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Structural and functional roles of small group-conserved amino acids present on helix-H7 in the β_2-adrenergic receptor

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ABSTRACT

Sequence analysis of the class A G protein-coupled receptors (GPCRs) reveals that most of the highly conserved sites are located in the transmembrane helices. A second level of conservation exists involving those residues that are conserved as a group characterized by small and/or weakly polar side chains (Ala, Gly, Ser, Cys, Thr). These positions can have group conservation levels of up to 99% across the class A GPCRs and have been implicated in mediating helix–helix interactions in membrane proteins. We have previously shown that mutation of group-conserved residues present on transmembrane helices H2–H4 in the β_2-adrenergic receptor (β_2-AR) can influence both receptor expression and function. We now target the group-conserved sites, Gly315$^{7.42}$ and Ser319$^{7.46}$, on H7 for structure-function analysis. Replacing Ser319$^{7.46}$ with smaller amino acids (Ala or Gly) did not influence the ability of the mutant receptors to bind to the antagonist dihydroalprenolol (DHA) but resulted in ~15–20% agonist-independent activity. Replacement of Ser319$^{7.46}$ with the larger amino acid leucine lowered the expression of the S319L mutant and its ability to bind DHA. Both the G315A and G315S mutants also exhibited agonist-independent signaling, while the G315L mutant did not show specific binding to DHA. These data indicate that Gly315$^{7.42}$ and Ser319$^{7.46}$ are stabilizing β_2-AR in an inactive conformation. We discuss our results in the context of van der Waals interactions of Gly315$^{7.42}$ with Trp286$^{6.48}$ and hydrogen bonding interactions of Ser319$^{7.46}$ with amino acids on H1–H2–H7 and with structural water.

1. Introduction

G protein-coupled receptors (GPCRs) share a seven transmembrane (TM) helix architecture and activate cognate G-proteins in response to agonist binding. The most extensively studied ligand-activated GPCR is the β_2-adrenergic receptor (β_2-AR), which mediates physiological responses to epinephrine and norepinephrine. The β_2-AR was the first ligand-activated GPCR to be cloned [1], and the first whose crystal structure was determined at high resolution [2]. The crystal structure of β_2-AR was solved with the inverse-agonist carazolol bound and provided a direct comparison with the crystal structure of the dark, inactive state of rhodopsin, the dim-light photoreceptor [3,4]. The two structures revealed the positions of amino acids that are conserved across the family of class A GPCRs, but have left largely unanswered questions concerning their contribution to stabilizing the structure of the inactive receptor or guiding the transition to an active receptor conformation.

Fig. 1 presents a two-dimensional representation of the β_2-AR sequence with the conserved amino acids highlighted by different colors. There are at least three levels of conservation that can be considered in understanding the structure and function of a given receptor or subfamily of receptors within the GPCR superfamily. The most important set of conserved residues in the class A GPCR family are the "signature" amino acids with sequence identities of >70%. There are 15 signature residues in the transmembrane helices (H1–H7) including the highly conserved (E/D)RY motif on H3 and NPxxY motif on H7.

The second level of conservation involves the "group-conserved" residues in the class A GPCR family with conservation of up to 99% when considered as a group of small and weakly polar residues (Ala,

Abbreviations: β_2-AR, β_2-adrenergic receptor; B_{max}, Binding maximum of the ligand for the receptor; COS-1, Monkey kidney cells; GPCRs, G protein-coupled receptors; HEK293, human embryonic kidney cells; [^{3}H] DHA, tritium labeled dihydroalprenolol; K_d, equilibrium dissociation constant of the ligand

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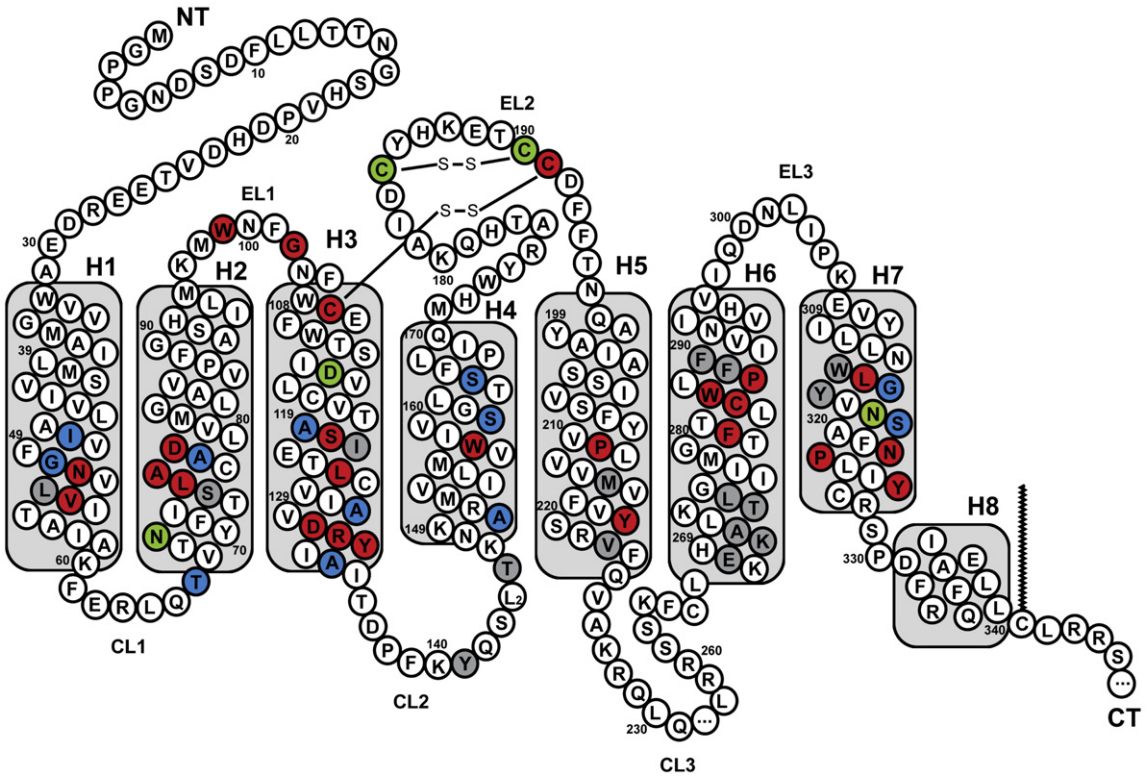

Fig. 1. Two-dimensional representation of the β_2-AR sequence showing amino acid conservation at the three different levels. The receptor has seven transmembrane helices (H1–H7) and a short amphipathic helix (H8) that lies on the cytoplasmic surface of the membrane. Amino acids are shown in single-letter codes. Red circles denote signature-conserved residues that include the (E/D)RY and NPxxY motifs, an asparagine (Asn51$^{1.50}$) on H1, aspartic acid on H2 (Asp79$^{2.50}$), three prolines on helices H5 (Pro211$^{5.50}$), H6 (Pro288$^{6.50}$) and H7 (Pro323$^{7.50}$) and several hydrophobic residues (Leu75$^{2.46}$,Trp158$^{4.50}$). Blue circles represent small and weakly polar group-conserved residues in the Class A GPCRs excluding the olfactory subfamily, and green circles subfamily-specific residues. The residues in gray are between 70% and 90% conserved in the amine receptor subfamily. CL, cytoplasmic loop; CT, C terminus; EL, extracellular loop; NT, N terminus.

Gly, Ser, Cys and Thr). These amino acids have been identified in membrane proteins as key determinants in helix–helix interactions [5,6]. We have previously suggested that H1–H4 form a tightly packed core on the basis of the location of the group-conserved positions [7]. The group-conserved residues present on H2, H3 and H4 in β_2-AR have both structural and functional roles [8]. The most significant changes in receptor expression and activity were observed upon replacement of the amino acids Ser161$^{4.53}$ and Ser165$^{4.57}$ on H4. (The amino acid numbering used in this manuscript incorporates the residue number from the receptor sequence (e.g. Ser165) and a residue number (e.g. 4.57) from a generic numbering system developed by Ballesteros and Weinstein [9]). Substitution at Ser161$^{4.53}$ and Ser165$^{4.57}$ by larger residues lowered the expression and activity of the receptor, but did not affect specific binding to the antagonist ligand dihydroalprenolol. Substitution of other group-conserved residues on helices H2–H4 by larger amino acids lowered receptor activity in the order Ala128$^{3.47}$, Ala76$^{2.47}$, Ser120$^{3.39}$, and Ala78$^{2.49}$ [8].

The third level of conservation includes those residues that have sequence identities of >90% in the amine subfamily. Each class A GPCR subfamily contains a set of residues that makes it uniquely able to respond to its own ligand. For example, Asp113$^{3.32}$ on H3 is 92% conserved in the amine subfamily. This residue is the counterion to the positively charged amine [10]. The next highest conservation (92%) is Asn318$^{7.45}$ on H7. Asn318$^{7.45}$ corresponds to Ser298$^{7.45}$ in rhodopsin, a residue that only has 50% conservation in the opsin subfamily. Other residues that are highly conserved within the amine subfamily are Asn69$^{2.40}$ (90%), Trp313$^{7.40}$ (88%) and Tyr316$^{7.43}$ (82%).

To fully understand the structure and activation mechanism of any class A GPCR one needs to define the roles of key residues at each level of amino acid conservation. Here, we target two group-conserved residues, Gly315$^{7.42}$ and Ser319$^{7.46}$, present on H7. These residues are of particular interest for several reasons. First, they are connected to two different functional microdomains [11–13], the conserved aromatic cluster on H6 and the NPxxY motif on H7. Second, they are flanked by residues that have very high sequence conservation in the amine subfamily of receptors: Trp313$^{7.40}$, Tyr316$^{7.43}$ and Asn318$^{7.45}$. Third, while being group-conserved throughout the class A GPCR family, they have very high sequence identity within the amine subfamily (69.2% glycine at position 7.42 and 98% serine at position 7.46). To determine the structural and functional roles of these two group-conserved residues on H7 in the β_2-AR, mutants were generated by site-directed mutagenesis and transiently expressed in COS-1 or HEK293S cells, and then ligand binding assays were performed using membrane preparations. To elucidate the effect of these mutations on G-protein signaling, cAMP levels were measured following stimulation by isoproterenol. We discuss the influence of mutation at Gly315$^{7.42}$ and Ser319$^{7.46}$ on receptor structure and stability.

2. Materials and methods

2.1. Materials

The β_2-AR ligands, alprenolol, isoproterenol, epinephrine and salbutamol were purchased from Sigma (St. Louis, MO, USA). Protease inhibitors and common chemicals were purchased either from Fisher (Ottawa, ON, Canada) or Sigma. The detergent n-dodecyl-β-D-maltoside (DDM) was purchased from Anatrace (Maumee, OH, USA). The monoclonal antibody, rho-1D4, was prepared by the Cell Culture Center (Minneapolis, MN) from a cell line provided by R.S. Molday (University of British Columbia, Vancouver, Canada). Fetal bovine serum was purchased from Sigma and DME High Glucose was from Invitrogen (Burlington, ON, Canada). The β_2-AR antagonist [^{3}H] DHA

was purchased from GE Healthcare (GE Healthcare Biosciences, Little Chalfont, UK). Synthetic oligonucleotides were purchased from Invitrogen (Carlsbad, CA, USA).

Buffers used were as follows: Phosphate-buffered saline (PBS) buffer: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4 (pH 7.4); Buffer A (Lysis buffer), 10 mM Tris–HCl, pH 7.4, containing protease inhibitors (1 mM EDTA, 10 μg/ml benzamidine, 10 μg/ml leupeptin, 20 μg/ml soybean trypsin inhibitor, 5 μg/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride); Buffer B (Storage buffer) 50 mM Tris–HCl, pH 7.4, 12.5 mM $MgCl_2$, containing protease inhibitors as in Buffer A; Buffer C (Binding buffer), 75 mM Tris–HCl, pH 7.4, 12.5 mM $MgCl_2$, containing protease inhibitors as in Buffer A. Buffer E (solubilization buffer), 20 mM Tris–HCl (pH 7.4), containing 500 mM NaCl, 10% glycerol, 1% DM and the protease inhibitors as in Buffer A.

2.2. Construction of mutant hamster β_2-AR genes

Mutant hamster β_2-AR genes in the plasmid expression vector, pMT4, were constructed using the Quick-Change mutagenesis kit (Stratagene, La Jolla, CA, USA) [8]. The DNA sequences of all the mutated genes were verified by automated DNA sequencing (MICB DNA Sequencing Facility, Winnipeg).

2.3. Cell culture and immunoblot analysis

The wild-type β_2-AR and mutant genes were expressed in COS-1 cells using a DEAE-dextran based transient transfection method [14,15]. For transient transfection of HEK293T cells using the plasmid pMT4, lipofectamine 2000 (Invitrogen) mediated transfection was used as described by the manufacturer. Membranes were prepared using Buffers A and B and as described previously [16]. The protein concentration in the resuspended membrane pellet was determined using a modified DC protein assay kit from Bio-Rad Laboratories (Hercules, CA). Approximately 2.5–5 μg of the total solubilized membrane protein were resolved by 10% SDS-PAGE and electro-blotted onto a nitrocellulose membrane. β_2-AR was visualized by immunodetection with the monoclonal antibody, rho-1D4 [16]. The band at approximately 65 kDa corresponds to the completely glycosylated receptor [14].

2.4. Radioligand binding assays

These were carried out in Buffer C, using 2–20 μg of membrane protein and as described earlier [16,17]. Saturation binding assays were carried out using 0.1–20 nM [^{3}H] DHA. Binding of [^{3}H] DHA in the presence of 10 μM alprenolol was used as a measure of nonspecific binding. Specific binding was derived by subtracting nonspecific binding from the total binding and is shown in Fig. 7 (supporting information). Competition binding assays were performed using 3 nM [^{3}H] DHA and different concentrations of unlabeled agonists (10^{-2}–10^{-9} M) and the reactions kept for 2 h at room temperature. Radioligand binding data obtained from competition curves were analyzed by non-linear regression analysis to determine the K_i and EC_{50} values using PRISM software version 4.03 (GraphPad Software Inc, San Diego, CA).

2.5. Immunofluorescence microscopy

HEK293T cells were seeded into six-well tissue culture plates containing sterilized poly-L-lysine (Sigma)-treated glass cover-slips and transiently transfected with wild-type β_2-AR or mutant DNA according to the aforementioned transfection protocols. Unless specified, all steps were carried out room temperature. Cells were fixed in 3.7% formaldehyde/1x PBS buffer for 15 min, then permeablized with 0.05% triton X-100/1x PBS buffer for 30 min. The cells were washed and blocked with 1x PBS buffer containing 2% bovine serum albumin (IgG and Protease free) for 1 h. β_2-AR was labeled for 90 min using a 1:1000 dilution of the mouse-anti-rho-1D4 monoclonal antibody (C-terminal tagged β_2-AR) and a 1:250 dilution of rabbit-anti-calnexin polyclonal antibody (Abcam, MA, USA; endoplasmic reticulum marker). The labeled receptor was washed and incubated with a fluorescent coupled secondary antibody using a 1:500 dilution of anti-mouse-FITC (Jackson ImmunoResearch labs, PA, USA) and anti-rabbit-Texas Red (Jackson ImmunoResearch Laboratories, PA, USA) for 60 min in the dark. The coverslip was washed twice, mounted with Prolong-antifade-gold (Invitrogen, Molecular probes, CA, USA) for 15 min and its edges sealed with nail-polish. Representative cells were selected and visualized using an Olympus BX61 microscope for cytoplasmic or plasma membrane localization.

2.6. Determination of receptor activation by cAMP assays

Functional characterization of the β_2-AR was carried out with HEK293S cells using a commercially available cAMP assay system (DiscoveRx HitHunter™ cAMP XS+, Freemont, CA). HEK293S cells were used as these cells show very small changes in endogenous cAMP upon stimulation with isoproterenol [8,17]. Measurements of cAMP levels were made according to the directions supplied by the manufacturer. Briefly, 44 h after transfection HEK293S cells (100,000 cells/well) were seeded in a 96 well plate and stimulated for 30 min with concentrations of agonist (−) isoproterenol ranging from 10^{-6} M to 10^{-12} M. Luminescence was read using a Flex Station 3 plate reader (Molecular Devices, CA, USA). The assays were carried out a minimum of three times, each in duplicate and the data were analyzed using PRISM software version 4.03 (GraphPad Software Inc, San Diego, CA). The cAMP values of the mutants expressed as relative luminescence units (RLUs) were normalized to that of wild type β_2-AR.

2.7. Occluded surface calculations

The occluded surface (OS) method for the analysis of packing interactions in proteins has been previously described [5,18]. The method yields packing values of individual amino acids within a protein. Briefly, a packing value is composed of two parameters, the OS area and the distribution of distances to occluded atoms. Using coordinates from a high resolution structure, a molecular dot surface is calculated for each residue using a 1.4-Å probe. The dot density is chosen such that each dot represents 0.215 Å^2 of the surface area. A normal is extended radially from each dot until it either intersects the van der Waals surface of a neighboring atom or reaches a length of 2.8 Å (the diameter of a water molecule). The OS is defined as that molecular surface area on the originating atom associated with normal that intersect with another atom surface as opposed to reaching the 2.8-Å limit. All other molecular surface area is considered non-occluded or exposed. The packing value (PV) relates the occluded surface to the total surface of the residue (sum of occluded and non-occluded areas). Division by the total molecular surface area normalizes the packing value to account for various sizes of amino acid residues. Structural waters are not included in the calculations, resulting in surface areas that are non-occluded.

2.8. Homology modeling

The homology model of G315L β_2-AR was created in Swiss PDBviewer [19] (http://www.expasy.org/spdbv/) by threading the G315L β_2-AR sequence onto the opsin structure (3CAP). The resulting structure was energy minimized *in vacuo* with the GROMOS96 43B1 parameter set without a reaction field. The energy computations were done with the GROMOS96 implementation of Swiss-PdbViewer.

3. Results

3.1. Influence of mutations on receptor expression and ligand binding

Table 1 presents the saturation binding data of the antagonist DHA for the wild-type β_2-AR and the Gly315$^{7.42}$ and Ser319$^{7.46}$ mutants. Two types of mutations were made. First, mutations were made to other group-conserved amino acids, for example, glycine to alanine or serine to glycine. The hypothesis is that these conservative substitutions will have a minimal effect on receptor folding and ligand binding. Second, mutations were made to a non-group conserved amino acid with a bulky side chain (i.e. leucine). Total binding, specific binding and non-specific binding of DHA were measured [16]. Specific binding is the difference between the observed total binding and non-specific binding. Binding of [^{3}H] DHA in the presence of 10 μM alprenolol was used as a measure of non-specific binding.

When compared to the wild-type β_2-AR, the G315A mutant showed a similar affinity for DHA. The G315S mutant exhibited a higher K_D and an increased amount of non-specific binding. High non-specific binding can be due to the improperly folded protein interfering with the radioligand assay, or else it can also be due to the mutation perturbing the ligand binding site of the receptor. In contrast to the receptors with these conservative substitutions, the G315L mutant failed to bind to DHA in a specific manner.

The binding affinity of DHA to the β_2-AR with mutations at the group-conserved site Ser319$^{7.46}$ was similar to the wild-type receptor for the conservative mutations S319A and S319G, but the K_D increased for the non-group conserved leucine substitution, S319L.

The level of receptor expression was quantified by immunoblots and saturation binding assays using DHA (SI Figs. 6 and 7). The B_{max} is higher than wild-type for the S319A and S319G mutants, and immunoblot analysis showed heterogeneous expression as indicated by the presence of three predominant bands in the molecular mass arrange of 45–65 kDa, with none of the low expressing mutants producing the ~65 kDa band (SI, Fig. 6). Previously, photocrosslinking of β_2-AR expressed in COS-1 cells showed that the band at ~65 kDa corresponds to the completely glycosylated receptor [14]. Furthermore, low expressing group-conserved mutants present in H2−H4 also did not show the band at ~65 kDa.

Cell surface expression of the receptor is an excellent indicator of correct protein folding. To elucidate the subcellular localization of the mutant receptors, immunofluorescence microscopy studies on the mutants were carried out in the HEK293T cells (Fig. 2). Immunofluorescence microscopy showed that the G315A, S319A and S319G mutants appear to be predominantly localized on the cell surface with only a small amount of receptor present intracellularly, presumably due to receptor that is in-transit (Fig. 2). By contrast, a significant amount of the G315S and S319L mutants appear to be retained in the intracellular compartments, while the G315L mutant is predominantly found localized internally in the cytoplasm (Fig. 2). Cell surface localization is a strong indicator of correct folding of the G315A, S319A and S319G mutants because misfolded membrane proteins are typically retained either in the Golgi and/or in the endoplasmic reticulum by the endoplasmic reticulum quality control system and targeted for degradation [20].

Table 1
Summary of DHA binding to the wild type β_2-AR and Gly315 or Ser319 mutant receptors. The values are expressed as the mean ± S.E (n = 3 to 5 experiments).

Receptor	K_d (nM)	B_{max} (pmol/mg)
Wild type	3.1 ± 0.3	18 ± 0.6
G315A	4.9 ± 0.7	7.8 ± 0.7
G315S[a]	25.3 ± 7.4	5.3 ± 0.1
G315L	ND[b]	
S319A	2.8 ± 0.3	48 ± 3.0
S319G	3.0 ± 0.2	42 ± 2.0
S319L[a]	20.6 ± 5.1	9.5 ± 1.5

[a] High non-specific binding (>20% of total binding).
[b] Not detected (no significant specific binding detected under the assay conditions).

3.2. Agonist competition assays and Gαs mediated signaling

Table 2 presents the binding of unlabelled agonists to the wild type β_2-AR and mutant receptors as determined by competition with 3 nM [^{3}H] DHA. We characterized the ligand binding properties of the wild-type β_2-AR and the G315A, S319A and S319G β_2-AR mutants with the following β_2-AR agonists: (−) isoproterenol (full agonist), epinephrine (natural, full agonist) and salbutamol (partial agonist). We could not determine the binding properties of the G315S, G315L and S319L mutants as these mutants showed high non-specific binding under our assay conditions. Since the affinity of DHA to the wild-type receptor is similar for the G315A, S319A and S319G mutants (see Table 1), we can estimate the binding affinities of the various agonists from the competition data in Table 2. Salbutamol has a lower affinity to the wild-type receptor than either epinephrine or isoproterenol; i.e. the concentration needed to displace DHA is 5–6 times greater for salbutamol. The S319A and S319G mutants showed slight decreases in affinity for all three agonists. The G315A substitution had the most marked effect in the competition assay, requiring a 200-fold increase in agonist concentration to displace the DHA antagonist (Table 2).

The agonist competition data for S319A and S319G are in agreement with the agonist activation of the wild-type and mutant receptors measured by cAMP assays (Fig. 3). For the measurements on the Gly315 mutants treated with isoproterenol in a dose-dependent manner, the relative activity decreased in the order G315A>G315S>G315L. For the Ser319 mutants, the relative activity decreased in the order S319A>S319G>S319L. In general, the basal activity of the mutant receptors was higher than that of the wild-type β_2-AR (indicated by arrows in Fig. 3). The mutants showed agonist independent or constitutive activity to various degrees, for the S319A and S319G it was 15–20% of wild type, while for the G315A and G315S it was 10–15% of wild type. An unexpected finding was the G315A mutant exhibited high agonist dependent cAMP production even though it had the lowest affinity for (−) isoproterenol of the mutants assayed in Table 2.

3.3. Molecular packing of Gly315$^{7.42}$

The crystal structure of β_2-AR shows Gly315$^{7.42}$ is tightly packed against Cys285$^{6.47}$ and Trp286$^{6.48}$ on H6 [2] (Fig. 4A). The packing interaction can be assessed by the method of occluded surfaces [5,18]. Occluded surface calculations show that the packing value for Gly315$^{7.42}$ is high (0.65) indicating that the small glycine side chain does not form a water binding site in β_2-AR. For comparison, the average packing value for amino acids in helices of membrane proteins is 0.44 [5]. In helical membrane proteins, glycine has the highest amino acid packing value (0.52), followed by proline (0.51) and alanine (0.49) [5].

The replacement of Gly315$^{7.42}$ with amino acids having larger side chains results in a steric clash with the side chain of Trp286$^{6.48}$ (packing value 0.56 [5]). Since the glycine side chain is part of the helix backbone, larger amino acids at position 7.42 can be accommodated by rotation of the Trp286$^{6.48}$ side chain, which may be coupled to the rotation of transmembrane helix H6. Fig. 4B shows a model of the G315Lβ_2-AR structure based on homology with the structure of active opsin. The leucine side chain at position 315 is well packed against Trp286$^{6.48}$. For comparison, Fig. 4C and D show the structures of inactive rhodopsin and active opsin. In the transition from rhodopsin to opsin, Trp265 moves away from Ala295, whose packing value decreases from 0.55 to 0.44. Displacement of the highly

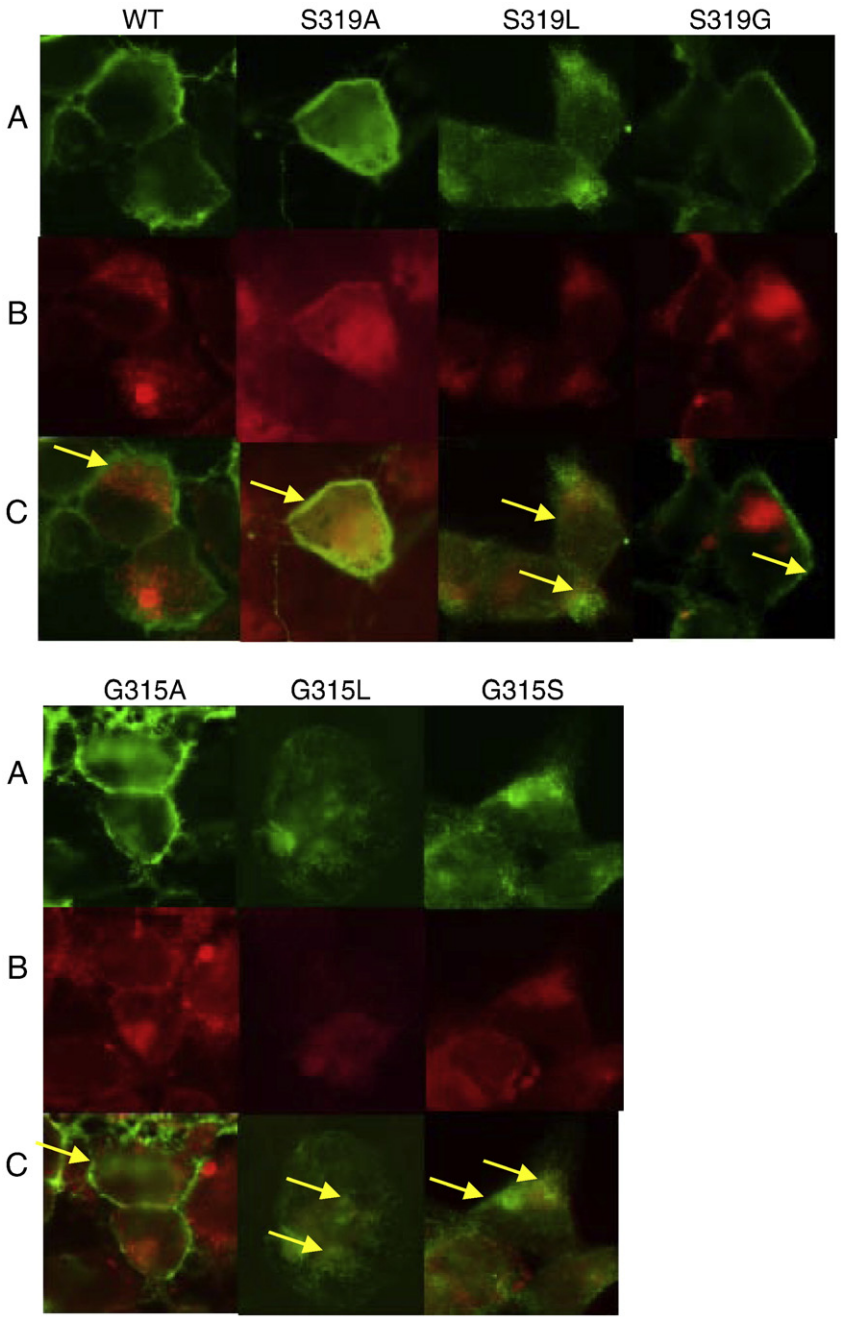

Fig. 2. Immunofluorescence microscopy showing localization of wild-type β_2-AR and the group-conserved mutants expressed in HEK293T cells. Immunofluorescence was performed with the mouse rho-1D4 antibody (**A**) and the endoplasmic reticulum marker, rabbit anti-calnexin antibody (**B**). The mouse rho-1D4 antibody was visualized with anti-mouse-FITC secondary antibody (green), and the rabbit anti-calnexin antibody was visualized with anti-rabbit-Texas Red secondary antibody (red). Overlays of (**A**) and (**B**) are shown in (**C**). Yellow arrows show the locations of receptors. In the wild-type, S319A, S319G and G315A mutants, the receptors were predominantly localized at the plasma membrane, whereas in G315S, G315L and S319L the receptors appear to be located in intracellular compartments.

conserved Trp286$^{6.48}$ and rotation of H6 would explain the constitutive activity observed for the Gly315$^{7.42}$ mutants.

3.4. Molecular packing of Ser319$^{7.46}$

Occluded surface calculations yield a smaller packing value (0.51) for Ser319$^{7.46}$ than for Gly315$^{7.42}$. Nevertheless, the packing value is higher than the average packing value for serine in membrane proteins (0.47) [5] and reflects the tightly hydrogen bonded network surrounding the Ser319$^{7.46}$ side chain (Fig. 5). The side chain of Ser319$^{7.46}$ forms hydrogen bonds with Asp79$^{2.50}$ (3.3 Å) and Tyr316$^{7.43}$ (3.1 Å). In addition, Ser319$^{7.46}$ hydrogen bonds with water 534 (3.1 Å), a structural water that also coordinates Asp79$^{2.50}$, Trp286$^{6.48}$, and Gly315$^{7.42}$ (Fig. 5B). Replacement of Ser319$^{7.46}$ with non-polar residues (S319A, S319G and S319L) results in loss of its hydrogen bonding interactions.

4. Discussion

We targeted Gly315$^{7.42}$ and Ser319$^{7.46}$ because of their position between two key functional microdomains in β_2-AR. Gly315$^{7.42}$ is in contact with Cys285$^{6.47}$ and Trp286$^{6.48}$ of the conserved CWxP motif on H6. Ser319$^{7.46}$ hydrogen bonds directly with the conserved Asn51$^{1.50}$ on H1 and Asp79$^{2.50}$ on H2, and bridges Trp286$^{6.48}$ and

Table 2
Summary of competition ligand binding of wild type β_2-AR and mutant receptors.

Receptor	Ligands (K_i) μM (95% confidence intervals)		
	Epinephrine	(−) Isoproterenol	Salbutamol
Wild type	4.9 (3.5–7.0)	4.2 (2.4–7.5)	25 (21–30)
G315A	1014 (372–2760)	658 (304–1237)	1616 (398–5680)
S319A	127 (65–246)	31 (17–56)	204 (85–488)
S319G	141 (68–293)	64 (30–135)	154 (60–394)

Data obtained from determinations of two or more independent transfections and analyzed by non-linear regression as described under methods. The 95% confidence intervals for each measurement are indicated in parenthesis. The binding of unlabeled agonists to β_2-AR was determined by competition with the antagonist 3 nM [^{3}H] DHA as the radioligand.

Asn322$^{7.49}$ of the conserved NPxxY motif on H7 through water mediated hydrogen bonds. Gly315$^{7.42}$ and Ser319$^{7.46}$ are also flanked by amino acids that have high sequence conservation within the amine subfamily of receptors (Trp313$^{7.40}$, Tyr316$^{7.43}$ and Asn318$^{7.45}$). As a result, these group conserved amino acids are strategically positioned between the ligand-binding site on the extracellular side of the receptor and the ionic lock on the cytoplasmic side of the receptor.

4.1. Gly315$^{7.42}$ functions as a molecular notch to stabilize Trp286$^{6.48}$ in an inactive position

Fig. 5A shows the close packing interaction of Gly315$^{7.42}$ with the indole ring of Trp286$^{6.48}$. These residues are 69% and 96% conserved in the amine receptor subfamily, respectively. We found that the conservative mutation of Gly315$^{7.42}$ to alanine exhibits agonist-independent cAMP production. The constitutive activity suggests that Gly315$^{7.42}$ is stabilizing Trp286$^{6.48}$ in an inactive conformation. This packing arrangement is similar to the packing of Ala295$^{7.42}$ against Trp265$^{6.48}$ in rhodopsin [21,22] where substitution of Ala295$^{7.42}$ with valine results in constitutive activity and leads to autosomal dominant congenital stationary night blindness [23]. A similarly close packing interaction between Ala291$^{7.42}$ and Trp252$^{6.48}$ is observed in the recent structure of the CXCR4 receptor, a member of the large subfamily of GPCRs that bind peptide ligands [24].

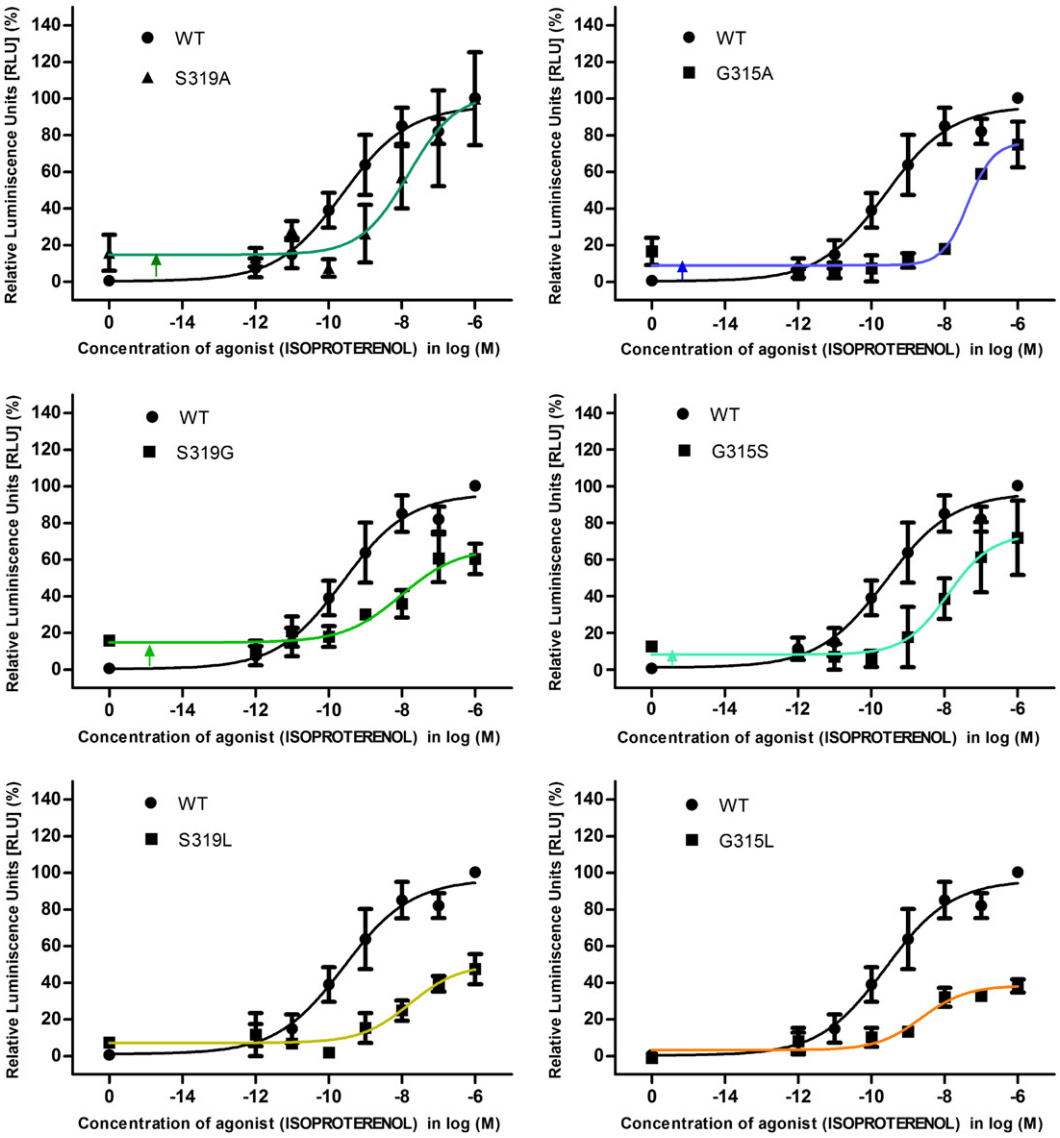

Fig. 3. Characterization of Gαs-mediated signaling of the wild-type and group-conserved helix H7 mutants of β_2-AR. Receptor activity was determined by measuring cAMP production using transiently transfected HEK293S cells. Shown are the basal (zero concentration) and agonist (isoproterenol) induced cAMP production of the wild-type receptor and the Gly315 and Ser319 mutants. Arrows indicate an increase in the basal activity of the mutants over the wild type receptor. The results are expressed as a percentage of the wild type β_2-AR activity, and are from at least three independent experiments performed in duplicate.

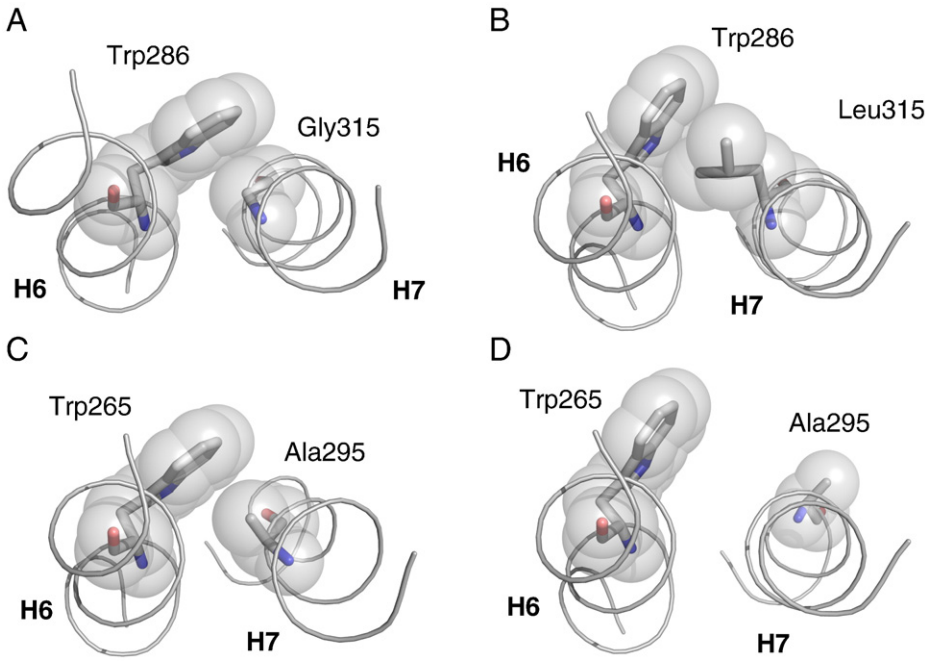

Fig. 4. Molecular packing of Gly315$^{7.42}$ and Leu315$^{7.42}$ in β_2-AR. (A) Crystal structure of β_2-AR (2RH1) showing van der Waals interaction of Trp286$^{6.48}$ and Gly315$^{7.42}$. (B) Homology model of G315L β_2-AR where the G315L β_2-AR sequence was threaded onto the structure of active opsin (3CAP) and the resulting structure was energy minimized with 1000 steps steepest descent. The inactive structure of rhodopsin (C) and the active structure of opsin (D) are shown for comparison.

Trp286$^{6.48}$ has been proposed to be part of the rotamer toggle switch involved in receptor activation [25], although the recent crystal structure of active opsin shows only a small displacement of Trp265$^{6.48}$ rather than a change in the side chain rotamer angle (see Fig. 4D). Evidence for large displacement of the Trp265$^{6.48}$ side chain in activated rhodopsin comes from NMR measurements showing the gain of a direct packing interaction between the retinal C19 methyl group and the Trp265$^{6.48}$ side chain, in concert with a loss of packing interactions with the retinal C18 and C20 methyl groups [21,26]. Substitution of larger residues at position 7.42 in the β_2-AR would be expected to displace the conserved tryptophan as suggested by computational studies of activated β_2-AR [27].

Gly315$^{7.42}$ is also in close contact with Cys285$^{6.47}$. Cysteine is highly conserved at position 6.47; mutation to serine in the β_2-AR shows normal ligand binding, but reduced activity [25,28], while mutation to threonine results in constitutive activity [25]. The mechanism of receptor activation in the C285T mutant may involve a steric clash of the β-branched threonine methyl group with Gly315$^{7.42}$ rather than a change in rotamer angle since the predominant conformer for threonine in α-helices is *gauche* + [25], which is the observed conformer for Cys285$^{6.47}$ in the carazolol-bound structure of β_2-AR. Moreover, the Cys285$^{6.47}$ side chain is more exposed to cysteine reactive reagents in a constitutively active mutant of β_2-AR [29] and upon agonist binding [30]. Together with our

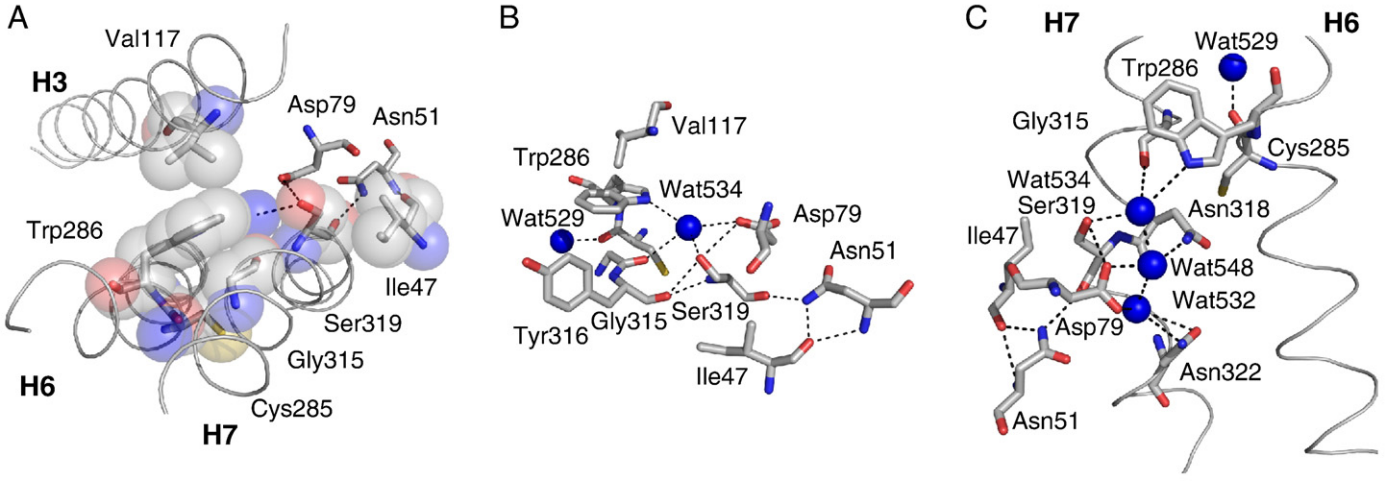

Fig. 5. Crystal structure of the β_2-AR in the region of Gly315$^{7.42}$ and Ser319$^{7.46}$. (**A**) Packing of Gly315$^{7.42}$ with Trp286$^{6.48}$. An increase in size of the amino acid at position 7.42 causes a steric clash with Trp286$^{6.48}$ on H6 and is predicted to displace the Trp286$^{6.48}$ side chain toward the extracellular surface. (**B**) Hydrogen bonding interactions of Ser319$^{7.46}$. The side chain hydroxyl group of Ser319$^{7.46}$ side chain is hydrogen bonded to the carboxyl C(O)OH of Asp79$^{2.50}$ (3.3 Å), to the backbone carbonyl of Tyr316$^{7.43}$ (3.1 Å) and to water 534 (3.1 Å), while the backbone carbonyl of Ser319$^{7.46}$ makes contact with the side chain NH of Asn51$^{1.50}$ (3.1 Å). Water 534 is shown hydrogen bonded to the backbone of Gly315$^{7.42}$. (**C**) Hydrogen bonding network connecting the indole NH of Trp286$^{6.48}$ to the amide side chain of Asn322$^{7.49}$. Water 534 in β_2-AR mediates hydrogen bonding between the Trp286$^{6.48}$ indole NH (3.48 Å), Ser319$^{7.46}$ CβOH (2.82 Å), Asn318$^{7.45}$ NH_2 (3.11 Å) and Gly315$^{7.42}$ (C=O) (3.38 Å). Water 534 is also hydrogen bonded to Wat548, which in turn is hydrogen bonded to Asn322 through Wat532. The same overall set of interactions appears to exist in the recent crystal structure of the CXCR4 receptor where cysteine occurs at position 7.46 and histidine occurs at position 7.45 [24]. The polar imidizole of histidine is located between the conserved indole ring of Trp6.48 and Cys7.46.

mutational results on Gly315$^{7.42}$, these cysteine accessibility studies are consistent with a more open structure in the active receptor being due to displacement of Trp286$^{6.48}$ from a molecular notch formed by Gly315$^{7.42}$.

4.2. Ser319$^{7.46}$ mediates hydrogen bonding interactions between Trp286$^{6.48}$ and Asn322$^{7.49}$

Ser319$^{7.46}$ is highly conserved (98%) in the amine subfamily and is in a key position between Asn51$^{1.50}$, Asp79$^{2.50}$, Trp286$^{6.48}$, and Asn322$^{7.49}$. These residues are all highly conserved across the class A GPCRs.

Both the backbone carbonyl and side chain hydroxyl groups of Ser319$^{7.46}$ form hydrogen bonds that have structural and functional importance. The Ser319$^{7.46}$ carbonyl does not form an *intra*-helical hydrogen bond due to its position one helix turn above Pro323$^{7.50}$ of the conserved NPxxY sequence. The backbone carbonyl of Ser319$^{7.46}$ forms an *inter*-helical hydrogen bond with the amide NH_2 functional group of Asn51$^{1.50}$, the most highly conserved residue in the class A GPCRs. Along with the backbone carbonyl at position 1.46, another group-conserved residue, this hydrogen bond functions to orient the conserved Asn51$^{1.50}$ side chain (Fig. 5B) [31].

The Ser319$^{7.46}$ side chain is in the center of a hydrogen-bonding network connecting Trp286$^{6.48}$ to Asn322$^{7.49}$. Asn322$^{7.49}$ is part of the conserved NPxxY motif on H7 that is involved in receptor activation. We found that the conservative mutation of Ser319$^{7.46}$ to alanine results in constitutive activity. Unlike the Gly315$^{7.42}$ to alanine mutation in β_2-AR and the Ala295$^{7.42}$ to valine mutation in rhodopsin that lead to constitutive activity, the S319A mutation does not increase the molecular volume of the group-conserved side chain, but rather removes its ability to hydrogen bond. Ser319$^{7.46}$ is hydrogen bonded to water 534, a conserved structural water within the transmembrane core of the receptor. In rhodopsin, water 2015 mediates interaction of Trp265$^{6.48}$, Ser298$^{7.45}$ and Tyr301$^{7.48}$. In both β_2-AR and rhodopsin, this conserved water is hydrogen bonded to several additional conserved water molecules that form a shell surrounding the side chain of Asn7.49. While the details of the structural changes between Trp6.48 and Asn7.49 have not been determined by crystallography or NMR spectroscopy in either the β_2-AR or rhodopsin, NMR measurements of rhodopsin show that displacement of Trp265$^{6.48}$ upon activation weakens the water-mediated hydrogen bond between the Trp265$^{6.48}$ indole NH and Asn302$^{7.49}$ amide side chain [21]. As a result, motion of Trp286$^{6.48}$ away from Gly315$^{7.42}$ in the β_2-AR upon agonist binding would be expected to alter the hydrogen bonding network illustrated in Fig. 5C.

The idea that conserved structural waters can act as allosteric regulators of GPCR function was introduced by Palczewski and colleagues [32]. These authors showed that the interior water molecules do not exchange with bulk solvent suggesting that structural waters represent an intrinsic part of the interfaces between helices H1, H2, H6 and H7. We propose that the structural waters in combination with the group-conserved residues on these helices (see Fig. 5A) create an interface that facilitates the rotation of H6 relative to H7 during activation.

4.3. Interplay between the three levels of amino acid conservation in class A GPCRs

Fig. 5 presents the core conserved transmembrane region of the β_2-AR to illustrate the interplay between the signature, group-conserved and subfamily specific amino acids. Trp286$^{6.48}$ is sandwiched between Gly315$^{7.42}$ and Val117$^{3.36}$. The residue at position 3.36 on H3 appears to play a role that parallels that of group-conserved residue 7.42 on H7. In rhodopsin, residue 3.36 is a glycine, which has very high conservation (95%) in the rhodopsin subfamily of receptors. Replacement of this glycine with larger residues results in dark activity of rhodopsin, where the activity is linearly correlated with the residue volume [33]. In a similar fashion, mutation of the residue at position 3.36 in receptors of the amine subfamily has been shown to modulate the receptor activity. The specific residue at this position in the 5HT4 and histamine H1 receptors has been associated with constitutive (basal) activity, and mutation can either increase or decrease this activity. For example, in the histamine H1 receptor substitution of the wild-type serine with cysteine or threonine increases activity, while substitution with alanine decreases activity [34]. Pardo and co-workers proposed that when Trp6.48 rotates upon ligand binding, Ser3.36 forms an interhelical hydrogen bond with Asn7.45 on H7 [34]. Asn7.45 has the second highest level of subfamily conservation in the amine receptors (92%). Threonine at position 3.36 has the highest constitutive activity and may be correlated with a preference for the $g+$ rotamer. In the 5HT4 receptor, the wild-type residue is threonine and substitution with alanine or serine decreases activity [35]. The role of the residue at position 3.36 extends beyond the amine and rhodopsin subfamilies to other class A GPCRs. Schwartz and colleagues concluded residue 3.36 often influences the function of the Trp6.48 rotamer toggle switch, but that it acts in a highly context-dependent manner [27]. In the gherlin peptide hormone receptor, mutation of the wild-type threonine residue at 3.36 to alanine was found to increase, rather than decrease, receptor activity [36]. Together, these results illustrate the interplay between the three levels of class A receptor conservation: signature conserved (Trp6.48, Asp2.50, Asn7.49), group-conserved (Cys6.47, Gly/Ala7.42, Ser/Ala7.46), subfamily conserved (Gly/Ser/Thr/Val3.36 and Asn7.45).

5. Conclusion

We show that the two group-conserved amino acid residues, Gly315$^{7.42}$ and Ser319$^{7.46}$ play important structural and functional roles in the β_2-AR. Gly315$^{7.42}$ stabilizes Trp286$^{6.48}$ in the inactive conformation, while Ser319$^{7.46}$ is in the center of a hydrogen bonding network that stretches from Trp286$^{6.48}$ to Asn322$^{7.49}$. The hydrogen-bonding interactions between Trp286$^{6.48}$ and Asn322$^{7.49}$ appear to be strongly coupled. The conservative mutation to alanine of either Gly315$^{7.42}$ or Ser319$^{7.46}$ results in an increase in the basal activity of the receptor. We propose that the group-conserved residues on H7 along with conserved structural waters create an extended interface between the transmembrane helices that facilitates the motion of H6 relative to H7 upon receptor activation. These changes would be encompassed within the global toggle switch mechanism proposed by Schwartz and colleagues [37].

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamem.2011.01.012.

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