Signaling by a Non-dissociated Complex of G Protein βγ and α Subunits Stimulated by a Receptor-independent Activator of G Protein Signaling, AGS8*

Chujun Yuan1, Motohiko Sato6,1, Stephen M. Lanier2,5, and Alan V. Smrcka3,6

From the Departments of 6Pharmacology and Physiology and of 4Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 and the 5Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112

Accumulating evidence suggests that heterotrimeric G protein activation may not require G protein subunit dissociation. Results presented here provide evidence for a subunit dissociation-independent mechanism for G protein activation by a receptor-independent activator of G protein signaling, AGS8. AGS8 is a member of the AGS group III family of AGS proteins thought to activate G protein signaling primarily through interactions with Gβγ subunits. Results are presented demonstrating that AGS8 binds to the effector and a subunit binding “hot spot” on Gβγ yet does not interfere with Gα subunit binding to Gβγ or phospholipase C β2 activation. AGS8 stimulates activation of phospholipase C β2 by heterotrimeric Gαβγ and forms a quaternary complex with Gαi1, Gβ1, Gγ2, and phospholipase C β2. AGS8 rescued phospholipase C β binding and regulation by an inactive β subunit with a mutation in the hot spot (β2(W99A)) that normally prevents binding and activation of phospholipase C β2. This demonstrates that, in the presence of AGS8, the hot spot is not used for Gβγ interactions with phospholipase C β2. Mutation of an alternate binding site for phospholipase C β2 in the amino-terminal coiled-coil region of Gβγ prevented AGS8-dependent phospholipase C binding and activation. These data implicate a mechanism for AGS8, and potentially other Gβγ binding proteins, for directing Gβγ signaling through alternative effector activation sites on Gβγ in the absence of subunit dissociation.

4 The abbreviations used are: α-NT, Gα subunit amino-terminal helix; bβγ, biotinylated βγ; AGS, activator of G protein signaling; GRK2, G protein-coupled receptor kinase 2; GST, glutathione S-transferase; PLC, phospholipase C; GDPγS, guanosine 5’-O-(thiotriphosphate); b, biotinylated; wt, wild type; tt, trypsin-treated.
example, activators of G protein signaling (AGS proteins), discovered in a yeast-based screen for receptor-independent activation of the pheromone pathway, are a group of proteins that stimulate G protein signaling (11, 12). The best characterized of the AGS proteins act through interactions with G protein \( \alpha \) subunits to dissociate the G protein heterotrimer either through nucleotide exchange-dependent (Class I AGS proteins) or -independent mechanisms (Class II AGS proteins). Here we explore the mechanism for G protein activation by a recently described AGS protein, AGS8, which binds to G protein \( \beta \gamma \) subunits (13). AGS8 forms a complex interacting with \( \text{G} \alpha \) and \( \text{G} \beta \gamma \) simultaneously, occupies the \( \text{G} \beta \gamma \) "hot spot," a critical effector binding and signal transfer region on \( \text{G} \beta \gamma \) (14–17), yet does not dissociate \( \text{G} \alpha \) from \( \text{G} \beta \gamma \) subunits. In complexes between \( \text{G} \alpha \), \( \beta \gamma \), and AGS8, a signal transfer region on \( \text{G} \beta \gamma \) that does not involve the hot spot is critical for signaling to phospholipase C (PLC) \( \beta \). This introduces a concept for G protein \( \beta \gamma \) subunit signaling where amino acids outside the \( \text{G} \alpha \)-\( \beta \gamma \) subunit interface drive \( \text{G} \beta \gamma \) signaling and provides a model for how non-dissociated G protein complexes can activate downstream targets and a potential modified alternative of the clam shell hypothesis.

**EXPERIMENTAL PROCEDURES**

**Materials—** Peptides (SIRK, SIRKALNILGYPDYD; SIGK, SIGKAFKILGYPDYD; and SIRK(L9A) SIRKALNILGYPDYD) were synthesized by Alpha Diagnostics International, purified by high-performance liquid chromatography to >90% purity, and their identity was confirmed by mass spectrometry analysis. Phosphatidylinositol 4,5-bisphosphate and phosphatidylethanolamine were obtained from Avanti Polar Lipids, nickel-nitritroliacetic acid-agarose was from Qiagen, GTP \( \gamma \S \) was from Sigma, glutathione-Sepharose and anti-GST antibody were from GE Healthcare, TetraLink\textsuperscript{TM} tetrameric avidin resin was from Promega (Madison, WI), anti-\( \text{G} \alpha \) antibody was from Oncogene.

**Purification of \( \beta \gamma \) and \( \alpha \) Subunits—** \( \text{G} \beta \gamma_{12} \) or biotinylated \( \text{G} \beta \gamma_{12} \) (b\( \text{G} \beta \gamma_{12} \)) subunits were purified from 2 liters of Sf-9 cells triply infected with His\textsubscript{C}-\( \text{G} \alpha \text{11} \), wild-type, or b\( \text{G} \beta \gamma \) subunits, and G\textsubscript{\( \gamma \)}\textsubscript{11} subunits essentially as described (18). All of the alanine-substituted \( \text{G} \beta \gamma \) subunit mutants used in this study were modified with a biotin acceptor peptide at the amino terminus and were biotinylated. These mutants were expressed with G\textsubscript{\( \gamma \)}\textsubscript{11} in 200 ml of SF9 cells and partially purified as described in a previous study (16). Experiments comparing alanine-substituted \( \text{G} \beta \gamma \) activities to wt \( \text{G} \beta \gamma \) utilized wt \( \text{G} \beta \gamma \) subunits partially purified in parallel with the mutants for direct comparison. Myristoylated \( \text{G} \alpha \text{11} \) was purified from *Escherichia coli* as previously described (19) and bound GTP\( \gamma \S \) (0.5 mol/mol of protein) was determined by an Amido Black protein assay.

**Expression and Purification of GST-tagged cAGS8—** The coding sequence of cAGS8 (A1359-W1730 (cDNA\#1–16)) was fused in-frame to GST in the pGEX-4T vector (GE Healthcare) and expressed and purified from *E. coli* BL21/DE3 cells as previously described (13) with some modification. Overnight cultures grown in Luria broth containing ampicillin (50 \( \mu \)g/ml) were diluted to an \( A_{600} \) equal to 0.5–0.6 and induced with 0.1 mm isopropyl 1-thio-\( \beta \)-D-galactopyranoside for 3 h at 30 °C. Fusion proteins from a 1-liter culture were purified following the previously described protocol except prior to elution the glutathione-Sepharose column was washed with 5 mm ATP and a denatured *E. coli* protein extract to remove contaminating GroEL. The resulting eluted protein was 80% pure and dialyzed against 50 mm HEPES, pH 7.5, 50 mm NaCl, 1 mm dithiothreitol, and protease inhibitors. Protein concentrations were determined by Amido Black protein assay, and purified proteins were snap frozen and stored at −80 °C.

**Protein Binding Assays—** GST-cAGS8 or GST was incubated with either purified G\textsubscript{\( \alpha \)}\text{11}, or b\( \text{G} \beta \gamma_{12} \), or various b\( \text{G} \beta \gamma \) mutants in 200 \( \mu \)l of binding buffer (20 mm HEPES, pH 8.0, 1 mm EDTA, 1 mm MgCl\textsubscript{2}, 0.1% C\textsubscript{12}E\textsubscript{10}, 1 mm dithiothreitol, 150 mm NaCl, 10 \( \mu \)m GDPS). The binding mixture was rotated at 4 °C overnight. 40 \( \mu \)l of 50% glutathione-Sepharose was added to the binding mixture, and the mixture was further rotated for 1 h. The glutathione matrix was washed with three rounds of centrifugation/washing in binding buffer. Identical conditions were used for avidin-agarose isolations of b\( \text{G} \beta \gamma_{12} \), except avidin-agarose was used. Proteins bound in precipitates were identified by immunoblotting following PAGE. In some cases immunoblots were quantitated by chemiluminescence imaging with a charge-coupled device camera. This has a much larger dynamic range than film and overcomes issues with signal saturation observed with x-ray film.

**Gel-filtration Chromatography—** Various combinations of GST-cAGS8, G\textsubscript{\( \alpha \)}\text{11}, G\textsubscript{\( \beta \gamma \)}\text{12}, and PLC\textsubscript{\( \beta \)} (100 nm each) were incubated in 500 \( \mu \)l of gel-filtration buffer (20 mm HEPES, pH 8.0, 1 mm EDTA, 1 mm MgCl\textsubscript{2}, 0.1% C\textsubscript{12}E\textsubscript{10}, 1 mm dithioreitol, 150 mm NaCl, 10 \( \mu \)m GDPS, and protease inhibitor mixture) at 4 °C for 2 h. Then the mixture was applied to tandem Superdex 75/200 columns (GE Healthcare) pre-equilibrated with gel-filtration buffer and resolved at a flow rate of 0.4 ml/min at 4 °C. 1-ml fractions were collected. An aliquot of each fraction (20 \( \mu \)l) was analyzed by SDS-PAGE on a 12% polyacrylamide gel and visualized by silver staining. No binding of any of the proteins to GST was detected in the gel-filtration assay (data not shown).

**GTP\( \gamma \S \) Binding Assay—** This assay was performed as described previously (20) except without membranes or receptors. Briefly, 120 nm G\textsubscript{\( \alpha \)}\text{11} (6 pmol), in the presence or absence of 180 nm GST-cAGS8 and with or without 240 nm G\textsubscript{\( \beta \gamma \)}\text{12} subunits, were incubated with 0.4 \( \mu \)m GTP\( \gamma \S \) ([\( ^{35} \)S]GTP\( \gamma \S \), ~5000 cpm/pmol specific radioactivity in the assay) in the presence of 2 \( \mu \)m GDP to suppress the basal rate of nucleotide exchange by G\textsubscript{\( \alpha \)}\text{11}. Bound [\( ^{35} \)S]GTP\( \gamma \S \) detected by binding to nitrocellulose filters.

**PLC Assay—** PLC assays were performed as described previously (21) and in the figure legends.

**Measurement of \( \text{G} \alpha \)-\( \beta \gamma \) Interactions by Flow Cytometry—** Equilibrium binding of fluorescein isothiocyanate-labeled myristoylated G\textsubscript{\( \alpha \)}\text{11} to b\( \text{G} \beta \gamma \) subunits was measured using flow cytometry as has been previously described (22–24). Nonspecific binding, determined by the simultaneous addition of 300 pm fluorescein isothiocyanate-labeled myristoylated G\textsubscript{\( \alpha \)}\text{11} and 50 nm myristoylated G\textsubscript{\( \alpha \)}\text{11} subunits to the b\( \text{G} \beta \gamma \) bound beads, was 10–20% of the total signal and was subtracted from the
Signaling by a Non-dissociated G Protein Complex

mean channel numbers from each experiment unless otherwise indicated.

Trypsin Digestion of Ga,i,–3 μg of Ga,i was mixed with 0.15 μg of 1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (New England Biolabs Inc.) and incubated in 100 μl of digestion buffer (20 mM Tris, pH 8.0, 0.6 mM EDTA, 5 mM MgCl2, 1 mM dithiothreitol, 70 mM NaCl, 10 μM GDP, 30 μM AlCl3, and 10 mM NaF) at 25 °C for 1 h. The digestion reaction was terminated by adding 5 mM 1-chloro-3-tosylamido-7-amino-2-heptanone. The digestion was analyzed by PAGE and visualization with Coomassie Blue staining. The protein concentration after digestion was determined with an Amido Black protein assay. To confirm that trypsin-treated Ga,i still bound to cAGS8, binding to GST-cAGS8 was analyzed in a GST pull-down assay as has been described in parallel with undigested Ga,i.

RESULTS

AGS8 Binds to a Site on Gβ1γ2 That Overlaps the Ga Binding Site Yet Does Not Affect Binding of Ga to Gβγ—The carboxy-terminal domain of AGS8 (amino acids A1359-W1730, cAGS8) binds to Gβγ and activates Gβγ signaling in yeast and in mammalian cells transfected with G protein subunits (13). To understand the molecular mechanism for how a protein can bind to Gβγ and simultaneously propagate Gβγ signaling, we investigated the molecular nature of the interaction between Gβγ and cAGS8. To initially identify and characterize a binding site for cAGS8 on Gβγ we used peptide competition and mutagenic analyses. The peptides SIGK and SIRK bind to a hot spot on Gβ1γ2 that interacts with multiple effectors, including PLCβ2, and corresponds to the Ga subunit switch II binding site (2, 14, 16, 25). SIGK and SIRK compete for binding of both α subunits and PLCβ2 to Gβ1γ2 (15, 23). These peptides also blocked binding of cAGS8 to Gβ1γ2, indicating the binding sites for the peptides, PLCβ2, Ga subunits and cAGS8 overlap on the surface of Gβ1γ2 (Fig. 1A). A control peptide that does not bind Gβ1γ2, SIRK (L9A) (15), had little effect on cAGS8-Gβ1γ2 interactions.

To identify specific amino acids within the Gβγ hot spot site required for cAGS8 interactions, GST-cAGS8 binding to a series of alanine-substituted bGβ1γ2 subunit mutants within the hot spot was tested (Fig. 1, B and C; see Fig. 10). Compared with wild-type Gβγ, Gβ1,W99A, Gβ1,D186A, Gβ1,M188A, and Gβ1,H311A (Gβ1,H311 is outside the hot spot and is a control) mutations did not affect binding to cAGS8, whereas Gβ1,K57A, Gβ1,Y59A, Gβ1,M101A, Gβ1,L17A, Gβ1,Y145A, and Gβ1,N230A mutations all significantly inhibited binding of Gβ1γ2 to cAGS8. These data complement the peptide competition data and clearly demonstrate that cAGS8 interacts with the hot spot on Gβ1γ2.

Many of these amino acids are required for activation of effectors, including PLCβ2, and are directly at the Ga-βγ binding interface (Fig. 10) (3, 26). Thus binding of cAGS8 to this region would be expected to preclude Ga subunit binding and effector activation. We used flow cytometry to analyze Ga-βγ interactions as previously described (22–24). As previously shown, the hot spot-binding peptide, SIGK, competes with Ga,i for binding to Gβ1γ2 in a concentration-dependent manner (Fig. 2A) consistent with its apparent Ki for Gβγ of ~1 μM (22); however, cAGS8 did not inhibit Ga binding to Gβγ, despite apparently binding to the same site as SIGK and Ga subunit switch II. The highest concentration of cAGS8 tested, 1 μM, was higher than concentrations shown to have maximal functional effects on Gβγ signaling (see Figs. 5B and 9C for examples) and higher than the concentration required for strong binding in the GST pulldown assay and formation of stable complexes by gel filtration.

cAGS8 Binds to Ga Subunits and Gβγ Simultaneously—SIGK peptide binds to the switch II binding site and competes for α subunit binding to Gβγ. How could cAGS8 occupy the same binding site on Gβγ yet not compete for Ga subunit binding to Gβγ? We hypothesized that, if cAGS8 bound both Gβγ and Ga simultaneously, then the complex would not be disrupted. It had previously been shown that Ga,i did not bind

FIGURE 1. cAGS8 binds to a domain on the Gβ subunit that overlaps with Ga binding. A, hot spot binding peptides block Gβγ-cAGS8 interactions. 300 nM GST-cAGS8 was incubated with 30 nM purified Gβγ, with or without 20 μM peptides in binding buffer. Proteins bound to GST-cAGS8 were detected with immunoblotting as described under "Experimental Procedures." Samples were also immunoblotted with anti-GST antibodies to validate loading equal amounts of protein (not shown). Data shown are representative of three independent experiments. This Western blot was quantified by chemiluminescence imaging; percent inhibition values relative to control were 20% (L9A), 89% (SIGK), and 89% (SIRK). B, GST pulldown assay showing the effect of various bGβ, hot spot mutants on bGβγ-cAGS8 interaction. 300 nM GST-cAGS8 protein was incubated with 30 nM wt bGβγ, or with 30 nM various bGβ mutants (all partially purified and quantitated in parallel (16)) in binding buffer. The bottom panel is a Western blot of the suspension prior to pulldown assay, demonstrating that equal amounts of each Gβγ mutant were included in each pulldown assay. Data shown are representative of three independent experiments. C, Western blots from two separate experiments as in B were quantified by chemiluminescence imaging, and the data were pooled and plotted relative to wt Gβγ binding. Data are mean ± S.E.
Signaling by a Non-dissociated G Protein Complex

4). As a control to demonstrate that Gαi is activated by AlF₄⁻ in this assay, the Gαᵢ₁ subunit was tested for AlF₄⁻ activation-dependent protection from trypsin digestion. In the absence of AlF₄⁻ treatment there is no detectable binding of Gαᵢ₁ pretreated with trypsin, because the inactive conformation of Gαᵢ₁ is not resistant to trypsin digestion and is degraded prior to binding, while a stable proteolytic fragment of Gαᵢ₁ is formed in the presence of trypsin and AlF₄⁻ that binds to GST-cAGS8 (27). This demonstrates that the Gαᵢ₁ subunit is activated by AlF₄⁻ treatment. The overall conclusion is that the AlF₄⁻-dependent activation does not significantly alter binding of Gαᵢ₁ to AGS8. GST-cAGS8 also did not affect the rate of binding of GTPγS to Gαᵢ₁ or Gβγᵢ₂, indicating that AGS8 is not a guanine nucleotide exchange factor (Fig. 2C). These data are consistent with the observation that the cAGS8-mediated activation of G-protein signaling in the yeast-based functional screen was independent of guanine nucleotide exchange (13).

To assess binding of cAGS8 to G protein heterotrimer, 30 nm Gαᵣ, GDP and 30 nm Gβᵣγᵢ₂ subunit were incubated together with varying concentrations of cAGS8 and compared with binding to either subunit alone. Gαᵣ and Gβᵣγᵢ₂ subunit bound simultaneously to substoichiometric concentrations of GST-cAGS8 (Fig. 3, lane 9, with 10 nm cAGS8 and 30 nm for each subunit), and binding as a function of GST-cAGS8 concentration was more efficient for both subunits together than for either of the subunits alone (Fig. 3A, lanes 6–9 compared with lanes 2–5, Fig. 3B, lanes 6–9 compared with lanes 2–5). The data in Fig. 3 (C and D) are compiled from quantitative analysis of chemiluminescence intensities from three separate binding experiments each. We could not estimate an accurate Kᵣ for complex formation from this analysis, but GST-cAGS8 consistently gave stronger binding signals with the heterotrimer than with either subunit alone. The simplest explanation for this data is that cAGS8 binds to a complex of α and βγ and that the affinity for this complex is greater than for either subunit alone.

To provide further evidence for formation of the ternary complex we performed gel-filtration chromatography to evaluate the size and composition of potential complexes between Gαᵢ₁-GDP, Gβᵣγᵢ₂, and GST-cAGS8 (Fig. 4). All of the individual proteins and the heterotrimer elute at approximately their predicted molecular masses, including GST-cAGS8, which elutes as an apparent monomer (monomeric molecular mass predicted to be 75 kDa). Incubation of GST-cAGS8 with either Gαᵢ₁-GDP or Gβᵣγᵢ₂ resulted in the appearance of cAGS8 and either Gαᵢ₁ or Gβᵣγᵢ₂ in an earlier fraction (fraction 24) than either of the G protein subunits alone (fraction 27) at a molecular weight indicative of formation of a 1:1 complex between GST-cAGS8 and either Gαᵢ₁ or Gβᵣγᵢ₂. The proportion of cAGS8 associated with a Gβᵣγᵢ₂ subunit complex appears to be greater than the proportion of cAGS8 with Gαᵣᵢ₁ present with higher affinity binding of cAGS8 to Gβᵣγᵢ₂ relative to Gαᵣᵢ₁. When cAGS8 was mixed with Gαᵣᵢ₁ and Gβᵣγᵢ₂ together, the three proteins eluted earlier (fraction 23) than the individual cAGS8-Gαᵣᵢ₁ or cAGS8-Gβᵣγᵢ₂ complexes at a position consistent with the predicted molecular weight of a 1:1:1 complex, indicating formation of a stable ternary complex containing GST-cAGS8, Gαᵣᵢ₁, and Gβᵣγᵢ₂.
cAGS8 Binds to a Rearranged Gαβγ Complex—Experiments were conducted to determine how the complex between GST-cAGS8 and the G protein heterotrimer is assembled. There are two interaction sites between Gα and Gβγ: the Gα switch II interacts with the hot spot on Gβ, and the amino-terminal helix of Gα (α-N) interacts with blade I of the Gβ propeller (2) (see purple helix, Fig 10A). We hypothesized that cAGS8 binding competes for a subunit switch II interactions with the hot spot, but interactions of Gα-NT with the Gβ blade region are maintained. In the complex, simultaneous Gα subunit interactions with cAGS8 bound to the hot spot and with Gβ blade 1 result in a bivalent interaction that might be strong enough to maintain a stable ternary complex. To test this idea Gα3 subunit missing the amino-terminal 21 amino acids (Gα(tt)) of the ∼30-amino acid Gα-NT was prepared by limited trypsin digestion. Trypsin treatment of the AlF₄⁻-activated Gα subunit results in specific cleavage at Arg-21 of Gα₃ (27, 28) and formation of a stable trypsin-resistant fragment, as discussed earlier. This trypsin-

digested Gα subunit still binds to cAGS8 (Fig. 2B), but, as has been previously reported, Gα(tt) did not bind Gβγ (Fig. 4B, top three panels). If interactions between Gα-NT with Gβγ are required for formation of the ternary complex then it would be expected that Gα(tt) would not form a complex with cAGS8 and Gβγ. Indeed when GST-cAGS8 was mixed with trypsintreated Gα₃ and Gβγ, only the GST-CAGS8-Gβγ complex was formed eluting in fraction 24, with no formation of the GST-CAGS8/heterotrimeric complex in fraction 23. This clearly demonstrates that cAGS8 is binding to a non-dissociated complex of Gαβγ requiring interaction of Gα-NT with Gβγ to maintain the complex.

Gα₁,β₁γ₂-CAGS8 Ternary Complex Is Signaling Competent—AGS8 activates G protein signaling in yeast, and studies in transfected COS7 cells showed that transfection of cAGS8 could stimulate PLCβ₂ activation in cells transfected with Gα/βγ, suggesting that AGS8 can stimulate Gβγ signaling from a G protein heterotrimer. Thus the data demonstrating that cAGS8 binds to the heterotrimer raises a central question: How can cAGS8 binding to Gβγ or Gαβγ cause activation of G protein signaling to allow propagation of signaling by Gβγ?

Based on the data presented so far a simple model where cAGS8 competes for two interaction sites between GcAGS8 and the G protein heterotrimer is assembled. There are conducted to determine how the complex between GST-cAGS8 and the G protein heterotrimer is assembled. There are two interaction sites between Gα and Gβγ: the Gα switch II interacts with the hot spot on Gβ, and the amino-terminal helix of Gα (α-N) interacts with blade I of the Gβ propeller (2) (see purple helix, Fig 10A). We hypothesized that cAGS8 binding competes for a subunit switch II interactions with the hot spot, but interactions of Gα-NT with the Gβ blade region are maintained. In the complex, simultaneous Gα subunit interactions with cAGS8 bound to the hot spot and with Gβ blade 1 result in a bivalent interaction that might be strong enough to maintain a stable ternary complex. To test this idea Gα₃ subunit missing the amino-terminal 21 amino acids (Gα(tt)) of the ∼30-amino acid Gα-NT was prepared by limited trypsin digestion. Trypsin treatment of the AlF₄⁻-activated Gα subunit results in specific cleavage at Arg-21 of Gα₃ (27, 28) and formation of a stable trypsin-resistant fragment, as discussed earlier. This trypsin-
occupies the hot spot and competes with Ga for binding and releasing free Gβγ cannot be correct. Additionally, either “free” Gβγ or the cAGS8–αβγ complex would have cAGS8 bound at a critical effector binding site and be predicted to be unable to signal to effectors such as PLCβ2, because cAGS8 binding to the Gβγ hot spot requires amino acids also required for PLCβ2 activation. To determine if cAGS8 could block signaling by Gβγ, cAGS8 was tested for inhibition of Gβγ-dependent PLC activation. cAGS8 at concentrations as high as 1 μM did not affect PLCβ2 activation by Gβ1γ2, whereas SIGK peptide, which also binds the hot spot, potently and effectively inhibited Gβ1γ2-dependent activation (Fig. 5A). Thus Gβ1γ2 with cAGS8 bound to a key signaling interface can still regulate a down-regulated (Fig. 5C).

To directly test if cAGS8 could activate signaling by a G protein heterotrimer, the ability of purified GST-cAGS8 to stimulate PLCβ2 activation in vitro by Ga12,13-GDP/Gβ1γ2 was assessed. In the presence of stoichiometric amounts of Ga12,13-GDP, Gβ1γ2-dependent PLCβ2 activation is completely inhibited (Fig. 5B). cAGS8 restored the ability of Gβ1γ2 to activate PLCβ2 in the presence of Ga12,13-GDP, in a concentration-dependent manner with an EC50 of ~30 nM (Fig. 5B). Because cAGS8 does not dissociate Ga from Gβγ, the data suggest that the heterotrimeric G protein complex can stimulate PLCβ2 when bound to cAGS8. This is surprising, because both Ga12 and cAGS8 bind to a region on Gβγ thought to be required for PLCβ2 activation (14, 16).

**Complex Formation between cAGS8, Ga12–GDP, Gβγ, and PLCβ2—**To directly determine if a complex can be formed between GST-cAGS8, Ga12–GDP, Gβγ, and PLCβ2, immobilized avidin was used to precipitate bGγ12, whereas SIGK peptide, which also binds the hot spot, potently and effectively inhibited Gβ1γ2-dependent activation (Fig. 5A). Thus Gβ1γ2 with cAGS8 bound to a key signaling interface can still regulate a downstream target in vitro.

To directly test if cAGS8 could activate signaling by a G protein heterotrimer, the ability of purified GST-cAGS8 to stimulate PLCβ2 activation in vitro by Ga12,13-GDP/Gβ1γ2 was assessed. In the presence of stoichiometric amounts of Ga12,13-GDP, Gβ1γ2-dependent PLCβ2 activation is completely inhibited (Fig. 5B). cAGS8 restored the ability of Gβ1γ2 to activate PLCβ2 in the presence of Ga12,13-GDP, in a concentration-dependent manner with an EC50 of ~30 nM (Fig. 5B). Because cAGS8 does not dissociate Ga from Gβγ, the data suggest that the heterotrimeric G protein complex can stimulate PLCβ2 when bound to cAGS8. This is surprising, because both Ga12 and cAGS8 bind to a region on Gβγ thought to be required for PLCβ2 activation (14, 16).

**Complex Formation between cAGS8, Ga12–GDP, Gβγ, and PLCβ2**—To directly determine if a complex can be formed between GST-cAGS8, Ga12–GDP, Gβγ, and PLCβ2, immobilized avidin was used to precipitate bGγ12, whereas SIGK peptide, which also binds the hot spot, potently and effectively inhibited Gβ1γ2-dependent activation (Fig. 5A). Thus Gβ1γ2 with cAGS8 bound to a key signaling interface can still regulate a downstream target in vitro.

To directly test if cAGS8 could activate signaling by a G protein heterotrimer, the ability of purified GST-cAGS8 to stimulate PLCβ2 activation in vitro by Ga12,13-GDP/Gβ1γ2 was assessed. In the presence of stoichiometric amounts of Ga12,13-GDP, Gβ1γ2-dependent PLCβ2 activation is completely inhibited (Fig. 5B). cAGS8 restored the ability of Gβ1γ2 to activate PLCβ2 in the presence of Ga12,13-GDP, in a concentration-dependent manner with an EC50 of ~30 nM (Fig. 5B). Because cAGS8 does not dissociate Ga from Gβγ, the data suggest that the heterotrimeric G protein complex can stimulate PLCβ2 when bound to cAGS8. This is surprising, because both Ga12 and cAGS8 bind to a region on Gβγ thought to be required for PLCβ2 activation (14, 16).

**Complex Formation between cAGS8, Ga12–GDP, Gβγ, and PLCβ2**—To directly determine if a complex can be formed between GST-cAGS8, Ga12–GDP, Gβγ, and PLCβ2, immobilized avidin was used to precipitate bGγ12, whereas SIGK peptide, which also binds the hot spot, potently and effectively inhibited Gβ1γ2-dependent activation (Fig. 5A). Thus Gβ1γ2 with cAGS8 bound to a key signaling interface can still regulate a downstream target in vitro.

To directly test if cAGS8 could activate signaling by a G protein heterotrimer, the ability of purified GST-cAGS8 to stimulate PLCβ2 activation in vitro by Ga12,13-GDP/Gβ1γ2 was assessed. In the presence of stoichiometric amounts of Ga12,13-GDP, Gβ1γ2-dependent PLCβ2 activation is completely inhibited (Fig. 5B). cAGS8 restored the ability of Gβ1γ2 to activate PLCβ2 in the presence of Ga12,13-GDP, in a concentration-dependent manner with an EC50 of ~30 nM (Fig. 5B). Because cAGS8 does not dissociate Ga from Gβγ, the data suggest that the heterotrimeric G protein complex can stimulate PLCβ2 when bound to cAGS8. This is surprising, because both Ga12 and cAGS8 bind to a region on Gβγ thought to be required for PLCβ2 activation (14, 16).

**Complex Formation between cAGS8, Ga12–GDP, Gβγ, and PLCβ2**—To directly determine if a complex can be formed between GST-cAGS8, Ga12–GDP, Gβγ, and PLCβ2, immobilized avidin was used to precipitate bGγ12, whereas SIGK peptide, which also binds the hot spot, potently and effectively inhibited Gβ1γ2-dependent activation (Fig. 5A). Thus Gβ1γ2 with cAGS8 bound to a key signaling interface can still regulate a downstream target in vitro.

To directly test if cAGS8 could activate signaling by a G protein heterotrimer, the ability of purified GST-cAGS8 to stimulate PLCβ2 activation in vitro by Ga12,13-GDP/Gβ1γ2 was assessed. In the presence of stoichiometric amounts of Ga12,13-GDP, Gβ1γ2-dependent PLCβ2 activation is completely inhibited (Fig. 5B). cAGS8 restored the ability of Gβ1γ2 to activate PLCβ2 in the presence of Ga12,13-GDP, in a concentration-dependent manner with an EC50 of ~30 nM (Fig. 5B). Because cAGS8 does not dissociate Ga from Gβγ, the data suggest that the heterotrimeric G protein complex can stimulate PLCβ2 when bound to cAGS8. This is surprising, because both Ga12 and cAGS8 bind to a region on Gβγ thought to be required for PLCβ2 activation (14, 16).

**Complex Formation between cAGS8, Ga12–GDP, Gβγ, and PLCβ2**—To directly determine if a complex can be formed between GST-cAGS8, Ga12–GDP, Gβγ, and PLCβ2, immobilized avidin was used to precipitate bGγ12, whereas SIGK peptide, which also binds the hot spot, potently and effectively inhibited Gβ1γ2-dependent activation (Fig. 5A). Thus Gβ1γ2 with cAGS8 bound to a key signaling interface can still regulate a downstream target in vitro.

To directly test if cAGS8 could activate signaling by a G protein heterotrimer, the ability of purified GST-cAGS8 to stimulate PLCβ2 activation in vitro by Ga12,13-GDP/Gβ1γ2 was assessed. In the presence of stoichiometric amounts of Ga12,13-GDP, Gβ1γ2-dependent PLCβ2 activation is completely inhibited (Fig. 5B). cAGS8 restored the ability of Gβ1γ2 to activate PLCβ2 in the presence of Ga12,13-GDP, in a concentration-dependent manner with an EC50 of ~30 nM (Fig. 5B). Because cAGS8 does not dissociate Ga from Gβγ, the data suggest that the heterotrimeric G protein complex can stimulate PLCβ2 when bound to cAGS8. This is surprising, because both Ga12 and cAGS8 bind to a region on Gβγ thought to be required for PLCβ2 activation (14, 16).

**Complex Formation between cAGS8, Ga12–GDP, Gβγ, and PLCβ2**—To directly determine if a complex can be formed between GST-cAGS8, Ga12–GDP, Gβγ, and PLCβ2, immobilized avidin was used to precipitate bGγ12, whereas SIGK peptide, which also binds the hot spot, potently and effectively inhibited Gβ1γ2-dependent activation (Fig. 5A). Thus Gβ1γ2 with cAGS8 bound to a key signaling interface can still regulate a downstream target in vitro.

To directly test if cAGS8 could activate signaling by a G protein heterotrimer, the ability of purified GST-cAGS8 to stimulate PLCβ2 activation in vitro by Ga12,13-GDP/Gβ1γ2 was assessed. In the presence of stoichiometric amounts of Ga12,13-GDP, Gβ1γ2-dependent PLCβ2 activation is completely inhibited (Fig. 5B). cAGS8 restored the ability of Gβ1γ2 to activate PLCβ2 in the presence of Ga12,13-GDP, in a concentration-dependent manner with an EC50 of ~30 nM (Fig. 5B). Because cAGS8 does not dissociate Ga from Gβγ, the data suggest that the heterotrimeric G protein complex can stimulate PLCβ2 when bound to cAGS8. This is surprising, because both Ga12 and cAGS8 bind to a region on Gβγ thought to be required for PLCβ2 activation (14, 16).

**Complex Formation between cAGS8, Ga12–GDP, Gβγ, and PLCβ2**—To directly determine if a complex can be formed between GST-cAGS8, Ga12–GDP, Gβγ, and PLCβ2, immobilized avidin was used to precipitate bGγ12, whereas SIGK peptide, which also binds the hot spot, potently and effectively inhibited Gβ1γ2-dependent activation (Fig. 5A). Thus Gβ1γ2 with cAGS8 bound to a key signaling interface can still regulate a downstream target in vitro.

To directly test if cAGS8 could activate signaling by a G protein heterotrimer, the ability of purified GST-cAGS8 to stimulate PLCβ2 activation in vitro by Ga12,13-GDP/Gβ1γ2 was assessed. In the presence of stoichiometric amounts of Ga12,13-GDP, Gβ1γ2-dependent PLCβ2 activation is completely inhibited (Fig. 5B). cAGS8 restored the ability of Gβ1γ2 to activate PLCβ2 in the presence of Ga12,13-GDP, in a concentration-dependent manner with an EC50 of ~30 nM (Fig. 5B). Because cAGS8 does not dissociate Ga from Gβγ, the data suggest that the heterotrimeric G protein complex can stimulate PLCβ2 when bound to cAGS8. This is surprising, because both Ga12 and cAGS8 bind to a region on Gβγ thought to be required for PLCβ2 activation (14, 16).
GST, or GST-cAGS8 (Fig. 6B, lanes 4 – 6). If Go11 subunit was added with PLCβ2, only Go11 bound to GST-cAGS8 (Fig. 6B, lane 7). If Gβ1γ2 and PLCβ2 were added, both Gβ1γ2 and its bound PLCβ2 were isolated with GST-cAGS8 (Fig. 6B, lane 8). Addition of Go11 did not prevent binding of PLCβ2 to this complex, and binding of Go11 was enhanced in the complex compared with binding of Go11 alone (Fig. 6B, lane 9). These data further confirm the existence of a quaternary complex between cAGS8, PLCβ2, Go11, and Gβ1γ2, with PLC interacting with the heterotrimer only when cAGS8 is present.

Finally we used gel filtration to demonstrate that cAGS8 promotes binding of PLCβ2 to the heterotrimer (Fig. 7). In these experiments PLCβ2 eluted as an apparent monomer. When PLCβ2 was mixed with Gβ1γ2, prior to gel filtration (PLCβ2 plus Gβ1γ2) both PLCβ2 and Gβγ eluted earlier than either protein alone, indicating complex formation. When PLCβ2 was mixed with the G protein heterotrimer (PLCβ2 plus Gβ1γ2 plus Go11), a small proportion of the PLCβ2 eluted at a higher molecular weight, but only Gβγ was associated with this complex indicating that PLC formed a stable complex with the free Gβ1γ2 in the mixture but not with the heterotrimer. In contrast, when GST-cAGS8 was added to the G protein heterotrimer (cAGS8 plus PLCβ2 plus Gβ1γ2 plus Go11), all of the proteins eluted at a much higher molecular weight than any of the individual proteins or complexes demonstrating formation of a stable complex between GST-cAGS8, Go11, Gβ1γ2, and PLCβ2. This demonstrates conclusively that bound cAGS8 promotes association of PLCβ2 with the cAGS8-Gα/βγ complex despite AGS8 binding to a key signaling surface on Gβγ.

**cAGS8 Rescues the Loss of Function of Gβγ(W99A)γ2 Mutant—**

The currently accepted model for activation of Gβγ signaling to downstream effectors is that separation of Gβγ from Gα uncovers the hot spot on Gβγ allowing for the binding of effectors to this region (3). In the presence of cAGS8, or both cAGS8 and Gα subunit, this surface would be expected to be occupied and the resulting complexes would be unable to signal downstream. Nevertheless, we demonstrated that both the cAGS8-Gβγ complex and the cAGS8-Gαβγ complex can bind and activate PLCβ2. To understand this we hypothesized that, in these complexes, the hot spot on Gβγ is no longer utilized for PLCβ2 activation, and a new activation site is exposed in the cAGS8-Gβγ complexes that can activate PLCβ2. To test this idea we mutated a hot spot for PLCβ2 activation, but not cAGS8 binding, was analyzed. It had been previously reported that Gβγ(W99A)γ2 is defective for PLCβ2 activation (14), but Gβγ(W99A)γ2 still bound efficiently to cAGS8 (Fig. 1C, see Fig 10A, *blue amino acid*). If a new binding/signal transfer surface for PLC activation was created in the presence of cAGS8, then binding of cAGS8 might rescue PLCβ2 binding and activation by Gβγ(W99A)γ2. First binding of PLCβ2 to bGβγγ2 and bGβγ(W99A)γ2 was tested (Fig. 8A). Mutation of Gβγ(W99A) to Ala significantly inhibited binding of PLCβ2, indicating that this amino acid, previously shown to be important for activation of PLCβ2, is also important for binding to PLCβ2 (Fig. 8A, lanes 6 and 7). Addition of cAGS8 rescued binding of PLCβ2 to bGβγγ2 to levels approaching binding to wt Gβγγ2 (Fig. 8A, compare lanes 6, 7, and 9); whereas the binding of PLCβ2 to wt Gβγγ2 did not change in the absence or presence of cAGS8 (Fig. 8A, compare lanes 6 and 8).

Next it was determined if cAGS8 could restore the ability of Gβγ(W99A)γ2 to activate PLCβ2 (Fig. 8B). Wild-type Gβγγ2 stimulated PLCβ2 12-fold, whereas mutation of W99 to Ala greatly impaired this activation. cAGS8 restored the capacity of Gβγ(W99A)γ2 to activate PLCβ2 to near wt Gβγγ2 levels.
cAGS8 itself did not stimulate PLCβ2, as described earlier and cAGS8 did not affect wt Gβ1γ2-dependent activation of PLCβ2. Together these data indicate that cAGS8 unmask and/or creates a new binding and signal transfer site for PLCβ2 that does not involve the hot spot on Gβγ.

**cAGS8 May Unmask a New Binding Surface at the Amino Terminus of Gγ—**The data support a model where AGS binds to the heterotrimer with simultaneous interactions with Gβγ at the hot spot and the Ga subunit and that the hot spot is no longer used for signal transfer to PLCβ2 activation, because it is occupied with cAGS8. To accommodate this model we propose that another site for effector interactions is unmasked on Gβγ that signals to downstream effectors. We recently presented evidence that PLCβ2 can bind to two distinct regions on Gβ, at the amino-terminal coiled-coil domain, and at the hot spot (29). In this earlier work it was proposed that association of PLCβ2 with the Gβγ-coiled region inhibited PLCβ2 activation.

To test whether the amino-terminal region on the Gβ subunit might participate in activation of PLCβ2 by the cAGS8-Gβγ complex, binding of PLCβ2 to a β subunit mutant that no longer interacts with PLCβ2 at the amino terminus (bGβ2, 23–27;γ2) (29) was tested in the presence or absence of cAGS8 (Fig. 9A, see Fig 10A, yellow amino acids). Addition of cAGS8 greatly inhibited the binding of PLCβ2 to the bGβ2, 23–27;γ2 (mutant, but not wild-type Gβ1γ2 (Fig. 9A, compare lanes 7, 9, and 10). The Gβ2, 23–27;γ2 mutation did not significantly alter interactions with PLCβ2 in the absence of cAGS8 (Fig. 9A, compare lane 7 and 8). This data were confirmed by gel-filtration analysis where, in contrast to data in Fig. 7 for the wild-type heterotrimer, cAGS8 did not promote binding of PLCβ2 to a heterotrimer containing bGβ2, 23–27;γ2 (compare Figs. 7 and 9B). Free bGβ2, 23–27;γ2 associates with PLCβ2, presumably through the hot spot, indicating that this mutant Gβ subunit protein is viable.

This indicates that the amino-terminal coiled-coil binding region on Gβ binds PLCβ2 when the hot spot is occupied by cAGS8. We next tested whether activation of PLCβ2 by cAGS8-Gβγ complex was affected by the amino-terminal mutation. We predicted that, if this alternate binding site for PLCβ2 was disabled, the Gβγ would no longer be able to activate PLCβ2 with cAGS8 bound to the hot spot. cAGS8 did not significantly inhibit wild-type Gβ1γ2-stimulated PLCβ2 activity (see also Fig. 5A). However, cAGS8 inhibited bGβ2, 23–27;γ2-stimulated PLCβ2 activity with an IC₅₀ of ~15 nM, indicating that the amino-terminal interaction region was in fact a second site required for stimulation of PLCβ2 activity by the cAGS8-Gβγ complex. This also suggests that the apparently inhibiting activity of the amino terminus toward PLCβ2 participates in activation of PLCβ2 upon cAGS8 binding.

**DISCUSSION**

Here we describe a mechanism for activation of G protein signaling that does not involve nucleotide exchange or subunit dissociation. Multiple assays of direct binding demonstrate that cAGS8 binds to the G protein heterotrimer and promotes binding of PLCβ2 to Gβγ subunits to form a quaternary signaling complex. Gel-filtration analysis demonstrates that the quaternary complex is stable, indicating the proteins in the complex interact with relatively high affinity. In addition, AGS8 modulated G protein-dependent PLC regulation with EC₅₀ or IC₅₀ values of ~30 nM (Figs. 5B and 9C). A key experiment, supporting the idea that subunit dissociation is not needed for signaling in the quaternary complex, demonstrated that cAGS8 could rescue the function of a mutant Gβγ (Gβγ,W99A) with the hot spot at the Ga/βγ interface disabled for PLCβ2 binding and activation. This experiment rules out the possibility that undetected free Gβγ might be generated upon AGS8 binding that somehow transiently reveals the hot spot for PLCβ2 binding.

**FIGURE 9.** The amino-terminal coiled-coil region of Gβγ is an alternate site required for PLCβ2 binding and activation in the presence of AGS8. A, AGS8 inhibits binding of Gβ1,(23–27)γ2 to PLCβ2. 30 nM bGβ2,γ2 or bGβ2, (23–27)γ2 mutant was mixed with 30 nM PLCβ2, and/or 100 nM GST-AgS8 in binding buffer. Pulldown assay with avidin-agarose was performed as described under “Experimental Procedures.” Data are representative of three independent experiments. Western blots of PLCβ2 from two separate experiments as in A were quantified by chemiluminescence imaging, and the data were pooled and calculated as a percentage of lane 7. PLCβ2 binding to wt Gβ1γ2 alone: lane 8, Gβ1,(23–27)γ2 plus PLCβ2 (97 ± 2%); lane 9, wt Gβ1γ2 plus PLCβ2 plus GST-AgS8 (98 ± 4%); lane 10, Gβ1,(23–27)γ2 plus PLCβ2 plus GST-AgS8 (12 ± 1%). Data are means ± S.E. B, Gβ1,(23–27)γ2 does not form a complex with PLCβ2 in the presence of cAGS8. Gel-filtration analysis was performed as described under “Experimental Procedures” and in legends for Figs. 4 and 7 except bGβ1,(23–27)γ2 was analyzed. C, AGS8 inhibited Gβ1,(23–27)γ2 activation of PLCβ2. PLC assays were performed as described in Fig. 5A with 1 ng of purified PLCβ2 assay, 30 nM Gβ1,(23–27)γ2, (α), or 30 nM wt Gβ1γ2 (β), in the presence of varying concentrations of AGS8. In this experiment basal PLC activity was 5.4 nmol/mg/min and with wt Gβ1γ2-stimulated activity was 25 μmol/mg/min. However, Gβ1,(23–27)γ2-stimulated activity was 42 μmol/mg/min (as previously reported, bGβ1,(23–27)γ2 stimulates PLC activity more effectively than wt Gβ1γ2, 29). Data are representative of experiments performed at least three times.
and activation. Clearly cAGS8 cannot be acting simply by dissociating Ga from Gβγ to expose the hot spot on Gβγ.

It was initially surprising that cAGS8 binding to the hot spot on Gβγ did not result in subunit dissociation, whereas the peptide SIGK that binds to an overlapping site promotes subunit dissociation (16, 22). These observations can be reconciled by the observation that AGS8, but not SIGK, also exhibits some dissociation (16, 22). These observations can be reconciled by the observation that AGS8, but not SIGK, also exhibits some dissociation (16, 22). These observations can be reconciled by the observation that AGS8, but not SIGK, also exhibits some dissociation (16, 22). These observations can be reconciled by the observation that AGS8, but not SIGK, also exhibits some dissociation (16, 22). These observations can be reconciled by the observation that AGS8, but not SIGK, also exhibits some dissociation (16, 22). These observations can be reconciled by the observation that AGS8, but not SIGK, also exhibits some dissociation (16, 22). These observations can be reconciled by the observation that AGS8, but not SIGK, also exhibits some dissociation (16, 22). These observations can be reconciled by the observation that AGS8, but not SIGK, also exhibits some dissociation (16, 22). These observations can be reconciled by the observation that AGS8, but not SIGK, also exhibits some dissociation (16, 22).

Because GβW99A disables the hot spot for PLCβ2 binding and activation, cAGS8 itself did not bind to or activate PLCβ2, and cAGS8 occupied the region on the Gβ subunit required for PLCβ2 binding. The only way that cAGS8 could rescue the binding and activation of PLCβ2 by GβW99A would be by creating a new binding site for PLCβ2 in the cAGS8-Gβγ complex. Site-directed mutagenesis has shown that there are contact surfaces outside of the hot spot that are important for effector recognition (29, 31).

A binding site for PLCβ2 in the amino-terminal coiled-coil region of the Gβγ subunits has previously been identified by chemical cross-linking and site-directed mutagenesis (29, 32). In those studies it was demonstrated that disabling this binding surface by site-directed mutagenesis resulted in increased Gβγ-dependent activation of PLCβ2 leading to the conclusion that this binding site for PLC was inhibitory while the binding site in the hot spot was stimulatory. Here it is demonstrated that the Gβγ coiled-coil region is required for binding and activation of PLCβ2 when AGS8 is bound. This suggests that this binding site is converted from an inhibitory to a stimulatory site in the presence of cAGS8. It is also possible that PLCβ2 binding and activation in the complex requires both binding to the Gβγ amino terminus and cAGS8, although no binding or activation of PLCβ2 by cAGS8 alone was detected. These data suggest a signaling mechanism for non-dissociated G protein complexes that uses binding sites outside the Gaβγ interface for effector activation.

Interestingly, the signal transfer surfaces of the Gβγ subunit in yeast, *Saccharomyces cerevisiae*, have not been shown to involve amino acids at the Gaβγ interface but rather have been mapped to a region in the amino-terminal coiled-coil at a position that is nearly identical to the region we described for PLC binding (33–35). In contrast to the hot spot region where Gγ subunits cannot directly contribute to binding, the more variable Gγ subunits could conceivably directly contribute to this contact interface. Thus some signaling specificity could be supplied by binding effectors to this region.

Mechanisms for activation of G protein signaling by group II AGS proteins are distinct from receptor-mediated mechanisms for G protein activation but involve an apparently straightforward subunit dissociation-based mechanism where the GPR motif of the AGS protein binds Ga-switch II and either promotes subunit dissociation or competes for Gβγ subunit binding, leading to accumulation of free Gβγ (22, 36, 37). Here we describe a model for Gβγ activation that does not involve revealing the hot spot on Gβγ for PLC binding but rather involves alternative utilization of binding sites outside the Gaβγ interface upon binding of the activator AGS8. This mechanism has significant implications for mechanisms of sig-
signaling by non-dissociated G protein complexes that probably extends beyond AGS8 regulation.

It is not clear from our studies how AGS8 is regulated. One possible mechanism is transcriptional up-regulation of AGS8 as was observed in cardiac myocytes subjected to ischemic stress (13). Another possibility is that AGS8 is regulated post-translationally by an upstream stimulus. It is important to emphasize that this study examined only a carboxyl-terminal portion of the full-length AGS8 protein. We elucidated how this G protein-binding domain of AGS8 can regulate heterotrimeric G protein function, but this carboxyl terminus may in turn be regulated by intramolecular interactions in full-length AGS8. Thus, it is important that we understand how this domain operates in the context of the full-length AGS8 protein. Full-length AGS8 has been difficult to express and purify. It has been expressed in COS7 cells and does not inhibit G protein function, although based on in vitro data, provides molecular details for how a G protein-binding domain of AGS8 can regulate heterotrimeric G protein function, but this carboxyl terminus may in turn be regulated by intramolecular interactions in full-length AGS8.

Acknowledgments—We thank Tabetha Bonacci for preparation of the G protein β subunit mutants and Dr. Elliott M. Ross for comments on the manuscript.

REFERENCES

1. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
2. Sprang, S. R. (1997) Annu. Rev. Biochem. 66, 639–678
3. Hamm, H. E. (1998) J. Biol. Chem. 273, 669–672

4. Levitzki, A., and Klein, S. (2002) Chembiochem. 3, 815–818
5. Bunemann, M., Frank, M., and Lohse, M. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 16077–16082
6. Frank, M., Thumer, L., Lohse, M. I., and Bunemann, M. (2005) J. Biol. Chem. 280, 24584–24590
7. Gales, C., Van Durm, J. J. I., Schaak, S., Pontier, S., Percherancier, Y., Audet, M., Paris, H., and Bouvier, M. (2006) Nat. Struct. Mol. Biol. 13, 778–786
8. Klein, S., Reuveni, H., and Levitzki, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3219–3223
9. Ross, E. M., and Wilkie, T. M. (2000) Annu. Rev. Biochem. 69, 795–827
10. Unger, O., Oner, S. S., Molinari, P., Ambrosio, C., Sayar, K., and Onaran, H. O. (2005) Mol. Pharmacol. 68, 720–728
11. Blumer, J. B., Cismowski, M. J., Sato, M., and Lanier, S. M. (2005) Trends Pharmacol. Sci. 26, 470–476
12. Sato, M., Blumer, J. B., Simon, V., and Lanier, S. M. (2006) Annu. Rev. Pharmacol. Toxicol. 46, 151–187
13. Sato, M., Cismowski, M. J., Toyota, E., Smrcka, A. V., Lucchesi, P. A., Chilian, W. M., and Lanier, S. M. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 797–802
14. Forst, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reuveni, E., Shkret, L. R., Rosal, R., Weng, G., Yang, C.-S., Iyengar, R., Miller, J. L., and Hamm, E. H. (1998) Science 280, 1271–1274
15. Scott, J. K., Huang, S. F., Gangadhar, B. P., Samoriski, G. M., Clapp, P., Gross, R. A., Taussig, R., and Smrcka, A. V. (2001) EMBO J. 20, 767–776
16. Davis, T., Bonacci, T. M., Sprang, S. R., and Smrcka, A. V. (2005) Biochemistry 44, 10593–10604
17. Bonacci, T. M., Mathews, J. L., Yuan, C., Lehmann, D. M., Malik, S., Wu, D., Font, J. L., Bidlack, J. M., and Smrcka, A. V. (2006) Science 312, 443–446
18. Kozasa, T., and Gilman, A. G. (1995) J. Biol. Chem. 270, 1734–1741
19. Mummy, S. M., and Linder, M. E. (1994) Methods Enzymol. 237, 254–268
20. Hartman IV, J. L., and Northup, J. K. (1999) J. Biol. Chem. 274, 22591–22597
21. Romoser, V., Ball, R., and Smrcka, A. V. (1996) J. Biol. Chem. 271, 25071–25078
22. Ghosh, M., Peterson, Y. K., Lanier, S. M., and Smrcka, A. V. (2003) J. Biol. Chem. 278, 34747–34750
23. Goubaua, F., Ghosh, M., Malik, S., Yang, J., Hinkle, P. M., Griendling, K. K., Neubig, R. R., and Smrck, A. V. (2003) J. Biol. Chem. 278, 19634–19641
24. Sarvayzyn, N. A., Remmers, A. E., and Neubig, R. R. (1998) J. Biol. Chem. 273, 7934–7940
25. Lambrecht, D. G., Sondek, J., Alkins, D. R., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) Nature 379, 311–319
26. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) Cell 83, 1047–1058
27. Neer, E. J., Pulsifer, L., and Wolf, L. G. (1988) J. Biol. Chem. 263, 8996–9000
28. Denker, B. M., Neer, E. J., and Schmidt, C. J. (1992) J. Biol. Chem. 267, 6272–6277
29. Bonacci, T. M., Ghosh, M., Malik, S., and Smrcka, A. V. (2005) J. Biol. Chem. 280, 10174–10181
30. Tesmer, V. M., Kawano, T., Shankaranarayanan, A., Kozasa, T., and Tesmer, J. J. G. (2005) Science 310, 1686–1690
31. Panchenko, M. P., Saxena, K., Li, Y., Charmecki, S., Sternweis, P. M., Smith, T. T., Gilman, A. G., Kozasa, T., and Neer, E. J. (1998) J. Biol. Chem. 273, 28298–28304
32. Yoshikawa, D. M., Bresciano, K., Hatwar, M., and Smrcka, A. V. (2001) J. Biol. Chem. 276, 11246–11251
33. Dohlmann, H. G. (2002) Annu. Rev. Physiol. 64, 129–152
34. Leeuw, T., Wu, C., Schrag, J. D., Whiteway, M., Thomas, D. Y., and Leberer, E. (1998) Nature 391, 191–195
35. Leberer, E., Dignard, D., Hougan, L., Thomas, D. Y., and Whiteway, M. (1992) EMBO J. 11, 4805–4813
36. Kimpel, R. J., Kimpel, M. E., Betts, L., Sondek, J., and Siderovski, D. P. (2002) Nature 416, 878–881
37. Peterson, Y. K., Bernard, M. L., Ma, H. Z., Hazard, S., Graber, S. G., and Lanier, S. M. (2000) J. Biol. Chem. 275, 33193–33196
38. Digby, G. J., Lober, R. M., Sethi, P. R., and Lambert, N. A. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 17789–17794
39. Riven, I., Iwaniar, S., and Reuveni, E. (2006) Neuron 51, 561–573