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# Structure-based design, synthesis, and pharmacologic evaluation tf peptide RGS4 inhibitors

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**Key words:** cyclic peptides; G-proteins; GTPase-activating proteins; protein–protein interactions; regulator of G-protein signaling

**Abstract:** Regulators of G-protein signaling (RGS) proteins form a multifunctional signaling family. A key role of RGS proteins is binding to the G-protein Gα-subunit and acting as GTPase-activating proteins (GAPs), thereby rapidly terminating G protein-coupled receptor (GPCR) signaling. Using the published RGS4–$G_{i\alpha}1$ X-ray structure we have designed and synthesized a series of cyclic peptides, modeled on the $G_{i\alpha}$ Switch I region, that inhibit RGS4 GAP activity. These compounds should prove useful for elucidating RGS-mediated activity and serve as a starting point for the development of a novel class of therapeutic agent.

**Abbreviations:** CHO, Chinese hamster ovary; GAP, GTPase-activating proteins; GPCR, G protein-coupled receptor; GST, glutathione S-transferase; RGS, regulator of G-protein signaling; RP-HPLC, reverse-phase high-performance liquid chromatography.

Regulators of G-protein signaling (RGS) proteins are a family of highly diverse, multifunctional signaling proteins. This distinct mammalian family was recognized when a 120-residue homologous domain (defined as the RGS domain) was described in several proteins at approximately the same time (1,2) and was demonstrated to be responsible for binding of the RGS protein to the G-protein α-subunit. Upon this binding, RGS proteins act as GTPase-activating proteins (GAPs) to limit the lifetime of bound guanosine triphosphate (GTP), thus inhibiting G-protein signaling by the rapid turnoff of the G protein-coupled receptor (GPCR) signaling pathway (3–5). Thus, RGS proteins represent a novel drug target, as inhibitors of RGS acceleration of GTP hydrolysis by $G_\alpha$ could, in principle, potentiate the effects of agonist GPCR ligands (6,7).

In 1997, Tesmer and colleagues (8) reported a 2.8 Å resolution crystal structure of the RGS protein, RGS4, complexed with $G_{i\alpha1}$-$Mg^{2+}$-GDP-$AlF_4^-$. In this complex the GDP-$AlF_4^-$ mimics the transition state of GTP during its hydrolysis to guanosine diphosphate (GDP), inducing the conformation of $G_\alpha$ thought to be stabilized by RGS-binding. The core domain of RGS4 forms a nine α-helix bundle that binds to the three distinct 'Switch' regions of $G_{i\alpha1}$, so named because they undergo large conformational changes during the transition from the inactive GDP-bound form of the $G_\alpha$ subunit to the active GTP-bound form (9). These conformational changes allow the Switch regions to contact the γ-phosphate of GTP and play an essential role in GTP hydrolysis by $G_\alpha$. The crystal structure of the complex reveals that RGS4 does not contribute catalytic residues that directly interact with either GDP or $AlF_4^-$. Instead, RGS4 appears to promote rapid hydrolysis of GTP primarily by stabilizing the Switch regions of $G_{i\alpha1}$ in the transition state conformation.

Analysis of the RGS4–$G_{i\alpha1}$ crystal structure provides opportunities for the structure-based design of inhibitors of the RGS interaction with Gα-subunits. Such inhibitors would be expected to block RGS GAP activity and thus increase the transduction efficiency of the associated Gα-subunit. These types of RGS inhibitors could then be used as potentiators of endogenous agonist function similar to the action of benzodiazepines at the ionotropic GABA-A receptor (10). We describe here initial results of structure-based inhibitors of RGS4 GAP activity that demonstrate the feasibility of this approach.

## Materials and Methods

### Solid phase peptide synthesis

All peptides were synthesized by solid-phase methods on an ABI Model 431A solid phase peptide synthesizer (Applied Biosystems, Foster City, CA, USA), using Fmoc-protected amino acids obtained from Advanced ChemTech (Louisville, KY, USA). Rink resin (Advanced ChemTech, Louisville, KY, USA) was used as the solid support for C-terminal carboxamide peptides and pre-loaded PEG-PS resin (Applied Biosystems) was used for the C-terminal carboxylic acid peptide. Peptide elongation on the peptide–resin involved treating resin with piperidine (Aldrich, Milwaukee, WI, USA) to cleave the Fmoc-protecting group, followed by coupling of the next amino acid with *o*-benzotriazol-1-yl-N,N,N′,N′-tetramethyl uronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) (Applied Biosystems). Trifluoroacetic acid/$H_2O$/dithioethane (9 : 0.5 : 0.5, v/v/v) 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.

### General method for disulfide cyclization of peptides

To obtain disulfide cyclized peptide, linear disulfhydryl-containing peptide was dissolved in a 1% (v/v) HOAc in $H_2O$ solution (saturated with $N_2$) at 5 °C (1 mg linear peptide/mL of aqueous HOAc solution). The pH of the peptide solution was raised to 8.5 using $NH_4OH$, followed by the addition of 4 mol equivalents of $K_3Fe(CN)_6$. The reaction mixture was stirred for 1 min, then quenched by adjusting the pH to 3.5 with HOAc. The mixture was then subjected to preparative RP-HPLC to afford the disulfide-cyclized peptide.

### General method for dithioether cyclization of peptides

To form dithioether-containing cyclic peptides, linear disulfhydryl peptide was added to dimethylformamide and maintained at 5 °C under a $N_2$ atmosphere (0.1 mg linear peptide/mL dimethylformamide). Five mole equivalents of potassium *tert*-butoxide were added to the peptide solution, followed by the addition of 2.5 mol equivalents of Br-$(CH_2)_n$-Br ($n = 1$, 2, or 3). The reaction was quenched with 2 mL HOAc after 2 h and the solvent was removed in vacuo. The residue was dissolved in water, filtered, and then subjected to preparative RP-HPLC to afford the alkyl dithioether-cyclized peptide.

All final product peptides were >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% trifluoroacetic acid (TFA) in water/0.1% TFA in acetonitrile by a gradient of 0–40% organic component in 40 min, and all peptides displayed the appropriate molecular weights as determined by mass spectrometry. Physicochemical data for product peptides are summarized in Table 1.

### Biological materials

[$\gamma$-$^{32}$P]GTP (30 Ci/mmol) was from New England Nuclear (NEN) Life Science Products (Boston, MA, USA). A Chinese

***Table 1.*** **Physicochemical data for peptide inhibitors of RGS4 GAP activity**

| Compound numbers | RP-HPLC elution time (min)[a] | Mass Spectrometry $[M + H]^+$ |
|---|---|---|
| **1** | 22.8 | 919.4 |
| **2** | 25.9 | 921.4 |
| **3** | 24.2 | 905.4 |
| **4** | 25.8 | 933.4 |
| **5** | 23.5 | 891.4 |
| **6** | 25.0 | 893.5 |
| **7** | 18.1 | 877.3 |
| **8** | 25.1 | 920.3 |

a. See Methods for RP-HPLC details.
RGS4, regulator of protein signaling; GAP, GTPase-activating proteins; RP-HPLC, reverse-phase high-performance liquid chromatography.

hamster ovary (CHO) cell line with stable expression of an Hemaglutinin (HA)-epitope tagged porcine $\alpha_{2a}$AR adrenoceptor ($\alpha_{2a}$AR-CHO, 10–20 pmol/mg) was cultured and cell membranes prepared as described (11). Glutathione S-transferase (GST) fusion protein containing rat RGS4 was prepared as described (12).

#### [$^{32}$P]GTPase assay

The ability of RGS proteins to stimulate steady-state [$^{32}$P]GTPase activity of receptor-stimulated G-proteins in CHO cell membranes expressing high (>5 pmol/mg) concentrations of $\alpha_{2a}$AR was recently described by Zhong *et al.* (13). To assess peptide inhibition of RGS-stimulated [$^{32}$P]GTPase, measurements were performed in a reaction mixture (100 μL) containing 0.2 mM ATP, 1 μM GDP, 50 units/mL creatine phosphokinase, 50 mM phosphocreatine, 20 mM NaCl, 2 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris/HCl, 1 mM dithiothreitol (DTT), and 0.1 μM [$^{32}$P]GTP (pH 7.6). All components were pre-incubated for *c.* 10 min on ice along with 4 μg of $\alpha_{2a}$AR-CHO membrane protein, the $\alpha_{2a}$AR adrenoceptor agonist, UK 14 304, (10 μM), 1–300 μM synthesized peptide, with or without 300 nM GST-RGS4 protein. The reaction was started by addition of [$\gamma$-$^{32}$P]GTP to the pre-incubation mixture and GTP hydrolysis was allowed to proceed for 10 min at 30 °C. The reaction was then terminated by adding 1 mL of 50% (w/v) ice-cold activated charcoal slurry in 20 mM phosphoric acid, followed by incubation on ice for 30 min. Reaction tubes were then centrifuged at 4000 × *g* for 20 min at 4 °C and 200 μL of supernatant fluid containing the free [$^{32}P_i$] was withdrawn and counted by liquid scintillation counting. Each peptide was tested in three separate experiments. Data were fit by nonlinear least-squares analysis in Graph Pad Prism 3.0 (San Diego, CA, USA) to the equation

$$Y = Y\max + (Y\min - Y\max)/(1 + 10^{\log IC_{50} - X})$$

where, $X$ is the logarithm of peptide concentration, $Y$ is the GTPase activity, $Y$max is the maximum and $Y$min is minimum GTPase activity. $Y$min was constrained to equal the GTPase activity in the absence of RGS4.

## Results and Discussion

From the crystal structure of the RGS4–$G_{i\alpha1}$-$Mg^{2+}$-GDP-$AlF_4^-$ complex (Protein Data Bank (PDB) file 1agr) it can be seen that the functional binding site for RGS4 on the surface of $G_{i\alpha1}$ is formed by residues in the three Switch regions of $G_{i\alpha1}$: residues 179–185 in Switch I, residues 204–213 in Switch II, and residues 235–237 in Switch III. Of these regions, Switch I of $G_{i\alpha1}$ interacts with three-fourths of the RGS4-binding pocket (8). Furthermore, two surface residues of Switch I (Thr182 and Gly183) appear to be essential for high-affinity G$\alpha$–RGS interaction (8,14). Therefore, the Switch I region was chosen as the starting point for the development of small peptide ligands designed to bind to the RGS4 protein and inhibit its GAP activity by preventing the RGS4–$G_{i\alpha1}$ interaction.

As depicted in Fig. 1, the RGS4-binding region of Switch I, residues 179–185, has the amino acid sequence Val-Lys-Thr-Thr-Gly-Ile-Val. Additionally, Glu186, which is conserved in most $G_\alpha$-subunits, is positioned such that it may interact with nearby positively charged residues of RGS4 (Arg172 and Arg167). Therefore, we chose the octa-peptide, Val$^{\mathbf{1}}$-Lys-Thr-Thr-Gly-Ile-Val-Glu$^{\mathbf{8}}$, as the starting point for design of analogs of the $G_{i\alpha1}$ Switch I region. As linear peptides based upon the RGS-binding region of Switch 1 would be expected to be very flexible, they would be unlikely to highly populate the desired native conformation observed in the RGS4–$G_{i\alpha1}$ X-ray structure. Indeed, the linear Switch I peptide has been prepared and was found to be devoid of inhibitory activity (K.-L. Lan and R. R. Neubig, unpublished data). Hence, our approach was to focus on cyclic peptide analogs, as cyclization is expected to reduce conformational freedom and enhance binding affinity by mimicking or inducing bound structure motifs. From the crystal structure (Fig. 1), it can be seen that the side chains of Thr181 and Val185 of $G_{i\alpha1}$ are pointing toward each other

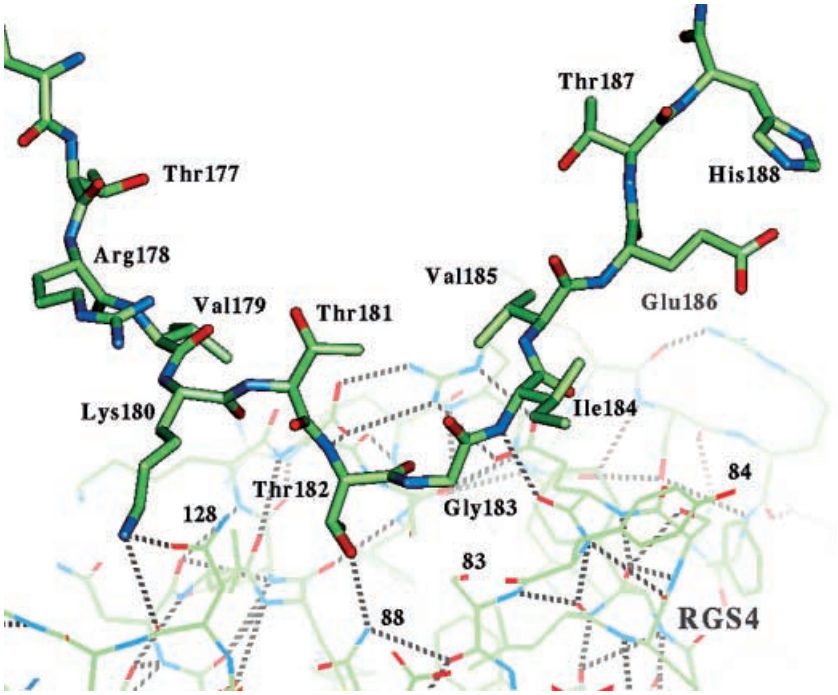
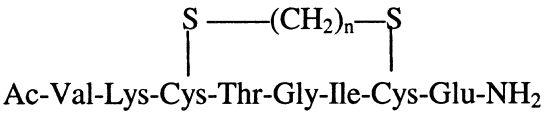



*Figure 1*. Interface between regulator of protein signaling (RGS4) (thin lines) and $G_{i\alpha 1}$ Switch I (thick lines) from X-ray structure of Tesmer *et al*. (8).

and have no direct interaction with RGS4. These residues are thus ideal candidates for substitution by amino acids that allow side chain–side chain cyclization. Accordingly, we designed an initial peptide in which cysteine is substituted for the corresponding Thr[3] and Val[7] residues in the model octa-peptide. Cyclization can be affected by linking these two Cys-residues through a disulfide or dithioether-bridge. Figure 2 depicts a model of the ethylene dithioether-containing cyclic analog, **1**, in the RGS4-binding site. Although cyclization via an ethylene dithioether provides the best spatial fit to the observed distance between the α carbons Thr181 and Val185 in the X-ray structure (8.665 Å in X-ray vs. 8.486 Å in the modeled ethylene dithioether) the optimal geometry for a small peptide ligand may differ from that of the corresponding region of the much larger Gα-subunit. Consequently a more structurally diverse set of peptide scaffolds was sampled for possible mimics of the $G_{i\alpha 1}$

Switch 1 region:




where, $n = 0$–3. Amino-terminal acetylation and C-terminal amidation were chosen to best mimic the corresponding region of $G_{i\alpha 1}$.

Figure 3 depicts the concentration dependence of the inhibition of RGS4 GAP activity exhibited by compound **1**–**5** and Table 2 summarizes the inhibitory potencies of these peptides. As seen in Table 2, compound **1**, the lead ethylene dithioether-containing analog, does indeed inhibit RGS

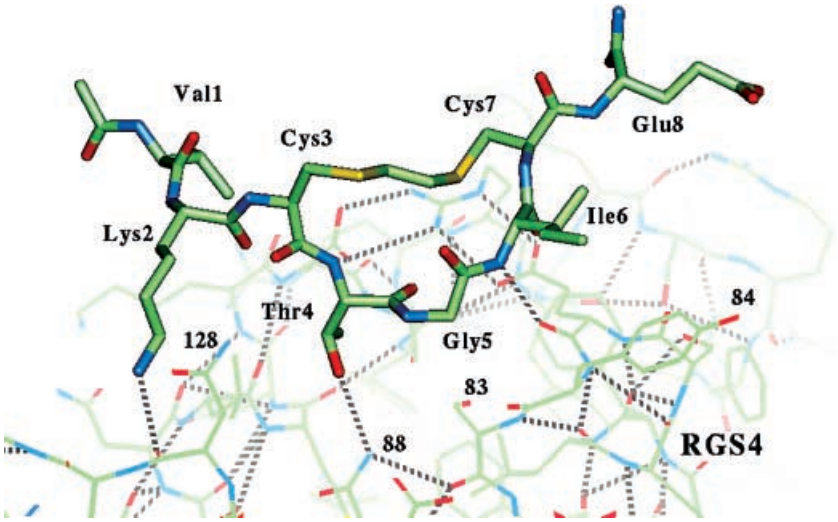



*Figure 2*. Interface between regulator of protein signaling (RGS4) (thin lines) and designed ethylene-bridged dithioether peptide, **1** (thick lines). Cysteine residues are indicated by their sequence positions, 3 and 7.

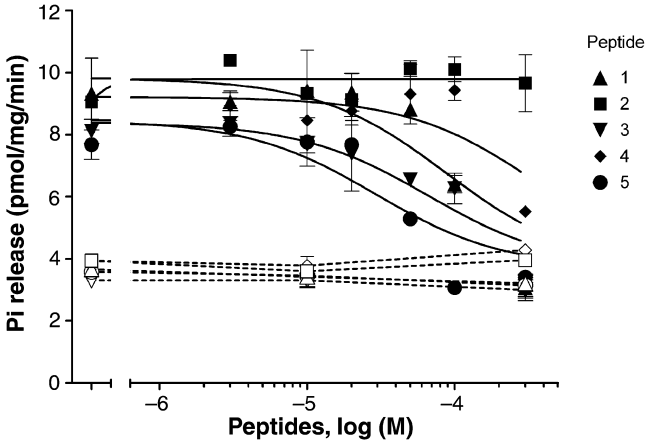



*Figure 3*. Inhibition by peptides **1–5** of regulator of protein signaling (RGS) stimulation of GTPase activity. Pi release as a function of peptide concentration in the presence (closed symbols) or absence (open symbols) of 300 nM glutathione S-transferase (GST)-RGS4 protein is shown.

***Table 2.*** **$IC_{50}$ values of peptide inhibitors of RGS4 GAP activity**

| Numbers | Structure | $IC_{50}$ ($\mu$M) ± SEM |
|---|---|---|
| **1** | Ac-Val-Lys-Cys-Thr-Gly-Ile-Cys-Glu-$NH_2$ (Et)$_a$ | 88 ± 12 |
| **2** | Ac-Val-Lys-Cys(Me)-Thr-Gly-Ile-Cys(Me)-Glu-$NH_2$ | ≫300 |
| **3** | Ac-Val-Lys-Cys-Thr-Gly-Ile-Cys-Glu-$NH_2$ (Me) | 79 ± 6 |
| **4** | Ac-Val-Lys-Cys-Thr-Gly-Ile-Cys-Glu-$NH_2$ (Pr) | 61% @ 300 $\mu$M |
| **5** | Ac-Val-Lys-Cys-Thr-Gly-Ile-Cys-Glu-$NH_2$ (SS) | 26 ± 2 |
| **6** | Ac-Val-Lys-Cys(SH)-Thr-Gly-Ile-Cys(SH)-Glu-$NH_2$ | ≫300 |
| **7** | H-Val-Lys-Cys-Thr-Gly-Ile-Cys-Glu-$NH_2$ (Et) | ≫300 |
| **8** | Ac-Val-Lys-Cys-Thr-Gly-Ile-Cys-Glu-OH (Et) | ≫300 |

a. Cyclization type between the two Cys-residues denoted in parentheses:
(SS), -SS-; (Me), -$SCH_2$S-; (Et), -$SCH_2CH_2$S-; (Pr), -$SCH_2CH_2CH_2$S-; RGS4, regulator of protein signaling; GAP, GTPase-activating proteins.

acceleration of $G_{i\alpha 1}$ GTPase activity, with an $IC_{50}$ of 88 μM. Compound **1** did not inhibit GTPase activity of receptor-membranes alone indicating that the effect is on RGS4 and not on receptor or G-protein. Consistent with the earlier observation that the linear octa-peptide corresponding to the Switch I region is inactive, compound **2**, the linear analog of **1** (with each Cys-sulfur converted to a methyl thioether) displays no inhibition of GTPase activity at the highest concentration tested, 300 μM. Compounds **3–5** further examine the effect of ring size in the cyclic RGS inhibitor series. As seen from Table 2, **3**, the methylene dithioether (dithioacetal), in which the ring is one carbon smaller, displays comparable inhibitory potency as **1**. In contrast **4**, the propylene dithioether, with ring size one carbon larger than **1**, displays considerably lower inhibitory potency (61% inhibition at 300 μM). Interestingly, **5**, the disulfide-containing analog, whose ring size is two carbon atoms smaller than the lead compound **1**, displays *c.* threefold higher potency ($IC_{50}$ = 26μM). To examine the possibility that the improved potency of **5** is due to the reduced, linear sulhydryl-containing species, **6**, the free sulfhydryl-containing precursor of **1**, **3–5**, was evaluated. As shown in Table 2, **6** displayed no RGS inhibitory activity at 300 μM, the highest concentration tested, indicating that contribution of the free sulfhydryl form of the peptide to the observed potency of **5** is unlikely. Finally, the design assumption that N-acetylation and C-terminal amidation represent the optimal starting point for analogs in this series was evaluated by preparing and testing the free amino containing analog, **7**, and the free carboxylate containing analog, **8**. As expected, neither of these analogs inhibited RGS GAP activity at 300 μM.

These results support the hypothesis that structure-based design of inhibitors of RGS protein GAP activity modeled upon the RGS-binding conformation of $G_{i\alpha 1}$ is a viable approach and, further, represent the first examples of any rationally designed RGS inhibitors. Efforts to improve potency within this series are in progress.

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