Pivotal Role of Extended Linker 2 in the Activation of Go by G Protein-coupled Receptor*

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Background: G protein-coupled receptors mainly signal through heterotrimeric G-proteins. The mechanisms of G-protein activation by GPCRs are not well understood. Previous studies have revealed a signal relay route from a GPCR via the C-terminal α5-helix of Go to the guanine nucleotide-binding pocket. Recent structural and biophysical studies uncover a role for the opening or rotating of the α-helical domain of Go during the activation of Go by a GPCR. Here we show that β-adrenergic receptors activate eight Goα mutant proteins (from a screen of 66 Goα mutants) that are unable to bind Gβγ subunits in cells. Five of these eight mutants are in the αF/Linker 2/β2 hinge region (extended Linker 2) that connects the Ras-like GTPase domain and the α-helical domain of Goα. This extended Linker 2 is the target site of a natural product inhibitor of Gq. Our data show that the extended Linker 2 is critical for Goα activation by GPCRs. We propose that a GPCR via its intracellular loop 2 directly interacts with the β2/β3 loop of Go to communicate to Linker 2, resulting in the opening and closing of the α-helical domain and the release of GDP during G-protein activation.

A structurally diverse repertoire of ligands, from photons to many hormones and neurotransmitters, activate G protein-coupled receptors (GPCRs)6 to elicit their physiological functions (1, 2). GPCRs comprise a large and diverse superfamily, and family members have been identified in organisms as evolutionarily distant as yeast and human. Heterotrimeric G-proteins (Goβγ) directly relay the signals from GPCRs (3–5). These G-proteins are composed of α, β, and γ subunits. The β and γ subunits are tightly associated and can be regarded as one functional unit. G-proteins function as molecular binary switches with their biological activity determined by the bound nucleotide (3–5). Upon ligand-binding, GPCRs increase the exchange of GDP bound on the Go subunit with GTP, in the presence of Gβγ subunits. This leads to the dissociation of the Go subunit from the Gβγ dimer resulting in two functional subunits (Goα and Gβγ). Both Goα and Gβγ subunits signal to various cellular pathways.

Over the past 30 years, great progress has been made in understanding the mechanisms by which heterotrimeric G-proteins regulate their downstream targets (6, 7). Recently a series of crystal structures of GPCRs in the inactive and active states, bound with antagonists, inverse agonists, or agonists, have elucidated the structural basis for the modulation and activation of GPCRs by ligands (1, 8, 9). A crystal structure of the complex of β2-adrenergic receptor and Go has revealed the structural changes in β2-adrenergic receptor and Go, the interacting regions, and residues between a GPCR and a G-protein (10). However, the molecular mechanisms by which GPCRs activate G-proteins are still not completely understood (11, 12).

A structural domain of Go subunits consists of two domains: a Ras-like GTPase domain and an α-helical domain (6) (Fig. 1, A and B). These two domains are linked by Linker 1 and Linker 2 (Fig. 1B). Between these two domains lies a deep cleft within which GDP or GTP is tightly bound (Fig. 1, A and B). The nucleotide is essentially occluded from the bulk solvent, leading to a proposal that the helical domain is the inhibitory barrier and provides the regulatory entry point by GPCRs or Gβγ subunits (13–16).

One of the major remaining problems in the biology of GPCR/G-protein signaling is to experimentally demonstrate whether GPCRs or Gβγ subunits play the catalytic exchange role in Go protein activation (17). In theory, the question could be straightforwardly answered with purified proteins of GPCRs, Go, and Gβγ subunits. However, purified GPCRs had no guanine-nucleotide exchange effect on Go in the absence of Gβγ subunits (18, 19). A GPCR could only activate Go in the presence of Gβγ (18, 19), leading some to believe that GPCRs were not the real catalysts, although they were required to initiate the activation event from agonist binding. Yet, purified Gβγ subunits alone also could not accelerate the guanine nucleotide exchange on Go subunits (3, 20). In fact, Gβγ subunits inhibit the basal nucleotide exchange activity of Go sub-

G protein-coupled receptors (GPCRs) relay extracellular signals mainly to heterotrimeric G-proteins (Goβγ) and they are the most successful drug targets. The mechanisms of G-protein activation by GPCRs are not well understood. Previous studies have revealed a signal relay route from a GPCR via the C-terminal α5-helix of Go to the guanine nucleotide-binding pocket. Recent structural and biophysical studies uncover a role for the opening or rotating of the α-helical domain of Go during the activation of Go by a GPCR. Here we show that β-adrenergic receptors activate eight Goα mutant proteins (from a screen of 66 Goα mutants) that are unable to bind Gβγ subunits in cells. Five of these eight mutants are in the αF/Linker 2/β2 hinge region (extended Linker 2) that connects the Ras-like GTPase domain and the α-helical domain of Goα. This extended Linker 2 is the target site of a natural product inhibitor of Gq. Our data show that the extended Linker 2 is critical for Goα activation by GPCRs. We propose that a GPCR via its intracellular loop 2 directly interacts with the β2/β3 loop of Go to communicate to Linker 2, resulting in the opening and closing of the α-helical domain and the release of GDP during G-protein activation.

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4 The abbreviations used are: GPCR, G protein-coupled receptor; DM, dodecyl-β-D-maltoside; MEF, mouse embryonic fibroblast; β-AR, β-adrenergic receptor; GEF, guanine-nucleotide exchange factor.

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units and behave as a guanine-nucleotide dissociation inhibitor (20). When the structure of Gβ was revealed to be similar to RCC1, a guanine-nucleotide exchange factor for the small GTPase Ran, Gβγ was proposed to be the real guanine-nucleotide exchange factor for Goα, whereas GPCRs served as a trigger (15, 21). Therefore, it remains a fundamental unsolved question: which one, a GPCR and/or Gβγ subunit, has the ability to catalyze the nucleotide exchange on Goα.

We reasoned that if GPCRs or Gβγ subunits are the nucleotide exchangers, we should be able to find mutants of GPCRs or Gβγ subunits that would accelerate the nucleotide exchange on Goα subunits in the absence of Gβγ or GPCRs, respectively. Alternatively, we might be able to find mutant Goα subunits that could be activated by GPCRs alone or by Gβγ alone. Here we describe our finding of a direct activation of some Goα mutant proteins by β-adrenergic receptors without Gβγ subunits. Although our data do not exclude a possible additional catalytic role for Gβγ, it clearly demonstrates that GPCRs by themselves have the catalytic ability to activate Goα subunits. Furthermore, from this systematic study, a pivotal role for the αF/Linker 2/β2 region (extended Linker 2) in Gβγ for 120 min at 4 °C. After centrifugation, the membrane pellet was resuspended into 50 ml of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2% dodecyl-β-d-maltoside (DM) and protease inhibitors). After centrifugation at 10,000 × g for 120 min at 4 °C, 1 ml of nickel-nitritotriacetic acid-agarose beads pre-equilibrated with extraction buffer was added to the supernatant. The mixture was gently agitated overnight at 4 °C. After washing 3 times with 10 ml of washing buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM imidazole, and 0.2 mM PMSF), Gβγ was eluted with 10 ml of elution buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, and 200 mM imidazole).

**EXPERIMENTAL PROCEDURES**

**G-protein Purification—Goα** wild-type and mutant proteins were purified from *Escherichia coli*. The pGEX-6P-Goα plasmid was transformed into bacteria strain BL21(DE3). One liter of bacterial culture was grown at room temperature until the absorbance at 600 nm was ~1. G-protein expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 18 h at room temperature. The bacterial pellet was resuspended in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1 mg/ml of lysozyme, and 0.2 mM PMSF) and incubated on ice for 30 min. After sonication, the lysate was spun down at 10,000 × g for 120 min at 4 °C. Glutathione-agarose resin (0.5 ml, from Sigma) was added to the supernatant after pre-cleared with glutathione beads. 500 µl of supernatant was mixed with 10 µg of Goα protein (with an N-terminal GST tag, attached to glutathione beads). After SDS-PAGE, Western blots were performed with anti-Goα and anti-Gβ antibodies (Santa Cruz Biotechnology, Inc.).

**Gβγ Proteins Were Purified from Insect Hi5 Cells**—One liter of Hi5 cell pellet was resuspended into 50 ml of lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and protease inhibitors: 10 µg/ml of leupeptin, 1 µg/ml of pepstatin A, 1 mM benzamidine, and 0.2 mM PMSF). After sonication, the lysate was spun down at 150,000 × g for 90 min at 4 °C. The membrane pellet was resuspended in 50 ml of lysis buffer. After homogenization, the lysate was centrifuged again. The final pellet was resuspended in 50 ml of extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2% dodecyl-β-D-maltoside (DM) and protease inhibitors). After centrifugation at 10,000 × g for 120 min at 4 °C, 1 ml of nickel-agarose resin (0.5 ml, from Sigma) was added to the supernatant. The mixture was gently agitated at 4 °C for 3 h. After washing twice with HEM buffer (20 mM Hepes, pH 8.0, 2 mM GDP, 1 mM DTT, 150 mM NaCl, and 1% DM), glutathione beads) were incubated in 100 µl of binding buffer (20 mM Hepes, pH 8.0, 2 mM GDP, 1 mM DTT, 150 mM NaCl, and 0.02% DM) with 100 nM Gβγ proteins overnight at 4 °C. After centrifugation, beads were washed three times with binding buffer and then eluted with binding buffer containing 10 mM reduced glutathione. After SDS-PAGE, Western blots were performed with anti-Goα and anti-Gβ antibodies.

**Size Exclusion Chromatography—**Size exclusion chromatography was used to examine the binding of Goα and its mutants to Gβγγγ in solution. Goα (1 µM) and Gβγ(C68S)γγ (2 µM) were mixed and incubated on ice for 30 min. Samples were loaded on a Superdex 200 column (GE Healthcare Life Sciences) equilibrated with 20 mM Hepes, pH 8.0, 50 mM NaCl, and 1 mM EDTA at a flow rate of 0.5 ml/min. The elution was monitored at 280 nm, and 0.8-ml fractions were collected for subsequent SDS-PAGE analysis.

**In Vivo cAMP Assay**—Cells were plated on 6-well plates and treated with 1 mM isobutylmethylxanthine for 30 min at 37 °C. After washing twice with HEM buffer (20 mM Hepes, pH 7.4, 135 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM NaHCO3, 0.1 mM Ro-20-1724, 0.5 unit/ml of adenosine deaminase, and 1 mM isobutylmethylxanthine), cells were treated with 0.1 mM, 10 mM, 100 mM, 1 µM, 10 µM, and 100 µM isoproterenol in HEM buffer for 5 min at 37 °C. After two additional washes with HEM buffer, cells were harvested and lysed with 0.5% Triton X-100 containing 1 mM isobutylmethylxanthine. The amount of cAMP was measured with a Direct Cyclic AMP Enzyme Immunoassay kit (Assay Designs, Inc.).

**In Vitro Adenylyl Cyclase Activation Assay**—Adenylyl cyclase activation assay of S9 membrane preparations was performed as previously described (22). Briefly, adenylyl cyclase isofrom V (dog) was recombinantly expressed in S9 cells, and the adenylyl cyclase-containing membranes were prepared. Goα protein was activated by adding a buffer consisting of 10 mM NaF, 10 mM MgCl2, 30 µM AlCl3, and incubated at 30 °C for 1 h. Activated Goα proteins with membrane preparations of adenylyl cyclase...
Activation of G-protein by GPCR

V were incubated in buffer of 50 mM Tris, pH 7.6, 2 mM isobutylmethylxanthine, 1 mM ATP, 10 mM MgCl₂, 20 mM creatine phosphate, 100 units/ml of creatine phosphokinase at 30 °C for 10 min. The samples were boiled for 3 min to stop the reaction. After spinning for 3 min at 16,000 × g, the supernatant was used for cAMP measurement with Direct Cyclic AMP Enzyme Immunoassay kit.

Purification of Turkey β₁-Adrenergic Receptor Proteins—Turkey β₁-adrenergic receptor protein was purified as described previously (23). Turkey β₁-adrenergic receptor (residues 34–424 with a mutation C116L) cDNA was subcloned into the pVL1393 vector. Hi5 cells were infected with the recombinant baculovirus carrying the turkey β₁-adrenergic receptor at a density of 2 × 10⁴/ml. Sixty-hours later, cells were harvested and resuspended in lysis buffer (50 mM Tris-HCl, pH 8, 1 mM EDTA, 10 µg/ml of leupeptin, 9 mM benzamidine, 5 µg/ml of pepstatin A, and 2 mM PMSF). After homogenization, the cell lysate was centrifuged at 2,000 × g for 10 min and the supernatant was centrifuged again at 150,000 × g at 4 °C for 1.5 h. The membrane pellet was resuspended in lysis buffer and homogenized again. After spinning down at 150,000 × g at 4 °C for 1.5 h, the final pellet was then resuspended in membrane extraction buffer (50 mM Tris-HCl, pH 8, 350 mM NaCl, 3 mM imidazole, 2% DM, 10 µg/ml of leupeptin, 9 mM benzamidine, 5 µg/ml of pepstatin A, and 1 mM PMSF) and rolled at 4 °C for 3 h. After centrifugation at 150,000 × g at 4 °C for 1 h, solubilized membrane proteins were incubated with nickel-nitrilotriacetic acid–agarose (Qiagen) overnight. After washing with buffer containing 50 mM Tris-HCl, pH 8, 350 mM NaCl, 3 mM imidazole, 0.1% DM, 10 µg/ml of leupeptin, 9 mM benzamidine, 5 µg/ml of pepstatin A, and 1 mM PMSF, the receptor protein was eluted down by an imidazole gradient.

GTPγS Loading Assay—Agonist-stimulated GTPγS loading to G-proteins was performed as previously described (19). Ga₃ (with or without 1 µM Gβγ) and turkey β₁-adrenergic receptor (20 nm) together with 10 µM alprenolol or isoprotanol in 200 µl of loading buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 0.02% DM, and 5 mM GDP) were incubated on ice for 20 min. After incubation at 30 °C (for wild-type and Ga₁E219Q or at room temperature (for Ga₃263 and Ga₃195) for 5 min, 100 nM GTPγS was added. At various times, 40-µl aliquots were removed and added to 1 ml of termination buffer (20 mM Tris, pH 8.0, 100 mM NaCl, and 25 mM MgCl₂, ice cold) and loaded onto nitrocellulose membrane (Schleicher & Schuell BioScience). After 3 washes with 1 ml of termination buffer, 4 ml of scintillation liquid were added to the membrane and [³⁵S]GTPγS loading. 5 mM GDP was used to determine nonspecific binding.

Ga₃ and Receptor Binding Assay—Different concentrations of Ga₃ proteins (with an N-terminal GST tag, attached to glutathione beads) were incubated in 100 µl of binding buffer with 80 nm β-AR proteins (with a FLAG tag) at 4 °C for 1 h. GST (600 nM) was used as control. After centrifugation, beads were washed three times with washing buffer. After SDS-PAGE, Western blots were performed with anti-Ga₃ and anti-FLAG M2 antibodies.

RESULTS AND DISCUSSION

Identification of Ga₃ Mutants Defective in Gβγ Binding—To identify Ga₃ subunits that could be activated by GPCRs in the absence of Gβγ subunits, we first characterized Ga₃ mutants that were defective in Gβγ binding. The crystal structures of Ga₃ and Gaαβγ have been solved (24, 25). The conformational differences between the free and Gβγ-bound forms of Gaα-GDP mainly involve residues that directly interact with Gβγ (24, 25). Based on the crystal structures of GaαGβ₁γ₁ and GaαGβ₂γ₂ complexes, residues in the N-terminal, Switch 1 (Arg-185 to Ile-193, encompassing Linker 2 region), and Switch II region of Gaα subunits are involved in contacting or binding of Gβγ (24, 25) (Fig. 1C, red residues). Furthermore, from the crystal structures of the Ras superfamily GTPases and their guanine nucleotide-exchange factors, such as Ras and Sos1, Arf1 and Sec7, Rac1 and Tiam1, EF-Tu and EF-Ts, as well as Ran and RCC1 (26–30), guanine nucleotide-exchange factors interact extensively with and remodel the Switch I and II regions of GTPases. Therefore, we have performed alanine scanning mutagenesis of every residue in these regions as well as some residues in Switch III and its adjacent regions (Table 1 and Fig. 1D).

First we identified Ga₃ mutants that could not bind Gβγ. We purified recombinant proteins of wild-type Ga₃ and 66 mutant Ga₃ proteins from E. coli (Table 1 and some examples shown in Fig. 2A). (Here we used the short spliced variant of bovine Ga₃. The difference between the short and long splice forms of Ga₃ is an insertion of 14 amino acids after residue Gly-70.) We also purified recombinant Gβ₁γ₁ proteins from insect Hi5 cells (Fig. 2A). To test the functionality of these purified Ga₃ mutant proteins, we performed in vitro GTPγS binding assays and adenylyl cyclase activation assays for all Ga₃ mutants (Table 1). Other than eight mutants (Table 1), all Ga₃ mutant proteins were able to bind to GTPγS, implying these purified proteins were stable and functional. Furthermore, using membrane preparations from Sf9 insect cells infected with recombinant baculoviruses carrying adenylyl cyclase type V, we performed in vitro adenylyl cyclase activation assays (Table 1, and some examples shown in Fig. 2). In addition to the 8 mutants that could not bind GTPγS in vitro, 4 more mutants could not activate adenylyl cyclase in vitro (Table 1). Three (A3n-265, Arg-266, and Trp-267) of these 4 residues are in the α3/β5 loop, which directly contacts the catalytic domain of adenylyl cyclase as revealed by the crystal structure of the complex of Ga₃ and the catalytic domains of adenylyl cyclase (31). Thus, most of the purified mutant Ga₃ proteins were stable and functional.

We next tested the interaction of these Ga₃ mutants with Gβγ. For the in vitro binding experiments, we incubated wild-type Ga₃ and mutant Ga₃ proteins with purified Gβ₁γ₂ proteins. Glutathione beads were used to pulldown Ga₃ proteins. Co-precipitation of Gβ₁γ₂ was detected with anti-Gβ antibody. As shown in Table 1 (and some examples in Fig. 2F), wild-type Ga₃ and 48 mutant Ga₃ proteins pulled down Gβ₁γ₂, 18 other mutant Ga₃ proteins (including Ga₃E195A, Ga₃K219A, or Ga₃W263A) did not bind Gβ₁γ₂. To confirm these binding data and show that the 18 mutant Ga₃ proteins could not interact with other Gβ subunits in addition to Gβ, we used membrane preparations of 293T cells as the source of Gβγ subunits (293T
cells at least express Gβ₁₂, Gβ₂₃, and Gβ₃.1). The results were the same as the binding experiments with purified Gβ₁₂ (Table 1 and some examples shown in Fig. 2G). As a third approach, we used size exclusion chromatography to verify the inability of some Gαₙ mutants binding to Gβ₁₂. As shown in Fig. 2H, wild-type Gαₙ, GαₙK219A, and Gβ₁₂ eluted as single major peaks from the size exclusion column, demonstrating the homogeneity of the subunit preparations. When combined with excess Gβ₁₂, wild-type Gαₙ showed a two-peaks elution profile: one was the free Gβ₁₂ and the other was the complex of Gαₙ-Gβ₁₂ with a shorter retention time as compared with the isolated wild-type Gαₙ (Fig. 2H). On the other hand, GαₙK219A...
Activation of G-protein by GPCR

| TABLE 1 |
|---|
| Summary of the characterization of 66 Gαs mutants |

| MUTANTS | GTPγS binding (%) | AC activation in vitro (%) | Gαγ binding (%) |
|---|---|---|---|
| Gαs | 100 | 100 | YES |
| D180A | 55 | 50 | 12X, 15X, 10X, 14X |
| Q181A | 40 | 40 | 20X, 30X, 18X, 14X |
| L183A | NO | NO | 4X, 4X, 3X, 3X |
| A184A | NO | NO | 2X, 5X, 4X, 4X, 2X, 3X |
| R185A | 10 | 10 | 5X, 16X, 8X, 20X |
| C186A | 105 | 100 | 12X, 20X, 25X, 20X |
| R187A | 43 | 49 | NO |
| V263A | NO | NO | YES |
| L189A | 100 | 207 | 30X, 5X, 10X, 2X |
| T190A | 41 | 30 | 5X, 2X, 9X, 2X |
| L189A/190A | 75 | 289 | NO |
| S191A | 40 | 110 | NO |
| G192A | 25 | 93 | 3X, 4X, 8X, 6X |
| I193A | 40 | 52 | NO |
| F214A | 85 | 76 | 1X, 12X, 29X, 17X |
| E195A | 35 | 52 | 3X, 4X, 7X, 6X |
| T218A | 70 | 149 | NO |
| K219A | 97 | 78 | 4X, 5X, 15X, 4X |
| P219A | NO | NO | 4X, 2X, 4X, 3X |
| G190A | 170 | 52 | 1X, 10X, 18X, 14X |
| V200A | 85 | 71 | 12X, 24X, 12X, 7X |
| D201A | 55 | 60 | 1X, 10X, 8X, 8X |
| K202A | 130 | 72 | 22X, 30X, 21X, 44X |
| V203A | 125 | 91 | 4X, 6X, 4X, 6X |
| N204A | 55 | 77 | 11X, 14X, 14X |
| F205A | NO | NO | NO |
| H206A | NO | NO | NO |
| M207A | 10 | 30 | 18X, 40X, 20X, 20X |
| F208A | 10 | 20 | 8X, 1X, 5X, 8X |
| D209A | 50 | 68 | NO |
| V210A | 140 | 57 | 6X, 15X, 4X, 7X |
| G211A | 85 | 38 | 10X, 24X, 9X, 14X |
| G212A | 55 | 35 | NO |
| Q213A | 40 | 140 | 3X, 4X, 3X |
| R214A | 15 | 107 | NO |
| D215A | 50 | 27 | NO |
| E216A | 110 | 156 | 20X, 20X, 20X |
| R217A | 70 | 190 | NO |
| R218A | 100 | 67 | 6X |
| C219A | 115 | 79 | 4X, 5X, 6X, 4X |
| W220A | 100 | 216 | NO |
| W220R | 65 | 14 | NO |
| E221A | 150 | 261 | NO |
| W220A/I221A | NO | NO | NO |
| C222A | 45 | 216 | NO |
| C223A | 65 | 261 | 8X, 6X, 21X, 10X |
| F224A | 80 | 45 | NO |
| N225A | 85 | 72 | 35X, 50X, 15X, 20X, 25X |
| N227A | 19 | 13 | NO |
| L258A | 50 | 68 | 9X, 8X, 5X, 6X |
| F259A | NO | NO | NO |
| K260A | 105 | 69 | 8X, 6X, 10X, 11X |
| S261A | 120 | 233 | NO |
| K262A | 30 | 428 | 13X, 7X, 5X, 3X |
| W263A | 40 | 35 | 13X, 13X, 21X, 24X |
| N264A | NO | NO | NO |
| L265A | 95 | 55 | NO |
| R266A | 20 | NO | NO |
| W267A | 45 | NO | NO |
| L268A | 110 | 117 | 3X, 3X, 6X, 8X |
| T270A | 105 | 97 | 20X, 17X, 21X, 7X |
| W263A/268 | NO | NO | NO |
| AR269A | NO | NO | NO |
| A231 | 20 | 146 | NO |
| A232 | 10 | 78 | NO |
| L326A/268A | 10 | 75 | 5X, 3X, 3X, 3X |

| Summary of the values obtained with wild-type Gαs. The increase of cAMP in cells is shown as the fold increase from each individual experiment. ΔN31 and AN38 are deletion mutants of the N-terminal 31 or 38 amino acids, respectively. |

- Investigated the activation of all 66 Gαs mutants by GPCRs in Gαs-deficient cells. Gαs-deficient mouse embryonic fibroblast (MEF) cells were derived from Gαs knock-out mouse embryos (32, 33). Because exon 2 of the Gαs gene was deleted, none of the two alternative spliced variants of Gαs were present in these Gαs−/− MEF cells (32, 33). We have made stable cell lines with these Gαs−/− MEF cells expressing Gαs and all 66 Gαs mutants (in pcDNA3.1/hygromycin vector). Stimulation of these cells with isoproterenol, which activates the endogenous Gαs-coupled β-adrenergic receptors, increased cellular cAMP accumulation in cells expressing wild-type and 37 mutant Gαs proteins (Table 1, and some examples shown in Fig. 4). Among the 18 Gαs mutants that could not interact with Gβγ, 8 mutants (Gαs182A, GαsL184A, GαsR185A, GαsE195A, GαsF198A, GαsK219A, GαsW263A, and GαsN23A/I26A/E27A/L30A/D33A) could still mediate receptor activation of adenyl cyclase leading to cAMP increase (Fig. 4, B, E, and green in Fig. 1D). As control, Gαs−/− MEF cells did not show any cAMP increase upon isoproterenol stimulation (Fig. 4). The functionality of these Gαs mutant proteins was also verified by cholera toxin, a direct activator of Gαs (Fig. 4). These cellular studies demonstrate that 8 Gαs mutants are able to functionally couple to β-adrenergic receptors, are activated by the receptors, and stimulate the downstream effector adenyl cyclase, leading to the cellular accumulation of cAMP, even though they could not bind to Gβγ.

Activation of Purified Gαs Mutants by Purified β-Adrenergic Receptors in Vitro in the Absence of Gβγ—To directly demonstrate that a GPCR has the capability to accelerate the guanine-nucleotide exchange on Gαs without Gβγ, we performed biochemical studies with purified recombinant proteins of a GPCR and Gαs mutants in vitro. We purified recombinant turkey β1-adrenergic receptors from insect Hi5 cells (23) (Fig. 5A). Gαs proteins were initially purified as GST fusion proteins and the GST tag was removed by protease cleavage (Fig. 5B). Among the 8 Gαs mutants identified above (able to be activated by β-AR in cells but unable to bind Gβγ), 5 (D182A, L184A, R185A, F198A, and N23A/I26A/E27A/L30A/D33A) were unstable after removal of the GST tag. Therefore, we examined the activation of the remaining 3 Gαs mutants (E195A, K219A, and W263A) by β-adrenergic receptor. The activation was monitored by the initial rate of GTPγS loading onto Gαs subunits. As reported previously, purified β-adrenergic receptors had no effect on the rate of GTPγS loading (~90 fmol/min) onto wild-type Gαs proteins in the absence of Gβγ subunits (19, 34) (Fig. 5C). The rate of GTPγS loading on Gαs was the same in the presence of isoproterenol (an agonist for β-adrenergic receptor) or alprenol (an antagonist). The initial rate of GTPγS loading to Gαs alone was similar to that of Gαs with β-adrenergic receptor in the presence of the antagonist alprenol. In contrast, in the presence of purified Gβγ proteins, isoproterenol increased the initial rate of GTPγS loading onto Gαs (~450 fmol/min) by 4-fold compared with that in the presence of alprenol (~115 fmol/min) (Fig. 5D). The fold-increase is similar to that reported in previous reconstituted systems, reflecting a relatively high basal nucleotide exchange rate of Gαs (19, 34).
FIGURE 2. Functional characterization of Gαs and its mutants. A, Coomassie Blue staining shows the purified Gαs proteins (some representatives), GST and Gβ1/γ2. B–E, in vitro activation assays of adenylyl cyclase by Gαs and its mutants (some representatives). F, G, in vitro binding of Gαs and representative mutants to Gβ, γ. H, size exclusion chromatography of heterotrimer formation. Upper panels: elution profiles are shown for wild-type Gαs alone, GαsK219A alone, Gβ1/γ2 alone, the mixture of wild-type Gαs/Gβ1, γ2, and the mixture of GαsK219A/Gβ1, γ2. Lower panels, SDS-PAGE analysis of the elution fractions. One representative experiment from three independent experiments is shown for each case. WB, Western blot.
Activation of G-protein by GPCR

With purified Ga1, we found that, without Gβγ subunits, isoproterenol increased the rate of GTPγS loading by about 2.4-fold compared to that with alprenolol (from ~39 fmol/min with alprenolol to ~95 fmol/min with isoproterenol) (Fig. 5E). Addition of Gβγ subunits did not significantly change the rates with either isoproterenol (~105 fmol/min) or alprenolol (~40 fmol/min) (Fig. 5F). Similar results were obtained with GaK219A and GaW263A mutants: without Gβγ subunits, isoproterenol increased the rate of GTPγS loading by 2~3-fold on GaK219A and GaW263A mutants, compared to that with alprenolol. Furthermore, the mutations did not seem to alter the interaction between Ga and β-AR (Fig. 5G). Moreover, increasing the concentrations of Gβγ enhanced the initial rate of GTPγS loading onto wild-type Ga, but not GaK219A (Fig. 5H). Additionally, increasing the concentrations of β-AR elevated the initial rate of GTPγS loading onto both wild-type Ga (in the presence of Gβγ) and GaK219A (in the absence of Gβγ) (Fig. 5H). These biochemical experiments clearly demonstrate that purified β1-adrenergic receptors can accelerate the guanine nucleotide exchange on Ga1, in the absence of Gβγ subunits. We should note that previous studies with rhodopsin and transducin had indicated that high concentrations of rhodopsin might activate transducin in the absence of Gβγ, and that Gβ1 or Gγ1 knock-out mice still had some light response (35~40). Thus, GPCRs possess the ability to catalyze the nucleotide exchange on Ga subunits.

Role of Extended Linker 2 in Ga Activation—How do the 8 Ga mutations (GaD182A, GaL184A, GaR185A, GaE195A, GaF198A, GaK219A, GaW263A, and GaN23A/I26A/E27A/L30A/D33A) alleviate the requirement of Gβγ for the activation of Ga by β-adrenergic receptors? When mapped onto the crystal structure of the complex of β1-AR and Ga3, 3 of these 8 residues (Asp-182, Leu-184, and Arg-185) are in αF helix (Fig. 6A and B). Two of the 8 residues (E195 and F198) are in αF helix (Fig. 6B). αF and α2 flank Linker 2, which connects the Ras-like GTPases domain and the α-helical domain of Ga3 (Figs. 6, A and B, and 1D). Also, within Linker 2, mutations of Arg-187, Leu-189/Thr-190, Ile-193, and Thr-196 all blocked β-AR induced cAMP increases in cells even though these mutants could bind to Gβγ, GTPγS, and activate adenylyl cyclase in vitro (Table 1). Hence, almost all residues in the extended Linker 2 are critical for Ga activation. Although some mutations (such as R185A and E195A) enable Ga activation by...
GPCR in the absence of Gβγ, other mutations (such as S191A and I193A) block Gα activation by GPCR in cells. When comparing the structures of inactive Gαs-GDP and active Gαs-GTPγS, or the structures of inactive Gαt-GDP and active Gαt-GTPγS, Linker 2 is moved toward the γ-phosphate, bringing the side chain oxygen of Thr-190 into the coordination sphere of the Mg2+ ion where it replaces one of the water molecules observed in the structure of the GDP form (24, 25). Furthermore, it has been shown that Linker 2 changes the conformation upon GPCR activation (41).
A critical role for the extended Linker 2 in Gα activation is consistent with the observation from the crystal structure of the complex of β2-AR and Gs, which shows a large rotation (~127°) of the α-helical domain relative to the Ras-like GTPase domain upon G-protein activation (Fig. 6, A and E) (10). αF/Linker 2, through the β2-strand, is connected to the β2/β3 loop, which interacts with intracellular loop 2 of β2-AR (Fig. 6F). Furthermore, experiments using various biophysical measurements
suggest a “clam-shell” like opening model for Ga activation by GPCRs, in which the helical domain opens away from the Ras-like GTPase domain (42–45) (Fig. 6D). Here the clustering of several Ga mutations critical for GPCR activation of Ga around the extended Linker 2 suggests a possibility that Linker 2 may serve as the “hinge” in this clam-shell model (Fig. 6D). Similarly, in the “rolling-top” model (in which the α-helical domain slides and rotates away along the sideways from the Ras-like GTPase domain) observed in the β2-AR-Gαs complex structure, this hinge is stretched (some residues in the linkers were disordered and thus were unmodeled in the structure) (10) (Fig. 6E). Mutations of residues in this hinge could make it easy to open the interface between the two domains of Ga or to enhance the interdomain motions. Preventing this interdomain opening by cross-linking inhibits GPCR-catalyzed G-protein activation (42). We should note that, in addition to Linker 2, Linker 1 also connects the Ras-like GTPase domain and the α-helical domain. Indeed a point mutation (G56P) in Linker 1 of transducin increased the basal exchange rate and exhibited some degrees of activation at high levels of rhodopsin in the absence of βγ (35). The role of Linker 1 in Ga protein activation by GPCRs requires future systematic investigation. Here we propose that a GPCR via its intracellular loop 2 directly interacts with the βγ loop of Ga to communicate to Linker 2 and αF, resulting in the rotation of the helical domain and the release of GDP (Fig. 6, E and F).

Linker 2 as the Target Site of a Natural Product Inhibitor for Gαs—The crystal structure of Gαs and a small molecule natural product inhibitor shows the inhibitor binds to a hydrophobic cleft and directly contacts Linker 2 (Fig. 6G) (46). This inhibitor, YM-254890, inhibits the guanine-nucleotide exchange reaction by preventing the GDP release (46). Also, this inhibitor blocks the AlF4−-induced conformational change in Gαs (46). It is proposed that this inhibitor stabilizes an inactive GDP-bound form (46). These structural data suggest that Linker 2 might be critical for G-protein activation, and that the Linker 2 region is a potential therapeutic targeting site.

Role of αN, α3, and Switch Regions in Ga Activation in Cells—In addition to the extended Linker 2, our data have uncovered some other residues that are essential for Ga activation in cells by β-arrestins. The N-terminal residues (Asn-23, Ile-26, Glu-27, Leu-30, and Asp-33) and Trp-263 are located at or close to the receptor-interacting surface (10, 15, 47, 48) (Fig. 6, A and C). Trp-263 is close to the αN/β1 loop. This region is analogous to GEF contact sites in other GTPases such as Ras and EF-Tu (26, 27). The crystal structure of β2-AR-Gαs has revealed that β2-AR interacts with the αN/β1 hinge; this may explain the role of the 5 N-terminal residues (Asn-23, Ile-26, Glu-27, Leu-30, and Asp-33) in the activation of Ga by GPCR.

Lys-219 is located within Switch II (Figs. 1D and 6C). Lys-206 of Ga (corresponding to Lys-219 in Ga) had been identified to be important for Ga activation by rhodopsin (49). Several residues near Lys-219 of Ga such as Gly-212 and Gln-213 are critical for triggering conformational changes in Switch II through an interaction with the γ-phosphate in GDP and for stabilizing the transition state for GDP hydrolysis, respectively (13, 14, 50, 51). Indeed, most Ga mutations in the Switch I and II regions (such as Ile-193, Thr-196, Asp-209, Gly-212, Arg-214, Asp-215, Arg-217, Arg-218, Trp-220, Ile-221, and Gln-222) could not be activated by β-arrestin receptors in cells, despite their ability to bind to Gβγ subunits, bind to GTPγS, and activate adenylyl cyclases in vitro (Table 1). Arg-187 and Thr-190 (in Switch I) as well as Gly-212 (in Switch II) contact the γ-phosphate of GDP (6). Together, these data demonstrate that αN, α3, Switch I and Switch II are critical for Ga activation by GPCRs.

The critical role of Switch I and II regions in Ga activation is similar to the activation of Ras-superfamily GTPases by their GEFs. GEFs for small GTPases have different structures (26–30). They contact GTPases at the same as well as different amino acid residues and induce different conformational changes on GTPases to drive out the GDP. However, they all utilize a two-sided attack to release positive charges (the Mg2+ ion and the invariant lysine residue in the P loop (phosphate-binding loop)) from their interaction with the phosphates of the nucleotide (52). These GEFs interact extensively with and remodel Switch I and II regions of GTPases, which form part of the binding pocket for Mg2+ and the γ-phosphate of GDP. Thus, although GPCRs are unusual GEFs because they do not contact these switch regions directly, they still require/remodel these switch regions for the activation of Ga.

Possible Role of Gβγ in Ga Activation—Our mutagenesis studies unexpectedly reveal a role for residues Trp-263 (in α3) and Asn-265 (in the α3-β5 loop) in Gβγ binding (Table 1). From the structure of the Ga3-GDP/Gβγ complex, this region is not directly involved in contacting with Gβγ. However, this region is right next to Switch III and forms an elaborate interdependent network of polar interactions with the Switch II region, which is critical for interacting with Gβγ (24). Although we did not address the role of Gβγ in the guanine-nucleotide exchange on Ga, we should mention that there were two models that proposed a catalytic role for Gβγ (15, 16, 21). Gβγ subunits contact the switch regions of Ga subunits (24, 25), and have a structure similar to RCC1 (30). Hence, Gβγ subunits would be more like GEFs if only Gβγ subunits could be proven to possess GEF activity without GPCRs. The role of Gβγ in Ga activation could be complex. Gβγ stabilizes GDP binding on Ga, thus serving as a guanine-nucleotide dissociation inhibitor.
Activation of G-protein by GPCR

GPCR activation could release the inhibition of Gβγ. However, the nucleotide exchange rate of Ga, in the absence of Gβγ, is still slower than that in the presence of GPCRs. This would indicate that, in addition to releasing Gβγ, GPCRs act on the nucleotide exchange on Ga. Gβγ subunits could serve an additional catalytic role to augment the complex formation between GPCRs and Ga subunits, similar to the role of ELMO in facilitating nucleotide exchange on Rac by Dock180 (53). Indeed, our data (Table 1, Fig. 5, E and F) show that, whereas Gaα/E195A could be activated by β-ARs in the absence of Gβγ, the extent of activation was suboptimal relative to wild-type Gaα in the presence of Gβγ. This implies that Gβγ somehow contributes to the activation process.

Previously two models were proposed for a catalytic role for Gβγ subunits in activating Ga subunits. In the “lever” model (15, 21), GPCRs cause a tilt (or a rotation) of Gβγ relative to Ga. The membrane proximal part (interacting with the Gaα N-terminal helix) of Gβγ moves closer to Ga. The opposite end of Gβγ moves away from Gaα and, at the same time, pulls along the interacting parts of Gaα. These interacting residues from Switch I and II of Gaα are part of the lip of the nucleotide binding pocket. Therefore, GDP can exit permitting exchange by GTP. In the second “gear shift” model (16), GPCRs also cause a tilt of the pocket. Therefore, GDP can exit permitting exchange by GTP. The exact role of Gβγ in the exchange reaction needs further investigation.

Conclusions—Upon ligand-binding, GPCRs initiate the exchange of GDP bound on Ga subunits of G-proteins with GTP. However, the detailed molecular mechanisms by which GPCRs activate G-proteins are not well understood. Here we reveal some new insights on this process. We demonstrate that a GPCR could activate some Gaα mutants in the absence of Gβγ, and that the extended Linker 2 in Gaα is critical for Gaα activation by GPCRs. These data will advance our understanding of this critical cellular signaling process.

The closed and open states of the cleft (the GDP/GTP binding site) are determined by relative pivoting of the Ras-GTPase domain and the α-helical domain at Linker 2. The closed state of the cleft is represented in all crystal structures of Gaα subunits. One of the open states of the cleft is captured in the crystal structures of the Gαα/E195A mutations in the absence of Gβγ. This implies that Gβγ somehow contributes to the activation process.

REFERENCES

1. Rosenbaum, D. M., Rasmussen, S. G., and Kobilka, B. K. (2009) The structure and function of G-protein-coupled receptors. Nature 459, 356–363
2. Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002) Seven-transmembrane receptors. Nat. Rev. Mol. Cell Biol. 3, 639–650
3. Gilman, A. G. (1987) G proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. 56, 615–649
4. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348, 125–132
5. Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) Diversity of G proteins in signal transduction. Science 252, 802–808
6. Sprang, S. R., Chen, Z., and Du, X. (2007) Structural basis of effector regulation and signal termination in heterotrimeric Gaα proteins. Adv. Protein Chem. 74, 1–65
7. Tesmer, J. J. (2010) The quest to understand heterotrimeric G protein signaling. Nat. Struct. Mol. Biol. 17, 650–652
8. Choe, H. W., Park, J. H., Kim, Y. J., and Ernst, O. P. (2011) Transmembrane signaling by GPCRs: insights from rhodopsin and opsin structures. Neuropharmacology 60, 52–57
9. Katritch, V., Cherezov, V., and Stevens, R. C. (2013) Structure-function of the G protein-coupled receptor superfamily. Annu. Rev. Pharmacol. Toxicol. 53, 531–556
10. Rasmussen, S. G., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T., Lyons, J. A., Caflrey, M., Gellman, S. H., Steyaert, J., Skiniotis, G., Weis, W. I., Sunahara, R. K., and Kobilka, B. K. (2011) Crystal structure of the β2 adrenergic receptor-G- protein complex. Nature 477, 549–555
11. Ramachandran, S., and Ceroni, R. A. (2006) How GPCRs hit the switch. Nat. Struct. Mol. Biol. 13, 756–757
12. Schwartz, T. W., and Sakmar, T. P. (2011) Structural biology: snapshot of a signalling complex. Nature 477, 540–541
13. Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993) The 2.2 Å crystal structure of transducin-α complexed with GTPγS. Nature 366, 654–663
14. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) Structures of active conformations of Gaα, and the mechanism of GTP hydrolysis. Science 265, 1405–1412
15. Iiri, T., Farfel, Z., and Bourne, H. R. (1998) G-protein diseases furnish a model for the turn-on switch. Nature 394, 35–38
16. Cherfils, J., and Chabre, M. (2003) Activation of G-protein Galpha subunits by receptors through Go-γβ and Gaα-Gy interactions. Trends Biochem. Sci. 28, 13–17
17. Bourne, H. R. (1997) How receptors talk to trimeric G proteins. Curr. Opin. Cell Biol. 9, 134–142
18. Fung, B. K. (1983) Characterization of transducin from bovine retinal rod outer segments: I. separation and reconstitution of the subunits. J. Biol. Chem. 258, 10495–10502
19. Florio, V. A., and Sternweis, P. C. (1989) Mechanisms of muscarinic receptor activation on G, in reconstituted phospholipid vesicles. J. Biol. Chem. 264, 3909–3915
20. Higashijima, T., Ferguson, K. M., Sternweis, P. C., Smigiel, M. D., and Gilman, A. G. (1987) Effects of Mg2+ and the βγ-subunit complex on the interactions of guanine nucleotides with G proteins. J. Biol. Chem. 262, 762–766
21. Rondard, P., Iiri, T., Srinivasan, S., Meng, E., Fujita, T., and Bourne, H. R. (2001) Mutant G protein α subunit activated by Gβγ—a model for receptor activation? Proc. Natl. Acad. Sci. U.S.A. 98, 6150–6155
22. Steegborn, C., Litvin, T. N., Hess, K. C., Capper, A. B., Taussig, R., Buck, J.
Levin, L. R., and Wu, H. (2005) A novel mechanism for adenylyl cyclase inhibition from the crystal structure of its complex with catechol estrogen. J. Biol. Chem. 280, 31754–31759

23. Huang, J., Chen, S., Zhang, J. I., and Huang, X. Y. (2013) Crystal structure of oligomeric β1-adrenergic G protein-coupled receptors in ligand-free basal state. Nat Struct Mol Biol 20, 419–425

24. Wall, M. A., Coleman, D. E., Lee, E., Iñiguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) The structure of the G protein heterotrimer Gα1β1γ2. Cell 83, 1047–1058

25. Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) The 2.0-Å crystal structure of a heterotrimeric G protein. Nature 379, 311–319

26. Kawashima, T., Berthet-Colominas, C., Wulff, M., Cusack, S., and Leberman, R. (1996) The structure of the Escherichia coli EF-Tu-EF-Ts complex at 2.5-Å resolution. Nature 379, 511–518

27. Boriack-Sjodin, P. A., Margarit, S. M., Bar-Sagi, D., and Kuriyan, J. (1998) The structural basis of the activation of Ras by Sos. Nature 394, 357–343

28. Goldberg, J. (1998) Structural basis for activation of ARF GTPase: mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. Cell 95, 237–248

29. Worthylake, D. K., Rossman, K. L., and Sondek, J. (2000) Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1. Nature 408, 682–688

30. Renault, L., Kuhlmann, J., Henkel, A., and Wittinghofer, A. (2001) Structural basis for guanine nucleotide exchange on Ran by the regulator of chromosome condensation (RCC1). Cell 105, 245–255

31. Tesmer, J. J., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) Crystal structure of the catalytic domain of adenyl cyclase in a complex with Gα5GTPγS (see comments). Science 278, 1907–1916

32. Bastepe, M., Gunes, Y., Perez-Villamil, B., Hunzelman, J., Weinstein, L. S., and Jüppner, H. (2002) Receptor-mediated adenylyl cyclase activation through XLa(s), the extra-large variant of the stimulatory G protein α-subunit. Mol. Endocrinol. 16, 1912–1919

33. Sun, Y., Huang, J., Xiang, Y., Bastepe, M., Jüppner, H., Kobilka, B. K., Zhang, J. J., and Huang, X. Y. (2007) Dosage-dependent switch from G protein-coupled to G protein-independent signaling by a GPCR. EMBO J. 26, 53–64

34. Pedersen, S. E., and Ross, E. M. (1982) Functional reconstitution of β-adrenergic receptors and the stimulatory GTP-binding protein of adenylyl cyclase. Proc. Natl. Acad. Sci. U.S.A. 79, 7228–7232.

35. Singh, G., Ramachandran, S., and Cerione, R. A. (2012) A constitutively active Gαi subunit provides insights into the mechanism of G protein activation. Biochemistry 51, 3232–3240

36. Phillips, W. J., Wong, S. C., and Cerione, R. A. (1992) Rhodopsin/transducin interactions: II. influence of the transducin-βγ subunit complex on the coupling of the transducin-α subunit to rhodopsin. J. Biol. Chem. 267, 17040–17046

37. Herrmann, R., Heck, M., Henklein, P., Hofmann, K. P., and Ernst, O. P. (2006) Signal transfer from GPCRs to G proteins: role of the Gαi terminal region in rhodopsin-transducin coupling. J. Biol. Chem. 281, 30234–30241

38. Lobanova, E. S., Finkelstein, S., Herrmann, R., Chen, Y. M., Kessler, C., Michaud, N. A., Trieu, L. H., Strissel, K. J., Burns, M. E., and Arshavsky, V. Y. (2008) Transducin γ-subunit sets expression levels of α- and β-subunits and is crucial for rod viability. J. Neurosci. 28, 3510–3520

39. Kolesnikov, A. V., Rikimaru, U., Hennig, A. K., Lukasiewicz, P. D., Flesler, S. I., Govardovskii, V. I., Kefalov, V. I., and Kisselev, O. G. (2011) G-protein βγ-complex is crucial for efficient signal amplification in vision. J. Neurosci. 31, 8067–8077

40. Nikonorov, S. S., Lyubarsky, A., Fina, M. E., Nikonova, E. S., Sengupta, A., Chinniah, C., Ding, X. Q., Smith, R. G., Pugh, E. N., Jr., Vardi, N., and Dhingra, A. (2013) Cones respond to light in the absence of transducin β subunit. J. Neurosci. 33, 5182–5194

41. Oldham, W. M., Van Eps, N., Preininger, A. M., Hubbell, W. L., and Hamm, H. E. (2007) Mapping allosteric connections from the receptor to the nucleotide-binding pocket of heterotrimeric G proteins. Proc. Natl. Acad. Sci. U.S.A. 104, 7927–7932

42. Van Eps, N., Preininger, A. M., Alexander, N., Kaya, A. I., Meier, S., Meiler, J., Hamm, H. E., and Hubbell, W. L. (2011) Interaction of a G protein with an activated receptor opens the interdomain interface in the α subunit. Proc. Natl. Acad. Sci. U.S.A. 108, 9420–9424

43. Chung, K. Y., Rasmussen, S. G., Liu, T., Li, S., DeVree, B. T., Chae, P. S., Calinski, D., Kobilka, B. K., Woods, V. L., Jr., and Sunahara, R. K. (2011) Conformational changes in the G protein Gαi induced by the β2 adrenergic receptor. Nature 477, 611–615

44. Westfield, G. H., Rasmussen, S. G., Su, M., Dutta, S., DeVree, B. T., Chung, K. Y., Calinski, D., Velez-Ruiz, G., Oleskie, A. N., Pardon, E., Chae, P. S., Liu, T., Li, S., Woods, V. L., Jr., Steyaert, J., Kobilka, B. K., Sunahara, R. K., and Skinisotis, G. (2011) Structural flexibility of the Gαi-α helical domain in the βγ-adrenoreceptor G protein complex. Proc. Natl. Acad. Sci. U.S.A. 108, 16086–16091

45. Jones, J. C., Jones, A. M., Temple, B. R., and Dohlman, H. G. (2012) Differences in intradomain and interdomain motion confer distinct activation properties to structurally similar Gα proteins. Proc. Natl. Acad. Sci. U.S.A. 109, 7275–7279

46. Nishimura, A., Kitano, K., Takasaki, J., Taniguchi, M., Mizuno, N., Tago, K., Hakoshima, T., and Itoh, H. (2010) Structural basis for the specific inhibition of heterotrimeric Gq protein by a small molecule. Proc. Natl. Acad. Sci. U.S.A. 107, 13666–13671

47. Grishina, G., and Berlot, C. H. (2000) A surface-exposed region of Gαi in which substitutions decrease receptor-mediated activation and increase receptor affinity. Mol. Pharmacol. 57, 1081–1092

48. Zhang, Z., Melia, T. J., He, F., Yuan, C., McGough, A., Schmid, M. F., and Wensel, T. G. (2004) How a G protein binds a membrane. J. Biol. Chem. 279, 33937–33945

49. Onrust, R., Herzmark, P., Chi, P., Garcia, P. D., Lichtarge, O., Kingsley, C., and Bourne, H. R. (1997) Receptor and βγ binding sites in the α subunit of the retinal G protein transducin. Science 275, 381–384

50. Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) Structural determinants for activation of the α-subunit of a heterotrimeric G protein. Nature 369, 621–628

51. Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) GTase mechanism of G proteins from the 1.7 Å crystal structure of transducin α-GDP-AIF-4. Nature 372, 276–279

52. Vetter, I. R., and Wittinghofer, A. (2001) The guanine nucleotide-binding switch in three dimensions. Science 294, 1299–1304

53. Brugnera, E., Haney, L., Grimsley, C., Lu, M., Walk, S. F., Tosello-Tramontini, A. C., Macara, I. G., Madhani, H., Fink, G. R., and Ravichandran, K. S. (2002) Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. Nat. Cell. Biol. 4, 574–582