AGS3 Inhibits GDP Dissociation from Gα Subunits of the G_{i1} Family and Rhodopsin-dependent Activation of Transducin*

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A number of recently discovered proteins that interact with the α subunits of G_{i} like G proteins contain homologous repeated sequences named G protein regulatory (GPR) motifs. Activator of G protein signaling 3 (AGS3), identified as an activator of the yeast pheromone pathway in the absence of the pheromone receptor, has a domain with four such repeats. To elucidate the potential mechanisms of regulation of G protein signaling by proteins containing GPR motifs, we examined the effects of the AGS3 GPR domain on the kinetics of guanine nucleotide exchange and GTP hydrolysis by G_{i1} and transducin-α (G_{α}). The AGS3 GPR domain markedly inhibited the rates of spontaneous guanosine 5’-O-(3-thiotriphosphate) (GTPγS) binding to G_{α} and rhodopsin-stimulated GTPγS binding to G_{α}. The full-length AGS3 GPR domain, AGS3-(463–650), was ~30-fold more potent than AGS3-(572–629), containing two AGS3 GPR motifs. The IC_{50} values for the AGS3-(463–650) inhibitory effects on G_{α} and transducin were 0.12 and 0.15 μM, respectively. Furthermore, AGS3-(463–650) and AGS3-(572–629) effectively blocked the GDP release from G_{α} and rhodopsin-induced dissociation of GDP from G_{α}. The potencies of AGS3-(572–629) and AGS3-(463–650) to suppress the GDP dissociation rates correlated with their ability to inhibit the rates of GTPγS binding. Consistent with the inhibition of nucleotide exchange, the AGS3 GPR domain slowed the rate of steady-state GTP hydrolysis by G_{α}. The catalytic rate of G_{α} GTP hydrolysis, measured under single turnover conditions, remained unchanged with the addition of AGS3-(463–650). Altogether, our results suggest that proteins containing GPR motifs, in addition to their potential role as G protein-coupled receptor-independent activators of G\_βγ signaling pathways, act as GDP dissociation inhibitors and negatively regulate the activation of a G protein by a G protein-coupled receptor.

Signal-activated G protein-coupled receptors (GPCRs) stimulate GDP/GTP exchange on the α subunits of G proteins. Following the activational interaction with receptors, GoGTP and G\_βγ are released to activate their targets, which include adenyl cyclases, phospholipases, phosphodiesterases, and ion channels (1–3). A novel class of GTPase-activating proteins (GAPs) for G proteins termed regulators of G protein signaling (RGS) has been identified (4–6). RGS proteins share a highly conserved RGS domain, which is responsible for the GAP function. Recently, cloning of proteins critical for glial cell development resulted in the identification of the first known Drosophila RGS protein, LOCO (7). The LOCO sequence revealed significant homology to RGS12 and RGS14 within the RGS domain and three additional regions B, C, and D (7). A yeast two-hybrid screen was carried out using G_{α} as bait in an attempt to confirm the interaction of LOCO with G_{α}. Interestingly, the D region, rather than the RGS domain of LOCO, was found to bind G_{α} (7). Sequence analysis of the D region revealed that it contained a segment of homology with four ~20-amino acid repeats present in the human mosaic protein, LGN. LGN has been previously identified as a G_{α},G_{βγ}-interacting protein using a yeast two-hybrid system (8). LGN is similar to the activator of G protein signaling 3 (AGS3), which was isolated in a functional screen for receptor-independent activators of heterotrimeric G protein signaling (9). Site-directed mutagenesis and protein interaction studies with AGS3 (9) indicated that the ~20-amino acid repeats common to AGS3, LGN, and LOCO were responsible for binding G_{α}. The ~20-amino acid repeats were termed the G protein regulatory (GPR) (9) or GoLOCO motif (10). The GPR motif was also identified in Purkinje cell protein-2 (Pcp2) and Rap1GAP, which were identified as G_{α} beta-interacting proteins in yeast-two hybrid screens (11, 12). These studies suggest that GPR-containing proteins, hereafter termed GPR proteins, are likely to represent a diverse family of proteins that modulate G protein signaling.

At present very little is known about the mechanisms and functions of GPR proteins. The yeast pheromone response pathway is mediated by G\_βγ subunits, and its GPCR-independent activation by AGS3 suggests that it may induce release of G\_βγ from G proteins (9). Pcp2 protein was shown to stimulate GDP release from G_{α} without affecting the k_{cat} for GTP hydrolysis, thus raising the possibility that GPR proteins may serve as guanine nucleotide exchange factors for G proteins (11). To date, no studies on the regulation of GPCR-mediated G protein activation by GPR proteins have been reported. In this study, we examined the effects of the AGS3 GPR domain (AGS3GPR) on the intrinsic guanine nucleotide exchange of G_{α}, and transducin-α (G_{α}) and on the rhodopsin-stimulated GDP/GTP exchange on the α subunits of G proteins. Following the activational interaction with receptors, GoGTP and G\_βγ are released to activate their targets, which include adenyl cyclases, phospholipases, phosphodiesterases, and ion channels (1–3). A novel class of GTPase-activating proteins (GAPs) for G proteins termed regulators of G protein signaling (RGS) has been identified (4–6). RGS proteins share a highly conserved RGS domain, which is responsible for the GAP function. Recently, cloning of proteins critical for glial cell development resulted in the identification of the first known Drosophila RGS protein, LOCO (7). The LOCO sequence revealed significant homology to RGS12 and RGS14 within the RGS domain and three additional regions B, C, and D (7). A yeast two-hybrid screen was carried out using G_{α} as bait in an attempt to confirm the interaction of LOCO with G_{α}. Interestingly, the D region, rather than the RGS domain of LOCO, was found to bind G_{α} (7). Sequence analysis of the D region revealed that it contained a segment of homology with four ~20-amino acid repeats present in the human mosaic protein, LGN. LGN has been previously identified as a G_{α},G_{βγ}-interacting protein using a yeast two-hybrid system (8). LGN is similar to the activator of G protein signaling 3 (AGS3), which was isolated in a functional screen for receptor-independent activators of heterotrimeric G protein signaling (9). Site-directed mutagenesis and protein interaction studies with AGS3 (9) indicated that the ~20-amino acid repeats common to AGS3, LGN, and LOCO were responsible for binding G_{α}. The ~20-amino acid repeats were termed the G protein regulatory (GPR) (9) or GoLOCO motif (10). The GPR motif was also identified in Purkinje cell protein-2 (Pcp2) and Rap1GAP, which were identified as G_{α} beta-interacting proteins in yeast-two hybrid screens (11, 12). These studies suggest that GPR-containing proteins, hereafter termed GPR proteins, are likely to represent a diverse family of proteins that modulate G protein signaling.

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nucleotide exchange of Gα. Our results suggest that AGS3GPR acts as a GDP dissociation inhibitor (GDI) and may block GPCR-dependent activation of G proteins from the Gα family.

**EXPERIMENTAL PROCEDURES**

**Materials**—[35S]GTPyS (1160 Ci/mol), [γ-32P]GTP (>3000 Ci/mol), and [γ-32P]GTP (>3000 Ci/mol) were purchased from Amer sham Pharmacia Biotech. Restriction enzymes were from New England Biolabs. Cloned Pfu DNA polymerase was from Stratagene. All other chemicals were from Sigma or Fisher. Bovine ROS membranes were prepared as described previously (13). Urea-stripped ROS (uROS) membranes were prepared according to protocol in Yamanaka et al. (14). Gβγ was purified according to Kleuss et al. (15). GαGDP was prepared as described (16). Gα was expressed in Escherichia coli and purified as described (17).

**Cloning and Expression of AGS3GPR Constructs**—A cDNA sequence corresponding to residues AGS3-(463–650) of rat AGS3 and containing GPR motifs III (aa 572–590) and IV (aa 606–624) was polymerase chain reaction amplified from the human retinal cDNA lambda gt10 library (provided by J. Nathans, The Johns Hopkins University) using the following primers: ACTAATCAGACCTTCTACAACTGCTCAACATC and ATCTCCCTGAGCTACTGACCTGTCATCATG. The primers were designed based on the cDNA sequence of a human homologue of AGS3 (GenBank™ accession number 5911952), identified using a Basic Local Alignment Search Tool search at NCBI (Bethesda, MD). In comparison to the rat AGS3-(572–629) sequence, the human sequence had a single homologous substitution, Glu-573→Asp. The polymerase chain reaction DNA fragment was digested with XbaI and XhoI and subcloned into a pGEX-KG vector (18) digested with the same enzymes. The sequence of an insert was confirmed by automated DNA sequencing at the University of Iowa DNA Core Facility. An additional construct, GST-AGS3-(463–650), was prepared as described. The GST-AGS3-(463–650) construct consists of the last 189 amino acids of rat AGS3 and includes all four GPR motifs (aa 470–489), GPR-II (aa 524–542), GPR-III (aa 572–590), and GPR-IV (aa 606–624). GST-AGS3-(572–629) and GST-AGS3-(463–650) were expressed in BL21 cells and purified using glutathione-agarose as described (20). The yields of soluble products were 15–20 mg/liter of culture.

**GTPyS Binding Assay**—Gα and Gα subunits (0.5 μM) alone or Gα mixed with 1 μM Gβγ and uROS membranes (5 μM rhodopsin) were incubated for 3 min at 25 °C in 0.5 ml of 20 mM Tris-HCl (pH 8.0) buffer containing 130 mM NaCl and 10 mM MgSO4 (buffer A) with or without the addition of varying concentrations of AGS3-(572–629) or AGS3-(463–650). Binding reactions were started by the addition of 5 μM [35S]GTPyS (0.1 μCi). Aliquots of 10 μl were sampled at the indicated times and passed through Whatman cellulose nitrate filters (0.45 μm). The filters were then washed three times with 1 ml of the same buffer and were transferred to 100 μl of 10% charcoal suspension, and 32Pi formation was measured using 700 Ci). Aliquots of 10 μl of 100 M GTP or GTP in buffer A for 1 h at 25 °C. Gα, Gαβγ complexed with [32P]GDP was prepared by incubating 1 μM Gα, 1 μM Gβγ, and uROS membranes (100 μM rhodopsin) with 2 μM [32P]GTP in buffer A for 40 min at 25 °C. uROS membranes were removed by centrifugation (30 min, 100,000 × g). Excess of unlabeled GTP (1 μM), uROS membranes (5 μM rhodopsin, only for Gα), and varying concentrations of AGS3-(572–629) or AGS3-(463–650) were added to monitor dissociation of [32P]GDP from Gα subunits. Aliquots were withdrawn at the indicated times and passed through Whatman cellulose nitrate filters (0.45 μm). The dissociation rates (koff) were calculated by fitting the experimental data to a single exponential decay function: %GDP bound = 100%e–kofft.

**GTPase Assays**—Urease-steady state GTPase activity measurements were initiated by mixing Gα (1 μM) with 10 μM [32P]GTP in 100 μl of buffer (buffer A). Aliquots (10 μl) were withdrawn at the indicated times and transferred to 100 μl of 7% perchloric acid. Nucleotides were precipitated using charcoal, 32P, and GTPγS formation was measured by liquid scintillation counting.

Single turnover GTPase activity measurements were carried out in suspensions of uROS membranes (5 μM rhodopsin) reconstituted with Gα (0.5 μM) and Gβγ (1 μM) essentially as described (21). The reaction was initiated by mixing bleached ROS membranes with 50 μM Gα (0.5 μM) and Gβγ (1 μM) at room temperature. The reaction rate (kcat) was measured as the rate of [32P]GTP hydrolysis by Gα, Gαβγ, and uROS membranes (5 μM rhodopsin).

**RESULTS**

**Effects of AGS3GPR on Guanine Nucleotide Binding to Gα**—Two AGS GPR polypeptides, AGS3-(463–629) and AGS3-(463–650), were utilized to investigate the modulation of guanine nucleotide binding to Gα and Gα. AGS3-(572–629) contains the GPR repeats III and IV of AGS3, whereas AGS3-(463–650) the rate of GTP nucleotide exchange. The GTPase activity of AGS3-(572–629) led to a dose-dependent increase in the GTP binding rates. In the presence of 15 μM AGS3-(572–629) the rate of GTP binding to Gα was inhibited (kapp = 0.018 min–1) (Fig. 1A). Addition of increasing concentrations of AGS3-(572–629) led to a dose-dependent increase in the GTP binding rates. The IC50 values for AGS3-GPR binding to Gα are as follows: 0.042 ± 0.001 μM, 0.032 ± 0.001 μM, and 0.033 ± 0.003 μM.

**[32P]GTP (−5 × 104 dpm/ml) in a total volume of 20 μl. The reaction was quenched by the addition of 100 μl of 7% perchloric acid. Nucleotides were then precipitated using charcoal, and [32P]GTP formation was measured by liquid scintillation counting.**

**Effects of AGS3 on Transducin and Gα**

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Effects of AGS3 on Transducin and \( G_i \)

GTP blocks the dissociation of GDP and, as a result, binding of \( G_i \) to GDP.\(^\text{Fig. 1} \)
The inhibition of GTP\(^\text{binding to } G_i \) shows that AGS3-(572–629) as a GDI correlated with its inhibition of GDP dissociation rates from \( G_i \).\(^\text{Fig. 2} \)

The rate of steady-state GTP hydrolysis by \( G_i \) was 0.028 \( \text{min}^{-1} \) in the presence of AGS3-(463–650), containing two and four GPR motifs, respectively, have been reported (25). Fig. 2 shows that AGS3-(572–629) was a more effective GDI than AGS3-(463–650).\(^\text{Fig. 2B} \)

Effects of AGS3GPR on Rhodopsin-catalyzed Nucleotide Exchange on \( G_i \)—AGS3 binds GDP very tightly and practically does not exchange nucleotides in the absence of \( R^\alpha \). The slow GTP\(^\text{S binding rate of } G_i \) was 0.001 \( \text{min}^{-1} \) in the presence of GDP (1 mM) and varying concentrations of AGS3-(572–629) (Fig. 4). AGS3-(572–629) produced an empty pocket for \( G_i \). The rate of GDP dissociation of \( G_i \) in the absence of AGS3GPR \((k_{\text{off}} = 0.35 \text{ min}^{-1}) \) (Fig. 2A) was higher than the GTP\(^\text{S binding rate in Fig. 1A. Although it is thought that GDP release is a rate-limiting step in the nucleotide exchange by Go subunits (24), rates of GDP dissociation considerably exceeding GTP\(^\text{S binding rates have also been reported (25). Fig. 2A shows that AGS3-(572–629) significantly slowed the rate of GDP release. The potency of AGS3-(572–629) as a GDI correlated with its inhibition of GTP\(^\text{S binding. Not surprisingly, the full AGS3 GPR domain, AGS3-(463–650), was a more effective GDI than AGS3-(572–629).}} \)

Effects of AGS3GPR on Steady-state and Single Turnover GTP Hydrolysis—The rate of steady-state GTP hydrolysis is limited by the rate of nucleotide exchange (26). Therefore, a steady-state GTPase assay provides an additional method of confirming the inhibition of \( G_i \) nucleotide exchange by AGS3GPR. The rate of steady-state GTP hydrolysis by \( G_i \) was 0.028 \( \text{min}^{-1} \) in the presence of AGS3-(463–650) (Fig. 5A). Reflecting the ability to inhibit nucleotide exchange, AGS3-(463–650) effectively suppressed the steady-state rate of GTP hydrolysis by \( G_i \) (Fig. 5A).

To test the possibility that AGS3GPR regulates a catalytic step of GTP hydrolysis \((k_{\text{cat}})\), transducin GTPase activity was measured under single turnover conditions (\([\text{GTP}] < [G_i]\)) (21). The \( k_{\text{cat}} \) for GTP hydrolysis by transducin reconstituted with uROS was 0.015 \( s^{-1} \) (Fig. 5B). Addition of 5 \( \mu M \) AGS3-(463–650) had no effect on GTP hydrolysis \((k_{\text{cat}} = 0.014 \ s^{-1})\).

**DISCUSSION**

Recent findings have identified a novel domain present singly or as multiple repeats in a variety of G protein-interacting proteins (7–12). The first indication that this domain mediates the interaction with Go subunits came from the analysis of the RGS protein LOCO from *Drosophila* (7). Subsequently this sequence motif containing 19 amino acid residues has been termed GoLOCO (10) or, more generally, a GPR motif (9). GPR proteins identified to date appear to specifically target Go subunits from the \( G_i \) family. GST pull-down experiments showed association of Pcp2 with \( G_i \) and \( G_s \) but not \( G_o \) (11). Similarly, Rap1GAP selectively interacted with \( G_i \) but not with \( G_s \) and \( G_o \) (12). AGS3 activated the *Saccharomyces cerevisiae* pheromone response in the \( G_o \) genetic background but not in yeast strains expressing *Gpa1*, \( G_o \), or \( G_o \) (9). The specificity of GPR proteins toward different conformations of \( G_o \) remains unclear. Pcp2 bound equally the GDP and GTP\(^\text{S conformations of } G_o \) (11). In contrast, AGS3 displayed preferential interaction with the GDP-bound Go subunits (9). Rap1GAP was shown to interact more avidly with \( G_o \) than \( G_s \) (12), whereas the \( N \)-terminally extended isoform of Rap1GAP, Rap1GAPII, preferentially bound the GTP-complexed \( G_o \) (19). Modulation of the Rap1GAP and Rap1GAPII activities by Go subunits suggests an interesting link via GPR motifs between G protein signaling and cascades involving small monomeric GTPases (12, 19). Currently, the mechanisms of regulation of G protein signaling by GPR proteins are not well understood. By dissociating heterotrimeric complexes \( G\beta\gamma \), GPR proteins could serve as GPCR-independent activators of G protein pathways that utilize \( G\beta\gamma \) subunits to transduce signals (9). Effects of GPR proteins on GPCR-dependent G protein signaling have not yet been investigated. To examine potential mechanisms of G protein regulation by GPR proteins, we analyzed the effects of AGS3GPR on the guanine nucleotide exchange of \( G_o \) and \( G_s \) Two AGS3 constructs, AGS3-(572–629) and AGS3-(463–650), containing two and four GPR motifs, respectively, have been utilized to assess the role of domain multiplicity. The AGS3 GPR constructs markedly inhibited spontaneous GTP\(^\text{S binding to } G_o \) because of its intrinsic nucleotide exchange. The full-length AGS3 GPR domain, AGS3-(463–650), was approximately 30-fold more potent than AGS3-(572–629), indicating the possibility that several Go subunits can simultaneously bind to a single AGS3 molecule. In addition, a better
Effects of AGS3 on Transducin and $G_i$

Fig. 3. Effects of AGS3GPR on the rhodopsin-induced GTP$\gamma$S binding to $G_\alpha$. A and B, the binding of GTP$\gamma$S to $G_\alpha$ (0.5 $\mu$M) with reconstituted G$_{i}$/$\beta$Y (1 $\mu$M) and uROS membranes (5 nM rhodopsin) in the absence ($\square$) or presence of AGS3-(572–629) (A) or AGS3-(463–650) (B) 15 $\mu$M, $\textbullet$, 10 $\mu$M, $\bigtriangleup$, 5 $\mu$M, $\blacktriangle$, 3 $\mu$M, $\blacktriangledown$, 0.5 $\mu$M. The binding reactions were initiated by the addition of 5 $\mu$M [35S]GTP$\gamma$S. The calculated $k_{eq}$ values (min$^{-1}$) are as follows: (A) 0.040 ± 0.001 ($\square$), 0.025 ± 0.001 ($\textbullet$), 0.025 ± 0.001 ($\bigtriangleup$), and 0.019 ± 0.001 ($\blacktriangle$); (B) 0.040 ± 0.001 ($\square$), 0.029 ± 0.001 ($\textbullet$), 0.022 ± 0.002 ($\bigtriangleup$), and 0.018 ± 0.001 ($\blacktriangle$). C, the rate constants ($k_{app}$) for GTP$\gamma$S binding to $G_\alpha$ are plotted as a function of AGS3-(572–629) (C) or AGS3-(463–650) (B) concentrations. The IC$_{50}$ values are 4.6 ± 0.9 ($\square$) and 0.15 ± 0.01 $\mu$M ($\bigtriangleup$).

Fig. 4. Effects of AGS3GPR on the rhodopsin-induced dissociation of GDP from $G_\alpha$. $G_\alpha$,$\beta$Y complexed with [32P]GDP was obtained as described under “Experimental Procedures. Excess of unlabeled GTP (1 mM), uROS membranes (5 nM rhodopsin), and varying concentrations of AGS3-(572–629) (C), 0.15 $\mu$M; $\bigtriangleup$, 0.5 $\mu$M; $\blacktriangle$, 3 $\mu$M) or AGS3-(463–650) (B) $\bigtriangleup$, 0.15 $\mu$M; $\blacktriangle$, 0.5 $\mu$M; $\blacktriangledown$, 3 $\mu$M were added to G$_{i}$/$\beta$Y$\gamma$DP (1 $\mu$M). Aliquots were withdrawn at the indicated times and passed through cellulose nitrate filters (0.45 $\mu$m). The $k_{off}$ (min$^{-1}$) values are as follows: (A) 0.76 ± 0.06 ($\square$), 0.28 ± 0.02 ($\bigtriangleup$), and 0.066 ± 0.008 ($\blacktriangle$); (B) 0.76 ± 0.06 ($\square$), 0.27 ± 0.02 ($\bigtriangleup$), 0.17 ± 0.03 ($\blacktriangle$), and 0.12 ± 0.01 ($\blacktriangledown$).

Fig. 5. Effects of AGS3GPR on steady-state and single turnover GTPase activity. A, steady-state GTPase activity measurements were carried out as described under “Experimental Procedures” using 1 $\mu$M $G_\alpha$ in the absence ($\square$) or presence of AGS3-(463–650) (C), 0.15 $\mu$M, $\bigtriangleup$, 0.3 $\mu$M; $\square$, 1 $\mu$M; $\bigtriangleup$, 3 $\mu$M). The rates of GTP hydrolysis (pmol of P/µmol of $G_\alpha$/min) are as follows: 0.028 ± 0.001 ($\square$), 0.018 ± 0.001 ($\bigtriangleup$), 0.010 ± 0.001 ($\blacktriangledown$), 0.006 ($\square$), and 0.004 ($\bigtriangleup$). B, single turnover GTPase activity measurements were carried out in suspensions of uROS membranes (5 $\mu$M rhodopsin) reconstituted with $G_\alpha$ (0.5 $\mu$M) and G$\beta$Y (1 $\mu$M) in the absence ($\square$) or presence of 5 $\mu$M AGS3-(463–650) ($\bigtriangleup$). The $k_{off}$ values are 0.015 ± 0.01 s$^{-1}$ ($\square$) and 0.014 ± 0.02 s$^{-1}$ ($\bigtriangleup$).

Effects of AGS3 on GPCR/G protein coupling by GPR proteins. AGS3-(572–629) and AGS3-(463–650) effectively inhibited activation of transducin by R* in the GTP$\gamma$S binding assay. Furthermore, AGS3GPR was capable of blocking the R*-induced GDP release from $G_\alpha$. By analogy to its effect on $G_\alpha$, AGS3GPR is likely to act directly on $G_\alpha$ as a GDI. Other factors may have contributed to the inhibitory effect of AGS3GPR on transducin activation. AGS3 is capable of dissociating $G_\alpha$ and G$\beta$Y (9), and this would also lead to an impairment in the G$_{i}$R* coupling. Furthermore, AGS3GPR and R* may compete for binding to $G_\alpha$, thus causing the inhibition. We did not find any evidence that AGS3GPR affects the $k_{off}$ for GTP hydrolysis by $G_\alpha$. Interestingly, some RGS proteins, such as RGS12 and RGS14, contain GPR motifs (10). These proteins might be capable of
very effective dual inhibition of G protein signaling at the level of activation by receptor and at the level of inactivation due to accelerated GTP hydrolysis.

Overall, our results demonstrate the capacity of GPR proteins for negative modulation of GPCR-dependent G protein activation. GPR proteins appear to have an interesting dual potential to regulate G protein signaling. AGS3 may act as a selective activator of Gbg-regulated effector systems independent of a receptor, while at the same time inhibiting the activation of G proteins by a GPCR.

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