechanism by which each RGS-box inhibits signaling, and the multidomain structure of several of these

proteins. The GPCR signaling regulator, GRK2, contains a conserved N-terminal RGS-box that does not stimulate GTPase activity, but rather sequesters $G\alpha$ subunits of the G_q family ([93]; see also in this series [94, 95]). The advantage of using the RGS-box of GRK2 is that it is selective for $G\alpha$ subunits of the G_q family, and it is a potent effector antagonist for G_q-coupled GPCRs (as described below under Application). However, a caveat of GRK2 is its multidomain architecture (Figure 3.1). The C-terminal fragment of GRK2 competitively binds $G\beta\gamma$ via its pleckstrin-homology (PH) domain [96], and the kinase domain phosphorylates and desensitizes GPCRs [97]; thus, it is important to employ a construct of GRK2 restricted to its RGS-box for discrimination of GPCR signaling through $G\alpha_q$.

RGS2, a founding member of the RGS protein family, stimulates the GTPase activity of $G\alpha$ subunits of the G_q family in biochemical assays [98, 99]. However, in receptor reconstitution and cellular assays, RGS2 acts as a negative regulator of both $G_{i/o}$- and G_q-coupled receptor signaling [99]. These findings suggest that assay conditions alter the G protein specificity of RGS2 from $G\alpha_q$ to both $G\alpha_q$ and $G\alpha_{i/o}$. Thus, when using RGS2 to discriminate GPCR signaling, pertussis toxin and/or expression of the $G\alpha_q$-specific GRK2RGS construct should be tested in parallel to support the findings obtained.

RGS4 stimulates the GTPase activity of $G\alpha$ subunits of the $G_{i/o}$ [84, 100] and G_q family [99, 101] via its RGS-box. Similar to RGS2, RGS4 contains little sequence beyond the RGS-box. However, RGS4 has been demonstrated to exert receptor-selective inhibitory activity via its amphipathic alpha-helical N-terminus [102]. In addition, the N-terminus also has been shown to confer high potency inhibition of

G_q-mediated receptor signaling *in vivo* [102]. Thus, RGS4 should be applied as a tool to discriminate Gα subunits in GPCR signaling with cognizance of the benefits and caveats associated with using either the full length or isolated RGS-box construct. As above, results should be supported by parallel experiments using pertussis toxin and expression of GRK2RGS.

The guanine nucleotide exchange factor (GEF) for Rho, p115 RhoGEF, contains an NH_2-terminal RGS-box and acts as a GTPase-accelerating protein (GAP) for Gα_{12} and Gα_{13} [103]. This GAP activity requires sequences flanking the RGS-box [104] and, thus, it is important to use a construct of p115 RhoGEF that contains the N-terminus of the protein in addition to the RGS-box. p115 RhoGEF also contains a tandem Dbl-homology domain (DH/RhoGEF) and PH domain C-terminal cassette (Figure 3.1) that exhibits RhoA-specific GEF activity [105] and could thus confound the use of p115 RhoGEF in discriminating $G_{12/13}$-coupled receptor signaling. Thus, full length constructs containing the C-terminal DH and PH domains should be avoided in targeting G$\alpha_{12/13}$-dependent signaling with p115 RhoGEF overexpression.

3.6 Application

1. *Specificity of RGS-box constructs for constitutively active mutants of Gα_q and Gα_{12} in COS-7 Cells*

To demonstrate the selectivity of RGS-box constructs as effector antagonists of specific Gα subunits in COS-7 cells, we utilized constitutively active (GTPase-deficient) mutants of Gα_q, Gα_{12}, and RhoB. Gα_qQL was expressed in COS-7 cells in the absence or presence of GRK2RGS, RGS2, RGS4, and p115RGS; marked

activation of endogenous PLC-β was observed with expression of $G\alpha_q$QL alone (Figure 3.2). GRK2RGS and RGS2 potently inhibited the activation of PLC-β by $G\alpha_q$QL (Figure 3.2). In contrast, RGS4 only partially inhibited the activation of PLC-β, and p115RGS did not alter $G\alpha_q$QL-stimulated PLC activity. These results suggest that GRK2RGS and RGS2 are effective inhibitors of $G\alpha_q$QL-mediated activation of PLC-β, and thus, are useful tools for discriminating G_q-coupled receptor signaling in COS-7 cells. Full-length RGS4 and the RGS-box of RGS4 have both been shown to inhibit $G\alpha_q$-promoted Ca^{2+} signaling in rat pancreatic acinar cells [102]; however, RGS4 does not appear to be as effective as GRK2RGS or RGS2 for discriminating $G\alpha_q$ signaling in COS-7 cells.

To examine the specificity of GRK2RGS, RGS2, RGS4, and p115RGS on $G\alpha_{12}$QL-mediated activation of PLC-ε, we co-transfected $G\alpha_{12}$QL and PLC-ε in the absence or presence of the RGS-box constructs, and examined the ability of each to attenuate inositol phosphate accumulation. $G\alpha_{12}$QL activated PLC-ε when both were co-expressed in COS-7 cells (Figure 3.2). p115RGS, but not GRK2RGS, RGS2, and RGS4, antagonized the activation of PLC-ε by $G\alpha_{12}$QL (Figure 3.2). These results suggest that p115RGS selectively inhibits Gα subunits of the $G\alpha_{12/13}$ family, whereas GRK2RGS, RGS2, and RGS4 have no effect on this pathway. Thus, p115RGS is a useful tool for discriminating $G\alpha_{12/13}$-dependent receptor signaling in COS-7 cells.

A control experiment was carried out to demonstrate that the RGS-box constructs do not indirectly affect the activation of PLC-ε by small GTPases of the Rho family. Wing and colleagues [52] have recently shown that the Rho subfamily GTPases, RhoA, RhoB, and RhoC, can each directly activate PLC-ε. Thus,

expression constructs for PLC-ε and constitutively active (GTPase-deficient) RhoB (RhoBGV) were co-tranfected in COS-7 cells in the absence and presence of GRK2RGS, RGS2, RGS4, and p115RGS. RhoBGV expression promoted PLC-ε-dependent increases in inositol phosphate accumulation in both the absence and presence of the RGS-box constructs (Figure 3.2), suggesting that these RGS-box constructs selectively inhibit Gα-promoted stimulation of PLC without affecting regulation by other activators.

2. RGS2 is a useful tool for discriminating $G_{i/o}$-mediated signaling by the LPA_1 receptor in COS-7 cells

The LPA_1 receptor couples to $G_{i/o}$ heterotrimers in COS-7 cells to activate an endogenous PLC in response to LPA [54]. This LPA-promoted response is pertussis toxin sensitive and is inhibited by coexpression of the Gβγ-binding PH domain of GRK2 (GRKct) (Figure 3.3B). Thus, to demonstrate the capacity of RGS-box constructs to discriminate $G_{i/o}$-mediated signaling in COS-7 cells, we coexpressed the LPA_1 receptor with and without GRK2RGS, RGS2, RGS4, and p115RGS, and quantified LPA-promoted inositol phosphate accumulation. RGS2, but not GRK2RGS, RGS4, or p115RGS, blocked the effects of LPA (Figure 3.3A). RGS4 is a potent GAP for $G\alpha_{i/o}$ subunits *in vitro* [84, 100]; thus, the lack of effect of RGS4 on LPA_1 receptor ($G_{i/o}$-coupled) signaling to PLC activation most likely reflects the receptor-selective nature of RGS4 activity (as observed and discussed below).

These results with LPA_1 receptor signaling demonstrate that RGS2 can inhibit $G_{i/o}$-mediated GPCR signaling in COS-7 cells, presumably by virtue of its GAP activity on $G\alpha_{i/o}$ subunits [99]. The ability of RGS2 to inhibit LPA_1 receptor signaling

is consistent with previous reports of activity on $G_{i/o}$-coupled pathways; for example, RGS2 has been shown to inhibit M2 mAChR-mediated (G_i-coupled) MAP kinase activation in COS cells [99]. This ability of RGS2 to inhibit $G_{i/o}$-mediated signaling could be applied to other $G_{i/o}$-coupled receptors in COS-7 and other cells, and should be kept in mind in light of reports that consider RGS2 as solely a negative regulator of $G\alpha_q$-mediated signaling (*e.g.*, [261] in this series).

3. RGS2 and GRK2RGS discriminate $G\alpha_q$-mediated signaling by the $P2Y_1$ and $S1P_3$ receptors in COS-7 cells

Both the purinergic receptor $P2Y_1$ and the sphinogine-1-phosphate receptor $S1P_3$ predominantly couple to $G\alpha$ subunits of the G_q family in COS-7 cells to stimulate endogenous PLC [54, 245]. To examine the capacity of RGS-box constructs to inhibit signaling by the $P2Y_1$ and $S1P_3$ receptors, we coexpressed each receptor in the absence or presence of GRK2RGS, RGS2, RGS4, and p115RGS, and measured inositol phosphate accumulation. Both GRK2RGS and RGS2 markedly inhibited agonist-promoted activation of PLC by the $P2Y_1$ and $S1P_3$ receptors, respectively (Figure 3.4A and Figure 3.4B). Conversely, RGS4 and p115RGS exhibited relatively little effect on receptor-promoted activation of endogenous PLC. Thus, GRK2RGS and RGS2, but not RGS4 or p115RGS, are useful tools for discriminating $G\alpha_q$ signaling in COS-7 cells. RGS4 is a potent GAP for $G\alpha_q$ signaling *in vivo*; however, the N-terminus of the protein can impose receptor-selective effects [102] as described above. In the next section, we provide an example of a $G\alpha_q$-coupled receptor that is inhibited by GRK2RGS, RGS2, and RGS4.

4. RGS2, GRK2RGS, and RGS4 inhibit $G\alpha_q$-mediated signaling by the LPA_2 receptor

Thus far in this chapter, GRK2RGS and RGS2, but not RGS4, have been shown to be useful tools for inhibiting $G\alpha_q$-mediated GPCR signaling in COS-7 cells. However, in the case of the lysophosphatidic acid receptor LPA_2, RGS4 as well as GRK2RGS and RGS2 effectively inhibited LPA-promoted inositol phosphate accumulation (Figure 3.5A). Similar to the results observed with the $P2Y_1$ and $S1P_3$ receptors, p115RGS did not inhibit PLC activation by the LPA_2 receptor. The expression level of HA-tagged GRK2RGS and RGS4 in the absence and presence of the LPA_2, $S1P_3$, or $P2Y_1$ receptor was examined by cell lysate immunoblotting using an anti-HA antibody. The expression level of GRK2RGS and RGS4 was not significantly altered in the presence of the receptors (Figure 3.5B), suggesting that the lack of action of RGS4 in inhibiting the $S1P_3$ and $P2Y_1$ receptors is not due to the inhibition of RGS4 expression. These results suggest that GRK2RGS, RGS2, and RGS4 discriminate $G\alpha_q$-mediated signaling by the LPA_2 receptor. These data also demonstrate the receptor-selective effects of full length RGS4 (*i.e.*, inhibition of LPA_2, but not $S1P_3$ nor $P2Y_1$, receptor signaling to PLC), and thus highlight the importance of using alternative reagents such as GRK2RGS or RGS2 to confirm observations of signaling inhibition (or lack thereof) using RGS4 in particular and $G\alpha_{i/o}$/$G\alpha_q$-specific RGS proteins in general.

5. p115RGS discriminates $G\alpha_{12/13}$-mediated signaling by the LPA_1 receptor in COS-7 cells

The LPA_1 receptor couples to $G\alpha_{12/13}$ to activate PLC-ε in COS-7 cells [54]. To assess the action of RGS-box constructs in discriminating GPCR-promoted activation of $G\alpha_{12/13}$, we coexpressed the LPA_1 receptor and PLC-ε in the absence and presence of the RGS proteins, and examined the capacity of the constructs to inhibit PLC-ε activation by the LPA_1 receptor. In the absence of the RGS-box constructs, the LPA_1 receptor promoted inositol phosphate accumulation when coexpressed with PLC-ε (Figure 3.6). In contrast, in the presence of p115RGS, but not GRK2RGS, RGS2, or RGS4, LPA-mediated activation of PLC-ε by the LPA_1 receptor was markedly inhibited. These results suggest that p115RGS is a useful tool for discriminating $G\alpha_{12/13}$ GPCR signaling in COS-7 cells.

3.7 Concluding Remarks

RGS proteins specific for $G_{i/o}$, G_q, $G_{12/13}$, and G_s Gα subunits have been described [23], and these RGS proteins can be utilized to delineate the G proteins involved in signaling pathways activated by GPCRs. In addition to Gα subunit selectivity, many RGS proteins also exhibit receptor selectivity, thus highlighting the importance of using additional RGS proteins or other inhibitors of Gα signaling (*e.g.*, GoLoco motif peptides; see in this series: [262]) when employing RGS-box constructs to delineate signaling pathways. For example, we have found that RGS4 demonstrates receptor selectivity in inhibiting $G\alpha_q$ signaling by the LPA_2, but not the $P2Y_1$ nor $S1P_3$ receptors. Thus, the lack of an effect of RGS4 overexpression on a

particular GPCR signal transduction pathway should not be misconstrued as a lack of involvement of G_q-class $G\alpha$ subunits in receptor/effector coupling.

RGS-box-independent regulation of GPCR signaling has also been demonstrated and should be considered when choosing an RGS protein as a tool to probe GPCR/effector coupling. The N-terminus of RGS2 inhibits $G\alpha_sQ227L$ and β_2-adrenergic receptor-stimulated cAMP accumulation in HEK293 cells expressing type V adenylyl cyclase [106]; see also in this series: [107]). Similarly, RGS16, a GAP for $G\alpha_{i/o}$ and $G\alpha_q$, binds to $G\alpha_{13}$ and inhibits $G\alpha_{13}$-mediated signal transduction via its amino terminus [108].

In this chapter, we have demonstrated the application of full length RGS proteins (RGS2, RGS4) and isolated RGS-boxes (GRK2RGS, p115RGS) to discriminate $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$ GPCR signaling in COS-7 cells. These constructs should prove useful for discerning the contributions of these three families of $G\alpha$ subunits to GPCR signaling in a variety of systems when applied with the appropriate controls.

3.8 Acknowledgements

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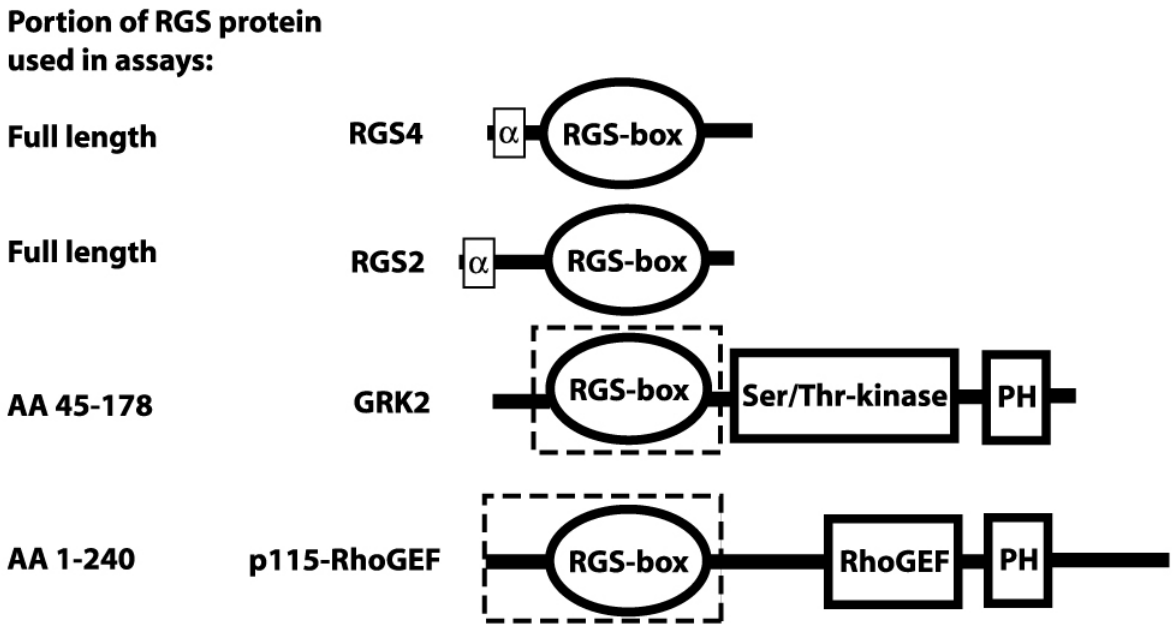

Figure 3.1. Domain architecture of GRK2, RGS2, RGS4, and p115 RhoGEF. The portion of each RGS protein used in the analyses described in this chapter is described to the left of the structures. Dashed lines around GRK2 and p115 RhoGEF highlight the regions of these proteins subcloned into expression vectors for this study.

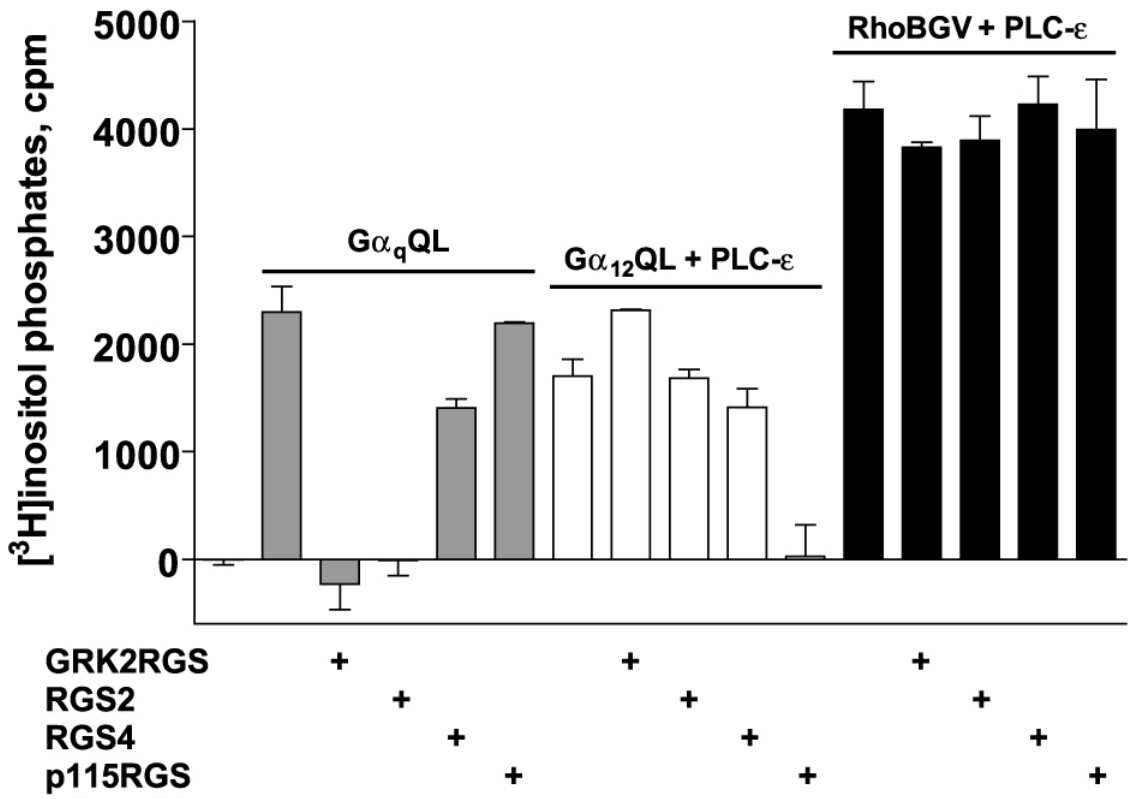

Figure 3.2. Effect of GRK2RGS, RGS2, RGS4, and p115RGS expression on activation of PLC isozymes by constitutively active, GTPase-deficient mutants of $G\alpha_q$, $G\alpha_{12}$, and RhoB. COS-7 cells were transfected with the indicated DNA (supplemented to 700 ng total DNA with empty vector) and [^{3}H]inositol phosphate accumulation was measured 36 h later as described in *Materials and Methods*. COS-7 cells were transfected with either 50 ng of empty pcDNA3 vector, $G\alpha_q$QL, $G\alpha_{12}$QL, or RhoBGV with or without PLC-ε (100 ng), and with or without GRK2RGS, RGS2, RGS4, or p115RGS (550 ng). [^{3}H]Inositol phosphate accumulation was quantified by incubation for 45 min with 10 mM LiCl. Data shown are mean ± S.D. for duplicate samples in one experiment. Basal levels of [^{3}H]inositol phosphate production in the presence of empty pcDNA3 vector alone (approximately 3000 cpm) were subtracted from the values presented.

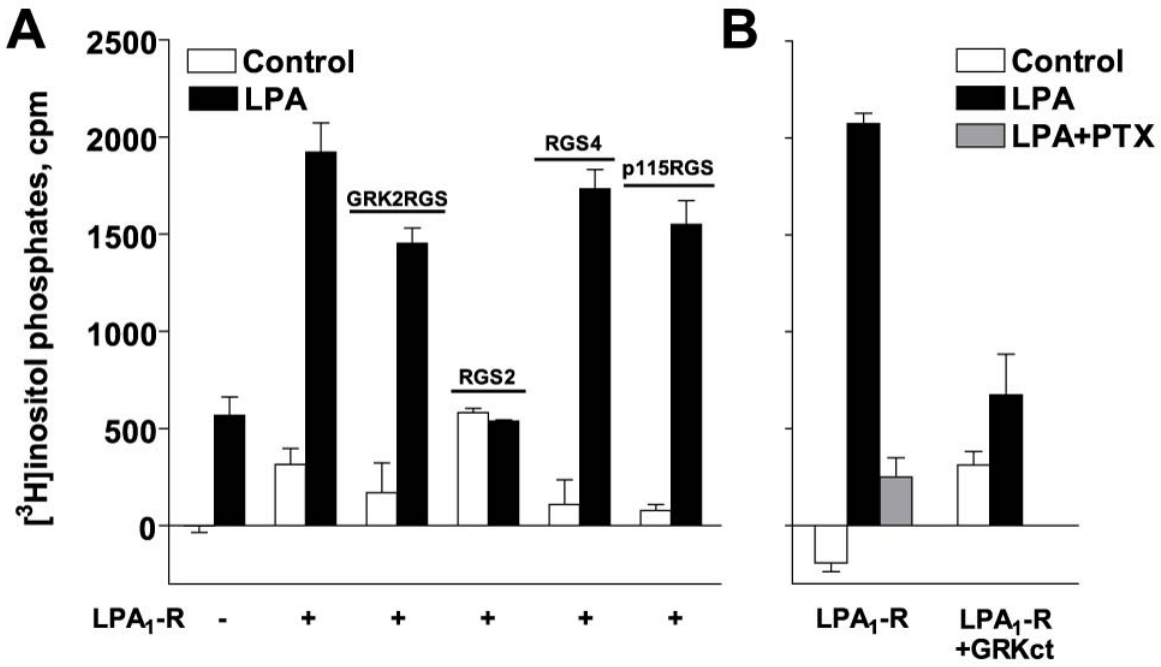

Figure 3.3. $G_{i/o}$-dependent stimulation of an endogenous PLC by the LPA_1 receptor is inhibited by RGS2. **A**, COS-7 cells were transfected with the indicated DNA (supplemented to 600 ng total DNA with empty vector), and [^{3}H]inositol phosphate accumulation was measured 36 h later as described in *Materials and Methods*. COS-7 cells were transfected with either 50 ng of empty pcDNA3 vector or the LPA_1 receptor (LPA_1-R), and with or without GRK2RGS, RGS2, RGS4, and p115RGS (550 ng). Cells were then incubated for 45 min with 10 mM LiCl in the absence of agonist (Control) or presence of 10 μM lysophosphatidic acid (LPA). Panel **B** shows a separate experiment (with the same y-axis scale) in which 50 ng of empty vector or the LPA_1 receptor was co-transfected with or without 300 ng of GRKct in COS-7 cells. Cells were labeled with [^{3}H]inositol in the presence or absence of 100 ng/mL pertussis toxin (PTX; List Biochemicals, Campbell, CA) for 12 h, followed by incubation for 30 min with 10 mM LiCl in the absence of agonist (Control) or presence of 10 μM LPA. Data shown are mean ± S.D. for duplicate samples in one experiment. Basal levels of [^{3}H]inositol phosphate production in the presence of empty pcDNA3 vector alone (approximately 2500 cpm) were subtracted from the values presented.

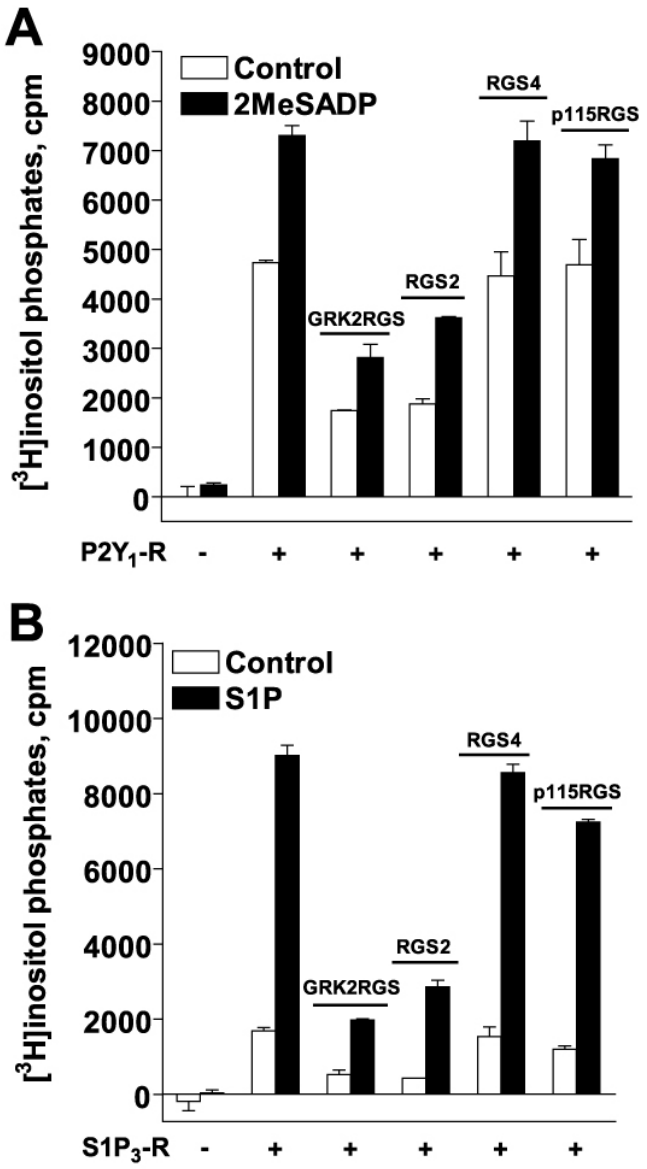

Figure 3.4. $G\alpha_q$-dependent stimulation of an endogenous PLC by the $P2Y_1$ and $S1P_3$ receptors is inhibited by GRK2RGS and RGS2. COS-7 cells were transfected with the indicated DNA [supplemented to 400 ng (**A**) or 600 ng (**B**)], and [^{3}H]inositol phosphate accumulation was measured 36 h later as described in *Materials and Methods*. **A**, COS-7 cells were transfected with either 50 ng of empty pcDNA3 vector or the $P2Y_1$ receptor ($P2Y_1$-R), and with or without GRK2RGS, RGS2, RGS4, and p115RGS (350 ng). Cells were then incubated for 45 min with 10 mM LiCl in the absence of agonist (Control) or presence of 10 μM 2MeSADP. [Note that overexpression of the $P2Y_1$ receptor results in increased [^{3}H]inositol phosphate

production in the absence of exogenous agonist application because of autocrine release by cells of the cognate adenine nucleotide agonist [245]. **B**, COS-7 cells were transfected with either 50 ng of empty pcDNA3 vector or the $S1P_3$ receptor ($S1P_3$-R), and with or without GRK2RGS, RGS2, RGS4, and p115RGS (550 ng). Cells were then incubated for 45 min with 10 mM LiCl in the absence of agonist (Control) or presence of 10 μM sphingosine-1-phosphate (S1P). Data shown are mean ± S.D. for duplicate samples in one experiment. Basal levels of inositol phosphate production in the presence of empty pcDNA3 vector alone (approximately 2500 cpm) were subtracted from the values presented.

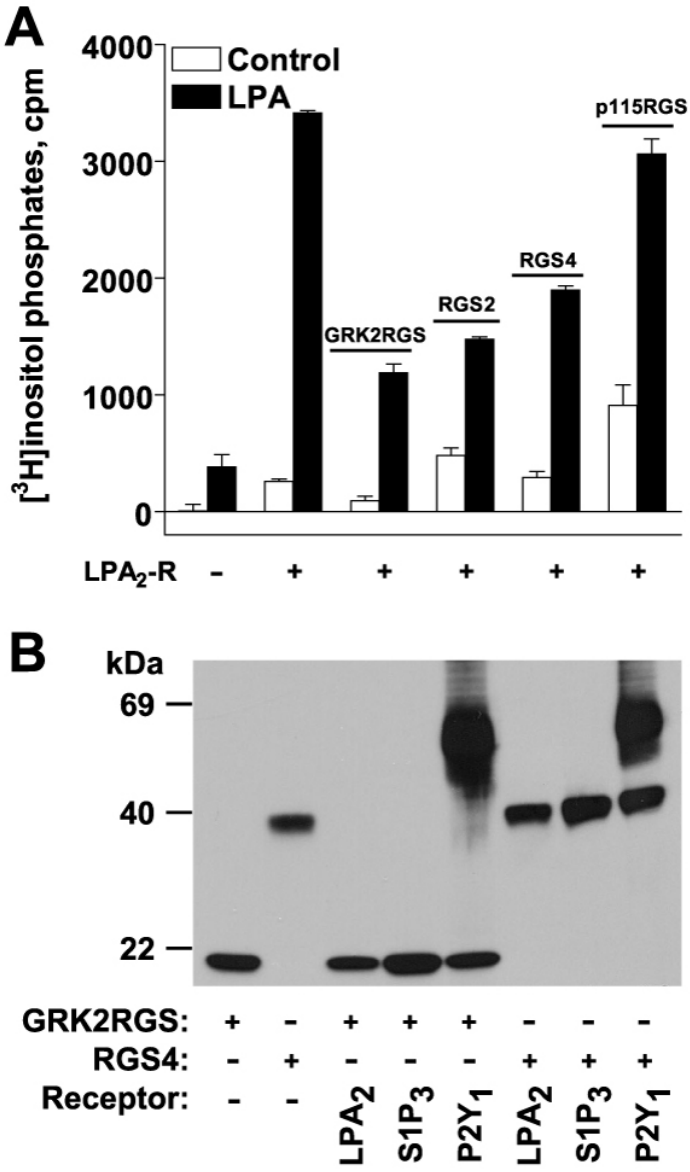

Figure 3.5. Gα$_q$-dependent stimulation of an endogenous PLC by the LPA$_2$ receptor is inhibited by GRK2RGS, RGS2, and RGS4. COS-7 cells were transfected with the indicated DNA (supplemented to 400 ng), and [^{3}H]inositol phosphate accumulation was measured 36 h later as described in *Materials and Methods*. **A**, COS-7 cells were transfected with either 50 ng of empty pcDNA3 vector or the LPA$_2$ receptor (LPA$_2$-R), and with or without GRK2RGS, RGS2, RGS4, and p115RGS (350 ng). Cells then were incubated for 45 min with 10 mM LiCl in the absence of agonist (Control) or presence of 10 μM LPA. Data shown are mean ± S.D. for duplicate samples in one experiment. Basal levels of [^{3}H]inositol phosphate production in the presence of empty pcDNA3 vector alone (approximately 2000 cpm)

were subtracted from the values presented. **B**, COS-7 cells were transfected with either 100 ng of empty pcDNA3 vector, the LPA_2 receptor, the $S1P_3$ receptor, or the $P2Y_1$ receptor with or without 550 ng of GRK2RGS or RGS4. COS-7 cells were lysed in 500 μL of lysis buffer (150 mM NaCl, 20 mM Tris·HCl pH 7.5, 2 mM EDTA, 1% Triton X-100, 0.1% protease inhibitors), sonicated for 5 min, diluted 1:1 in 2.5x Laemmli sample buffer, and boiled for 5 min. Proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose, and detected by a rat monoclonal anti-HA-peroxidase high affinity antibody (clone 3F10; Roche Diagnostics Corporation, Indianapolis, IN) and enhanced chemiluminescence (Amersham Biosciences). Note that, of the three receptors co-transfected with HA-tagged GRK2RGS or HA-tagged RGS4, the LPA_2 and $S1P_3$ receptors were not epitope-tagged; however, the purinergic $P2Y_1$ receptor was N-terminally HA-tagged and thus appears in the anti-HA immunoblot as a broad band centered about 60 kDa.

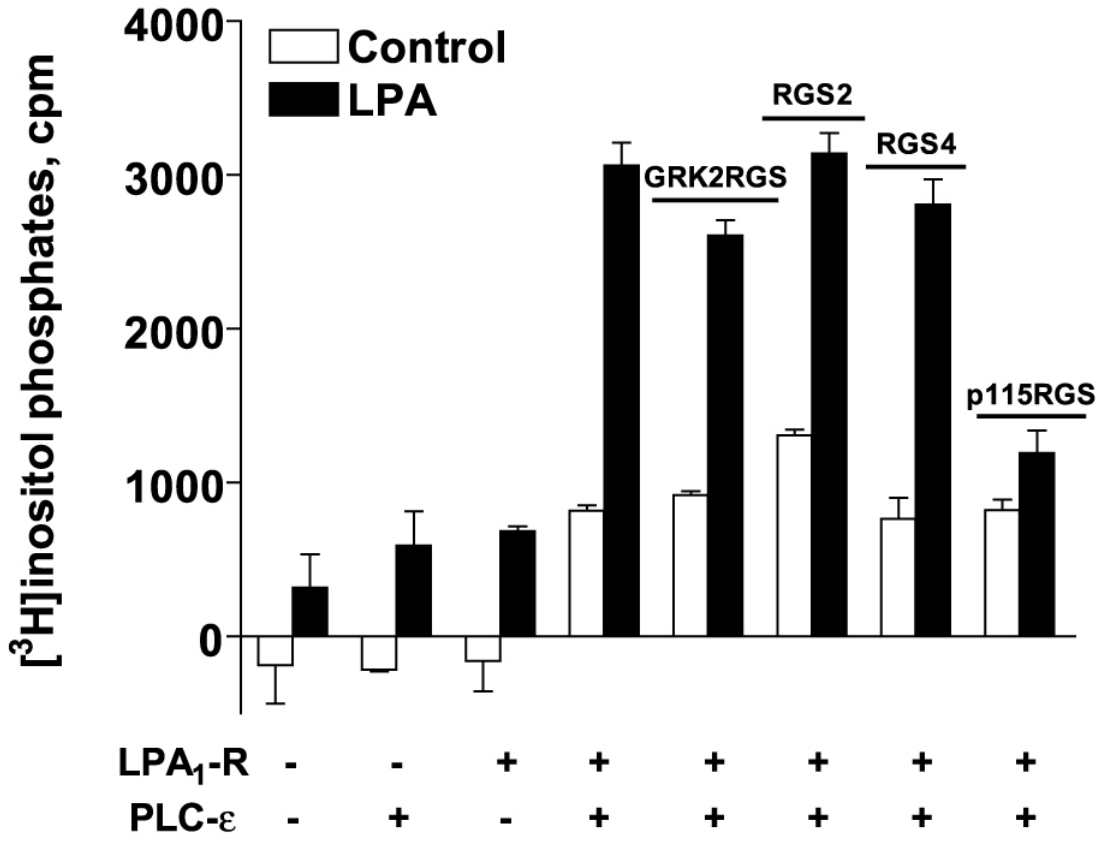

Figure 3.6. Inhibition of LPA_1 receptor-mediated activation of PLC-ε by the RGS domain of p115 RhoGEF. COS-7 cells were transfected with the indicated DNA (supplemented to 700 ng total DNA with empty vector), and [^{3}H]inositol phosphate accumulation was measured 36 h later as described in *Materials and Methods*. COS-7 cells were transfected with either 50 ng of empty pcDNA3 vector or the LPA_1 receptor (LPA_1-R), with or without PLC-ε (100 ng), and with or without individual RGS-box expression vectors (550 ng) as indicated. Cells then were incubated for 45 min with 10 mM LiCl in the absence of agonist (control) or presence of 10 μM LPA. Data shown are mean ± S.D. for duplicate samples in one experiment. Basal levels of inositol phosphate production in the presence of empty pcDNA3 vector alone (approximately 3000 cpm) were subtracted from the values presented.

CHAPTER 4

SELECTIVE ROLE FOR RGS12 AS A RAS/RAF/MEK SCAFFOLD IN NERVE GROWTH FACTOR-MEDIATED DIFFERENTIATION

4.1 Abstract

Regulator of G-protein signaling (RGS) proteins accelerate GTP hydrolysis by heterotrimeric G-protein alpha subunits and thus inhibit signaling by many G protein-coupled receptors. Several RGS proteins have a multidomain architecture that adds further complexity to their roles in cell signaling in addition to their GTPase-accelerating activity. RGS12 contains a tandem repeat of Ras-binding domains but, to date, the role of this protein in Ras-mediated signal transduction has not been reported. Here we show that RGS12 associates with the nerve growth factor (NGF) receptor tyrosine kinase TrkA, activated H-Ras, B-Raf, and MEK2 and facilitates their coordinated signaling to prolonged ERK activation. RGS12 is required for NGF-mediated neurite outgrowth of PC12 cells, but not outgrowth stimulated by basic fibroblast growth factor. These data suggest that RGS12 may play a critical, and receptor-selective, role in coordinating Ras-dependent signals that are required for promoting and/or maintaining neuronal differentiation.

4.2 Introduction

Extracellular stimuli, such as neurotransmitters, hormones, chemokines, inflammatory mediators, odorants, and light, are recognized by G protein-coupled receptors (GPCRs), one of the most abundant and diverse protein families in the

nervous system [263]. Intercellular communication via GPCRs and G-protein-mediated signaling pathways in the nervous system is crucial for normal brain development and the regulation of adult neural processes [263]. Thus, defining the molecular determinants that control GPCR signaling is important to understanding the normal development and physiology of the nervous system and the pathophysiology of nervous system-related disorders.

One level of regulation on GPCR signaling is mediated by the action of "regulators of G-protein signaling" (RGS) proteins. RGS proteins accelerate $G\alpha$-mediated GTP hydrolysis and profoundly inhibit signaling by many GPCRs [23]. For example, mice lacking RGS9 show enhanced responses to both photonic stimulation and morphine administration, suggesting that RGS9 is a potent negative regulator of both rhodopsin and opioid receptor signal transduction *in vivo* [264, 265]. In general, however, contributions by RGS proteins to the physiological control of specific receptor-mediated signal transduction cascades are only now beginning to be elucidated. The identification of multidomain RGS proteins (reviewed in [112, 266]) has added further complexity to the potential cell signaling roles that RGS proteins play in addition to their inhibition of GPCR signaling via $G\alpha$-directed GTPase-accelerating activity.

RGS12, a member of the R12-subfamily of RGS proteins, is an example of a multidomain RGS protein with numerous signaling regulatory elements [115]. In addition to a central RGS domain, RGS12 contains a PDZ (PSD-95/Discs-large/ZO-1) homology domain, a phosphotyrosine-binding (PTB) domain, tandem Ras-binding domains (RBDs), and a GoLoco ($G\alpha_{i/o}$-Loco) interaction motif [111, 151, 155]. The

PTB domain recruits RGS12 in a phosphotyrosine-dependent manner to the SNARE-binding region of the $Ca_v2.2$ calcium channel [138], thereby determining the rate of desensitization of $GABA_B$-receptor-mediated calcium current inhibition in dorsal root ganglia (DRG) neurons [137]. In contrast to the PTB domain, relatively little is known about the *in vivo* function of other domains within mammalian RGS12. Recently, we examined the spatio-temporal expression patterns of RGS12 during mouse development. The full-length form of RGS12 is expressed predominantly in trigeminal and DRG neurons and muscle in the E14.5 mouse [267], suggestive of a role for mammalian RGS12 in myo- and neurogenesis.

Loco, the *Drosophila* ortholog of mammalian RGS12, is essential for multiple processes in fly development including dorsal/ventral axis formation, neuroblast asymmetric cell division, and nurse cell dumping [161, 162]. The *loco* gene is expressed in lateral glial cells in the developing embryonic central nervous system and is required for correct glial cell differentiation [117]. Normal glial-glial cell contacts are absent in *loco*-deficient flies, which results in a loss of the blood-brain barrier, and subsequent gross locomotor defects in surviving mutants. Relatively little is known about the specific molecular mechanisms underlying glial cell development. Recently, Loco, the GPCR Moody, and the Gα subunits Gαi and Gαo have been found expressed in surface glia; these four proteins are thought to act as part of a common signaling pathway critical for blood-brain barrier formation [163, 164].

The requirement for Loco in glial cell development and for normal locomotor capabilities suggests that mammalian RGS12 may also play a critical role in glial cell

differentiation. Here we demonstrate that RGS12 acts as a scaffold for the ERK mitogen-activated protein kinase (MAPK) cascade *in vivo,* facilitating the formation of a Ras/Raf/MEK/ERK multiprotein complex. Furthermore, RGS12 interacts with the nerve growth factor (NGF)-receptor TrkA and siRNA-mediated downregulation of endogenous RGS12 selectively inhibits NGF-induced neuronal differentiation of PC12 cells. These results suggest that RGS12 may function in promoting a differentiated phenotype by organizing a TrkA, Ras, Raf, MEK, and ERK signal transduction complex.

4.3 Results

RGS12 Interacts with Components of the Mitogen-Activated Protein Kinase Cascade

Beyond two, distinct $G\alpha_i$-subunit interaction domains (a central RGS domain and a C-terminal GoLoco motif [111, 159]), RGS12 has a number of additional protein motifs reminiscent of signal transduction adaptors, scaffolds, and effectors (Figure 4.1A): N-terminal PDZ and PTB domains and a tandem repeat of RBDs [111, 137, 151, 159]. To identify additional protein interactors of RGS12, we performed separate yeast two-hybrid screens of mouse embryo and mouse brain cDNA libraries using an N-terminal span encompassing the PDZ and PTB domains and an internal span encompassing the RGS domain and tandem RBDs (Figure 4.1A). Two interactors from the mouse brain library were obtained with the PDZ/PTB domain bait and validated (Figure 4.1B) as bait-specific: the C-terminal tail of mouse SAP90/PSD-95-associated protein-3 (SAPAP3/Dlgap3; [268]) and the C-terminal tail

of MEK2, a middle-tier protein kinase ("MAP2K") within the extracellular signal-regulated kinase (ERK) cascade [269].

Both SAPAP3 and MEK2 end in canonical class I PDZ-binding targets (SAPAP3: –Q-T-R-L-COOH; MEK2: –R-T-A-V-COOH), suggesting that both interactors bind the RGS12 N-terminus via its PDZ domain. We previously established that the RGS12 PDZ domain recognizes class I PDZ-binding targets ending in the general consensus of Thr-x-[Leu/Val]-COOH [111]. Surface plasmon resonance biosensor studies indicated that the final 16 amino-acids of MEK2 were sufficient to bind directly to the RGS12 PDZ domain *in vitro* (Figure 4.2). The interaction with MEK2 was specific for the PDZ domain of RGS12, as cellular co-immunoprecipitation was only seen between full-length MEK2 and protein fragments containing the RGS12 PDZ domain (Figure 4.1D); neither the first PDZ domain of the *Drosophila* phototransduction scaffold InaD nor the PTB domain of mouse Numb were capable of co-immunoprecipitating MEK2. We also assessed the specificity of RGS12 for MEK2 versus other MAP2K members, and found that RGS12 selectively interacts with MEK2 in cellular co-immunoprecipitation experiments (data not shown).

In a separate yeast two-hybrid screen of a mouse embryo cDNA library using an RGS12 fragment from the RGS domain to the second RBD, we identified a fragment of A-Raf (aa 36-153) that spans the RBD and cysteine-rich C1 (or "CRD") domains which reside N-terminal to the catalytic kinase domain [270] (Figure 4.1C). Subsequent cellular co-immunoprecipitation studies showed that full-length A-Raf *and* B-Raf (Figures 4.1E and 4.1F), but not c-Raf-1 (Figure 4.3), associate with full-

length RGS12; indeed, the interaction with B-Raf is observed upon expressing a minimal fragment of RGS12 containing solely the tandem RBDs (Figure 4.1F). Similar to A-Raf, residues within the N-terminus of B-Raf appear to mediate binding to RGS12, as fragments of B-Raf spanning the first 374 amino-acids efficiently co-immunoprecipitated with full-length RGS12 (Figure 4.4).

RGS12 Preferentially Binds to Activated H-Ras in Mammalian Cells

The association of RGS12 with Raf and MEK proteins (two tiers of the Ras-regulated ERK signaling cascade), coupled with the presence of two Ras-binding domains (RBDs) within RGS12, led us to test whether RGS12 also interacts directly with Ras-family GTPases. RBDs are found in Ras effector proteins such as RalGDS, phosphoinositide-3' kinase, and the Raf kinases that bind preferentially to activated Ras GTPases [141]. We therefore screened several wildtype and constitutively-activated Ras family GTPases for RGS12 association. Full-length RGS12 preferentially co-immunoprecipitated with H-Ras, and not K-, M-, or R-Ras isoforms. Furthermore, this interaction was specific to the activated form (Figure 4.5A and B). In some instances, we observed binding of RGS12 to activated N-Ras (Figure 4.5A); however, when detected, this interaction was substantially weaker than that observed with H-Ras. The association between RGS12 and active H-Ras appears to be mediated solely by the tandem RBD region of RGS12 (Figure 4.5C). The interaction between full-length RGS12 and activated H-Ras is diminished by point mutation of His-995, within the first RBD of RGS12, to Leu (H995L; Figure 4.5D), a position analogous to the loss-of-function R89L mutation that abrogates c-Raf-1 binding to H-Ras [271] (Figure 4.6). However, the H995L mutation to RGS12 does

not interfere with its interaction with B-Raf (Figure 4.5E), indicating that the H995L variant of RGS12 is properly folded. The RGS12 paralog RGS14 is reported to interact with isoforms of Rap GTPase [152-154]; however, no interaction of RGS12 with Rap1A, Rap1B, nor Rap2B was observed in co-immunoprecipitation experiments (Figure 4.7).

RGS12 Coordinates and Enhances Signaling to ERK Activation

The ability of RGS12 to interact with activated H-Ras and the first two tiers of the Ras-regulated ERK signaling cascade suggests a role for this protein as a MAPK scaffold facilitating receptor signaling to ERK activation. We previously demonstrated that RGS12 associates with the PDGFβ receptor in an agonist-independent fashion [18]; therefore, we measured endogenous PDGFβR signaling in CHO-K1 cells [272] in the presence of increasing RGS12 expression levels. RGS12 expression modulated PDGF-BB-stimulated activation of ERK2 phosphorylation in a concentration-dependent, biphasic manner (Figure 4.8A) – namely, an increase in phospho-ERK2 levels upon low RGS12 expression, transitioning to a decrease to basal phospho-ERK2 levels upon higher RGS12 expression. These results are reminiscent of "combinatorial inhibition": a hallmark of signaling scaffolds that arises because, at low scaffold concentrations, signaling components are in excess and formation of a functional complex is likely to occur, but when scaffold is in excess, non-functional complexes with less than the full complement of components become more prevalent [197, 198]. This biphasic effect of RGS12 expression was selective for PDGFβR signaling in the CHO-K1 cells tested, as no inhibition of EGF-mediated

ERK2 activation was seen even at the highest levels of ecotopic RGS12 expression (data not shown).

To investigate the interaction of RGS12 with multiple components of the Ras/MAPK signaling pathway, we co-expressed RGS12 and activated Ras GTPases with Raf kinase isoforms A-Raf, B-Raf, or c-Raf-1, and examined the ability of RGS12 to bind to Ras. RGS12 does not interact with activated R-Ras (Figure 4.5A); however, activated H-Ras and R-Ras both interact with all three Raf isoforms (reviewed in [273] and our own unpublished observations). Activated R-Ras did not co-immunoprecipitate with RGS12 in the absence or presence of any of the three Raf kinases (Figure 4.8B). In contrast, the amount of H-Ras bound to RGS12 increased upon concomitant expression of B-Raf, but not A-Raf nor c-Raf-1 (Figure 4.8B), suggesting that these binary interactions (H-Ras/RGS12, RGS12/B-Raf, and H-Ras/B-Raf) might be mutually supportive and/or a trimeric complex of all three proteins exists.

In addition to H-Ras and B-Raf, RGS12 interacts with MEK2 (Figure 4.1B and D); thus, we examined whether a complex of all four proteins might assemble in cells. As shown in Figure 4.8C and D, activated H-Ras (G12V mutant), B-Raf, and MEK2 were each detected in RGS12 immunoprecipitates upon co-expression. Simultaneous expression of MEK2 with either activated H-Ras or B-Raf had no effect on the amount of H-Ras or B-Raf that co-immunoprecipitated with RGS12 (Figure 4.8C). In contrast, co-expression of activated H-Ras enhanced the amount of both B-Raf and MEK2 associating with RGS12 (Figure 4.8C). Amounts of co-immunoprecipitating H-Ras, B-Raf, and MEK2 were additionally enhanced when all

four proteins were simultaneously co-expressed in HEK 293T cells (Figure 4.8C & D). These results suggest that the binding of activated H-Ras to RGS12 facilitates assembly of a multiprotein complex on RGS12 that functionally links H-Ras with the first two-tiers of the MAPK cascade (B-Raf and MEK2).

Several MAPK scaffold proteins have been found to also form macromolecular complexes with the third-tier kinase ERK (*e.g.*, KSR, MP1, and β-arrestin-2; reviewed in [274]. As RGS12 interacts with H-Ras·GTP, B-Raf, and MEK2, we reasoned that RGS12 may also assemble a MAPK complex containing the third-tier kinase as well. We therefore ectopically expressed full-length RGS12 and ERK1, and carried out additional co-immunoprecipitation studies in HEK 293T cells. An interaction between RGS12 and ERK1 was not observed when only those two proteins were co-expressed (Figure 4.8D); however, when co-expressed with activated H-Ras, B-Raf, and MEK2, ERK1 was seen to co-immunoprecipitate with RGS12 (Figure 4.8D). These findings further support the hypothesis that RGS12 acts as a scaffold to assemble multiple components of the Ras-activated MAPK cascade.

Loss of RGS12 Inhibits NGF-Mediated Neurite Outgrowth in PC12 Cells

To define the role of RGS12 as a MAPK scaffold, we generated antibodies to identify cell lines that express RGS12 endogenously. Rabbit antisera raised against both the N-terminus of RGS12 and RGS domain confirmed expression of full-length RGS12 in PC12 rat pheochromocytoma cells (Figure 4.9). RGS12 co-immunoprecipitated with both endogenous H-Ras and B-Raf in PC12 cells (Figure 4.10A), but not with Rap1. These results suggest that a multiprotein complex of

RGS12 with specific MAPK pathway members exists between endogenous components in PC12 cells.

Nerve growth factor (NGF) stimulation of the NGF receptor, TrkA, causes terminal differentiation, growth inhibition, and neurite formation in PC12 cells [275, 276]. NGF induces rapid and sustained activation of both Ras and ERK, and inhibition of either Ras or ERK blocks neurite induction [277]. Thus, NGF-induced neurite formation is mediated by Ras activation of the ERK MAPK cascade. To address a possible role for RGS12 in NGF-induced neurite formation, we employed rat RGS12 directed-siRNA to suppress endogenous RGS12 expression. A pool of four individual duplexes efficiently reduced RGS12 expression (Figure 4.11), and upon separation, all four individual oligonucleotides were found to efficiently knockdown expression of RGS12 (Figure 4.11 and Figure 4.10D inset). RNAi suppression of RGS12 expression impaired NGF-mediated neurite formation when compared to cells treated with control siRNA (Figure 4.10B); this led to a significant reduction in the average length of NGF-promoted neurites over 48 hr compared to cells transfected with non-specific siRNA ($p < 0.0001$) (Figure 4.10D). To highlight this inhibition, cells were also stained (Figure 4.10C) with a marker specific for neuronal differention, β-tubulin isoform III [221]. These results suggest that RGS12 positively regulates NGF signaling during PC12 cell neuritogenesis.

Prolonged ERK Activation by NGF is Reduced Upon RGS12 Depletion

Prolonged ERK activation promotes PC12 cell differentiation, whereas transient ERK activation promotes PC12 growth [226, 278, 279]. We thus examined the effect of RGS12 knockdown on ERK activation kinetics in PC12 cells stimulated

with NGF. The duration of ERK activation was shortened by RGS12 knockdown (Figure 4.12A), suggesting a mechanistic explanation for the impairment in NGF-mediated neurite formation seen upon RGS12 depletion (Figure 4.10).

Subcellular localization of MAPK scaffolds, and the proteins that they coordinate, is critical for their function [202]. We previously established that epitope-tagged RGS12 localizes to intracellular punta in primary guinea-pig airway smooth muscle cells and regulates PDGFβR trafficking and PDGF-mediated activation of the MAPK cascade [18]. Similarly, in PC12 cells, YFP-RGS12 and RGS12-YFP fusion proteins both localize to punctate structures, in comparison to YFP alone (Figure 4.12B). To determine whether these punctate structures were endocytic vesicles, we stained the cells with the early endosomal and late endosomal markers, EEA1 and LAMP1, respectively [280, 281]. RGS12-YFP showed co-localization with EEA1 (Figure 4.12C), as well as with LAMP1 (Figure 4.12D), suggesting that overexpressed RGS12 is endosomal.

To explore the expression pattern of endogenous RGS12 protein, we used subcellular fractionation following NGF stimulation of PC12 cells. Detection of RGS12 first occurs in the crude membrane fraction (see the 0 min timepoint in Figure 4.12E), but RGS12 subsequently becomes enriched in both the cytosolic and nuclear fractions after NGF stimulation, with a concomitant decrease in the membrane fraction (Figure 4.12E). In contrast, we observed little change in the localization or protein levels of nuclear and membrane markers (lamin A/C and pan-cadherin, respectively; [282, 283]), suggesting that the movement of endogenous RGS12 from membrane to cytosolic and nuclear fractions is specific.

RGS12 Forms a Multiprotein Complex Containing TrkA, H-Ras, B-Raf, and MEK2

MAPK pathway scaffolds have been identified that promote formation of stable complexes containing receptors [181, 274]. For example, β-arrestin-2 forms a complex with the angiotensin II type 1a receptor and component kinases of the ERK MAPK cascade [28]. RGS12 interacts with activated H-Ras, B-Raf, and MEK2 (Figures 4.1, 4.5, and 4.8), and appears to regulate, and redistribute upon, NGF signaling in PC12 cells (Figures 4.10 and 4.12). Given these findings, we hypothesized that RGS12 may also associate with the primary NGF receptor in PC12 cells, namely TrkA [284]. To test this hypothesis, we transiently expressed full-length RGS12 and TrkA in HEK 293T cells and assayed for protein complex formation (Figure 4.13). RGS12 co-immunoprecipitated with the TrkA receptor, but not with the fibroblast growth factor receptor (FGFR) (Figure 4.13A). Additionally, co-expression of TrkA, but not FGFR, redistributed YFP-RGS12 out of endosomes (Figure 4.13B,C), suggesting that RGS12 subcellular localization is regulated specifically by TrkA. A multiprotein complex containing RGS12, TrkA, activated H-Ras, B-Raf, and MEK2 was detected by co-immunoprecipitation (Figure 4.13D). Taken together, these findings suggest that RGS12 associates with TrkA and potentially tethers the Ras-Raf-MEK signaling module to this receptor.

We did not detect an interaction between RGS12 and FGFR; thus, we investigated whether RGS12 might selectively regulate NGF-mediated but not basic fibroblast growth factor (bFGF)-mediated signaling in PC12 cells. Basic FGF can reproduce the entire spectrum of PC12 cell responses known to be elicited by NGF

including neurite outgrowth [222]. Whereas knockdown of RGS12 attenuated NGF-promoted neurite outgrowth (Figure 4.10), we observed no significant change in neurite outgrowth mediated by bFGF ($p > 0.05$; Figure 4.13E). Consistent with this result, no change in RGS12 subcellular localization was observed upon stimulation of PC12 cells with bFGF for up to 120 min (Figure 4.13E), in contrast to the movement seen upon NGF stimulation (Figure 4.12E). These data strongly suggest that RGS12 is critical for NGF-mediated, but not bFGF-mediated, neurite outgrowth and this may be due, at least in part, to a specific interaction between RGS12 and the NGF receptor TrkA.

4.4 Discussion

The Raf-MEK-ERK cascade is often depicted as a linear signaling pathway that traverses the cytoplasm to connect receptor activation at the plasma membrane with transcriptional effects in the nucleus. However, regulation of this pathway is complex – this kinase cascade lies downstream of a variety of receptors and is involved in many different biological processes [269]. Specificity in receptor-promoted signaling to ERK activation and spatio-temporal regulation of these signals are both achieved by scaffolding proteins. Several MAPK scaffolds have now been identified, with the *S. cerevisiae* protein Ste5 serving as the archetype, coordinating the kinases Ste11 (MAP3K), Ste7 (MAP2K), and Fus3 (MAPK) for efficient, pheromone-dependent activation of the MAPK cascade in haploid yeast [167, 175]. Another well-characterized ERK scaffold, kinase suppressor of Ras (KSR), binds c-Raf-1, MEK1/2, ERK1/2, and other proteins [274] and is thought critical to Ras function given that KSR1-deficient fibroblasts are impaired in both Ras-mediated

ERK activation and cell transformation [285]. Our data suggest that RGS12, analogous to these established functions of Ste5 and KSR, sustains NGF-mediated ERK activation in PC12 cells by forming a signaling complex containing TrkA, H-Ras, B-Raf, MEK2, and ERK proteins.

MAPK scaffolds not only tether proteins together, but also specify their subcellular localization and guide their ultimate output. In tethering Ste11, Ste7, and Fus3 through independent binding sites [178], Ste5 recruits these kinases to the plasma membrane – the site at which upstream proteins participate in pheromone-dependent activation of the MAPK cascade [179]. In this way, Ste5 not only increases signaling efficiency through the MAPK cascade, but also generates specificity by insulating the pheromone response pathway from other parallel pathways (nutritional cues, osmotic stress) that use some or all of these same signaling components [175]. The ubiquity of these signaling components is even more dramatic in mammalian cell signaling, as underscored by the fact that over 160 substrates for the final-tier kinases ERK1/2 have now been described. The majority of these ERK substrates are found in the nucleus; however, numerous others exist in the cytosol and at other organelles, including endosomes [286, 287]. Scaffold-mediated regulation of the utilization of particular MAPK substrates contributes to the distinct, and at times opposing, cellular events that are regulated by the ubiquitous MAPK cascade. In this context, our results provide several lines of evidence that suggest RGS12 is a selective scaffold within PC12 cells that regulates prolonged ERK activation downstream of particular inputs and components: *e.g.*, NGF *versus* bFGF, activated H-Ras *versus* related GTPases, and B-Raf *versus* c-Raf-1.

In contrast to its robust association with activated H-Ras, RGS12 was not found to bind significantly to other Ras isoforms. As H-, N-, and K-Ras in particular are highly homologous, it is likely that their unique hypervariable linker domain sequences and differential post-translational modifications, known to sequester these Ras family members to distinct cellular locales [288], may dictate, in part, the functional differences seen among Ras isoforms for RGS12 association. Ras signaling from internal membrane compartments is well documented, with one well-described mechanism involving endocytosis [289], whereby EGF promotes internalization of active H-Ras, EGFR, and Ras effectors into endosomes. Similarly, the scaffold protein MP1, which binds to MEK1 and enhances ERK1 activation, localizes EGF-promoted Ras/MAPK signaling to late endosomes [202]; MP1 is dispensable for transient EGFR activation of ERK at the plasma membrane, but essential for delayed activation of ERK on late endosomes. The enhancement of MAPK activation by MP1 is strictly dependent on the endosomal localization of the complex, as the artificial mislocalization of MP1 to the plasma membrane abolishes its positive effect. Thus, compartmentalizing Ras at endosomes appears to provide a means for trafficking and promoting MAPK signaling. These results, and our findings that RGS12 localizes to endocytic vesicles and binds multiple MAPK cascade components in addition to activated H-Ras, suggest that RGS12 may be a novel endosomal Ras effector scaffold.

In PC12 cells, transient ERK activation promotes cell proliferation, whereas prolonged ERK activation promotes neuronal differentiation [224, 226, 278, 279]. Furthermore, endosomal localization of MAPK signaling components appear to be

critical for these differences, suggesting that endosomes may serve as the sites that generate persistent, rather than transient, signaling through Ras. Our findings are that RGS12 localizes to endosomes and depletion of RGS12 attenuates prolonged activation of ERK and cellular differentiation of PC12 cells. It has previously been demonstrated that proteins involved in NGF-mediated neurite outgrowth localize to endosomes, and this localization is critical for their participation in neuronal differentiation of PC12 cells [290, 291]. Isolated clathrin-coated vesicles from NGF-treated PC12 cells were found to contain NGF bound to TrkA together with activated signaling proteins of the Ras/MAPK pathway [290]. Additionally, neuronal differentiation is seen to be stimulated by catalytically-active Trk receptors specifically trafficked to endocytic vesicles; if NGF internalization in PC12 cells is inhibited by dominant negative dynamin, neurite outgrowth is blunted [291]. These results suggest that RGS12, by binding TrkA and components of the Ras/MAPK cascade, might serve to coordinate this endosomal MAPK signaling required for prolonged ERK activation and neuritogenesis.

The most compelling context for this new data regarding RGS12 as a putative endosomal scaffold for TrkA-activated MAPK signaling is in the known response of primary neurons to trophic factors. NGF signals through TrkA to promote the differentiation, survival, and maintenance of primary neurons [5, 292]. For neurotrophins to induce these pleiotropic effects, the signal must be communicated from axon terminals to cell bodies. One hypothesis suggests that “signaling endosomes” containing NGF-bound TrkA are formed at the axon terminal and traffic in a retrograde fashion to the cell body where they initiate local signal transduction

cascades [293]; such retrograde transport and signaling of neurotrophins like NGF is thought to be critical to the development and maintenance of the nervous system [5]. Retrograde-transported NGF-containing vesicles are found to contain active components of the ERK pathway *in vivo* [294]. Thus, it is possible that RGS12 represents a key component of the NGF-derived signaling endosome.

In addition to their originally described roles as Gα GAPs and negative regulators of GPCR signaling [266], RGS proteins increasingly appear to serve as scaffolds that integrate signaling from diverse receptors and steer pathway output to divergent cytoplasmic signaling networks [112]. Our observation that RGS12 preferentially binds to activated H-Ras is in line with yeast two-hybrid data that Loco (the fly ortholog of RGS12) interacts with *Drosophila* Ras1 [165], suggesting that RGS12 might constitute an evolutionarily-conserved molecular link that integrates receptor tyrosine kinase signaling with GPCR signaling in both vertebrates and non-vertebrates. Interestingly, the localization of Loco in surface glia is punctate throughout the cytoplasm [164], similar to our own observations in PC12 cells, HEK 293T cells, primary airway smooth muscle cells, and primary DRG neurons (this report, [18], and unpublished results [M.D.W., M. Diversé-Pierluissi, and D.P.S.]). Although the mechanism by which RGS12 localizes to endosomes is unknown, it is possible that the Gα_i-binding activity of the C-terminal GoLoco motif plays a role; we have previously reported a loss-of-function mutation in the GoLoco motif that mislocalizes RGS12 to the nucleus [18]. These findings, as well as those described herein, suggest that the multiple functional domains found within RGS12 may cooperate to define the spatial and temporal nature of a RGS12-coordinated

signaling output initiated from receptor tyrosine kinases and both monomeric and heterotrimeric G-protein subunits.

4.5 Materials and Methods

Cell Culture and Transfection

All cells were cultured at 37°C in 5% CO_2 humidified air. HEK 293T and COS-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. HEK 293T and COS-7 cell cultures, seeded the day before at 800,000 and 160,000 cells per well, respectively of a 6-well dish, were transfected with a total of 1.5 µg plasmid DNA using Fugene 6 (Roche) at a ratio of 3:1 (Fugene:DNA). Empty pcDNA3.1 vector DNA was used to maintain a constant total amount of DNA per well. PC12 cells were maintained in DMEM supplemented with 10% horse serum/5% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Depending on the length of the assay, PC12 cells were seeded at 260,000 or 130,000 cells per well in a 12-well culture dish, and grown for 24 hr. Cells were transfected with a total of 300 ng plasmid DNA per well of a 12-well dish using LipofectAMINE 2000 (L2K) (Invitrogen) at a ratio of 7:1 (L2K:DNA). CHO-K1 cells were maintained in EMEM supplemented with non-essential amino-acids, 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. CHO-K1 cells were seeded at 1.6 x 10^6 cells per 100 mm dish, and grown for 24 hr prior to transfection with a total of 12 µg DNA using Fugene 6 at a ratio of 3:1 (Fugene:DNA).

Immunofluorescence Microscopy

PC12 or HEK 293T cells were plated on PDL-coated chamber slides or coverslips (BD Biocoat) and then transfected the next day and treated as described in the figure legends. Cells were subsequently fixed with 4% (w/v) paraformaldehyde, permeabilized with 100% methanol (-20°C) for 30 s, washed three times with PBS containing 1% (w/v) nonfat dry milk, 150 mM sodium acetate, pH 7, and then blocked in 1% nonfat dry milk/PBS for 15 min. Cells were incubated with primary antibodies for 1 hr at room temperature, or overnight at 4°C, then incubated with either Alexa Fluor 488 or 594 (Invitrogen) secondary antibodies for 1 hr at room temperature in the dark. Cells were then stained with the nuclear stain 7-aminoactinomycin D (7-AAD) [295] (Invitrogen) as per manufacturer's specifications. Cells were then washed four times with PBS, and Fluorsave anti-fade reagent (Chemicon, Temecula, CA) was added to each coverslip before mounting. Confocal images were collected using a Fluoview 300 laser scanning confocal imager (Olympus, Tokyo, Japan) on an IX70 fluorescence microscope with a PlanApo 60x oil objective (Olympus). Fluorescent images, X-Y sections at 0.28 μm, were collected sequentially at 800 x 600 resolution with 2x optical zoom.

Subcellular Fractionation

Subcellular fractionation was performed as described [296], with minor modifications. PC12 cells were grown to 90% confluence in a 100 mm dish, and rinsed two times with PBS. All subsequent fractionation steps were performed at 4°C. Cells were lysed in 2 ml of lysis buffer: 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 300 mM sucrose, 0.05% (v/v) Triton X-100, 17 μg/ml calpain inhibitor I, 7 μg/ml

calpain inhibitor II, 2 mM Na_3VO_4, and protease inhibitors (Roche). Cells were then homogenized by 28 strokes with a 1 ml Wheaton Tenbroeck Tissue Grinder (Fisher). Lysate was then fractionated into nuclear, crude cytoskeletal, cytosol, and membrane fractions. Cells were centrifuged at 800 *x g* for 6 min at 4°C. Supernatant was removed and set aside on ice. Pellet was washed with 1 ml buffer and the 800 *x g* spin repeated twice. Supernatant was discarded, and resultant nuclear fraction pellet was resuspended in 150 μl lysis buffer. The post-nuclear supernatant was centrifuged at 16,300 *x g* for 25 min at 4°C. Supernatant was removed and set aside on ice as membrane and cytosolic material. Pellet was resuspended in 1 ml buffer and re-centrifuged. Supernatant was discarded, and resultant crude cytoskeletal fraction pellet was resuspended in 150 μl lysis buffer. Membrane and cytosolic material was then centrifuged for 18 hr at 48,000 rpm (~100,000 *x g*) at 4°C in a TLA-55 rotor (Beckman Coulter, Inc., Fullerton, CA). Supernatant (cytosolic fraction) was removed and placed in a fresh tube. The membrane fraction pellet was resuspended in 150 μl lysis buffer. All fractions were sonicated in an ice water bath for 5 min, and equalized with a BCA Protein Assay (Pierce).

Immunoprecipitation and Western Blotting

If fractionation was not required, cells were simply lysed 48 hr post-transfection in cold lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1% (v/v) Triton X-100, protease inhibitors; or for phospho-protein detection: 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM NaPPi, 200 μM Na_3VO_4, 1% (v/v) Triton-X-100, protease inhibitors). Cell lysates were sonicated in an ice water bath for 5 min and then centrifuged at 13,000 x *g* for 20 min at 4°C. A

portion of the supernatant was removed and mixed 1:1 with 5x Laemmli sample buffer ("lysate" samples). Remaining supernatant was combined with appropriate antibody and rotated overnight at 4°C. Protein A/G agarose (Santa Cruz) was added 12 hr later. Each sample was then rotated at 4°C for 2 hr, washed four times with 1 ml lysis buffer, eluted with 2.5x Laemmli sample buffer, boiled for 5 min, subjected to SDS-PAGE and transferred to nitrocellulose. Western blotting was performed using aforementioned primary antibodies, secondary anti-mouse or -rabbit IgG antibody-HRP conjugates (GE Healthcare), and enhanced chemiluminescence (ECL™, ECL Plus™, or SuperSignal® West Femto from GE Healthcare and Pierce, respectively).

Surface Plasmon Resonance (SPR) Biosensor Measurements

SPR binding assays were performed at 25°C on a BIAcore 3000 (BIAcore Inc., Piscataway, NJ) at the UNC Department of Pharmacology Protein Core Facility. N-terminally biotinylated, synthetic peptides of the MEK2 C-terminus (wildtype: RTLRLKQPSTPTRTAV-COOH; "triple-alanine" tail mutant: RTLRLKQPSTPTRAAA-COOH) were separately bound to streptavidin-coated sensor surfaces (Sensor Chip SA, BIAcore) as per manufacturer's instructions to a density of 400 resonance units (RU). Binding analyses were performed using HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Surfactant P20) as the running buffer. Purified His_6-RGS12-PDZ/PTB or GST-RGS12-PDZ fusion proteins [138] were diluted in HBS-EP buffer and injected at a 20 μl/min flow rate over all test flow-cell surfaces simultaneously using the KINJECT command. Surface regeneration was performed, when necessary, using 20 μl injections of 1 M NaCl/50 mM NaOH at a flow rate of 20 μl/min. Binding curves were generated using BIAevaluation software

version 3.0 (BIAcore Inc.) and plotted using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA).

Plasmids, Antibodies, Agonists, and other Materials

cDNA encoding human MEK1 and MEK2 were subcloned in frame with an N-terminal FLAG-tag into pCMV2 (Eastman Kodak). pcDNA3.1-based expression constructs encoding N-terminal myc-His_6 tagged full-length RGS12 and tandem RBD domain region (aa 961-1105 of GenBank AAC53176) were generated by heterostagger PCR cloning [297]. Site-directed mutagenesis was performed using the QuikChange system (Stratagene, La Jolla, CA) to generate the histidine-995-to-leucine mutant of RGS12 and the glycine-12-to-valine mutant of Rap2B. Rat RGS12 was subcloned in-frame with either an N-terminal or a C-terminal YFP-epitope tag into pEYFP-C1 or pEYFP-N1 (Clontech), respectively. 7-aminoactinomycin D (7-AAD) and all Alexa Fluor conjugates were from Invitrogen (Carlsbad, CA). Receptor agonists were obtained as follows: Nerve Growth Factor 2.5S (mouse NGF) (Roche), human epidermal growth factor (Sigma), human basic fibroblast growth factor (Sigma), and human platelet derived growth factor BB homodimer (Upstate).

Yeast Two-Hybrid Screens

Yeast two-hybrid screening with rat RGS12 baits was performed at the Molecular Interaction Facility of the University of Wisconsin (Madison) Biotechnology Center. To confirm initial positives, pairwise mating of yeast expressing bait or prey plasmids was performed in interaction-selection medium (histidine/adenine dropout plus 1 mM 3AT). Yeast were subsequently lysed and incubated for several hr at 37^{o}C with chlorophenyl red-β-D-galactopyranoside as chromogenic substrate for the β-

galactosidase reporter of bait/prey interaction. Test baits included empty vector (pBUTE) and the following proteins cloned in-frame with the GAL4 DNA-binding domain: mouse RGS12 PDZ/PTB domain N-terminus (aa 1-369), rat RGS12 spanning the RGS domain and both RBDs (aa 702-1115), the EF-hand or coiled-coil domains of intersectin, and the human SKP1 homolog p19. Test prey included empty GAL4 activation domain vector (pGADC1), MEK2 C-terminus (aa 324-401), SAPAP3/Dlgap3 C-terminus (aa 814-977), an N-terminal portion of the MAP3K kinase A-Raf (aa 36-153), mouse Epsin, and human F-box 5 protein. The latter two prey serve as positive controls for interaction with the ITSN EF-hand domain and p19 baits, respectively [298, 299].

Small Interfering RNA (siRNA) Transfection

PC12 cells were plated the day before transfection at 130,000 or 260,000 per well in 12-well culture dishes coated with poly-D-lysine (PDL; BD Biocoat, Franklin Lakes, NJ) and then transfected with 150 nM siRNA using L2K according to the manufacturer's protocol. Rat RGS12 siRNAs (both SMARTpool and constituent individual duplexes) were from Dharmacon Research (Lafayette, CO). The non-specific siRNA (5'-CUA CGU CCA GGA GCG CAC C -3') was used as a negative control [300].

PC12 Differentiation Assays

PC12 cells were transiently transfected with non-specific siRNA or RGS12 siRNA for 24 hr, and then treated with 100 ng/ml NGF or bFGF (in 1% serum-containing medium) for an additional 48 hr. Brightfield images were obtained using an Olympus IX70 with sideport Q-Fire CCD camera. To quantitate differences in neurite length,

more than 100 cells were counted per condition in three independent experiments using the ImageJ Measure feature (http://rsb.info.nih.gov/ij/). These measurements were imported into Prism 4.0 (GraphPad, San Diego, CA) and length was converted into μm.

4.6 Acknowledgements

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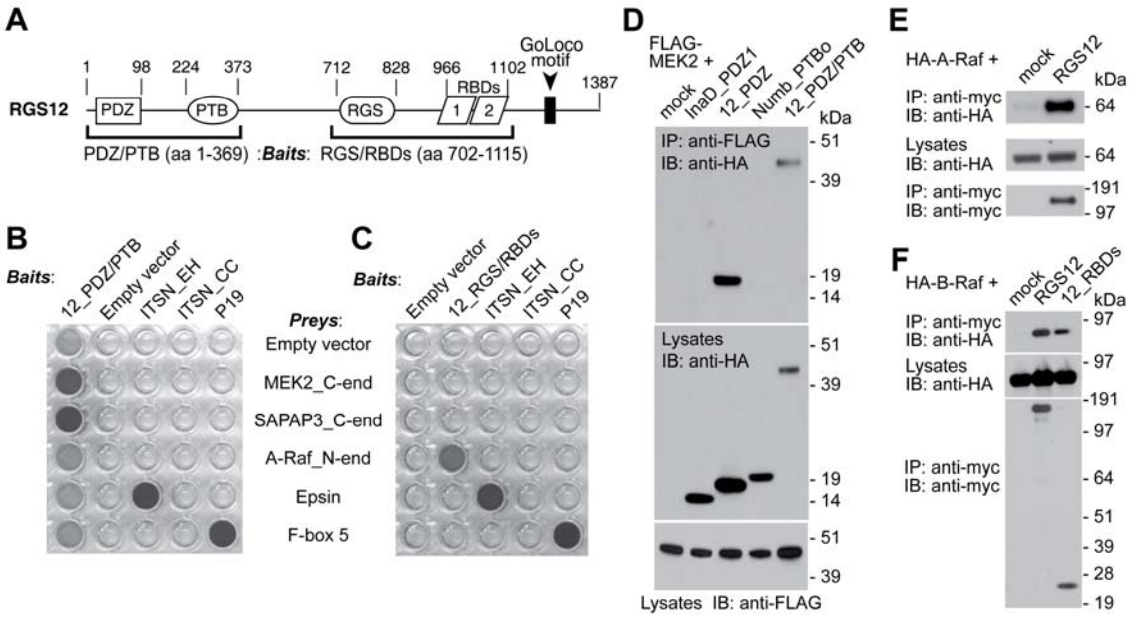

Figure 4.1. MAP3K and MAP2K components of the mitogen-activated protein kinase (MAPK) cascade identified as RGS12 interactors. (**A**) Multidomain architecture of RGS12. Numbering denotes amino acid boundaries of protein domains within rat RGS12. Square brackets denote regions of RGS12 used as baits in separate yeast two-hybrid screens against a mouse embryo/brain cDNA library. (**B, C**) Pairwise mating of yeast, expressing indicated bait or prey plasmids, was performed as described in *Materials and Methods* to confirm initial positives from the yeast two-hybrid screen. Yeast were incubated for 7 hr (panel B) or 16 hr (panel C) at 37^{o}C with chlorophenyl red-β-D-galactopyranoside to detect the β-galactosidase reporter of bait/prey interaction. (**D**) HEK 293T cells were transiently co-transfected with FLAG-MEK2 and empty vector ("mock"), or the indicated HA-tagged protein domain expression constructs. Anti-FLAG antibody was used to immunoprecipitate ("IP") MEK2 from resultant cell lysates. Co-immunoprecipitating proteins and total cell lysates were separately resolved by SDS-PAGE and immunoblotted ("IB") with anti-FLAG and anti-HA antibodies as indicated. (**E**) HA-A-Raf was co-transfected with empty vector or full-length, myc-RGS12 in COS-7 cells. (**F**) HA-B-Raf was co-transfected with empty vector, full-length myc-RGS12, or the isolated tandem RBDs of RGS12 ("12_RBDs") in COS-7 cells. In both panels E and F, anti-myc antibody was used to immunoprecipitate RGS12. Co-immunoprecipitating proteins and total cell lysates were then immunoblotted for expression with anti-myc and anti-HA antibodies.

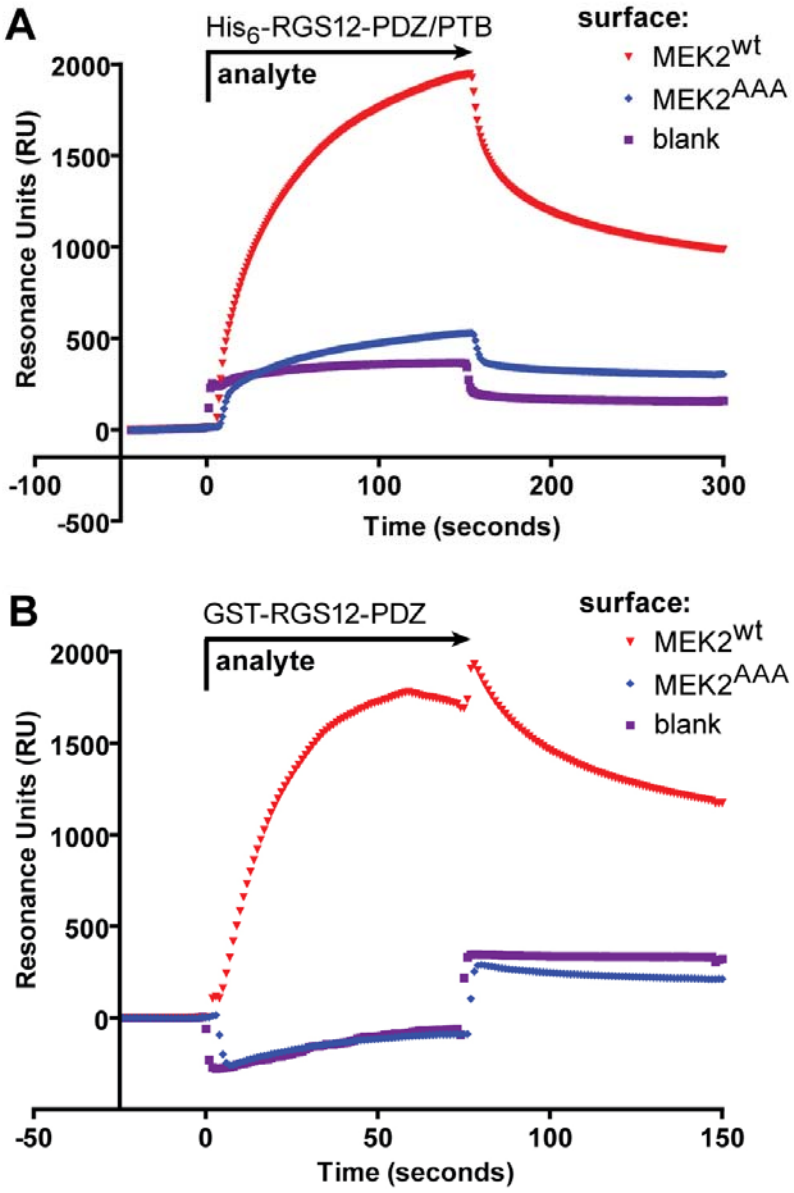

Figure 4.2. RGS12-derived PDZ/PTB tandem and PDZ domain fusion proteins specifically interact with the wildtype MEK2 C-terminal tail *in vitro*. Simultaneous SPR measurements of binding to a biotinylated peptide comprising the last 16 amino acids of MEK2 (wt), to the same peptide but ending with triple-alanine (AAA), and to a blank flow cell after injection (time 0 s, flow rate 20 μl/min) of 50 μl of 5 μM His_6-RGS12-PDZ/PTB fusion protein (panel **A**) or 50 μl of 5 μM GST-RGS12-PDZ fusion protein (panel **B**) at 25°C.

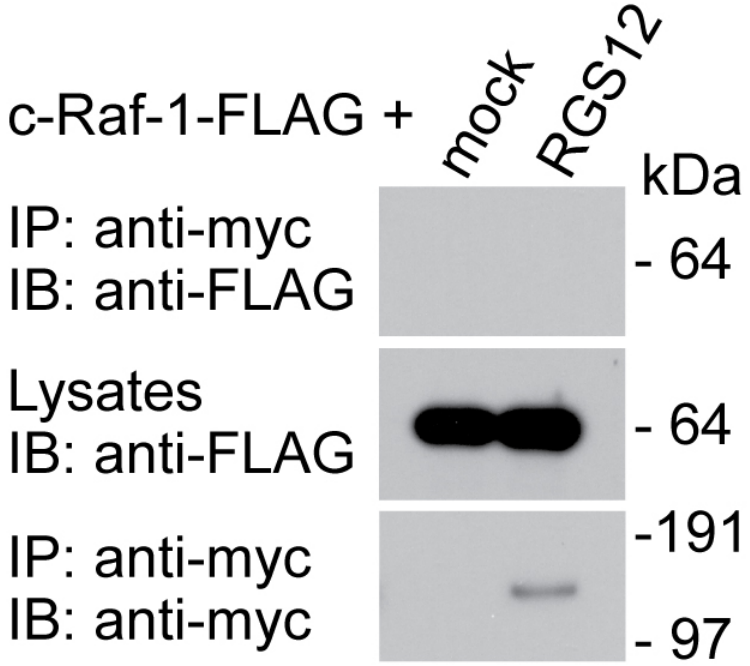

Figure 4.3. c-Raf-1 does not co-immunoprecipitate with RGS12. FLAG-epitope tagged c-Raf-1 and myc-epitope tagged, full-length RGS12 were transiently co-expressed in HEK 293T cells. Cell lysates obtained 48 hr after transfection were immunoprecipitated (IP) with anti-myc antibody; IP samples and cell lysates were subsequently immunoblotted (IB) as indicated above.

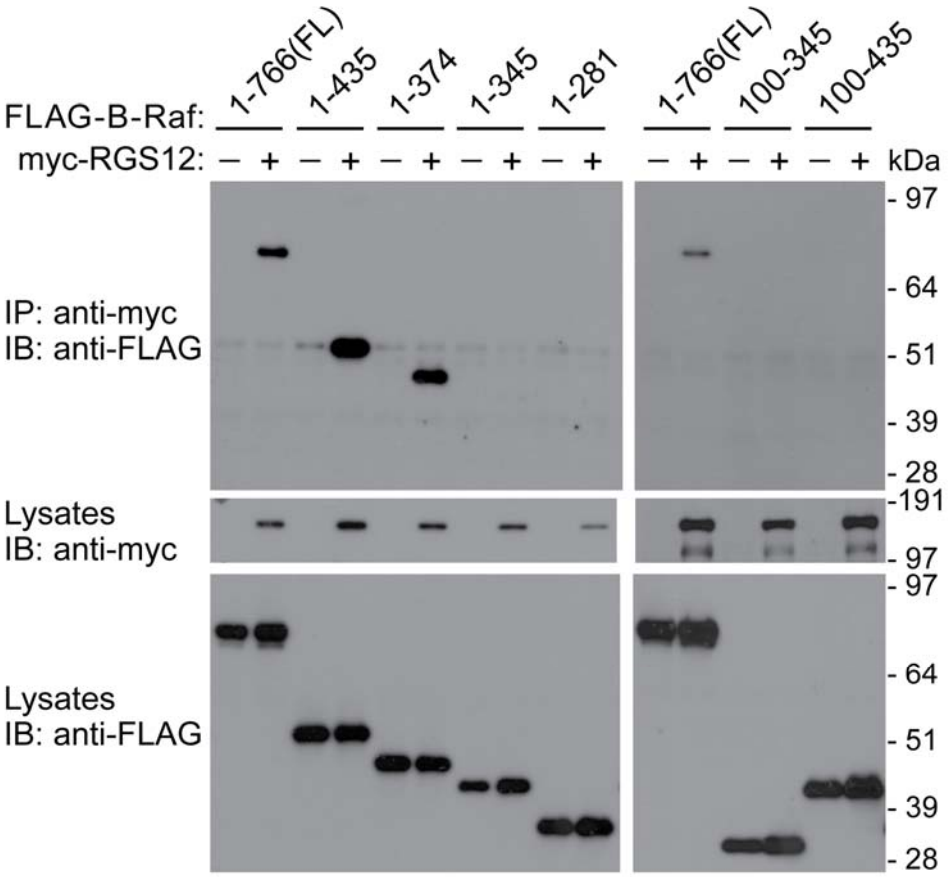

Figure 4.4. The N-terminal 374 amino-acids of B-Raf co-immunoprecipitate with RGS12. FLAG-epitope tagged B-Raf truncation mutants and myc-epitope tagged, full-length RGS12 were transiently co-expressed in HEK 293T cells. Cell lysates obtained 48 hr after transfection were immunoprecipitated (IP) with anti-myc antibody; IP samples and cell lysates were subsequently immunoblotted (IB) as indicated above.

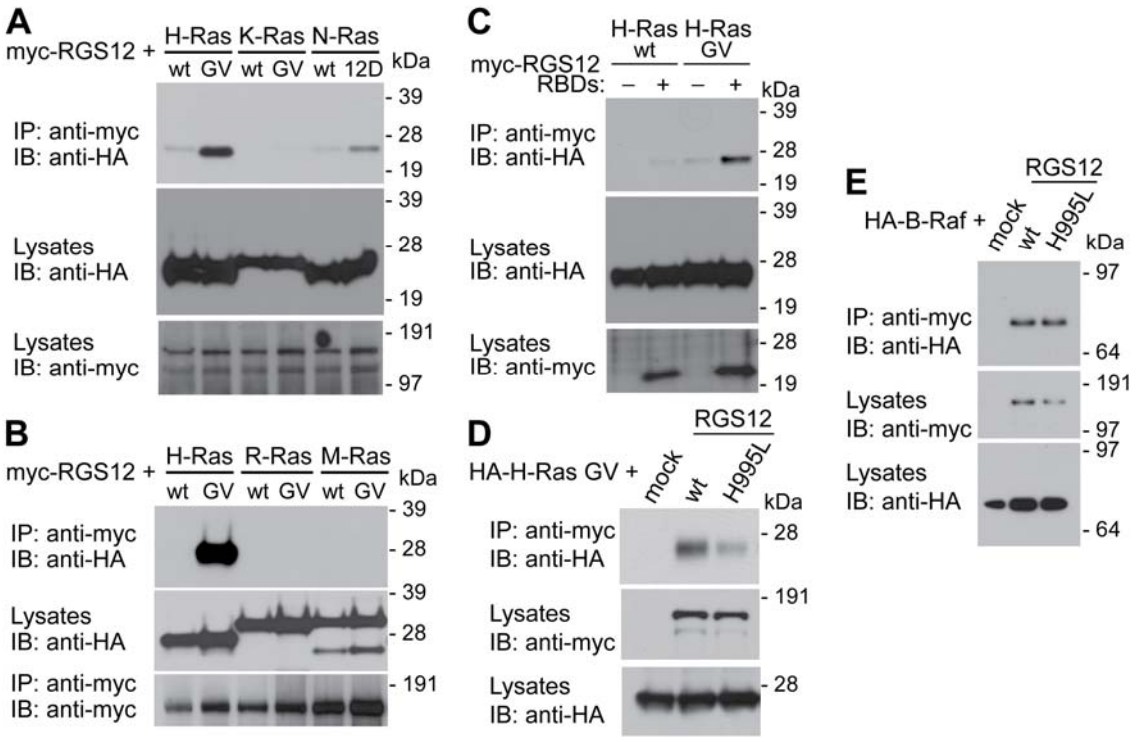

Figure 4.5. Full-length RGS12 and its isolated tandem RBDs preferentially interact with activated H-Ras. (**A, B**) HEK 293T cells were transiently co-transfected with full-length, myc-RGS12 and the indicated HA-Ras plasmids. Anti-myc antibody was used to immunoprecipitate RGS12 and anti-HA antibody was used to detect associated HA-Ras proteins via immunoblotting. (**C**) Wildtype or activated (G12V) HA-H-Ras was co-expressed with empty vector or myc-tagged, isolated tandem RBD region of RGS12 in HEK 293T cells. Lysates were immunoprecipitated and immunoblotted as described in panels A and B. (**D, E**) Activated (G12V) HA-H-Ras (D) or HA-B-Raf (E) was co-expressed with empty vector, full-length wildtype myc-RGS12 (wt), or histidine-995-to-leucine mutant myc-RGS12 (H995L) in HEK 293T cells. Lysates were immunoprecipitated and immunoblotted as described in panels A and B.

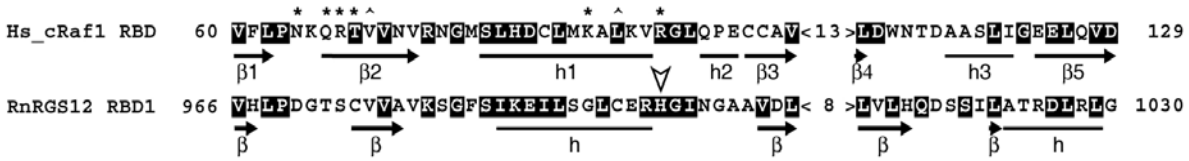

Figure 4.6. Predicted structural similarity between the Ras-binding domain (RBD) of human c-Raf-1 with the first, N-terminal RBD of rat RGS12. Comparison of known secondary structure of human c-Raf-1 (Protein Data Bank id 1GUA) with the predicted secondary structure of the first RBD within rat RGS12, the latter as derived from the 3D-PSSM protein fold recognition server (http://www.sbg.bio.ic.ac.uk/3dpssm/). Asterisks (*) and carats (^) denote c-Raf-1 residues involved in polar or hydrophobic interactions, respectively, with the effector-binding domain of bound Ras-family GTPase (PDB 1GUA). β, beta-strand secondary structure; h, alpha- or 3_{10}-helical structure. Open arrowhead denotes position of histidine-995 residue mutated to leucine within rat RGS12 to create a loss-of-function for Ras GTPase binding.

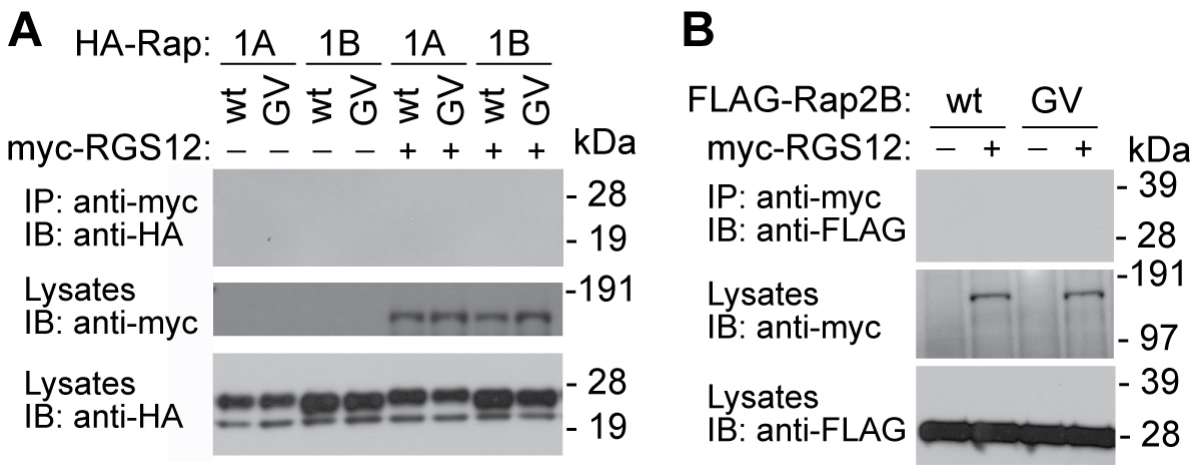

Figure 4.7. Rap-family GTPases do not co-immunoprecipitate with RGS12. Wildtype (wt) and constitutively-activated (GV), HA-epitope tagged Rap1A and Rap1B (panel **A**) or FLAG-epitope tagged Rap2B (panel **B**) were transiently co-expressed in HEK 293T cells along with myc-epitope tagged, full-length RGS12. Cell lysates obtained 48 hr after transfection were immunoprecipitated (IP) with anti-myc antibody; IP samples and cell lysates were subsequently immunoblotted (IB) as indicated above.

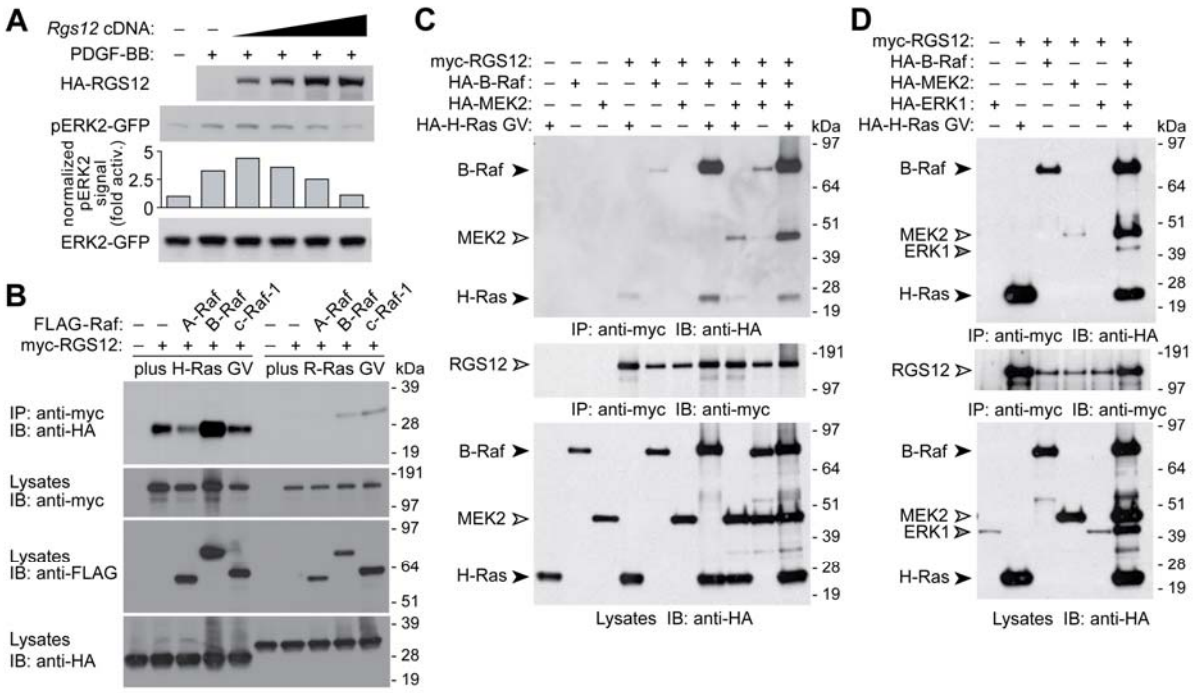

Figure 4.8. RGS12 expression coordinates a Ras/Raf/MEK/ERK complex and enhances signaling to ERK. (**A**) CHO-K1 cells expressing endogenous PDGFβR were transiently transfected with the reporter construct ERK2-GFP and either empty vector or increasing amounts of full-length, HA-RGS12 expression vector. Cells were serum starved overnight, treated with PDGF-BB (10 ng/ml) for 5 min, and resultant ERK2 activation determined by immunoblotting cell lysates with an anti-phospho-ERK1/2 antibody. Scion Image was used for densitometric quantitation of pERK2 and total ERK2-GFP levels to generate the bar graph of normalized pERK2 signal (i.e., fold activation above basal signal in the absence of PDGF-BB). *Bottom panel*: Total ERK2-GFP levels were immunoblotted with anti-ERK1/2 antibody. (**B**) HA-tagged, activated H-Ras (G12V) (*left*) or R-Ras (G38V) (*right*) was co-transfected with empty vector, with full-length myc-RGS12, or with full-length myc-RGS12 and FLAG-A-Raf, B-Raf-FLAG, or c-Raf-1-FLAG expression vectors in HEK 293T cells. Anti-myc immunoprecipitates were immunoblotted for co-precipitated HA-Ras proteins with an anti-HA antibody. Total lysates were immunoblotted with anti-myc, anti-FLAG, and anti-HA antibodies to confirm expression. Image shown is representative of three experiments; R-Ras interaction with RGS12 upon B-Raf or c-Raf-1 co-expression was weak and variable between the three experiments. (**C**) HEK 293T cells were transiently transfected with plasmids encoding full-length myc-RGS12, HA-B-Raf, HA-MEK2, and HA-H-Ras G12V in various combinations as

indicated. Anti-myc immunoprecipitates were immunoblotted for co-precipitated HA-B-Raf, HA-MEK2, and HA-H-Ras GV using an anti-HA antibody. Expression of each construct was confirmed by immunoblotting immunoprecipitated proteins with anti-myc antibody, and total lysates with anti-HA antibody. (**D**) HEK 293T cells were transiently transfected as in panel C but with the addition of HA-ERK1 expression vector where indicated. Detection of expression and co-immunoprecipitation was as described for panel C. *Note*: To highlight the increase in complex formation observed when multiple Ras/MAPK components are co-transfected in panel C, we present a lighter exposure that reveals only a faint band representing HA-MEK2 co-immunoprecipitation with myc-RGS12. In contrast, panel D presents a darker exposure to highlight the co-immunoprecipitation of HA-ERK1, which in turn displays the RGS12-MEK2 interaction better than panel C.

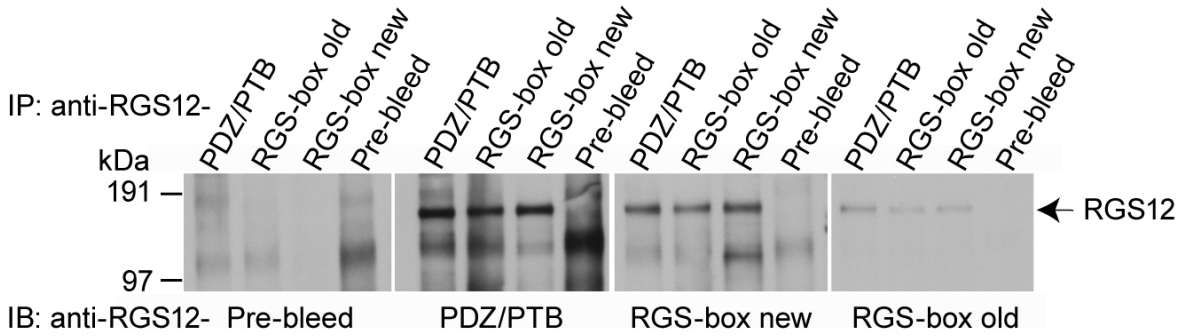

Figure 4.9. Validation of anti-RGS12 rabbit antisera and demonstration of endogenous RGS12 expression in PC12 rat pheochromocytoma cells. Rabbits were immunized with recombinant protein spanning the first 440 amino-acids of rat RGS12 (encompassing the N-terminal PDZ and PTB domains: "PDZ/PTB") or spanning residues 664-885 (encompassing the central RGS-box). Resultant antisera (or serum from pre-immunized rabbits ["pre-bleed"]) were used to immunoprecipitate (IP) proteins from PC12 cell lysate as indicated; immunoprecipitating proteins were resolved by SDS-PAGE, electroblotted onto nitrocellulose, and subsequently immunoblotted with indicated antisera, as per methods described in *Materials and Methods*. Note that "RGS-box old" describes rabbit antiserum previously described [137], but subsequently exhausted and hence replaced via a new round of rabbit immunization using the same immunogen.

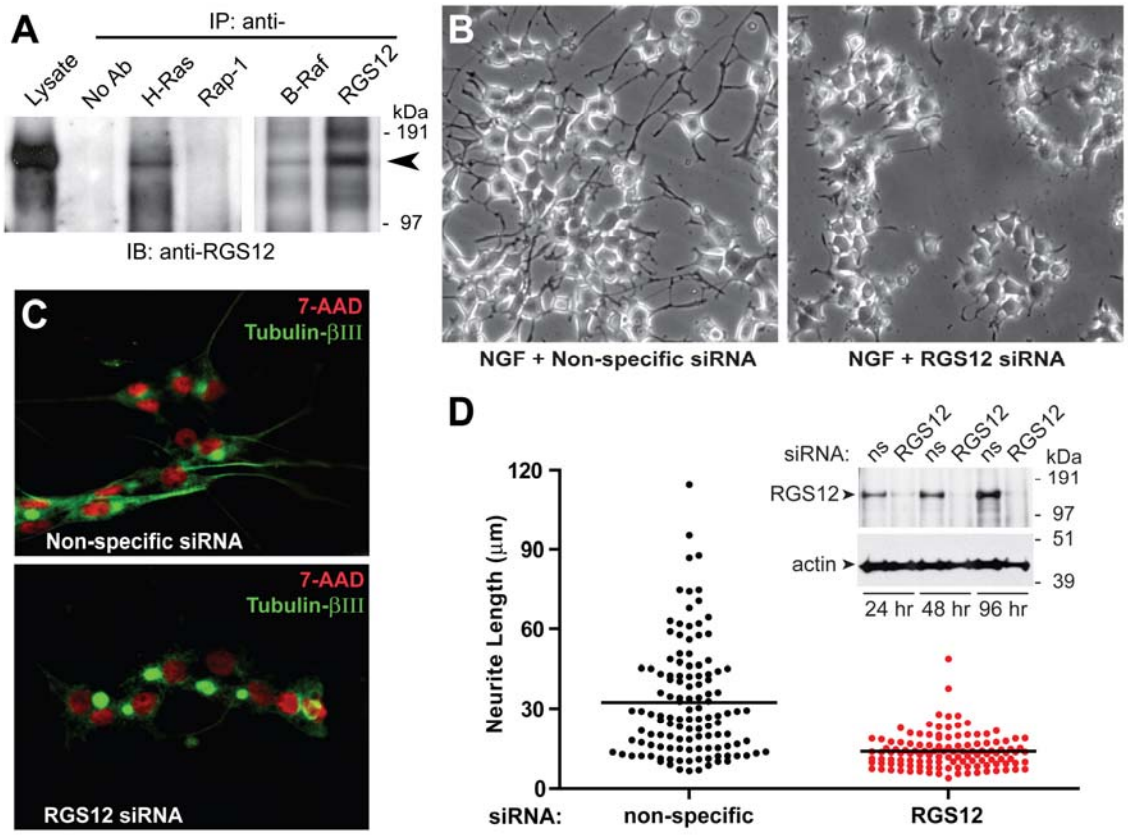

Figure 4.10. RGS12 is critical for NGF-mediated neurite outgrowth in PC12 cells. (**A**) PC12 cell lysates were immunoprecipitated with beads alone (No Ab), or anti-H-Ras, anti-Rap-1, anti-B-Raf, or anti-RGS12 antibodies. After SDS-PAGE, anti-RGS12 antibody was used to detect associated endogenous RGS12. (**B**) PC12 cells were transfected with non-specific siRNA or RGS12 siRNA for 24 h, and then treated with 100 ng/ml NGF for an additional 48 hr. Brightfield images were obtained as described in *Materials and Methods*. (**C**) PC12 cells were transfected and treated with NGF as described in panel B. Cells were fixed, permeabilized, and stained as described in *Materials and Methods* with the differentiation marker anti-tubulin-βIII (*green*) and the nuclear stain 7-aminoactinomycin D (7-AAD; *red*) prior to analysis by confocal microscopy. (**D**) PC12 cells were transfected, treated with NGF, and imaged as described in panel B. Neurite lengths were measured for more than 100 cells per condition in three independent experiments as described in *Materials and Methods*. The average of each condition is represented by a black line in the dot plots. Neurite length in non-specific siRNA- *versus* RGS12 siRNA-transfected cell cultures were significantly different ($p < 0.0001$; Student's *t* test). *Inset*: PC12 cells were transfected with non-specific siRNA or RGS12 siRNA, and lysed 24, 48, and 96 hr post-transfection. Cell lysates were then immunoblotted for expression of both RGS12 and actin.

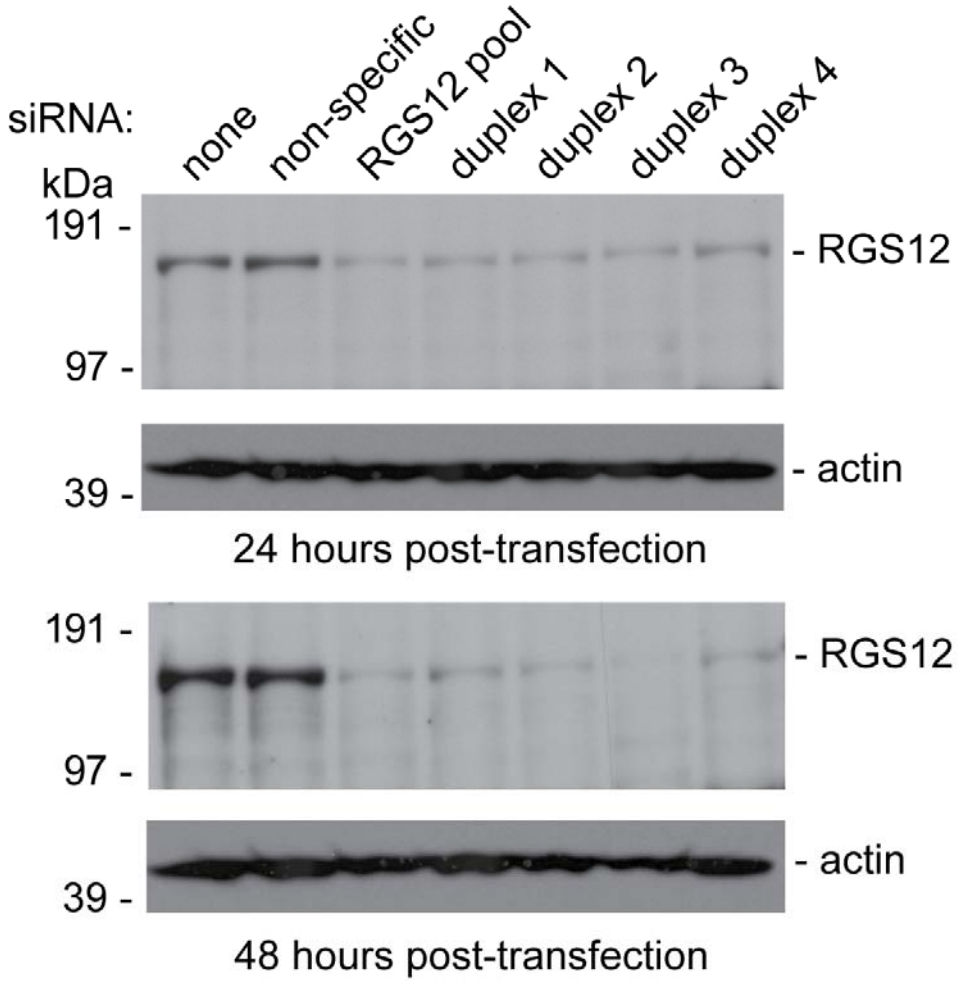

Figure 4.11. Four distinct siRNA oligonucleotide duplexes each result in knockdown of endogenous RGS12 expression. PC12 cells were transiently transfected with siRNAs as described in *Materials and Methods* and resultant cell lysates (obtained 24 or 48 hr later) were immunoblotted for RGS12 and actin as indicated. “RGS12 pool” refers to Dharmacon Research’s SMARTpool designed against the rat *Rgs12* mRNA, comprised of four siRNA oligonucleotide duplexes targetting the following specific mRNA sequences: duplex 1 (5’-GGC CGA AAC UUG ACU CUA A-3’); duplex 2 (5’-GAA CAC UAG GCA AGU CUA A-3’); duplex 3 (5’- GCA ACA GGG UGC UUG UAG U-3’); duplex 4 (5’- CAU GAC AGU UUA CAG GCU A-3’).

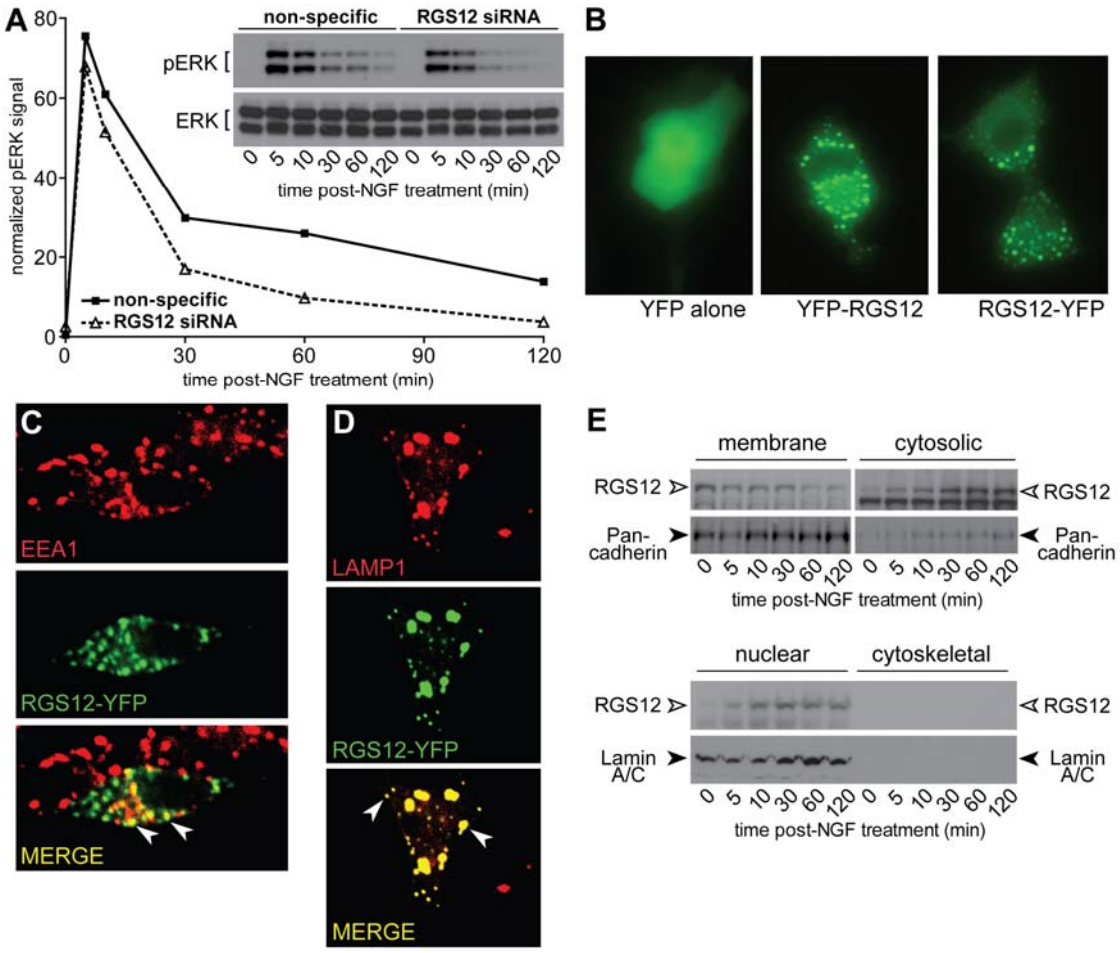

Figure 4.12. Prolonged ERK activation by NGF is reduced upon RGS12 depletion, expressed RGS12 is punctate and co-incident with endosomal markers, and translocation of endogenous RGS12 from membranes is regulated by NGF. (**A**) PC12 cells were transfected with non-specific or RGS12 siRNA oligonucleotides, and stimulated with NGF. ERK activation (pERK) and total ERK expression was determined by immunoblot analyses (*inset*) with either anti-phospho-ERK1/2 or anti-ERK1/2 antibodies at the indicated times after NGF treatment. Scion Image was used for densitometric quantitation of pERK1/2 and total ERK1/2 levels. (**B**) PC12 cells were transfected with pEYFP-N1 empty vector, YFP-RGS12, or RGS12-YFP expression vectors for 24 hr, and then treated with 100 ng/ml NGF for an additional 48 hr prior to epifluorescence imaging. (**C, D**) PC12 cells were transfected with RGS12-YFP (*green*) for 24 hr, and then treated with 100 ng/ml NGF for an additional 48 hr. Cells were fixed, permeabilized, and stained with the early endosomal marker EEA1 (panel C; *red*) or the late endosomal marker LAMP1 (panel D; *red*) prior to imaging by confocal microscopy. Colocalization of RGS12-YFP and the endosomal markers is shown in *yellow*. White arrows indicate colocalization of RGS12-YFP with EEA1 or LAMP1 on individual puncta. (**E**) PC12

cells were stimulated with NGF at the indicated timepoints. Cells were then fractionated into membrane, cytosolic, nuclear, and cytoskeletal fractions as described in *Materials and Methods*. Proteins were resolved by SDS-PAGE and immunoblotted with anti-RGS12 antibody, or antibodies against the nuclear envelope marker Lamin A/C or the plasma membrane marker anti-pan-cadherin.

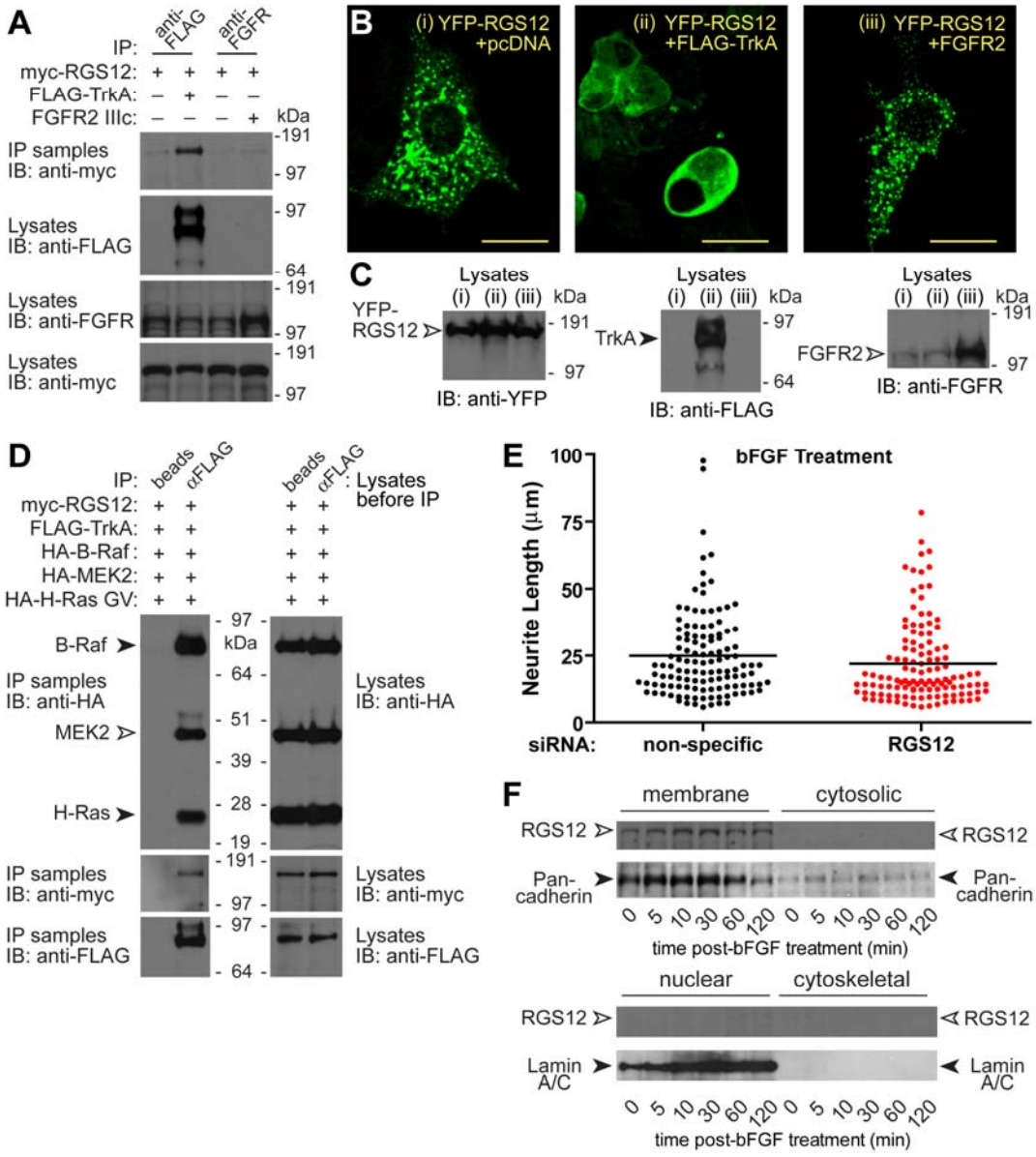

Figure 4.13. RGS12 forms a multiprotein complex containing TrkA and shows selectivity for TrkA signaling *versus* FGFR signaling. (**A**) HEK 293T cells were transiently co-transfected with full-length myc-RGS12, and either empty vector, FLAG-TrkA, or FGFR2 IIIc expression plasmids. Anti-FLAG or anti-FGFR antibody was used to immunoprecipitate FLAG-TrkA or FGFR2 IIIc, respectively. After SDS-PAGE, anti-myc antibody was used to detect associated myc-RGS12 protein. Total cell lysates were also immunoblotted to verify expression. (**B**) HEK 293T cells were co-transfected with YFP-RGS12 and either (i) pcDNA3.1 empty vector, (ii) FLAG-

TrkA, or (iii) FGFR2 expression vectors and imaged by confocal microscopy; scale bar represents 20 μm. (**C**) HEK 293T cells were transfected as described in panel B. 72 hr post-transfection, cells were lysed and immunoblotted to verify expression of YFP-RGS12, FLAG-TrkA, and FGFR2. (**D**) HEK 293T cells were transiently transfected with plasmids encoding full-length myc-RGS12, FLAG-TrkA, HA-B-Raf, HA-MEK2, and HA-H-Ras G12V. Cell lysates were immunoprecipitated with beads alone or with anti-FLAG antibody. After SDS-PAGE, anti-HA and anti-myc antibodies were used to detect associated myc-RGS12, HA-B-Raf, HA-MEK2, and HA-H-Ras G12V. Expression of each construct was confirmed by immunoblotting total lysates with anti-HA, anti-myc, and anti-FLAG antibodies. (**E**) PC12 cells were transfected with non-specific siRNA or RGS12 siRNA for 24 h, and then treated with 100 ng/ml basic fibroblastic growth factor (bFGF) for an additional 48 hr. Differences in neurite length were quantitated as described in Fig. 4D. Neurite lengths in non-specific siRNA-transfected cells were not significantly different from those of RGS12 siRNA-transfected cells ($p > 0.05$; Student's *t* test). (**F**) PC12 cells were stimulated with bFGF at the indicated timepoints. Cells were then fractionated into membrane, cytosolic, nuclear, and cytoskeletal fractions as in Fig. 5E. Proteins were resolved by SDS-PAGE and immunoblotted with anti-RGS12 antibody, or antibodies against the nuclear envelope marker Lamin A/C or the plasma membrane marker anti-pan-cadherin.

Table 4.1: Plasmid Sources

Plasmid(s)	Source
Wildtype and constitutively-activated (GV) mutants of triple hemagglutinin (3x HA) tagged human H-Ras, M-Ras, R-Ras, Rap1A, and Rap1B in pcDNA3.1	UMR cDNA Resource Center (www.cdna.org)
Human MEK1 and MEK2 in pUSEamp	Upstate Cell Signaling Solutions (Charlottesville, VA)
Wildtype and activated mutants of HA-tagged H-Ras, K-Ras, and N-Ras in pCGN	Dr. Channing Der (UNC-Chapel Hill)
FGFR2 isoform IIIc in pBABE	Dr. Channing Der (UNC-Chapel Hill)
3xHA-human A-Raf in modified pACTAG2	Dr. D. Anderson (Univ. of Saskatchewan)
HA-tagged human B-Raf and human c-Raf-1-FLAG in pcDNA3	Dr. K.-L. Guan (Univ. of Michigan, Ann Arbor)
Human A-Raf with an N-terminal FLAG-epitope tag in pCMV-FLAG-6b	Dr. Jeffrey Frost (Univ. of Texas Health Science Center, Houston)
N-terminal FLAG constructs of B-Raf deletion mutants in pCMV-FLAG-6b	Dr. Jeffrey Frost (Univ. of Texas Health Science Center, Houston)
Human B-Raf with a C-terminal FLAG-epitope tag in pLNCX	Dr. Deborah Morrison (NCI, Frederick, MD)
Rat TrkA with an N-terminal FLAG epitope tag in pcDNA3	Dr. Francis Lee (Weill Medical College of Cornell University)
Wildtype FLAG-tagged Rap2B in pCMV2	Dr. Lawrence Quilliam (IUPUI)
N-terminally HA-tagged InaD-PDZ1, Numb PTBo, full-length RGS12, RGS12-PDZ/PTB, and RGS12-PDZ expression vectors	[18]
Rat ERK2 in the GFP fusion vector pEGFP-N1	[194]

Table 4.2: Antibody Sources

Antibody (antibodies)	**Source**
Anti-HA-horseradish peroxidase (HRP) 3F10, anti-HA 12CA5, and anti-myc 9E10	Roche
Anti-myc-HRP 4A6 and anti-Rap-1	Upstate
Anti-RGS12 (A-14), -B-Raf (C-19), -EGFR (1005), -FGFR (C-17), -A-Raf (C-20), -MEK2 (A-1), -Lamin A/C (346), and -goat IgG HRP	Santa Cruz Biotechnology
Anti-tubulin-βIII, -β-actin, -pan-cadherin, -M2 FLAG HRP, and -M2 FLAG	Sigma (St. Louis, MO)
Anti-p44/42 ERK MAP kinase and -phospho-p44/42 (Thr202/Tyr204) ERK MAP kinase	Cell Signaling Technology (Danvers, MA)
Anti-rabbit IgG HRP and anti-mouse IgG HRP	GE Healthcare (Piscataway, NJ)
Anti-Ras OP-40	Calbiochem (San Diego, CA)
Anti-H-Ras 146	Dr. Adrienne Cox (UNC-Chapel Hill)
Anti-EEA1	BD Biosciences (San Jose, CA)
Anti-lysosomal-associated membrane protein-1 (LAMP1) H4A3 mouse antibody	Developmental Studies Hybridoma Bank (Univ. of Iowa, Iowa City, IA)
Anti-RGS12-PDZ/PTB and anti-RGS12-RGS-box	[266]

CHAPTER 5

FUTURE DIRECTIONS

Signal transduction pathways emanating from both GPCRs and RTKs regulate many fundamental cellular processes, including cell proliferation, survival, differentiation, apoptosis, motility, and metabolism. The basic mechanisms of these pathways are well established; however, how each pathway processes a myriad of specific inputs into diverse biological outputs remains an enigma. Recently, a number of scaffold/nexus proteins have been identified, and their roles in regulating GCPR and RTK signaling are now emerging. What is becoming apparent is that scaffolds coordinate signal transduction using different mechanisms. For example, some scaffolds provide a simple platform for the construction of signaling networks by separating individual components into discrete units; whereas others augment the kinetics and amplitude of the signal, mediate cross-talk with other pathways, target signaling complexes to specific subcellular localizations, and provide regulated inhibition. Versatility is further increased by the dynamic regulatory processes placed on nexus proteins that affect their assembly and turnover, as well as the influence of 'meta-scaffolds', such as MORG1, that connect different scaffolding complexes to regulate signaling [274].

The future aims of this research are to continue to identify and characterize interactions of RGS12, and to build upon the foundation established by this work to dissect the individual contributions of such interactions to complex physiological processes. The first step in this process is to make use of the insights gained

through determining the various binding partners and regulators of RGS12 to ascertain the physiological roles of RGS12's MAPK scaffold activity. My work in PC12 cells provides biological evidence for RGS12 as a MAPK scaffold, and it is likely that RGS12 acts as a scaffold in primary DRG neurons to regulate NGF and/or $GABA_B$ receptor signaling.

RGS12 is required for NGF-mediated neurite outgrowth and prolonged ERK activation in PC12 cells, but it is not clear whether this directly relates to its proposed scaffold role. My hypothesis is that the scaffold function of RGS12 plays a critical role in these processes, and numerous experiments can be performed to test this hypothesis. As RGS12 binds to MEK2 and A-Raf/B-Raf, it will be interesting to examine whether knockdown of either of these gives a similar phenotype (*i.e.*, reduced duration of ERK activation and reduced neurite length) to knockdown of RGS12. Overexpression of RGS12 biphasically regulates PDGFβR-induced ERK activity in CHO-K1 cells, consistent with the "combinatorial inhibition" paradigm: at low scaffold concentrations, signaling components are in excess and formation of a functional complex is less likely to occur, but when scaffold is in excess, nonfunctional complexes with less than the full complement of components becomes more prevalent [197, 198]. Does RGS12 overexpression biphasically regulate TrkA-induced ERK activation and neurite outgrowth in PC12 cells? Additionally, to provide a clear link between the role of RGS12 as a scaffold protein and its function in neurite outgrowth and the regulation of ERK activation kinetics, it will be important to demonstrate that RGS12 mutants, crippled in their binding to particular MAPK pathway components, do not enhance ERK activity when moderately

overexpressed, and to test such RGS12 mutants in complementation experiments in conjunction with siRNA-mediated knockdown of endogenous RGS12 (*i.e.*, compare the effects of the mutants to those of wildtype RGS12).

RGS12 contains two regions that regulate Gα signaling (*i.e.*, the RGS domain and the GoLoco motif); however, my findings in PC12 cells, and the conclusions that I draw from these results, do not yet provide an explanation of how Gα signaling integrates into this signaling network. Do the two Gα binding regions play a role in RGS12's scaffold function, and if so, what role? Is it possible, albeit unlikely, that both regions are nonfunctional in the context of NGF-mediated neurite outgrowth in PC12 cells? One possibility is that the $G\alpha_i$-binding activity of the C-terminal GoLoco motif plays a role in localizing RGS12 to specific membrane compartments, as we have shown in airway smooth muscle cells that a loss-of-function mutation in the GoLoco motif mislocalizes RGS12 to the nucleus [18]. An intriguing possibility is that RGS12 facilitates signal integration between RTK and GPCR signaling pathways in PC12 cells; in particular, the RGS domain of RGS12 might negatively regulate those $G_{i/o}$-coupled GPCRs that promote PC12 cell proliferation, thus enabling NGF to promote differentiation more efficiently. In this regard, one well-described endogenous receptor that promotes MAPK activation in PC12 cells by coupling to $G\alpha_{i/o}$ subunits is the α_{2A}-adrenergic receptor [301]. Specifically, activation of the α_{2A}-adrenergic receptor by UK14304 (a subtype selective full-agonist [302]) leads to Gβγ-mediated activation of the Ras-ERK pathway [303, 304]. As described above, one mechanism of cross-talk may be that RGS12 simultaneously inhibits signaling by the α_{2A}-adrenergic receptor while promoting NGF signaling, with the net result

being enhanced neuronal differentiation. In line with this, knockdown of RGS12 would effectively remove negative regulation of the α_{2A}-adrenergic receptor and positive regulation of the TrkA receptor, with the net result being enhanced proliferation. Therefore, one would predict that α_{2A}-adrenergic receptor-promoted ERK activation would be enhanced in the absence of RGS12 in PC12 cells.

Loss of RGS12 expression inhibits neuritogenesis in PC12 cells; thus, RGS12 appears to be critical for this process. It is possible that RGS12 is the primary scaffold in PC12 cells for enhancing NGF-promoted MAPK signaling; however, it may also work in concert with other scaffolds involved in MAPK coordination to promote neurite outgrowth. The scaffold proteins B-KSR1 and DYRK1A both regulate neuronal differentiation of PC12 cells [187, 228], and scaffold-scaffold interactions have been identified: *e.g.*, MP1 binds to the MAPK scaffold MORG1 [305]. These results suggest that distinct scaffolds regulate similar processes, and that MAPK scaffolds may orchestrate the formation of complete modules to enhance signaling efficiency. Orchestration of large 'meta-scaffold' complexes may also be a way in which scaffolds are able to regulate proteins that they do not directly bind. For example, a number of studies in PC12 cells describe the small GTPase Rap1 as the critical GTPase responsible for NGF-mediated prolonged ERK activation and neurite outgrowth in PC12 cells (reviewed in [306]); however, it is clear that Ras function is also important, as microinjection of a Ras-neutralizing antibody blocks differentiation of PC12 cells [307]. RGS12 binds to H-Ras, and not to Rap isoforms (as described in Chapter 4); thus, one might question how inhibition of RGS12 reduces NGF-mediated neuronal differentiation if it does not bind to a critical protein involved in

neurite outgrowth. One possibility is that RGS12 forms a 'meta-scaffold' with an as-yet unidentified scaffold protein that binds to Rap1, and in the larger complex, regulates Rap1 signaling. Another possibility is that too much emphasis has been placed on the role of Rap in neurite outgrowth and, in reality, the biological outcome results from both Ras and Rap signaling in PC12 cells [227]. Since critical findings have emerged from the use of controversial reagents such as "dominant-negative" Rap1 (S17N), which is actually incapable of inhibiting Rap1 activation by the Rap-specific GEF C3G *in vitro* and *in vivo* [308, 309], the biological role of Rap1 in PC12 differentiation requires careful consideration.

We identified B-Raf as a binding partner for RGS12 (Chapter 4); however, it is not clear whether B-Raf directly associates with RGS12. The interaction does not appear to be indirect through H-Ras, as the H995L mutation in RBD1 of RGS12 that disrupts binding to H-Ras does not inhibit binding to B-Raf (Chapter 4). Interestingly, RGS12 has been found in a protein complex with 14-3-3 [310], an adaptor protein that is essential for Raf activation [311], and 14-3-3 interacts with B-Raf [312]; thus, 14-3-3 may serve as a bridge between RGS12 and B-Raf. Further studies examining the structural determinants of the RGS12/B-Raf interaction, as well as whether RGS12 directly interacts with 14-3-3, will be required to address this issue.

The function of many MAPK scaffolds is regulated by protein modifications such as phosphorylation, *e.g.*, JIP-1 and KSR [200, 201]. For instance, KSR is phosphorylated by C-TAK1 (Cdc25C-associated kinase 1) which creates a 14-3-3 binding site on serine-392 [201]. Interestingly, the phosphorylation of KSR, and subsequent binding to 14-3-3, regulates not only the localization of KSR, but also its

function as a MAPK scaffold. In quiescent cells, KSR is bound to 14-3-3 which localizes the protein predominantly in the cytosol. However, upon activation by a mitogen such as EGF, phosphoserine-392 on KSR is dephosphorylated, releasing 14-3-3, and allowing KSR to translocate to the membrane where it coordinates Raf-MEK-ERK signaling. It is possible that the function and/or localization of RGS12 is regulated by phosphorylation; however, this remains to be determined.

It is hypothesized that the release of 14-3-3 by KSR exposes the cysteine-rich C1 domain of KSR, which is critical for membrane localization. Additionally, exposure of this region may reveal an ERK docking site (*i.e.*, the polypeptide sequence FxF; [313]), thus providing one explanation for activation-promoted interaction between KSR and ERK. I did not observe a constitutive interaction between RGS12 and ERK (Chapter 4); however, ERK was observed in RGS12 immunoprecipitates upon coexpression of H-Ras (G12V), B-Raf, and MEK2. One possibility is that an activation event is required to reveal an ERK binding site on RGS12. Analogous to KSR, RGS12 may constitutively bind 14-3-3 in a manner that prevents ERK binding, and upon activation, 14-3-3 may be released to expose an ERK binding region.

The mechanism(s) by which RGS12 localization is regulated are unknown. RGS12 localizes to endocytic vesicles when overexpressed in multiple cell types, including DRG neurons, airway smooth muscle cells, COS-7 cells, HEK 293T cells, and PC12 cells (Chapter 4, [18], and unpublished results). Coexpression of a subset of interacting proteins promotes translocation of RGS12 to the plasma membrane, namely TrkA (Chapter 4) and $G\alpha_{i1}$ (data not shown). In contrast, some protein

partners such as H-Ras (G12V) and $G\alpha_o$ (data not shown) do not promote translocation. However, H-Ras does appear to alter the expression levels of RGS12. In both HEK 293T and PC12 cells, the expression of YFP-RGS12 is dramatically increased, as assessed by fluorescence microscopy and western blotting. Full-length RGS12 is susceptible to calpain-mediated proteolysis, and may be subject to ubiquitinylation (unpublished data); thus, it is possible that activated H-Ras, for example, stabilizes the normally labile RGS12 preventing degradation. Strikingly, in PC12 cells, NGF promotes translocation of endogenous RGS12 out of the membrane fraction, and into the cytosolic and nuclear fractions. The significance of this shuttling is unknown, as proteins involved in NGF-mediated neurite outgrowth localize to endosomes, and this localization is critical for their participation in neuronal differentiation [290, 291]. It is possible that the translocation of RGS12 into the cytosol or nucleus is important for its scaffold function. KSR has been found to cycle through the nucleus in a manner that is dependent on its interaction with MEK [314]. Intriguingly, the yeast scaffold protein Ste5 must cycle through the nucleus to be a functional MAPK scaffold, although the exact mechanism by which Ste5 is affected by nuclear shuttling is unknown [175].

The movement of RGS12 between the plasma membrane, the cytosol, and the nucleus may play a role in spatial and temporal regulation of ERK activation. For instance, it is plausible that the localization of RGS12 promotes differential ERK substrate selection, and in this way, determines whether the activation of ERK is transient or prolonged. Numerous MAPK scaffolds have been identified that spatially and temporally regulate ERK activation, and it is possible that RGS12 regulates ERK

activation in a similar manner. MP1, a MAPK scaffold for MEK1 and ERK1, facilitates MEK1 activation of ERK1 [182], and its function is dependent on binding p14, a protein found on the cytoplasmic side of late endosomes [202]. MP1 is required not for transient EGFR activation of ERK at the plasma membrane but for delayed activation of ERK on late endosomes. β-arrestin, as described previously, is a MAPK scaffold that facilitates c-Raf-1 activation of MEK1 and ERK2 [28], and can spatially regulate MAPK signaling by specifying substrate selection. Specifically, β-arrestin-associated ERK is sequestered in the cytosol, such that ERK regulates cytosolic and not nuclear substrates [195, 196]. Sef is a Golgi-associated MEK/ERK scaffold that binds activated MEK and promotes its phosphorylation of ERK [203]. Similar to β-arrestin, Sef forms a complex with activated MEK and ERK and prevents activated ERK translocation to the nucleus, thus preventing ERK phosphorylation of the Elk-1 transcription factor. Instead, Sef-associated ERK promotes phosphorylation of cytosolic p90RSK2. These and other MAPK scaffolds influence the spatio-temporal activation of ERK, and thereby regulate ERK activation of distinct substrates. The importance of temporal differences in ERK activation is supported by observations that prolonged ERK activation promotes PC12 cell differentiation, whereas transient ERK activation promotes PC12 cell proliferation [226, 278, 279]. As described in Chapter 4, we hypothesize that RGS12 functions as a MAPK scaffold that regulates ERK function in endosomes and that may promote differential ERK substrate selection due to its endosomal location. In this scenario, RGS12 may function to restrict ERK activation to the cytoplasm and alter ERK substrate utilization.

Recently, we identified p90RSK1 as a binding partner for RGS12 via transient co-transfection and co-immunoprecipitation experiments (data not shown), and the interaction appears to be mediated by the PDZ domain of RGS12, as predicted by Snow *et al.* [111]. p90RSK1, as well as p90RSK2, p90RSK3, and p90RSK4, contain C-terminal motifs that are Class I PDZ docking sites, *i.e.*, STTL, STAL, STRL, and STGL, respectively; thus, it is likely that the PDZ domain of RGS12 binds to additional p90RSK isoforms. Although endogenous RGS12 localizes to both the cytoplasm and nucleus following NGF treatment of PC12 cells, it is possible that RGS12 selectively restricts ERK activation to the cytoplasm by binding to, and promoting phosphorylation of, cytosolic p90RSK. In line with this, knockdown of RGS12 expression would reduce cytoplasmic p90RSK phosphorylation, and enhance nuclear Elk-1 phosphorylation. In preliminary experiments, I have observed a reduction in NGF-promoted p90RSK1 phosphorylation in RGS12-depleted PC12 cells (data not shown), suggesting that RGS12 may normally restrict ERK activation to the cytoplasm. Intriguingly, p90RSK1, -2, and -3 kinases are activated by NGF treatment of PC12 cells [315], and constitutively active p90RSK1 promotes differentiation of PC12 cells [316]. These findings suggest that p90RSK1 plays a key role in the differentiation process in PC12 cells. Whether RGS12 binds to p90RSK1 in PC12 cells and regulates its activation, requires further clarification.

With the findings that RGS12 is present in a complex with numerous proteins involved in MAPK signaling (*i.e.*, TrkA, Ras, Raf, MEK, ERK, p90RSK, and 14-3-3); is critical for a MAPK cascade-promoted biological process in PC12 cells (*i.e.*, neuronal differentiation); and is expressed in trigeminal and DRG neurons in the

E14.5 mouse [267]; it is possible that RGS12 regulates a MAPK-mediated process in primary neurons. Snider and colleagues have demonstrated that the Ras-Raf-ERK cascade is crucial for axon elongation in embryonic $Bax^{-/-}$ DRG cultures [317]. Specifically, they found that overexpression of activated H-Ras, Raf-1, or ERK led to axon growth that was comparable to NGF-treated cells. It will be interesting to examine whether knockdown of RGS12 in these primary DRG cultures inhibits NGF-promoted axon growth, analogous to the inhibition of neurite outgrowth observed in PC12 cells.

In addition to its potential role in axonal growth, RGS12 may act as a scaffold to organize MAPK cascade members involved in $GABA_B$ receptor-mediated activation of the MAPK pathway. It has previously been established that RGS12 is specifically involved in establishing the rate of desensitization of $GABA_B$ receptor-mediated, voltage-independent inhibition of presynaptic $Ca_v2.2$ channels in embryonic chick DRG neurons [137]. Recently, Diversé-Pierluissi and colleagues characterized several aspects of the signaling pathway that underlie $GABA_B$ receptor-mediated $Ca_v2.2$ channel inhibition and ERK2 activation in chick DRG and rat hippocampal neurons. The tyrosine kinase c-Src, pre-associated with the $Ca_v2.2$ channel [138, 318], is activated within 20 seconds of $GABA_B$ receptor activation. Ca^{2+}-dependent activation and recruitment of other signaling components to the $Ca_v2.2$ channel quickly follow, including the Ca^{2+}-dependent tyrosine kinase PYK-2, the adaptor protein ShcC, the Ras exchange factor Sos (son-of-sevenless), and Ras GTPase(s) [318]. Interestingly, RGS12 is also recruited to the $Ca_v2.2$ channel in a similar time-frame (20 - 60 seconds) following $GABA_B$ receptor activation [137].

Originally, it was hypothesized that RGS12 desensitizes $GABA_B$ receptor signaling by virtue of its $G\alpha_{i/o}$-directed GAP activity [137]. However, as detailed in Chapter 4, we have now identified several novel binding partners for RGS12 including H-Ras, A-/B-Raf, and MEK2. Thus, it is possible that RGS12, beyond accelerating $G\alpha$·GTP hydrolysis, may also act as a scaffold protein to facilitate the assembly and regulation of the Ras/Raf/MAPK cascade at the $Ca_v2.2$ channel. Future experiments are required to further our understanding of the specific involvement of RGS12 and its binding partners in controlling neuronal responses to $GABA_B$ receptor activation. For instance, microinjection of recombinant full-length RGS12, but not other RGS proteins such as RGS14, into DRG neuron cell bodies dramatically accelerates the time to termination of GABA-mediated $Ca_v2.2$ channel inhibition [137]. If RGS12 regulates MAPK cascade signaling at the channel by promoting formation of a multiprotein complex, then one would expect that microinjection of loss-of-function mutants of RGS12 that are incapable of binding MAPK members may differentially modulate the rate of desensitization of $GABA_B$ receptor-mediated inhibition of the $Ca_v2.2$ channel.

GABA is the main inhibitory neurotransmitter in the central nervous system (CNS), and this inhibitory action arises via activation of $GABA_A$ and $GABA_B$ receptors [319-322]. GABA plays an important role in controlling the level of sensory information by modifying primary-afferent mediated transmission to the dorsal horn [322], and dysfunction of the GABAergic system leads to a clinical expression of central pain. Fortunately, the selective $GABA_B$ receptor agonist, baclofen (β-p-chlorophenyl-GABA), has been shown to suppress neuropathic pain [323] and this

effect, at least in part, occurs via inhibition of the $Ca_v2.2$ calcium channel [324]. Hence, the design of novel therapeutic strategies that activate $GABA_B$ receptors, inhibit $Ca_v2.2$ channels, or inhibit proteins like RGS12 that desensitize $Ca_v2.2$ channel inhibition by the $GABA_B$ receptor could have a high likelihood of success for future pain pharmacotherapy. The interactions described in Chapter 4 have furthered our understanding of the molecular determinants of RGS12 function and thus, will provide valuable insight necessary for rational drug design or high-throughput molecular screening for RGS12 inhibitors.

My findings in Chapter 4 also suggest that RGS12 may play a role in NGF-promoted pain. Although NGF was originally identified as a survival factor for neurons in the developing nervous system [325], it is now well-established that NGF has a crucial role in the generation of pain in adults (reviewed in [326]). NGF expression is elevated in inflammed and injured tissues, and stimulation of TrkA on nociceptors activates and potentiates pain signaling [326]. Moreover, inhibition of NGF function and signaling reduces pain and hyperalgesia in murine models of pain [327-330], including models of neuropathic pain [331-336]. Intriguingly, agents that inhibit NGF are proving to be effective in murine models of pain in which traditional analgesic drugs either produce severe side effects or have poor efficacy. For instance, although non-steroidal anti-inflammatory drugs (NSAIDs) and opiates are generally effective analgesics for many pain conditions, their use is limited by their efficacy and tolerability [337]. As such, several pharmaceutical companies are developing drugs to antagonize NGF [326].

NGF activates MAPK signaling in sensory neurons, and this activation has been implicated in NGF-promoted hyperalgesia [338-340]. Activated MAPKs such as ERK are present in NGF-TrkA signaling endosomes that are transported in a retrograde fashion to the cell bodies of sensory neurons [341]; thus, it is possible that activation of downstream transcription factors by these MAPKs contributes to the transcriptional changes in nociceptors that are associated with peripheral and central sensitization, such as rapid post-translational changes in the transient receptor potential vanilloid receptor 1 cation channel [342]. RGS12 localizes to endosomes, and positively regulates NGF-TrkA neuronal differentiation in PC12 cells. Therefore, RGS12 may be a component of signaling endosomes, and enhance NGF signaling in sensory neurons. If this is the case, inhibition of RGS12 may prove to be an effective pain therapeutic, similar to agents that antagonize NGF.

In addition to pain modulation, RGS12 may play a role in Ras- and B-Raf transformation and oncogenesis. We identified B-Raf as an RGS12-interacting protein suggesting that RGS12 may be an important regulator of B-Raf function (Chapter 4). B-Raf is the main kinase that is responsible for coupling Ras to MEK in cells (reviewed in [343]), and this isoform is mutationally activated in 66% of malignant melanomas and in 7% of human cancers [344]. Over 80% of B-Raf missense mutations correspond to a V600E amino-acid substitution in the B-Raf kinase domain. Interestingly, we have established that RGS12 binds to this frequently occurring activated mutant of B-Raf (data not shown). Similarly, RGS12 binds to activated H-Ras. Ras is the most frequently mutated oncogene in human cancers, and is suggested to be involved in oncogenesis. Thus, there is

considerable interest and effort in targeting Ras signaling for the development of novel approaches for cancer treatment [345, 346]. RGS12 appears to regulate both Ras and B-Raf signaling; however, defining the role of RGS12 in normal and neoplastic cells will greatly facilitate future strategies targeting Ras and mutant B-Raf signaling pathways.

RGS12 is endogenously expressed in Rat-1 rat fibroblast, RIE-1 rat intestinal and ROSE rat ovarian epithelial cells (data not shown), which are three well-established cell lines for the study of Ras signaling and transformation [347-351]. Thus, it will be interesting to examine whether suppression of RGS12 expression in these cells impairs H-Ras-mediated growth transformation. Additionally, we found expression of RGS12 in B-Raf mutation (V600E)-positive colorectal carcinoma human tumor lines (*i.e.*, COLO 205 and Caco-2; data not shown). The transformed growth of these cell lines is dependent upon the continued expression of mutant B-Raf [352-354], and as such, inhibition of the Raf-MEK-ERK pathway impairs the growth of these lines [355]. Since RGS12 interacts with activated B-Raf (data not shown) and regulates MAPK signaling (Chapter 4), it is possible that RGS12 may associate with mutant B-Raf in these cells, and influence the ability of B-Raf (V600E) to promote growth of colorectal carcinomas. If this is the case, depletion of RGS12 expression would likely result in impaired growth *in vitro* (*i.e.*, soft agar colony formation) and *in vivo* (*i.e.*, tumor formation or regression in nude mice), as well as reduced ERK activity. Intriguingly, it was recently shown that the *RGS12* gene has high coding micronucleotide repeat instability, and frameshift mutations in *RGS12*

were identified in 29% of colorectal tumors with high levels of microsatellite instability [356].

As described in Chapter 1, Loco, the *Drosophila melanogaster* orthologue of mammalian RGS12, is essential for glial cell development and subsequent blood-brain barrier formation [117]. Although relatively little is known about the specific molecular mechanisms underlying glial cell development, Loco, the GPCR Moody, and the Gα subunits $G\alpha_i$ and $G\alpha_o$ have been found expressed in surface glia, and these four proteins are thought to act as part of a common signaling pathway critical for blood-brain barrier formation [163, 164]. These findings suggest that the regulation of Gα signaling by Loco may be important for glial cell development. However, it is highly likely that the MAPK scaffold function of RGS12 also plays a role in glial cell development. For instance, Loco interacts with *Drosophila* Ras1 [165]; thus, it would be intriguing to examine whether Loco containing a point mutation in the first RBD (a mutation analogous to the loss-of-function H995L mutation that abrogates RGS12 binding to H-Ras; Chapter 4) alters glial cell differentiation.

The requirement for Loco in glial cell development and for normal locomotor capabilities suggests that mammalian RGS12 may also play a critical role in glial cell differentiation. Recently, the spatio-temporal expression patterns of RGS12 during mouse development were examined, and it was demonstrated that RGS12 is expressed predominantly in trigeminal and DRG neurons and muscle in the E14.5 mouse [267], suggestive of a role for mammalian *Rgs12* in neuro- and myogenesis. *Rgs12*-deficient mice have not been described to date, but elucidating the signaling

pathway(s) that RGS12 regulates will help to determine and define the role of the protein in the developing nervous system. Identification and characterization of additional players that modulate glial cell differentiation in the developing brain will provide new functional insights into the molecular basis of glial cell development, and will contribute in significant ways to understanding their potential function in CNS-related developmental disabilities such as cerebral palsy [357] and infantile spasms ("West syndrome"; [358, 359]).

RGS12 appears to represent a signaling nexus that facilitates convergence and cross-regulation of RTK, heterotrimeric G-protein, and Ras signaling (Figure 5.1). Investigations of the capacity of RGS12 to assemble novel signaling scaffolds will further our understanding of how highly-specific biological outcomes derive from seemingly-universal intracellular signaling components; in the particular case of RGS12, such knowledge will potentially lead to novel drug discovery targets for cancer treatment and modulation of nociception.

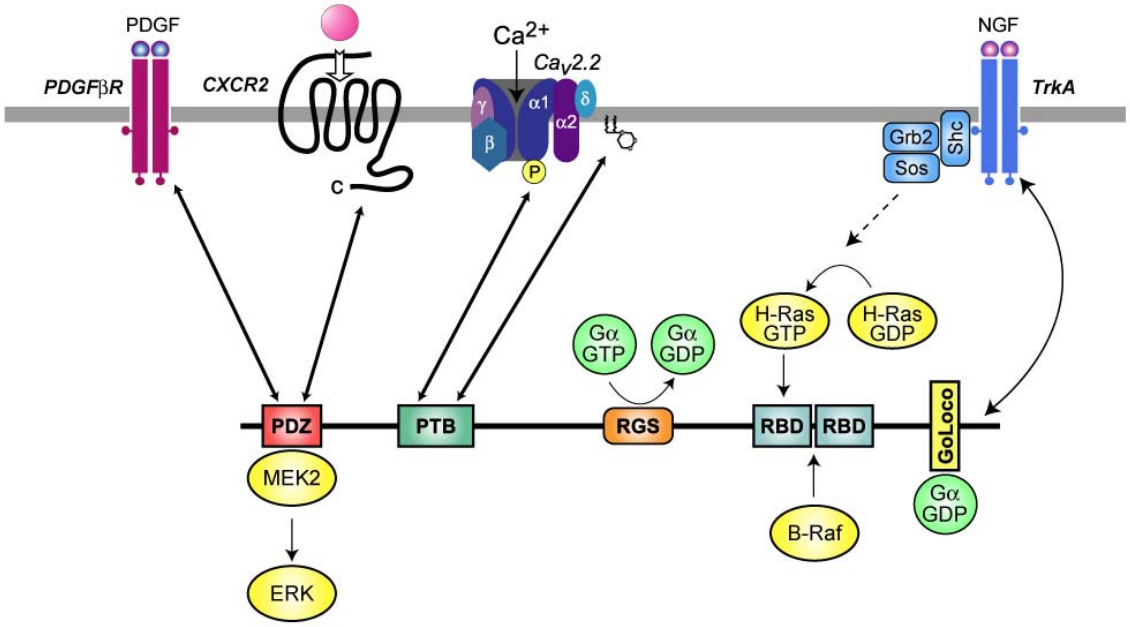

Figure 5.1. RGS12 and its interactions with components of RTK, GPCR, ion channel, and GTPase signal transduction pathways. In addition to the interactions described in Figure 1.5, RGS12 interacts with the NGF receptor TrkA, and regulates NGF-mediated neurite outgrowth. MEK2 associates with the N-terminal PDZ domain. B-Raf and activated H-Ras associate with the isolated tandem RBDs. ERK does not directly associate with RGS12, but has been found in a RGS12 protein complex.

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