A Single Mutation Asp^{229} → Ser Confers upon $G_s\alpha$ the Ability To Interact with Regulators of G Protein Signaling†

Michael Natochin and Nikolai O. Artemyev*

Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

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ABSTRACT: RGS proteins (regulators of G protein signaling) are GTPase activating proteins (GAPs) for G_i and G_q families of heterotrimeric G proteins but have not been found to interact with $G_s\alpha$. The $G_s\alpha$ residue Asp229 has been suggested to be responsible for the inability of RGS proteins to interact with $G_s\alpha$ [Natochin, M., and Artemyev, N. O. (1998) *J. Biol. Chem. 273,* 4300−4303]. To test this hypothesis, we have investigated the possibility of generating an interaction between $G_s\alpha$ and RGS proteins by substituting $G_s\alpha$ Asp229 with Ser and replacing the potential $G_s\alpha$ Asp229 contact residues in RGS16, Glu129 and Asn131, by Ala and Ser, respectively. RGS16 and its mutants failed to interact with $G_s\alpha$. A single mutation of $G_s\alpha$, Asp229Ser, rendered the $G_s\alpha$ subunit with the ability to interact with RGS16 and RGS4. Like RGS protein binding to G_i and G_q α-subunits, RGS16 preferentially recognized the AlF_4^--bound conformation of $G_s\alpha$ Asp229Ser. In a single-turnover assay, RGS16 maximally stimulated GTPase activity of $G_s\alpha$ Asp229Ser by ∼5-fold with an EC_{50} value of 7.5 μM. Our findings demonstrate that Asp229 of $G_s\alpha$ represents a major barrier for $G_s\alpha$ interaction with known RGS proteins.

Recently identified RGS proteins[1] function as GTPase activating proteins (GAPs) for α-subunits of heterotrimeric G proteins (*1*−*5*). They inhibit signaling by members of the G_i and G_q families (*6*−*10*). A major component of the RGS protein GAP activity is the ability to preferentially bind to the transition-state conformation mimicked by the AlF_4^--bound Gα (*7*, *9*). The first crystal structure of a complex of a RGS protein with $G\alpha\cdot AlF_4^-$ has suggested that the stabilization of switch regions is the key mechanism for the acceleration of Gα GTPase activity (*11*). This conclusion was further supported by biochemical evidence (*12*−*14*). Surprisingly, no RGS protein has been shown to serve as a GAP for $G_s\alpha$ (*5*). For example, RGS4 and GAIP did not stimulate GTPase activity of $G_s\alpha$ in in vitro experiments with reconstituted purified proteins (*9*) and did not attenuate $G_s\alpha$ signaling under in vivo conditions (*10*). The backbone conformations of the switch I and switch II regions in $G_i\alpha$ and $G_s\alpha$ are practically identical. This suggests that the differences in the primary structures of $G_s\alpha$ and $G_i\alpha$ are responsible for the specificity of RGS proteins (*11*, *15*). Analysis of $G_t\alpha$ mutants in which six RGS contact residues, corresponding to the residues that differ between $G_i\alpha$ and $G_s\alpha$, were replaced by $G_s\alpha$ residues has elucidated the key role of the Gα residue at position 202 of $G_t\alpha$ (*16*). The $G_t\alpha$ Ser202Asp substitution fully disrupted the $G_t\alpha$−RGS interaction, whereas other mutations had little or no effect (*16*). Attempts to rescue the interaction between the $G_t\alpha$ mutant and RGS protein using mutations in RGS have been unsuccessful (*16*). On the basis of these data, we hypothesized that residue Asp229 of $G_s\alpha$, which corresponds to the $G_t\alpha$ Ser202, is the primary cause for the failure of $G_s\alpha$ to interact with RGS proteins.

Here, we test this hypothesis by introducing a substitution of Asp229Ser into $G_s\alpha$ and by examining the ability of RGS16 (*17*−*19*) and RGS4 to accelerate GTPase activity of the mutant $G_s\alpha$. We also examined the possibility that wild-type $G_s\alpha$ may interact with RGS16 mutants containing substitutions of the potential $G_s\alpha$ Asp229 contact residues. These contact residues in RGS16 are likely to be analogous to those in RGS4 identified from the crystal structure (*11*), since RGS16 and RGS4 are highly homologous.

EXPERIMENTAL PROCEDURES

Materials. GTP and GTPγS were from Boehringer Mannheim. [γ-^{32}P]GTP (>5000 Ci/mmol) was from Amersham Pharmacia Biotech. TPCK-treated trypsin was from Worthington. All other chemicals were from Sigma.

Preparation of Rod Outer Segment (ROS) Membranes, $G_t\alpha\beta\gamma$, and RGS16. Bovine ROS membranes were prepared as previously described (*20*). Urea-washed ROS membranes (uROS) were prepared according to the protocol in ref *21*. $G_t\alpha\beta\gamma$ was prepared by the procedure described in ref *22*. GST−RGS16 and RGS16 were prepared and purified as previously described (*19*).

Cloning and Site-Directed Mutagenesis of $G_s\alpha$ and RGS16. The short splice form of bovine $G_s\alpha$ was subcloned into the $pHis_6$-tagged vector for bacterial expression in *Escherichia*

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* Address correspondence to this author. Telephone: (319) 335-7864. Fax: (319) 335-7330. E-mail: Nikolai-Artemyev@uiowa.edu.

[1] Abbreviations: RGS proteins, regulators of G protein signaling; GAP, GTPase activating protein; AlF_4^--bound Gα or $G\alpha\cdot AlF_4^-$, GαGDP activated with addition of 30 μM $AlCl_3$ and 10 mM NaF, respectively; ROS, rod outer segment(s); uROS, urea-washed ROS membranes.

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coli BL21(DE3) cells (*23*) using PCR amplification with the following primers: 5′-AGAAGTCCATGGGCTGTCTCG-GAAACAGCAAG and 3′-ATATATAAGCTTAGAGCAG-CTCATACTGACGGAG (the cloning restriction sites, *Nco*I and *Hin*dIII, are underlined). The $G_s\alpha$ Asp229Ser mutation (the numbering is according to the long splice variant) was introduced by PCR amplification using the 5′-primer AACTTCCATATGTTTGACGTGGGCGGCCAGCGC**TCT**-GAACGC (the mutated codon is bold) and the 3′-primer shown above. The PCR product was digested with *Hin*dIII and ligated into the pHis$_6$-$G_s\alpha$ cut with *Hin*cII and *Hin*dIII. Expression and purification of $G_s\alpha$, $G_s\alpha$ Asp229Ser, and rat $G_i\alpha_1$ were performed as described in ref *23*. Mutations of human RGS16 (previously referred to as hRGSr), Glu129Ala and Glu129Ala/Asn131Ser, were generated using PCR amplifications from the pGEX-KG-RGS16 template (*19*) in a manner similar to that described in ref *14*. cDNA for human RGS4 (*2*) in the pGEX-KG vector was kindly provided by R. A. Fisher (University of Iowa, Iowa City, IA). RGS4, RGS16, and its mutants were expressed in DH5α *E. coli* cells, and the GST portion was removed as described previously (*14*, *19*). All sequences were verified by automated DNA sequencing at the University of Iowa DNA Core Facility.

Trypsin-Protection Assay. $G_s\alpha$ or $G_s\alpha$ Asp229Ser (1 mg/mL) was incubated for 30 min at 25 °C in 50 mM Tris-HCl buffer (pH 8.0), containing 50 mM NaCl, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. Where indicated, either 100 μM GDP, 100 μM GTPγS, or 100 μM GDP/30 μM $AlCl_3$/10 mM NaF was included in the incubation buffer. Trypsin was added at a concentration of 25 μg/mL followed by further incubation for 15 min at 25 °C. The reactions were stopped by addition of the SDS−PAGE sample buffer followed by boiling the samples.

Single-Turnover GTPase Assay. $G_s\alpha$ or $G_s\alpha$ Asp229Ser (1 μM final concentration) was incubated for 5 min at 25 °C in 50 mM HEPES buffer (pH 8.0) containing 5 mM EDTA, 5 mM 2-mercaptoethanol, and 0.1% polyoxyethylene ether W-1. Then, 2 μM [γ-^{32}P]GTP (10000−20000 cpm/pmol) was added, and the mixtures were further incubated for 15 min at 25 °C. At that moment, the samples were cooled to 4 °C and the GTPase reactions were initiated by addition of 20 mM $MgSO_4$ and 200 μM GTP. The reactions were quenched by addition of 100 μL of 7% perchloric acid and analyzed for [^{32}P]P_i production as described previously (*19*).

Competition between $G_s\alpha$·AlF4$^-$ or $G_s\alpha$ Asp229Ser·AlF4$^-$ for Stimulation of $G_t\alpha$ GTPase Activity by RGS16. Transducin (0.4 μM $G_t\alpha\beta\gamma$) GTPase activity was measured in the reconstituted system with uROS membranes (5 μM rhodopsin) in the presence or in the absence of 0.1 μM RGS16 in 50 mM HEPES buffer (pH 8.0) containing 50 mM NaCl, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. Where indicated, $G_s\alpha$ or $G_s\alpha$ Asp229Ser (5 μM) in the AlF_4^--bound conformation was added to the reaction mixtures. Single-turnover $G_t\alpha$ GTPase activity measurements were carried out as described in ref *19*.

Other Methods. The extent of binding between RGS16 and $G_s\alpha$ or $G_s\alpha$ Asp229Ser was analyzed using protein precipitation by glutathione−agarose beads containing immobilized GST−RGS16 as previously described (*16*). Protein concentrations were determined by the method of Bradford (*24*) using IgG as a standard or using calculated extinction coefficients at 280 nm. SDS−PAGE was performed by the method of Laemmli (*25*) in 12% acrylamide gels. Fitting of the experimental data was performed with nonlinear least-squares criteria using GraphPad Prizm (version 2) software.

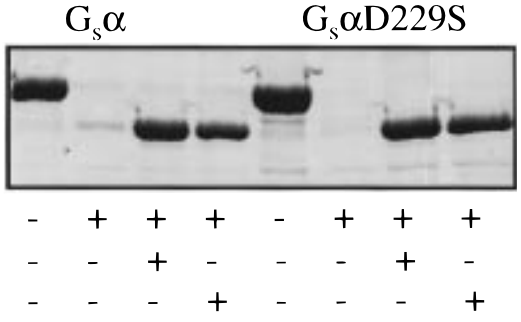

FIGURE 1: Trypsin-protection test for $G_s\alpha$ and the $G_s\alpha$ Asp229Ser mutant. SDS−polyacrylamide gel (12%) stained with Coomassie Blue. $G_s\alpha$ and $G_s\alpha$ Asp229Ser (1 mg/mL) were treated with trypsin (25 μg/mL) for 15 min at 25 °C in the absence or presence of 100 μM GDP, 100 μM GTPγS, or 100 μM GDP/30 μM $AlCl_3$/10 mM NaF.

RESULTS

Expression of the $G_s\alpha$ Asp229Ser Mutant. Expression of $G_s\alpha$ and $G_s\alpha$ Asp229Ser produced equivalent amounts of soluble protein with typical yields of 10 mg/L of culture. In a single-turnover GTPase assay, $G_s\alpha$ and $G_s\alpha$ Asp229Ser had similar basal GTPase activities (1.3−1.5 min^{-1}, 4 °C). Both $G_s\alpha$ and $G_s\alpha$ Asp229Ser were fully capable of undergoing an activating conformational change upon binding of GTPγS or AlF_4^- as can be seen in the trypsin-protection assay (Figure 1).

Binding of RGS16 to Different Conformations of $G_s\alpha$ or $G_s\alpha$ Asp229Ser. RGS16 has been shown to bind tightly to the AlF_4^- conformations of Gα and very weakly to GαGTPγS or GαGDP (*19*, *26*). The extent of binding between RGS16 and $G_s\alpha$ or $G_s\alpha$ Asp229Ser was analyzed using protein precipitation by glutathione−agarose beads containing immobilized GST−RGS16. The assay showed no detectable binding between RGS16 and $G_s\alpha$ regardless of the latter's conformation. In contrast, the GST−RGS16-loaded beads coprecipitated $G_s\alpha$ Asp229Ser in the AlF_4^--bound conformation, pointing out a specific interaction between RGS16 and the $G_s\alpha$ mutant (Figure 2A). The control experiment shows coprecipitation of $G_i\alpha_1$ by RGS16 under the same experimental conditions (Figure 2B).

On the basis of the RGS4−$G_i\alpha_1$ crystal structure (*11*), $G_t\alpha$ Ser202 (Ser206 of $G_i\alpha_1$) likely contacts Glu129 and Asn131 of RGS16 (Glu126 and Asn128 in RGS4, respectively). A charge repulsion between Asp229 of $G_s\alpha$ and the Glu residue in RGS16 and RGS4 proteins may therefore lead to a very low affinity of $G_s\alpha$ for these RGS domains. To investigate this possibility, we examined if the RGS16 Glu129Ala mutant is capable of binding $G_s\alpha$ using the precipitation assay. While RGS16−Glu129Ala exhibited intact binding to $G_i\alpha_1$, it failed to bind $G_s\alpha$ (not shown). Asn131 of RGS16 is critical for its interaction with Gα and may only be functionally substituted by Ser (*14*). The double RGS16 mutant, Glu129Ala/Asn131Ser, also showed no detectable binding to $G_s\alpha$ (not shown).

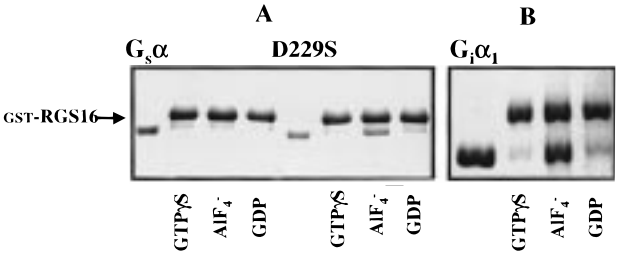

FIGURE 2: Binding of $G_s\alpha$ and the $G_s\alpha$ Asp229Ser mutant to GST−RGS16. SDS−polyacrylamide gel (12%) stained with Coomassie Blue. $G_s\alpha$ and $G_s\alpha$ Asp229Ser (A) or $G_i\alpha_1$ (B) (2 μM final concentration) was mixed with glutathione−agarose retaining ~10 μg of GST−RGS16 in 200 μL of 20 mM HEPES buffer (pH 7.6) containing 100 mM NaCl and 5 mM $MgCl_2$. Where indicated, the buffer contained 50 μM GTPγS, 50 μM GDP, or 50 μM GDP/30 μM $AlCl_3$/10 mM NaF. After incubation for 20 min at 25 °C, the agarose beads were spun down and washed three times with 1 mL of the buffer. The bound proteins were eluted with a sample buffer for SDS−PAGE.

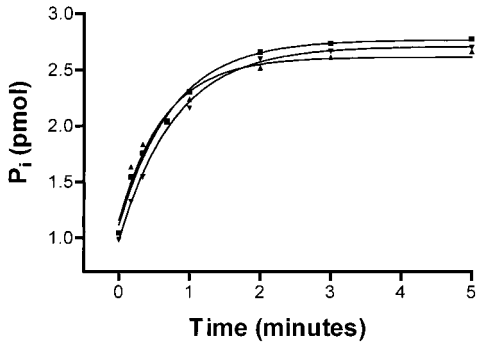

FIGURE 3: Effects of RGS16 and RGS16−Glu129Ala on GTPase activity of $G_s\alpha$. The time course of GTP hydrolysis by $G_s\alpha$ alone (■) or in the presence of 12 μM RGS16 (▲) or 12 μM RGS16−Glu129Ala (▼). The results of a typical experiment, which was repeated three times, are shown. Symbols indicate k_{cat} values as follows: (■) 1.50, (▲) 1.54, and (▼) 1.30 min^{-1}.

Effects of RGS16 and RGS4 on GTPase Activity of $G_s\alpha$ and $G_s\alpha$ Asp229Ser. The ability of RGS16 and RGS4 to stimulate GTPase activity of $G_s\alpha$ and $G_s\alpha$ Asp229Ser was tested using a single-turnover assay (*6*). In control experiments, RGS16 and RGS4 potently stimulated the GTPase activity of $G_i\alpha$. The single-turnover GTP hydrolysis by $G_i\alpha$ in the presence of 1 μM RGS16 or RGS4 was completed in less than 10 s, precluding accurate calculation of the k_{cat} values (not shown). Figure 3 shows that the GTPase activity of $G_s\alpha$ (k_{cat} = 1.50 min^{-1}) was unaffected in the presence of RGS16 even at concentrations as high as 12 μM (k_{cat} = 1.54 min^{-1}). Furthermore, the GTPase activity of $G_s\alpha$ was insensitive to high concentrations of the RGS16 Glu129Ala (Figure 3) and Glu129Ala/Asn131Ser (not shown) mutants as well. Likewise, RGS4 had no effect on the GTPase activity of $G_s\alpha$ (not shown), thus confirming previous findings (*6*). However, addition of RGS16 or RGS4 to the $G_s\alpha$ Asp229Ser mutant resulted in a substantial acceleration of the GTPase rate. In the presence of 12 μM RGS16 or 12 μM RGS4, the GTPase activity of $G_s\alpha$ Asp229Ser was enhanced by ~4−4.5-fold (Figure 4A,B). Our estimate for the EC_{50} value of the maximal acceleration of $G_s\alpha$ Asp229Ser GTPase activity by RGS16 is 7.5 ± 0.9 μM, and an estimate for the V_{max} value is 7.5 ± 0.2 min^{-1} (Figure 5).

Effects of the AlF_4^--Bound Conformations of $G_s\alpha$ or $G_s\alpha$ Asp229Ser on Stimulation of Transducin GTPase Activity

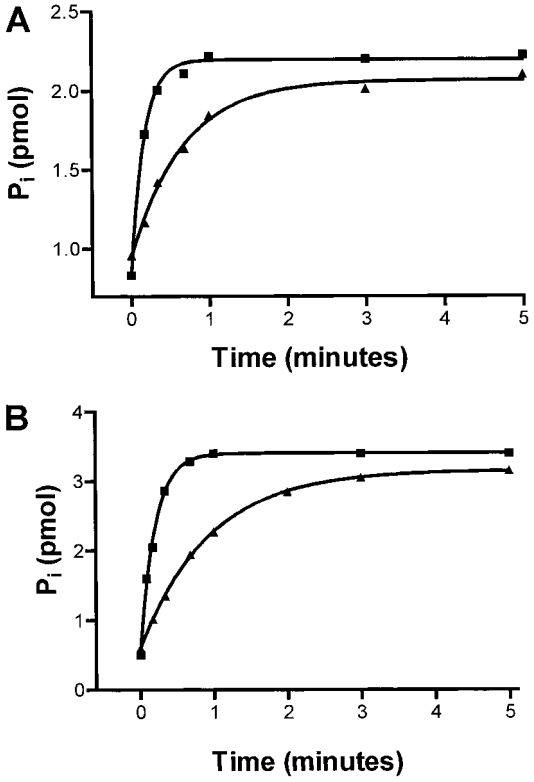

FIGURE 4: Effects of RGS16 and RGS4 on GTPase activity of $G_s\alpha$ Asp229Ser. The time course of GTP hydrolysis by $G_s\alpha$ Asp229Ser in the absence (▲) or in the presence (■) of 12 μM RGS16 (A) or 12 μM RGS4 (B). The results are representative of three experiments. Symbols indicate k_{cat} values as follows: (A) (▲) 1.34 and (■) 6.0 min^{-1} and (B) (▲) 1.21 and (■) 4.8 min^{-1}.

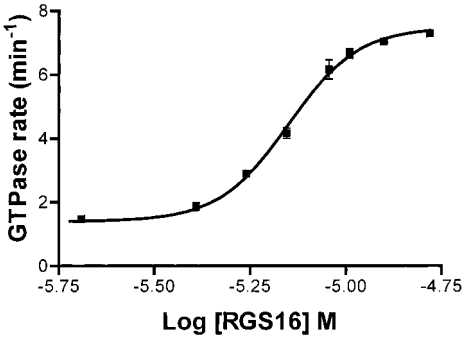

FIGURE 5: Dose dependence of the GAP effect of RGS16 on $G_s\alpha$ Asp229Ser. The GTPase rate constants for $G_s\alpha$ Asp229Ser were determined in the presence of RGS16 at increasing concentrations. The calculated EC_{50} value is 7.5 ± 0.9 μM, and the V_{max} value is 7.5 ± 0.2 min^{-1}.

by RGS16. A potential weak interaction between $G_s\alpha$ and RGS16 could have been missed in the coprecipitation experiments with these two proteins. Competition assays may represent a more sensitive test for examining weak interactions. We have tested whether the AlF_4^--bound conformations of $G_s\alpha$ and $G_s\alpha$ Asp229Ser can compete with $G_t\alpha$ for binding to RGS16 and block the stimulation of $G_t\alpha$ in a single-turnover GTPase assay. Neither $G_s\alpha$·AlF_4^- nor $G_s\alpha$ Asp229Ser·AlF_4^- had any appreciable effect on the basal GTPase activity of transducin (not shown). RGS16 at 0.1 μM caused ~50% of the maximal stimulation of $G_t\alpha$ GTPase activity (Figure 6). $G_s\alpha$ Asp229Ser·AlF4$^-$ (5 μM) effectively, by 45%, inhibited the stimulation of $G_t\alpha$ GTPase activity by RGS16 (Figure 6). In contrast, the same

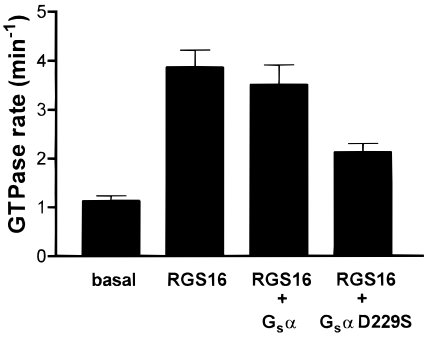

FIGURE 6: Effects of $G_s\alpha$ and $G_s\alpha$ Asp229Ser complexed with AlF_4^- on the stimulation of $G_t\alpha$ GTPase activity by RGS16. The GTPase activity of transducin (0.4 μM) reconstituted with uROS membranes (5 μM rhodopsin) was measured in the presence of 0.1 μM RGS16 with or without addition of the AlF4$^-$-bound conformations of $G_s\alpha$ or $G_s\alpha$ Asp229Ser at 5 μM.

concentrations of $G_s\alpha$ failed to significantly affect the $G_t\alpha$ GTPase stimulation by RGS16 (Figure 6), suggesting that $G_s\alpha$ has no physiologically relevant affinity for RGS16.

DISCUSSION

Thus far, the specificity of known RGS proteins has been well established for two families of heterotrimeric G proteins, G_i and G_q (*5*, *9*, *10*). Although an inhibitory effect of RGS3T on cAMP accumulation in the G_s signaling cascade has been reported (*27*), no GAP activity of RGS3T toward $G_s\alpha$ has been detected under in vitro conditions (*5*). This raises the possibility that this effect was not mediated by direct interaction of RGS3T with $G_s\alpha$, or perhaps, the manifestation of the effect required an unknown adapter protein. The crystal structures of different Gα subunits and the structure of the complex between RGS4 and $G_i\alpha_1$ have provided structural data that supports the biochemical evidence of RGS specificity for G_i and G_q (*11*). The six RGS contact residues that are different between $G_i\alpha$ and $G_s\alpha$ are likely to be important structural determinants of RGS−Gα interactions (*11*). Interestingly, only one position in the Gα sequence has been identified as absolutely critical for the selectivity of RGS for $G_i\alpha$ versus $G_s\alpha$. A substitution of Ser202 of $G_t\alpha$ by the corresponding Asp in $G_s\alpha$ ($G_s\alpha$ Asp229) led to a complete loss of RGS binding to $G_t\alpha$ (*16*). Initially, this finding raised our expectation that an RGS protein that would be a GAP for $G_s\alpha$ can be identified. Indeed, Ser206 of $G_i\alpha$ ($G_t\alpha$ Ser202) makes a limited number of contacts with RGS4, particularly with Glu126 (RGS16 Glu129) and Asn128 (RGS16 Asn131) (*11*). Furthermore, the Glu126 of RGS4, which could potentially have the most adverse effect for the RGS−Gα interaction when the Ser residue in Gα is replaced by Asp, is not strongly conserved in the RGS superfamily. However, the interaction of RGS16 with the $G_t\alpha$ Ser202Asp mutant was not rescued by the Glu129Ala mutant of RGS16 (*16*). Moreover, the double RGS16 mutant, Glu129Ala/Asn131Ser, also failed to bind to $G_t\alpha$ Ser202Asp and stimulate its GTPase activity (M. Natochin and N. O. Artemyev, unpublished observation). We have not pursued further substitutions of the RGS16 Glu129 residue, since a number of RGS proteins with different residues at the corresponding position have been recently tested for their ability to interact with $G_s\alpha$. RGS1, RGS7, and RGS12, containing Gln, Ala, and Pro, respectively, in the position corresponding to RGS16 Glu129, were incapable of stimulating $G_s\alpha$ GTPase activity (*7*, *28*, *29*). These results coupled with our data suggest that $G_s\alpha$ Asp229 may represent a major obstacle for the $G_s\alpha$−RGS interaction and that this obstacle is difficult to overcome by substitutions in RGS proteins. In agreement with existing data, we show that RGS16 even at very high concentrations does not bind to $G_s\alpha$. Similarly, the RGS16 Glu129Ala and Glu129Ala/Asp131Ser mutants exhibited no detectable affinity for $G_s\alpha$. However, a single mutation of $G_s\alpha$, Asp229Ser, conferred upon $G_s\alpha$ the ability to bind RGS16, as seen using the RGS16−$G_s\alpha$ mutant coprecipitation assay. In addition, the AlF_4^--bound $G_s\alpha$ Asp229Ser inhibited stimulation of $G_t\alpha$ GTPase activity by RGS16. As has been demonstrated for $G_t\alpha$, RGS16 preferentially recognized the AlF_4^--bound $G_s\alpha$ Asp229Ser. In a single-turnover assay, both RGS16 and RGS4 maximally accelerated the $G_s\alpha$ Asp229Ser GTPase rate by ~5-fold. A relatively high EC_{50} value for the RGS16 GAP effect on $G_s\alpha$ Asp229Ser (~7.5 μM) is nonetheless comparable to the half-maximal concentrations required for stimulation of $G_t\alpha$ GTPase by an RGS protein such as GAIP (*30*).

What are the potential implications of finding that a point mutation makes $G_s\alpha$ a target for RGS proteins? Although no naturally occurring mutations of $G_s\alpha$ Asp229 have been described, it is conceivable that they may exist and cause pathological conditions by targeting RGS to $G_s\alpha$. In addition, our results highlight the possibility that some as yet undescribed RGS-like proteins may interact with $G_s\alpha$ and stimulate its GTPase activity. An interesting possibility is a novel protein kinase A-anchoring protein, D-AKAP2, which contains a putative RGS-like domain (*31*). D-AKAP2 shares with other RGS proteins conserved hydrophobic residues, but lacks conservation of the charged residues within the RGS domain (*31*). The $G_i\alpha_1$ Ser206 contact residues of RGS4, Glu126 and Asn128, are replaced in D-AKAP2 by Pro and Gly, respectively. It is attractive to speculate that the Pro and Gly residues could introduce a bend into the D-AKAP2 RGS domain that accommodates $G_s\alpha$ Asp229. Finally, the $G_s\alpha$ Asp229Ser mutant may become a valuable tool for studying $G_s\alpha$ signaling in vivo. While this mutation is unlikely to interfere with $G_s\alpha$−adenylyl cyclase interaction (*15*, *32*), it places $G_s\alpha$ signaling under the control of RGS proteins.

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