Receptor-regulated Interaction of Activator of G-protein Signaling-4 and Gαi[*][s]

Sukru Sadik Oner[‡]**, Ellen M. Maher**[‡]**, Billy Breton**[§]**, Michel Bouvier**[§1]**, and Joe B. Blumer**[‡2]

From the [‡]*Department of Cell and Molecular Pharmacology, Medical University of South Carolina, Charleston, South Carolina 29425 and the* [§]*Department of Biochemistry, Institute for Research in Immunology and Cancer, Université de Montréal, Montreal, Quebec H3C 3J7, Canada*

Activator of G-protein signaling-4 (AGS4), via its three G-protein regulatory motifs, is well positioned to modulate G-protein signal processing by virtue of its ability to bind Gαi-GDP subunits free of Gβγ. Apart from initial observations on the biochemical activity of the G-protein regulatory motifs of AGS4, very little is known about the nature of the AGS4-G-protein interaction, how this interaction is regulated, or where the interaction takes place. As an initial approach to these questions, we evaluated the interaction of AGS4 with Gαi1 in living cells using bioluminescence resonance energy transfer (BRET). AGS4 and Gαi1 reciprocally tagged with either *Renilla* luciferase (RLuc) or yellow fluorescent protein (YFP) demonstrated saturable, specific BRET signals. BRET signals observed between AGS4-RLuc and Gαi1-YFP were reduced by G-protein-coupled receptor activation, and this agonist-induced reduction in BRET was blocked by pertussis toxin. In addition, specific BRET signals were observed for AGS4-RLuc and α2-adrenergic receptor-Venus, which were Gαi-dependent and reduced by agonist, indicating that AGS4-Gαi complexes are receptor-proximal. These data suggest that AGS4-Gαi complexes directly couple to a G-protein-coupled receptor and may serve as substrates for agonist-induced G-protein activation.

Activators of G-protein signaling (AGS)[3] proteins were identified using a yeast-based functional screen of mammalian cDNA libraries for cDNAs that activated G-protein signaling in the absence of a GPCR (1–4). Group II AGS proteins all contain at least one G-protein regulatory (GPR) motif (3, 5) (also termed the GoLoco motif (6)), a 20–25-amino acid motif that binds and stabilizes the GDP-bound conformation of Gαi/o/t

and competes with Gβγ for Gα binding (reviewed in Ref. 5). Proteins with multiple GPR motifs can bind to multiple Gα subunits simultaneously, which presents a unique opportunity to act as a scaffold to organize a signaling complex (7, 8).

Functional studies indicate crucial roles for GPR proteins beginning with the original observations in model organisms describing a role for GPR proteins and their interaction with G-proteins in asymmetric cell division (5, 9). Additional functional studies with GPR proteins indicate further functional diversity with roles observed in blood pressure control, fat deposition and energy expenditure, neuronal outgrowth, drug addiction and relapse behavior, autophagy, G-protein-coupled inwardly rectifying potassium channel regulation, and transport of membrane proteins to the cell surface (10–18). These observations indicate crucial functionality of the GPR motif in biological systems and implicate Gαi-GPR complexes in the regulation of G-protein signaling in unexpected, albeit poorly understood ways. In the context of the group II AGS proteins, which contain multiple GPR motifs, many outstanding questions remain to be addressed. Chief among them is what regulates the formation and disassembly of GPR-Gαi complexes? Is their interaction with G-protein influenced by GPCR activation or other signals?

AGS4 was identified in the yeast based functional screen for receptor-independent G-protein activators from a human prostate leiomyosarcoma cDNA library (1), and apart from initial reports describing its interaction with Gαi subunits from cell lysates and purified proteins *in vitro*, little is known regarding its interaction with Gαi in the intact cell or how this interaction is regulated. Although AGS3 and AGS5/Leu-Gly-Asn repeat-enriched protein (LGN) both contain seven tetratricopeptide repeats upstream of their four GPR motifs and are broadly expressed in many tissues, AGS4 has a much different domain organization with a proline-rich N terminus followed by three GPR motifs that are somewhat dissimilar as compared with AGS3 and AGS5/LGN (1, 19). AGS4 also appears to be more restricted in its expression to the immune system.[4] In this study, we report the interaction of AGS4 with Gαi in the intact cell as determined by bioluminescence resonance energy transfer (BRET) and its regulation by cell surface GPCRs. In addition, the data indicate that AGS4-Gαi complexes are receptor-proximal as specific BRET signals were observed between AGS4 and the α2A-adrenergic receptor (α2A-AR), which were Gαi-dependent and reduced by an α2A-AR agonist. These data suggest that

AGS4-Gα_i complexes directly couple to a GPCR and may serve as substrates for receptor-induced G-protein activation.

EXPERIMENTAL PROCEDURES

Materials—Polyethyleneimine (PEI) (25 kDa molecular mass, linear form), was obtained from Polysciences, Inc. Benzyl-coelenterazine was obtained from NanoLight Technology (Pinetop, AZ). UK14304, rauwolscine HCl, and pertussis toxin were purchased from Sigma. Materials for cell culture were purchased from Invitrogen. Gray 96-well Optiplates were obtained from PerkinElmer Life Sciences. Anti-Gα_{i1} and anti-*Renilla* luciferase were purchased from Millipore (Billerica, MA). Anti-Gα_{i3} and Gα_s were kindly provided by Dr. Thomas Gettys (Pennington Biomedical Research Center, Baton Rouge, LA). AGS4 GPR mutants, Gα_{i1}-YFP-G202T, Gα_{i1}-YFP-Q204L, and Gα_{i1}-YFP-N149I were generated by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). Gα_{i3}, Gα_{i3}-G202T, and Gα_{i3}-Q204L cDNAs were obtained from the cDNA Resource Center (University of Missouri, Columbus, MO). pcDNA3.1-Gα_{i1}-YFP was generated by Dr. Gibson (20) and provided by Gregory G. Tall (University of Rochester School of Medicine and Dentistry). Construction of expression vectors for α_{2A}-AR, β_2-AR, and μ-opioid receptor (MOR) were previously described (21–23). MOR-YFP plasmid was kindly provided by Dr. Lakshmi A. Devi (Mount Sinai Medical Center, New York, NY). All other reagents and materials were obtained as described elsewhere (22, 24).

Cell Culture and Transfection—HEK-293 cells were maintained in Dulbecco's minimal essential medium (high glucose, without phenol red) containing 5% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were grown in the presence of 5% CO_2 at 37 °C in a humidified incubator. For transfection, 8×10^5 cells/well were seeded on 6-well plates and cultured overnight at 37 °C. BRET donor and acceptor plasmids were used for transfection with PEI (1 mg/ml in distilled H_2O) at a DNA:PEI ratio of 1:4. PEI and plasmid DNA were diluted in separate tubes with 100 μl of serum-free medium. DNA and PEI solutions were vortexed at maximum speed for 3–5 s and incubated for 15 min at room temperature prior to addition to the cells. Cells were incubated for 48 h prior to collection for experiments. Cell lysates and immunoblotting were performed as described previously (24).

BRET—Initial experiments were performed to optimize the BRET system for AGS4-Gα_{i1} interactions and to ensure the specificity of observed signals. All studies involved saturation BRET analysis, altering donor/acceptor ratios and/or time course analysis. Forty-eight hours after transfection, the cells were washed once with phosphate-buffered saline and harvested with Tyrode's solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.37 mM NaH_2PO_4, 24 mM $NaHCO_3$, 10 mM HEPES, pH 7.4, and 0.1% glucose (w/v)). Cells were distributed in triplicate at 1×10^5 cells/well into gray 96-well plates. Fluorescence and luminescence signals were measured with a TriStar LB 941 plate reader (Berthold Technologies, Oak Ridge, TN). Total fluorescence (excitation, 485 nm; emission, 535 nm) was first measured to quantify acceptor expression. The luciferase substrate coelenterazine H (5 μM final concentration) was then added, and luminescence was measured (donor, 480 ± 20

nm; acceptor, 530 ± 20 nm). Net BRET values were determined by first calculating the 530 ± 20:480 ± 20 nm ratio and then subtracting the background BRET signal determined from cells transfected with the *Renilla* luciferase (RLuc) expression vector phRLuc$_{N3}$ alone. Spectral measurements were conducted with the protocol described above using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). BRET saturation curves and statistical analyses were measured using GraphPad Prism (GraphPad Software, San Diego, CA). Data were analyzed by analysis of variance with significant differences between groups determined by Tukey's post-hoc test.

RESULTS AND DISCUSSION

Key questions in the field and for AGS4 in particular are what regulates the formation and disassembly of AGS4-Gα_i complexes and is the AGS4-G-protein interaction influenced by GPCR activation or other signals? As an initial approach to address these questions, we used BRET with contingent binding proteins tagged with RLuc or yellow fluorescent protein (YFP). The enzymatic oxidation by luciferase of substrates such as coelenterazine and subsequent non-radiative emission can excite YFP if the two proteins are in close proximity (< 100 Å). The strength of the BRET signal for two proteins fused with RLuc and YFP, respectively, depends upon distance between the fluorophores, their relative orientations, and the relative expression levels of donor (RLuc) and acceptor (YFP). Specific interactions exhibit saturation of BRET signals using a constant amount of the luciferase donor and increasing amounts of the YFP acceptor (25). AGS4 was tagged with either YFP or RLuc, and YFP or RLuc was inserted into the loop connecting helices αB and αC of the helical domain in Gα_{i1}, which confers nucleotide binding and hydrolysis properties similar to the untagged protein (20, 22).

Analysis of different AGS4 and Gα_i BRET donor/acceptor pair combinations revealed that the AGS4-RLuc-Gα_{i1}-YFP (YFP at position 122) BRET pair yielded the strongest BRET signal and the highest signal-to-noise ratio (data not shown). The higher BRET signals detected by C-terminal tagged AGS4-RLuc and Gα_{i1}-YFP likely reflect the ability of the donor AGS4-RLuc to simultaneously bind more than one acceptor Gα_{i1}-YFP due to the presence of three GPR motifs in AGS4. Robust, specific, and saturable BRET signals were observed between AGS4-RLuc and Gα_{i1}-YFP (Fig. 1, *A* and *B*). BRET signals were not observed in cells expressing AGS4-Q/A, which contains Gln-Ala mutations in each of the three GPR motifs in AGS4, rendering it unable to bind Gα_i (26) (Fig. 1, *A* and *B*), thus confirming the specificity of the BRET signal. AGS4-RLuc-Gα_{i1}-YFP BRET signals were also decreased following co-expression of G$\beta\gamma$ subunits, consistent with the ability of G$\beta\gamma$ subunits to compete with GPR motifs for Gα binding (8, 27) (Fig. 1*B*). AGS4-RLuc-Gα_i-YFP BRET signals were markedly reduced upon introduction of the Q204L mutation in Gα_i-YFP, which alters GTP hydrolysis consistent with the known preference of GPR motifs for GDP-bound Gα_i subunits. The N149I mutation, which renders Gα_i incapable of binding GPR motifs (28), also predictably reduced the AGS4-Gα_i BRET signal (Fig. 1*C*). Interestingly, treatment of cells with pertussis toxin, which ADP-ribosylates a cysteine residue in G$\alpha_{i/o}$ subunits four amino acids from the C terminus and renders Gα incapable of
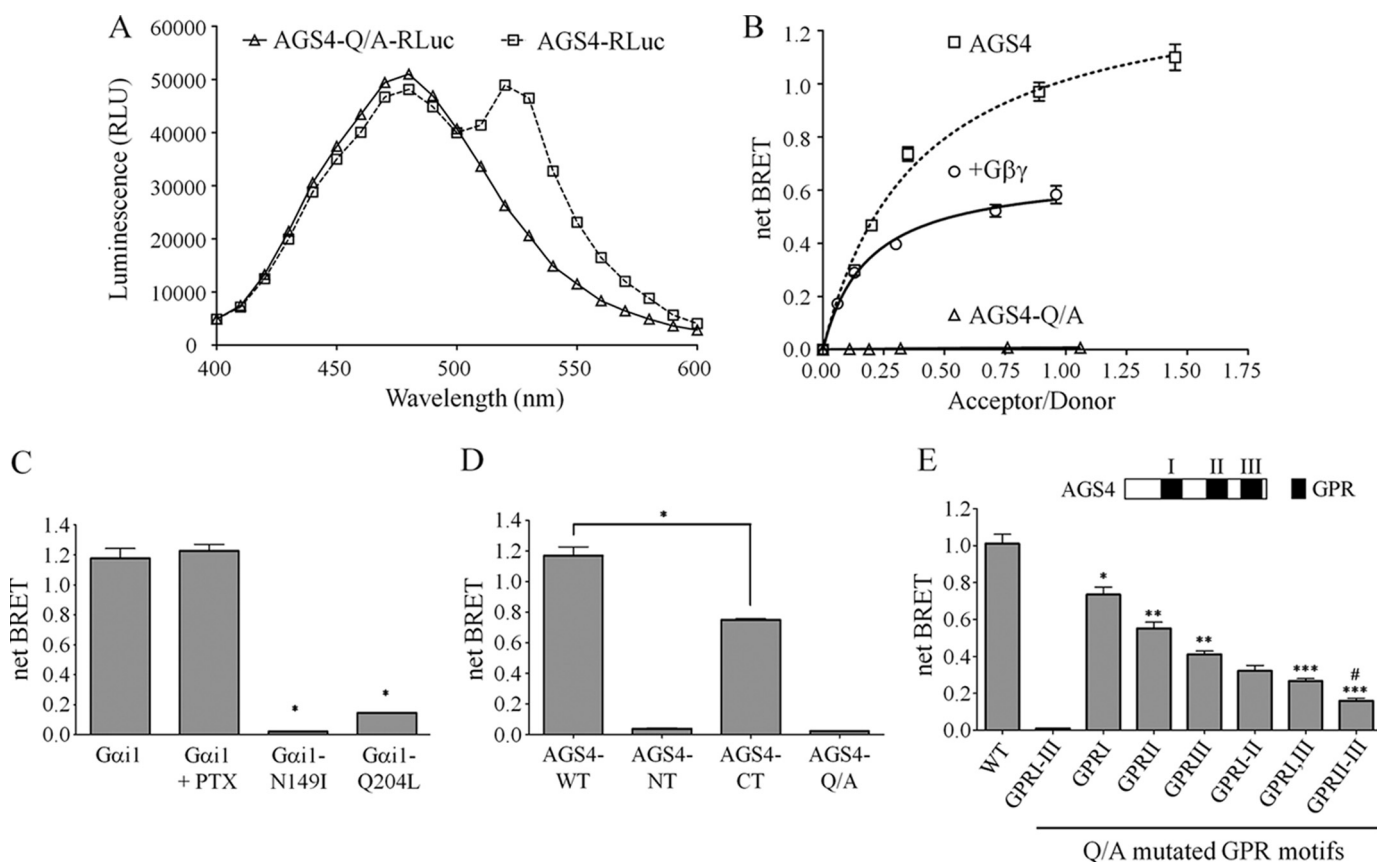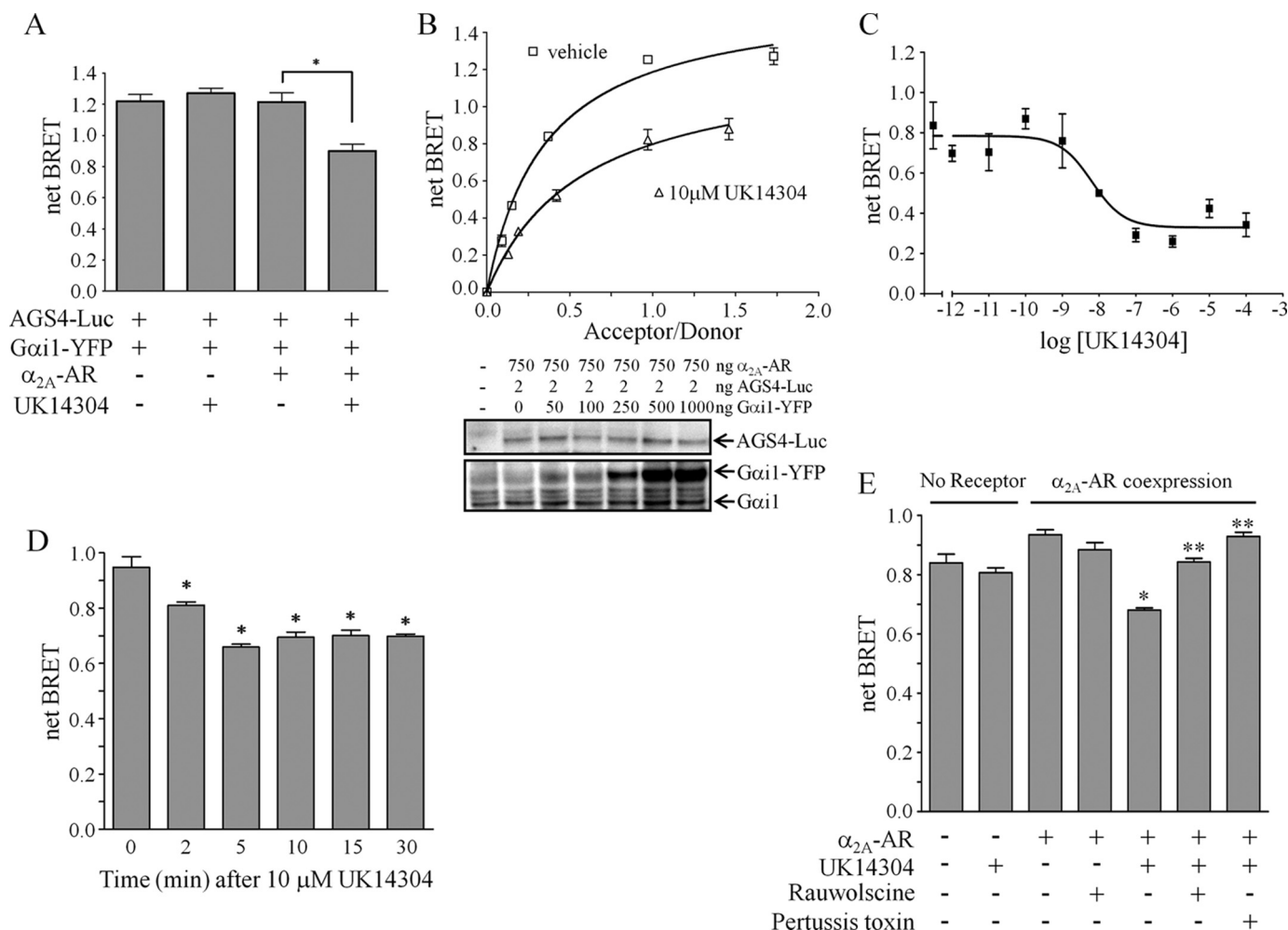
FIGURE 1. **Interaction of AGS4 and Gα_i in living cells using BRET.** *A,* emission spectra for luminescence in HEK cells transfected with 10 ng of phRLuc-AGS4 or phRLuc-AGS4-Q/A and 750 ng of pcDNA3-Gα_i-YFP. *RLU,* relative luminescence units. *B,* HEK cells were transfected with a fixed amount (2 ng) of phRLuc-AGS4 (*open squares*) or phRLuc-AGS4-Q/A (*open triangles*) and increasing amounts of pcDNA3-Gα_i-YFP (0–750 ng) without or with 500 ng of pcDNA3-Gβ_1 and 500 ng of pcDNA3-Gγ_2 (*G$\beta\gamma$, open circles*), and BRET signals were measured as described under "Experimental Procedures." *C,* net BRET signals generated from HEK cells transfected with 2 ng of phRLuc$_{N3}$-AGS4 and 750 ng of pcDNA3-Gα_{i1}-YFP in the presence or absence of 100 ng/ml pertussis toxin (*PTX*) pretreatment for 18 h or of Gα_{i1}-N149I-YFP or Gα_{i1}-Q204L-YFP. Mean acceptor/donor ratios are: Gα_{i1}, 2.2; Gα_i + pertussis toxin, 1.6; Gα_{i1}-N149I, 1.6; Gα_{i1}-Q204L, 2.3. *, $p < 0.05$ as compared with Gα_{i1}. *D,* net BRET signals generated from HEK cells transfected with 2 ng of phRLuc$_{N3}$-AGS4, AGS4-NT (Met[1]–Ser[56]), AGS4-CT (Leu[57]–Cys[160]), or AGS4-Q/A and 750 ng of pcDNA3-Gα_{i1}-YFP. Mean acceptor/donor ratios are: AGS4, 2.2; AGS4-NT, 1.8; AGS4-CT, 1.5; AGS4-Q/A, 1.6. *, $p < 0.05$ as compared with AGS4-RLuc. *WT,* wild type. *E,* net BRET signals generated from HEK cells transfected with 750 ng of pcDNA3-Gα_{i1}-YFP and 2 ng of phRLuc$_{N3}$ containing AGS4 or AGS4 variants with single residue mutations in each of the three AGS4-GPR motifs (GPRI-Q80A; GPRII-Q122A; GPRIII-Q151A) that inhibit GPR motif-Gα_i binding. Mean acceptor/donor ratios are: wild type, 1.6; GPRI–III, 1.3; GPRI, 1.6; GPRII, 1.3; GPRIII, 1.4; GPRI–II, 1.1, GPRI,III, 1.2; GPRII–III, 1.0. *, $p < 0.05$ as compared with AGS4-WT. **, $p < 0.05$ as compared with GPRI. ***, $p < 0.05$ as compared with GPRI–III. #, $p < 0.05$ as compared with GPRI–II. All data presented are representative of 3–9 experiments, and individual 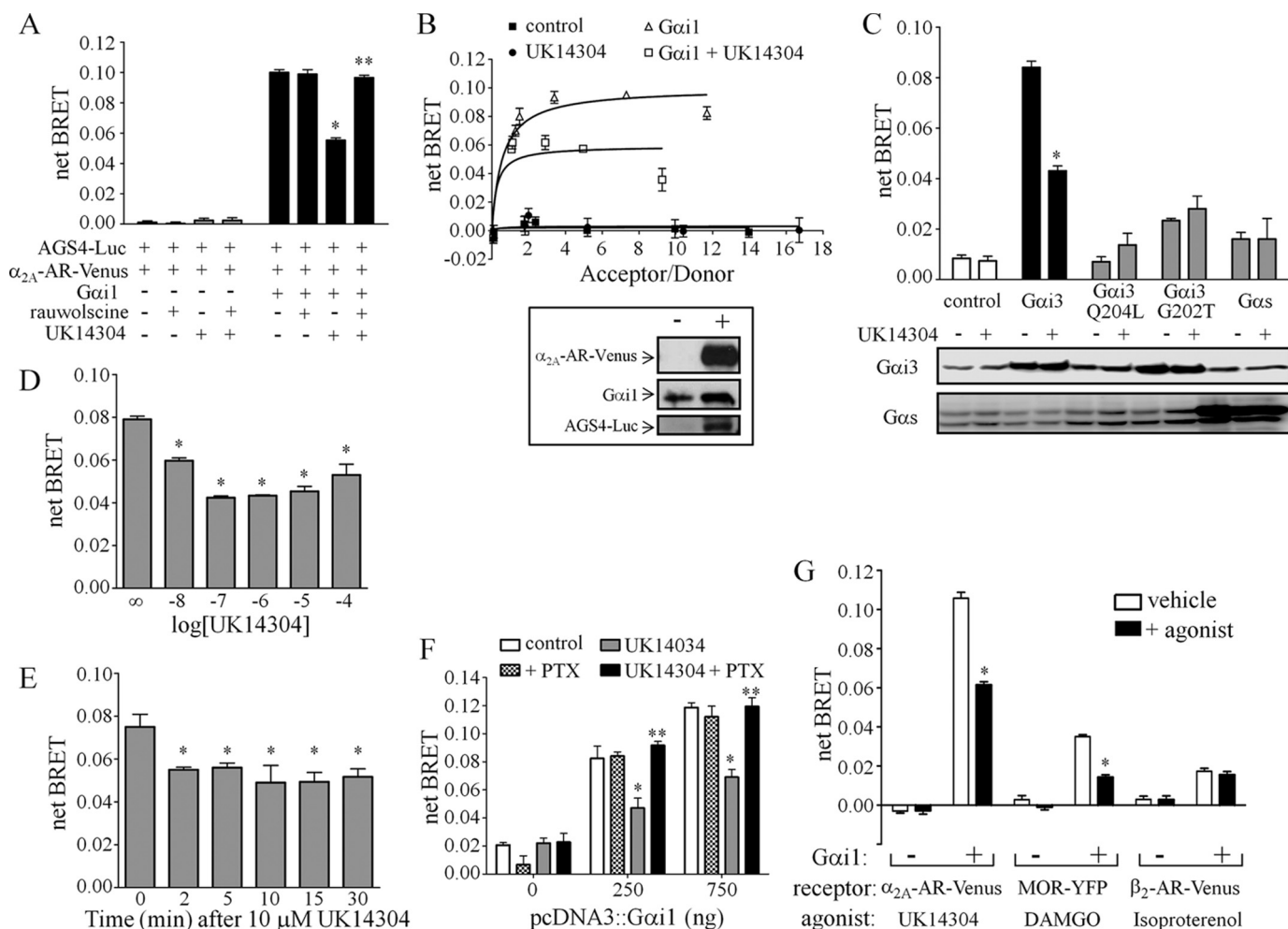values were the average of triplicate determinations. BRET saturation curves were fitted using a non-linear regression equation assuming one-site binding. *Error bars* in *B–E* indicate S.E.

being activated by GPCRs, had no effect AGS4-RLuc-Gα_i-YFP BRET signals (Fig. 1*C*).

Truncations of AGS4 revealed that the AGS4-RLuc-Gα_{i1}-YFP BRET signals require the AGS4 C-terminal GPR domain (AGS4-CT-Leu[57]–Cys[160]) (Fig. 1*D*). However, although no BRET signals were observed between the AGS4 N terminus (AGS4-NT-Met[1]–Ser[56]) and Gα_i-YFP, the reduction in the overall magnitude of the BRET signals of AGS4-CT-RLuc as compared with full-length AGS4-RLuc suggests that the N terminus of AGS4 may influence the ability of AGS4 to interact with Gα_i, consistent with the idea that residues outside of the core GPR motif may influence the GPR-Gα_i interaction (1, 19, 26, 29).

As an initial approach to define the relative influence of each of the GPR motifs in AGS4 on Gα_i binding, we tested AGS4-RLuc constructs with the Q/A substitution in each of the AGS4-GPR motifs (Q80A, Q122A, and Q151A) in AGS4-RLuc-Gα_i-YFP BRET experiments (Fig. 1*E*). These data are interesting in a number of aspects. First, individually mutated

AGS4-GPR motifs show a progressive decrease in Gα_{i1}-YFP BRET signals with GPRIII-Q/A having the most significant effect. In addition, AGS4-RLuc constructs with mutations in GPRIII in combination with either GPRI (GPRI,III-Q/A) or GPRII (GPRII–III-Q/A) have significantly lower BRET signals with Gα_{i1}-YFP than AGS4-Q/A mutations in the first two GPR motifs (GPRI–II-Q/A). Taken together these data suggest that GPR-III is important for Gα_i binding in cells. Alternatively, the decreased BRET signals generated in GPR-III Q/A mutants may reflect the proximity of the C-terminal RLuc tag to GPR-III and increased resonance energy transfer to Gα_i-YFP. Secondly, Q/A mutations in both GPR-I and GPR-III of AGS4 (GPRI,III) still yield significant Gα_i-YFP BRET signals with GPR-II as the sole GPR motif, suggesting that in the intact cell, GPR-II does indeed contribute to Gα_i binding consistent with earlier observations (1). This is in contrast to a previous report in which a glutathione *S*-transferase fusion protein containing AGS4-GPRII was essentially inactive as a guanine nucleotide dissociation inhibitor for Gα_{i1} unless Ala[121] was changed to Asp (19).

FIGURE 3. **AGS4 forms a Gα_i-dependent complex with GPCRs that is regulated by agonist.** *A*, net BRET signals generated from HEK cells transfected with 2 ng of phRLuc_{N3}-AGS4 and 500 ng of pcDNA3-α_{2A}-AR-Venus in the presence or absence of 750 ng of pcDNA3-Gα_{i1}. Cells were treated with vehicle (−), the α_2-AR agonist UK14304 (1 μM), and/or the α_2-AR antagonist rauwolscine (10 μM) for 15 min and processed for BRET as described under "Experimental Procedures." *, $p < 0.05$ as compared with vehicle treatment. *B*, BRET saturation signals from HEK cells transfected with a fixed amount (2 ng) of phRLuc_{N3}-AGS4 and increasing amounts of pcDNA3-α_{2A}-AR-Venus (0–1 μg) in the presence or absence of 750 ng of pcDNA3-Gα_{i1}. Cells were treated with vehicle or 10 μM UK14304 as indicated in the figure for 15 min and processed for BRET measurements. *Bottom panel*, representative immunoblot of HEK cells (50 μg protein/lane) untransfected (−) or transfected (+) with 250 ng of pcDNA3-α_{2A}-AR-Venus (IB: Venus), 500 ng of pcDNA3-Gα_{i1}, and 2 ng of phRLuc_{N3}-AGS4. *C*, net BRET signals generated from HEK cells transfected with 2 ng of phRLuc_{N3}-AGS4, 500 ng of pcDNA3-α_{2A}-AR-Venus, and 750 ng of pcDNA3-Gα_{i3}, Gα_{i3}-Q204L, Gα_{i3}-G202T, or Gα_s. Cells were treated with vehicle or 10 μM UK14304 as indicated in the figure for 15 min and processed for BRET measurements. *, $p < 0.05$ as compared with vehicle treatment. *Bottom panel*, Gα_{i3} and Gα_s immunoblot (75 μg of protein lysate per lane). *D* and *E*, HEK cells were transfected with 2 ng of phRLuc_{N3}-AGS4, 500 ng of pcDNA3-α_{2A}-AR-Venus, and 750 ng of pcDNA3-Gα_{i1}. Cells were incubated with increasing amounts of UK14304 (10^{-8}-10^{-4} M) for 15 min (*D*) or treated with 10 μM UK14304 for 0–30 min (*E*) and processed for BRET measurements. *, $p < 0.05$ as compared with vehicle treatment (*D*) or 0 time point (*E*). *F*, HEK cells were transfected with 2 ng of phRLuc_{N3}-AGS4, 500 ng of pcDNA3-α_{2A}-AR-Venus, and increasing amounts of pcDNA3-Gα_{i1} (0–750 ng). Cells were incubated in the presence or absence of 100 ng/ml pertussis toxin (*PTX*) for 18 h and then treated with vehicle or 10 μM UK14304 for 15 min. *, $p < 0.05$ as compared with control in each group. **, $p < 0.05$ as compared with UK14304 treatment in each group. *G*, HEK cells were transfected with 2 ng of phRLuc_{N3}-AGS4 and 500 ng of pcDNA3-α_{2A}-AR-Venus, MOR-YFP, or β_2-AR-Venus in the absence or presence of 750 ng of pcDNA3-Gα_{i1} as indicated in the figure. Cells were then treated with 10 μM UK14304, 10 μM [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (*DAMGO*), or 10 μM isoproterenol for 15 min as indicated in the figure. *, $p < 0.05$ as compared with vehicle treatment for each group. All data presented are representative of 4–10 experiments, and individual values were the average of triplicate determinations. *Error bars* in *A–G* indicate S.E.

cell surface. Although AGS4 is primarily localized to the cytosol (supplemental Fig. S1) (1), co-expression with Gα_i resulted in a dramatic recruitment of AGS4-GFP to the cell cortex (supplemental Fig. S1), suggesting that AGS4-Gα_i complexes are at least within the same subcellular compartment as GPCRs, *i.e.* at the cell surface. Gα_i-mediated recruitment of AGS4-GFP to the cell cortex was not observed for AGS4-Q/A-GFP, nor in the context of the GTP hydrolysis-deficient Gα_{i3}-Q204L variant (supplemental Fig. S1). As AGS4-RLuc-Gα_i-YFP BRET signals were dramatically reduced or absent in the context of the Gα_i-Q204L and

AGS4-Q/A variants (Fig. 1), the co-localization of wild-type AGS4 and Gα_i suggest that their co-localization at the cell surface likely results from their interaction. We then measured agonist-induced changes in AGS4-RLuc and Gα_{i1}-YFP distribution in crude membrane and cytosol fractions (supplemental Fig. S2). Receptor activation decreased the amount of AGS4-RLuc in the membrane fraction with a concomitant increase in the cytosol fraction as measured by both luminescence and immunoblotting (supplemental Fig. S2A), whereas Gα_{i1}-YFP distribution was unaltered (supplemental Fig. S2B). These data suggest that AGS4 and Gα_i physi-

cally dissociate after receptor activation, with AGS4 moving into the cytosol and Gα$_i$ remaining at the plasma membrane.

We then sought to determine whether AGS4 and Gα$_i$ were actually forming a complex with receptors. Under basal conditions, specific BRET signals were not observed between AGS4-RLuc and α$_{2A}$-AR-Venus (Fig. 3, *A* and *B*). However, when co-expressed with Gα$_i$, dramatic and specific BRET signals were observed (Fig. 3, *A* and *B*).[6] We did not observe significant alterations in either the basal or the agonist-induced changes in BRET observed between AGS4-RLuc and α$_{2A}$-AR-Venus when either Gα$_{i1}$ or Gα$_{i3}$ was used (data not shown). BRET signals were not observed between AGS4-RLuc and α$_{2A}$-AR-Venus in the context of the Gα$_i$-Q204L or Gα$_i$-G202T mutations or Gα$_s$ (Fig. 3*C*), nor were they observed with the AGS4-Q/A-RLuc variant, which cannot bind Gα$_i$ (data not shown), indicating that the AGS4-Gα$_i$ interaction is required for the BRET signals observed between AGS4-RLuc and α$_{2A}$-AR-Venus. In addition, the Gα$_i$-dependent AGS4-RLuc-α$_{2A}$-AR-Venus BRET signals were reduced by ~40–50% upon treatment with the α$_2$-AR agonist UK14304 (Fig. 3, *A* and *B*). As was observed for the agonist-regulated BRET signals between AGS4-RLuc and Gα$_{i1}$-YFP (Fig. 2), the reductions in Gα$_i$-dependent BRET signals between AGS4-RLuc and α$_{2A}$-AR-Venus exhibited were dose-dependent and occurred on a similar timescale (Fig. 3, *D* and *E*). The agonist-mediated reduction in AGS4-RLuc-α$_{2A}$-AR-Venus BRET was blocked by the antagonist rauwolscine (Fig. 3*A*) and by pertussis toxin pretreatment (Fig. 3*F*). Similar Gα$_i$-dependent and agonist-induced reductions in BRET signals were also observed between AGS4-RLuc and MOR-YFP but were not observed with AGS4-RLuc and β$_2$-AR-Venus, which is primarily a Gα$_s$-coupled receptor (Fig. 3*G*).

In the case of AGS4-RLuc-Gα$_i$-YFP BRET signals, receptor activation resulted in a reduction in BRET that could result either due to dissociation of AGS4-RLuc-Giα-YFP complexes or from a conformational rearrangement of the complex. The observation that receptor activation leads to a redistribution of AGS4 from the plasma membrane to the cytosol supports the first possibility. The reduction in agonist-modulated AGS4-RLuc-Gα$_i$-YFP BRET signal suggests that the agonist-regulated effect either occurs in a spatially restricted manner (*e.g.* at the plasma membrane) or is the result of second messengers. It is also possible that the agonist-induced reductions in BRET may arise from subunit exchange between the Gα$_i$-YFP complexed with AGS4-RLuc and wild-type, endogenous (*i.e.* untagged) Gα$_i$ from Gαβγ heterotrimers or that "free" Gβγ released from activated Gαβγ heterotrimers competes with AGS4-RLuc for Gα$_i$-YFP binding. Regarding the latter scenario, although data indicate that increased expression of Gβγ subunits does indeed reduce AGS4-RLuc-Gα$_i$-YFP BRET (Fig. 1*B*), this is likely due to an overall reduction in the amount of AGS4-RLuc-Gα$_i$-YFP complexes being formed in the presence of excess Gβγ rather than the release of free Gβγ subunits occurring via receptor activation.

The data therefore suggest that AGS4-Gα$_i$ complexes are directly coupled to and are regulated by a GPCR. The agonist-induced reduction in Gα$_i$-dependent BRET signals between AGS4-RLuc and α$_{2A}$-AR-Venus (Fig. 3) and the agonist-induced reductions in AGS4-RLuc-Gα$_i$-YFP (Fig. 2) are consistent with nucleotide exchange occurring on Gα$_i$ while complexed with AGS4-RLuc and suggest that once bound to GTP, the Gα$_i$ subunit dissociates from AGS4-RLuc, resulting in the decrease in BRET signals in both cases. The pertussis toxin blockade of these agonist-regulated BRET signals suggests that the mechanism of activation of an AGS4-Gα$_i$ complex is similar to that of receptor-mediated activation of Gαβγ heterotrimers and that GPCRs provide one mode of regulation of AGS4-Gα$_i$ complexes. As AGS4 and other proteins containing more than one GPR motif can bind multiple Gα$_i$ subunits simultaneously (7, 8, 19), these data have far reaching implications for G-protein signal processing and may provide cells with additional flexibility to modulate signal efficiency, specificity, duration, and location in ways previously unappreciated.

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[6] Attempts to isolate an α$_{2A}$-AR-Venus-Gα$_i$-AGS4-RLuc complex by co-immunoprecipitation revealed α$_{2A}$-AR-Venus-Gα$_i$ complexes (data not shown); however, the presence of AGS4-RLuc within this larger complex was inconsistent, suggesting either that the interaction of AGS4-Gα$_i$ complexes with α$_{2A}$-AR is too transient to be consistently observed by co-immunoprecipitation or that the complex is sensitive to the conditions required to solubilize the receptor from the membrane.

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