Identification and Characterization of AGS4

A PROTEIN CONTAINING THREE G-PROTEIN REGULATORY MOTIFS THAT REGULATE THE ACTIVATION STATE OF Gα*

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Activators of G-protein signaling 1–3 (AGS1–3) were identified in a functional screen of mammalian cDNAs that activated G-protein signaling in the absence of a receptor. We report the isolation and characterization of an additional AGS protein (AGS4) from a human prostate leiomyosarcoma cDNA library. AGS4 is identical to G18.1b, which is encoded by a gene within the major histocompatibility class III region of chromosome 6. The activity of AGS4 in the yeast-based functional screen was selective for Gα2/Gα3 and independent of guanine-nucleotide exchange by Gα. RNA blots indicated enrichment of AGS4/G18.1b mRNA in heart, placenta, lung, and liver. Immunocytochemistry with AGS4/G18.1b-specific antisera indicated a predominant nonhomogeneous, extranuclear distribution within the cell following expression in COS7 or Chinese hamster ovary cells. AGS4/G18.1b contains three G-protein regulatory motifs downstream of an amino terminus domain with multiple prolines. Glutathione S-transferase (GST)-AGS4/G18.1b fusion proteins interacted with purified Gαi, and peptides derived from each of the G-protein regulatory motifs inhibited guanosine 5′-3-O-(thio)triphosphate (GTPγS) binding to purified Gαi. AGS4/G18.1b was also selected by Gα3 in COS7 cell lysates following cell transfection. However, AGS4/G18.1b did not alter the generation of inositol phosphates in COS7 cells cotransfected with the Gβγ-regulated effector phospholipase C-β2. These data suggest either that an additional signal is required to position AGS4/G18.1b in the proper cellular location, or that it can promote heterotrimer and promote subunit dissociation or that AGS4 serves as an alternative binding partner for Gαi independent of Gβγ participating in G-protein signaling events that are independent of classical G-protein-coupled receptors at the cell surface.

Although heterotrimeric G-proteins are transducers for signals recognized by the superfamily of G-protein-coupled receptors (GPCRs)1 at the plasma membrane, several observations suggest that the G-protein subunits serve a broader role in cellular function. For example, the activation state of G-protein is influenced by accessory proteins or signal regulators distinct from GPCRs, whereas other accessory proteins may serve as alternative binding partners for G-protein subunits independent of heterotrimer formation (1). In addition, G-protein subunits and such accessory proteins may be located in intracellular domains as well as at the plasma membrane (2–5). Such observations have broad conceptual and functional implications as they also suggest that G-proteins are processing intracellular signals that do not involve a GPCR at the cell surface (1, 6, 7), as recently demonstrated for the role of G-proteins and such accessory proteins in asymmetric positioning of the mitotic spindle in the one-cell Caenorhabditis elegans embryo (8, 9).

We previously identified three such signal regulators in a yeast-based functional screen, which allowed for rapid screening of mammalian cDNAs that activated the pheromone response pathway in the absence of a GPCR (6, 7). The proteins were defined functionally as activators of G-protein signaling 1, 2 and 3 (AGS1, AGS2, and AGS3). These three proteins do not exhibit sequence homology, and they each activated G-protein signaling by different mechanisms (6, 7, 10–12). To further extend these concepts and to potentially identify additional post-receptor regulators of G-protein signaling, we used the yeast-based system to screen a human prostate leiomyosarcoma cDNA library. This study reports on the characterization of AGS4, which was one of the cDNAs isolated in the screen. AGS4 is identical to G18.1b, which is encoded by a gene within the major histocompatibility complex class III region of chromosome 6. The activity of AGS4 in the yeast-based functional screen was selective for Gα2/Gα3 and independent of guanine nucleotide exchange by Gαi.

AGS4/G18.1b contains three GPR motifs downstream of an amino terminus domain with multiple prolines. The GPR motif is a 20–25-amino-acid domain that binds to Gαi and competes for binding of Gβγ to Gα (1, 7, 10, 11, 13–16). Recent studies suggest that the GPR motif can actively promote subunit dissociation (18). Despite clear differences in key residues within the GPR consensus motif, protein interaction studies with a GST-AGS4/G18.1b fusion protein indicated direct binding of Gαi, and peptides derived from the GPR motifs inhibited GTPγS binding to purified Gαi. These data provide further structural insight regarding the binding pocket for the GPR motif on Gαi. AGS4/G18.1b did not alter the gener-

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1 The abbreviations used are: GPCR, G-protein-coupled receptor; GPR, G-protein regulatory; AGS, activators of G-protein signaling; GAP, GTPase-activating protein; PLC, Phospholipase C.

2 The GPR motif was also identified as the GoLOCO motif (17).
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EXPERIMENTAL PROCEDURES

Materials—[α-32P]ATP (3000 Ci/mmol), inositol, myo-[2-3H(ND)] (25 Ci/mmol), and [35S]GTP-S (1250 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Human MTN and human immune system MTN blots were purchased from Clontech. Gαi3, and Gα1 were purified from SF9 insect cells infected with recombinant virus and kindly provided by Dr. Stephen Graber (West Virginia University School of Medicine, Morgantown, WV). GPR peptides were synthesized and purified by Bio-Synthesis, Inc. (Lewisville, TX), and peptides were mass-verified by matrix-assisted laser desorption ionization mass spectrometry. The amino terminus of the peptides was acetylated, and the carboxyl terminus was amidated. Gαi3 and Gα1 antisera were a kind gift from Dr. Thomas Gettys (Pennington Biomedical Research Institute). All other materials were obtained as described elsewhere (5, 10, 11, 19).

Cloning and Expression of AGS4/G18.1b cDNA—A human fetal lung AGS4/G18.1b cDNA (ATCC 162838R) was amplified by polymerase chain reaction and subcloned into pTOPO and subsequently pGEX4T for expression as a GST fusion protein. The subcloned cDNA exhibited two nucleotide differences from the GenBank™ coding region sequence for G18.1b (G-41 instead of A; G-63 instead of A). The Gly-41 difference resulted in a change in the encoded amino acid from Glu to Arg. It is not clear whether these nucleotide differences represent a polymorphism in the human population. Preliminary studies indicated that both Gln-14 and Arg-14 variants behaved similarly in the yeast-based functional screen. An amino terminus fragment (Met-1-Ser-56) and a carboxyl terminus segment (Leu-57–Cys-116) of AGS4/G18.1b were generated as GST fusion proteins by polymerase chain reaction using AGS4/G18.1b cDNA as the template. GST fusion proteins were generated, purified, and eluted for protein interaction studies as described previously (10).

AGS4/G18.1b Antisera—Antipeptide antibodies were generated by immunizing rabbits with two synthetic peptides (P1, Cys-Pro-6–Ser-28; P2, Cys-116–Leu-135). Peptides were synthesized by the Louisiana State University Health Sciences Center (LSUHSC) peptide synthesis facility, and rabbits were immunized through the LSUHSC antibody production facility. Each antisera was characterized by analysis of varying amounts of GST-AGS4/G18.1b fusion proteins and/or extracts from COS7 cells transfected with pcDNA3::AGS4/G18.1b to determine optimal conditions for immunoblotting. The P1 antisera exhibited the highest specificity and titer and was affinity-purified by AminoLink Plus immobilization kit obtained from Pierce and was used in subsequent experiments. Purified antibodies (AAbs = 1.04) were used at dilutions of 1:500 or 1:500 for immunoblotting and immunocytochemistry, respectively. Multiple series of experiments (different fixation procedures, incubation times, and amounts of antibody) were performed to determine the optimal conditions for signal detection by immunocytochemistry and to verify the specificity of observed signals. No immunofluorescent signal was detected in control cells transfected with the pcDNA3 vector or in the absence of primary antibody. Each field of cells examined by microscopy contained both transfected and nontransfected cells, providing additional internal controls for observed signals. Images were generated and evaluated as described previously (5).

Miscellaneous Procedures—The functional screen in Saccharomyces cerevisiae was conducted as described previously using a yeast strain, which contains Gα1 in place of the yeast Gα subunit Gap1 (6, 7). Human MTN blot and Human Immune System MTN blot II (Clontech) were prehybridized and hybridized in phosphate buffer and washed as described previously (19) with subsequent exposure to XAR-5 film for 8–10 days at –70 °C.

For GTPγS binding assays, purified Gα (100 nm) was preincubated with varying amounts of the GPR peptides for 20 min at 24 °C. Binding assays (duplicate determinations) were initiated by the addition of 0.5 μM[35S]GTPγS (4.0 × 106 dpm/pmol), and incubations (total volume = 50 μl) continued for 30 min at 24 °C. Both preincubination and GTPγS binding assays were conducted in binding buffer containing 50 mM HEPES-HEC, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 2 mM MgCl2, 50 μM adenosine triphosphate, and 0.5 mg/ml bovine serum albumin and then processed as described previously (11).

For preparation of cell lysates for immunoblotting, confluent 100-mm dishes of cells were washed twice with cell washing solution (137 mM NaCl, 2.6 mM KCl, 1.8 mM KH2PO4, 10 mM Na2HPO4, pH 7.4) and resuspended in lysis buffer (5 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, pH 7.4) by homogenization with a 26/3 gauge needle. Following a 1-h incubation at 4 °C, the cell homogenate was centrifuged at 100,000 × g for 30 min at 4 °C to generate a crude membrane pellet and a 100,000 × g supernatant containing cytosol. Cell membrane pellets were washed three times with intervening homogenization in membrane buffer (50 mM Tris, 0.6 mM EDTA, 5 mM MgCl2, pH 7.4) and pelleting at 100,000 × g. Immunoprecipitation, cell transfections, and measurements of inositol phosphates were performed as described (10, 20, 21).

For protein interaction assays, rat brain was homogenized in 3 ml/g of tissue of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40). The tissue homogenate was centrifuged at 27,000 × g for 30 min after a 1-h incubation on ice. Supernatants were collected and spun at 100,000 × g for 1 h to generate a detergent-soluble fraction. The interaction of GST-AGS4/G18.1b fusion proteins with purified G-proteins was assessed by protein interaction experiments using tissue/cell lysates as described previously (10). All purified G-proteins used in these studies were isolated in the GDP-bound form, and G-protein interaction assays contained an additional 10 μM GDP. Protein concentrations were determined by Bio-Rad protein assay.

FIG. 1. Properties of AGS4 in the yeast-based system with different genetic backgrounds. A, selectivity of AGS4/G18.1b for G-proteins. Cells from each Gα integrated strain carrying pYES2:AGS4/G18.1b were grown on glucose (Glu) medium containing (+His) or lacking (−His) histidine or galactose (Gal) medium lacking histidine. Growth on medium lacking histidine was conditional for activation of the pheromone response pathway. The −His medium contained 1 mM aminotriazole. B, pYES2:AGS4/G18.1b transformants of strain CY1316/1183 and its derivatives were grown on glucose medium containing or lacking histidine and galactose medium lacking histidine. Gα1 was expressed via introduction of plasmid pR51183 (6). Medium lacking histidine contained 1 mM AT, Gα1-G204A: CY1316/1183 with Gly204 of Gap1-Gα1 changed to alanine; Gαi3 + AGS4: CY1316/1183 constitutively expressing human RGS4; 48B: CY1316/1183 carrying a genomic deletion of Gβ6; and Ste5. The two forms of Gα1, E10K AND D229S used in A contain mutations that are required for effective coupling to yeast Gβγ and are functionally similar, although the E10K mutant exhibits somewhat tighter coupling to Gβγ. Ste5 refers to a scaffolding protein in the MAP kinase cascade, and Ste20 is a p21-activated protein kinase. WT, wild type.
RESULTS AND DISCUSSION

As part of a broad strategy to identify post-receptor regulators of G-protein signaling, we used a yeast-based functional screen designed to rapidly screen cDNA libraries for such a bioactivity. The functional screen (6, 7) was based upon the pheromone response pathway in *S. cerevisiae* and used a strain of yeast that lacked the pheromone receptor, a typical GPCR that interfaces via Gβγ to a MAP kinase cascade. The yeast strain was further modified by replacing the yeast Gα subunit Gpa1 with human Gαi3 and rendering growth dependent upon activation of the pheromone response pathway. This yeast strain was used to screen a human prostate leiomyosarcoma cDNA library in a galactose-inducible vector pYES2 for entities that promoted growth in galactose-specific manner. cDNAs isolated in this

**FIG. 2. AGS4/G18.1b sequence and tissue distribution of human AGS4/G18.1b mRNA.** A, amino acid sequence of AGS4/G18.1b. GPR domains I, II, and III are underlined. B, Clontech multiple-human tissue and immune system blots containing 2 μg of poly(A)+ RNA per lane were hybridized with a 492-nucleotide radiolabeled probe corresponding to the coding region of AGS4/G18.1b.-sk m, skeletal muscle. The arrows indicate AGS4/G18.1b mRNA.

**FIG. 3. Subcellular localization of AGS4/G18.1b.** A, COS7 cells (100-mm plates) were transfected with 10 μg of pcDNA3 (cont) or pcDNA3::AGS4/G18.1b and processed for immunoblotting with affinity-purified antibody as described under “Experimental Procedures.” Each lane contained 25 μg of protein. B, Chinese hamster ovary (CHO) cells (100 mm plates) were transfected with 10 μg of pcDNA3::AGS4/G18.1b and processed for immunocytochemistry as described under “Experimental Procedures.” Left panel, fluorescent signal (green). Right panel, differential interference contrast image. DNA (blue) was visualized by 4',6-diamidino-2-phenylindole staining. Data in A and B are representative of 3 independent experiments.
screen that required the presence of G\(\gamma\) for activity were termed activators of G-protein signaling (AGS) as described previously for AGS1–3 (6, 7). This study reports the identification and characterization of AGS4 and indicates that the yeast-based system may be used to identify disease-specific signal regulators that target various entities within the signal transduction cascade.

The selectivity of AGS4 for different G-proteins was first determined by evaluating the activity of the cDNA in yeast expressing G\(i_3\), G\(s\), or G\(q_16\) in place of Gpa1. AGS4 effectively promoted growth in the G\(i_3\) genetic background but did not activate G\(s\) or G\(q_16\) (Fig. 1A). Epistasis analysis indicated that the bioactivity of AGS4 required the presence of G\(\gamma\) (Fig. 1B), and as there were no changes in the amount of G\(\alpha\) or G\(\gamma\) upon expression of AGS4, this protein effectively behaved as a receptor-independent activator of G-proteins. AGS4 expression also promoted growth in a yeast strain expressing G\(q_2\), and this activation was not antagonized by overexpression of RGS4, a GTPase-activating protein (GAP) for G\(i_3\) (Fig. 1B). Activity was also observed in a strain expressing G\(q_2\)-G204A, a mutant G\(q_2\) that is deficient in exchanging GDP for GTP (6, 7) (Fig. 1B). These data suggest that the activation of G-protein signaling is occurring in the absence of nucleotide exchange and suggest that AGS4 is influencing subunit interactions in a manner that increases the availability of G\(\gamma\) for activation of the MAP kinase cascade and promotion of growth. AGS4 may actively promote dissociation of G\(\alpha\) and G\(\gamma\), as reported for a consensus GPR peptide (18), or it may impede the reassociation of G\(\alpha\)GDP with G\(\gamma\) within the G-protein activation/deactivation cycle (1).

Sequence analysis of AGS4 indicated that it was identical to G18.1b (NM_022107;BC018724). AGS4/G18.1b consists of 160 amino acids (molecular weight, 17,864) (Fig. 2A) and is encoded by guest on July 25, 2018http://www.jbc.org/Downloaded from
by a gene (8 exons) within the major histocompatibility complex class III region (chromosome 6, open reading frame 9). The major histocompatibility complex class III region contains genes that encode proteins of diverse function and is of particular interest in terms of disease susceptibility. The related protein in mouse (AAH21942; BC021942) exhibits 88% sequence identity with its human counterpart. Blastp searches did not reveal any obvious candidate orthologs in *C. elegans*, *S. cerevisiae*, or *Drosophila melanogaster*.

Although initially AGS4/G18.1b was a “predicted” protein based upon genome sequence, both the identification of AGS4/G18.1b expressed sequence tags and subsequent RNA blots (Fig. 2B) indicate that it is indeed generated as a transcript. AGS4/G18.1b mRNA (~1.4 kb) was expressed in heart, placenta, lung, and liver. An immune system RNA blot indicated expression of AGS4/G18.1b in spleen, lymph node, peripheral blood leukocytes, and bone marrow. Immunoblotting of COS7 cells transfected with AGS4/G18.1b cDNAs indicated that the protein was primarily found in the 100,000 × g supernatant (Fig. 3A). Immunocytochemistry in COS7 and Chinese hamster ovary AGS4/G18.1b transfectants indicated a nonhomogeneous distribution often enriched around the nucleus or adjacent to the plasma membrane (Fig. 3B). A faint signal is also seen in the nucleus. These data suggest a regulated localization of

![Diagram](Fig. 6. Influence of GST-AGS4/G18.1b and GPR peptides derived from AGS4/G18.1b, PCP2, RapIGAP-II, and *C. elegans* GPR-1/2 on nucleotide binding to purified Gα and Gα.)

A, GTPγS binding to Gα (100 nM) was measured in the presence of increasing concentrations of GST, GST-AGS4/G18.1b, and AGS3-GPR (Pro-463–Ser-650). The diamond symbol indicates the results obtained with 1 μM truncated GST-AGS4/G18.1b (Leu-57–Cys-160) containing the three GPR motifs. Data are expressed as the percentage of specific binding observed in the absence of the peptides and are presented as the mean ± S.E. of two experiments with duplicate determinations. B, alignment of the AGS4/G18.1b peptides with the GPR consensus peptide and GPR motifs in PCP2, *C. elegans* (CE) GPR-1/2, and Rap1GAP11. The latter three proteins each contain one GPR motif. Residues in red indicate identical amino acids or conserved substitutions as compared with the GPR consensus sequence. Note that in AGS4/G18.1b GPR-II, the second set of hydrophobic amino acids (Ile-111, Leu-112 in the first half of the consensus sequence) is shifted one residue to the right as compared with the other GPR peptides. Note also that Gin-143 in GPR11 is shifted to the left one residue as compared with the other GPR peptides and that CE GPR-1/2 contains an additional hydrophobic residue (Phe-432) that stretches the second set of hydrophobic amino acids in the first half of the consensus sequence toward the downstream DDQR residues. The lowercase t in the *C. elegans* GPR-1/2 peptide is not part of the actual GPR1/2 sequence. C, GTPγS binding to Gα (100 nM) or Gα (100 nM) was measured in the presence of increasing concentrations of the GPR peptides as described under “Experimental Procedures.” Data are expressed as the percentage of specific binding observed in the absence of the peptides and are presented as the mean ± S.E. of two experiments with duplicate determinations.
AGS4/G18.1b within the cell. AGS4/G18.1b was also identified by mass spectrometry (22, 23) within a detergent-resistant membrane skeleton of neutrophil cell-surface membranes and in immunoprecipitates of major histocompatibility complex class I molecules. The fragment of AGS4/G18.1b in the latter immunoprecipitates was actually phosphorylated at Ser-59, which is just upstream of the first GPR motif and analogous to the site of phosphorylation in RGS14 upstream of the GPR motif, where it was suggested to influence the affinity of the interaction of the GPR motif with G-protein (24). Additional sites of phosphorylation may occur within the GPR motif itself, influencing the interaction with G-protein and providing a regulatory mechanism for signal input or subcellular location (25).

G18.1b was one of six mammalian proteins identified by a motif searching strategy based upon a consensus GPR motif in AGS3 (6, 17). Each of these proteins (Rap1GAP1, LGN, RGS12, RGS14, PCP2, and G18.1b) contained at least one GPR motif. Three core GPR motifs are present at the carboxy-terminal region of AGS4/G18.1b (GPR-I Thr-62–Arg-81, GPR-II Arg-104–Arg-123, GPR-III Gln-133–Arg-152). Four GPR motifs are found in AGS3 and LGN, where at least for AGS3, it appears that each of the four GPR motifs can simultaneously bind G\_a GDP free of G\_i (10, 26). Structure-function studies with a consensus GPR peptide (15) and the crystal structure of the RGS14-GPR/GoLoco peptide complexed with G\_a (16) provide important structural information on key residues required for interaction with and regulation of G\_a. The functionality of the AGS4/G18.1b GPR motifs is not defined, nor is it known whether they exhibit selectivity for different G-proteins.

The GPR domains in AGS4/G18.1b exhibit some nonconservative substitutions in amino acids when compared with a conserved GPR motif, which may influence the interaction with Ga subunits. To address these issues, we evaluated the interaction of AGS4/G18.1b with G-proteins by two experimental studies. The first approach involved the generation of GST-AGS4/G18.1b fusion proteins and protein interaction studies. The second approach involved the synthesis of peptides corresponding to each of the GPR motifs and the characterization of their ability to interact with G-proteins. Protein interaction studies with purified Ga subunits indicated that GST-AGS4/G18.1b interacted with G\_a versus G\_\alpha (Fig. 4B). The interaction between AGS4/G18.1b also occurred in COS-7 cells cotransfected with AGS4/G18.1b and G\_a, where AGS4/G18.1b was coimmunoprecipitated with G\_\alpha from cell lysates (Fig. 5). The AGS4/G18.1b GST fusion protein also inhibited GTP\_S binding to purified G-protein (Fig. 6A).

To define the regions of the protein that interacted with G-protein, we generated GST-AGS4/G18.1b fusion protein constructs containing the first 56 amino acid residues or the carboxy terminal 104 amino acid residues, which contained the three GPR motifs (Fig. 4, A and B). Only the carboxy terminal construct bound to G\_a (Fig. 4, B and C). To further address these issues, we generated three GPR peptides based upon the amino acid sequence of GPR motifs within AGS4/G18.1b and tested them for their ability to influence GTP\_S binding to purified G-protein (GPR-I, Ser-59–Gln-88; GPR-II, Leu-101–Leu-128; GPR-III, Gly-131–Thr-159). We compared the bioactivity of the AGS4/G18.1b GPR peptides with the GPR consensus peptide (11) and peptides derived from the GPR motifs in PCP2 (Ala-60–Ser-87), Rap1GAP1 (Ser-25–Pro-50), and the C. elegans protein GPR-1/2 (Asn-421–Ala-447). Alignment of the AGS4/G18.1b GPR motifs with the consensus sequence indicated differences in key residues within GPR-II and III (Fig. 6B). The GPR consensus residues are fairly well conserved in GPR-I, whereas both the second and third motifs have disruptions in the spatial relationship of key residues. Nevertheless, each of the peptides inhibited GTP\_S binding to G\_a (IC\_50; GPR-I 0.35 \( \mu M \) > GPR-II 0.82 \( \mu M \) > GPR-III 3.6 \( \mu M \) (Fig. 6C, left panel)). Each of the GPR peptides from AGS4/G18.1b exhibited much lower affinity in nucleotide binding assays with G\_a. The GPR consensus peptide was included to provide a standard for comparison. A similar selectivity for G\_a versus G\_\alpha was observed for GPR peptides from Rap1GAP1, PCP2, and C. elegans GPR-1/2 (Fig. 6C). The rank order of potency for the latter peptides in GTP\_S binding assays with G\_a was GPR-I/2-GPR > PCP2-GPR > Rap1GAP1-GPR (IC\_50; GPR-I/2-GPR 0.41 \( \mu M \) > PCP2-GPR 0.75 \( \mu M \) > Rap1GAP1-GPR 5.5 \( \mu M \)). Of note is that the rank order for potency in GTP\_S binding assays for GPR-I versus GPR-I/2 and PCP2-GPR versus Rap1GAP1-GPR differed with G\_a and G\_\alpha (Fig. 6C). A similar IC\_50 for GPR peptides was observed at a 10-fold lower concentration of G\_\alpha.

GPR-II contains an alanine (Ala-121) in place of an arginine residue, which was suggested to be critical for positioning of the arginine residue at the end of the core GPR motif within the nucleotide binding pocket of G\_a (16). However, the GPR-II peptide from AGS4/G18.1b exhibits an IC\_50 in guanine nucleotide binding assays that is similar to that of GPR-I, which contains the conserved arginine residue at the corresponding location (Glu-79). The retention of affinity in GPR-II with Ala-121 in place of the consensus Asp may relate to the spatial relationship between the 2nd set of hydrophobic residues (Ile-111, Leu-112 in the first half of the consensus sequence) and Gln-122, which is altered by one less residue when compared with the GPR consensus sequence and the GPR-I peptide. Thus, the change in this spatial relationship may minimize the influence of the Ala-121 substitution in terms of bioactivity of the GPR peptide such that affinity is maintained. Such subtle shifts in the organization of the key residues within a GPR motif can influence selectivity for different G-proteins (11, 15), as suggested by Kimple et al. (16) for residues outside of the core GPR motif. Further analysis of the structure activity relationships for GPR peptides and molecular modeling strategies may lead to the generation of a synthetic GPR peptide that selectively targets Ga subunits other than G\_a. Indeed, the reversal in the relative potencies for GPR peptides (Fig. 6C) (GPR-I versus GPR-II, Rap1GAP1-GPR versus PCP2-GPR) in GTP\_S binding assays with G\_\alpha (GPR-I > GPR-II; PCP2-GPR > Rap1GAP1-GPR) versus G\_a (GPR-II > GPR-I, Rap1GAP1-GPR > PCP2-GPR) supports this possibility.

The amino-terminal 56 amino acids of AGS4/G18.1b upstream of the three GPR motifs contain several proline residues (Fig. 2A). Repetitive proline-rich sequences are found in many

X. Cao and S. M. Lanier, unpublished data.

A report (33) published while the current study was under review indicated that the an AGS4-GST fusion protein (Pro-100–Leu-140), which contains GPR-II, the spacer between GPR-II, and the first 8 amino acids of the core GPR motif in GPR-III, was relatively inactive unless Ala-121 was converted to aspartate. This contrasts with the bioactivity of the GPR-II peptide reported in the present study. A GPR-II peptide containing a glutamate in place of Ala-121 exhibited only a modest decrease in the IC\_50 (see Footnote 4). These data suggest that the ability of the GPR-II motif to bind G-protein and the relative importance of the Ala-121 residue may be influenced by regions outside of the core GPR motif or the binding of G-proteins to other GPR motifs in the intact protein.
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proteins and may function as docking sites for signaling modules. In this regard, AGS4/G18.1b, AGS3, and LGN are similar in that each of the proteins contain multiple GPR motifs downstream of an apparent regulatory domain (tetrameric peptide repeats for AGS3 and LGN) (10) that regulates the subcellular location of the protein and/or possibly influences the interaction of the GPR motifs with G-proteins via interaction with binding partners (5, 19, 25, 27–30). The presence of multiple GPR motifs allow the protein to bind more than one Gα subunit (25) along with whatever other proteins may complex at the amino-terminal region of the proteins (25, 27). Such complexing of proteins may be key for turning on or off the signal or play a role in the spatial dynamics of signaling events. The GPR motif, by virtue of its ability to stabilize the GDP-bound conformation of Gα, presents an opportunity for selective control of Gα- and Gβγ-regulated effector systems. Of particular note, such motifs can function as binding partners for Gα independent of heterotrimer formation and provide a mechanism for G-proteins to regulate events distinct from the role they play in transducing signals from G-protein-coupled receptors at the cell surface.

As an initial approach to these questions, we screened a number of cell lines for expression of endogenous AGS4 so that we could evaluate various aspects of signal processing. To date, we have not identified any cell line that expresses endogenous AGS4. Thus, we used a transient transfection system to explore the potential effects of a GPR protein on the activity of a Gβγ-regulated effector. Although a GPR peptide promotes dissociation of heterotrimeric G-protein subunits in vitro (18), it is not clear whether this would occur in a cell model. Phospholipase C (PLC)-β2 is directly regulated by Gβγ. Activation of the Gα/Gβγ-coupled α2AR-adrenergic receptor in this system increases inositol phosphates in cells cotransfected with PLC-β2, and this effect is blocked by pretreatment of cells with pertussis toxin, which blocks receptor-mediated activation of Gαi (Fig. 7, left panel). Thus, if AGS4/G18.1b expression in COS-7 cells promoted subunit dissociation, we would expect to see activation of PLC-β2. Transfection of COS-7 cells with PLC-β2 and Gβγ clearly increased inositol phosphates (Fig. 7, right panel) when compared with the values obtained upon transfection with PLC-β2 as reported previously (20). However, transfection of AGS4/G18.1b with PLC-β2 did not increase the level of inositol phosphates above that observed with PLC-β2 (Fig. 7). Immunoblotting of transfected cell lysates indicated the expression of each transfected cDNA and similar levels of PLC-β2 expression whether or not the enzyme was cotransfected with other plasmids (Fig. 7, inset). These data suggest the following possibilities: 1) AGS4/G18.1b interacts with a subpopulation of Gαi that exist free of Gβγ, 2) by virtue of its predominant location in cytosol, AGS4/G18.1b requires a signal for translocation to the appropriate membrane domain to effect inositol subunit interactions; and/or 3) AGS4/G18.1b is not expressed at quantities sufficient to generate an amount of free Gβγ that equals the amount achieved by simple transfection with Gβγ plasmids.

From a broader perspective, each protein that contains a GPR motif likely uses the motif for a different functional mission. With this perspective, to fully define the functional role of each GPR-containing protein, one has to move beyond traditional questions or experimental paradigms that are often posed in the context of GPCRs. Each functional mission for a GPR protein probably relates to its expression in the proper cellular context and to non-GPR motifs that position it in the right place at the right time or serve as a sensor for incoming signals that influence the interaction of the GPR motifs with G-proteins. This appears to be the case for the AGS3/LGN ortholog PINS (partner of inscuteable) in Drosophila melanogaster and for GPR-1/2 in C. elegans (8, 9, 28, 30–32). Intrinsic signals result in the apical cortex positioning of PINS via an interaction with InsCuteable and G-proteins, which is then involved in spindle positioning for asymmetric division (30). In C. elegans, the posterior enrichment of GPR-1/2 in the one-cell embryo requires an interaction with LIN-5, and these two proteins, along with the G-proteins GPA-16 and GOA-1, regulate spindle...
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