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Helix dipole movement and conformational variability contribute to allosteric GDP release in $G\alpha_i$ subunits^{†,‡}

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Abstract

Heterotrimeric G proteins ($G\alpha\beta\gamma$) transmit signals from activated G protein coupled receptors (GPCRs) to downstream effectors through a guanine nucleotide signaling cycle. Numerous studies indicate that the carboxy-terminal $\alpha 5$ helix of $G\alpha$ subunits participate in $G\alpha$ -receptor binding, and previous EPR studies suggest this receptor-mediated interaction induces a rotation and translation of the $\alpha 5$ helix of the $G\alpha$ subunit [Oldham *et al.*, Nat. Struct. Mol. Biol., 13: 772-7 (2006)]. Based on this result, an engineered disulfide bond was designed to constrain the $\alpha 5$ helix of $G\alpha_{i1}$ into its EPR-measured receptor-associated conformation through the introduction of cysteines at positions 56 in the $\alpha 1$ helix and 333 in the $\alpha 5$ helix (I56C/Q333C $G\alpha_{i1}$). A functional mimetic of the EPR-measured $\alpha 5$ helix dipole movement upon receptor association was additionally created by introduction of a positive charge at the amino-terminus of this helix, D328R $G\alpha_{i1}$. Both proteins exhibit dramatically elevated basal nucleotide exchange. The 2.9 Å resolution crystal structure of the I56C/Q333C $G\alpha_{i1}$ in complex with GDP-AlF₄⁻ reveals the shift of the $\alpha 5$ helix toward the guanine nucleotide-binding site that is anticipated by EPR measurements. The structure of the I56C/Q333C $G\alpha_{i1}$ subunit further revealed altered positions for the switch regions and throughout the $G\alpha_{i1}$ subunit, accompanied by significantly elevated crystallographic temperature factors. Combined with previous evidence in the literature, the structural analysis supports the critical role of electrostatics of the $\alpha 5$ helix dipole and overall conformational variability during nucleotide release.

$G\alpha$ subunits of heterotrimeric G proteins ($G\alpha\beta\gamma$) are members of a family of over 100 different guanine nucleotide binding proteins identified in eukaryotic cells. These G proteins transmit signals through a guanine nucleotide signaling cycle, with the protein-bound guanine

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nucleotide regulating the activation state of the $G\alpha$ subunit (1–4). Structural and biochemical studies of $G\alpha$ and small G proteins indicate that these proteins encode the information about their activation state in the conformation of loops at the protein surface. Aptly known as the switch regions, these regions switch between conformations in response to the identity of the bound nucleotide (5). As a result, the binding affinities for both upstream and downstream signaling proteins are dramatically affected.

GTP-bound G proteins can readily activate downstream pathways through direct protein-protein interactions. Hydrolysis of GTP to GDP by the $G\alpha$ subunit returns the protein back to its inactive, $G\beta\gamma$ -bound state. The uncatalyzed rate of nucleotide exchange is slow, but is dramatically enhanced by the interaction with an activated guanine-nucleotide exchange factor (GEF). For small G proteins, a soluble GEF catalyzes the release of GDP, while for heterotrimeric G proteins, a 7-transmembrane helix G protein coupled receptor (GPCR) acts as a GEF to catalyze nucleotide release.

Unlike many of the small G proteins which have a single domain and a guanine nucleotide binding site that is accessible to solvent, the guanine nucleotide in the $G\alpha$ subunits of heterotrimeric G proteins is nestled tightly between a Ras-like GTPase domain and a helical domain (6–11), with no obvious exit tunnel for the nucleotide. As a result, the rate limiting step of heterotrimeric G protein signaling is release of GDP catalyzed by an activated GPCR (R^* ; (12)), which physiologically requires the formation of a transient $G\alpha\beta\gamma$ - R^* complex. The structure determination of R^* in complex with its cognate $G\alpha\beta\gamma$ heterotrimer in the nucleotide-free state would provide the most accurate information about the mechanism of nucleotide exchange for this system. Since current experimental limitations make this a daunting undertaking, the investigation of R^* -catalyzed nucleotide exchange in G proteins has used alternative, low-resolution techniques. Many of the experiments have pointed to methods that can be used to stabilize $G\alpha$ in its R^* -bound conformation and provide new avenues to reveal mechanisms of nucleotide exchange in $G\alpha$.

The first hints of the mechanism of activation of $G\alpha$ subunits by GPCRs came through mapping of the binding interface of the complex. The C-terminus of $G\alpha$ subunits has long been known to interact with activated GPCRs. As early as the late 1980's, screening of $G\alpha$ -derived peptides revealed that peptides corresponding to the C-terminal $\alpha 5$ helix of $G\alpha$ could bind to receptors with high affinity and mimic G protein stabilization of the active state of the receptor (13–15). Later studies employed photoactivatable crosslinkers to show that light-activated rhodopsin could crosslink to the C-terminus of $G\alpha_t$ (16). The use of chemical crosslinkers confirmed the binding of receptor to the C-terminal residues of the $G\alpha$ subunit (17), and additionally identified residues within the amino terminus of $G\alpha_t$ that crosslinked to activated rhodopsin. Mapping of the receptor-G protein interaction using peptides corresponding to the cytoplasmic domain of the dopamine D_2 receptor further verified the interaction between receptor and the C-terminus of the $G\alpha$ subunit (18). As a complement to the peptide studies, C-terminal truncations in both $G\alpha_i$ and $G\alpha_o$ proteins have been shown to reduce affinity for GPCR, demonstrating the importance of this region in receptor affinity (19,20). The C-terminus may also be important for receptor selectivity as chimeric $G\alpha$ subunits that replace C-terminal residues in one $G\alpha$ subtype for another affect receptor selectivity (21–23). Further, mutations within the carboxy terminal region of $G\alpha$ subunits alter receptor-G protein coupling (24–26). A fluorescence-quenching approach revealed the binding site for a peptide derived from the carboxy terminus of $G\alpha$ maps to a hydrophobic region on helix VI in activated rhodopsin (27,28), demonstrating the utility of such approaches to provide detailed structural information, especially in the absence of a structure of an activated GPCR-G protein complex. Excitingly, a recent structure of opsin determined in complex with a peptide derived from the C-terminus of $G\alpha_t$ revealed the specific contacts between receptor and the last 10 amino acids of the $G\alpha$ subunit (29).

However, the carboxy terminus is not the sole region of $G\alpha$ that contacts the receptor; other studies have also implicated the $\alpha 4$ - $\beta 6$ loop (30,31) and the amino terminus (13,17,30,32–35). Together with the C-terminus, these regions can be mapped onto the structure of the $G\alpha_{i1}$ subunit and form a contiguous, three-dimensional surface as would be anticipated for a protein binding interface (30,36) (Figure 1a).

After the receptor binds its cognate G protein, the signal is transmitted from the receptor-binding site to the nucleotide-binding pocket of the $G\alpha$ subunit. Electron paramagnetic resonance (EPR) spectroscopy measurements are consistent with the R^* - $G\alpha$ interaction causing a rotation and translation of the $\alpha 5$ helix of the $G\alpha$ subunit toward the base of the guanine nucleotide (37) (Figure 1b). The insertion of a five amino acid flexible linker at the start of the C-terminal helix decouples receptor binding from the nucleotide release process (37), suggesting that the rigid-body roto-translation of the C-terminal helix changes the conformation of the $\beta 6$ - $\alpha 5$ linker containing the conserved TCAT sequence motif, which directly contacts the guanine ring of the nucleotide, to achieve receptor-mediated allosteric nucleotide release (38). In addition, the structure of opsin in complex with a $G\alpha$ -mimicking peptide (29) cannot predict the magnitude of the rigid body movement of the C-terminal $\alpha 5$ helix toward the GDP-binding pocket during receptor association. However, this co-structure indicates an additional conformational change of the $\alpha 5$ helix of $G\alpha$ is required to prevent steric clash of the $G\alpha$ protein with the membrane upon receptor binding. This additional conformational change was proposed as a 40° tilt of the $\alpha 5$ helix of the $G\alpha$ subunit relative to the remainder of the G protein. A less drastic change might involve the introduction of a small bend after the C-terminus of the $\alpha 5$ helix. This would prevent the exposure of hydrophobic side chains along the $\alpha 5$ helix to solvent and is consistent with both the crystal structure and the EPR spectroscopy measurements, which shows decreased mobility for residues along the $\alpha 5$ helix (residues 330, 331, 334, 340, 342, 344 and 349 in a $G\alpha_i$ protein show decreased mobility) upon receptor activation (37).

In light of the importance of receptor-catalyzed nucleotide exchange in the $G\alpha$ subunit, the changes in inter-residue distances measured by EPR spectroscopy during receptor activation (37) were used to develop a conceptual model of $G\alpha_{i1}$ conformation upon binding activated receptors (R^*). This EPR data suggests that the roto-translation of the $\alpha 5$ helix plays a critical role in receptor-mediated G protein activation, which could be communicated to the nucleotide binding site via global structural effects, as well as communicated through specific electrostatic effects directed at the base of the $\alpha 5$ helix, which comes into closer proximity to the bound nucleotide upon roto-translation of the $\alpha 5$ helix. The roto-translation of the $\alpha 5$ helix indicated by EPR studies which accompany receptor activation (Figure 1b), when mapped onto existing crystal structures, results in a close proximity between side chains of Gln 333 (in the $\alpha 5$ helix) and Ile 56 suggesting that an engineered disulfide bond between these residues could stabilize the structural changes that are present when $G\alpha$ is bound to receptor (37). The Gln at residue 333 is not conserved, and position 56 typically contains a hydrophobic Ile residue at this position (Leu in $G\alpha_s$).

A rigid-body roto-translation of the $\alpha 5$ helix would also be expected to move the positive dipole of the $\alpha 5$ helix towards the guanine ring of the bound nucleotide upon receptor activation. To accentuate this effect, a positive charge was introduced into the base of the $\alpha 5$ helix of $G\alpha_{i1}$ at residue 328, a residue conserved among $G\alpha_i$ family members (D328R $G\alpha_{i1}$, Figure 1b). This residue is next to the TCAT motif involved in nucleotide binding (residues 324–327). The I56C/Q333C $G\alpha_{i1}$ and the D328R $G\alpha_{i1}$ proteins (as well as other mutant proteins for control experiments) were constructed; the proteins expressed at levels similar to wild-type $G\alpha_{i1}$ and could be purified using similar protocols.

EXPERIMENTAL PROCEDURES

Construction, expression and purification of rat G α_{i1} mutant proteins

G α_{i1} mutants were created using the Quikchange site-directed mutagenesis kit (Stratagene). The I56C/Q333C double mutant used primers 5'-AAGCAGATGAAAATTTGTCACGAGGCTGGC (I56C forward), 5'-GCCAGCCTCGTGACAAAATTTTCATCTGCTT (I56C reverse), 5'-ACGAAGAATGTGTGTTTTGTGTTTCGATGCT (Q333C forward) and 5'-AGCATCGAACACAAAACAC-ACATTCTTCGT (Q333C reverse). The D328R mutant used primers 5'-TTCACTTGCGCCACGCGCACGAAGAATGTGCAG (forward) and 5'-CTGCACATTCTTCGTGCGCGTGGCGCAAGTGAA (reverse); D328A, (forward) 5'-CACTTCACTTGCGCCACGGCTACGAAGAATGTGCAG- 3', (reverse) 5'-CTGCACATTCTTCGTAGCCGTGGCGCAAGTGAAGTG- 3'; D328N (forward) 5'-CACTTCACTTGCGCCACGAATACGAAGAATGTGCAG- 3', (reverse) 5'-CTGCACATTCTTCGTATTTCGTGGCGCAAGTGAAGTG-3', K330D (forward) 5'-CTTGCGCCACGGATACGGATAATGTGCAGTTTGTG-3'(reverse) 5'-CACAAACTGCACATTATCCGTATCCGTGGCGCAAG- 3'. All constructs contain an internal hexahistidine affinity tag inserted between residue 119 and residue 120 in the helical domain (39). Proteins were expressed and purified as described (39).

Nucleotide exchange of GDP as measured by intrinsic tryptophan fluorescence

The basal rate of GTP γ S binding was determined by monitoring the relative increase in the intrinsic fluorescence (λ_{ex} = 300 nm, λ_{em} = 345 nm) on a Varian Cary Eclipse fluorescence spectrophotometer of 500 nM wild-type or mutant G α_{i1} subunits in 10 mM MOPS (pH 7.2), 130 mM NaCl and 2 mM MgCl₂ for 40 min at 21°C after the addition of 10 μ M GTP γ S (37). Receptor-catalyzed exchange was performed on G α subunits reconstituted with G $\beta_1\gamma_1$ (500 nM each) in the presence of 500 nM light activated rhodopsin from ureawashed Rod Outer Segments (ROS). Data were an average of three independent experiments and were normalized to the baseline (0%) and the fluorescence maximum (100%). The exchange rate was determined by fitting the data to an exponential association curve using Prism 4.0 (GraphPad Software).

Nucleotide exchange of GDP and GDP-AlF₄⁻ as measured by BODIPY-GTP γ S fluorescence

Basal nucleotide exchange was measured as fold-increase in emission of 1 μ M BODIPY-GTP γ S (λ_{ex} = 490 nm, λ_{em} = 515 nm) in buffer containing 50mM Tris, 100mM NaCl, 1mM MgCl₂, 10 μ M GDP, pH 7.5, at 21°C, in the presence and absence of 75 μ M AlF₄⁻ before and after the addition of G α_{i1} subunits (200 nM) to the cuvette containing BODIPY-GTP γ S.

Rhodopsin binding assay and metarhodopsin II stabilization

The ability of labeled G α subunits to bind rhodopsin in urea-washed ROS membranes was determined as described (35). G α_{i1} (5 μ M) with G $\beta\gamma$ (10 μ M) and rhodopsin (50 μ M) in a buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl and 1 mM MgCl₂ were incubated in the dark, after light activation, and after light activation with the addition of GTP γ S (100 μ M) for 30 minutes incubation at 4°C. Membranes were separated by centrifugation at 20,000 \times g for 1 hour, and the supernatants removed from pellets. For the dark fraction, the reaction was protected from light during centrifugation, and supernatant was removed in dim red light. The isolated fractions were boiled, visualized on Coomassie-stained SDS-PAGE, and quantified by densitometry using BioRad Multimager (37) by comparison of the amount of 37 kDa G α in either pellet or supernatant to the total amount of G α subunits in both fractions, and expressed as a percent of the total. Data are the average of 3 independent experiments. Stabilization of metarhodopsin II by increasing the concentration of G α proteins (as detailed in Supplementary

Figure 2) was performed essentially as described in (40) using an Olis DW2000 spectrophotometer before and after light activation of rhodopsin-G protein complexes.

Crystallization, data collection, structure determination, and refinement

Purified wild-type and I56C/Q333C $G\alpha_{i1}$ were exchanged into crystallization buffer (20 mM HEPES pH 8.0, 1 mM EDTA, 10 mM $MgCl_2$, 20 μ M GDP, 16 mM NaF, and 40 μ M $AlCl_3$) and concentrated to 10 mg/mL prior to crystallization. Crystals of the $G\alpha_{i1}$ I56C/Q333C double mutant were grown at 18°C by the hanging drop vapor diffusion method using 2 μ L of 10 mg/mL protein and 2 μ L of reservoir solution (16% PEG 3350, 240 mM $Li(CH_3COO)$, 0.1 M bis-Tris pH 6.5, and 10 mM $SrCl_2$). Oxidizing agents were not added to the protein solution, and inclusion of DTT or β -mercaptoethanol inhibited crystal growth. For accurate comparison, crystals of the wild-type $G\alpha_{i1}$ with a lipid modification were grown in the same space group using a modified reservoir solution containing 12% PEG 3350, 0.1 M $Ca(CH_3COO)_2$, and 0.1 M bis-Tris, pH 6.5. Crystals formed within three days and belong to the tetragonal space group $P4_32_12$ with unit cell dimensions $a=b=79.7$ Å, $c=114.5$ Å, $\alpha=\beta=\gamma=90^\circ$. Crystals were cryo-cooled in reservoir solution containing 25% glycerol. Data for the I56C/Q333C $G\alpha_{i1}$ subunit were collected at 93 K at wavelengths of 1.0 Å and 1.77 Å (Table 2) at the Advanced Photon Source IMCA-CAT beamline 17-ID on an ADSC CCD detector. Data for the wild-type protein were collected at the Advanced Photon Source LS-CAT ID-21-F at 93 K using a wavelength of 0.98 Å on a MAR CCD detector. Data were processed using the HKL (41) and CCP4 (42) suites of programs. Initial phases were calculated by molecular replacement using the program PHASER (43) using wild-type GDP- AlF_4^- -bound $G\alpha_{i1}$ as the search model (accession code 1GFI; (6)). Sulfur-SAD phases resulting in a figure of merit of 0.32 were determined using SHARP (44). The molecular replacement and S-SAD phases were combined using SIGMAA (42). Model building was performed in O (45), and refinement performed in CNS (46), REFMAC (42,47), and PHENIX (48).

Structural analyses

All structural comparisons of the GDP- AlF_4^- -bound I56C/Q333C $G\alpha_{i1}$ subunit structure were performed against wild-type protein crystallized in a binary complex with GDP- AlF_4^- in both the same space group with the same crystallographic packing ($P4_32_12$) and space group $P3_221$ (PDBID 1GFI). Root mean squared deviations of C_α positions were calculated in O (45) and temperature factors were calculated in CNS (46). Figures and movies were made using PyMOL (DeLano Scientific LLC) and LSQMAN (49).

Surface plasmon resonance (SPR) biosensor measurements

SPR binding assays were performed at 25°C on a BIAcore 3000. N-terminally biotinylated versions of peptides KB-752 (SRVTWYDFLMEDTKSR; (50)) and KB-1753 (SSRGYYHGIWVGEEGRLSR; (51)), were diluted to 0.1 μ g/ml in BIA running buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, and 0.005 % NP40], and coupled to separate flow cells of streptavidin biosensor chips (Biacore SA chips; GE Healthcare) using the MANUAL INJECT command to a surface density of approximately 750 resonance units. Prior to injection, the $G\alpha$ subunits were diluted to a range of final concentrations used to establish K_D in BIA running buffer containing either 100 μ M GDP or 100 μ M GDP, 30 μ M $AlCl_3$, and 10 mM NaF. 40 μ L of 10–50 μ M $G\alpha$ subunits were then simultaneously injected over flow cells at 10 μ L/min followed by a 200 second dissociation phase. Signal from binding to a non- $G\alpha$ interacting, biotinylated peptide (C-terminal tail of mNOTCH1: PSQITHIPEAFK) was subtracted from all binding curves to correct for non-specific binding and buffer shifts created during injection. Surfaces were regenerated between each injection with two pulses of 10 μ L of regeneration buffer (500 mM NaCl and 25 mM NaOH) at 20 μ L/min. Binding curves and kinetic analyses were conducted using BIAevaluation software version 3.0 and plotted

using GraphPad Prism version 4.0b. Binding affinities were calculated using simultaneous association (k_a) and dissociation (k_d) rates derived from generated sensorgram curves.

RESULTS

Identification of the I56C/Q333C and D328R G α_{i1} subunits as functional mimetics of R*-bound G α_{i1}

As structural and functional mimetics of the receptor-activated form of G α , I56C/Q333C and D328R G α_{i1} proteins should exchange nucleotide at an accelerated rate, even in the absence of receptor, as compared to wild-type G α subunits. To further characterize the mutant G α subunits, the ability of each mutant to bind to activated receptors was compared to wild-type G α_{i1} .

(1) Rates of nucleotide exchange—The first experiment to evaluate if the I56C/Q333C and D328R G α_{i1} subunits mimic R*-bound G α_{i1} was the measurement of the rate of nucleotide exchange. Rhodopsin is the cognate receptor for G $_t$, which is a member of the G $_i$ family of proteins. Rhodopsin can also efficiently catalyze nucleotide exchange in G α_{i1} subunits (37, 52). Additionally, G α_{i1} subunits can stabilize extra metarhodopsin II formation in a dose-dependent manner (Supplementary Figure 2). These studies are consistent with a functional interaction between these G α_{i1} proteins and rhodopsin. The uncatalyzed rate of nucleotide exchange for the I56C/Q333C (0.013 sec⁻¹) and D328R (0.018 sec⁻¹) G α_{i1} subunits are both increased relative to wild type under both uncatalyzed (0.002 sec⁻¹) and rhodopsin-catalyzed (0.008 sec⁻¹) conditions (Figure 2 a–c, Table 1), consistent with the structural change in the I56C/Q333C G α_{i1} subunit and the electrostatic change in the D328R G α_{i1} subunit functionally mimicking receptor-bound G α . Both mutant G α_{i1} subunits can associate with receptor (see section 2, below) and the addition of activated rhodopsin to either mutant G α_{i1} did not further enhance nucleotide exchange; this suggests that these mutant G α_{i1} proteins are maximally activated in the absence of R*. To ensure rates of exchange in the D328R G α_{i1} protein were not a non-specific effect of mutation we expressed control proteins D328N, D328A and K220D G α_{i1} . The D328N and K330D mutations did not dramatically perturb nucleotide exchange as compared to wild type, and while the D328A mutant did exhibit increased basal exchange rates, it was not to the extent seen in either I56C/Q333C or D328R G α_{i1} . In addition, the increases in exchange due to individual cysteine mutations in the background of a cysteine-depleted G α_{i1} protein (37) at residues 56 and 333 (Supplementary Figure 3), when added together, accounts for only half of the fold-increase observed in the I56C/Q333C G α_{i1} double mutant (Figure 2). Finally, unlike the I56C/Q333C or D328R G α_{i1} proteins, nucleotide exchange in all of the control mutant G α_{i1} subunits exhibited accelerated nucleotide exchange in the presence of activated receptor to accelerate nucleotide exchange, as does wild-type G α_{i1} protein.

The structure of the complex between bound GDP and AlF $_4^-$ in the nucleotide binding pocket of G α has been correlated to the transition state associated with GTP hydrolysis in G α proteins. The formation of a complex between bound GDP and AlF $_4^-$ stabilizes the bound nucleotide through additional hydrogen-bonds from the nucleotide binding pocket to the GDP-AlF $_4^-$ complex. To determine if the propensity for nucleotide release in the receptor-bound mimetics of G α_{i1} subunits was sufficient to overcome these stabilizing forces and release GDP-AlF $_4^-$, fluorescently labeled GTP γ S (BODIPY-GTP γ S) was used to monitor nucleotide release by measuring the increase in BODIPY-GTP γ S emission after the addition of G α subunits. GDP-AlF $_4^-$ -bound wild type and I56C/Q333C G α_{i1} proteins both exhibit decreased binding of BODIPY-GTP γ S when compared to their GDP-bound counterparts. This suggests additional contacts mediated by binding of AlF $_4^-$ stabilizes bound GDP, reducing BODIPY-GTP γ S binding in wild type and G α_{i1} I56C/Q333C subunits (Figure 2d). Surprisingly, both GDP-

AlF_4^- and GDP-bound D328R $\text{G}\alpha_{i1}$ subunits bound BODIPY-GTP γ S to a similar extent (Figure 2d) suggesting that AlF_4^- is not sufficient to stabilize nucleotide binding in the D328R $\text{G}\alpha_{i1}$ protein. These results suggest that electrostatic perturbations at the base of the $\alpha 5$ helix exert distinct effects on both the affinity and duration of nucleotide binding.

(2) Binding to membrane-bound receptors—The ability of the GDP- $\text{G}\alpha\beta\gamma$ subunits containing either wild-type, I56C/Q333C, or D328R $\text{G}\alpha_{i1}$ to bind to rhodopsin-containing rod outer segment membranes in the dark, after light activation, and after addition of GTP γ S to light activated complexes was investigated (Figure 3). Light activation significantly enhanced the membrane association of both wild-type and I56C/Q333C $\text{G}\alpha_{i1}$ to a similar extent. Consistent with this being a more dramatic mimetic of receptor activated protein, D328R $\text{G}\alpha_{i1}$ subunit had an even higher level of interaction with membrane bound receptors after light activation than did wild-type or I56C/Q333C $\text{G}\alpha_{i1}$ proteins. As expected, subsequent addition of GTP γ S dissociated the rhodopsin- $\text{G}\alpha\beta\gamma$ complex for all three $\text{G}\alpha_{i1}$ subunits, resulting in predominantly soluble GTP γ S-bound $\text{G}\alpha_{i1}$ subunits.

Identification of global structural changes in the I56C/Q333C $\text{G}\alpha_{i1}$ by X-ray crystallography

To determine if the physical constraint of the $\alpha 5$ helix by the disulfide bond in the I56C/Q333C $\text{G}\alpha_{i1}$ subunit propagated further structural changes, crystal structure of the I56C/Q333C $\text{G}\alpha_{i1}$ was determined in complex with GDP- AlF_4^- to 2.9 Å resolution (Table 2). The structure of the I56C/Q333C $\text{G}\alpha_{i1}$ is similar to wild-type $\text{G}\alpha_{i1}$ (Figure 1a); however the superposition of the I56C/Q333C $\text{G}\alpha_{i1}$ with wildtype protein results in root mean squared (RMS) deviation of 0.6 Å, which is higher than would be anticipated for the introduction of two cysteines. Excitingly, significant structural changes are located in regions of the GTPase domain previously identified for their importance in receptor binding and nucleotide binding, sensing, and exchange (Table 3).

The first statistically significant movement is at the site of introduction of the two cysteines. The initial σ_A -weighted $2|F_o| - |F_c|$ maps of the I56C/Q333C $\text{G}\alpha_{i1}$ showed clear density between Cys 56 and Cys 333 indicating the presence of a disulfide bond (Figure 4a). This disulfide bond constrains the position of the $\alpha 5$ helix (residues 328–345) towards the guanine nucleotide-binding site along the helical axis (Figure 4b; average C_α displacement of 0.6 Å and a maximal C_α displacement of 0.8 Å over the 17 residue helix) and shifts the position of the preceding $\beta 6$ - $\alpha 5$ loop. The disulfide bond further constrains the four C-terminal residues of the $\alpha 1$ helix (residues 46–57) towards the $\alpha 5$ helix with an average displacement of 1.0 Å and a maximal displacement of 1.5 Å. This shifts positions of the C_α atoms of the $\alpha 1$ - αA loop (residues 59–64) an average of 1.5 Å (Figure 4b). There is also a significant, albeit smaller, effect on the positions of the C_α atoms in the preceding P-loop (residues 41–45, Figure 4c). In the P-loop, a conserved glycine motif allows the backbone amide nitrogens to make hydrogen-bonding contacts with the nucleotide phosphates while the conserved Glu43 orients Arg 178 to further stabilize bound nucleotide. The C_α positions of the P-loop are shifted slightly toward the nucleotide with an average displacement of 0.65 Å and maximum displacement of 0.8 Å; however, the hydrogen-bonding interactions to phosphate and AlF_4^- are not significantly altered, and the side chains of Glu 43 and Arg 178 do not change conformation such that the latter remains within hydrogen-bonding contact of the phosphates. While AlF_4^- binding stabilizes the I56C/Q333C $\text{G}\alpha_{i1}$ structure in a conformation similar to that observed for the wild type $\text{G}\alpha_{i1}$ under the same conditions, the subtle differences between the two structures in the P-loop indicate a propensity towards conformational adaptability in the phosphate-binding region which may aid in nucleotide release. These subtle changes are detailed in figure 4f, the RMSD plot for this structure (as compared to wild-type protein). Figure 4f demonstrates that while subtle changes are present throughout the protein, there are particular regions which are significantly altered as compared to wild type, including residues in the $\alpha 1$ - αA linker region

(residues 56–64), the α B helix (residues 98–102), β 2 and β 3 strands (190–195), switch II region (residue 215–217) and the α 4/ β 6 loop (311–314). These differences are not related to differences in space group between the two proteins, as these changes are still noted when comparing I56C/Q333C $G\alpha_{i1}$ GDP-AlF₄⁻ to wild type $G\alpha_{i1}$ GDP-AlF₄⁻ crystallized in the same space group.

While shifts in the positions of the α 5 and α 1 helices (containing residues 333 and 56, respectively) and their associated connecting loops were anticipated, several additional regions of the I56C/Q333C $G\alpha_{i1}$ have unexpectedly altered positions when compared to wild-type $G\alpha_{i1}$ (Table 3, Figure 4b–e). These regions include the β 2– β 3 hairpin (residues 188–197, Figure 4b, d), the β 3– α 2 loop (Figure 4d), the α 2 helix (Switch II, residues 202–218, Figure 4d, e), and the β 4– α 3 loop (Switch III, residues 234–240). Each of these regions has previously been shown to have important functions connected with nucleotide sensing and exchange; the switch regions in particular exhibit distinct conformations in response to the identity of the bound guanine nucleotide, thereby altering the affinity of $G\alpha$ for $G\beta\gamma$ and other binding partners.

In addition to visible changes in atomic positions, parameters such as crystallographic temperature factors provide a measure of relative mobility in crystallographic models, and these are generally used to identify regions of increased mobility in a given structure. A comparison of temperature factors between the wild-type and I56C/Q333C $G\alpha_{i1}$ proteins crystallized in the same space group and at similar resolutions (3.0 Å for wild-type and 2.9 Å for the I56C/Q333C $G\alpha_{i1}$) shows an average increase of 31 Å² for the I56C/Q333C $G\alpha_{i1}$ as compared to wild type (Supplementary Figure 1). This is consistent with the I56C/Q333C $G\alpha_{i1}$ subunit having increased mobility and flexibility compared with wild-type $G\alpha_{i1}$. The increase in temperature factors is even greater in several key regions of the protein, reaching 50 Å² above wild type in the α 1 and α 5 helices and the switch regions.

Verification of structural changes of the switch regions of the I56C/Q333C and D328R $G\alpha_{i1}$ mutants by binding to nucleotide-state selective peptides

The ability of wild-type, I56C/Q333C and D328R $G\alpha_{i1}$ subunits to bind to peptides selective for the activation state of $G\alpha_i$ proteins were analyzed by surface plasmon resonance (Figure 5). The peptides KB-1753 and KB-752 discriminate between active, GDPAlF₄⁻-bound and inactive, GDP-bound $G\alpha_{i1}$ subunits (50,51). We found these peptides bind to wild-type and mutant $G\alpha_{i1}$ subunits with the same selectivity (Figure 5) but with different binding kinetics and affinities (Table 4). The peptide KB-752, selective for GDP-bound $G\alpha$ subunits, has been shown to bind to $G\alpha_{i1}$ at the switch II region (50). KB-752 has an 8-fold higher affinity for GDP-bound wild-type $G\alpha_{i1}$ subunits than it does for GDP-bound I56C/Q333C $G\alpha_{i1}$ subunits, suggesting that the switch II region in the GDP-bound I56C/Q333C $G\alpha_{i1}$ likely adopts a different conformation than is observed for GDP-bound wild-type $G\alpha_{i1}$ subunits. This suggests that the altered position of switch II observed in the crystal structure is also present in solution. This peptide binds to GDP-bound D328R on the order of that seen in wild-type $G\alpha_{i1}$ subunits, consistent with these proteins sharing a similar switch II geometry where the peptide binds. Conversely, GDP-AlF₄⁻-bound I56C/Q333C and D328R $G\alpha_{i1}$ subunits bind peptide KB-1753 (selective for activated $G\alpha_{i1}$ subunits (51)) with a 3–4 fold higher affinity than wild-type $G\alpha_{i1}$ -GDP-AlF₄⁻. This suggests that GDP-AlF₄⁻ bound I56C/Q333C $G\alpha_{i1}$ and D328R $G\alpha_{i1}$ subunits readily accommodates binding of the peptide selective for activated subunits.

DISCUSSION

In the rate-determining step of G protein signaling, activated GPCRs catalyze the release of GDP in the $G\alpha$ subunit of heterotrimeric G proteins (12). $G\alpha$ lacking bound nucleotide is unstable in solution; in vivo $G\alpha$ remains bound to R* until GTP binding, which result in predominantly soluble $G\alpha$ -GTP subunits, capable of activating downstream effectors. Previous

studies have mapped the binding site for the receptor onto a contiguous surface of the $G\alpha$ subunit (30,36) (Figure 1a), identified the three-dimensional constraints underlying the interaction between the C-terminus of the $G\alpha$ subunit and receptor (29), and have characterized a rigid body movement of the $\alpha 5$ helix of $G\alpha$ that accompanies receptor-catalyzed allosteric GDP release (37,53). The latter study set the stage for the current work, where the known conformational changes in the $\alpha 5$ helix were used as a starting point to mimic the R^* -bound conformation of $G\alpha$ through a disulfide bond, or by replicating the movement of the $\alpha 5$ helix dipole.

The I56C/Q333C $G\alpha_{i1}$ subunit is a structural and functional mimic of the R^* -bound $G\alpha$ subunit

An anticipated hallmark of an R^* -bound $G\alpha$ subunit is a dramatically decreased ability to bind to GDP, and a conformation predisposed to binding GTP. Enhanced nucleotide exchange in both mutant $G\alpha_{i1}$ subunits occurs in the absence of receptor and is consistent with both of these mutant subunits acting as functional mimics of the R^* -bound $G\alpha$ subunit. In the I56C/Q333C $G\alpha_{i1}$ variant, this is a result of structural changes that are anticipated for an R^* -bound $G\alpha$ subunit based on previous EPR studies. Even in the presence of $GDP\text{-}AlF_4^-$, the crystal structure of the I56C/Q333C $G\alpha_{i1}$ subunit reveals altered positions for the switch regions, which shows switch II and switch III each have a conformation that is intermediate between those observed for the active (GTP-bound) and inactive (GDP-bound) forms of the $G\alpha$ subunit.

This structure of the I56C/Q333C $G\alpha_{i1}$ subunit also exhibits increased temperature factors relative to wild-type structures. Crystallographic temperature factors give a relative assessment of the average displacement of any given atom in a structure, which is usually interpreted as mobility. Elevated temperature factors throughout the molecule may also reflect a greater number of substructures in the ensemble, resulting in increased frequency of conformations facilitating nucleotide release. Temperature factors are generally limited to a range of 0 \AA^2 to 100 \AA^2 in soluble proteins, with a higher number correlating with increased motions. A 31 \AA^2 average increase in crystallographic temperature factors is observed in the I56C/Q333C $G\alpha_{i1}$ as compared to wild-type crystallized in the same space group and at similar resolution (2.9 \AA for the I56C/Q333C $G\alpha_{i1}$ subunit and 3.0 \AA for the wild-type). These temperature factors are elevated to an even greater extent in the $\alpha 1$ and $\alpha 5$ helices and the switch I and switch II regions of the I56C/Q333C $G\alpha_{i1}$, where they are an average of 50 \AA^2 above wild-type. This suggests that the I56C/Q333C variant is more conformationally variable than wild-type, especially in these four regions. An increase in protein mobility in $G\alpha_{i1}$ proteins during nucleotide exchange has previously been noted in EPR experiments (53), while NMR spectra of nucleotide-free chimeric $G\alpha_t$ show significant line-broadening, which has been interpreted as an increase in protein dynamics (57). Increased dynamics may facilitate exit tunnel formation by moving the protein conformation into a higher energy landscape that can more easily convert the structure of the $G\alpha$ subunit into the open state.

Increased flexibility in switch II and altered the P-loop conformations may also play a role in nucleotide exchange dynamics. The increased intrinsic nucleotide exchange rates of GDP-bound $G\alpha_i$ subunits compared to $G\alpha_t$ subunits is a distinctive feature of $G\alpha_i$ proteins. Another distinctive feature of $G\alpha_i$ subunits is the disorder in switch II regions, as compared to the more ordered switch II regions of $G\alpha_t$ -GDP proteins. These data are consistent with increased flexibility of this region contributing to increased nucleotide exchange in $G\alpha_i$ proteins.

There are a number of statistically significant structural changes clustered in the GTPase domain of the I56C/Q333C $G\alpha_{i1}$ subunit (Figure 4f and Table 3). Many of these regions are implicated in nucleotide sensing, binding, exchange, or receptor interaction. The region linking the helical domain and GTPase domain, which in this structure shows a marked shift in position relative to wild-type, is predicted to lie at some distance from the regions of $G\alpha$ already known

to interact with receptors, such as the C-terminus and $\alpha 4$ - $\beta 6$ loop. Consistent with an allosteric mechanism of receptor-mediated G-protein activation, this region has been linked to specificity of receptor coupling, as mutation of the residue of $G\alpha_q$ homologous to residue 61 in the linker region of $G\alpha_{i1}$ can alter the specificity of receptor coupling (55). The high RMSD deviation in this region, in addition to other regions known to be involved in receptor contacts, is consistent with an overall picture of specific structural changes and overall protein flexibility, which contribute to receptor-mediated G protein activation. While this data points to factors which may play a role in nucleotide release in $G\alpha_i$ proteins, further studies are currently underway to determine which of these conformational changes contribute to GDP release and which are compensatory shifts reflecting the fact that the protein is stabilized in its $GDP\text{-}AlF_4^-$ transition state.

Forces contributing to allosteric GDP release in G α subunits: the D328R G α_{i1} subunit is a functional mimic of the R*-bound G α subunit

Structural and functional characterization of the D328R $G\alpha_{i1}$ subunits suggest electrostatics from movement of the $\alpha 5$ helix dipole may contribute to increased nucleotide release. Shoemaker et al (54) points out the stabilizing role of a negatively charged residue (such as the native Asp at position 328) at the N-terminus of an α -helix with a positively charged helix dipole (Figure 1b). The negative charge can neutralize and stabilize the positively charged helix dipole. Mutation of Asp328 to positively charged Arg would be predicted to perturb this helix cap and de-shield the amide nitrogen. Consistent with this idea, the D328R mutant exhibits markedly increased nucleotide exchange while D328N has activity similar to wild type, suggesting that the Asn can participate in stabilization of the helix dipole. Substitution of D328 with Ala would not be predicted to have the same effect, since the amide nitrogen would be de-shielded. Indeed, the D328A mutant exhibits somewhat elevated nucleotide exchange as compared to wild type.

The use of electrostatics to initiate nucleotide release is a novel proposal for the mechanism of nucleotide exchange in G proteins. These results, combined with previous EPR studies, strongly suggest that the movement of the helix dipole during receptor binding contributes to decreased affinity of $G\alpha$ for GDP by altering the local electrostatic distribution around the nucleotide. It is possible that even subtle changes in the electric field may act to reorient the GDP as a prerequisite to nucleotide release. In the absence of stabilizing contacts between a third phosphate or AlF_4^- , even complexation with AlF_4^- cannot overcome the electrostatic perturbation of the D to R mutation, allowing $GDP\text{-}AlF_4^-$ to be exchanged for BODIPY-GTP γ S. A decreased affinity for GDP in D328R $G\alpha_{i1}$ subunits may result in a $G\alpha_i$ subunit exquisitely poised to bind GTP. Similarly, a nucleotide-free $G\alpha_{i1}$ subunit is anticipated to bind to activated receptors with high affinity, and indeed D328R $G\alpha_{i1}$ subunits display enhanced association with membranes containing activated receptors (Figure 3). This is consistent with ability of activated receptors to stabilize $G\alpha$ subunits in the nucleotide-free state. Although $G\alpha_{i1}$ subunits are known to bind to BODIPY-labeled GTP γ S analog at a slower rate (and with relatively lower affinity) than unlabeled GTP γ S (56), this reagent provides a useful tool to distinguish differences in nucleotide exchange between subunits which demonstrate elevated intrinsic nucleotide exchange.

Peptide binding studies are another tool which can be used to tease out differences between wildtype, D328R and I56C/Q333C $G\alpha_{i1}$ subunits. The KB752 peptide which has been shown to bind to switch II region of inactive $G\alpha_{i1}$ subunits binds to wild-type and D328R $G\alpha_{i1}$ subunits with a similar affinity, not especially surprising given the mutation at residue 328 is in the $\alpha 5$ helix, removed from the switch II region. However, the I56C/Q333C mutation in $G\alpha_{i1}$ subunits does substantially reduce the KB752 binding, indicating changes in the relative positions of these two helices can be allosterically communicated to the switch II region. On the other hand,

both D328R and I56C/Q333C $G\alpha_{i1}$ GDP-AlF₄⁻ subunits demonstrate a 3–4 fold enhancement in binding affinities to the KB1752 peptide (selective for activated subunits) relative to wild-type $G\alpha_i$. This would be consistent with a conformation for both mutant proteins which favors GDP release and readily adopts the activated conformation, as would be expected for receptor-bound mimetics of activated $G\alpha$ proteins. However, the differing binding affinities for the mutant subunits to peptides selective for the inactive state, as well as differences in ability of these proteins to exchange GDP-AlF₄⁻ for BODIPY-GTP γ S, together suggest these mutations exert their effects on nucleotide release through distinct mechanisms.

Mechanisms of GEF-catalyzed GDP release from G proteins

For small G proteins with soluble GEFs, the GEFs bind across the switch I and switch II regions, and the conformational change induced by this interaction with switch II weakens GDP binding in two ways (5,58). In the case of an Arf-GEF, the Sec7 domain utilizes the carboxylate from a conserved acidic residue the FG loop of the GEF to form a new hydrogen bond with a lysine in the phosphate-binding P-loop of the G protein, thereby introducing an electrostatic repulsion between the β -phosphate and negatively charged carboxylate group, and decreasing nucleotide affinity. Second, the binding of the Mg²⁺ ion in the nucleotide binding pocket is abrogated by steric interference with the new position of switch II. While GPCRs act as GEFs on heterotrimeric G proteins, they do not bind to the switch II region, which is in contact with G $\beta\gamma$. Therefore, a different mechanism appears to have evolved to facilitate nucleotide exchange in $G\alpha$ subunits. Here, nucleotide exchange is initiated with a roto-translation of the $\alpha 5$ helix, which exerts a coulombic effect that contributes to a reduction in its GDP-binding affinity. While the structure presented here is in complex with GDP-AlF₄⁻, there are hints that this coulombic effect pulls the phosphates of the guanine nucleotide away from the P-loop. Other previously identified structural elements distributed throughout the GTPase domain respond to this roto-translation and nucleotide shift with subtle structural changes (Figure 6, Supplementary Movie 1).

The direction of nucleotide egress from the binding site has been debated using terms such as ‘front door,’ referring to egress toward the guanine ring, and ‘back door,’ referring to egress toward the phosphate corridor (59). After the initial roto-translation of the $\alpha 5$ helix, the negatively charged guanine ring may be attracted by a positively charged helix dipole, pulling the base of the guanine nucleotide toward the front door. Such an attraction would likely reorient the nucleotide and weaken the protein-phosphate contacts by pulling the phosphates away from the P-loop. Exit via the front door would require a conformational change in the TCAT motif, the side chain of Arg 176 (on the αF - $\beta 2$ loop) and the side chain of Asp 272 (on the $\alpha 4$ helix).

Alternatively, the reorganization of the P-loop and decreased interactions with phosphates may allow nucleotide release through a back door route (Supplementary Movie 3), with the positively charged dipole shielded from bound nucleotide through helix capping via negatively charged D328. Exit via the back door requires a change in the position of the main chain of switch II and P-loop regions. Hints as to the nature of conformational changes near the back door exit route come from the I56C/Q333C $G\alpha_{i1}$ GDP-AlF₄⁻ structure. Intriguingly, the position of the switch II region the I56C/Q333C $G\alpha_{i1}$ structure can be modeled as a continuous motion into the dramatic conformational change observed in the analysis of the complex between the KB-752 and D2N peptides and the $G\alpha_{i1}$ subunit (31) (supplementary movie 2). While the KB-752 peptide does not exhibit sequence similarity to $G\alpha$ binding regions on any known GPCRs, conformational changes of the switch II region upon the binding of KB-752 appear to correlate with increased nucleotide exchange activity in $G\alpha$ subunits (50). In addition to the likely movement of the switch II region during back door nucleotide exchange, the structure of the I56C/Q333C $G\alpha_{i1}$ suggests that a subtle shift in phosphate binding residues

allows the γ -phosphate binding pocket to adopt a more open position (Figure 4c, Supplementary Movie 3). A larger conformational change may occur in the P-loop region during physiological GDP release than that seen in the GDP- AlF_4^- -bound I56C/Q333C $\text{G}\alpha_{i1}$ subunit.

The results presented here do not unambiguously point to exit through either the front or back door, and egress through either route would require a unique arrangement of structural elements of the $\text{G}\alpha$ subunit that has not yet been observed in any published $\text{G}\alpha$ subunit crystal structure. Physiological nucleotide release from a front door exit route would likely involve the TCAT motif, αA - β2 loop, and α4 helix, while a back door egress would likely involve switch II and the P-loop. These data reported here do not discriminate the conformational movements required for GDP egress from those movements that are important for decreasing nucleotide affinity, which is a prerequisite for nucleotide release. If GTP, $\text{GTP}\gamma\text{S}$, or GDP- AlF_4^- is bound in the active site, the additional stabilization through protein interactions to the third phosphate (or AlF_4^-) likely prevents the reorientation of the nucleotide, thus blocking the conformational changes that would be required for nucleotide egress. In the absence of the stabilizing influence imparted by a third phosphate or AlF_4^- , GDP-bound subunits may exhibit greater overall conformational flexibility, especially in the switch II and P-loop regions, which are involved in binding AlF_4^- or the γ phosphate of GTP. The lack of these additional contacts may contribute to allosteric changes at both the front door and the back door of the nucleotide binding site.

The $\text{G}\alpha$ subunit differs from small G proteins in that receptor activation results in association of heterotrimeric $\text{G}\alpha\beta\gamma$ with receptors. The $\text{G}\beta\gamma$ subunits have been implicated in receptor-mediated nucleotide exchange, and a number of proposals have suggested how these subunits affect GDP release (31,60). While this study cannot address whether $\text{G}\beta\gamma$ -mediated structural changes follow the initial recognition event between the GPCR and the $\text{G}\alpha$ subunit, the contribution of the roto-translation of the α5 helix to nucleotide exchange does not preclude the involvement of $\text{G}\beta\gamma$ in GDP release.

Conclusion

Physiological nucleotide exchange in $\text{G}\alpha$ is likely initiated through a receptor-catalyzed roto-translation of the α5 helix. The data presented here suggest that following receptor binding the change in position of the α5 helix may be transmitted into subtle positional, electrostatic, and dynamic changes throughout $\text{G}\alpha$ that may contribute to the efficiency of G protein activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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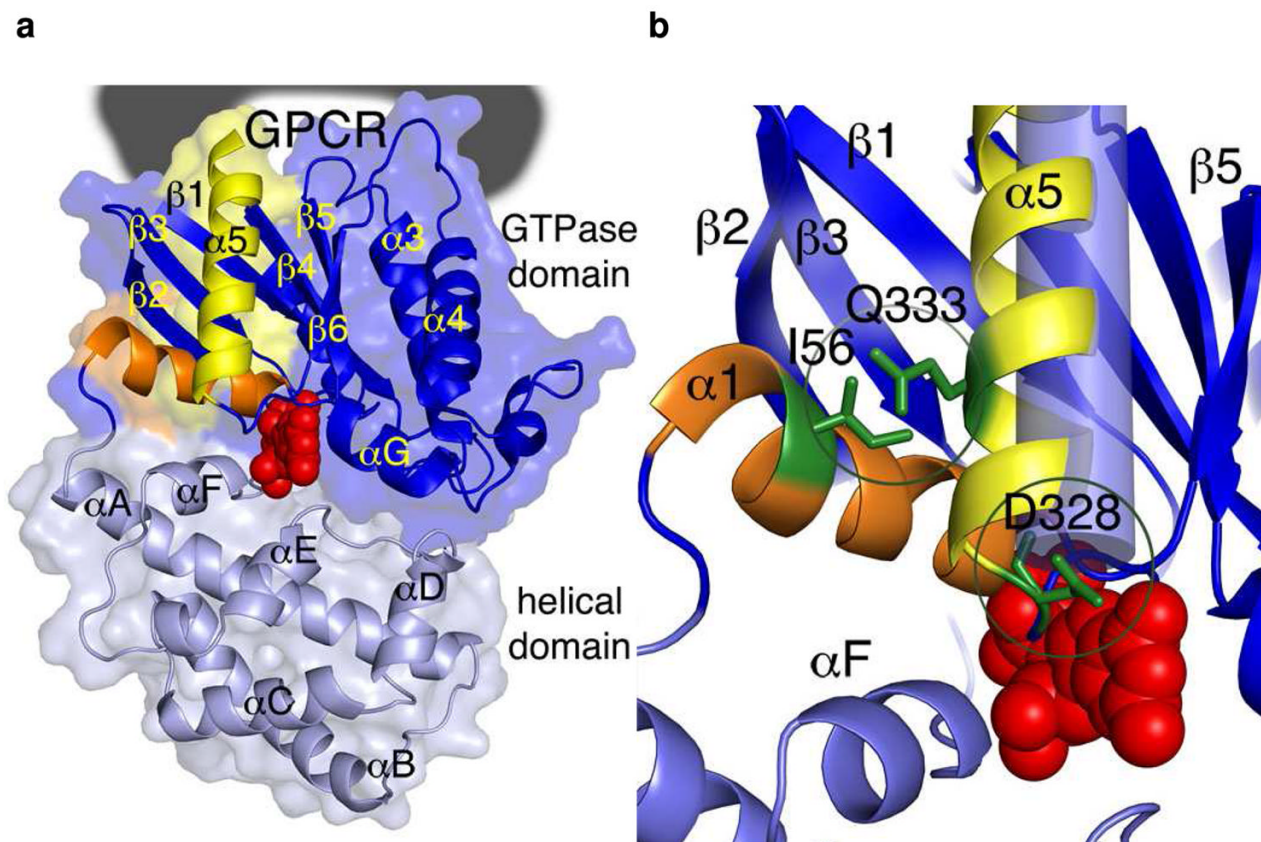


Figure 1. Design of the $G\alpha_{i1}$ mutants. **(a)** Location of GPCR binding site on $G\alpha_{i1}$. The GTPase domain is shown in *dark blue* and the helical domain is shown in *grey*. The $\alpha 5$ helix is highlighted in *yellow*, the $\alpha 1$ helix is *orange*, and the GDP molecule is shown in a space-filling model colored *red*. The location of the GPCR binding site is indicated with a *black donut*. The GPCR is predicted to induce a conformational change that pushes the $\alpha 5$ helix toward the helical domain (down in this representation). **(b)** Design of $G\alpha_{i1}$ mutations. A close-up view of helices $\alpha 1$ and $\alpha 5$ highlighting the locations of point mutations I56C/Q333C, and D328R. The I56C/Q333C double mutation is predicted to form a disulfide bond that constrains the $\alpha 5$ helix into its receptor-activated conformation, which is modeled as a *blue cylinder*. The D328R mutation is located at the N-terminus of the $\alpha 5$ helix and designed to mimic the movement of the $\alpha 5$ helix dipole. This charge reversal mutation was designed to maximize the effect of the electrostatics.

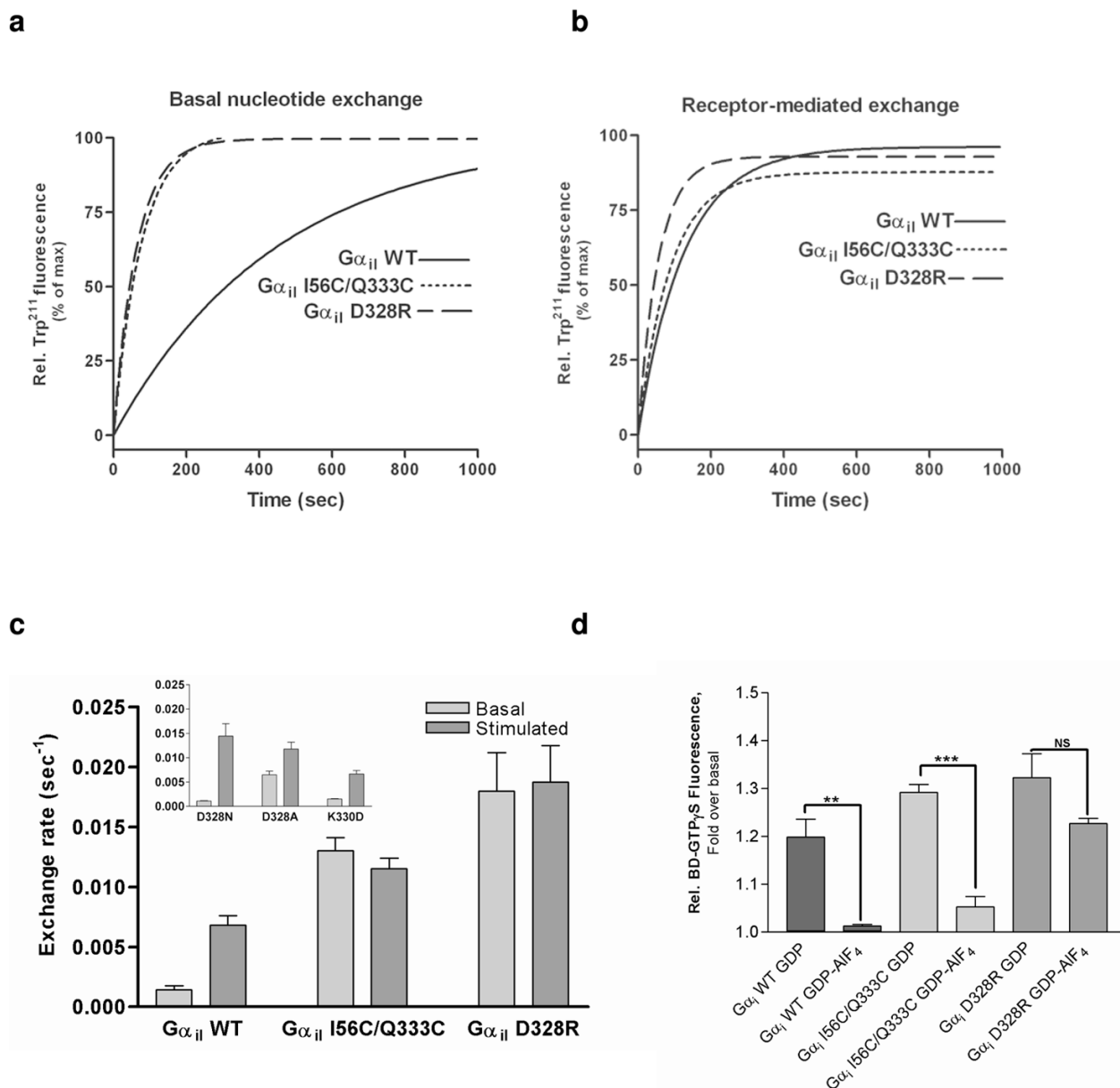
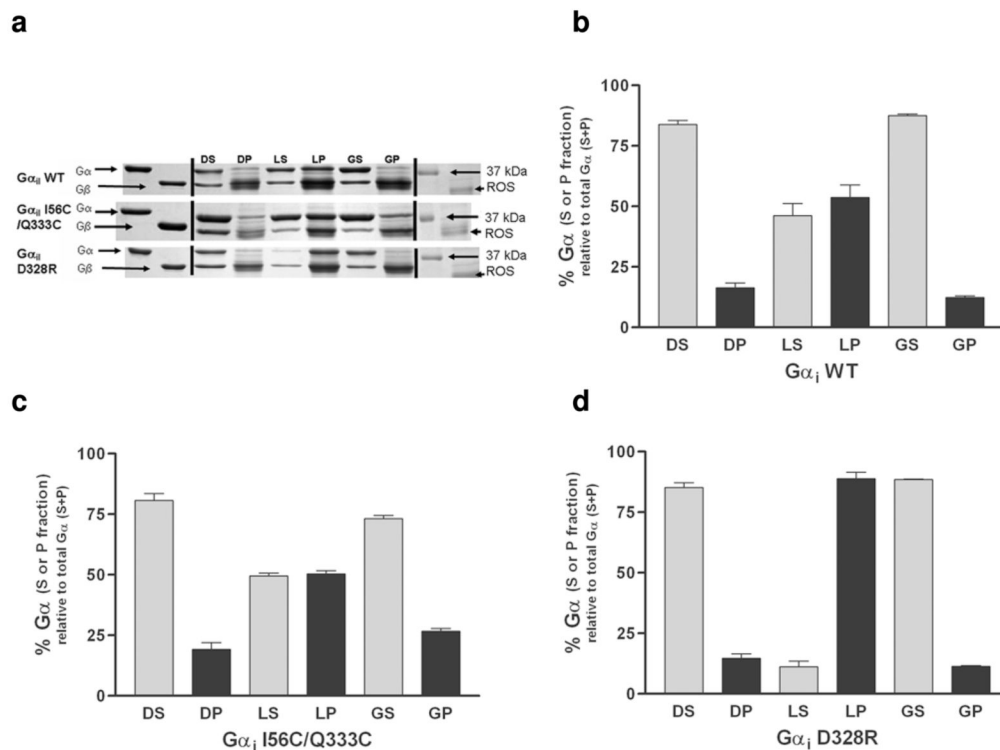


Figure 2.

Elevated rates of basal nucleotide exchange in $G\alpha_{i1}$ mutants. **(a)** Basal Exchange. The basal rate of GTP- γ S binding was determined by monitoring the relative increase in the intrinsic Trp²¹¹ fluorescence. Basal exchange for the wild-type is shown with a *solid* line (bottom trace), basal exchange for the I56C/Q333C double mutant is shown with a *small-dashed* line (-----), and basal exchange for the D328R mutant is shown with a *large-dashed* line (——). **(b)** Receptor catalyzed nucleotide exchange. Nucleotide exchange for the receptor-catalyzed wild-type and mutant $G\alpha_{i1}$ subunits was determined by monitoring the relative increase in the intrinsic Trp²¹¹ fluorescence performed in presence of 500 nM rhodopsin. The addition of rhodopsin does not significantly affect the rate of nucleotide exchange in the I56C/Q333C or D328R $G\alpha_{i1}$ subunits but catalyzes a 4-fold rate enhancement for wild-type $G\alpha_{i1}$ subunits ($p=0.0005$). **(c)** Quantitation of the initial rates of basal and rhodopsin-catalyzed nucleotide exchange monitored by the relative increase in the intrinsic Trp²¹¹ fluorescence for wild-type

and mutant $G\alpha_{i1}$ subunits. **(d)** Basal nucleotide exchange of GDP- or GDP- AlF_4^- -bound $G\alpha_{i1}$ subunits as measured by the increase in emission from BODIPY-labeled $GTP\gamma S$ upon addition of GDP-bound $G\alpha_{i1}$ or GDP- AlF_4^- -bound $G\alpha_{i1}$ subunits. Relative increase was plotted as percentage of basal BODIPY- $GTP\gamma S$ fluorescence prior to addition of $G\alpha$ subunits. Data are the average of three independent experiments (** $p = 0.0027$ and $p=0.0001$).

**Figure 3.**

Membrane localization and rhodopsin binding of the wild-type and mutant G α_{i1} subunits. **(a)** Representative Coomassie stained SDS-PAGE analysis of wild-type (WT; top panel) I56C/Q333C (middle panel) and D328R (bottom panel) G α_{i1} subunits reconstituted with excess G $\beta\gamma$ prior to binding to ROS in the dark, light, and after light activation followed by addition of GTP γ S. DS, supernatant from dark sample; DP, pellet fraction from dark sample; LS, supernatant from light sample; LP, pellet from light sample; GS, supernatant from light and GTP γ S activated sample; GP, pellet from light and GTP γ S activated sample. **(b)** **(c)** and **(d)** Quantitation of membrane binding. Each measurement is the average of three independent experiments. The D328R G α_{i1} subunit showed a significant enhancement in binding to membrane fractions upon light activation as compared to either wild-type or I56C/Q333C G α_{i1} subunits (** $p = 0.0035$ and $p=0.0002$, respectively).

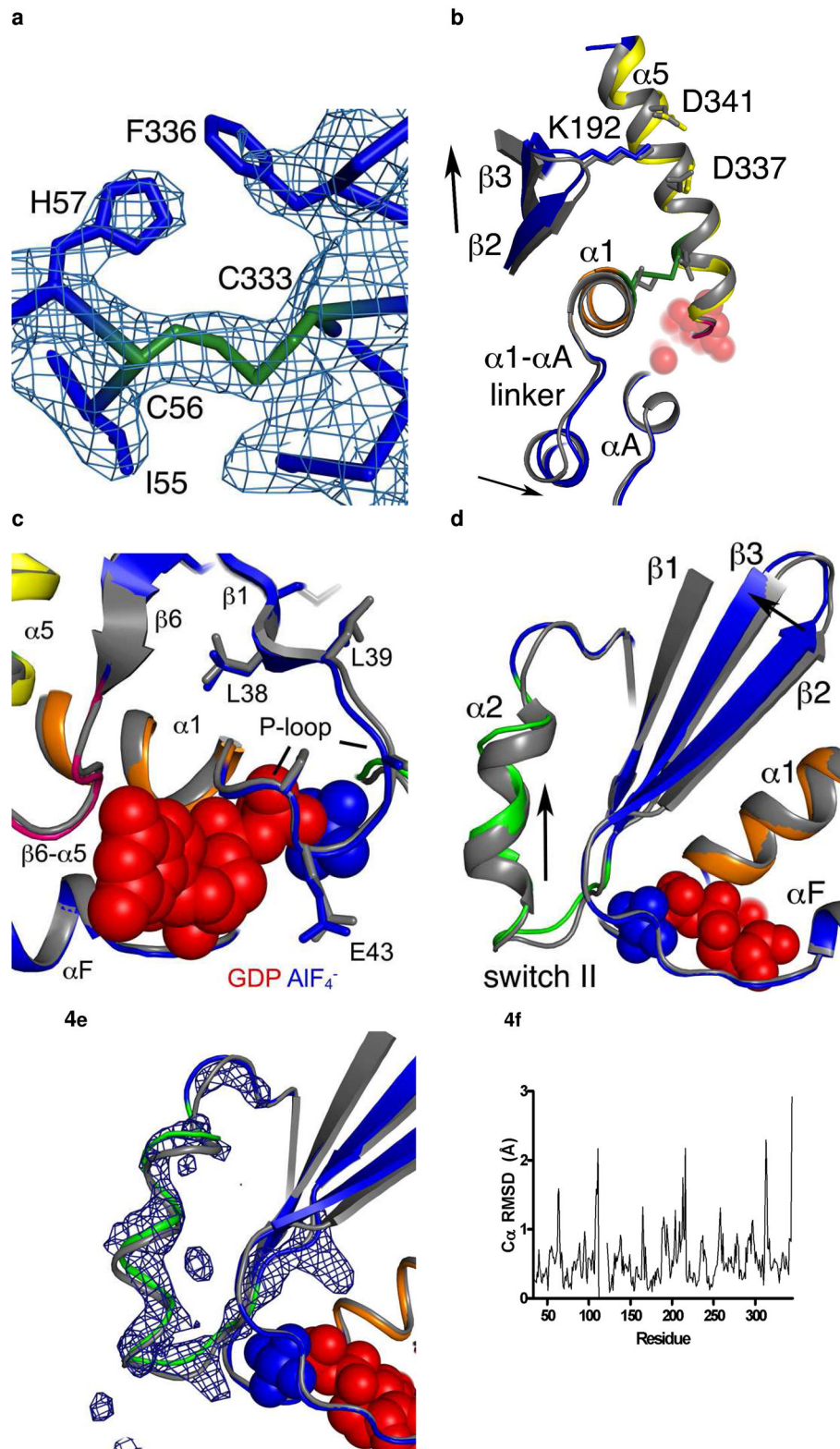


Figure 4.

Structural changes in the I56C/Q333C $G\alpha_{i1}$. All comparisons are made between the I56C/Q333C $G\alpha_{i1}$ subunit and the wild-type $G\alpha_{i1}$ subunit crystallized in complex with GDP- AlF_4^- . Coloring for panels (b) - (d) is the same as in Figure 1. (a) $2|F_o| - |F_c|$ density (*blue mesh*) contoured at 1.2σ shows a connection between the side chains at positions 56 and 333 and is consistent with the formation of a disulfide bond. (b) Movement of the $\alpha 5$ helix, the $\beta 2$ - $\beta 3$ hairpin and the $\alpha 1$ - αA linker that connects the GTPase (*dark blue*) and helical domains (*cyan*). (c) Movement of the P-loop. (d) Movement of the $\beta 2$ - $\beta 3$ hairpin away from the $\alpha 1$ helix toward the $\beta 1$ strand. The hairpin directly connects the second linker (residues 177–182) and switch II (residues 202–215; *light green*). (e) $|F_o| - |F_c|$ simulated annealing omit density calculated using CNS and contoured at 3.0σ after the removal of all atoms between residues 202–217 ($\alpha 2$ helix and switch II) highlights the shift between the I56C/Q333C (*blue/green*) and wild type (*grey*) $G\alpha_{i1}$. The GDP molecule is in *red* and the AlF_4^- is in *blue*. (f) RMSD of each alpha carbon atom in I56C/Q333C $G\alpha_{i1}$ structure as compared to wild-type $G\alpha_{i1}$ (PDB 1GFI, selected regions overlaid in 4a-e).

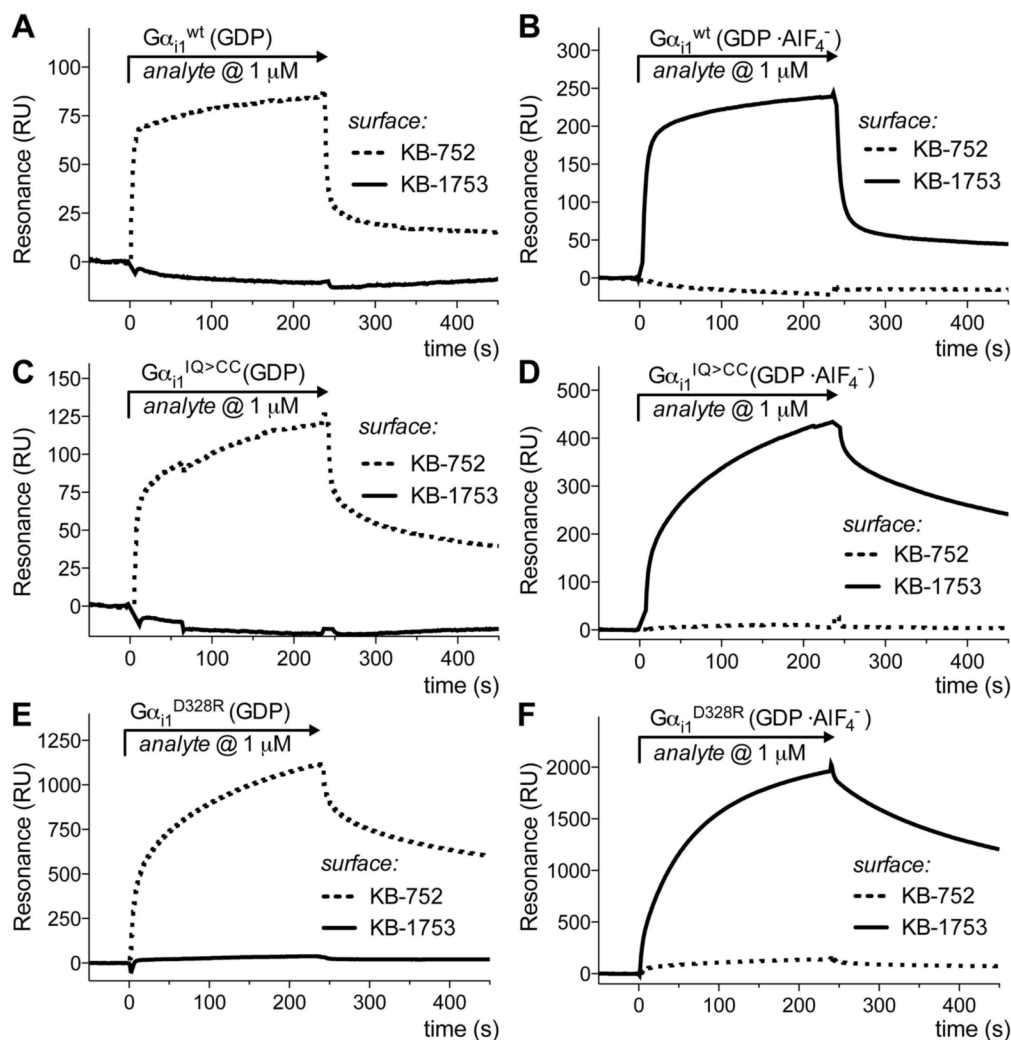


Figure 5.

Surface plasmon resonance analysis of the binding of $G\alpha_{i1}$ subunits to nucleotide-state selective peptides. Surface plasmon resonance demonstrates peptides KB-752 and KB-1753 discriminate between activation state of $G\alpha_{i1}$ proteins. In each graph, the *dashed* line represents binding of the indicated $G\alpha$ subunit to immobilized peptide KB-752, selective for inactive (GDP-bound) $G\alpha$ subunits, while the *solid* line represents binding to immobilized peptide KB-1753, selective for activated (GTP-bound) $G\alpha$ subunit. (a) Wild-type $G\alpha_{i1}$ in the presence of GDP; (b) Wild-type $G\alpha_{i1}$ in the presence of GDP·AlF₄⁻; (c) I56C/Q333C $G\alpha_{i1}$ in the presence of GDP; (d) I56C/Q333C $G\alpha_{i1}$ in the presence of GDP·AlF₄⁻. (e) D328R $G\alpha_{i1}$ in the presence of GDP; (f) D328R $G\alpha_{i1}$ in the presence of GDP·AlF₄⁻.

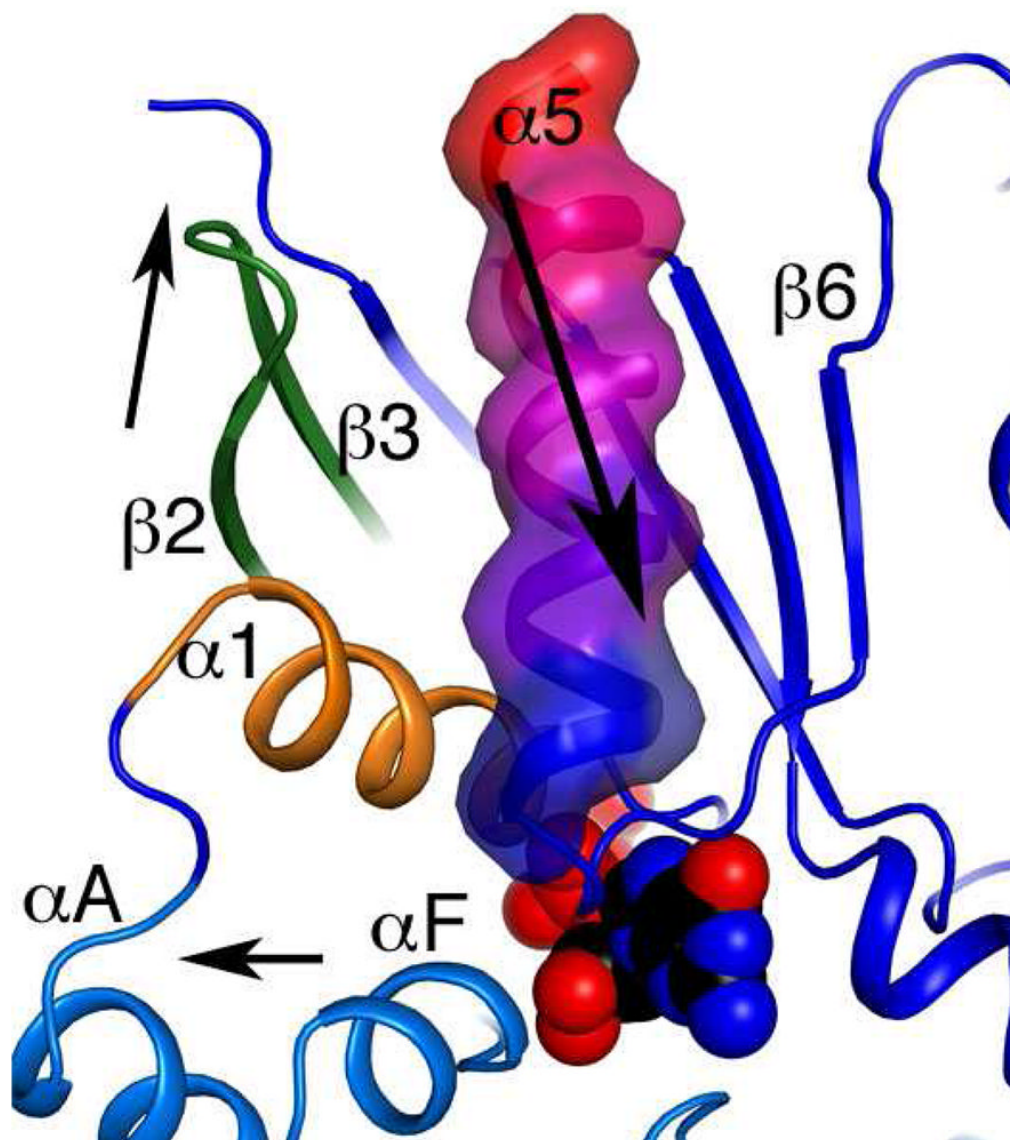


Figure 6. Proposed mechanism of receptor-catalyzed GDP release by $G\alpha_{i1}$ mediated by movement of the $\alpha 5$ helix. Crystallographic and biochemical data implicate the receptor-induced movement of the $\alpha 5$ helix toward the bound GDP molecule in destabilization of guanine ring through coulombic attraction to the positive helix dipole. The movement of the $\alpha 5$ is coupled with a translation in the $\alpha 1$ helix (*orange*), the $\beta 2$ - $\beta 3$ hairpin (*dark green*), and the αF helix (*cyan*). These concerted motions may be the first step of opening a tunnel to the guanine nucleotide binding pocket and allowing exchange.

Table 1

Summary of nucleotide exchange rates

Crystallographic data collection, phasing, and refinement statistics. Values in parenthesis indicate statistics for the highest resolution shell. $^aR_{\text{sym}} = \sum |I_i - \langle I \rangle| / \sum I_i$ where I is intensity, " I " is the i^{th} measurement, and $\langle I \rangle$ is the weighted mean of I . $^bR_{\text{cryst}}, R_{\text{free}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum F_{\text{obs}}$.

	Basal	Rhodopsin-catalyzed
I56C/Q333C $G\alpha_i$	$0.013 \pm 0.001 \text{ sec}^{-1}$	$0.012 \pm 0.0008 \text{ sec}^{-1}$
D328R $G\alpha_i$	$0.018 \pm 0.003 \text{ sec}^{-1}$	$0.019 \pm 0.003 \text{ sec}^{-1}$
Wild type $G\alpha_i$	$0.0021 \pm 0.0001 \text{ sec}^{-1}$	$0.0081 \pm 0.0009 \text{ sec}^{-1}$

Table 2

Data collection, phasing and refinement statistics

GDP exchange rates in sec^{-1} for wild-type, I56C/Q333C, and D328R $G\alpha_{i1}$ subunits. The exchange rate was determined by fitting the data to an exponential association equation $F\lambda = F\lambda_{\text{max}}(1 - e^{-kt})$. The value of k is given in $\text{sec}^{-1} \pm \text{SEM}$.

	I56C/Q333C	Anomalous S I56C/Q333C	Wild-type
Wavelength	1.0 Å	1.77 Å	0.98 Å
Resolution	2.9 Å	3.5 Å	3.0 Å (3.11–3.0 Å)
Completeness	96.8 (80.6)	100.0 (100.0)	91.4 (92.6)
I/σ	20.5 (1.9)	62.9 (1.8)	18.1 (3.6)
R_{sym}^a	0.05 (0.30)	0.090 (0.30)	0.08 (0.38)
Figure of Merit	—	0.32 (17 sites)	—
Model refinement statistics			
R_{cryst}^b	0.249		
R_{free}^b	0.293		
RMSD bonds	0.016 Å		
RMSD angles	1.7°		

Table 3

Summary of structural features of I56C/Q333C G α_{i1}
 Summary of structural changes observed in I56C/Q333C G α_{i1} .

Structural Element	Residues	Functional Role	Structural Change
P-loop	41–45	Phosphate-binding loop highly conserved in G proteins	0.7 Å shift of G45 C $_{\alpha}$ toward the phosphates
$\alpha 1$ helix	44–58	Walker A motif and connection to linker/ $\beta 2$ - $\beta 3$ loop	Translation toward $\alpha 5$ helix and nucleotide-binding pocket by 1.0 Å
$\alpha 1$ - αA loop	59–64	Linker region between GTPase and helical domains	Unfolding of αA helix at the $\alpha 1$ - αA linker loop by 1.5 Å
$\beta 2$ - $\beta 3$ hairpin	188–197	Connects $\alpha 1/\alpha 5$ with Switch II and $\beta 1$	Translation toward $\beta 1$ strand by 1.3 Å; moves with $\alpha 5$ helix residues
$\beta 3$ - $\alpha 4$ loop	202–207	Part of Switch II; pulls away in GEF peptide and G $\beta\gamma$ -bound structures	Average 0.5 Å and maximum 1.25 Å movement away from AlF $^{4-}$
$\alpha 2$ helix	207–215	Switch II, GEF peptide and G $\beta\gamma$ binding site	Unfolding and destabilization; pulls away from phosphate pocket by 0.8 Å
$\alpha 4$ helix	294–299	$\alpha 4$ helix upstream of $\alpha 4$ - $\beta 6$ loop involved in receptor interaction	Average 0.9 Å movement relative to wild type
$\alpha 4$ - $\beta 6$ loop	312–316	Known receptor contact region	Average 1.3 Å movement relative to wild type
$\beta 4$ - $\alpha 3$ loop	234–238	Switch III, known to move in activating peptide structure	Pulls away from Switch II by 0.8 Å
$\beta 6$ - $\alpha 5$ loop	324–329	TCAT motif directly interacts and stabilizes guanine ring	0.5 Å movement towards the guanine ring of C $_{\alpha}$ and side chains
$\alpha 5$ helix	329–345	Known receptor contact and exchange mediator	Translation toward nucleotide-binding pocket by 0.6 Å

TABLE 4

Comparison of k_{on} , k_{off} , and K_d values for wild-type, I56C/Q333C and D328R $G\alpha_{i1}$. Values are from an average of 4 trials (wild type) or 2 trials (I56C/Q333C and D328R).

KB-752 (selective for $G\alpha_i$-GDP)	k_{on} (1/M • s)	k_{off} (s^{-1})	K_d (μM)
WT $G\alpha_i$ GDP	21,100 \pm 5,800	0.06 \pm 0.01	2.84 \pm 0.81
I56C/Q333C $G\alpha_i$ GDP	7,750 \pm 250	0.22 \pm 0.03	28.2 \pm 2.9
D328R $G\alpha_i$ GDP	8,560 \pm 650	0.01 \pm 0.001	1.28 \pm 0.02
KB-1753 (selective for $G\alpha_i$-GDP•AlF₄)			
WT $G\alpha_i$ GDP •AlF ₄ ⁻	22,900 \pm 2,300	0.025 \pm 0.001	1.1 \pm 0.1
I56C/Q333C $G\alpha_i$ GDP•AlF ₄ ⁻	16,800 \pm 1,700	0.006 \pm 0.0008	0.36 \pm 0.01
D328R $G\alpha_i$ GDP•AlF ₄ ⁻	8,810 \pm 1,390	0.003 \pm 0.0004	0.26 \pm 0.04