

# Role of group-conserved residues in the helical core of $\beta_2$ -adrenergic receptor

Prashen Chelikani\*<sup>†</sup>, Viktor Hornak<sup>‡</sup>, Markus Eilers<sup>‡</sup>, Phillip J. Reeves<sup>§</sup>, Steven O. Smith<sup>‡</sup>, Uttam L. RajBhandary\*, and H. Gobind Khorana\*<sup>†¶</sup>

Departments of \*Biology and <sup>†</sup>Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139; <sup>‡</sup>Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY 11794; and <sup>§</sup>Department of Biological Sciences, University of Essex, Essex CO4 3SQ, United Kingdom

Contributed by H. Gobind Khorana, March 9, 2007 (sent for review February 8, 2007)

**G** protein-coupled receptors (GPCRs) belonging to class A contain several highly conserved (>90%) amino acids in their transmembrane helices. Results of mutational studies of these highly conserved residues suggest a common mechanism for locking GPCRs in an inactive conformation and for their subsequent activation upon ligand binding. Recently, a second set of sites in the transmembrane helices has been identified in which amino acids with small side chains, such as Gly, Ala, Ser, Thr, and Cys, are highly conserved (>90%) when considered as a group. These group-conserved residues have not been recognized as having essential structural or functional roles. To determine the role of group-conserved residues in the  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR), amino acid replacements guided by molecular modeling were carried out at key positions in transmembrane helices H2–H4. The most significant changes in receptor expression and activity were observed upon replacement of the amino acids Ser-161 and Ser-165 in H4. Substitution at these sites by larger residues lowered the expression and activity of the receptor but did not affect specific binding to the antagonist ligand dihydroalprenolol. A second site mutation, V114A, rescued the low expression of the S165V mutant. Substitution of other group-conserved residues in H2–H4 by larger amino acids lowered receptor activity in the order Ala-128, Ala-76, Ser-120, and Ala-78. Together these data provide comprehensive analysis of group-conserved residues in a class A GPCR and allow insights into the roles of these residues in GPCR structure and function.

rhodopsin | G protein-coupled receptors | helix packing | site-directed mutagenesis | molecular modeling

**G** protein-coupled receptors (GPCRs) contain seven transmembrane helices and mediate signal transduction in response to a wide variety of extracellular stimuli. The large GPCR family is subdivided into five classes on the basis of sequence conservation. Although the  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR) and the visual pigment rhodopsin are well-characterized members of class A GPCRs, rhodopsin is the only GPCR for which high-resolution structural information has been obtained by protein crystallography (1, 2) and by NMR spectroscopy (3, 4). The most extensively studied ligand-activated GPCR is  $\beta_2$ -AR, which mediates physiological responses to adrenaline and noradrenaline and, therefore, plays a significant role in the regulation of the cardiovascular system.

The class A GPCRs are made up of >1,000 unique sequences that are subdivided into several subclasses, which include the opsin, amine, peptide, and olfactory receptors. The class A receptors have been analyzed extensively in regard to structure and function with a focus on those amino acids with sequence identities of >90%, such as the conserved Asn at position 1.50<sup>||</sup> (100%) in H1, Asp at position 2.50 (93%) in H2, and the signature D/ERY and NPxxY motifs in helices H3 and H7, respectively. In addition to these polar residues, there are several conserved hydrophobic amino acids at positions 2.46 (98%), 4.50 (98%), 6.50 (98%), and 5.50 (91%). In all there are  $\approx$ 20 sites in class A GPCR sequences that have amino acid identities of >75%.

Recently, a second set of sites in the transmembrane helices has been identified in which small (Gly, Ala) and weakly polar (Ser, Thr, Cys) amino acids are highly conserved (>90%) when considered as a group (6). Structural analysis of helical membrane proteins has shown that small and weakly polar amino acids are highly likely to mediate helix–helix interactions. Thus, the group-conserved amino acids with small side chains in the class A GPCRs are likely to play a role in stabilizing receptor structure (e.g., in determining the proper fold of the protein) and in receptor function (e.g., in facilitating rigid body motion of the transmembrane helices that are thought to be involved in the activation mechanism).

Mutational studies on rhodopsin suggest that the group-conserved residues have both structural and functional roles. For example, the amino acids at positions 4.53 and 4.57 are small residues that are highly conserved in the class A GPCR family. These sites contain alanine in rhodopsin, and their mutation to leucine prevents folding to form a functional receptor (7). In contrast, the amino acid at position 3.36, glycine, is strictly conserved in the opsin subclasses of GPCRs. Mutations at this site to amino acids with larger molecular volumes lead to activity in the dark (8). In rhodopsin, the retinal chromophore intercalates between Gly-121 (3.36) in H3 and Trp-265 (6.48) in H6. The close packing of H3 and H6 locks the receptor off in the dark, and motion of Trp-265 (6.48) induced by retinal isomerization leads to motion of H6 and receptor activation. Thus, the small residue at position 3.36 plays both a structural and functional role in the opsin subclass of GPCRs.

In this paper, we focus on the roles of the group-conserved amino acids in H2, H3, and H4 in  $\beta_2$ -AR structure and function. The identification of the group-conserved residues in class A GPCRs (6), along with recent mutational studies of these residues in rhodopsin (P.J.R., M.E., P.C., V.H., U.L.R., H.G.K., and S.O.S., unpublished data), has led to the conclusion that transmembrane helices H2–H4 form a stable core, whereas receptor activation involves rigid body motion of helices H5–H7. Our strategy is to replace the group-conserved residues with amino acids containing both small and large molecular volumes and to study the effect of these replacements on receptor expression, activity, and binding of the antagonist dihydroalprenolol (DHA).

Author contributions: P.C., S.O.S., U.L.R., and H.G.K. designed research; P.C. and V.H. performed research; P.C., V.H., M.E., P.J.R., S.O.S., U.L.R., and H.G.K. analyzed data; and P.C., S.O.S., U.L.R., and H.G.K. wrote the paper.

The authors declare no conflict of interest.

Abbreviations:  $\beta_2$ -AR,  $\beta_2$ -adrenergic receptor; EL2, extracellular loop-2; DHA, dihydroalprenolol; GPCR, G protein-coupled receptor.

<sup>†</sup>To whom correspondence should be addressed. E-mail: khorana@mit.edu.

<sup>||</sup>The nomenclature used to describe amino acid positions in GPCRs follows the convention established by Ballesteros and Weinstein (5).

This article contains supporting information online at [www.pnas.org/cgi/content/full/0702024104/DC1](http://www.pnas.org/cgi/content/full/0702024104/DC1)

© 2007 by The National Academy of Sciences of the USA

**Table 1. Summary of ligand binding properties of wild-type  $\beta_2$ -AR and mutant receptors**

Receptor	Transmembrane helix	$K_d$ , nM	95% confidence intervals	$B_{max}$ , pmol/mg
Wild type		2.1	1.70–2.40	18 ± 2.2
A76S	II	1.2	0.92–1.39	12 ± 0.5
A76L	II	1.4	1.10–1.67	14 ± 0.4
A76V	II	1.4	1.00–1.28	9.1 ± 0.2
A78S	II	1.5	1.14–1.73	17 ± 1.1
A78L	II	0.9	0.47–1.39	9.0 ± 0.6
A78V	II	1.4	1.14–1.73	19 ± 0.4
S120A	III	2.3	1.77–2.96	28 ± 1.2
S120L	III	1.5	0.81–2.14	4.3 ± 0.3
S120V	III	1.6	0.58–2.62	4.9 ± 0.5
A128S	III	2.2	1.58–2.92	28 ± 1.4
A128L	III	3.2	1.00–5.39	4.8 ± 0.5
A128V	III	1.6	1.31–1.83	16 ± 0.5
S161A	IV	2.7	1.98–3.55	33 ± 2.0
S161L	IV	ND		ND
S161V*	IV	2.0	0.80–3.14	2.7 ± 0.4
S165A	IV	4.7	3.54–5.98	15 ± 0.8
S165L	IV	ND		ND
S165V	IV	1.2	0.68–1.71	6.7 ± 0.4

The values are expressed as the mean ± SE ( $n = 3$ –5 experiments). ND, not detected (no significant specific binding detected under the assay conditions). \*High nonspecific binding (15–20% of total binding).

In general, the conservative mutations, serine-to-alanine or alanine-to-serine, did not change activity of  $\beta_2$ -AR in response to agonist binding, and in many cases, these mutations actually increased the level of receptor expression. The most dramatic effects were observed for nonconservative mutations of Ser-161 and Ser-165, comprising an SxxxS motif in H4. Although both valine and leucine substitutions at positions 161 and 165 lower the expression and activity of the receptor, they do not affect binding of DHA. This result suggests a structural role for Ser-161 and Ser-165 in  $\beta_2$ -AR. These findings contrast with those observed for mutations of Ser-203 and Ser-207 in H5, which comprise another conserved SxxxS motif in the  $\beta$ -adrenergic receptor subfamily and are known to have a functional role in ligand binding (9, 10). Using mutation at a second site, V114A, we were able to rescue expression of the poorly expressed S165V mutant to wild-type level. In contrast to a previous report (9), we observe that Ser-120 on H3 can be mutated to alanine, and the resulting receptor is expressed at wild-type levels. Finally, replacement of other group conserved sites in H2–H4 with valine and leucine lowered receptor activity in the order Ala-128, Ala-76, Ser-120, and Ala-78.

## Results

**Expression and Ligand Binding Properties of Group-Conserved Mutants in H2–H4.** One main structural difference between aryloxypropranolamine  $\beta_2$ -AR antagonists, such as alprenolol, and aryloxyethanolamine  $\beta_2$ -AR agonists, such as isoproterenol, is that the distance between the aryl moiety and the amino group is shorter in the agonists, by a  $-\text{OCH}_2-$  linkage. In the current study, the ligand binding properties of the  $\beta_2$ -AR mutants were measured by using the antagonist DHA (Tables 1 and 2). The level of receptor expression is qualitatively gauged by immunoblots and quantified by saturation binding assays using DHA. In general, the binding of DHA to  $\beta_2$ -AR with mutations at the group-conserved sites was similar to the wild-type receptor. In fact, in contrast to the finding in a previous report (9), we observed that mutations of Ser-120 in H3 are tolerated with

**Table 2. Summary of ligand binding properties of single and compensatory mutant receptors**

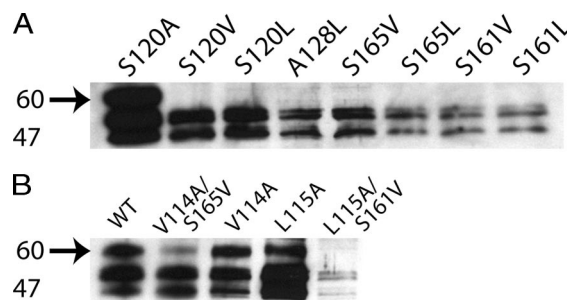
Receptor	Transmembrane helix	$K_d$ , nM	95% confidence intervals	$B_{max}$ , pmol/mg
Wild type		2.1	1.70–2.40	18 ± 2.2
V114A	III	19	17.0–21.1	39 ± 1.6
S165V/V114A	IV/III	9.0	8.64–9.38	17 ± 0.6
S165V	IV	1.2	0.68–1.71	6.7 ± 0.4
L115A	III	2.4	2.06–2.89	24 ± 1.2
S161V/L115A*	IV/III	4.8	0.81–8.90	4.5 ± 0.7
S161V*	IV	2.0	0.80–3.14	2.7 ± 0.4

The values are expressed as the mean ± SE ( $n = 3$ –5 experiments). \*High nonspecific binding (15–20% of total binding).

regard to expression (Fig. 1A) and the ability of the mutant receptors to bind ligand (Table 1). Only the S161L and S165L mutants lacked the ability to bind ligand in a specific manner. The S161V and the S165V mutant receptors were very poorly expressed (as reflected by the low  $B_{max}$  values in Table 1 and the weak intensity in the immunoblot analysis in Fig. 1A) and were used for the isolation of compensatory mutants (see below).

An interesting observation from the saturation binding data is that  $B_{max}$  is higher, in general, in the mutants containing small amino acid replacements. For example, the mutants S120A, A128S, S161A, V114A, and L115A show  $B_{max}$  values in the range of 24–39 pmol/mg, whereas the mutants A76S, A78S, and S165A show  $B_{max}$  values similar to that of wild-type  $\beta_2$ -AR. Immunoblotting showed heterogeneous expression of the wild-type and the mutant  $\beta_2$ -ARs, as indicated by the presence of three predominant bands in the molecular mass range of 45–65 kDa, with none of the low-expressing receptors producing the  $\approx 65$  kDa band (Fig. 1A). Photocrosslinking of  $\beta_2$ -AR expressed in COS-1 cells was previously used to show that the band at  $\approx 65$  kDa corresponds to the completely glycosylated receptor (11).

**G $\alpha_s$  Mediated Signaling of the Group-Conserved Mutants.** The effect of  $\beta_2$ -AR mutations on G protein activation was measured by isoproterenol-stimulated cAMP accumulation in HEK293S cells. Transiently transfected HEK293S cells were induced with 10  $\mu\text{M}$  (–)-isoproterenol for 30 min, and the G $\alpha_s$ -mediated cAMP production was measured as described (12). Fig. 2 shows that the agonist-induced activity of the group-conserved mutants containing small amino acid replacements (e.g., A76S, A128S) was either similar to or higher than that of the wild-type  $\beta_2$ -AR. In contrast, there was generally a loss of activity in mutants



**Fig. 1.** Immunoblot analysis of  $\beta_2$ -AR expressed in COS-1 cells. (A) S120A and low-expressing  $\beta_2$ -AR mutants ( $\approx 5$   $\mu\text{g}$  of solubilized membrane protein was loaded). (B) Wild-type  $\beta_2$ -AR, V114A, L115A, and the compensatory mutants ( $\approx 2.5$   $\mu\text{g}$  of solubilized membrane protein was loaded). The arrows indicate the position of the fully glycosylated receptor. Mobility of molecular mass standards (kDa) is indicated next to the gel.



tation, the double mutant S111G/S165V showed the phenotype characteristic of the single mutant S165V and not that of the wild-type receptor (data not shown). However, the double mutant V114A/S165V was expressed at wild-type levels (Table 2), and immunoblotting showed that a part of it migrated as a fully glycosylated receptor (Fig. 1B). Thus the V114A mutation compensates for the reduced expression level of the S165V mutant. The V114A mutation reduced the binding affinity of the receptor for DHA from a  $K_d$  of 2.1 nM for the wild-type receptor to 19 nM for the mutant. The double mutant V114A/S165V exhibited a  $K_d$  of 9 nM, a value that is intermediate between those found for the wild-type receptor and the V114A mutant. Thus, the S165V mutant in turn compensates, at least partially, for the decreased affinity of the V114A mutant for the antagonist DHA.

**Agonist Competition Assays.** Val-114 (3.33) is a highly conserved amino acid (96%) in the adrenergic receptor family ( $\alpha$  and  $\beta$ ) and is in close proximity to Ser-165 in the model of the ligand-free receptor. The V114A mutation decreased the affinity of  $\beta_2$ -AR for [ $^3$ H]DHA by 9-fold but sustained high-level expression (Table 1 and Fig. 1B). These observations are consistent with Val-114 having a functional role by forming part of the ligand-binding site for DHA and a structural role by being involved in close H3-H4 packing (Fig. 3). To investigate the possibility that Val-114 is important for ligand binding, we carried out competition radioligand binding assays using the catecholamine agonists, isoproterenol, epinephrine and norepinephrine, which differ only in the number of methyl substituents attached to the amine. The V114A mutation reduced by 200- to 300-fold the affinity for isoproterenol and epinephrine while causing only a 3-fold decrease in affinity for norepinephrine compared with the wild type  $\beta_2$ -AR (SI Table 3). These results suggest that Val-114 is present in the ligand-binding pocket of the receptor and influences the position of bound ligand (see Discussion). The weak affinity of the V114A mutant for isoproterenol (SI Table 3) likely explains the finding that the agonist-stimulated activity of the V114A mutant as measured by cAMP accumulation is 4-fold lower than that of the wild type  $\beta_2$ -AR under our assay conditions (Fig. 2D).

**Molecular Modeling of  $\beta_2$ -AR.** Molecular models of  $\beta_2$ -AR were constructed to facilitate structural comparison of rhodopsin and  $\beta_2$ -AR and to illuminate possible roles for the group-conserved sites in the two receptors. The sequence alignment is presented in SI Fig. 4. The structure of the ligand-free  $\beta_2$ -AR was based on homology with the crystal structure of rhodopsin, except for the second extracellular loop (EL2) that was constructed *de novo*. EL2 forms a part of the retinal binding site in rhodopsin and likely forms part of the ligand-binding site in the  $\beta_2$ -AR. However, there is virtually no homology between the EL2 sequences in these receptors. Noda *et al.* (11) concluded on the basis of alanine mutations and DTT sensitivity that two unique disulfide bonds constrain the position of EL2 in  $\beta_2$ -AR, one between Cys-106 and Cys-191 and the other between Cys-184 and Cys-190. Furse and Lybrand (15) found that a *de novo* model containing EL2 having these two disulfide links was favored over an EL2 model constructed on the basis of homology with rhodopsin. Fig. 3 shows the ligand-free  $\beta_2$ -AR structure. In A, we highlight the position of group-conserved residues Ala-78 (2.49), Ser-161 (4.53), and Ser-165 (4.57). This figure also shows the proximity of the H2-H4 core to the ligand-binding site and the positions of highly conserved binding site residues Val-114 (3.33) and Asp-113 (3.32) on H3. One of the functions of conserved Val-114 may be to orient the Asp-113 side chain for interaction with ligand. In B, we highlight the interactions of H3 with helices H5, H6, and H7, which are thought to move upon receptor activation. Group-conserved residues Ser-120 and Ala-128 are

oriented toward H7 and H5, respectively. A structural model of  $\beta_2$ -AR containing the bound antagonist alprenolol (SI Fig. 5) was based on the model of the ligand-free  $\beta_2$ -AR. The alprenolol was manually docked into the binding site; the orientation of the antagonist was guided primarily by position of the amine (near Asp-113) and the aromatic ring (near Ser-203 and Ser-207).

## Discussion

**Structural Role of the Group-Conserved Residues in the  $\beta_2$ -AR.** Identification of the group-conserved residues in class A GPCRs led to the conclusion that the transmembrane helices H1-H4 form a stable core containing the specific residues 1.46, 2.47, 2.49, 4.53, and 4.57 that mediate helix interactions within this core (6). In  $\beta_2$ -AR, residue 1.46 is an isoleucine, which is not a group-conserved amino acid, suggesting that the packing core involves only helices H2-H4. Fig. 3A illustrates that close packing of H2, H3, and H4 in  $\beta_2$ -AR allows an interhelical hydrogen bond to form between Trp-158 (4.50) and Ser-74 (2.45). These residues are highly conserved (97% and 88%, respectively) in the amine subfamily of class A GPCRs. In rhodopsin, substitution with leucine at each of the three group-conserved sites within the H2-H4 core (i.e., positions 2.49, 4.53, and 4.57) is detrimental to chromophore regeneration with 11-*cis* retinal and formation of a stable pigment. Interestingly, substitution of position 2.47 in the H1-H2 interface with leucine is tolerated in rhodopsin without effecting the ability to form the rhodopsin pigment or the conversion to the active metarhodopsin II intermediate (P.J.R., M.E., P.C., V.H., U.L.R., H.G.K., and S.O.S., unpublished results). In  $\beta_2$ -AR, replacement of Ala-78 (2.49) with either serine or valine is tolerated. However, A78L mutation decreases the level of expression as illustrated by a low  $B_{max}$  value, consistent with the idea that Ala-78 contributes to the H2-H4 core packing. The observation that all of the expressed and folded mutants at positions 2.47 and 2.49 had similar affinities for [ $^3$ H]DHA eliminates a role for these residues in ligand binding.

Recent studies in membrane proteins have shown that sequence motifs containing small (Gly, Ala) and weakly polar (Ser, Thr, Cys) residues can mediate the interaction of  $\alpha$ -helical transmembrane helices. In  $\beta_2$ -AR, group-conserved residues Ser-161 and Ser-165 in H4 form an SxxxS packing motif; however, the structural or functional significance of this motif is unknown. Our mutational studies show that the  $\beta_2$ -AR mutants containing the smaller amino acids (i.e., alanine) at positions Ser-161 (4.53) and Ser-165 (4.57) are expressed at high levels and bind ligand with near wild-type affinity, whereas substitution with valines lowers the expression of the mutant receptors. The bulkier amino acid leucine is not tolerated well at either position, with S161L and S165L mutants showing no significant specific binding to the antagonist DHA. Furthermore, the successful rescue of the poorly expressed S165V mutant by the V114A mutation supports the argument that helices H3 and H4 are in close proximity in this region of  $\beta_2$ -AR. These results suggest that Ser-161 and Ser-165 in  $\beta_2$ -AR have a structural role in the tight packing of helices H3 and H4.

**Comparison of Roles of SxxxS Motifs in the  $\beta_2$ -AR.** It has been previously shown that Ser-203 (5.42), Ser-204 (5.43), and Ser 207 (5.46) conserved in H5 of  $\beta_2$ -AR are directly involved in agonist binding and receptor activation (9, 10). Our model of the inactive receptor (ligand-free or alprenolol-bound) places Ser-203 and Ser-207 in the interface with transmembrane helix H3, hydrogen bonding with Thr-118 on H3 and Thr-164 on H4 (SI Fig. 5). This placement is consistent with the observation that SxxxS motifs often mediate helix interactions by forming interhelical hydrogen bonds (13). It is also consistent with extensive mutational studies of Ser-207 by Ambrosio *et al.* (16) suggesting that this serine is *not* exposed to the polar binding crevice in the ligand-

free receptor. We propose that the initial binding of the agonist to  $\beta_2$ -AR leads to rotation of H5 so that the two serines in H5 are exposed to the ligand-binding site and interact with the hydroxyl-groups in the catechol ring. The same mechanism appears to be present in the visual pigment rhodopsin. Ser-207 in  $\beta_2$ -AR corresponds to His-211 in rhodopsin, whereas Thr-118 (3.37) corresponds to Glu-122 in rhodopsin. Glu-122 in rhodopsin forms an interhelical hydrogen bond with the backbone carbonyl of His-211 in the inactive receptor. In the active receptor, H5 changes orientation and a new hydrogen bonding interaction is formed between the side chains of Glu-122 and His-211 (3).

The two conserved serines on H4 (Ser-161 and Ser-165), which also correspond to an SxxxS motif, do not appear to play functional roles in the  $\beta_2$ -AR. Rather, they are involved primarily in helix packing and thereby maintaining the stability of the protein. Thus, the two conserved SxxxS motifs (S203xxxS207 and S161xxxS165) have different roles in the  $\beta_2$ -AR.

**Role of Group Conserved Residues in Receptor Activation.** Transmembrane helix H3 in rhodopsin and  $\beta_2$ -AR plays a central role in receptor activation (Fig. 3). In addition to the group-conserved residues that facilitate the formation of the H2–H4 core, there are group-conserved residues on H3 (Ser-120 and Ala-128) that are oriented away from the H2–H4 core toward H5–H7. Helices H5–H7 are generally thought to change orientation upon GPCR activation and consequently, the group-conserved sites on H3 facing these helices may be involved in receptor function.

Ser-120 (3.39) is one of the highly conserved serines in the amine family (100% conserved among 387 sequences of amine family receptors). However, its role in receptor function was not known. Strader *et al.* (9) showed that the S120A mutation in hamster  $\beta_2$ -AR resulted in no protein detectable by immunoblot analysis. To elucidate the role of Ser-120 (3.39) in  $\beta_2$ -AR structure-function, we mutated it to alanine, leucine, and valine. Surprisingly, S120A behaves exactly like wild-type  $\beta_2$ -AR in terms of ligand binding and the amount of protein observed on immunoblots. S120L and S120V are also tolerated based on the binding of the antagonist [<sup>3</sup>H]DHA, although the levels of receptor expression are 4- to 5-fold lower than that of the wild-type  $\beta_2$ -AR (Table 1). Thus, Ser-120 (3.39) in  $\beta_2$ -AR might be serving a role similar to that of Ala-124 rather than that of Gly-121 in rhodopsin, in which the A124L mutant is tolerated but not the G121L mutant. In our ligand-free  $\beta_2$ -AR model (Fig. 3B), Ser-120 is situated in the interface with H6 and H7. The serine side chain is in close proximity to Phe-282 on H6 and also interacts with Asn-318 on H7. Mutation of Phe-282 in  $\beta_2$ -AR has been shown to promote constitutive activity of the receptor (17), whereas Asn-318 is predicted to interact with Trp-286 (6.48). Motion of tryptophan at position 6.48 is generally thought to be associated with receptor activation (4, 18). Furthermore, Ser-120 in  $\beta_2$ -AR is present in the part of H3, which is also referred to as the “switch region,” which was suggested to be involved in active state isomerization (19). Not surprisingly, our results from the cAMP assays suggest that the Ser-120 mutants display a slightly higher level of agonist-independent basal activity compared with the wild type  $\beta_2$ -AR, however, the mechanism underlying this slight increase in activity needs further study.

Ala-128 (3.47) in  $\beta_2$ -AR corresponds to Ala-132 in rhodopsin and is 1 aa away from the highly conserved E/DRY motif at the cytoplasmic end of H3 in GPCRs. In both receptors, mutation of alanine to serine, valine, and leucine are well tolerated, although the A128L  $\beta_2$ -AR mutant shows reduced expression levels. Interestingly, both A128V and A128L mutants show very low levels of agonist-induced activity (Fig. 2C). In our ligand-free  $\beta_2$ -AR model, Ala-128 (3.47) is present in the H3–H5 interface positioned between Val-214 and Val-218 on H5. As indicated above, it is thought

that agonist binding results in motion of H5. Our mutational data suggest that the larger side chains at this position (3.47) might prevent H5 from adopting an active orientation.

**Role of Val-114 (3.33) in Ligand Binding.** Val-114 (3.33) in  $\beta_2$ -AR is found to be highly conserved (96%) in the adrenergic receptor family (including the  $\alpha$  and  $\beta$  receptors) and is at a critical position relative to H4, H5, and the ligand-binding site(s) (Fig. 3). Val-114 (3.33) is located between Asp-113 (3.32), an amino acid essential for electrostatic interaction with the amine-group common to the ligands in the amine receptor subfamily (20), and the serine residues of the two conserved SxxxS motifs. As a result, the side chain of Val-114, is positioned to be in van der Waals contact with the bound ligand. On the basis of the results presented above, the methyl groups on the receptor agonists (SI Table 3) contribute to the binding energy of the ligand in the wild-type receptor. Mutation of Val-114 to alanine decreases agonist binding with the largest effect being on the ligands with amine methyl groups. Given the central position of Val-114, the V114A mutation must change the structure of the ligand-binding site. The methyl groups on the amine ligand may directly interact with Val-114 in the wild-type receptor or the  $\beta$ -branched side chain of Val-114 may orient the Asp-113 side chain. It is also possible that Val-114 may serve to orient the ligand to maximize hydrophobic interactions with other nonpolar amino acids and hydrogen bonding contacts with polar amino acids in the binding site. For instance, the low activity of the V114A mutant with bound isoproterenol may reflect the loss of interaction with the conserved serines on H5. Interestingly, we find that the double mutant S165V/V114A is able to rescue, at least partially, the affinity of  $\beta_2$ -AR for the antagonist DHA.

Together, the results presented above indicate that an understanding of the mechanism of activation of the  $\beta_2$ -AR and other class A GPCRs will require knowledge of the interplay between highly conserved signature residues, the group-conserved residues, and those residues that are specific to a receptor subfamily or type of ligand.

## Methods

Synthetic oligonucleotides were purchased from Invitrogen (Carlsbad, CA). The  $\beta_2$ -AR antagonists, [<sup>3</sup>H]DHA and [<sup>3</sup>H]CGP 12177, were purchased from Amersham (Little Chalfont, U.K.). Protease inhibitors and common chemicals were purchased either from Sigma (St. Louis, MO) or Invitrogen. Restriction enzymes were purchased from NEB (Boston, MA). Buffers used were as follows: PBS buffer, 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.4); Buffer A (lysis buffer), 10 mM Tris·HCl, pH 7.4, containing protease inhibitors (1 mM EDTA, 10  $\mu$ g/ml benzamide, 10  $\mu$ g/ml leupeptin, 20  $\mu$ g/ml soybean trypsin inhibitor, 5  $\mu$ g/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride); Buffer B (storage buffer), 50 mM Tris·HCl, pH 7.4, 12.5 mM MgCl<sub>2</sub>, containing protease inhibitors as in Buffer A; Buffer C (binding buffer), 75 mM Tris·HCl, pH 7.4, 12.5 mM MgCl<sub>2</sub>, containing protease inhibitors as in Buffer A.

**Construction of Mutant  $\beta_2$ -AR Genes.** Amino acid substitutions were introduced into the synthetic  $\beta_2$ -AR gene carried by the pMT4 expression vector (21) by using the Quick-Change mutagenesis kit (Stratagene, La Jolla, CA). Compensatory mutations for the S161V and S165V mutants were introduced by using the S161V or S165V mutant, respectively, as the template DNA. DNA sequences of the mutated genes were verified by dideoxynucleotide chain-termination sequencing (MIT Biopolymers Laboratory, Cambridge, MA).

**Cell Culture and Immunoblot Analysis.** The wild-type  $\beta_2$ -AR gene was expressed in COS-1 cells by using a DEAE-dextran-based

transient transfection method (22). For transient transfection of HEK293S cells, the plasmid pMT4 was cotransfected with pRSVTag plasmid, a gift from Jeremy Nathans (The Johns Hopkins University, Baltimore, MD), and Lipofectamine 2000 (Invitrogen) mediated transfection was used as described by the manufacturer. Membranes were prepared as described in ref. 21. The protein concentration in the resuspended membrane pellet was determined by using a modified DC protein assay kit from Bio-Rad Laboratories (Hercules, CA). Solubilized protein (2.5 or 5  $\mu\text{g}$ ) was resolved by 10% SDS/PAGE and electroblotted onto a nitrocellulose membrane.  $\beta_2$ -AR was visualized by immunodetection with the monoclonal antibody rho-1D4.

**Radioligand Binding Assays.** These were carried out as previously described (21). Competition binding assays were performed by using 2 nM [ $^3\text{H}$ ]DHA and different concentrations of unlabeled agonists ( $10^{-2}$  to  $10^{-9}$  M) and the reactions were carried out for 2 h at room temperature. Binding was terminated by filtering under vacuum on GF/A filters (Millipore, Bedford, MA). Filter-bound radioactivity was measured by using a liquid scintillation counter. Equilibrium dissociation constants ( $K_d$ ) were determined from saturation isotherms. Radioligand binding data obtained from competition curves were analyzed by nonlinear regression analysis to determine the  $\text{EC}_{50}$  values and  $K_i$  values by using PRISM software version 4.03 (GraphPad Software, San Diego, CA).

**cAMP Assays.** Functional characterization of  $\beta_2$ -AR was carried out by using a commercially available cAMP assay system

(Amersham Biosciences) following the directions supplied by the manufacturer, other details are as described in ref. 12. The cAMP values were normalized to the total membrane protein in each assay.

**Molecular Modeling of the  $\beta_2$ -Adrenoreceptor.** A homology model of  $\beta_2$  adrenergic receptor was constructed by using Modeller (version 8v2) (23). The high-resolution (2.2 Å) crystal structure of rhodopsin (1) was used as a template with the sequences (Opsd\_bovin and Adrb2\_mesau) aligned according to GPCR database (<http://www.gpcr.org/7tm>) (24). The EL2 was built *de novo* by using loop modeling facility in Modeller (24) incorporating restraints due to two disulfide bonds (26). The resulting structure was further refined by the use of the all-atom force-field ff94 (27) and Amber simulation package by running short (200 ps) molecular dynamics with weak positional restraints (0.5 kcal/mol per Å) on  $\alpha$ -carbons in the transmembrane region, followed by complete minimization. Alprenolol was manually docked into the binding site and refined by short molecular dynamics (100 ps) and minimization.

The assistance of Ms. Judy Carlin in the preparation of this manuscript is gratefully acknowledged. This work was supported by National Institutes of Health Grants EY11716 (to H.G.K.) and GM41412 (to S.O.S.) and National Science Foundation Grant EIA-0225609 (to H.G.K.). P.C. is supported by an American Heart Association Post-doctoral fellowship.

- Okada T, Sugihara M, Bondar AN, Elstner M, Entel P, Buss V (2004) *J Mol Biol* 342:571–583.
- Li J, Edwards PC, Burghammer M, Villa C, Schertler GF (2004) *J Mol Biol* 343:1409–1438.
- Patel AB, Crocker E, Reeves PJ, Getmanova EV, Eilers M, Khorana HG, Smith SO (2005) *J Mol Biol* 347:803–812.
- Patel AB, Crocker E, Eilers M, Hirshfeld A, Sheves M, Smith SO (2004) *Proc Natl Acad Sci USA* 101:10048–10053.
- Ballesteros JA, Weinstein H (1995) *Methods Neurosci* 25:366–428.
- Liu W, Eilers M, Patel AB, Smith SO (2004) *J Mol Biol* 337:713–729.
- Hwa J, Garriga P, Liu X, Khorana HG (1997) *Proc Natl Acad Sci USA* 94:10571–10576.
- Han M, Lin SW, Smith SO, Sakmar TP (1996) *J Biol Chem* 271:32330–32336.
- Strader CD, Candelore MR, Hill WS, Sigal IS, Dixon RA (1989) *J Biol Chem* 264:13572–13578.
- Liapakis G, Ballesteros JA, Papachristou S, Chan WC, Chen X, Javitch JA (2000) *J Biol Chem* 275:37779–37788.
- Noda K, Saad Y, Graham RM, Karnik SS (1994) *J Biol Chem* 269:6743–6752.
- Kim JM, Hwa J, Garriga P, Reeves PJ, Rajbhandary UL, Khorana HG (2005) *Biochemistry* 44:2284–2292.
- Adamian L, Liang J (2002) *Proteins* 47:209–218.
- Stojanovic A, Hwang I, Khorana HG, Hwa J (2003) *J Biol Chem* 278:39020–39028.
- Furse KE, Lybrand TP (2003) *J Med Chem* 46:4450–4462.
- Ambrosio C, Molinari P, Fanelli F, Chuman Y, Sbraccia M, Ugur O, Costa T (2005) *J Biol Chem* 280:23464–23474.
- Chen S, Lin F, Xu M, Riek RP, Novotny J, Graham RM (2002) *Biochemistry* 41:6045–6053.
- Shi L, Liapakis G, Xu R, Guarnieri F, Ballesteros JA, Javitch JA (2002) *J Biol Chem* 277:40989–40996.
- Perez DM, Karnik SS (2005) *Pharmacol Rev* 57:147–161.
- Strader CD, Sigal IS, Register RB, Candelore MR, Rands E, Dixon RA (1987) *Proc Natl Acad Sci USA* 84:4384–4388.
- Chelikani P, Reeves PJ, Rajbhandary UL, Khorana HG (2006) *Protein Sci* 15:1433–1440.
- Oprian DD, Molday RS, Kaufman RJ, Khorana HG (1987) *Proc Natl Acad Sci USA* 84:8874–8878.
- Marti-Renom MA, Stuart AC, Fiser A, Sanchez R, Melo F, Sali A (2000) *Annu Rev Biophys Biomol Struct* 29:291–325.
- Horn F, Bettler E, Oliveira L, Campagne F, Cohen FE, Vriend G (2003) *Nucleic Acids Res* 31:294–297.
- Fiser A, Do RK, Sali A (2000) *Protein Sci* 9:1753–1773.
- Dohlman HG, Caron MG, Deblasi A, Frielle T, Lefkowitz RJ (1990) *Biochemistry* 29:2335–2342.
- Cornell WD, Cieplak P, Bayly CI, Gould IR, Merz KM, Ferguson DM, Spellmeyer DC, Fox T, Caldwell JW, Kollman PA (1995) *J Am Chem Soc* 117:5179–5197.