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Recent Progress in Understanding the Conformational Mechanism of Heterotrimeric G Protein Activation

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Abstract

Heterotrimeric G proteins are key intracellular coordinators that receive signals from cells through activation of cognate G protein-coupled receptors (GPCRs). The details of their atomic interactions and structural mechanisms have been described by many biochemical and biophysical studies. Specifically, a framework for understanding conformational changes in the receptor upon ligand binding and associated G protein activation was provided by description of the crystal structure of the β2-adrenoceptor-Gs complex in 2011. This review focused on recent findings in the conformational dynamics of G proteins and GPCRs during activation processes.

Key Words: G protein, G protein-coupled receptor, Structure, Dynamics

OVERVIEW OF HETEROTRIMERIC G PROTEINS

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are signal transducers that play a crucial role in mediating downstream signal transduction of G protein-coupled receptors (GPCRs) (Ross and Gilman, 1980). G proteins contain three subunits, α, β, and γ. In their inactive state, the Gα subunit binds to guanosine diphosphate (GDP) to form a stable complex with Gβγ partners. When agonist-activated GPCRs couple to the GDP-bound form of G proteins, GDP is replaced by guanosine triphosphate (GTP), which induces dissociation of the Gα subunit from GPCR and Gβγ subunits (Fig. 1A). The GTP-bound Gα subunit or the Gβγ subunits transduce signals through interaction with downstream effectors (Hamm, 1998). Finally, the signal is terminated by the intrinsic GTPase catalytic activity of the Gα subunit, which hydrolyzes GTP to GDP, enabling recruitment of Gβγ subunits to form inactive heterotrimers (Fig. 1A).

There are 21 known isoforms encoded by 16 Gα subunit genes, 6 documented Gβ subunits encoded by 5 genes, and 12 reported Gγ subunits in human (Simon *et al*., 1991; Downes and Gautam, 1999). These subunits can thus combine to form approximately 700 potential Gαβγ heterotrimers, which contributes to the selectivity as well as the specificity of both GPCRs and effector systems (Fletcher *et al*., 1998; Richardson and Robishaw, 1999). Despite this, G proteins are typically grouped into only four main classes on the basis of sequence similarity of the Gα subunit: Gαs, Gαi/o, Gαq/11, and Gα12/13 (Moreira, 2014). This classification also defines both the specificity and selectivity of effectors and receptors.

The Gs family includes 2 isoforms, Gs and Golf, which signal via stimulation of second messengers such as cAMP, as well as Src tyrosine kinase and protein kinase A (Neves *et al*., 2002). The Gi/o family is the largest subgroup, consisting of 8 members including Gi1, Gi2, Gi3, GoA, GoB, Gz, and Gt, which inhibit adenylyl cyclase activity resulting in a decrease in intracellular cAMP levels (Neer, 1995). The Gq/11 family is composed of 5 isoforms: Gq, G11, G14, G15, and G16. The most important effector for Gq signaling is phospholipase C-β (PLC-β); this pathway produces the intracellular messengers inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 subsequently induces the release of calcium from the intracellular reservoir, while DAG recruits and activates protein kinase C. The G12/13 subgroup contains only 2 members, G12 and G13, which mediate activation of RhoGTPase nucleotide exchange factors (Siehler, 2009).

OVERVIEW OF G PROTEIN STRUCTURES

In the 1990s, the structures of Gα subunits in GTP- and GDP-bound forms were described as either a monomer or a

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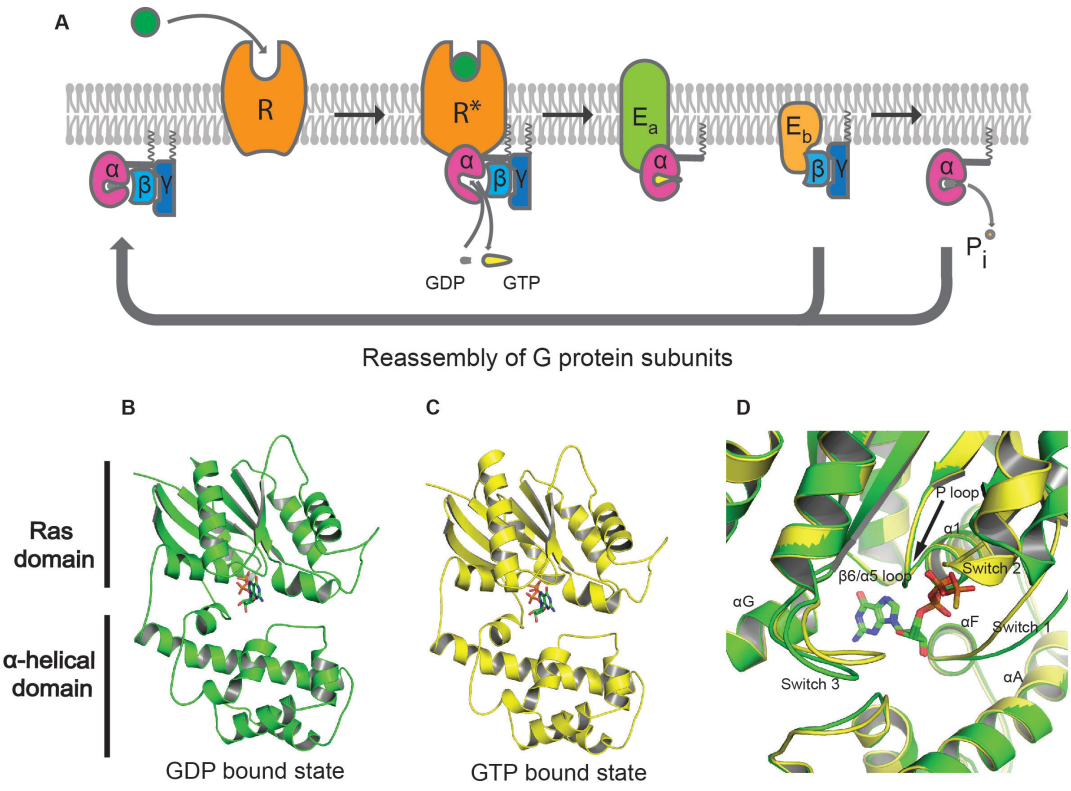

Fig. 1. G protein structure and activation process. (A) The GPCR-mediated G protein activation cycle. (B and C) The representative structures of GDP-bound inactive state of Gα subunit (green, PDB: 1TAG) (B) and GTP-bound active state of Gα subunit (yellow, PDB: 1TND) (C). The GDP and GTPγS are shown as sticks. (D) Structural comparison near the nucleotide-binding pocket between GDP-bound inactive (green) and GTP-bound active (yellow) states.

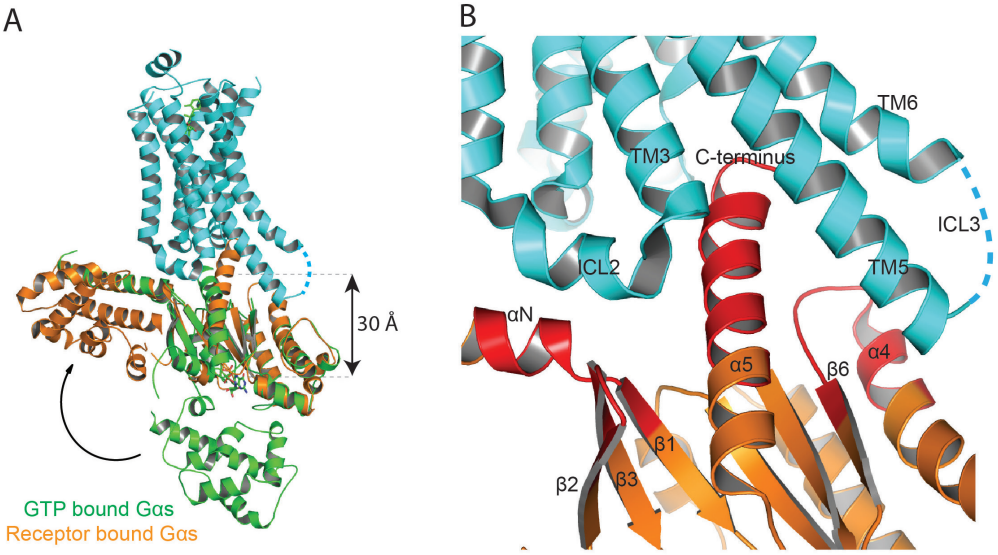

Fig. 2. The structure of GPCR-G protein complex. (A) Structural comparison between β2AR-bound nucleotide-free Gαs subunit (PDB: 3SN6) and the GTPγS-bound Gαs subunit (1AZT). β2AR is colored as cyan, nucleotide-free Gαs as orange, and GTPγS-bound Gαs as green, respectively. GTPγS is shown as stick. The superimposition of Ras domains from each states shows the displacement of AH domain. (B) GPCR and G protein interfaces. The binding regions of Gα subunit with receptor are shown in red which constitute of αN/β1 hinge, α4/β6 loop, and C-terminus of α5 helix.

Table 1. Summary of published papers described in this review

Authors	Journal	Method	Main findings
Shim *et al*.	2013, *J. Biol. Chem.* **288**, 32449	Computer simulation	This study described a structural model of cannabinoid CB1 receptor and Gi protein complex showing the important role of the α5 helix in G protein signaling.
Yao and Grant	2013, *Biophys. J*. **105**, L08	MD simulation	This study revealed the intrinsic flexibility of the AH domain in the nucleotide-free state of Gα.
Mnpotra *et al*.	2014, *J. Biol. Chem*. **289**, 20259	Cross-linking and simulations	This study presented a model of the complex between cannabinoid CB2 receptor and Gi protein.
Alexander *et al*.	2014, *Nat. Struct. Mol. Biol.* **21**, 56	Modeling	This study described the formation and structural basis of the Rho-Gi heterotrimer complex and the role of the α5 helix in G protein activation.
Kaya *et al*.	2014, *J. Biol. Chem.* **289**, 24475	Crystallography, binding assay	This study described the role of hydrophobic interactions between the α5 helix, β2-β3 strands, and α1 helix.
Dror *et al*.	2015, *Science* **348**, 1361	MD simulation, DEER spectroscopy	This study found that the AH domain is spontaneously separated in the native state of GDP-bound Gα, as well as investigated the important role of the α5 helix, subsequently β6/α5, in G protein activation.
Flock *et al*.	2015, *Nature* **524**, 173	Analysis	This study provided a universal mechanism for Gα activation by GPCRs.
Sun *et al*.	2015, *Nat. Struct. Mol. Biol.* **22**, 686	Alanine-scanning mutagenesis	This study described the role of distinct residues in the stability of GDP, GTP, and receptor-bound states of Gi protein.
Yao *et al*.	2016, *J. Biol. Chem.* **291**, 4742	Computer simulation	This study provided the role of the β1 strand in activation of G proteins by GPCRs.
Goricanec *et al*.	2016, *Proc. Natl. Acad. Sci. U.S.A.* **113**, E3629	NMR, SAXS, and MD simulations	This study indicated the highly dynamic flexibility of apo and GDP-bound states relating to activation by GPCR.
DeVree *et al*.	2016, *Nature* **535**, 182	Radio-ligand binding assay	This study suggested that coupling to G proteins allosterically stabilized the ligand-binding pocket of a GPCR.
Pachov *et al*.	2016, *J. Chem. Theory Comput.* **12**, 946	Computer simulation	This study mentioned that interactions between αN with ICL2 facilitates nucleotide exchange by weakening a salt bridge between the P-loop and Switch 1 through β1 strand.

Gαβγ heterotrimer (Noel *et al*., 1993; Coleman *et al*., 1994; Lambright *et al*., 1994; Sondek *et al*., 1994; Mixon *et al*., 1995; Wall *et al*., 1995; Lambright *et al*., 1996; Lutz *et al*., 2007; Nishimura *et al*., 2010). These studies revealed that the nucleotide-binding pocket is tightly sandwiched between two domains of the Gα subunit, the Ras-like (Ras) domain and the α-helical (AH) domain (Fig. 1B, 1C). In its inactive state, GDP is stabilized by interactions between the phosphate groups of GDP and the P-loop, the α1 helix, and switch 1 of the Gα subunit, and interactions between the guanine ring of GDP and the αG helix and strands β4-β6 of the Gα subunit (Fig. 1D). In the GTP-bound active state, the γ-phosphate group dynamically contacts the switches 1, 2, and 3, which helps to stabilize these highly flexible regions (Jonesa *et al*., 2012) (Fig. 1D).

Various biochemical and biophysical studies have investigated GPCR-mediated G protein activation (Preininger *et al*., 2013; Moreira, 2014; Duc *et al*., 2015). In 2011, the high-resolution X-ray crystal structure of the GPCR-G protein complex was first revealed using the β_2-adrenoceptor-Gs complex (β_2AR-Gs) as a model system (Rasmussen *et al*., 2011b). This structure provided fundamental information about the structural mechanism of GPCR-mediated G protein activation. It showed that the nucleotide-binding pocket opens via movement of the AH domain of the Gα subunit, and, furthermore, revealed the interfaces between GPCRs and G proteins (Fig. 2A). There are three major contact sites between β_2AR and Gs: 1) the C-terminus of Gαs contacting transmembranes (TMs) 3, 5, and 6 and the intracellular loop (ICL) 2 of β_2AR, 2) the hydrophobic region surrounded by the αN/β1 hinge and β2/β3 loops of Gαs contacting ICL2 of β_2AR, and 3) part of the α4 helix, α4/β6 loop, and β6 of Gαs contacting ICL3 of β_2AR (Fig. 2B). Knowledge regarding the structure of β_2AR-Gs heralded a new era in G protein studies and accelerated other biochemical and biophysical studies on the conformational mechanisms of G protein regulation. This review focuses on recent progress in this field with two main topics: 1) GPCR-mediated allosteric conformational changes of G proteins, 2) G protein-mediated allosteric modulation of GPCRs.

GPCR-MEDIATED ALLOSTERIC CONFORMATIONAL CHANGES OF G PROTEINS

The distance between GPCR-G protein contact sites and the nucleotide-binding pocket is approximately 30 Å (Fig. 2A), and therefore an allosterical regulation induced by GPCRs

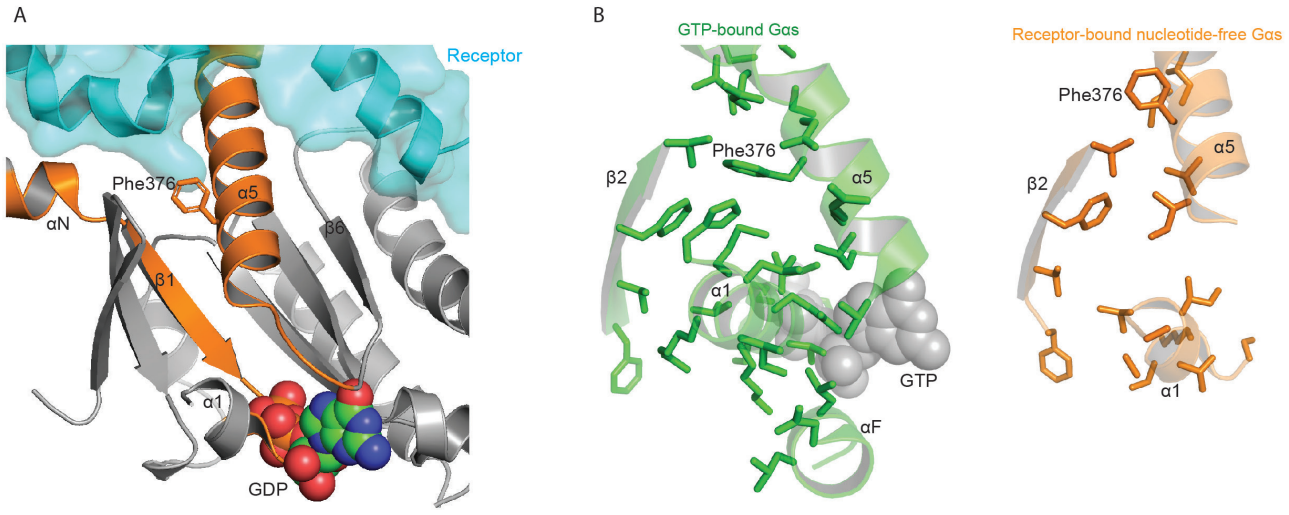

Fig. 3. The allosteric regulation of G protein by GPCR (A) Connections between GPCR-G protein interfaces and the nucleotide-binding pocket based on β_2AR-Gs complex (PDB: 3SN6). β_2AR is shown as cyan surface and Gsα as ribbon, respectively (PDB: 3SN6). GDP is shown as spheres. The β1 and α5, are colored as orange, described as bridges between GPCR-G protein interfaces and the nucleotide-binding pocket. The Phe376, is equivalent with Phe336 of Gαi, is shown as stick. (B) Molecular interactions around the α1 helix in the nucleotide-free state (orange, PDB: 3SN6) or GTP-bound state (green, PDB: 1AZT) of Gαs subunit. GTP is shown as gray spheres.

should be existed to transform signal from the binding sites to the nucleotide-binding pocket to trigger the release of GDP from Gα subunit. A number of recent studies sought to define the allosteric conformational changes in G proteins upon GPCR binding using *in silico*, biochemical, and biophysical approaches (Table 1).

The C-terminus of the Gα subunit is the major GPCR contact site (Fig. 2B), and therefore the interaction between a GPCR and the C-terminus of the Gα subunit may induce conformational changes allosterically in Gα through the α5 helix. Recent modeling and experimental studies predicted the critical role of the α5 helix in G protein activation by GPCRs. Using a combination of mutagenesis and MD simulation, Shim and colleagues first described the molecular basis of cannabinoid CB1 receptor coupling to heterotrimeric Gαi$\beta\gamma$ proteins (Shim *et al*., 2013). This study described tight interactions between CB1 receptor and the C-terminal of the α5 helix of Gαi, as well as emphasized the crucial role of these interactions in G protein activation. Alexander *et al*. (2014) applied Rosetta-based sampling and energy analysis to provide a structural mechanism for rhodopsin-mediated GDP release from Gi, and observed a 5.7-Å translation and 63° rotation of the α5 helix. More recently, a long time-scale MD simulation by Dror *et al*. also observed a 60° rotation of the α5 helix with the removal of GDP (Dror *et al*., 2015).

The displacement or rotation of the α5 helix appears to be linked to the perturbation of intramolecular interactions in the Gα subunit, which would facilitate GDP release (Fig. 3A). Dror *et al*. (2015)'s study suggested that α5 displacement upon receptor binding increases the flexibility of the guanine ring-contacting the β6/α5 loop, thus perturbing contact between GDP and the Ras domain. Alexander *et al*. (2014)'s study also suggested that G protein activation is associated with rearrangement of the intramolecular interaction between the α5 helix, β6/α5 loop, α1 helix, and αG helix. Subsequent experimental mutagenesis studies of the same group indicated that residue F336 in the α5 helix of Gαi1 is crucially important in G protein activation because its mutation increases the rate of spontaneous GDP release (Kaya *et al*., 2014). The proposed mechanism involves F336 acting as a relay transmitting conformational changes from the C-terminus via a hydrophobic interaction with strands β2, β3 and α1 helix (Fig. 3A). More recently, Sun *et al*. (2015) revealed the particular importance of this residue and its surrounding contacts with the α1 helix, β1, and β2 strands in GPCR-G protein complex formation, as well as its role in the stability of GDP-bound Gαi.

A comprehensive analysis of available Gα crystal structures further emphasized the role of the α5 helix as a bridge for GPCR-mediated allosteric GDP release and suggested the α1 helix as a "hub"; the α1 helix links various important functional regions of Gα including the N-terminus of the α5 helix, AH domain, and GDP through universally conserved residues (Flock *et al*., 2015) (Fig. 3B). In this model, the mechanism of allosteric activation is triggered by movement of the α5 helix, subsequently breaking the contacts between the α5 and α1 helices leading to an increased flexibility in the α1 helix. The contacts between the α1 helix and AH domain as well as GDP are disrupted, thereby GDP affinity is weakened, which promotes GDP release together with AH domain separation. Importantly, this study indicated that the residues involved in these contacts are highly conserved across all Gα proteins suggesting that the above-mentioned mechanism is likely to be universal throughout Gα proteins. An experimental study also suggested the crucial role of the interaction between the α1 and α5 helices in GPCR-mediated G protein activation since cross-linking between these two helices, which restricts free movement or translocation of the α5 helix, impeded G protein coupling to the receptor (Kaya *et al*., 2014).

The hydrophobic region surrounded by the αN/β1 hinge and the β2/β3 loop of Gαs is another major contact site with

receptors (Fig. 3A), and the interaction of receptors with this region may induce allosteric conformational changes at the nucleotide-binding pocket through the β1 strand. A crosslinking study together with MD simulation data indicated the high conservation of Phe139 in ICL2 of cannabinoid CB2 receptor anchors in a hydrophobic triad formed by residues from the αN/β1 hinge, β2/β3 loop, and α5 helix of Gαi1 (Mnpotra *et al*., 2014). The authors also suggested that the interactions between ICL2 of CB2 receptor and the hydrophobic pocket in the Gαi1 act as the key "registration" for complex formation. Recent MD simulations demonstrate new evidence for an "alternate allosteric route" through the β1 strand (Fig. 3A) (Yao *et al*., 2016). The role of the β1 strand is involved in the paths from receptor to the Ras-AH interface which expressed different favored routes in distinct states of Gα (apo, GDP-, and GTP-bound form). Indeed, the MD simulations of L32A of Gαt, a highly conserved residue in the β1 strand, displayed an enhanced domain displacement and increased nucleotide exchange rate as well as G protein activation suggesting the functional relevance of this allosteric mutation. Furthermore, analysis of the structural dynamic of mutations in these regions manifests the novel role of the β1 strand together with β2, β3, P-loop, and Switch 1 in the modulation of domain opening that is critical for nucleotide exchange. Most recently, Pachov *et al*. (2016) also suggests that ICL2 of the receptor interacts with the N terminus of β1, subsequently weakening the interaction of the P loop with the nucleotide.

When GDP or GTP is bound to G proteins, Ras and AH domains are in the "close state" based on the X-ray crystal structures (Fig. 1). The interface between the two domains is comprised of interactions between the α1, αA, and αF helices and the linker 1 (α1/αA loop) and between the αG and αA helices, the β4/α3 loop, the αD/αE loop, and the switch 1 (Fig. 1D). It is noteworthy that the residues responsible for the interdomain interactions are highly conserved in all Gα family proteins (Flock *et al*., 2015; Sun *et al*., 2015). Once activated by GPCRs, they turn to the "open state" or nucleotide-free state by movement of the AH domain away from the Ras domain (Rasmussen *et al*., 2011b) (Fig. 2A). It has not been clear until recently if the domain displacement is cause or consequence of nucleotide release.

Surprisingly, Dror *et al*. (2015)'s simulation study showed that the AH domain fluctuates spontaneously between closed and open states relative to the Ras domain in the microsecond time scale even in the absence of GPCRs while GDP is still bound to the G protein. The spontaneous motion of the AH domain in the ensemble of native states was also mentioned in previous MD simulations (Jonesa *et al*., 2012; Yao and Grant, 2013). The contacts of GDP with the AH domain were occasionally disrupted and reformed, which leads to highly dynamic movement of this domain even in the receptor-free or GDP-bound inactive state, however, this spontaneous separation is not sufficient to trigger GDP release (Dror *et al*., 2015). More recently, Goricanec *et al*. (2016) indicated that the opening of the nucleotide binding pocket is more populated in the GDP-bound state compared to the GTP-bound state by a combination of NMR, small angle X-ray scattering (SAXS), circular dichroism (CD), and fluorescence spectroscopy. The data also showed that GDP binds to the Ras domain, but does not dock into the AH domain at the same time indicating weak contacts between the GDP and AH domain. GDP binding to the Ras domain has also been observed in a simulation with deletion of the AH domain (Markby *et al*., 1993). The simulations mimicking the α5 distal C-terminus in the receptor-bound conformation showed that GDP is dissociated from binding sites within microseconds; this study indicated that activation of G protein by GPCR triggers GDP release primarily by reducing GDP affinity with the Ras domain rather than by Ras-AH domain separation (Dror *et al*., 2015). These studies suggest that, upon GPCR binding, the weakening between GDP and the Ras domain is the major factor in GDP release.

Taken together, these combined studies show structural dynamics and conformational relevance of distinct states containing GDP, GTP, and receptor-bound Gα, allowing us to gain insight into the activation process of G proteins which start from the GDP-bound form and progress to the nucleotide-free state or receptor bound form and finally to the GTP bound form or active state (Fig. 1A). The activation mechanism suggests the involvement of receptor induced allosteric conformational changes in the Gα subunit through two major interactive sites, which was clearly identified previously (Duc *et al*., 2015). This includes the relocation and binding of the C terminus of Gα to the receptor or formation of interactions between the αN/β1 hinge and the receptor, and the respective signal is subsequently transmitted either via a combination of the α5 helix, β6/α5 loop, and α1 helix, or via strand β1 to P-loop to destabilize the nucleotide-binding site and allow GDP release, as well as displacement of the helical domain.

G PROTEIN-MEDIATED ALLOSTERIC MODULATION OF GPCRS

It has been known that agonist-bound receptors adopt multiple conformations equilibrated between inactive and active states (Nygaard *et al*., 2013; Manglik *et al*., 2015). In addition, biophysical and pharmacological studies suggest that the active conformation in intracellular regions of GPCRs is stabilized by nucleotide-free Gα or by G protein mimetic nanobodies (Rasmussen *et al*., 2011a, 2011b; Kruse *et al*., 2013; Huang *et al*., 2015). Recently, it has been newly proposed that G protein coupling or nanobody interaction at the cytoplasmic side allosterically induces conformational changes in the orthosteric ligand-binding sites leading to the enhancement of agonist-binding affinity, which results in the stable ternary complex formation of agonist, receptor, and G proteins (DeVree *et al*., 2016; Staus *et al*., 2016).

The modeling study suggests the existence of an inverse correlation between the ligand binding site and the G protein binding interface (Kolan *et al*., 2014). This study indicated that, upon coupling, the G protein leads to contraction of the ligand binding pocket which positively correlated to the expansion of the G protein binding cavity. Previously, Louet and colleagues used normal mode analysis (NMA) to predict collective motions of agonist-bound β_2AR both in complexes with G proteins and in the uncoupled conformation (Louet *et al*., 2013). In this model, the receptor adopts only one major motion in the presence of G proteins which relates to an anti-symmetric rotation of both its extra and intra-cellular regions; the authors proposed that the overall dynamic conformations of the β_2AR-Gs complex might be controlled by the G protein rather than by the receptor.

DeVree *et al*. (2016) have used a radioligand binding assay to investigate the ligand association and dissociation induced

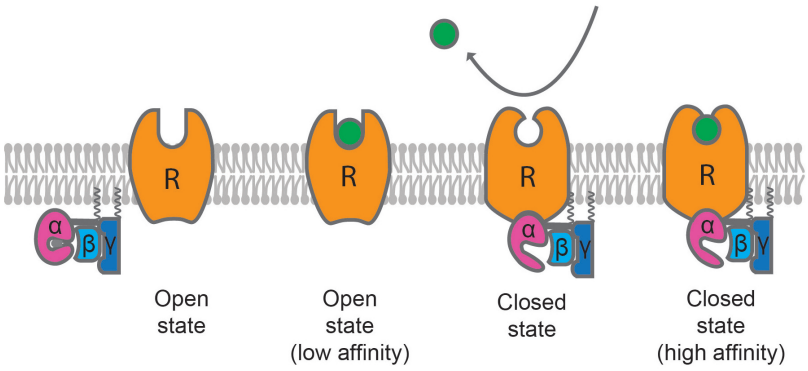

Fig. 4. The allosteric regulation of GPCR by G protein.

by G protein engagement in GPCRs. They found that Gs protein can form a complex with β_2AR even in the absence of an agonist, indicating the existence of basal receptor activity. Particularly, nucleotide-free Gs stabilized the conformation of the β_2AR in a "closed state", in which state the ligand inserting route is closed at the extracellular side and the association with an antagonist, full agonist, as well as partial agonist is restricted (Fig. 4). This study also illustrated that G protein coupling induced the stabilized conformation that restrains the agonist in the binding pocket thus enhancing its initially observed affinity or impairing agonist dissociation from the binding site (Fig. 4). In general, TM domains in the intracellular region undergo an outward movement in active-state GPCRs to open a docking cavity for the C-terminus of Gα. This large displacement of the TM domain is associated with inward structural changes on extracellular regions to form a "lid-like structure" that impairs the dissociation of an agonist from the orthosteric ligand-binding site.

Together, these data suggest that coupling to G protein and subsequent nucleotide release is sufficient to promote stabilization of the active state of the receptor or "a closed receptor conformation", preventing ligand access to and/or exit from the orthosteric ligand-binding site. Despite structural variance, the stabilization of G proteins in structural changes of GPCRs might be shared throughout GCPRs. Similar findings were observed in several families of GPCRs including the muscarinic receptor, the opioid receptor, and the ghrelin receptor (Mary *et al*., 2012; DeVree *et al*., 2016). Previously, Mary *et al*. (2012) also suggested that heterotrimeric Gq protein coupling to the ghrelin receptor subsequently stabilized receptor conformation. Interestingly, this conformation was not regulated by addition of an inverse agonist suggesting the restriction of the ligand binding site for an inverse agonist (Mary *et al*., 2012).

PERSPECTIVES

The high-resolution crystal structure of β_2AR-Gs provides an excellent model to carry out a large number of computational and biochemical/biophysical studies in order to understand the conformational mechanism of G protein activation. Combined with previous findings over the last thirty years, these studies provide us with more details about the structural mechanism of the G protein activation cycle. However, there are still more questions to be answered to develop a concrete model for the G protein activation processes.

Although many structural and functional assays have been used to indicate several critical regions in either the Gα subunit or GPCR that are responsible for selectivity, understanding how various ligand-induced conformational changes in GPCRs allow recognition of specific cognate G proteins still remains challenging. Several studies have reported models of GPCR-G protein complexes by using β_2AR-Gs structures as a model (Shim *et al*., 2013; Alexander *et al*., 2014; Mnpotra *et al*., 2014), which provides more information about novel residues that are extremely important in GPCRs-G protein selectivity. It has been reported that receptor oligomerization can regulate coupling ability and selectivity of GPCRs to G proteins (Moreno *et al*., 2011; Ellenbroek, 2013; Ferré *et al*., 2014; Navarro *et al*., 2016). However, the exact mechanism underlying GPCR/G protein specificity is still ambiguous and will require further investigation.

Another question yet to be answered is the conformational sequence of G protein activation by GPCRs. Two major regions in the Gα subunit are involved in the GPCR interaction: the C-terminus of the α5 helix and αN/β1 hinge as described in this review. However, we still do not have definitive answers on the conformational steps of GPCR-G protein activation or which regions have a major role in the initial release of GDP.

Besides G proteins, arrestins also have important roles in GPCR signaling in relation to G protein-independent signal transduction. Recently, great achievements have been made to understand the structural mechanism of the GPCR-mediated arrestin activation process (Park *et al*., 2016) increasing our understanding of the structural mechanism of functionally biased GPCR signaling. However, we still do not know the conformational switch that distinguishes G protein- or arrestin-dependent GPCR signaling, or how the ligand induces specific conformational changes to selectively recognize G proteins or arrestins. Interestingly, the recent crystal structures of activated rhodopsin bound to the finger loop peptide of arrestin (Szczepek *et al*., 2014) or visual arrestin (Kang *et al*., 2015) also indicate the outward movement of TM6 as seen in the β_2AR-Gs structure. It is not clear if the structural changes upon arrestin engagement into intracellular regions results in stabilization of the extracellular domain to form a "lid-like structure" over the orthosteric or a closed receptor conformation as described for G proteins. Hopefully, the rapid development of technology and a collaboration of experimental approaches and computational modeling will allow it to be possible to solve

these problems in the near future.

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