Interaction of human retinal RGS with G-protein α-subunits

Michael Natochin[a], Valery M. Lipkin[b], Nikolai O. Artemyev[a,*]

[a] *Department of Physiology and Biophysics, University of Iowa College of Medicine, 5-660 BSB, Iowa City, IA 52242, USA*
[b] *Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow 117871, Russia*

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Abstract A novel family of RGS proteins negatively regulates signaling via heterotrimeric G-proteins by accelerating the GTPase activity of G-protein α subunits. We have investigated interaction of human retinal RGS protein (hRGSr) with in vitro translated $G_{t\alpha}$ subunits: $G_{t\alpha}$, $G_{i\alpha1}$, $G_{o\alpha}$ and $G_{s\alpha}$. hRGSr binds well to $G_{t\alpha}$, $G_{i\alpha1}$ and $G_{o\alpha}$ in the presence of AlF_4^-, but does not interact with $G_{s\alpha}$. The N- and C-terminally truncated G_α subunits interact with hRGSr similarly to the intact G_α polypeptides. Analysis of interaction between hRGSr and $G_{o\alpha}$/$G_{s\alpha}$ chimeras suggests that a region of $G_{o\alpha}$, $G_{o\alpha}$22–212, contains major structural determinants for binding to RGS proteins.

Key words: G-protein; Transducin; Regulator of G-protein signaling; Retina

1. Introduction

Recently, a novel protein family termed regulators of G-protein signaling (RGS) has been discovered [1–3]. RGS proteins in their capacity as GTPase activating proteins negatively regulate signaling via heterotrimeric G-proteins by rapidly converting the active GTP bound conformation of G-protein α subunits (G_αGTP) to the inactive G_αGDP conformation. Currently characterized RGS proteins interact with and stimulate GTPase activity of at least two families of G-proteins, G_i and G_q [4–7]. A highly homologous RGS-domain provides a relatively broad specificity of different RGS-proteins towards members of the two G-protein classes in vitro. Specific functional targets of RGS proteins in vivo may be defined by their differential tissue expression patterns, subcellular distribution and diverse functional domains outside the RGS segment [8,9]. The mechanism of RGS protein action involves stabilization of the transition state during GTP hydrolysis which is thought to be mimicked by the AlF_4^--bound conformation of G_α subunits [5,10]. Retina-specific RGS may be involved in the complex regulation of GTPase activity of the visual G-protein, transducin [11–14]. Two different retinal RGS proteins have been shown to stimulate GTP hydrolysis by transducin [13,14]. In vertebrate photoreceptor cells, light-activated rhodopsin induces the GDP/GTP exchange on the α-subunit of transducin ($G_{t\alpha}$). $G_{t\alpha}$GTP activates the effector enzyme, cGMP phosphodiesterase (PDE), by relieving the inhibitory constraint imposed by two identical inhibitory P_γ subunits of PDE [15,16]. The GTPase activity of transducin controls re-inhibition of PDE activity by the P_γ subunits and is the key reaction in the signal turn-off mechanism.

Here, we study the interaction of human retinal RGS (hRGSr) protein with different types of G-proteins and elucidate regions of G_α comprising the RGS-G_α interface using $G_{o\alpha}$/$G_{s\alpha}$ chimeric proteins.

2. Materials and methods

2.1. Materials

[^{35}S]methionine (>1000 Ci/mmol) was purchased from Amersham. Trypsin was obtained from Worthington Biochem. Corp. All other reagents were acquired from Sigma.

2.2. Cloning and expression of hRGSr

A human homologue of mouse retinal RGS [13], A28-RGS14p (the GenBank accession number U70426), was PCR amplified from the human retinal cDNA λgt10 library (gift from Dr. J. Nathans, Johns Hopkins University) using the following primers: ATACTCTAGACATGTGCCGCACCCTGGC(5′); ATGCCTCGAGACTAGGTGTGTGAGG (3′). The PCR product (620 bp) was digested with *Xba*I and *Xho*I (the restriction sites are underlined) and subcloned into the pGEX-KG vector [17] for GST-hRGSr fusion protein expression. The DNA sequence was verified by automated DNA sequencing at the University of Iowa DNA Core Facility. Expression, purification and functional characterization of hRGSr is described in details elsewhere (J. Biol. Chem., in press).

2.3. Preparation of ROS membranes and transducin

Bovine ROS membranes were prepared as previously described [18]. Urea washed ROS membranes were prepared according to protocol in [19]. Transducin, $G_{t\alpha\beta\gamma}$, was extracted from ROS membranes using GTP as previously described [20].

2.4. Assay of transducin GTPase activity

Single turnover GTPase activity measurements were carried out essentially as described in Ref. [21]. The reaction was initiated by mixing bleached ROS membranes with 200 nM [γ-^{32}P]GTP ($\sim 5\times10^4$ dpm/pmol) in a total volume of 20 μl. The reaction was quenched by addition of 100 μl of 7% perchloric acid. Nucleotides were then precipitated using charcoal, and $^{32}P_i$ formation was measured by liquid scintillation counting.

2.5. In vitro transcription-translation of G_α subunits

In vitro transcription-translations were carried out in the T_NT coupled reticulocyte lysate system (Promega) according to the manufacture's recommendations. Recombinant plasmids were added to the translation mixture at the concentration of 20 μg/ml. The pGEM2 vectors (Promega) containing $G_{o\alpha}$ and $G_{s\alpha}$ (short splicing form) cDNAs and 9 $G_{o\alpha}$/$G_{s\alpha}$ chimeras [22] ($G_{o\alpha}$1–212/$G_{s\alpha}$221–380/; $G_{o\alpha}$1–272/$G_{s\alpha}$281–380; $G_{o\alpha}$1–298/$G_{s\alpha}$323–380; $G_{o\alpha}$1–212/$G_{s\alpha}$221–280/$G_{o\alpha}$273–354; $G_{s\alpha}$1–220/$G_{o\alpha}$213–354; $G_{s\alpha}$1–280/$G_{o\alpha}$273–354; $G_{s\alpha}$1–321/$G_{o\alpha}$300–354; $G_{s\alpha}$1–220/$G_{o\alpha}$213–272/$G_{s\alpha}$281–380; $G_{s\alpha}$1–280/$G_{o\alpha}$273–298/$G_{s\alpha}$323–380) were transcribed with SP6 RNA polymerase (Stratagene). Vectors pHis$_6$$G_{i\alpha1}$ and pHis$_6$$G_{t\alpha}$ [23] containing rat $G_{i\alpha1}$ and bovine $G_{t\alpha}$ cDNAs were transcribed using T7 RNA polymerase (Stratagene). Transcription-translations were carried out for 2 h at room temperature with addition of 1 μCi [^{35}S]methionine. TPCK-treated trypsin (1 μg) was added to 2 μl aliquots of translation mixtures, which were then incubated for 30 min at room temperature.

*Corresponding author. Fax: +1 (319) 335-7330.
E-mail: nikolai-artemyev@uiowa.edu

Abbreviations: G_α, heterotrimeric GTP-binding protein α-subunit; PDE, photoreceptor cGMP phosphodiesterase; $G_{t\alpha\beta\gamma}$, rod G-protein, transducin; RGS proteins, regulators of G-protein signaling; hRGSr, human retinal RGS protein

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Proteolysis was stopped by addition of 5 μg of soybean trypsin inhibitor. Where indicated, 30 μM $AlCl_3$ and 10 mM NaF (AlF_4^-) were added prior to trypsin digestion.

2.6. Binding of G_α subunits to hRGSr

GST-hRGSr (10 μg) immobilized on glutathione-agarose beads (2 mg protein/ml agarose) was incubated with translation mixtures (15 μl final volume) for 20 min at room temperature followed by three washes with 1 ml of 20 mM Tris-HCl buffer (pH 8.0), containing 100 mM NaCl and 10 mM $MgCl_2$. Where indicated, AlF_4^- was added to translation mixtures and to the washing buffer. The bound proteins were separated by SDS-PAGE in 12% SDS-polyacrylamide gels. The gels were soaked in Amplify reagent (Amersham), dried and exposed to Fuji X-ray film.

3. Results

3.1. hRGSr interacts with members of the G_i family

GST-hRGSr fusion protein expressed in *E. coli* was fully functional. It activated the GTPase activity of transducin reconstituted with urea-washed rod outer segment membranes by up to ~10-fold to a rate of 0.22 s^{-1} (Fig. 1A). The transducin GTPase rate constants were determined in the presence of increasing concentrations of GST-hRGSr. An EC_{50} value of 85 nM was calculated for stimulation of transducin GTPase activity by GST-hRGSr (Fig. 1B). To study the interaction between hRGSr and different G-proteins we investigated the ability of GST-hRGSr fusion protein immobilized on a glutathione-agarose to co-precipitate the in vitro translated AlF_4^--bound conformations of G_α subunits: $G_{t\alpha}$, $G_{i\alpha1}$, $G_{o\alpha}$ and $G_{s\alpha}$. First, we confirmed that the in vitro translated G_α subunits are functionally active and capable of undergoing a conformational change upon binding to AlF_4^-. Binding of AlF_4^- to G_αGDP subunits leads to a protection of their switch II regions from the tryptic cleavage producing stable G_α polypeptides lacking N-terminal or both, N- and C-terminal fragments [24,25]. Addition of AlF_4^- to all in vitro translated G_α subunits resulted in protection from trypsin digestion and led to accumulation of the polypeptides with expected molecular weights: $G_{t\alpha}$, 32 kDa; $G_{i\alpha1}$, 33 kDa; $G_{o\alpha}$, 37 kDa; and $G_{s\alpha}$, 38 kDa (Fig. 2A). Fig. 2B shows that hRGSr did not bind G_αGDP subunits, but efficiently precipitated the AlF_4^--bound conformations of $G_{t\alpha}$, $G_{i\alpha1}$, and $G_{o\alpha}$. hRGSr showed no detectable interaction with $G_{s\alpha}$.

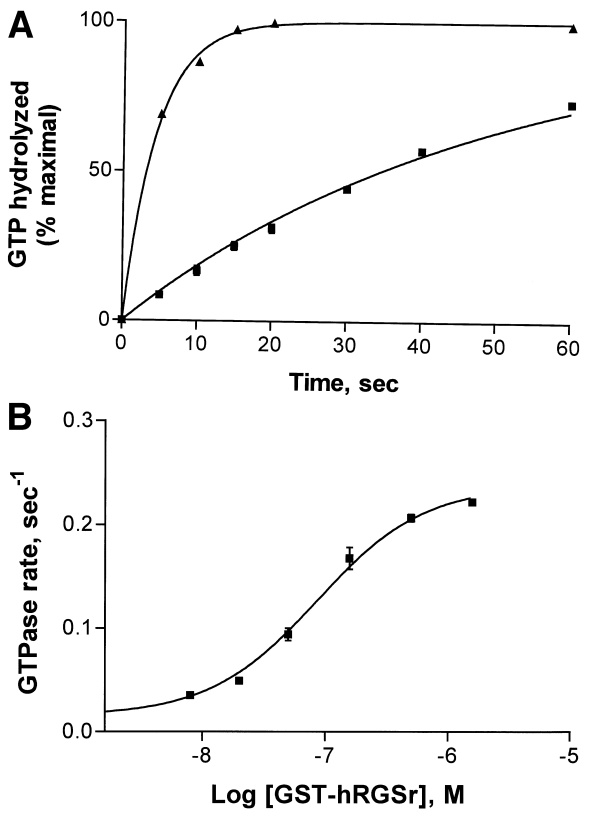

Fig. 1. Stimulation of transducin GTPase activity by GST-hRGSr. (A) The time course of GTP hydrolysis in suspensions of urea-washed ROS membranes was determined as described in Section 2. The reaction mixtures contained 5 μM rhodopsin reconstituted with 0.4 μM $G_{t\alpha\beta\gamma}$ alone (■), or with 0.4 μM $G_{t\alpha\beta\gamma}$ and 3 μM GST-hRGSr (▲). The calculated GTPase rate constants are: ■, 0.020 s^{-1}; ▲, 0.22 s^{-1}. (B) The transducin GTPase rate constants in suspensions of urea-washed ROS membranes (5 μM rhodopsin) reconstituted with 0.4 μM $G_{t\alpha\beta\gamma}$ were determined in the presence of increasing concentrations of GST-hRGSr.

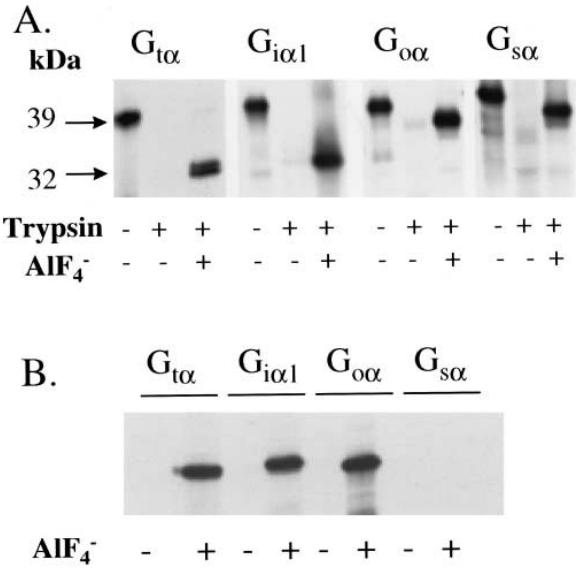

Fig. 2. Interaction of hRGSr with Gα subunits. Fluorograms of the ^{35}S-labeled G_α subunits. (A) The trypsin-protection test for Gα subunits. In vitro translated $G_{t\alpha}$, $G_{i\alpha1}$, $G_{o\alpha}$ and $G_{s\alpha}$ were treated with trypsin in the absence or presence of AlF_4^- as described in Section 2. (B) The ^{35}S-labeled G_α subunits were precipitated with GST-hRGSr immobilized on glutathione agarose beads in the absence or in the presence of AlF_4^-. The bound proteins were analyzed by SDS-PAGE in 12% polyacrylamide gels.

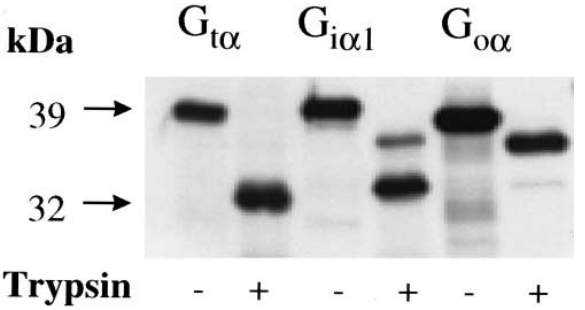

Fig. 3. Effect of tryptic digestion on binding of G_α subunits to hRGSr. In vitro translated $G_{t\alpha}$, $G_{i\alpha1}$ and $G_{o\alpha}$ were precipitated with the GST-hRGSr bound beads before and after cleavage with trypsin in the presence of AlF_4^-. The bound proteins were analyzed using fluorography of 12% polyacrylamide gels.

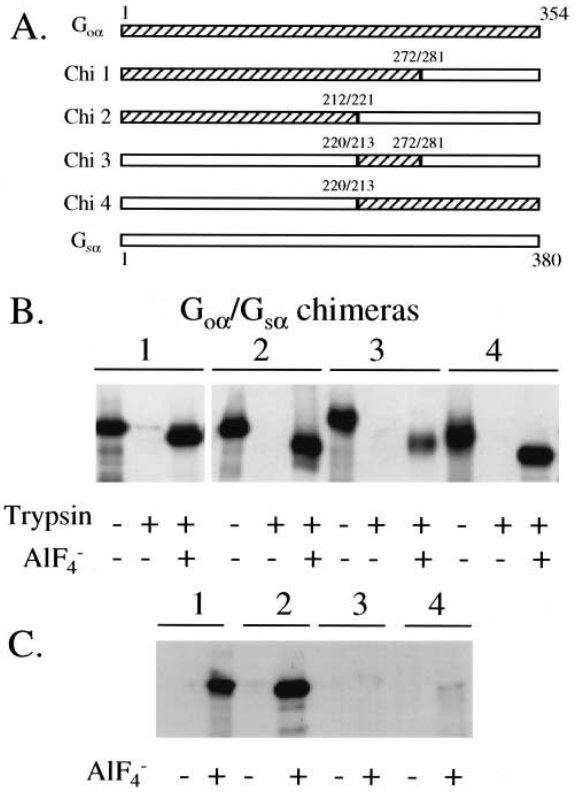

Fig. 4. Interaction of hRGSr with $G_{o\alpha}/G_{s\alpha}$ chimeras. (A) Schematic representation of the $G_{o\alpha}/G_{s\alpha}$ chimeras resistant to trypsin proteolysis in the presence of AlF_4^-. (B) Trypsin protection test of the $G_{o\alpha}/G_{s\alpha}$ chimeras. (C) Fluorogram of the in vitro translated $G_{o\alpha}/G_{s\alpha}$ chimeras precipitated with the GST-hRGSr bound beads in the absence or in the presence of AlF_4^-.

3.2. *Role of the N- and C-terminal regions of G_α subunits in interaction with hRGSr*

To determine if the N- and C-terminal regions of G_α subunits participate in the interaction with hRGSr, we compared the efficiency of precipitation of intact G_α subunits by GST-hRGSr bound agarose beads with that of trypsin digested G_α subunits. Trypsin cleaves off 18 N-terminal and 40 C-terminal amino acid residues of $G_{t\alpha}$ producing a stable 32 kDa polypeptide [24,25]. The amount of the 32 kDa tryptic fragment of $G_{t\alpha}$ precipitated by hRGSr was comparable to that of the intact 39 kDa $G_{t\alpha}$ polypeptide (Fig. 3). Next, we used endoproteinase Lys-C which cleaves off 25 N-terminal amino acid residues of $G_{t\alpha}$ [26]. Binding of the 36 kDa endoproteinase Lys-C fragment of $G_{t\alpha}$ to hRGSr was equivalent to the binding of the 32 kDa tryptic fragment (not shown), suggesting that the 25 N-terminal residues of $G_{t\alpha}$ are not involved in the interaction with hRGSr. Similar results were obtained using $G_{i\alpha1}$ and $G_{o\alpha}$. The tryptic patterns of $G_{t\alpha}$ and $G_{i\alpha1}$ are very similar, and it appears that trypsin cleaves $G_{i\alpha1}GDPAlF_4^-$ at both, N- and C-terminal sites. $G_{o\alpha}GDPAlF_4^-$ is cleaved by trypsin only at the N-terminal site, resulting in truncation of 21 amino terminal residues [25]. Tryptic fragments of $G_{i\alpha1}$ (33 kDa) and $G_{o\alpha}$ (37 kDa) did not reveal significantly decreased interaction with hRGSr (Fig. 3).

3.3. *Binding of $G_{o\alpha}/G_{s\alpha}$ chimeric proteins to hRGSr*

Chimeric $G_{o\alpha}/G_{s\alpha}$ proteins were utilized for mapping of the G_α regions involved in interaction with RGS proteins. We took advantage of the fact that $G_{s\alpha}$ showed no interaction with hRGSr in our assay. Nine $G_{o\alpha}/G_{s\alpha}$ chimeras were prepared as described previously [22]. The trypsin protection assay was utilized to ensure proper folding and activity of the chimeric proteins. Only four out of nine chimeric proteins were functionally active using this assay (Fig. 4A and B). These $G_{o\alpha}/G_{s\alpha}$ chimeras were tested for binding to hRGSr. Replacement of the 82 C-terminal amino acid residues of $G_{o\alpha}$ for the corresponding C-terminal portion of $G_{s\alpha}$ in chimera 1 caused no reduction in the amount of G-protein bound to RGS (Fig. 4C). A more extensive substitution of the $G_{o\alpha}$ C-terminal part for the $G_{s\alpha}$ sequence (chimera 2) also had no significant effect on the interaction between hRGS and the chimeric G_α (Fig. 4C). In contrast, chimera 3, which contained residues 213–272 of $G_{o\alpha}$, did not bind hRGSr, whereas chimera 4 with a relatively large C-terminal portion of $G_{o\alpha}$ interacted with hRGSr very weakly (Fig. 4C). Our data suggest that the sequence $G_{o\alpha}22$–212 contains major structural determinants for binding to RGS proteins, while the rest of the molecule only weakly contributes to this interaction.

4. Discussion

A novel protein family of negative regulators of G-protein signaling (RGS) has been recently identified [1–3,8]. A growing number of studies have demonstrated that members of this family, GAIP, RGS4, RGS1, RGS10 and others, stimulate GTPase activity of G-proteins from G_i and G_q families [4–7]. Two retina-specific RGS proteins, RGSr and RET-RGS have been recently discovered [13,14]. Both proteins were found to bind transducin and accelerate its GTPase activity [13,14]. The specificity of retinal RGS proteins for different types of G_α subunits has not been investigated. We have expressed a novel human homologue of mouse RGSr [13] and studied binding of hRGSr to in vitro translated G_α subunits, $G_{t\alpha}$, $G_{i\alpha1}$, $G_{o\alpha}$ and $G_{s\alpha}$. Our data demonstrate that hRGSr binds well to members of the G_i family in the AlF_4^-, but not GDP-bound conformation. This rather broad specificity of RGSr towards G_i-like G-proteins is similar to that of other characterized RGS proteins [8]. hRGSr did not interact with $G_{s\alpha}$. To date, no RGS protein specific for $G_{s\alpha}$ has been described.

Recent study has demonstrated that the C-terminus of $G_{i\alpha3}$ is important for its interaction with GAIP, a member of the RGS family [27]. We found that $G_{t\alpha}$ and $G_{i\alpha1}$, cleaved at N- and C-terminal sites with trypsin, and the N-terminally truncated $G_{o\alpha}$ showed no significant alteration in binding to hRGS. The C-terminal fragments of $G_{t\alpha}$ and $G_{i\alpha1}$ have extensive interactions with the core molecules based on the crystal structures of these G-proteins [28,29], and thus, may remain in association with them. The data, therefore, only indicate that N-terminal regions of $G_{t\alpha}$, $G_{i\alpha1}$ and $G_{o\alpha}$ are not essential for the interaction with RGS. However, further analysis of the hRGSr interaction with $G_{o\alpha}/G_{s\alpha}$ chimeras suggests that the $G_{o\alpha}$ sequence, $G_{o\alpha}$22-212, contains major RGS binding domains. Previous findings have established that many RGS proteins interact strongly with the AlF_4^- conformation but only weakly with the GTPγS conformation of G_α subunits. The crystal structures of $G_{t\alpha}$ and $G_{i\alpha1}$ in the AlF_4^- and GTPγS-bound conformations are different only in the switch I and switch II regions [29,30] making them likely candidates for binding to RGS. Supporting this idea, the

$G_{o\alpha}$22–212 sequence includes the switch I region and most of the switch II region of $G_{o\alpha}$

At the time of the preparation of this manuscript Tesmer et al. [31] reported a crystal structure of RGS4 bound to AlF_4^- activated $G_{i\alpha1}$. The crystal structure reveals the RGS4 binding site formed by the three switch regions of $G_{i\alpha1}$: I-residues 179–185; II-residues 204–213; and III-residues 235–237. Our results are consistent with the crystal structure because the $G_{o\alpha}$1–212 sequence contains most of the amino acid residues implicated in the RGS4/$G_{i\alpha1}$ interface. Additional interactions between the switch III region and RGS4 protein are not extensive, and the contribution of the switch III regions to the overall affinity of the RGS/G_α interaction may not be very significant. Interestingly, Tesmer et al. [31] observed a second binding site between RGS4 and $G_{i\alpha1}$ which involves the extended N and C termini of $G_{i\alpha1}$ contacting a neighboring RGS4 molecule in the crystal. Our data support the assertion that this binding site could be an artifact of the crystal packing [31].

Binding of RGS proteins to the switch regions of G-proteins validate the notion that RGS proteins may serve as antagonists for some effectors [31]. RGS4 has been shown to block activation of phospholipase Cβ by $G_{q\alpha}$GTPγS [7]. hRGSr, however, does not block activation of rod PDE by $G_{t\alpha}$GTP or $G_{t\alpha}$GTPγS (N.O.A., personal communication). As shown here, $G_{t\alpha}$ and $G_{o\alpha}$ bind hRGSr comparably well. In contrast, $G_{o\alpha}$ has a 10^4-fold lower affinity for Pγ than $G_{t\alpha}$ [32]. This indicates that the hRGSr and effector binding surfaces on $G_{t\alpha}$ are different, although they may partially overlap.

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