Selective Interaction of AGS3 with G-proteins and the Influence of AGS3 on the Activation State of G-proteins*

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AGS3 (activator of G-protein signaling 3) was isolated in a yeast-based functional screen for receptor-independent activators of heterotrimeric G-proteins. As an initial approach to define the role of AGS3 in mammalian signal processing, we defined the AGS3 subdomains involved in G-protein interaction, its selectivity for G-proteins, and its influence on the activation state of G-protein. Immunoblot analysis with AGS3 antisera indicated interaction in rat brain, the neuronal-like cell lines PC12 and NG108-15, as well as the smooth muscle cell line DDT1-MF2. Immunofluorescence studies and confocal imaging indicated that AGS3 was predominantly cytoplasmic and enriched in microdomains of the cell. AGS3 coimmunoprecipitated with Go/i3 from cell and tissue lysates, indicating that a subpopulation of AGS3 and Go/i exist as a complex in the cell. The coimmunoprecipitation of AGS3 and Go/i was dependent upon the conformation of Go/i (GDP \(\rightarrow\) GTP\(S\) (guanosine 5'-3-O-\((\text{thio})\text{triphosphate})). The regions of AGS3 that bound Go/i were localized to four amino acid repeats (G-protein regulatory motif (GPR)) in the carboxyl terminus (Pro\(463\)-Ser\(469\)), each of which were capable of binding Go/i. AGS3-GPR domains selectively interacted with Go/i in tissue and cell lysates and with purified Go/i/Go\(i3\). Subsequent experiments with purified Go/i and Go\(i3\) indicated that the carboxy-terminal region containing the four GPR motifs actually bound more than one Go/i subunit at the same time. The AGS3-GPR domains effectively competed with Go/\(i\beta\gamma\) for binding to Go\(i(GDP)\) and blocked GTP\(S\) binding to Go\(i1\). AGS3 and related proteins provide unexpected mechanisms for coordination of G-protein signaling pathways.

Signal processing via heterotrimeric G-protein genera-

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1 The abbreviations used are: AGS, activator of G-protein signaling; TPR, tetratricopeptide repeat motif; GPR, G-protein regulatory motif; PINS, Partner of Inscuteable; CWS, cell washing solution; GST, glutathione S-transferase; CHO, Chinese hamster ovary; GTP\(S\), guanosine 5'-3-O-(\text{thio})triphosphate.

2 The GPR motif was also termed the GoLoco motif (6).
early stages of development (9, 10). PINS is part of a multiprotein complex that is translocated from the cytosol to one pole of the dividing neuroblast. In this article, we report the existence of an AGS3-Ga complex within the cell, define the Ga-interacting domains of AGS3, and determine the selectivity of AGS3 for different Ga subunits. AGS3, which preferentially binds to GaGDP, can bind multiple Ga subunits and hence may function as a scaffolding protein to provide spatially and temporally discrete signaling events. AGS3 and Gbg actually competed with each other for interaction with GaGDP, and AGS3 inhibited guanine nucleotide exchange on Gai1. The properties of the AGS3-Ga interactions add unexpected dimensions to signal processing by G-protein-regulated signaling systems.

EXPERIMENTAL PROCEDURES

Materials—[35S]GTPγS (1250 Ci/mmol) was purchased from PerkinElmer Life Sciences. Tissue culture supplies were obtained from JRH Bioscience (Lenexa, KS). Acrylamide, bisacrylamide, Bio-Rad protein assay kits, and sodium dodecyl sulfate were purchased from Bio-Rad. Ecoscint A was purchased from National Diagnostics (Manville, NJ). Guanosine diphosphate, guanosine triphosphate, and Thesit (polyoxyethylene-9-lauryl ether) were obtained from Rche Molecular Biochemicals. Polyvinylidene difluoride membranes were obtained from Pall Gelman Sciences (Ann Arbor, MI). Gammabind G-Sepharose was obtained from Amersham Pharmacia Biotech, and nitrocellulose BA85 filters were purchased from Schleicher & Schuell. Poly-L-lysine, normal goat serum, biotinylated goat anti-rabbit IgG, and Extravidin fluorescein isothiocyanate were purchased from Sigma.

Fig. 1. Schematic representation of AGS3 and related proteins. Full-length rat AGS3 (AAF08683) was aligned with the human Lgn protein (AA526387), the C. elegans protein (CEAAA9185) by PILEUP (University of Wisconsin GCG program), and visual inspection of the amino acid sequence similarity and identity from the four indicated below the four sequences by 1 or residue, respectively. The shaded and lined sequences represent TPR I–VII and a repeated segment of amino acids (GPR I–IV). The amino-terminal half of AGS3 contains six TPRs, as defined by SMART analysis (7), that exist as a cluster of two (Ser43–Gln116, Gly183–Ile336) and four motifs with a spacer of ~60 amino acids between the two clusters. Visual inspection of the spacer region between the two clusters of TPR motifs indicates the likely existence of an additional spacer motif that lies between two of the clusters. PINS contains three GPRs with highest homology to GPRs I, III, and IV of AGS3. AGS3 and Ga interactions add unexpected dimensions to signal processing by Ga-protein-regulated signaling systems.
by Drs. Paul Goldsmith, Andrew Shenker, and Allen Spiegel (17). All other materials were obtained as described elsewhere (4, 18).

Generation of AGS3 Subdomains—AGS3 subdomains were generated as glutathione fusion proteins by polymerase chain reaction using the full-length cDNA of AGS3 as a template. Primers were designed to add BamHI and EcoRI sites to the 5' and 3' ends, respectively, of AGS3 subdomains to fuse the AGS3 open reading frame with the reading frame of glutathione S-transferase contained in the pGEX-1T vector. The polymerase chain reactions were generally performed using 250 nM primers and 125 pmol template DNA in a total volume of 50 μl. Cycles were 1 × 3 min at 94 °C; 30 × 1.5 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C; and 1 × 10 min at 72 °C. Primers used to generate specific constructs were as follows.

TPR (Met1–Ile462) 5'-GGGATCCATGGGACCCGCTTCTGTCTGG 3'-GGGATCCCATAGATACCCTGGGACCCGCTTCTGG

GPR (Pro300–Ser650) 5'-CGGATCCACCATGCGTCTCGTTTCTCAGATACCCGTGCGAGGCACCTG 3'-CGGATCCACCATGCGTCTCGTTTCTCAGATACCCGTGCGAGGCACCTG

GPR (Pro300–Glu501) 5'-CGGATCCACCATGCGTCTCGTTTCTCAGATACCCGTGCGAGGCACCTG 3'-CGGATCCACCATGCGTCTCGTTTCTCAGATACCCGTGCGAGGCACCTG

GPR (Pro300–Glu501) 5'-CGGATCCACCATGCGTCTCGTTTCTCAGATACCCGTGCGAGGCACCTG 3'-CGGATCCACCATGCGTCTCGTTTCTCAGATACCCGTGCGAGGCACCTG

AGS3 subdomains were generated by introducing subdomains to fuse the AGS3 open reading frame with the reading frame of glutathione S-transferase contained in the pGEX-1T vector. The polymerase chain reactions were generally performed using 250 nM primers and 125 pmol template DNA in a total volume of 50 μl. Cycles were 1 × 3 min at 94 °C; 30 × 1.5 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C; and 1 × 10 min at 72 °C. Primers used to generate specific constructs were as follows.

TPR (Met1–Ile462) 5'-GGGATCCATGGGACCCGCTTCTGTCTGG 3'-GGGATCCCATAGATACCCTGGGACCCGCTTCTGG

GPR (Pro300–Ser650) 5'-CGGATCCACCATGCGTCTCGTTTCTCAGATACCCGTGCGAGGCACCTG 3'-CGGATCCACCATGCGTCTCGTTTCTCAGATACCCGTGCGAGGCACCTG

GPR (Pro300–Glu501) 5'-CGGATCCACCATGCGTCTCGTTTCTCAGATACCCGTGCGAGGCACCTG 3'-CGGATCCACCATGCGTCTCGTTTCTCAGATACCCGTGCGAGGCACCTG

Immunofluorescence—DDT, MF2 control cells and DDT, MF2 cells stably transfected with AGS3 were plated onto coverslips (18-mm round no. 1) precoated with 0.01% polylysine and allowed to grow to 60% confluence. Coverslips were then rinsed with 3 × 5 ml of cell washing solution (CWS) (127 mM NaCl, 2.6 mM KCl, 1.8 mM KH2PO4, 10 mM Na2HPO4) followed by 3 × 5 min incubations with CWS containing 0.1 M glycine. Coverslips were then incubated for 10 min prior to loading on denaturing 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes for immunoblotting.

Protein Interaction Assays—The interaction of AGS3 with G-proteins was assessed by both communoprecipitation and protein interaction experiments using tissue/cell lysates or purified G-proteins. Protein Extracts—Cell lysates were prepared as described elsewhere (4, 18). For analysis of the interaction of AGS3-GPR with multiple G-protein subunits, Gzα1 (200 nm) was incubated with Gzα1 (50 nm) in the presence or absence of the AGS3-GPR GST fusion protein (250 nm) in 250 μl of buffer A (20 mM Tris, pH 7.5, 70 mM NaCl, 1 mM dithiothreitol, 0.05 mM EDTA, 0.01% Thesit) for 1 h at 4 °C. Gzα1 (1:500) was added, and the incubation was continued for 3 h at 4 °C. Protein complexes were isolated and evaluated by immunoblotting as described above.

For analysis of the interaction of AGS3 with G-protein subunits were conducted as described previously (4, 18). All purified G-proteins used in these studies were isolated in the GDP-bound form. Unless indicated otherwise, all G-protein interaction assays contained 10 μM GDP. The AGS3-GST fusion proteins were expressed in and purified from bacteria using a glutathione affinity matrix. The AGS3-GST fusion proteins were eluted from the matrix with glutathione and desalted by centrifugation (Centricon YM-3; Millipore, Bedford, MA). For interaction assays with cell/tissue lysates, the AGS3-GST fusion protein (100–300 ng) was incubated with purified G-protein (50–100 ng) or cell/tissue lysate (~4 μg of protein/ml) for 1 h at 24 °C in a total volume of 250 μl. 12.5 μl of packed glutathione-Sepharose slurry was added and the mixture rotated at 4 °C for 20 min, after which the affinity matrix was pelleted and washed three times with 500 μl of incubation buffer. Proteins retained on the matrix were solubilized in 2× Laemmli loading buffer and separated by electrophoresis on denaturing 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes for immunoblotting. Each blot was checked by Amido Black staining to verify equal loading of fusion proteins.

Immunofluorescence—DDT, MF2 control cells and DDT, MF2 cells stably transfected with AGS3 were plated onto coverslips (18-mm round no. 1) precoated with 0.01% polylysine and allowed to grow to 60% confluence. Coverslips were then rinsed with 3 × 5 ml of cell washing solution (CWS) (127 mM NaCl, 2.6 mM KCl, 1.8 mM KH2PO4, 10 mM Na2HPO4) and fixed in 4% paraformaldehyde for 10 min. Fixed cells were then incubated overnight at 4 °C. Cell protein complexes were captured by adding Gammabind G-Sepharose (12.5 μl packed volume) and continuing the incubation for 30 min at 4 °C. The mixture was then microcentrifuged at 4 °C and the pellets washed (3 × 500 μl of incubation buffer) and resuspended in 2× Laemmli buffer. Resuspended samples were placed in a boiling water bath for 5 min and microcentrifuged for 10 min prior to loading on denaturing 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes for immunoblotting.

Protein interaction assays using purified G-protein subunits were conducted as described previously (4, 18). All purified G-proteins used in these studies were isolated in the GDP-bound form. Unless indicated otherwise, all G-protein interaction assays contained 10 μM GDP. The AGS3-GST fusion proteins were expressed in and purified from bacteria using a glutathione affinity matrix. The AGS3-GST fusion proteins were eluted from the matrix with glutathione and desalted by centrifugation (Centricon YM-3; Millipore, Bedford, MA). For interaction assays with cell/tissue lysates, the AGS3-GST fusion protein (100–300 ng) was incubated with purified G-protein (50–100 ng) or cell/tissue lysate (~4 μg of protein/ml) for 1 h at 24 °C in a total volume of 250 μl. 12.5 μl of packed glutathione-Sepharose slurry was added and the mixture rotated at 4 °C for 20 min, after which the affinity matrix was pelleted and washed three times with 500 μl of incubation buffer. Proteins retained on the matrix were solubilized in 2× Laemmli loading buffer and separated by electrophoresis on denaturing 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes for immunoblotting. Each blot was checked by Amido Black staining to verify equal loading of fusion proteins.
cience signals were detected in nontransfected DDT1-MF2 cells as expected from the relative strengths of the signals for control and AGS3-transfected cells observed by immunoblotting. No immunofluorescent signal was detected in control or AGS3 transfectants in the absence of any primary antibody.

Nucleotide Binding Assays—Nucleotide binding assays were conducted by a modification of a described previously techniques (2, 20). G-proteins (100 nM) were preincubated with varying amounts of AGS3 subdomains or GST controls for 15 min at 24°C (binding buffer = 50 mM Hepes-HCl, pH 7.5, 1 mM EDTA, 2 mM MgCl2, 1 mM dithiothreitol, 50 μM adenosine triphosphate, 10 μg/ml bovine serum albumin) prior to addition of 0.5–1 μM GTP-γ-S (4.0 × 10^5 dpm/ml); the final incubation volume was 50 μl. Samples were incubated with GTP-γ-S at 24°C for 30 min. Incubated reactions were terminated by rapid filtration through nitrocellulose filters (Schleicher & Schuell BA85) with four 4-ml washes of stop buffer (50 mM Tris-HCl, 5 mM MgCl2, 1 mM EDTA, pH 7.4, 4°C). Radioactivity bound to the filters was determined by liquid scintillation counting.

Additional Methods—DDT1-MF2 cells were stably transfected with pcDNA3.AGS3 by DNA/calcium phosphate coprecipitation (21). For antipeptide antisera, AGS3 peptides (P-32 Thr306–Ile436 and P-22 Asp528–Gly550) were synthesized and conjugated for generation of rabbit antipeptide antibodies indicated expression of AGS3 (Fig. 2). The same immunoreactive M r ~74,000 species was observed with two different antibodies (P-32, P-22) generated against peptides derived from different regions of the protein (Fig. 2).

Fractionation of tissues/cells expressing AGS3 indicated that AGS3 is enriched in the 100,000 × g supernatant consistent with a major distribution of AGS3 in the cytosol (Fig. 3A). A similar fractionation of AGS3 was observed in DDT1-MF2 cells stably transfected with AGS3 (Fig. 3A). The subcellular localization of AGS3 was also addressed by immunofluorescence analysis following stable expression of AGS3 in the DDT1-MF2 cell line (Fig. 3B). Confocal microscopy was used to generate an image approximately through the middle plane of the cell. The immunofluorescent image indicates that AGS3 is predominantly cytosolic (Fig. 3B), as suggested by immunoblot analysis of the 100,000 × g supernatant from cell lysates illustrated in Fig. 3A. Within the cell, the AGS3 signal is often punctate and occasionally enriched in microdomains of the cell.

We previously reported that the carboxyl-terminal 74 amino acids of AGS3 were active in the yeast functional screen and that this peptide fragment directly bound to Goi (4). We thus asked if full-length AGS3 was complexed with Goi in lysates of rat brain or DDT1-MF2 cells stably transfected with AGS3. As AGS3 preferentially regulated Goi3 in lysates of brain lysate Goi3 was immunoprecipitated with a Goi3 carboxyl terminus antibody. Immunoblots of membrane transfers containing Goi3 immunoprecipitates indicated that AGS3 coimmunoprecipitated with Goi3 in a nucleotide-dependent manner (Fig. 4). The absence of Gβ in the GTPγS-treated samples provided internal controls for G-protein activation and subunit dissociation by added GTPγS/Mg2+. Immunoprecipitation experiments were also conducted with the AGS3 antisera P-32. Although AGS3 was effectively immunoprecipitated by the P-32 antisera in each cell/tissue extract, coimmunoprecipitation of Goi3 was variable, which may reflect lower immunoprecipitation efficiency for P-32 and/or a masking of the P-32 epitope in the AGS3-Goi3 complex (data not shown). Nevertheless, these data indicated that a subpopulation of Goi3 and AGS3 exists as a complex in the cell and that this interaction is regulated by nucleotide binding to Goi.

AGS3 Domains That Interact with G-proteins—The interaction between AGS3 and G-proteins was further explored in vitro binding assays to define the regions of AGS3 actually involved in binding to Goi. We generated the amino-terminal half of AGS3 (AGS3-TPR, Met1–Ile462) and the COOH-terminal

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3 M. L. Bernard and S. M. Lanier, unpublished observations.
AGS3 and G-protein Interactions

The preceding data also suggested that AGS3 is capable of binding multiple Gα subunits. To address this issue, we asked if a GST-AGS3 fusion protein containing GPRs I–IV indeed bound more than one Gα, at the same time. A GST-AGS3 fusion protein containing GPRs I–IV was incubated with a mixture of Gαq and Gα12. Samples were then immunoprecipitated with antisera directed against the carboxyl terminus of Gα12. In the presence of AGS3, Gα12 was also found in the Gα12 immunoprecipitate (Fig. 6). Gαq was not found in the Gα12 immunoprecipitate in the absence of AGS3 (Fig. 6). These data clearly indicate that AGS3 is capable of binding more than one Gα subunit consistent with a putative role of AGS3 as a scaffolding protein within a larger signal transduction complex.

Selectivity of AGS3 for G-proteins—The preceding data clearly established the interaction of AGS3 with Gα within the cell and defined the regions of AGS3 involved in G-protein binding. We then asked if the interaction of AGS3 with Gα was selective for different G-protein families. We approached this question using crude tissue/cell lysates and purified Gα subunits. The GST-AGS3-GPR fusion protein was incubated with rat brain lysate and bound proteins identified by immunoblotting with Gα specific antisera. AGS3-GPR effectively bound Gα1/2, but not Gαq, Gα11, or Gβγ (Fig. 7A). Based upon the comparison of the signal intensity in the input versus sample lane, it is estimated that AGS3-GPR binds ~20-% of the total Gα protein in the lysate sample. Similar results were obtained in DDT, MF2 cell lysates. Each of the protein interaction experiments in the tissue/cell lysates were done in the presence of GDP which would stabilize heterotrimeric Gαβγ; however, immunoblotting with G-protein β subunit antisera indicated that AGS3 was complexed with Gαq in the absence of Gβ (Fig. 7A). Thus, either AGS3 effectively promoted subunit dissociation or there is a population of Gαq that exists free of Gβγ. The selectivity of AGS3 for different G-proteins was also observed using purified Go subunits. AGS3 bound to Gα1/2 and purified Go, but it did not interact with Gαq and weakly bound Gα11 and Gα10 (Fig. 7B). A similar profile of AGS3 selectivity for Go subunits was also observed in a yeast functional assay (4). Comparison of the relative intensities of the bound Go versus input Go for Gα11/Gαq and Go, (Fig. 7B) indicated a higher apparent affinity of AGS3 for Gi versus Gq/Gi, which may account for the inability of AGS3 to interact with Gα11 and Gα11 in brain lysates (Fig. 7A).

AGS3 and G-protein Activation—As both AGS3 and Gβγ interact with the GDP-bound conformation of Gα, the two proteins may actually compete with each other for interaction with Gα and thus AGS3 would essentially promote subunit dissociation in the absence of nucleotide exchange. This issue was addressed by determining the influence of Gβγ on the interaction of AGS3 with Gα. We first compared the ability of AGS3 to interact with purified Gα versus heterotrimeric Gα (Fig. 8A). At equimolar concentrations of purified Gα and heterotrimeric Gα, AGS3 bound equivalent amounts of Gα. As observed with the AGS3-Gα complex isolated from tissue/cell lysates, Gδγ was not present in the AGS3-Gα complex isolated from purified heterotrimeric Gα, indicating that AGS3 effectively dissociated Gδ from Gβγ. We thus asked if Gβγ would interfere with formation of the AGS3-Gα complex. In these experiments, Gα was first incubated with equimolar or excess Gβγ to generate heterotrimeric Gα, prior to exposure of the complex to AGS3. The interaction of AGS3 with Gα was not altered by Gβγ at concentrations equivalent to Gα, as observed in the experiments using heterotrimeric Gα (Fig. 8B), but it was completely blocked by 10-fold higher concentrations of Gβγ (Fig. 8B), indicating that AGS3 and Gβγ are effectively competing with each other for binding to GαGDP.
The interaction of AGS3 with Gaα may actually stabilize the GDP-bound or nucleotide-free conformation of Gaα and "free up" Gbg for downstream signaling. Indeed, this conjecture would account for the biological activity of AGS3 in the yeast functional assay (4), where Gbg is responsible for subsequent activation of the pheromone response pathway. To address this issue, we asked if AGS3 influenced the guanine nucleotide binding properties of Gaαi. AGS3-GPR blocked the binding of GTPγS to Gaαi (IC50 ~ 0.1 μM) (Fig. 9A). We had previously identified key amino acid residues in GPR-IV that disrupted binding of AGS3-CT to Gaαi (4), and we then examined the effect of this series of AGS3-CT mutants on GTPγS binding to Gaαi1. The AGS3-CT peptides containing GPR mutations that resulted in a loss of binding to Gaαi in protein interaction assays (F609R, R624F) (4) were also ineffective at inhibiting GTPγS binding to Gaαi1 (Fig. 9B). These data, and the results obtained in protein

![Figure 4](http://www.jbc.org/)

**Fig. 4. Coimmunoprecipitation of AGS3 and Gaα3.** Rat brain (2.5 mg; A) and DDT-AGS3 cell (1.25 mg; B) lysates were preincubated with 30 μM GDP or 30 μM GTPγS/25 mM MgCl2 at 24 °C for 30 min. Lysates were then precleared and Gaα3 immunoprecipitated as described under “Experimental Procedures.” Membrane transfers were first blotted with AGS3 P-32 antisera and then stripped and sequentially reprobed with Gaα and Gβ antisera. Input lanes contained 5 μl (A) or 20 μl (B) of the Nonidet P-40 lysate used for immunoprecipitation. The aberrant migration of Gα and Gβ in the input lane in B is due to the larger amount of 1% Nonidet P-40 in the samples. The data are representative of two to four experiments. The input lane contains one-tenth of the lysate volume used for immunoprecipitation. IP, immunoprecipitation; IB, immunoblot. Antipeptide antisera recognizing AGS3 were generated as described under “Experimental Procedures.” P-32 antisera was purified on a peptide affinity matrix and used for immunoblotting at a concentration of 1.0 μg/ml. DDT-AGS3, DDT-T-MF2 cells stably transfected with rat AGS3.

![Figure 5](http://www.jbc.org/)

**Fig. 5. AGS3 domains interacting with G-proteins.** Subdomains of AGS3 (A) were generated as GST fusion proteins and purified following expression in bacteria for protein interaction studies as described under “Experimental Procedures.” The left panels in B and C are Coomassie Blue-stained gels indicating the migration of GST and each of the fusion proteins (1 μg/ml) used for protein interaction studies. Right panels of B and C, lysates were prepared from DDT-T-MF2 cells and 1 μg of lysate protein was incubated with 300 nm GST-AGS3 fusion proteins as described previously (4). Membrane transfers of bound proteins were probed with G-protein subunit antisera. B, TPR, Met1–Ile462; GPR, Pro463–Ser650; CT, Met577–Ser650. The Roman numbers in C correspond to the GPR domains in A: GPR-I, Pro463–Glu501; GPR-II, Ser516–Leu555; GPR-III, Gly563–Thr602; GPR-IV, Thr602–Ser650. Similar results were obtained in 3–5 individual experiments using different batches of lysate. The input lane contains one-tenth of the lysate volume used in each individual interaction assay.

The interaction of AGS3 with Gaα may actually stabilize the GDP-bound or nucleotide-free conformation of Gaα and “free up” Gβγ for downstream signaling. Indeed, this conjecture would account for the biological activity of AGS3 in the yeast functional assay (4), where Gβγ is responsible for subsequent activation of the pheromone response pathway. To address this issue, we asked if AGS3 influenced the guanine nucleotide binding properties of Gaα. AGS3-GPR blocked the binding of GTPγS to Gaα1 (IC50 ~ 0.1 μM) (Fig. 9A). We had previously identified key amino acid residues in GPR-IV that disrupted binding of AGS3-CT to Gaα1 (4), and we then examined the effect of this series of AGS3-CT mutants on GTPγS to Gaα1. The AGS3-CT peptides containing GPR mutations that resulted in a loss of binding to Gaα in protein interaction assays (F609R, R624F) (4) were also ineffective at inhibiting GTPγS binding to Gaα1 (Fig. 9B). These data, and the results obtained in protein
interaction experiments where AGS3 preferentially binds Ga$_{GDP}$ versus Ga$_{GTP-S}$, suggest that AGS3 actually stabilizes the Ga$_{GDP}$ or nucleotide-free conformation and functions as an inhibitor of guanine nucleotide exchange on Ga. These biochemical data are consistent with the functional properties of AGS3 in _S. cerevisiae_ in that the action of AGS3 did not require the generation of Ga$_{GTP}$ and it was not antagonized by overexpression of the GTPase activating protein RGS4 (4).

**DISCUSSION**

A large number of diverse signaling mechanisms within the cell utilize guanine nucleotide-binding proteins as a molecular switch to process biological signals. Due to the central place of these events in signal propagation, several mechanisms have evolved to turn this switch on and off. Such mechanisms include the regulation of guanine nucleotide exchange (e.g. guanine nucleotide exchange factors, guanine nucleotide dissociation inhibitors), hydrolysis (GTPase-activating proteins) and the subcellular targeting of G-proteins themselves. In general, signal processing by G-protein-regulated systems, as is the case for single membrane span receptors, likely operates within the context of a dynamic signal transduction complex. Such a multiprotein complex may provide coordinated and integrated functionality for heterotrimeric G-protein signaling systems, which process a myriad of external stimuli via G-protein-coupled receptors. Within such a complex there are likely accessory proteins distinct from receptor, G-protein and effector that influence various aspects of signal propagation. Such proteins may: 1) determine the specific pathway that the signal travels, 2) provide a cell-specific mechanism for signal amplification, 3) influence the population of activated G-protein/effector within the cell independent of receptor activation, 4) be “effectors” subject to receptor regulation providing attractive targets for cross-talk between diverse signaling systems, 5) provide alternative modes of input to G-protein-regulated signaling pathways independent of a classical G-protein-coupled receptor, and/or 6) serve as scaffolding proteins to organize a signal transduction complex.

AGS3 is one of three mammalian cDNAs isolated in an expression cloning system in _S. cerevisiae_ as receptor-independent activators of heterotrimeric G-protein signaling (3–5). Epistasis experiments in the yeast system indicated that the three cDNAs activated the pheromone response pathway at the level of G-protein, and the proteins were therefore termed activators of G-protein signaling (AGS1–3). Both cellular and/or _in vitro_ studies indicated that these proteins exhibited selectivity for G-proteins and used different mechanisms to activate G-protein signaling. AGS1 is a novel Ras-related protein that directly increases GTP$_S$ binding to Ga (3, 5). AGS2 is identical to mouse Tctex1, a protein that exists as a light

**FIG. 6. Interaction of AGS3 with multiple Ga subunits.** Ga$_{o2}$ (200 nM) was incubated with Ga$_{o3}$ (50 nM) in the presence or absence of the AGS3-GPR GST-fusion protein (250 nM). Samples were immunoprecipitated with Ga$_{o3}$ antisera and processed for SDS-PAGE as described under “Experimental Procedures.” Membrane transfers were immunoblotted with the indicated antisera. The arrows to the left indicate the migration of the indicated proteins. Similar amounts of Ga$_{o3}$ antisera were pelleted in each lane as indicated by detection with secondary antibody. Input lanes represent one-tenth of the the sample processed for immunoprecipitation. This experiment is representative of three such experiments.

**FIG. 7. Selective interaction of AGS3 with G-proteins from brain lysates and with purified G-proteins.** One milligram of lysate protein from rat brain was incubated with 300 nM GST-AGS3 fusion (TPR Met$^1$–Ile$^{462}$, GPR Pro$^{463}$–Ser$^{650}$, CT Met$^{577}$–Ser$^{650}$) as described under “Experimental Procedures”. Membrane transfers of bound proteins were probed with the indicated antisera. B) Recombinant Ga$_o$ (100 nM) or purified Ga$_o$ (100 nM) were incubated with 300 nM GST or AGS3-GPR. All interactions were done in the presence of 10 μM GDP. The input lanes represent one-tenth of the G-protein used in each interaction assay. Membrane transfers of bound proteins were probed with the indicated antisera with intervening stripping of the blot as described under “Experimental Procedures.” The Ga$_o$ antibody exhibits some cross-reactivity with Ga$_o$ that likely accounts for the broad immunoreactive band observed in the input lane in panel A. Similar results were obtained in two to three separate experiments.

$^4$ These data contrast with the properties of AGS1, isolated in the same functional screen, in that the action of AGS1 is blocked by overexpression of the GTPase activating protein RGS4 (4).
chain component of the cytoplasmic motor protein dynein and subserves as yet undefined functions in cell signaling pathways.

The two domain structure (TPR and GPR motifs) of AGS3 is highly conserved. TPR motifs serve a range of functions for diverse proteins (9, 10, 22, 23). The TPR domains of Rap85, which contains an organization of TPR motifs most closely related to the AGS3 TPR domains, are involved in clustering of nicotinic receptors at the neuromuscular junction (23). Studies with the D. melanogaster AGS3/LGN homolog PINS suggest a role for the TPR domains in trafficking of AGS3 within the cell (9, 10). In D. melanogaster PINS binds to Inscuteable, and this interaction is required for placement of key proteins (Inscuteable and Bazooka) involved in polarization and proper orientation of mitotic spindles of neuroblasts during asymmetric cell division of neuroblasts (9,10). Goi/Gog, is apparently complexed with the PINS/Inscuteable complex, where it presumably plays a signaling function. In mammalian tissues, AGS3 is expressed at highest levels in brain where it is primarily found in a 100,000 × g supernatant following homogenization. AGS3 may oscillate between cytosol and membrane compartments as observed for PINS (AGS3/LGN ortholog) in D. melanogaster.

AGS3 selectively binds to Goi in the presence of GDP, and protein interaction assays with AGS3-GPR indicate that the AGS3-GPR-Goi complex is free of Gβγ suggesting the following possibilities. First, AGS3 binds to G-protein heterotrimer (Goβγ) and actively promotes subunit dissociation, while maintaining Goi in the GDP-bound state. Second, AGS3 “catches” a transient nucleotide-free conformation of Goi, and this interaction is stabilized by binding of GDP to Goi with AGS3 replacing Gβγ as a Goi binding partner. Third, during “basal” cycling of the Goi through its various states of activation/inactivation, there is a period when Goi is free of Gβγ, allowing AGS3 to bind Goi and exclude rebinding of Gβγ. Each possibility could account for the activity of AGS3 in the yeast functional screen, where the pheromone response pathway is activated by Gβγ (4), and each is consistent with the biochemical data indicating that Gβγ and AGS3 compete with each other for interaction with Goi. Thus, the activity of AGS3 as a receptor-independent activator of G-protein signaling may actually involve dissociation of Goi and Gβγ in the absence of nucleotide exchange “releasing” Gβγ from Goi GDP to activate downstream effectors. In such a scenario, Goi bound to AGS3 may be functionally inert and signal termination would require dissociation of AGS3 and Goi GDP with rebinding of Gβγ and Goi GDP. Each of these scenarios are also of note relative to the role of AGS3 (PINS)-Goi/Goi complexes in neuroblast processing in D. melanogaster and the defect in orientation of the mitotic spindle observed in the absence of PINS (9,10). In the latter situation, interaction of AGS3 (PINS) with Goi GDP/Goi GDP, could “release” Gβγ for effector regulation, which may involve the apparent localization of Gβγ to microtubules and/or mitotic spindles (24, 25). The detailed analysis of the interaction of AGS3 with Goi presented in this report should greatly facilitate efforts to further
define the role of AGS3 in signal processing in mammalian systems.

Another possible explanation for the detection of AGS3-Gα complexes that do not contain Gβγ is that there is a population of Gαi in the cell that exists free of Gβγ. In such a case, AGS3 may regulate the activation state of Gα in much the same way as does Gβγ. AGS3-Gα complexes may be regulated by unexpected modes of signal input, which promote nucleotide exchange on Gα motifs indicate that this motif actually behaves as an inhibitor of guanine nucleotide exchange on heterotrimeric G-proteins (28).

The functional and biochemical studies with the AGS3-GPR protein was reported to activate brain G-protein by accelerating as does the Purkinje cell-specific protein Pcp2. The latter protein domains are found in proteins that have apparently different discrete structure that binds to G-protein subunits.5 The GPR motif is also found in other proteins. AGS3 may actually be complexed with a mixture of Gα1,3-

As noted earlier, the GPR motif is also found in other proteins that interact with or regulate G-protein α subunits, and one would hypothesize that the GPR motif has evolved to serve as an anchor for proteins to bind to Gα subunits.5 The GPR domains are found in proteins that have apparently different effects on the activation state of G-protein. The GTPase-activating proteins RGS12 and RGS14 both contain a GPR motif, as does the Purkinje cell-specific protein Pcp2. The latter protein was reported to activate brain G-protein by accelerating guanine nucleotide exchange on heterotrimeric G-proteins (28). The functional and biochemical studies with the AGS3-GPR motifs indicate that this motif actually behaves as an inhibitor of guanine nucleotide exchange on Gα. As a relatively small discrete structure that binds to G-protein α subunits, the GPR motif may serve as a template for rational design of peptides/small molecules that directly influence the activation state of G-protein.

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