

# Chemokine receptors and other G protein-coupled receptors

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## Purpose of review

Class A G protein-coupled receptors (GPCRs), including the chemokine receptors, CCR5 and CXCR4, share a seven transmembrane-spanning  $\alpha$ -helix architecture that accommodates signal propagation from across biological membranes. CXCR4 and CCR5 are utilized as co-receptors during HIV viral entry and, therefore, crystal structures of GPCRs aid in the understanding of their function in viral entry.

## Recent findings

Recent progress in structure determination of class A GPCRs, which include vertebrate and invertebrate rhodopsin as well as adrenergic and adenosine receptors, provides molecular templates for how this diverse group of transmembrane receptors functions. Each of these GPCRs differs in how specific ligands bind to the transmembrane core, underscoring that additional structures of GPCRs from other subfamilies are needed to facilitate rational drug design. More recent studies also indicate a need to consider the more complex character of GPCRs, such as their oligomerization and dynamics.

## Summary

Recently, the atomic structures of invertebrate rhodopsin,  $\beta_1$ -adrenergic and  $\beta_2$ -adrenergic receptors and the  $A_{2A}$ -adenosine receptor have been solved via X-ray crystallography. The impact that these structures have on the biochemistry of viral entry and signal transduction is discussed. Because the chemokine receptors have proven refractory to structural studies thus far, further structural study of the chemokine receptors will be essential to understanding ligand binding, activation and function as co-receptors during viral entry.

## Keywords

CCR5 and CXCR4, G protein-coupled receptor, GPCR, membrane protein crystallography, rhodopsin

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## Introduction

G protein-coupled receptors (GPCRs) represent approximately 3% of the genes in the human genome and are responsible for the senses of sight, smell, and taste as well as chemotaxis, blood pressure, and regulation of metabolism [1]. Being the largest group of transmembrane cellular receptors and having the ability to transduce signal across the plasma membrane, the GPCR superfamily represents an ideal drug target and approximately 50% of all nonantimicrobial therapeutics currently in use target GPCRs or GPCR-mediated pathways [2]. In short, GPCRs are activated by the binding of an agonist, which can be a small molecule, a peptide, an entire protein, or even a photon of light. This binding event initiates structural changes distal to the binding site on its cytoplasmic loops and tail, which bind to and catalyze the exchange of GDP on the  $\alpha$  subunit of the heterotrimeric G protein [3] (Fig. 1a). The  $\alpha$  and  $\beta\gamma$  subunits then dissociate and bind

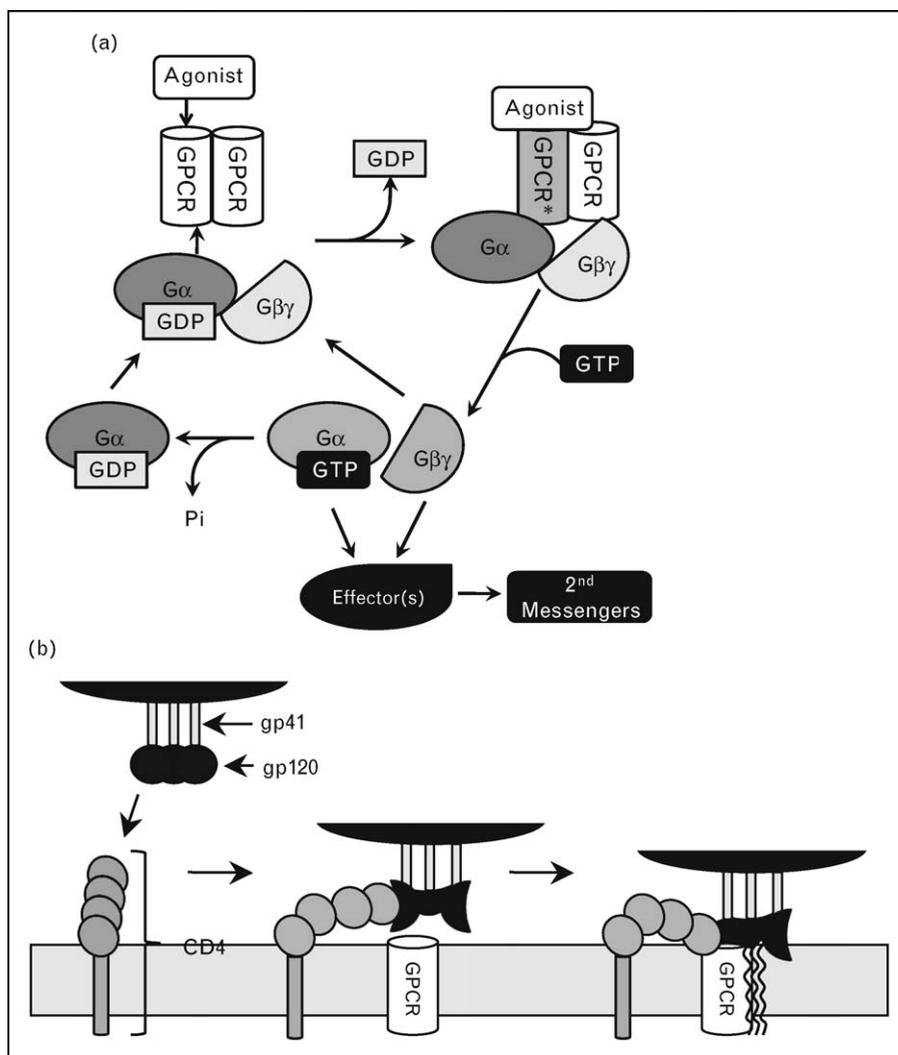
to cellular effector enzymes, which act to produce second messenger molecules such as cAMP or  $IP_3$ , which activate other cellular enzymes, ultimately leading to a cellular response. A further layer of complexity in the activation process is added by heterodimerization and homodimerization of GPCRs [4]. Many studies utilizing fluorescence and bioluminescence techniques as well as cross-linking data appear to suggest that GPCRs exist as dimers or higher-order oligomers [5]. The chemokine receptors have been shown to function as both homodimers and heterodimers, and dimerization has been shown to affect viral entry [6–8]. Although some studies indicate that a GPCR monomer is sufficient to activate G protein [9,10], the footprint of the regions in heterotrimeric G proteins shown to interact with GPCRs is larger than the footprint of a GPCR monomer, and atomic force microscopy images of intact rod outer segments reveal that rhodopsin exists as ordered arrays of dimers arranged as rows of higher-order oligomers [11,12].

## G protein-coupled receptors in HIV infection

The chemokine family of GPCRs are expressed in the membranes of leukocytes, and the differences in the expression patterns among specific subtypes of leukocytes have been shown to be the basis for tropism of HIV-1 subtypes [13]. These subtypes utilize CCR5 (R5), CXCR4 (X4), or both (R5X4) as coreceptor during the infection process. However, the primary route of infection is through the CCR5 receptor, whereas progression to development of AIDS often is associated with the

emergence of viruses that can utilize CXCR4. In the large majority of cases, X4 tropism is undetectable until later stages of infection. A mutation within the CCR5 receptor results in a nonfunctional truncated GPCR (CCR5- $\Delta$ 32) that is not expressed on the cell membrane [14]. Persons expressing one of these mutant alleles often show delayed progression of disease, whereas those who are homozygous for the mutant allele are nearly completely resistant to infection. Thus, the CCR5 receptor represents a viable route for the inhibition of viral entry.

**Figure 1 Schematic representation of how G protein-coupled receptors function in signal transduction and HIV-1 viral entry**



(a) The G protein-coupled receptor (GPCR): heterotrimeric G protein signaling mechanism. Upon agonist binding to the receptor, the GPCR undergoes a structural change, freeing it to form a ternary complex with a heterotrimeric G protein. Formation of the ternary complex induces the release of the bound GDP from the  $\alpha$  subunit and a GTP molecule binds to the empty nucleotide-binding site. This event causes the  $\alpha$  and  $\beta\gamma$  subunits to dissociate and each is free to then bind to cellular effector enzymes such as adenylyl cyclase or phospholipases. These effectors produce second messenger compounds that activate downstream signaling proteins, ultimately resulting in a cellular response. Hydrolysis of the bound GTP on the  $\alpha$  subunit results in inactivation and this GDP-bound  $\alpha$  binds to a free  $\beta\gamma$  subunit, thus reforming the inactive heterotrimer. (b) GPCRs as co-receptors for viral entry. Trimers of the Env protein are expressed upon the surface of the viral envelope. The gp120 subunit of Env binds to the CD4 cellular receptor on a leukocyte. This event serves to tether the virus to the membrane and induces structural changes within gp120, which makes it possible to bind to the chemokine (CXCR4 or CCR5) GPCRs. Upon GPCR binding, the gp41 subunit undergoes structural changes and inserts helices into the membrane, initializing fusion of the viral membrane with the cellular membrane.

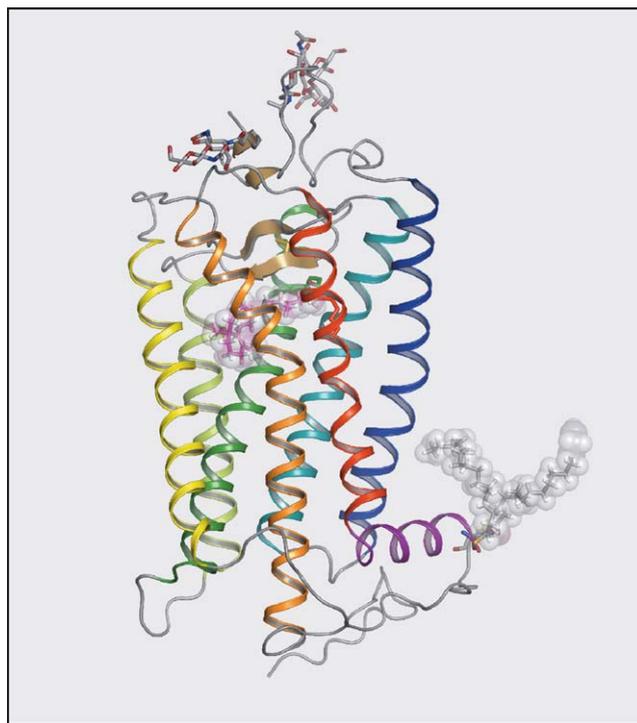
In fact, several viral entry inhibitors (maraviroc and viciroc), which are antagonists to the CCR5 receptor, are in clinical use or in clinical trials [15]. These compounds can block HIV replication but ultimately resistance develops, both as a consequence of emergence of viruses that can utilize the CXCR4 coreceptor but more interestingly by development of the ability to bind to antagonist-bound CCR5 receptor or by binding to an alternate portion of the receptor. Small-molecule ligands of CXCR4 have also been developed, but have not been successful therapeutics because of severe side-effects [13,16]. Although the chemokine receptors physiologically work to mediate proinflammatory signals, in the case of HIV infection they have been subverted into providing a docking site for the HIV virus during viral entry and membrane fusion [13]. In short, this process begins with gp120 protein binding the CD4 cell surface receptor (Fig. 1b). This binding exposes the hypervariable V3 loop, freeing it to interact with CXCR4 or CCR5, and differences in this loop largely form the basis for viral tropism [17,18]. This binding induces structural changes within the gp41 subunit, initiating the membrane fusion process where HIV and cell membrane fusion occurs.

### Structural studies of G protein-coupled receptors and membrane protein crystallization

The crystallization of integral membrane proteins is a difficult task; of the almost 55 000 structures deposited in the Protein Data Bank, less than 1% are membrane proteins, and of that 1% only approximately 50 structures of mammalian membrane proteins have been solved [19]. Membrane protein structure determination is especially complicated by low expression amounts and difficulties in protein crystallization. In the case of rhodopsin, native protein is easily purified from the outer segments of the rod cells of the retina. Most membrane proteins cannot be simply purified from natural tissue and must be produced in heterologous expression systems such as mammalian cell culture, baculovirus-infected insect cells, or in yeast. Several novel expression methods have been proposed for production of GPCRs, including the transgenic expression of GPCRs within the retinas of mice or *Xenopus laevis*, in-vitro transcription/translation or by directed evolution in *Escherichia coli* [20–23].

Once the difficulties of membrane protein expression are addressed, crystallization conditions must be determined. In developing crystallization conditions for a membrane protein, additional crystallization reagents must be screened including types of detergent, inclusion of lipids, and even the methodology for protein crystallization. Often, the effects of small-molecule additives must also be screened to achieve well diffracting protein crystals [24]. In the determination of the photoactivated

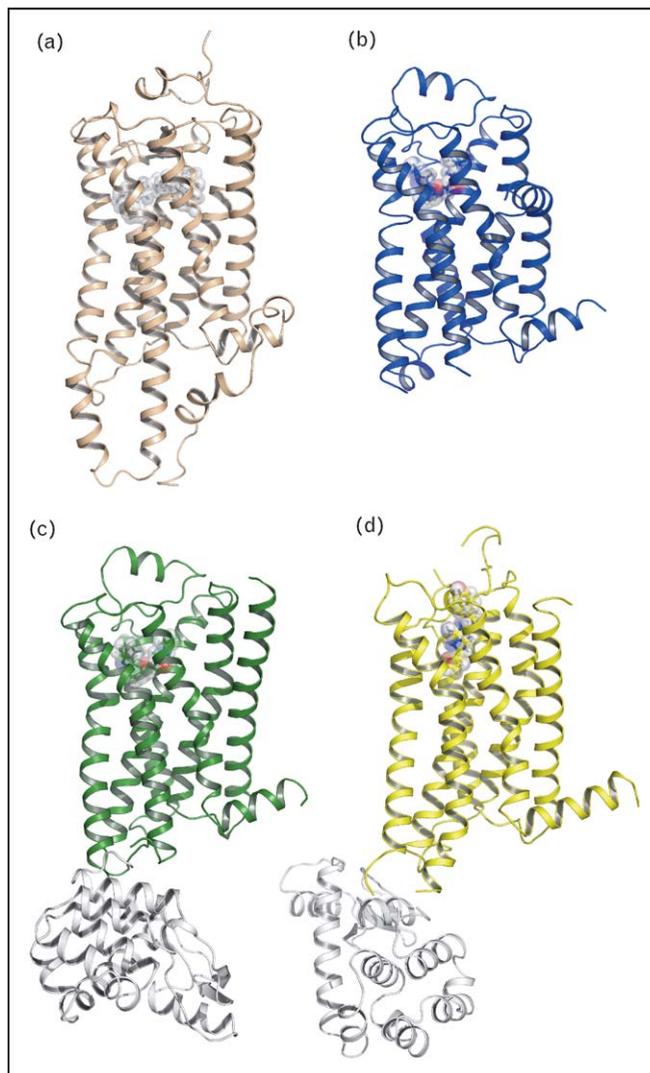
**Figure 2** Atomic structure of rhodopsin, a prototypical G protein-coupled receptor



Helices are colored according to their primary sequence: helix-I, blue; helix-II, blue-green; helix-III, green; helix-IV, lime-green; helix-V, yellow; helix-VI, orange; helix-VII, red; cytoplasmic helix-8, purple. Chromophore is colored as transparent hot-pink surface. The initial crystal structure revealed the topology of these individual helices and the conformation of the chromophore allowing a structural understanding of previously determined biochemical and biophysical studies of rhodopsin. Later improvements in resolution revealed the presence of ordered waters within the transmembrane region as well as the complete polypeptide chain. The region above the receptor is the extracellular face and below is the cytoplasmic (G protein interacting) face (PDB ID:1U19).

structure of rhodopsin, optimization of the rhodopsin crystals was critical in the success of the structure determination [25].

The structures of several GPCRs have been determined via X-ray crystallography. These include rhodopsin (Fig. 2), the  $\beta_1$ -adrenergic and  $\beta_2$ -adrenergic receptors, and most recently, the structure of the  $A_{2A}$  adenosine receptor [26,27–29] (Fig. 3). However, no structure exists for any of the chemokine receptors. Several shared motifs within GPCRs are conserved with high homology even among very distantly related GPCRs [30]. These include the ‘ionic lock’ (D/E)RY and NPXXY motifs that serve to constrain the GPCR in an inactive orientation until agonist binding [31,32]. In an inactive receptor, the Arg of the (D/E)RY motif that is located at the beginning of the second cytoplasmic loop makes a salt bridge with an Asp or Glu residue at the end of the third cytoplasmic loop, thus constraining the cytoplasmic loops in an inactive conformation. Upon activation, this motif becomes disrupted and

**Figure 3 Representative structures of several G protein-coupled receptors**

All structures are in either the ground (inactive) state or have inverse agonist bound. (a) The 2.6 Å structure of invertebrate (squid) rhodopsin (PDB ID:2Z73). (b) The 2.8 Å structure of the  $\beta_1$ -adrenergic receptor (PDB ID:2VT4). (c) The 2.4 Å structure of the T4 lysozyme- $\beta_2$ -adrenergic receptor fusion (PDB ID:2RH1). (d) The 2.6 Å structure of the T4 lysozyme- $A_{2A}$  adenosine receptor (PDB ID:3EML). Although each of these receptors has evolved to bind different ligands and G proteins, the overall seven transmembrane helix architecture and topology have been retained. All GPCRs are shown in the same orientation as rhodopsin in Fig. 1.

the cytoplasmic loops are freed to interact with and trigger nucleotide exchange in heterotrimeric G protein  $\alpha$  subunits, which ultimately results in a cellular response.

### Rhodopsin crystal structures

Bovine rhodopsin was the first GPCR to have its atomic structure solved and the 2.2 Å structure of rhodopsin remains the highest resolution structure of any GPCR solved to date (Fig. 2). In fact, there are almost 20 rhodopsin structures within the Protein Data Bank, including nine ground state structures, two structures of early photointermediates, photoactivated rhodopsin, two invertebrate (squid) rhodopsin structures (Fig. 3a),

and most recently two structures of the inactive apoprotein opsin, the end product of the phototransduction cascade [26,33–35,36<sup>••</sup>,37–39]. The structures of rhodopsin allowed a framework upon which the large volume of biochemical and biophysical data on rhodopsin and rhodopsin activation could be viewed [40]. It should be noted that rhodopsin remains the only GPCR that has been crystallized and solved in its native state, as all of the other GPCR structures solved to date have required extensive modifications to the primary amino acid sequence in order to yield diffracting crystals. Furthermore, the rhodopsin structure or a derivative thereof has served as a molecular replacement model for solving all GPCR structures to date.

Structures for squid rhodopsin have also been determined [33,36<sup>••</sup>]. Although this GPCR binds to  $G_q$  rather than  $G_t$  heterotrimeric G proteins and does not require hydrolysis of chromophore for regeneration, the topology and transmembrane regions are quite similar to rhodopsin as well as to the other GPCR structures. The notable difference seen in the squid structures is a large extension of the C-III loop, which must likely move in order for heterotrimeric G protein to bind and nucleotide exchange to occur. Crystallization of squid rhodopsin required proteolysis to remove a large C-terminal domain from protein purified from squid retina.

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### Recent G protein-coupled receptor structures and strategies for structure determination

The structure determination of the  $\beta_2$ -adrenergic receptor (Fig. 3c) required extensive study and protein engineering in order to yield well diffracting crystals. Initial studies utilizing an antibody fragment in complex with receptor diffracted poorly and failed to yield a structure for the entire receptor [41,42]. Through a painstaking process, a series of truncations were introduced within the third cytoplasmic loop of the receptor and replaced with the amino acid sequence for T4 lysozyme [28<sup>••</sup>,43]. Further mutations were also made to increase the amount of protein produced in cell culture. These mutant receptors were expressed in insect cell culture and tested to ascertain that they bound agonists and antagonists at binding affinities similar to those of wild-type protein. They were purified and crystallized utilizing a modification of the lipid cubic phase crystallization methodology in which protein is reconstituted into a lipid matrix and protein crystals grow within this matrix [44,45]. This process culminated in the determination of the structure of the  $\beta_2$ -adrenergic receptor in complex with the inverse agonist, carazolol bound. This methodology has also proven to be successful in the determination of the 2.6 Å structure of the  $A_{2A}$  adenosine receptor (Fig. 3d) in complex with an inverse agonist as well as an additional  $\beta_2$ -adrenergic receptor structure [29<sup>••</sup>,46]. In all of these structures, the (D/E)RY motif has been disrupted because of interactions with the T4 lysozyme in the third cytoplasmic loop.

The determination of the structure of the  $\beta_1$ -adrenergic receptor (Fig. 3b) also required a large amount of mutational analysis and protein engineering, although their approach was quite different [27<sup>••</sup>]. The authors chose to utilize the thermal stability of their mutant receptors as a read-out of their stability and thus crystallizability [47]. A series of mutations in the turkey  $\beta_1$ -adrenergic receptor were designed utilizing a homology model based on the rhodopsin crystal structures, which would likely result in higher thermal stability. These mutants were expressed in insect cell culture, assayed for ligand binding, and

ultimately resulted in the determination of the 2.7 Å structure in complex with the antagonist, cyanopindolol.

The determination of these recent structures required two technological developments: the use of microfluidic/small volume crystallization technology and microfocused X-ray beam lines. Microfluidic technology allows crystallization of protein utilizing a fraction of the protein needed for traditional screening techniques [48,49]. Microfocused beamlines provide the highly focused and collimated X-rays, which are necessary to collect data from tiny crystals that cannot be collected at a traditional synchrotron source [50,51]. The use of a microfocus beamline was critical in the structure determination of the recent adrenergic and adenosine receptors.

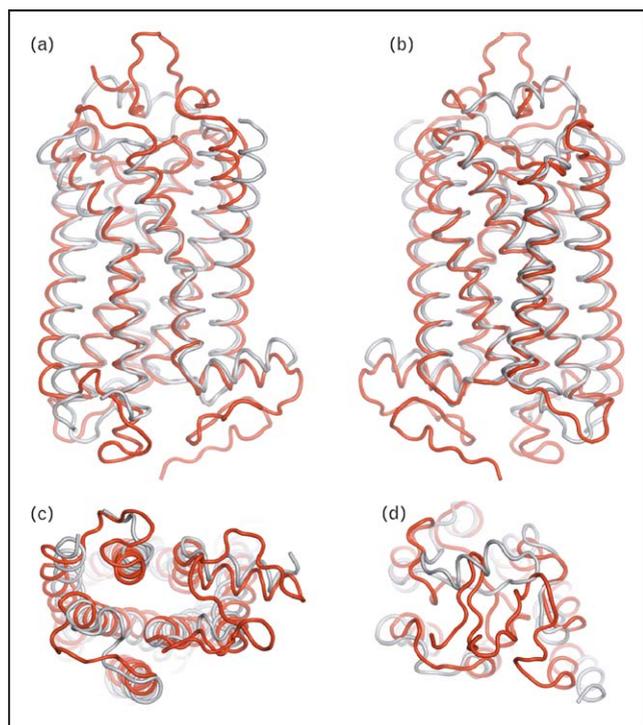
Although no structure of an intact CCR5 receptor has been determined, some informative structural data exist. Sulfation of exposed tyrosine residues on the extracellular face of cytokine receptors has been shown to be necessary for ligand binding as well as for viral entry [52]. A structure of an antibody that contained a sulfated tyrosine in complex with gp120 was solved [53<sup>••</sup>]. Although the antibody has no homology with the N terminus of CCR5, the insertion of a sulfotyrosine from the antibody into a binding pocket on gp120 is probably similar to the mode of gp120 binding sulfotyrosines on the CXCR4 and CCR5 receptors; NMR and molecular docking studies appear to confirm this. Structures of neutralizing antibodies bound to gp120 may also recapitulate some aspects of the receptor-binding site as well [54].

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### Homology of G protein-coupled receptors

Structures of rhodopsin and other GPCRs present a valuable template for the production of homology models of other GPCRs [55]. In examining the structures of GPCRs of known structure, it is apparent that their structural homology is high; they share similar arrangements of their transmembrane helices and topology, and even the positions of several water molecules bound within the transmembrane region (Figs 3 and 4). However, with the exception of functionally important conserved motifs, the level of sequence homology is relatively low [30]. In all of the GPCR structures solved to date, there are significant similarities in the location of the ligand-binding sites, but there are distinct differences in the residues that interact with ligand. In the  $\beta_1$ -adrenergic and  $\beta_2$ -adrenergic receptors that both bind epinephrine, but have different affinities for norepinephrine, the residues that line the ligand-binding pocket are identical, revealing that residues that do not directly interact with the ligand-binding site may play a role in ligand selectivity. In the case of the chemokine

**Figure 4 Structural similarities between ground state rhodopsin and the  $\beta_2$ -adrenergic receptor**



Rhodopsin is represented depicted in red and the  $\beta_2$ -adrenergic receptor depicted in light grey. The T4 lysozyme fusion has been removed from  $\beta_2$ -adrenergic receptor for clarity. The high degree of structural similarity within the transmembrane regions of the two receptors is very high and root-mean-squared deviations this region are only 3.3 Å. (a and b) Two views of the receptors as viewed in the plane of the plasma membrane. The cytoplasmic loops (c) follow different routes in each receptor and the C-terminal tail is not observed in the adrenergic receptor structure. The extracellular face (d) is also significantly different as this face of the receptor has different functions in each receptor; a diffusible hydrophilic ligand must enter adrenergic receptor, whereas the region surrounding the chromophore has evolved to shield it from the extracellular environment.

receptors, and all other class A GPCRs, the ligand-binding site is located close to the extracellular face, and thus the signal arising from ligand binding must be transferred across the plasma membrane to G protein.

### Homology modeling and ligand binding

Several studies have shown reasonable hypothetical models based on the rhodopsin structural backbone and biochemical data for the binding of chemokine receptor as well as CCR5 in complex with small-molecule antagonists, which have been shown to impair viral entry [56]. One approach for improving homology models has been the coupling of site-directed mutations within CCR5 and CXCR4 and their effects on ligand binding [57,58]. Further work has extended CXCR4 and CCR5 homology models as targets for virtual ligand screening of therapeutics, but docking ligands against homology

models relies on the accuracy of the homology model and were only partially able to identify compounds that are known to bind receptor [59,60]. Now that several GPCR structures have been determined, these structures can be utilized to further improve upon these homology models derived from the rhodopsin structure. However, given the large amount of structural similarities seen between the adrenergic receptors and rhodopsin, it is likely that more closely related GPCRs would make better templates. Although these homology models can suggest how CXCR4 or CCR5 may function or how therapeutics bind to the receptor, these models are unlikely to be as useful for the *de novo* design of therapeutic agents or the understanding of the fundamental basis of the gp120 and co-receptor interactions as actual crystal structures of CXCR4 or CCR5.

### Conclusion

In order to fully understand the functions of co-receptor in the viral entry process, further structural studies are needed. Structures of any GPCR significantly increase our understanding of GPCR activation, G protein coupling, and ligand binding. Structures of CXCR4, CCR5, and other cytokine receptors are needed to fully understand the role that these GPCRs play in viral entry as well as in signal transduction. Apart from the structures of the receptors themselves, structures of the receptor interacting with gp120 or fragments containing the V3 loops are needed to understand the structural basis of viral tropism and would aid in the understanding of the roles that these GPCRs play in viral fusion. The use of techniques and technologies that allow production of GPCRs in cell culture, the screening of thousands of crystallization conditions with only a small amount of protein, and use of microfocus X-ray sources represent promising methodologies for determining GPCR structure and allowing a structural understanding of the large volume of biochemistry concerning cytokine receptors and their role in viral entry.

### Acknowledgements

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- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 163).

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