Unraveling the structural basis of GPCR activation and inactivation

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By comparing ligand-free G protein–coupled receptor (GPCR) structures with those of receptors bound to inverse agonists, agonists and signaling effectors, two recent papers refine the understanding of GPCR activation. One group also reported the oligomeric assembly of the β1 adrenergic receptor in its ligand-free form, raising the question of the role of oligomers in receptor activation.

GPCRs form the largest family of proteins involved in the detection and translation of signaling information across biological membranes. They bind a vast diversity of signaling molecules, including hormones, neurotransmitters, chemokines, lipid mediators, ions, tastants and odorants. Ligand binding promotes or stabilizes specific conformations that lead to the engagement and activation of signaling effectors that include but are not limited to the heterotrimeric G proteins. Despite more than three decades of intense studies, the receptors' conformational changes leading to the activation of signaling cascades have not been fully characterized.

Classically, drugs acting on GPCRs are classified into agonists, inverse agonists and neutral antagonists. Whereas agonists promote an 'active conformation' of the receptor, leading to increased signaling, inverse agonists inhibit the spontaneous signaling activity by inducing or stabilizing an 'inactive conformation'. Neutral antagonists prevent binding of either agonists or inverse agonists but do not influence the equilibrium between active and inactive conformations. A major challenge to understanding receptor activation and inhibition is obtaining the structures of receptors in their ligand-free state as well as bound to either agonists or inverse agonists. In recent years, structural studies have started to shed some light on the transitions controlling receptor activity[1]. However, all crystal structures for GPCRs binding diffusible ligands were obtained in their inverse agonist– or agonist-bound states, which makes it difficult to draw conclusions about the transitions from the basal state (**Fig. 1**).

In their recent study in *Nature Structural & Molecular Biology*, Huang and colleagues[2] report the first crystal structure for a GPCR that binds a diffusible hormone in its ligand-free state. This was achieved by using a thermostabilized mutant form of the turkey β1 adrenergic receptor known as the β1AR(m23), containing a number of mutations and deletions that confer greater stability[3]. The crystal was obtained in a lipid membrane–like environment and the structure solved with a resolution of 3.5 Å.

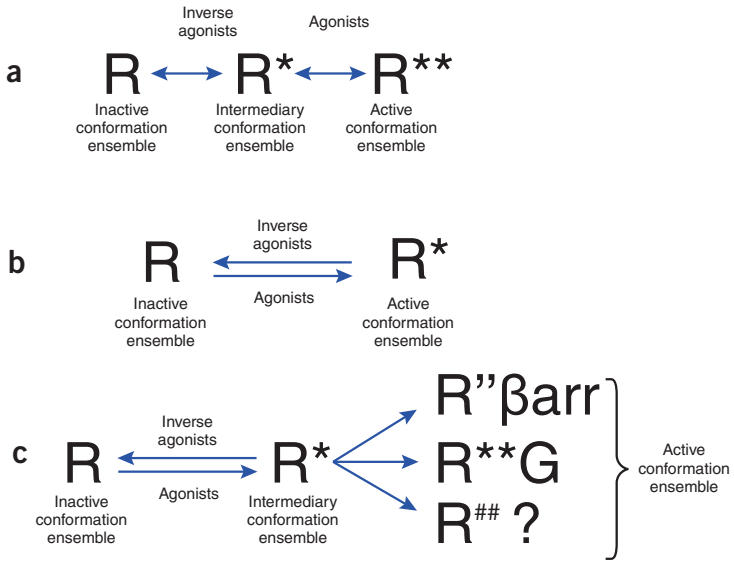

Figure 1 Alternative models describing the transition between active and inactive states in GPCRs. (**a**) Three-state model: at resting state, the receptor is in an intermediate state (R*), showing some level of activity. Inverse agonists favor the transition toward inactive conformations, whereas agonists favor fully active conformations (R**). (**b**) Two-state model: at the resting state, the receptor is largely in inactive conformations (R*) that are stabilized by inverse agonists; agonists favor the active conformations. (**c**) Three-state model: at resting state, the receptor is largely in inactive conformations. Agonists favor intermediate states (R*), and the fully activated states (R**, $R^{\#\#}$ and R") are promoted by signaling effectors. The data reported by Huang and colleagues[2] support the two-state model illustrated in **b**. In combination with other crystallographic and NMR studies, the three-state model illustrated in **c** can be proposed. R, GPCR; βarr, β-arrestin; G, G protein–coupled receptor kinase.

The overall structure was very similar to those previously reported for the same receptor bound to the inverse agonist cyanopindolol[3,4]. This indicates that the ligand-free basal state of the receptor is largely in an inactive conformation that is not different from the conformation stabilized by the inverse agonists. In particular, transmembrane domains TMV, TMVI and TMVII, whose movements play important parts in the receptor activation process[5,6], are in a restrained position characteristic of an inactive conformation. The 'ionic lock' salt bridge between the highly conserved D(E)R3.50Y motif and the E/D6.30 residues, considered a trademark of the inactive conformation of rhodospsin, is found in the closed position in the ligand-free β1AR(m23). It should be noted that the closed ionic lock is not a universal feature, as it was seen open in some β1AR structures[7] as well as in crystals of the closely related β2AR obtained in the presence of inverse agonists. Whether these different states of the ionic lock represent dynamic transition states toward an active conformation that were captured only in a subset of the available structures remains to be determined.

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The similarities between the ligand-free and the inverse agonist–bound forms of the receptor could indicate that under basal conditions the receptor does not adopt intermediate conformations between inactive and active conformations (**Fig. 1b** compared with **Fig. 1a**); instead, a large fraction of the receptor would remain in an inactive conformation. The receptor's constitutive activity in cells in the absence of agonist would therefore result from a small fraction of the receptor spontaneously reaching an active conformation that has a high energy and low stability in the absence of ligand and hence cannot be captured in crystallographic studies. However, it is possible that intermediate states were not observed by Huang and colleagues[2] because the mutations in β1AR (m23) may have stabilized an inactive conformation similar to the one stabilized by inverse agonists (**Fig. 1b**). Indeed, β1AR (m23) binds inverse agonists with wild-type affinity but has a weaker affinity for agonists and does not display any constitutive signaling activity.

In another recent study, Kobilka and colleagues[8] used NMR spectroscopy to assess the dynamics of β2AR activation, and their analyses also revealed that ligand-free and inverse agonist–bound receptors share very similar conformations. Because they used a mutant form of the receptor (β2AR-Δ5M-L272M) that shows a modest increase in agonist-binding affinity and therefore should not favor an inactive conformation, the data support the notion that the ligand-free conformations are similar to the inactive conformations stabilized by inverse agonists. (**Fig. 1b**). Yet, from molecular dynamics analyses based on the crystal structure of the β2AR, the authors suggest that at least two inactive conformations exist (inactive and alternative), both highly dynamic and existing with the ionic lock in both open and closed positions[8], consistent with previous crystallographic observations[7]. Thus, an ensemble of inactive conformations exists, and the substrates can be bound by inverse agonists without resulting in major conformational rearrangements.

The lack of major differences between the ligand-free and the inverse agonist–bound structures observed by both groups may not be surprising, given the relatively small differences between the latter and agonist-bound structures in most GPCR crystal structures solved to date (reviewed in ref. 1). In fact, the major structural changes characteristic of a fully activated receptor could only be detected when β2AR was cocrystallized with the G protein or a G protein–mimicking nanobody[6]. Albeit to a lesser extent, a similar effect of the G protein was also seen by NMR by Kobilka and colleagues[8]. These observations suggest that, in the presence of agonist alone, the receptor transitions between different substates of intermediate conformations and that association of a G protein stabilizes a fully active conformation (**Fig. 1c**). GPCRs can engage with different downstream effectors in addition to G proteins, such as G protein–coupled receptor kinases and β-arrestins. Thus, the above observations open the intriguing possibility that the association of agonist-bound receptor with different effectors may result in different active conformations. This may explain the phenomenon of ligand-biased signaling, whereby different ligands differentially activate distinct subsets of downstream signaling effectors engaged by a given receptor[9–11]. The association of the effector would complete the conformational change toward a fully active conformation and reciprocally increase the affinity of the receptor for the particular agonist favoring this specific conformation (**Fig. 1c**).

Another important observation by Huang and colleagues[2] is that in a lipid-like environment the β1AR crystallized as an oligomeric complex. Each crystallographic unit had two β1AR molecules and the receptor packed in a linear oligomeric array. This is consistent with the large body of biophysical and biochemical evidence indicating that many GPCRs including the βARs can form dimers and possibly larger oligomeric complexes[12,13]. Recently, crystals of other GPCRs also revealed potential dimer interfaces, including rhodopsin[14], chemokine CXC type-4 receptor (CXCR4)[15], μ opioid receptor (μOR)[16] and κOR[17]. Huang and colleagues[2] observed two distinct dimer interfaces: the first, involving transmembrane segments TM1-TM2 and helix H8, was also observed in the rhodopsin, μOR and κOR crystals, which suggests a common mechanism of dimerization for many GPCRs. The second interface (involving TM4, TM5 and intracellular loop 2 (ICL2)) was different from those previously seen in rhodopsin, the CXCR4 and the μOR (involving TM5 and TM6). Whether these differences reflect distinct modalities of oligomerization for different GPCRs or an artifact of crystallization remains to be determined, but it should be noted that Huang and colleagues[2] validated both observed dimer interfaces by using cross-linking in membrane preparations. The detection of dimers in the ligand-free state supports the notion that the receptor may be a constitutive dimer.

The roles of dimerization and oligomerization in GPCR activation remain a topic of intense research and debate (**Fig. 2**). For some class-C GPCRs such as the GABA and glutamate receptors, an asymmetric mode of activation whereby only one protomer within a dimer can activate the G protein has been proposed[18]. Huang and colleagues[2] did not experimentally assess the role of dimerization in the activity state of β1AR, but *in silico* modeling revealed that one heterotrimeric G protein could be docked under the β1AR dimer formed through the TM1-TM2-H8 interface but not under the TM4-TM5-ICL2 dimer[2]. As a result, only two G proteins could be docked under a tetramer in an asymmetric configuration, but the relevance of such arrangements in

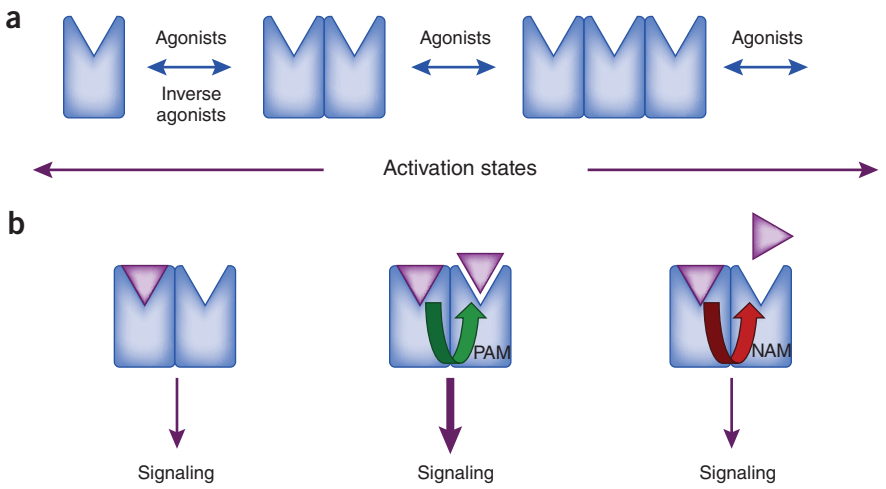

Figure 2 Oligomeric states and activation of GPCRs. (**a**) Possible equilibrium between monomeric, dimeric and multimeric states of GPCRs. The existence of dimeric and higher-order oligomeric states is supported by biochemical, biophysical and structural evidence, but the dynamic equilibrium between those states and its link with receptor activation remains poorly understood. (**b**) The oligomeric state can modulate the signaling activity of the receptor. Depending on the receptor (blue) and ligands (purple) considered, both positive (PAM) and negative (NAM) allosteric modulation have been proposed to result from receptor dimerization.

controlling G-protein activation remains an open question (**Fig. 2a**). Reconstitution in lipid nanodiscs has clearly demonstrated that a β2AR monomer is sufficient to activate a G protein *in vitro*[19], but one could speculate that the assembly into dimers or larger oligomers could regulate such activation. Consistent with this idea, FRET studies following reconstitution of the β2AR into a model lipid bilayer indicated that the receptor can form tetramers that are stabilized upon binding of inverse agonist, whereas the addition of G proteins destabilizes the oligomer, thus indicating that the tetramer may represent an inactive form of the receptor[20]. Furthermore, both positive and negative allosteric modulation of ligand binding to various GPCRs have been attributed to dimerization[13] (**Fig. 2b**).

In conclusion, the studies by Huang and colleagues[2] and Kobilka and colleagues[8] contribute to an increasingly clear picture of the molecular events that take place during GPCR activation and inhibition. Yet, future studies will be needed to define the conformational substates within the inactive and active conformation ensembles and to determine the role that receptor oligomerization may have in those ensembles.

COMPETING FINANCIAL INTERESTS
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From pseudo-ceRNAs to circ-ceRNAs: a tale of cross-talk and competition

Riccardo Taulli, Cristian Loretelli & Pier Paolo Pandolfi

RNA is believed to have been the first reservoir of genetic information, but despite its ancient history, RNA continues to fascinate and is only now beginning to be understood in its entire variety and communication modality. New discoveries include the pseudogene RNA network regulating *PTEN* transcription and translation and the identification of circular RNAs as a new class of competing endogenous RNA molecules that sequester microRNAs to suppress their function.

For decades, RNA was considered to be merely a 'carrier' of genetic information, but in the past 15 years unexpected discoveries in the epigenetic landscape, from microRNAs (miRNAs) to pseudogenes and the recently described long noncoding RNAs (lncRNAs), have completely revolutionized this simplistic view. miRNAs are small RNA molecules of 20–22 nucleotides that post-transcriptionally negatively modulate the translation and stability of target RNA molecules containing miRNA-responsive elements (MREs) in their sequence[1]. LncRNAs and pseudogenes are longer and surprisingly more numerous than protein-encoding genes and are extremely versatile in their mechanism of action[2,3].

Although one miRNA can potentially regulate hundreds of different mRNAs (each one in turn containing several MREs) the majority of these transcripts are actively expressed and translated, which supports the existence of several mechanisms that counteract miRNA regulation to achieve homeostasis. Probably the most ancestral and intuitive example among these regulatory mechanisms is the cross-talk and functional competition among different RNA molecules, as epitomized by the interplay between miRNAs and competing endogenous RNAs (ceRNAs)[4] (described below).

Three recent papers have now shed light on an intertwined system of interaction between noncoding RNA molecules and mRNAs from protein-coding genes. Johnsson *et al.*[5] have identified a pseudogene network able to regulate phosphatase and tensin homolog (*PTEN*) at both the transcriptional and the post-transcriptional level. The *PTEN* pseudogene (PTENpg1) locus encodes three different lncRNA molecules: two functional antisense RNAs (asRNAs) and one sense PTENpg1. PTENpg1 asRNAα acts in *trans*, localizes to the *PTEN* promoter and inhibits *PTEN* transcription by recruiting epigenetic repressor complexes, whereas PTENpg1 asRNAβ, which is partially complementary to PTENpg1 sense, promotes stabilization of PTENpg1 sense by binding its 5′ end. PTENpg1 sense was previously shown to act as a ceRNA for *PTEN*[6] through its ability to compete for several miRNAs that also target *PTEN*. Thereby, the stabilization of PTENpg1 sense by PTENpg1 asRNAβ can regulate its ceRNA activity[6] (**Fig. 1a**), perhaps in a tissue-specific manner, which further highlights the regulatory activity on antisense and pseudogene RNAs species.

Knowledge of the endogenous competition between noncoding RNAs recently expanded with the discovery of a new, highly prevalent class of conserved RNA molecules called circular RNAs (circRNAs or ciRS)[7,8]. The majority of circRNAs overlap with coding genes, often at the protein-coding portion of the mRNAs, and arise from circularized splicing of the reverse ends

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