Peptide Inhibitors of Regulator of G Protein Signaling 4 (RGS4): Rational and Combinatorial Approaches

by

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List of Abbreviations

A1   Adenosine Receptor type 1

AC   Adenylyl cyclase

ACh   Acetylcholine

AD   Alzheimer's Disease

ANOVA   Analysis of Variance

αAR alpha-Adrenergic Receptor

βAR   beta-Adrenergic Receptor

AngII   Angiotensin II

AT1   Angiotensin Receptor type 1

CAM   Calmodulin

cAMP   cyclic Adenosine Monophosphate

cGMP   cyclic Guanosine Monophosphate

D1   Dopamine Receptor type 1

D2   Dopamine Receptor type 2

DA   Dopamine

DEP   Dishevelled/EGL-10/Pleckstrin

DIEA   N,N'-diisopropylethylamine

DIX   Dimerization domain

DMSO	Dimethlysulfoxide
DOR	Delta Opioid Receptor
DPDPE	[D-Pen2,5]-enkephalin
DTT	Dithiothreitol
ERK	Extracellular Regulated MAPK
ES	Embryonic Stem
GABA	Gamma-Aminobutryic Acid
GAIP	G-Alpha Interacting Protein
GAP	GTPase Accelerating Protein
GDI	Guanine-nucleotide Dissociation Inhibitors
GDP	Guanine Diphosphate
GGL	Gγ-like
GIP	Glucose-dependent Insulinotropic Peptide
GIPC	GAIP-interacting protein, C terminus
GIPN	GAIP Interacting Protein N terminus
GIP-R	Glucose-dependent Insulinotropic Peptide Receptor
GIRK	G protein-gated inwardly rectifying potassium
GnRH	Gonadotropin Releasing Hormone
GPCR	G Protein-Coupled Receptors
GRK	G Protein-Coupled Receptor Kinase
GST	Glutathione S-Transferase
GTP	Guanosine Triphosphate
HBTU	o-benzotriazol-1-yl-N,N,N',N'-tetramethyl uronium hexafluorophosphate

HEK	Human Embryonic Kidney
HOBT	1-hydroxybenzotriazole
HPLC	High-Performance Liquid Chromatography
5-HT	Hydroxytryptamine or serotonin
5-HT1a	Serotonin Receptor type 1a
5-HT2	Serotonin Receptor type 2
HTS	High Throughput Screen
KOR	Kappa Opioid Receptor
LARG	Leukemia-Associated Rho GEF
M1	Muscarinic Receptor type 1
M2	Muscarinic Receptor type 2
M3	Muscarinic Receptor type 3
M4	Muscarinic Receptor type 3
MAPK	Mitogen-Activated Protein Kinase
MOR	Mu Opioid Receptor
NEM	N-Ethylmaleimide
NMDA	N-Methyl-D-aspartic Acid Receptor
NMP	N-Methylpyrrolidone
NO	Nitric Oxide
OBOC	One-Bead, One-Compound
ODN	Oligodeoxynucleotides
6-OHDA	6-Hydroxydopamine
PAG	Periaqueductal Gray

PBM	Peripheral Blood Mononuclear
PDEγ	Phosphodiesterase γ
PDZ	PSD95, Dlg and Z0-1/2
Pen	Penicillamine
PFC	Prefrontal Cortex
PIP3	Phosphatidylinositol-3,4,5,-trisphosphate
PKG	Protein Kinase G
PP2A	protein phosphatase 2A
PPI	protein-protein interaction
PTB	phosphotyrosine-binding
PTX	Pertussis Toxin
R7BP	RGS7 Binding Protein
R9AP	RGS9 Anchoring Protein
RBD	Ras-binding domains
RGS	Regulator of G Protein-Signaling
RH	RGS homology
RhoGEF	Rho Guanine nucleotide Exchange Factor
RP-HPLC	Reverse-Phase High-Performance Liquid Chromatography
SAR	Structure- Activity Relationship
SNX	Sorting Nexin
SRB-RGS	Steroid-Receptor-Binding RGS (RGS3)
TIPS	Triisopropylsilane
TFA	Trifluoroacetic Acid

Chapter 1

Introduction

1a. RGS proteins modulate cellular signaling.

G Protein-Coupled Receptors (GPCRs) are a family of over 800 proteins that contain seven transmembrane regions (Pierce, et al. 2002). When activated by an agonist, such as a hormone, a neurotransmitter, a drug, or a photon of light, a GPCR stimulates exchange of GTP for GDP on the Gα subunit of the heterotrimeric G protein, which then undergoes an activating conformational change that allows it and its associated βγ subunit to interact with effector proteins (Figure 1.1) (Hepler. 1999). This can result in a number of downstream signaling events that can cause changes in membrane polarization, changes in neurotransmitter or hormone release, gene transcription or other cellular events (Pierce, et al. 2002). The Gα subunit inactivates itself by hydrolyzing GTP to GDP and this allows reassociation with Gβγ and the receptor. Unlike GTPase accelerating proteins (GAPs) for small G-Proteins, regulators of G protein signaling (RGS) proteins bind to the activated Gα protein and stabilize the transition state for GTP hydrolysis without directly interacting with the nucleotide (Tesmer, et al. 1997, Berman, et al.

1996a). This accelerates GTP hydrolysis and inactivation of the G-protein, and inhibits cell responses to GPCR signaling.

In 1982, Chan and Otte found a protein in *Saccharomyces cerevisiae*, Sst2, that when mutated resulted in increased sensitivity to pheromone responses through a yeast GPCR using a growth arrest read-out (Chan & Otte. 1982). It was then found that Sst2 was not affecting Gpa1 (the yeast Gα protein) stability or anything downstream of Gpa1 (Dohlman, et al. 1995). Another study showed that overexpression of Sst2 caused decreased pheromone signaling and that Sst2 could be isolated in complex with Gpa1, thus demonstrating that Sst2 regulates signaling by acting on the G-protein. It was noticed that Sst2 had homology with other mammalian proteins, some of which were known to be GAPs (Dohlman, et al. 1996). Soon new RGS proteins were identified, and it was found that putting these proteins into yeast blunted signal transduction and reduced pheromone sensitivity in yeast lacking Sst2 (Druey, et al. 1996). Hence Sst2 became the founding member of a family of RGS proteins. At the same time, Egl 10, the C. elegans RGS protein (Koelle & Horvitz 1996), and G-alpha interacting protein (GAIP) (DeVries, et al. 1995) were discovered independently.
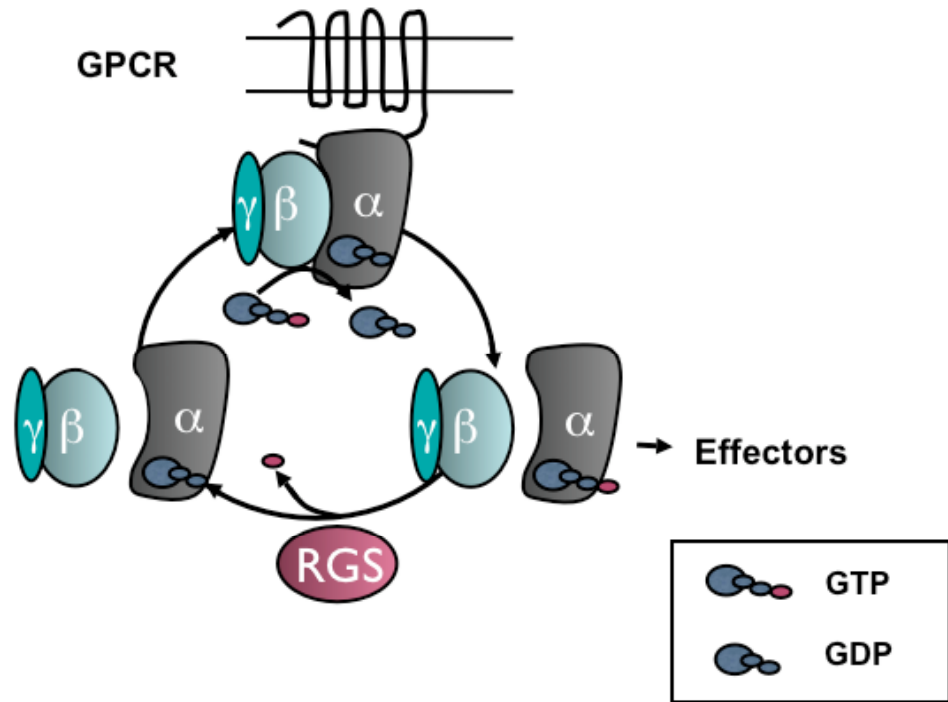
Figure 1.1: The G Protein cycle. When activated by an agonist, a GPCR stimulates exchange of GTP for GDP on the Gα subunit of the heterotrimeric G protein, which results in an activating conformational change allowing both the α and the βγ subunits to interact with effector proteins. The Gα subunit inactivates itself by hydrolyzing GTP to GDP and this allows reassociation with Gβγ and the receptor. RGS proteins accelerate GTP hydrolysis and inactivation of the G protein.

The RGS proteins are divided into several families. There have been over 30 RGS proteins identified (Abramow-Newerly, et al. 2006), which are divided into families based on the homology of the 120 amino acid RGS domain as well as the presence or absence of other domains (Figure 1.2) (Hepler. 1999). Members of the R4 (containing RGS1, RGS2, RGS3, RGS4, RGS8, RGS13, RGS16 and RGS18) and the RZ (containing RGS19 (also called GAIP), RGS20 (also called RGSZ1) and RGS17 (also called RGSZ2)) families primarily contain the RGS domain, however the R4 family also has a short amphipathic N-terminus (Bernstein, et al. 2000, Bernstein, et al. 2004) that plays a role in membrane targeting and/or receptor specificity (Bernstein, et al. 2000, Bernstein, et al. 2004, Gu, et al. 2007a). On the other hand, RZ family members contain a string of cysteines in the N-terminus that can be palmitoylated (Hiol, et al. 2003, Nunn, et al. 2006) (Figure 1.2). Hence the N-termini of both families are important for membrane targeting.

Other RGS families have accessory domains. RGS6, RGS7, RGS9 and RGS11 are R7 family members and have a Dishevelled/EGL-10/Pleckstrin (DEP) domain which is thought to mediate RGS-receptor interactions (Ballon, et al. 2006, Chen & Hamm. 2006), and a Gγ-like (GGL) domain which binds Gβ5 (Witherow & Slepak. 2003). RGS9 has two splice variants, with RGS9-2 having an extra 191 amino acids in the C-terminus (Rahman, et al. 1999). It is interesting that the different splice variants have strikingly distinct expression patterns: RGS9-1 is only expressed in the retina, while RGS9-2 is expressed in the brain, mostly in the striatum, but not the retina (Rahman, et al. 1999). The R12 family includes RGS10, RGS12 and RGS14 and these family

members have PSD95, Dlg and Z0-1/2 (PDZ) domains involved in protein-protein interactions (Snow, et al. 2002), phosphotyrosine-binding domains (PTB), Ras-binding (RBD) domains and GoLoco motifs which are guanine-nucleotide dissociation inhibitors (GDIs). The fact that both GDI and GAP domains for $G\alpha_i$ exist on the same protein may have interesting implications for the function of R12 family members. The significance of this has yet to be fully evaluated. Members of the E/RA family, Axin and Conductin, contain a glycogen synthase kinase3-β binding (GSK3β) domain, a β-catenin binding (Cat) site, a protein phosphatase 2A homology region (PP2A), and a dimerization (DIX) domain (Hollinger & Hepler. 2004). Other proteins that contain RGS homology (RH, rather than true RGS) domains include members of the G protein receptor kinase (GRK), sorting nexin (SNX), axin, and Rho guanine-nucleotide exchange factor (RhoGEF) families. Non-RGS domains in RGS proteins regulate other steps in the G Protein cycle (with GDI and GEF domains), mediate protein-protein interactions, post-translational modifications, and cellular localization. This can result in specific and complex actions of these proteins. It can be speculated that multiple actions of a single protein could allow for targeted effects and pathway integration.
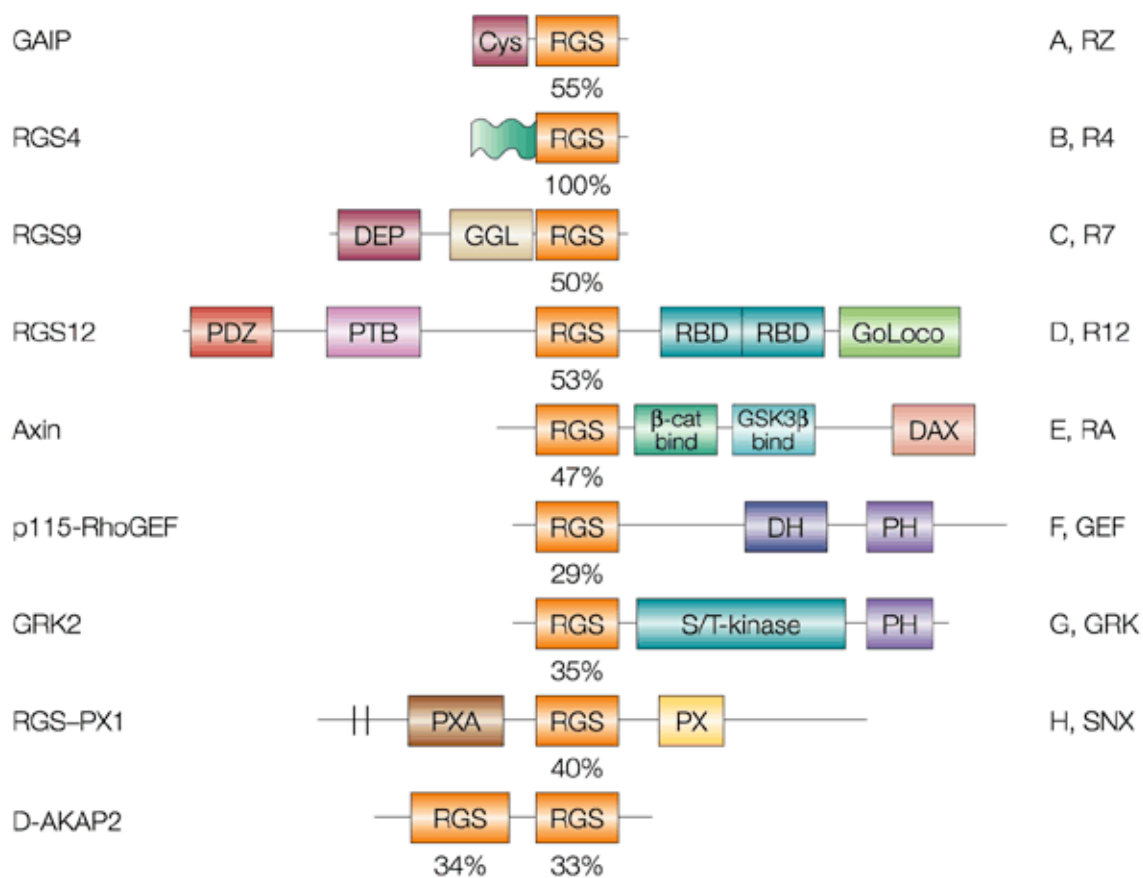
Figure 1.2: RGS families. From (Neubig & Siderovski. 2002). Prototypical RGS members are on the left and family designation is on the right. RGS families are characterized by homology of the RGS domain as well as presence of other domains.

Structural insights have been gained from X-ray crystalograph. Shortly after the discovery of RGS proteins, Tesmer et al. solved the first RGS crystal structure in 1997. This structure of RGS4 bound to AlF_4-activated $G\alpha_{i1}$ has been very useful for understanding how RGS proteins interact with $G\alpha$ subunits to stabilize the transition state of GTP hydrolysis without directly interacting with the nucleotide. The RGS protein contacts the three switch regions of the $G\alpha$ subunit that mediate the binding to and hydrolysis of the GTP. Since the elucidation of this structure, several other RGS structures, both NMR and X-ray, have been determined. These include RGS proteins alone or in complex with associated proteins for 18 different RGS proteins in 7 families (Cheever, et al. 2008a, Cheever, et al. 2008b, Chen, et al. 2001, Chen, et al. 2003, Chen, et al. 2005, Fedorov, et al. 2007, Higman, et al. 2006a, Higman, et al. 2006b, Higman, et al. 2006c, Lodowski, et al. 2005, Lodowski, et al. 2006, Longenecker, et al. 2001, Moy, et al. 1999, Moy, et al. 2000, Rhee, et al. 2006, Slep, et al. 2001, Slep, et al. 2008, Soundararajan, et al. 2008, Spink, et al. 2000, Sterne-Marr, et al. 2003, Tesmer, et al. 1997, Tesmer, et al. 2005, de Alba, et al. 1999).
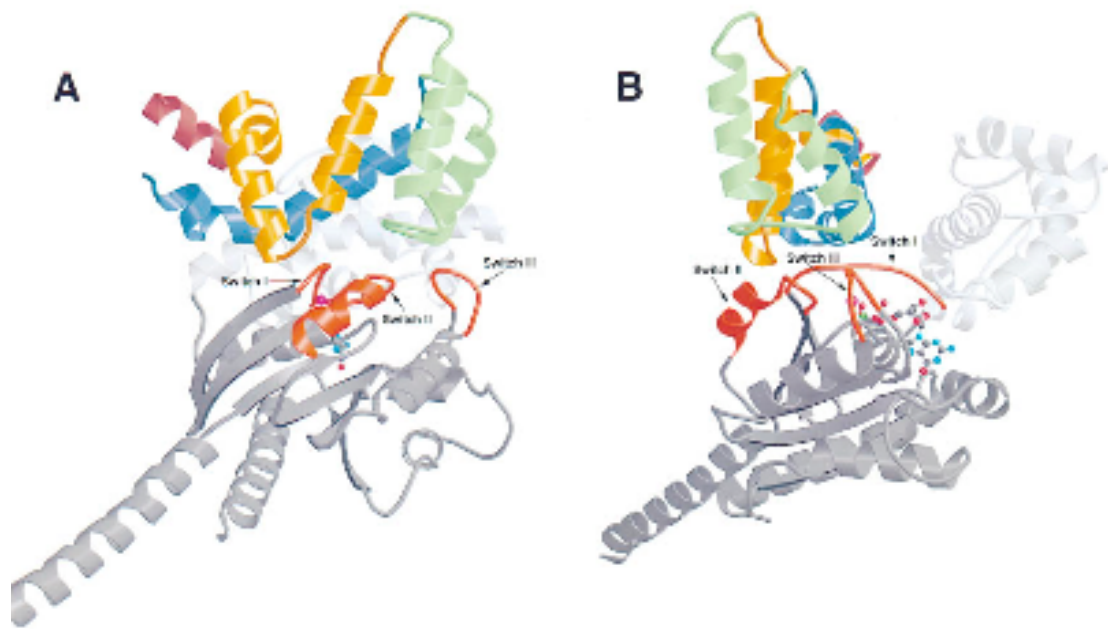
Figure 1.3: Structure of RGS4 bound to $G\alpha_{i1}$-AlF$_4^-$. From (Tesmer et al. 1997). A) The RGS protein is on top and is colored, while $G\alpha_{i1}$ is on bottom in grey. The three switch regions are in orange. B) The same structure rotated 90°.

RGS proteins have G Protein selectivity. There are 23 $G\alpha$ proteins divided into four families: $G\alpha_i$, $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$ based on amino acid homology as well as functional similarities (Nurnberg, et al. 1995). Among other things, the $G\alpha_i$ family inhibits adenylyl cyclase (AC) activity while $G\alpha_s$ stimulates AC. One of the many functions of the $G\alpha_q$ family is its involvement in calcium mobilization, and the $G\alpha_{12}$ family activates small G-proteins (Neves, et al. 2002). By selectively regulating G-proteins, RGS proteins can selectively regulate different signaling pathways (Table 1.1). Thus, although there is significant overlap, there is some G protein selectivity with RGS proteins. Since many GPCRs can activate more then one $G\alpha$ subunit (Kenakin. 2007), one could speculate that this selectivity may be physiologically useful for selectively modulating one pathway over another.

RGS family	Gα subunits	Reference
RZ	$G\alpha_{i/o/z}$, $G\alpha_q$	De Vries, et al. 1995, Berman, et al. 1996b, Hepler, et al. 1997, Wang, et al. 1998
R4	$G\alpha_{i/o}$, $G\alpha_q$	Berman, et al. 1996b, Hepler, et al. 1997, Heximer, et al. 1999
R7	$G\alpha_{i/o}$	(Hooks, et al. 2003, Lan, et al. 2000
R12	$G\alpha_{i/o}$	(Hooks, et al. 2003,
RA	ND	
GEF	$G\alpha_{12/13}$	Hains, et al. 2004
SNX	maybe $G\alpha_s$	Zheng, et al. 2001

Table 1.1: Table of RGS-Gα selectivity. Different RGS families will act as GAPs for different Gα subunits.

RGS proteins also have receptor selectivity. Even more so than with G protein selectivity, receptor selectivity can make RGS proteins very targeted and selective signaling modulators. There are many examples in the literature of RGS-receptor selectivity, but there is still a lot to learn about which RGSs are involved in which pathways.

The muscarinic receptors have been the subject of multiple selectivity studies. RGS1, RGS3, RGS4 and RGS16 have been found to selectively regulate signaling by muscarinic receptors over cholecystokinin (for RGS1, RGS4, and RGS16) bombesin (for RGS4) or angiotensin AT1a receptors (for RGS3) (Wang, et al. 2002, Xu, et al. 1999). RGS3 has been shown to mediate both M2 and M3 muscarinic receptor signaling, while RGS2, RGS4, RGS5 and RGS16 have been shown to regulate M2 but not M3 signaling (Anger, et al. 2007). This is not supported by biochemical data showing that RGS2 directly interacts with the M3 but not the M2 third intercellular loop (Bernstein, et al. 2004). Not all RGS proteins can regulate muscarinic signaling. It has been shown that RGS5 and RGS9 fail to regulate muscarinic signaling (through non-selective muscarinic receptor activation or M2-selective activation respectively) but inhibit angiotensin- or D_2-mediated signaling respectively (Cabrera-Vera, et al. 2004, Wang, et al. 2002). This is in contrast to RGS8, which can regulate signaling by both D_2 and M4 receptors, but not by adenosine receptors (Benians, et al. 2005).

RGS selectivity is also seen with the opioid receptors. Morphine induces pigment aggregation in Xenopus melanophore cells. Overexpression of RGS2 but not RGS1, RGS3 or RGS4 caused a rightward shift in the morphine dose-response curve in this system (Potenza, et al. 1999). In COS-7 cells, RGS19/GAIP is selective for the

nociceptin receptor over the mu, delta or kappa opioid receptors (MOR, DOR or KOR) (Xie, et al. 2005). RGS selectivity is not limited to the above systems. RGS proteins regulate adrenaline activated α_{2a}AR-Gα_o fusion protein GTPase activity with the following order of potency: RGS16>RGS1>RGS19/GAIP (Hoffmann, et al. 2001). In another study, RGS4, RGS10 and RGS20/RGSZ1 were found to inhibit 5-hydroxytryptamine (5-HT or serotonin) 1A receptor but not dopamine D_2 receptor-mediated signaling (Ghavami, et al. 2004). And finally, RGS1, RGS2, RGS3, and RGS4 inhibit AT1 receptor signaling, whereas only RGS3 and to a lesser extent RGS4, inhibit endothelin-1 receptor signaling (Cho, et al. 2003).

There is a lot of information regarding which RGS proteins can regulate which receptors in expression systems, however very few are supported with direct biochemical evaluation. There is also a great need for further evaluation of which RGS proteins regulate which signaling pathways in endogenous systems. There are limited examples of this, which will be discussed below in regard to receptor interactions and in regard to pathophysiology.

Receptor selectivity can be achieved through direct binding of RGS proteins to receptors or through indirect interactions via scaffolds. There are examples of both methods in the literature. In some cases, the scaffolds mediate receptor interactions, or they can regulate cellular localization or stability of the RGS. Examples are given in Table 1.2.

RGS	Scaffold	Receptor	Reference
RGS2	Direct binding	M1, $\alpha_{1\alpha}$AR, β_2AR	Bernstein, et al. 2004, Hague, et al. 2005, Roy, et al. 2006
RGS12	Direct binding	interleukin-8 receptor	Snow, et al. 2002
RGS20 RGS17	ND. Small ubiquitin like modifier is required	MOR	Rodriguez-Munoz, et al. 2007a
RGS19	GAIP-interacting protein, C terminus (GIPC)	D2	Jeanneteau, et al. 2004
RGS9	ND. DEP domain required	D2	Kovoor, et al. 2005
RGS4	Homer 2	M1	Shin, et al. 2003
RGS1 RGS2 RGS4 RGS16 RGS19	Spinophilin	αAR (in the case of RGS2 and RGS4)	Wang, et al. 2005, Liu, et al. 2006
RGS1 RGS2 RGS4 RGS19	Neurabin	Does NOT bind α_{1b}AR, D2, M3, cholecystokinin receptors	Wang, et al. 2007b
RGS9	R9 anchoring protein (R9AP)	Necessary for RGS9 stability and activity	Hu, et al. 2003a, Hu & Wensel. 2002, Keresztes, et al. 2004,
R7 family	Gβ5	Necessary for R7 family stability and activity	Cabrera, et al. 1998, Chen, et al. 2003, Kovoor, et al. 2000, Makino, et al. 1999, Snow, et al. 1998, Song, et al. 2007
R7 family	R7 binding protein (R7BP)	Lipid rafts, also necessary for stability and function	Nini, et al. 2007, Anderson, et al. 2007, Drenan, et al. 2005, Drenan, et al. 2006, Martemyanov, et al. 2005
RGS9	α actinin	NMDA	Bouhamdan, et al. 2006

Table 1.2: RGS proteins regulate GPCRs through direct interactions or through scaffolds. Examples of RGS proteins regulating or binding to receptors are shown. ND: not determined.

RGS	Effector	RGS function	References
RGS2 RGS3 RGS4 RGS10 RGS 13	AC	All inhibit $G\alpha_s$-mediated AC activation. RGS2 inhibits forskolin-mediated AC activation. RGS2 directly binds $G\alpha_s$ and AC	Chatterjee, et al. 1997, Ghavami, et al. 2004, Johnson & Druey. 2002, Roy, et al. 2003, Salim, et al. 2003, Scheschonka, et al. 2000, Sinnarajah, et al. 2001, Tseng & Zhang. 1998, Ko, et al. 2001, Roy, et al. 2006, Tseng & Zhang. 1998
RGS1 RGS2 RGS3 RGS4 RGS5 RGS7 RGS8	GIRK	RGSs mediate the kinetics of channel opening and closing as well as basal currents. RGS4 binds the channel directly.	Doupnik, et al. 1997, Herlitze, et al. 1999, Inanobe, et al. 2001, Saitoh, et al. 1997, Saitoh, et al. 1999, Keren-Raifman, et al. 2001, Jaen & Doupnik. 2006
RGS2 RGS4 RGS10 RGS12	Calcium Channels	RGSs mediate the kinetics of channel opening and closing. RGS12 binds the channel directly.	Jeong & Ikeda. 1998, Jeong & Ikeda. 2000, Mark, et al. 2000, Melliti, et al. 2001, Schiff, et al. 2000, Richman, et al. 2005
RGS9	phosphodiesterase γ (PDEγ)	PDEγ increases the affinity of $G\alpha_t$ for RGS9. $G\alpha_t$ activates PDEγ	Skiba, et al. 2000
RGS2 RGS3 RGS4 RGS10	PLCβ	RGSs inhibit PLCβ.	Cunningham, et al. 2001, Hepler, et al. 1997, Heximer, et al. 1997, Saugstad, et al. 1998

Table 1.3: RGS proteins regulate effector proteins. Examples are shown of RGS proteins regulating effector proteins.

RGS proteins can directly interact with effectors to mediate GPCR signaling.
In addition to regulating G protein signaling by acting as a GAP for Gα subunits, RGS proteins can regulate effector activity. See Table 1.3 for details.

RGS proteins may be regulated by phospholipids and calmodulin.
Phosphatidic acid binds to the RGS4 N-terminus and inhibits its GAP activity (Ouyang, et al. 2003, Tu & Wilkie. 2004). Phosphatidylinositol-3,4,5,-trisphosphate (PIP3) inhibits the modulatory capacity of RGS4 on GIRK currents in myocytes, but is reversed by direct binding of Ca^{2+}/calmodulin (CAM) to RGS4 (Popov, et al. 2000, Ishii, et al. 2002). Both PIP3 and CAM were shown to compete for the same positively charged site on the RGS domain, which is distinct from the Gα binding site and the phosphatidic acid binding site (Ishii, et al. 2005a, Tu & Wilkie. 2004). It has been suggested that competition between CAM and PIP3 could result in the oscillatory Ca^{2+} signaling seen in pancreatic acinar cells (Luo, et al. 2001). According to the suggested model, PIP3 inhibition of RGS4 would prevent inhibition of PLCβ. This would lead to increased Ca^{2+} levels which would lead to increased Ca^{2+}/CAM interaction with RGS4, thus preventing PIP3 inhibition and completing the cycle (Abramow-Newerly, et al. 2006). This model is supported by the fact that a GAP deficient mutant of RGS4 still binds CAM and can prevent GIRK channel relaxation caused by Ca^{2+} mediated hyperpolarization in myocytes (Ishii, et al. 2001).

RGS-insensitive Gα proteins are useful tools for understanding endogenous RGS function. DiBello et al, using a yeast model, found a mutation in Gpa1 that

phenocopied Sst2 null yeast (DiBello, et al. 1998). It was later found that the homologous mutant in $G\alpha_{i1}$ (G183S) and $G\alpha_o$ (G184S) abolished RGS GAP activity and markedly decreased RGS affinity for the G-proteins (Lan, et al. 1998). RGS-insensitive mutants of $G\alpha$ subunits represent powerful tools for studying the endogenous RGS function without the need for overexpressing RGS proteins (although G-proteins with the mutation need to be expressed). It is also a good tool for understanding global RGS action in a system where the individual RGSs responsible are not known and possibly redundant. The identity of the G protein responsible for an effect can also be investigated. This system has been used to show that endogenous RGS proteins regulate norepinephrine inhibition of N-type calcium channels in rat sympathetic neurons (Jeong & Ikeda. 2000), adenosine-induced presynaptic inhibition in hippocampal neurons through $G\alpha_o$ (Chen & Lambert. 2000), dopamine-mediated GTPγS binding, agonist-mediated Ca^{2+} responses in CHO cells expressing the D_{2S} receptor (Boutet-Robinet, et al. 2003), lysophosphatidic acid-mediated inhibition of AC in ovarian cancer cells (Hurst, et al. 2008) and GABA(B) receptor-dependent desensitization of GIRK currents in human embryonic kidney (HEK) cells (Mutneja, et al. 2005).

This system has been used to measure the effects of RGS proteins in modulating MOR-mediated signaling in C6 cells overexpressing both the receptor and RGS-sensitive or RGS-insensitive $G\alpha_o$. Pertussis toxin (PTX, which selectively inactivates $G\alpha_{i/o}$)-insensitive mutants of $G\alpha_o$ were used to eliminate the contributions of endogenous G proteins. Using this system, the contribution of that endogenous RGS proteins to regulate MOR-mediated AC inhibition and mitogen-activated protein kinase (MAPK) pathway activation were revealed (Clark, et al. 2003). Endogenous RGSs were also shown to

regulate AC supersensitization, which accompanies withdrawal of agonist treatment (Clark, et al. 2004) and MOR desensitization and leads to the development of tolerance (Clark & Traynor. 2005). Thus RGS proteins are involved in more than simply acute $G\alpha_o$ signal transduction. They can also regulate the effects of chronic agonist treatment and may be important in drug dependence, tolerance and withdrawal.

The RGS-insensitive mutations have been used in embryonic stem (ES) cells as well. A1 and M2 bradycardic responses were increased in mouse ES cells expressing the $G\alpha_o$ RGS-insensitive mutant instead of wild type $G\alpha_o$ at endogenous levels. Whereas only M2 responses were enhanced in the $G\alpha_{i2}$ RGS-insensitive ES cell line (Fu, et al. 2006). This shows both receptor and $G\alpha$ selectivity of endogenous RGS proteins.

1b. RGS proteins in pathophysiology

RGS proteins are important in cardiovascular signaling. At least 15 different RGS proteins have been shown to be expressed at the mRNA level in the heart with expression levels varying with disease state and developmental stage (Doupnik, et al. 2001, Kardestuncer, et al. 1998, Mittmann, et al. 2002). Functional data are lacking for many of the RGS proteins, but the following outlines the importance of some of the more studied RGS proteins in regulating cardiovascular function.

Hypertension. Hypertension is estimated to affect more then 72 million people in the US alone, and is a factor for the development of more serious heart disease (Rosamond, et al. 2008). Hypertension is unique in that it is perhaps the only disease for which such a strong connection to a single RGS (RGS2) has been demonstrated to date;

there is data on the genetic, cellular and animal levels in both mice and in humans for RGS2 involvement in blood pressure regulation. Compared to normotensive patients, RGS2 mRNA is lower in the peripheral blood mononuclear cells (PBM). Interestingly, there is an increased incidence of a C1114G polymorphism, and there is increased AT II stimulated calcium signaling and ERK activation in fibroblasts from hypertensive patients (Semplicini, et al. 2006). Other RGS2 polymorphisms have also been associated with hypertension in humans (Riddle, et al. 2006, Yang, et al. 2005) with one producing a mutant protein that has decreased expression and function (Bodenstein, et al. 2007). Taken together, these data support a strong role of RGS2 in the regulation of peripheral blood pressure.

Studies in mice have been performed to try to elucidate the mechanism of these effects. The angiotensin receptor is $G\alpha_q$-coupled and is activated by angiotensin II (AngII), a potent vasoconstrictor. AngII increases RGS2 expression through a phospholipase A2-dependent mechanism in vascular smooth muscle cells, which may be a negative feedback mechanism (Grant, et al. 2000, Li, et al. 2005, Xie, et al. 2007). Increased angiotensin signaling is thought to be responsible for the hypertensive phenotype seen in the RGS2-deficient mice as this phenotype can be reversed with AT_1 antagonists (Heximer, et al. 2003) and RGS2-deficient mice are hyper-responsive to AngII (Hercule, et al. 2007). In addition to the hypertension seen in the RGS2 knock-out mice, decreased RGS2 mRNA has been associated with hypertension in rats (Grayson, et al. 2007).

RGS2 is also involved in the nitric oxide (NO)-stimulated cGMP pathway, which mediates the regulation of blood pressure. cGMP-activated protein kinase G (PKG) can

phosphorylate RGS2 and enhance RGS2 GAP activity. It also increases membrane localization, inhibition of thrombin receptor protease-activated receptor-1 signaling (Tang, et al. 2003) and RGS2 protein stability (Osei-Owusu, et al. 2007). It has also been found that RGS2 deficient mice are less sensitive to the vasodilating effects of the NO donor sodium nitroprusside (Sun, et al. 2005), suggesting that RGS2 mediates the vasodilating effects of NO.

These data support the notion that decreased RGS2 function (with knock-out in mice or with human polymorphisms) causes hypertension through ablating the endogenous NO signaling. Increased angiotensin signaling also plays a role. Whether or not there is a relationship between the increased AngII signaling and the decreased NO-mediated vasodilation is unclear, but there has been some suggestion that AngII can regulate endothelial nitric oxide synthase (Hennington, et al. 1998, Li, et al. 2007, Nakashima, et al. 2006, Ritter, et al. 2003, Suzuki, et al. 2006).

In contrast to what is seen with RGS2, RGS5-deficient mice have hypotension. These mice have dilated aortas and increased signaling through spingosine 1 phosphate or in response to sodium nitroprusside. This suggests that RGS5 could be a potential anti-hypertension target (Cho, et al. 2008). A three gene region of chromosome 1 that includes RGS5 has been associated with hypertension in humans (Chang, et al. 2007). In a cDNA screen, RGS5 was found to be upregulated in stroke-prone hypertensive rats compared to wild type (Kirsch, et al. 2001). RGS5 has been shown to be expressed in arteries but not veins (Adams, et al. 2000, Li, et al. 2004), and atrial expression is increased in mice overexpressing the β_2AR (Jean-Baptiste, et al. 2005).

Cardiac Hypertrophy. Cardiac hypertrophy is a compensatory mechanism by which the heart increases size in an attempt to handle increased stress. Depending on the stress, this response may be beneficial or it could be detrimental and over time lead to heart failure. Many forms of hypertrophy result in increased expression of embryonic genes, and this has been used as an indicator of clinical severity, as well as an experimental read-out (Hunter & Chien. 1999).

Changes in RGS levels have been observed in models of cardiac hypertrophy. RGS2 expression is upregulated by phenylephrine treatment (Gan, et al. 2005, Zou, et al. 2006) and also by adenovirus containing a constitutively active $G\alpha_q$ (Q209L) in cultured myocytes (Hao, et al. 2006). In the phenylephrine model, RGS2 upregulation is blocked by adenosine receptor agonists, which are known to prevent hypertrophy (Gan, et al. 2005). It has also been shown that RGS3 and RGS4 mRNA levels are increased in pulmonary artery-banded mice and also in growth factor-treated cultured myocytes (Zhang, et al. 1998). Aortic RGS2, RGS4 and RGS5 expression in an aortic banding model of hypertrophy was found to vary over time (Wang, et al. 2007a).

Compensatory changes in RGS expression are suggestive of their role in the development of cardiac hypertrophy, but it does not prove that there is a role, or reveal the mechanism. To address these questions, recombinant RGSs can be introduced to myocytes or mice. Adenovirus overexpression of RGS2 protects cells from increased size and fetal gene expression by phenylephrine (Zou, et al. 2006) while decreases in RGS2 expression with interfering RNA exacerbated hypertrophy in myocytes (Zhang, et al. 2006). RGS4 overexpression blocked phenylephrine and endothelin induction of fetal genes and cell growth in isolated myocytes (Tamirisa, et al. 1999), reduced transaortic

constriction-induced hypertrophy which increased mortality in mice (Rogers, et al. 1999), prevented hypertrophy in $G\alpha_q$ overexpressing mice (Rogers, et al. 2001), and prevented hypertrophy in a transcription factor overexpression model (Harris, et al. 2004). Thus it appears that RGS4 blocks many forms of hypertrophy, both beneficial and detrimental.

$G\alpha_{i2}$ RGS-insensitive mutant knock-in. Mice that express the RGS-insensitive mutation of $G\alpha_{i2}$ instead of the wild type protein develop cardiac hypertrophy (Huang, et al. 2006), consistent with other models described above. Studies performed in isolated perfused hearts from these mice confirmed what was observed in ES cells, where the mutation increases sensitivity to carbachol-mediated bradycardia. It was also found that these isolated hearts were more sensitive to carbachol-induced arrhythmias (Fu, et al. 2007), consistent with the notion that RGS proteins may tightly regulate muscarinic signaling, as mentioned previously.

RGS proteins are important in neurological disease. There is an extensive literature describing the roles of RGS proteins in neuronal function and disease. Several RGS proteins have been shown to be expressed in the brain, and in many cases they display very distinct expression patterns (Gold, et al. 1997, Grafstein-Dunn, et al. 2001, Ingi & Aoki. 2002, Krumins, et al. 2004).

Psychosis. Schizophrenia is characterized by delusions, hallucinations, social withdrawal, attention and cognitive defects and is treated with drugs that antagonize the D_2, serotonin, and other receptors (Hardman & Limbird. 2001). Several groups have found an association between RGS4 polymorphisms and schizophrenia (Bakker, et al. 2007, Chen, et al. 2004, Chowdari, et al. 2002, Levitt, et al. 2006, Li & He. 2006, Morris,

et al. 2004, Williams, et al. 2004, Winantea, et al. 2006) while other studies have failed to detect an association (Guo, et al. 2006, Ishiguro, et al. 2007, Puri, et al. 2007, Rizig, et al. 2006). Although it is not clear whether RGS4 is associated with the development of schizophrenia, recent evidence suggests that RGS2 and RGS5 variants may be associated with the severity of the disease in affected individuals (Campbell, et al. 2008). Similarly, allelic variations in RGS4 have been shown to influence brain development in humans, which may impact predisposition for psychosis (Buckholtz, et al. 2007). RGS4 polymorphisms have also been shown to be associated with bipolar disorder in humans (Cordeiro, et al. 2005).

To study the effects of RGS4 on signaling in schizophrenia, Gu et al did electrophysiology on pyramidal neurons from the prefrontal cortex (PFC) from rats. Application of an RGS specific antibody in the patch clamp pipette increased 5-HT1A– but not D4-mediated inhibition of NMDA currents. These data suggest that RGS4 may protect against NMDA hypofunction, which is associated with schizophrenia (Gu, et al. 2007b).

RGS proteins not only play a role in the manifestation and severity of psychosis, but can also influence responses to antipsychotic drugs. Mice lacking RGS9 experience drug-induced dyskinesia when given quinpirole (a D2-like selective agonist) after dopamine (DA) depletion with reserpine (Kovoor, et al. 2005). This is consistent with the DA supersensitivity and increased D_2 high affinity states seen in schizophrenia (Seeman, et al. 2006). Also, RGS2 polymorphisms in human are associated with worsening of parkinsonian symptoms from antipsychotic treatment (Greenbaum, et al. 2007).

RGSs have also been implicated in other neurological abnormalities. Mice lacking RGS2 have increased anxiety and decreased male aggression compared to wild type mice. It has been suggested that these behaviors may be due to the decreased dendritic spine density observed in the hippocampal CA1 neurons and decreased electrical activity of these neurons. Thus it has been suggested that RGS2 plays a role in the proper development of these neurons (Oliveira-Dos-Santos, et al. 2000).

Neurodegenerative diseases. Parkinson's Disease (PD) is caused by loss of DA neurons innervating the striatum. This is accompanied by increased acetylcholine (ACh) levels, and results in motor symptoms. The increase in ACh is thought to be due to a loss of DA activation of inhibitory D_2 receptors on ACh releasing neurons and subsequent inhibition of Cav2 Ca^{2+} channels leading to increased ACh release. However, Ding et al suggests that it is actually M4 autoreceptors regulated by RGS4, which cause an increase in ACh release. They show with electrophysiology studies in isolated cholinergic interneurons from 6-hydroxydopamine (6-OHDA which selectively ablates DA neurons) lesioned, reserpine-treated and untreated mice that DA depletion decreased oxotremorine-M (a muscarinic agonist) but not quinpirole-mediated Ca^{2+} currents. DA depletion also leads to increased RGS4 expression in these cells and inclusion of RGS4 in the patch clamp pipette mimicked the effect of DA depletion in cells from untreated mice (Ding, et al. 2006). These data suggest that RGS4 may inhibit the inhibitory M4 receptor. Thus RGS4 activity in the DA depletion model results in increased Ca^{2+} channel activity, increased ACh release, and exacerbated motor symptoms. These data also support the notion that RGS4 could be a therapeutic target for the treatment of Parkinson's disease.

Like PD, AD (Alzheimer's Disease) is a neurodegenerative disease that is associated with a progressive and premature onset of dementia, eventually leading to fatality. This correlates with the appearance of β-amyloid plaques, neurofibrillary tangles and loss of neurons (Masters, et al. 2006). In studies performed on brains from deceased AD and normal age matched patients, RGS4 and $G\alpha_q$ protein levels were found to be lower in the parietal cortex (53 and 40% respectively) while membrane-bound protein levels were unchanged. These changes also correlated with changes in carbachol binding (Muma, et al. 2003).

Morphine treatment of pain. Morphine and other opiates are of great clinical importance for the treatment of pain. However, their use is limited due to the development of tolerance and their related abuse liability (Rozenfeld, et al. 2007). Perhaps the most impressive illustration of the importance of RGS proteins in opioid receptor signaling is the phenotype of the RGS9-deficient mice. These mice have increased morphine reward, analgesia, delayed tolerance and exacerbated dependence and withdrawal symptoms (Rodriguez-Munoz, et al. 2007b).

The Garzon lab has several studies on the effects of antisense oligodeoxynucleotides (ODN) of RGSs in mice. They found that knock-down of RGS9 but not RGS2 increased the potency and duration of action of morphine analgesia (Garzon, et al. 2001) due to regulation of the MOR but not the DOR (Sanchez-Blazquez, et al. 2003). They have also looked at tolerance in this system. RGS9 knock-down mice had less tolerance after 4 daily morphine treatments (Garzon, et al. 2001). RGS14 ODN knock-down decreased MOR internalization by altering its phosphorylation in periaqueductal gray (PAG). These mice also had decreased tolerance (Rodriguez-Munoz,

et al. 2007b). Knock-down of RGS20/RGSZ1 and RGS17/RGSZ2 increased morphine analgesia, tolerance and tachyphylaxis (Sanchez-Blazquez, et al. 2005). This was not seen with DOR agonists DPDPE ([D-Pen2,5]-enkephalin), or [D-Ala2] deltorphin II (Garzon, et al. 2005b). Hence RGS proteins, particularly RGS9, regulate morphine analgesia and tolerance and are an attractive therapeutic target.

RGS proteins are important for endocrine function. It is well established that G protein signaling is important for proper endocrine function (Lania, et al. 2006, Melien. 2007). It is therefore reasonable to consider the roles of RGS proteins in theses systems.

Diabetes. Type II diabetes develops when obesity and sedentary lifestyle results in decreased insulin sensitivity. The prevalence of obesity and diabetes is growing at an alarming rate and has reached epidemic levels (Smyth & Heron. 2006). RGS2 inhibited glucose-dependent insulinotropic peptide receptor (GIP-R, a $G\alpha_s$ coupled GPCR)-mediated insulin release and was shown to bind $G\alpha_s$. RGS2 mRNA levels were increased with GIP treatment in betaTC3 cells (Tseng & Zhang. 1998).

But the most impressive demonstration of the involvement of RGS proteins in diabetes is the phenotype of the $G\alpha_{i2}$ G184S knock-in mice. These mice have reduced weight gain and are protected from insulin resistance when fed a high fat diet compared to wild type mice (Huang, et al. 2008). The signaling pathways and RGS proteins involved were not identified, but this demonstrates the global importance of RGS proteins in the development of diabetes. Since these mice also have hypertrophy, hyperactivity, and increased neutrophil count (Huang, et al. 2006), a global RGS inhibitor

may not be desirable. However, if the RGS proteins involved in preventing insulin resistance could be identified, they would be attractive therapeutic targets.

Reproductive hormones. RGS proteins have also been shown to be important in hormone receptor signaling. RGS3 but not RGS1, RGS2 or RGS4 inhibited gonadotropin releasing hormone (GnRH) mediated IP3 signaling through $G\alpha_q$ in COS-1 cells, and RGS3 is expressed in a gonadotropic cell line (Neill, et al. 1997). RGS3 has also been shown to inhibit GnRH mediated luteinizing hormone secretion from cultured rat pituitary cells (Neill, et al. 2001). RGS10 has also been shown to regulate this receptor (Castro-Fernandez & Conn. 2002). RGS3 but not RGS10 regulate signaling by follicle-stimulating hormone and luteinizing hormone receptor (Castro-Fernandez, et al. 2004). The estrogen receptor α was shown to be regulated by RGS3 (also called steroid-receptor-binding RGS, SRB-RGS) in a transcriptional read-out and this was due to a direct interaction (Ikeda, et al. 2001). This is an interesting observation as the estrogen receptor α is not a GPCR.

Other hormones. RGS2 regulates parathyroid hormone and thyroid stimulating hormone-mediated signaling (Eszlinger, et al. 2004, Thirunavukkarasu, et al. 2002). RGS4, but not RGS7, RGS9 or RGS19 has been shown to inhibit thyrotropin releasing hormone receptor-mediated IP formation in HEK cells (Harder, et al. 2001). RGS4 is also important in melatonin receptor signaling. RGS4 knock-down with siRNA in CHO cells expressing the melatonin receptor attenuated receptor desensitization without affecting forskolin response (Witt-Enderby, et al. 2004). RGS4 and RGS10 blocked melatonin inhibition of androgen receptor reporter gene activity. This was shown to be through $G\alpha_q$ and $G\alpha_i$ mediated signaling, respectively (Rimler, et al. 2007). And finally,

overexpression of the RGS-insensitive $G\alpha_q$ in rats caused increased oxytocin and corticosterone responses with 5-HT2A/2C stimulation compared to overexpression of wild type $G\alpha_q$ (Shi, et al. 2006).

RGS proteins regulate immune function. RGS1 mRNA is found in monocytes (Denecke, et al. 1999), RGS1, RGS3 and RGS13 mRNA are in B-cells (Moratz, et al. 2000, Reif & Cyster. 2000, Shi, et al. 2002) and RGS2, RGS3, RGS4, RGS14 and RGS16 are in T-cells (Beadling, et al. 1999, Cho, et al. 2000, Johnson & Druey. 2002, Sato, et al. 2002). Natural killer cells contain RGS1, RGS2, RGS5, RGS8, RGS16, and RGS18 (Kveberg, et al. 2005).

In B-cells, RGS1 inhibits platelet activating factor mediated increases in intracellular Ca^{+2} and stromal-derived factor-1-induced cell migration (Moratz, et al. 2000). RGS1 or RGS3 overexpression diminishes chemotaxis toward certain chemokines (Reif & Cyster. 2000), and RGS1 overexpression in progenitor B cells impairs chemotaxis and adhesion. It is thought that this is involved in B-cell development as RGS1 levels increase as the B-cells mature (Le, et al. 2005). On the other hand, mice lacking RGS1 have B-cells that are overresponsive to chemokines and have improper desensitization (Moratz, et al. 2004). When injected into a wild type host, more B lymphocytes from RGS1 deficient mice home to the lymph nodes compared to cells isolated from wild type mice (Han, et al. 2005). RGS13 also impairs chemokine signaling (Shi, et al. 2002), and knock-down of RGS1 and/or RGS13 in a lymphoma cell line increases chemokine responsiveness (Han, et al. 2006b). In transfected 293T cells, wild type RGS14, but not a GAP deficient mutant, inhibited interleukin 8 mediated ERK

activation. Both the wild type and the mutant RGS14 inhibited $G\alpha_{13}$ mediated serum response element activation (Cho, et al. 2000).

T-cells from RGS2 deficient mice have lower levels of the growth factor interleukin-2 and reduced proliferation. This correlates with impaired antivirus responses in theses mice (Oliveira-Dos-Santos, et al. 2000). In T-cells, there is a correlation between decreased expression of RGS1, RGS9 and RGS16 and increased migration (Agenes, et al. 2005). Transgenic mice overexpressing RGS16 have T cells with impaired lung recruitment in response to inhaled allergen, but increased T-cell activation (Lippert, et al. 2003).

Using RGS13 deficient mice, it was shown that RGS13 inhibits antigen induced mast cell activation and deficient mice had increased anaphylaxis. Interestingly, this was independent of its GAP function and not mediated by a GPCR (Bansal, et al. 2008). In lymphoid cells, RGS1, RGS3 and RGS4 inhibit migration towards chemoattractants (Bowman, et al. 1998).

The Ga_{i2} G184S knock-in mice mentioned above have enlarged spleens and increased neutrophil counts (Huang, et al. 2006). What this means for immunity is an interesting question for future work.

1c RGS inhibition.

It has been estimated that approximately 40% of all clinically used drugs target GPCRs (Eglen, et al. 2007). However, selectively activating the GPCR of interest is problematic due to the high homology between related receptors. As a result, many drugs

have unintended effects at non-target GPCRs and may result in various side effects. Another problem is that the same GPCR can activate multiple signaling pathways, as mentioned above. Because RGS proteins have limited G protein selectivity, receptor selectivity, and distinct expression patterns, it has been suggested that selective RGS inhibitors could potentiate a single signaling pathway by a certain GPCR in a specific area of the body. This could result in very targeted effects which would cause fewer side effects (Neubig. 2002, Traynor & Neubig. 2005, Zhong & Neubig. 2001).

Individual examples of where an RGS inhibitor would be useful are outlined above and include treatments for hypertension (Cho, et al. 2008), PD (Ding, et al. 2006), pain (Rodriguez-Munoz, et al. 2007b), cocaine reward (Rahman, et al. 2003), asthma (Druey. 2003), and diabetes (Huang, et al. 2008, Usui, et al. 2004). Although not discussed in detail here, RGS proteins have also been suggested as useful cancer targets (Boss, et al. 2007, Heo, et al. 2006).

For these reasons, the Neubig and Mosberg labs have been interested in targeting RGS proteins. RGS4 was chosen for the initial efforts because it is a prototypical member of the R4 family and the first with a crystal structure that could be used for rational design (Tesmer, et al. 1997). The goal of this project was to identify new and more potent inhibitors of RGS4 than our lead compound.

There are three approaches to developing an inhibitor to a protein. The first approach is rational design based on a structure. For this, the crystal structure of RGS4 bound to $G\alpha_{i1}$ was used (Tesmer, et al. 1997). The strategy employed in these studies was to create a peptide that blocks the RGS4-Gα interaction by mimicking a piece of the G

protein. Initially, two peptides with the sequences of two of the three switch regions on the Gα protein were purchased but they were found to be inactive. It was then determined that a structurally constrained analog of the switch 1 region would be a better approach. The resultant peptide, **YJ34** (*Ac*-Val-Lys-c[Cys-Thr-Gly-Cys]-Glu-*NH₂*, S-S) and a series of related peptides represented the first published RGS4 inhibitors (Jin, et al. 2004). Mechanistic and structure-activity relationship (SAR) studies on **YJ34** will be discussed in Chapter 2.

The second approach is high-throughput screening (HTS). For this approach a large number (thousands or millions) of compounds are screened for activity. The advantage of this approach over the rational approach is that a large number of compounds can be tested more rapidly. As with all HTS-related screening endeavors, there is considerable time and effort committed to evaluating the hits; true hits have to be distinguished from false positives, and the mechanisms have to be determined (Keseru & Makara. 2006). This method has been used to identify small molecule inhibitors of RGS4. In a screen in yeast, some compounds were identified, but no structures were published (Young, et al. 2004). Recently, in the Neubig lab, Roman et al (2007) identified CCG-4986, a small molecule RGS4 inhibitor (Roman, et al. 2007). The method used for this study will be mentioned in Chapter 4.

The third approach is computational. *In silico* screening of virtual libraries has the advantage of being much faster and affordable than physically screening compounds once an appropriate algorithm has been established. But there are challenges to screening for inhibitors of a protein-protein interaction, and although there have been advances (Headd, et al. 2007), this approach was not chosen for this study.

The plan for this project was to utilize a combination of rational and combinatorial design in order to identify novel peptide inhibitors. There are features of **YJ34** that were found to be necessary for function (Chapter 2). These structures were constrained in the library, while the other positions were randomized to afford a 2.5-million peptide library, which will be discussed in detail in Chapters 3 and 4. The hope was that by using a focused library, the likelihood of identifying peptides that had the same mechanism of action as **YJ34** would be increased.

There are several methods that can be employed in a peptide library approach. The first approach utilizes positional scanning peptide libraries, which are a series of soluble peptide sub-libraries where one position is fixed while the other positions are randomized. There is one sub-library for each amino acid at each position, and the top few sub-libraries at each position are chosen. Peptides made that combine the best amino for each position are then synthesized (Pinilla, et al. 1992). Many have used this approach successfully and it has been especially useful in identifying protease substrates (Choe, et al. 2006, Cuerrier, et al. 2007, Diamond. 2007, Schmid, et al. 2007). In order to employ this method, a solution based high-throughput assay for testing peptide activity would be needed.

A second peptide library method is phage display. For this approach, DNA encoding peptides are fused to bacteriophage coat proteins. Bacteriopage libraries are screened by virtue of their capacity to bind a tethered target (Cesareni. 1992). Although others have had success with it in the past (McLafferty, et al. 1993), there were concerns about proper formation of the disulfide bond within the peptide library. Another

disadvantage of this kind of library is that unnatural amino acids and N- and C-terminal modifiers cannot be incorporated.

The method chosen for this project was a one-bead, one-compound (OBOC) library. This kind of library is synthesized and screened on beads and it is designed such that each bead has only one sequence and the beads that bind a fluorescent protein are isolated (Lam, et al. 1991). The isolated beads are then sequenced by Edman degradation to afford the hit sequences. This method was chosen with the assumption that the screening would be more straightforward than a soluble peptide library screen. Also, since the library is synthetic, N- and C-terminal modifiers could be incorporated, an option that is not possible with a phage display approach. The details and results of this will be discussed in Chapters 3 and 4.

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Chapter 2

Mechanism of Action and Structural Requirements of Constrained Peptide Inhibitors of RGS Proteins

Abstract:

Regulators of G Protein Signaling (RGS) accelerate GTP hydrolysis by Gα subunits and profoundly inhibit signaling by G protein-coupled receptors. The distinct expression patterns and pathophysiologic regulation of RGS proteins suggest that inhibitors may have therapeutic potential. The Mosberg and Neubig labs previously reported the design of a constrained peptide inhibitor of RGS4 (**YJ34**: *Ac*-Val-Lys-[Cys-Thr-Gly-Ile-Cys]-Glu-*NH₂*, S-S) based on the structure of the Gα$_i$ switch 1 region but its mechanism of action was not established. In this Chapter, it is shown that **YJ34** inhibits RGS4 activity in a single-turnover GTPase accelerating protein (GAP) assay. Furthermore, a mutation in Gα (G183S) that disrupts binding of RGS4 to the Gα subunit also disrupts activity of the Gα mimetic peptide, suggesting that **YJ34** binds the RGS protein in the same manner that Gα does. Also, **YJ34** shows selectivity for RGS4 and RGS8 vs. RGS7 and structure activity relationships illustrate key features for RGS inhibition. Finally, the capacity of the methylene dithioether-bridged peptide inhibitor, **YJ33,** to modulate muscarinic receptor-regulated potassium currents in atrial myocytes is demonstrated. These data support the proposed mechanism of action of peptide RGS

inhibitors, demonstrate their action in native cells, and provide a starting point for the design of RGS inhibitory drugs.

Materials and Methods:

Materials: Fmoc-protected amino acids and Rink amide resin were purchased from Advanced ChemTech (Louisville, KY). Pre-loaded PEG-PS resin and peptide grade synthesis chemicals were purchased from Applied Biosystems (Foster City, CA). $\gamma[^{32}P]$GTP (10 mCi/ml) was purchased from Amersham (Piscataway, NJ), or from Perkin Elmer (Boston, MA) and diluted in unlabeled GTP to the desired level of radioactivity.

Protein Expression and Purification: His_6-$G\alpha_o$ (rat), His_6-RGS4 (rat), GST-RGS7box (human, nucleotides 915-1359) and GST-RGS8 (rat, nucleotides 315-857) were expressed and purified according to previous protocols (Lan, et al. 1998, Lan, et al. 2000, Lee, et al. 1994). Hualing Zhong made some of the proteins used.

Peptide Synthesis: Peptides were synthesized and cyclized as described previously (Jin, et al. 2004). Resin was treated with piperidine (Aldrich, Milwaukee, WI, USA) to cleave the Fmoc-protecting group, then the first amino acid was coupled with o-benzotriazol-1-yl-N,N,N',N'-tetramethyl uronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) (Applied Biosystems). Trifluoroacetic acid (TFA)/water/ dithioethane (90:5:5) or TFA/phenol/water/triisopropylsilane (TIPS) (88:5:5:2) was used to cleave the linear peptide from the resin and simultaneously remove the side chain

protecting groups. The peptide solution was filtered from the resin and then subjected to preparative reverse-phase high-performance liquid chromatography (RP-HPLC) to afford the linear disulfhydryl-containing peptide with a purity of at least 90%. Yafei Jin, Katarzyna Sobczyk-Kojiro, Joe Musleh, Eric Schneider, Liangcai Gu or myself synthesized the YJ, KSK, JM, ES, GU, and BR peptides respectively.

Cyclization of Linear Peptides: For disulfide formation, linear disulfhydryl-containing peptides were dissolved (1mg/ml) in 1% acetic acid, 0.1 % trifluoroacetic acid, 2M urea in N_2 saturated water on ice. The pH of the peptide solution was raised to 8.5 using NH_4OH, followed by the addition of 4 mol equivalents of $K_3Fe(CN)_6$. The reaction mixture was stirred for 1 min, and quenched with acetic acid to a pH 3.5 or less. The mixture was then subjected to HPLC.

To form dithioether or dithiomethyl-containing cyclic peptides, a linear disulfhydryl peptide was added to dimethylformamide on ice under a N_2 atmosphere (0.1 mg linear peptide/mL dimethylformamide). Five mole equivalents of potassium tert-butoxide were added to the peptide solution, followed by the addition of 2.5 mol equivalents of $Br-(CH_2)_n-Br$ (n=1 or 2). The reaction was quenched with 2 mL acetic acid after 2 h and the solvent was removed in vacuo. The residue was dissolved in water, filtered, and then subjected to HPLC.

All final product peptides were at least 95% pure as assessed by RP-HPLC on a Vydac 218TP C-18 column (The Nest Group, Southboro, MA, USA) using the solvent system 0.1% TFA in water/0.1% TFA in acetonitrile by a gradient of 0–90% organic

component in 90 min. All peptides displayed the appropriate molecular weights as determined by mass spectrometry.

RGS-Stimulated GTPase: Single turnover GTP hydrolysis measurements with and without RGS were based on Lan, et al. (1998) and adapted to a 96 well plate format. Briefly, 200 to 800 nM $G\alpha_o$ was loaded with a 2-3 fold molar excess of $\gamma[^{32}P]GTP$ in 20 mM Hepes, 20 mM EDTA pH 8.0 for 5 or 20 minutes at room temperature and then cooled on ice. In some cases, the loaded $G\alpha_o$ was gel filtered through a 1 ml G-25 sephadex spin column to remove unbound $\gamma[^{32}P]GTP$. The loaded $G\alpha_o$ was then added to equal volumes of ice-cold initiation buffer (20 mM Hepes, 40 mM $MgCl_2$ pH 8.0 and 5-20 μM unlabeled GTP) containing RGS and/or peptide. The concentration of RGS protein (15-500 nM) was varied to keep the uninhibited rate of GTP hydrolysis less than 5.5 min^{-1}. This variation in RGS concentration was needed because of different activities of the protein preparations, different activities of the various RGS proteins against $G\alpha_o$, and different concentrations of $G\alpha_o$ used. After incubation for various times on ice, the reaction was quenched with 5% activated charcoal in buffer containing 20 mM sodium phosphate buffer (pH 2.0). After 20 minutes, the charcoal was centrifuged and the supernatant counted in a Perkin Elmer TopCount 96 well plate counter by Cerenkov counting. The amount of $[^{32}P]$ P_i released at each time point was fit to an exponential function:

$$[^{32}P] \text{ Pi counts}(t) = \text{counts}_{(t=0)} + \text{counts}_{(t=30 \text{ min})}*(1-e^{-kt})$$

Where counts $_{t=0}$ represents the counts per minute (cpm) obtained before the addition of initiation buffer, counts $_{t=30}$ represents the cpm at 30 minutes, and k is the rate constant,

which was calculated using GraphPad Prism (San Diego, CA). Fitting constraints included setting counts$_{(t=0)}$ for each curve to the average of the counts$_{(t=0)}$ for the experiment, and setting counts$_{(t=30\ min)}$ to the same value for all curves in an experiment. Peptide activity is determined from the percent decrease in RGS stimulated GTPase rate constant.

Electrophysiology: All animal studies were performed by Masaru Ishii according to the guidelines of the ethical committee of Osaka University Graduate School of Medicine. Single rat atrial myocytes were enzymatically isolated from hearts removed from adult male Wister-Kyoto rats as described elsewhere (Ishii, et al. 2001). Briefly, rats were deeply anesthetized by intraperitoneal injection of pentobarbital. A cannula was inserted into the aorta, and the heart was perfused in a retrograde manner through the coronary arteries. The heart was digested by collagenase (Boehringer Mannheim, Ingelheim, Germany) in nominally Ca^{2+}-free solution at 37°C for 10 min. Dissociated myocytes were seeded on glass coverslips (15 mm diameter) which had been coated with poly-D-lysine (Sigma, Milwaukee, WI) kept in a humidified environment of 0.5% CO_2 at 37°C, and cultured with medium M199 (PAA laboratories, Etobicoke, Ontario, Canada) containing gentamycin and kanamycin (25 mg/L each) for 2-4 days. Muscarinic-receptor regulated KG channel (GIRK) currents in atrial myocytes were measured using whole-cell mode patch clamp method as previously described (Ishii, et al. 2001). The whole-cell currents were measured at room temperature by a patch-clamp amplifier (Axon 200A, Molecular Devices, Sunnyvale, California) and recorded on videocassette tape with a PCM converter system (VR-10B, Instrutech). Data was analyzed with commercially

available software (Patch Analyst Pro, MT Corporation, Los Angeles, California) after a low-pass-filtration at 1 kHz (-3 dB) by an eightpole Bessel filter, sampled at 5 kHz. The control bathing solution contained (in mmol/L): 115 NaCl, 20 KCl, 1.8 $CaCl_2$, 0.53 $MgCl_2$, 5.5 glucose, and 5.5 Hepes-NaOH, pH 7.4. The pipette (internal) solution contained (in mmol/L): 150 KCl, 5 EGTA, 1 $MgCl_2$, 3 K_2ATP, 0.1 Na_2GTP, and 5 Hepes-KOH (pH 7.3). The ACh-induced GIRK currents were obtained by digitally subtracting currents recorded under control conditions from those recorded in the presence of ACh. Three parameters of RGS action on GIRK currents, i.e., time course of onset (k_{on}) and offset (k_{off}) of K_G current and degree of relaxation (I_{ins}/I_{max}), were determined (Ishii, et al. 2001). Peptide **YJ33** was applied intracellularly through the patch capillary electrode. Peptide **YJ33** was first dissolved into DMSO as 15 mM stock, and then diluted at 1/100 into patch electrode internal solution (final internal solution contained 150 μM peptides and 1% (v/v) DMSO).

Statistical Analysis: Data are expressed as mean ± S.E.M. and analyzed by either a 2-tailed unpaired t-test or a one-way ANOVA with a Dunnett's post-test. Significance is indicated as follows: $*p<0.05$, $**p<0.01$, $***p<0.001$.

Results:

YJ34 Inhibits RGS4 GAP activity in a single turnover GTPase assay. It has been previously shown that **YJ34**, a constrained peptide mimetic of the switch 1 region of $G\alpha_i$ (Table 2.1), inhibits RGS4 activity in a steady state membrane receptor GTPase

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assay (Jin, et al. 2004). To determine whether the results seen previously are due to **YJ34** activity on the RGS4 protein or whether it has its activity on the receptor or other proteins or lipids in the membrane preparation, **YJ34** activity was measured in a single turnover GTPase assays with purified proteins. RGS4-enhanced hydrolysis of $G\alpha_o$-bound $\gamma[^{32}P]GTP$ was measured in the presence and absence of **YJ34**. RGS4-stimulated $G\alpha_o$ GTPase activity is inhibited by **YJ34** in a dose-dependent manner (Figure 2.1) with an IC_{50} of 9 μM. It should be noted that **YJ34** was tested up to only 40 μM (i.e. less than half its aqueous solubility limit) in order to avoid aggregation. Consistent with previous results in a membrane GTPase assay (Jin, et al. 2004), the methylene dithioether-bridged peptide **YJ33**, was active but had lower potency than did the disulfide-bridged peptide **YJ34** (Table 2.1).
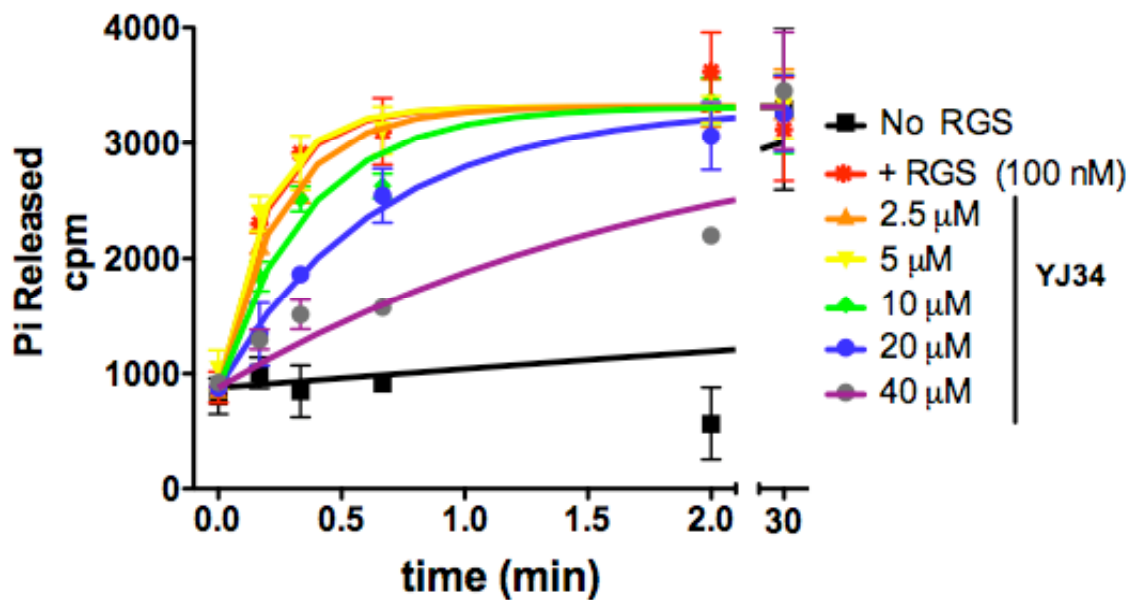
Figure 2.1: **YJ34** inhibits RGS4 in a single turnover GTPase assay. The rate of GTP hydrolysis was measured with no RGS, 100 nM RGS4, or 100 nM RGS4 with increasing concentrations of **YJ34**. This graph is representative of 4 experiments done in duplicate.

Name	Sequence	% Inhibition (100 μM unless otherwise noted)
Gα	…^{180}Val-Lys-*Thr*-Thr-Gly-Ile-*Val*-Glu^{187}…	
YJ34	Ac-Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-GluNH$_2$ (S-S)	75 ± 3 (40 μM)
YJ33	Ac-Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-GluNH$_2$ (S-CH$_2$-S)	25 ± 7
	Gly Substitutions:	
BR2	Ac-Val-Lys-c[Cys-Thr-**Ser**-Ile-Cys]-GluNH$_2$ (S-S)	-10 ± 8
YJ37	Ac-Val-Lys-c[Cys-Thr-**D-Ser**-Ile-Cys]-GluNH$_2$ (S-S)	59 ± 4 (30 μM)
YJ47	Ac-Val-Lys-c[Cys-Thr-**D-Pro**-Ile-Cys]-GluNH$_2$ (S-S)	1 ± 14
	N-terminal modifications:	
BR13	**NH$_2$**-Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-GluNH$_2$ (S-S)	2 ± 8
YJ41	Ac-__-Lys-c[Cys-Thr-Gly-Ile-Cys]-GluNH$_2$ (S-S)	8 ± 11
BR7	**Arg**-Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-GluNH$_2$ (S-S)	4 ± 9
BR1	**Arg-Thr-Arg**-Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-GluNH$_2$ (S-S)	13 ± 10
	C-terminal modifications:	
YJ49	Ac-Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-Glu**OH** (S-S)	1 ± 11
YJ42	Ac-Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-__-NH$_2$ (S-S)	3 ± 29
	Thr substitutions:	
YJ45	Ac-Val-Lys-c[Cys-**Ser**-Gly-Ile-Cys]-GluNH$_2$ (S-S)	73 ± 3
YJ46	Ac-Val-Lys-c[Cys-**Ala**-Gly-Ile-Cys]-GluNH$_2$ (S-S)	53 ± 10
BR8	Ac-Val-Lys-c[Cys-**Lys**-Gly-Ile-Cys]-GluNH$_2$ (S-S)	19 ± 15
	Cys substitutions:	
BR5	Ac-Val-Lys-c[**Pen**-Thr-Gly-Ile-Cys]-GluNH$_2$ (S-S)	-9 ± 10
BR6	Ac-Val-Lys-c[Cys-Thr-Gly-Ile-**Pen**]-GluNH$_2$ (S-S)	-2 ± 2
KSK99A	Ac-Val-Lys-c[Cys-Thr-Gly-Ile-**D-Cys**]-GluNH$_2$ (S-S)	10 ± 17
	Ile substitutions:	
GU1	Ac-Val-Lys-c[Cys-Thr-Gly-**Met**-Cys]-GluNH$_2$ (S-S)	22 ± 9
GU2	Ac-Val-Lys-c[Cys-Thr-Gly-**Met**-Cys]-Glu-NH$_2$ (S- CH$_2$CH$_2$-S)	61 ± 11
YJ36	Ac-Val-Lys-c[Cys-Thr-Gly-**Phe**-Cys]-GluNH$_2$ (S-S)	38 ± 10
ES2	Ac-Val-Lys-c[Cys-Thr-Gly-**Phe**-Cys]-GluNH$_2$ (S- CH$_2$CH$_2$-S)	52 ± 12 (30 μM)

Table 2.1: Sequences and RGS4 inhibition of various peptide analogs of **YJ34**. (S-S), (S-CH$_2$-S), (S-CH$_2$CH$_2$-S), indicate cyclization via disulfide, methylene dithioether, and ethylene dithioether, respectively.

Gly183 is critical for peptide-mediated inhibition of RGS4 GAP activity. It is possible that **YJ34** could bind to either the Gα subunit or RGS4. In order to determine the target of the peptide inhibitors, **BR2** was made to mimic the RGS insensitive G183S mutant of Gα$_i$. If **YJ34** binds to RGS4 in the same manner that the switch 1 region of Gα$_i$ binds, then a mutation that disrupts this binding in the Gα$_i$ subunit should also disrupt binding of **YJ34**. As predicted, **BR2** does not inhibit RGS4 activity at a concentration that is nearly 4 times greater then **YJ34** (150 μM), (Figure 2.2 and 2.3A), suggesting that **YJ34** binds to RGS4 as designed, by mimicking the Gα subunit. **BR2** does not bind, perhaps due to a direct steric clash of the Ser side chain with RGS4 or through conformational effects at the β-turn. **YJ37** was also prepared, using D-Ser in place of the Gly, since D-Ser (unlike L-Ser) is compatible with the β-turn present in the Gα switch 1-RGS4 contact. Interestingly, **YJ37** displayed similar inhibitory properties as **YJ34** (Table 2.1, and Figure 2.3A). A D-Pro analog, **YJ47**, was ineffective at inhibiting RGS4 activation of Gα$_o$, confirming that the structure of the constrained peptide is important (Table 2.1 and Figure 2.3A). This is not surprising since Gly183 is important for Gα$_i$-RGS4 interactions (Lan, et al. 1998).

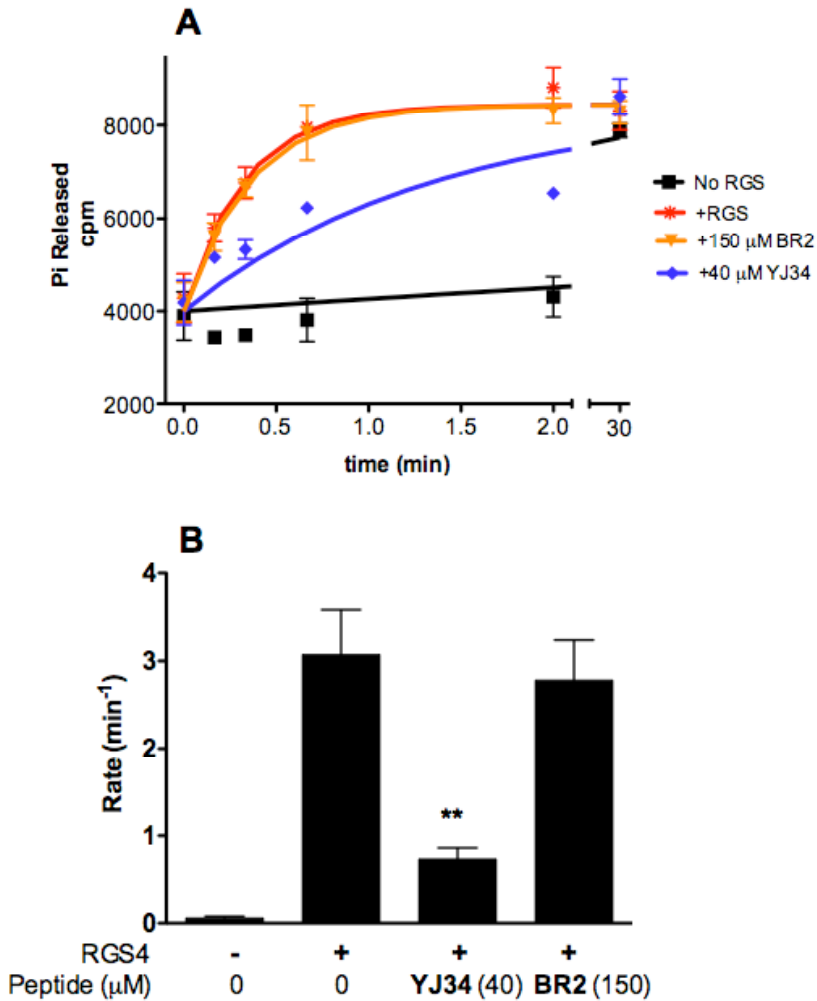Figure 2.2: Gly183 is required for peptide activity at RGS4. A) Single turnover GTPase assays were preformed as described with no RGS, 100 nM RGS4, 100 nM RGS4 with 40 µM **YJ34**, and 100 nM RGS4 with 150 µM **BR2**. This graph is representative of 2 experiments done in duplicate. B) Rates were calculated from the data in *A* as described. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to RGS4 alone.

Structure activity relationship results. In order to better understand which structural components of **YJ34** contribute to its activity, and in hope of identifying more potent inhibitors, several additional modifications to the structure of **YJ34** were examined. The inhibition of RGS4 GAP activity by each peptide at 100 μM concentration was measured using the single turnover GTPase assay and the data are summarized in Table 2.1. By comparison, **YJ34** inhibits RGS4 stimulated GTPase activity by 75 ± 3 percent at 40 μM (p=0.0004 compared to control).

In **YJ34**, incorporation of the N-terminal acetyl and C-terminal carboxamide was chosen to best correspond to the internal 'parent' sequence in Gα_i. In order to examine whether such uncharged termini and the octapeptide framework of **YJ34** are optimal, **BR13**, **YJ41**, **BR7**, **BR1**, **YJ49**, and **YJ42** were prepared. As shown in Table 2.1, analogs with a free N-terminal amine (**BR13**) or a C-terminal carboxylate (**YJ49**) were inactive (Table 2.1 and Figure 2.3B). Truncation of the peptide from either the amino terminal (**YJ41**) or carboxy terminal (**YJ42**) end also abolishes activity (Table 2.1 and Figure 2.3B). N-terminal elongation of the peptide by either the previous 1 or 3 amino acids in the sequence of Gα_{i1} (**BR7** and **BR1**, respectively) also resulted in complete loss of activity (Table 2.1 and Figure 2.3B). These suggest that the peptide length and the absence of charged termini are critical for peptide activity.

The Thr182 of Gα_i makes several contacts with RGS4 including interactions with polar (Glu87, Asp163) and nonpolar (Leu159) side chains (Tesmer, et al. 1997). It is therefore not surprising that changing the Thr of **YJ34** to Ala, lacking the polar hydroxyl group, decreases activity (**YJ46**, 53 ± 10 percent inhibition of RGS activity at 100 μM, Table 2.1 and Figure 2.3C), while the more conservative Ser substitution has intermediate

activity (**YJ45**, 73 ± 3 percent inhibition of RGS4 activity at 100 μM, Table 2.1 and Figure 2.3C). Substitution of Lys for Thr, with the intention of facilitating a salt bridge with Glu87 of RGS4, was unsuccessful, demonstrating very low inhibitory activity (**BR8**, Table 2.1 and Figure 2.3C)

The structure/conformation of the peptide cycle is also important for activity. In agreement with earlier studies (Jin, et al. 2004), increasing the cycle size from the disulfide of **YJ34** to the methylene dithioether bridge of **YJ33** reduces potency. The latter peptide inhibited RGS4 activity by 25 ± 7 percent at 100 μM. Thus the estimated IC_{50} for **YJ33** is approximately 300 μM, significantly less potent than the disulfide-bonded **YJ34** (Figure 2.3 and Table 2.1). Substitution of either disulfide bridge Cys by the more rigid penicillamine (Pen) resulted in complete loss of activity (**BR5**, **BR6**, Table 2.1 and Figure 2.3C). Substitution of the second Cys with D-Cys also resulted in a complete loss of peptide activity (**KSK99A**, Table 2.1 and Figure 2.3C). Linear peptides have no activity (Jin, et al. 2004, data not shown). The Ile184 residue of $G\alpha_i$ appears to form a Van der Waals contact with Tyr84 of RGS4. It was found that other hydrophobic residues at this position (**GU1, GU2**, **YJ36**, and **ES2)** also had some activity (Table 2.1 and Figure 2.3C). In particular, peptides with substitution of Met (**GU1** and **GU2)** inhibit RGS4 activity by 22 ± 9 and 61 ± 11 percent at 100 μM (Table 2.1 and Figure 2.3C), and Phe **(YJ36** and **ES2)** inhibits 38 ± 10 and 52 ± 12 at 100 μM and 30 μM respectively. Interestingly, the ethylene dithioether-bridged **GU2** and **YJ36** are more active then the disulfide-bridged **GU1** and **ES2**. The opposite is true for the Ile peptides (Jin, et al. 2004).
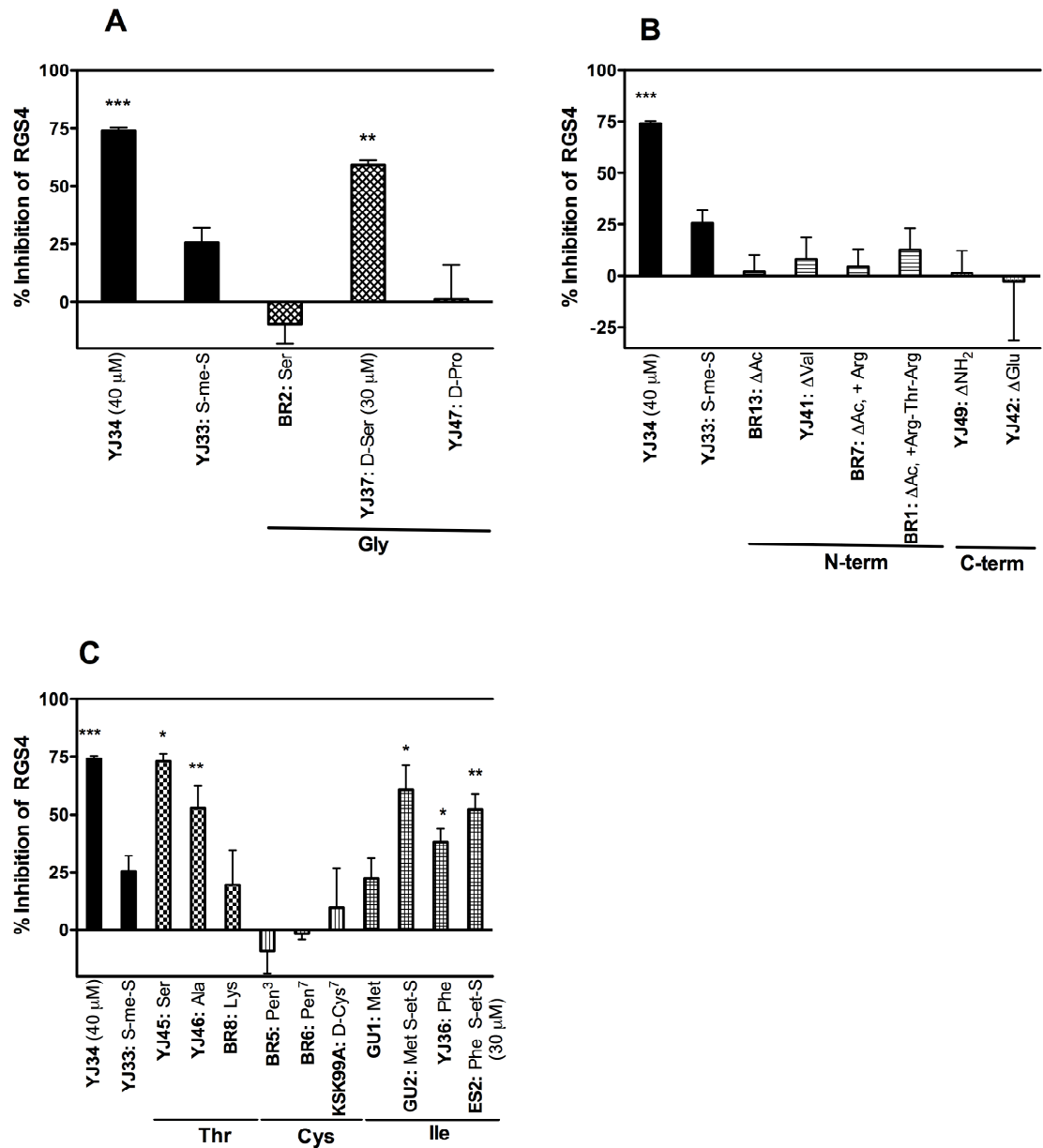
Figure 2.3: Inhibition of RGS4 by analogs of **YJ34**. Peptides (from Table 2.1) were tested in a single turnover GTPase assay at 100 μM (unless otherwise indicated) and percent inhibition of RGS4-stimulated GTP hydrolysis rate is shown. The modifications from **YJ34** at the indicated position are shown after the peptide name. Inhibition of RGS activity by peptides with modifications from **YJ34** at the Gly position (*A*), at the N or C termini (*B*), or at the Thr, Cys or Ile (*C*) positions was determined. These graphs are the average of experiments done in duplicate, triplicate or quadruplicate (mean ± S.E.M., n =2-8). *p<0.05, **p<0.01, ***p<0.001 compared to RGS4 alone.

YJ34 and YJ33 show RGS specificity. The concentration-dependent effect of **YJ34** on the rate of RGS-stimulated GTP hydrolysis was also measured for RGS7 and RGS8. The IC_{50} of **YJ34** on RGS4, RGS7 and RGS8 is 9 μM, 43 μM and 11 μM respectively (Figure 2.4A). RGS4 and RGS8 are approximately 4-fold more sensitive to **YJ34** than RGS7. It has previously been demonstrated that longer bridges within the same sequence as **YJ34** behave as less potent peptides inhibitors of RGS4 (Jin, et al. 2004). The methylene dithioether bridged peptide, **YJ33**, is the second most potent peptide with this sequence and appears to be selective for RGS4 over RGS8 but has no effect on RGS7 at 100 μM (Figure 2.4B). Interestingly, the ability of an RGS to be inhibited by **YJ34** correlates with that RGS protein's catalytic efficiency for $G\alpha_{i1}$ and $G\alpha_o$ (RGS4=RGS8>RGS7) (Lan, et al. 2000).
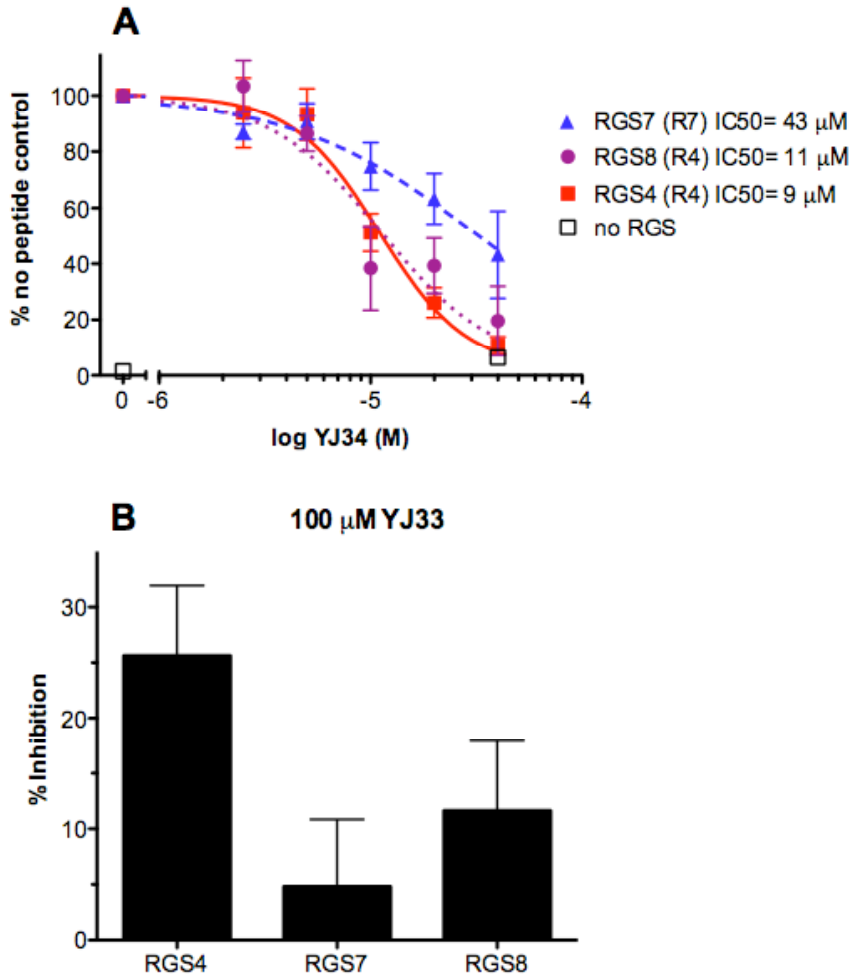
Figure 2.4: **YJ34** and **YJ33** have greater activity for RGS4 and RGS8 than RGS7. *A,* Rate constants were calculated as described from Fig. 1, for no RGS, RGS4, RGS7, and RGS8 with various concentrations of **YJ34**. These graphs are the average of three (RGS4, no RGS at 40 μM **YJ34**), four (RGS7 and RGS8) or five (no RGS, 0 μM **YJ34**) experiments done in duplicate (mean ± S.E.M). *p<0.05, **p<0.01, ***p<0.001 compared to RGS4. *B,* Percent inhibition of the rate of RGS4, RGS7 and RGS8 stimulated GTP hydrolysis by 100 μM **YJ33** was measured. This is the average of two to eight experiments done in triplicate (mean ± S.E.M.) *p<0.05, **p<0.01, ***p<0.001 compared to RGS4.

Peptide 2 inhibits RGS regulation of GIRK currents in cardiac myocytes. To determine whether the peptide inhibitors act in a physiological system and to demonstrate a role of RGS proteins in ion channel regulation, the well-characterized muscarinic GIRK currents in atrial myocytes and a whole-cell patch-clamp pipette to deliver the peptides to the intracellular space was used. **YJ34** had no effect on GIRK currents (not shown), presumably due to residual reducing equivalents in the cell that reduced the essential disulfide bond. The methylene bridged peptide **YJ33**, however, did reduce the degree of current relaxation (Figure 2.5A and Ca) and slowed the rate of onset and offset of the muscarinic response (Figure 2.5B, Cb and Cd). In some experiments (Figure 2.5Ab and Bb) the effect was not seen. Overall, however, there was a statistically significant effect of peptide **YJ33** on all measures of RGS function (Figure 2.5C).
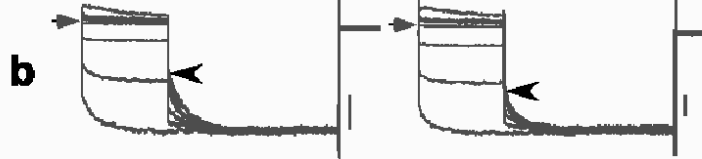
Figure 2.5: Inhibition of RGS effects on GIRK currents in atrial myocytes by **YJ33**. *A*, Representative tracings of currents evoked by 0.1 (left) or 1 (right) µM acetylcholine in the presence (a and b) or absence (c) of 150 µM **YJ33**, 1% DMSO. Sections a and b show the variation of responses to **YJ33**. Currents at -100 mV were recorded after prepulses at -100 to + 40 mV in steps of 20 mV. Baseline currents at 0 µM acetylcholine were subtracted out. Arrows indicate the end point of the instantaneous and the start point of the relaxing components of the currents. Vertical bars represent 500 pA, and horizontal bars indicate 1 sec. *B*, Representative tracings of deactivation (left) and activation (right) of GIRK currents in the presence (a and b) and absence (c) of 150 µM **YJ33**, 1% DMSO. Sections a and b show the variation of responses to peptide **YJ33**. Arrows indicate the endpoint of deactivation or activation and the horizontal bar indicates 10 s. *C*, I_{Ins}/I_{max}, (a), $T_{1/2\ (deactivation)}$ (b) and $T_{1/2\ (activation)}$ (c) were calculated in the presence (left) and absence (right) of 150 µM **YJ33** from A and B and are shown for individual cells. The mean is indicated with open circles (n=5-10 cells).

A

YJ33 150 μM (+1% (v/v) DMSO)

effect (++) ACh 0.1 μM   ACh 1 μM

a

500 pA

1 sec

effect (+/-)

b

1% (v/v) DMSO

c

B

ACh 0.1 μM

a

10 sec

b

c
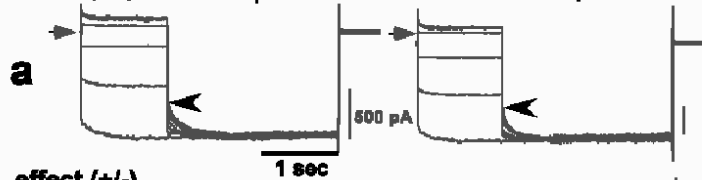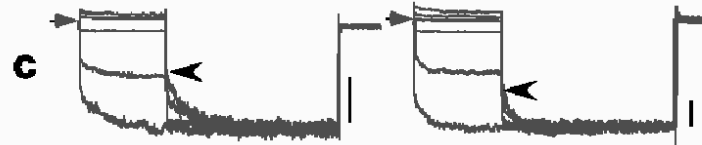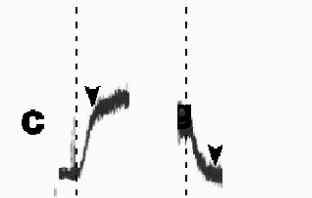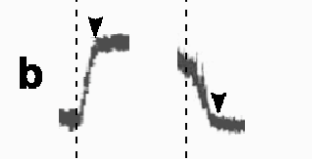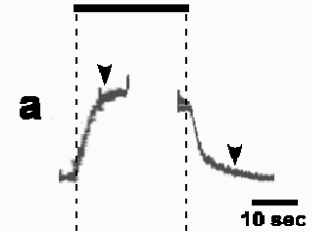
C

a I_{Ins} / I_{max}
at +40 mV pre-pulse

p < 0.01

1

0.8

0.6

0.4

0.2

0

blocker   control

b $T_{1/2}$ (deactivation)

p < 0.01

6

5

4

3

2

1

0

[sec]   blocker   control

c $T_{1/2}$ (activation)

p < 0.05

3

2.5

2

1.5

1

0.5

0

[sec]   blocker   control

74
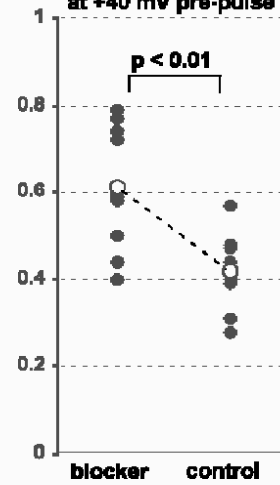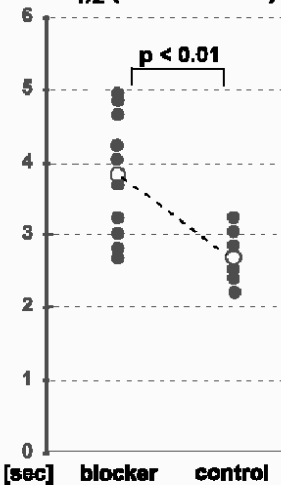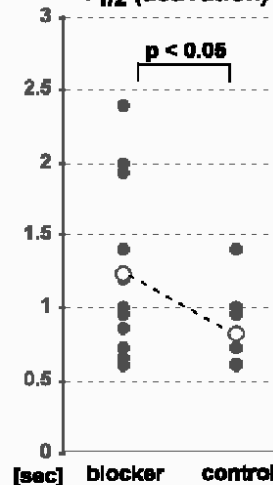
Discussion:

Mechanism of action. YJ34 was designed based on the crystal structure of the RGS4-Gα$_i$ complex in the presence of GDP•AlF$_4^-$. The peptide attempts to mimic the switch 1 region of Gα$_i$, a constrained loop that makes considerable contacts with RGS4. It was designed to bind to RGS4 and competitively inhibit Gα/RGS interactions and thus inhibit RGS4 activity. It was previously shown that **YJ34** inhibits RGS4 activity in a steady state GTPase assay (Jin, et al. 2004) using cell membranes. In the present study, it is demonstrated that **YJ34** also inhibits RGS4 activity in a single turnover GTPase assay using purified proteins. The results of these assays suggest that **YJ34** is interacting directly with RGS4 protein rather than with other proteins or lipids found in the membrane preparation, although data in Figure 2.1 is not inconsistent with **YJ34** being a Gα$_o$ inhibitor. Since **YJ34** had little to no effect on the GTP hydrolysis by Gα (Figure 2.4A, Jin, et al. 2004), is concluded that **YJ34** is most likely acting directly on the RGS protein. This is supported by data that will be discussed in Chapter 3 showing that YJ34 on beads does bind RGS4.

To determine whether the mode of interaction of **YJ34** with RGS truly mimics the Gα subunit interaction, **BR2** was designed to mimic the switch 1 region of the RGS-insensitive G183S mutant of Gα$_i$ (Table 2.1). The Gly to Ser mutation in the Gα subunit prevents RGS4 binding (Lan, et al. 1998). It is therefore hypothesized that if **YJ34** bound to RGS4 the same way as switch 1 of Gα$_i$, then a Gly to Ser modification would prevent peptide inhibition of RGS4 GAP activity. At almost 4 times the maximal concentration of **YJ34** (150 μM), **BR2** does not inhibit RGS4 activity (Figure 2.2), indicating that the Gly

in both Gα and in **YJ34** is essential for binding to RGS4. These data further support the notion that **YJ34** mimics the switch 1 region of Gα to block RGS4 activity on Gα$_o$.

Also in support of the proposed mode of action, **YJ34** exhibits significant RGS subtype selectivity. Because **YJ34** has little direct effect on the catalytic activity of Gα$_o$ alone (Figure 2.4 and Jin, et al. 2004), and because it displays selectivity for certain RGS proteins (Figure 2.4A), it is believed that the peptide inhibitor is directly binding to and inhibiting the RGS protein, rather than to the Gα protein. Although it is less potent, **YJ33** appears to have similar RGS selectivity and likely shares a similar mechanism of action (Figure 2.4B).

In the present study, it was found that **YJ34** had an IC$_{50}$ of 9 μM against RGS4 (Figure 2.4A) while in the previous report, in a membrane steady state GTPase assay, it was less potent with an IC$_{50}$ of 26 ± 2 μM (Jin, et al. 2004). **YJ34** inhibited RGS4 81% at 40 μM in a capillary electrophoresis assay with Gα$_o$ (Jameson, et al. 2005), which is in good agreement with the $75 \pm 3\%$ inhibition seen here (Table 2.1). For RGS8, 40 μM **YJ34** inhibited 38 % and 74 ± 23 % in the capillary electrophoresis and in the single turnover GTPase assays, respectively (Jameson, et al. 2005 and Figure 2.4A). Both assays using purified proteins give similar results. However there are several differences between these systems and the membrane-based steady state GTPase assay that may contribute to the disparate activities observed. These include the presence of other proteins (including endogenous RGS proteins), various G proteins, the presence of membranes and different temperatures (30 ^{o}C in the steady state assay and ice cold for the single turnover assay). In addition, the steady state and the single turnover GTPase assays measure different aspects of the GTP cycle and may thus complicate a direct

comparison. The difference is even greater for **YJ33** (Table 2.1). Here it is shown that RGS4 is inhibited 24 ± 5 percent by 100 μM **YJ33** for an IC_{50} of about 300 μM compared to an IC_{50} of 79 ± 6 μM in the steady state GTPase assay (Jin, et al. 2004).

Structure-Activity Relationships of Peptide Inhibitors. Although Val179 and Glu186 of Gα_i do not appear to make direct contacts with RGS4 (Tesmer, et al. 1997), these residues, with their N- and C-terminal charges blocked, appear to be necessary for peptide activity (Table 2.1). Perhaps the absence of constraints from the rest of Gα allows these residues and modifiers to make contacts with RGS4 that the switch 1 of Gα_i could not normally make. It is likely that the charges on the free N- or C-termini interfere with binding to RGS4 via adverse electrostatic interactions and the terminal acetyl and amide groups of **YJ34** prevent these negative interactions.

The Thr182 of Gα_i makes contacts with several residues in RGS4 including Glu87 and Asn88, (Tesmer, et al. 1997). It is therefore not surprising that changing the Thr of **YJ34** to Ala decreases its activity as an RGS4 inhibitor (**YJ46**, Table 2.1 and Figure 2.3C), while a more conservative Ser substitution retains considerable activity (**YJ45**, Table 2.1 and Figure 2.3C). Glu87 and Asn88 of RGS4 form hydrogen bonds with the hydroxyl group on Thr182 of Gα_i (Tesmer, et al. 1997). It is expected that similar interactions are made with the hydroxyl group of Ser in **YJ45**. It was proposed that a Lys in this position might interact with Glu87 on RGS4 and enhance its inhibitory effect, however the Lys substitution for Thr abolishes activity (**BR8**, Table 2.1 and Figure 2.3C). It is interesting to note that Gα_{12} has a Lys at this position in switch 1, and is not a substrate for RGS4 GAP activity.

Ile184 of Gα$_i$ may form a Van der Waals contact with Tyr84 of RGS4. It is therefore not surprising that a peptide with a Met at this position still retains activity as an RGS inhibitor (Table 2.1 and Figure 2.3C). It is interesting that **GU2,** with an ethylene dithioether bridge, is more active than **GU1** with a disulfide bridge. This is also the case with Phe substitutions for Ile (**YJ26** and **ES2**, Table 2.1 and Figure 2.3C). The opposite is true for the peptides with the Ile at this position (Jin, et al. 2004). Perhaps the alternate conformation of the ethylene bridge positions the Met to make better contact with the RGS4 protein. However, neither peptide is as potent as **YJ34**.

Peptide RGS inhibitor effects on cardiac GIRK current. This inhibition of RGS-dependent phenomena in atrial myocytes by peptide **YJ33** is the first demonstration of the actions of an RGS inhibitor in a physiological system. The magnitude of the effect is only modest but is consistent with the IC$_{50}$ of this peptide at RGS4. The 25% inhibition of *in vitro* single turnover GAP activity seen at 100 μM **YJ33** (Figure 2.4B) and the 25-35% inhibition of k$_{on}$, k$_{off}$ and I$_{ins}$/I$_{max}$ seen in the patch clamp studies (Figure 2.5C) with 150 μM **YJ33** are both consistent with a K$_i$ of about 300 μM. The RGS proteins that are known to be expressed in rat atrial myocytes are RGS2, RGS3, RGS4, RGS6, RGS10, RGS17/RGSZ2, and RGS19/GAIP (Doupnik, et al. 2001). Since GIRK currents are primarily regulated by Gα$_i$, (Sowell, et al. 1997) and RGS2, RGS6, and RGS17/RGSZ2, act primarily on Gα$_q$, Gα$_o$, and Gα$_z$, respectively, RGS3, RGS4, RGS10, and RGS19/GAIP are the best candidates for controlling the kinetics of GIRK currents. Peptide **YJ33** inhibits RGS4 and the sequence similarity of the RGS domain of these other RGS proteins is quite high (43-61%) and greater than that for 7 (35%); it is likely

that peptide **YJ33** may inhibit multiple RGS proteins that may be involved in $G\alpha_i$ and GIRK current regulation.

Although the Mosberg and Neubig labs are the first to design and synthesize peptide inhibitors of RGS proteins, peptide inhibitors have been made against numerous biological targets including ribonucleotide reductase, (Cooperman, et al. 2005) protein kinases (Hines, et al. 2005) and bacterial membranes as antibacterial peptides (Rezansoff, et al. 2005). Here the modest specificity of the constrained peptide RGS inhibitors **YJ34** and **YJ33** (RGS4 ≥ RGS8 > RGS7) are shown, evidence for the predicted mechanism of the peptide is provided, and activity in a native cell system are shown. Additional work will clearly be needed to enhance the potency of the peptides, more completely define the RGS specificity, and to develop RGS inhibitors that are cell-permeable for more general use beyond patch clamp approaches.

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Chapter 3

Novel Peptide Ligands of RGS4 from a Focused One-Bead, One-Compound Library

Abstract:

Chapter 2 discusses the first RGS inhibitor, **YJ34** (*Ac*-Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-Glu-*NH₂*, S-S) (Jin, et al. 2004). This peptide was designed to mimic the switch 1 region of the Gα subunit and was modeled from the RGS4-Gα$_{i1}$ crystal structure (Tesmer, et al. 1997). It has a sequence similar to the switch 1 region with 2 amino acid substitutions (Thr181 to Cys and Val185 to Cys) to incorporate a disulfide bridge, which constrains the peptide in the correct conformation (Jin, et al. 2004, Roof, et al. 2006, Chapter 2). This peptide has an IC$_{50}$ of 26 μM in a membrane based steady state GTPase assay (Jin, et al. 2004), a 9 μM IC$_{50}$ in a purified protein single turnover GTPase assay (Roof, et al. 2006 Chapter 2), inhibits RGS4 81% at 40 μM in a capillary electrophoresis assay (Jameson, et al. 2005), and an analog inhibits RGS modulation of GIRK current kinetics in atrial myocytes (Roof, et al. 2006, Chapter 2). It was found that **BR2** (*Ac*-Val-Lys-c[Cys-Thr-Ser-Ile-Cys]-Glu-*NH₂*, S-S), a peptide that mimics the RGS-insensitive G184S mutation in the Gα protein is inactive, suggesting that the peptide binds to RGS4 as designed (i.e. in the same way the Gα switch 1 binds). SAR studies on this peptide showed that the following features were necessary for peptide activity: The N-terminal

acetyl group, C-terminal amide, the Gly at position 5, and Cys at positions 3 and 7 with a disulfide bridge (Roof, et al. 2006, Chapter 2).

YJ34 is a useful tool for studying RGS function, and in this Chapter, efforts to improve its potency with the use of a focused OBOC peptide library to screen for RGS4 inhibitors is reported. The library was restricted to peptides containing the structural elements listed above that have been shown to be required for the function of **YJ34**. The other 5 amino acids were randomized among the 19 natural amino acids (except Cys) to afford 2.5 million possible peptide sequences. The library was synthesized and screened for RGS4 binding using beads with only one peptide sequence each. However, approximately half of the peptides on each bead had an acetyl group, because it was important for **YJ34** function, and half had a free N-terminus, so that the hits could be sequenced by Edman degradation. From this screen, seven peptide sequences that bound to RGS4 were identified. Two analogs of peptide **2**, **2ad** (*Ac*-Gly-Thr-c[Cys-Phe-Gly-Thr-Cys]-Trp-*NH₂*, S-S) and **2nd** (Gly-Thr-c[Cys-Phe-Gly-Thr-Cys]-Trp-*NH₂*, S-S) are novel inhibitors of RGS4.

Materials and Methods:

Materials: Fmoc-protected amino acids and Rink amide resin were purchased from Advanced ChemTech. Fmoc protected and acetylated amino acid purity were verified by HPLC. Tentagel Resin was purchased from ChemImpex or Rapp-Polymer, and Alexa Fluor label from Invitrogen. Peptide synthesis grade chemicals were purchased from Applied Biosystems. $\gamma[^{32}P]$GTP (10 mCi/ml) was purchased from Perkin Elmer and

diluted with unlabeled GTP to the desired level of radioactivity. Amylose resin was purchased from New England Biolabs and the Ni-NTA resin from Qiagen.

Protein Expression, Purification and Labeling: His$_6$-Gα_o (rat), and RGS4Δ18N (rat), were expressed and purified according to previous protocols (Lan, et al. 1998, Lan, et al. 2000, Lee, et al. 1994, Roman, et al. 2007). Mbp-His$_6$-RGS4Δ51N (human), Mbp-His$_6$-RGS16 (human) Mbp-His$_6$-RGS19Δ11C (human) Mbp-His$_6$-RGS7 RGS domain (human, nucleotides 915-1359), and the mutant Mbp-His$_6$-RGS4Δ51N lacking all seven cysteines (called "-7C") were in Gateway pMAL vectors and were expressed using a similar protocol as the RGS4Δ18N construct and purified over an amylose column followed by a Ni-NTA column according to the manufacturer's protocol. In some cases this was followed by a size exclusion column as necessary. The mutagenesis was done using the "QuickChange Multi Site-Directed Mutagenesis Kit" from Stratagene according to the manufacturer's protocol. David Roman made some of the Gα_o and RGS4Δ51N used. He produced the -7C mutant, RGS16 and RGS7. Levi Blazer made the RGS19. Labeling of RGS4 with succinimide ester fluorophores (Alexa Fluor 568 and Alexa Fluor 532) was done according to the manufacturer's protocol with approximately 2- to 3-fold excess fluorophores and was done by either David Roman or myself.

OBOC Peptide Library Synthesis: The protocol was based on previous reports (Cabilly. 1998, Lam, et al. 1991) using a manual "mix and split" synthesis. TentaGel amide resin (10 g of 130 μm sized beads with a substitution level of 300 pmole/bead) was swelled in NMP (N-methylpyrrolidone) and divided by volume into 19, 8 ml

polypropylene filter columns. To each column a different Fmoc-protected amino acid dissolved in NMP was added; all natural amino acids were used at the random positions except for cys. This was followed by a solution with a 3-fold excess HBTU/HOBt plus DIEA in NMP and the mixture was shaken for 1 hour. Then the beads were rinsed thoroughly in NMP. Unreacted amines on the resin were acetylated with a 20-fold excess of acetic anhydride with DIEA and HOBt in NMP. After a negative bromophenol blue test, the beads were pooled and treated with 50% piperidine in NMP. The splitting, coupling, pooling, and deprotection (but not the acetylation) steps were repeated for the randomized positions. For the non-random positions, the resin was not split and Fmoc-Cys(trt)-OH (positions 3 or 7) or Fmoc-Gly-OH (position 5) was coupled to the whole batch. In the final coupling step, a 1:1 mixture of acetylated and Fmoc-protected amino acids was used. To remove the side chain protecting groups without cleaving the peptide from the resin (based on a control peptide and Cabilly. 1998), all the beads were pooled and treated with 50 ml ice-cold 88:5:5:2 TFA:phenol:water:TIPS and shaken for 3 hours. (Treatment of test peptides with deprotection conditions resulted in no detectable cleaved peptide by HPLC.) After thorough washing (including a wash in 10% DIEA), the resin was added to 7.8 L oxidation solution (20% DMSO, 5% Acetic acid, in water pH 6.0 purged of air with N_2) and shaken for 48 hours.

Library Screening: Beads were washed and plated in a single layer (on average about 1,500 beads per well) in 96 well plates in buffer (1% BSA in 20 mM Hepes pH 8.0) and incubated with 25 nM RGS4Δ18N-Alexa Fluor 568. Wells containing **YJ34** on beads were included in every scan. Fluorescence was imaged on a Typhoon 9200 Gel Imager

belonging to Gus Rosania, using an excitation of 532 nm and an emission of 610 nm and quantified with the accompanying ImageQuant software. To do this, a 12x8 grid was placed over the image such that each grid block contained only one well. Grid blocks containing an object with intensity more than 5-10 fold over the most intense object in the wells containing **YJ34** were collected and pooled. The image was visually inspected and any objects that were clearly not beads, such as dust or other debris, were excluded. Pooled beads were washed, diluted and re-screened against 10 nM RGS4Δ18N-Alexa Fluor 568. The process was repeated until a single bead per well was obtained. The top 20 most fluorescent beads were then isolated with a needle under a dissecting microscope and sent for sequencing by Edman degradation at the Biomedical Research Core Facilities at the University of Michigan.

Synthesis of individual Peptides: Soluble peptides (for GAP assays) were synthesized by myself or Anjanette Turbiak on cleavable Rink resin and cyclized as described previously (Jin, et al. 2004, Roof, et al. 2006, Chapter 2). Peptide purity (at least 95%) and solubility were verified by HPLC and correct mass was verified by MS analysis (Roof, et al. 2006, Chapter 2). **YJ34** and **BR2** bead bound controls were synthesized by myself on 130 μm Tentagel resin using the same protocol as the library except no "mix and split" steps were incorporated. Peptides on beads (for the FACScan assay) were synthesized by Katarzyna Sobczyk-Kojiro using a LabMate apparatus (Advanced Chem Tech) and cyclized manually using the similar chemistries as for the library except no "mix and split" randomization steps were incorporated, and 20 μm

Tentagel resin was used. The deprotection mixture was 83:5:5:5:2 TFA:thioanisole:phenol:TIPS and an Ellman test was performed to ensure complete oxidation.

Binding of test peptides: Unreacted 130 μm TentaGel amide resin or resin containing **YJ34** or **BR2** was put into wells of a 96 well plate with 5 mg/well in 0.5 ml buffer (1% BSA in 20 mM Hepes pH 8.0) for 1 hour. The supernatant was removed and various concentrations of Alexa Fluor 568 labeled RGS4Δ18N were added in 200 μl followed by incubation for 15 min. After a wash with 500 μl buffer, fluorescence was measured in black Costar 96 well plates in a Victor^2 fluorescence plate reader with an excitation filter at 560 and an emissions filter at 595. Samples were measured in duplicate.

FACScan Flow Cytometry: Peptide beads (about 5×10^3 per sample for a maximum of 5 nmoles peptide) were washed then incubated with 25 nM RGS4Δ51N-Alexa Fluor 532 in 300 μl for at least 15 min at room temperature under foil. The Becton Dickinson FACScan, kindly shared by Kathleen Collens, was gated and the laser intensity set in CellQuest such that control beads (acetylated TentaGel resin) were gated and had low but measurable fluorescence. No compensation was set. Fluorescence of RGS4Δ51N-Alexa Fluor 532 bound to acetylated beads was about ~60 % of YJ34 beads and was considered background and subtracted from all samples. Samples were read in duplicate.

RGS-Stimulated GTPase: Single turnover GTP hydrolysis measurements with and without RGS were performed as described previously (Roof, et al. 2006, Chapter 2).

Modeling: Peptides were modeled by Ira Pogozheva using Quanta by modifying the residues from the $G\alpha_{i1}$ switch 1 in the RGS4-$G\alpha_{i1}$ (PDB: 1agr) crystal structure (Tesmer, et al. 1997). Upon residue substitution and formation of the disulfide bond, energy minimization of each peptide with all hydrogen atoms added was performed using the Quanta/CHARMm simulation package with dielectric constant (ε)=10 and the Adopted-Basis Newton Raphson method (50 steps). To model the peptide-RGS complex, each minimized peptide with the hydrogens removed was substituted for the corresponding $G\alpha_i$ fragment from the $G\alpha_i$-RGS4 complex. Images were prepared using PyMol for OS X (http://www.pymol.org).

Statistical Analysis: Data are expressed as mean $\pm$ S.E.M (or $\pm$ S.D for n=2) and analyzed by either a t-test (GAP data) or a one-way ANOVA (Victor based test binding and FACScan data). A Bonferroni post-test was done on the FACScan data. Significance is indicated as follows: $*p<0.05$, $**p<0.01$, $***p<0.001$.

Results:

OBOC Library Design and Screening: It was found in Chapter 2 that some features were necessary for activity of **YJ34**: The N-terminal acetyl group, the C-terminal amide, the Gly at position 5, and Cys at positions 3 and 7 with their side chains linked via a disulfide bridge. However, some of the other 5 positions can be varied (Roof, et al.

2006, Chapter 2). As seen in Table 3.1, the features necessary for **YJ34** function were constrained in the library, while the other positions were randomized. All natural amino acids except cysteine were used in the other 5 positions to give 2.5 million possible sequences. Using 10 g of 130 μm TentaGel beads resulted in approximately 7.9 million beads. Thus each sequence would have been on 3 beads in the library on average. In an OBOC library, each bead has only one amino acid sequence. However, for this library, approximately half of the peptides on each bead were acetylated, since that was necessary for the function of **YJ34**, and half had a free N-terminus, to allow for sequencing by Edman degradation. The beads had a substitution level of approximately 300 pmoles, resulting in about 150 pmoles of free-amine-containing peptides for sequencing. This was found to be sufficient on test peptides.

Another control included ensuring that **YJ34** on beads bound to RGS4 (Figure 3.1). Although TentaGel resin is known not to be cleavable with TFA treatment (Cabilly. 1998), it was verified that deprotection conditions did not remove test peptides from resin (as discussed in the methods). Tentagel resin was chosen because it has been used extensively and has good mechanical stability and has a uniform size. Unfortunately, it has green autofluorescence (Olivos, et al. 2003), so a red dye (Alexa Fluor 568) was chosen for RGS4 labeling for the bead screen. Other investigators have found non-specific binding to be problematic with this type of resin (Anna Mapp, personal communication). Indeed, high non-specific binding to empty resin was found, but this was not observed with resin containing **BR2**, which is an inactive peptide that mimics the RGS-insensitive mutation in the Gα protein (Roof, et al. 2006, Chapter 2) (Figure 3.1).

To screen the library, the beads were treated with Alexa Fluor 568 labeled RGS4Δ18N and imaged in a Typhoon Gel Imager as shown in Figure 3.2. Twenty hits (numbered 1-20) were isolated and sequenced by Edman degradation (Appendix A). Unfortunately, 6 of the hits (numbers **7**, **8**, **12**, **13**, **15** and **20**) yielded only partial sequences due to inconclusive amino acid assignments by Edman degradation, leaving 14 fully sequenced hits (hit numbers **1**, **2**, **3**, **4**, **5**, **6**, **9**, **10**, **11**, **14**, **16**, **17**, **18**, and **19**) for further evaluation (Appendix A, Table 3.2).

Name	Sequence	Bridge
Gα$_i$ switch 1	…V-K-T-T-G-I-V-E…	none
YJ34	*Ac*-V-K-[**C**-T-**G**-I-**C**]-E-*NH$_2$*	(S-S)
Library	*Ac*-X^1-X^2-[**C**-X^3-**G**-X^4-**C**]-X^5-*NH*-- X^1-X^2-[**C**-X^3-**G**-X^4-**C**]-X^5-*NH*--	(S-S)

Table 3.1: OBOC library design. The key features of **YJ34** were constrained in the library. The library had the Gly at position 5, the Cys at positions 3 and 7 with a disulfide bonds, and a C-terminal amide. The other 5 amino acid positions were randomized such that each bead had only one sequence. Half of the peptides on each bead had an acetyl group and the other half had a free N-terminus. There were approximately 300 pmoles peptide on each bead, 2.5 million sequences and 7.9 million beads in the library.
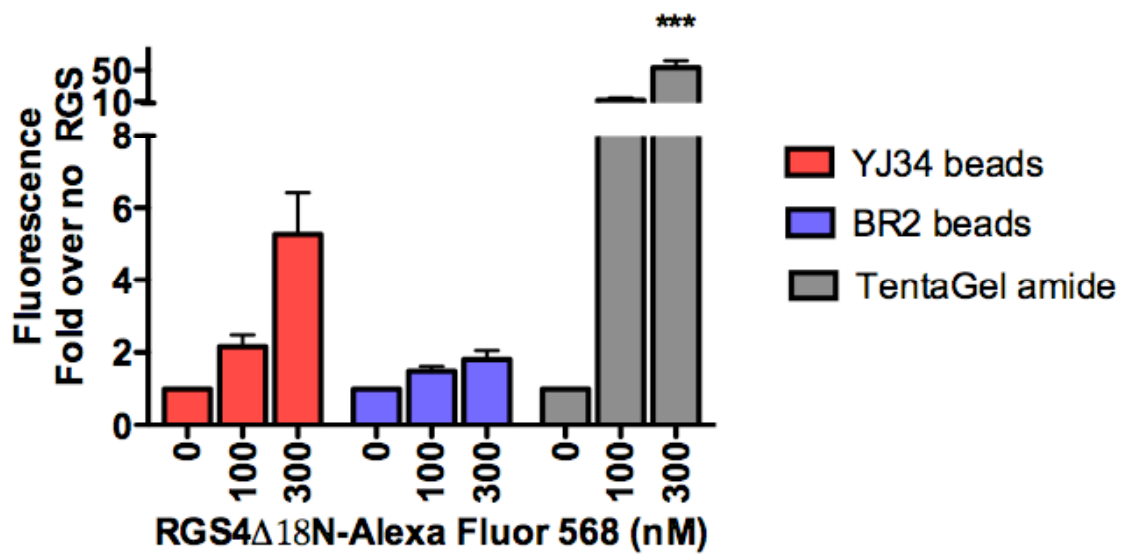
Figure 3.1: RGS4Δ18N-Alexa Fluor 568 binds **YJ34** on beads but not **BR2** on beads. Binding of RGS4 to beads was measured as described. The experiment was done in duplicate. (mean ± S.D., n =2). *p<0.05, **p<0.01, ***p<0.001 compared to no RGS4Δ18N-Alexa Fluor 568.
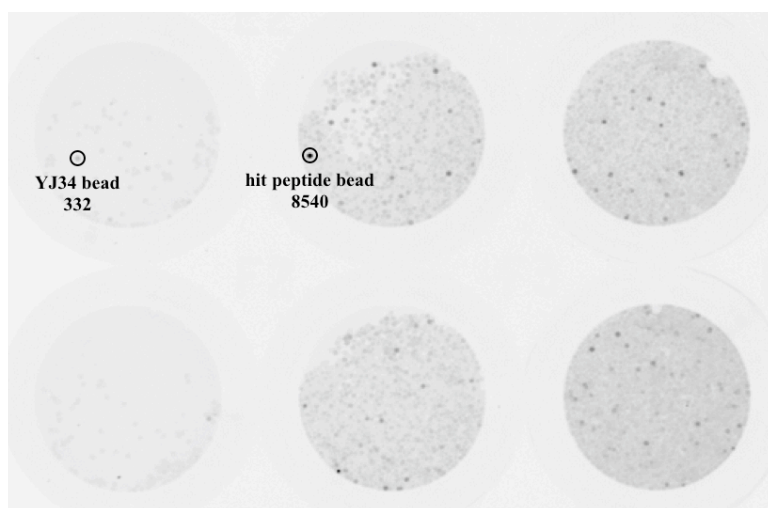
Figure 3.2: Screening of the library. The library was screened as described. The left two wells contain **YJ34** on beads while the right four wells contain library beads. Fluorescence was quantified and represented in arbitrary units by the number below the circled bead.

Hit verification: Each of the 14 sequenced hit peptides was resynthesized on 20 μm TentaGel beads. Since both were present in the library, free N-terminal, disufide bridged (nd) and acetylated, disulfide bridged (ad) versions of each sequence were made. Interaction with Alexa Fluor 532 labeled RGS4Δ51N was detected in a FACScan Flow Cytometer (Figure 3.3). The RGS4Δ51N protein lacking the amphipathic helix in the N-terminus was used to ensure that the verified hits bound to the RGS domain of RGS4. Using 2.5-fold increased binding to RGS4Δ51N compared to **YJ34** on beads as a cut-off, it was found that some of the initial hits were false positives, but hit numbers **1**, **2**, **3**, **4**, **9**, **16**, and **19** (Figure 3.3) were verified and were chosen for further evaluation. The hits identified were structurally different from each other and our lead compound. Both the free N-terminal and the acetylated versions of the verified hits bound RGS4Δ51N. This is in contrast to **YJ34**, where only the acetylated version has activity in the GAP assay (Roof, et al. 2006, Chapter 2).

YJ34	*Ac*-VKc[CTGIC]E-*NH₂*, S-S
hit 1	YNc[CQGEC]E-*NH₂*, S-S
hit 2	GTc[CFGTC]W-*NH₂*, S-S
hit 3	LVc[CKGYC]Q-*NH₂*, S-S
hit 4	KVc[CMGGC]T-*NH₂*, S-S
hit 9	YWc[CKGLC]K-*NH₂*, S-S
hit 16	KLc[CHGYC]H-*NH₂*, S-S
hit 19	KHc[CYGFC]K-*NH₂*, S-S

Table 3.2: The verified hits. Peptides from Figure 3.3 that bind RGS4Δ51N-Alexa Fluor 532 at least 2.5-fold more than YJ34 are shown. Note that both acetylated, disulfide bridged (ad) and free N-terminal, disulfide bridged (nd) peptides were chosen for further evaluation.

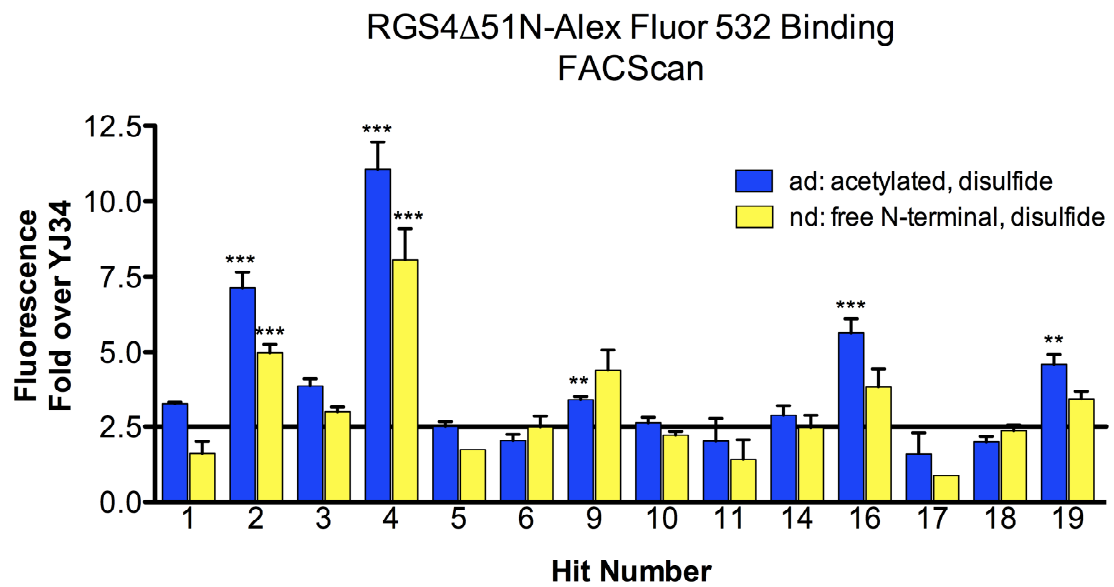RGS4Δ51N-Alex Fluor 532 Binding
FACScan

Figure 3.3: Hit verification. The 14 hits for which complete sequences were obtained were resynthesized on beads and tested for binding to RGS4Δ15N-Alexa Fluor 532 as described (mean ± S.E.M.(or S.D when n=2), n≥2). *p<0.05, **p<0.01, ***p<0.001 compared to YJ34 on beads.

Peptide activity in GAP assays: Peptides were tested in a single turnover GTPase assay at 50 μM except where limited by solubility. Effects on the rate of RGS stimulated $G\alpha_o$ GTPase activity was measured in a single turnover assay with purified proteins and peptides in solution (Figure 3.4). Hit **16** was not evaluated because of signs of aggregation. Only peptides **2nd** (the free N-terminal, disulfide bridged hit **2**) and **2a**d (the acetylated, disulfide bridged peptide **2**) had statistically significant inhibition of RGS activity (48 ± 7 percent inhibition at 50 μM and 30 ± 12 percent inhibition at 25 μM respectively). Based on these values, both peptides have an estimated IC_{50} of about 50 μM. Other peptides that had some activity included **1nd** (33 ± 15 percent inhibition at 50 μM) and **4n**d (33 ± 16 percent inhibition at 50 μM). The activity of hit **2** was investigated further (Figure 3.5).

Linear as well as disulfide bridged versions of peptide **2** (**2nl**: free N-terminal linear and **2al**: acetylated linear) were tested in the GAP assay since cyclization yields may have been less than 100% in the synthesis of the library leaving some linear peptides present on each bead, which may have contributed to the observed fluorescence of the bead (Figure 3.5A). Consistent with previous results for **YJ34**, the linear peptides were inactive. RGS selectivity was also investigated. Interestingly, peptides **2ad** and **2nd** were inactive against RGS7, RGS16 and RGS19 (Figure 3.5B). RGS16, like RGS4, is in the R4 family, while RGS7 and RGS19 are in different families (R7 and RZ respectively) and have lower homology with RGS4. It is worth noting that different concentrations of RGS proteins were needed to obtain comparable hydrolysis rates in the absence of peptide for Figure 3.5B. It is not thought that the higher concentrations of other RGSs

compared to RGS4 explain the lack of activity, however. It is expected that higher concentrations are needed due to either presence of inactive protein (as concentration measurements are determined by Bradford assays) or lower intrinsic activity of the RGSs on $G\alpha_o$. In the former case, inactive protein would not affect peptide activity. In the later case, it would be expected that, selectivity aside, ability of the peptide to inhibit would correlate with RGS activity, which has been equalized by the way the experiment was set up (ie. by having different RGS concentrations). Thus it is thought that peptide **2** has RGS selectivity.

Recently, CCG-4986, a small molecule inhibitor of RGS4 was identified and found to interact with RGS4 through cysteine modification on RGS4 (Kimple, et al. 2007, Roman, et al. 2007, Roman, unpublished observations). For this reason, the activity of our original peptide, **YJ34** as well as hit **2** was evaluated with an RGS4Δ51N mutant lacking all 7 cysteines (-7Cys) (Figure 3.5C). The -7C mutant was inhibited by **2ad**, **2nd** and **YJ34**. There was no statistically significant difference between the activity on wild type and the -7C RGS4Δ51N for **2ad**, **2nd** or **YJ34** (59 ± 4, 27 ± 7, 60 ± 11 percent inhibition for **2ad**, **2nd** and **YJ34** respectively). This indicates that hit **2** is not a cysteine modifier.
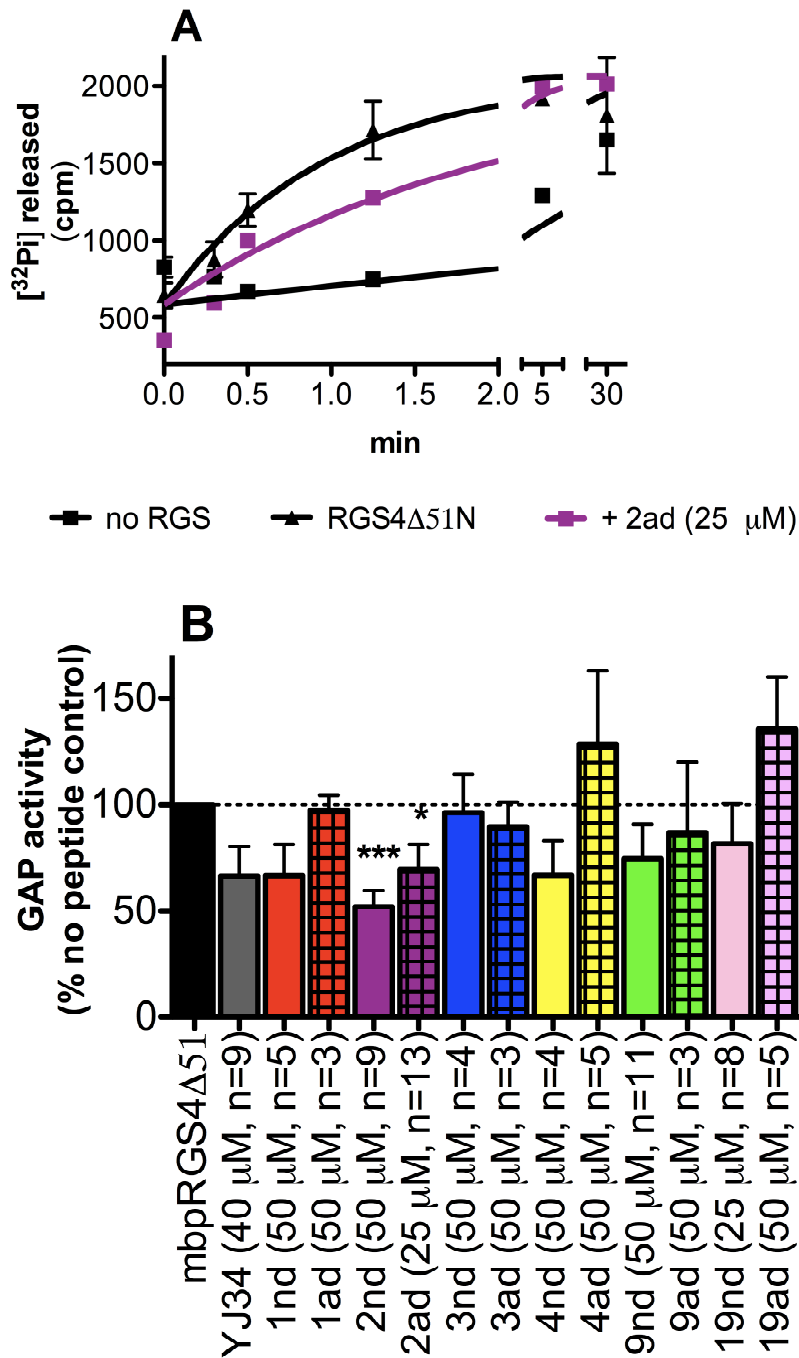
Figure 3.4: Activity of hit peptides in a single turnover GAP assay. A) Representative time course. B) Rates were calculated based on the time courses, and percent decrease in the rate of RGS stimulated GTP hydrolysis by peptides was measured (mean ± S.E.M., n ≥3). *p<0.05, **p<0.01, ***p<0.001 compared to no peptide.
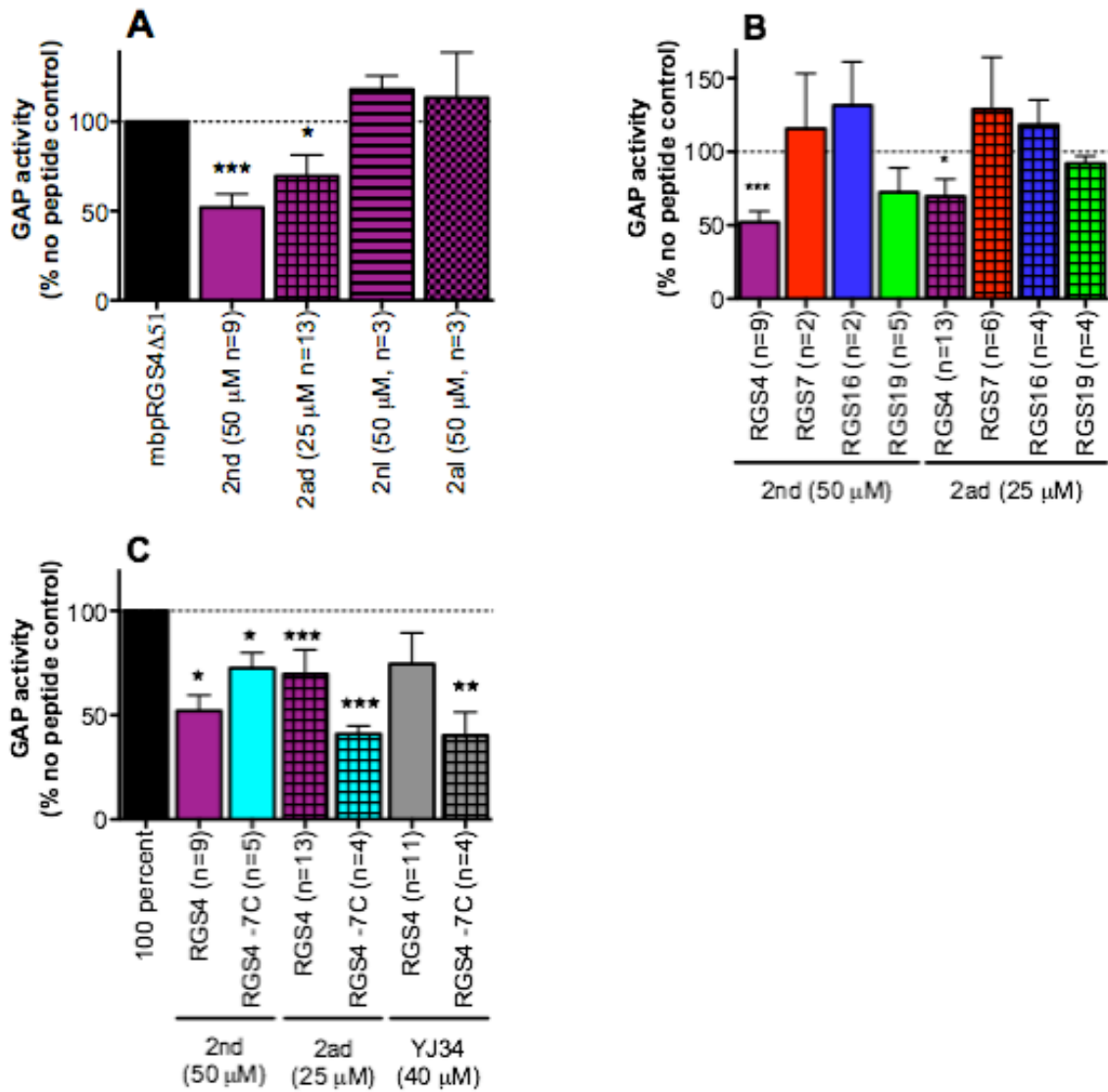
Figure 3.5: Functional activity of peptide **2**. A) Disulfide-bridged (**2nd** and **2ad**), but not linear (**2nl** and **2al**) peptides inhibit RGS4Δ51N GAP activity. B) Peptides **2nd** and **2ad** are selective for RGS4Δ51N (75-125 nM) over RGS7box (500 nM), RGS16 (1.5 μM) or RGS19 (200 nM). C) Activity of **2nd** and **2ad** on wild type and -7C mutant (200 nM) RGS4 (mean ± S.E.M. (or S.D when n=2), n ≥2). *p<0.05, **p<0.01, ***p<0.001 compared to no peptide.

Model of 2ad binding. Based on the crystal structure of RGS4 bound to $G\alpha_{i1}$ (Tesmer, et al. 1997), Thr182 of $G\alpha_i$ interacts with several residues in RGS4 including Asn88, Asp163 and Leu159. The corresponding residue in **YJ34**, Thr[4], was modeled to interact with RGS4 in the same manner. However, there is a Phe at this position in peptide **2** and it is unlikely that this bulkier, non-polar side chain interacts with RGS in the same manner as the Thr[4] side chain of **YJ34** (Figure 3.6A). However, if the peptide were rotated around the central Gly, then the Thr in position 6 would fit into the pocket that Thr182 of switch 1 binds, and the Thr[4] of **YJ34** is thought to bind. This would also position the Phe[4] of hit **2** above Tyr84 in RGS4 where Ile185 in switch 1 is (Figure 3.6B). These two amino acid interactions are reminiscent of **YJ36** (*Ac*-Val-Lys-c[Cys-Thr-Gly-Phe-Cys]-Glu-*NH₂*, S-S, Roof, et al. 2006, Chapter 2), which has a Thr at position 4 and a Phe at position 6. **YJ36** inhibited RGS4 GAP activity nearly 40% at 100 μM (Roof, et al. 2006, Chapter 2). Thus it is proposed that hit **2** interacts with RGS4 in the switch 1 binding site in an antiparallel orientation (Figure 3.6B). Also in Chapter 2, peptide **GU1**, (*Ac*-Val-Lys-[Cys-Thr-Gly-Met-Cys]-Glu-*NH₂*, S-S) with a Met at the Ile position of **YJ34**, inhibited RGS4 22 ± 9 percent. This may indicate that hit **4nd**, which inhibited RGS4 33 ± 16 percent at 50 μM and with a Met in position 4 and a Gly at position 6, also binds in the reverse orientation. It is interesting that **ES2**, and **GU2**, the ethylene dithioether bridged versions of **YJ36**, and **GU1** respectively, are more active than **YJ36** (52 ± 12 percent inhibition at 30 μM for **ES2** vs. 38 ± 10 at 100 μM for **YJ36**) and **GU1**

(61 ± 11 percent inhibition at 100 μM for **GU2** vs. 22 ± 9 at 100 μM for **GU1**). This raises the possibility that increasing the bridge length of **2ad** or **2nd** might increase activity. Based on our model, the Trp^8 also makes some contacts with Asn128 and Leu159.
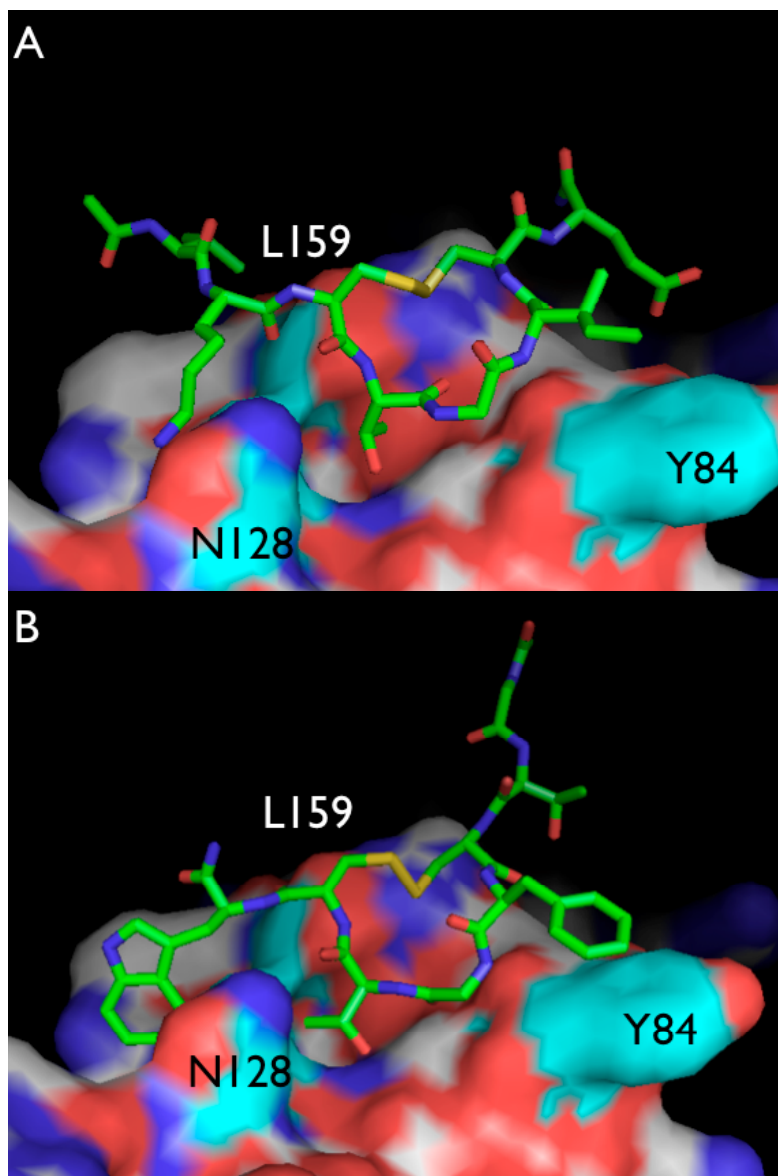
Figure 3.6: Model of **YJ34** and **2ad** in the switch 1 binding site of RGS4. Amino acids in RGS4 mentioned in the text are in cyan. A) **YJ34** is modeled to bind RGS4 the same way the switch 1 region of Gα$_i$ binds. B) **2ad** is modeled in the same pocket but in the reverse orientation.

Discussion:

A focused OBOC peptide library was screened for binding to a fluorescently labeled RGS4Δ18N. Twenty hits were selected and sequenced, yielding 14 complete sequences that were resynthesized. The hit peptides were verified for binding to RGS4Δ51N, and seven were chosen for further evaluation. From these seven hits, one peptide sequence (in both free amide and acetylated form) was found to inhibit RGS4Δ51N GAP activity in the single turnover GAP assay. Thus the focused library approach was a success; hit peptides were identified that appear to have the desired mode of binding to RGS4, and the desired activity.

As shown in Figure 3.3, both the free N-terminal and the acetylated versions of most verified hit peptides bind RGS4Δ51N, with hit **9** being the exception. This is in contrast to the **YJ34** series where only the acetylated version inhibits RGS4 activity (Roof, et al. 2006, Chapter 2). It is possible that some of these peptides do not interact with RGS4 the same way **YJ34** does. In fact, the observation that many of them do not have an effect on GAP activity would suggest that this is the case, and that these analogs interact with RGS in alternate ways. RGS4 has been shown to have an allosteric site where PIP3 and calmodulin are known to bind (Ishii, et al. 2005). Calmodulin binding alone does not affect GAP activity, but blocks the inhibition by PIP3 (Ishii, et al. 2005). It is therefore possible that some of the peptides isolated in the library bind RGS4 at this site without affecting RGS4 GAP activity. Alternatively, these peptides may bind other, perhaps as of yet unidentified, protein-protein interaction sites. The fact that the sequences obtained in the library are so divergent from the sequence of **YJ34** also supports the possibility that many of the hit peptides do not bind at the **YJ34** binding site.

In fact, it is hard to find any clear clusters based on the homology of the varied positions. It is therefore speculated that RGS4 may have several peptide (and therefore endogenous protein or lipid) binding sites. The physiological significance of these sites on RGS4 would be an interesting topic for future work.

As in the **YJ34** series, linear versions of hit **2** are inactive. Also, as in the **YJ34** series, hit **2** is most active on RGS4. It is thought that CCG-4986 inhibits through cysteine modification (Kimple, et al. 2007, Roman, et al. 2007, Roman, unpublished observation). Because of the apparent sensitivity of RGS4 to redox manipulations, and because of cysteines in the peptides, it was examined whether a similar mechanism might exist here. Hence, peptides **2ad**, **2nd** and **YJ34** were tested against the -7C mutant. The observation that none of the peptides differentiated between wild type and the -7C mutant supports a mechanism of action like that of **YJ34**, rather than via a covalent interaction with RGS4 cysteines.

The side chain order and ring size of **2ad** and **2nd** are the same as **YJ36** within the cycle. However, the backbone is different. The fact that both have activity opens up the possibility for non-peptide analogs such as β-amino acid and peptoid analogs. Peptidomimetics have the advantage of not being protease substrates and increased stability makes them more useful in cells and in vivo (Fear, et al. 2007). Thus peptidomimetics are an interesting avenue for future investigations.

Future directions could also include verification of the peptide **2**-RGS4 model, which could be done by modifying either the peptide or the RGS4. It could be hypothesized that substitution of Tyr84 by something charged would disrupt **2nd** and **2ad** activity. It could also be expected that a mutation of Asn128, which is known to be

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important for interactions with the Gα subunit and RGS GAP activity (Natochin, et al. 1998), would disrupt peptide activity. Also, based on the **YJ36** data in Chapter 2 and in Roof et al (2006), it could be hypothesized that an ethylene bridged peptide would be more potent than a disulfide bridged version of hit **2**.

Thus the focused library was a success; new peptides were identified which are modeled to bind in the **YJ34** binding site of RGS4, and have **YJ34**-like activities. And although the potency or **YJ34** was not improved on, structural insights were gained, which can aid in the design of future peptides and peptidomimetics.

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Chapter 4

Observations from a Focused One-Bead, One Compound Library

Introduction:

As discussed in Chapter 1, RGS proteins are interesting therapeutic targets. Also as discussed in Chapter 1 and Chapter 3, a focused OBOC library was utilized to screen for RGS4 inhibitors. Here the observations made in Chapter 3 are extended to include a second hit from the library that did not have significantly inhibit RGS4 GAP activity, but disrupts RGS4-Gα_o binding. Thus the focused library approach had mixed success as one peptide, peptide **2**, has the intended action and is modeled to bind the intended site on RGS4, while another peptide, peptide **9**, appears to have a different mechanism.

Materials and Methods:

Materials: Fmoc-protected amino acids and Rink amide resin were purchased from Advanced ChemTech. Peptide synthesis grade chemicals were purchased from Applied Biosystems. Avidin coated microspheres were purchased from Luminex.

Protein Expression, Purification and Labeling Proteins were expressed, purified and labeled as described in Chapters 2 and 3. Most of the mutagenesis was done by David Roman using the "QuickChange Multi Site-Directed Mutagenesis Kit" from Stratagene according to the manufacturer's protocol. To cleave RGS4 form the Mbp-His$_6$ construct (for MS analysis), Mbp-His$_6$-RGS4Δ51N was treated with 15% Mbp-His$_6$-Tev protease S219V that was prepared with the help of David Roman (Lucast, et al. 2001) for 22 hours at 4°C and purified over a Ni-NTA column.

Peptide Synthesis: Soluble peptides (for GAP assays) were synthesized by myself or Anjanette Turbiak on cleavable Rink resin and cyclized as described previously (Jin, et al. 2004, Roof, et al. 2006, Chapter 2, Chapter 3). Peptide purity (at least 95%) and solubility were verified by HPLC and correct mass was verified by MS analysis (Roof, et al. 2006, Chapter 2, Chapter 3).

FCPIA (Flow Cytometry Protein Interaction Assay): The FCPIA was performed as previously published (Roman, et al. 2007). For the peptide wash experiments, biotin-RGS on avidin beads was treated with peptide for 10 min and then washed three times. The beads were then added to the Gα_o-Alexa Fluor 532 as described in Roman et al (2007). For the peptide pretreatment experiment, the 10 min peptide incubation was followed by addition of a second peptide or DMSO for 15 min before the wash. The final concentrations are stated in the Figure legends. Samuel Clements did the RGS selectivity experiment.

Mass Spectrometry: Total mass MS of 5 μM RGS4Δ51N was analyzed on a MicroMX MALDI MS instrument in positive ion mode with an accuracy of ± 0.1% after pretreatment with DMSO or 250 μM **9nd**. Alternatively, samples cut from a gel were treated with 1.5 μg trypsin in 100 mM ammonium bicarbonate pH 8.0-8.5 in enough buffer to cover the gel piece. This was incubated overnight at 37 $^{\circ}$C, extracted with 50% Acetonitrile/0.1% aqueous TFA, and then subject to LC-MS/MS on a Q-TOF Premier Mass Spectrometer. Both were performed at the Biomedical Research Core Facilities at the University of Michigan.

Results and Discussion:

The hit peptides reported in Chapter 3 were tested in the FCPIA assay. This measures the interaction between biotinylated RGS4 on avidin beads with Alexa Fluor 532 labeled $G\alpha_o$ in a Luminex flow cytometer (Roman, et al. 2007). Peptides were tested at 50 μM except where limited by solubility (Table 4.1). Peptides **2nd**, **9nd**, **9ad**, **19nd** and **19ad** (Table 4.1) all had statistically significant inhibition of the RGS4-$G\alpha_o$ interaction, with hit **9** being the most active (80 ± 5 and 37 ± 3 percent inhibition for **9nd** and **9ad**, respectively) (Figure 4.1). It is interesting to note that **YJ34**, a known inhibitor of RGS GAP activity (Jameson, et al. 2005, Jin, et al. 2004, Roof, et al. 2006), failed to disrupt the RGS4-$G\alpha_o$ interaction (-17 ± 4 percent inhibition), as will be discussed further in Chapter 5.
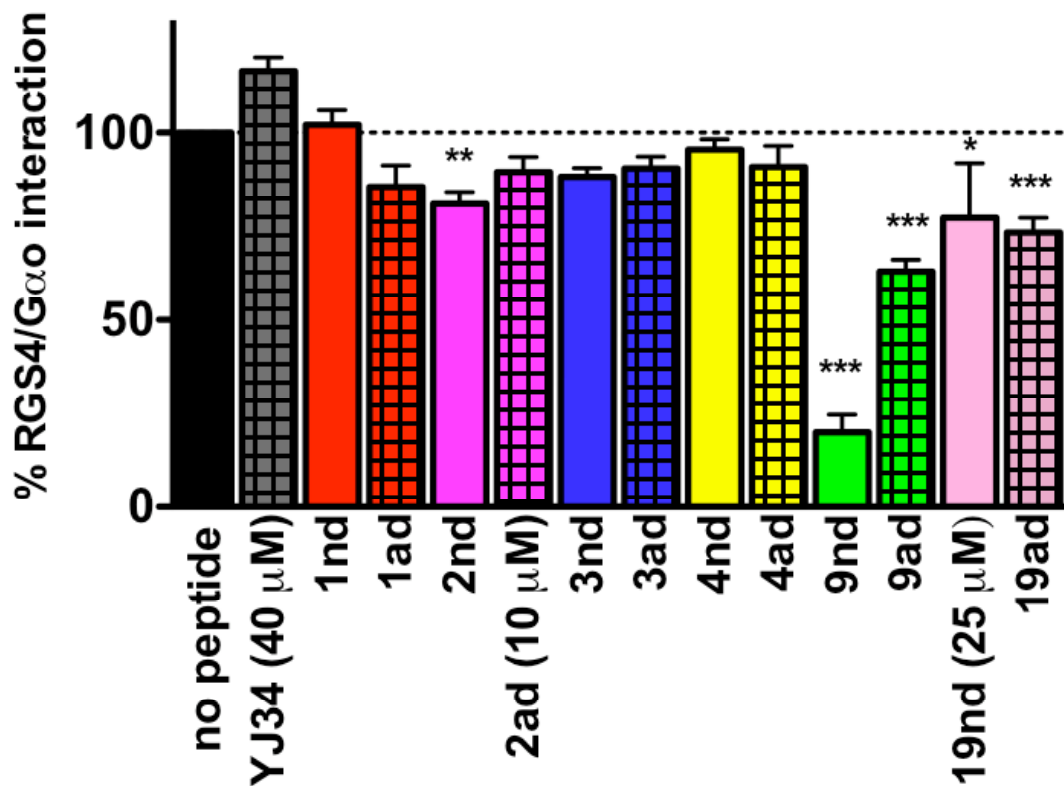
Figure 4.1: Hit peptides in the FCPIA. Hit peptides from Chapter 3 were tested at 50 µM unless otherwise indicated for ability to disrupt the RGS4-$G\alpha_o$ interaction as described using 25 nM $G\alpha_o$-Alexa Fluor-532 and 5 nM wild type RGS4Δ51N-biotin (mean ± S.E.M., n≥3) *p<0.05, **p<0.01, ***p<0.001 compared to no peptide.

Name	Sequence	cyclization	Buffer Solubility (μM)	ALogP	% Inhibition of RGS4-$G\alpha_o$
YJ34	Ac–VKCTGICE–NH$_2$	S-S	80	-2.58	-17 ± 4 (40 μM)
1nd	YNCQGECK–NH$_2$	S-S	≥ 420	-2.8	2 ± 4
1ad	Ac–YNCQGECK–NH$_2$	S-S	123	-2.37	14 ± 6
2nd	**GTCFGTCW–NH$_2$**	**S-S**	**464**	**-0.37**	**19 ± 3****
2ad	Ac–GTCFGTCW–NH$_2$	S-S	30	0.08	10 ± 4 (10 μM)
3nd	LVCKGYCQ–NH$_2$	S-S	≥ 470	-0.37	12 ± 2
3ad	Ac–LVCKGYCQ–NH$_2$	S-S	427	0.17	10 ± 3
4nd	KVCMGGCT–NH$_2$	S-S	≥ 470	-2.02	4 ± 3
4ad	Ac–KVCMGGCT–NH$_2$	S-S	459	-2.09	9 ± 6
9nl	YWCKGLCK–NH$_2$	Linear	465	1.46	-1 ± 10
9al	Ac–YWCKGLCK–NH$_2$	Linear	460	1.66	-2 ± 4
9nd	**YWCKGLCK–NH$_2$**	**S-S**	**463**	**0.48**	**80 ± 5*****
9ad	**Ac–YWCKGLCK–NH$_2$**	**S-S**	**464**	**1.04**	**37 ± 3*****
9nm	YWCKGLCK–NH$_2$	S-me-S	≥ 470	0.53	3 ± 0.1 (100 μM)
19nd	**KHCYGFCY–NH$_2$**	**S-S**	**low**	**0.94**	**23 ± 15 (25 μM) ***
19ad	**Ac–KHCYGFCY–NH$_2$**	**S-S**	**421**	**1.24**	**27 ± 4*****

Table 4.1: <u>Hit peptide names, sequences and physical properties</u>. Solubility in buffer was determined experimentally by HPLC. ALogP calculations are from www.vcclab.org, (Tetko, et al. 2005). Percent inhibition (50 μM unless otherwise stated) is from Figure 4.1, (mean ± S.E.M., n≥3) *p<0.05, **p<0.01, ***p<0.001 compared to no peptide. Peptides with significant inhibition in the FCPIA are in bold.

In Figure 4.2A, it is shown that the IC_{50} values for **9nd** are 28 μM, 43 μM, 66 μM, 145 μM and 175 μM on RGS4, RGS8, RGS16, RGS19 and RGS7 respectively ($logIC_{50}$ of -4.56 ± 0.02, -4.36 ± 0.02, -4.18 ± 0.03, -3.84 ± 0.05 and -3.76 ± 0.08 for RGS4, RGS8, RGS16, RGS19 and RGS7 respectively). RGS4, RGS8 and RGS16 are all in the same family (R4) and are the most homologous to RGS4. RGS19 and RGS7 are in the RZ and R7 families respectively. Linear as well as bridged versions of peptide **9** were tested since cyclization yields would most likely have been less then 100% in the synthesis of the library leaving some linear peptides present on each bead. The linear peptides were found to be inactive (-1 ± 10 and -2 ± 4 percent inhibition for **9nl** and **9al** respectively) (Figure 4.2C). This may be due to a lack of structural rigidity necessary for the correct interaction of the peptide with the RGS protein.

As discussed in Chapter 3, a small molecule inhibitor of RGS4 was identified in a FCPIA screen and found to interact with RGS4 through cysteine modification (Kimple, et al. 2007, Roman, et al. 2007, Roman, personal communication). For this reason it was desirable to determine if peptide **9nd** acts in a similar manner. It was found that when biotin-RGS4 on avidin beads was treated with **9nd** and then washed, the peptide still inhibited RGS4-$G\alpha_o$ interactions (75 ± 11 percent). Inclusion of DTT in the wash buffer significantly decreased **9nd** activity (15 ± 4 percent inhibition) (Figure 4.3A). This suggests that the peptide may bind irreversibly through a disulfide bridge. To further investigate this possibility, a methylene dithioether bridged peptide, **9nm** (Table 4.1), was synthesized and found to be inactive (3 ± 0.1 percent inhibition) (Figure 4.3A). Since the methylene dithioether bridged peptide would be incapable of forming a disulfide bridge with RGS4, it is suggested that **9nd** may form a disulfide bridge with RGS4 and

that this bridge may be necessary for its function. Although it is also possible that the structural change from the increased bridge length was responsible for the loss of activity of **9nm** compared to **9nd**. The same pattern was seen with RGS8. The activity of **9nd** was lost with washing only if DTT was included in the buffer and **9nm** had only a small effect on RGS8 activity (64 ± 2, 50 ± 3, 10 ± 3, and 18 ± 1 percent inhibition for **9nd** no wash, **9nd** wash, **9nd** DTT wash and **9nm** respectively) (Figure 4.3B). Although **YJ34** does not affect the interaction of RGS4 with $G\alpha_o$, it was reasoned that if **9nd** was working by binding to the switch 1 binding site of RGS4, its activity could be blocked with **YJ34**. In Figure 4.3C, **YJ34** failed to block the activity of **9nd** suggesting that binding to the **YJ34** site on RGS4 is not necessary for **9nd** function, or that the covalent binder can compete with **YJ34** for binding. Peptide **9mn** also failed to block **9nd** binding, which is consistent with **9nm** not binding to RGS4 (Figure 4.3C).
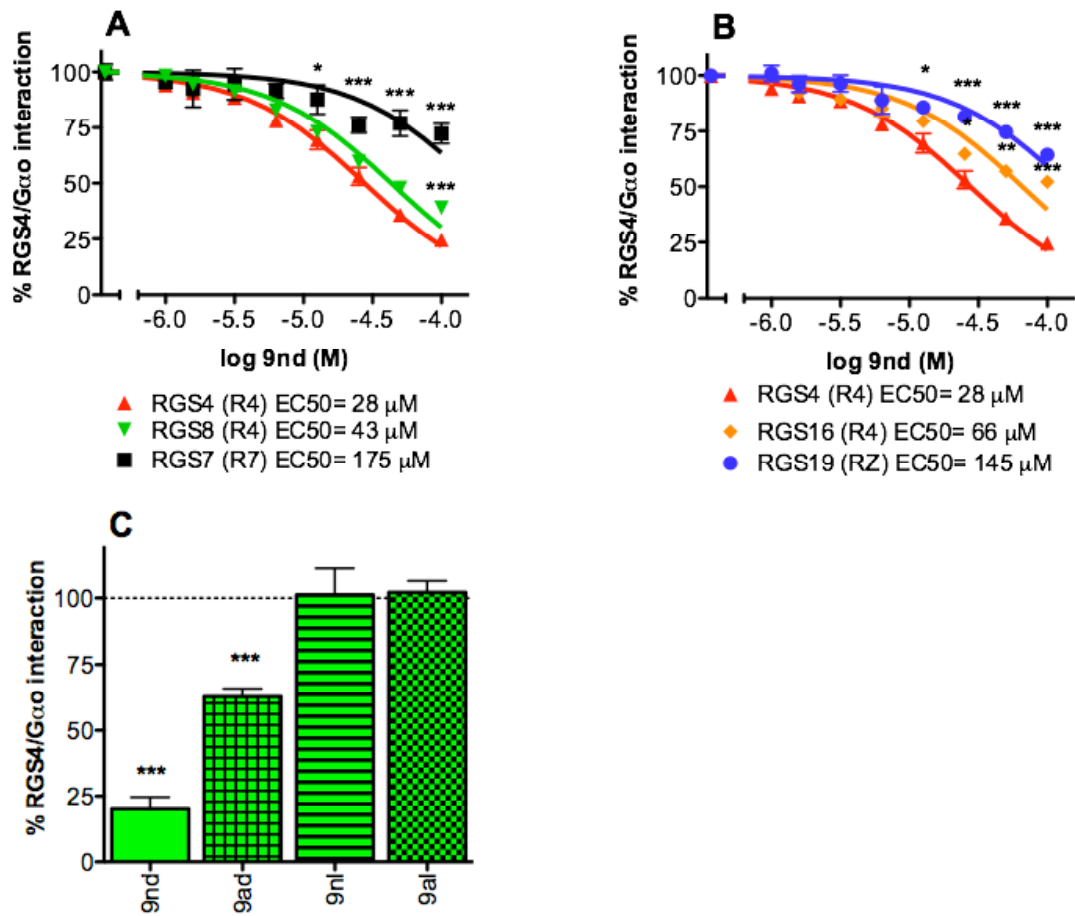
Figure 4.2: Characterization of hit **9**. A and B) Dose response curves for **9nd** in the FCPIA with different RGS proteins (RGS Family), (mean ± S.E.M., n=3) *p<0.05, **p<0.01, ***p<0.001 compared to RGS4 at the same **9nd** concentration. C) Peptides were tested at 50 μM as in Figure 4.1, (mean ± S.E.M., n≥3) *p<0.05, **p<0.01, ***p<0.001 compared to no peptide.
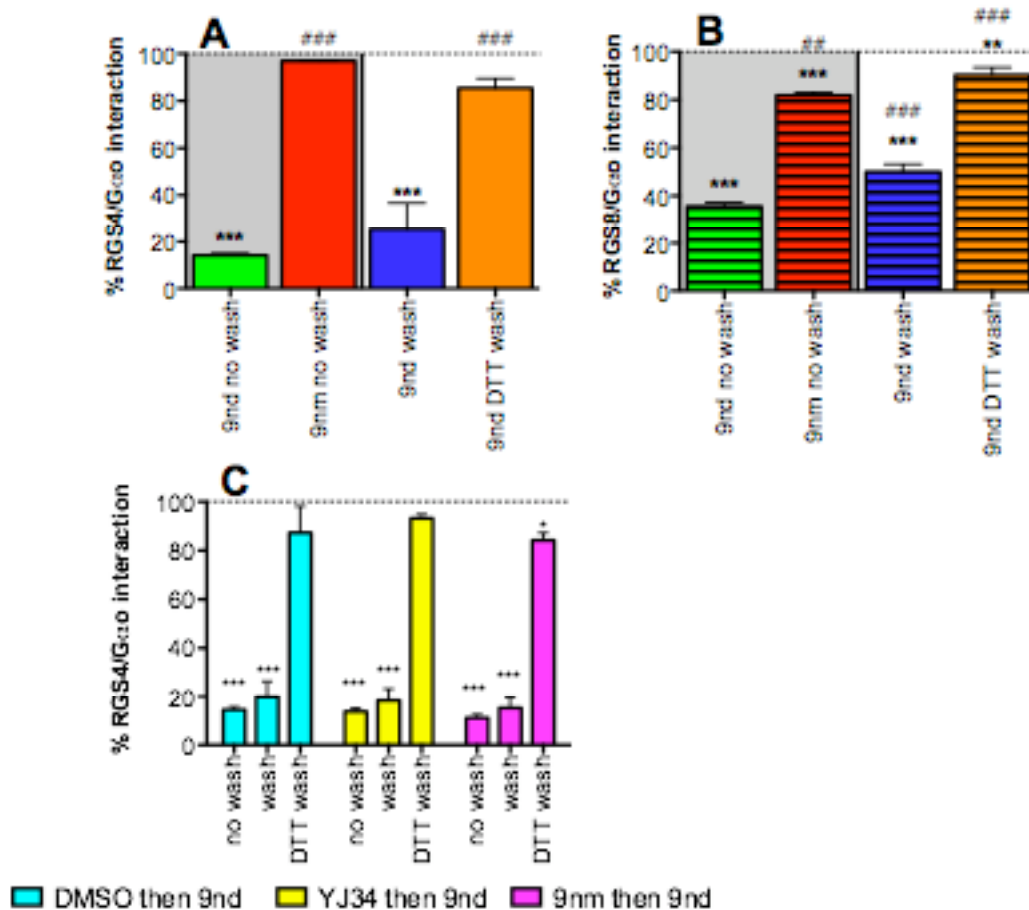
Figure 4.3: Irreversibility experiments. RGS-biotin on avidin beads was treated with **9nd** (100 μM) or **9nm** (100 μM) and then washed in buffer (with or without 1 mM DTT) or not washed and put in the FCPIA using RGS4Δ51N (A) or RGS8 (B) (mean ± S.E.M., n≥2) *p<0.05, **p<0.01, ***p<0.001 compared to no peptide (100%); #p<0.05, ##p<0.01, ###p<0.001 compared to **9nd** no wash. C) RGS4Δ51N-biotin on avidin beads was treated with DMSO or peptide (20 μM **YJ34** or 50 μM **9nm**). After the pre-incubation, the samples were treated with DMSO or **9nd** (50 μM) (mean ± S.E.M., n=3) *p<0.05, **p<0.01, ***p<0.001 compared RGS4 treated with DMSO then DMSO (100%); #p<0.05, ##p<0.01, ###p<0.001 compared to RGS4 treated with DMSO then 9nd (50 μM).

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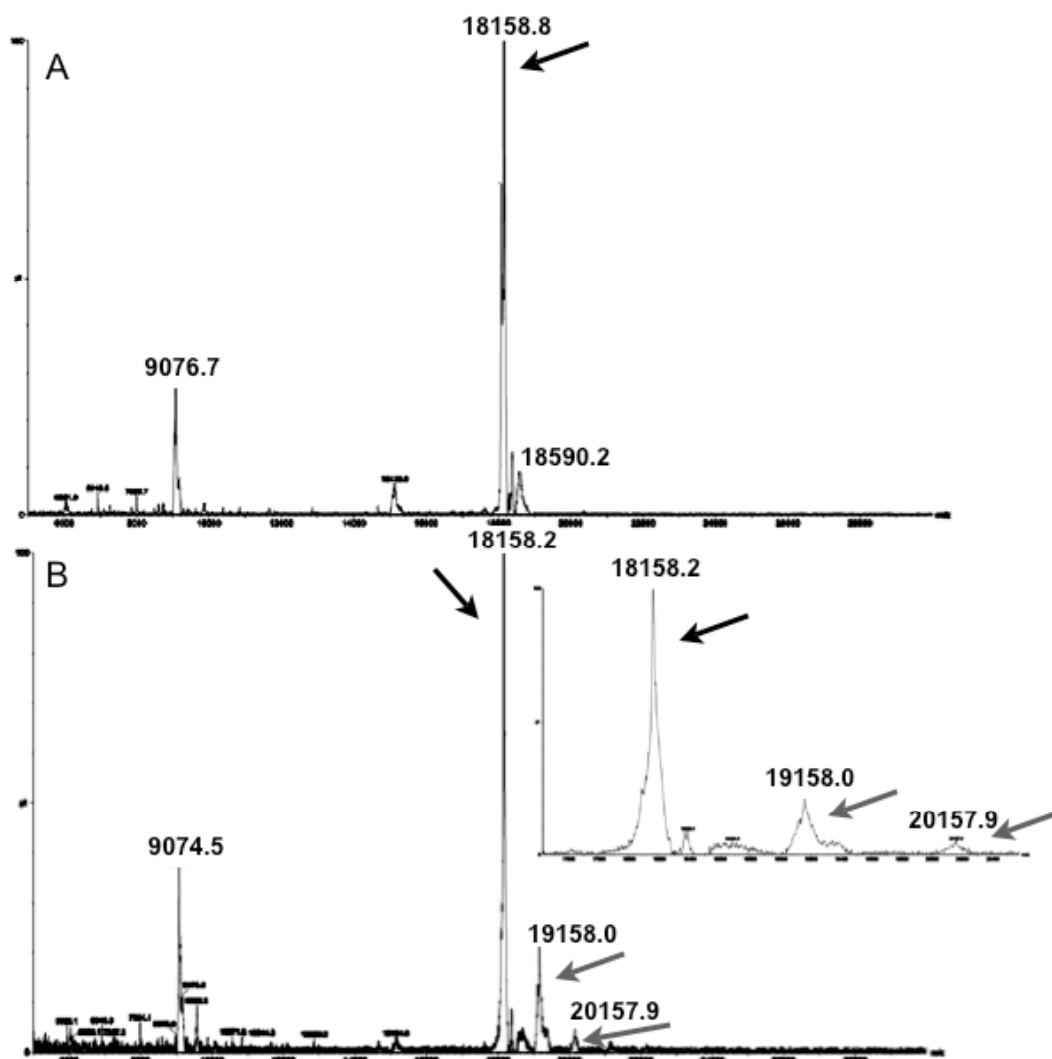
Figure 4.4: MS analysis of RGS4 with and without **9nd** pretreatment. RGS4Δ51N was cleaved from the mbpHis$_6$ construct and subject to MALDI MS after pretreatment with DMSO (A) or **9nd** (250 μM) (B). Both spectra show the region between 4,000 and 30,000 m/z and have the peak at 18158.8 or 18158.2 m/z for A and B respectively, which corresponds to the RGS4 protein (black arrows). The spectrum in B also has a peak at 19158 m/z that corresponds to a peptide adduct (grey arrows). There is a very small peak at 20157.9 that may correspond to 2 peptide adducts (grey arrows). The inset is a blow-up of the region between 17500 and 20500 m/z.

A

71 **95** **148** **132**

B

```
                                        71              *** **          95
RGS4   vsqeevkkwa eslenlishe cglaafkafl kseyseenid fwiscleeykk ikspsklspk
RGS8   lsteeatrwa dsfdvlllshk ygvaafrafl ktefseenle fwlaceefkk trstaklvsk
RGS16  sfsedvlgwr esfqslllnsk ngvaafhafl ktefseenle fwlaceefkk irsatklasr
RGS19  pspeevqswa qsfgklmhsp agrsvfrafl rteyseenml fwlaceelka eanqhvvdek
RGS7   psqqrvkrwg fgmdealkdp vgreqflkfl esefssenlr fwlavedlkr -rpirevpsr

         * *** *  *  *          132              148
RGS4   akklynefis vqatkevnld sctreetsrn mleptitcfd eaqkkifnlm ekdsyrrflk
RGS8   ahrlfeefvd vqaprevnid fqtreatrkn lqepsltcfd qaqgkvhslm ekdsyprflr
RGS16  ahhifdeyir seapkevnid hetreltktn lqaatpscfd vaqgktrtlm ekdsyprflk
RGS19  arliyedyvs ilspkevsld srvreginkk mqepsahtfd daqlqiytlm hrdsyprfls
RGS7   vqelwqefla pgapsainld sksydkttqn vkepgrytfe daqehiyklm ksdsyprfir

              183           197                              204
RGS4   srsyldlvnp sscgaekqkg akssadcas- ------------ ------------l vpqca
RGS8   skmyldlls- ---------- ---------- ------------ ------------- -----
RGS16  spayrdlaaq as-------- atsasgssp- ------------ ------------a epsht
RGS19  sptyralllq gpsqssse-- ---------- ------------ ------------- ----a
RGS7   ssayqellqa krksgnsmdr rtslekfaqn vgrnlpifpc hknctptlra stnll
```

Figure 4.5: RGS alignment. A) The structure of the RGS4 domain (Tesmer, et al. 1997) with the cysteines highlighted. B) An alignment of the RGS proteins used in Figure 4.3 compared to the RGS4Δ51N construct. The RGS proteins ar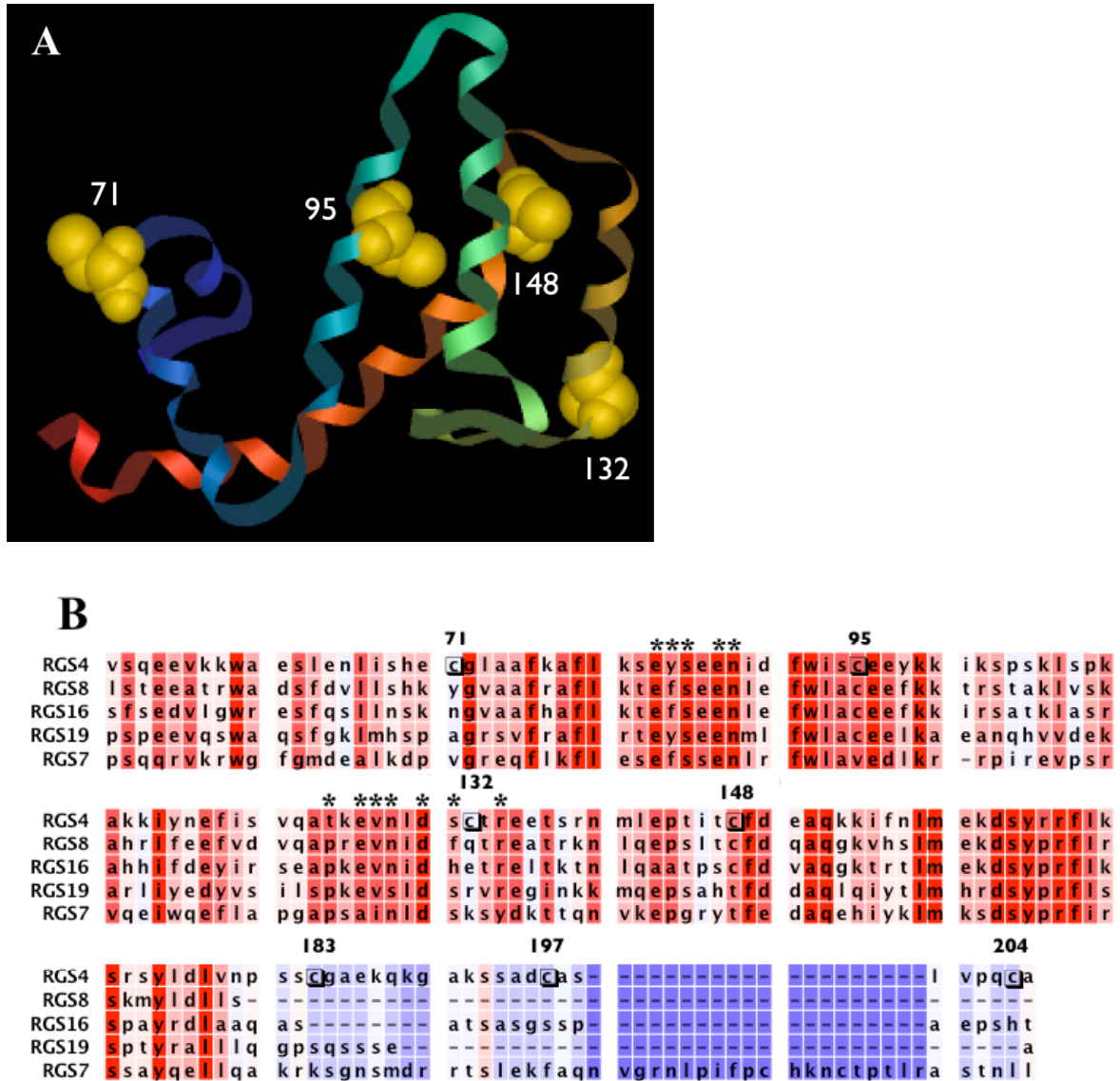e listed in order of inhibition by **9nd**. The red residues are conserved and the blue are not. The stared residues are important for Gα interactions and the cysteines in RGS4 are boxed.

The formation of a covalent adduct between **9nd** and RGS4 was confirmed with MS analysis (Figure 4.4). An adduct to the protein (following Tev-protease cleavage from the MbpHis$_6$ construct) that is consistent with the mass of **9nd** binding through a disulfide bridge was observed by MS (observed: $\Delta 999.2 \pm 1$; expected: $\Delta 998.5$). No such adduct was observed with DMSO treated RGS4Δ51N. There is also a small peak that may be a two peptide adduct (observed: $\Delta 1999.1 \pm 2$; expected $\Delta 1997.0$).

Since **9nd** forms an irreversible, DTT-sensitive bond with RGS, it was suspected that it binds covalently to a cysteine in the protein. Indeed, removal of all 7 cysteines (termed the "-7C" mutant) from RGS4Δ51N abolished **9nd** activity (logIC$_{50}$ of -3.24 $\pm$ 0.14 for -7C vs. -4.36 $\pm$ 0.02 for wt) (Figure 4.6, Table 4.2). In Figure 4.5, the RGS proteins tested in Figure 4.2A are aligned with the N-terminal truncated RGS4 used in these studies. It was hypothesized that Cys95 or Cys148 in RGS4 was involved in **9nd** activity because they are conserved with RGS8 and RGS16, which are also inhibited by **9nd**. However, neither the C95A nor the C148A mutations diminished **9nd** activity (logIC$_{50}$ -4.71 $\pm$ 0.0.5 and -4.27 $\pm$ 0.05 for C95A and C148A respectively) (Figure 4.6A, Table 4.2). All mutants bound Gα_o in an AMF-dependent manner, although some had decreased affinity compared to wild type (Table 4.2). With the assumption that **9nd** would have to bind within the RGS domain to inhibit Gα_o binding, C71A and C132A mutations were also tested. The C71A mutation did not affect **9nd** activity (logIC$_{50}$ -4.34 $\pm$ 0.06). The C132A mutation did decrease **9nd** activity, but only partially (logIC$_{50}$ -3.86 $\pm$ 0.07) (Figure 4.6B, Table 4.2). Thus no single cysteine within the RGS domain is completely necessary for **9nd** activity.

In an alternative approach, cysteines were added back to the -7C mutant to determine whether **9nd** activity could be restored. No single A to C mutation within the RGS domain of the -7C mutant restored **9nd** activity (Figure 4.6C, Figure 4.6D, Table 4.2). This suggests that either **9nd** can inhibit RGS4 by binding to any one of multiple cysteines, or that its actions are through a cysteine in the C-terminus. To evaluate the later possibility, mutants lacking all cysteines in either the RGS domain or the C-terminus were tested. It was found that the C-terminal cysteines were not involved in **9nd** activity (logIC$_{50}$ -4.5 ± 0.2), but that removal of all cysteines from the RGS domain only partially blocked **9nd** activity (logIC$_{50}$ -3.8 ± 0.2) (Figure 4.6E, Table 4.2). These observations suggest that **9nd** has a complex mechanism, as no single cysteine is either necessary or sufficient for its actions. Thus it is concluded that **9nd** is a non-selective cysteine modifier. This would suggest that RGS4 is more sensitive to covalent redox manipulations then the other RGS proteins tested.

MS was also used in attempt to identify important cysteines for the activity of **9nd**. Following Tev-protease cleavage from the MbpHis$_6$ construct, RGS4Δ51N was treated with DMSO or **9nd** and subjected to trypsin digest. The fragments were analyzed by LC-MS/MS analysis. However, no fragments with an adduct could be identified (data not shown). It is suspected that if **9nd** were interacting with multiple cysteines in RGS4, then no single cysteine adduct would be abundant enough to be observed.
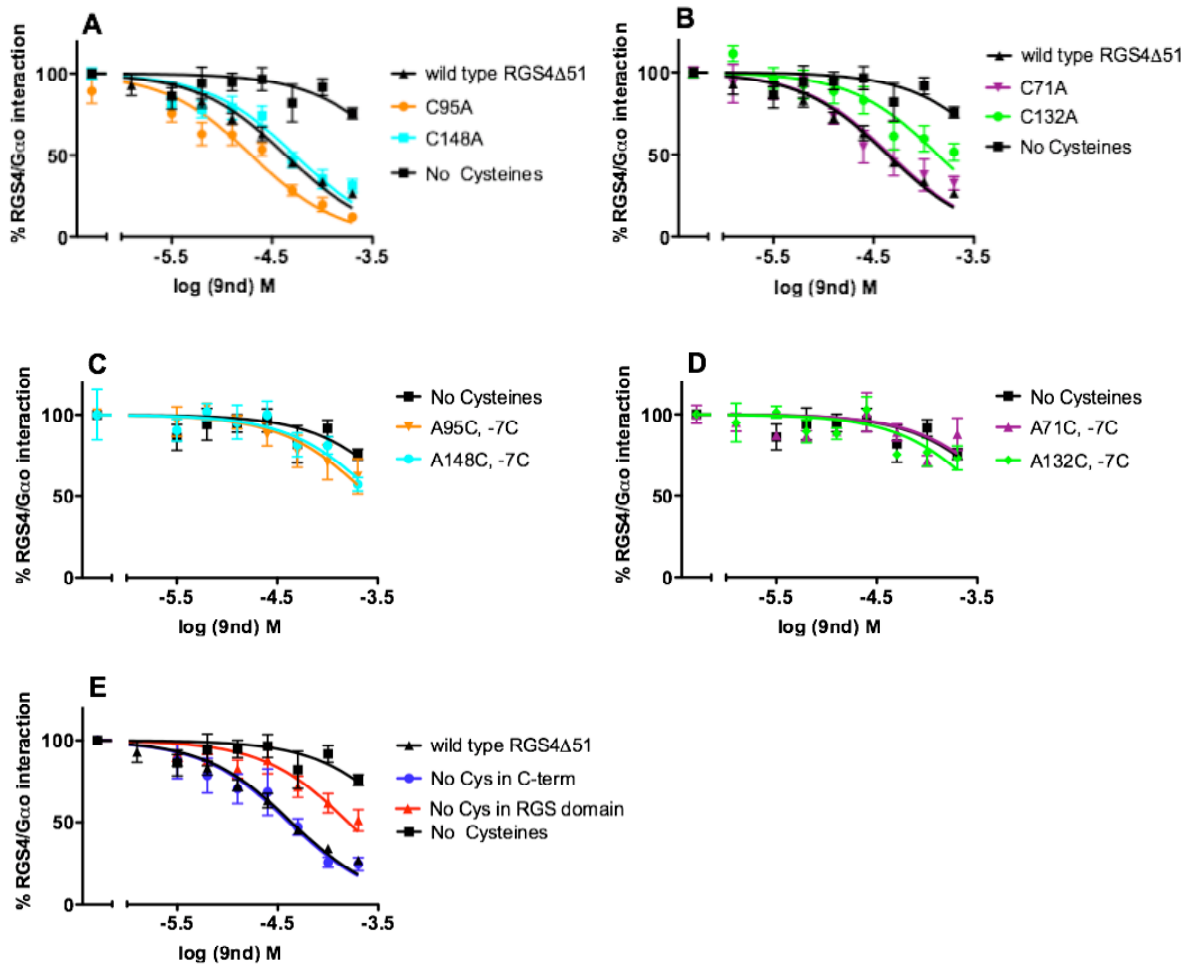
Figure 4.6: Dose curves of **9nd** on various RGS4Δ51N cysteine mutants. Various concentrations of **9nd** were tested for disruption of the C95A (5 nM), or C148A (5nM) (A), C71A (5nM) or C148A (5nM) (B), A95C in the -7C (5nM), or A132C in the -7C (5nM) (C), A71C in the -7C (5nM), or A148C in the -7C (5nM) (D) or the mutant with no cysteines in the domain (20 nM) or the C-terminus (20 nM) (E) RGS4Δ51N-Gα_o interaction in the FCPIA (mean ± S.E.M., n≥3). See Table 4.2 for statistics.

Mutant	$G\alpha_o$ affinity (nM)	9nd $logIC_{50}$ (M)
wild type	11.6 ± 1.4	-4.36 ± 0.03###
C95A	8.17 ± 1.5	-4.71 ± 0.05###
C148A	6.84 ± 1	-4.27 ± 0.05#
C71A	12.2 ± 1.8	-4.34 ± 0.06##
C132A	7.9 ± 3.1	-3.86 ± 0.07
-7C	8.3 ± 1	-3.24 ± 0.14***
A95C, -7C	12 ± 2	-3.58 ± 0.09**
A148C, -7C	5.7 ± 0.2	-3.51 ± 0.09**
A71C, -7C	9.2 ± 2	-3.20 ± 0.13**
A132C, -7C	12.5 ± 1	-3.41 ± 0.09**
no Cys in RGS domain	3.9 ± 0.8	-3.80 ± 0.06*
no Cys in C-terminus	5.0 ± 0.7	-4.39 ± 0.07##

Table 4.2: Mutant RGS4Δ51N $G\alpha_o$ affinities and **9nd** $logIC_{50}$s. $G\alpha_o$ affinities were calculated in the FCPIA as described (Roman, et al. 2007) and $logIC_{50}$ data is from Figure 4.5. (mean ± S.E.M., n≥3) *p<0.05, **p<0.01, ***p<0.001 compared to wild type RGS4Δ51N; #p<0.05, ##p<0.01, ###p<0.001 compared to -7C RGS4Δ51N.

Conclusions:

Here the identification and characterization of a peptide RGS4 inhibitor with a novel, yet unknown, mechanism is reported. This peptide, **9nd**, binds RGS proteins covalently through a disulfide bridge to random cysteines. This peptide raises some interesting points regarding the focused screen discussed in Chapter 3. First, it is interesting that although the library was focused to include features necessary for **YJ34** activity, a peptide was isolated that clearly works through a different mechanism. Thus there was mixed success with the focused approach. The library was biased towards peptides that would have the same mechanism as the lead compound. However, this bias is by no means a guarantee; there is no reason to suspect that a peptide like hit **2** would have been found from a completely random library.

Another interesting observation is that RGS4 is preferentially inhibited by the cysteine modifier peptide over other RGS proteins. This could be because the peptide is selective for RGS4, or because RGS4 is particularly susceptible to cysteine modification. This latter possibility is supported by the observation that a small molecule inhibitor of RGS4, CCG-4986, that was identified in an FCPIA screen appears to inhibit RGS4 through covalent modification of cysteines while having no activity against RGS8 (Kimple, et al. 2007, Roman, et al. 2007, Roman, unpublished observation). Also, RGS4 is more sensitive to inhibition with N-ethylmaleimide, (a cysteine modifier) then RGS8 (David Roman, personal communication). Unlike **9nd**, CCG-4986 appears to selectively modify 1 or 2 cysteines in the RGS4 (Roman, et al. 2007, Roman, unpublished observation). This cysteine selectivity may be why CCG-4986 has more RGS selectivity

compared to **9nd**. In future efforts, it may be possible to exploit this susceptibility of RGS4 to cysteine modification if a cycteine reactive peptide were also a selective, high affinity binder to a particular pocket on the RGS.

A third observation is that there is a correlation between peptide logP and activity in the FCPIA (Table 4.1). The order of activity in the FCPIA is hit **9**>hit **19**>hit **2**> the rest of the hits, while the order of hydrophobicity is hit **19**>hit **9**>hit **3**>hit **2**> the rest of the hits. Given the considerable error in computational logP calculations (Thompson, et al. 2006), it is reasonable to suggest that the most hydrophobic peptides have the most activity in general. This also tells us something about RGS4. These data suggest that RGS4 binding sites have hydrophobic surfaces and investigators should be mindful of this when choosing libraries for future screens.

Although attempts to improve on existing RGS4 inhibitors failed, it is hoped that this experience will result in improved screens in the future.

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Chapter 5

Conclusions

Inhibition of protein-protein interactions (PPI) is particularly challenging. Unlike enzymes and receptors that have well defined pockets, protein-protein interfaces tend to be large and flat (Whitty & Kumaravel. 2006). Because of this, many PPI have been declared "non-amenable" to inhibition based on computational measurements (Fry & Vassilev. 2005). In fact, the RGS4-Gα_o interaction falls in this category (David Fry, personal communication).

However, some "hot spots" have been found in protein-protein interfaces, involving a limited number of residues that are responsible for the majority of the interaction energy. Others have taken advantage of this to successfully create peptide and small molecule inhibitors of PPIs. For example, Thanos et al. found a small molecule that was capable of binding to interleukin-2 in such a way that prevented its receptor interaction. Even though it was much smaller, the molecule was able to target the same critical "hot spots" that the receptor contacts (Thanos, et al. 2006). Another example is the screen done by the Smrcka lab for peptide inhibitors of Gβγ. Although the hit sequences were diverse, they all bound the same site on the protein, and one of the peptides tested inhibited some but not all functions of the Gβγ subunits (Scott, et al. 2001).

Another encouraging point is that computational approaches such as those used in Fry and Vassilev (2005) are based on rigid structures of proteins. Others have shown that protein interfaces are adaptable and in multiple cases small molecules bind in induced pockets that are not observed in the structures of the protein alone or in complex with its natural protein binding partner (Wells & McClendon. 2007 and references therein). Thus these interactions could not be rationally predicted in spite of the existence of a structure of the target protein and could only come from experimental screening. Hence, although this screen of RGS4 inhibitors was an ambitious project, there are ample precedents for success.

A relatively uncommon approach for HTS was taken with the focused library. Although there are examples of those who have used the focused approach (Chang, et al. 2008, Laird & Blake. 2004, Whiting, et al. 2006), screening of a random library is far more common. Placing limitations on the structures screened can increase the chances of identifying an inhibitor with the desired mechanism of action, but it can also prevent identification of potentially more interesting or more potent compounds that act with unique mechanisms. In retrospect, it can also be speculated that the library was not constrained enough as peptides were found that clearly work through different mechanisms than the lead. On the other hand, no peptides were found that were more potent than the lead, so it could be argued that a more random screen would have been more appropriate. Attempts to identify small molecule inhibitors of the RGS-Gα_o interaction are underway in the Neubig lab using libraries containing more chemical space (Roman, et al. 2007).

One potential reason that so many peptides were identified that were not as effective as **YJ34** is the fact that 4 versions of each sequence were on each bead in the library (acetylated and free N-terminal, disulfide and linear). The expectation when the library was designed was that only the acetylated disulfide bridged peptide would have activity, and the free N-terminal and linear peptides would contribute nothing to the RGS interaction, since only the acetylated disulfide bridged version of **YJ34** inhibits RGS4 GAP activity (Jin et al, 2004, Roof et al, 2006, Chapter 2). This hypothesis was based on the assumption that many hit peptides would have a **YJ34**-like mechanism, as the library was designed**.** However, it was found that for most of the peptides, if one version displayed significant binding capacity to RGS4 in the FACScan assay, all four did (Appendix B). A possible explanation for this observation is that if all four versions of a sequence are capable of binding RGS4, then the library bead containing that sequence has the potential to be much more fluorescent than if only one version binds. Hence, it appears that sequences were inadvertently selected for based on the ability of all four versions to contribute to the RGS4 interaction, which, based on **YJ34** analog data in Chapter 2, should be incompatible with a **YJ34**-like mode of binding. Thus it is suspected that these peptides do not bind RGS4 the way **YJ34** binds and this screen may have actually selected against peptides with a **YJ34**-like mechanism. This is in contrast to the intended library bias. These competing mechanisms may have hurt the chances of identifying more potent RGS4 inhibitors.

In the single example of a verified hit peptide that inhibits RGS4 GAP activity, it is suspected that the peptide binds in the reverse orientation compared to **YJ34** and the switch 1 region of Gα. This orientation, based on modeling, puts the free N-terminus of

the peptide away from the RGS4 protein. One could speculate that this orientation and not the **YJ34**-like orientation is compatible with the free N-terminal peptide binding to RGS4. Hence beads with both free N-terminal and acetylated peptides would have increased binding to RGS4 if they bound in the reverse orientation compared to peptides binding in the **YJ34**-like orientation. Had the screen been performed using only acetylated peptides, perhaps the identified hits would bind in the **YJ34** orientation. In that scenario, another method for identifying hit sequences would have to be employed. Sophisticated "topically segregated" tags have been used in the past by others with OBOC libraries (Hwang, et al. 2004, Liu, et al. 2002, Wang, et al. 2004). Such an approach could be used should an OBOC library be screened in the future.

Several hit peptides were identified that bound RGS4 in the FACScan assay that did not inhibit RGS4 activity in the GAP or FCPIA assays. Aside from the interesting observation that the focused library resulted in several hits without the predicted mechanism of action (and therefore, presumably, without the predicted mode of binding to RGS4), this also raised the question of where on RGS4 the peptides were binding. As discussed in chapter 4, there are known allosteric binding sites on the RGS domain of RGS4. There is also the interesting possibility that these peptides bind unidentified protein-protein interaction sites on RGS4. As discussed in Chapter 1, RGS4 has physiological roles beyond being a GAP protein, and these peptides could be used as tools for identifying other RGS4 binding partners within a cell. It would be an exciting project for a future student to see what can be immunoprecipitated out of cell lysates with RGS4, and what interactions are lost with peptide pretreatment. It would be exciting to

have a new PPI and an inhibitor of it. This student would immediately have a tool for evaluating the function of the newly discovered interacting proteins.

There appears to be disconnects between peptide activity in the FACS scan assay, the GAP assay and the FCPIA assay. It is easy to imagine peptides binding to RGS4 but not inhibiting its function. These peptides would bind RGS4 in the FACScan assay but not inhibit RGS4 activity in the GAP or FCPIA assays. In fact, peptide **3nd** (Leu-Val-c[Cys-Lys-Gly-Tyr-Cys]-Gln-*NH₂*, S-S) binding to RGS4 has been confirmed in an assay that measures the change in the intrinsic fluorescence of unlabeled RGS4Δ18N with peptide binding (Appendix C). Peptide **3nd** has a Kd of 55 ± 3 nM in this assay, but does not inhibit RGS4 activity in the GAP or FCPIA activity (Chapter 3 and Chapter 4).

The discrepancy between peptide activity in the FCPIA and the GAP assays is harder to explain. It is possible that a peptide could block the activity of RGS4 on $G\alpha_o$ without disrupting the binding of RGS4 to $G\alpha_o$. This may be why peptide **2** is so much more active at inhibiting RGS4 in the GAP assay than in the FCPIA assay. But how could a peptide block the $G\alpha_o$ interaction without impairing GAP activity of RGS4? The answer might have to do with differences in the temperatures of the experiments (4° C for the GAP assay and 25° for the FCPIA, as described in Chapter 2 and Chapter 4). There may also be a difference in the structure of RGS4 bound to AMF ($AlCl_3$, $MgCl_2$, NaF and GDP, a transition state mimic) activated $G\alpha_o$ (as is bound to RGS4 in the FCPIA assay) compared to GTP activated $G\alpha_o$ (as is bound to RGS4 in the GAP assay). The peptide that inhibited RGS4 the most in the FCPIA, **9nd**, did inhibit RGS4 in the GAP assay, although to a lesser extent than the inhibition in the FCPIA and it failed to reach

statistical significance. This may suggest subtle differences in assay conditions, and not a fundamental flaw in the assay or the peptide is responsible for the disparity.

Other observations from this screening experience that may be helpful in the future have to do with the physical properties of RGS4. The Neubig lab has now identified both a peptide and at least one small molecule inhibitor of RGS4 that binds irreversibly through cysteine modification (Chapter 4, David Roman, personal communication, Kimple, et al. 2007, Roman, et al. 2007). As discussed in Chapter 4, RGS4 appears to be particularly sensitive to cysteine modifications. Whether or not there are physiological implications of this phenomenon is unknown. The -7C RGS4Δ51N has both GAP and FCPIA activity. But as cysteines are important for palmitoylation (Srinivasa, et al. 1998) one could speculate that this modification may play a role in RGS4 actions or regulation *in vivo*. Regardless, care needs to be taken when screening against RGS4 (or other RGSs) in the future. In fact, the Neubig lab is currently screening against the -7C RGS4Δ51N mutant.

A second observation regarding the physical properties of RGS4 that may be useful in future screens, is the observation that there appears to be a correlation between hydrophobicity of the peptides and the activity in the FCPIA. It is tempting to speculate that Van de Waals interactions between RGS4 and $G\alpha_o$ are more important than non-hydrophobic interactions. However, there is no data to suggest that **9nd** is binding to the $G\alpha_o$ interface of RGS4. This may reflect an overall hydrophobicity of RGS4, or it could mean that there are hydrophobic allosteric sites on RGS4. What this means for RGS actions and protein interactions *in vivo* is yet to be determined. However, knowing this,

future screens may benefit from the inclusions of hydrophobic compounds, as long as the hydrophobicity does not preclude *in vivo* usefulness.

In conclusion, overall, this focused library approach was a success, but there are lessons to be learned here that may increase the likelihood of success of future screens.

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Appendix A: Complete List of Hit-Bead Sequencing Results

hit 1	Tyr-Asn-Cys-Gln-Gly-Glu-Cys-Lys
hit 2	Gly-Thr-Cys-Phe-Gly-Thr-Cys-Trp
hit 3	Leu-Val-Cys-Lys-Gly-Tyr-Cys-Gln
hit 4	Lys-Val-Cys-Met-Gly-Gly-Cys-Thr
hit 5	Lys-Trp-Cys-Ala-Gly-Met-Cys-Met
hit 6	Phe-Pro-Cys-Leu-Gly-Ile-Cys-Tyr
hit 7	___-Asn-Cys-Try-Gly-Phe-Cys-Lys
hit 8	___-Phe-Cys-Phe-Gly-Asn-Cys-Trp
hit 9	Tyr-Trp-Cys-Lys-Gly-Leu-Cys-Lys
hit 10	Ser-Val-Cys-Phe-Gly-Leu-Cys-Tyr
hit 11	Ile-Lys-Cys-Arg-Gly-Ile-Cys-Ser
hit 12	Gly-Asn-Cys-Gln-Gly-Val-Cys-___
hit 13	Pro-Arg-Cys-Leu-Gly-___-Cys-___
hit 14	Val-Phe-Cys-Ala-Gly-Ala-Cys-Arg
hit 15	Arg-Ile-Cys-Gly-Gly-___Cys-Phe

hit 16	Lys-Leu-Cys-His-Gly-Tyr-Cys-His
hit 17	Pro-Arg-Cys-Leu-Gly-Ala-Cys-Arg
hit 18	Trp-Ala-Cys-Arg-Gly-Val-Cys-Tyr
hit 19	Lys-His-Cys-Tyr-Gly-Phe-Cys-Tyr
hit 20	Val-Ile-Cys-Tyr-Gly-Gln-Cys-Gly

Hit peptide-beads were sent for sequencing by Edman degradation at the University of Michigan biomedical research core facilities. It should be noted that no sequence data can be obtained for cysteines without prior derivatization. Hence, when no amino acid assignments were obtained at positions 3 and 7, they were designated to be cysteines because of the way the library was synthesized (see Chapter 3). C-terminal modifications and cyclization status also cannot be determined with Edman degradation, but all hits should have had a C-terminal amide because of the type of resin used in the library (see Chapter 3). Inconclusive amino acid assignments are represented by ___.

Appendix B: Linear and Cyclized Peptide FACScan
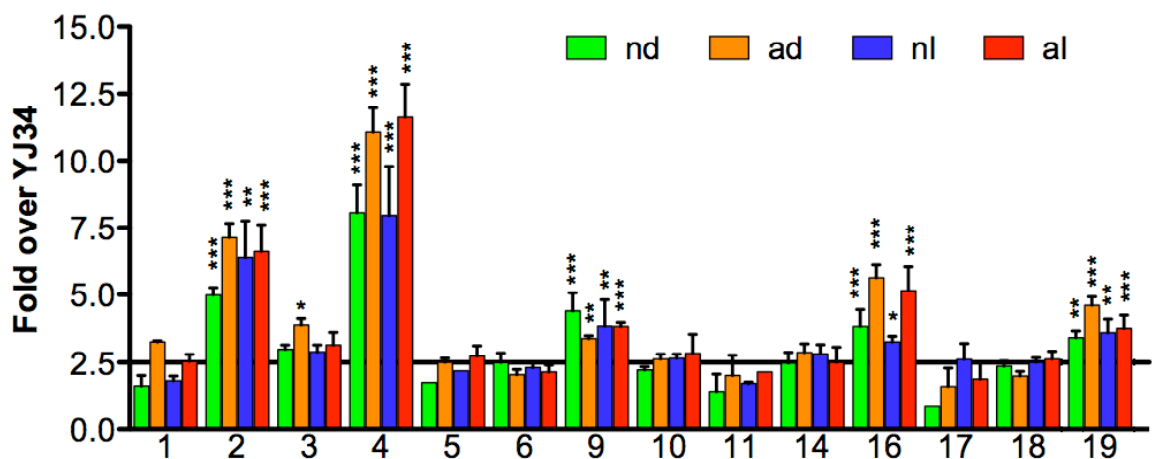
All four versions (nd: free N-terminal, disulfide bridged; ad: acetylated, disulfide bridged; nl: free N-terminal, linear; al: acetylated, linear) of the 14 hits for which complete sequences were obtained were resynthesized on beads and tested for binding to RGS4Δ15N-Alexa Fluor-532 as described in Chapter 3 (mean ± S.E.M. (S.D. when n=2), n≥2). *p<0.05, **p<0.01, ***p<0.001 compared to **YJ34** on beads.

Appendix C: Peptide 3nd Changes the Intrinsic Fluorescence of RGS4
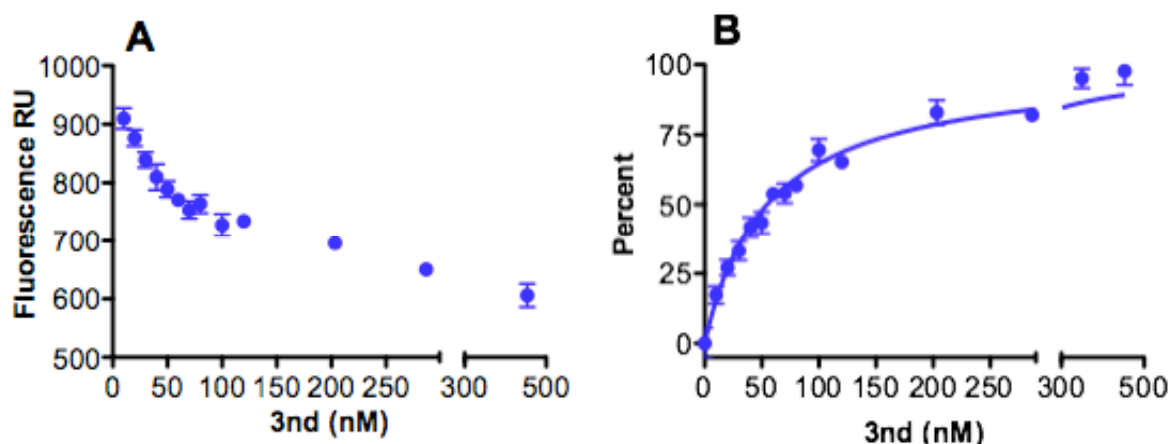
The intrinsic fluorescence of 200 nM unlabeled (no Mbp, His$_6$ or fluorophores, prepared as described in Roman et al (2007)) RGS4Δ18N was measured in 600 μl buffer A (filtered 20 mM Hepes, 100 mM NaCl, pH 8.0, at room temperature) in a 10x2 quartz cuvette. A Photon Technology International AlphaScan Spectrofluorometer with 4 nm slits was used with an excitation wavelength of 285 nm and an emission of 340 nM. Increasing concentrations of **3nd** (from a 6 μM stock in buffer A with 0.7% DMSO) was added. The change in fluorescence seen with addition of the same amount of peptide to buffer (with no RGS) was subtracted from each point. For each experiment, the average of 10 readings was taken 3 times for each sample. A) A representative decrease in

fluorescence with peptide addition. B) The average of three experiments normalized and inverted. The data was analyzed using a one fit binding curve with the Bmax constrained to 100% using Graphpad Prism. From this the Kd was calculated to be 55 ± 3 nM. Complete data was not obtained for any other peptide.

Appendix D: Complete Table of Hit Peptide Names

Name	Hit#	Sequence	Cyclization	FW	Solubility (μM in buffer)
YJ34	-	Ac-VKCTGICE-NH$_2$	S-S	1005	80
BR14	Hit #1	YNCQGECK-NH$_2$	Linear	1286	364
BR15	Hit #1	Ac-YNCQGECK-NH$_2$	Linear	1328	≥ 470
BR24	Hit #1	YNCQGECK-NH$_2$	S-S	1284	≥ 420
BR25	Hit #1	Ac-YNCQGECK-NH$_2$	S-S	1326	123
BR20	Hit #2	GTCFGTCW-NH$_2$	Linear	873	356
BR21	Hit #2	Ac-GTCFGTCW-NH$_2$	Linear	916	237
AJTL63	Hit #2	GTCFGTCW-NH$_2$	S-S	871	464
AJTL65	Hit #2	Ac-GTCFGTCW-NH$_2$	S-S	914	30
BR18	Hit #3	LVCKGYCQ-NH$_2$	Linear	1026	≥ 470
BR19	Hit #3	Ac-LVCKGYCQ-NH$_2$	Linear	1069	408
AJTL48	Hit #3	LVCKGYCQ-NH$_2$	S-S	1024	≥ 470
AJTL49	Hit #3	Ac-LVCKGYCQ-NH$_2$	S-S	1067	427
BR22	Hit #4	KVCMGGCT-NH$_2$	Linear	796	361
BR23	Hit #4	Ac-KVCMGGCT-NH$_2$	Linear	839	≥ 470
AJTL69	Hit #4	KVCMGGCT-NH$_2$	S-S	794	≥ 470
AJTL71	Hit #4	Ac-KVCMGGCT-NH$_2$	S-S	837	459
BR16	Hit #5	KWCAGMCV-NH$_2$	Linear	1041	344
BR17	Hit #5	Ac-KWCAGMCV-NH$_2$	Linear	1082	223
BR26	Hit #5	KWCAGMCV-NH$_2$	S-S	1039	≥ 470
BR27	Hit #5	Ac-KWCAGMCV-NH$_2$	S-S	1081	141
AJTL68	Hit #6	FPCLGICY-NH$_2$	Linear	914	431
AJTL67	Hit #6	Ac-FPCLGICY-NH$_2$	Linear	955	190
AJTL97	Hit #6	FPCLGICY-NH$_2$	S-S	912	≥ 470
AJTL89	Hit #6	Ac-FPCLGICY-NH$_2$	S-S	954	63
AJTL83	Hit #9	YWCKGLCK-NH$_2$	Linear	1227	465
AJTL82	Hit #9	Ac-YWCKGLCK-NH$_2$	Linear	1268	460
ALTJ93	Hit #9	YWCKGLCK-NH$_2$	S-S	1225	463
AJTL91	Hit #9	Ac-YWCKGLCK-NH$_2$	S-S	1267	464
BR32	Hit #9	YWCKGLCK-NH$_2$	S-me-S	1354	≥ 470
AJTL85	Hit #10	SVCFGLCY-NH$_2$	Linear	890	441
AJTL84	Hit #10	Ac-SVCFGLCY-NH$_2$	Linear	931	340

AJTL99	Hit #10	SVCFGLCY-NH$_2$	S-S	888	297
AJT105	Hit #11	Ac-IKCRGICS-NH$_2$	Linear	816	ND
AJT115	Hit #11	Ac-IKCRGICS-NH$_2$	S-S	814	ND
AJT120	Hit #14	VFCAGACR-NH$_2$	Linear	824	ND
AJT119	Hit #14	Ac-VFCAGACR-NH$_2$	Linear	866	ND
AJT127	Hit #14	VFCAGACR-NH$_2$	S-S	822	ND
AJT125	Hit #14	Ac-VFCAGACR-NH$_2$	S-S	864	ND
BR30	Hit #16	KLCHGYCH-NH$_2$	S-S	1186	400
BR33	Hit #19	KHCYGFCY-NH$_2$	Linear	1246	good
BR34	Hit #19	Ac-KHCYGFCY-NH$_2$	Linear	1289	good
BR31	Hit #19	KHCYGFCY-NH$_2$	S-S	1233	low
BR35	Hit #19	Ac-KHCYGFCY-NH$_2$	S-S	1287	421

This table contains the names of peptides as they are referred to in laboratory notebooks. Solubility in pH 8.0 buffer was performed as described in Chapter 3 by myself (for "BR" peptides) or Anjanette Turbiak (for "AJ" pepides). FW masses include TFA salts. Please note that some of the "AJT" or "AJTL" peptides may also be referred to as "AJ" peptides. ND: Not determined.