A Point Mutation in Go and Gai Blocks Interaction with Regulator of G Protein Signaling Proteins*

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Regulator of G protein-signaling (RGS) proteins accelerate GTP hydrolysis by Go subunits and are thought to be responsible for rapid deactivation of enzymes and ion channels controlled by G proteins. We wanted to identify and characterize Gi-family subunits that were insensitive to RGS action. Based on a glycine to serine mutation in the yeast Go subunit Gpa1s that prevents deactivation by Sst2 (DiBello, P. R., Garrison, T. R., Apanovitch, D. M., Hoffman, G., Shuey, D. J., Mason, K., Cockett, M. L., and Dohlman, H. G. (1998) J. Biol. Chem. 273, 5780–5784), site-directed mutagenesis of go and ai was done. G184S go and G188S ai show kinetics of GDP release and GTP hydrolysis similar to wild type. In contrast, GTP hydrolysis by the G → S mutant proteins is not stimulated by RGS4 or by a truncated RGS7. Quantitative flow cytometry binding studies show IC50 values of 30 and 96 nM, respectively, for aluminum fluoride-activated wild type go and G188S ai, to compete with fluorescein isothiocyanate-αi binding to glutathione S-transferase-RGS4. The G → S mutant proteins showed a greater than 30–100-fold lower affinity for RGS4. Thus, we have defined the mechanism of a point mutation in go and ai that prevents RGS binding and GTPase activating activity. These mutant subunits should be useful in biochemical or expression studies to evaluate the role of endogenous RGS proteins in Gi function.

Receptor-mediated activation of heterotrimeric guanine nucleotide-binding proteins initiates signals elicited by numerous hormone, neurotransmitter, and sensory stimuli (1). Receptors activate G proteins by stimulating the release of GDP from the α subunit, allowing GTP to bind and to induce dissociation of the G protein α and βγ subunits, which interact with effector proteins to modulate cellular responses (2–5).

The duration and strength of receptor-generated physiological responses are regulated by the rate at which GTP is hydrolyzed by the α subunit (6, 7). It has been known for some time that the physiological turn-off of some G protein-mediated signals is faster than would be predicted from the in vitro GTPase activity of isolated G protein subunits (8, 9). The solution to this paradox appears to reside in the newly recognized family of regulator of G protein signaling (RGS) proteins, first identified genetically in the yeast Saccharomyces cerevisiae and in the nematode, Caenorhabditis elegans (10–13). At least 19 RGS protein cDNAs have been identified in mammalian tissues, all sharing a homologous carboxyl-terminal region of ~120 amino acid residues termed the RGS domain (13–15). Biochemical studies with go and ai family of G proteins demonstrated that RGS4 and Gα-interacting protein (GAIP) act as GTPase accelerating proteins (GAPs) (16, 17), which could account for inhibition of G protein-mediated responses (15). GAP activity of Sst2 for Gpa1 has also been recently demonstrated (18). The mechanism by which GTPase activity is enhanced by RGS appears to be the stabilization of the transition state conformation of Gα for nucleotide hydrolysis (19, 20). RGS4 also directly inhibits the interaction of the GTPγS-bound αi subunit with phospholipase Cβ, presumably by binding to the effector region of activated go (16).

A mutant yeast Gα subunit, Gpa1s, was recently identified in a screen for novel strains showing the “supersensitive to pheromone” (sst) phenotype. It has a single glycine to serine mutation and escapes from negative regulation by the RGS protein, Sst2 (21). Since many RGS proteins affect Gγ-family G proteins, and the crystal structure of the RGS4–go complex was recently reported, we wanted to see if the corresponding G → S mutation in ai would produce insensitivity to RGS. A major objective was to obtain a detailed biochemical and mechanistic analysis of this newly identified class of mutations.

We report that G go and αi subunit G → S mutants are insensitive to RGS protein activation by two different RGS proteins. Quantitative flow cytometry studies demonstrated that a >30–100-fold reduction in affinity for RGS for the α subunit transition state is the mechanism of the insensitivity. In future studies, these mutant αi subunits should be useful for evaluating the role of endogenous RGS proteins in the kinetics and function of Gi family members. Given the existence of nearly 20 RGS proteins of which at least 5 act on Gi-family proteins, it would be difficult to inactivate all of them to determine the physiological role of endogenous RGS proteins in Gi signaling. Thus, a Gi αi subunit insensitive to RGS proteins can be used to assess the combined role of all RGS proteins in Gi function in vivo.

**Experimental Procedures**

DNA Construction and Mutagenesis—The G184S mutation in the go sequence was introduced by the megaprimer polymerase chain reaction mutagenesis technique using mutagenic antisense primer 5′-GGTTTC-TACGATCGAAGTTGTTTGAC-3′ as described (22, 23). The G183S

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‡‡‡‡ The abbreviations used are: RGS, regulator of G protein signaling; FITC, fluorescein 5-isothiocyanate; GAP, GTPase activating protein; GST, glutathione S-transferase; GTPγS, guanosine 5′-O-(3-thiotriphosphate).
mutation in α1 was constructed by overlap-extension polymerase chain reaction using sense and antisense mutagenic primers 5'-AGTGA-
AAAAACGTCATTTTGAAAAACC-3' and 5'-GTTTGGCAACATTGAG-
CGTTGTTTACT-3', respectively. The coding region of rat RGS4 was amplified by polymerase chain reaction originated in Expression Sequence Tag and respectively obtained from The Institute of Genome Research and cloned into the pGEX-2T expression vector. A restriction fragment comprising nucleo-
tides 913–1358 of the complete human RGS7 cDNA was cloned into the GST expression vector pgGSTtag. The expressed protein contained the RGS domain (nucleotides 985–1341) but only a portion of the long amino terminus of RGS7.

**Purification of His-tagged Subunits and GST-RGS Proteins—** All Ga subunits in this paper were expressed in *Escherichia coli* and purified as amino-terminal His6 constructs by a modification of the method of Lee et al. (24). The GST-RGS4 and -RGS7 fusion proteins were purified as described (25). The bacterial supernatant was incubated with glutathione-agarose beads (Amersham Pharmacia Biotech) at 4 °C overnight. After washing with phosphate-buffered saline, the GST-RGS4 was eluted with 10 mM glutathione in phosphate-buffered saline and dia-
lized against 50 mM Tris and 1 mM EDTA, pH 7.4. The fusion proteins were cleaved by incubation overnight at 4 °C with 10 units of throm-
bin/mg of fusion protein followed by incubation with glutathione-agarose
to remove GST and any uncleaved GST-RGS4.

**GAP Assays—** [γ-32P]GTP (1 μM) was allowed to bind to 50 nM α1, for 20 min at 25 °C or 50 nM α2 for 15 min at 30 °C. After lowering the temperature to 4 °C, the hydrolysis reaction was started by the addition of MgSO4 and GTP-S to final concentrations of 15 mM and 200 μM in the presence or absence of 100 nM RGS4 or RGS7. Aliquots (50 μl) were diluted in 1 ml of 15% (w/v) charcoal solution (50 mM NaH2PO4, pH 2.0, 3 °C) at the indicated time points. The amount of [γ-32P]Pi released at each time point was fit to an exponential function, cpm(t) = cpm0 + Δcpm × (1 - e-kt).

**Binding of α to GST-RGS4-agarose—** Wild-type or mutant GDP-bound α (1 μM) was mixed with GST-RGS4 fusion protein (1 μM) bound to glutathione-agarose beads (5 × 106 beads/m) in a final volume of 100 μl of HEDML buffer (50 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM dithio-
threitol, 10 mM MgSO4, 200 mM deionized Lubrol) with 0.05% bovine serum albumin in the presence or absence of 20 μM AlCl3 and 10 mM NaF (HEDML/AF). After 2 h, the beads were washed three times with 1 ml of ice-cold HEDML/AF buffer with bovine serum albumin, and bound products were separated by SDS-polyacrylamide gel electro-
phoresis and visualized by Coomassie Blue stain.

**Competition Binding of the α Subunit to GST-RGS4—** Labeling of purified Hisα, with fluorescein isothiocyanate (FITC) was conducted as described (26). Glutathione-agarose beads (Amersham) with sizes between 35 and 75 μM were prepared for flow cytometry by filtering through stainless steel Tyler sieves in HEDML buffer. Two nm GST or
glutathione-RGS4 fusion protein was bound to the beads (5 x 109/m) for 20 min at room temperature in HEDML buffer. Beads were then washed and incubated with 4 nm FITC-α1, with the indicated amounts of unla-
beled α-subunit in 10 μl of HEDML/AF buffer at room temperature for 2.5 h. The amount of FITC-α1 bound to the GST-RGS4 was quantitated on a Becton Dickinson FACScan as described (26). Data are presented as a fraction of the control fluorescence after subtracting nonspecific binding (~20% of the total). Results were fit to a one-site competition binding function, and the IC50 values for the competitors were calculated using Prism version 2.01 for Windows 95 (Graphpad Software, San Diego, CA).

**RESULTS AND DISCUSSION**

**Nucleotide Binding to and RGS-stimulated Hydrolysis by a Subunit—** The time course of nucleotide binding to α subunits was determined by use of the fluorescent nucleotide derivative, methylanthraniloyl GTP/γS as described (27). Similar rates of binding were seen for both the G → S mutants and the wild
type proteins. Rate constants were 0.20 ± 0.06 and 0.21 ± 0.08 min−1 for αo and 0.049 ± 0.002 and 0.12 ± 0.03 min−1 for α1, wild type and mutant, respectively. The kcat values for wild type and G → S mutant proteins in the absence of RGS were very similar (Fig. 1 and Table I). In the presence of 100 nM RGS4, the reaction was competed by the first time point for wild type αo and α1 (Fig. 1). Even at 4 °C, the reaction was too fast to measure with a rate constant greater than 5 min−1.

![Fig. 1. Effect of RGS proteins on single-turnover GTP hydrolysis by wild type (WT) and mutant α subunits.](image308x536 to 553x731)

**TABLE I**

| Subunit       | Control kcat | +RGS4 kcat | +RGS7 kcat |
|---------------|--------------|------------|------------|
| Wild type αo  | 0.10 ± 0.01  | >5         | ND         |
| G184S αo      | 0.12 ± 0.01  | 0.14 ± 0.01 | ND         |
| Wild type α1  | 0.23 ± 0.02 (5) | >5        | 2.0 ± 0.2 |
| G183S α1      | 0.16 ± 0.02 (4) | 0.22 ± 0.04 | 0.16 ± 0.02 |

*ND*, not determined.

There was no effect of RGS4 on the kcat of either G → S mutant (Fig. 1, B and D, and Table I). The RGS domain fragment of RGS4 (100 nM) increased the kcat of wild type α1 by ~9-fold, whereas there was no effect on the α1 G → S (Fig. 1C and Table I).

**GAP Activity at Increasing Concentrations of RGS4—** To determine if GAP activity could be restored at higher concentra-
tions of RGS4, GTP hydrolysis at 1 min was measured with 50 nM Ga subunit and 0–3 μM RGS4. The EC50 values for RGS4 were 5 ± 1 and 10 ± 4 μM for wild type αo and α1, respectively (Fig. 2). There was only a slight increase in GTP hydrolyzed by either G → S mutant α subunit, even at the maximum concentration of RGS4 (Fig. 2).

**Reduced GAP Activity Is Due to Reduced Affinity—** To determine whether the marked reduction in sensitivity of mutant α to RG54 was due to decreased binding, we tested co-precipitation of α and RGS4. In the absence of AlF4−, GAP-bound α subunit did not bind to GST-RGS4 (Fig. 3). In the presence of AlF4−, wild type αo and α1 showed substantial binding to GST-RGS4 immobilized on glutathione-agarose beads.3 In con-

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3The apparent excess of bound Ga over GST-RGS4 protein was due to incomplete elution of the GST-RGS4 from the beads in this experiment.
60–150 nM; see Fig. 4). As observed for the effects of RGS4 on His6 mean maximal GTP hydrolysis determined at 30 min. Data represent the mean ± S.E. of three experiments, each done in duplicate.

Fig. 2. GAP activity at increasing concentrations of RGS4. Single-turnover GTP hydrolysis by wild type and mutant α subunits was measured at 1 and 30 min after the addition of MgSO4 and GTPS with increasing concentrations of RGS4. Conditions were the same as in Fig. 1. The amount of GTP hydrolyzed at 1 min was normalized to maximal GTP hydrolysis determined at 30 min. Data represent the mean ± S.E. of three experiments, each done in duplicate.

Fig. 3. Mutant α does not bind to RGS4. GST-RGS4 fusion protein (1 μm) was bound to glutathione-agarose beads (5×10^6 beads/ml) and incubated with 1 μM GDP-bound α. The reaction was done in 100 μl of HEDML buffer with 0.05% bovine serum albumin in the presence or absence of AIF4. Top, the total amount of α used in each reaction is shown. Bottom, pellets were prepared as described under “Experimental Procedures.” The bound proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining. A representative result from three separate experiments is shown. WT, wild type.

Fig. 4. Affinity of α subunits for GST-RGS4. Binding of FITC-αo was measured by flow cytometry as described under “Experimental Procedures.” Samples contained 4 nM FITC-αS, 2 nM GST-RGS4 or glutathione-agarose beads, and the indicated concentrations of unlabeled α subunits. All incubations were done in the presence of AIF4 at room temperature for 2.5 h. Data are presented as a fraction of control with nonspecific binding (~20% of total) subtracted. Experiments have been replicated twice in duplicate. The data for wild type (WT) proteins were fit to one-site competition function.

30–100-fold lower than that of wild type.

Generality of G → S Mutation in Ga-RGS Interactions—In this report, we identify a G → S point mutation in the conformationally flexible “switch I” region of αo and αS, that completely eliminates the interaction of these α subunit with RGS proteins. This mutation should be useful in determining the role of endogenous RGS proteins in the physiological functioning of Ga proteins. This is especially important for Ga, since the kinetics of Ga-mediated regulation of effectors (e.g. adenylyl cyclase (28) and potassium channels (9)) are faster than expected for the in vitro GTP hydrolysis rates in the absence of RGS proteins. Deactivation of potassium currents was recently shown to be accelerated by overexpressed RGS1, 3, or 4 in oocytes and Chinese hamster ovary cells (29, 30). These data show that exogenous RGS proteins can alter Ga protein function, but the question of whether normal cellular concentrations of RGS proteins alter the kinetics of ion channel function has not been addressed experimentally. With the α subunit mutation described here, it is now possible to directly address that question.

This class of G → S mutations was first described in yeast by DiBello et al. (21). They identified, in a genetic screen for the supersensitive phenotype, a G → S mutation in the yeast α subunit (Gpa1<sup>ins</sup>) that resulted in insensitivity to 1) the functional effects of the yeast RGS protein, Sts2, and 2) the biochemical effects of the Ga-interacting protein. The loss of function due to these G → S mutations is selective, since our mutant α subunits retain nearly normal intrinsic GTPase activity and kinetics of GDP release. The corresponding mutation in αo prevented the RGS7-mediated reduction of phospholipase C activation in co-transfection studies (21). These latter data also demonstrate that the G → S mutation in αo does not disrupt effector coupling. In preliminary data, a myristoylated mutant G → S αo inhibited forskolin stimulated type IV adenylyl cyclase activity. Thus, mutating this glycine residue has profound and consistent effects on four different α subunits and their interactions with four different RGS proteins.

Structural Basis of Glycine to Serine Effects—In the crystal
structure of the α1-RGS4 complex (19), the switch I region of α interacts with three of the four different segments of the RGS consensus domain, but there are also contacts with switch II and switch III. Glycine 183 is located in the switch I region of α1, forming a turn just before the β1 strand (31, 33). Interestingly, Natochin and Artemyev (32) recently found that the mutation of Ser-202 in switch II of transducin prevents interaction with retinal specific RGS. Glycine 183 provides a substantial contribution to the buried surface area between RGS protein. Introduction of the hydroxymethyl side chain of RGS4 (see Ref. 21 for details). There is direct contact of glycine 183 with the same residue found in the Gpa1 sst, suggesting that these mutations of Ser-202 in switch II of transducin prevents interaction with retinal specific RGS. Glycine 183 provides a significant contribution to the buried surface area between RGS and RGS proteins. Introduction of the hydroxymethyl side chain of serine would sterically hinder the formation of a tight complex of α and RGS. Also, threonine 182, which is directly adjacent to glycine 183 mutated in the α1 G → S mutant, exhibits the greatest change in accessibility of any residue upon forming the α1-RGS4 complex (19). Thus the G → S mutation may also disrupt local protein conformation and prevent threonine 182 from interacting with the highly conserved residues in RGS.

The glycine at position 183 in α1 is highly conserved among all subunits. There are only two exceptions; in α7 of Dictyostelium discoideum and in open reading frame B0207.3 of C. elegans it is replaced by a serine (34, 35). Interestingly, this is the same residue found in the Gpa1 sst, suggesting that these proteins may be naturally occurring sst variants that are insensitive to modulation by RGS proteins. The contact site in RGS is similarly conserved. Either serine or cysteine is present at the position equivalent to 85 in RGS4 where the Ga glycine interacts. Druey and Kehrl (36) recently showed that modification of asparagine 88 and leucine 159 in RGS4 disrupted α subunit binding and GAP activity. In the three-dimensional structure, both residues are very close to serine 85 where glycine 183 in Ga makes contact (19).

Binding of a Subunits to GST-RGS4—Both in co-precipitation and fluorescence competition studies, the affinity of the α1-RG-bound mutant α subunits for RGS4 are dramatically reduced. With the flow cytometry method, we obtained quantitative measures of subunit affinities. The IC50 values of wild type α and α1 (30 and 96 nM, respectively) are similar to the KD of 45 nM determined by surface plasmon resonance for transducin binding to retinal-specific RGS (37). Our IC50 is significantly higher than the IC50 or direct binding

In summary, our data show a dramatic disruption in both RGS binding and RGS-mediated GAP activity when the glycine in the switch I region of α1 or α is mutated to serine. The effect of the G → S mutation is both specific in that it only disrupts RGS binding and quite general in the range of α subunit and RGS proteins that it effects. The introduction of this mutation into G protein α subunits can be used in conjunction with expression or transgenic animal studies to evaluate the physiological role of endogenous RGS proteins in the function of a given G protein.

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