Influence of Cytosolic AGS3 on Receptor−G Protein Coupling†

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ABSTRACT: Activator of G protein signaling 3 (AGS3) activates the G$\beta\gamma$ mating pathway in yeast in a manner that is independent of heptahelical receptors. It competes with G$\beta\gamma$ subunits to bind GDP-bound Gi/oα subunits via four repeated G protein regulatory (GPR) domains in the carboxyl-terminal half of the molecule. However, little is known about the functional role of AGS3 in cellular signaling. Here the effect of AGS3 on receptor−G protein coupling was examined in an Sf9 cell membrane-based reconstitution system. A GST−AGS3-GPR fusion protein containing the four individual AGS3-GPR domains inhibits receptor coupling to Gα subunits as effectively as native AGS3 and more effectively than GST fusion proteins containing the individual AGS3-GPR domains. While none of the GPR domains distinguished among the three Giα subunits, both individual and full-length GPR domains interacted more weakly with Goα than with Giα. Cytosolic AGS3, but not membrane-associated AGS3, can interact with Giα subunits and disrupt their receptor coupling. Immunoblotting studies reveal that cytosolic AGS3 can remove Giα subunits from the membrane and sequester Giα subunits in the cytosol. These findings suggest that AGS3 may downregulate heterotrimeric G protein signaling by interfering with receptor coupling.

Heterotrimeric G proteins, located on the cytoplasmic face of the plasma membrane, are widely used to transduce extracellular signals through heptahelical GPCRs[1] to intracellular signaling networks. According to the widely accepted ternary complex model (*1*−*3*), agonist-occupied GPCRs stimulate the release of GDP and binding of GTP to the Gα subunit of a heterotrimer. The conformational changes associated with GTP binding dissociate the ternary complex, and both the GTP-bound Gα subunit and G$\beta\gamma$ dimer are then free to modulate downstream effectors, including enzymes and ion channels. This signaling is in part terminated by GTP hydrolysis and subunit reassociation. A diverse array of proteins distinct from GPCRs, G proteins, and effectors are now known to regulate this signaling process at multiple levels (*4*−*7*). Among the newest family of accessory proteins are the activators of G protein signaling, or AGS proteins (*8*, *9*). A member of this family, AGS3, belongs to a class of proteins containing G protein regulatory (GPR) motifs (*9*), also known as GoLoco motifs (*10*). AGS3 has four repeated GPR sequences in its carboxyl-terminal half and seven tetratricopeptide repeat (TPR) sequences in its amino-terminal half. AGS3, via its GPR domains, selectively interacts with the GDP-bound conformation of α-subunits of the Gi/o family (*9*, *11*−*15*) and effectively competes with G$\beta\gamma$ subunits for binding to Gα (*13*).

Studies in *Drosophila melanogaster*, *Caenorhabditis elegans*, and several cultured cell lines indicate that GPR-containing proteins related to AGS3 are involved in cell polarity and cell division (*16*−*20*), but little is known about the influence of AGS3 and related proteins on signal processing by GPCRs. Since AGS3 can potentially disrupt G protein heterotrimers and stabilize the GDP-bound conformation of Gα subunits, we hypothesized that AGS3 might interfere with receptor−G protein coupling. To examine the effect of AGS3 on receptor−G protein coupling, we used an Sf9 cell membrane-based reconstitution system. Relatively few GPCRs expressed in Sf9 cells are coupled to endogenous G proteins (*21*−*24*). Reconstitution of the expressed receptors with appropriate, purified, exogenous G proteins couples the majority of the expressed receptors. This functional receptor−G protein coupling is readily detected as an enhanced level of agonist binding using a radiolabeled agonist at concentrations near the high-affinity K_D of the receptor (*21*, *24*). The abilities of cytosolic AGS3, membrane-associated AGS3, and individual GPR domains to interfere with receptor−G protein coupling were assessed in reconstitution assays.

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[1] Abbreviations: 5-HT, 5-hydroxytryptamine; AGS3, activator of G protein signaling 3; CHAPS, 3-[(3-cholamidopropyl)dimethylammonil]-1-propanesulfonic acid; GDI, guanine nucleotide dissociation inhibitor; GoLoco motif, Gi/o-Loco interaction motif; GPCRs, G protein-coupled receptors; GPR, G protein regulatory; GST, glutathione *S*-transferase; MOI, multiplicity of infection; Pins, partner of inscuteable; PVDF, polyvinylidene fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Sf9, *Spodoptera frugiperda*; TPR, tetratricopeptide repeat.

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In this study, we report that cytosolic AGS3, but not membrane-associated AGS3, interferes with 5-HT_{1A} receptor−Gi/o protein coupling. In interactions with Gi/oα subunits, the GPR domain alone is as effective as the native AGS3 protein. Although all four individual AGS3-GPR domains bind Giα more tightly than Goα, they do not distinguish among the three Giα subunits. Taken together, our results demonstrate that AGS3 affects receptor−G protein coupling by dissociating Gα subunits from the membrane and sequestering Gα subunits in the cytosol.

EXPERIMENTAL PROCEDURES

Expression and Purification of Proteins. Recombinant $Gi_{1,2,3}\alpha$ and Goα and $G\beta_1\gamma_2$ subunits were purified after expression in Sf9 cells using recombinant baculoviruses as described previously (*25*, *26*). Cytosolic AGS3 was prepared as a 100000*g* extract from Sf9 cells infected at an MOI of 2 with recombinant baculoviruses encoding AGS3 (*9*). The infected Sf9 cells were then cultured, harvested, and fractionated as previously described (*21*) except that harvested cells were thawed in 5 times (rather than 15 times) their wet weight of ice-cold homogenization buffer [10 mM Tris-HCl (pH 8.0, 4 °C), 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 20 μg/mL benzamidine, and aprotinin, leupeptin, and pepstatin A (2 μg/mL each)]. The 100000*g* crude cytosol was concentrated to a final protein concentration of 5−7 mg/mL in a pressurized Amicon cell using a YM-30 membrane. Control cytosol was prepared from uninfected Sf9 cells in the same fashion. The GST−AGS3 fusion proteins containing all four AGS3-GPR domains, GST−AGS3-GPR (Pro^{463}−Ser^{650}), or individual AGS3-GPR domains, GST−AGS3-GPR-I (Pro^{463}−Glu^{501}), GST−AGS3-GPR-II (Ser^{516}−Leu^{555}), GST−AGS3-GPR-III (Gly^{563}−Thr^{602}), and GST−AGS3-GPR-IV (Thr^{602}−Ser^{650}), were expressed and purified as previously described (*13*). Protein concentrations were determined with the bicinchonic acid method (Pierce Chemicals).

Preparation of Sf9 Cell Membranes Containing Expressed 5-HT_{1A} Receptors. To prepare membranes containing 5-HT_{1A} receptors, Sf9 cells were infected with recombinant baculoviruses expressing the 5-HT_{1A} receptor at an MOI of 2. To prepare membranes containing both 5-HT_{1A} receptors and AGS3, Sf9 cells were co-infected with recombinant baculoviruses expressing the 5-HT_{1A} receptor and AGS3 at MOIs of 1 and 3, respectively. Sf9 cells were cultured and harvested; membranes were prepared and analyzed to determine 5-HT_{1A} receptor numbers as described previously (*21*) except that membranes coexpressing AGS3 were washed once rather than thrice during preparation.

Reconstitution of Sf9 Cell Membranes with Purified G Proteins. Reconstitution was performed as previously described (*21*). Briefly, 50 μg of membrane protein and 1−2 μL containing the desired amount of G protein heterotrimer were resuspended in reconstitution buffer with 0.04% CHAPS (Calbiochem) prior to the addition of the indicated additional components. The mixtures were incubated at 25 °C for 15 min and then held on ice until the start of the binding assay, or pelleted in a refrigerated microcentrifuge at 12 000 rpm and 4 °C for 10 min for the immunoblotting assay.

To allow soluble, cytosolic AGS3 or exogenous G protein heterotrimers to associate with membranes, membranes containing 5-HT_{1A} receptors were first incubated (25 °C for 15 min) with cytosol containing AGS3 or purified G protein heterotrimers, respectively. The membranes were then pelleted in a refrigerated microcentrifuge at 12 000 rpm and 4 °C for 10 min and washed thrice with 100 μL of reconstitution buffer. The resulting membranes were then used in a second reconstitution with the indicated additional components as described above.

[^{3}H]-5-HT Binding Assay. Just prior to the start of the binding assay, the reconstitution mixture was diluted 10-fold with binding assay buffer [50 mM Tris-HCl, 5 mM $MgCl_2$, and 0.5 mM EDTA (pH 7.5)] such that the desired amount of membranes (∼15 μg/assay tube) was contained in 50−100 μL. High-affinity agonist binding was assessed with 1−2 nM [^{3}H]-5-HT (25.5 Ci/mmol, Perkin-Elmer), which is near the high-affinity K_D of the 5-HT_{1A} receptor, in a final volume of 150−300 μL. Nonspecific binding was assessed in the presence of a 1 million-fold excess of unlabeled 5-HT. Incubation was performed at 25 °C for 1.5 h in a temperature-controlled shaker and terminated by filtration over Whatman GF/C filters (Brandel Inc.) using a Brandel cell harvester (Brandel Inc.). The filters were rinsed thrice with 4 mL of ice-cold washing buffer [50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM EDTA, and 0.01% sodium azide (pH 7.5)], placed in 4.5 mL of CytoScint (ICN Pharmaceuticals), and counted to constant error in a scintillation counter. The binding data were analyzed using GraphPad PRISM (GraphPad Software).

Preparation of Rat Brain Membranes. One rat brain was homogenized on ice in a Dounce glass homogenizer with 40 mL of lysis buffer [5 mM Tris (pH 7.4), 5 mM EDTA, 5 mM EGTA, and protease inhibitors] for eight strokes. The homogenate was centrifuged at 1000*g* for 10 min to remove debris. The resulting supernatant was centrifuged at 40000*g* for 30 min at 4 °C. The resulting pellet was homogenized (in 40 mL) and centrifuged (at 40000*g*) four times: twice in high-salt buffer [50 mM Tris (pH 7.4), 0.6 mM EDTA, 5 mM $MgCl_2$, and 500 mM NaCl] and twice in no-salt buffer [50 mM Tris (pH 7.4) and 0.6 mM EDTA]. The resulting pellet was resuspended in 1 mL of no-salt buffer using a glass homogenizer.

Immunoblot Analysis. To determine the amount of AGS3 in membrane or cytosolic fractions, 0.5 μg of protein was resolved by 12% SDS−PAGE and transferred to a PVDF membrane (Millipore). The membrane was probed with AGS3 antisera P-32 (*13*) and visualized with the ProtoBlot AP immunoblotting system (Promega). The levels of AGS3 were estimated by comparing the band intensities with known amounts of purified GST−AGS3-GPR (Pro^{463}−Ser^{650}) fusion protein standard using the FluorChem 8000 system (Alpha Innotech Corp.). To determine the amounts of protein that associated with membranes during reconstitution, the reconstitution mixtures were pelleted and washed three times as described above. The washed pellets or $^1/_{10}$ volume of the first supernatant was electrophoresed, transferred to PVDF membranes, and probed with anti-$G\alpha_{i1/2}$ (Calbiochem), anti-Gβ antibody (NEN Products), and AGS3 antisera P-32 as indicated in the text. The antibody detection and visualization were performed with a ProtoBlot AP kit as described above. Band intensities were analyzed with the FluorChem 8000 system (Alpha Innotech Corp.) and quantified within the linear range of a standard curve prepared from known

quantities of Gi1α or GST−AGS3-GPR (Pro^{463}−Ser^{650}) fusion protein.

To perform the fractionation experiments depicted in Figure 5, rat brain membranes were incubated with homogenization buffer [10 mM Tris-HCl (pH 8.0, 4 °C), 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 20 μg/mL benzamidine, and aprotinin, leupeptin, and pepstatin A (2 μg/mL each)], control cytosol, or AGS3-transfected cytosol for 30 min at room temperature, rotating. The incubation mixture was then centrifuged at 100000*g* for 30 min at 4 °C. The resulting supernatant was termed the 100000*g* supernatant (S). The remaining pellet was resuspended in no-salt buffer, centrifuged at 12 000 rpm in a microcentrifuge for 30 min, and designated (P). Samples were then mixed with Laemmlli buffer and boiled prior to being loaded on 10% SDS−PAGE gels. Proteins were transferred to PVDF, immunoblotted with Gα specific anitsera 976, and visualized with ECL reagents (PerkinElmer Life Sciences, Inc., Boston, MA).

RESULTS

Membrane-Expressed AGS3 Does Not Interfere with Receptor−Gi Protein Coupling. Protein−protein interaction studies indicate that AGS3 can bind GDP-containing Giα subunits (*9*, *13*), and thus, membrane-localized AGS3 might disrupt G protein heterotrimers by interactions with Giα and thereby interfere with their coupling to receptors. To test this possibility, membranes were prepared from Sf9 cells expressing 5-HT_{1A} receptors alone or coexpressing AGS3. In both crude membrane preparations, 5-HT_{1A} receptors were expressed at a level of 3.5−5.5 pmol/mg of membrane protein as determined by saturation radioligand binding. In preparations coexpressing AGS3, AGS3 was present in the membranes at a 518-fold molar excess over 5-HT_{1A} receptors as quantitated by immunoblotting.

Membrane preparations with and without coexpressed AGS3 were reconstituted with increasing amounts of Gi1 heterotrimers to stabilize the high-affinity agonist binding state of the receptors. As shown in Figure 1A, the high-affinity [^{3}H]-5-HT binding to both membranes reached the maximum level at a Gi1 concentration of ∼85 nM. Interestingly, there was no right-ward curve shift observed for the membranes with coexpressed AGS3 even though it was present in a 1.2-fold molar excess over the highest concentration of G protein used. The EC_{50} values for Gi1 were not significantly different between the two membrane preparations, 6.07 ± 0.58 nM for the membranes expressing 5-HT_{1A} receptors alone and 6.53 ± 1.44 nM for the membranes with coexpressed AGS3 ($p > 0.05$, Student's *t* test). These results suggest that the membrane-expressed AGS3 does not interfere with receptor−Gi protein coupling. Additional evidence is shown in Figure 1B. Reconstitution with Gi1 did not alter the amount of AGS3 in the membrane fraction (panel B, lane 7 vs lane 8, $p > 0.05$, one-sample *t* test, $n = 3$), and equivalent amounts of Gi1 associated with membranes expressing or not expressing AGS3 (panel B, lane 4 vs lane 8, $p > 0.05$, one-sample *t* test, $n = 3$). Taken together, these results indicate that even when present in excess over receptors and G proteins, membrane-expressed AGS3 does not interfere with receptor−Gi1 protein coupling.

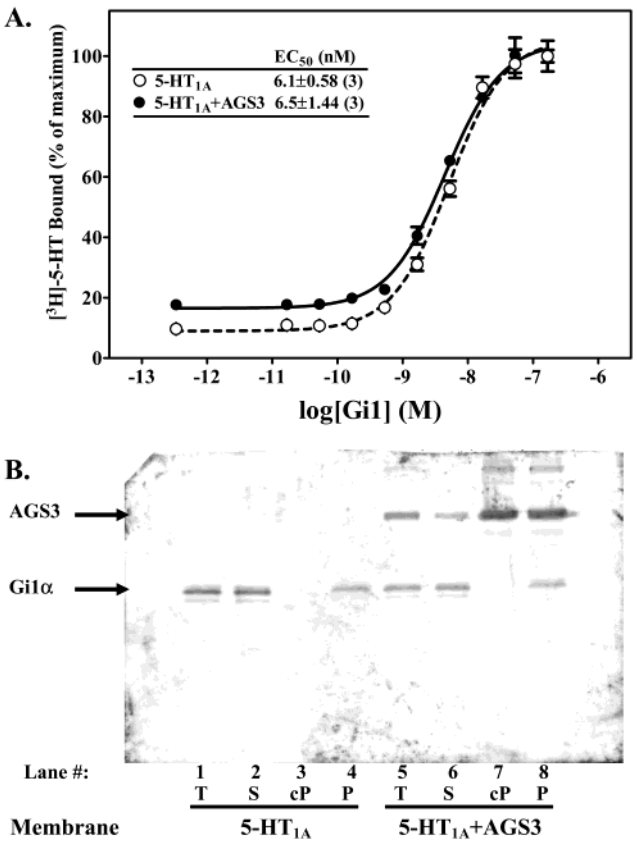

FIGURE 1: Effect of membrane-expressed AGS3 on receptor−G protein coupling and association of Gi1α with the membrane. (A) Sf9 cell membranes expressing 5-HT_{1A} receptors (110 fmol) or coexpressing AGS3 (110 fmol of receptor, 57 pmol of AGS3) were reconstituted with increasing concentrations of Gi1 heterotrimers (2−42 pmol) in 17 μL. Following dilution into binding buffer, high-affinity agonist binding was assessed with 1 nM [^{3}H]-5-HT and normalized as the percent of maximal binding achieved with a saturating amount of Gi1 (85 nM). Final conditions in the binding assay were 0.2 nM receptor with or without 104 nM AGS3 and the indicated concentrations of Gi1 in 150 μL. Data are means ± standard error of the mean from three independent experiments. The EC_{50} values for Gi1 were not significantly different for membranes expressing 5-HT_{1A} receptors or coexpressing AGS3 ($p > 0.05$, Student's *t* test). (B) Sf9 cell membranes (50 μg, ∼225 fmol of receptor) expressing 5-HT_{1A} receptors (lanes 1−4) or coexpressing AGS3 (lanes 5−8) were reconstituted with 50 pmol of Gi1 heterotrimers in a volume of 20 μL. The amounts of Gi1α and AGS3 associated with the soluble and membrane fractions were estimated by immunoblotting. For each membrane condition, the lanes contained the following: T, 10% of the reconstitution before pelleting; S, 10% of the supernatant after pelleting in a refrigerated microcentrifuge; cP, entire membrane pellet (after washing three times) from control membranes without reconstitution with Gi1; P, entire membrane pellet (after washing three times) from membranes reconstituted with Gi1. An estimated 2.5 pmol of Gi1α and 90 pmol of AGS3 remained associated with the membranes as determined by densitometry in comparison with standards. In three separate experiments, there was no significant difference in either the amount of AGS3 associated with the membrane in the presence or absence of Gi1 or the amount of Gi1α associated with the membrane in the presence or absence of AGS3.

Cytosolic AGS3 Interferes with Receptor−Gi Protein Coupling by Sequestering Giα and Blocking Its Membrane Association during Reconstitution. Although a subpopulation is found loosely associated with a membrane fraction, AGS3 is primarily localized in the 100000*g* supernatant of native tissue lysates and various transfected cell lines (*13*, *15*, *20*).

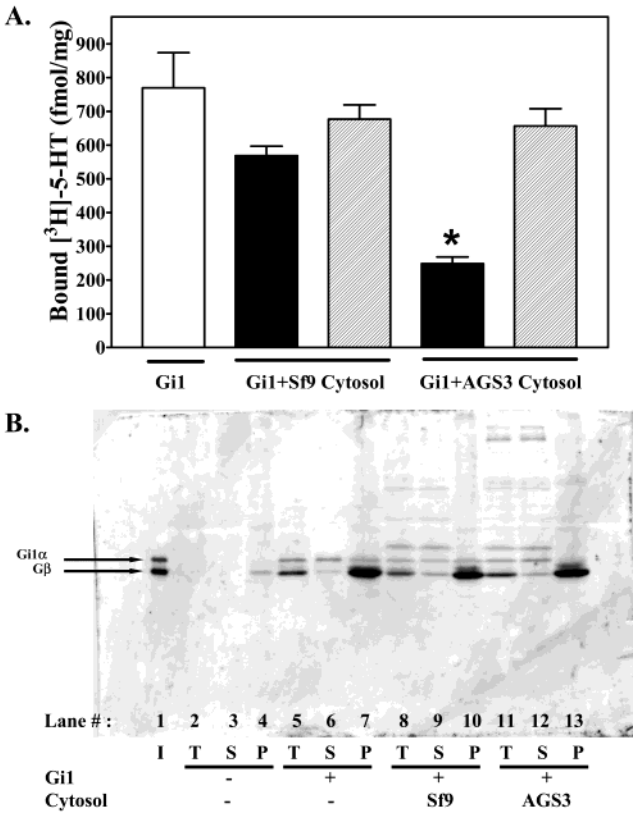

FIGURE 2: Effect of cytosolic AGS3 on receptor−G protein coupling and association of Gi1 with the membrane. (A) Sf9 cell membranes expressing 5-HT_{1A} receptors (655 fmol) were reconstituted with 2.75 pmol of Gi1 heterotrimers and 71 μg of crude cytosol from Sf9 cells expressing AGS3 (23 pmol) or from uninfected Sf9 cells in 32 μL. Following dilution into binding buffer, high-affinity agonist binding was assessed with 1 nM [^{3}H]-5-HT. Final conditions in the binding assay were 1 nM receptor and 4.2 nM Gi1 with or without 35 nM AGS3 in 200 μL. The level of binding to membranes in the absence of exogenous G proteins was 360 ± 8 fmol/mg in the experiment depicted (data not shown). The white bar represents control binding to membranes reconstituted with Gi1 without either Sf9 cell cytosol. The black bars represent binding to membranes reconstituted with Gi1 in the presence of the indicated cytosol fractions. The striped bars represent data for cytosol fractions that were boiled prior to reconstitution. Data are means ± standard deviation of triplicate determinations from a representative experiment that was repeated three times with similar results. Only the nonboiled AGS3 cytosol was different from the Gi1 control (asterisk, $p < 0.01$, Tukey's test). (B) Membranes expressing 5-HT_{1A} receptors were reconstituted as described for panel A with the indicated components. The number of G protein subunits associated with the soluble and membrane fractions was estimated by immunoblotting. The lane labeled I contained 1 pmol of Gi1 heterotrimer (40 ng of Gi1α and 36 ng of Gβ1) as a standard. For each reconstitution, the lanes contained the following: T, 10% of the reconstitution before pelleting; S, 10% of the supernatant after pelleting in a refrigerated microcentrifuge; P, entire membrane pellet (after washing three times). Immunoblots were repeated three times with similar results.

To determine if cytosolic AGS3 interferes with receptor−Gi protein coupling, a 100000*g* crude cytosol preparation was obtained from Sf9 cells infected with a recombinant baculovirus expressing AGS3. As a control, a similar cytosol preparation was obtained from uninfected Sf9 cells. As shown in Figure 2A, the cytosol preparation containing AGS3 significantly inhibited 5-HT_{1A} receptor−Gi1 protein coupling but lost its activity after being heated at 99 °C for 5 min. In contrast, the control cytosol preparation, reconstituted at the same total protein concentration, did not alter agonist binding significantly, demonstrating the specificity of the AGS3 effect.

Cytosolic AGS3 may interfere with receptor−G protein coupling by inhibiting the membrane association of heterotrimeric G proteins or by stabilizing a conformation of Giα-GDP that is not capable of interacting with receptors. We addressed this issue by first determining the effect of cytosolic AGS3 on the membrane association of Gi1α and G$\beta\gamma$ subunits in immunoblotting experiments. Cytosolic AGS3 blocks the membrane association of Gi1α subunits, but has no effect on the membrane association of G$\beta\gamma$ subunits (Figure 2B, lane 10 vs lane 13). These data indicate that cytosolic AGS3 sequesters Gi1α subunits during reconstitution and prevents their association with the membrane, thus interfering with receptor coupling to G proteins.

Cytosolic AGS3 Associates with the Membrane and Loses Its Ability To Interfere with Receptor−G Protein Coupling. A portion of the cytosolic AGS3 associates with Sf9 cell membranes in a concentration-dependent manner regardless of the presence of receptors in the membrane (data not shown). Although the nature of this association is not understood, to rule out the possibility that the inactivity of the membrane-associated AGS3 expressed in Sf9 cells shown in Figure 1 was due to denaturation or aggregation, it was important to know if the cytosolic AGS3 retains its ability to interfere with receptor−G protein coupling following membrane association. To accomplish this, membranes expressing 5-HT_{1A} receptors were first preincubated with or without sufficient cytosol containing AGS3 to allow an excess of AGS3 over receptors to associate with the membrane. After a thorough washing, the membranes were reconstituted with or without Gi1 heterotrimers and receptor coupling was assessed by assessing high-affinity agonist binding.

As shown in Figure 3A, equivalent amounts of AGS3 remained associated with the membranes following the preincubation whether the membranes were subsequently reconstituted with Gi1 (compare AGS3 band in lanes 5 and 6). Figure 3A also shows that the preincubation with the AGS3-containing cytosol did not alter ($p > 0.05$, one-sample *t* test, $n = 3$) the amount of Gi1α that associated with the membranes during reconstitution (compare the Gi1α band in lanes 4 and 6). Quantitation of the immunoblots indicates that the AGS3 that remained associated with the membranes was in 29-fold molar excess over receptors and 69-fold molar excess over reconstituted Gi1α. As shown in Figure 3B, the partition of cytosolic AGS3 to the membrane did not alter high-affinity agonist binding to the receptors following reconstitution with Gi1. Taken together, the results shown in Figures 2 and 3 suggest that although cytosolic AGS3 can prevent receptor−G protein coupling during reconstitution, it does not do so once it associates with the membrane.

Cytosolic AGS3 Interacts with Membrane-Associated Gi1α Subunits. In a physiological setting, G proteins are associated with the inner face of the plasma membrane. To understand if cytosolic AGS3 could interact with membrane-associated Giα subunits and interfere with their coupling to receptors, Sf9 cell membranes expressing 5-HT_{1A} receptors were first reconstituted with Gi1 heterotrimers to allow their association with the membrane. The membranes were washed three times to remove unassociated Gi1 heterotrimers and incubated with

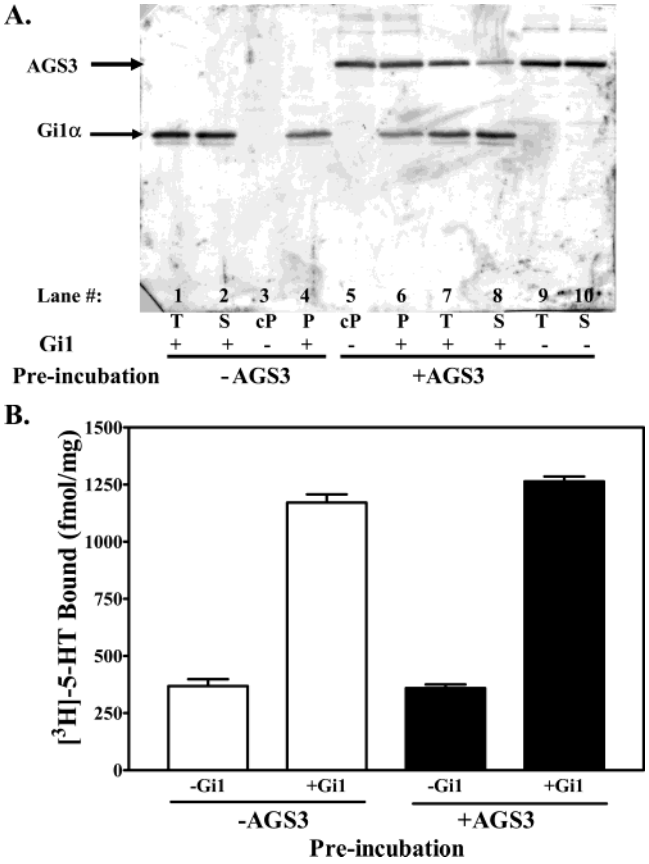

FIGURE 3: Association of cytosolic AGS3 with membranes and its effect on receptor−G protein coupling. Sf9 cell membranes expressing 5-HT_{1A} receptors (655 fmol) were preincubated with or without cytosolic AGS3 (64 pmol) in 40 μL to allow AGS3 to associate with the membranes. Following preincubation, the membranes were pelleted, washed, and resuspended. Preincubated membranes were reconstituted with or without 5.5 pmol of Gi1 heterotrimers in 33 μL. (A) The amounts of Gi1α and AGS3 associated with the soluble and membrane fractions from membranes preincubated and reconstituted under the indicated conditions were estimated by immunoblotting. The lanes contained the following: T, 10% of the reconstitution before pelleting; S, 10% of the supernatant after pelleting in a refrigerated microcentrifuge; cP and P, entire membrane pellet (after washing three times). Lanes 9 and 10 represent 10% of the total and supernatant from the first preincubation with AGS3 cytosol and should not be compared with lanes 7 and 8, respectively, which are following the second incubation with Gi1. An estimated 19 pmol of AGS3 (lanes 5 and 6) and 275 fmol of Gi1α (lanes 4 and 6) remained associated with the membrane pellets. In three separate experiments, there was no significant difference in either the amount of AGS3 associated with the membrane in the presence or absence of Gi1 or the amount of Gi1α associated with the membrane in the presence or absence of AGS3. (B) Membranes treated as described for panel A were diluted in binding buffer, and high-affinity agonist binding was assessed with 1.0 nM [^{3}H]-5-HT. The bars correspond to lanes 3−6 in panel A. Final conditions in the binding assay were 1 nM receptor with or without 8.5 nM Gi1 and with or without 29 nM AGS3 in 200 μL. Data are means ± standard deviation of triplicate determinations from a representative experiment that was repeated three times with similar results.

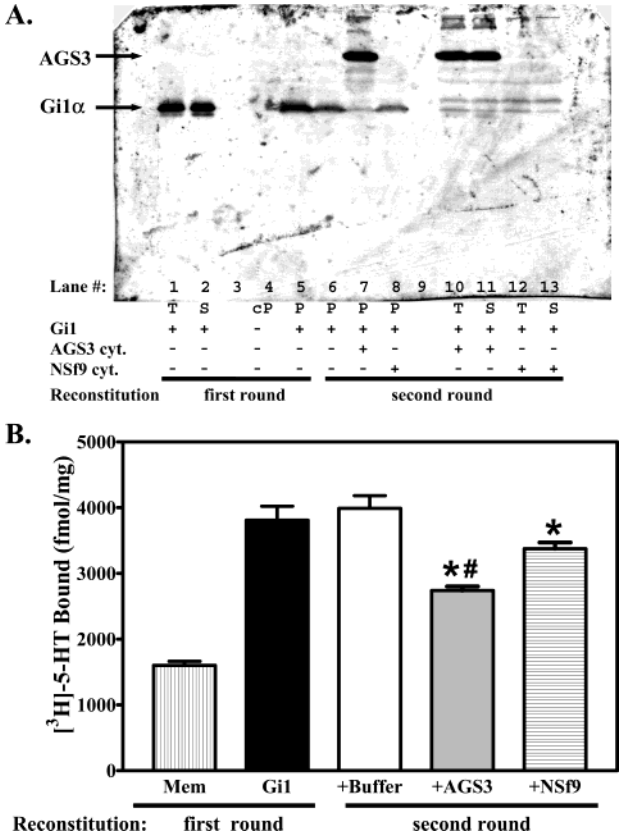

FIGURE 4: Effect of cytosolic AGS3 on membrane-associated Gi1. Sf9 cell membranes expressing 5-HT_{1A} receptors (620 fmol) were reconstituted with or without 35 pmol of Gi1 heterotrimers in 45 μL. After pelleting and washing had been carried out, an estimated 1.75 pmol of Gi1α remained associated with the membranes reconstituted with Gi1. Membranes reconstituted with Gi1 were incubated a second time with buffer, 245 μg of cytosolic proteins from Sf9 cells expressing AGS3 (+AGS3, 78 pmol total), or 245 μg of cytosolic proteins from uninfected Sf9 cells (+NSf9) in a volume of 45 μL. (A) The amounts of Gi1α and AGS3 associated with the soluble and membrane fractions from membranes reconstituted as described above were estimated by immunoblotting. The lanes contained the following: T, 10% of the reconstitution before pelleting; S, 10% of the supernatant after pelleting in a refrigerated microcentrifuge; cP and P, entire membrane pellet (after washing three times). In four separate experiments, the amount of Gi1α (expressed as picomoles per milligram of membrane protein) in lane 7 was smaller than that in lanes 5, 6, and 8 ($p < 0.05$, Tukey's test), while the amount in lanes 5 and 6 did not differ from one another ($p > 0.05$, Tukey's test). (B) Membranes treated as described above were diluted into binding buffer, and high-affinity agonist binding was assessed with 1.5 nM [^{3}H]-5-HT. Final conditions in the binding assay were 0.6 nM receptor in the presence or absence of 2 nM Gi1. The vertically striped bar represents binding to control membranes without exogenous G proteins, while the black bar represents binding to membranes after a single reconstitution with Gi1. The white, gray, and horizontally striped bars represent binding to membranes after the second incubation in the presence of buffer, cytosol containing 84 nM AGS3 (final concentration), and control cytosol from uninfected Sf9 cells, respectively. Binding in the Gi1 controls (black and white bars) is not significantly different ($p > 0.05$, Tukey's test). The asterisk indicates a p of <0.01 vs +Buffer (Tukey's test), while the pound sign indicates a p of <0.05 vs +NSf9 (Tukey's test). Data are means ± standard deviation of triplicate determinations from a representative experiment repeated three times with similar results.

crude Sf9 cell cytosol containing expressed AGS3 or with an equivalent amount of Sf9 cell cytosol from uninfected cells or buffer as controls. As an additional control, membranes were reconstituted with or without Gi1 and not subjected to a second incubation. The amount of Gi1α that remained in the membranes following these treatments was determined by immunoblotting. The lanes from a representative immunoblot repeated four times are shown in Figure 4A. As shown in lane 4, no Gi1α was detected in the membrane pellet without reconstitution with exogenous Gi1. As shown in lane 5, an estimated 1.75 pmol of Gi1α subunits

associated with 33 μg of membrane protein following the first round reconstitution with Gi1 heterotrimers. Pelleting, washing, and a second reconstitution with either control buffer (lane 6) or 245 μg of cytosolic proteins from normal Sf9 cells (lane 8) did not significantly decrease the amount of membrane-associated Gi1α subunits, indicating a strong association of G proteins with the membrane. Interestingly, lane 7 shows a significant decrease in the amount of membrane-associated Gi1α following the addition of the AGS3-containing cytosol in the second reconstitution ($p < 0.05$, Tukey's test, $n = 4$).

The high-affinity agonist binding to membranes following these treatments from a representative experiment repeated three times with similar results is shown in the bar graph in Figure 4B. The binding data are consistent with the immunoblotting data, and also indicate that the association of G proteins with the membrane during reconstitution is quite strong. The level of control binding to membranes lacking exogenous G proteins (vertically striped bar in Figure 4B) was lower than those of all other groups ($p < 0.01$, Tukey's test, $n = 3$). Pelleting, washing, and the second incubation with control buffer did not lower the level of high-affinity agonist binding, which reflects receptor−G protein coupling (compare the white and black bars in Figure 4B). However, incubation with both control and AGS3-containing cytosol led to a significant decrease ($p < 0.01$, Tukey's test, $n = 3$) in the level of high-affinity agonist binding (gray and horizontally striped bars in Figure 4B). In titration experiments, the IC_{50} for inhibition of high-affinity agonist binding of the AGS3 cytosol was significantly lower than the IC_{50} of the control cytosol (4.6 $\pm$ 0.7 and 34.6 $\pm$ 6.8 ng/μL, respectively, $n = 2$, $p < 0.05$, data not shown), indicating the significant contribution of the cytosolic AGS3 protein. Thus, the results show that cytosolic AGS3 can dissociate Gi1α subunits from the membrane and interfere with receptor coupling. The nature of the inhibitory activity in the normal Sf9 cell cytosol is unknown but may represent an endogenous GPR-containing protein. The AGS3 antibody cross-reacts with several faint bands present in the normal Sf9 cytosol (Figure 4, lanes 12 and 13). Though it did not reach the level of statistical significance, the normal Sf9 cell cytosol did slightly reduce the level of high-affinity agonist binding, and this effect was reversed upon boiling (Figure 2A). To demonstrate that cytosolic AGS3 is capable of dissociating endogenous G proteins from a native membrane, a preparation of rat brain membranes was incubated in the presence of buffer, 40 μg of control cytosol from uninfected Sf9 cells, and 40 μg of cytosol from Sf9 cells expressing AGS3 (0.3 pmol/μg of protein). As shown in Figure 5, only the AGS3-containing cytosol was able to redistribute a significant amount of Gi/oα to the 100000*g* supernatant following fractionation of the incubation mixtures. Clearly, AGS3 is capable of extracting Gi/oα subunits from their native membrane environment.

Selective Inhibition of Receptor−Gi Protein Coupling by GST−AGS3-GPR Fusion Proteins. The general domain structure of mammalian AGS3 is shown in Figure 6A. The carboxyl-terminal GPR domain of AGS3, which consists of four homologous repeated motifs, is known to bind Giα subunits (*9*, *13*), but little is known about possible functional roles for the amino-terminal TPR domain. The role of each individual GPR motif and their relative selectivity for specific

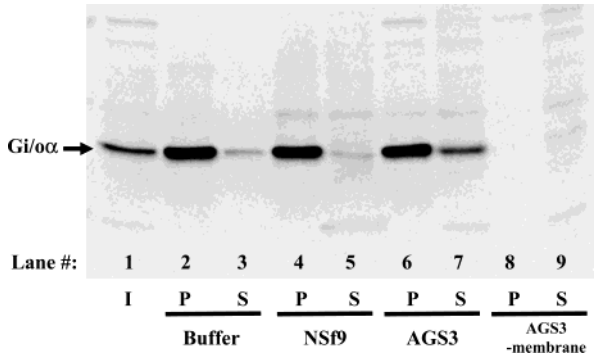

FIGURE 5: Effect of cytosolic AGS3 on the subcellular distribution of Gi/oα from native rat brain membranes. Rat brain membranes (10 μg) were incubated in 50 μL with buffer or in 40 μg of crude cytosol protein from uninfected Sf9 cells (NSf9) or from Sf9 cells expressing AGS3 (AGS3) at 0.3 pmol/μg. After incubation, the mixtures were fractionated into a 100000*g* supernatant (S) and pellet (P). Lane 1 contained 2.5 μg of membrane protein without incubation. Lanes 8 and 9 (AGS3-membrane) contained supernatant and pellet fractions from 40 μg of AGS3 containing cytosol incubated without the addition of membranes. After SDS−PAGE, membrane transfers were immunoblotted for G proteins using the Gα specific antisera 976. The immunoblot is representative of results obtained in four independent experiments.

G proteins are also poorly understood. The latter issues were addressed using GST fusion proteins that contain all four GPR motifs or individual GPR motifs. The GST−AGS3-GPR (Pro^{463}−Ser^{650}) fusion protein inhibited 5-HT_{1A} receptor coupling to all four G proteins that were tested (Figure 6B). The IC_{50} for Go is significantly different from the values obtained with Gi ($p < 0.05$, Neuman-Keuls test). The native cytosolic AGS3 protein exhibited an IC_{50} of 29 $\pm$ 6 nM ($n = 3$) for Gi1 (data not shown), indicating that the presence of amino-terminal TPR domains did not alter the apparent affinity of the GPR domains for Gi.

Each of the four individual AGS3-GPR domains inhibited 5-HT_{1A} receptor−G protein coupling (Figure 6C), although not to the same extent as the complete AGS3-GPR domain ($p < 0.05$, Tukey's test). Two-way ANOVA of the inhibition of 5-HT_{1A} receptor coupling to different G proteins by individual AGS3-GPR domains indicates that all four AGS3-GPR domains interact more weakly with Go than with Gi proteins ($p < 0.01$), while there is no significant difference among individual AGS3-GPR domains for their interactions with Gi/o proteins ($p > 0.05$). These results suggest that the individual GPR domains do not differ from one another in their interactions with Gα subunits and that all of them interact more weakly with Goα than with the Giα subunits.

DISCUSSION

The recent discoveries of diverse proteins that interact with various components of heterotrimeric G protein-mediated signaling pathways provide additional insights into the regulation of this signaling process. In particular, the identification of the AGS family of proteins suggests that inputs other than GPCRs are capable of activating these pathways. AGS3 was initially identified as a receptor-independent activator for G protein signaling in a yeast-based functional screen (*9*). Sequence analysis reveals that AGS3 has four repeated GPR motifs in the carboxyl-terminal half of the protein. This GPR sequence is conserved among various proteins from different species. Although these

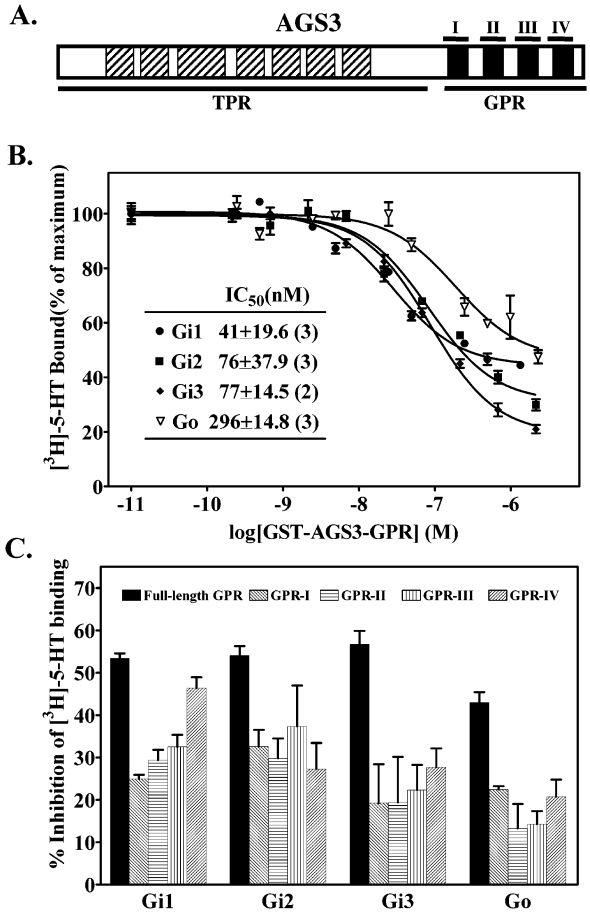

FIGURE 6: Effect of GST−AGS3-GPR domains on receptor−G protein coupling. (A) The general domain structure of mammalian AGS3 is shown with striped boxes for the amino-terminal TPR domains and gray boxes for the carboxyl-terminal GPR domains known to interact with G protein α-subunits. (B) Sf9 cell membranes expressing 5-HT_{1A} receptors (187 fmol) were reconstituted with the indicated Gi/o proteins (4.25 pmol) in 17 μL. Increasing concentrations (0.1−1.163 pmol) of GST−AGS3-GPR (Pro^{463}−Ser^{650}) fusion protein containing all four individual AGS3-GPR domains were added to the reconstitution mixtures. Following dilution with binding buffer, high-affinity agonist binding was assessed with 1 nM [^{3}H]-5-HT binding and expressed as the percent maximum binding in the absence of the GST−AGS3-GPR (Pro^{463}−Ser^{650}) fusion protein for each G protein. Final conditions in the binding assay were 0.4 nM receptor, 8 nM G protein, and the indicated concentrations (0.2−2.281 nM) of the GST−AGS3-GPR fusion protein in 150 μL. The data shown are means ± standard deviation of triplicate determinations from a representative experiment, and the IC_{50} values are means from the indicated number of independent experiments. The IC_{50} for Go is significantly different from the others ($p < 0.05$, Tukey's test). (C) Sf9 cell membranes expressing 5-HT_{1A} receptors (655 fmol) were reconstituted with 9 pmol of individual Gi/o proteins and 250 pmol of the indicated GST−AGS3 fusion proteins in 17 μL. Following dilution into binding buffer, high-affinity agonist binding was assessed with 2 nM [^{3}H]-5-HT. The bars represent the percent inhibition by the GST−AGS3 fusion proteins relative to the maximum binding observed after reconstitution with the indicated G proteins in the absence of GST−AGS3 fusion proteins. Data are means ± standard error of the mean from two independent experiments. For each G protein, the degree of inhibition by the complete AGS3-GPR domain is greater than any individual AGS3-GPR domains ($p < 0.05$, Tukey's test). Two-way ANOVA indicates that there is no significant difference among individual AGS3-GPR domains for their interactions with G proteins, although the degree of inhibition with all four AGS3-GPR domains is least with Go ($p < 0.01$).

proteins have diverse functions, they share the ability to bind α-subunits of the Gi family via their GPR domains. Biochemical studies from different groups have demonstrated that the GPR motif acts as a GDI to stabilize Giα subunits in a GDP-bound form (*9*, *11*−*15*, *27*−*29*).

Recent studies indicate that GPR-containing proteins are involved in cell polarity and cell division (*16*−*20*), but little is known about the physiological role of AGS3 in GPCR signaling. The effects of AGS3 on receptor−G protein coupling and potential mechanisms were the subjects of this study. We hypothesized that because G$\alpha\beta\gamma$ heterotrimers are required on the cytoplasmic face of the plasma membrane for coupling with heptahelical GPCRs, AGS3 might interfere with receptor−Gi protein coupling since AGS3 can disrupt G protein heterotrimers by competing with G$\beta\gamma$ to bind GDP-bound Giα. The majority of cellular AGS3 is found in the cytosol, but a small portion is associated with membrane fractions (*13*, *15*, *20*). In a cellular context, either fraction might be capable of disrupting G protein heterotrimers and interfere with receptor coupling. Our results indicate that membrane-localized AGS3 does not appear to interact with Gα subunits and has no effect on the reconstitution of receptor−G protein coupling (Figure 1). To exclude the possibility that the membrane-expressed AGS3 failed to block receptor coupling because it was aggregated or denatured, we demonstrated that cytosolic AGS3, which has the ability to block reconstitution of receptor−G protein coupling (Figure 2), lost its ability to do so following association with the membrane fraction (Figure 3). Our data do not exclude the possibility of a signaling mechanism that would allow membrane-associated AGS3 to interact with Gα subunits and modulate receptor coupling. Although it is not well understood how AGS3 associates with the membrane, the amino-terminal TPR domains have been implicated in subcellular localization (*11*) perhaps through an as yet undefined mammalian binding partner. Interestingly, the subcellular localization of a short form of AGS3, lacking the TPR domains, may be determined in part by a regulated interaction with Giα (*11*). However, Yu and colleagues (*30*) reported that both carboxyl-terminal GPR and amino-terminal TPR domains are required for the apical membrane targeting of Pins, an AGS3/LGN ortholog from *Drosophila*.

The localization of G protein heterotrimers at the plasma membrane involves a complex interplay among several processes, including post-translational modifications, subunit assembly, and direct protein−membrane interactions (*31*−*40*). It is clear that assembly with G$\beta\gamma$ is required for localization of Giα at the plasma membrane (*33*, *35*, *37*). A recent model proposes that all three G protein subunits are synthesized in the cytosol on free polysomes and that Gα and G$\beta\gamma$ associate on the cytosolic face of the Golgi prior to transport from an endosome to the plasma membrane (*37*). Since AGS3 can compete with G$\beta\gamma$ subunits to bind Giα subunits, cytosolic AGS3 might disturb the assembly of G$\beta\gamma$ with Giα subunits and thereby decrease the amount of G protein heterotrimer at the plasma membrane. As reviewed by Geyer and Wittinghofer (*41*), the GDIs for small G proteins generally form complexes with small G proteins in the cytosol and prevent their translocation to the membrane. Our results suggest the possibility of a similar role for AGS3 with heterotrimeric G proteins. In reconstitution assays, AGS3 in a cytosol fraction binds Giα subunits and prevents the membrane association of Giα, but not G$\beta\gamma$ (Figure 2).

Cytosolic AGS3 actually interacted with membrane-associated Giα subunits, dissociating them from the membrane and interfering with receptor coupling (Figure 4). While disruption of G protein heterotrimers is sufficient to inhibit receptor coupling in a reconstitution system, in a cellular context there must be an appropriate signal regulating the access of AGS3 to membrane-localized G proteins. In the few existing studies on cellular functions of related GPR-containing proteins, it is clear that the distributions and functions of these proteins are regulated by signals during the cell cycle (*18*−*20*).

The AGS3 carboxyl-terminal domain containing four repeated GPR motifs is the region responsible for binding Giα subunits (*13*). The carboxyl-terminal GPR domain is as potent as full-length AGS3 in interactions with Giα subunits. Our results also confirm a weak interaction between AGS3-GPR and Goα subunits (*12*, *13*, *15*). While it is tempting to interpret the reduced apparent affinity for Goα in terms of selectivity, it is perhaps more likely that the reduced affinity ensures that only appropriate amounts of AGS3 and Goα form complexes in a cellular setting. For example, in brain membranes, Goα is by far the most abundant Gα subunit (*42*, *43*).

It is clear that amino acids within both the GPR domain and the Gα subunit contribute to the selectivity of the interaction. Peterson et al. (*27*) have defined critical amino acids within the core GPR domain and demonstrated differential effects on interactions with Giα versus Goα, while the crystal structure of $G\alpha_{i1}$-GDP bound to the RGS14 GPR motif peptide (*44*) and functional studies with chimeric G proteins (*45*) demonstrate that the helical domains of Gα subunits contribute to the selectivity of Gα−GPR interactions. However, for proteins such as Pins, LGN, and AGS3 that contain multiple GPR motifs, the possibility of selectivity for different Gi/oα subunits has not been previously addressed. Interestingly, our data indicate that although all four individual AGS3 GPR motifs interact more strongly with Giα than with Goα, they do not discriminate among the three Giα subunits (Figure 6B). Each AGS3-GPR motif can bind Giα subunits, and the complete GPR domain actually binds multiple subunits at the same time, suggesting a possible scaffolding function for the complete AGS3-GPR domain (*13*). Our results are consistent with this interpretation and show that each AGS3-GPR domain can interfere with receptor−G protein coupling but that at equal concentrations [near the IC_{50} for the GST−AGS3-GPR (Pro^{463}−Ser^{650}) fusion protein], the complete GPR domain inhibits coupling to a greater extent than any individual GPR domain (Figure 6C).

In summary, our data show that AGS3 can interfere with receptor−G protein coupling by disrupting Gα$\beta\gamma$ heterotrimers in the cytosol as well as by removing Gα subunits from the plasma membrane. The disruption of G protein heterotrimers might also serve to initiate G$\beta\gamma$-mediated signaling events. Similarly, there may be as yet unappreciated roles in cellular signaling for the AGS3−Gα-GDP complex. Interestingly, membrane-associated AGS3 appears not to interact with Gα subunits, although our study does not exclude the possibility that such interactions may require an initiating signal.

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