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RGS14 is a biochemically diverse protein which integrates G proteins and the MAPKinase signaling cascade

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An Abstract of a Thesis submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

RGS14 is a biochemically diverse protein which integrates G proteins and the MAPKinase Signaling Cascade

Donald Patrick Cowan

Heterotrimeric G-proteins link extracellular neurotransmitter and hormone receptors to intracellular signaling cascades. In conventional models of G-protein signaling, GPCR's act as guanine nucleotide exchange factors (GEFs) to activate signaling events, and a family of proteins called the regulators of G-protein coupled signaling (RGS) function to negatively modulate G-protein signaling to effectively shut down G-protein signaling cascades. Of particular interest is RGS14, a complex RGS protein that contains a conserved RGS domain, tandem Ras/Rap binding domains (RBD) and a GoLoco/GPR (GL) motif. RGS14 is known to bind activated GTP-bound Gαi and Gio at its RGS domain, Rap2a at its RBD domains, and inactivated GDP-bound Gαi1/3 at its GL domain. Recent data in our lab has shown RGS14 also binds H-Ras, Raf kinases and Ric8a, a known cytosolic GEF. These new data link RGS14 to the MAPKinase cell proliferation pathway and suggest that RGS14 is acting as a novel protein scaffold to regulate local Gα and Raf kinase activity in a non-receptor mediated manner. Early work in our lab has shown RGS14 has the capacity to bind Gαi and activated H-Ras at the same time, but not Gαi and Raf-1 at the same time, suggesting that RGS14 may be acting as a protein switch to regulate Raf kinase activity inside the cell. My data shows that purified RGS14 inhibits Raf-1 mediated phosphorylation of MEK-1/2 in vitro. Additionally, our lab has also previously shown phosphorylation of RGS14 to plays a role in the protein's function. My work has shown RGS14 to be phosphorylated at an unknown site when recruited to the plasma membrane by Gαi. My data suggests the unknown phosphorylated site is likely within the first 213 amino acids of RGS14, or the required binding site for the kinase lies within this region. These findings elucidate the diverse biochemical roles for and potential mechanisms of RGS14 in non-receptor G protein signaling cascades.

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List of Abbreviations

cAMP- cyclic adenosine monophosphate

ERK2- Mitogen activated kinase 1

GEF- guanine nucleotide exchange factor

GDP- guanine nucleotide diphosphate

GPCR- G protein coupled receptor

GRK- G protein coupled receptor kinase

GTP- guanine nucleotide triphosphate

PI- phosphatidylinositol

PLCβ- 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta

PKA-cAMP dependent protein kinase A

PKC-cAMP dependent protein kinase C

PKG- GMP-dependent protein kinase

RGS- Regulators of G protein signaling

CHAPTER I: Introduction

1.1 G-protein coupled receptors (GPCRs)

Cells in advanced multi-cellular organisms transmit signals to each other in a variety of ways, one of which is through membrane bound cell-surface receptors. Among membrane bound receptors is the family of receptors known as the G-protein coupled receptors (GPCRs), seven-transmembrane receptors, or heptahelical receptors. The human genome contains approximately 800 distinct GPCRs which are all related by their seven transmembrane regions and signaling through bound G-proteins (Fredriksson, Lagerstrom et al. 2003). The total number of functional G-protein coupled receptors in the human genome is likely much higher due to splice variants and editing isoforms, with the true number almost impossible to determine (Kroeze, Sheffler et al. 2003).

The GPCR class of cell receptors contains a very diverse group of proteins capable of binding many different types of small molecules, known as ligands. Peptides, hormones, amino acids, biogenic amines, lipid mediators, sensory stimuli (taste, olfactory, and visual) all act as natural agonists/ligands at specific target GPCRs (Hill 2006). GPCRs may be specific for a particular ligand, though there are many cases where a GPCR can bind multiple ligands. Alternatively, one ligand can activate multiple GPCRs. The GPCR super family is divided into six subclasses A-F, which are further divided into specific sub-families (Fredriksson, Lagerstrom et al. 2003).

The primary role of GPCRs is to transmit extra-cellular signals to the intra-cellular signaling machinery and this is done through their linked G-proteins. The first step in the signaling process is the binding of an extra-cellular ligand to the extra cellular regions of the GPCR. Binding of the ligand to the extra-cellular domain results in a conformational switch of the GPCR causing activation of its linked G-protein. The G-protein activation is the result of guanine nucleotide exchange factor (GEF) activity of

GPCRs acting to exchange free GTP for G-protein bound GDP, releasing the G-protein for further downstream signaling (Abramowitz, Iyengar et al. 1979; J Codina 1983; Hill 2006) The signaling consequences depend on the type of G protein which is coupled to the GPCR and a vast array of different intracellular conditions. The types of G-proteins and signaling consequences of the different G-proteins will be discussed in section **1.3**.

1.2 Clinical Relevance of GPCRs

GPCRs are one of the main mechanisms by which cells transmit signals to each other to convey a very broad spectrum of physiological responses. Somatic cells in the body express many varieties of GPCRs simultaneously (Deupi, Kobilka et al. 2007). The GPCRs which are expressed on each cell can be members of the same family or any combination of GPCRs. Due to the extreme prevalence of GPCRs and the specificity for their ligands GPCRs make for excellent drug targets (Bortolato A 2009). Current estimates indicate upwards of 45%-50% of current clinical drug targets are GPCR family members (Lagerstrom and Schioth 2008). GPCRs regulate pain, cardiovascular function, the immune system, metabolic functions, and virtually every aspect of cellular regulation of the body (Johnson and Druey 2002; Insel, Tang et al. 2007; Premont and Gainetdinov 2007; Brinks and Eckhart 2010). Therefore GPCRs make excellent drug targets for a wide range of ages and diseases (Lagerstrom and Schioth 2008).

Additional practical factors lend benefit to using GPCRs as drug targets. The location of GPCRs on the surface of the cell makes them accessible to many pharmacological ligands that may not otherwise be able to pass through the plasma membrane. Specificity of action is another desirable trait for a drug target. The wider the range of action for a drug the greater chance the drug has to cause undesirable side effects, therefore specific action to a narrow range of cells is desirable.

For example, a commonly used medication is the anti-histamine benadryl. Benadryl acts widely on the histamine class of cell receptors as an antagonist, which causes both the desired inhibition effect on the H1 receptor, but also crosses the blood brain barrier to cause drowsiness. On the other hand, fexofenadine (Allegra©), does not cross the blood brain barrier and also acts as a specific H1 antagonist, resulting in desired anti-allergenic effects without the side effects of interacting at neuronal muscarinic receptors. Specific tissue distribution of GPCRs allows drugs to target specific tissues while leaving surrounding tissues unaffected (Insel, Tang et al. 2007). By using known ligands for GPCRs molecular chemists and biologists can use these compounds as a building block for developing molecules to target specific receptors.

Most drug discovery research is dedicated to finding ligands to either bind and activate G-proteins or bind to inhibit their natural functions. Clinical strategies to modulate GPCR signaling function through alternative methods, such as proteins which regulate G-protein activity, is a relatively new idea which is poorly understood but has great potential. With modern molecular biology techniques, expansive drug libraries and over 50 billion annual worldwide sales, the importance of GPCR signaling pathways in modern drug development has much room to grow (Lundstrom 2006).

1.3 Conventional GPCR signaling cascades and G proteins

Upon ligand binding to a GPCR a conformational switch occurs which results in the release and activation of the GPCR bound G protein (Hepler and Gilman 1992). The GPCR is then able to bind and activate another G protein or become inactive and internalize before recycling back to the surface of the plasma membrane. GPCRs regulate cellular function primarily through two pathways: the cyclic adenosine monophosphate (cAMP) pathway and the phosphatidlyinositol (IP) signaling pathway.

The G protein exists as a heterotrimer consisting of three subunits: the alpha subunit (Gα) and the obligate beta-gamma (Gβγ) dimer. Gα and/or Gβγ subunits will then go on to act on their downstream effectors to produce a wide variety of cellular results. There are four families of Gα proteins: Gαs, Gαi, Gαq/11 and Gα12/13 and each has their own specific downstream effectors and are classified based on their sequence homology (Kristiansen 2004). Gαs activates adenylyl cyclase (AC), Gαi inhibits AC, Gαq/11 activates 1-phosphatidylinositol-4,5-bisphosphate phospholipase C beta (PLCβ) and Gα12/13 regulate RHO-GEFs; however all have similar activation/inactivation cycles.

G protein activation is achieved through the receptor catalyzed exchange of free guanine triphosphate (GTP) for the bound guanine diphosphate (GDP) (Hamm 1998). This activity is known as guanine nucleotide exchange (GEF) function and plays a very important role in cellular signaling cascades. The now separated Gα and Gβγ subunits go on to their downstream effectors with inactivation of signaling occurring through intrinsic Gα GTPase activity. Gα hydrolyzes the terminal phosphate on GTP, returning the G protein to the GDP bound state, which results in the reassociation of the heterotrimer and cessation of G protein signaling activity.

It should be noted that GPCRs have also been shown to initiate signaling cascades independently from G proteins. A prominent mechanism of GPCR-G protein independent signaling is transactivation of receptor tyrosine kinases and their subsequent signaling cascades by GPCRs (Blesen, Hawes et al. 1995; Daaka, Pitcher et al. 1997). To date, two mechanisms of RTK activation by GPCRs have been discovered. The first mechanism is GPCR stimulation resulting in the activation of metalloproteinases. The metalloproteinase activation results in ectodomain shedding of transmembrane RTK ligand precursor, which then activates its sister RTK. A second method of non-G protein GPCR activation of RTKs involves GPCR association with a protein complex bound to the

RTK or the phosphorylation of transactivated RTK by tyrosine kinases which are downstream of GPCR signaling (Almendro, Garcia-Recio et al. 2010).

Another of the most widely studied G protein independent signaling pathways is the β_2-adrenergic receptor activation via GPCRs and arrestins. The β_2-adrenergic receptor, a GPCR, has been shown to activate the mitogen activated protein kinase 1 (ERK2) pathway after arrestin-mediated uncoupling of G protein mediated signaling (Daaka, Luttrell et al. 1998). In this activation pathway GPCRs are phosphorylated by GPCR kinases (GRKs), increasing β_2-adrenergic receptor affinity for β-arrestin, resulting in ERK phosphorylation (Gurevich and Gurevich 2008; Song, Coffa et al. 2009). Free arrestin 2 and 3 have been shown to activate JNK3 mediated by ASK1 in addition to binding other members of the ERK cascade such as ERK1/2 and p38 (Song, Coffa et al. 2009). The β_2-adrenergic receptor has also been shown to induce inhibition of renal Na+/H+ exchangers through association of Na+/H+ exchanger regulatory factor (Hall, Premont et al. 1998).

1.4 Regulators of G protein signaling

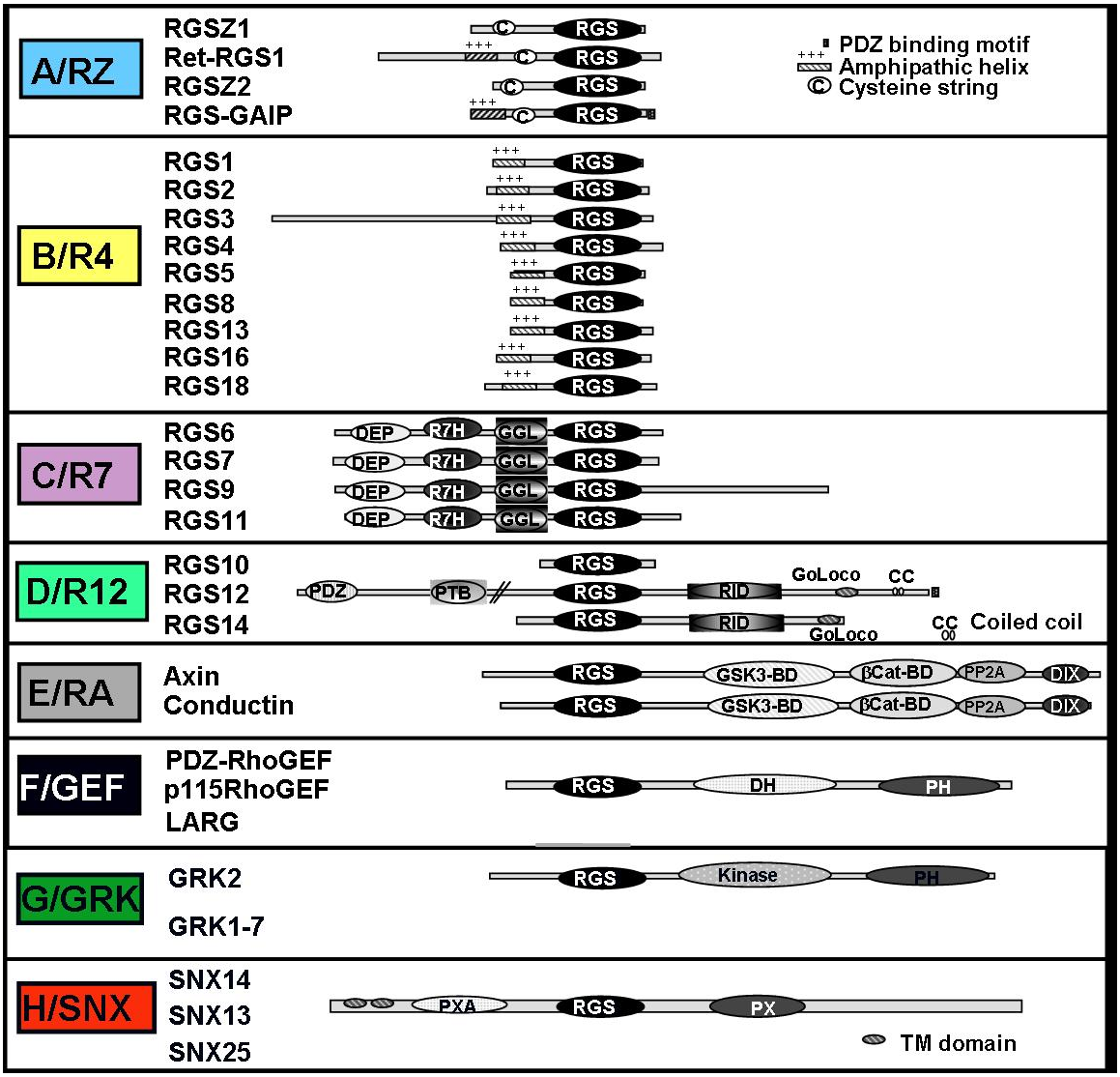
The Regulators of G-protein coupled signaling (RGS) are a large family of proteins which are related via a conserved RGS domain (**Fig 1.1)**. The RGS domain of RGS proteins acts as a GTPase activating protein (GAP) on activated Gα subunits to catalyze the hydrolysis of GTP to GDP (Henry R. Bourne 1991; De Vries, Zheng et al. 2000). Currently over 30 members of the RGS protein family have been identified in mammals and are divided into eight subfamilies (De Vries, Zheng et al. 2000; Ross and Wilkie 2000; Hollinger and Hepler 2002; Siderovski DP 2005).

The RGS super family is defined by a conserved 130 amino acid RGS domain. RGS proteins range from small and simple, like RGS 10, to complex multi-domain

proteins such as RGS 12. RGS proteins catalyze the conversion of GTP to GDP on G alpha subunits by binding the switch region of G alpha and stabilizing the transition state of G alpha during GTP hydrolysis, thereby increasing the rate of G alpha inactivation (Tesmer, Berman et al. 1997; Srinivasa, Watson et al. 1998). RGS proteins have also been reported to act as effector antagonists which bind and directly inhibit effector signaling (Hepler, Berman et al. 1997; Usui H 2000).

RGS family members contain additional domains which confer a variety of additional binding and signaling properties (Hepler 1999; Siderovski, Strockbine et al. 1999; Scott 2000; Hollinger and Hepler 2002). Members of the RGS family have been shown to bind both monomeric GTPases and ion channels to affect their signaling functions (Kozasa T 1998; S Traver 2000; Bender, Nasrollahzadeh et al. 2008; Cifelli, Rose et al. 2008). Members of the D/R12 subfamily, RGS12 and RGS14, have been shown to integrate G protein, RGS, and RTK signaling pathways (Xiaojing Lou 2001; Willard, Willard et al. 2007; Shu, Ramineni et al. 2010). The specificity RGS proteins as drug targets could be attributed to their specificity of expression within cells, rather than their specificity for specific receptors or GPCRs.

Figure 1-1. The RGS family of proteins. RGS proteins are divided into 8 sub families based on their RGS domain sequence homology and function. Families are named A-H or referred to via one of the representative proteins within a family (e.g. RGS12 for the D/R12 family). Functional and binding domains of the RGS proteins are shown. Figure adapted from (Hollinger and Hepler 2002).


A/RZ
RGSZ1
Ret-RGS1
RGSZ2
RGS-GAIP
RGS
PDZ binding motif
Amphipathic helix
Cysteine string
B/R4
RGS1
RGS2
RGS3
RGS4
RGS5
RGS8
RGS13
RGS16
RGS18
C/R7
RGS6
RGS7
RGS9
RGS11
DEP
R7H
GGL
D/R12
RGS10
RGS12
RGS14
PDZ
PTB
RID
GoLoco
CC
Coiled coil
E/RA
Axin
Conductin
GSK3-BD
βCat-BD
PP2A
DIX
F/GEF
PDZ-RhoGEF
p115RhoGEF
LARG
DH
PH
G/GRK
GRK2
GRK1-7
Kinase
H/SNX
SNX14
SNX13
SNX25
PXA
PX
TM domain

1.4.1 The functional domains and families of RGS proteins

The non-RGS domains of RGS proteins add a wide range of complexity to the signaling functions within the RGS super family. The nomenclature of the RGS family of proteins is either an alphabetical A-H designation or an abbreviation using a representative member of the sub family (e.g. RGS12 representing the D/R12 family) (De Vries, Zheng et al. 2000; Ross and Wilkie 2000). RGS proteins in the A/RZ and B/R4 families consist of RGS domains flanked by short and variable N and C terminal domains. Due to the small nature of these families of RGS proteins, their main function is to bind activated Gα subunits to act as GAPs, though there is some data to support additional non-GAP roles for these families (Tinker 2006). Larger, complex, multi-domain RGS protein families include members of the C/R7, D/R12, E/RA, F/GEF, G/GRK, and H/SNX and range in size from 60kDa to 160kDa. RGS proteins in families C-H have been linked to modulation of GPCR signaling, GRK signaling and G protein signaling cascades (Hepler 1999; Siderovski DP 2005; Willars 2006).

While the canonical role of RGS proteins is to serve as GAPs, they can also serve as effector antagonists for Gα (Hepler, Berman et al. 1997; Yan, Chi et al. 1997). In fact, the primary role of the RGS domain in the G/GRK subfamily is to block Gq/llα signaling with no obvious GAP activity for Gα (Carman, Parent et al. 1999). Complex RGS proteins also contain well characterized domains which confer additional signaling properties. RGS12 and RGS14 of the D/R12 family contain GoLoco and RAP binding domains (RBD) and in the case of RGS12 additional PDZ and PTB domains. These domains give RGS12 and RGS14 the capacity to bind Gαi-GDP, Ras/Rap GTPases, Raf kinases and RGS12 the capacity to bind ion channels (Carman, Parent et al. 1999; Schiff, Siderovski et al. 2000; Hollinger and Hepler 2002; Richman, Diversé-Pierluissi et al. 2004; Siderovski DP 2005; Mittal and Linder 2006; Shu, Ramineni et al. 2010).

Novel binding partners are still being discovered for each subfamily of RGS proteins and the complete functional capacity of RGS proteins has yet to be fully elucidated. Models being developed by several labs in the last few years have suggested RGS proteins may also be acting as protein scaffolds to integrate a wide range of cellular signaling machinery. RGS proteins may be acting as the primary scaffolds themselves, or as part of a larger complex of proteins linked with receptors and G proteins. Functioning as scaffolds RGS proteins may link GPCR/G protein signaling events into additional pathways and cellular signaling events.

1.4.2 RGS proteins as drug targets

As mentioned previously, GPCRs now represent a majority of current drug targets which are being brought to market, with some estimates reaching as high as 45% -50% of new drugs (Johnson JA 2003). As such, the demand to find novel mechanisms to specifically modulate GPCR pathways is very high. RGS proteins provide a spectrum of targets which modify an impressive array of cellular pathways. Current work on RGS drug targets is still in its early stages; however several RGS members have been suggested as potential drug targets for therapeutics. While some data exist for a role for RGS proteins in cancer, the two main areas of interest so far for RGS proteins as drug targets are in cardiovascular diseases and in the central nervous system (Ogier-Denis, Pattingre et al. 2000; Neubig and Siderovski 2002; Riddle, Schwartzman et al. 2005).

RGS proteins are widely expressed in cardiac tissue, with members of the R4, R7, R12 and RL families all being found in the heart (Riddle, Schwartzman et al. 2005). GPCRs play a critical role in the cardiovascular system, notably by regulating vasoconstriction coupled to Gq signaling cascades through ion channels. Nearly half of the known RGs proteins exhibit some GAP activity toward Gq and could therefore have

potential therapeutic influence over vascular tone. RGS2 GAP activity for Gq antagonizes G1-mediated vasoconstriction, showing both some Gq selectivity and potent GAP activity (Heximer, Srinivasa et al. 1999; Heximer, Knutsen et al. 2003). RGS4 has been shown to speed the activation and deactivation of G-protein gated K^+ ion channels, which are responsible for acetylcholine mediated bradycardia (Leaney, Milligan et al. 2000). An additional role of RGS4 involved in cardiac hypertrophy (the thickening of the heart muscle) has been characterized by Muslin and co-workers (Rogers, Tamirisa et al. 1999; Tamirisa, Blumer et al. 1999; Rogers, Tsirka et al. 2001).

RGS proteins are also widely expressed in neuronal tissue and play critical roles in the signal transduction of many GPCRs and G protein signaling events in the brain (Doupnik, Davidson et al. 1997; Zerangue and Jan 1998; De Vries, Zheng et al. 2000; Ross and Wilkie 2000; Zachariou, Hooks et al. 2008; Traynor 2010). RGS7 has been recently shown to be recruited to G protein-coupled inwardly rectifying potassium channels (GIRKs) and modify their actions in GABA neurons with unknown functional consequences (Xie, Allen et al. 2010). Exciting new data from our own lab shows RGS14 KO mice to have enhanced learning and memory compared to wild type mice, with implications involving synaptic plasticity (Lee, Simons et al. 2010). While no drugs are currently on the market which target RGS proteins, the potential for new drugs is an area of intense interest (Neubig and Siderovski 2002).

1.5 RGS14

RGS14 is a member of the D/R12 family of RGS proteins and is among the larger and more complex RGS proteins. RGS14 is approximately 62kDa and contains a conserved RGS domain on its C-terminus, tandem Ras/Rap binding domains (RBD1 and RBD2), and a GoLoco/GPR motif on its C-terminus. Originally characterized as a Rap

binding protein which preferentially regulates the GTPase activity of Gαo (Snow, Antonio et al. 1997; S Traver 2000), RGS14 was subsequently found to bind activated GTP-bound Gαi/o on its RGS domain to act as a non-selective GAP (Cho, Kozasa et al. 2000; Hollinger, Taylor et al. 2001). Inactivated GDP-bound Gαi1/3 is bound at RGS14's GoLoco/GPR domain to act as a selective guanine nucleotide exchange inhibitor (GDI) (Hollinger, Taylor et al. 2001; Kimple, De Vries et al. 2001; Traver, Splingard et al. 2004). GDI's function to inhibit guanine nucleotide exchange (from bound GDP to GTP), serving as signaling regulators in GTP signaling cascades and protein complexes. Both Rap1 and Rap2 have been shown to bind RGS14 at its RBD1 domain (S Traver 2000; Mittal and Linder 2006). Data in our own lab have shown RGS14 to be recruited to the plasma membrane by Gαi1/3 (Shu 2007) and by activated Rap2a and H-Ras (Shu 2010).

Although much is known biochemically about RGS14, its role in biological systems is still somewhat unclear. RGS14 gene knockout mice were originally reported to be mitotic lethal at early embryonic stages (Martin-McCaffrey, Willard et al. 2004). Embryonic lethality refers to a protein or factor which is required for further development of the embryo. A deletion or mutation of an embryonic lethal protein typically results in death of the embryo. Later studies have linked RGS14 with spindle fiber formation and localization to the centrosome, but its role in this regard has not been fully studied and confirmed (Martin-McCaffrey, Willard et al. 2004; Cho and Kehrl 2007). RGS14 has also been shown to modulate calcium channel signaling through Gi/o linked receptors in a biological assay, i.e. purified RGS14 accelerates the M2 muscarinic (M2-Ach)-stimulated steady-state GTPase activity of Go and Gi in cell membranes (Hepler, Cladman et al. 2005).

The mRNA of RGS14 is found in many human cells and tissues; however, it is highly enriched in the brain and in particular the hippocampus (S Traver 2000;

Hollinger, Taylor et al. 2001). Current studies in our lab have shown native RGS14 to be enriched in the CA2 region of the hippocampus, a relatively unstudied region of the hippocampus which is thought to modulate learning and memory (Lee, Simons et al. 2010). It should also be noted RGS14's binding partners H-Ras, Rap2a, Gαi1/3 and Gαo are also found in hippocampal neurons and post-synaptic densities and have been shown to have roles in dendritic spine formation (Manabe, Aiba et al. 2000; S Traver 2000; Zhu, Qin et al. 2002; Peng, Kim et al. 2004; Manuel, Maria et al. 2006). RGS12, RGS14's closest relative, has been shown to bind and modulate the activity of N-type and L-type Ca^{++} receptors in neurons where RGS14 is also found (Schiff, Siderovski et al. 2000; Richman, Tombler et al. 2004; Richman, Strock et al. 2005).

Post synaptic plasticity, the rearrangement of postsynaptic dendritic proteins and membranes post excitatory simulation, is thought to be a key pathway through which learning, memory and neuronal development are regulated in neurons(Sheng and Kim 2002; Carlisle and Kennedy 2005; Ethell and Pasquale 2005). Exciting new data from our own lab has shown loss of the RGs14 gene/protein (i.e. RGS14-KO) in mice leads to significantly enhanced learning and memory (Lee, Simons et al. 2010).

More recently RGS14 has been shown to bind members of the Ras and Raf protein families on its R1 and R2 domains. H-Ras, C-RAF, and B-Raf were shown to co-immunoprecipitate with RGS14 when over-expressed in HeLa cells with poorly understood functional consequences (Willard, Willard et al. 2009; Shu, Ramineni et al. 2010). Due to H-Ras and Raf kinases being heavily involved in the MAPkinase signaling cascade, the authors did examine RGS14's effects on several well known RTKs which, when activated, activate H-Ras and Rafs. The authors found that RGS14 selectively inhibits PDGF stimulated ERK1/2 phosphorylation (a marker of Raf kinase activity), but not EGF stimulated ERK1/2 phosphorylation (Shu, Ramineni et al. 2010). In addition to the ERK1/2 studies, the authors showed Raf and Gαi binding to be mutually exclusive,

leading the authors to hypothesize that RGS14 was acting as a scaffolding protein/switch to regulate Raf kinase, and consequently, MAPkinase signaling (Shu, Ramineni et al. 2010).

1.5.1 Unconventional G protein signaling and RGS14

Unconventional G protein signaling can be described as signaling events which occur through G proteins which are not linked through GPCRs. Examples of unconventional G protein signaling include proteins which interact with Gα in similar manners as RGS14 are prevalent in the literature (Hampoelz and Knoblich 2004; Blumer, Cismowski et al. 2005; Wilkie and Kinch 2005; Blumer, Smrcka et al. 2007). One example of receptor independent G protein regulation is the activators of G protein signaling (AGS) proteins. AGS proteins have been shown to modulate mitotic spindle formation mediated by Gα proteins, play roles in NMDA signaling in rat brain extract, and in the case of AGS3, function in drug seeking behavior in rats (Blumer, Cismowski et al. 2005; Siderovski DP 2005).

AGS proteins function in a similar manner as canonical heterotrimeric G protein signaling, although the exact mechanism of AGS activation is not currently known. The GoLoco domain on AGS proteins substitute as the Gα binding domain and cytosolic guanine nucleotide exchange factors (GEFs) regulate the Gα protein binding states. In this manner GoLoco proteins have been shown to regulate cell division, neuronal development, and synaptic plasticity (Hampoelz and Knoblich 2004; Blumer, Cismowski et al. 2005).

A required element for Gα protein GDP to GTP exchange is a GEF, which is typically a GPCR; however other proteins also function as GEFs. One example of a GEF which acts on Gα subunits is Ric8a. Ric8a is a cytosolic GEF enriched in the brain and

involved in mitotic spindle formation. The deletion of Ric8a has been shown to be embryonic lethal (Miller and Rand 2000). Ric8a is a known cytosolic GEF of Gα and data by F. Shu and C. Vellano in our lab shows transfected RGS14 binds and translocates from the cytosol to the plasma membrane when co-transfected with Ric8a and Gαi1 (Miller and Rand 2000). Additional studies in our lab show Ric8a binding on RGS14 truncation mutants to be dependent on the first 213 amino acid residues of RGS14, which includes the RGS domain.

As mentioned previously, RGS14 binds inactive-GDP bound Gαi1/3 at its GL/GPR domain to function as a GDI. When Gαi1/3 is co-expressed with RGS14 in HeLa cells both RGS14 and Gαi1/3 co-localize to the plasma membrane (Shu, Ramineni et al. 2007). RGS14 has thus been shown to bind and interact with all necessary elements to function as a non-receptor G protein signaling scaffold.

1.5.2 RGS14 and the MAPkinase signaling cascade

The MAPkinase signaling cascade is one of the most heavily studied and important signaling cascades involved cell growth/survival, neuronal development and synaptic plasticity (Seger and Krebs 1995). The MAPkinase cascade has also been heavily implicated in various forms of human cancers (Roberts and Der 2007). G proteins and receptor tyrosine kinases (RTKs) can activate the MAPkinase pathway by distinctive pathways; either through GPCRs or downstream effectors of RTK activation (Igishi and Gutkind 1998; Avruch, Khokhlatchev et al. 2001; Goldsmith and Dhanasekaran 2007). RTK's are a class of cell surface receptors that contain a tyrosine kinase domain which phosphorylates proteins on a tyrosine residue. RTK's bind hormones, growth factors, and cytokines resulting in receptor auto-phosphorylation and

subsequent activation of internal signaling pathways with a wide array of cellular responses. MAPkinase signaling cascades begin with an agonist binding and activating a RTK, GPCR or other receptor. In the case of RTK signaling H-Ras is activated by GEF activity to exchange GDP for GTP and in turn H-Ras activates Raf kinase via binding of the Raf regulatory domain (Chong, Vikis et al. 2003). GPCR's activate the MAPkinase pathway through Gαq or Gαi/o upstream activation which leads to PKC activation of Raf kinase (Enrique 2007). Both pathways result in Raf activation and the subsequent phosphorylation of MEK, which then phosphorylates ERK. ERK phosphorylation results in ERK translocation to the nucleus and a wide range of transcriptional responses. Substrates of ERK include the transcription factors Elk-1, c-Myc, c-Jun, c-Fos and C/EBP beta (Davis 1995).

One upstream activator of the MAPKinase signaling cascade is the platelet derived growth factor receptor (PDGF-R), a member of the tyrosine kinase growth factor receptor super family. As would be expected of a MAPKinase cascade stimulator, PDGF-R is associated with cell proliferation, cellular differentiation, cell growth and development, and has been associated with vascular smooth cell growth and human cancers (Andrae, Gallini et al. 2008). Uncontrolled angiogenesis is one of the major characteristics of human cancers and PDGF has been the subject of several clinical trials (Homsi J 2007).

Recent data published by our lab shows that expression of RGS14 selectively inhibits PDGF stimulated ERK1/2 phosphorylation in HeLa cells and we find that this RGS14-mediated inhibition is dependent on H-Ras binding and is reversed by co-expression of Giα1 (Shu, Ramineni et al. 2010). Additional reports on RGS14 regulation of MAPkinase members show RGS14 is a selective H-Ras isoform binding protein (Willard, Willard et al. 2009). RGS14 has also been shown to attenuate Gi/o linked muscarinic receptor mediated ERK1/2 phosphorylation (Traver, Splingard et al. 2004).

RGS14 has been uniquely shown to bind both G proteins and members of the MAPKinase signaling cascade, positioning it to possibly integrate the two signaling pathways.

1.5.3 RGS14 is regulated by phosphorylation

Early work attempting to characterize RGS14's activity towards GDP-bound Gαi1 resulted in the discovery of several sites on RGS14 which are phosphorylated by PKA (Hollinger, Ramineni et al. 2003). RGS14 was found to be phosphorylated on Threonine 494 which resulted in potentiated GDI activity towards Gαi1. An additional PKA phosphorylation site was shown at serine 289 and RGS14 is known to be phosphorylated by ERK at Serine 52 (Hollinger and Hepler 2004). These data suggest RGS14 is likely regulated by phosphorylation at additional sites with unknown signaling consequences.

1.6 Overall Hypothesis Guiding this Research

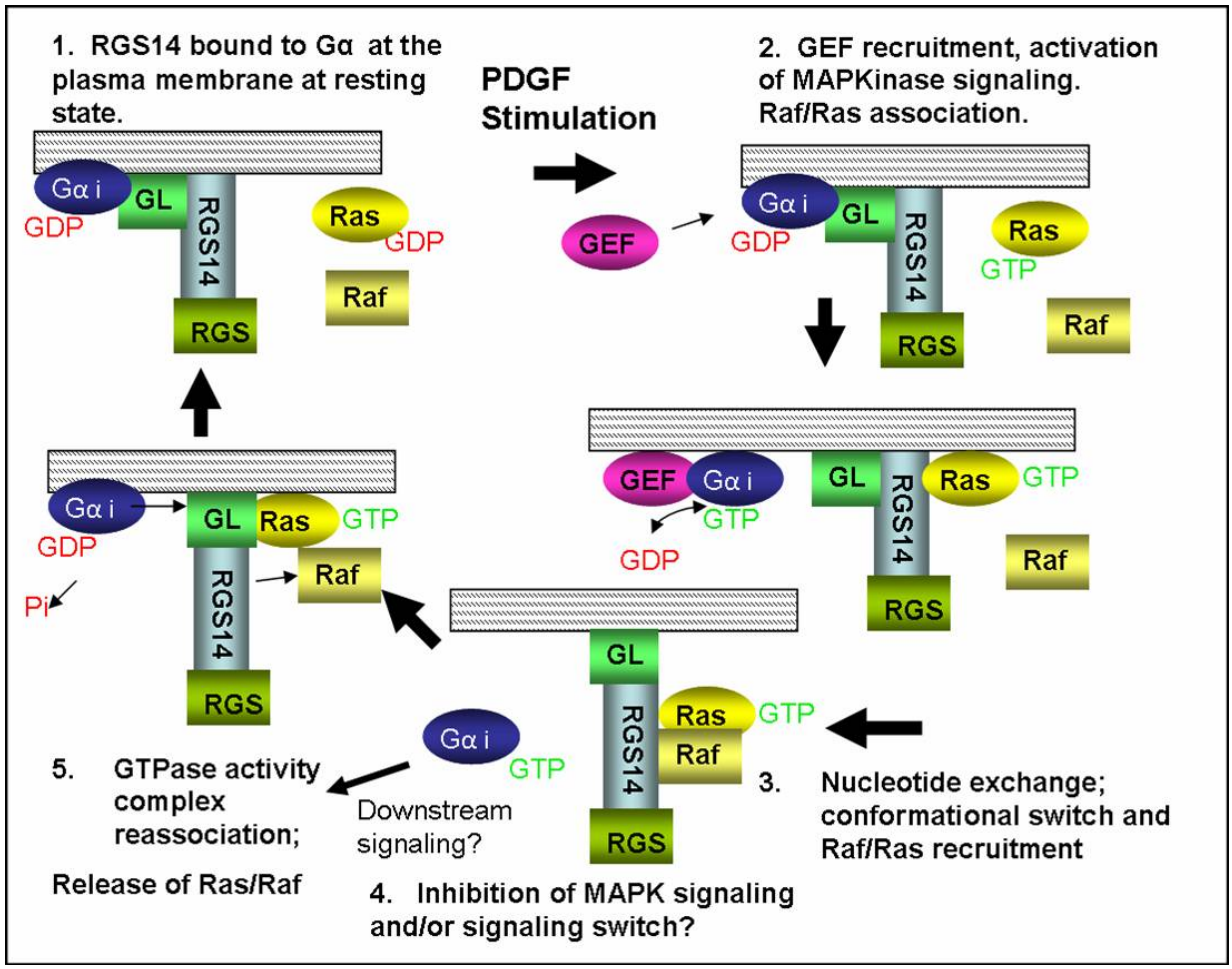
RGS14 is a complex protein with multiple binding domains and many known biochemical functions, yet its physiological function in biological systems remains unclear. While some labs have postulated RGS14 plays a role in mitotic spindle formation and cell division in mitotic competent cells, additional functions in non-dividing mature neurons must also occur. Our lab and others have published data showing RGS14 to bind and regulate members of the MAPkinase signaling pathway with functional consequences. Recent data in our lab has shown RGS14 to play a role in synaptic plasticity in the hippocampus. Not fully known are the functional consequences and regulatory mechanisms of RGS14 binding to its many partners and how these proteins interact when in complex with each other. The aim of this work is to provide

insight into the biochemical functions and regulations that RGS14 undergoes when bound to H-Ras, Raf kinase, and Gαi.

Central to the research presented is the hypothesis that RGS14 acts as a signaling scaffold/switch to integrate Gαi, H-Ras, and Raf kinase interactions. In this model, RGS14 bound to Gαi1-GDP at the plasma membrane serves as the basal state of our model **(Fig 1-2, step 1)**. A growth factor, such as PDGF, would stimulate a cytosolic GEF to exchange GTP for GDP on Gαi1 and activate the signaling cascade. Gαi1 may then bind and activate unknown downstream effector molecules with unknown signaling consequences **(Fig 1-2 step 2)**. The third step of our model includes the recruitment of free Ras and Raf to bind RGS14 which inhibits MAPKinase signaling **(Fig 1-2 step 3)**. Intrinsic GTPase activity of RGS14 would catalyze the hydrolysis of Gαi1-GTP to Gαi-1-GDP, resulting in the release of Ras/Raf and a return to basal resting state **(Fig 1-2 step 4-5)**.

The second aim of my research project was to determine how phosphorylation may regulate RGS14 sub-cellular localization and signaling functions. Previous work by our lab has shown RGS14 to be highly regulated by phosphorylation. A novel observation by Fengjue Shu in our lab led us to believe RGS14 was phosphorylated when in complex with Gαi1/3 with unknown functional or regulatory consequences. The knowledge gained in these studies will be useful to fully characterize RGS14 and its role in cellular biology.

Figure 1-2 Proposed model for non-receptor RGS14 regulation of Gαi1-GDP and MAPkinase signaling cascades. 1. RGS14 at resting state bound to the plasma membrane via its GL domain(GL=GoLoco domain of RGS14, RGS=RGS domain) 2. Extracellular or intracellular signaling cascade results in GEF activation and subsequent exchange of GTP for GDP on Gαi1. Map kinase signaling proteins activated through same signaling mechanisms. 3. GTP exchange on Gαi1 results in Gαi1 dissociation from RGS14 and subsequent Ras/Raf association/binding with RGS14. 4. Intrinsic GTPase activity of RGS14 cleaves terminal phosphate on GTP bound to Gαi1. Reassociation of Gαi1 to RGS14 results in a conformational shift releasing Ras/Raf. 5. Return to baseline state with GDP- Gαi1 bound to RGS14 at the plasma membrane.


1. RGS14 bound to Gα at the plasma membrane at resting state.
PDGF Stimulation
2. GEF recruitment, activation of MAPKinase signaling. Raf/Ras association.
Gα i
GL
RGS14
RGS
GDP
Ras
GTP
Raf
GEF
Pi
3. Nucleotide exchange; conformational switch and Raf/Ras recruitment
4. Inhibition of MAPK signaling and/or signaling switch?
5. GTPase activity complex reassociation;
Release of Ras/Raf
Downstream signaling?

Chapter II: RGS14 regulation of Raf kinase activity and MAP kinase signaling cascades

2.1 Introduction

A growing body of evidence suggests G proteins can function independent from GPCRs to modulate a wide array of signaling functions inside the cell (Cismowski 2006; Blumer, Smrcka et al. 2007). In non-receptor G protein signaling cascades, scaffolding proteins function to bind Gα proteins separate from Gβγ and GPCRs. Once G proteins have become bound additional proteins are recruited to function as GAPs and GEFs on bound G proteins to regulate various cellular signaling cascades.

The MAPkinase signaling cascade is one of the most studied and well understood pathways involved in cellular growth and proliferation (Benjamin and Jones 1994; Seger and Krebs 1995; J T Lee Jr1 and J A McCubrey1 2002; Roux and Blenis 2004). Mutations of several members of the MAPkinase cascade have been shown to lead directly to cancer, most notably and well characterized being the constitutively activated H-Ras mutation (Bos 1989; Adjei 2001).

Recent data published in our lab has shown RGS14 to bind several members of the MAPkinase cascade, including B-Raf, C-Raf/Raf-1 and active H-Ras (Shu, Ramineni et al. 2010). In the same study, RGS14 was found to down-regulate PDGF directed ERK phosphorylation through Ras/Raf inhibition. Of particular importance, PDGF signaling inhibition was dependent on H-Ras binding to RGS14 and is reversed by Gαi1 co-expression. Also noted in these studies were that Raf and Ras binding facilitate each others' binding to RGS14, while Gαi1 disrupts Raf binding (Shu, Ramineni et al. 2010).

When put together these findings suggest that RGS14 is acting as a signaling scaffold to mediate G protein and MAPkinase signaling cascades. RGS14, along with its close relative RGS12, are the only two proteins which contain both an RGS and GoLoco domain and the capacity to bind G proteins at two distinct locations. The ability of RGS14 to bind non-active GDP-bound Gαi1 on one domain and regulate the life span of

activated GTP-bound Gαi1 at its RGS domain has the potential for a novel method of G protein signaling regulation.

The goals of these studies were to expand upon previous data obtained in our lab and to further elucidate the roles RGS14 is playing in MAPkinase signaling. The first aim of my research was to determine how RGS14 interacts with Ras and Raf to inhibit ERK phosphorylation previously reported in PDGF signaling. Future studies will determine the different binding conformations RGS14 may undertake with its various binding partners.

2.2 Experimental Procedures

Materials

Recombinant RGS14 Full Length penta-His , recombinant RGS14 Truncation AA 1-415 penta-His, recombinant full length RGS2 his tagged were constructed from cDNA kindly provided by D. P. Siderovski (University of North Carolina, Chapel Hill, NC) as described in (Hollinger, Taylor et al. 2001). Glutamine-Glutamine (EE) tagged Gαi1 in pcDNA 3.1 was purchased from UMR cDNA Resource Center (Rolla, Missouri). HA-cRaf in pcDNA 3.1 was a gift from Dr. Deborah Anderson (Saskatchewan Cancer Agency). Raf-1 Kinase Assay kit for Chemiluminescence Detection was purchased from Upstate (#17-360). H-Ras V12-GST protein was purchased from Cytoskeleton (#GV12G01). Raf-1 Recombinant protein is a truncated version of the full length Raf-1 protein with the regulatory domain cleaved and was purchased from Novus Biologicals (#5894) Anti-Mitogen Activated Protein Kinase Kinase (MEK, MAPKK) was used in kinase assays and purchased from Sigma (#M5795).

Methods

Cell Culture and Transfection HeLa cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution at 37°C with 5% CO2. Cells were grown to 80-90% confluency and transfected using the Lipofectamine 2000© protocol method. A mixture of recombinant DNA (0.4µg to 4µg dependent on well size) and 2µl-60µl of Lipofectamine (per well) were incubated together for 30 minutes in Opti-MEM prior to transfection. Cells were then washed 2x with Opti-MEM and the DNA/Lipofectamine mixture was added drop-wise to be incubated 5-6 hours. After

incubation period Opti-MEM was aspirated and growth media was added for an overnight incubation.

Purified Protein Expression

Thioredoxin and hexa-histidine tagged RGS14 (TxH6-RGS14) and hexa-histidine tagged amino acids 1-299 [H6-R14(RGS/RR)] and His-tagged RGS2 were expressed in BL21DE3 cells. The cells were grown to mid-log phase and protein production was induced with 1 mm IPTG for two hours. Cells were lysed using the French Press method, supernatant recovered, loaded to a Ni21 HiTrap affinity column (Amersham Pharmacia, NJ, USA), and purified by FPLC. Proteins were eluted with an imidazole gradient from 20 to 200 mM in 150 mM NaCl and 50 mM HEPES as previously described in (Hollinger, Taylor et al. 2001).

Raf Kinase Activation Assay

Raf kinase assays were performed per Upstate protocol for pure protein assay (Upstate cat #17-360). All mixing step were on ice; reagent stability was ensured by aliquoting to limit freeze thaw cycles. Briefly, 10µl of Magnesium/ATP cocktail was added to a 100µl tube and mixed with 0.05µg truncated (constitutively activated) Raf-1 kinase. Assay dilution buffer I (ADBI, Cat #20-108) was then added to bring total reaction volume per tube to 19ul. 0.5 µg of inactivated MEK was then added and mixed in to begin the reaction. Tubes were placed at 30°C for 30 minutes with shaking to ensure proper mixture of reagents. The assay is stopped by adding 20µl 2x sample buffer and boiled for 5 minutes. Samples were run on an agarose gel immediately or stored at -20°C for use at a later date. Protein immunoblots were performed for both

phosphorylated MEK and total MEK (anti-phospho MEK1 (Ser218/222)/MEK2 (Ser222/226) cat # 07-461). Results are normalized to total MEK within each assay.

For RGS14 purified protein inhibition assays, the protocol is exactly as above with one exception: prior to MEK1 addition, various concentrations of purified RGS14 were added to the reaction mixture. The mixture was then incubated for 30 minutes at 30°C with shaking prior to addition of un-activated MEK1. Reaction was stopped as previously described. Total volumes were kept constant with RGS14 buffer.

For assays run with cell lysates, the protocol was as outlined above; however, 10µl of transfected cell lysate was added to the reaction mixture (in RGS14 purified protein buffer) for 30 minutes at 30°C prior to MEK1 kinase addition. ADBI added was equal to 9µl and RGS14 buffer was used to make the reaction volumes equal, with a total reaction volume not to exceed 50µl. MEK1 (non-activated) was then added to the pre-incubated mixture for 30 minutes at 30°C with shaking and reaction was stopped as previously shown. All results are compared using densitometry analysis of total MEK to phospho-MEK (NIH Image J software, freeware version 1.40g).

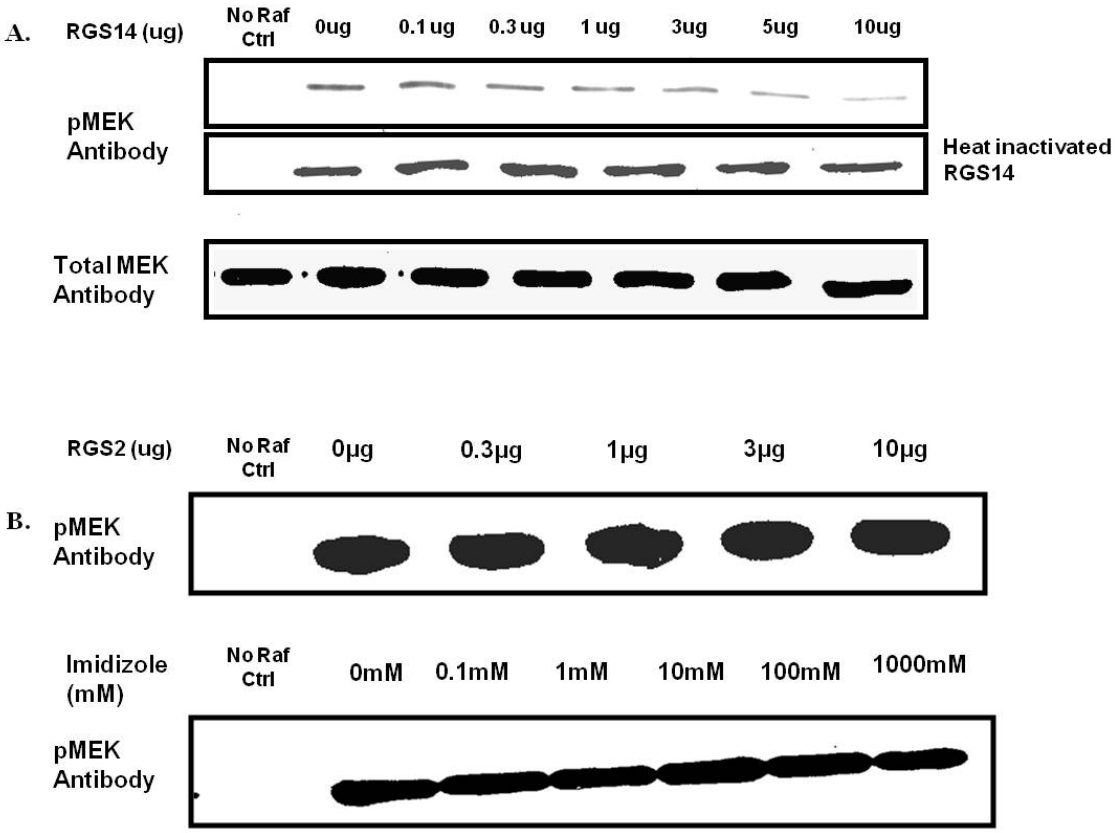
2.2 Results

2.2.1 Purified RGS14 inhibits Raf kinase activation of MEK

RGS14 has been shown to inhibit PDGF signaling in cell culture but the exact mechanism of ERK phosphorylation inhibition remains unknown (Shu, Ramineni et al. 2010). RGS14 could inhibit ERK1/2 phosphorylation through several mechanisms, i.e. by either direct Raf binding and inhibition, or inhibition of the Raf activator H-Ras. Our first study was to determine if direct binding of purified RGS14 to Raf-1 could inhibit Raf kinase activity in vitro (**Fig 2.1**). To explore direct RGS14 inhibition we used MEK1/2 phosphorylation levels as an output of Raf activity. In our initial studies we added purified His-tagged RGS14 to truncated, active Raf kinase, incubated for 30 minutes, and then added MEK substrate for 30 minutes. Reactions were stopped and protein was run on an SDS PAGE. The resulting gels were immunoblotted for total MEK and phospho MEK.

Our studies show purified RGS14 inhibits activated Raf kinase as measured by phosphorylated MEK/total MEK. A representative experiment is shown **(Fig 2.1)**. Increasing concentrations of RGS14 decrease the amount of phospho MEK stimulated by the Raf kinase domain. Multiple experiments were performed (n = 5) and the data normalized to Raf kinase activity alone equal to 100% and quantified using densitometry **(Fig 2.1)** I found that RGS14 directly inhibited Raf kinase activity by nearly 90%. Half-maximal MEK phosphorylation inhibition was observed with 2-4 ug of RGS14 added to 0.01 ug Raf.

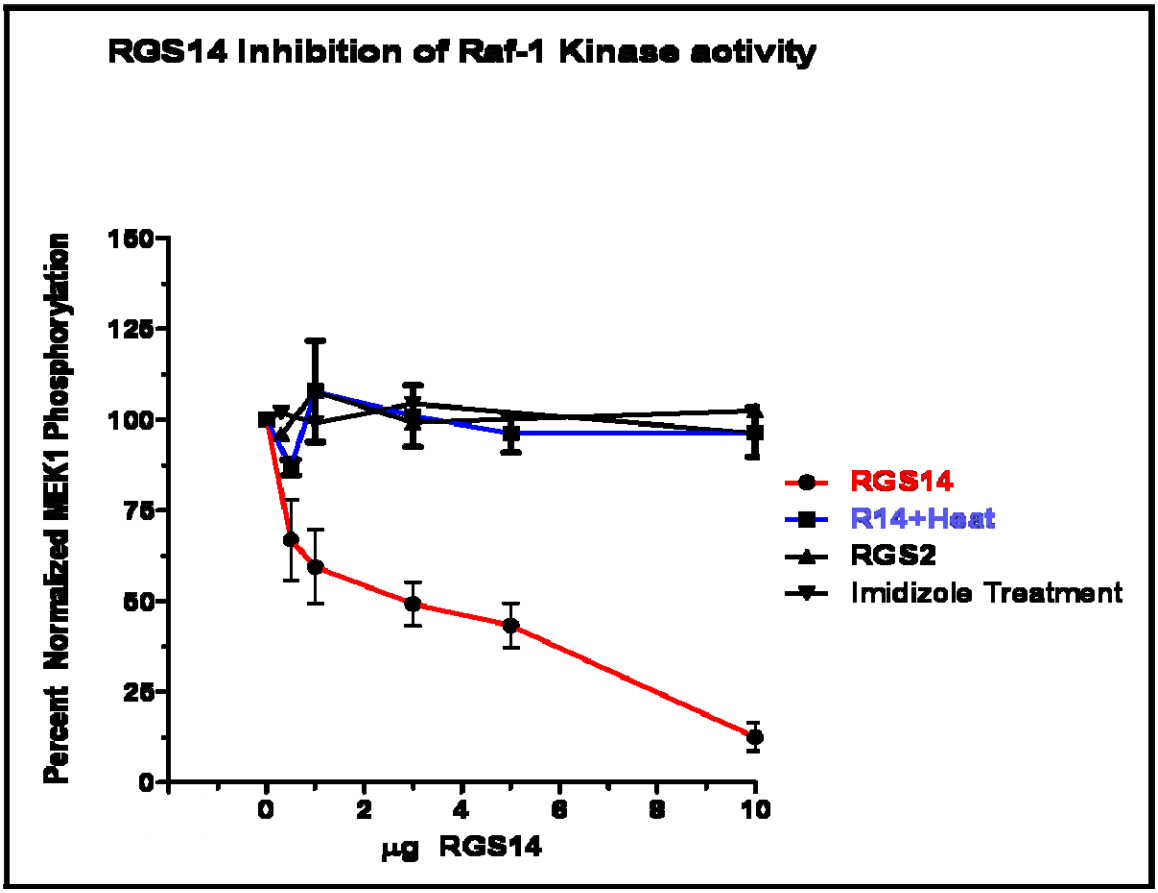
Figure 2-1 A) Purified RGS14 inhibits Raf kinase mediated phosphorylation of MEK. Increasing concentrations of purified His-tagged RGS14 were added to MEK in the presence of free phosphate and Raf kinase at shown amounts. Negative controls for this experiment were wells containing only MEK with no Raf kinase added. Heat inactivated (ΔRGS14, 5 min 100°C) does not inhibit MEK phosphorylation by Raf kinase in the same assay. B) Neither RGS2 nor imidizole treatment at shown concentrations inhibit MEK phosphorylation by Raf kinase in the same Raf kinase assay. These experiments were carried out five times and a representative experiment is shown (N=5).


A.
RGS14 (ug)
No Raf Ctrl
0ug
0.1 ug
0.3 ug
1 ug
3ug
5ug
10ug
pMEK Antibody
Heat inactivated RGS14
Total MEK Antibody
RGS2 (ug)
No Raf Ctrl
0µg
0.3µg
1µg
3µg
10µg
B.
pMEK Antibody
Imidizole (mM)
No Raf Ctrl
0mM
0.1mM
1mM
10mM
100mM
1000mM
pMEK Antibody

2.2.2 RGS14 specifically inhibits Raf-1 kinase activity in vitro.

The second set of experiments (**Fig 2-1 B)** we conducted to confirm RGS14 specific inhibition of MEK phosphorylation. In these experiments we confirmed the results found in **Figure 2-1 A**. As additional controls I also tested the specificity of RGS14 inhibition by testing an additional RGS protein RGS2 in our assay. I also tested the effects of imidizole at concentrations equal to amounts present in the RGS14 buffer. We find that, like heat-killed RGS14, imidizole treatment does not inhibit Raf kinase capacity to phosphorylate MEK. Representative immunoblots from a single experiment are shown (**Fig 2-1 B**). Multiple experiments were performed (n=5), the data quantified by densitometry, and pooled (**Fig 2-2**). It appears that very low amounts of RGS14 are needed to inhibit Raf kinase activity, under 2μg RGS protein can reach 40% inhibition of 1 μg Raf protein. With 10μg RGS14 inhibiting nearly 90% of MEK phosphorylation . By contrast RGS2, imidazole buffer or heat-inactivated RGS14 did not inhibit Raf kinase activity. Data was normalized by setting MEK kinase phosphorylated with Raf kinase as 100% phosphorylation.

Figure 2-2 RGS14 specifically inhibits Raf-1 kinase activity in vitro. RGS14 inhibits Raf kinase directed MEK phosphorylation in vitro. RGS2 in the same amount of RGS14 was added and resulted in no decrease in normalized phosphorylation levels. Heat killed RGS14 (ΔRGS14), and imidizole treated Raf kinase does not inhibit MEK phosphorylation. Experiments were performed as described in Fig 2-1, data was pooled from 5 experiments (n=5). Data normalized to MEK phosphorylation by Raf kinase set to 100% phosphorylation.


RGS14 Inhibition of Raf-1 Kinase activity
Percent Normalized MEK1 Phosphorylation
150
125
100
75
50
25
0
0
2
4
6
8
10
µg RGS14
RGS14
R14+Heat
RGS2
Imidizole Treatment

2.3 Discussion

The studies in the previous section clearly show for the first time that RGS14 acts as a novel inhibitor of Raf kinase activity (in vitro). These data suggests RGS14 may be inhibiting ERK phosphorylation by binding the upstream activator Raf kinase or MEK kinase to inhibit Raf phosphorylation. **Figure 2-2** also gives evidence to the validity of steps three and four of our proposed model in **Figure 1-2.** The next steps of this aim would have been to test the other configurations necessary for our model to be true.

Early efforts into testing RGS14 inhibition or Raf kinase activity were not successful due to a lack of RGS14 pre-incubation with truncated Raf kinase. One plausible explanations is Raf kinase has a higher affinity for its substrate MEK and pre-incubation is required for sufficient RGS14/Raf complex to form. Another possible explanation is Raf kinase can rapidly phosphorylate enough MEK to render our measurement methods unable to differentiate between various concentrations of RGS14.

It should be noted the Raf kinase used in these experiments is a truncation mutant with the regulatory domain of Raf removed (this domain lies in the first 306 amino acid residues). The regulatory domain of Raf kinase functions to inhibit the kinase domain when the protein is in an inactive state, upon Raf kinase activation the regulatory domain undergoes a conformational shift which reveals the kinase domain(Baccarini 2005). The deletion of the regulatory domain leaves Raf kinase in a constitutively active conformation that is useful for our experiments; however, there are drawbacks to using a truncated mutant. The regulatory domain and the surrounding amino acid residues may contain important binding regions necessary for Raf to function in its complete cellular role. Removal of this region may lead to unknown binding and functional consequences. Our data suggests RGS14 can directly bind and inhibit Raf kinase at a region different from its regulatory domain.

Of additional note are the relatively low concentrations of RGS14 required for MEK1/2 phosphorylation inhibition. One explanation could be RGS14 is a highly specific inhibitor of Raf-1 kinase activity, which our limited data suggests. Additional factors may also increase RGS14 affinity towards Raf kinase, such as the presence of H-Ras, another known RGS14 binding partner.

These studies were only to be the beginning of a more thorough investigation into the role of RGS14 and its MAPkinase binding partners. In the second series of Raf kinase assay experiments I began to incorporate cell lysates transfected with various constructs into our Raf kinase assay. To begin, I tested full length c-Raf to see if lysates could produce MEK phosphorylation, which they did (data not shown). The next step was to see if RGS14 could inhibit full length c-Raf from phosphorylating MEK. Data collected by Chris Vellano in our lab suggests RGS14 does indeed inhibit full length c-Raf, but questions still remain regarding how these interactions are further regulated by RGS14's binding partners. H-Ras and Gαi1 would be added to our kinase assays to determine if either plays a role in enhancing or mitigating RGS14's inhibition of ERK phosphorylation.

Chapter 3: RGS14 regulation by phosphorylation

3.1 Introduction

Phosphorylation plays a key role in the regulation of many biological systems. RGS proteins are highly regulated by phosphorylation, with a wide range of functional consequences. RGS2 is phosphorylated by PKC at an unknown residue to decrease its GAP activity and phosphorylated by GMP-dependent protein kinase alpha (PKGI-α) to increase GAP activity (Cunningham, Waldo et al. 2001; Tang, Wang et al. 2003). RGS4 is translocated to the plasma membrane from the cytosol when phosphorylated by protein kinase G(PKG) (Pedram, Razandi et al. 2000). In addition to the previous examples, RGS's 7, 9, 10, 16, 18, and 19 have all been shown to exhibit regulation through phosphorylation with functional consequences spanning from binding partners to GAP or GDI activity (Benzing, Yaffe et al. 2000; Ogier-Denis, Pattingre et al. 2000; Balasubramanian, Levay et al. 2001; Burgon, Lee et al. 2001; Chen, Wang et al. 2001; Derrien and Druey 2001; Sokal, Hu et al. 2003; Garcia, Prabhakar et al. 2004). Recent data has linked phosphorylated RGS domains of several RGS family members with regulation of hypertension (Brinks and Eckhart 2010). It should also be noted that RGS proteins have been shown to be modified by both glycosylation and palmitoylation (Tu, Wang et al. 1997; Castro-Fernandez, Janovick et al. 2002; Garzon, Rodriguez-Munoz et al. 2005).

RGS14 has been previously shown by our lab to be phosphorylated by cAMP-dependent Protein Kinase A (PKA) at Ser258 and Thr494 (Hollinger, Ramineni et al. 2003), and by Erk1/2 at Ser52 (Hollinger, 2005?). When both Ser258 and Thr494 are mutated to alanine residues, the incorporation of phosphate into RGS14 by PKA is effectively eliminated. Additionally, mimicking the phosphorylation of Thr494, which is located adjacent to the GoLoco/GPR motif, enhances RGS14s GDI activity on Gαi1, while not affecting GAP activity (Hollinger, Ramineni et al. 2003).

Previous unpublished observations in the Hepler lab led us to believe RGS14 was being phosphorylated when co-expressed with Gαi1 or Gαi3. When recombinant RGS14 and Gαi1/3 were expressed in HeLa cells and visualized via western blot, an additional higher molecular weight RGS14 band would appear approximately 2kD higher than expected, consistent with a phosphorylation event. In addition to the extra band, RGS14 would be recruited to the plasma membrane from the cytosol and co-localize with Gαi1/3.

Given the previous work in our lab and others, we hypothesized that RGS14 contains one or more additional sites which are phosphorylated with unknown signaling, binding and cell localization ramifications. The goals of this research were to determine the specific site(s) on RGS14 which are phosphorylated when in complex with Gαi1/3, to identify the kinase(s) responsible and, eventually, to determine what consequences this modification has on RGs14 function.

3.2 Experimental Procedures

Materials Henrietta Lacks cell line (HeLa cells) were obtained from ATCC (Manassass VA). Dubelco's Modified Eagles Medium (DMEM) was purchased from CellGro. OptiMEM cell culture medium was purchased from Invitrogen/CellGro. Lipofectamine2000 transfection reagent (Invitrogen# 11668-019) and penicillin/streptomycin solution were purchased from Invitrogen. Fetal Bovine Solution (FBS) was purchased from Atlanta Biologicals, Atlanta Ga, nitrocellulose membranes, Bio-Rad (Hercules Ca), enhanced chemiluminescence agent(ECL), anti-Flag M2 antibody, anti-EE antibody, and anti-Flag m2 agarose beads were purchased from Sigma. O-glycosidase was purchased from QA Bio (E-G001). Ellagic Acid was purchased from (#3058, Tocris Bioscience Ellisville, Missouri)

Cell Culture and Transfection HeLa cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution at 37°C with 5% CO2. Cells were grown to 80-90% confluency and transfected using the Lipofectamine 2000© protocol method. A mixture of recombinant DNA (0.4µg to 4µg dependent on well size) and 2µl-60µl of Lipofectamine (per well) was incubated together for 30 minutes in Opti-MEM prior to transfection. Cells were then washed 2x with Opti-MEM and the DNA/Lipofectamine mixture was added drop wise to be incubated 5-6 hours. After incubation period Opti-MEM was aspirated and growth media was added for an overnight incubation. cDNAs used in transfections were: Flag-RGS14 in pcDNA 3.1, Δc-213, Δ213-490, Δ490-n Flag-RGS14 in pcDNA 3.1, EE Gα i1 in pcDNA 3.1, HA-cRaf in pcDNA 3.1, HA-H-Ras in pcDNA3.1, HA-H-Ras G12V in pcDNA 3.1

Plasmids and antibodies

RGS14 cDNA used in this study was derived from rattus norvegicus (Genbank accession number U92279). Gl-Glu epitope (EE) tagged recombinant Gαi1/pcDNA3.1 plasmid was purchased from UMR cDNA Resource Center (Rolla, Missouri). The expression plasmids encoding RGS14 full length, RGS14 deletion mutants coding for amino acids 1-213, 213-544, 213-490, and 444-544 cloned in frame into the pcDNA3.1 (Invitrogen) were prepared by F. Shu as described in (Shu, Ramineni et al. 2007).

Anit-FLAG antibody, Alexa 488-conjugated goat anti-rabbit and Alexa546-conjugated goat anti-mouse antibodies were purchased from Invitrogen. Anti-EE antibody was purchased from BD Biosciences. Monoclonal anti-HA horseradish peroxidase (HRP) conjugate antibody and monoclonal anti-HA TRITC (Rhodamine) conjugate antibody were also purchased from Sigma. Anti-Flag M2 antibody affinity gel was purchased from Sigma.

Immunoblot analysis

Samples were resolved by SDS-PAGE on 4-20% Tris-Glycine gels, followed by transfer to nitrocellulose membranes. The membranes were incubated in blocking buffer (2% nonfat dry milk, 50mM NaCl, 20mM HEPES, 0.1% Tween 20) for 30 min and then incubated with primary antibody for either 1 h at RT or overnight at 4°C. Next, the membranes were washed three times in blocking buffer and incubated with either a fluorescent- or HRP-conjugated secondary antibody for 30 min. Membranes were washed three times and finally visualized using the Odyssey imaging system (Li-Cor) or via ECL reagent followed by exposure to film.

Immunoprecipitation Assays

For non-denaturing immunoprecipitation assays of transfected proteins, transfected cells were washed 3x in cold PBS and lysed in buffer containing 150mM NaCl, 50mM Tris-HCl (pH 8.0), 1mM EDTA, 1mM EGTA, 10mM MgCl2, 50µg/ml Aprotinin, 100µg/mL Leupeptin, 1um phenylmethylsulfonyl fluoride (PMSF) and 1% TritonX-100. Cells were scraped and lysate was collected in a 1.5mL tube. Lysates were spun at max RPM on a bench top centrifuge to remove cellular debris. Each lysate was incubated with 30µl/mL FLAG M2 beads for 2 hours at 4 C with rotation in a 1.5mL tube. After incubation lysates were spun for 1 minute at >12,000 RPM, supernatant removed and washed with 1mL cold TBS. Cell were washed a total of 4 times. After final wash step, supernatant was removed and beads were resuspended in 100ul 2x sample buffer. The final sample to be run on gels was collected after a final spin at >12,000 RPM in order to remove beads from the gel run. Samples were then run on acrylamide gels for western blot analysis.

Denatured Immunoprecipitation Assay

Cells were grown on desired plates and perform transfections as previously described. In these experiments 6 P150 plates were grown and transfected. Cells were washed two times with 5mL of cold PBS and then washed with 5mL IP wash buffer. Cells were then lysed on plates in 1% NP-40 lysis buffer with protease and phosphatase inhibitors over ice for 2 minutes. Cells lysates were collected with a cell scraper and placed in 1.5ml tube and rotated for 20 minutes at 4°C. Cell lysates were then added to microcentrifuge tubes and spun at 13,000g for 10 minutes at 4°C. Supernatant was then transfered to new tubes and aliquoted. After dilution the minimal final volume is minimally 400ul per

tube so supernatant can mix properly on rotation. For the denatured immunopreciptations ~0.5% SDS was added to the lysis buffer (2.5µl 20% SDS per 100µl lysate). Next, 5µl of 100mM beta mercaptoethanol/100µl was added to the original lysate to a final concentration of 5mM beta mercaptoethanol. Lysates were mixed well and heated at 100°C for 5 minutes to stop reaction. Lysates were then run on a SDS-PAGE for analysis.

Phosphatase Assay

Cells were transfected with plasmid(s) and harvested as described in the non-denaturing immunoprecipitation assay using the same buffer with the exception that phosphatase inhibitors were removed (150mM NaCl, 50mM Tris-HCl (pH 8.0), 1mM EDTA, 1mM EGTA, 10mM MgCl2, 50µg/ml Aprotinin, 100µg/mL Leupeptin, 1um phenylmethylsulfonyl fluoride (PMSF) and 1% TritonX-100). 2µl phosphatase (New England Biosciences) was added to 40ul lysate for 1 hour at 30°C. Reaction was stopped with the addition of 2x reducing Sample Buffer, 5 minutes boiling in a water bath. RGS14-Flag was visualized with a western blot analysis.

O-glycosidase Assay

Cells were transfected with plasmid(s) and harvested as described in the non-denaturing immunoprecipitation assay. The same buffers were used as previously described with the exception that phosphatase inhibitors were removed 150mM NaCl, 50mM Tris-HCl (pH 8.0), 1mM EDTA, 1mM EGTA, 10mM MgCl2, 50µg/ml Aprotinin, 100µg/mL Leupeptin, 1um phenylmethylsulfonyl fluoride (PMSF) and 1% TritonX-100). Next 1µl of 10x glycoprotein denaturing buffer was added to 10-20µg potential glycoprotein (from

cell lysate). The reaction mixture was made to a total volume of 20µl by adding 2µl reaction buffer, 2µl 10% NP40, 2µl Neuramidase, 10 µl H20 and 1µl, 2µl and 6ul O-glycosidase. Final mixture was incubated for 4 hours at 37°C. Reaction was stopped with the addition of 20µl 2x reducing Sample Buffer, 5 minute boil in water bath and visualization of RGS14-Flag via western blot analysis.

Kinase inhibition Assays

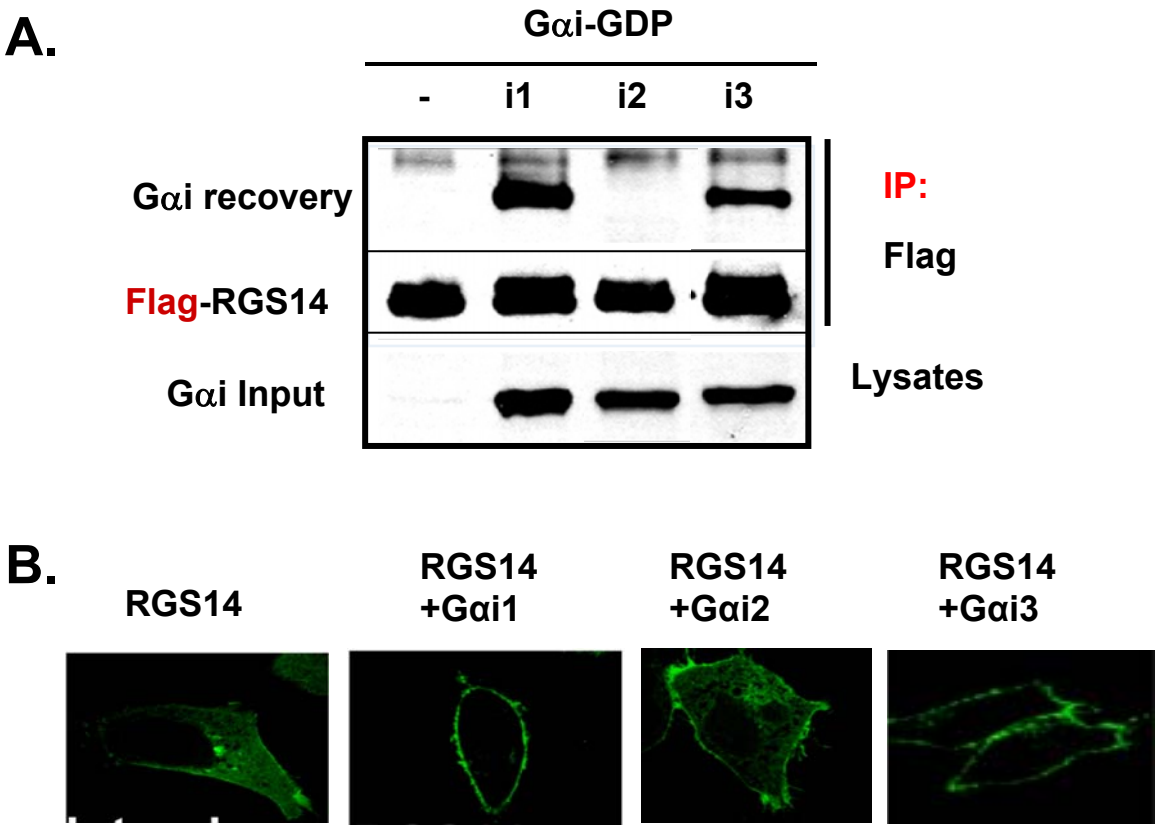
HeLa cells were transfected as previously described. After plasmid incubation time, kinase inhibitors were added to growth media in various concentrations and incubated overnight. Cells were washed and harvested using 200µl 2x sample buffer, 100°C for five minutes, and immediately loaded onto SDS-PAGE for western blot analysis.

3.3 Results

3.3.1 RGS14 forms a stable complex with Gαi1/3 when co-transfected into HeLa cells and this complex is translocated from the cytosol to the plasma membrane.

RGS14 interactions with its known binding partners Gαi1, and Gαi3 were assessed using recombinant protein in HeLa cell lines. These studies were also repeated in Cos-7 cell lines with similar results (data not shown). RGS14 was co-transfected into HeLa cells with Gαi1, Gαi2 or Gαi13 plasmid were immunoprecipitated using FLAG M2 beads (denatured and visualized via western blot) and show both Gαi1 and Gαi3 binding, but not Gαi2 binding **Fig 3-1 A**. These observations reported here are consistent with Gαi1/3-GDP binding and forming a complex with RGS14 at its GoLoco/GPR domain as reported in previous studies (Kimple, De Vries et al. 2001; Hollinger, Ramineni et al. 2003; Mittal and Linder 2004). RGS14-Gα complexes were translocated to the plasma membrane as shown by confocal microscopy in **Fig 3-1 B**.

Figure 3-1 RGS14 forms a stable complex with Gαi1/3 at the plasma membrane. (A) Both Gαi1 and Gαi3, but not Gαi2, co-immunoprecipitate in stable complexes with Flag tagged RGS14. (F. Shu, repeated by D. Cowan) RGS14 with a Flag epitope was transfected in HeLa cell lines in the presence of three isotypes of Gαi. Gαi1, Gαi2, and Gαi3 were co-transfected with Flag-RGS14 and immunoprecipitated with M2 flag beads. Immunoprecipitated beads were denatured, run on a SDS page gel and immunoblotted for either Flag-HRP or EE. Cell lysates (non-immunopreciptated) are also shown to confirm Gα presence in transfection. (B) RGS14-Flag translocated from the cytosol to the plasma membrane when co-expressed with Gαi1 and Gαi3 (anti-Flag confocal microscopy, F. Shu)

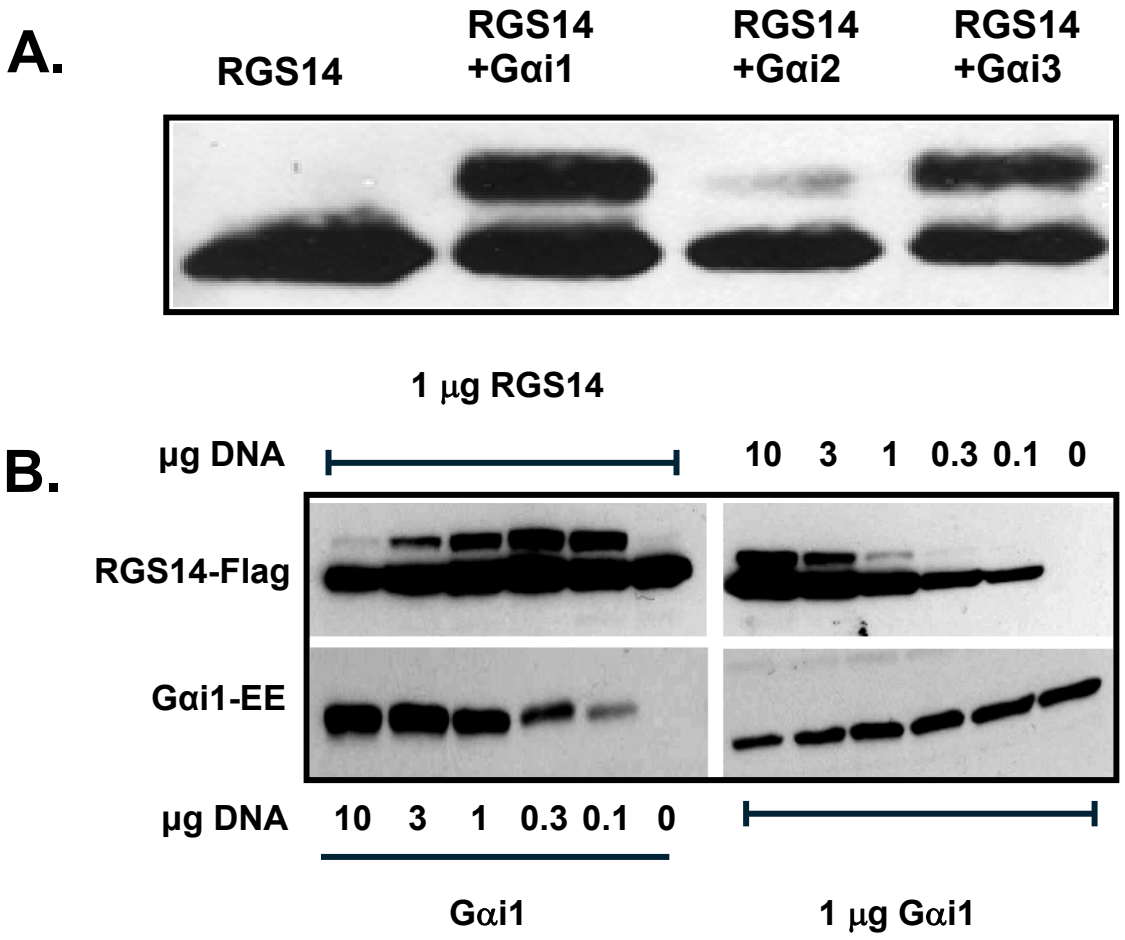

A.
Gαi-GDP
-
i1
i2
i3
Gαi recovery
Flag-RGS14
Gαi Input
IP:
Flag
Lysates
B.
RGS14
RGS14
+Gαi1
RGS14
+Gαi2
RGS14
+Gαi3

3.3.2 RGS14 is biochemically modified when in complex with Gαi1/3

Previous observations in our lab (F. Shu, unpublished) showed that RGS14 co-transfected with Gαi1 or Gαi3 appears as two bands on immunoblot analysis instead of the expected single band **(Fig 3-2 A)**. The higher molecular weight band is approximately 64kDa as opposed to the RGS14 predicted band of 62kDa. Studies were undertaken to characterize this biochemical modification on RGS14 that appears when co-transfected with GDP-Gαi1/3. It should also be noted that RGS14 appears to have a similar modification when co-transfected with its binding partners Rap2a and H-Ras (data not shown). RGS14 does not show high levels of modification when co-transfected with Gαi2, leading our group to believe RGS14 was modified when translocated to the plasma membrane Giα1 and/or Giα3 (and also perhaps Rap2 or H-Ras)

Varying concentrations of both RGS14 and Gαi1 show RGS14 modification is dependent on Gαi1 concentration **(Fig 3-2 B)**. High concentrations of Gαi1 protein levels relative to RGS14 protein levels show a decreasing level of RGS14 modification. An additional observation made during these studies was high concentrations of RGS14 and Gαi1 plasmid resulted in cell death. Either plasmid alone in high concentrations did not elicit cell death. It is possible excessive concentrations of RGS14 and Gαi1 together cause disruptions in normal cell cycle progression.

Figure 3-2 RGS14 is biochemically modified when co-expressed with Gαi1/3. (A) RGS14 is modified when co-expressed with Gαi1/3, but not with Gαi2. Modification results in a band approximately 2kd larger than the unmodified RGS14 band. RGS14 with a Flag epitope was transfected in HeLa cells alone or with Gαi1, Gαi2, or Gαi3 and lysed in SB. Cell lysates were immediately run on a SDS Page Gel and immunoblotted for anti-Flag-HRP. (B) Co-Transfection of varying concentrations of RGS14 and Gαi1 show modification of the RGS14 is dependent on Gαi1 concentration. RGS14 with a Flag epitope and Gαi1 were transfected in HeLa cells alone or co-transfected with varying concentrations of Gαi1 and RGS14 respectively.


A.
RGS14
RGS14 +Gαi1
RGS14 +Gαi2
RGS14 +Gαi3
1 μg RGS14
B.
μg DNA
10 3 1 0.3 0.1 0
RGS14-Flag
Gαi1-EE
μg DNA
10 3 1 0.3 0.1 0
Gαi1
1 μg Gαi1

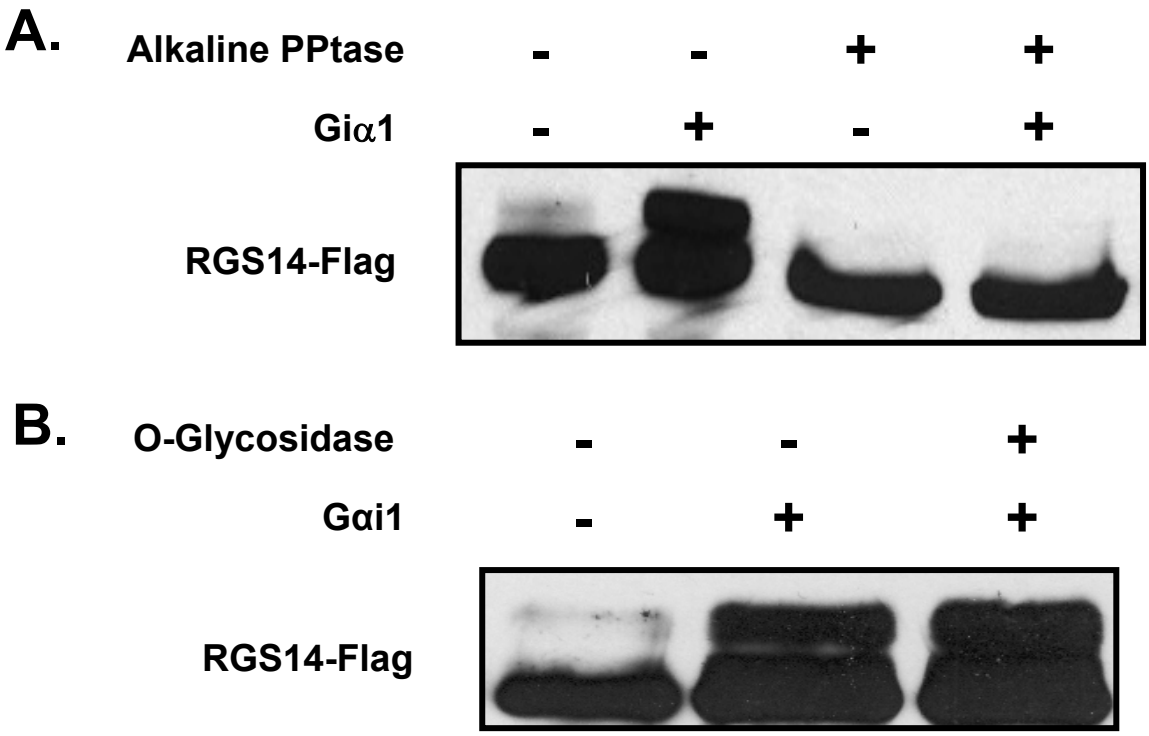
3.3.3 RGS14 is modified by phosphorylation when co-transfected with Gαi1

RGS14 phosphorylation studies were designed to identify what cellular factor(s) might be causing the biochemical modification of RGS14 when co-expressed with Gαi1. As previously reported, RGS14 is regulated by phosphorylation to enhance its GDI activity towards GDP-bound Gαi1 and Gαi3 (REF). We hypothesized RGS14 might also be regulated by phosphorylation on additional sites for additional regulation of its function(s). Other proteins have also been known to produce a higher molecular weight band in tandem with their predicted band, henceforth to be described as a doublet. One such protein is the Na+/H+-exchanger regulatory factor which is phosphorylated on serine 289 by G protein-coupled receptor kinase 6A (GRK6A) (Hall, Spurney et al. 1999). The phosphorylated residue S289 resides next to a proline residue and it is hypothesized that a phosphorylation next to a proline can lead in a modification of protein structure resulting in the protein becoming 'kinked', and thusly migrate through a SDS-PAGE gel more slowly (Hall, Spurney et al. 1999). To test this hypothesis RGS14 and Gαi1 were co-transfected as described previously, harvested in Co-IP buffer, and treated for 1 hour with alkaline phosphatase, a non-specific phosphatase. In figure **3-3 A** we show alkaline phosphatase treatment totally eliminates the higher molecular weight upper band. By contrast, treatment with alkaline phosphatase has little or no effect on RGS14 not co-transfected with Gαi1.

Glycosylation is the addition of saccharides to produce glycans attached to proteins and other organic molecules. The addition of glycosylation products has been shown to modify proteins causing molecular weight shifts as seen on acrylamide gels similar to our observations with RGS14 and Gαi1 (Hall, Premont et al. 1998). O-linked glycosylation is a post-translational modification which proteins may undergo. Proteins are glycosylated by O-N-acetylglucosamine at either serine or threonine residues which

may otherwise be phosphorylated by serine/Threonine kinases. Proteins which are glycosylated on their serine/threonines can not be phosphorylated on the same protein, and vise versa. It is important for us to show O-linked glycosylation was not the cause of our biochemical modification or if it was possibly involved in RGS14 regulation, previously unknown. As a control we tested to see if an inhibitor of glycosylation had any effect on the RGS14 doublet (Hall, Premont et al. 1998) . I found that treatment of HeLa cell lysates co-transfected with RGS14/Gαi1 and incubated for 1 hour with inhibitors of O-linked glycosylation show no reduction in RGS14 modification as determined by immunoblot analysis **(Fig 3-3 B).**

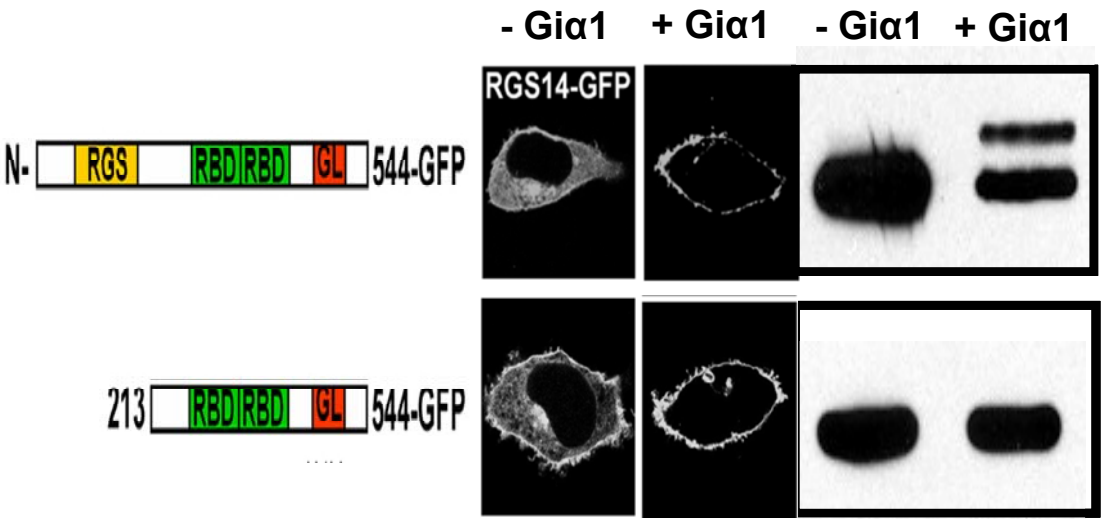
Figure 3-3 RGS14 modification is reversed with alkaline phosphatase treatment but not by inhibitors of O-linked glycosylation. (A) HeLa cell lysates containing both transfected RGS14 and Gαi1 were treated with alkaline phosphatase for 30 minutes. Alkaline phosphatase treatment shows removal of the RGS14 modification. (B) Under the same conditions 30 minutes of O-Glycosidase treatment does not remove RGS14 modification.


A.
Alkaline PPtase
- - + +
Giα1
- + - +
RGS14-Flag
B.
O-Glycosidase
- - +
Gαi1
- + +
RGS14-Flag

3.3.4 The Amino (N-)terminal region of RGS14 is required for Gαi1 modification of RGs14

The next step in our studies was to map the region of RGS14 that is critical to modification. Using constructs available in our lab, a series of co-transfections were performed with various regions of RGS14 eliminated. Constructs tested were ΔRGS14 amino acid residues 213-544, ΔRGS14 213-490 amino acid residues and ΔRGS14 amino acid residues 490-544 in addition to full length RGS14 (some data not shown). All constructs which had the first 212 amino acid residues removed did not contain the additional upper band. The N-terminal region of RGS14, including the first 212 amino acid residues coding for the RGS domain, are required for Gαi1/3 directed RGS14 modification **(Fig 3-4).** Using confocal microscopy we determined elimination of the N terminal region does not affect Gαi1-mediated RGS14 translocation to the plasma membrane. As previously mentioned the GoLoco/GPR directs plasma membrane targeting of proteins when bound to Gαi1 **(Fig 3.4).**

Figure 3-4. The N terminus of RGS14 is required for Gαi1/3 modification of RGS14. Truncation constructs of RGS14 were created removing the various domains of RGS14. Removal of the N terminal (first 213 amino acid residues), which includes the RGS domain, eliminates modification when co-expressed with Gαi1. However, removal of this N terminus does not effect Gαi1-directed RGS14/Gαi1 co-localization to the plasma membrane as visualized by confocal microscopy. HeLa cells were transfected with either RGS14 full length or RGS14 (Flag epitope tagged) with the first 213 amino acid residues removed in the presence or absence of Gαi1. Cell lysates were then run on SDS PAGE and immunoblotted with anti-Flag HRP. For fluorescent images HeLa cells were transfected with RGS14 full length or RGS14 truncation mutant with a GFP tag in the presence or absence of Gαi1.


- Giα1
+ Giα1
- Giα1
+ Giα1
RGS14-GFP
N-
RGS
RBD
RBD
GL
544-GFP
213
RBD
RBD
GL
544-GFP

3.3.5 Mutation of known phosphorylation sites and pretreatment with potential kinase inhibitors does not eliminate RGS14 modification.

RGS14 has been shown to be phosphorylated at several amino acid residues, including Serine 52 and Threonine 494 (Hollinger, Ramineni et al. 2003; Hollinger and Hepler 2004). Previous studies have shown that targeted phosphorylation at Ser/Thr residues adjacent to prolines recruit peptidyl-prolyl cis-trans isomerases (PINs) to induce an isomer "kink" at the Phospho-proline site. The molecular structure resulting from PIN activity moves slower through acrylamide gels, resulting in a marked molecular weight shift much larger than predicted by simple addition of a phosphate (Lu, Liou et al. 2002). Such a shift is observed when RGS14 and Gαi1 are co-transfected. Based on previous research I postulated that the observed shift may be due to phosphorylation at Ser/Thr next to a proline. In order to test my hypothesis, I decided to mutate all serines next to prolines to alanines in the first 213 amino acid residues of RGS14. The potential phosphorylation sites were chosen based on truncation mutant data collected earlier **(Fig 3-4).**

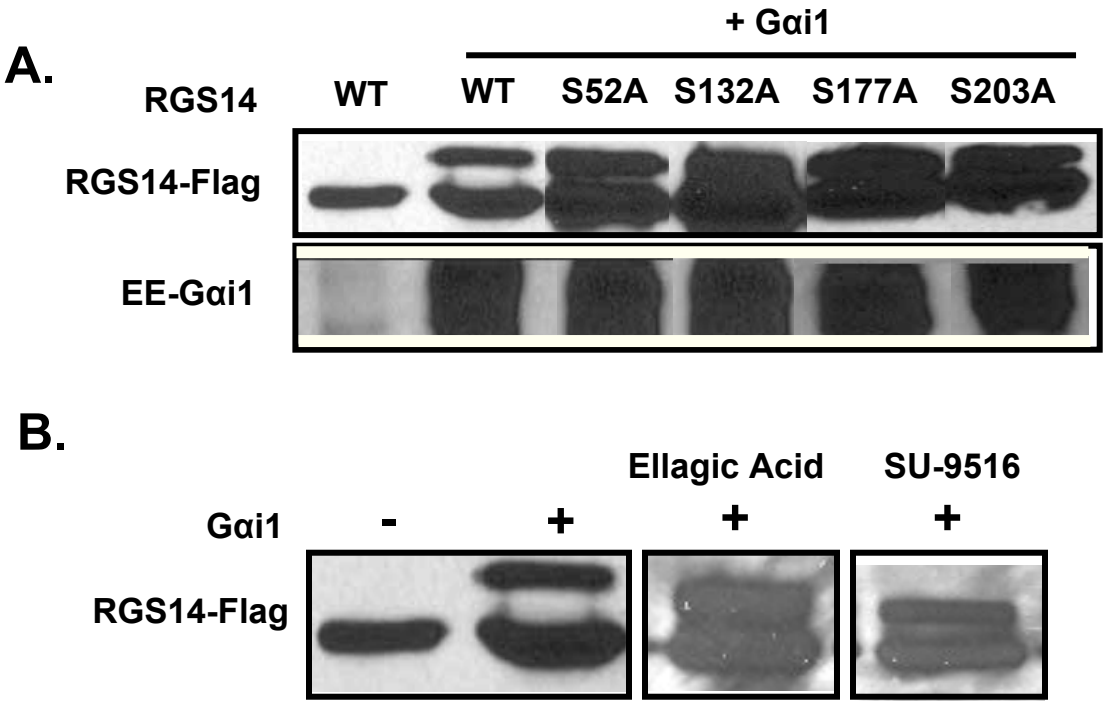
Site directed mutagenesis was performed using Stratagene QuikChange Site Directed Mutagenesis Kit on three sites containing SP motifs as well as the known ERK phosphorylation site on Serine 52. Plasmids created were then sequenced through Sigma Aldrich to confirm Serine to Alanine mutations. The plasmids were then transfected into HeLa cells as previously described in the presence or absence of Gαi1. I found that none of the mutant RGS14 plasmids we tested had their RGS14 phosphorylation eliminated through Serine to Alanine mutations **(Fig 3-5 A).**

An additional goal of these studies was to determine the kinase(s) that are responsible for RGS14 phosphorylation. Previous publications reported RGS14 is phosphorylated at T494 by PKA and S52 by ERK (Hollinger, Ramineni et al. 2003;

Hollinger S 2004). We decided to include inhibitors for both known kinases of RGS14 and inhibitors for a wide range of potential kinases. Potential kinases were screened for two criteria before being tested in our assay: 1) likelihood of expression in cells expressing RGS14 and, 2) consensus sequence homology on RGS14 (whether or not RGS14 contained a consensus sequence in its exons). Several kinase inhibitors were tested which covered a wide spectrum of potential kinase inhibition. Ellagic acid, a naturally occurring kinase inhibitor, has inhibitory effects on Casein Kinase II (CKII) and protein kinase A (PKA) (IC50 40, 2900 nM respectively). Small molecule inhibitor SU9516 inhibits cyclin dependent kinase(s) 1, 2 and 4(CDK1,2,4), platelet derived growth factor receptor (PDGFR) and endothelial growth factor receptor (EGFR) (IC_{50} values are 0.022, 0.04, >10, >10, 18 and >100 μM for cdk2, cdk1, cdk4, PKC, p38, PDGFR and EGFR respectively). Treatment of HeLa cells co-transfected with both RGS14 and Gαi1 with kinase inhibitors Ellagic Acid(400μM) and SU 9516(400μM) overnight do not eliminate RGS14 phosphorylation leading to large molecular weight shifts**(Fig 3-5 B)**.

HeLa cells were transfected as previously described in other sections with RGS14 and Gαi1. After 5 hour incubation with plasmid, cells were washed three times with warm PBS and kinase inhibitors were added at varying concentrations (1 nM, 3 nM, 10 nM, 30 nM, 100 nM, and 400 nM) overnight. Only the maximum concentration of 400 nM is shown in data figure **3-5 B**. Cells were harvested with 2x sample buffer the next morning and run on SDS-PAGE. Cells were immunoblotted with anti-Flag-HRP.

Figure 3-5. Mutation of phosphorylation sites for known kinases and treatment of cells with inhibitors of candidate kinases do not prevent RGS14 modification. (A) Mutation of candidate phosphorylation sites that interact with peptidyl-prolyl cis-trans isomerase (Ser/Thr-Pro) on RGS14 do not eliminate RGS14 modification when RGS14 point mutants were co-transfected with Gαi1 in HeLa cells. (B) Treatment of HeLa cells with 400 nM Ellagic acid, a selective Casein kinase and PKA inhibitor (at 40nM concentrations), does not prevent RGS14 modification. In addition treatment of cells with 400 nM SU-9516, an inhibitor of candidate kinases cyclin dependent kinases CDK1, CDK2, CDK4 and PKC, does not prevent RGS14 modification.


A.
+ Gαi1
RGS14
WT
WT
S52A
S132A
S177A
S203A
RGS14-Flag
EE-Gαi1
B.
Ellagic Acid
SU-9516
Gαi1
-
+
+
+
RGS14-Flag

3.4 Discussion

RGS14 is still a poorly understood protein with regard to its function in biological systems. In an attempt to elucidate some of its biochemical roles, we undertook studies to determine how RGS14 is biochemically modified following interaction with its Gαi1 binding partner via unknown post-translation modifications and mechanisms. In the studies outlined in this chapter, I have shown that RGS14 is modified by a phosphorylation event, when co-expressed with Gαi1. As of now modificiation has unidentified functional consequences. These studies also test several kinases and phosphorylation sites which, alone, are not singly responsible for RGS14 modification.

Several alternative hypotheses exist for the outcomes described in the above sections. In **Fig 3-2** we show RGS14 modification while co-expressed with Gαi1. One explanation for RGS14 modification could be plasma membrane recruitment of RGS14 by Gαi1-GDP results in RGS14 becoming a substrate for an (unknown) kinase. Additional proteins could also be recruited or required for RGS14 modification, with unknown signaling outcomes. Many proteins have been shown to become phosphorylated when recruited to the plasma membrane, including Ras and Raf, both binding partners of RGS14 (Avruch, Khokhlatchev et al. 2001; Mahon, Hawrysh et al. 2005). Phosphorylation leads to conformational and/or binding alterations on Ras and Raf kinase, effecting their downstream signaling behaviors. It is also hypothesized that the many distinct roles for the ERK phosphorylation pathway are somewhat regulated by cellular localization (Zehorai, Yao et al. 2010). It is possible RGS14 is a protein which is responsible for some localized effects involved in MAPkinase signaling. RGS14 recruitment to the plasma membrane when expressed with Gαi1 and subsequent binding of c-RAF could partially explain how some cellular localization pathways may work.

In **Fig 3-3** we show alkaline phosphatase removing the 'upper' band of RGS14 on western blot. This modification is possibly a phosphorylation on RGS14; however the phosphorylation of an unknown protein may also be required for the RGS14 modification to retain its structure. A known binding partner of RGS14, such as Raf or Ras, may have a phosphorylation site which is required for modification of RGS14. It is possible alkaline phosphatase treatment removed a required phosphate on an associated protein which resulted in the loss of RGS14 modification. It could be possible RGS14 is recruited to the plasma membrane by Gαi1 and upon membrane localization further modified by a known or unknown binding partner.

O-linked glycosylation has been linked by several groups to LTP in hippocampal AMPA receptor trafficking and synaptic plasticity (Jiang, Suppiramaniam et al. 2006; Berta, Weifei et al. 2008; Kanno, Yaguchi et al. 2010). Recent work in our own lab by S. Lee in our lab and others has shown that RGS14 is likely involved with synaptic plasticity in the brain and could potentially be modified by glycosylating enzymes (Lee, Simons et al. 2010). My data indicates O-glycosylation is not important for post RGS14 'doublet' modification but may play other roles in RGS14 biochemistry. However, I did not have a positive control to confirm O-glycoslyation worked in my assay and this would need to be addressed in any subsequent experiments.

Figure 3-4 shows that the first 212 amino acid residues are required for RGS14 modification. It is important to note that the RGS domain of RGS14 is contained in the first 213 amino acids of the protein. RGS14's RGS domain is responsible for activated Gαi1-GTP binding and is a different region from the Gαi1-GDP binding on the GL domain of RGS14. It is possible that a GEF exchanging the Gαi1-GDP for Gαi1-GTP on the GL domain results in the same Gαi1 subunit re-binding RGS14 at its RGS domain. These interactions could be playing a role in the modification of RGS14 which we note in

these studies. It is unknown at this time whether RGS14 can actively bind two Gαi1 proteins simultaneously.

The hypothesis we tested in **Figure 3-5** is that a single amino acid residue and a single phosphorylation event are responsible for RGS14 modification. The residues we chose to modify included serine-proline motif's which we felt would be likely candidate regions for eliciting such a dramatic molecular weight shift due to the molecular configuration prolines exist in (sharp angles). It is possible the phosphorylation responsible for RGS14 modification is not in the region truncated and the RGS domain and the C-terminus region is required for kinase binding. Another possible conclusion is the C-terminus region is required for an associated protein to bind and modify RGS14. Further studies will need to be performed to determine the exact site on RGS14 required for modification. The most likely candidate experiments would be mass spectrometry analysis, additional truncation mutants or site-directed mutagenesis.

RGS14 contains the consensus sequences for several kinases which are also found to be highly enriched in the brain. CKII is a serine/threonine kinase which has been highly conserved through evolution and is a critical player in cell cycle progression and highly enriched in the brain (Blanquet 1998; Blanquet 2000). In addition to its cell cycle roles CKII has been linked to synaptic plasticity in the hippocampus and RGS14 contains its consensus sequence (S/T)-X-X-(E/D) (Blanquet 2000; Kimura and Matsuki 2008). Studies outlined in **Figure 3-5** would lead us to conclude CKII is not the kinase responsible for RGS14 modification observed from Gαi1 co-expression. In addition to CKII, inhibitors of PKA, PKC, and CDK's 1, 2, 4, and 5 were tested to determine if any of these had an effect the Gαi-dependent phosphorylation of RGS14. The CDK family of kinases is largely responsible for cell cycle regulation and is also found to be enriched in the brain. More recently a member of the CDK family, CDK 5 has been linked to synaptic plasticity (Marco, Florian et al. 2006; Ammar and James 2007). None of the tested

kinases eliminated RGS14/Gαi1 interaction in our assay. Roscovatine, a CDK 5 specific inhibitor, was tested in the same HeLa cell assay (results not shown), but no effect on doublet formation was observed. No tested kinases are involved in RGS14/Gαi1 modification or kinase inhibitors added at a later point in the expression of transfected cells do not reverse or prevent RGS14/Gαi1 modification.

It is certainly possible our kinase assay would not effectively show whether or not the inhibited kinase was actually responsible for direct modification or as a required element for modification. Kinase inhibition was only introduced after RGS14 and Gαi1 were incubated for five hours in a live cell culture. If modification was already present when inhibitor was added the inhibition would not necessarily reverse the modification. Attempts were made to keep inhibitor present during all steps of transfection but were not successful due to the cells low tolerance for inhibition of kinases required for cell cycle progression. If these studies were to be continued, a different transfection reagent would be necessary which does not require cell division for plasmid incorporation.

In summary, RGS14 is modified when co-expressed with Gαi1. In addition, the first 213 amino acids of RGS14 are required for modification. Several candidate kinases and phosphorylation sites were tested and shown to be non-critical for modification in our assays. Additional proteomic and mass spectrometry studies need to be conducted to determine if any functional consequences are the result of RGS14 modification via Gαi1 co-expression. Proteomics involving RGS14 and known binding partners of RGS14 would be the most likely candidate experimental options. We would ideally test RGS14/Gαi1 complexes in functional assays with known RGS14 binding partners.

Chapter 4: Conclusion

The studies in the previous two chapters elucidate biochemical mechanisms of RGS14 regulation using both cell based assays and those with purified proteins. We have found evidence that full length RGS14 directly inhibits Raf-1 kinase phosphorylation of Raf-1 substrate MEK, likely through direct binding and inhibition of Raf-1 kinase **(Fig 2-1, 2-2)**. My data supports and expands upon the working hypothesis of our lab that RGS14 acts as a signaling scaffold. In our model RGS14 utilizes its RGS domain, GL domain and Ras/Raf binding domains to integrate G protein and MAPkinase signaling cascades.

A cellular role for RGS14 could also be supported by these data. As mentioned previously, RGS14 is highly enriched in the brain and is thought to be involved in both cell division of non-neuronal cells, and in synaptic plasticity in mature neurons. The MAPkinase cascade has been repeatedly linked to growth in a variety of cells in the body and our data suggests RGS14 may be modulating RTK signaling through MAPkinase proteins. It is possible RGS14 could be acting as a cell signaling checkpoint to moderate neuron signaling and/or growth during development. My unpublished observations while working with RGS14 and Gαi1 in cells suggests co-expression of large amounts of RGS14 and Gαi1 protein disrupts HeLa cell growth and causes cells to become detached and die (Cho and Kehrl 2007). Given RGS14s published roles in mitotic spindle formation these observations make some sense, yet remain unexplored. Studies were planned for cell cycle synchronization to determine if RGS14 phosphorylation occurs in cell-cycle dependent manner, but were halted due to technical difficulties.

Our studies do not discern if RGS14 is inhibiting Raf-1 activity through direct binding and modulation as opposed to passive binding and inhibition of the kinase domain. Studies with full length Raf protein may help elucidate the mechanism by which RGS14 inhibits Raf-1 phosphorylation of MEK. Full length Raf, which contains

the regulatory domain, may behave differently than the constitutively activated form used in our experiments. RGS14 could be inhibiting Raf-1 activity through allosteric regulation and not direct inhibition/binding of the kinase domain on Raf-1 kinase. An additional explanation could be that RGS14's large size inhibits the ability of Raf-1 kinase substrates to bind Raf. H-Ras may also play a role in RGS14's activity with Raf-1 kinase, possibly through binding of RGS14's RBD domain in order to recruit Raf-1 kinase to the complex. Recruitment of the Raf-1 kinase in this scenario would not be for Raf activation, but rather for RGS14 inhibition. Possible experiments to elucidate these roles could involve BRET/FRET microscopy and proteomics to determine different binding conformations and cellular localization of candidate proteins.

We have also shown that RGS14 is biochemically modified, likely through phosphorylation, when co-expressed with Gαi1 **(Fig 3-2).** This modification requires the C terminal region of RGS14, including the RGS domain **(Fig 3-4).** Several candidate kinases have been tested to determine if they may play a role in RGS14 modification with Gαi1. Neither PKA, PKC, CKII, nor members of the CDK family seem to be critical for modification in the assays tested.

Potential roles for modification in our proposed model could include recruitment of kinase(s) to the plasma membrane or the RGS14/Gα complex. More complexly, a conformational shift caused by a phosphorylation event could potentially recruit other proteins which may then recruit kinases to the complex. In this scenario a phosphorylation on RGS14 could recruit a required binding partner for kinase activation.

A large amount of effort was put into obtaining a sample of protein containing modified RGS14 with the intention of using this specimen for mass spectrometry analysis. The RGS14/Gαi1 'doublet' interaction is very easily repeated using our cell based assay. However in order to utilize mass spectrometry analysis, larger amounts of a more purified sample were required. Initial attempts at immunoprecipitation assays met

with very little success, as doublet formation would not survive for the entirety of our protocol or any freezing cycles. A committee member suggested using an alternative, denaturing, immunoprecipitation protocol to attempt to denature any phosphatases that may be still active during a normal immunoprecipitation assay. These efforts proved more successful and I was able to obtain a sample for analysis at the Emory Proteomics Core Facility. Our samples did indeed contain RGS14 in both upper and lower bands; however, neither sample contained phosphorylation sites consistent with our findings. In fact, only the lower band had a single phosphorylated residue and it was not one of the published phosphorylation sites. Given the evidence presented in this thesis, direct phosphorylation of RGS14 by Gαi1 seems unlikely. One possibility is the phosphorylation of an associated protein is required for RGS14/Gαi1 complex stability. Associated protein phosphorylation loss and subsequent loss of the RGS14/Gαi1 complex could explain our alkaline phosphatase data **(Fig 3-3)**.

We believe a more concentrated sample may provide insight into RGS14/Gαi1 modification, yet will remain technically difficult. Additional studies using 2D gel electrophoresis to further characterize RGS14/Gαi1 modification also showed some early success, yet further studies will need to be performed in order to fully utilize this tool.

These studies represent the beginning efforts in a greater task of fully understanding RGS14's role in MAPkinase and phosphorylational control. Ongoing studies in our lab with Ric-8A, RGS14, Raf, Ras and Gαi1 are showing promise for validating the model proposed in **Figure 2-2.** Data submitted and in revision by C. Vellano has shown Ric-8A to both bind and translocate with RGS14 to the plasma membrane when co-expressed with Gαi1. Additionally, Ric-8A has been shown to confer GEF activity on RGS14 in single turnover GTP assays. The Ric-8A studies provide strong evidence to support early steps in our proposed model and together with data in the

above chapters helps to validate our hypothesis of RGS14 acting as a protein scaffold to integrate G protein and MAPkinase signaling.

RGS14 has also been shown by our lab to be phosphorylated when co-expressed with H-Ras and Rap2a in addition to Gαi1. These data may be significant in regard to the role RGS14 is playing in the C2 region of the hippocampus. RGS14 could be an important intermediary to regulate cellular growth signals. Phosphorylational control could act as a regulatory mechanism to control RGS14's cellular localization in response to cellular signals. Upon phosphorylation by several different binding partners, RGS14 could be recruited to the plasma membrane to serve either as a negative regulator of Gαi1 signaling or as a scaffold for MAPkinase signaling proteins.

Future experiments for providing further evidence for each additional step of our proposed model were and are planned. Early pure protein binding studies have been done using Fast Protein Liquid Chromatography (FPLC) showing that the stable RGS14/Gαi1 complex becomes dissociated when Ric-8A is added (C. Vellano and C. Yates, in review). Complementary studies involving cell assays would be carried out in parallel to the in vitro assays. Cells will be transfected with the PDGF receptor tagged with the FLAG epitope (Flag-PDGF) along with YFP-Ric8A, Giα-EE, H-Ras, Raf-1 and HA-RGS14. Cells will be untreated or treated with PDGF for various times (0-120 min) and fixed for staining. We will look for co-localization of YFP-Ric8A, H-Ras, and Raf-1 and HA-RGS14 with the Flag-PDGF receptor by confocal microscopy either at the cell surface or in internalized vesicles.

While the exact role of RGS14 may still be unknown in cells, each new piece of data collected provides insight to the many functions in which this very interesting protein is involved. In conclusion, we have shown purified RGS14 directly inhibits Raf kinase activity, the first time an RGS protein has shown direct inhibition of a purified MAPkinase signaling pathway component in an in vitro assay. This finding further

characterizes the roles RGS14 may be playing in cells. Additionally, we have shown RGS14 becomes modified when co-expressed with Gαi1 with unknown signaling consequences. The findings reported here will provide the groundwork for future studies which may fully elucidate the role RGS14 plays in biological systems.

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