Thermodynamic Characterization of the Binding of Activator of G Protein Signaling 3 (AGS3) and Peptides Derived from AGS3 with Ga11

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Activator of G protein signaling 3 (AGS3) is a guanine nucleotide dissociation inhibitor (GDI) that contains four G protein regulatory (GPR) or GoLoco motifs in its C-terminal domain. The entire C-terminal domain (AGS3-C) as well as certain peptides corresponding to individual GPR motifs of AGS3 bound to Gaq and inhibited the binding of GTP by stabilizing the GDP-bound conformation of Gaq. The stoichiometry, free energy, enthalpy, and dissociation constant for binding of AGS3-C to Gaq were determined using isothermal titration calorimetry. AGS3-C possesses two apparent high affinity (Keq ~ 20 nM) and two apparent low affinity (Keq ~ 300 nM) binding sites for Gaq. Untypical deletion of the C-terminal GPR motif from AGS3-C, the remaining sites were approximately equivalent with respect to their affinity (Keq ~ 400 nM) for Gaq. Peptides corresponding to each of the four GPR motifs of AGS3 (referred to as GPR1, GPR2, GPR3, and GPR4, respectively, going from N to C terminus) bound to Gaq with Keq values in the range of 1–8 µM. Although GPR1, GPR2, and GPR4 inhibited the binding of the fluorescent GTP analog BODIPY-FL-guanosine 5’-3-O-(thiotriphosphate to Gaq, GPR3 did not. However, addition of N- and C-terminal flanking residues to the GPR3 GoLoco core increased its affinity for Goq and conferred GDI activity similar to that of AGS3-C itself. Similar increases were observed for extended GPR2 and extended GPR1 peptides. Thus, while the tertiary structure of AGS3 may affect the affinity and activity of the GPR motifs contained within its sequence, residues outside of the GPR motifs strongly potentiate their binding and GDI activity toward Gaq, even though the amino acid sequences of these residues are not conserved among the GPR repeats.

The α subunits of heterotrimeric G proteins (Ga) act as molecular switches to modulate intracellular signaling pathways. In the classical G protein signaling cycle, agonist-bound G protein-coupled receptors catalyze the exchange of GDP for GTP at the catalytic site of Ga. Thus activated, Ga subunits subsequently dissociate from the heterodimeric complex of Gβ and Gγ subunits (Gβγ) (1). GTP-bound Ga subunits modulate the activity of effector enzymes, including adenyl cyclase, phospholipase Cβ, and nucleotide exchange factors for the small GTPase, Rho (2–4). Signaling is terminated by the intrinsic GTPase activity of Ga; upon hydrolysis of GTP, Ga-GDP reassociates with Gβγ to form the inactive heterotrimer. Several molecules can regulate the G protein reaction cycle by modulating the rate of GTP hydrolysis or nucleotide exchange.

The most familiar of these are regulators of G protein signaling (RGSs), which increase the GTPase activity of Ga subunits (5). In recent years, a new class of regulators has been discovered, typified by activator of G protein signaling 3 (AGS3). Like Gβγ, AGS3 selectively binds to the GDP-bound form of Ga and acts as a guanine nucleotide dissociation inhibitor (GDI) (6, 7). AGS3 is a 650-residue protein from Rattus norvegicus first identified in a yeast expression screen as a receptor-independent activator of Gβγ-dependent signaling (7, 8). The N-terminal half of AGS3 contains seven tetratricopeptide repeats, which have been shown to act as protein interaction domains in multiprotein complexes (9). The C-terminal half of AGS3 (AGS3-C) contains a series of four G protein regulatory (GPR) motifs (6, 7, 10). AGS3-related proteins, having similar arrangements of tetratricopeptide repeat-rich and GPR-rich domains, include the human protein LGN, Drosophila protein partner of inscuteable (PINS), and a related protein from Caenorhabditis elegans (7, 11, 12).

The GoLoco motif was first identified in the Drosophila protein “loco,” the homolog of mammalian RGS12 (13). GoLoco motifs occur either singly or as tandem repeats in proteins that interact with Gα and Gγ class α subunits (6, 14). Both RGS12 and RGS14 contain one GoLoco motif as do Purkinje cell protein-2 (Pcp2) and Rap1GAP (10, 15, 16). GPR and GoLoco motifs are one and the same.

AGS3 binds specifically to the GDP-bound forms of all three Gαα isoforms, to Gαq, and weakly to Gα12 and Gα13 (6, 7, 10, 12) but exhibits GDI activity only toward Gαq and Gα12 (6, 12, 14). GPR-containing proteins Pcp2 and Rap1GAP also display GDI activity toward Gαq (10). None of the GPR/GoLoco-containing proteins that have been characterized have GDI activity toward Gα12 (10, 14). By binding to Ga-GDP, AGS3 blocks association of Ga with Gβγ (12, 14) and thereby prevents G protein

1 The abbreviations used are: RGS, regulator of G protein signaling; AGS3, activator of G protein signaling 3; AGS3-C, C-terminal half (residues 465–650) of AGS3; GDI, guanine nucleotide dissociation inhibitor; PINS, partner of inscuteable; ITC, isothermal titration calorimetry; GPR, G protein regulatory; GPRex, extended GPR peptide; GST, glutathione S-transferase; GTP-γ-S, guanosine 5’-3-O-(thiotriphosphate; Pcp2, Purkinje cell protein-2; GAP, GTPase-activating protein; DTT, dithiothreitol.

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activation through G protein-coupled receptors, which act only upon G protein heterotrimers. Because both PINS and AGS3 can activate Gαi/γ-mediated signaling in the absence of G protein-coupled receptor activation, it has been suggested that they serve as “receptor-independent activators” of G protein signaling (7, 17). These proteins, together with the recently identified cytosolic Go exchange factor RIC-8 (18, 19), may participate in novel receptor-independent G protein-regulated events such as asymmetric cell division and mitotic spindle formation (20–23).

The GPR motifs of AGS3-C are necessary and sufficient for the GDI activity of AGS3 (6, 12). Indeed a fully active splice variant of AGS3 expressed in cardiac muscle consists only of the GPR-containing C-terminal domain of AGS3 (24). Peptides that correspond to the GPR motif of RGS12 and a synthetic peptide that bears the consensus amino acid sequence of the four GPR motifs in AGS3 have potent GDI activity (10, 11, 15). Mutagenic scanning experiments have revealed a conserved functional core within GPR repeats corresponding to the sequence FFXLLXXXXXXXMX(D/E)QR that is required for GDI activity (16). Experiments with chimeric constructs of Gαi1 and Gα1 identified the switch regions and helical domain of Gα as GoLoco binding sites (25). The recently determined crystal structure of the complex between a peptide that contains the GoLoco motif of RGS14 and the GDP-bound form of Gα1 reveals the structural basis of GDI activity and specificity (26).

The GoLoco peptide reorganizes and stabilizes the switch regions of Gαi1, and the arginine residue located in the conserved Asp-Gln-Arg (DQR) triad of the GoLoco motif is inserted into the active site of Gαi1 and interacts with the β phosphate of GDP. Resides C-terminal to the GoLoco motif interact with non-conserved residues in the helical domain of Gαi1 and are therefore proposed to be determinants of specificity (26).

Inasmuch as a single GoLoco/GPR motif can possess GDI activity, the biochemical advantage conferred by proteins such as AGS3, which possess multiple copies of GPR motifs in tandem repeats, is worthy of investigation. The presumption that each of the GPR motifs in AGS3 possesses GDI activity has received experimental support, suggesting that AGS3 and its homologs might serve as scaffolds for GDP-bound Gα in a manner analogous to the interactions of RGS proteins with Gαi. The GPR consensus peptide of AGS3 is required for GDI function, even when inserted into the second GPR motif of the GoLoco peptide derived from RGS14 (26) and the GPR consensus peptide (11). The GPR motif of AGS3 possesses GDI activity has been extended with native N- and C-terminal flanking residues (Fig. 1B) were a gift from Dr. Stephen M. Lanier (Department of Pharmacology, Louisiana State University Health Sciences Center). These peptides were expressed as N-terminal fusion proteins with glutathione S-transferase (GST) (12). The fusion proteins corresponding to the extended forms of the first three GPR motifs are referred to as GPR1ex, GPR2ex, and GPR3ex. The fusion proteins corresponding to the extended forms of the first three GPR motifs are referred to as GPR1ex (Pro(D/E)Glu), GPR2ex (Ser(L/D)Leu), and GPR3ex (Glu(Thr/Val)), respectively.

**AGS3 Expression Plasmids—**The C-terminal domain of rat AGS3, residues 465–650, subcloned as an N-terminal His, fusion protein in the expression vector pQE30 was a gift from Dr. Stephen M. Lanier. This domain contains four GPR motifs: GPR1, residues 470–527; GPR2, residues 524–542; GPR3, residues 572–590; and GPR4, residues 606–624. AGS3-C was cloned into the pDEST-15 destination vector as a GST fusion protein using the Gateway cloning system (Invitrogen). A tobacco etch virus protease cleavage site was inserted between coding regions for GST and AGS3-C. Cleavage by tobacco etch virus protease introduces an extra glycine residue at the N terminus. Two deletion mutants of AGS3 were also created: AGS3-A34 encompasses the first two (N-terminal) GPR domains of AGS3, residues 465–548; and AGS3-A34 includes the first three GPR domains, residues 465–597. Both mutants were created by inserting stop codons at the desired C terminus of the open reading frame of AGS3 using the QuickChange™ directed mutagenesis kit (Stratagene).

**Expression and Purification of Recombinant Proteins—**Recombinant Gα1 was expressed and purified as described previously (27). AGS3 and the deletion mutants of AGS3 were expressed in transformed BL21 (DE3) strains of Escherichia coli cells as GST fusion proteins. Cells were grown in 1–3 liters of LB medium at 37 °C to A600nm of ~0.9 and induced with 200 μM isopropyl-β-D-thiogalactopyranoside at 30 °C for 5 h for expression of recombinant proteins. Induced cells were harvested by centrifugation, flash frozen in liquid nitrogen, and stored at −80 °C. Frozen cells were thawed and resuspended in lysin buffer (50 mM Na+ HEPES, pH 7.5, 100 mM NaCl, 5 mM diithiothreitol (DTT), and 1× protease inhibitor mixture PTE (1000×); TTT contains 25 mg/ml phenylmethylsulfonyl fluoride, 21 mg/ml N°-tosyl-l-lysine chloromethyl ketone, and 21 mg/ml N°-tosyl-l-phenylalanine chloromethyl ketone dissolved in 1:2 (v/v) solution of dimethyl sulfoxide and isopropanol) containing 3 mg/ml hen egg white lysozyme for 30 min at 4 °C with continuous stirring followed by sonication for 5 min on ice (5-s pulse and 5-s idle for 30 s) at 0 °C. The cell lysate was centrifuged at 35,000 rpm for 40 min at 4 °C in a Beckman Ti 45 rotor. Clear supernatant was filtered using a 0.45-μm syringe filter and loaded on glutathione-Sepharose 4B resin (Amersham BioSciences). The resin was washed with lysin buffer, and the GST-tagged proteins were eluted with elution buffer (20 mM Tris,
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pH 8.0, 100 mM NaCl, 5 mM DTT, 25 mM reduced glutathione, and 1× PTT). The purified GST fusion proteins were cleaved using 10 μg of recombinant tobacco etch virus protease/mg of fusion protein at 4 °C overnight and dialyzed against low salt buffer (20 mM Tris, pH 8.0, 2 mM DTT, 1 mM EDTA, 1× PTT, 5% glycerol). The protein was loaded on a Hi-Trap Q™ column (Amersham Biosciences) and eluted with a 75-ml linear gradient of 100–750 mM NaCl. The proteins were eluted at 150–250 mM NaCl concentration. Fractions containing AGS3 or its deletion mutants were pooled, concentrated, and loaded on tandem Superdex™ 200 and 75 gel filtration columns (Amersham Biosciences) equilibrated with low salt buffer supplemented with 100 mM NaCl. The peak fractions were pooled, concentrated, and stored at 4 °C. Protein concentration was estimated by the Bradford assay using the Bio-Rad protein assay kit. GST fusion proteins with extended GPR peptides were estimated using gel filtration standards (Bio-Rad).

Preparation and Purification of AGS3-Ga1-GDP Complexes—The complex of Ga1-GDP with AGS3-C was formed by incubating the proteins together in molar ratio (Ga1:AGS3-C) exceeding 4:1 to ensure saturation of AGS3-C. Molar ratios exceeding 3:1 and 2:1 were used for complex formation of Ga1 with AGS3-C and AGS3-C, respectively. In a typical experiment, 100 μl of 500 μM AGS3-C was mixed with 500 μl of 350 μM Ga1 in buffer containing 50 mM Tris, pH 8.0, 2 mM DTT, 1 mM EDTA, and 1 mM GDP and incubated on ice for 2 h. The resulting complex was separated at 4 °C on tandemly connected Superdex 200 and 75 gel filtration columns (Amersham Biosciences) at a flow rate of 0.4 ml/min with 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2 mM DTT, and 1× PTT as the running buffer. The apparent molecular weights of the complexes were estimated using gel filtration standards (Bio-Rad).

Isothermal Titration Calorimetry—Isothermal titration calorimetry was performed at 20 °C (293 K) using a MicroCal VP-ITC (MicroCal, Northampton, MA) calorimeter. Protein samples were dissolved in assay buffer (20 mM Tris, pH 8.0, 2 mM DTT, and 10 μM GDP). Because peptides could not be dialyzed due to their lower molecular weight, lyophilized peptides were dissolved directly in titration buffer. Samples were centrifuged at 13,000 rpm in a bench-top microcentrifuge (Forma Scientific) for 5 min before loading in the sample cell or syringe. Contents of the sample cell were stirred continuously at 300 rpm during the experiment. A typical titration of Ga1, with GPR peptides involved 25–30 injections at 3-min intervals of 8–10 μl of peptide solution (1 mM) into a sample cell containing 1.4 ml of Ga1 (45–50 μM). For the titration of Ga1 with AGS3-C or its deletion mutants, Ga1 (125–200 μM) was injected into the syringe and a sample cell containing AGS3-C (5–10 μM) the injection volume was decreased from 5–6 μl, and the number of injections was increased to 45–50. For the GST fusion extended GPR peptides, Ga1 (175–225 μM) was injected (35 injections of 8 μl) into the sample cell containing GFPex fusion peptide (20–25 μM), and NaCl (100 mM) was added to the titration buffer to avoid nonspecific interactions with GST. The heats of dilution of the titration buffer were subtracted from the titration data for base-line correction.

The base-line-corrected data were analyzed with MicroCal Origin™ 5.0 software to determine the enthalpy (ΔH), association constant (Kd), and stoichiometry of binding (N). Thermal titration data were fit to one or more of the three association models available in the software: “single set of identical sites,” “two sets of independent sites,” and “sequential binding.” The models were compared by visual inspection of the fitted curves and by comparing the x2 values obtained after the computation. The model resulting in the lowest value of x2 was considered the best model to describe the molecular mechanism of binding. Free energy change (ΔG) and entropy change (ΔS) were calculated from ΔH and Kd using standard free energy relationships. Several titrations were performed (two to four) for each sample set to evaluate reproducibility.

Nucleotide Exchange Assays—The rate of guanine nucleotide exchange on Ga1 was assayed by monitoring the rate at which the fluorescent GTP analog BODIPY-FL-GTP-S replaces bound GDP in the catalytic site of Ga1. (30) Binding of the fluorescent nucleotide analog is accompanied by an increase in fluorescence of the BODIPY moiety. Ga1 (final concentration, 200 nM) was injected into a assay buffer (50 mM Tris, pH 8.0, 1 mM EDTA, and 10 mM MgCl2) was incubated in the presence or absence of AGS3-C, AGS3-C, or GDP, GPR peptides, or GST fusion Ga1 (final concentration, 10 μM). All assays were performed at 20 °C. Typically, fluorescence intensity was recorded at 30-s intervals with 10-s averaging time over a period of 1 h after mixing of the data sets were measured for each experiment- and the base-line fluorescence (intensity at time t = 0) was subtracted from the data sets. The data were averaged and smoothed using five-point adjacent averaging. The data were fit to a first order exponential association model, Y = Y1(1 – e(−kt)), where k is the rate constant (s−1) and Y1 and Y2 represent concentrations of BODIPY-FL-GTP-S bound to Ga1 at time t and at maximum saturation, respectively. Initial rates were estimated by linear approximation to the change in fluorescence intensity during the first 10 min after initiation of the exchange reaction.

Circular Dichroism Spectroscopy—CD spectra were measured with a Jasco Model J715 spectropolarimeter (Jasco Inc., Easton, MD) using a 0.05-cm path length cylindrical cell. AGS3-C was dialyzed overnight in 10 mM Tris, pH 8.0, and lyophilized GPR consensus peptide was dissolved directly in the buffer. Spectra were recorded of 8 and 16 μM AGS3-C and of 100 μM GPR consensus peptide. CD spectra were measured at 25 °C with a 1-nm spectral bandwidth, scan speed of 50 nm/min, and a response time of 1 s. Data were collected at 0.1-nm intervals, and 15 accumulations were averaged to obtain each spectrum. The spectra of AGS3-C at the two different concentrations were averaged to obtain the final spectrum. CD data were smoothed by the Savitzky-Golay method using the program provided by Jasco and k = \( k = \frac{1}{e} \) was calculated (in units of cm−1·residue−1) at 1-nm intervals. The CD spectra over the range of 250–190 nm were analyzed for fractional contents of secondary structures using CDPRO software CONTINLL (31), SELCON3 (32), and DSSR (33) with a reference set containing 43 proteins.

RESULTS

Stoichiometry and Affinity of the Binding of AGS3-C and Its Deletion Mutants to Ga1—AGS3-C (residues 465–650) contains four tandemly repeated GPR motifs, each of which has been shown in immunoprecipitation assays to be capable of binding to Ga1 (10), suggesting that a single AGS3-C domain can bind up to four molecules of the α subunit. We used gel filtration chromatography to estimate the stoichiometry of the interaction between AGS3-C and Ga1. Upon incubation together in the presence of GDP, Ga1, and AGS3-C formed a stable complex. The complex could be purified by gel filtration chromatography (Fig. 2) and eluted with an apparent molecular mass of 207 kDa, consistent with four Ga1 subunits bound to one mole of AGS3-C (molecular mass, ~180 kDa). Hence it appears that the each of the GPR motifs binds to Ga1. To further test this hypothesis, C-terminal deletion mutants of AGS3-C were created that contain the first two (AGS3-Δ34) or the first three (AGS3-Δ4) GPR motifs, respectively (see “Materials and Methods”). Both deletion mutants formed complexes with Ga1 in the presence of GDP and could be separated from their constituents by gel filtration chromatography. An esti-

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2 M. Bernard, Y. Peterson, and S. M. Lanier, personal communication.
molecular masses of the complexes by gel filtration using standards of known molecular mass. The estimated molecular masses for both of these complexes were ~20 kDa higher than the masses expected for the predicted complexes.

We then used ITC to precisely determine the stoichiometry and affinity of the interaction between AGS3-C and Go_{11}. The dependence of heat evolved upon titration of Go_{11}-GDP into a solution containing AGS3-C (Fig. 3A) yielded an overall stoichiometry of 4:3 (Go_{11}-AGS3-C), consistent with a 4:1 stoichiometry of binding. Because the amino acid sequences of the GPR repeats differ from each other, the most general model for binding of Go_{11} to AGS3-C would require four association constants. If some of the affinity constants are of similar magnitude, their individual values cannot be accurately determined from the titration data. On the other hand, binding of Go_{11} to AGS3-C was not well approximated by a model in which a single set of identical binding sites is present (Fig. 3A) such as the one set of identical sites model for AGS3-C. The titration profiles of AGS3-C (open circles), AGS3-Δ4 (open triangles), and AGS3-Δ34 (closed squares) with Go_{11} are superimposed. Nonlinear least squares fit to the “two sets of independent sites” model for AGS3-C is shown as a solid line and the “single set of identical sites” models used to fit the ITC profile of AGS3-Δ4 and AGS3-Δ34 are shown as dotted and dashed lines, respectively.

molecules of Go_{11}-GDP (Table I), respectively, which again is consistent with the gel filtration data. However, unlike the binding of AGS3-C to Go_{11}, the titration data for both of the deletion mutants was most consistent with a model in which a single set of identical binding sites is present (Fig. 3B). Although the deletion mutants share a similar binding mechanism, AGS3-Δ34 had almost 3-fold greater affinity for Go_{11} than did AGS3-Δ4. The apparent change in the Go_{11} binding

![Fig. 2](image)

**Fig. 2.** Purification of Go_{11}-AGS3 complex using gel filtration chromatography. Upper panel, Go_{11} and AGS3 were mixed at a molar ratio >4:1 (see "Materials and Methods") and separated on a tandemly connected Superdex 200/Superdex 75 gel filtration column. The profile of absorbance at 280 nm shows three distinct peaks from left to right: the AGS3-Go_{11}-GDP complex, unbound Go_{11}-GDP, and free GDP. The lower panel shows the SDS-PAGE separation of different fractions after gel filtration. The elution volumes of the fractions are indicated at the top of each lane. The 40-kDa upper band corresponds to Go_{11}, and the 20-kDa lower band corresponds to AGS3-C. The molecular masses of the proteins in each band were verified by mass spectroscopy.

![Fig. 3](image)

**Fig. 3.** ITC analysis of the binding of AGS3-C and its deletion mutants to Go_{11}. A, ITC profile for the binding of Go_{11} to AGS3-C. Aliquots (6 μl) of 0.2 mM Go_{11} were injected into an ITC cell containing 1.4 ml of 5.4 μM AGS3-C. Nonlinear least squares fit using the “two independent sets of sites” model resulted in the fit shown here. B, the titration profiles of AGS3-C (closed circles), AGS3-Δ4 (open triangles), and AGS3-Δ34 (closed squares) with Go_{11} are superimposed. Nonlinear least squares fit to the “two sets of independent sites” model for AGS3-C is shown as a solid line and the “single set of identical sites” models used to fit the ITC profile of AGS3-Δ4 and AGS3-Δ34 are shown as dotted and dashed lines, respectively.
Mechanism of AGS3-C upon deletion of one or two of its C-terminal GPR domains could reflect an alteration of the tertiary structure or structural environment of the remaining GPR repeats. Alternatively, the four repeats may differ substantially in their affinity for Go11-GDP.

The AGS3-C constructs used in these experiments inhibit the GDP exchange activity of Go11 as expected from previous studies (Fig. 4A). The apparent association rate for the binding of a fluorescent non-hydrolyzable GTP analog (BODIPY-FL-GTPγS) to Go11 was measured in the presence of AGS3-C and the two C-terminal truncation mutants. In keeping with earlier reports (14), the GDP exchange rate of Go11 was reduced 50% at 100 nM AGS3-C (Fig. 4A). Deletion of one or two GPR repeats from AGS3-C did not have a severe effect on nucleotide exchange activity. At the same concentration, AGS3-C does not result in loss of its GDI activity.

Thermodynamic Analysis of the Binding of GPR Peptides to Go11—The apparent differences in the binding models for AGS3-C and that of its truncation mutants could reflect differences in the affinities of individual GPR motifs for Go11. These might arise from sequence variation at positions other than those that are conserved and critical for function (Fig. 1A) (16).

To investigate this possibility, we sought to determine the contribution of each GPR motif to the binding of AGS3-C to Go11. Peptides corresponding to each of the four GPR motifs in AGS3-C were monitored at 510 nm for 60 min. The black curve represents BODIPY-FL-GTPγS binding in the presence of Go11 only.

**Fig. 4. Effect of AGS3-C and its truncation mutants on the kinetics of BODIPY-FL-GTPγS binding to Go11.** A, time course of BODIPY-FL-GTPγS (1 μM) binding to Go11 (200 nM) in the absence (black) or presence (red, 50 nM; green, 100 nM; blue, 500 nM; cyan, 1 μM) of AGS3-C. Reactions were initiated by addition of preincubated AGS3-Go11 complex to a cuvette containing BODIPY-FL-GTPγS, and fluorescence was monitored at 510 nm for 60 min. B, comparison of the time course of inhibition of BODIPY-FL-GTPγS binding to Go11 (200 nM) by 100 nM AGS3-C (red), 100 nM AGS3-Δ4 (green), and 100 nM AGS3-Δ34 (blue). The black curve represents BODIPY-FL-GTPγS binding in the presence of Go11 only.

**Fig. 5. ITC profile showing the titration of Go11 with GPR4.** Aliquots (10 μl) of 0.5 mM GPR4 were injected into an ITC cell containing 1.4 ml of 45 μM Go11. Nonlinear least squares fit using the "one set of identical sites" model yielded a dissociation constant (Kd) of 1.9 μM, enthalpy (ΔH) of −6.11 kcal/mol, and stoichiometry (N) of 1.09.

| AGS3   | N   | Kd  | ΔH   | ΔG  | ΔS  |
|--------|-----|-----|------|-----|-----|
| Strong sites | 2.13 ± 0.11 | 19 ± 8 | −8.13 ± 0.06 | −10.30 | 7.41 |
| Weak sites  | 2.21 ± 0.12 | 326 ± 10 | −8.69 ± 0.16 | −8.67 | −3.48 |
| AGS3-Δ4 | 3.07 ± 0.01 | 384 ± 10 | −8.96 ± 0.02 | −8.57 | 5.49 |
| AGS3-Δ34 | 2.03 ± 0.01 | 137 ± 8  | −9.98 ± 0.04 | −9.17 | −2.76 |

Thermodynamic parameters of the binding of AGS3-C and its deletion mutants to Go11-GDP

**Table I**

Thermodynamic parameters for the binding of AGS3-C and its deletion mutants with Go11 were determined using ITC at 20 °C in 20 mM Tris, pH 8.0, 2 mM DTT, and 10 μM GDP. A binding model that assumes two sets of independent sites described the titration data for AGS3-C. The two different types of sites are referred to as strong sites and weak sites in the table. Titration data for AGS3-Δ4 and AGS3-Δ34 were fit to a single set of identical sites model. Kd, ΔH, ΔG, ΔS, and N represent the dissociation constant, enthalpy, free energy, entropy, and stoichiometry, respectively, and the units corresponding to these parameters are shown in the table.

**Table I**

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AGS3 were synthesized, and the binding of these peptides to Go11 was analyzed by ITC (Fig. 5). The interaction of the previously described GPR consensus peptide (11) with Go11 was also analyzed. The titration data for the GPR peptides are best described by a single binding site model. Each of the peptides bound to Go11 with a stoichiometry of 1:1. The dissociation constant (Kd) for the peptides ranged from 1–8 μM, and the enthalpy of binding ranged from −3.5 to −9.8 kcal/mol (Table II). The differences in the enthalpy changes were balanced by entropic terms such that binding free energies of individual peptides differed by no more than 1.2 kcal/mol (Table II). The affinity of the consensus peptide for Go11 was somewhat higher than that of the four GPR peptides. The average free energy of binding for the four GPR peptides was −7.3 kcal/mol, which is significantly less in absolute value than the average free energy of binding per site for AGS3-C (−10.3 kcal/mol) and for AGS3Δ4 and AGS3Δ34 (−8.6 and −9.2 kcal/mol, respectively). Since sequence variation among the AGS3-C GPR motifs appears to have little effect on their affinity for Go11, it is possible that residues outside of the GPR motif are involved in binding or that the three-dimensional structure of AGS3-C contributes to the stability of the complex.

**GDI Activity of GPR Peptides**—To investigate the possibility that GPR peptides differ in GDI activity, the nucleotide exchange rate of Go11 was determined in the presence of each peptide. Peptides were present in the assay at a concentration of 10 μM, which exceeds the Kd of GPR1, which has the lowest affinity of the four for Go11. Under these conditions, all the GPR peptides with the exception of GPR3 demonstrated nucleotide exchange inhibitory activity (Fig. 6A). Relative GDI activity of the peptides followed the rank order: GPR consensus > GPR1 > GPR2 > GPR4 > GPR3. GPR consensus, GPR1, GPR2, GPR3, GPR4, and GPR5 were expressed as N-terminally GST-tagged fusion proteins. The proteins were purified to near homogeneity by glutathione affinity chromatography and used without further purification. Because GST-GPR4ex was rapidly degraded despite the presence of protease inhibitors, it was not tested for Go11 binding or GDI activity. GST-GPR3ex was a potent GDI toward Go11, which is consistent with its remaining activity in the absence of protease inhibitors.

**Affinity and GDI Activity of Extended GPR Peptides**—In light of the observation that residues extending C-terminal to the GoLoco motif of RGS14 contribute to Ga specificity and GDI activity (26), we investigated the effect of residues outside of the GPR motifs of AGS3. The extended peptides (Fig. 1B) were expressed as N-terminally GST-tagged fusion proteins. The proteins were purified to near homogeneity by glutathione affinity chromatography and used without further purification. Because GST-GPR4ex was rapidly degraded despite the presence of protease inhibitors, it was not tested for Go11 binding or GDI activity. GST-GPR3ex was a potent GDI toward Go11, which is consistent with its remaining activity in the absence of protease inhibitors.

**Table II**

| Peptide       | N  | Kd, μM | ΔH, kcal mol⁻¹ | ΔG, kcal mol⁻¹ | ΔS, cal mol⁻¹ K⁻¹ |
|---------------|----|--------|----------------|----------------|------------------|
| GPR1          | 1.25 ± 0.01 | 8.18 ± 0.57 | −9.87 ± 0.17 | −8.80 | −10.49 | 12.82 |
| GPR2          | 1.05 ± 0.02 | 3.29 ± 0.19 | −3.57 ± 0.09 | −7.33 | −5.21 | 5.21 |
| GPR3          | 1.02 ± 0.01 | 2.98 ± 0.13 | −5.12 ± 0.04 | −7.38 | 7.22 | 7.22 |
| GPR4          | 1.09 ± 0.01 | 1.90 ± 0.08 | −6.11 ± 0.03 | −7.65 | 5.21 | 5.21 |
| GPR consensus | 1.13 ± 0.01 | 1.02 ± 0.14 | −7.32 ± 0.10 | −8.01 | 2.39 | 2.39 |
| GPR1ex        | 0.74 ± 0.01 | 251 ± 5 | −6.62 ± 0.02 | −8.82 | 0.66 | 0.66 |
| GPR2ex        | 0.88 ± 0.01 | 65 ± 4 | −4.49 ± 0.02 | −9.58 | 17.37 | 17.37 |
| GPR3ex        | 0.98 ± 0.01 | 268 ± 7 | −10.34 ± 0.02 | −8.78 | −5.32 | −5.32 |

**Fig. 6. Inhibition of BODIPY-FL-GTPγS binding to Go11 by GPR peptides derived from AGS3.** A. Go11 (200 nM) was incubated with 10 μM GPR1 (red), GPR2 (green), GPR3 (blue), GPR4 (cyan), and GPR consensus (magenta) for 10 min. Fluorescence intensity was monitored at 510 nm. The black curve represents the binding of BODIPY-FL-GTPγS with Go11 in the absence of any peptide. B, histogram showing the GDI activity of different peptides at 10 μM concentration. Fractional exchange at any time point was calculated as a ratio of fluorescence intensity of the sample with peptide to that without peptide. Percentage of inhibition = (1 – fractional exchange) × 100. The peptides can be arranged in descending order of activity as GPR consensus > GPR1 > GPR2 > GPR4 > GPR3, GPR consensus, GPR1, GPR2, GPR3, GPR4, and GPR5.
were mixed with 1/H9262 also bound with 10 that for the core GPR peptides. The extended GPR peptides BODIPY-FL-GTP order with respect to G greater than that of GPR3. For the extended peptides, the rank in contrast, the binding enthalpy of GPR3ex was substantially was also marked by a large increase in the entropy of binding. The greater affinity of GPR1ex relative to GPR1 did the smaller peptides that contain only few (approximately three) flanking residues in addition to the core GoLoco consensus motif (Table II). Binding constants for these extended peptides were within the range measured for AGS3 itself. Interestingly, much like GPR2, which had the lowest enthalpy of binding among the GPR peptides, GPR2ex also had a low enthalpy of binding, and binding was to a great extent driven by entropy. The greater affinity of GPR1ex relative to GPR1 was also marked by a large increase in the entropy of binding. In contrast, the binding enthalpy of GPR3ex was substantially greater than that of GPR3. For the extended peptides, the rank order with respect to Gαi1 affinity followed that of GDI activity. GST itself had no affinity or GDI activity toward Gαi1 (data not shown).

Solution Structure of AGS3 and a GPR Peptide—To compare the content of secondary structure in GPR peptides and AGS3, CD spectra were obtained for the GPR consensus peptide and AGS3-C in the wavelength range of 190–250 nm. Analysis of the CD spectra (Fig. 8) indicated higher α-helical content in AGS3-C (20.4% or ∼38 residues of 186 residues) than in the GPR consensus peptide (8% or ∼2 residues of 28 residues), suggesting that AGS3-C could have native helical structures that are in the proper conformation to interact with Gαi1. The analysis also suggested that AGS3-C and the GPR peptide have approximately equal fractions of turn (∼25%) and disordered structure (∼33%), but the latter has a higher content of β-strand structure (33.5%) than AGS3-C (22.2%).

Discussion

The residues that confer GDI activity in GPR/GoLoco motifs have been defined by mutagenesis (16), and the structure of a GoLoco repeat bound to Gαi1-GDP provides insight into its mechanism of action (20). Several proteins such as AGS3 possess multiple GPR repeats, and therefore the question arises how these repeats function biochemically as an ensemble.

Using isothermal titration calorimetry and gel filtration analysis, we demonstrated that AGS3-C could bind up to four molecules of Gαi1, equal to the number of GPR motifs present in its amino acid sequence. Successive deletion of GPR motifs from the C terminus of AGS3-C did not abrogate the Gαi1 binding activity of the remaining motifs. Hence GPR repeats can function independently within the context of the AGS3-C scaffold. Indeed the naturally occurring splice variant of AGS3 known as AGS3-SHORT contains three complete GPR motifs and is known to be a functionally active molecule (24). Nevertheless the ITC data for AGS3-C and its truncation mutants suggest that the structural context of the GPR repeats within AGS3-C may affect their affinity for Gαi1. Surprisingly AGS3-C and its truncation mutants had nearly equal potency as GDI when tested at 100 nM concentration, which is close to their average Kd as determined by ITC.

Although all conformed to the GPR consensus sequence, the four GPR repeats otherwise differed in sequence from each other, and therefore AGS3-C must have four non-equivalent binding sites. These sites could function independently or with some degree of cooperativity. The experimental errors inherent in the ITC measurements do not allow thermodynamic parameters to be extracted for each binding site in the most general four-site model. However, the binding data clearly did not fit the simplest model in which four identical, independent sites are assumed. Although the titration data could be fit to a more general sequential four-site binding model (eight free parameters), the uncertainties associated with derived constants did not justify a model of this complexity, and a simple two-site model (six free parameters) gave an adequate fit to the binding data. From this latter analysis, two high affinity (Kd ~ 20 nM) and two low affinity (Kd ~ 300 nM) binding sites for Gαi1 could be derived. However, this binding model cannot be considered a complete or quantitative thermodynamic description of the interaction between AGS3-C and Gαi1. The key observation is that the four binding sites in the context of AGS3-C were not equivalent even though the GPR peptides bound to Gαi1 with approximately equal affinity (see below). Successive deletion of the two C-terminal GPR motifs from AGS3 destroyed the biphase character of Gαi1 binding. The truncated constructs bound Gαi1 with lower affinity, and binding could be defined by
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a single association constant. The apparent change in binding mechanism upon truncation may reflect the loss of tertiary structure that is present in AGS3-C as well as the loss of specific Gol1-binding elements.

The GPR motif of RGS14 adopts a helical conformation while bound to Gol1 (26). However, CD and 1H NMR analysis (data not shown) of the consensus GPR peptide showed little evidence of α-helical structure. The CD spectrum of AGS3-C indicated that a modest fraction of the molecule is helical, so that it is possible that, within the context of AGS3-C, the GPR motifs adopt the secondary structure consistent with the requirements of the Gol1 binding site. This may account for some small fraction of the 2.5 kcal/mol (per binding site) increase in binding energy for GPR repeats in AGS3-C relative to that for individual GPR peptides.

It was evident that the sequences that flank the GPR consensus motif include residues that contribute substantially to Gol1 binding energy. These additional residues augmented GDI activity as well, and for GPR3, they were required. The affinity of any one of the extended GPR peptides for Gol1 was comparable to that of the high or low affinity sites in AGS3-C. Even the GPR consensus peptide, which is longer by a few residues at both termini than that of the other four GPR peptides, had a greater free energy of binding to Gol1. The dissociation constants derived for the binding of AGS3-C to Gol1 may therefore simply represent the distribution of affinities of the individual extended GPR motifs. Nevertheless the possibility that the tertiary organization of the Gol1-binding motifs in AGS3-C influences their affinity for Gol1 cannot be dismissed. That AGS3-A34 bound to Gol1 more strongly than did AGS3-A3 suggests steric interference between sites in the latter, which may be relieved by truncation of the third motif. More rigorous structural and thermodynamic analysis of the AGS3-C-Gol1 binding interaction will be required to address these questions in depth.

While it is clear that residues outside of the GoLoco motif contribute binding energy toward Gol1, it is not apparent whether the N- or C-terminal residues are equally important. The structure of the complex between Gol1 and the GoLoco motif of RGS14 reveals a substantial interface between the helical domain of Gol1 and the residues that extend from the C-terminus of the GoLoco motif (26). In contrast, the N-terminal boundary of the GoLoco motif almost extends beyond the surface of the Ras-like domain of Gol1. Hence it is probable that it is the C-terminal flanking residues of GPRex peptides that contribute the additional binding energy toward Gol1. It is remarkable that these residues are not conserved among the four GPR repeats in AGS3-C (Fig. 1B). Again the structures of the complexes between extended peptides and Gol1 should reveal the structural basis for their contribution to binding.

Residues that flank the GPR/GoLoco motif not only confer specificity, as demonstrated for RGS14 (26), but may be critical for GDI activity as our data indicated for GPR3 of AGS3-C. Apparently the binding mode of GPR3 is nonproductive for GDI activity (26), which has been shown to be important for GDI activity (16), and 3) occurrence of a pair of proline residues immediately following the catalytic arginine that binds the β-phosphate of GDP in the Gol1-GDP complex (26). Whether these or other factors account for the inactivity of GPR3, residues that flank this motif are compensatory to the extent that GPR3ex exhibits potent GDI activity.

Several protein families have evolved multiple GoLoco motifs, although only a single GoLoco motif is necessary and sufficient for biochemical activity. Molecules such as AGS3 could act as scaffolding molecules (24, 35) to bring several Gα subunits together and thereby enhance the efficiency of signaling. The strong sites in AGS3-C differ from the weak sites by 1.5 kcal/mol in binding free energy for Gol1 (15-fold difference in Kd) suggesting that a nearly linear response in GDI activity is possible over a −10−500 nM range in effective Gol1 concentration. The broad dynamic range of AGS3-C might play a critical role in the mechanism by which it inhibits Gol1 signaling in vivo.

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