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Nanobodies to Study G Protein–Coupled Receptor Structure and Function

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nanobody, G protein–coupled receptor, conformational plasticity, receptor activation, crystallographic chaperone, intrabody

Abstract

Ligand-induced activation of G protein–coupled receptors (GPCRs) is a key mechanism permitting communication between cells and organs. Enormous progress has recently elucidated the structural and dynamic features of GPCR transmembrane signaling. Nanobodies, the recombinant antigen-binding fragments of camelid heavy-chain-only antibodies, have emerged as important research tools to lock GPCRs in particular conformational states. Active-state stabilizing nanobodies have elucidated several agonist-bound structures of hormone-activated GPCRs and have provided insight into the dynamic character of receptors. Nanobodies have also been used to stabilize transient GPCR transmembrane signaling complexes, yielding the first structural insights into GPCR signal transduction across the cellular membrane. Beyond their *in vitro* uses, nanobodies have served as conformational biosensors in living systems and have provided novel ways to modulate GPCR function. Here, we highlight several examples of how nanobodies have enabled the study of GPCR function and give insights into potential future uses of these important tools.

G protein-coupled receptors (GPCRs):

a large eukaryotic protein family of membrane receptors that sense molecules outside the cell and activate inside signal transduction pathways

Arrestins: a family of regulatory proteins that arrest GPCR signaling and are responsible for G protein-independent signaling

G protein-coupled receptor kinase

(GRK): a specialized set of kinases that recognize and phosphorylate activated GPCRs

Nanobody (Nb):

recombinant single-domain antibody fragment that contains the unique structural and functional properties of naturally occurring heavy-chain-only antibodies in camelids

INTRODUCTION

The G protein-coupled receptor (GPCR) superfamily comprises more than 800 distinct human cell surface receptors that share a common seven-transmembrane α -helical fold (1–3). As highly versatile membrane sensors, GPCRs respond to a variety of extracellular signals, including photons, ions, sensory stimuli, lipids, neurotransmitters, hormones, and large proteins (4). These receptors convert various signaling cues into cellular responses by engaging with intracellular G proteins, β -arrestins, and other downstream effectors (5, 6). About 450 human sensory GPCRs mediate olfaction, taste, light perception, or pheromone signaling (7). The remaining approximately 350 nonsensory GPCRs mediate cell-to-cell signaling and are the targets for a large fraction of clinically used drugs (8, 9), although only a minority of these receptors are exploited therapeutically. About 120 human receptors are still orphan receptors with unknown endogenous ligands and/or function (10).

GPCRs relay diverse extracellular signals into cells by activating associated heterotrimeric G proteins. Agonist binding causes the formation of an active transmembrane signaling complex and results in the exchange of bound GDP for GTP by the G_{α} subunit of the G protein and the concomitant dissociation and release of the G_{α} and $G_{\beta\gamma}$ subunits (11, 12). The separate G_{α} and $G_{\beta\gamma}$ subunits then promote the production of and consequent signaling by second-messenger systems, such as those involving cyclic AMP (13), diacylglycerol (14), or calcium (15). Signaling is terminated by phosphorylation of the cytoplasmic loops and the C-terminal tail of the receptor by GPCR kinases (GRKs). Receptor phosphorylation triggers a cascade of events that include the recruitment of arrestins, uncoupling of GPCRs from heterotrimeric G proteins, receptor internalization, and activation of G protein-independent signal transduction pathways (16, 17). In recent years, evidence has accumulated that GPCRs can form homo- and heterodimers as well as higher-order oligomeric assemblies under physiological conditions (18, 19). It is now well accepted that family C GPCRs form constitutive homo- or heteromers (20).

Researchers have made remarkable progress in the field of GPCR structural biology during the past decade, with more than 100 GPCR structures reported for more than 30 unique receptor subtypes (21). Several obstacles to generating diffraction-quality crystals of GPCRs have been overcome by innovative methods such as recombinant expression systems (22), protein engineering (23–27), lipid-based crystallography (28), and novel methods for collecting X-ray diffraction data (29, 30). These methodological innovations have been combined with novel reagents, including compounds tailored for crystallography (31) and detergents that improve receptor stability (32). Most GPCR structures solved to date represent the most energetically stable, inactive, antagonist-bound conformations. These structures have increased our understanding of ligand binding and selectivity (33). However, a mechanistic understanding of GPCR signaling requires structural insights into the active state as well as other ligand-specific states of the receptor responsible for the broad array of GPCR functions. This review focuses on the instrumental role of nanobodies as tools to study the structural and dynamic features of GPCR transmembrane signaling.

GPCR SIGNALING COMPLEXITY AND CONFORMATIONAL PLASTICITY

Views on activation of GPCRs have evolved considerably over the past 20 years (34). The earliest models consisted of two receptor conformations: an inactive state in equilibrium with an active state (35). In these binary models, receptor signaling correlates directly with this equilibrium. Agonists and partial agonists shift the equilibrium toward the active state and thereby induce activation of heterotrimeric G proteins. Inverse agonists shift the equilibrium to the inactive state and suppress basal activation of G proteins. Although many aspects of GPCR function can be explained by a

CONFORMATIONAL PLASTICITY OF COMMON DRUG TARGETS

Protein structures elucidated through X-ray crystallography give the impression that proteins are rigid entities. Although static structures are known for many proteins, the functions of proteins are governed ultimately by their dynamic character (133). Allostery, the coupling between ligand binding and protein conformational change, is at the heart of biological networks, and conformational flexibility is key to the function and pharmacology of the majority of current and future drug targets including GPCRs, ion channels, nuclear receptors, and kinases. Agonists, inverse agonists, and biased ligands bind different structural conformations of the same GPCR (37). Allosteric transitions also occur in many therapeutic ion channels (134). A typical ion channel shuttles between open and closed states, and the conformational transition between open and closed states is referred to as gating. Channel opening is triggered by specific stimuli, such as the voltage or pH difference across the cell membrane, and modulated by the binding of ligands (135). In the kinase family, allosteric druggable pockets other than the ATP binding site are formed when these enzymes adopt specific conformations (136). Allosteric effects also govern the biological activity of nuclear receptors (137).

simple two-state model, accumulating evidence supports more complex multistate behavior (see the sidebar, Conformational Plasticity of Common Drug Targets) (36). Many GPCRs are capable of signaling through multiple intracellular pathways. These include canonical second-messenger signaling pathways mediated by G protein activation as well as G protein-independent pathways. Ligands acting on a given GPCR can induce varying levels of response (37). Some ligands, termed biased agonists, are capable of preferentially activating one signaling pathway among many (38). In addition to ligand-directed activation of a broad range of signaling responses, many other elements can influence GPCR function, including changes in cell membrane lipid composition, tension, and fluidity (39–41) as well as membrane voltage (42). The function of GPCRs is also regulated by pH (43) and common ions such as sodium (44). This broad complexity in function has suggested that GPCRs sample a continuum of conformational ensembles rather than discrete on and off states. A wide variety of biophysical studies—spanning X-ray crystallography, NMR spectroscopy (45–48), double electron-electron resonance (DEER) spectroscopy (49), single-molecule fluorescence (50, 51), and electron microscopy (52–55)—have confirmed and further examined the role of this conformational complexity in GPCR function.

Ligand Efficacy and Biased Signaling is Dependent on GPCR Conformational Complexity

Although highly complex, the conformational dynamics of GPCR signaling can be conceptualized by free-energy landscapes that provide a schematic relation between structure, dynamics, function, and ligand efficacy (56). The relative populations of different states are indicated by the depth of each energy well, and the rate of transition between different states is illustrated by the height of the energy barriers. Recent single-molecule fluorescence (50, 51), NMR (48), DEER spectroscopy (49), and molecular dynamics experiments (57) on the β_2 -adrenergic receptor (β_2 AR), a prototypical class A GPCR, indicate that the ligand-free transmembrane core of the receptor is highly flexible and exists in separate inactive conformations that exchange within hundreds of microseconds. Basal activity most likely results from a small fraction of activated receptors that exist within the conformational ensemble of the ligand-free receptor (**Figure 1**). Surprisingly, agonist binding does not reduce this conformational heterogeneity but rather increases conformational dynamics with states that exchange on a millisecond timescale (58). It thus appears that ligands bind particular

Biased ligand:

ligands that engage some signaling pathways selectively while avoiding, or even inactivating, other pathways mediated by the same receptor

Ligand efficacy: the efficacy of a drug is determined by its ability to induce a quantifiable biological response

β_2 AR: β_2 -adrenergic receptor

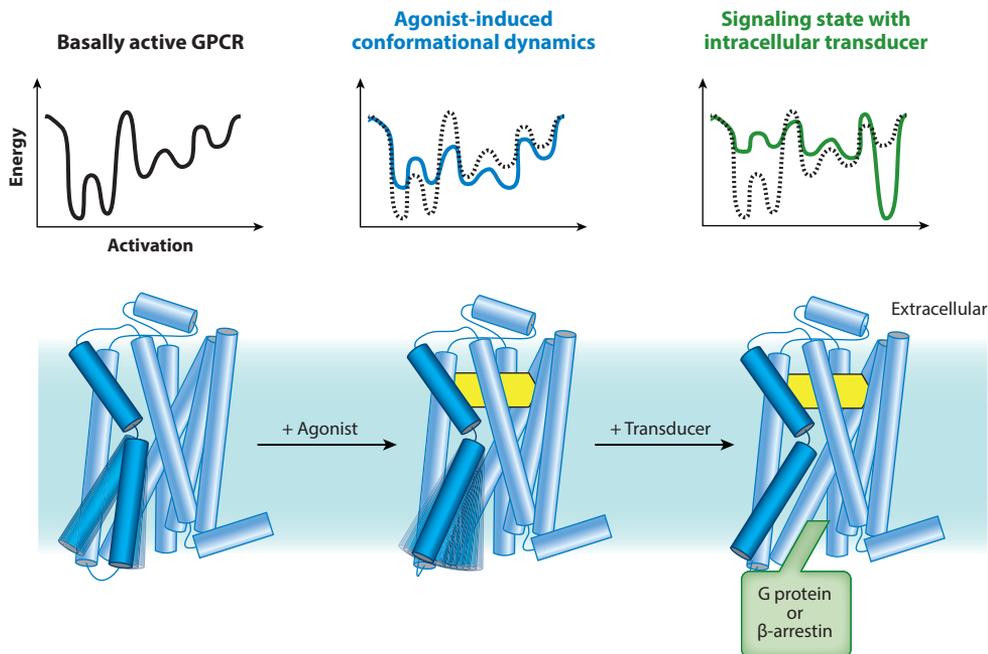


Figure 1

Conformational complexity in G protein–coupled receptor (GPCR) function. Unliganded and antagonist-bound GPCRs are conformationally dynamic. Agonists further induce receptor dynamics to varying degrees, and the crystallographically observed active state is stabilized only in the presence of a G protein or an active state–stabilizing chaperone (49). Energy diagrams illustrate the conformational complexity of GPCRs and highlight the inherent difficulty in capturing agonist-bound receptors in conformationally homogeneous crystallographic lattices.

conformations of the conformational ensemble preferentially, at the expense of conformational states from the pool of possible conformations for which the ligand has a lower affinity, creating a bias in the reference ensemble by mass action (59). Proteomics-based studies have also demonstrated that different ligands stabilize qualitatively different receptor conformational ensembles (60).

NANOBODY-BASED STABILIZATION OF GPCR CONFORMATIONS

The intrinsic conformational heterogeneity of GPCRs presented a major hurdle in obtaining the first crystal structures of hormone-activated receptors (61). Most GPCR structures solved to date have relied on high-affinity, slowly dissociating antagonists or the introduction of mutations to limit this inherent conformational flexibility. As most agonists are predicted to induce even further GPCR conformational heterogeneity (58), it is not surprising that the majority of structures solved to date represent inactive conformations (33). In an effort to stabilize the active conformation of agonist-bound GPCRs, we and others have used recombinant camelid single-domain antibody fragments called nanobodies.

Nanobodies: Single-Domain Antibodies that Bind Conformational Epitopes

In addition to conventional heterotetrameric antibodies, camelids (e.g., llamas) also produce functional heavy-chain-only antibodies devoid of light chains (62). The identical heavy chains of these

Camelids:

the biological family Camelidae comprises camels (*Camelus dromedarius* and *Camelus bactrianus*), llamas (*Lama glama* and *Lama guanicoe*), and vicuñas (*Vicugna vicugna* and *Vicugna pacos*)

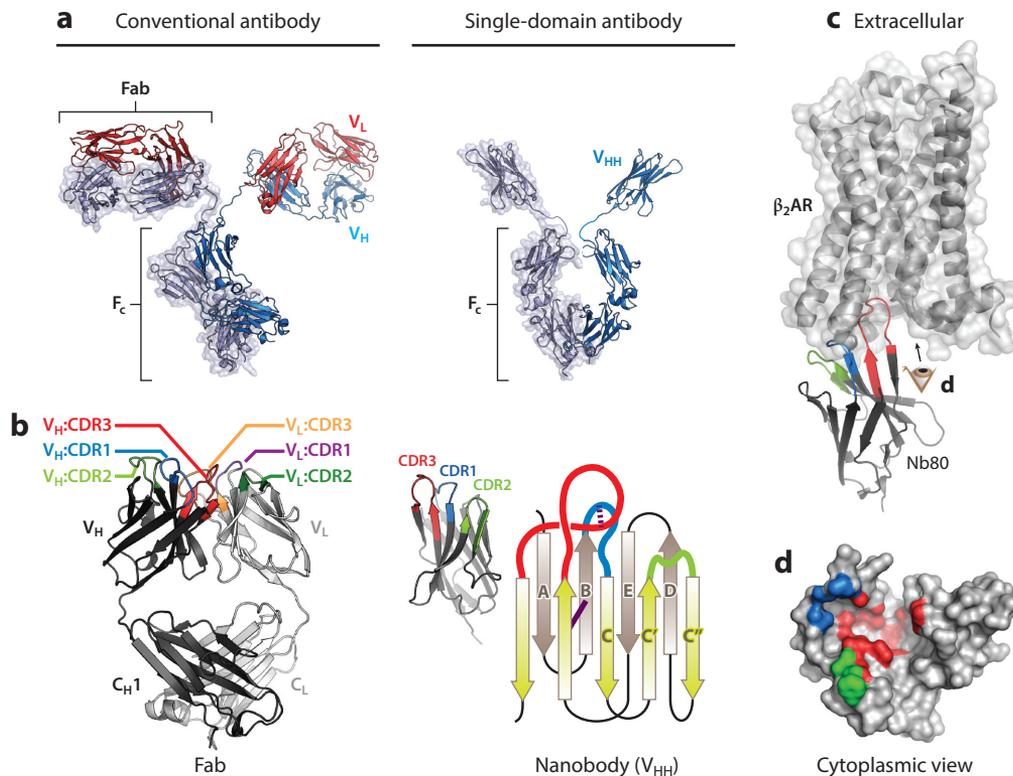


Figure 2

Nanobody structure and function and comparison to conventional antibodies. (a) Comparison of conventional antibodies to camelid single-domain antibodies. Conventional antibodies are heterotetrameric molecules consisting of two heavy chains (V_H) and two light chains (V_L) with a conserved domain called the crystallizable fragment (F_c). Variable loops responsible for antigen binding are within the distal tips of the Fab domain. Camelid single-chain antibodies contain a single immunoglobulin domain (V_{HH}) that binds antigens individually. (b) Comparison of the minimal binding domain of conventional antibodies (Fab) and single-domain antibodies (V_{HH} or nanobody). The antigen-binding region of a Fab is composed of six complementarity-determining regions (CDRs), with three in each V_H and V_L . Correct V_H/V_L pairing is required for antigen binding. In contrast, nanobodies contain three CDRs, and the single immunoglobulin fold is sufficient for antigen binding. The nanobody immunoglobulin fold is built from a pair of antiparallel β sheets with a conserved disulfide bond (solid purple line). The CDRs originate from loops between individual strands. Many nanobodies contain an extra interloop disulfide bond that restricts the flexibility of CDR1 and CDR3 (dotted purple line). (c) The prolate structure of the nanobody forms a convex paratope surface, which allows it to access antigenic cavities. In the β_2 -adrenergic receptor-Nanobody80 ($\beta_2AR \cdot Nb80$) complex shown here [Protein Data Bank (PDB) ID: 3P0G], CDR3 of Nb80 inserts into the cytoplasmic surface of active β_2AR , with additional interactions made by CDR1 and CDR2. The resulting β_2AR epitope recognized by Nb80, viewed from the cytoplasmic surface (eye symbol), is displayed in panel d. Note that each CDR binds different regions of the complex three-dimensional epitope that is discontinuous in β_2AR sequence. CDRs and framework residues in this figure have been defined according to the International ImMunoGeneTics Information System (IMGT) (138).

homodimeric antibodies consist of one variable domain and two constant domains (Figure 2). Nanobodies, the recombinant variable domains of the heavy-chain antibodies, are monomeric single-domain proteins encoded by single-gene fragments and harbor the full antigen-binding capacity of the parental antibody (63). Nanobodies are produced easily in microorganisms (64–67), mammalian cell lines (68), and plants (69, 70) and exhibit superior stability compared to recombinant derivatives of conventional antibodies such as single-chain variable fragments (71).

Single-chain variable fragment (scFv):

the paired V_H and V_L domains of conventional antibodies tethered via an oligopeptide

Conformational epitope: the surface making contact with the nanobody that is composed of discontinuous amino acid residues brought together by the protein folding of the antigen

Antigen-specific nanobodies are obtained by immunization of a camelid followed by cloning the variable V_{HH} gene repertoire from peripheral blood lymphocytes upon induction of a sufficient immune response. Nanobodies of desired function are then obtained by selection through one of many combinatorial biology methods, which include phage display (72), yeast display (73), and ribosome display (74, 75). Because the full immune repertoire of antigen-specific heavy-chain antibodies in a given llama is approximately 1×10^6 , libraries covering the complete nanobody repertoire of an immunized animal can be derived from the total number of B cells contained in a 50-mL sample of blood.

Nanobodies have gained significant traction as alternatives to conventional antibodies. Because of their compact prolate shape, nanobodies expose a convex paratope and have access to cavities or clefts on the surfaces of proteins that are inaccessible to conventional antibodies (76). These cryptic epitopes can often be recognized readily by the long complementarity-determining region 3 (CDR3) loop of the nanobody (77). Perhaps most importantly, camelids immunized with natively folded proteins produce nanobodies that can bind conformational epitopes, which are often composed of discontinuous amino acid segments and occur only within the fully native protein (**Figure 2**). Nanobodies have been developed to trap unstable structural intermediates along the fibrillation pathway of amyloidogenic proteins (78, 79). Multidomain proteins are more rigid in complex with a nanobody than the multidomain protein is by itself (80). In complex with a nanobody, the total amount of structured polypeptide increases, thus providing a much better starting point for the crystallization of intrinsically unfolded proteins (79, 81). Nanobodies can also be used to stabilize the protomers of larger protein assemblies (82).

Building on their unique properties, several laboratories have demonstrated that nanobodies are highly useful reagents for examining dynamic biological systems. Nanobodies have been used to crystallize flexible membrane proteins (83–87), transient multiprotein assemblies (88–90), and individual substates of conformationally complex proteins (91–95). Although nanobodies may select high-energy, low-population conformations of a dynamic protein, the probability of an *in vivo* matured nanobody inducing a non-native conformation of the antigen is low. Immature B cells require engagement of displayed antibodies with antigen to proliferate and differentiate during clonal selection (96). Antibodies that induce non-native conformations of the antigen pay a substantial energetic penalty in this process, and B cell clones displaying such antibodies will have a significantly lower probability of proliferation and differentiation into mature antibody-secreting B lymphocytes. This notion is supported by recent experiments that identified several nanobodies raised by immunization against the prokaryotic lactose permease transporter LacY. Although each nanobody stabilizes a unique conformation of LacY, the stabilized states are within the broad conformational ensemble of the transporter. This observation gives credence to the notion that nanobodies bind antigens primarily by conformational selection and not induced fit (97).

Active State-Stabilizing Nanobodies

The first structural insights into GPCR activation came from the light-activated GPCR rhodopsin. Rhodopsin is activated by absorption of a photon, which causes the isomerization of 11-*cis*-retinal to all-*trans*-retinal. Upon activation, rhodopsin converts rapidly to metarhodopsin II, which is the conformational state capable of activating the G protein transducin (98). Hydrolysis of the Schiff base between rhodopsin and retinal occurs spontaneously, and subsequent dissociation of retinal leaves the apoprotein opsin. To stabilize the active conformation of opsin for crystallography, previous studies utilized low pH (99, 100) and the C-terminal peptide of transducin (101). Subsequent efforts elucidated the structure of retinal-bound metarhodopsin II, both alone and in complex with the C-terminal peptide of transducin (102, 103).

Although these structures revealed considerable insight into receptor activation, the strategies used to capture the active conformation of rhodopsin were not easily transferable to other GPCRs. Most hormone-activated GPCRs are too dynamic to crystallize in the presence of agonist alone. Spectroscopic studies highlighted above demonstrated that a large fraction of agonist-bound β_2 AR exists in an inactive conformation (104); indeed, our efforts to crystallize the β_2 AR covalently bound to an engineered agonist resulted in an inactive conformation of the receptor (105). Similarly, other groups have pursued strategies to conformationally stabilize active state–receptors by introducing mutations at key positions (106, 107). These strategies have succeeded in determining the structures of agonist-occupied receptor, but the receptor conformation remains in the inactive states (108).

Building on the unique features of nanobodies outlined above, our labs initially explored the use of these single-domain antibodies as chaperones for stabilizing the active, agonist-bound conformation of the β_2 AR. We posited that these nanobodies may function as structural surrogates of native signaling partners (109), most notably for the heterotrimeric G protein. In our efforts to generate such nanobodies, we immunized llamas with agonist-bound β_2 AR reconstituted into lipid vesicles (72). The entire repertoire of *in vivo* matured nanobodies was cloned and subjected to phage display to identify conformational antibodies that recognized agonist-bound β_2 AR selectively. Several such nanobodies were identified and one clone, Nanobody80 (Nb80), was characterized extensively by spectroscopic and pharmacological assays. Binding of Nb80 to fluorophore-labeled β_2 AR induced a change in signal consistent with receptor activation, as was observed previously with the heterotrimeric G protein G_s (104). In pharmacological assays, Nb80 recapitulated a key feature of GPCR allostery. Researchers have previously shown that G proteins allosterically increase the affinity of agonists at a receptor (11, 110). In the presence of Nb80, purified and reconstituted β_2 AR demonstrated an increase in the affinity of the agonist isoproterenol, similar to what occurs in the presence of G_s (**Figure 3**). These lines of evidence suggested strongly that Nb80 stabilizes an active-state conformation of β_2 AR comparable to that in the G_s complex. Subsequent identification of nanobodies with similar properties at the M_2 muscarinic receptor (M2R) (92) and the μ -opioid receptor (μ OR) (94) have demonstrated that this approach is generalizable and can likely be extended to the broader GPCR family (**Figure 3**).

M2R: M_2 muscarinic receptor

μ OR: μ -opioid receptor

Nanobodies Facilitate Crystallization of Active-State GPCRs

The first structure of active β_2 AR was determined using Nb80 and a high-affinity, slowly dissociating agonist BI167107 (91). This structure revealed several important features of GPCR activation. Since this initial use of Nb80 to stabilize the β_2 AR active state, several nanobodies have been derived to obtain active-state structures of several GPCRs. Although Nb80 successfully enabled crystallization of the β_2 AR bound to BI167107, crystallization of the same receptor bound to its low-affinity endogenous agonist adrenaline remained challenging. To facilitate crystallization with low-affinity agonists, Nb80 was affinity matured using yeast surface display and novel conformational selection strategies. The resulting affinity-matured nanobody, Nb6B9, displayed slower dissociation kinetics at the β_2 AR and was used successfully to capture the structure of β_2 AR bound to adrenaline (93). Nanobodies raised against M2R (92), μ OR (94), and the viral chemokine receptor US28 (95) demonstrated subsequently that active state–stabilizing nanobodies are a robustly general tool for crystallizing agonist-bound receptors. This holds true even for constitutively active GPCRs such as US28, in which the presence of a nanobody (Nb7) improved the diffraction quality significantly compared to the US28·CX3CL1 complex alone (95).

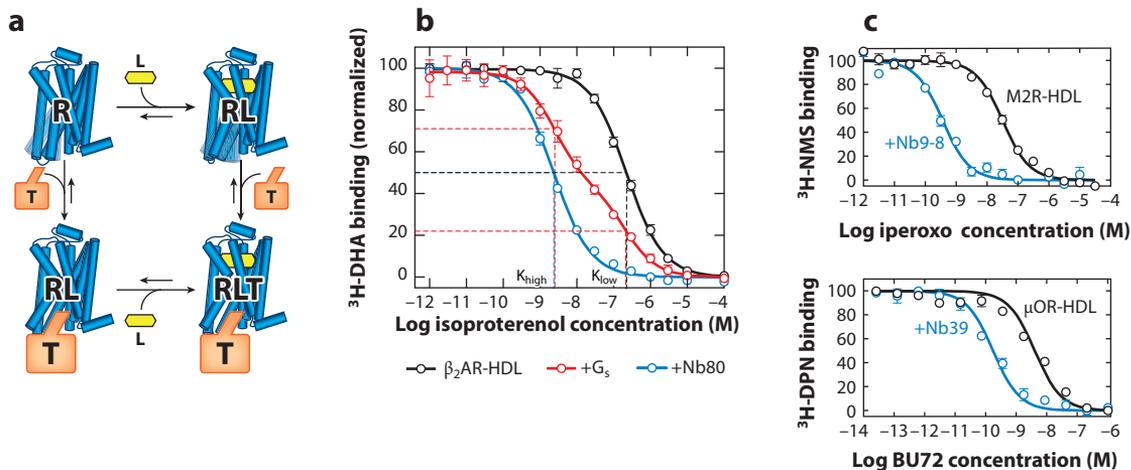


Figure 3

Active state-stabilizing nanobodies recapitulate the G protein-coupled receptor ternary complex model. (a) In the ternary complex model, agonist (L) binding results in increased affinity for the intracellular transducer, most commonly one of the heterotrimeric G proteins. Conversely, binding of a transducer (T) to the receptor (R) induces a reciprocal increase in agonist affinity. (b) The β_2 -adrenergic receptor (β_2 AR) reconstituted into high-density lipoprotein (HDL) particles shows a monophasic competition curve resulting from its affinity for the agonist isoproterenol. Addition of G_s results in a biphasic curve, with a fraction of receptor displaying an increase in agonist affinity induced by the G protein. Nanobody80 (Nb80), an active state-stabilizing nanobody for the β_2 AR, induces a similar high-affinity agonist state. (c) Similar nanobodies were identified subsequently for the M_2 muscarinic receptor (M_2 R) (Nb9-8) and the μ -opioid receptor (μ OR) (Nb39). In each case, active state-stabilizing nanobodies increase the affinity of agonists. Other abbreviations: 3 H-DHA, 3 H-dihydroalprenolol; 3 H-DPN, 3 H-diprenorphine; 3 H-NMS, 3 H-N-methylscopolamine.

In each case, active state-stabilizing nanobodies bind to the intracellular domain of the receptor in a cavity similar to that occupied by the G protein α subunit and arrestin (54) (Figure 4). The nanobody CDRs engage with the intracellular loops of the receptor, and the net binding energy stabilizes the receptor in an active conformation. These structures have revealed several conserved features of activated GPCRs, the most notable being a 9–11-Å outward displacement of transmembrane helix 6 (TM6) accompanied by slight inward movement of TM5 and a rearrangement of the NPxxY motif in TM7 (Figure 4). In each active-state structure, the nanobody stabilizes a conformation of the receptor where two key tyrosine residues, Y^{5.58} and Y^{7.53}, interact via a conserved hydrogen-bonding network mediated by a water molecule (see Reference 111 for a recent update of the generic Ballesteros-Weinstein numbering of GPCR residues). These structures also reveal a recurring rearrangement of a cluster of conserved hydrophobic and aromatic residues including I/L/V^{3.40}, P^{5.50}, and F^{6.44}, called the transmission switch (112), that connects the intracellular nanobody-binding domain to the orthosteric ligand-binding site (Figure 4). Although the overall structural changes are conserved in the intracellular domain, each receptor displays unique modes of agonist recognition in the orthosteric-binding pocket. Activation of the β_2 AR and μ OR is driven by subtle changes in the orthosteric pocket induced by agonists (91, 92). In comparison, activation of the M_2 R by the agonist iperoxo is driven by a dramatic closure of the orthosteric ligand-binding pocket and a pivoting motion of TM6 (94).

Based on the above successes, we anticipate that nanobody-assisted receptor crystallization will continue to offer new insights into GPCR function. Most previous inactive-state GPCR crystal structures have utilized receptor fusions to a highly stable soluble protein that facilitates crystal lattice contacts (23, 24). In the case of the M_2 R, μ OR, and US28, the active state-stabilizing

Ballesteros-Weinstein number:
generic GPCR
transmembrane
residue number in
X.YY format; X is the
helix number and YY
is the residue position
relative to the most
conserved residue in
helix X, designated
X.50

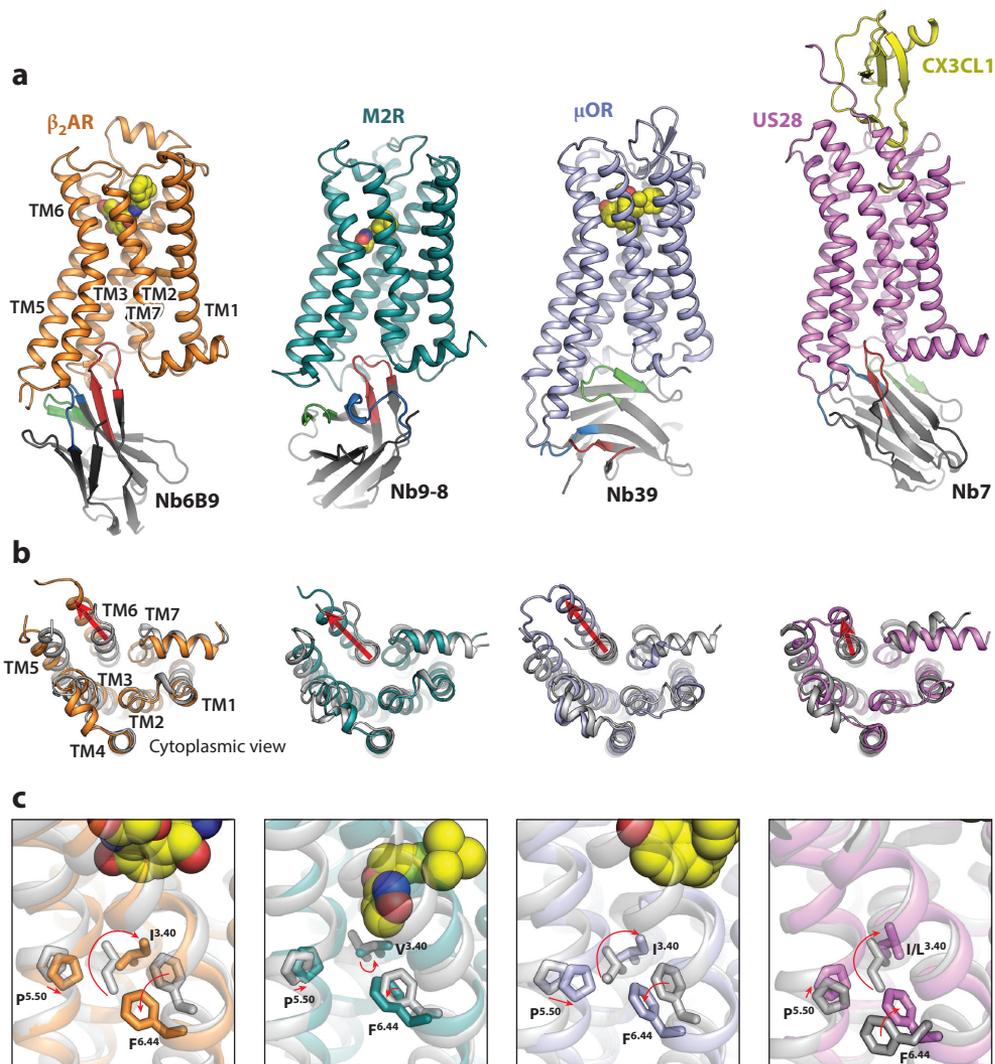


Figure 4

Structural basis of G protein–coupled receptor (GPCR) activation revealed by nanobody–assisted crystallography. (a) Active-state structures of agonist-bound, nanobody-stabilized GPCRs. Each nanobody binds the intracellular surface of the receptor in a unique orientation. Whereas complementarity-determining region 3 (CDR3) (red) most commonly extends into the core of the receptor, Nanobody39 (Nb39) uses framework residues, CDR1, and CDR2 to engage with the μ -opioid receptor (μ OR). (b) Cytoplasmic view comparing inactive [grey; Protein Data Bank (PDB) IDs: 2RH1, 3UON, 4DKL, 4XT1] and active receptors (colored; PDB IDs: 4LDE, 4MQS, 5C1M, 4MBS). Key conserved features include an outward displacement of transmembrane helix 6 (TM6), an inward movement of TM5, and a rearrangement of TM7. (c) Active-state GPCR structures reveal a conserved mode of allostery between the ligand-binding pocket and the cytoplasmic domain. The agonists (yellow spheres) induce conformational changes in the ligand-binding pocket, which are relayed via a conserved set of transmission switch residues (F^{6.44}, P^{5.50}, and I/L^{3.40}) that undergo similar rearrangements upon activation.

nanobody provides sufficient hydrophilic contact surface area to facilitate receptor crystallization, thereby obviating the need for extensive receptor engineering. Unlike stabilization by G proteins, nanobody-based stabilization of GPCRs is not dependent on low-affinity interactions or complicated by nucleotides required for G protein function. As a result, nanobodies provide an efficient means to add hydrophilic lattice contacts simultaneously and to conformationally stabilize biologically interesting GPCR conformations.

GPCR Dynamics Revealed by Nanobodies

Multiple lines of pharmacological, biochemical, and spectroscopic evidence suggest that GPCRs adopt a multitude of conformations that interchange rapidly on timescales ranging from microseconds to hundreds of milliseconds. Determining how ligands alter this conformational landscape will be critical to a full understanding of GPCR allostery. Whereas crystallographic studies provide a high-resolution view of distinct conformations within this landscape, they provide static descriptions of highly dynamic behavior. Several recent spectroscopic studies have started to examine the complex conformational landscape of GPCR function. Early fluorescence studies established that different ligands induce unique conformations of the β_2 AR (113, 114). Ligand-specific conformations of β_2 AR have been examined subsequently by more sophisticated spectroscopic techniques, including single-molecule fluorescence spectroscopy (50, 51), ^{13}C -dimethyllysine spectroscopy (115), ^{19}F -NMR spectroscopy (47, 116), and ^{13}C - ϵ -methionine NMR spectroscopy (117). These studies have established clearly that different agonists can induce unique spectroscopic signatures in the β_2 AR, further supporting the hypothesis that a given GPCR can adopt unique conformations in the presence of different agonists.

As GPCRs are likely highly dynamic in both the presence and absence of agonists, spectroscopic studies of the receptor alone do not allow precise correlation of spectroscopic signals to specific structural states. Agonists may increase the population of the active state but rarely allow sufficient conformational stabilization to uniquely assign a given signal to the conformation observed by crystallographic study. Our groups and others have therefore utilized active state-stabilizing nanobodies to assign spectroscopic signals to specific receptor conformations. Using Nb80, we demonstrated previously that the β_2 AR achieves the crystallographically observed conformation only in the presence of both a high-affinity agonist and the nanobody. These experiments were performed using several complementary techniques, including ^{13}C - ϵ -methionine NMR (48), ^{19}F -NMR (49, 118, 119), and DEER spectroscopy (49), with additional evidence provided by molecular dynamics simulations (105). In each case, addition of Nb80 provided a useful control to determine signals originating from a fully active β_2 AR. As anticipated, both NMR and DEER experiments revealed that binding of Nb80 stabilizes a single active conformation with minimal residual β_2 AR dynamics. A similar approach has even been applied successfully to assess the fully active conformation of a turkey β_1 AR mutant thermostabilized in the inactive conformation (45). In this case, addition of Nb80 provided sufficient binding energy to overcome inactive-state conformational stabilization, and the authors were able to use backbone signals obtained in the presence of Nb80 to examine the active conformation of the turkey β_1 AR. In a more extreme example, we observed that ^{13}C -dimethyllysine spectroscopy of the μ OR revealed minimal changes in the cytoplasmic domain with agonists alone (46). Conformational changes associated with receptor activation were observed only in the presence of a μ OR active state-stabilizing nanobody, Nb33. The last example highlights the necessity of assigning fully active states with either signaling proteins or active state-stabilizing nanobodies during spectroscopic studies (Figure 5). In the absence of such controls, it remains challenging, if not impossible, to fully assess the dynamic range of the receptor in a spectroscopic experiment.

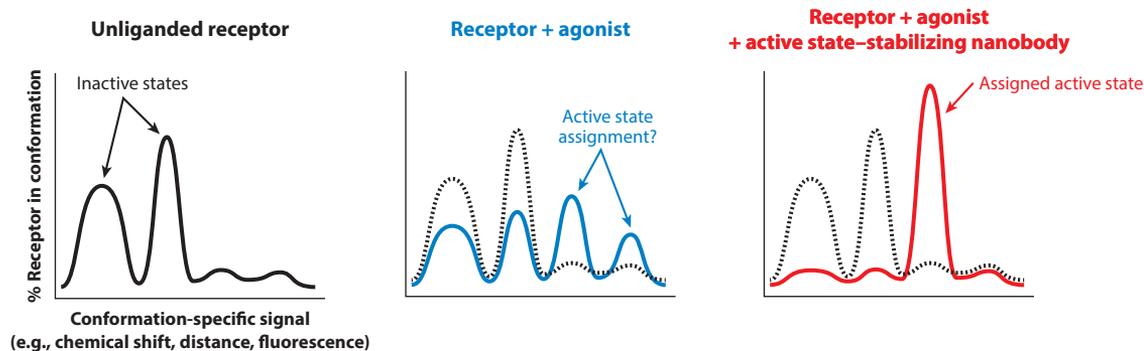


Figure 5

Nanobody-based assignment of G protein-coupled receptor conformational states. In many spectroscopic experiments, distinct signals arise from unique conformations of a given receptor. In the absence of ligand, multiple signals reflect conformational heterogeneity. Addition of agonists is associated with increased conformational heterogeneity and the presence of new signals arising from a greater number of conformations. Assignment of a specific signal to a crystallographically observed conformation or a pharmacologically characterized state is challenging (*middle panel*). Active state-stabilizing nanobodies can conformationally stabilize the receptor, thereby allowing clear assignment of signals and further interpretation of spectroscopic data.

STABILIZATION OF GPCR SIGNALING COMPLEXES

Active state-stabilizing nanobodies have revealed several conserved features of GPCR activation. However, the most extensive insight into receptor-mediated activation of G protein signaling required a structure of a GPCR bound to its cognate G protein. The structure of the agonist-bound $\beta_2\text{AR}\cdot\text{G}_s$ complex was instrumental in understanding the cooperative interactions across the membrane between agonist, receptor, and G protein that cause agonist binding at the extracellular side to activate the cytosolic G_α subunit (90). Because of the difficulty in stabilizing multiple conformationally dynamic proteins, each requiring disparate biochemical conditions for stability, the crystallization of this complex necessitated a highly multidisciplinary approach (61). Several inventive steps were needed, including biochemical optimization for purification of a nucleotide-free complex, identification of an ultrahigh-affinity agonist, and development of new detergents (30) and mesophase lipids (120), as well as new GPCR protein engineering (121). Despite these innovations, single-particle electron microscopy data of the purified complex suggested that the α -helical domain of the G_α subunit is conformationally heterogeneous in the complex (53).

To compensate for this heterogeneity, we attempted to identify nanobodies that would rigidify the $\beta_2\text{AR}\cdot\text{G}_s$ complex. Llamas were immunized with the agonist-bound $\beta_2\text{AR}\cdot\text{G}_s$ complex cross-linked with bis(sulfosuccinimidyl)glutarate (72). Nanobodies were selected for binding to the nucleotide-free complex reconstituted into high-density lipoprotein particles (also known as Nanodiscs). Panning identified several binders, and two nanobodies called Nb35 and Nb37 were selected for their ability to selectively bind the $\beta_2\text{AR}\cdot\text{G}_s$ complex but not the receptor alone. Most importantly, Nb35 and Nb37 prevented dissociation of the nucleotide-free complex by the nonhydrolyzable GTP analog $\text{GTP}\gamma\text{S}$ (90). With the use of Nb37, electron microscopy of the $\beta_2\text{AR}\cdot\text{G}_s$ complex revealed the conformational flexibility of the G_α α -helical domain more convincingly (53). Addition of Nb35 to the T4L- $\beta_2\text{AR}\cdot\text{G}_s$ complex resulted in a marked improvement in crystallogensis. The resulting crystals diffracted to 2.9 Å and yielded the structure of the first GPCR-G protein complex (90). In the final refined structure, Nb35 bound at the interface of the G_α and the G_β subunits, thereby stabilizing the two domains. The nanobody also provided key lattice contacts to adjacent repeating units.

NANOBODIES ILLUMINATE GPCR FUNCTION IN LIVING CELLS

As highlighted in the sections above, structural and biophysical studies over the past 15 years have yielded significant insight into the molecular details of GPCR function. Indeed, researchers have determined over 100 crystal structures of 30 unique GPCRs in complex with small molecule ligands, peptides, or antibodies (21). Recent efforts have elucidated the first structures of GPCRs in complex with important intracellular signaling proteins (52, 54, 90). Accompanying these structures are numerous biophysical, biochemical, and pharmacological studies that have examined the complexity of GPCR signaling. More in-depth understanding of GPCR signaling, however, requires correlation of these molecular and structural insights to the function of receptors in native living systems. Because nanobodies can be functionally expressed in the cytoplasm of eukaryotic cells (122, 123), recent studies have utilized active state–stabilizing nanobodies to probe conformational changes in receptors within living cells to correlate structure with signaling more fully. In the first example of such an approach, Irannejad and coworkers (124) fused Nb80 to green fluorescent protein (GFP) to follow the activation of the β_2 AR by the agonist isoproterenol. The same study also utilized a Nb37-GFP fusion, which recognizes nucleotide-free G_s in the agonist-bound β_2 AR- G_s signaling complex (53), to probe the activation of the β_2 AR and its cognate G protein directly (124). By fusing Nb80 or Nb37 to GFP and expressing it as a genetically encoded intrabody at a suitably low concentration in the cytoplasm, the authors could show that isoproterenol promotes receptor and G protein activation in the plasma membrane. Intriguingly, the same reporters also demonstrated that β_2 AR can activate G proteins in the early endosome membrane. Thus, internalized receptors can apparently also contribute to the overall cellular cyclic AMP response within several minutes after agonist application.

In a similar set of studies, nanobodies that bind selectively to active (agonist-bound) or inactive (antagonist-bound) β_2 AR were generated and characterized (125). When expressed as intrabodies, these nanobodies inhibit G protein activation, GRK-mediated receptor phosphorylation, β -arrestin recruitment, and receptor internalization to varying extents. These functional effects were likely due to either steric blockade of downstream effector interactions or stabilization of specific receptor conformations that do not support effector coupling. In addition to imaging applications, intrabodies may provide novel ways of modulating GPCR signaling with exquisite selectivity for a given receptor and signaling pathway. As more conformation-selective nanobodies become available that are specific for other GPCRs, G proteins, or arrestin, they will likely find broad utility in such applications examining GPCR signaling. Ultimately, these nanobodies may be useful in imaging and modulating GPCR signaling within transgenic animals, thereby allowing correlation of structure and function at the whole-organism level.

THERAPEUTIC TARGETING OF GPCRs USING NANOBODIES

As discussed above, nanobodies are highly useful tools to understand GPCR signaling. Several studies have also demonstrated that nanobodies directed against GPCRs may have therapeutic potential. For example, picomolar-affinity nanobodies have been identified that inhibit CXCR4-mediated signaling competitively and antagonize the chemoattractant effect of the CXCR4 agonist CXCL12 (126). These nanobodies bind extracellular loops of human CXCR4 and display strong antiretroviral activity against T cell-tropic and dual-tropic HIV-1 strains. Additionally, these antibodies induce stem cell mobilization *in vivo*, perhaps providing utility as alternatives for currently approved small-molecule drugs such as plerixafor. Antagonist nanobodies that bind other chemokine receptors, including CXCR2 or CXCR7, have also been described (127, 128). As nanobodies are only approximately 15 kDa, they may provide better penetration into tumors, the central nervous system, or other bodily compartments difficult to access by conventional

antibodies. Depending on the desired indication, half-life extension methods such as PEGylation (129) or fusion to serum albumin (130) could be used for tailoring the half-life of nanobodies and increase their therapeutic window depending on the clinical indication. Also, their small size (gene and domain) and monomeric and soluble behavior render single-domain antibodies ideal building blocks to generate multivalent or multispecific constructs with subsequent fusion to an Fc, enzyme, or toxin (131). Finally, nanobodies that constrain GPCRs in specific, biologically relevant conformations may enable the binding of small-molecule fragments that would otherwise not be able to gain a foothold on the flexible target (132).

SUMMARY POINTS

1. Camelid heavy-chain-only antibody fragments (nanobodies) show promise as tools for stabilizing discrete GPCR conformations and as chaperones for crystallogensis.
2. Several structures of agonist-bound GPCRs in the active state have been obtained by nanobody-assisted X-ray crystallography. These structures provide insight into ligand-induced receptor activation.
3. Nanobodies were instrumental in solving the structure of the agonist-bound $\beta_2\text{AR}\cdot\text{G}_s$ transmembrane signaling complex.
4. Most nanobodies can be functionally expressed as genetically encoded intrabodies within a eukaryotic cell. This makes nanobodies ideal tools to correlate structural or dynamic features observed *in vitro* by biophysical measurements with functional observations from living cells.
5. Characterizing GPCR conformational states may facilitate the development of more selective drugs capable of modulating a specific signaling pathway, thereby improving therapeutic activity and minimizing undesirable side effects.

FUTURE ISSUES

1. GPCRs signal through multiple intracellular pathways, and some ligands can induce selective signaling down one pathway. It remains to be seen whether nanobodies that stabilize ligand-specific receptor conformations can be identified.
2. Nanobodies stabilizing the $\beta_2\text{AR}\cdot\text{G}_s$ complex have provided highly useful tools for structural studies as well as imaging GPCR signaling. Nanobody-based stabilization of GPCR complexes with other G proteins, GRKs, or β -arrestin would be valuable to study the structural and functional features of GPCR transmembrane signaling.
3. Evidence has accumulated that GPCRs can form dimers or multimers. Nanobodies that bind monomers or multimers selectively would allow investigation of oligomerization and its effects on ligand recognition, receptor signaling, and intracellular trafficking.
4. Allosteric, the coupling between ligand binding and protein conformational change, is key to the function and pharmacology of many drug targets. Nanobodies that constrain target proteins in specific, biologically relevant conformations may ultimately prove to be of great value to the pharmaceutical industry as next-generation biologicals or as tools that enable the discovery of conformer-selective drugs.

DISCLOSURE STATEMENT

A.M. is a founder of Ab Initio Biotherapeutics. B.K.K. serves as an advisor for Ab Initio Biotherapeutics and is a founder of ConfoRx. J.S. is a founder of Confo Therapeutics.

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