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

by

Rebecca A. Roof

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Doctorial Committee:

Professor Richard R. Neubig, Co-Chair
Professor Henry I. Mosberg, Co-Chair
Associate Professor Anna K. Mapp
Associate Professor John J. G. Tesmer
Assistant Professor Roger K. Sunahara
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<td>OBOC</td>
<td>One-Bead, One-Compound</td>
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<td>6-OHDA</td>
<td>6-Hydroxydopamine</td>
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<td>PAG</td>
<td>Periaqueductal Gray</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PBM</td>
<td>Peripheral Blood Mononuclear</td>
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<td>PDEγ</td>
<td>Phosphodiesterase γ</td>
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<tr>
<td>PDZ</td>
<td>PSD95, Dlg and Z0-1/2</td>
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<td>Pen</td>
<td>Penicillamine</td>
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<td>PFC</td>
<td>Prefrontal Cortex</td>
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<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5,-trisphosphate</td>
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<tr>
<td>PKG</td>
<td>Protein Kinase G</td>
</tr>
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<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PPI</td>
<td>protein-protein interaction</td>
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<tr>
<td>PTB</td>
<td>phosphotyrosine-binding</td>
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<tr>
<td>PTX</td>
<td>Pertussis Toxin</td>
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<td>R7BP</td>
<td>RGS7 Binding Protein</td>
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<td>R9AP</td>
<td>RGS9 Anchoring Protein</td>
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<td>RBD</td>
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<td>RGS</td>
<td>Regulator of G Protein-Signaling</td>
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<td>RH</td>
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<td>RhoGEF</td>
<td>Rho Guanine nucleotide Exchange Factor</td>
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<td>RP-HPLC</td>
<td>Reverse-Phase High-Performance Liquid Chromatography</td>
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<td>SAR</td>
<td>Structure- Activity Relationship</td>
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<td>Steroid-Receptor-Binding RGS (RGS3)</td>
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<td>Triisopropylsilane</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
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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 (GDI). The fact that both GDI and GAP domains for Gαi 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.
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 Gα proteins divided into four families: Gαᵢ, Gαₛ, Gαᵣ, and Gα₁₂ based on amino acid homology as well as functional similarities (Nurnberg, et al. 1995). Among other things, the Gαᵢ family inhibits adenylyl cyclase (AC) activity while Gαₛ stimulates AC. One of the many functions of the Gαᵣ family is its involvement in calcium mobilization, and the Gα₁₂ 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 than one Gα subunit (Kenakin. 2007), one could speculate that this selectivity may be physiologically useful for selectively modulating one pathway over another.
<table>
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<tr>
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<th>Gα subunits</th>
<th>Reference</th>
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<td>R12</td>
<td>Gαi/o</td>
<td>(Hooks, et al. 2003,</td>
</tr>
<tr>
<td>RA</td>
<td>ND</td>
<td></td>
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<td>GEF</td>
<td>Gα12/13</td>
<td>Hains, et al. 2004</td>
</tr>
<tr>
<td>SNX</td>
<td>maybe Gαs</td>
<td>Zheng, et al. 2001</td>
</tr>
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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 D2-mediated signaling respectively (Cabrera-Vera, et al. 2004, Wang, et al. 2002). This is in contrast to RGS8, which can regulate signaling by both D2 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 $\alpha_{2a}$AR-$\mathrm{G}\alpha_{o}$ 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.
<table>
<thead>
<tr>
<th>RGS</th>
<th>Scaffold</th>
<th>Receptor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGS12</td>
<td>Direct binding</td>
<td>interleukin-8 receptor</td>
<td>Snow, et al. 2002</td>
</tr>
<tr>
<td>RGS20</td>
<td>ND. Small ubiquitin like modifier is required</td>
<td>MOR</td>
<td>Rodriguez-Munoz, et al. 2007a</td>
</tr>
<tr>
<td>RGS17</td>
<td>GAIP-interacting protein, C terminus (GIPC)</td>
<td>D2</td>
<td>Jeanneteau, et al. 2004</td>
</tr>
<tr>
<td>RGS19</td>
<td>ND. DEP domain required</td>
<td>D2</td>
<td>Kovoor, et al. 2005</td>
</tr>
<tr>
<td>RGS4</td>
<td>Homer 2</td>
<td>M1</td>
<td>Shin, et al. 2003</td>
</tr>
<tr>
<td>RGS2</td>
<td>Neurabin</td>
<td>Does NOT bind α₁bAR, D2, M3, cholecystokinin receptors</td>
<td>Wang, et al. 2007b</td>
</tr>
<tr>
<td>RGS9</td>
<td>α actinin</td>
<td>NMDA</td>
<td>Bouhamdan, et al. 2006</td>
</tr>
</tbody>
</table>

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.
<table>
<thead>
<tr>
<th>RGS</th>
<th>Effector</th>
<th>RGS function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGS10</td>
<td></td>
<td>AC</td>
<td></td>
</tr>
<tr>
<td>RGS 13</td>
<td></td>
<td>AC</td>
<td></td>
</tr>
<tr>
<td>RGS2</td>
<td></td>
<td>RGSs bind the channel directly.</td>
<td>et al. 1999, Keren-Raifman, et al. 2001, Jaen &amp; Doupnik. 2006</td>
</tr>
<tr>
<td>RGS3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGS4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGS5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGS7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGS8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGS10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Channels</td>
<td>channel directly.</td>
<td>al. 2000, Richman, et al. 2005</td>
</tr>
<tr>
<td>RGS9</td>
<td>phosphodiesterase γ (PDEγ)</td>
<td>PDEγ increases the affinity of Go_t for RGS9. Go_t activates PDEγ</td>
<td>Skiba, et al. 2000</td>
</tr>
<tr>
<td>RGS3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGS4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGS10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 Ca²⁺/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 Ca²⁺ 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 Ca²⁺ levels which would lead to increased Ca²⁺/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 Ca²⁺ 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 Gα\textsubscript{i1} (G183S) and Gα\textsubscript{o} (G184S) abolished RGS GAP activity and markedly
decreased RGS affinity for the G-proteins (Lan, et al. 1998). RGS-insensitive mutants of
Gα 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 Gα\textsubscript{o} (Chen & Lambert.
2000), dopamine-mediated GTP\textsubscript{γ}S binding, agonist-mediated Ca\textsuperscript{2+} responses in CHO
cells expressing the D\textsubscript{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 Gα\textsubscript{o}. Pertussis toxin (PTX, which selectively inactivates Gα\textsubscript{i/o})-
isinsensitive mutants of Gα\textsubscript{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 Gαq-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 AT1 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 β2AR (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 Gαq 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.

**Gαi2 RGS-insensitive mutant knock-in.** Mice that express the RGS-insensitive mutation of Gαi2 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 D2, 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, Winant, et al. 2006) while other studies have failed to
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 D2 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 D₂ receptors on ACh releasing neurons and subsequent inhibition of Cav2 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Gαq 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 these 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 Gαs coupled GPCR)-mediated insulin release and was shown to bind Gαs. 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 Gαi2 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 Goq 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
RGS4, but not RGS7, RGS9 or RGS19 has been shown to inhibit thyrotropin releasing
cellular 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 Goq
and Goi mediated signaling, respectively (Rimler, et al. 2007). And finally,
overexpression of the RGS-insensitive Gαq in rats caused increased oxytocin and corticosterone responses with 5-HT2A/2C stimulation compared to overexpression of wild type Gαq (Shi, et al. 2006).


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 $\alpha_{13}$ 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 these 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 $\alpha_{12}$ 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\(\alpha_i\) was used (Tesmer, et al. 1997). The strategy employed in these studies was to create a peptide that blocks the RGS4-G\(\alpha\) interaction by mimicking a piece of the G
protein. Initially, two peptides with the sequences of two of the three switch regions on the G$\alpha$ 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-$NH_2$, 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. Bacteriophage 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 Gαi 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). \( \gamma^{[32]} \)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 (HOBr) (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 N₂ saturated water on ice. The pH of the peptide solution was raised to 8.5 using NH₄OH, followed by the addition of 4 mol equivalents of K₃Fe(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 N₂ 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-(CH₂)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 Gαo was loaded with a 2-3 fold molar excess of γ[^32P]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 Gαo was gel filtered through a 1 ml G-25 sephadex spin column to remove unbound γ[^32P]GTP. The loaded Gαo was then added to equal volumes of ice-cold initiation buffer (20 mM Hepes, 40 mM MgCl₂ 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 Gαo, and different concentrations of Gαo 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[^32P] P₁ released at each time point was fit to an exponential function:

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

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\(_{t=30\text{ min}}\) 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 CaCl₂, 0.53 MgCl₂, 5.5 glucose, and 5.5 Hepes-NaOH, pH 7.4. The pipette (internal) solution contained (in mmol/L): 150 KCl, 5 EGTA, 1 MgCl₂, 3 K₂ATP, 0.1 Na₂GTP, 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 K₉ 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 Gαᵢ (Table 2.1), inhibits RGS4 activity in a steady state membrane receptor GTPase
assay (Jin, et al. 2004). To determine whether the results seen previously are due to \textbf{YJ34} activity on the RGS4 protein or whether it has its activity on the receptor or other proteins or lipids in the membrane preparation, \textbf{YJ34} activity was measured in a single turnover GTPase assays with purified proteins. RGS4-enhanced hydrolysis of G\textsubscript{\alpha}\textsubscript{o}-bound \(\gamma^{[32P]}\text{GTP}\) was measured in the presence and absence of \textbf{YJ34}. RGS4-stimulated G\textsubscript{\alpha}\textsubscript{o} GTPase activity is inhibited by \textbf{YJ34} in a dose-dependent manner (Figure 2.1) with an IC\textsubscript{50} of 9 \(\mu\text{M}\). It should be noted that \textbf{YJ34} was tested up to only 40 \(\mu\text{M}\) (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 \textbf{YJ33}, was active but had lower potency than did the disulfide-bridged peptide \textbf{YJ34} (Table 2.1).
Figure 2.1: \textbf{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 \textbf{YJ34}. This graph is representative of 4 experiments done in duplicate.
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 Ga subunit or RGS4. In order to determine the target of the peptide inhibitors, BR2 was made to mimic the RGS insensitive G183S mutant of Ga. If YJ34 binds to RGS4 in the same manner that the switch 1 region of Ga binds, then a mutation that disrupts this binding in the Ga subunit should also disrupt binding of YJ34. As predicted, BR2 does not inhibit RGS4 activity at a concentration that is nearly 4 times greater than YJ34 (150 µM), (Figure 2.2 and 2.3A), suggesting that YJ34 binds to RGS4 as designed, by mimicking the Ga 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 Ga 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 Ga, 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 Ga-RGS4 interactions (Lan, et al. 1998).
Figure 2.2: Gly183 is required for peptide activity at RGS4. A) Single turnover GTPase assays were performed 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_i (**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 IC50 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 Gαi 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 than 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 IC₅₀ 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 Gα₁₁ and Gαₒ (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 sec. C, $I_{\text{ins}}/I_{\text{max}}$, (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).
**Discussion:**

**Mechanism of action.** YJ34 was designed based on the crystal structure of the RGS4-Gαi complex in the presence of GDP•AlF₄. The peptide attempts to mimic the switch 1 region of Gαi, 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 Gαo 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 Gαi (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 Gαi, 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 Gαo.

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 Gαo 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 IC₅₀ 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 IC₅₀ of 26 ± 2 µM (Jin, et al. 2004). **YJ34** inhibited RGS4 81% at 40 µM in a capillary electrophoresis assay with Gαo (Jameson, et al. 2005), which is in good agreement with the 75 ± 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 °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 Goi 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 IC50 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 Goi, (Sowell, et al. 1997) and RGS2, RGS6, and RGS17/RGSZ2, act primarily on Goq, Goi, and Gox, 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** ($RGS4 \geq RGS8 > RGS7$) 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.
LITERATURE CITED


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-NH$_2$, S-S) (Jin, et al. 2004). This peptide was designed to mimic the switch 1 region of the $G_{\alpha}$ subunit and was modeled from the RGS4-$G_{\alpha_{i1}}$ 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 $\mu$M in a membrane based steady state GTPase assay (Jin, et al. 2004), a 9 $\mu$M IC$_{50}$ in a purified protein single turnover GTPase assay (Roof, et al. 2006 Chapter 2), inhibits RGS4 81% at 40 $\mu$M 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-NH$_2$, S-S), a peptide that mimics the RGS-insensitive G184S mutation in the $G_{\alpha}$ protein is inactive, suggesting that the peptide binds to RGS4 as designed (i.e. in the same way the $G_{\alpha}$ 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. γ[³²P]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$_6$-Go$_o$ (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 Go$_o$ 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 N₂) 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 \textbf{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.

\textbf{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). \textbf{YJ34} and \textbf{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 Victor² 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 x 10³ 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 Gαi1 switch 1 in the RGS4-Gαi1 (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 Gαi fragment from the Gαi-RGS4 complex. Images were prepared using PyMol for OS X (http://www.pymol.org).

**Statistical Analysis:** Data are expressed as mean ± S.E.M (or ± 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.
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).
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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Bridge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ga1 switch 1</td>
<td>…V-K-T-T-G-I-V-E…</td>
<td>none</td>
</tr>
<tr>
<td>YJ34</td>
<td>Ac-V-K-[C-T-G-I-C]-E-NH₂</td>
<td>(S-S)</td>
</tr>
<tr>
<td>Library</td>
<td>Ac-X₁-X₂-[C-X³-G-X⁴-C]-X⁵-NH₂-X₁-X₂-[C-X³-G-X⁴-C]-X⁵-NH₂</td>
<td>(S-S)</td>
</tr>
</tbody>
</table>
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 \textbf{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, disulfide 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).
<table>
<thead>
<tr>
<th></th>
<th>Ac-VKc[CTGIC]E-NH₂, S-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>hit 1</td>
<td>YNc[CQGEC]E-NH₂, S-S</td>
</tr>
<tr>
<td>hit 2</td>
<td>GTc[CFGTC]W-NH₂, S-S</td>
</tr>
<tr>
<td>hit 3</td>
<td>LVc[CKGYC]Q-NH₂, S-S</td>
</tr>
<tr>
<td>hit 4</td>
<td>KVc[CMGGC]T-NH₂, S-S</td>
</tr>
<tr>
<td>hit 9</td>
<td>YWc[CKGLC]K-NH₂, S-S</td>
</tr>
<tr>
<td>hit 16</td>
<td>KLc[CHGYC]H-NH₂, S-S</td>
</tr>
<tr>
<td>hit 19</td>
<td>KHc[CYGFC]K-NH₂, S-S</td>
</tr>
</tbody>
</table>

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.
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 Gαo 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 2ad (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 IC₅₀ of about 50 µM. Other peptides that had some activity included 1nd (33 ± 15 percent inhibition at 50 µM) and 4nd (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 $\alpha_o$. 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-NH\(_2\), 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 \(\mu\)M (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-NH\(_2\), S-S) with a Met at the Ile position of YJ34, inhibited RGS4 22 \(\pm\) 9 percent. This may indicate that hit 4nd, which inhibited RGS4 33 \(\pm\) 16 percent at 50 \(\mu\)M 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 \(\pm\) 12 percent inhibition at 30 \(\mu\)M for ES2 vs. 38 \(\pm\) 10 at 100 \(\mu\)M 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 Trp8 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 Goᵢ 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
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.


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-Gαo 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 °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 Gαo 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-Gαo 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-Gαo 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-Gαo interaction as described using 25 nM Gαo-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.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>cyclization</th>
<th>Buffer Solubility (µM)</th>
<th>ALogP</th>
<th>% Inhibition of RGS4-Gαo</th>
</tr>
</thead>
<tbody>
<tr>
<td>YI34</td>
<td>Ac–VKCTGICE-NH₂</td>
<td>S-S</td>
<td>80</td>
<td>-2.58</td>
<td>-17 ± 4 (40 µM)</td>
</tr>
<tr>
<td>1nd</td>
<td>YNCQGECK-NH₂</td>
<td>S-S</td>
<td>≥ 420</td>
<td>-2.8</td>
<td>2 ± 4</td>
</tr>
<tr>
<td>1ad</td>
<td>Ac–YNCQGECK-NH₂</td>
<td>S-S</td>
<td>123</td>
<td>-2.37</td>
<td>14 ± 6</td>
</tr>
<tr>
<td>2nd</td>
<td>GTCFGTCW-NH₂</td>
<td>S-S</td>
<td>464</td>
<td>-0.37</td>
<td>19 ± 3**</td>
</tr>
<tr>
<td>2ad</td>
<td>Ac–GTCFGTCW-NH₂</td>
<td>S-S</td>
<td>30</td>
<td>0.08</td>
<td>10 ± 4 (10 µM)</td>
</tr>
<tr>
<td>3nd</td>
<td>LVCKGYCQ-NH₂</td>
<td>S-S</td>
<td>≥ 470</td>
<td>-0.37</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>3ad</td>
<td>Ac–LVCKGYCQ-NH₂</td>
<td>S-S</td>
<td>427</td>
<td>0.17</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>4nd</td>
<td>KVCMGGCT-NH₂</td>
<td>S-S</td>
<td>≥ 470</td>
<td>-2.02</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>4ad</td>
<td>Ac–KVCMGGCT-NH₂</td>
<td>S-S</td>
<td>459</td>
<td>-2.09</td>
<td>9 ± 6</td>
</tr>
<tr>
<td>9nl</td>
<td>YWCKGLCK-NH₂</td>
<td>Linear</td>
<td>465</td>
<td>1.46</td>
<td>-1 ± 10</td>
</tr>
<tr>
<td>9al</td>
<td>Ac–YWCKGLCK-NH₂</td>
<td>Linear</td>
<td>460</td>
<td>1.66</td>
<td>-2 ± 4</td>
</tr>
<tr>
<td>9nd</td>
<td>YWCKGLCK-NH₂</td>
<td>Linear</td>
<td>463</td>
<td>0.48</td>
<td>80 ± 5***</td>
</tr>
<tr>
<td>9ad</td>
<td>Ac–YWCKGLCK-NH₂</td>
<td>S-S</td>
<td>464</td>
<td>1.04</td>
<td>37 ± 3***</td>
</tr>
<tr>
<td>9nm</td>
<td>YWCKGLCK-NH₂</td>
<td>S-me-S</td>
<td>≥ 470</td>
<td>0.53</td>
<td>3 ± 0.1 (100 µM)</td>
</tr>
<tr>
<td>19nd</td>
<td>KHCGFCY-NH₂</td>
<td>S-S</td>
<td>low</td>
<td>0.94</td>
<td>23 ± 15 (25 µM) *</td>
</tr>
<tr>
<td>19ad</td>
<td>Ac–KHCGFCY-NH₂</td>
<td>S-S</td>
<td>421</td>
<td>1.24</td>
<td>27 ± 4***</td>
</tr>
</tbody>
</table>

Table 4.1: Hit peptide names, sequences and physical properties. 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 than 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 Gαo, 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).
Figure 4.4: MS analysis of RGS4 with and without 9nd pretreatment. RGS4Δ51N was cleaved from the mbpHis6 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.
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 are 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 MbpHis6 construct) that is consistent with the mass of 9nd binding through a disulfide bridge was observed by MS (observed: ∆999.2 ± 1; expected: ∆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: ∆1999.1 ± 2; expected Δ1997.0).

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 (logIC50 of -3.24 ± 0.14 for -7C vs. -4.36 ± 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 (logIC50 -4.71 ± 0.0.5 and -4.27 ± 0.05 for C95A and C148A respectively) (Figure 4.6A, Table 4.2). All mutants bound Gαo 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 Gαo binding, C71A and C132A mutations were also tested. The C71A mutation did not affect 9nd activity (logIC50 -4.34 ± 0.06). The C132A mutation did decrease 9nd activity, but only partially (logIC50 -3.86 ± 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 than 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-Gαo interaction in the FCP1A (mean ± S.E.M., n≥3). See Table 4.2 for statistics.
Table 4.2: Mutant RGS4Δ51N Gαo affinities and 9nd logIC50s. Gαo affinities were calculated in the FCIPIA as described (Roman, et al. 2007) and logIC50 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 $9^{nd}$. In future efforts, it may be possible to exploit this susceptibility of RGS4 to cysteine modification if a cysteine 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.
LITERATURE CITED


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-Gαo 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-Gαo 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 \textbf{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 \textbf{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 \textbf{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 \textbf{YJ34} analog data in Chapter 2, should be incompatible with a \textbf{YJ34}-like mode of binding. Thus it is suspected that these peptides do not bind RGS4 the way \textbf{YJ34} binds and this screen may have actually selected against peptides with a \textbf{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 \textbf{YJ34} and the switch 1 region of G\alpha. 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 Gαo without disrupting the binding of RGS4 to Gαo. 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 Gαo 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 (AlCl₃, MgCl₂, NaF and GDP, a transition state mimic) activated Gαo (as is bound to RGS4 in the FCPIA assay) compared to GTP activated Gαo (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 Gαo are more important than non-hydrophobic interactions. However, there is no data to suggest that 9nd is binding to the Gαo 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.
LITERATURE CITED


### Appendix A: Complete List of Hit-Bead Sequencing Results

<table>
<thead>
<tr>
<th>Hit</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tyr-Asn-Cys-Gln-Gly-Glu-Cys-Lys</td>
</tr>
<tr>
<td>2</td>
<td>Gly-Thr-Cys-Phe-Gly-Thr-Cys-Trp</td>
</tr>
<tr>
<td>3</td>
<td>Leu-Val-Cys-Lys-Gly-Tyr-Cys-Gln</td>
</tr>
<tr>
<td>4</td>
<td>Lys-Val-Cys-Met-Gly-Gly-Cys-Thr</td>
</tr>
<tr>
<td>5</td>
<td>Lys-Trp-Cys-Ala-Gly-Met-Cys-Met</td>
</tr>
<tr>
<td>6</td>
<td>Phe-Pro-Cys-Leu-Gly-Ile-Cys-Tyr</td>
</tr>
<tr>
<td>7</td>
<td>___-Asn-Cys-Try-Gly-Phe-Cys-Lys</td>
</tr>
<tr>
<td>8</td>
<td>___-Phe-Cys-Phe-Gly-Asn-Cys-Trp</td>
</tr>
<tr>
<td>9</td>
<td>Tyr-Trp-Cys-Lys-Gly-Leu-Cys-Lys</td>
</tr>
<tr>
<td>10</td>
<td>Ser-Val-Cys-Phe-Gly-Leu-Cys-Tyr</td>
</tr>
<tr>
<td>11</td>
<td>Ile-Lys-Cys-Arg-Gly-Ile-Cys-Ser</td>
</tr>
<tr>
<td>12</td>
<td>Gly-Asn-Cys-Gln-Gly-Val-Cys-___</td>
</tr>
<tr>
<td>13</td>
<td>Pro-Arg-Cys-Leu-Gly-<em><strong>-Cys-</strong></em></td>
</tr>
<tr>
<td>14</td>
<td>Val-Phe-Cys-Ala-Gly-Ala-Cys-Arg</td>
</tr>
<tr>
<td>15</td>
<td>Arg-Ile-Cys-Gly-Gly-___Cys-Phe</td>
</tr>
<tr>
<td>Hit</td>
<td>Sequence</td>
</tr>
<tr>
<td>------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>16</td>
<td>Lys-Leu-Cys-His-Gly-Tyr-Cys-His</td>
</tr>
<tr>
<td>17</td>
<td>Pro-Arg-Cys-Leu-Gly-Ala-Cys-Arg</td>
</tr>
<tr>
<td>18</td>
<td>Trp-Ala-Cys-Arg-Gly-Val-Cys-Tyr</td>
</tr>
<tr>
<td>19</td>
<td>Lys-His-Cys-Tyr-Gly-Phe-Cys-Tyr</td>
</tr>
<tr>
<td>20</td>
<td>Val-Ile-Cys-Tyr-Gly-Gln-Cys-Gly</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Hit#</th>
<th>Sequence</th>
<th>Cyclization</th>
<th>FW</th>
<th>Solubility (µM in buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YJ34</td>
<td>-</td>
<td>Ac-VKCTGICE-NH₂</td>
<td>S-S</td>
<td>1005</td>
<td>80</td>
</tr>
<tr>
<td>BR14</td>
<td>Hit #1</td>
<td>YNCQGECK-NH₂</td>
<td>Linear</td>
<td>1286</td>
<td>364</td>
</tr>
<tr>
<td>BR15</td>
<td>Hit #1</td>
<td>Ac-YNCOGECK-NH₂</td>
<td>Linear</td>
<td>1328</td>
<td>≥ 470</td>
</tr>
<tr>
<td>BR24</td>
<td>Hit #1</td>
<td>YNCQGECK-NH₂</td>
<td>S-S</td>
<td>1284</td>
<td>≥ 420</td>
</tr>
<tr>
<td>BR25</td>
<td>Hit #1</td>
<td>Ac-YNCOGECK-NH₂</td>
<td>S-S</td>
<td>1326</td>
<td>123</td>
</tr>
<tr>
<td>BR20</td>
<td>Hit #2</td>
<td>GTCFGTCW-NH₂</td>
<td>Linear</td>
<td>873</td>
<td>356</td>
</tr>
<tr>
<td>BR21</td>
<td>Hit #2</td>
<td>Ac-GTCFGTCW-NH₂</td>
<td>Linear</td>
<td>916</td>
<td>237</td>
</tr>
<tr>
<td>AJTL63</td>
<td>Hit #2</td>
<td>GTCFGTCW-NH₂</td>
<td>S-S</td>
<td>871</td>
<td>464</td>
</tr>
<tr>
<td>AJTL65</td>
<td>Hit #2</td>
<td>Ac-GTCFGTCW-NH₂</td>
<td>S-S</td>
<td>914</td>
<td>30</td>
</tr>
<tr>
<td>BR18</td>
<td>Hit #3</td>
<td>LVCKGYCQ-NH₂</td>
<td>Linear</td>
<td>1026</td>
<td>≥ 470</td>
</tr>
<tr>
<td>BR19</td>
<td>Hit #3</td>
<td>Ac-LVCKGYCQ-NH₂</td>
<td>Linear</td>
<td>1069</td>
<td>408</td>
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<tr>
<td>AJTL48</td>
<td>Hit #3</td>
<td>LVCKGYCQ-NH₂</td>
<td>S-S</td>
<td>1024</td>
<td>≥ 470</td>
</tr>
<tr>
<td>AJTL49</td>
<td>Hit #3</td>
<td>Ac-LVCKGYCQ-NH₂</td>
<td>S-S</td>
<td>1067</td>
<td>427</td>
</tr>
<tr>
<td>BR22</td>
<td>Hit #4</td>
<td>KVCMGCT-NH₂</td>
<td>Linear</td>
<td>796</td>
<td>361</td>
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<tr>
<td>BR23</td>
<td>Hit #4</td>
<td>Ac-KVCMGCT-NH₂</td>
<td>Linear</td>
<td>839</td>
<td>≥ 470</td>
</tr>
<tr>
<td>AJTL69</td>
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<td>KVCMGCT-NH₂</td>
<td>S-S</td>
<td>794</td>
<td>≥ 470</td>
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<td>AJTL71</td>
<td>Hit #4</td>
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<td>S-S</td>
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</tr>
<tr>
<td>BR16</td>
<td>Hit #5</td>
<td>KWCAVMCV-NH₂</td>
<td>Linear</td>
<td>1041</td>
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<tr>
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<td>Ac-KWCAVMCV-NH₂</td>
<td>Linear</td>
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<td>BR26</td>
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<td>S-S</td>
<td>1039</td>
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<tr>
<td>BR27</td>
<td>Hit #5</td>
<td>Ac-KWCAVMCV-NH₂</td>
<td>S-S</td>
<td>1081</td>
<td>141</td>
</tr>
<tr>
<td>AJTL68</td>
<td>Hit #6</td>
<td>FPCGLICY-NH₂</td>
<td>Linear</td>
<td>914</td>
<td>431</td>
</tr>
<tr>
<td>AJTL67</td>
<td>Hit #6</td>
<td>Ac-FPCGLICY-NH₂</td>
<td>Linear</td>
<td>955</td>
<td>190</td>
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<tr>
<td>AJTL97</td>
<td>Hit #6</td>
<td>FPCGLICY-NH₂</td>
<td>S-S</td>
<td>912</td>
<td>≥ 470</td>
</tr>
<tr>
<td>AJTL89</td>
<td>Hit #6</td>
<td>Ac-FPCGLICY-NH₂</td>
<td>S-S</td>
<td>954</td>
<td>63</td>
</tr>
<tr>
<td>AJTL83</td>
<td>Hit #9</td>
<td>YWCKGLKC-NH₂</td>
<td>Linear</td>
<td>1227</td>
<td>465</td>
</tr>
<tr>
<td>AJTL82</td>
<td>Hit #9</td>
<td>Ac-YWCKGLKC-NH₂</td>
<td>Linear</td>
<td>1268</td>
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<tr>
<td>ALTJ93</td>
<td>Hit #9</td>
<td>YWCKGLKC-NH₂</td>
<td>S-S</td>
<td>1225</td>
<td>463</td>
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<tr>
<td>AJTL91</td>
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<td>Ac-YWCKGLKC-NH₂</td>
<td>S-S</td>
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<td>464</td>
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<tr>
<td>BR32</td>
<td>Hit #9</td>
<td>YWCKGLKC-NH₂</td>
<td>S-me-S</td>
<td>1354</td>
<td>≥ 470</td>
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<tr>
<td>AJTL85</td>
<td>Hit #10</td>
<td>SVCDFGLCY-NH₂</td>
<td>Linear</td>
<td>890</td>
<td>441</td>
</tr>
<tr>
<td>AJTL84</td>
<td>Hit #10</td>
<td>Ac-SVCDFGLCY-NH₂</td>
<td>Linear</td>
<td>931</td>
<td>340</td>
</tr>
</tbody>
</table>
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” peptides). 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.