A Conserved Hydrophobic Core in Gαi1 Regulates G Protein Activation and Release from Activated Receptor*

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G protein-coupled receptor-mediated heterotrimeric G protein activation is a major mode of signal transduction in the cell. Previously, we and other groups reported that the α5 helix of Gαi1, especially the hydrophobic interactions in this region, plays a key role during nucleotide release and G protein activation. To further investigate the effect of this hydrophobic core, we disrupted it in Gαi1 by inserting 4 alanine amino acids into the α5 helix between residues Gln133 and Phe334 (Ins4A). This extends the length of the α5 helix without disturbing the βα-α5 loop interactions. This mutant has high basal nucleotide exchange activity yet no receptor-mediated activation of nucleotide exchange. By using structural approaches, we show that this mutant loses critical hydrophobic interactions, leading to significant rearrangements of side chain residues His57, Phe189, Phe191, and Phe336; it also disturbs the rotation of the α5 helix and the π–π interaction between His57 and Phe189. In addition, the insertion mutant abolishes G protein release from the activated receptor after nucleotide binding. Our biochemical and computational data indicate that the interactions between α5, α1, and β2-β3 are not only vital for GDP release during G protein activation, but they are also necessary for proper GTP binding (or GDP rebinding). Thus, our studies suggest that this hydrophobic interface is critical for accurate rearrangement of the α5 helix for G protein release from the receptor after GTP binding.

Heterotrimeric G proteins, composed of α, β, and γ subunits, act as molecular switches that turn on intracellular signaling cascades in response to the activation of G protein-coupled receptors by extracellular stimuli. Therefore, G proteins have a critical role in many different cellular responses (1–6). The Gα subunit binds GDP and forms a tight complex with the Gβγ subunits. Activated G protein-coupled receptors can catalyze the exchange of GDP for GTP, which leads to the dissociation of the receptor-G protein complex into isolated receptor and Gα and Gβγ subunits. Both the Gα and Gβγ subunits can then stimulate or inhibit downstream effectors. Signal propagation ceases after the Gα subunit hydrolyzes GTP, returns to the inactive state, and rebinds to the Gβγ subunit, regenerating the GDP-bound heterotrimeric state.

Previous studies showed that the activated receptor directly interacts with the G protein by binding to the C-terminal α5 helix of Gα, inducing a rigid body rotation and translation that pull this helix into a hydrophobic pocket on the receptor (7, 8). This leads to the rearrangement of the interfaces between helices α5, α1, and the β2-β3 strands and between α5 and the β6-α5 loop (1, 7, 9–11). Residue Phe336 in the α5 helix is highly conserved in small (12, 13) and large GTPases (14) in both the animal and plant kingdoms (15–18). Our in silico results predicted that Phe336 is the most energetically important residue both in maintaining the basal state and in promoting the receptor-bound conformation (6). Our proposed mechanism involves Phe336 acting as a relay to transmit conformational changes via strands β2 and β3 and helix α1 to the phosphate-binding loop (5, 6). These studies are supported by recently published computational studies (11, 19, 20). Another critical computational paper from Dror et al. (21) used molecular dynamic simulations to suggest that the key events in receptor-mediated G protein activation and GDP release are due to the structural rearrangements of the β6-α5 loop. This is one of the two identified signal transmission pathways from the receptor to the GDP binding site (21).

To critically examine the roles of these two possible routes of communication with the nucleotide binding site, we inserted a 4-amino acid linker into the α5 helix of Gαi1 between residues Gln333 and Phe334. This insert should disrupt the hydrophobic core (Phe336, His57, Phe189, and Phe191) and mimic the receptor-bound state while leaving the β6-α5 loop interactions intact (Fig. 1, A and B). Mutant Gαi1 subunits were analyzed for their ability to interact with light-activated rhodopsin (R+) to exchange nucleotides in both the basal and receptor-bound states and for the structural changes mediated by this insertion. In this study, Gαi1 was used to replace the visual G protein found in rods, Gαt1. Gαt1 shows very close homology with Gαi1, is activated by rhodopsin as well as Gαi1 (22), and is much more easily expressed in Escherichia coli.

Our findings support the role of the hydrophobic interaction between α5, the β2-β3 strands, and the α1 helix during activation and nucleotide release. We also uncovered an unexpected

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dependence on these hydrophobic interactions for promoting G protein release from the receptor-G protein complex.

Results

Biochemical Characterization and Functional Properties of Ins4A-Gα1 Protein—To examine the two activation routes of G protein activation, we inserted 4 alanines between residues Gln333 and Phe334 of the α5 helix, with this variant termed Ins4A (Fig. 1, A and B). This insertion is proposed to perturb the interactions between the critical Phe336 and both the α1 helix and the β2-β3 strands while leaving the β6-α5 loop intact. We tested how this insertion, which should mimic the rotation of the α5 helix toward the receptor in the R*-Gαβγ complex, affects both the critical structural interactions between α5 and α1 and β1-β3 and the functions of basal and receptor-mediated nucleotide exchange rates.

Ins4A displayed a highly increased basal exchange rate, as monitored by the relative increase in the intrinsic tryptophan (Trp211) fluorescence of Gα1, compared with WT protein (Fig. 2A, light gray). However, in receptor-mediated activation, the Ins4A mutant showed a significantly decreased nucleotide exchange rate compared with WT (dark gray).

One potential explanation for these data would be that the mutant does not interact with the receptor properly. To test this idea, we conducted a membrane binding assay with light-activated rhodopsin in rod outer segments (ROS).2 The data show normal levels of Ins4A interaction with R* and the capability to bind ROS membrane as well as the WT protein (Fig. 2B). However, the addition of the GTPγS non-hydrolyzable nucleotide analog does not induce dissociation of the complex even at high concentration (0.5 μM) (Fig. 2B, black arrows). We repeated this experiment in the presence of 1 mM GDP, and once again, the mutant did not release from the ROS membrane. Densitometric calculations of membrane binding show that the mutant is not responsive to nucleotide (Fig. 2C).

Accordingly, an alternative possibility is that the Ins4A mutant might not properly dock its C terminus to R* to transmit the activation signal to the nucleotide binding region. Using extra-Meta II (eMII) to measure the high affinity state of the receptor shows that there is normal eMII induced by increasing concentrations of heterotrimeric Gt binding (Fig. 2D), implying normal interaction between the α5 C-terminal helix and active receptor. Thus, the ability of Gt to induce a high affinity state was similar between WT and Ins4A mutant (Fig. 2D). To confirm the nucleotide sensitivity in the membrane binding experiment (Fig. 2B), the eMII assay was repeated in the presence of a high concentration of GDP (0.5 mM). Even this high concentration of GDP did not inhibit eMII in Ins4A, although it did effectively inhibit it in the WT protein (compare Fig. 2, D and E). This result confirms the membrane binding results and also shows that the C terminus of Ins4A properly interacts with and induces the high affinity state of R* similar to WT.

Guanine Nucleotide Interactions with Ins4A Protein—There are several scenarios that might explain how the Ins4A protein could bind the receptor with similar affinity to WT yet lack receptor-mediated nucleotide exchange or nucleotide-dependent membrane release activity (Fig. 3A): 1) the helical domain opening does not take place properly, so GDP cannot release; 2) the β6-α5 loop does not properly trigger GDP release, as suggested by Dror et al. (21); 3) GDP can release normally, but GTP, GTP, or GTPγS cannot rebind to the empty nucleotide-binding pocket; or 4) nucleotide exchange happens normally, but the G protein cannot release from the receptor.

To distinguish between these possibilities, we measured receptor-mediated GDP release and GTPγS binding using BODIPY-labeled nucleotides. To measure GDP release from the G protein, the Go subunit was incubated with BODIPY-GDP, and then Gβγ was added as described under “Experimental Procedures.” After 2 min, light-activated rhodopsin was added (Fig. 3B, first arrow). The data show that WT Gα1, β1, γ1 releases labeled GDP very quickly after interaction with R* (Fig. 3B, black circles), whereas Ins4A-B1,γ1 releases GDP almost 100-fold more slowly (Fig. 3B, gray trace, and Table 2). The BODIPY-GDP dissociation rate constants were calculated to be ~3.52 and 0.042 min⁻¹ for WT and the Ins4A mutant, respectively. To test whether GDP was still able to access the nucleotide binding region, we added excess unlabeled GDP and monitored BODIPY-GDP release. Unlabeled GDP can compete with the BODIPY nucleotide (Fig. 3B, second arrow). BODIPY-GDP release was faster in the presence of unlabeled GDP (dissociation rate, ~0.755 min⁻¹); this is probably due to the affinity difference between these two GDP nucleotides.

GTPγS binding was also monitored by using BODIPY-GTPγS (Fig. 3C). Like GDP release, the Ins4A insertion mutant also affects GTPγS binding. The data show that labeled GTPγS interaction with the mutant was ~30-fold slower than with the
Structure of a Gα C-terminal Insertion Mutant

WT protein (Table 1); the binding rate reflects GDP release as well as labeled GTPγS interaction. The GTPγS binding rate constants were calculated to be 0.913 and 0.031 min⁻¹ for WT and the Ins4A insertion mutant, respectively. These results indicate that the insertion of an extra helical turn in α5 dramatically affects receptor-mediated GDP release; however, GDP can still be released from the nucleotide binding pocket, and both GDP and GTPγS can access the pocket.

Examination of Conformational Changes in Functionally Important Regions Mediated by Receptor and GTPγS—To examine local environmental changes within specific regions of the Gα subunit, we used a Gαi1 protein lacking six solvent-exposed cysteines as a background for the introduction of cysteine residues at sites of interest. We selected three positions in the Gαi1 subunit that are critical for G protein function (7). Leu273 (Leu296 in Gαi) is a sensor of the presence of the guanine ring of guanine nucleotides, Lys349 (Arg389 in Gαi) is a sensor of receptor binding, and Lys330 (Glu370 in Gαi) senses rotation and disorder in α5 in the presence of R* (8) (Fig. 4, A and C). These positions were mutated to Cys and labeled with the Alexa Fluor 594C5-maleimide probe. The fluorescent intensity was measured after a 40-min incubation with either GDP, GTPγS, receptor, or receptor plus GTPγS. Each result was normalized to the fluorescence of its wild type G protein (Fig. 4D, black bars). To determine the relative changes in those regions in the basal state, we compared the fluorescence intensity in GDP- and GTPγS-bound states.

The -fold change in emission intensity of Ins4A in the presence of GDP (black bars) or GTPγS (gray bars) with the indicated labeled residues, as compared with the environment of the same labeled residue in WT are shown in Fig. 4B. The extreme C-terminal region (Lys349) showed relatively low fluorescence intensity compared with the WT protein in both GDP and GTPγS bound states, which indicates a highly polar environment. This highly polar environment might be due to the more exposed location induced by the extra 4 alanine residues in the α5 helix. Other mutants were similar to WT.

Next, we evaluated the conformational changes of the same regions in the heterotrimeric G protein (black bars) in the presence of active receptor (gray bars) and after the addition of GTPγS (black shaded bars) (Fig. 4D). The decreased emission intensity from labeled Leu273 upon receptor activation indicates an increased polar environment for the probe in both WT and the insertion mutant, consistent with the effect of nucleotide release from the binding pocket after receptor interaction. After GTPγS incubation, the fluorescence intensity came back to its GDP bound level in both proteins, indicating nucleotide binding and domain closing.

Residue Lys330 is located at the beginning of the α5 helix; it senses rotation of the helix (7) and disorder in presence of active...
receptor (8). The local environment of this residue indicated low solvent exposure in both WT and Ins4A after receptor interaction, indicating that it establishes new contact interactions that were absent in the heterotrimeric structure (Fig. 4D). These results are consistent with previous EPR studies (7). However, unlike WT, the mutant fluorescence intensity did not fully return to its heterotrimeric state after GTPγS incubation, indicating a perturbation in this region.

The extreme C terminus of Goα is disordered or absent in most crystal structures of isolated Goα or the Goβγ heterotrimer (23–26). It is a known receptor contact site that undergoes a receptor-mediated conformational change. Comparison of the fluorescence intensity of the Alexa Fluor label inserted in the C-terminal region at Lys349 in wild type versus the Ins4A-Goαi1βγ1, suggests that this residue is in a similar environment before receptor activation. Upon binding to the light-activated rhodopsin, the -fold change in intensity indicates an immobilization of the probe for both wild type and Ins4A-Goαi1βγ1, consistent with the expected interactions at the receptor-G protein interface (Fig. 4D, right). As expected, the strong fluorescence intensity of Lys349 disappeared in the GTPγS-bound WT G protein (Fig. 4D), whereas in the mutant, the signal did not change, consistent with the membrane binding and eMII results (Fig. 2B).

**Amino Acid Identity and the Hydrophobic Core Is Important for Rearrangement of the α5 Helix after Nucleotide Binding**—To test whether the functional properties of the Ins4A protein are due to the longer α5 helix or due to disruption of the hydrophobic core, we replaced the 4-alanine insertion of Ins4A with a duplication of the 4 adjacent wild type residues (from Phe334 to Asp337), terming the variant Ins4X (Fig. 1B). This change reestablishes the hydrophobic core around Phe336 (α5) in the presence of an insertion while altering the length of α5 to be the same as Ins4A. To investigate the function of the Ins4X protein, we evaluated its nucleotide exchange rates and membrane binding properties. Unlike Ins4A, Ins4X exhibited basal and receptor-mediated nucleotide exchange rates (Fig. 5A) and membrane binding (Fig. 5B) similar to wild type Goαi1βγ1. The Ins4X protein dissociated from ROS membrane after incubation with active receptor and GTPγS, similar to WT (Fig. 2B). As shown in Fig. 5B, unlike Ins4A, Ins4X released from the ROS membrane completely after incubation with nucleotide. This result suggests that the effect of Ins4A on G protein function is not due to the increase in length of the C terminus. Instead, it suggests that the amino acid identity and the establishment of the hydrophobic core play critical roles for proper rearrangement of the α5 helix and Goα subunit release from the receptor after nucleotide binding.

**X-ray Structures of the Ins4A Mutant**—To probe the structural basis for the biochemical properties of the Ins4A variant, the crystal structure in the GTPγS-bound state was determined.
at 2.7 Å resolution (Table 2). After insertion of the 4 alanines between Gln333 and Phe334, the helix rotates 60° starting from the insertion point (Fig. 6A, labeled in red). This rotation relocates Phe336 to a position similar to that observed for the homologous residue (Phe376) in the 2-adrenergic receptor (2AR)-Gs complex structure (8) (Fig. 6B, compare WT GTP\textsuperscript{S} (brown), Ins4A (cyan), and 2AR-Gs (green) (PDB entry 3SN6 (8)). Although attempted, we could not crystallize either the GDP-bound or nucleotide-free Ins4A protein.

The 4-Ala insert completely repositions the network of interactions between Phe\textsuperscript{336} (5), Phe\textsuperscript{189}, Phe\textsuperscript{191}, Phe\textsuperscript{196} (2-3), and His\textsuperscript{57} (a1). It also disturbs the π-π interaction between His\textsuperscript{57} and Phe\textsuperscript{189} (Fig. 6, C and D). In Ins4A, almost the entire β2-β3 strands move away from the α5 helix compared with the WT structure (Fig. 6, C and D). The relative Cα distances between insertion mutant and WT proteins in Phe\textsuperscript{189}, Phe\textsuperscript{191}, Lys\textsuperscript{192}, and Phe\textsuperscript{196} are 1.5, 2.3, 3.8, and 1 Å, respectively, whereas the overall root mean square deviation between WT G\textsubscript{αi1} and Ins4A was 0.79 Å (304 Cα atoms aligned totally).

Crystallized Ins4A has GTP\textsuperscript{S} bound, and the guanine nucleotide holds the GTPase and helical domains together in the structure. Therefore, we did not expect to see any signifi-
cant differences between the WT and mutant structure in the nucleotide contact regions. However, we identified an interesting feature in this structure. In the structure of Ins4A, the side chain of His57 (localized on the end of the α1 helix) flips from pointing inside to outside of the core, probably due to the lost network of interactions between Phe189 (α5), Phe196 (β2), and Phe191 (β2). The relative Cα distances in the α1 helix for residues Ile45, Ile46, His57, and Glu108 are 0.5, 0.9, 1.3, and 1.2 Å, respectively, between the Ins4A insertion mutant and the WT protein, with the end of the α1 helix moving away from α5. This structural rearrangement of the end of the α1 helix and His57 were predicted in our Rho-G complex model (Fig. 6F; gray) (19). The β2AR-Gαs complex structure (Fig. 6E), lacking the end of the α1 helix.

Structural Features of the Ins4A-β1γ1 Mutant—In the β2AR-Gαs complex crystal structure (8), the Gβγ subunit does not make any contact with the receptor and does not undergo statistically significant conformational changes upon complex formation, although, because of the low resolution of that structure, some real changes might not have been statistically significant. To evaluate any possible role of the Gβγ subunits in the biochemical properties seen in the Ins4A mutant, we determined the crystal structure of the heterotrimeric Ins4A-β1γ1 mutant in the GDP-bound state to 1.9 Å resolution (Table 2 and Fig. 7A). The Ins4A-β1γ1 structure shows a similar α5 helix rotation pattern as the isolated Ins4A bound to GTPγS (Fig. 7A, teal). However, there was no dramatic displacement of the α1 helix and β2-β3 regions (Fig. 7A). The relative Cα distances between mutant (Fig. 7A, teal) and WT (yellow) heterotrimeric structures in His57, Phe189, Phe191, Lys192, and Phe196 residues are 0.5, 0.5, 1.4, 1.4, and 0.6 Å, respectively. This might be due to the effect of the crystal packing. Fig. 7, B and C, shows that the α5 helix, β2-β3 strands, and α1 helix interact with a symmetric molecule of the Gβγ subunit, which might block or limit the displacement of the β2-β3 strands and α1 helix. The Ins4A-β1γ1 heterotrimeric structure also shows significant differences at the αN (Fig. 7D) and α2 helices and the Gβγ subunits (Fig. 7E) compared with the WT structure. There is not any

**Table 2**

| Crystallographic data collection and refinement statistics |
|-----------------------------------------------------------|
| **Ins4A-1G11**-GTPγS-Mg2+ | **Ins4A-1G11β1γ1**-GDP |
|---------------------------|-------------------------|
| PDB accession code        | 5KDL                    |
| Data Collection and        | 5KDO                    |
| Processing*                |                         |
| Beamline                   | 27-ID-F                 |
| Space groups               | P2₁                    |
| Cell dimensions: x, y, z (Å)| 61.7, 77.3, 71.1         |
| α, β, γ (degrees)          | 90, 99.8, 90            |
| Resolution (Å)             | 0.8-2.8 (2.8-2.7)        |
| Total Reflections          | 217,114                 |
| Unique Reflections         | 19,135                  |
| Rmerge (%)                 | 8.8 (51.7)              |
| Rwork (%)                  | 5.6 (33.0)              |
| Complteness (%)            | 99.5 (97.4)             |

*Numbers in parentheses indicate statistics for the highest shell.

**Figure 6. Structural features of GTPγS-bound Ins4A mutant protein.** A, schematic representation of Glu333, Phe189, and Phe196 residues in the α5 helix of Ins4A. The 4-alanine insertion region is represented in salmon color. B, comparison of the α5 helix and Phe196 residue location between WT Gαs, (PDB entry 1GIA (26), brown), Ins4A (cyan), and β2AR-Gαs complex structure (PDB entry 3SN6 (8), green). C, comparison of the α5 helix and β1-β6 strands between WT Gαs, (brown) and Ins4A (cyan) (D) The effect of α5 helix rotation and the connection between Phe196 and the β2-β3 strands and the α1 helix. Shown is a comparison of α5, β2-β3, and α1 regions between WT Gαs, (brown) and Ins4A (cyan). This structure shows significant rearrangement of side chains in His57, Phe189, Phe191, and Phe196 and disturbed π-π interaction between His57 and Phe189 (E); comparison of β2AR-Gαs complex structure (green) and WT Gαs, β1, γ1 (brown) (F); relative position of residue His57 and the α1 helix between WT Gαs, (brown), Ins4A (cyan), and rhodopsin-G protein model (gray) (19).
direct interaction between the 4-Ala insertion site and these regions. Therefore, these structural differences might be allosteric effects of the insertion region. Another possibility is that all three heterotrimeric structures, \( \alpha \), \( \beta_2 \), \( \gamma_1 \), \( \beta_1 \), \( \gamma_2 \), and \( \alpha \) regions between WT \( \alpha \), \( \beta_2 \), and \( \gamma_1 \) contain the same \( \alpha \) but different \( \beta_2 \) and \( \gamma_1 \) subtypes, which might affect the heterotrimeric structures in specific regions. The root mean square deviation of the WT \( \beta_1 \gamma_2 \) (PDB entry 1GP2 (24)) and Ins4A-\( \beta_1 \gamma_1 \) was 0.82 and 1.2 Å (with a total of 329 and 697 Ca atoms aligned, respectively).

**The Effect of the 4-Alanine Insertion on \( \alpha \) Helix Interface Binding Energy**—To investigate the effect of the extra helical turn of \( \alpha_5 \) on the \( \alpha \) helix computationally, we calculated interaction energy scores for all residues within the \( \alpha \) helix in both the WT and heterotrimeric \( \alpha \) \( \beta_1 \gamma_2 \) structures, which could propagate the conformational changes necessary for G protein activation and nucleotide exchange. The \( \Delta \Delta G \) calculations predicted and support the crystallographic data.

**TABLE 3**

| G protein \( \alpha \) subunit \( \alpha \) helix interface energetic predictions |
|--------------------------------|
| Entity | Amino acid | Energy | Z score | Energy | Z score | Energy | Z score |
|--------|------------|--------|---------|--------|---------|--------|---------|
| \( \beta_1 \) | Leu\(^{38} \) | 1.1 | 75 | 1.2 | 281 | 1.4 | 53 | 2.0 | 17 |
| \( P \)-loop | Gly\(^{40} \) | 0.8 | 103 | 1.1 | 89 | 1.9 | 6 | 1.9 | 35 |
| \( \alpha_1 \) | Lys\(^{43} \) | 1.3 | 53 | 1.0 | 179 | 1.1 | 192 | 1.0 | 10 |
| \( \alpha_1 \) | Ser\(^{47} \) | 0.7 | 61 | 0.5 | 34 | 0.9 | 118 | 1.6 | 25 |
| \( \alpha_1 \) | Thr\(^{48} \) | 0.9 | 52 | 0.8 | 154 | 0.7 | 27 | 0.9 | 27 |
| \( \alpha_1 \) | Ile\(^{49} \) | 1.0 | 38 | 1.0 | 179 | 1.1 | 192 | 1.0 | 10 |
| \( \alpha_1 \) | Val\(^{50} \) | 0.7 | 61 | 1.5 | 34 | 1.7 | 266 | 1.6 | 25 |
| \( \alpha_1 \) | Glu\(^{52} \) | 1.2 | 25 | 1.4 | 462 | 1.0 | 116 | 1.4 | 14 |
| \( \alpha_1 \) | Met\(^{53} \) | 0.6 | 71 | 2.3 | 101 | 2.2 | 441 | 2.5 | 24 |
| \( \alpha_1 \) | Lys\(^{54} \) | 1.8 | 122 | 2.3 | 101 | 2.2 | 441 | 2.5 | 24 |
| \( \alpha_1 \) | Ile\(^{55} \) | 1.4 | 170 | 1.0 | 263 | 1.0 | 158 | 1.1 | 35 |
| \( \alpha_1 \) | Ile\(^{56} \) | 1.0 | 85 | 1.1 | 57 | 1.0 | 158 | 1.1 | 35 |
| \( \alpha_1 \) | His\(^{57} \) | 1.3 | 28 | 1.6 | 133 | 1.5 | 127 | 1.7 | 34 |
| Linker 1 | Gly\(^{60} \) | 0.7 | 71 | 0.9 | 339 | 0.9 | 67 |
| Linker 1 | Tyr\(^{61} \) | 1.0 | 125 | 1.5 | 70 | 0.8 | 208 | 1.0 | 9 |
| \( \alpha_A \) | Gln\(^{64} \) | 0.8 | 104 | 0.5 | 9 | 0.8 | 384 | 0.8 | 52 |
| \( \alpha_A \) | Leu\(^{75} \) | 0.9 | 51 | 1.0 | 279 | 1.0 | 104 | 1.0 | 23 |
| \( \beta_2 \) | Phe\(^{109} \) | 1.0 | 13 | 1.4 | 114 | 1.5 | 229 | 1.4 | 28 |
| \( \beta_2 \) | Phe\(^{111} \) | 0.5 | 73 | 0.5 | 73 | 0.5 | 73 | 0.5 | 73 |
| \( \beta_3 \) | Met\(^{118} \) | 0.8 | 84 | 1.0 | 3 | 1.0 | 24 |
| \( \beta_5 \) | Asp\(^{208} \) | 1.3 | 53 | 1.3 | 88 | 1.3 | 124 | 1.6 | 38 |
| \( \beta_5 \) | The\(^{218} \) | 0.5 | 21 | 0.8 | 100 | 0.8 | 346 | 0.8 | 16 |
| \( \alpha_5 \) | Val\(^{322} \) | 0.6 | 11 | 0.7 | 189 | 0.7 | 397 | 0.8 | 35 |
| \( \alpha_5 \) | Phe\(^{326} \) | 0.9 | 736 | 0.9 | 736 | 0.9 | 736 | 0.9 | 736 |
We did not see any major differences at the N terminus of the α1 helix, Leu66–Ile69, compared with the WT protein structure. However, starting from Val50, significant differences were identified between mutant and WT proteins. The predicted ΔΔG values of Gln52, Met53, Ile56 and His57 residues, which play a major role in interaction with and stabilization of the α5 helix in the GDP-bound state, were decreased compared with WT (11, 19, 20, 27). The total interaction energy score was ~4 Rosetta energy units (REUs) in the Ins4A compared with 5.5 REUs in the WT protein (Table 3).

There are two critical stabilizing routes between the α1 and α5 helices in the GDP-bound state. To look at the individual residue-residue interactions and distinguish between these two pathways, we used Rosetta to predict the network energy scores between all amino acid pairs in our structural models and protein crystals. The first route is between Gln52 (α1) and Ile56 (α1) with Thr329 (α5). Previously, Kapoor et al. (28) showed that the T329A mutation causes high Gaα1 activity. The pairwise interaction scores were calculated between Gln52 (α1) and Thr329 (α5) and between Ile56 (α1) and Thr329 (α5) as 0.5 and 0.2 REUs, respectively. The second pathway is between Met53 (α1) and His57 (α1) with Val332 (α5) and Phe336 (α5), a part of the hydrophobic core between α5, α1, and β2-β3 strands (Fig. 8). The structural rearrangement at the end of the α1 helix also affects linker 1 and the beginning of the α5 helix. The ΔΔG values calculated at Gly60 (linker 1) decreased from 0.7 to under 0.5; at Tyr61, it was changed from 1.5 to 1.0 REU compared with WT protein. In Gln65 (αA), it increased from 0.5 to 0.8 REUs, as it approached linker 1. In the heterotrimeric structures, we observed a similar pattern between Ins4A and WT but with smaller margins.

Discussion

Two receptor-mediated G protein activation routes have been hypothesized. In the first, binding of the receptor to the C terminus of Gaα is thought to trigger conformational changes that can be transmitted to the nucleotide-binding pocket via outward rotation and translation of the α5 helix and distortion of the β6-α5 loop, a key site of interaction with the guanine ring (8, 21, 29–31). In the second pathway, the receptor-dependent α5 rotation and translation destabilizes the hydrophobic interactions between the α5 and α1 helices and the β2-β3 strands, which weaken both phosphate and purine binding sites of nucleotide (10, 11, 20, 27, 28). In the two proposed activation pathways, the extreme C terminus of the α5 helix facilitates both receptor-G protein interaction and G protein activation (2, 8, 9, 32, 33). To separate these two pathways and to further investigate the effect of the hydrophobic core between α5, α1, and β2-β3 strands, we inserted a 4-Ala linker between Gln333 and Phe334 in the α5 helix.

Our data show that the Ins4A mutant caused high basal nucleotide exchange, as anticipated from previous studies (9–11). The Ins4A-GTPγS crystal structure showed that, starting from Gln333, the α5 helix is displaced by an extra helical turn, which partially mimics the effect of the receptor on the G protein. Indeed, Phe336 of the α5 helix, which we previously showed was a critical residue for forming a hydrophilic core in the Ga subunit, is localized at a position similar to where it is localized in the β2AR-Gs complex structure.

The α5 helix is protected and surrounded with mostly hydrophobic interactions by six β-strands (β1–β6) and one α helix (α1). The effects of α5 helix rotation on the β6-strands are clearly observed in the Ins4A structure compared with WT protein. The relative positions of the β5 and β6 strands are not affected by the rotation, and these two strands almost perfectly superimpose with the WT structure. However, there are significant and progressive differences in the β-strands N-terminal to β4. This is most dramatically observed in the β2-β3 strands. This rotation completely repositions the network of interactions between Phe336 (α5); Phe189, Phe191, and Phe196 (β2-β3); and Met53 and His57 (α1), including disturbing the π–π interaction between His57 and Phe189. The conformational changes in this region mimic the receptor-bound state (10, 11, 20, 27, 34). This result supports the second route of G protein activation (see above), which was proposed in our previous study (11) and was recently supported by Flock et al. (20) and Sun et al. (27) via using evolutionary analysis and alanine scanning approaches, respectively.

In the β2AR-Gs complex structure, the α1 helix, starting from Met65 (Met66 in Gaα), is not ordered (8). In the rhodopsin-Gs complex model, it was predicted that the end of the α1 helix would move away from α5, and most of the residues (from Gln52 to His57) would lose contact with the α5 helix after GDP release and helical domain opening (19). The Ins4A mutant structure confirmed this prediction, although we could only crystallize the GTPγS-bound state, which holds the GTPase and helical domains together. Given that it is GTPγS-bound, significant differences between WT and mutant structures in the nucleotide contact regions, such as the P-loop and β6-α5 loop, were not expected. However, it appears that the reorganization between the α5 helix and β2-β3 strands is enough to trigger the α1 rearrangement although the β6-α5 loop and first helical turn of the α5 helix are still intact.

In contrast to its high constitutive activity in the basal state, the Ins4A mutant showed very little receptor-mediated nucleotide exchange activity. This, we believe, is due to the effect of the Gβγ subunit. In the basal state, without Gβγ, the Ga subunit does not require a large displacement of α5 and the β6-α5
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loop to release GDP from the binding pocket. Perturbation of β2-β3, α1, and the Mg$^{2+}$ binding regions is sufficient to trigger GDP release (10, 11, 28). However, in the heterotrimeric G protein, the Gβγ subunit interacts with Switch II and the phosphate binding region, reducing the dynamics of this region. Gβγ binding significantly limits nucleotide exchange of the G protein in the absence of receptor (11, 28, 35). When the receptor interacts with the heterotrimeric G protein, it rotates the α5 helix and initiates the nucleotide release mechanism.

The Ins4A mutant shows similar receptor binding capability compared with WT protein. The nucleotide binding and release data show that the Ala insertion significantly affects the G protein nucleotide interaction. The heterotrimeric Ins4A mutant releases GDP almost 100-fold more slowly than WT in the presence of activated receptor. Comparison with the GTPγS binding kinetics allows us to conclude that GDP release is the main affected event. However, even with a very slow nucleotide exchange rate, GTPγS can still access the nucleotide binding pocket. However, release from the receptor-G protein complex is abolished even in the presence of high concentrations of either GDP or GTPγS. This indicates that disrupting the hydrophobic core not only affects nucleotide interaction within the Gα subunit in the receptor G protein complex but also that the G protein can no longer release from the receptor complex.

How does the heterotrimeric G protein bind normally to the receptor and interact with the nucleotide but not release from the receptor complex? The β6-α5 loop directly interacts with the guanine ring of the nucleotide, and it is the only direct way to connect the nucleotide binding region to the receptor through the α5 helix. Within the β6-α5 loop resides a conserved TCAT motif that mediates key contacts with the guanine ring of GDP that are believed to stabilize the binding of GDP within Gα. This indicates that disrupting the hydrophobic core not only affects nucleotide interaction with the Gα subunit but also that the G protein can no longer release from the receptor complex.

Thus, receptor contacts to the Gα C terminus communicate structural changes through the α5 helix, which may modulate the conformation of the β6-α5 loop, ultimately resulting in the release of GDP. The N terminus of the α5 helix is unfolded in the β2AR-Gs complex structure (8). Recently, Dror et al. suggested that the structural rearrangements in the β6-α5 loop are the key events in G protein activation and GDP release (21). To examine environmental changes around this region, we fluorescently labeled residues Leu273 (αG) and K330 (αS) and showed that the N terminus of the α5 helix does not properly refold in the presence of nucleotide. This result indicates that either the 4-alanine insertion creates a buffer due to the extra length of the α5 helix between the receptor and nucleotide binding site of Gα subunit, or it disturbs the nucleotide-dependent rearrangement of the N terminus of the α5 helix (residues 368–371 in Gα and 328–331 in Gαi).

To address the question of whether slow nucleotide exchange and receptor release are caused by the increased length of the C-terminal helix, the repeated set of 4 WT amino acid residues was inserted back into the same region (Ins4X; Fig. 1B). This restores the hydrophobic core around Phe236 (αS) while maintaining the longer α5 helix. Notably, Ins4X showed similar basal and receptor-mediated nucleotide exchange activity to WT. In addition, it recovered its receptor release activity after guanine nucleotide incubation. This is consistent with previous studies (9, 31); Natochin et al. (31) showed that an 11-amino acid insertion above the hydrophobic core region (between Ile543 and Ile544) did not affect G protein-receptor binding and G protein activation compared with the WT protein. This indicates that it is not the length of the C terminus but rather maintaining the hydrophobic core interactions that is critical to complete the receptor-mediated G protein activation cycle.

In summary, the Ins4A crystal structure showed how a5 rotates significantly changes the conformation of β2-β3 and the α1 helix. Phe236 is probably making direct hydrophobic contacts with Phe191 and Met53, and it may also communicate with Phe189 indirectly. Residues Met53, His57, and Phe191 interact with Phe189 through a π-π interaction between residues His57 and Phe189. In addition, Gln53, Ile55, and Ile56 in the α1 helix also interact with Thr329 and Gln333 in the α5 helix.

This network not only plays a major role during G protein activation but also influences proper rearrangement of the N terminus of the α5 helix to allow release of Gα from the activated receptor after nucleotide binding. Thus, this study highlights changes through the G protein for receptor-mediated GDP release and G protein activation but also the reverse communication from GDP binding to release of the G protein from the activated receptor. How G proteins influence the ligand binding of receptor, leading to a high affinity ligand binding and, in the case of rhodopsin, Meta II stabilization, is currently unknown. This study provides the first clue that rearrangement of the N terminus of the α5 helix and re-engagement of the hydrophobic core are important elements of that signaling back to the receptor.

This mechanism might be generalizable for many receptor-G protein combinations; indeed, all residues of this hydrophobic core and the N terminus of the α5 helix are highly conserved in heterotrimeric G proteins. Our results support and experimentally demonstrate that the structural rearrangements of this region complete the G protein activation cycle. Although different receptor-mediated G protein activation models are presented as opposing mechanisms (4, 7, 9, 11, 20, 21, 27, 39), they may play complementary roles in the overall action of activated receptors. However, further studies are needed to identify the sequence of events involved in receptor-mediated G protein activation in molecular detail.

Experimental Procedures

Materials—The TSKgel G2000SW and G3000SW columns, GDP, and GTPγS were purchased from Sigma. BODIPY-GDP and β-GTPγS were purchased from Thermo Fisher Scientific. All other reagents and chemicals were of the highest available purity.

Construction, Expression, and Purification of Proteins—In this study, recombinant Gαi was used for all experiments instead of visual Gα protein (Gαi), given that Gαi is a very close homolog of Gαq yet is more easily expressed in E. coli. Briefly, the pSV277 expression vector encoding Gαi1 with an N-terminal His tag served as the template for introducing amino acid insertions between residues Gln233 and Phe334 by using the
QuikChange system (Stratagene). The 4-Ala insertion (Ins4A-Gα11) mutant used primers 5'-GTA ACCTG GAC TGTCATGC GAA CTG AAT AAC C3' (forward) and 5'-GTT ATT CTT TAT TGC TGC TGC TGC GAT GAC (reverse). The Phe-Val-Phenylalanyl insertion (Ins4X-Gα11) mutant used primers 5'-CGG AAT GTG CAGGT TTT GTG TTC GTT GTG TTC GAT C3' (forward) and 5'-GCG ATC GAA CAC AAA ATC GAA CAC AAA CTC GAC TTT TTT CG3' (reverse). All mutations were confirmed by DNA sequencing (GenHunter Corp.). The WT and the mutant constructs were expressed and purified as described previously (22). The purified proteins were cleaved with thrombin (Sigma; 0.5 units/mg final concentration) for 16 h at 4 °C to remove the N-terminal His tag. The samples were then loaded onto a nickel-nitritotriacetic acid column to separate the proteins from the cleaved His tag and any uncleaved fraction. For further purification, the protein solutions were loaded onto a size exclusion chromatography column (TSKgel G3000SW) to separate the proteins into uncomplexed subunits using a TSKgel G3000SW size exclusion chromatography column equilibrated with buffer containing 20 mM HEPES (pH 7.2), 100 mM NaCl, 1 mM MgCl2, 20 μM GDP (or 1 μM GTPyS), 1 mM DTT, and 100 μM PMSF. SDS-PAGE was used to test the purity of the proteins. Urea-washed ROS membranes containing dark-adapted rhodopsin and Gβγ subunits were prepared as described previously (40, 41). Protein concentrations were determined spectrophotometrically (41) and by a Bradford assay (42).

Preparation of Urea-washed ROS Membranes and Gβγγi1—Urea-washed ROS membranes and Gβγγi1 were prepared from bovine retina as described previously (40, 43).

Nucleotide Exchange Assays—The basal rate of GTPyS binding was determined by monitoring the relative increase in the intrinsic tryptophan (Trp) fluorescence (λex = 290 nm, λem = 340 nm) of Gα11 (200 nM) in buffer containing 50 mM Tris (pH 7.2), 100 mM NaCl, and 1 mM MgCl2 for at least 60 min at 21 °C after the addition of 10 μM GTPyS. Receptor-mediated nucleotide exchange was determined with Gβγγi1 (400 nM) in the presence of 50 mM rhodopsin at 21 °C for 60 min after the addition of GTPyS. The data were normalized to the baseline and maximum fluorescence and then fit to the exponential association equation (y = ymax × (1 - e-kx)) to calculate the rate constant (k) as described previously (44). For nucleotide exchange experiments with BODIPY nucleotides, the fluorescence was monitored at λex = 490 nm and λem = 510 nm with 5-nm slit widths as described (45). All experiments were performed in a buffer containing 50 mM Tris (pH 7.2), 100 mM NaCl, 1 mM MgCl2, and 1 mM DTT at 21 °C. To measure the GDP release from the G protein, the Gαi1 subunit was incubated with BODIPY-GDP in the absence of unlabeled GDP, Gβγγi1 subunit, or receptor for 90 min at room temperature to exchange GDP with BODIPY nucleotide. After 1.5 h, a 2-fold excess of Gβγγi1 was added and incubated for 15 min to suppress the nucleotide exchange. BODIPY-GDP-bound heterotrimeric G protein was recorded as the basal signal. After 2.5 min, Gβγγi1 subunit was added to initiate the exchange reaction. The kinetic data were plotted and fit to a one-phase association function. Data represent the averages from 8–10 experiments.

Protein Labeling—A cysteine-reduced Gα11 protein (C3S, C66A, C214S, C305S, C325I, C351I) was labeled as described previously using a 10-fold molar excess of Alexa Fluor 594C5-maleimide (A1) (Invitrogen), with a labeling time of 3–5 h in 50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM MgCl2, and 20 μM GDP (44). The proteins were purified via size exclusion purification, and the fractions were screened by intrinsic Trp fluorescence to ensure the functional integrity of the labeled proteins. Labeling efficiency was determined from comparison of A350 with protein concentration, as determined by Bradford, and found to be between 0.5 and 0.75 mol of label/mol of protein, depending on the location of the residue (44, 45).

Membrane Binding Assay—The ability of mutant Gα11 subunits to bind R* in urea-washed ROS membranes was determined as described previously (7). Each sample was evaluated by comparison of the amount of Gα11 subunit within the pellet (P) or supernatant (S) with the total amount of Gα11 subunit (P + S) in both treatments expressed as a percentage of the total Gα11 protein. Data represent the average of at least five experiments.

Protein Crystallization, Data Collection, and Structure Determination—Purified GTPyS-bound Ins4A-Gα11 subunits were exchanged into crystallization buffer (50 mM HEPES (pH 8.0), 1 mM EDTA, 10 mM MgSO4, 5 mM DTT, 20 μM GTPyS) using a TSKgel G3000SW size exclusion chromatography column. Appropriate fractions were pooled as described above, and SDS-PAGE was used to assess the purity of the proteins. Crystals were grown using the hanging drop vapor diffusion method at 21 °C by equilibrating a 1:1 ratio of protein (10 mg/ml in crystallization buffer) and reservoir solution (12–16% PEG 2000 monomethyl ether, 18% 2-propanol, and 100 mM MES (pH 6.0)) against a reservoir solution. Crystals appeared after 15 days and grew in the primitive monoclinic space group P21.

For Ins4A-Gα11βγγi1 crystallization, separately purified and concentrated Ins4A-Gα11 and WT Gβγγi1 subunits were mixed in a 1:1 molar ratio and incubated for 30 min at 25 °C. The heterotrimeric G protein complex was purified away from uncomplexed subunits using a G3000SW size exclusion chromatography column equilibrated with buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM DTT, and 200 μM GDP. Appropriate fractions were pooled, and the post-translational palmitoylation of the Gβγγi1 subunit was removed by incubating with 10 units of endoproteinase Lys-C in 50 mM Tris (pH 7.5; 150 mM NaCl) for 24 h at 4 °C (25). The protein complex was subjected to an additional step of size exclusion chromatography using a G3000SW column, as described above. Fractions were analyzed by SDS-PAGE to provide a guide to appropriate pooling of the purified heterotrimer. Heterotrimeric complex was crystallized using the hanging drop vapor diffusion method at 21 °C by equilibrating the protein (10 mg/ml in 20 mM HEPES (pH 7.5), 150 mM NaCl, 200 μM GDP, 1 mM EDTA) in a 1:1 ratio with reservoir solution against a reservoir solution containing 19–24% PEG 8000, 1–5% 2-propanol, 1% n-octyl-β-D-glucopyranoside, 100 mM HEPES (pH
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7.0), and 100 mM NaOAc (pH 6.4). Crystals appeared after 5 days and grew in the primitive tetragonal space group P4₃.

Both Ins4A-Gα₁₋GTPγS and Ins4A-Gα₁₋β₁γ₁, crystals were cryo-protected prior to data collection by briefly soaking in stabilization solution containing 18% glycerol and cryo-cooled by plunging into liquid nitrogen. Data sets were collected at the Life Sciences Collaborative Access Team (21-ID-G) of the Advanced Photon Source at Argonne National Laboratory at −180 °C using a wavelength of 0.98 Å on a MAR CCD detector. Data were scaled using HKL2000 (46), truncated and converted using CCP4 (47), and processed using Phenix suites (48). Crystallographic data collection and refinement statistics are reported in Table 2. Criteria for data cutoffs were a combination of Rsyn and I/σ, which both rose to unacceptable levels if the resolution were extended for either data set. The structures of Ins4A-Gα₁₋GTPγS-Mg²⁺ and Ins4A-Gα₁₋β₁γ₁-GDP complexes were determined by molecular replacement using 1GIA (WT Gα₁₋GTPγS-Mg²⁺) (26) and 1GJP2 (WT Gα₁₋β₁γ₂-GDP) (24) as search models for Phaser-MR (49) in the Phenix suite (48). Because PDB entries 1GIA and 1GJP2 were deposited prior to the requirement for deposition of structural factors, R-free reflections were randomly selected for Ins4A-Gα₁₋GTPγS-Mg²⁺ and Ins4A-Gα₁₋β₁γ₁-GDP. As a result, the free-R is of limited utility. Model building was performed in Coot (50) using composite omit maps calculated in Phenix (48) to minimize model bias. Refinement was conducted using both Refmac (51) and Phenix (48), with the final rounds of refinement performed using Phenix (48). In the final model, the regions corresponding to amino acids 1–33 and 348–354 (corresponding to WT numbers) in Ins4A-Gα₁₋β₁γ₁ were used to model the Gα₁ subunit, and purified heterotrimeric G protein for both biochemical assays and crystallization, performed protein labeling, performed all biochemical assays and analyzed results, performed crystallization trials, optimized crystallization conditions, collected diffraction data, solved and refined the structures, and wrote the manuscript. A. D. L. prepared and analyzed Rosetta energy and pairwise interaction calculations and wrote the manuscript with A. I. K. J. M. supervised Rosetta energy and pairwise interaction calculations and assisted with manuscript preparation. H. E. H. initiated and planned the project, supervised the research, and wrote the manuscript with A. I. K.

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Author Contributions—A. I. K. prepared mutant constructs, expressed and purified G protein α subunit and purified heterotrimeric G protein for both biochemical assays and crystallization, performed protein labeling, performed all biochemical assays and analyzed results, performed crystallization trials, optimized crystallization conditions, collected diffraction data, solved and refined the structures, and wrote the manuscript. A. D. L. performed and analyzed Rosetta energy and pairwise interaction calculations and wrote the manuscript with A. I. K. J. M. supervised Rosetta energy and pairwise interaction calculations and assisted with manuscript preparation. H. E. H. initiated and planned the project, supervised the research, and wrote the manuscript with A. I. K.
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