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Allosteric Inhibition of the Regulator of G Protein Signaling–Gα Protein–Protein Interaction by CCG-4986

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ABSTRACT

Regulator of G protein signaling (RGS) proteins act to temporally modulate the activity of G protein subunits after G protein-coupled receptor activation. RGS proteins exert their effect by directly binding to the activated Gα subunit of the G protein, catalyzing the accelerated hydrolysis of GTP and returning the G protein to its inactive, heterotrimeric form. In previous studies, we have sought to inhibit this GTPase-accelerating protein activity of the RGS protein by using small molecules. In this study, we investigated the mechanism of CCG-4986 [methyl-*N*-[(4-chlorophenyl)sulfonyl]-4-nitro-benzenesulfinimidoate], a previously reported small-molecule RGS inhibitor. Here, we find that CCG-4986 inhibits RGS4 function through the covalent modification of two spatially distinct cysteine residues on RGS4. We confirm that modification of Cys132, located near the RGS/Gα interaction surface, modestly inhibits Gα binding and GTPase acceleration. In addition, we report that modification of Cys148, a residue located on the opposite face of RGS4, can disrupt RGS/Gα interaction through an allosteric mechanism that almost completely inhibits the Gα–RGS protein–protein interaction. These findings demonstrate three important points: 1) the modification of the Cys148 allosteric site results in significant changes to the RGS interaction surface with Gα; 2) this identifies a "hot spot" on RGS4 for binding of small molecules and triggering an allosteric change that may be significantly more effective than targeting the actual protein-protein interaction surface; and 3) because of the modification of a positional equivalent of Cys148 in RGS8 by CCG-4986, lack of inhibition indicates that RGS proteins exhibit fundamental differences in their responses to small-molecule ligands.

Introduction

Regulator of G protein signaling (RGS) proteins provide a critical point of control for many cellular processes and signaling cascades initiated by inhibitory G proteins. Although many traditional pharmacotherapies target G protein-coupled receptors directly, RGS proteins may represent a unique and untapped target for modulating these signaling events (Zhong and Neubig, 2001; Traynor and Neubig, 2005).

During G protein-coupled receptor activation, GDP is exchanged for GTP on Gα subunits, which releases the Gα and $\beta\gamma$ subunits to modulate their effectors, such as adenylyl cyclase, ion channels, and phospholipase C (Gilman, 1987). Gα subunits possess intrinsic GTPase activity that hydrolyzes the bound GTP. The GDP-bound Gα subunit is inactive and can reassociate with $\beta\gamma$ subunits, resulting in termination of signaling. RGS proteins are GTPase-activating proteins (GAPs) that markedly increase the rate of the Gα subunit's GTP hydrolysis and function to reduce the magnitude and duration of Gα and $\beta\gamma$ signals (Berman et al., 1996a,b; Hepler et al., 1997; Srinivasa et al., 1998; Mukhopadhyay and Ross, 1999).

Structurally, RGS4 is one of the simplest RGS proteins. It contains the RGS homology domain (RH or "box") and an N-terminal amphipathic helix. Functional elements within the RGS4 RH domain have been termed the A- and B-sites (Zhong and Neubig, 2001). The A-site contains the surface of the RGS protein that interacts with Gα subunits, whereas the B-site is located where endogenous lipid modulators and calmodulin bind to regulate RGS4 function (Popov et al., 2000).

One of our goals, which we share with other researchers, has been to explore RGS proteins as drug targets. To that

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ABBREVIATIONS: RGS, regulator of G protein signaling; CCG-4986, methyl-*N*-[(4-chlorophenyl)sulfonyl]-4-nitro-benzenesulfinimidoate; DTT, dithiothreitol; GAP, GTPase-activating protein; FCPIA, flow cytometry-based protein interaction assay; MFI, median fluorescence intensity; LC, liquid chromatography; MS, mass spectrometry; AMF, NaF, $MgCl_2$, and $AlCl_3$.

end, we have undertaken an approach to high-throughput screening that examines the RGS/Gα protein-protein interaction directly. Targeting of protein-protein interactions presents unique challenges dependent on the nature and physical features of the individual proteins in the complex and the nature of the interaction (Wells et al., 2003; Arkin and Wells, 2004; Pagliaro et al., 2004; Blazer and Neubig, 2009). The implementation of a flow cytometry-based protein interaction assay (FCPIA) led to our discovery of a small-molecule RGS4 inhibitor, CCG-4986 [methyl-*N*-[(4-chlorophenyl)sulfonyl]-4-nitro-benzenesulfinimidoate] (Roman et al., 2007b). In efforts to characterize the mechanism of RGS4 inhibition by CCG-4986, our group (Roman et al., 2007a) and Kimple et al. (2007) discovered that CCG-4986 worked through a mechanism involving the covalent modification of a cysteine residue.

The apparent covalent modification of RGS4 by CCG-4986 led us to examine the RGS4 structure for potential sites of modification that could alter RGS4 function. The RGS4 construct used in these studies lacks the first 50 amino acids (designated Δ51RGS4). This protein lacks the amphipathic amino-terminal helix that is disordered in the X-ray crystal structure (Tesmer et al., 1997), and the deletion of this helix facilitates efficient expression of soluble protein from *Escherichia coli*. The Δ51RGS4 contains seven cysteine residues, at positions 71*, 95*, 132*, 148*, 184, 197, and 201 (the asterisk indicates cysteines within the RH domain). Our first candidates were Cys71 and Cys132, because they are divergent in RGS8, an RGS that is not functionally inhibited by CCG-4986. Of these residues, Cys132 seems to be the more obvious candidate, because it resides adjacent to the A-site of the RGS protein, which contains the Gα-interacting face (Fig. 1). However, in contrast with results published by Kimple et al. (2007), we discovered two distinct modes of inhibition for CCG-4986 at RGS4, one by which Gα_o binding is inhibited by modification of Cys132, and another unique and more potent mode of allosteric inhibition, facilitated by modification of Cys148.

In these studies we test the hypothesis that RGS4 function can be significantly inhibited by the modification of a cysteine residue located distal to the RGS/Gα interaction face at an allosteric site containing Cys148. Our results identify a dual mode of action for RGS4 inhibition: CCG-4986 inhibits Gα_o binding to RGS4 through modification of Cys132 and exhibits a more remarkable noncompetitive, allosteric mode of inhibition by CCG-4986 modification of Cys148 that is unique to RGS4.

Materials and Methods

Chemicals of reagent grade or better quality were purchased from Sigma-Aldrich (St. Louis, MO), Thermo Fisher Scientific (Waltham, MA), or Acros Organics (Geel, Belgium). Avidin-coated microspheres were purchased from Luminex (Austin, TX). [^{35}S]GTPγS was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Mutagenesis kits (QuikChange) were purchased from Stratagene (La Jolla, CA).

Protein Expression and Purification. Gα_o was purified as described previously (Lee et al., 1994). In brief, rat Gα_o was purified from *Escherichia coli* BL-21(DE3) by using a polyhistidine (6×His) N-terminal tag. Lysates were purified first by using nickel nitrilotriacetic acid resin followed by Q-Sepharose ion-exchange chromatography using a DuoFlow instrument (Bio-Rad Laboratories, Hercules, CA). Protein purity was >95%, and the concentration of active G protein was determined by using GTPγS binding as described previously (Sternweis and Robishaw, 1984). RGS4 and point mutants were purified from rat RGS4 expressed as a maltose-binding protein fusion at the N terminus. This construct contains, in a single open reading frame, the maltose-binding protein, a 10×His tag, a tobacco etch virus protease recognition site, and Δ51-RGS4, which is rat RGS4 that has had the first 51 amino acids truncated. The protein is expressed as a fusion in BL-21(DE3) *E. coli* and was purified by using an amylose column. After elution with 10 mM maltose, samples of protein fractions were assessed for purity by using SDS-polyacrylamide gel electrophoresis. RGS4 used in fluorescence-based GTPase experiments was purified as a native Δ18RGS4 construct as described previously (Krumins et al., 2004; Roman et al., 2007b). A region of human RGS8 comprising the RGS homology domain (Leu60 → Ser191, as from GenBank accession number AAG45337), was cloned into pQE-80 and expressed in *E. coli* with the following primers: sense: 5′-CGCGGATCCCTCAAGAGATTATCGACAGA-3′; antisense: 5′-CTCGTCGACCTACTAGGACAGCAGATCTAAGTACA-3′. The protein was purified as described previously (Soundararajan et al., 2008).

Fluorescent Labeling of Gα_o. Purified Gα_o was chemically labeled with Alexa Fluor 532 C_5-maleimide (mol. wt. 812.88; Invitrogen, Carlsbad, CA) at a 5:1 fluorophore/protein ratio. The reaction was completed in buffer containing 50 mM HEPES and 100 mM NaCl at pH 7.5. In brief, 1 mg of Alexa Fluor 532 was resuspended in 100 μl of dimethyl sulfoxide. Thirty microliters (375 nmol) of the Alexa Fluor solution was added to 3 mg (75 nmol) of purified Gα_o (at 1.5 mg/ml), and the solution was incubated at 4°C in the dark for 1.5 h. The reaction was quenched with 1 mM DTT (final) for 30 min. Excess fluorophore was removed by diluting the reaction to 15 ml with reaction buffer, then concentrating the protein in a 15-ml Amicon concentrator (Millipore Corporation, Billerica, MA) to 200 μl, followed by resuspension in 15 ml of buffer. This concentration-dilution process was repeated four times. The activity and effective concentration of Gα_o was determined after labeling by using [^{35}S]GTPS binding (Sternweis and Robishaw, 1984).

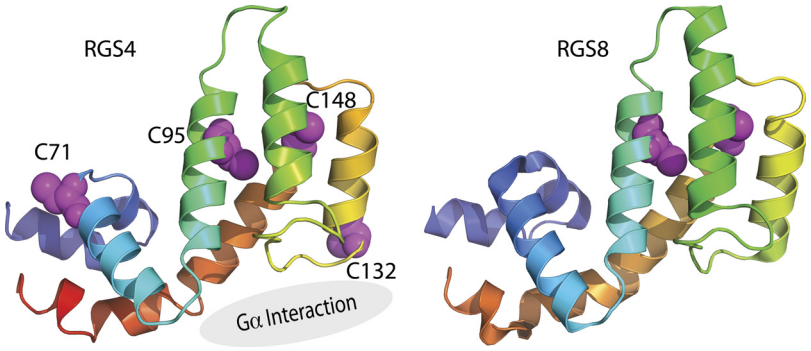

Fig. 1. Ribbon diagram of the RGS homology domain of RGS4 (left) and RGS8 (right) extracted from Protein Data Bank entries 1agr and 2ihd, respectively, with cysteine residues indicated by magenta space-filling spheres.

Chemical Biotinylation of Purified RGS Proteins. RGS proteins were biotinylated with amine-reactive biotinamidohexanoic acid *N*-hydroxysuccinimide ester (mol. wt., 454.54; Sigma-Aldrich) in a 3:1 (biotin/RGS) stoichiometry as described previously (Roman et al., 2007b).

FCPIA. Bead-based protein-protein interaction experiments were performed as described previously with the Luminex 200 instrument (Luminex) (Roman et al., 2007b). Saturation binding experiments in the presence of CCG-4986 used 10 μM GDP as a control to determine nonspecific binding and AMF to determine total binding. CCG-4986 (10 μM) was used in some binding experiments to determine the effect of the inhibitor on RGS4 binding to Gα_o.

Site-Directed Mutagenesis. RGS4 single and multiple point mutants were carried out with the Stratagene QuikChange and QuikChange multikits, respectively. Constructs generated using the QuikChange multikit require only one oligonucleotide; the A-to-C mutations were made in a Cysless RGS construct, and these add-backs were made individually by using standard QuikChange protocols with two primers each. The oligonucleotides for mutations were as follows: C71A, GCT GGA AAA CCT GAT TAA CCA TGA AGC TGG ACT GGC AGC T; C95A, GAA CAT TGA CTT CTG GAT CAG CGC TGA GGA GTA CAA GAA AAT CAA; C132A, GAG GTG AAC CTG GAT TCT GCC ACC AGA GAG GAG ACA AG; C148A, GTT AGA GCC CAC GAT AAC CGC TTT TGA TGA AGC CCA GAA G; C183A, CCA ATC CTT CCA GCG CCG GGG CAG AGA AGC; C197A, CCA AGA GTT CTG CAG ACG CCA CTT CCC TAG TCC CTC; C204A, TCC CTA GTC CCT CAG GCT GCC AAG CTT GGC AC; C95A, GAA CAT TGA CTT CTG GAT CAG CGC TGA GGA GTA CAA GAA AAT CAA; A71C sense, GCT GGA AAA CCT GAT TAA CCA TGA ATG TGG ACT GGC AGC; A71C antisense, GCT GCC AGT CCA CAT TCA TGG TTA ATC AGG TTT TCC AGC; A95C sense, CAT TGA CTT CTG GAT CAG CTG TGA GGA GTA CAA GAA AAT C; A95C antisense, GAT TTT CTT GTA CTC CTC ACA GCT GAT CCA GAA GTC AAT G; A132C sense, GAG GTG AAC CTG GAT TCT TGC ACC AGA GAG GAG ACA AGC; A132C antisense, GCT TGT CTC CTC TCT GGT GCA AGA ATC CAG GTT CAC CTC; A148C sense, GTT AGA GCC CAC GAT AAC CTG TTT TGA TGA AGC CCA GAA G; and A148C antisense, CTT CTG GGC TTC ATC AAA ACA GGT TAT CGT GGG CTC TAA C. All reactions were performed per the manufacturer's protocol, and resulting mutations were verified through bidirectional sequencing at the University of Michigan DNA Sequencing Core Facility.

Single-Turnover GTPase Assay. Single-turnover GTP hydrolysis measurements with and without RGS were performed by using protocols published previously by Lan et al. (2000) as adapted by Roof et al. (2008). For experiments assessing CCG-4986 inhibition, 10 μM CCG-4986 was used. The concentration of RGS protein was 50 to 100 μM. Controlling the RGS concentration was necessary because of different observed catalytic activity, probably caused by differing levels of inactive protein in each protein preparation.

Mass Spectrometry of RGS8. RGS8 (50 pmol) pretreated with CCG-4986 (100 μM; 1 h) was subject to reverse-phase high-performance LC using a Shimadzu (Kyoto, Japan) UFLC liquid chromatograph and a Phenomenex (Torrance, CA) Jupiter C18 column (5 μm; 300 A; 150 $\times$ 1.00 mm). Protein was eluted with a gradient from 20 to 80% acetonitrile in 0.05% formic acid and analyzed with a Shimadzu LCMS-IT-TOF mass spectrometer. Data were analyzed and deconvoluted by using the MagTran software package.

Results

Reversibility of CCG-4986 Inhibition. Initial experiments focused on the characterization of the mechanism of CCG-4986 inhibition of RGS4. After treatment of bead-bound RGS4 with CCG-4986 and subsequent washing with buffer, complete inhibition of RGS4/Gα_o was still observed, indicating potential irreversibility of the inhibition. It is noteworthy that when CCG-4986-treated RGS4 was washed with buffer containing the reducing agent DTT the ability of RGS4 to bind Gα_o was re-established, demonstrating the reversibility of the inhibition in a reducing environment (Fig. 2).

Examination of Divergent Cysteine Residues. Cys71 and Cys132 are absent in RGS8 (Fig. 1), which is not inhibited by CCG-4986 (Roman et al., 2007b). Consequently, these two residues were mutated to alanine, their ability to bind and GAP Gα_o was confirmed (Fig. 5), and the effect of CCG-4986 was evaluated. This experiment revealed that Cys132 plays a role in CCG-4986 inhibition, as shown by the rightward shift of the dose-response curve, whereas Cys71 is not required for inhibition (Fig. 3).

No Single Cysteine Accounts for Full Inhibition. Cysteine-to-alanine point mutants for the four cysteine residues in the RGS4 box were generated and then tested for sensitivity to inhibition by CCG-4986. Although C132A did show the greatest loss of potency, no single cysteine residue seems responsible for the full measure of CCG-4986 inhibition of RGS4-Gα_o (Fig. 3; Table 1).

Residues Required for Inhibition. The complete lack of effect of CCG-4986 on the "Cysless" RGS4 revealed that one or more cysteine residues were required for inhibition of RGS4 (Fig. 4). Individual cysteine residues were then inserted back into the Cysless RGS4 protein, and these mutants were examined for sensitivity to CCG-4986 (Fig. 5). Mutant RGS4 variants that have had individual native cysteine residues inserted back into the Cysless RGS4 construct are referred to as "add-back mutants" and indicated as A[residue no.]C, indicating the alanine at the given position was mutated to the natively occurring cysteine. Mutant RGS4 variants with cysteine residues mutated to alanine are designated C[residue no.]A. These data indicate that Cys71 cannot mediate CCG-4986 inhibition of RGS4, whereas Cys95 and Cys132 restore modest inhibitory effects. The greatest inhibition by CCG-4986 was seen in the A148C protein, which was nearly as sensitive as the wild-type RGS4. CCG-4986 (100 μM) produced >70% inhibition of Gα_o-RGS4 binding, which is significantly more than that seen with the other mutants and is most similar to effects on wild-type RGS4, which is inhibited 86% by 100 μM CCG-4986. It is noteworthy that a mutant containing Cys148 and Cys132 completely restored inhibition by CCG-4986, generating a dose-response identical to wild-type RGS4 (Fig. 4).

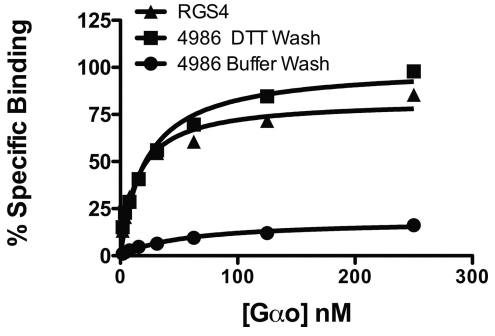

Fig. 2. Reversibility of CCG-4986 inhibition in the presence of the reducing agent dithiothreitol. Immobilized, biotinylated RGS4 on avidin microspheres was treated with CCCG-4986 and subsequently washed with buffer (●) or buffer containing DTT (■). Binding to increasing concentrations of Alexa Fluor 532-labeled Gα_o was measured by FCPIA. RGS4 binding to Gαo before washing is also shown (▲). Data points are from duplicate measures from three independent experiments (n = 3).

GTPase Activation by RGS Mutants and Inhibition by CCG-4986. Each of the mutant RGS4 proteins (at 200 nM) exhibited GAP activity toward $G\alpha_o$ in the single-turnover GTPase assay. Increases in rate varied somewhat between mutants as shown in Table 1. The increase in GTPase rate expressed as fold over $G\alpha_o$-catalyzed hydrolysis is as follows: RGS4 (20-fold), C132A (20-fold), C148A (27-fold), A132C (10-fold), A148C (40-fold), and Cysless RGS4 (3.7-fold). The range for inhibition by 100 μM CCG-4986 was 14% inhibition at the Cysless RGS4 to 90% inhibition at the C132A mutant (Table 1).

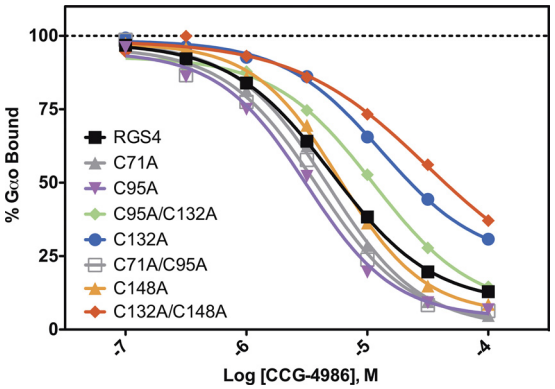

Fig. 3. Inhibition of RGS/$G\alpha_o$ interaction by CCG-4986 at cysteine point mutants. Dose-response curves for CCG-4986 at LumAvidin microspheres were coupled to 10 nM concentration of biotinylated constructs preincubated with varying concentrations of CCG-4986 before the addition of 50 nM AMF-activated $G\alpha_o$. Indicated values are IC_{50} ± S.E.M., with measurements made in duplicate in three independent experiments (n = 3).

TABLE 1
GTPase stimulation by RGS4 mutants

	Rate ± S.E.M. (n = 3)		
	Without Inhibition	+100 μM CCG-4986	Inhibition
	$\times 10^{-2}/s$		%
$G\alpha_o$ alone	0.11 ± 0.02		
RGS4	2.4 ± 1.4	0.75 ± 0.48	69
C132A	2.2 ± 0.26	0.22 ± 0.01	90
C148A	1.4 ± 0.6	0.19 ± 0.05	86
A132C	1.2 ± 0.15	0.34 ± 0.1	72
A148C	4.5 ± 0.48	1.1 ± 0.39	76
Cysless	0.42 ± 0.06	0.36 ± 0.03	14

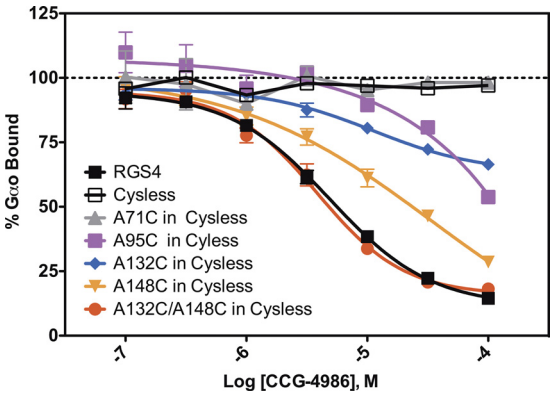

Fig. 4. Inhibition of RGS/$G\alpha_o$ interaction by CCG-4986 at cysteine "add-back" constructs. These mutants have had specific cysteine residues added back to the Cysless RGS4 construct. Dose-response curves for CCG-4986 at LumAvidin microspheres were coupled to 10 nM concentration of biotinylated constructs preincubated with varying concentrations of CCG-4986 before the addition of 50 nM AMF-activated $G\alpha_o$.

Allosteric Inhibition of $G\alpha_o$ Binding. The effect of CCG-4986 on $G\alpha_o$ binding to cysteine add-back mutants (Cys132 and Cys148 in the Cysless RGS4 background) and on wild-type RGS4 were examined by using a $G\alpha_o$ saturation binding experiment. Saturation isotherms revealed a potential mixed mode of inhibition for wild-type RGS4, exhibiting a decrease in B_{max} and a reduction in $G\alpha_o$ affinity in the presence of CCG-4986 (Fig. 5). Inhibition of $G\alpha_o$ binding through modification of Cys132, located adjacent to the $G\alpha_o$ binding site on RGS4, demonstrated a reduction in $G\alpha_o$ affinity and a very modest decrease in B_{max}, suggesting a competitive mode of inhibition. It is noteworthy that modification of Cys148 by CCG-4986 resulted in a remarkable decrease in B_{max}, but no significant alteration of $G\alpha_o$ affinity, consistent with a noncompetitive or allosteric mechanism of inhibition (Fig. 5).

Modification of RGS8 by CCG-4986. The ability of CCG-4986 to modify RGS8 was investigated by mass spectrometry. RGS8 was treated with 100 μM CCG-4986 for 1 h and then subjected to LC/MS using the IT-TOF instrument. The data indicate that CCG-4986 can indeed modify RGS8 on both cysteine residues (Fig. 6). Unmodified (mol. wt. 16,929), singly modified (mol. wt. 17,082), and doubly modified (mol. wt. 17,235) RGS8 proteins were apparent using MS, based on the reported molecular weight (153) of the CCG-4986 adduct reported previously (Kimple et al., 2007).

Discussion

Because of their unique role in temporally regulating G protein-mediated signals, RGS proteins have emerged as an attractive drug target with considerable effort focused on developing exogenous ligands to modulate their activity (Nieuwenhuijsen et al., 2003; Young et al., 2004; Roof et al., 2006, 2007, 2008, 2009; Roman et al., 2007b). Advantages to targeting RGS proteins include their often unique tissue distributions and the presence of accessory domains of some RGS families that can provide targets for modulating dis-

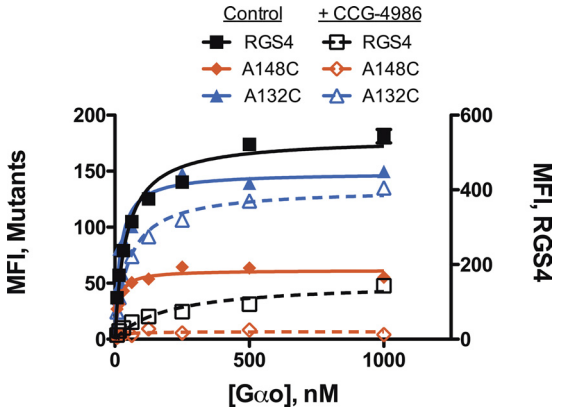

Fig. 5. Saturation binding of $G\alpha_o$ to wild-type RGS4 and point mutants in the presence and absence of 10 μM CCG-4986. Biotinylated RGS4 proteins were incubated with avidin-coated beads and incubated for 30 min in the presence of varying concentrations of AF523-labeled $G\alpha_o$ with or without the addition of 10 μM CCG-4986. The y-axis indicates median fluorescence intensity (MFI) in the AF532 channel of 100 bead-events collected with the Luminex Cytometer. Specific binding is shown. Nonspecific binding was determined by using GDP instead of AMF/GDP. The K_D and B_{max} values, respectively, for each data set are: RGS4, 42 nM, 538 MFI; RGS4 + 4986, 195 nM, 51 MFI; A132C, 19 nM, 149 MFI; A132C + 4986, 49 nM, 135 MFI; A148C, 13 nM, 62 MFI; and A148C + 4986, 19 nM, 7 MFI.

crete RGS-effector interactions (for review, see Hollinger and Hepler, 2002).

Rational design, such as the cyclic octapeptide developed by Jin et al. (2004a,b), focused on blocking the RGS/Gα interaction surface directly, which resulted in a peptide inhibitor that bound to RGS4, mimicking the switch 1 region of $G\alpha_{i1}$. Although this effort was successful, examination of the crystal structure of RGS4 bound to $G\alpha_{i1}$ demonstrates a relatively large protein interaction face lacking distinct pockets that would seem not amenable to targeting with a small molecule as opposed to a peptide (Tesmer et al., 1997). In fact, computational programs that examine protein structures for "druggable" pockets routinely assign low scores to the area of RGS4 at the interaction face, indicating a less than optimal target for small molecules (R. R. Neubig, unpublished observation).

Our efforts to develop a small-molecule RGS inhibitor identified CCG-4986 as an inhibitor of both the RGS4/$G\alpha_o$ protein-protein interaction and RGS4 GAP activity in vitro (Roman et al., 2007b). Further characterization of this phenomenon revealed that CCG-4986 inhibition of RGS4/$G\alpha_o$ binding could be abrogated by DTT, but not by a wash buffer solution lacking reducing agent (Fig. 2). This finding drew our focus on cysteine residues that had the potential to be irreversibly modified by CCG-4986 and resulted in inhibition of both RGS4 GAP activity and binding to Gα subunits. A similar set of studies was undertaken by Kimple et al. (2007).

Initial examination of the crystal structure (Protein Data Bank ID code 1AGR) of RGS4 bound to $G\alpha_i$, and the revelation of the irreversibility of CCG-4986 binding led to a hypothesis that Cys132 was the likely mediator of RGS4 inhibition by CCG-4986, as reported by Kimple et al. (2007).

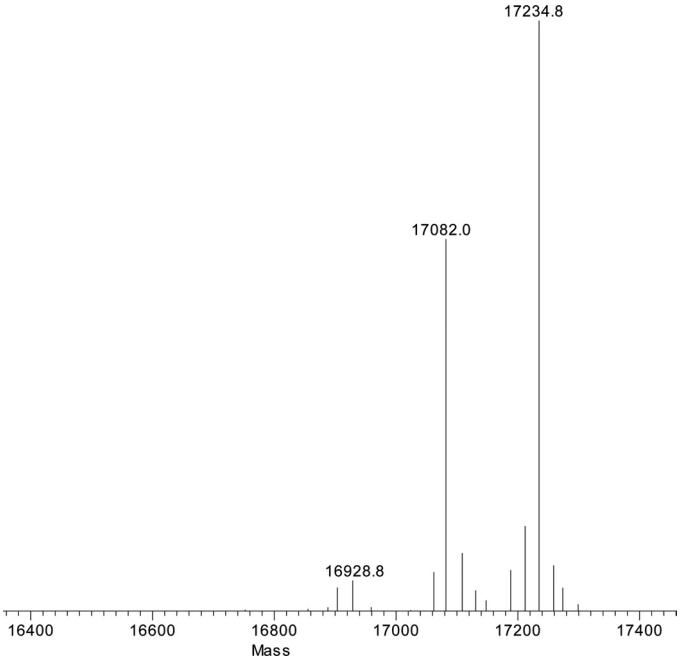

Fig. 6. LC/MS analysis of CCG-4986-treated RGS8. Purified human RGS8 was incubated with 100 μM CCG-4986 for 1 h at room temperature. RGS8 (50 pmol) was subject to reversed-phase high-performance LC using a C18 column. The deconvoluted chromatogram shows unmodified protein, a singly adducted RGS8 (+153 mol. wt.), and the doubly modified RGS8 (+306 mol. wt.), indicating that both of the cysteines present in RGS8 can be modified by CCG-4986.

However, our data demonstrate the presence of more than one sensitive cysteine residue on RGS4 that can be modified to alter the function of RGS4. These residues and features of the protein structure surrounding them can potentially be exploited for the development of tools or pharmacological agents that target RGS proteins.

Our findings demonstrate the presence of an allosteric modulatory site for CCG-4986 binding on RGS4 that is distant from the $G\alpha_o$ interaction surface. The presence of this allosteric site is exciting in that it may provide a more "druggable" pocket on RGS4. It also indicates that a second site near Cys148 may have features more amenable to targeting with small molecules.

This study demonstrates that the small-molecule RGS4 inhibitor CCG-4986 has a unique, dual mode of inhibition that includes a dominant allosteric component. A great portion of the total inhibition by CCG-4986 is caused by modification at Cys148, in an allosteric site, whereas a smaller level of inhibition (approximately 30%) is caused by direct competition of CCG-4986 at Cys132 near the $G\alpha_o$ binding site on RGS4. We have been able to dissect these two mechanisms by using a series of point mutants and a flow cytometry-based method that has been increasingly implemented for studying RGS proteins (Gu et al., 2007; Roof et al., 2008, 2009; Shankaranarayanan et al., 2008; Roman et al., 2009). We recognize that CCG-4986 modifies a number of cysteine residues on RGS4; however, the lack of inhibition of RGS8 despite the presence of CCG-4986 modification demonstrates differences in the conformational changes that occur in RGS4 after binding of the inhibitor at the allosteric site marked by Cys148. Most strikingly, the mechanism of allosteric inhibition and conformational changes that displace $G\alpha_o$ from binding RGS4 seems to be unique, because a cysteine in the position of Cys148 is conserved between RGS4 and RGS8, yet RGS8 is not inhibited by CCG-4986 (Roman et al., 2007b). In addition, RGS16 possesses a cysteine residue in an equivalent position, yet it is not inhibited by CCG-4986 (Kimple et al., 2007). If the mechanism of inhibition was simply nonspecific modification of Cys148 and loss of protein function, one would predict that RGS8 and RGS16 could be inhibited by modification at Cys148 as well. Indeed, mass spectrometry data indicate that RGS8 is modified at Cys148 (Fig. 6); however, our previous data indicate that RGS8 binding to $G\alpha_o$ is not inhibited by CCG-4986 (Roman et al., 2007b). Because RGS8 modification by CCG-4986 does not result in inhibition of binding, it is reasonable to hypothesize that RGS8 does not undergo the conformational change that displaces $G\alpha_o$ from its protein interaction face. This reinforces the hypothesis that RGS4, when modified by CCG-4986 at the allosteric site containing Cys148, undergoes a unique conformational change that prevents $G\alpha_o$ binding and subsequent GAP activity.

It is noteworthy that our data and observations by others (L. Blazer, personal communication) indicate that the RGS/Gα binding interaction is probably more difficult to inhibit than RGS GTPase activity. This difference may be caused by the higher affinity of RGS4 for $G\alpha_o$ afforded by AlF_4^-, compared with relatively low affinity of the binding partners in the presence of GTP as is the case in the single-turnover GTPase assays. It is also possible that finer perturbations of the RGS structure may disrupt GAP activity more than the protein-protein interaction that occurs over a relatively large contact surface area.

The pharmacological data support the role of modification of Cys148 as an allosteric site that affects the protein-protein interaction with $G\alpha_o$. The action of CCG-4986 at this site affords far more potent inhibition than the inhibition mediated by Cys132 located near the protein-protein interaction face. In addition, the consequence of the modification at Cys148 seems to be unique to RGS4, unlike modification of an equivalent to Cys132 engineered into RGS8, as demonstrated previously (Roman et al., 2007b).

This study describes a novel allosteric binding site and mechanism of inhibition for RGS4 that could be exploited for the development of new tools and potential therapeutics focused on modulating RGS activity. In addition, it highlights a unique allosteric site-induced conformational change in RGS4 that results in inhibition. We also present differences between two close members of the RGS4 family (RGS4 and RGS8) that share a high level of sequence identity and secondary structure, but exhibit dramatically different outcomes after modification by the small-molecule CCG-4986. The allosteric site and unique mechanism at RGS4 could provide a new avenue for targeting RGS proteins. In addition, this site could represent a novel regulatory site on some RGS proteins that is used for modulating RGS interactions with effectors such as G proteins.

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