Stabilization of the GDP-bound Conformation of Giα by a Peptide Derived from the G-protein Regulatory Motif of AGS3*

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The G-protein regulatory (GPR) motif in AGS3 was recently identified as a region for protein binding to heterotrimeric G-protein α subunits. To define the properties of this ~20-amino acid motif, we designed a GPR consensus peptide and determined its influence on the activation state of G-protein and receptor coupling to G-protein. The GPR peptide sequence (28 amino acids) encompassed the consensus sequence defined by the four GPR motifs conserved in the family of AGS3 proteins. The GPR consensus peptide effectively prevented the binding of AGS3 to Gia1,2 in protein interaction assays, inhibited guanosine 5′-O-(3-thiotriphosphate) binding to Gia, and stabilized the GDP-bound conformation of Gia. The GPR peptide had little effect on nucleotide binding to Go or brain G-protein indicating selective regulation of Gia. Thus, the GPR peptide functions as a guanine nucleotide dissociation inhibitor for Gia. The GPR consensus peptide also blocked receptor coupling to Giaβγ indicating that although the AGS3-GPR peptide stabilized the GDP-bound conformation of Gia, this conformation of Gia1,GDP was not recognized by a G-protein-coupled receptor. The AGS3-GPR motif presents an opportunity for selective control of Gia- and Giaβγ-regulated effector systems, and the GPR motif allows for alternative modes of signal input to G-protein signaling systems.

The G-protein regulatory (GPR)† motif or GoLOCO repeat is a ~20-amino acid domain found in several proteins that interact with and/or regulate G-proteins (1, 2). Such proteins include the activator of G-protein AGS3, the AGS3-related protein PINS in Drosophila melanogaster, two members of the RGS family of proteins, and three proteins (LGN, Pcp2, and Rap1GAP) isolated in yeast two-hybrid screens using Gia or Gαo as bait. Rat AGS3 was isolated in a yeast-based functional screen designed to identify receptor-independent activators of heterotrimeric G-protein signaling (1). The AGS3-related protein PINS is required for asymmetric cell division of neuroblasts in D. melanogaster, where it is found complexed with Gi/Go (3, 4), but neither the signal input nor output for this complex is known. Some insight as to how PINS may regulate Gi/Go is provided by studies with AGS3 (1). In the yeast-based system, AGS3 selectively activated Gia2 and Gia3. The action of AGS3 as a G-protein activator in the yeast-based system was independent of nucleotide exchange as it was not antagonized by overexpression of RGS4, and it was still observed following replacement of Gia2 with Gia2-G204A, a mutant that is deficient in making the transition to the GTP-bound state (1, 5). Both of these manipulations effectively prevent receptor-mediated activation of G-protein signaling in the yeast system and block the action of AGS1, which was isolated in the same screen and apparently behaves as a guanine nucleotide exchange factor for heterotrimeric G-proteins (5, 6). These data indicate that the interaction of AGS3 with G-protein influences a unique control mechanism within the activation/deactivation cycle of heterotrimeric G-proteins. AGS3 exists as a 650-amino acid protein enriched in brain and a 166-amino acid protein (AGS3-SHORT) enriched in heart (1,2). The 650-amino acid protein consists of two functional domains defined by a series of seven amino-terminal tetra-tricopeptide repeats (TPR) and four carboxyl-terminal GPR motifs. Site-directed mutagenesis, protein interaction studies, and subcellular localization experiments indicated that the GPR motifs of AGS3 were likely responsible for binding G-protein, whereas the TPR domain is a site for binding of regulatory proteins (1, 3, 4,2,3 AGS3 preferentially binds to Gia in the presence of GDP (1). AGS3-GPR effectively competed with Giaβγ subunits for binding to Gia and inhibited guanosine 5′-O-(3-thiotriphosphate) (GTPγS) binding to Gia1.2 Such an activity likely has significance in a number of aspects of G-protein-mediated signaling events and presents a novel opportunity to control the basal activity of G-protein signaling, as well as influence receptor-mediated activation of G-protein. These observations also raise many interesting questions relative to basic aspects of G-protein structure/function and alternative modes of regulation and functional roles for G-protein signaling systems in the cell. To address these issues, we generated a series of peptides based upon the consensus GPR motif in AGS3 and evaluated their effects on the nucleotide binding properties of Gia. A 28-amino acid GPR peptide effectively blocked the interaction of AGS3 with Gia and inhibited GTPγS binding to GDI, guanine nucleotide dissociation inhibitor; HT, hydroxytryptamine; GST, glutathione S-transferase; CHAPS, 3-[3-cholamido-
propl]dimethylammonio]-1-propanesulfonic acid.

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§ The abbreviations used are: GPR, G-protein regulatory; TPR, tetra-tricopeptide repeats; GTPγS, guanosine 5′-O-(3-thiotriphosphate); GDI, guanine nucleotide dissociation inhibitor; HT, hydroxytryptamine; GST, glutathione S-transferase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
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Gio by a mechanism that involved stabilization of the GDP-bound conformation of Gio. The GPR consensus peptide also blocked receptor coupling to Goβγ indicating that although the AGS3-GPR peptide stabilized the GDP-bound conformation of Gio, this conformation of Gio GDP was not recognized by a G-protein-coupled receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—[35S]-GTPγS (1250 Ci/mmol), [3H]-GDP (29.6 Ci/mmol) and [3H]-5-hydroxy tryptamine (HT) (21.8 Ci/mmol) were purchased from PerkinElmer Life Sciences. Peptides were synthesized and purified by Bio-Synthesis, Inc. (Lewisville, TX), and peptide mass was determined by matrix-assisted laser desorption ionization mass spectrometry. GDP, GTPγS, and [3H]-HT were obtained from Sigma. Acrylamide, bisacrylamide, protein assay kits, and sodium dodecyl sulfate were purchased from Bio-Rad. Eosostatin A was purchased from National Diagnostics (Manville, NJ). CytoScint was purchased from ICN Biomedicals (Costa Mesa, CA). Thesit (polyoxyethylene-9-lauryl ether) was obtained from Roche Molecular Biochemicals. Polyvinylidene difluoride membranes were obtained from Pall Gelman Sciences (Ann Arbor, MI). Nitrotetrazolium BA85 filters were purchased from Schleicher & Schuell (Keene, NH). Whatman GF/C FP200 filters were purchased from Brandel Inc. (Gaithersburg, MD). Purified bovine brain G-protein was kindly provided by Dr. John Hildebrandt (Department of Pharmacology, Medical University of South Carolina) (7). All other materials were obtained as described elsewhere (1, 8).

**Protein Interaction Assays**—The GPR domain of AGS3 (Pro465-Ser509) containing the four GPR motifs was generated as a glutathione S-transferase fusion protein by polymerase chain reaction using the full-length cDNA of AGS3 as a template. The AGS3-Pro465-Ser509 fragment was cloned into the pQE-30 vector (Qiagen, Valencia, CA) to generate an amino-terminal His-tagged protein. His-tagged AGS3 was expressed and purified from bacteria using a nickel affinity matrix (ProBond™ resin; Invitrogen, Carlsbad, CA). The His-tagged AGS3 was eluted from the matrix with imidazole and desalted by centrifugation with the GST fusion protein (1). The interaction of GST-AGS3-GPR and His-tagged AGS3-GPR with G-proteins was assessed by protein interaction experiments using purified G-protein as described previously (1). Giα3-1 and Goα were purified from Sf9 insect cells infected with recombinant virus as described (8). All purified G-proteins used in these studies were incubated in the GDP-bound form, and G-protein interaction assays contained 10 μM GDP.

A separate series of protein interaction experiments were designed to determine whether the Gio complexed with AGS3 contained bound GDP. Gio1 (100 nM) was loaded with [3H]-GDP (0.5 μM; 2 × 104 dpm/pmol) by incubation for 20 min at 24 °C in binding buffer (50 mM Hepes-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 50 μM adenosine triphosphate, and 10 μM bovine serum albumin). The [3H]-GDP-loaded Gio1 was incubated with 300 nM GST or GST-AGS3-GPR in a total volume of 5 μl (final concentration, 100 μM). Reactions were terminated at specified time points by rapid filtration through nitrotetrazolium filters (BA85, Schleicher & Schuell) with 4 × 4 ml washes of stop buffer. Radioactivity bound to the filters was determined by liquid scintillation counting. Nonspecific binding was defined by 100 μM GDP.

**High Affinity Agonist Binding—**Sf9 cells membranes expressing 5-HT1A receptors were reconstituted with Goβγ, and high affinity agonist binding was measured with [3H]-5-HT as described previously (8, 10). Membrane aliquots (100 μg of membrane protein, 85 nm receptor) were preincubated for 15 min at 25 °C with G-proteins (2125 nm Goβγ) with or without G-protein in a total volume of 17 μl (reconstitution buffer, 5 mM NaH2PO4, 5 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 500 mM GTPγS, 0.4% CHAPS, pH 7.5). The reconstituted receptors were then diluted 10-fold with binding buffer (50 mM Tris-HCl, 5 mM MgCl2, 0.5 mM EDTA, pH 7.5), and 50 μl were added to binding tubes (total volume = 150 μl) containing 2 nm [3H]-5-HT. The final concentrations of receptor, G-protein, and peptide in the binding tubes were 2.8 nm, 70.8 nm, and 114 μM, respectively. Nonspecific binding was determined in the presence of 100 μM 5-HT. Binding reactions were incubated at 25 °C for 1.5 h and terminated by filtration over Whatman GF/C FP200 filters using a Brandel cell harvester. The filters were rinsed thrice with 4 ml of ice-cold washing buffer (50 mM Tris-Cl, 5 mM MgCl2, 0.5 mM EDTA, 0.01% sodium azide, pH 7.5, at 4 °C), placed in 4.5 ml of CytoScint, and counted to constant error in a scintillation counter.

**RESULTS AND DISCUSSION**

The 20-amino acid GPR motif is repeated four times in AGS3-related proteins, with the exception of the three repeats found in the Drosophila protein PINS (Fig. 1). Alignment of the four GPR repeats from five species revealed a GPR consensus sequence (Fig. 1). The GPR consensus sequence is characterized by the presence of an amino acid cluster (Phe-Phe), Leu/Met10, Leu/Ile11, Gln15, Ser/Ala16, Arg18 Met/Leu19 and the Asp-Asp-Glu-Arg sequence at the carboxy end of the motif. Helical wheel and Chou-Fasman analysis indicated that this region is capable of existing as an amphipathic helix. Each of the GPR motifs illustrated in Fig. 1 possesses a varying number of Proline residues just after and in

![Fig. 1. Alignment of the GPR motifs found in AGS3 and related proteins.](Image)

The overall domain structure of AGS3 (650-amino acid protein) is indicated at the top of the figure. The hashed boxes represent the TPR domain. The GPR domains of rat AGS3 (AAF08683), human AGS3 (CA05951), D. melanogaster PINS protein (AAF36967), the Cae norhabditis elegans protein (CE/AA81387), and the Tetradon nigro viridis (taide) fish protein (AA023748) are all aligned by PILEUP (Ge netics Computer Group Wisconsin Package) and visual adjustment. A consensus amino acid was defined by the presence of an amino acid or closely related residue in all four GPR repeats.
GPR Motifs and Ga Regulation

Fig. 2. Influence of GPR peptides on the interaction of GST-AGS3-GPR with Gα and GTPγS binding to Gia. A and B, the carboxyl region of AGS3 (Pro153-Ser222) containing all four GPR repeats was generated as a His-tagged (H) GST fusion (B) protein for protein interaction assays as described under "Experimental Procedures." All interactions were done in the presence of 10 μM GDP, and the input lanes represent control GST or GST in the presence of 100 μM GDP. The inhibitory effect of the GPR consensus peptide on GTPγS binding was selective for Gα and the GPR consensus peptide (Fig. 2, 75 nm). The inhibition of GTPγS binding by the GPR consensus peptide was diminished in the presence of 100 μM GTPγS. The peptide concentration was 10 μM. B, Gia2 (100 nm) was loaded with 1H-GDP and incubated with 300 nm GST or GST-AGS3-GPR in the presence and absence of 10 μM GPR consensus peptide and processed for protein interactions as described under "Experimental Procedures." Data are expressed as the percent of specific binding observed in control samples for each time point that did not receive 100 μM GTPγS. The peptide concentration was 10 μM. B, Gia2 (100 nm) was loaded with 1H-GDP and incubated with 300 nm GST or GST-AGS3-GPR in the presence and absence of 10 μM GPR consensus peptide and processed for protein interactions as described under "Experimental Procedures." The proteins bound to the glutathione affinity matrix were eluted, and the amount of bound GDP was measured by liquid scintillation spectroscopy. Data in A and B are presented as the means ± S.E. derived from three experiments.

The stimulation of the GDP-bound conformation of Gia by the GPR consensus peptide indicates that the AGS3-GPR motif can influence subunit interactions by interfering with Gβγ binding to Gia. This apparent effect may account for the results obtained in protein interaction assays using GST-AGS3-GPR and brain lysates, where Gβγ is absent from the AGS3-Gia complex (1). The influence of the GPR motif on subunit interactions would have significant implications for signal processing. First, interaction of the AGS3-GPR motif with Giα could release Gαβγ for regulation of downstream signaling events, while stabilizing Giα(GDP) binding (2). Such a mode of signal input may be of utility where there is a need for selective regulation of Gβγ-sensitive effectors. The time frame for termination of such a signaling event (i.e., reassociation of Gβγ with Giα(GDP)) likely differs from that of a more typical signaling event in which there has been an exchange of nucleotide bound to Giα, and signal termination involves GTP hydrolysis along with Giα(GDP) reassociation. A second implication of stabilization of Giα(GDP) by a GPR domain is related to receptor G-protein coupling. We addressed this issue experimentally using a membrane assay system where receptor-G-protein coupling is reflected as high affinity binding of agonists. The high affinity binding of agonist observed upon reconstitution of the mem-

some cases before the core consensus sequence, which may exert an important influence within the overall organization of the four GPR motifs. As part of an effort to define the structural basis of the interaction of AGS3 with Gia and the functional consequences of this interaction, we asked whether a consensus sequence peptide effectively interacted with Gia.

The core GPR consensus sequence was bracketed by additional residues (three amino terminus, five carboxyl terminus) derived from AGS3-GPR-I, and the carboxyl terminus was amidated (Fig. 1). The 28-amino acid GPR consensus peptide completely blocked the binding of Gia1 or Gia2 to GST-AGS3-GPR with an IC50 of ~200 nM (Fig. 2A and B). The GPR consensus peptide also inhibited GTPγS binding to Gia1 and Gia2 (IC50 ~200 nM) (Fig. 2, C and D) consistent with the preferential binding of AGS3 to Gia in the presence of GDP (1). The inhibitory effect of the GPR consensus peptide on GTPγS binding was selective for Gia as it only minimally affected nucleotide binding to Gαα or brain G-protein (Fig. 2D). The activity of the GPR consensus peptide in both the protein interaction assays and GTPγS binding assays was lost upon substitution of Phe for the highly conserved Arg23.4 Similar results were obtained when these amino acid substitutions were made in the context of GST-AGS3 fusion protein, which contained the terminal 74 amino acids of AGS3 including part of GPR-III and all of GPR-IV (Fig. 1).5

We then addressed the mechanism by which the GPR consensus peptide inhibited GTPγS binding to Gia2 and determined the effect of the GPR motif on receptor coupling to G-protein. The inhibition of GTPγS binding by the GPR consensus peptide may reflect a reduction in the rate of nucleotide exchange. Indeed, the rate of GDP dissociation was markedly diminished in the presence of the GPR consensus peptide (Fig. 3A). The R23F mutation, which eliminated the effectiveness of the peptide to block interaction of AGS3 with Gia and GTPγS binding to Gia2, also did not alter GDP dissociation (Fig. 3A). The inhibition of GDP dissociation by the GPR consensus peptide suggests that the GPR motif stabilizes the GDP-bound conformation of Gia. To address this issue we evaluated the interaction of GST-AGS3-GPR with Gia2, which had been preloaded with 1H-GDP. Subsequent analysis of the G-protein complexed with AGS3-GPR on the glutathione affinity matrix indicated that the nucleotide binding site of G-protein bound to AGS3 indeed contained GDP (Fig. 3B). Giα(GDP) binding to AGS3-GPR was blocked by the GPR consensus peptide (Fig. 3B) consistent with the ability of this peptide to inhibit interaction of GST-AGS3-GPR with Gia1/2 (Fig. 2, A and B).

4 Y. K. Peterson and S. M. Lanier, unpublished observations.

5 M. Natochin, B. Lester, Y. K. Peterson, M. L. Bernard, S. M. Lanier, and N. O. Artemyev, submitted for publication.
brane-bound 5-HT₁ receptor with Giαβγ was inhibited by addition of the GPR peptide (Fig. 4). This action of the GPR peptide was not observed with the R23F peptide and was selective for Gi versus Gα (Fig. 4).

The influence of the single amino acid substitution on the bioactivity of the GPR both within the context of a short peptide and a GST fusion protein containing an additional 74 amino acids of AGS3 sequence strongly suggest a relatively discrete and specific surface interaction with Giα (Fig. 2) (1). Helical wheel projections and 3D models indicated that when the GPR consensus peptide is fixed in an α helical conformation, the Phe⁸, Ala¹², Gln¹⁵, Met¹⁹, and Arg⁲³ residues are on the same face of the helix. On this face of the helix is a hydrophobic sector defined by Phe⁸, Ala¹², and Met¹⁹ that is bound by polar residues, which may involve in charge pairing to residues in Giα. As was the case for the R23F substitution, disruption of this hydrophobic sector by substitution of Arg for Phe⁸ also resulted in a loss of activity for the GST-AGS3-GPR fusion protein in GTPγS binding and protein interaction assays (1). Thus, either extension (R23F substitution) or shortening (P8R) of the hydrophobic sector on this face of the helix resulted in a loss of bioactivity for the GPR motif. In contrast, strengthening of this hydrophobic sector by substitution of Ala for Gln¹⁵ did not alter the activity of the GPR peptide. These data indicate an important role for a spatially constrained hydrophobic stretch of −16.6 Å that is key for peptide interaction with Giα.

The inability of receptor to productively couple to GαGDP-GPR is of interest. The GαGDP conformation stabilized by the GPR peptide may differ from that stabilized by Gβγ in such a manner that the receptor cannot recognize Gα. Indeed, the orientations of the amino and carboxyl domains of Giα1, which are important interaction sites with receptor, are quite different in the GαGDP and GαGDPβγ structures (11–13). In addition to such differences in the structural orientation of Giα domains interacting with receptor, it is likely that receptor contact points on Gβγ also play a role in receptor-mediated activation of guanine nucleotide exchange (14–18). Alternatively, the receptor may indeed interact with the GαGDP-GPR complex, but this interaction stabilizes a receptor conformation with low affinity for agonist (19). Ultimately, one may think of the GαGDP-GPR complex as a type of dimeric G-protein, and it is not clear what might provide signal input to such a complex.

Although the GPR motif is present in several proteins that interact with Gα and/or regulate nucleotide binding/hydrolysis (1, 2), these proteins have different and often opposing effects on the activation state of G-protein (20, 21).² Pcp2, which contains two GPR motifs based upon this consensus sequence, actually appears to increase the dissociation of GDP from Gα (21). Thus, there are either subtle differences in this motif or other residues outside of this motif that play a key role in the specific functional output gendered by interaction of the GPR motif with Gα. Of note is the selective effects of the AGS3-GPR peptide for Gβγ versus Gα in both nucleotide binding assays and the analysis of receptor coupling to G-proteins. Further dissection of the structural basis for this selectivity will provide clues as to the site of interaction of the GPR peptide with Giα and the mechanism by which it stabilizes the GDP-bound conformation. One prominent area of sequence divergence between Goc and Gα encompasses switch IV, a region implicated in the formation of GαGDP multimers (11).

The role of AGS3 as a GDI is an unexpected concept for heterotrimeric G-proteins, although such proteins serve similar regulatory roles for Ras-related G-proteins. Proteins containing the AGS3-GPR motif may promote dissociation of Gα and Gβγ in the absence of nucleotide exchange and present an opportunity for selective control of Gα- and Gβγ-regulated effector systems. GPR-containing proteins likely play a role in regulating basal activity of G-protein signaling systems in the cell and provide alternative modes of signal input to G-protein signaling systems that may either augment, complement, or antagonize G-protein activation by GPCRs.

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⁶ H. Ma, M. L. Bernard, S. M. Lanier, and S. G. Graber, unpublished observations.