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

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Regulators of G protein signaling accelerate GTP hydrolysis by Gα subunits and profoundly inhibit signaling by G protein-coupled receptors. The distinct expression patterns and pathophysiologic regulation of regulators of G protein signaling proteins suggest that inhibitors may have therapeutic potential. We previously reported the design, mechanistic evaluation, and structure–activity relationships of a disulfide-containing cyclic peptide inhibitor of RGS4, YJ34 (*Ac*-Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-Glu-*NH₂*, S-S) (Roof *et al.*, Chem Biol Drug Des, 67, 2006, 266). Using a focused one-bead, one-compound peptide library that contains features known to be necessary for the activity of YJ34, we now identify peptides that bind to RGS4. Six peptides showed confirmed binding to RGS4 by flow cytometry. Two analogs of peptide 2 (Gly-Thr-c[Cys-Phe-Gly-Thr-Cys]-Trp-*NH₂*, S-S with a free or acetylated *N*-terminus) inhibited RGS4-stimulated Gα₀ GTPase activity at 25–50 μM. They selectively inhibit RGS4 but not RGS7, RGS16, and RGS19. Their inhibition of RGS4 does not depend on cysteine-modification of RGS4, as they do not lose activity when all cysteines are removed from RGS4. Peptide 2 has been modeled to fit in the same binding pocket predicted for YJ34 but in the reverse orientation.

Key words: focused library, one-bead one-compound library, protein–protein interaction inhibitors, regulators of G-protein signaling, structure–activity relationship

Abbreviations used: ANOVA, analysis of variance; DIEA, *N*,*N*-diisopropylethylamine; DMSO, dimethylsulfoxide; GAP, GTPase accelerating protein; GPCR, G protein-coupled receptor; HBTU, 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3,-tetramethylaminium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; NMP, *N*-methylpyrrolidone; OBOC, one-bead, one-compound; PPI, protein–protein interaction; RGS, regulators of G protein signaling; TFA, trifluoroacetic acid; TIPS, triisopropylsilane

Regulators of G Protein Signaling (RGS) proteins are GTPase accelerating proteins (GAPs) for Gα subunits (1). They diminish G protein-coupled receptor (GPCR) signaling by binding to the activated Gα protein and stabilizing the transition state for GTP hydrolysis (2). Because RGS proteins have Gα specificity (1), receptor specificity (3–6), and unique expression patterns (7), it has been suggested that RGS inhibitors could selectively potentiate GPCR signaling in specific tissues or brain regions (8–10). Others have suggested that RGS inhibitors would be useful treatments for hypertension (11), Parkinson's Disease (12), pain (13), cocaine reward (14), asthma (15), diabetes (16,17), and cancer (18,19).

There have been over 30 RGS proteins identified (20), which are divided into eight families (RZ, R4, R7, R12, RA, GEF, GRK, and SNX families) based on the homology of the 120 amino acid RGS domain as well as the presence or absence of other domains (1). The R4 family contains the RGS domain as well as an amphipathic *N*-terminus (21,22) that plays a role in membrane targeting and/or receptor specificity (21–23). RGS4 is the prototypical member of the R4 family. It has roles in cardiovascular and central nervous system signaling and was the starting point for our RGS inhibition efforts.

We previously reported the first RGS inhibitor, **YJ34** (*Ac*-Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-Glu-*NH₂*, S-S) (24). This peptide was designed to mimic the switch 1 region of the Gα subunit and was modeled from the RGS4-Gα$_{i1}$ crystal structure (25). It has a sequence similar to the switch 1 region with two amino acid substitutions (Thr181 to Cys and Val185 to Cys) to incorporate a disulfide bridge, which constrains the peptide in the correct conformation (24,26). This peptide has an IC$_{50}$ of 26 μM in a membrane-based steady state GTPase assay (24), a 9 μM IC$_{50}$ in a purified protein single turnover GTPase assay (26), inhibits RGS4 81% at 40 μM in a capillary electrophoresis assay (27), and an analog inhibits RGS modulation of G-protein inwardly rectifying potassium current kinetics in atrial myocytes (26). It has been found that **BR2** (*Ac*-Val-Lys-c[Cys-Thr-Ser-Ile-Cys]-Glu-*NH₂*, S-S), a peptide that mimics the RGS-insensitive G184S mutation in the Gα protein, is inactive, suggesting that **YJ34** binds to RGS4 as designed (i.e., in the same way that the Gα switch 1 binds). Structure–activity relationship 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 residues at positions 3 and 7 linked by a disulfide bridge (26).

Peptide **YJ34** is a useful tool for studying RGS function, but we wanted to find other, hopefully more potent, RGS inhibitors. We report here the use of a focused one-bead, one-compound (OBOC) (28) peptide library to screen for RGS4 inhibitors. The library was restricted to contain peptides with the features mentioned above that are required for the function of **YJ34**. The other five 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 containing only one peptide sequence each. Half of the peptides on each bead had an acetyl group, because it is important for peptide **YJ34** function, while half had a free N-terminus, to allow sequencing by Edman degradation. From this screen, we identified seven peptide sequences that bound RGS4. 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 (Louisville, KY, USA). Fmoc protected and acetylated amino acid purity was verified by high performance liquid chromatography (HPLC). Tentagel Resin was purchased from ChemImpex (Wood Dale, IL, USA) or Rapp-Polymer (Tubingen, Germany), and Alexa Fluor label from Invitrogen (Carlsbad, CA, USA). Peptide synthesis grade chemicals were purchased from either Applied Biosystems (Foster City, CA, USA) or Sigma-Aldrich (St Louis, MO, USA). $\gamma[^{32}P]GTP$ (10 mCi/mL) was purchased from Perkin Elmer (Boston, MA, USA) 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-$G\alpha_o$ (rat) and RGS4Δ18N (rat), were expressed and purified according to previous protocols (29–32). 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 RGS4Δ18 construct and purified over an amylose column followed by an 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 carried out using the 'QuickChange Multi Site-Directed Mutagenesis Kit' from Stratagene according to the manufacturer's protocol. Labeling of RGS4 with succinimide ester fluorophores (Alexa Fluor 568 and Alexa Fluor 532) was performed according to the manufacturer's protocol using approximately twofold to threefold excess fluorophore.

OBOC peptide library synthesis

The protocol was based on previous reports (28,33) using a manual 'mix and split' synthesis. TentaGel amide resin (10 g of 130 μm-sized beads with a substitution level of 300 pmol) was swelled in N-methylpyrrolidone (NMP) and divided by volume into 19, 8.0-mL polypropylene filter columns (Alltech, Deerfield, IL, USA). To each column, a solution of a different Fmoc-protected amino acid in NMP was added; all natural amino acids were used except cysteine. This was followed by a solution containing a threefold excess 2-(1H-Benzotriazole-1-yl)-1,1,3,3,-tetramethyl-aminium hexafluorophosphate/1-hydroxybenzotria-zole (HBTU/HOBt) and 2 M N,N'-diisopropylethylamine (DIEA) in NMP and the mixture was shaken for 1 h at room temperature. Then the beads were rinsed thoroughly with NMP. Unreacted amino groups 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 for free amines, 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 (position 3 or 7) or Fmoc-Gly-OH (position 5) was coupled to the whole batch of beads. 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 (33), all the beads were pooled and treated with 50 mL ice-cold 88:5:5:2 trifluoroacetic acid (TFA):phenol:water:triisopropylsilane (TIPS) and shaken for 3 h at room temperature. Treatment of test peptides with deprotection reagents resulted in no detectable cleaved peptide as checked by HPLC. After thorough washing (including a wash in 10% DIEA to neutralize obtained TFA salts), the resin was added to 7.8-L oxidation solution [20% Dimethylsulfoxide (DMSO), 5% Acetic acid, in water pH 6.0 purged of air with N_2] and shaken for 48 h.

Library screening

Beads were washed and plated in a single layer (on an average, approximately 1500 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 beads with **YJ34** were included in every scan as a control. Fluorescence was imaged on a Typhoon 9200 Gel Imager using an excitation of 532 nm and an emission of 610 nm and the fluorescence was quantified with the accompanying ImageQuant software. To do this, a 12 × 8 grid was placed over the image such that each grid block contained only one well. Grid blocks that contained an object with intensity more than 5- to 10-fold over the most intense object in the wells containing **YJ34** were collected and pooled. The image was visually inspected and any objects that were clearly not beads, such as dust or other debris, were excluded. Pooled beads were washed with buffer, diluted, and re-screened against 10 nM RGS4Δ18N-Alexa Fluor 568. The process was repeated with lower RGS4 concentrations until a single bead per well was obtained. The top 20 hits were then isolated with a needle under a dissecting microscope and sent for sequencing by Edman degradation at the Biomedical Research Core Facilities at the University of Michigan.

Synthesis of individual peptides

Soluble (not bead-bound) peptides (for GAP assays) were synthesized on Rink resin, cleaved from the resin and cyclized as described previously (24,26). Peptide purity (at least 95%) and solubility were verified by HPLC and correct mass was verified by mass spectrometry analysis (26). **YJ34** and **BR2** bead-bound controls were synthesized on 130 μm Tentagel resin using the same protocol as the library except that no 'mix and split' steps were incorporated. Hit peptides on beads (for the FACScan assays) were synthesized on 20 μm Tentagel resin using a LabMate apparatus (Advanced ChemTech) and cyclized manually using the similar chemistries as for the library with no 'mix and split' randomization. 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 per well in 0.5 mL buffer (1% BSA in 20 mM Hepes pH 8.0) for 1 h. The supernatant was removed and various concentrations of Alexa Fluor 568 labeled RGS4Δ18N were added in 200 μL followed by incubation for 15 min. After a wash with 500 μL buffer, fluorescence was measured in black Costar 96-well plates in a Victor[2] fluorescence plate reader with an excitation filter at 560 and an emissions filter at 595. Samples were measured in duplicate.

FACScan flow cytometry

Peptide beads (about 5×10^3 per sample for a maximum of 5 nmol peptide) were washed and 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 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 (about 60% of **YJ34** beads) 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 (26).

Modeling

Peptides were modeled using Quanta (Accelrys Inc., San Diego, CA, USA) by modifying the residues from the Gα_{i1} switch 1 in the RGS4-Gα_{i1} (PDB entry: 1agr) crystal structure (25). 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 (Accelrys) 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 Gα_i fragment from the Gα_i-RGS4 complex. Images were prepared using PyMol for OS X (http://www.pymol.org).

Statistical analysis

Data are expressed as mean ± SEM (or ± SD 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 carried out on the FACScan data. Significance is indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Results and Discussion

OBOC library design and screening

It has been found previously that several structural features were necessary for activity of **YJ34**: the N-terminal acetyl group, the C-terminal amide, Gly at position 5, and Cys at positions 3 and 7 with their side chains linked via a disulfide bridge. However, the other five positions can be varied (26). As seen in Table 1, the features necessary for **YJ34** function were constrained in the library, while the other positions were randomized. All natural amino acids except Cys were used in the other five positions to give 2.5 million possible sequences. Using 10 g of 130 μm TentaGel beads provides approximately 7.9 million beads; thus each sequence would have

Table 1: OBOC library design

Name	Sequence	Bridge
Gα_i switch I	…Val-Lys-Thr-Thr-Gly-Ile-Val-Glu…	None
YJ34	*Ac*-Val-Lys-[**Cys**-Thr-**Gly**-Ile-**Cys**]-Glu-*NH$_2$*	**(S-S)**
Library	*Ac*-Xxx^1-Xxx^2-[Cys-Xxx^3-Gly-Xxx^4-Cys]-Xxx^5-*NH*– ⬤ Xxx^1-Xxx^2-[Cys-Xxx^3-Gly-Xxx^4-Cys]-Xxx^5-*NH*–⬤	(S-S)

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 five 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 300 pmol peptide on each bead, 2.5 million sequences, and 7.9 million beads in the library.
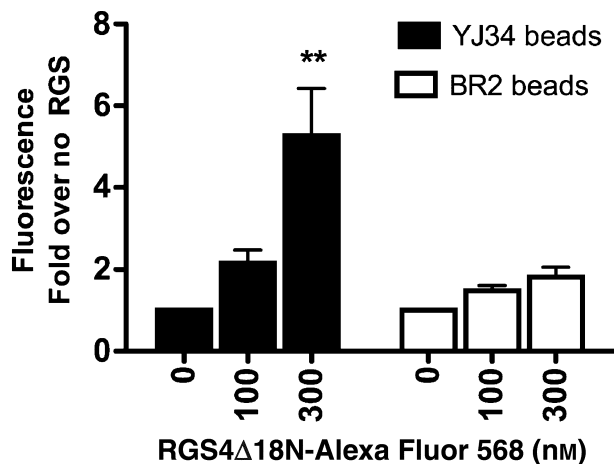
Figure 1: Alexa Fluor 568 labeled RGS4Δ18N binds peptide **YJ34** (filled bars) on beads but not peptide **BR2** (open bars) on beads. Binding of RGS4 to beads was measured as described. The experiment was carried out in duplicate (mean ± SD, $n = 2$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with no RGS4Δ18N-Alexa Fluor 568.

been present on three beads in the library, on an average. In an OBOC library, each bead has only one amino acid sequence. However, for this library, half of the peptides on each bead were acetylated, as this is necessary for the function of **YJ34**, and half had a free N-terminus, to allow sequencing by Edman degradation. The beads had a substitution level of 300 pmol per bead, resulting in about 150 pmol of free-amine-containing peptides for sequencing. This was found to be sufficient for sequencing of test peptides (data not shown). In addition, as a control, we ensured that **YJ34** on beads bound to RGS4 (Figure 1). Although TentaGel resin is known not to be cleavable with TFA treatment (33), it was verified that deprotection conditions did not remove test peptides from resin (see 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 (34), and so a red dye (Alexa Fluor 568) was chosen for RGS4 labeling for the library screen. Others have found non-specific binding to be problematic with this resin (Anna Mapp, personal communication, 2008). We did find high non-specific binding to unreacted resin (data not shown), but this was not observed with acetylated resin or with resin containing **BR2** [Ac-Val-Lys-c[Cys-Thr-Ser-Ile-Cys]-Glu-NH$_2$, S-S, which is an inactive peptide that mimics the RGS insensitive mutation in the Gα protein (26)].

To screen the library, the beads were incubated with Alexa Fluor 568 labeled RGS4Δ18N and imaged in a Typhoon Gel Imager as shown in Figure 2. The top 20 most fluorescent beads were isolated and sequenced by Edman degradation. Unfortunately, six of the hits yielded only partial sequences, leaving 14 fully sequenced hits for further evaluation.

Hit verification

Each of the 14 sequenced hit peptides was resynthesized on 20 μm TentaGel beads. As both were present in the library, free N-terminal, disulfide bridged (nd) and acetylated, disulfide bridged (ad) versions of each sequence were synthesized. Interaction with Alexa Fluor 532 labeled RGS4Δ51N was detected in a FACScan Flow Cytometer (Figure 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 of RGS4Δ51N-Alexa Fluor 532 compared with **YJ34** on beads as a cut-off, it was found that some of the initial hits were false positives, but peptides **1**, **2**, **3**, **4**, **7**, **11**, and **14** (Figure 3, Table 2) were verified and were chosen for further evaluation. The hits were structurally divergent from 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 (26).
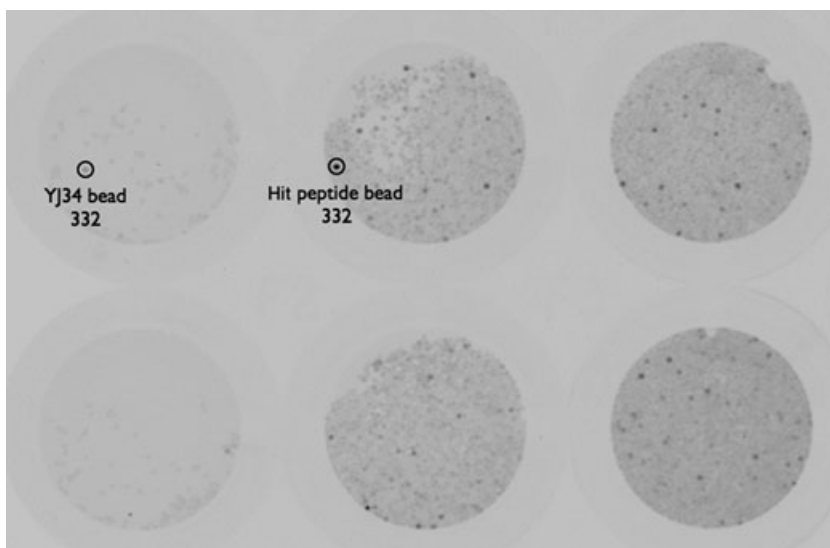
Figure 2: Screening of the library. The library was screened for binding of RGS4 as described. The left two wells contain peptide **YJ34** on beads while the right four wells contain library beads. Fluorescence was quantified and represented by the number below the circled beads.
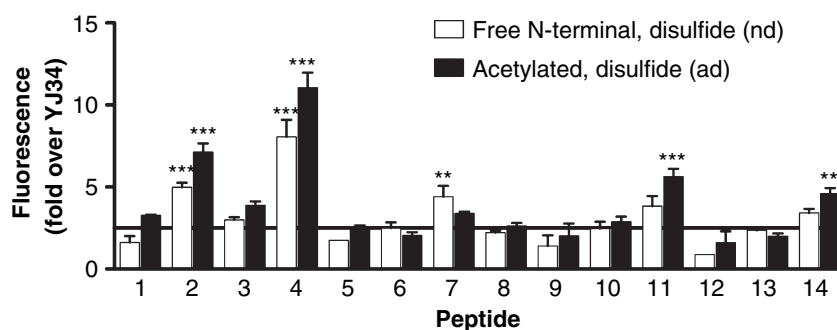
Figure 3: Hit verification. The 14 hits for which complete sequences were obtained were resynthesized on beads and tested for binding to RGS4Δ51N-Alexa Fluor 532 as described [mean ± SEM (or SD where $n = 2$), $n \geq 2$]. Acetylated peptides are the filled bars and the free *N*-terminal peptides are in the open bars. *p < 0.05, **p < 0.01, ***p < 0.001 compared with **YJ34** on beads.

Table 2: The verified hits

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

Peptides from Figure 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.

Peptide activity in GAP assays

Peptides were tested at 50 μM except for **YJ34**, **2ad**, and **14nd**, which were limited by solubility. Effects on the rate of RGS-stimulated Gαo GTPase activity were measured in a single turnover assay with purified proteins and peptides in solution (Figure 4). Hit **11** 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 RGS4 activity (48 ± 7% inhibition at 50 μM and 30 ± 12% inhibition at 25 μM, respectively). Based on these values, both peptides have an estimated IC$_{50}$ of about 50 μM. Other peptides that had some activity included **1nd** (33 ± 15% inhibition at 50 μM) and **4nd** (33 ± 16% inhibition at 50 μM, Figure 4). The activity of hit **2** was investigated further in Figure 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, as 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 5A). Consistent with previous results for **YJ34**, the linear peptides were inactive. Regulators of G protein signaling selectivity was also investigated. Interestingly, peptides **2ad** and **2nd** were inactive against RGS7, RGS16, and RGS19 (Figure 5B). The 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.
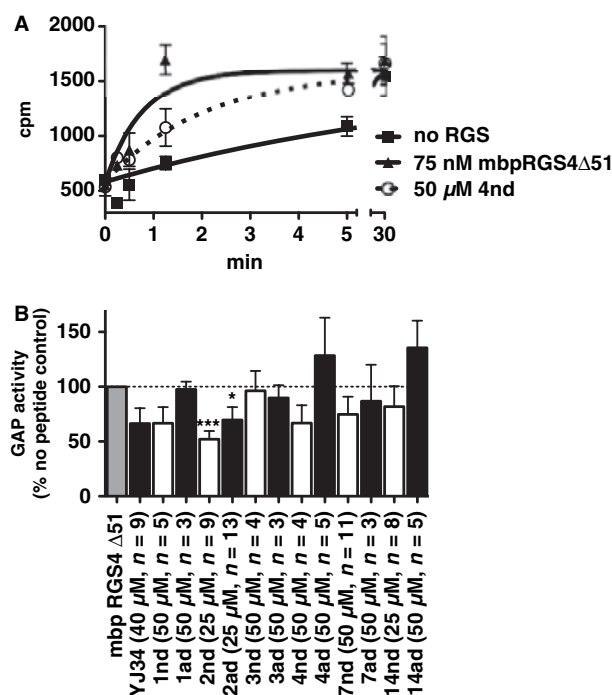
Figure 4: Activity of hit peptides in a single turnover GAP assay. (A) Representative time course. (B) Rates were calculated based on the time courses, and percent decrease in the rate of RGS-stimulated GTP hydrolysis by peptides was measured (mean ± SEM, $n \geq 3$). *p < 0.05, **p < 0.01, ***p < 0.001 compared with no peptide.

Recently, CCG-4986, a small molecule inhibitor of RGS4 was identified and found to interact with RGS4 through cysteine modification [(32,35), David Roman, unpublished observation]. For this reason, the activity of our original peptide, **YJ34**, as well as that of hit **2** was evaluated with an RGS4Δ51N mutant lacking all seven cysteines (-7C) (Figure 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% inhibition for **2ad**, **2nd**, and **YJ34**, respectively). This indicates that hit **2** does not inhibit RGS4 through cysteine modification.
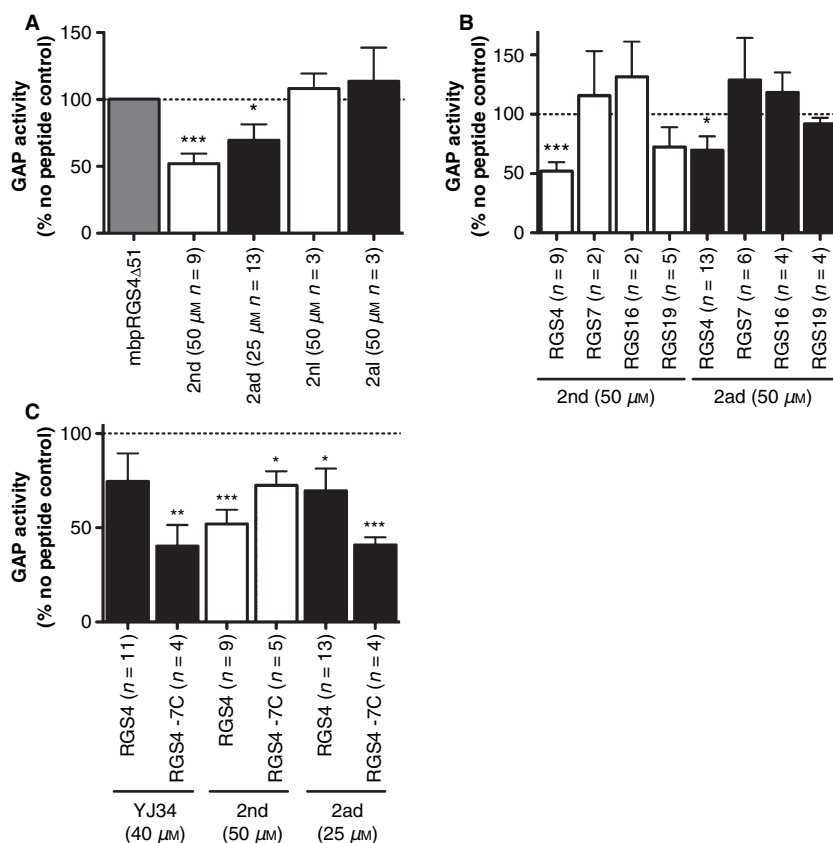
Figure 5: Functional activity of peptide **2**. (A) Disulfide-bridged (**2nd** and **2ad**), but not linear (**2nl** and **2al**) peptides inhibit RGS4Δ51N GAP activity. (B) Peptides **2nd** and **2ad** are selective for RGS4Δ51N (75–125 nM) over RGS7box (500 nM), RGS16 (1.5 μM) or RGS19 (200 nM). (C) Activity of **2nd** and **2ad** on wild type and -7C mutant (200 nM) RGS4 [mean ± SEM, (or SD where $n = 2$), $n \geq 2$]. *p < 0.05, **p < 0.01, ***p < 0.001 compared with no peptide.

Conclusions

Inhibition of protein–protein interactions (PPIs) is particularly challenging. Unlike enzymes and receptors that have well-defined pockets, protein–protein interfaces tend to be large and flat (36). Because of this, many PPIs have been declared 'non-amenable' to inhibition by drug-like molecules based on computational measurements (37). In fact, the RGS4-Gα_o interaction falls in this category (David Fry, personal communication, 2006). However, some protein–protein interfaces have been found to have 'hot spots', which are 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 (38–40). In addition, computational approaches such as those used in Fry and Vassilev (37) are based on rigid crystal 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 crystal structures of the protein alone or in complex with its natural protein-binding partner [(41) 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 was an ambitious project, there are ample precedents for success. In fact, the above-mentioned small molecule inhibitor of RGS4, CCG-4986, was found in a screen to block the RGS4-Gαo interaction (32). Inhibitors of RGS4 were also found in a yeast-based screen, but unfortunately, no structures were reported (42).

Of the seven RGS4-binding peptides found in our OBOC peptide library screen, one peptide sequence (in both free amide and acetylated form) was found to inhibit RGS4 GAP activity in the single turnover GAP assay. Although the potency of the new compounds is no greater then the starting peptide, we have identified new peptides that we believe have the desired mode of binding to RGS4, and the desired RGS4 inhibitor activity.

Based on the crystal structure of RGS4 bound to Gα_i (25), Thr182 of Gα_i forms hydrogen-bonding and hydrophobic interactions with several residues in RGS4 including Asn88, Asp163, Ser164, and Leu159. The corresponding residue in **YJ34**, Thr[4], may similarly interact with RGS4 residues. 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 does the Thr[4] side chain of **YJ34** (Figure 6A). However, if the peptide were rotated around the central Gly (i.e., oriented as a retrobinder in the **YJ34** binding site), then the Thr in position 6 would be placed into the pocket where Thr182 of switch 1 binds and the Thr[4] of **YJ34** is predicted to bind. This would also position the Phe[4] of hit **2** above Tyr84 in RGS4 where Ile185 from switch 1 is located. These two amino acid interactions are reminiscent of a previously identified **YJ34** analog, [Ac-Val-Lys-[Cys-Thr-Gly-Phe-Cys]-Glu-NH₂, S-S, peptide **20** in Roof et al. (26)], which has a Thr at position 4 and a Phe at position 6. This peptide inhibited RGS4 GAP activity by nearly 40% at 100 μM (26). Thus, we propose that hit **2** interacts with RGS4 in the switch 1 binding site in an inverse orientation (Figure 6). Based on our model, the Trp[8] residue of **2** also makes
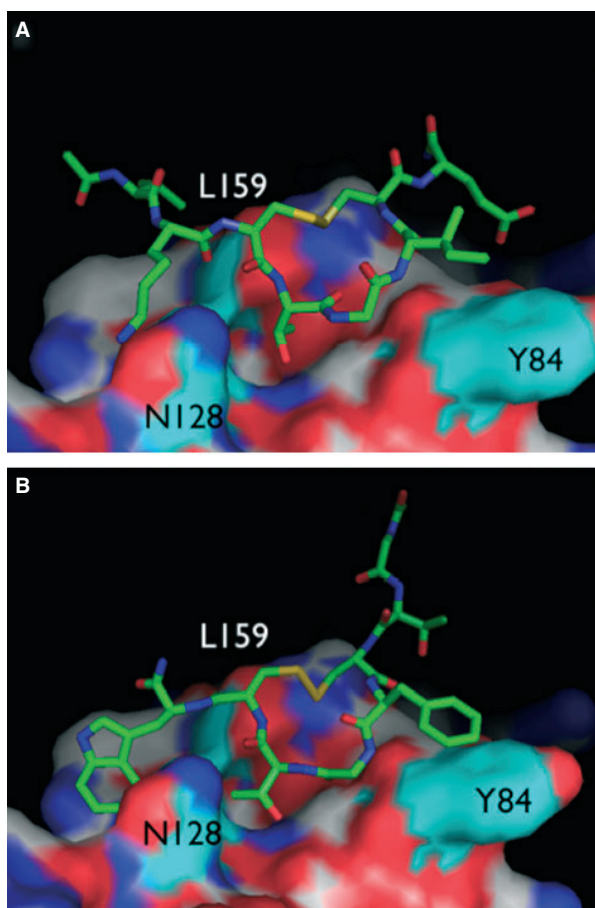
Figure 6: Model of **YJ34** and **2ad** in the switch 1 binding site of RGS4. Amino acids in RGS4 mentioned in the text are in cyan. (A) **YJ34** is modeled to bind RGS4 the same way the switch 1 region of Gα$_i$ binds. (B) **2ad** is modeled in the same pocket but in the reverse orientation.

favorable hydrophobic interactions with Leu159. On the contrary, because of the inverse position of the peptide **2** in the binding site, its N-terminus likely occupies a position of **YJ34** C-terminus, while C-terminal amide of peptide **2** is positioned similarly to **YJ34** N-terminus. The presence of positively charged Lys155 and Lys162 of RGS4 near N-terminus of **YJ34** may explain the poor activity of its non-acetylated version. However, the proposed different location of the peptide **2** N-terminus would not require its acetylation.

Although it is believed that hit **2** binds the **YJ34** binding site, it is possible that some of the other peptides do not. In fact, the observation that many of them do not have an effect on GAP activity would suggest that this may be the case and that these analogs interact with RGS in alternate ways. This may explain why peptide **4**, for example, bound RGS4 so well in the FACScan assay (Figure 3) while having little effect on RGS4 GAP activity (Figure 4). The RGS4 has been shown to have an allosteric site where phosphatidylinositol-3,4,5-trisphosphate (PIP3) and calmodulin are known to bind (43). Calmodulin binding alone does not affect GAP activity, but blocks

the inhibition by PIP3 (43). 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 structural 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.

As in the **YJ34** series, linear versions of hit **2** are inactive. In addition, as in the **YJ34** series, hit **2** is most active on RGS4 compared with RGS7. It is thought that CCG-4986 inhibits RGS4 through cysteine modification [(32,35), David Roman, personal communication, 2008]. Because of the apparent sensitivity of RGS4 to cysteine modifications, 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 -7Cys RGS4Δ51N 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 in peptide **20** in Roof *et al.* (26) 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 this increased stability makes them more useful in cells and *in vivo* (44), making them an interesting avenue for future investigations.

Acknowledgments

References

1. Hepler J.R. (1999) Emerging roles for RGS proteins in cell signalling. Trends Pharmacol Sci;9:376–382.
2. Berman D.M., Kozasa T., Gilman A.G. (1996) The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. J Biol Chem;44:27209–27212.

3. Wang Q., Liu M., Mullah B., Siderovski D.P., Neubig R.R. (2002) Receptor-selective effects of endogenous RGS3 and RGS5 to regulate mitogen-activated protein kinase activation in rat vascular smooth muscle cells. J Biol Chem;28:24949–24958.

4. Xu X., Zeng W., Popov S., Berman D.M., Davignon I., Yu K., Yowe D., Offermanns S., Muallem S., Wilkie T.M. (1999) RGS proteins determine signaling specificity of Gq-coupled receptors. J Biol Chem;6:3549–3556.

5. Hague C., Bernstein L.S., Ramineni S., Chen Z., Minneman K.P., Hepler J.R. (2005) Selective inhibition of alpha1A-adrenergic receptor signaling by RGS2 association with the receptor third intracellular loop. J Biol Chem;29:27289–27295.

6. Krumins A.M., Barker S.A., Huang C., Sunahara R.K., Yu K., Wilkie T.M., Gold S.J., Mumby S.M. (2004) Differentially regulated expression of endogenous RGS4 and RGS7. J Biol Chem;4:2593–2599.

7. Gold S.J., Ni Y.G., Dohlman H.G., Nestler E.J. (1997) Regulators of G-protein signaling (RGS) proteins: region-specific expression of nine subtypes in rat brain. J Neurosci;20:8024–8037.

8. Traynor J.R., Neubig R.R. (2005) Regulators of G protein signaling and drugs of abuse. Mol Interv;1:30–41.

9. Neubig R.R. (2002) Regulators of G protein signaling (RGS proteins): novel central nervous system drug targets. J Pept Res;6:312–316.

10. Zhong H., Neubig R.R. (2001) Regulator of G protein signaling proteins: novel multifunctional drug targets. J Pharmacol Exp Ther;3:837–845.

11. Cho H., Park C., Hwang I.Y., Han S.B., Schimel D., Despres D., Kehrl J.H. (2008) Rgs5 targeting leads to chronic low blood pressure and a lean body habitus. Mol Cell Biol;28:2590–2597.

12. Ding J., Guzman J.N., Tkatch T., Chen S., Goldberg J.A., Ebert P.J., Levitt P., Wilson C.J., Hamm H.E., Surmeier D.J. (2006) RGS4-dependent attenuation of M4 autoreceptor function in striatal cholinergic interneurons following dopamine depletion. Nat Neurosci;6:832–842.

13. Rodriguez-Munoz M., de la Torre-Madrid E., Gaitan G., Sanchez-Blazquez P., Garzon J. (2007) RGS14 prevents morphine from internalizing Mu-opioid receptors in periaqueductal gray neurons. Cell Signal;12:2558–2571.

14. Rahman Z., Schwarz J., Gold S.J., Zachariou V., Wein M.N., Choi K.H., Kovoor A. et al. (2003) RGS9 modulates dopamine signaling in the basal ganglia. Neuron;6:941–952.

15. Druey K.M. (2003) Regulators of G protein signalling: potential targets for treatment of allergic inflammatory diseases such as asthma. Expert Opin Ther Targets;4:475–484.

16. Usui I., Imamura T., Satoh H., Huang J., Babendure J.L., Hupfeld C.J., Olefsky J.M. (2004) GRK2 is an endogenous protein inhibitor of the insulin signaling pathway for glucose transport stimulation. EMBO J;14:2821–2829.

17. Huang X., Charbeneau R.A., Fu Y., Kaur K., Gerin I., MacDougald O.A., Neubig R.R. (2008) Resistance to diet-induced obesity and improved insulin sensitivity in mice with a regulator of G protein signaling-insensitive G184S Gnai2 allele. Diabetes;1:77–85.

18. Heo K., Ha S.H., Chae Y.C., Lee S., Oh Y.S., Kim Y.H., Kim S.H., Kim J.H., Mizoguchi A., Itoh T.J., Kwon H.M., Ryu S.H., Suh P.G. (2006) RGS2 promotes formation of neurites by stimulating microtubule polymerization. Cell Signal;12:2182–2192.

19. Boss C.N., Grunebach F., Brauer K., Hantschel M., Mirakaj V., Weinschenk T., Stevanovic S., Rammensee H.G., Brossart P. (2007) Identification and characterization of T-cell epitopes deduced from RGS5, a novel broadly expressed tumor antigen. Clin Cancer Res;11:3347–3355.

20. Abramow-Newerly M., Roy A.A., Nunn C., Chidiac P. (2006) RGS proteins have a signalling complex: interactions between RGS proteins and GPCRs, effectors, and auxiliary proteins. Cell Signal;5:579–591.

21. Bernstein L.S., Grillo A.A., Loranger S.S., Linder M.E. (2000) RGS4 binds to membranes through an amphipathic alpha-helix. J Biol Chem;24:18520–18526.

22. Bernstein L.S., Ramineni S., Hague C., Cladman W., Chidiac P., Levey A.I., Hepler J.R. (2004) RGS2 binds directly and selectively to the M1 muscarinic acetylcholine receptor third intracellular loop to modulate Gq∕11alpha signaling. J Biol Chem;20:21248–21256.

23. Gu S., He J., Ho W.T., Ramineni S., Thal D.M., Natesh R., Tesmer J.J., Hepler J.R., Heximer S.P. (2007) Unique hydrophobic extension of the RGS2 amphipathic helix domain imparts increased plasma membrane binding and function relative to other RGS R4∕B subfamily members. J Biol Chem;45:33064–33075.

24. Jin Y., Zhong H., Omnaas J.R., Neubig R.R., Mosberg H.I. (2004) Structure-based design, synthesis, and pharmacologic evaluation tf peptide RGS4 inhibitors. J Pept Res;2:141–146.

25. Tesmer J.J., Berman D.M., Gilman A.G., Sprang S.R. (1997) Structure of RGS4 bound to AlF4-activated G(i alpha1): stabilization of the transition state for GTP hydrolysis. Cell;2:251–261.

26. Roof R.A., Jin Y., Roman D.L., Sunahara R.K., Ishii M., Mosberg H.I., Neubig R.R. (2006) Mechanism of action and structural requirements of constrained peptide inhibitors of RGS proteins. Chem Biol Drug Des;4:266–274.

27. Jameson E.E., Roof R.A., Whorton M.R., Mosberg H.I., Sunahara R.K., Neubig R.R., Kennedy R.T. (2005) Real-time detection of basal and stimulated G protein GTPase activity using fluorescent GTP analogues. J Biol Chem;9:7712–7719.

28. Lam K.S., Salmon S.E., Hersh E.M., Hruby V.J., Kazmierski W.M., Knapp R.J. (1991) A new type of synthetic peptide library for identifying ligand-binding activity. Nature;6348:82–84.

29. Lan K.L., Sarvazyan N.A., Taussig R., Mackenzie R.G., DiBello P.R., Dohlman H.G., Neubig R.R. (1998) A point mutation in Galphao and Galphai1 blocks interaction with regulator of G protein signaling proteins. J Biol Chem;21:12794–12797.

30. Lan K.L., Zhong H., Nanamori M., Neubig R.R. (2000) Rapid kinetics of regulator of G-protein signaling (RGS)-mediated Galphai and Galphao deactivation. Galpha specificity of RGS4 AND RGS7. J Biol Chem;43:33497–33503.

31. Lee E., Linder M.E., Gilman A.G. (1994) Expression of G-protein alpha subunits in Escherichia coli. Methods Enzymol;237:146–164.

32. Roman D.L., Talbot J.N., Roof R.A., Sunahara R.K., Traynor J.R., Neubig R.R. (2007) Identification of small-molecule inhibitors of RGS4 using a high-throughput flow cytometry protein interaction assay. Mol Pharmacol;1:169–175.

33. Cabilly S. (1998) Combinatorial Peptide Library Protocols. Totowa, NJ: Humana Press.

34. Olivos H.J., Bachhawat-Sikder K., Kodadek T. (2003) Quantum dots as a visual aid for screening bead-bound combinatorial libraries. Chembiochem;11:1242–1245.

35. Kimple A.J., Willard F.S., Giguere P.M., Johnston C.A., Mocanu V., Siderovski D.P. (2007) The RGS protein inhibitor CCG-4986 is a covalent modifier of the RGS4 Galpha-interaction face. Biochem Biophys Acta;9:1213–1220.

36. Whitty A., Kumaravel G. (2006) Between a rock and a hard place? Nat Chem Biol;3:112–118.

37. Fry D.C., Vassilev L.T. (2005) Targeting protein–protein interactions for cancer therapy. J Mol Med;12:955–963.

38. Scott J.K., Huang S.F., Gangadhar B.P., Samoriski G.M., Clapp P., Gross R.A., Taussig R., Smrcka A.V. (2001) Evidence that a protein–protein interaction 'hot spot' on heterotrimeric G protein betagamma subunits is used for recognition of a subclass of effectors. EMBO J;4:767–776.

39. Trosset J.Y., Dalvit C., Knapp S., Fasolini M., Veronesi M., Mantegani S., Gianellini L.M., Catana C., Sundstrom M., Stouten P.F., Moll J.K. (2006) Inhibition of protein–protein interactions: the discovery of druglike beta-catenin inhibitors by combining virtual and biophysical screening. Proteins;1:60–67.

40. Thanos C.D., DeLano W.L., Wells J.A. (2006) Hot-spot mimicry of a cytokine receptor by a small molecule. Proc Natl Acad Sci USA;42:15422–15427.

41. Wells J.A., McClendon C.L. (2007) Reaching for high-hanging fruit in drug discovery at protein–protein interfaces. Nature;7172:1001–1009.

42. Young K.H., Wang Y., Bender C., Ajit S., Ramirez F., Gilbert A., Nieuwenhuijsen B.W. (2004) Yeast-based screening for inhibitors of RGS proteins. Methods Enzymol;389:277–301.

43. Ishii M., Fujita S., Yamada M., Hosaka Y., Kurachi Y. (2005) Phosphatidylinositol 3,4,5-trisphosphate and Ca2+/calmodulin competitively bind to the regulators of G-protein-signalling (RGS) domain of RGS4 and reciprocally regulate its action. Biochem J;385:65–73.

44. Fear G., Komarnytsky S., Raskin I. (2007) Protease inhibitors and their peptidomimetic derivatives as potential drugs. Pharmacol Ther;2:354–368.