CHAPTER FIVE

The Role of Hydrophobic Amino Acids in the Structure and Function of the Rhodopsin Family of G Protein-Coupled Receptors

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Contents

Abstract

Recent advances in crystallization methods have permitted to resolve the molecular structure of several members of the rhodopsin family of G protein-coupled receptors (GPCRs). Comparison among these structures revealed a number of conserved polar and charged residues implicated in the receptor transduction pathways. These residues function as micro-switches in the process of receptor activation and has been the object of study of many research groups. However, hydrophobic forces, usually underappreciated, also play a major role in GPCR function. Conserved hydrophobic residues contribute significantly to receptor activation, G protein coupling, and oligomerization

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processes. This review focuses on the impact of the hydrophobic amino acids observed in the structure of class A GPCRs necessary for their function. This information represents a fundamental piece to complete a holistic view of the GPCR signal transduction machinery.

1. INTRODUCTION

Cells are able to detect and respond to chemical signals present in their external environments. These actions are regulated by different classes of plasma membrane proteins, being the superfamily of G protein-coupled receptors (GPCRs) one of the largest and most studied. GPCRs are present in almost every eukaryotic organism, including fungi and plants. They are highly diversified in mammalian genomes with current estimates of about 1000 genes (2–3% of the human proteome; Fredriksson & Schioth, 2005). Based on sequence similarity methods, GPCRs are classified into three major families or classes (A, B, and C) (Vroling et al., 2011). GPCRs transduce external signals such diverse as photons, odors, pheromones, biogenic amines, neuro-peptides, proteases, glycoprotein hormones, and ions, among others, into the interior of the cell. The response is operated through second messenger cascades controlled by different heterotrimeric guanine nucleotide-binding proteins (G proteins) coupled at their intracellular (ICL) regions (Oldham & Hamm, 2008). However, increasing evidence indicates that GPCRs may also signal through G protein-independent pathways (Sun, McGarrigle, & Huang, 2007). Considering the vast amount of cellular processes regulated by the GPCRs system (Smit et al., 2007), it constitutes one of the most important pharmaceutical targets, as around 30% of marketed drugs act through this superfamily of proteins (Imming, Sinning, & Meyer, 2006).

2. THE STRUCTURE OF GPCRs

Recent advances in experimental crystallization techniques (Day et al., 2007; Serrano-Vega, Magnani, Shibata, & Tate, 2008) have permitted to elucidate the crystal structure of several members of the class A GPCRs in different conformational states, either bound to agonists, antagonists, inverse agonists, or the G protein (see Katritch, Cherezov, & Stevens, 2012 for a recent review). Interestingly, despite their overall low-sequence identity (Gonzalez, Cordomi, Caltabiano, Campillo, & Pardo, 2012; Mobarec, Sanchez, & Filizola, 2009), all class A GPCRs display a highly

conserved molecular architecture (Liapakis, Cordomi, & Pardo, 2012). This architecture is characterized by the presence of seven α-helical transmembrane (7TM) segments, which span the cell membrane, connected to each other by three extracellular (ECL) and three ICL loops, and a disulfide bridge between ECL2 and TM3. The N-terminal region is located toward the ECL side of the membrane, whereas the C-terminal region, containing a short α-helix lying perpendicular to the membrane plane (Hx8), faces the ICL milieu. Analysis of the known crystal structures of GPCRs shows that ligand binding mostly occurs in a main cavity located between the ECL segments of TMs 3, 5, 6, and 7 (rhodopsin, β_1-, β_2-, H_1-, D3-, A_{2A}, μ-OR, neurotensin, and CXCR4 bound to the CVX15 cyclic peptide) or in a minor binding cavity located between the ECL segments of TMs 1, 2, 3, and 7 (CXCR4 bound to the antagonist small molecule IT1t) (Rosenkilde, Benned-Jensen, Frimurer, & Schwartz, 2010).

Major sequence and structural divergences among GPCRs come from the N- and C-terminal regions, ICL3, and ECL2 (Peeters, van Westen, Li, & Ijzerman, 2011). ECL2 forms, in the majority of receptors, part of the ligand-binding cavity (Bokoch et al., 2010; de la Fuente et al., 2010; Massotte & Kieffer, 2005). In rhodopsin, the N-terminal region (formed by two β-strands) and ECL2 (two β-strands) block the access of the ECL ligand to the core of the receptor (Palczewski et al., 2000). Similarly, in the $S1P_1$ receptor, the N-terminus (contains a short α-helix) covers half the binding pocket and ECL2 (formed by a family-specific disulfide bridge within ECL2, but lacking the conserved disulfide bridge between TM3 and ECL2) covers the other half (Hanson et al., 2012). In these cases, retinal (Hildebrand et al., 2009; Park, Scheerer, Hofmann, Choe, & Ernst, 2008) and sphingosine-1-phosphate (Hanson et al., 2012) may gain access to the binding pocket from the lipid bilayer. In contrast, in aminergic (Rosenbaum et al., 2007; Warne et al., 2008), dopaminergic (Chien et al., 2010), histaminergic (Shimamura et al., 2011), adenosine (Jaakola et al., 2008), opioids (Manglik et al., 2012), and chemokine (Wu et al., 2010) receptors, ECL2 adopts different spatial conformations that maintain the binding site rather accessible from the ECL environment. Moreover, in contrast to other GPCRs, the N-terminal domain of melanocortin receptors plays a significant role in their constitutive, ligand-independent, activity (Ersoy et al., 2012). Thus, each receptor subfamily has probably developed, during evolution, a specific N-terminus/ECL2 to adjust the structural characteristics of its cognate ligands, and to modulate the ligand binding/unbinding events (Dror et al., 2011; Gonzalez, Perez-Acle, Pardo, & Deupi, 2011; Hurst et al., 2010).

Importantly, it has recently been reported that a small cavity (vestibule) present at the entrance of the ligand-binding cavity controls the extent of receptor movement to govern a hierarchical order of G protein coupling (Bock et al., 2012). On the other hand, ICL3 is highly variable in length (from few amino acids in rhodopsin to hundreds in muscarinic acetylcholine receptors), which has been related to the modulation of G protein signals (Ritter & Hall, 2009). The C-terminal tail also varies in length and plays a key role in downstream signaling cascade activation (Blattermann et al., 2012; Schroder et al., 2009).

3. SEQUENCE ANALYSES OF THE 7TM SEGMENTS OF THE RHODOPSIN FAMILY OF GPCRs

In general, sequence comparison among class A GPCRs in the TMs region revealed high variability on the ECL side and increasing conservation toward the cytoplasmic side (Mirzadegan, Benko, Filipek, & Palczewski, 2003). The asymmetry in the conservation profiles between the ECL ligand-binding region and the ICL G-interacting region reflects the functional modularity and distinctive evolutionary constraints between these two regions (Deupi et al., 2007). In addition, there are a number of highly conserved residues and short-sequence motifs readily identifiable: $N^{1.50}$ in TM1, $LxxxD^{2.50}$ in TM2, $(E/D)R^{3.50}Y$ in TM3, $W^{4.50}$ in TM4, $P^{5.50}$ in TM5, $CWxP^{6.50}$ in TM6, and $(N/D)P^{7.50}xxY$ in TM7. These residues are employed to define a common numbering scheme for class A GPCRs that allow easy comparison among residues in the 7TM segments of different receptors (Ballesteros & Weinstein, 1995). Most important, many of these conserved residues function as micro-switches in the process of receptor activation (see Nygaard, Frimurer, Holst, Rosenkilde, & Schwartz, 2009; Rosenbaum, Rasmussen, & Kobilka, 2009; Smit et al., 2007 for reviews). Thus, there is a vast amount of information regarding these conserved polar/charged residues that are relevant in the mechanism of receptor activation (Ballesteros et al., 2001; Govaerts, Blanpain, et al., 2001; Govaerts, Lefort, et al., 2001; Jongejan et al., 2005; Pellissier et al., 2009; Prioleau, Visiers, Ebersole, Weinstein, & Sealfon, 2002; Scheer, Fanelli, Costa, De Benedetti, & Cotecchia, 1996; Shi et al., 2002; Springael et al., 2007; Urizar et al., 2005). However, highly conserved hydrophobic amino acids also play an important role in receptor activation, G protein coupling, and receptor oligomerization. In the rest of this review, we are going to focus on the role of these hydrophobic amino acids in the structure and function of class A GPCRs.

4. IMPORTANCE OF THE HIGHLY CONSERVED HYDROPHOBIC AMINO ACID AT POSITION 3.40 IN THE PROCESS OF AGONIST-INDUCED RECEPTOR ACTIVATION

The crystal structure of a nanobody-stabilized active state of the β_2-adrenergic receptor bound to the BI-167107 agonist (Rasmussen, Choi, et al., 2011) shows hydrogen bonding interactions with $S^{5.42}$ and $S^{5.46}$ (Fig. 5.1). These interactions stabilize a receptor conformation that includes a 2.1 Å inward movement of TM5 at position 5.46 and 1.4 Å inward movement of the conserved $P^{5.50}$ relative to the inactive, carazolol-bound structure (Rosenbaum et al., 2007). $P^{5.50}$ in TM5 (conserved in 77% of the rhodopsin-like sequences) induces a local opening of the helix (*proline-unwinding*, in contrast to *proline-kink;* Deupi et al., 2004; Sansuk et al., 2011). This key distortion is stabilized in the known crystal structures by a bulky hydrophobic side chain at position 3.40 (Fig. 5.1), highly conserved in the whole Class A GPCR family (I:40%, V:25%, L:11%). Mutation of $I^{3.40}$ to either Ala or Gly, that is, removing the bulky side chain at this position, abolishes the constitutive activity of the histamine H_1 receptor, the effect of constitutive-activity increasing mutations, as well as the histamine-induced receptor activation (Sansuk et al., 2011). Moreover, mutations at position 3.40 in rhodopsin are related to retinitis

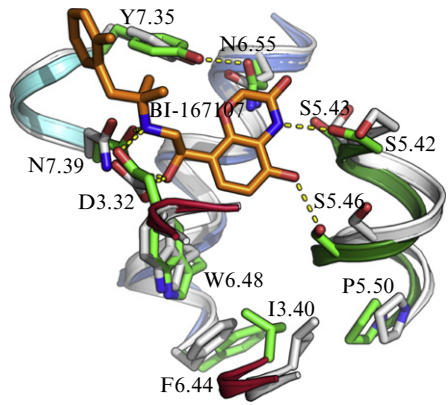

Figure 5.1 Detailed view of the β_2-adrenergic receptor bound to the full agonist BI-167107 (in orange). The hydrogen bond interaction between full agonists and $S^{5.46}$ stabilizes a receptor conformation that includes an inward movement of TM5 relative to the inactive (shown in white for comparison purposes), carazolol-bound structure. The color code of the helices is TMs 1 in white, 2 in yellow, 3 in red, 4 in grey, 5 in green, 6 in dark blue, and 7 in light blue.

pigmentosa, poor retinal binding, misfolding, reduced expression, decreased time in the MII state, and reduced transducin activation (Madabushi et al., 2004). Thus, the inward movement of $P^{5.50}$ upon agonist binding repositions $I^{3.40}$ and $F^{6.44}$, which contributes to a rotation and outward movement of TM6 for receptor activation (Rasmussen, Choi, et al., 2011) (see below).

5. THE HYDROPHOBIC CAGES OF ARGININE OF THE (D/E)RY MOTIF IN TM3, TYROSINE OF THE (N/D)PxxY MOTIF IN TM7, AND TYROSINE IN TM5

The publication of the crystal structure of the ligand-free opsin (Park et al., 2008), which contains several distinctive features of the active state as it has been confirmed in the recent structure of the β_2-adrenergic receptor bound to Gs (Rasmussen, DeVree, et al., 2011), showed that during the process of receptor activation, the ICL part of TM6 tilts outward, TM5 nears TM6, and $R^{3.50}$ within the (D/E)RY motif in TM3 adopts an extended conformation pointing toward the protein core, to interact with the highly conserved $Y^{5.58}$ in TM5 and $Y^{7.53}$ of the (N/D)PxxY motif in TM7 (Fig. 5.2). As shown in the original publication of the opsin structure, these conformational changes disrupt the ionic interaction between $R^{3.50}$ with negatively charged side chains at positions 3.49 in TM3 and 6.30 in TM6 (Fig. 5.2A and C) and facilitates the interaction between $K^{5.66}$ in TM5 and $E^{6.30}$ in TM6 (Fig. 5.2B and D).

Thus, GPCRs are maintained in the inactive conformation through these polar interactions between side chains. It has been suggested that conserved hydrophobic amino acids in the environment of these key polar residues form hydrophobic cages, which also restrain GPCRs in inactive conformations (Ballesteros et al., 1998). The hydrophobic amino acids at positions 3.46 (I:57%, L:17%, M:13%, V:10%) and 6.37 (L:36%, V:23%, I:20%, M:5%) form the arginine cage that restrains $R^{3.50}$ to adopt the extended conformation pointing toward the protein core (Fig. 5.2C). The outward movement of TM6, necessary for receptor activation, also moves the side chain at position 6.37, opening a small cavity to accommodate the extended conformation of $R^{3.50}$ (Fig. 5.2D). Accordingly, removal of these interfering bulky constrains by A or G replacement leads to constitutive activity in a number of cases (Baranski et al., 1999; Laue et al., 1995; Ringkananont et al., 2006). Similarly, the hydrophobic amino acids at positions 2.46 (L:90%, M:4%, I:3%, V:1%) and 6.40 (V:39%, I:27%, L:16%, M:5%) form the tyrosine cage that keeps $Y^{7.53}$ away from the bundle (Fig. 5.2C). Again movement of TM6 allows $Y^{7.53}$ to change conformation

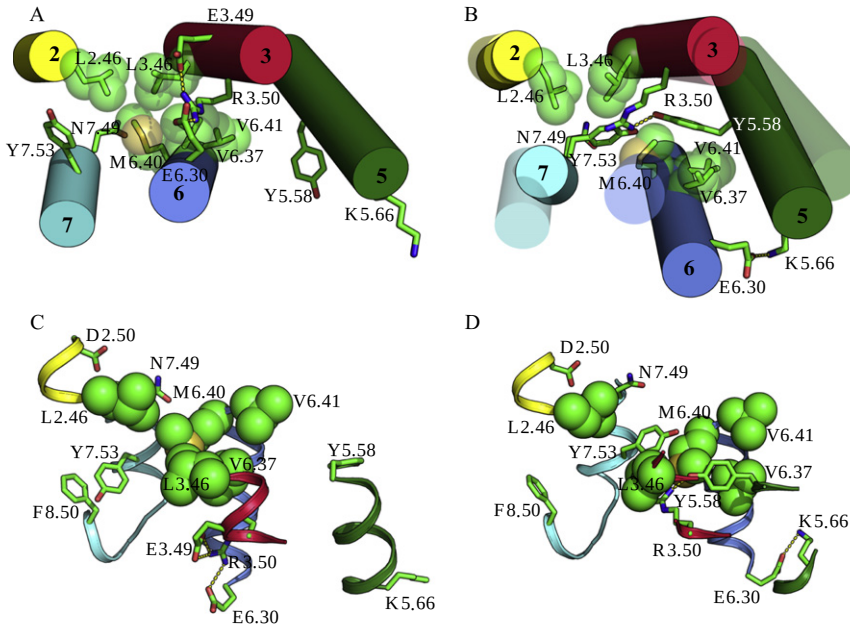

Figure 5.2 Comparison of (A, C) the crystal structure of inactive rhodopsin (1GZM) with (B, D) the crystal structure of the ligand-free opsin (3CAP), which contains distinctive features of the active state, in views parallel (C, D) and perpendicular (A, B) to the membrane. Panel B shows the positions of TMs 2, 3, 5–7 in rhodopsin (transparent cylinders) and opsin (opaque cylinders) for comparison purposes. The color code of the helices is as in Fig. 5.1. (See Color Insert.)

pointing toward the protein core (Fig. 5.2D). Removal (mutation to A or G) of the bulky and β- or γ-branched amino acids at positions 2.46 in rhodopsin (Madabushi et al., 2004) and the TSH receptor (Urizar et al., 2005) and 6.40 in rhodopsin (Han, Lin, Minkova, Smith, & Sakmar, 1996), serotonin $5HT_{2A}R$ (Shapiro, Kristiansen, Weiner, Kroeze, & Roth, 2002), and histamine H_1 receptors (Bakker et al., 2008) induces constitutive activity. Finally, statistical analysis shows that GPCRs mainly contain hydrophobic amino acids at position 6.41 (V:39%, L:23%, I:11%, M:9%, A:6%, T:6%, F:3%) as a polar side chain at this position could trigger the conformational change of $Y^{5.58}$ toward the bundle (Fig. 5.2C and D).

The recent crystal structure of the $M^{6.40}Y$ rhodopsin mutant has suggested the molecular basis for the constitutive activity of 6.40 substitutions (Deupi et al., 2012). The tyrosine of the $M^{6.40}Y$ mutant forms specific interactions with $Y^{5.58}$ in TM5, $Y^{7.53}$ of the (N/D)PxxY motif, and $R^{3.50}$ of the (E/D)RY motif in the active conformation of the receptor.

6. THE TRANSMEMBRANE AQUEOUS CHANNEL IS INTERRUPTED BY A LAYER OF HYDROPHOBIC RESIDUES

The majority of the TM regions deviate from ideal α-helices, showing structural anomalies like kinks and bulges (Gonzalez et al., 2012). These distortions are energetically stabilized through complementary intra- and inter-helical interactions involving polar side chains, backbone carbonyls, and, in some cases, specific structural and functional water molecules embedded in the TM bundle (Angel, Chance, & Palczewski, 2009; Angel, Gupta, Jastrzebska, Palczewski, & Chance, 2009; Li, Edwards, Burghammer, Villa, & Schertler, 2004; Pardo, Deupi, Dolker, Lopez-Rodriguez, & Campillo, 2007). The recent crystal structure of the M2 muscarinic acetylcholine receptor has shown the presence of a continuous aqueous channel extending from the ECL surface to a depth of approximately the middle of the membrane (Haga et al., 2012). As shown in the original publication of the M2 receptor, this channel is separated from the ICL part by highly conserved hydrophobic amino acids at positions 2.46 (L:90%, M:4%, I:3%, V:1%), 3.43 (L:73%, I:10%, V:6%, M:5%), and 6.40 (V:39%, I:27%, L:16%, M:5%).

7. THE ROLE OF HIGHLY CONSERVED HYDROPHOBIC RESIDUES IN G PROTEIN BINDING

The formation of the complex between the active conformation of the receptor and the heterotrimeric G protein triggers GDP release from the Gα-subunit, GTP binding to the Gα-subunit, and dissociation of the Gβγ-subunits (Chung et al., 2011), which finally leads to a cascade of signals depending on the G protein type. Noteworthy, more than 800 known GPCRs can bind 17 different Gα-subunits, which have been grouped into four different classes ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$) (Simon, Strathmann, & Gautam, 1991). To date, the crystal structures of the ligand-free opsin (Scheerer et al., 2008), metarhodopsin II (Choe et al., 2011), and the constitutively active rhodopsin mutant $E^{3.28}Q$ (Standfuss et al., 2011) in complex with a peptide derived from the carboxy terminus of the α-subunit of the G protein transducin, together with the structure of the β_2-adrenergic receptor bound to Gs (Rasmussen, DeVree, et al., 2011) have been released. These structures have shown that the C-terminal α5 helix of Gα binds to the ICL cavity that is opened by the movement of the cytoplasmic end of TM6

away from TM3 and toward TM5 (see above). The C-terminal α5 helix of the α-subunit interacts with the extended conformation of $R^{3.50}$, the short loop connecting TM7 and Hx8, and the inner side of the cytoplasmic TMs 5 and 6 (Fig. 5.3C). Previous biochemical studies had shown that the last 11 residues of this C-terminal α5 helix play an important role in receptor binding and selectivity (Conklin, Farfel, Lustig, Julius, & Bourne, 1993; Conklin et al., 1996; Garcia, Onrust, Bell, Sakmar, & Bourne, 1995; Hsu & Luo, 2007; Martin, Rens-Domiano, Schatz, & Hamm, 1996; Natochin, Muradov, McEntaffer, & Artemyev, 2000). The G protein family possesses highly conserved hydrophobic residues at positions *i-2* (relative to the final amino acid), *i-7*, *i-11*, and *i-12* (Fig. 5.3B). Notably, these conserved

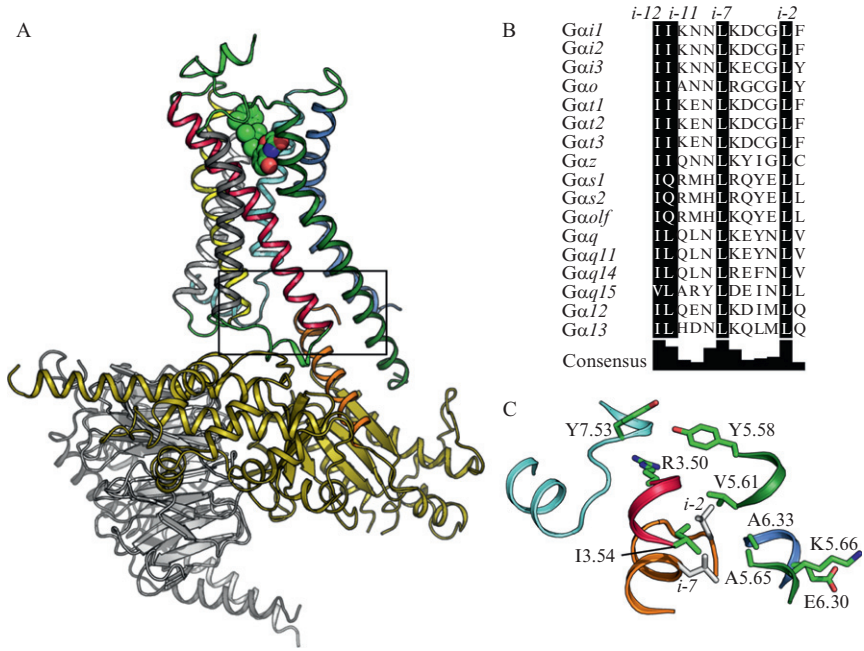

Figure 5.3 (A) Crystal structure of the β_2-adrenergic receptor in complex with the Gs heterotrimer (α-subunit in olive, β-subunit in white, and γ-subunit in grey). The C-terminal α5 helix of the α-subunit is shown in orange. The color code of TMs of β_2 is as in Fig. 5.1. The rectangle shows the part of the complex depicted in (C). (B) Sequence alignment of the C-terminal part of the α5 helix of the α-subunit. Highly conserved residues are shown in black. Percentage of conservation is shown by the histogram. (C) Detailed view of the interaction between the C-terminal α5 helix of the α-subunit (in orange) with the short loop connecting TM7 and Hx8 (light blue), TM3 (red), and the inner side of the cytoplasmic TMs 5 (green) and 6 (blue).

residues of the G protein family at positions *i-2* and *i-7* form hydrophobic–hydrophobic interactions with highly conserved hydrophobic amino acids at positions 3.54 (I:46%, V:39%, L:7%, A:3%, M:2%) in TM3, 5.61 (I:55%, V:13%, L:11%, M:9%) and 5.65 (L:48%, A:16%, V:11%, I:10%) in TM5, and position 6.33 (A:33%, V:16%, L:12%, T:11%, I:7%) in TM6 (Fig. 5.3C). Combined mutagenesis and molecular modeling experiments showed that hydrophobic interactions were critical for rhodopsin activation and/or efficient interaction with transducin (Bosch-Presegue et al., 2011). Thus, it seems reasonable to assume that the mode of recognition of the G protein by the other members of the GPCR family resembles this structure found for the β_2-adrenergic receptor bound to Gs.

8. THE ROLE OF HIGHLY CONSERVED HYDROPHOBIC RESIDUES IN RECEPTOR OLIGOMERIZATION

GPCRs have been classically described as monomeric transmembrane receptors that form a ternary complex: a ligand, the GPCR, and its associated G protein. This is compatible with observations that monomeric rhodopsin and β_2-adrenergic receptor are capable of activating G proteins (Ernst, Gramse, Kolbe, Hofmann, & Heck, 2007; Whorton et al., 2007). Nevertheless, it is now well accepted that many GPCRs have been observed to oligomerize in cells (Ferre et al., 2009; Pin et al., 2007). It has been shown that receptor activation is modulated by allosteric communication between protomers of dopamine class A GPCR dimers (Han, Moreira, Urizar, Weinstein, & Javitch, 2009). The minimal signaling unit, two receptors and a single G protein, is maximally activated by agonist binding to a single protomer. Inverse agonist binding to the second protomer enhances signaling, whereas agonist binding to the second protomer blunts signaling. Moreover, binding of agonists or the G protein to β_2 regulates receptor oligomerization (Fung et al., 2009). Cysteine cross-linking experiments have suggested that receptor oligomerization involves hydrophobic interactions via the surfaces of TMs 1, 4, and/or 5 (Guo, Shi, Filizola, Weinstein, & Javitch, 2005; Guo et al., 2008; Klco, Lassere, & Baranski, 2003). Nevertheless, electrostatic interactions of the ICL domains are key in the formation of receptor heteromers (Navarro et al., 2010).

The recent structure of μ-OR shows receptor molecules associated into pairs through two different interfaces (Fig. 5.4A) (Manglik et al., 2012). The first interface is via TMs 1 and 2 and Hx8 (Fig. 5.4B), and the second interface comprises TMs 5 and 6 (Fig. 5.4C). The central part of TM1 contains a

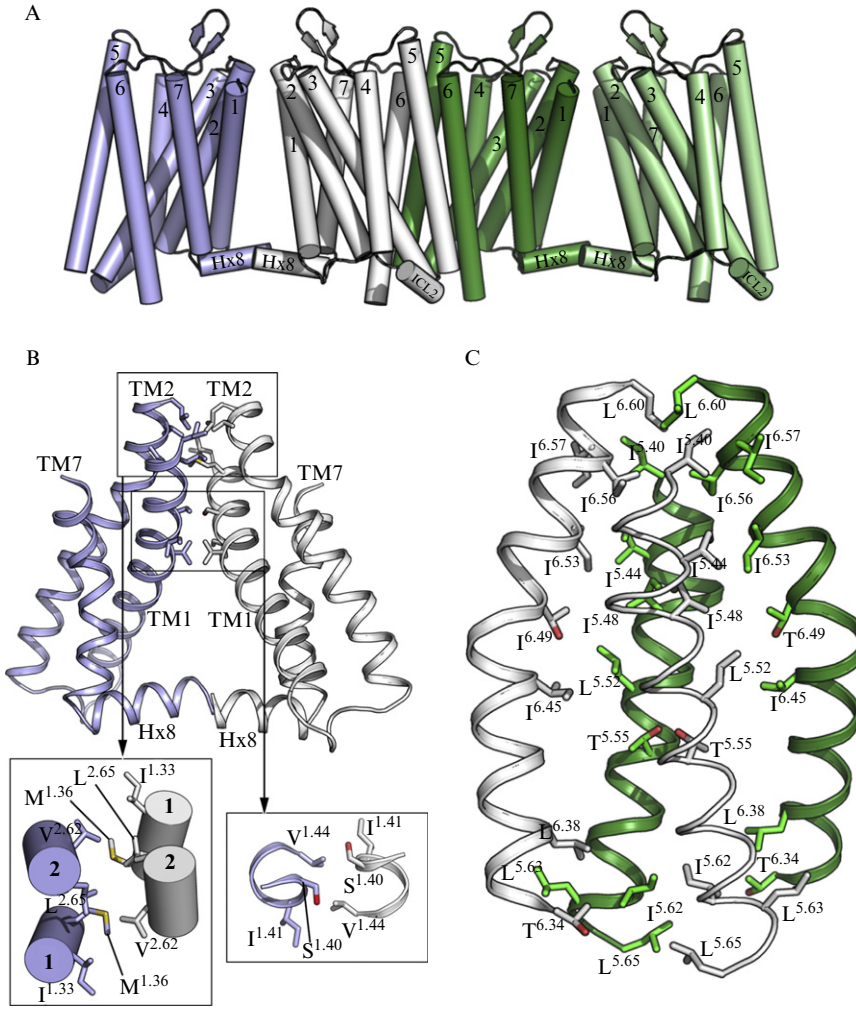

Figure 5.4 The structure of the μ-opioid receptor tetramer. (A) The four different protomers are shown in light blue, white, green, and light green. (B) Residues involved in receptor dimerization via the surfaces of TMs 1 and 2. The protomers shown correspond to the ones depicted in light blue and white in (A). (C) Residues involved in receptor dimerization via the surfaces of TMs 5 and 6. The protomers shown correspond to the ones depicted in white and green in (A).

symmetric domain for receptor dimerization involving the side chains of $S^{1.40}$, $I^{1.41}$, and $V^{1.44}$ (Fig. 5.4B), while the ECL part of TM1 ($I^{1.33}$ and $M^{1.36}$) forms hydrophobic–hydrophobic interactions with the ECL part of TM2 ($V^{1.33}$ and $M^{1.36}$) (Fig. 5.4B). A more extensive network of

hydrophobic interactions both at the ECL and ICL sites drives receptor dimerization via the TM5–6 interface (Fig. 5.4C). Importantly, most class A nonolfactory GPCRs contain hydrophobic amino acids at these positions (not shown), with the only exception of 5.63 that contains Lys/Arg in 36% of the sequences (this part of TM5 is exposed to the ICL environment). This pattern of conservation suggests that the other members of the GPCR family can also form homo- and hetero-oligomerization via these TMs 1–2/Hx8 and TMs 5–6 interfaces.

9. CONCLUSIONS

Class A GPCRs are characterized by a number of highly conserved charged and polar residues located within the TM region. Mutagenesis studies indicate that most, if not all, of these amino acids are involved in maintaining the three dimensional structure of the receptor and in the processes of receptor activation. Analysis of the available crystal structures of class A GPCRs has shown that highly conserved hydrophobic amino acids also play an important role in receptor activation, G protein coupling, and receptor oligomerization.

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