Molecular Basis of Cannabinoid CB1 Receptor Coupling to the G Protein Heterotrimer Gαiβγ

IDENTIFICATION OF KEY CB1 CONTACTS WITH THE C-TERMINAL HELIX α5 OF Gαi*

Received for publication, May 27, 2013, and in revised form, September 17, 2013 Published, JBC Papers in Press, October 3, 2013, DOI 10.1074/jbc.M113.489153

Joong-Youn Shim†, Kwang H. Ahn§, and Debra A. Kendall§

From the †J. L. Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, North Carolina 27707 and the §Department of Pharmaceutical Sciences, University of Connecticut, Storrs, Connecticut 06269

Background: The molecular basis of CB1 coupling to its cognate G protein is unknown.

Results: Using an approach combining mutagenesis and molecular dynamics simulations, we identified CB1 residues critical for G protein signaling.

Conclusion: Tight interactions between CB1 and the C-terminal helix α5 of Gαi are crucial for G protein signaling.

Significance: This is the first reported molecular description of CB1 receptor coupling at the receptor-Gi interface.

The cannabinoid (CB1) receptor is a member of the rhodopsin-like G protein-coupled receptor superfamily. The human CB1 receptor, which is among the most expressed receptors in the brain, has been implicated in several disease states, including drug addiction, anxiety, depression, obesity, and chronic pain. Different classes of CB1 agonists evoke signaling pathways through the activation of specific subtypes of G proteins. The molecular basis of CB1 receptor coupling to its cognate G protein is unknown. As a first step toward understanding CB1 receptor-mediated G protein signaling, we have constructed a ternary complex structural model of the CB1 receptor and Gi heterotrimer (CB1-Gi), guided by the x-ray structure of the β2-adrenergic receptor (β2-AR) in complex with Gs (β2-AR-Gs), through 824-ns duration molecular dynamics simulations in a fully hydrated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine bilayer environment. We identified a group of residues at the juxtaparamembrane regions of the intracellular loops 2 and 3 (IC2 and IC3) of the CB1 receptor, including Ile-218, Tyr-224, Asp-338, Arg-340, and Thr-344, as potential key contacts with the extreme C-terminal helix α5 of Gαi. Ala mutations of these residues at the receptor-Gi interface resulted in little G protein coupling activity, consistent with the present model of the CB1-Gi complex, which suggests tight interactions between CB1 and the extreme C-terminal helix α5 of Gαi. The model also suggests that unique conformational changes in the extreme C-terminal helix α5 of Gα play a crucial role in the receptor-mediated G protein activation.

A superfamily of human G protein-coupled receptors (GPCRs) includes more than 800 members (1), among which the rhodopsin class GPCR is the largest with ~680 members (2). They are known to be some of the most important drug targets (3). A GPCR is coupled to its cognate heterotrimeric Gαβγ protein, the Gα subunit of which is composed of two domains, a Ras-like nucleotide-binding domain (GαRs) that interacts not only with the receptor but also with the Gβ subunit and an α-helical domain (GαAH) that covers the bound nucleotide on GαRs (4). When a GPCR is activated by an appropriate signal, it binds the G protein and catalyzes the release of guanosine diphosphate (GDP) from the nucleotide-binding pocket. The nucleotide-free Gα binds guanosine triphosphate (GTP), and the resulting Gα-GTP dissociates from the receptor and Gβγ. The freed G protein subunits regulate adenylate cyclase, ion channels, phospholipase C, or the guanine nucleotide exchange factor RhoGEF activity (5). The Gα protein hydrolyzes the GTP to GDP and reassociates with the βγ dimer and the receptor to complete a cycle of G protein activation (6). In parallel with G protein signaling, a portion of activated receptors is simultaneously phosphorylated by GPCR kinase and binds to β-arrestin, initiating desensitization, internalization, and β-arrestin signaling (7, 8).

Due to the nature of their structural dependence on intact cell membranes, elucidation of the function of integral membrane GPCRs has been hampered by the lack of high quality structural data. Recent publication of the x-ray crystal structures of some GPCRs has helped greatly to remedy this situation. With early x-ray structures of GPCRs in the inactive state, the overall transmembrane (TM) topology indicative of common structure and functional elements of GPCRs (9) was found to be conserved (for a review, see Ref. 10). With more recent x-ray GPCR structures in the active state, we now have a general view of GPCR activation, where an intracellular (IC) opening of the sixth TM helix (TM6) of the activated receptor is important for G protein interaction (for a review, see Ref. 11 and references therein). Moreover, the x-ray structure of the active β2-adrenergic receptor (β2-AR) in complex with the nucleotide-free active state of Gαi (β2-AR-Gi) (12) allows us to have a general view of the molecular assembly of the GPCR-G protein complex.

* The abbreviations used are: GPCR, G protein coupled receptor; HU210, (−)-11-hydroxydihydrolopetaly-Δ5-tetrahydrocannabinol; GuRs, Ras-like nucleotide-binding domain of Gαi; GαAH, α-helical domain of Gαi; TM, transmembrane; H8, TM helix 8; IC, intracellular; ICN, intracellular loop n; EC, extracellular; β2-AR, β2-adrenergic receptor; GtCT, Gt C-terminal peptide; MD, molecular dynamics; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PAL, palmitoyl moiety; RMSD, root mean square deviation; GTPγS, guanosine 5′-O-(thiotriphosphate).
protein complex, providing critical structural insight into how GPCRs interact with G proteins during the G protein activation cycle. Compared with the active state of the β2-AR alone (13), the receptor structure in the β2-AR-Gi complex shows little change in the TM bundle but some noticeable changes in the IC region. These are an additional outward movement (~3 Å) of the IC end of TM6 and a helical extension of TM5 toward the IC face in response to the coupled G protein. This provides a tight interaction especially with the extreme C terminus of Ga at the end of helix αC of Ga, the segment important for the receptor-mediated G protein activation (6, 14). One striking molecular feature in the x-ray structure of the β2-AR-Gi complex is that GaAHH is completely displaced from GaRas and moves in a direction toward the N terminus of Ga near the IC membrane surface. Such displacement was predicted from the x-ray structure of Ga (4) and experimentally confirmed by site-directed spin labeling and deuterium exchange mass spectroscopy studies (15). GPCR-mediated GDP-GTP exchange, where GDP release is the rate-determining step (16), is the key event in the G protein activation cycle. Despite the x-ray structure of the β2-AR-Gi complex (12), it remains uncertain how the tightly bound GDP is released from the inactive G protein as a result of the binding of the activated receptor.

The human CB1 (brain cannabinoid) receptor is associated with neurons in the brain, spinal cord, and the peripheral nervous system (17), where it primarily activates the pertussis toxin-sensitive inhibitory Gi/o protein and is among the most expressed receptors in the brain (18). It has been implicated in several disease states, including drug addiction, anxiety, depression, obesity, and chronic pain (19). CB1 exhibits modest basal activity in the absence of ligand. Nonspecific binding was determined with 100 mM GDP, and 0.1% BSA. The basal GTPγS binding was measured in the presence of 1 μM unlabeled ligand. Reactions were terminated by filtration with a Brandel cell harvester through Whatman GF/C filter paper (Brandel Inc., Gaithersburg, MD) followed by four washes with ice-cold TME buffer to remove unbound radioactivity. Radioactivity was measured by liquid scintillation counting.

**GTPγS Binding Assay—**Approximately 5 μg of membrane preparations from HEK293 cells expressing CB1 receptors were incubated for 60 min at 30 °C in a total volume of 500 μl of GTPγS binding assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 0.2 mM EGTA, and 100 mM NaCl) with unlabeled CP55940 (147.9 Ci/mmol; PerkinElmer Life Sciences) or [3H]SRI141716A (43 Ci/mmol; PerkinElmer Life Sciences) in a total volume of 200 or 500 μl of TME buffer (25 mM Tris-HCl, 5 mM MgCl2, and 1 mM EDTA, pH 7.4) containing 0.1% fatty acid-free bovine serum albumin. At least nine radiolabeled-ligand concentrations were used to determine Kd values of the receptors. Nonspecific binding was determined in the presence of 1 μM unlabeled ligand. Reactions were terminated by filtration with a Brandel cell harvester through Whatman GF/C filter paper (Brandel Inc., Gaithersburg, MD) followed by four washes with ice-cold TME buffer to remove unbound radioactivity. Radioactivity was measured by liquid scintillation counting.

**Data Analysis—**All ligand binding assays and GTPγS binding assays were carried out in duplicate. Data are presented as the mean ± S.E. value except for EC50 values, which are the median

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3 The Ballesteros-Weinstein numbering (94) is used (in superscript) for the CB1 receptor to indicate the relative position of amino acid residues within the TM helical bundle; for loop residues, only the loop positions are indicated. G protein residues are also indicated by superscript.

4 Because the CB1 mutational data are cross-species (e.g. human and mouse), the numbering scheme for mutated residues is translated into the numbering that would apply in humans.
with the corresponding 95% confidence limits, from at least three independent experiments. For the saturation radioligand binding assay, the $K_d$ and $B_{\text{max}}$ values were calculated by non-linear regression using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). $E_{\text{cat}}$ values for the GTPγS binding assays were calculated using a sigmoidal dose-response relationship. Statistical significance between the wild-type and mutant receptors was assessed by analysis of variance, followed by Bonferroni’s post hoc test. $p$ values of $<0.05$ were considered to be statistically significant.

**Structural Model of the CB1 Receptor**—We chose the previously published active state model of the CB1 receptor (27) as a starting structure of CB1. In this CB1 receptor model, Glu-133 was neutralized, whereas Asp-163 remained charged, because the charged Asp-163 appears to be required for receptor activation in many diffusible ligand GPCRs (28). Similarly, the side chain of Asp-213 of the CB1 receptor was neutralized, because the neutralization of the corresponding residue in $\beta_2$AR and rhodopsin is crucial for receptor activation and G protein interaction (29, 30). All other ionizable residues were in their ionization state at physiological conditions. For the N-terminal end, the first 10 residues from Asn-112 at the start of TM1 were retained. For the C-terminal end, the palmitoyl moiety (PAL) coordinated to Cys-415 was included. An acetyl group and an N-methyl group were attached to the N terminus and the C terminus, respectively, of the CB1 receptor. Briefly, the active state model of the CB1 receptor (27) was obtained by a smooth conversion of the receptor from an early stage of the cannabionoid agonist (−)-11-hydroxydihydropyridine-6-tetrahydrocannabinol (HU210)-bound state (31) to the active form using some of the key molecular features of the active state of GPCRs (13, 32–34). We created five sets of distances between four residues at similar positions; for the IC half of TM3, TM5, TM6, and TM7 in the x-ray structure of the active state for $\beta_2$AR (13) and performed the distance-constrained MD simulations. To avoid any abrupt changes in the structure, we obtained the targeted distances over five short distance-constrained MD simulations (3 ns each) using adaptive biasing force (35). When the targeted distances were reached, we gradually released the applied force ($k = 100$) by scaling down the force by 80% over 20 short simulations (~5 ns each). We finally performed the simulation without any constraint for over 150 ns until the protein structure was fully converged.

Considering that IC3 of active GPCRs appears to be highly structured, forming parallel TM5 and TM6 at the IC end (13, 36), we refined the resulting active state model of the CB1 receptor to build a highly helical IC3 structure using the secondary structural information from an NMR study of the peptide (Lys-300 to Thr-344) corresponding to IC3 of the CB1 receptor (37). We chose one of the snapshots of the CB1 receptor model near the end of the simulation, where the helical structure of TM5 was extended to Lys-315 of the N-terminal region of IC3 with a kink at Ala-301, and performed a constrained MD simulation by applying torsional constraints of an ideal helix ($\phi = -57^\circ$ and $\psi = -47^\circ$) for (i) Lys-300-His-304 to correct the helical gap at Asn-112, (ii) Ser-316 and Ile-317 to extend TM5, and (iii) Gln-334-Ile-339 to extend TM6. During 25 ns of the simulation, the initially applied constraints ($k = 100$) were gradually released. We continued the simulation without constraint for ~80 ns. The final snapshot from the simulation was us ed as the starting structure for the construction of a model of the CB1-Gi complex. All of the above mentioned simulations of the receptor embedded in a fully hydrated POPC lipid bilayer were performed at 310 K in the constant pressure (NPT) ensemble. The detailed setup of the simulations is described under “Simulations.”

**Structural Model of Gi**—We chose the x-ray structure of the GDP-bound inactive state of Ga$_i$β$_i$γ$_i$ (Protein Data Bank code 1GP2) (38) as a starting structure and made the following protein modifications on the Ga and G$_i$ proteins. For Ga$_i$, (i) we manually added the missing residues Cys-3$^{\text{Gai}}$ and Thr-4$^{\text{Gai}}$ to the N-terminal end residue Leu-5$^{\text{Gai}}$ in preparation for lipid modification, because it is known that the N-terminal Cys-3$^{\text{Gai}}$ is myristoylated or palmitoylated in the lipid bilayer (39); (ii) we replaced Ala-98$^{\text{Gai}}$ by a Ser residue to generate the human Ga sequence; and (iii) we also added the extreme C-terminal segment (Lys-349$^{\text{Gai}}$–Phe-354$^{\text{Gai}}$), which was missing in the x-ray structure (38), using the coordinates of the corresponding segment (Lys-345–Phe-350) in the x-ray structure of the G$_i$ C-terminal peptide (G$_i$CT) (Protein Data Bank code 3DBQ) (36). For G$_i$γ, we added the missing C-terminal segment (i.e. Arg-62–Leu-71) using the coordinates from the x-ray structure of G$_i$β$_i$γ$_i$ (Protein Data Bank code 1OMW) (40). After completion of the protein modifications, the extreme N-terminal and C-terminal end residues of each protein were capped as follows. (i) For Ga$_i$, the N terminus was acetylated, whereas the C terminus was retained as a free carboxylate form due to its importance in interacting with the receptor (6). (ii) For G$_i$β$_i$, an acetyl group was attached to the N terminus, whereas an N-methyl group was attached to the C terminus. (iii) For G$_i$γ, an acetyl group was attached to the N terminus, whereas a methoxy group was attached to the C terminus in preparation for lipid modification. The resulting G$_i$ protein was solvated using the SOLVATE plug-in in VMD (41) and put into a water box of ~100 × 120 × 110 Å$^3$. After removing solvent water molecules within 1 Å of the crystal water molecules, we carried out an energy minimization of 2,500 iterations, followed by a short MD simulation of ~3 ns, during which the backbone atoms of the G$_i$ protein were fixed. The GDP-bound G$_i$ protein was then extracted from the water box and used as the starting structure for the construction of a model of the CB1-G$_i$ complex (see below).

**Construction of a Model of the CB1-G$_i$ Complex**—The GDP-bound G$_i$ protein was docked to the HU210-bound CB1 receptor embedded in a fully hydrated POPC bilayer using the following two sequential steps. In Step 1, the x-ray structure of the $\beta_2$AR-G$_i$ complex (12), as a docking template, was superimposed onto the CB1 receptor with respect to the sequence-aligned TM helical backbone atoms, using the highly conserved TM residues as published previously (42). In Step 2, the GDP-bound Ga$_i$ was then fitted to the G$_i$ of $\beta_2$AR-G$_i$, superposed on the CB1 receptor from Step 1 with respect to the backbone atoms of the commonly conserved secondary structures in the GoRas domain. We used SuperPose (43) for superposition. A PAL was added to Cys-3$^{\text{Gai}}$ of Ga$_i$ N terminus (Ga$_i$NT) and a
geranylgeranyl moiety was added to Cys-68 Gly of GyCT (39), such that both lipid moieties were positioned just below the IC membrane layer. Any water molecule and POPC molecule within 1.5 Å of the newly added G, and the following lipid modifications was removed from the system of the CB1-G complex model. Sodium chloride molecules were used to ionize (0.15 m) and neutralize the system to satisfy electrostatic calculations. A total of ~219,600 atoms, including two proteins (CB1 and G), two bound ligands (HU210 and GDP), ~51,450 water molecules, ~360 POPC molecules, 73 Na+, and 72 Cl–, resulted in a system of the CB1-G complex in a simulation box of ~90 × 130 × 190 Å3 (Fig. 1A). At the end of this stage, the area per lipid in the system was ~60 Å2. Because it has been demonstrated that sodium chloride contributes to a compression of POPC membranes by ~10% (44, 45), this value appeared to be in agreement with the experimentally measured values for a salt-free POPC bilayer in the range of 63–68 Å2 of the liquid crystalline phase (46, 47), which is the most biologically relevant phase (48).

The system of the CB1-G complex model in a fully hydrated POPC bilayer environment was carefully equilibrated using a sequence of steps. In Step 1, to relax unfavorable steric conflict in the side chains at the interface of the CB1 receptor and the G protein, the system of the CB1-G complex was initially subjected to an energy minimization of 2,500 iterations and followed by a simulation at 310 K for a duration of 6 ns only for the regions of His-219IC2–Thr-229IC2 and Thr-313IC3–Asp-333IC3 of the CB1 receptor and the Go i C-terminal residues Ile-344G,–Phe-354G, whereas the protein backbone atoms of the rest of the proteins were constrained. The Go i C-terminal residues Phe-66G–Cys-68G were also allowed to move freely. In Step 2, we removed the backbone constraints for the CB1 receptor but retained the backbone constraints for the G protein. We newly added a group of distance and torsion constraints to approximately maintain some key intermolecular interactions at the receptor-Go i C-terminal interface, as determined in the x-ray structure of the β2AR-Gi complex (12), which appeared commonly applicable to the CB1-G complex. These constrained distances between mostly conserved residues of the CB1 receptor and Go i (Fig. 1B) included Arg-3075,71–Asp-341Go i, Lys-225IC2–Asn-347Go i, Arg-2143,50–Cys-351Go i (backbone), and Thr-3446,36–Gly-352Go i (backbone). We applied additional intramolecular distance constraints: Arg-2143,50–Tyr-2945,58, Tyr-224IC2–Asp-213,49, and Ser-1523,39–Asp-213,49 for the CB1 receptor and Glu-318Go i–Lys-345Go i, Glu-28Go i–Arg-32Go i, and Asn-347Go i–Asp-350Go i for the G protein. We also applied torsional constraints for Ile-344Go i–Phe-354Go i to maintain the backbone conformation as found in the x-ray structure of the β2AR-Gi complex (12). The system was simulated for 25 ns. In Step 3, we removed the backbone constraints for the G protein but retained the distances and torsional constraints for an additional 52 ns of the simulation, during which the initially applied constraints (k = 40) were gradually released. In Step 4, we removed all of the constraints and continued the simulation for an additional 824 ns. In this final step, the entire system was free to equilibrate, and the lateral area of the box was kept fixed while the z dimension of the box was allowed to move freely in the NPT ensemble. The structures taken every 400 ps of the simulation were used for the analysis.

To validate the present model of the CB1-G complex, a second model with slightly changed orientation of G, relative to CB1 was constructed using the CB1-G complex in the initial stage of the 824 ns simulation by the protein-protein docking program ZDOCK (version 3.0.2) (49). To locate the extreme C-terminal region of Go i in close proximity to the IC region of the CB1 receptor, the Go residues Lys–194Go i–Leu–194Go i, Ser–293Go i–Glu–318Go i, and Asp–341Go i–Phe–354Go i and the CB1 residues Asp–2133,49–Lys–226IC2, Ile–2975,61–Leu–3456,37, and Arg–4007,56–Asp–4031,48 at the CB1/Go i interface were used as the contact residues. In this model, GDP was removed from the nucleotide binding site to obtain an intermediate state, where the CB1-Gi interactions are expected to be highly dynamic (50). This model was simulated for 300 ns.

Simulations—All simulations were performed using the NAMD simulation package (version 2.7 Linux-x86_64) (51), initially using CHARMM22 force field parameters for the proteins (52) and CHARMM27 force field parameters for the lipids (53) and later switching to CHARMM36 force field parameters for proteins with the ϕ/ψ angle cross-term map correction (52, 54, 55) and lipids (56) and the TIP3P water model (57). The topology definitions and the parameters for the palmitoylated Cys, including the parameters around the bond connecting the Cys residues and the carbonyl carbon of PAL, as used in the literature (58), were found in the NAMD Parameter Topology Repository site. To describe geranylgeranylated Cys-68 Gly of Gy in the MD simulations using the CHARMM force field, we determined missing parameters, including the angle parameter for S–CH2–CH2(= C) and the torsional parameters for S–CH2–C=H(= C) using MeSCH2CH=C(Me)2 obtained by ab initio MP2/6–31G* level calculations. The temperature was maintained at 310 K through the use of Langevin dynamics (59) with a damping coefficient of 1/ps. The pressure was maintained at 1 atm by using the Nosé–Hoover method (60) with the modifications as described in the NAMD user’s guide. The van der Waals interactions were switched at 10 Å and zero smoothly at 12 Å. Electrostatic interactions were treated using the particle mesh Ewald method (61). A pair list for calculating the van der Waals and electrostatic interactions was set to 13.5 Å and updated every 10 steps. A multiple time-stepping integration scheme, the impulse-based Verlet-I reversible reference system propagation algorithm method (62), was used to efficiently compute full electrostatics. The time step size for integration of each step of the simulation was 1 fs.

RESULTS

Structural Convergence of the CB1-Gi Complex—Fig. 1A shows the resulting model of the CB1-Gi complex at the end of an 824-ns simulation. To measure the structural stability of the present model of the CB1-G complex, the RMSDs have been calculated over the performed simulation. As shown in Fig. 2A, the RMSD values of the CB1-G complex are moderately increased for the initial ~250 ns of the simulation, due to the positional adjustment of the individual proteins to relieve unfavorable steric contacts present as a result of imposing a structure similar to the β2AR-Gi complex at the beginning of the
Key CB1 Contacts with the C-terminal Helix $\alpha_5$ of $G_{\alpha_i}$

**A**

![Diagram of CB1-Gi complex]

**B**

| Chain | Residue | Sequence |
|-------|---------|----------|
| CB1   | 283     | VSLGSFETADRSYSLHPLAKVRIVTPRKAKVAFSCGM |
| $\beta_2$AR | 120 | SIETLCVIADRYFAILSPFKYQSSLTEKARVILMWN |
| rhodopsin | 124 | ALHSLLVIAERYVVKPMSPNRFGLIEHA1MGVATM |

**FIGURE 1.** Simulation system of the CB1-Gi complex (the CB1 receptor in red, $G_{\alpha_i}$ in green, $G_{\beta\gamma}$ in blue, and Gy in orange). PALs, which are covalently bonded to Cys-415 of the CB1 receptor and Cys-324 of $G_{\alpha_i}$ and the geranylgeranyl moiety (GER), are represented by green sticks. Lipids and water molecules are represented as lines, whereas ions (Na$^+$ in yellow and Cl$^-$ in cyan) are represented as spheres. Lipid and water hydrogen atoms are omitted for clarity. The system at 824 ns of the simulation is shown. Lipids and water hydrogen atoms are omitted for clarity. The system at 824 ns of the simulation is shown.

A simulation. Increased RMSD values at the end of the simulation relative to those at the start of the simulation indicate noticeable changes in orientation of the individual proteins. Little change in the RMSD values after 725 ns of the simulation, however, suggests that the protein-protein interactions in the CB1-Gi complex become optimized at the interface. After 400 ns of the simulation, the RMSD values of $G_{\beta}$ are somewhat increased due to the displacement of its N-terminal helix $\alpha_N$ resulting from the flexibility of the segment (Cys-25$^{G\beta}$-Thr-29$^{G\beta}$) that links helix $\alpha_N$ to helix $\alpha_2$. At around 600 ns of the simulation, the RMSD values of the CB1-Gi complex are noticeably increased, mainly due to the increased RMSD values of

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**Note:** The figure and table content are provided as an example of how the text is structured for natural reading. The actual scientific content would require specific analysis and interpretation beyond the scope of this preview.
Key CB1 Contacts with the C-terminal Helix $\alpha_5$ of G$\alpha_i$

FIGURE 2. A, RMSD values of the proteins in the CB1-Gi complex (black), the CB1 receptor (red), G$\alpha_i$ (green), G$\beta$ (blue), and G$\gamma$ (orange), calculated by root mean square fitting to the initial coordinates with respect to the backbone Ca atoms of the TM helical residues of the CB1 receptor and the helical and sheet residues of G$\alpha_i$. Shown are the RMSD values of HU210 (cyan) bound to the CB1 receptor and GDP (magenta) bound to G$\alpha_i$, calculated with respect to the initial coordinates after fitting to the heavy atoms. B, the tilt angle of helix $\alpha_5$ of the G$\alpha_i$ subunit during the simulation, estimated by the angle created by two vectors: the one by the backbone Ca atoms of the sequence Asn-331$^{\text{331}}$–Ala-338$^{\text{338}}$ and the other by the vector of the backbone Ca atoms of the sequence Thr-340$^{\text{340}}$–Ser-342$^{\text{342}}$. C, comparison of the CB1-Gi complex with the known X-ray structures. (A) RMSD values of G$\alpha_i$ (green dots) were scaled up by multiplying the actual values in Å by 6. C, contact numbers of G$\alpha_i$ to the CB1 receptor are: the whole receptor (black), IC1 ($\pm$ 8 residues; Leu-136$^{\text{136}}$–Ser-158$^{\text{158}}$), IC2 ($\pm$ 8 residues; Ala-211$^{\text{211}}$–Phe-237$^{\text{237}}$) (green), IC3 ($\pm$ 8 residues; Ala-301$^{\text{301}}$–Ala-342$^{\text{342}}$) (blue), and H8 (Asn-393$^{\text{393}}$–Cys-415$^{\text{415}}$) (orange). Similarly, contact numbers of the CB1 receptor to G$\alpha_i$ are: G$\alpha_i$ (cyan) and G$\beta$ (magenta). Contact numbers of G$\alpha_i$ C terminus (Thr-340$^{\text{340}}$–Phe-354$^{\text{354}}$) to the CB1 receptor are: IC2 ($\pm$ 8 residues; Ala-211$^{\text{211}}$–Phe-237$^{\text{237}}$) (green dots) and IC3 ($\pm$ 8 residues; Ala-301$^{\text{301}}$–Ala-342$^{\text{342}}$) (blue dots). A criterion of 4.0 Å was used between non-bonded atoms. The contact numbers, with the S.D. value in parentheses, averaged over the last 20.0 ns of the simulation are as follows. Contact numbers of G$\alpha_i$ were 102 (5) for the whole receptor, 14 (2) for IC1, 30 (2) for IC2, 40 (4) for IC3, and 12 (2) for H8; contact numbers for the CB1 receptor were 84 (5) for G$\alpha_i$, 18 (2) for G$\beta$, and 0 for G$\gamma$. G$\alpha_i$. It appears that the RMSD values of G$\alpha_i$ are closely associated with the tilt angle change in helix $\alpha_5$ of G$\alpha_i$ (Fig. 2B). This suggests that the conformation of helix $\alpha_5$ influences the orientation relation to G$\alpha_i$ and determines the degree of the interaction with the receptor (see “Tilt in Helix $\alpha_5$ of G$\alpha_i$”). High RMSD values for HU210 (>2 Å) are caused by the rotameric angle change in the ligand’s C3 side chain which led to its end carbon being positioned between Val-204$^{\text{204}}$–Val-282$^{\text{282}}$ (see under “Molecular Features of the Active CB1 Receptor in the CB1-G$\alpha_i$ Complex”), whereas low RMSD values for GDP (<1 Å) are due to little change in GDP tightly bound to the nucleotide-binding pocket.

The second model became structurally stable after 200 ns of the simulation, as indicated by the RMSD values maintained at ~4 Å without any noticeable change. As shown in Fig. 3A, the initial orientation of G$\alpha_i$ relative to CB1 in the second model is different from that of the present model of the CB1-G$\alpha_i$ complex. However, the final orientation of G$\alpha_i$ in the second model becomes quite similar to that in the present model of the CB1-G$\alpha_i$ complex, supporting the validity of the orientation of G$\alpha_i$ relative to CB1 in the present model of the CB1-G$\alpha_i$ complex. It appears that the initial orientation of G$\alpha_i$ in the second model is better than that in the present CB1-G$\alpha_i$ complex in achieving the converged orientation.

Comparison of the CB1-G$\alpha_i$ Complex with the Known X-ray Structures—To evaluate the present model of the CB1-G$\alpha_i$ assembly, we compared it with the x-ray structure of the $\beta$AR-G$\alpha_i$ complex (12) (Fig. 3B). The overall orientation of G$\alpha_i$s relative to the receptor in the CB1-G$\alpha_i$ complex is maintained as in the $\beta$AR-G$\alpha_i$ complex. Interestingly, the position of the extreme C-terminal helix $\alpha_5$ (Thr-329$^{\text{329}}$–Phe-354$^{\text{354}}$) of G$\alpha_i$ in the CB1-G$\alpha_i$ complex is tilted away from TM6 compared with the corresponding helix of G$\alpha_i$ (Fig. 3B). We also compared the present model of the CB1-G$\alpha_i$ complex with the x-ray structure of the activated rhodopsin (metarhodopsin II) in complex with the C-terminal peptide of G$\gamma$ (metarhodopsin II-G$\alpha_i$CT) (Protein Data Bank code 3PQR) (63). The position of the extreme C-terminal helix $\alpha_5$ of G$\alpha_i$ in the CB1-G$\alpha_i$ complex is remarkably similar to that of the corresponding G$\alpha_i$CT peptide (ile-340C$^{\text{340}}$–Phe-350G$^{\text{350}}$) (Fig. 3C), suggesting that the orientation of the G$\alpha_i$ subunit relative to the receptor in the CB1-G$\alpha_i$ complex is similar to that of the corresponding G$\alpha_i$ subunit in the metarhodopsin II-G$\alpha_i$ complex. The outward movement of TM6 is small in the CB1-G$\alpha_i$ complex and in the metarhodopsin II-G$\alpha_i$CT complex (Fig. 3C), compared with the $\beta$AR-G$\alpha_i$ complex (Fig. 3B), suggesting that the position of the extreme C-terminal helix $\alpha_5$ of G$\alpha_i$ is sensitive to the receptor conformation (see “Tilt in Helix $\alpha_5$ of G$\alpha_i$”). Further comparisons with the x-ray structures of G$\alpha_i$s proteins reveal that helix $\alpha_5$ of G$\alpha_i$ in the CB1-G$\alpha_i$ complex is aligned slightly better with helix $\alpha_5$ in the GTP$\gamma$S-bound active state of G$\alpha_i$ (64) than with helix $\alpha_5$ in the nucleotide-free $\beta$AR-G$\alpha_i$ complex, suggesting that the conformation of helix $\alpha_5$ is associated with nucleotide binding to G$\alpha_i$s (12). Interestingly, a close examination reveals that helix $\alpha_5$ in the GTP$\gamma$S-bound active state G$\alpha_i$s (64) is tilted in the middle of the helix at Asp-378$^{\text{378}}$ and Cys-379$^{\text{379}}$, the residues equivalent to Ala-338$^{\text{338}}$ and Val-339$^{\text{339}}$ of G$\alpha_i$, tilted in the present CB1-G$\alpha_i$ complex at the middle stage of the simulation (Fig. 2B).

Contact Number Analysis of the CB1-G$\alpha_i$ Complex—As shown in Fig. 2C, the number of contacts of the CB1 receptor to G$\alpha_i$s is in the order IC3 (~40) > IC2 (~30) > IC1 (~14) > H8 (~12), suggesting key roles of IC2 and IC3 of the receptor in G$\alpha_i$s coupling. Similarly, the number of contacts of G$\alpha_i$s to the CB1 receptor is mainly with G$\alpha_i$ (~84), low with G$\beta$ (~18), and none with G$\gamma$. The number of contacts between the receptor and G$\alpha_i$s is temporarily increased for the initial ~250 ns of the simulation and then decreased to a value slightly higher than the initial value. Temporal increases in the contact number between the CB1 receptor and G$\alpha_i$s at the early stage (100–250 ns) of the simulation (Fig. 2C), which are closely related to the observed temporal increases in RMSD values of the CB1-G$\alpha_i$ complex (Fig. 2A), are mainly attributed to the temporarily increased contacts between the CB1 receptor and G$\beta$ (Fig. 2C). Because the main candidate regions of the receptor in contact with G$\beta$ would be IC1 and H8, it appears that IC1 and H8 try to maximize the local contacts to G$\beta$ in response to the docked G protein at the early stage of the simulation, but these contacts become few as the
FIGURE 3. A, the orientation of G_i (green surface) relative to CB1 (red ribbon) in the present CB1-G_i complex in comparison with the orientation of G_i (magenta surface) relative to CB1 (pink ribbon) in the second model, viewed from the extracellular side, at the initial and final stages of the simulations. We superimposed these complex models with respect to the backbone Cα atoms of the TM helical residues of CB1. The extracellular half of CB1 and the Gβγ subunits of G_i are omitted for clarity. B, comparisons of the present CB1-G_i complex structure (CB1 in red, Gα in green, Gβ in blue, and Gγ in orange) with the x-ray structure of the β2AR-Gs complex (12) (β2AR in cyan and Gs in yellow). C, comparisons of the present CB1-G_i complex structure (CB1 in red, Gα in green, Gβ in blue, and Gγ in orange) with the x-ray structure of metarhodopsin in complex with GtCT (63) (metarhodopsin in gray and GtCT in light gray). In B and C, we superimposed these structures with respect to the backbone Cα atoms of the TM helical residues of the receptors whenever the receptor was available. The structures on the right are the receptor-G protein interfaces marked by the blue dotted area viewed from the IC side by rotating 90° about the x axis. D, a sequence of conformational changes in helix α5 of Gα_i influenced directly by IC2 and indirectly by IC3 of the CB1 receptor during the present simulation of the CB1-G_i complex. Solid arrows indicate the conformational changes of IC2 that affect the conformation of the C-terminal half of helix α5 and helix αN of Gα_i, whereas dotted arrows indicate the conformational changes of IC3 that affect the conformation of the C-terminal half of helix α5 and helix αN of Gα_i. Color coding for snapshots of CB1-G_i at different times of the simulation is: 50 ns (blue), 400 ns (red), 600 ns (orange), and 800 ns (green). E, superposition of the present model of the inactive state of the CB1-G_i complex (CB1 in red and Gα in green) on the active state of the β2AR-Gs complex (12) (β2AR in cyan and Gs in yellow). The cyan arrows indicate the conformational changes in the CB1 receptor required for achieving the active state of CB1-G_i as observed in the β2AR-Gs complex, by rotating Gα_iRas as indicated by the yellow arrows. TM1, TM2, and TM7 of the receptors are omitted for clarity.
interactions between IC2 and IC3 of the receptor and $\alpha_i$ are fully established after 300 ns of the simulation. The number of contacts between IC2 and IC3 of the receptor and $\alpha_i$ is little changed, suggesting that the interactions between IC2 and IC3 of the receptor and $\alpha_i$ are important for the CB1–G complex. A transient decrease in the contact number between the CB1 receptor and $\alpha_i$ right after 600 ns of the simulation resulted from the formation of an IC3 helical segment (Glu-323$^{\alpha_i}$–Ala-335$^{\alpha_i}$) that moves toward the IC membrane surface (Fig. 3).

**Molecular Features of the Active CB1 Receptor in the CB1–G Complex**—To confirm the active state of the CB1 receptor in the CB1–G complex, we monitored the breaking of the ionic lock Arg-214$^{\alpha_i}$/Asp-338$^{\alpha_i}$ along with inward movements of Tyr-294$^{\alpha_i}$ and Tyr-397$^{\alpha_i}$ (Fig. 4A), key features of the GPCR active state (13, 65–68). The ionic lock Arg-214$^{\alpha_i}$/Asp-338$^{\alpha_i}$ stays broken (Cα–Cα distance, ~15 Å) throughout the simulation, similarly as those in the x-ray structures of metarhodopsin II (63) and β2AR (12). Whereas Arg$^{\alpha_i}$ is conserved in β2AR as part of the DRY motif, the residue corresponding to Lys-345$^{\alpha_i}$ of $\alpha_i$ is Arg-385$^{\alpha_i}$ in Go$_i$. It is shown that Arg-214$^{\alpha_i}$ released from Asp-338$^{\alpha_i}$ is confined to the region created by Thr-210$^{\alpha_i}$ and Tyr-294$^{\alpha_i}$ of the CB1 receptor and Cys-351$^{\alpha_i}$ and Leu-353$^{\alpha_i}$ of Go$_i$. Arg-214$^{\alpha_i}$ maintains a tight interaction with Tyr-294$^{\alpha_i}$ through a hydrogen bond, as indicated by the distance between the side chain nitrogen of Arg-214$^{\alpha_i}$ and the side chain oxygen of Tyr-294$^{\alpha_i}$ (~3 Å) (Fig. 4A). In contrast, Arg-214$^{\alpha_i}$ forms a loose hydrogen bond with Tyr-397$^{\alpha_i}$, as indicated by fluctuations in the distance between Arg-214$^{\alpha_i}$ and Tyr-397$^{\alpha_i}$ (Fig. 4A). A close examination reveals that when Tyr-397$^{\alpha_i}$ stays away from Arg-214$^{\alpha_i}$, it is deeply inserted into the TM core and appears to play a key role in establishing an extensive water-mediated hydrogen-bonding network in the IC half of the TM core formed by TM2/TM3/TM7 residues mainly of the (S/N)LAXAD and NPXXY motifs. Assuming that the deeply inserted Tyr-397$^{\alpha_i}$ represents the receptor in a fully activated state, as proposed in a recent study (68), Tyr-397$^{\alpha_i}$ appears to be an important molecular switch for the formation of a water channel that possibly stabilizes the active state of the receptor (69).

Substitution of Thr-210$^{\alpha_i}$ of the CB1 receptor with Ile and Ala generates constitutively active and inactive receptor mutants, respectively (25). A recent study proposed that mutations of Thr-210$^{\alpha_i}$ greatly alter the pattern of the salt bridges at the IC side of the receptor and the TM helical packing, leading to stabilization or destabilization of the wild type receptor activity (70). Thr-210$^{\alpha_i}$ in the present model of the CB1–G complex appears to play an important role in stabilizing Arg-214$^{\alpha_i}$ by forming an extensive water-mediated hydrogen-bonding network with Ser-152$^{\alpha_i}$, Asp-213$^{\alpha_i}$, Arg-214$^{\alpha_i}$, and Tyr-294$^{\alpha_i}$. However, such an interaction cannot explain why the elimination of the hydrogen bond-forming side chain oxygen of Thr-210$^{\alpha_i}$ by the Ile mutation induces receptor activation. Comparison of the present receptor model with the previously published inactive receptor model stabilized by the inverse agonist SR141716A (71) reveals that the space once occupied by Leu-345$^{\alpha_i}$, a TM6 residue in close contact with Thr-210$^{\alpha_i}$ at the inactive state of the receptor, is now occupied

**FIGURE 4.** A, geometries of the DRY motif in the CB1 receptor: the Cα–Cα distance of Arg-214$^{\alpha_i}$/Asp-338$^{\alpha_i}$ (black), the side chain distance of Arg-214$^{\alpha_i}$/Asp-338$^{\alpha_i}$–Tyr-294$^{\alpha_i}$/Tyr-397$^{\alpha_i}$ (red), the side chain distance of Arg-214$^{\alpha_i}$/Asp-338$^{\alpha_i}$–Tyr-294$^{\alpha_i}$/Tyr-397$^{\alpha_i}$ (green), and the side chain distance of Thr-210$^{\alpha_i}$/Thr-344$^{\alpha_i}$/Asp-350$^{\alpha_i}$ (blue). Rotameric angles, the ψ angle (black) (the rotation around the C3–C1 bond in the C3 dimethylheptyl chain) of Hu210, the χ1 angle (red) (the rotation around the Cα–Cβ bond) of Trp-356$^{\alpha_i}$ in the CB1 receptor, and the χ2 angle (green) (the rotation around the Cβ–Cγ bond) of Trp-356$^{\alpha_i}$ in the CB1 receptor, Hu210 (in space-filling representation) at 0, 100, 200, 300, 400, 500, 600, 700, and 800 ns of the simulation is also shown to demonstrate the conformational dynamics of its flexible C3 alkyl chain of Hu210. C, distances between the terminal C atom of the C3 dimethylheptyl chain and the Cβ atom in Val-282$^{\alpha_i}$ (black), the Cα atom in Leu-286$^{\alpha_i}$ (red), and the Cβ atom in Val-204$^{\alpha_i}$ (green) of the CB1 receptor. The inward bulge in TMS (cyan) of β2AR caused by tight hydrogen bonding between Ser-2035$^{\alpha_i}$, Ser-2044$^{\alpha_i}$, and Ser-2074$^{\alpha_i}$ and BI-167107 (the rotation around the C3–C2 angle) of Trp-205$^{\alpha_i}$ (the rotation around the Cα–Cβ bond) of Trp-215$^{\alpha_i}$ (the rotation around the Cβ–Cγ bond) of Trp-215$^{\alpha_i}$ (the rotation around the C3–C2 angle) of Trp-205$^{\alpha_i}$ (the rotation around the Cα–Cβ bond) of Trp-215$^{\alpha_i}$ (the rotation around the Cβ–Cγ bond) of Trp-215$^{\alpha_i}$ (the rotation around the C3–C2 angle) of Trp-205$^{\alpha_i}$ (the rotation around the Cα–Cβ bond)

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by Tyr-294\textsuperscript{575–585} in the present receptor model. Thus, it appears that the bulky Ile in place of Thr-210\textsuperscript{46–46} causes an unfavorable interaction that facilitates an outward movement of the IC end of TM6, leading to the breakage of the ionic lock.

Mutation of the highly conserved Asp-163\textsuperscript{2,50} of the (S/N/L)\textsuperscript{AXAD} motif in the CB1 receptor to an Asn or a Glu exhibited significant disruption of G protein coupling (72, 73). In the present study, these highly conserved residues, Asp-163\textsuperscript{2,50} and Asn-393\textsuperscript{7–49}, appear to play key roles in the formation of the water channel, possibly stabilizing the active state of the receptor (69). The distance between the side chain oxygen of Asp-163\textsuperscript{2,50} and the side chain nitrogen of Asn-393\textsuperscript{7–49} is <3 Å occasionally but ~6 Å most times during the simulation. It is possible that individual residues are important for the formation of the water channel, but a tight interaction between these residues is not required for G protein coupling. In support, Roche et al. (73) showed that the D163\textsuperscript{2,50}N/N393\textsuperscript{7–49}D mutation behaved similarly to the D163\textsuperscript{2,50}N/N393\textsuperscript{7–49}M mutant, indicating that Asp-163\textsuperscript{2,50} and Asn-393\textsuperscript{7–49} do not interact cooperatively for G protein coupling. Interestingly, the corresponding Asp-79\textsuperscript{5,50} and Asn-322\textsuperscript{7–49} of β2AR form a hydrogen bond according to the x-ray structure of the β2AR complex (12).

Throughout the simulation, the χ1 rotameric angle (the rotation around the Cα–Cβ bond) of Trp-356\textsuperscript{6,48} remained as trans (Fig. 4B), as was proposed to be preferred for the active state GPCRs (74, 75). It is interesting to see that the τ1 angle (the rotation around the C3–C1’ bond) in the C3 alkylic chain of the ligand changes from trans to gauche at 100 ns of the simulation (Fig. 4B), causing increases in the RMSDs. This only slightly affects the χ2 rotameric angle (the rotation around the Cβ–Cγ bond) in Trp-356\textsuperscript{6,48} but moves HU210 deep into the TM core, leading the C3 alkylic chain moving deep inside the core toward TM5. A group of water molecules, which stabilizes the extracellular half of the TM3/TM5/TM6 interhelical region according to the Trp-356\textsuperscript{6,48} toggle switch in the early stage of the activated state (31), is now completely eliminated, possibly due to the replacement of the water molecules by the ligand C3 alkylic chain. Its driving force is interaction with a hydrophobic pocket created by Val-204\textsuperscript{5,40} on TM3 and Val-282\textsuperscript{5,46} and Leu-286\textsuperscript{5,50} on TM5, as demonstrated by the close distances between the C3 alkylic chain of the ligand and Val-204\textsuperscript{5,40} and Val-282\textsuperscript{5,46} (Fig. 4C). The resulting hydrophobic interaction between the C3 alkylic chain of the ligand and TM5 appears to be important for transferring the molecular signal of the ligand. As shown in Fig. 4C, occupying a similar region in the TM core as HU210, the agonist BI-167107 interacts tightly through hydrogen bonding with a group of Ser residues on TM5 of β2AR, which induces a bulge in the middle of TM5. In contrast, hydrophobic interactions between HU210 and Val-282\textsuperscript{5,46} and Leu-286\textsuperscript{5,50} on TM5 of the CB1 receptor appear not strong enough to induce such a bulge. It should be noted that the highly conserved Pro\textsuperscript{5,50} in many GPCRs, equivalent to Leu-286\textsuperscript{5,50} in the CB1 receptor, has been proposed to play a key role in receptor activation (76).

**Structural Properties of the CB1-Gi Complex—**The current model of the CB1-Gi complex reveals that the N- and C-terminal regions, including helix αN, loop αC–β1, and helix αG, and other regions, including loop β2–β3, helix αG, loop αC–β6 and strand β6’, of the Gα subunit, form major contacts with the receptor. Thus, these segments appear to be crucial for transferring the molecular signal from the activated receptor to the nucleotide-binding pocket. In support, most of these segments have been demonstrated to be important for GPCR-G protein interactions leading to GDP release (6, 77–79). Because our simulation analyses indicate that IC2 and IC3 play key roles in Gi coupling, detailed structural analyses of these regions in contact with Gα are described as follows. According to the present model of the CB1-Gi complex, IC2 of the CB1 receptor, which spans 11 residues (His-219\textsuperscript{IC2–Thr-229\textsuperscript{IC2}}) and contains one helical segment (Ala-223\textsuperscript{IC2–Ile-227\textsuperscript{IC2}}), forms one of the major contacts to Gαi (Fig. 2C). A hydrophobic patch, formed by Pro-221\textsuperscript{IC3}, Leu-222\textsuperscript{IC2}, and the aliphatic hydrocarbon portions of Arg-220\textsuperscript{IC2} and Lys-225\textsuperscript{IC2}, is positioned toward Gαi, providing critical contacts mainly to the N- and C-terminal segments, including helix αN (Arg-32\textsuperscript{Gαi}, loop β2–β3, Lys-192\textsuperscript{Gαi}, Asp-193\textsuperscript{Gαi}, and Leu-194\textsuperscript{Gαi}) and helix αC (Thr-340\textsuperscript{Gαi}, Ile-343\textsuperscript{Gαi}, and Ile-344\textsuperscript{Gαi}). Leu-222IC2, the last residue of a consensus DRYXX(V/I)xxPL motif in many GPCRs, which has been proposed to be crucial for G protein coupling (80), is at the center of the hydrophobic patch. It has been reported that L222IC2A or L222IC2P mutations in the CB1 receptor exhibited coupling preference to Gαi over Gs, whereas the L222IC2F mutation exhibited coupling preference to Gβ over Gαi (81). Notably, Ile-218\textsuperscript{3,54} forms a tight hydrophobic cluster, not only with Ile-297\textsuperscript{5,61}, Lys-300\textsuperscript{5,64}, and Leu-341\textsuperscript{6,33} of the receptor but also with Ile-344\textsuperscript{Gαi}, Leu-348\textsuperscript{Gαi}, and Leu-353\textsuperscript{Gαi} of Gαi (Fig. 5A). Considering that both Ile-218\textsuperscript{3,54} and Ile-297\textsuperscript{5,61} are highly conserved residues in the rhodopsin family of GPCRs (82) and that the corresponding residues in all subtypes of Gα proteins to Leu-348\textsuperscript{Gαi} and Leu-353\textsuperscript{Gαi} are highly conserved (Fig. 1B), it appears that Ile-218\textsuperscript{3,54} plays a crucial role in receptor–G protein coupling. Of interest, Tyr-224\textsuperscript{IC2} forms two hydrogen bonds to Asp-350\textsuperscript{Gαi}, which appear to be tight, as indicated by the close distances maintained during the MD simulation (Fig. 4D). According to the present model of the CB1-Gi complex, IC3 forms the largest contact with Gi (Fig. 2C). As TM5 and TM6 of the CB1 receptor are extended to include a few N- and C-terminal IC3 residues, the extent of IC3 of the CB1 receptor is reduced to the sequence spanning 21 residues (Ile-309\textsuperscript{IC3–Arg-336\textsuperscript{IC3}}), among which the C-terminal residues Glu-323\textsuperscript{IC3–Ala-335\textsuperscript{IC3}} form a helical segment near the start of TM6. The helical content of IC3 of the CB1 receptor in the present model is similar to that of the IC3 peptide determined by NMR (37), although the NMR-determined helix (Ile-309\textsuperscript{5,72–Ser-316\textsuperscript{IC3}}) right next to the N-terminal helix is not seen in the present IC3 structure, possibly because of the presence of the G protein which affects the IC3 conformation. Extensive intramolecular hydrophobic interactions within IC3 lead to two distinct hydrophobic clusters, including the first one near the TM region (Ile-297\textsuperscript{6,61}, Leu-298\textsuperscript{5,62}, Ala-301\textsuperscript{5,65}, Leu-341\textsuperscript{6,33}, Ala-342\textsuperscript{5,34}, Leu-345\textsuperscript{6,37}, Val-346\textsuperscript{3,38}, and Leu-349\textsuperscript{6,41}) and the second one in the middle of IC3 (Ala-305\textsuperscript{5,69}, Val-306\textsuperscript{5,70}, Ile-308\textsuperscript{7,72}, Ile-309\textsuperscript{IC3}, Arg-331\textsuperscript{IC3}, Pro-333\textsuperscript{IC3}, Ala-335\textsuperscript{IC3}, and Ile-339\textsuperscript{IC3}). In particular, the highly conserved Ile-297\textsuperscript{6,61}, part of the first hydrophobic cluster, is in close proximity not only to Arg-214\textsuperscript{5,50} of the DRY motif, Ile-218\textsuperscript{3,54}, but also to Leu-353\textsuperscript{Gαi}, suggesting an important role of Ile-297\textsuperscript{6,61} in receptor-G cou-
Key CB1 Contacts with the C-terminal Helix $\alpha_5$ of G$\alpha_i$

![Image of Key CB1 Contacts with the C-terminal Helix $\alpha_5$ of G$\alpha_i$](image_url)

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**FIGURE 5.** (A) Hydrophobic interactions of Ile-218$^{5,54}$, Residue Ile-297$^{5,61}$, Lys-300$^{5,61}$, and Leu-341$^{5,53}$, which interact with Ile-218$^{5,54}$, are represented by red space-filling, whereas the G$\alpha_C$-terminal residues Ile-344$^{\text{Gu}}$, Leu-348$^{\text{Gu}}$, and Leu-353$^{\text{Gu}}$, which interact with Ile-218$^{5,54}$, are represented by green space-filling. Receptor residues Ala-211$^{5,47}$, Arg-214$^{5,50}$, Ala-293$^{5,57}$, Lys-300$^{5,61}$, Ala-301$^{5,65}$, Leu-341$^{5,53}$, and Leu-345$^{5,56}$, which form an extensive hydrophobic cluster with Ile-218$^{5,54}$, together with Ile-297$^{5,61}$, are represented by space-filling (red). For clarity, only side chains are shown. **(B)** Charge/hydrogen-bonding interactions between the CB1 receptor and the extreme C terminus of G$\alpha_i$, at the end of helix $\alpha_5$, including Tyr-224$^{E,2}$, Asp-350$^{E,4}$, Asp-338$^{E,4}$, Lys-344$^{\text{Gu}}$, Arg-340$^{E,3}$, Phe-354$^{\text{Gu}}$, Thr-344$^{E,6}$, Leu-353$^{\text{Gu}}$, and Arg-400$^{E,7}$. These interactions are represented by dotted lines. Only the side chains without hydrogen atoms of these residues (in stick representations) are represented for clarity. C, hydrophobic interactions of Thr-344$^{E,6}$ with Arg-214$^{E,5}$, Leu-341$^{E,5}$, Leu-345$^{E,5}$, Ile-348$^{E,4}$, and Arg-400$^{E,7}$ of the receptor and hydrogen-bonding interaction with the backbone carboxyl oxygen atom of Leu-353$^{\text{Gu}}$, $D$, interactions of Ile-218$^{5,54}$, Tyr-224$^{E,2}$, Asp-338$^{E,4}$, Arg-400$^{E,7}$, Leu-341$^{E,5}$, and Thr-344$^{E,6}$ of the CB1 receptor with the extreme C terminus of G$\alpha_i$, at the end of helix $\alpha_5$, viewed from the start of the helix $\alpha_5$ to the receptor IC face. We have examined these receptor residues by site-directed mutagenesis studies. The receptor is shown in a red volume, whereas the G$\alpha_i$ is shown in a green schematic. The extreme C-terminal helix $\alpha_5$ (Thr-299$^{5,56}$–Phe-354$^{5,57}$) of G$\alpha_i$ in the CB1-Gi complex is also shown in a helical wheel representation created by the helical wheel plotting program (available on the RZ Lab Web site), where hydrophobic residues are shown as diamonds, potentially negatively charged residues as triangles, and potentially positively charged residues as pentagons. Color coding for residues is as follows: from green for the most hydrophobic residue to yellow for the least hydrophobic residue and red for the hydrophilic residues.

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Interesting to see that Thr-344$^{E,6}$ and Arg-400$^{E,7}$, located near the IC membrane surface, appear to help securely lock the extreme C-terminal carboxylate of Phe-354 of G$\alpha_i$ (Figs. 4E and 5B). Arg-340$^{E,3}$ and Arg-400$^{E,7}$ of the CB1 receptor each form two hydrogen bonds to the C-terminal carboxylate of Phe-354$^{\text{Gu}}$, whereas Thr-344$^{E,6}$ forms a hydrogen bond with the backbone carboxyl oxygen atom of Leu-353$^{\text{Gu}}$. The side chain alkyl moiety of Thr-344$^{E,6}$ also forms close contacts with the side chain alkyl moieties of Arg-214$^{E,5}$, Leu-341$^{E,5}$, Leu-345$^{E,5}$, Ile-348$^{E,4}$, and Arg-400$^{E,7}$ of the receptor and Leu-353$^{\text{Gu}}$ (Fig. 5C). The importance of Arg-400$^{E,7}$ of the CB1 receptor in G protein coupling has been tested by the H8 (Arg-400 to Glu-416) peptide (84). The removal of Arg-400$^{E,7}$ from the peptide resulted in a 6-fold reduction in G$\alpha_i$ affinity and an almost complete loss of the inhibitory effect on adenylyl cyclase activity.

**Tilt in Helix $\alpha_5$ of G$\alpha_i$**—According to the present MD simulation of the CB1-Gi complex, the conformation of helix $\alpha_5$ of G$\alpha_i$, was altered as follows: a straight form was observed in the...
TABLE 1
Impact of CB1 amino acid mutation on ligand binding and G protein coupling

| Ligand binding | Basal* | GTPyS binding |
|----------------|--------|---------------|
|                | EC50   | Fmax          |                | EC50   | Fmax          |
|                | fmol/mg|               |                | fmol/mg|               |
| CP55940        |        |               |                |        |               |
| Wild type      | 2.9 ± 0.7| 3,637 ± 406  | 24 ± 0.7       | 3,318 ± 340| 46.8 ± 0.9***| ND  |
| I2183.54A      | 6.3 ± 0.9| 5,833 ± 256  | 14.8 ± 0.8     | 5,997 ± 278| 49.7 ± 0.7**  | ND  |
| H219IC2A       | 3.9 ± 0.9| 6,070 ± 259  | 38.8 ± 2.8     | 6,283 ± 297| 53.4 ± 1.5**  | ND  |
| Y224IC3A       | ND      | ND            | ND             | ND      | ND             | ND  |
| D3386.30A      | 0.3 ± 0.3| 1,497 ± 208  | 12.4 ± 0.8     | 1,531 ± 217| 10.9 ± 0.7***| ND  |
| I3396.31A      | 9.8 ± 0.9| 8,047 ± 208  | 18.8 ± 0.8     | 8,107 ± 257| 12.2 ± 1.4**  | ND  |
| R3406.93A      | 7.8 ± 0.9| 8,071 ± 208  | 15.5 ± 0.8     | 8,126 ± 257| 12.3 ± 1.5**  | ND  |
| L3416.33A      | 0.3 ± 0.3| 1,497 ± 208  | 12.4 ± 0.8     | 1,531 ± 217| 10.9 ± 0.7***| ND  |
| T3446.36A      | 9.8 ± 0.9| 8,047 ± 208  | 18.8 ± 0.8     | 8,107 ± 257| 12.2 ± 1.4**  | ND  |
| SR141716A      | 2.9 ± 0.7| 3,637 ± 406  | 24 ± 0.7       | 3,318 ± 340| 46.8 ± 0.9***| ND  |
| Kd             |        |               |                |        |               |
| Bmax           |        |               |                |        |               |
| CP55940-induced |       |               |                |        |               |

*For comparison, the basal level of GTPyS binding for mock-transfected cell membrane is 44.3 ± 1.4 fmol/mg.

initial stage (~100 ns) of the simulation; a tilted form with the tilt angle ~20° during the next 500 ns of the simulation; a transient stage at ~600 ns of the simulation where the tilt angle of helix α5 of Gαi reached its largest value (~30°); and a straight form in the late stage of the simulation (Fig. 2B). To understand what causes these conformational changes in helix α5 of Gαi during the simulation, we superimposed the snapshots at 50 ns (i.e. straight helix α5), 400 ns (i.e. tilted helix α5), 600 ns (i.e. highly tilted helix α5), and 800 ns (i.e. straight helix α5) of the simulation with respect to the TM helices of the receptor (Fig. 3D). The snapshot at 400 ns in comparison with that at 50 ns reveals that IC2 of the receptor alters its conformation and moves close to helix α5 of Gαi. This causes the C-terminal half of helix α5 and the N terminus of Gαi to move away from IC2, whereas the N-terminal half of helix α5 moves very little due to the minute effect the IC3 conformation has on Gαi (helix α5). As a result, the C-terminal half of helix α5 becomes tilted toward TM6 (Fig. 3D).

The snapshot at 600 ns in comparison with the snapshot at 400 ns reveals that IC2 moves closer to helix α5 of Gαi, which causes the C-terminal half of helix α5 and the N terminus of Gαi to move further away from IC2, whereas an altered IC3 conformation pushes Gαi (helix α5) away. This causes the N-terminal half of helix α5 to move toward IC2 (Fig. 3D). As a result, the tilt angle of helix α5 of Gαi becomes larger (~30°). At 600 ns of the simulation, IC2 moves slightly away from helix α5 of Gαi, which causes the C-terminal half of helix α5 and the N terminus of Gαi to move toward IC2. In contrast, IC3 undergoes a significant conformational change in which the C-terminal helical segment (Glu-323 IC3–Ala-335 IC3) moves close to the IC membrane surface. This allows Gαi (helix α5) to be relaxed and the N-terminal half of helix α5 of Gαi to move away from IC2. As a result, helix α5 of Gαi becomes straight (Fig. 3D). Overall, the position of helix α5 of Gαi is sensitive to the conformations of IC2 and IC3 of the CB1 receptor. It appears that IC2 of the CB1 receptor directly affects both the C-terminal side of helix α5 and the N terminus of Gαi, whereas IC3 of the CB1 receptor indirectly affects the N-terminal side of helix α5 through interactions with helix α5 of Gαi.

Site-directed Mutagenesis Studies of I2183.54A, H219IC2A, P221IC2A, Y224IC2A, D3386.30A, I3396.31A, R3406.32A, L3416.33A, and T3446.36A of the CB1 Receptor—The results from the simulation of the CB1-Gi complex were examined by site-directed mutagenesis studies. We selected a group of eight residues predicted to be crucial in receptor-G protein coupling. I2183.54, P221IC2, Y224IC2, D3386.30, I3396.31, R3406.32, L3416.33, and T3446.36 of the CB1 receptor were of particular interest due to their tight interactions with the extreme C-terminal helix α5, which forms the key contact surface for the activated receptor (85). We also selected a couple of residues, including His-219IC2 and Ile-3396.31, predicted to be little involved in receptor-G protein coupling. Thus, we assessed the impact of these mutations on ligand binding properties by saturation binding experiments using the CB1 agonist [3H]CP55940 and the inverse agonist [3H]SR141716A. As shown in Table 1 and Fig. 6, all eight mutant receptors displayed Kd values for [3H]CP55940 (Kd values of 1.1, 1.2, 3.3, 1.3, 1.6, 1.6, 0.9, and 2.9 nM for the I2183.54A, H219IC2A, P221IC2A, Y224IC2A, D3386.30A, I3396.31A, R3406.32A, L3416.33A, and T3446.36A receptors, respectively) comparable with that of the wild-type receptor (Kd value of 1.8 nM). The Kd values of [3H]SR141716A for the I2183.54A, H219IC2A, P221IC2A, D3386.30A, I3396.31A, R3406.32A, L3416.33A, and T3446.36A receptors, respectively, were also not significantly different from that of the wild-type receptor (Kd value of 2.1 nM). No significant differences were observed in the Bmax values of any of the receptors tested. In contrast, the Y224IC2A mutant receptor displayed no detectable binding to either [3H]CP55940 or [3H]SR141716A. These ligand binding data (with the exception of Y224IC2A mutant) suggest that all of the mutant receptors have levels of expression comparable with that of the wild-type and retain the ligand binding affinity of the wild-type CB1. To directly evaluate the importance of these nine residues in G protein coupling, [35S]GTPyS binding assays were performed for the I2183.54A, H219IC2A, P221IC2A, Y224IC2A, D3386.30A, I3396.31A, R3406.32A, L3416.33A, and T3446.36A receptors. This assay monitors the level of G protein coupling activity by determining the extent of binding of the nonhydrolyzable
analog of GTP to Go subunits. Because the CB1 receptor is known to couple to pertussis toxin-sensitive inhibitory G (G_i/o) protein, the [35S]GTPγS binding assay is suitable for evaluating the G protein association with this receptor (86).

First, we evaluated the impact that mutating these residues had on levels of [35S]GTPγS binding in the absence of ligand. The basal levels of G protein coupling activity for the I218A, H219IC2A, Y224IC2A, D3386.30A, I3396.31A, R3406.32A, L3416.33A, and T3446.36A receptors were 48.6, 50.7, 43.4, 49.1, 50.5, 45.5, 48.7, and 46.8 fmol/mg, respectively, which are substantially lower than that of the wild type (60.2 fmol/mg) and statistically not much different from the level of a mock-transfected sample (44.3 fmol/mg). In contrast, the basal level of G protein coupling activity for the P221IC2A receptor was 57.0 fmol/mg, which is comparable with the level of the wild-type receptor. It is important to note that the residual activity (~44 fmol/mg) in a mock-transfected sample is non-CB1-mediated and has also been observed by others (75, 87). Thus, all mutant receptors except for the P221IC2A receptor showed little or no CB1-mediated basal [35S]GTPγS binding. Because all of the mutants except the Y224IC2A receptor retained the wild type-like ligand binding properties for CP55940, we further evaluated the importance of the residues in agonist-promoted [35S]GTPγS binding to membranes expressing the wild-type and mutant receptors. Table 1 shows the levels of CP55940-induced [35S]GTPγS binding for the wild-type and mutant receptors. Although both the L3416.33A and the T3446.36A receptors showed virtually no
agonist-induced stimulation of \(^{[35]}\text{S}\)GTP\(\gamma\)S binding, the I218\(^{3,54}\)A, H219\(^{1C2}\)A, P221\(^{1C2}\)A, D338\(^{6,30}\)A, I339\(^{6,31}\)A, and R340\(^{6,32}\)A mutant receptors displayed an EC\(_{50}\) value of 4.0, 1.7, 2.9, 5.6, 1.9, and 7.3 nM, respectively, which is comparable with that of the wild-type (3.0 nM). However, a substantial reduction in \(E_{\text{max}}\) was observed for the I218\(^{3,54}\)A (\(E_{\text{max}} = 68.4 \text{ fmol/mg}\)), H219\(^{1C2}\)A (\(E_{\text{max}} = 66.3 \text{ fmol/mg}\)), D338\(^{6,30}\)A (\(E_{\text{max}} = 62.0 \text{ fmol/mg}\)), and R340\(^{6,32}\)A (\(E_{\text{max}} = 65.8 \text{ fmol/mg}\)) receptors relative to the wild type (\(E_{\text{max}} = 106.0 \text{ fmol/mg}\)). These results suggest that little G protein coupling activity occurs for these mutant receptors, but for the I218\(^{3,54}\)A, H219\(^{1C2}\)A, D338\(^{6,30}\)A, and R340\(^{6,32}\)A receptors, the concentration of CP55940 required to obtain that level of coupling activity is comparable with that required by the wild-type receptor. Some intermediate reduction in \(E_{\text{max}}\) values was observed for the I339\(^{6,31}\)A receptor (\(E_{\text{max}} = 78.8 \text{ fmol/mg}\)), suggesting that a partially impaired G protein coupling activity occurs for this mutant receptor. Note that CP55940 treatment showed no change in the mock-transfected sample, indicating that the CP55940-induced \(^{[35]}\text{S}\)GTP\(\gamma\)S is CB1 receptor-mediated (data not shown). Consistent with computational modeling results, these experimental data suggest that these residues may be G protein contact residues.

**DISCUSSION**

We experimentally evaluated the importance of key residues for G protein interaction. Our mutational study of the I218\(^{3,54}\)A, H219\(^{1C2}\)A, D338\(^{6,30}\)A, R340\(^{6,32}\)A, L341\(^{6,33}\)A, and T344\(^{6,36}\)A receptors indicates that these residues play key roles in CB1-mediated G protein coupling (Table 1 and Fig. 6), consistent with the present model of the CB1-G\(_i\) complex. Tyr-224\(^{1C2}\) may also be involved in G protein coupling, but because the Y224\(^{1C2}\)A receptor did not bind the agonist CP55940, we cannot assess whether the C-terminal helix \(\alpha_s\) is involved in a trafficking problem and a ligand binding defect. All of these receptor residues except for His-219\(^{1C2}\) form an interface with residues that are on different parts of the receptor. Among the residues examined by the present mutagenesis studies, Tyr-224\(^{1C2}\), Asp-338\(^{6,30}\), Arg-340\(^{6,32}\), and Thr-344\(^{6,36}\) with helix \(\alpha_s\) (Thr-329\(^{G_G}\)–Phe-354\(^{G_G}\)) (Fig. 5D). Considering that the extreme C-terminal helix \(\alpha_s\) of G\(_{s, G}\) forms the key contact surface for the activated receptor (85), Ala mutation of these residues in the receptor appears to eliminate crucial contacts with G\(_{s, G}\) and thereby greatly impair G protein coupling activity. That is what we observed in the present study (Table 1 and Fig. 6). Given that a shift in binding affinity for CP55940 (an agonist) or SR141716A (an inverse agonist) is expected if G protein coupling is altered, we are surprised to see that these mutant receptors bind the ligands like the wild-type receptor despite significantly disrupted G\(_i\) coupling activity (Table 1 and Fig. 6). Because these mutant receptors retain the wild-type ligand binding profile, it is not likely that they disrupt the receptor conformation such that the ligand-binding pocket is unfavorably modified. Instead, as in the wild-type receptor, the agonist-bound mutant receptors show the characteristic features of the activated receptor, including the breaking of the ionic lock and the outward movement of the IC end of TM6. Because the CB1 receptor binds G\(_i\) through multiple contacts not only with the C-terminal segment but also with other parts (helix \(\alpha_{sw}\), loop \(\beta_1\)–\(\alpha_1\) (P-loop), helix \(\alpha_1\), and strand \(\beta_6\)), the activated mutant receptors can still bind some G\(_i\) but much less due to the loss of a few key contacts for helix \(\alpha_s\). We cannot rule out the possibility that the mutant receptors bind G\(_i\) preferentially because its activity is not adequately detected by the \(^{[35]}\text{S}\)GTP\(\gamma\)S assay, which is much more sensitive to G\(_i\) than G\(_s\). However, according to the present CB1-G\(_i\) complex model, Arg-385\(^{G_G}\), Leu-388\(^{G_G}\), and Leu-393\(^{G_G}\) of G\(_i\), the corresponding residues that interact with Asp-338\(^{6,30}\), Arg-340\(^{6,32}\), Leu-341\(^{6,33}\), and Thr-344\(^{6,36}\), are highly conserved, suggesting that these mutant CB1 receptors may impair G protein coupling equally in G\(_i\) and G\(_s\).

The finding that His-219\(^{1C2}\) also plays a key role in CB1-mediated G protein coupling (Table 1 and Fig. 6) is unexpected because this residue maintains little contact with G\(_i\) in the present model of the CB1-G\(_i\) complex. A close examination, however, reveals that H219\(^{1C2}\) at the junction of TM3 and IC2 forms a hydrophobic cluster with Ile-216\(^{3,52}\), Ala-223\(^{1C2}\), and the aliphatic hydrocarbon portion of Arg-220\(^{1C2}\), suggesting its indirect role in CB1-mediated G protein coupling. Our observation that the P221\(^{1C2}\)A receptor does not affect CB1-mediated G protein coupling to a large extent (Table 1 and Fig. 6) is also unexpected because this residue forms close contacts with Leu-194\(^{G_G}\), Thr-340\(^{G_G}\), Ile-343\(^{G_G}\), and Asn-347\(^{G_G}\) on the C-terminal helix \(\alpha_s\) based on the present model of the CB1-G\(_i\) complex. This observation is also supported by the x-ray structure of the \(\beta_2\)-AR-G\(_i\) complex (12), wherein the equivalent Pro-138\(^{1C2}\) of \(\beta_2\)-AR forms close contacts with Ile-383\(^{G_G}\) and Gln-384\(^{G_G}\) on the C-terminal helix \(\alpha_s\). Thus, an Ala mutation of Pro-221\(^{1C2}\) would be expected to greatly destabilize the helical conformation of IC2 of the receptor (88). This disrupts CB1-mediated G protein coupling as a result of interference with the CB1-G\(_i\) interaction. Given the conservation of this position shared by Pro and Ala residues in class A GPCRs, the Ala mutation of Pro-221\(^{1C2}\) in the CB1 receptor appears to be a permissive mutation, retaining G protein coupling (Table 1). In support of this observation, the Ala mutation of the equivalent Pro-131\(^{1C2}\) of the M1 muscarinic acetylcholine receptor was found to little impair G protein coupling (80). Given that IC2 of \(\beta_2\)-AR forms an L-shaped non-helical conformation in the inactive state (89) but a helical conformation in the active state (12, 13), it appears that the helical conformation of IC2 is maintained in the P221\(^{1C2}\)A mutant receptor due to the propensity of IC2 to form a helix in the activated receptor upon association with G protein coupling.

Among the residues examined by the present mutagenesis studies, intermolecular interactions of Tyr-224\(^{1C2}\), Asp-338\(^{6,30}\), Arg-340\(^{6,32}\), and Thr-344\(^{6,36}\) with helix \(\alpha_s\) (Thr-329\(^{G_G}\)–Phe-354\(^{G_G}\)) of G\(_{s, G}\) through hydrogen bond/charge are unexpectedly tight, as indicated by the close interresidual distances maintained during the simulation (Fig. 4, D and E), given that hydrogen bond/charge interactions are weakened in the presence of solvent (90). A detailed examination of the CB1-G\(_i\) complex model reveals that most of these residues are partially buried by surrounding residues and that their accessibility to solvent is limited. Asp-338\(^{6,30}\) is unique in that it forms the intramolecular ionic lock with Arg-340\(^{6,32}\). Thus, its Ala mutation would lead to the breaking of the ionic lock and thereby shifting the equilibrium toward the active state, suggesting that Asp-
Key CB1 Contacts with the C-terminal Helix $\alpha_5$ of G$\alpha_i$

A $\beta_{338\,6.30}$ is involved in receptor activation. Interestingly, however, the D338$^{6.30}$N mutation of the CB1 receptor resulted in a decrease in the maximum response of agonist-induced activation without affecting CP55940 binding affinity relative to the wild type (91). Consistent with this, we found that the D338$^{6.30}$A mutation did not alter CP55940 binding affinity but caused an ~70% decrease in the $E_{\text{max}}$ value (Table 1). Thus, it is possible that the Asp-3386.30 → Ala mutation does not result in a fully active conformation of the receptor, because it cannot couple to G protein, supporting the present CB1-Gi complex model. Among the residues examined by the present mutagenesis studies, Ile-218$^{3.54}$, Leu-341$^{6.33}$, and Thr-344$^{6.36}$ are unique in that they form tight hydrophobic interactions at the receptor-Ga C-terminal interface (Fig. 5, A and C). Thus, it is possible that their Ala mutations would alter the receptor conformation, inducing a steric occlusion in the receptor-Ga C-terminal binding interface rather than a direct contact. The significant decrease of coupling by L341$^{6.33}$A and the complete loss of coupling by L341$^{6.33}$A and T344$^{6.36}$A, compared with other examined Ala mutations (Table 1 and Fig. 6), would support this idea.

The G protein cycle is thought to proceed via the following sequential events: (i) the GDP-bound heterotrimeric Ga$\beta\gamma$ protein forms a complex with the activated receptor; (ii) the GDP release from the nucleotide-binding pocket leads to the nucleotide-free Ga$\beta\gamma$-receptor complex; (iii) the GTP (or GTP$\gamma$S) is added to the “empty complex”; and (iv) the GTP-bound Ga$\alpha$ is dissociated from the Ga$\beta\gamma$ subunits and the receptor (6). Our finding that the position of the extreme C-terminal helix $\alpha_5$ of Ga$\alpha$ in the CB1-Gi complex is remarkably similar to that of the corresponding G$\alpha_i$CT peptide in the metarhodopsin II-G$\alpha_i$CT complex (63) (Fig. 3C) suggests that our system of the CB1-Gi complex is at the same stage as the metarhodopsin II-G$\alpha_i$CT complex, which is in the initial stage of the complex formation between the activated receptor and the G protein.

Given that recent electron paramagnetic resonance spectroscopy (EPR) studies suggest that ~10 residues from the extreme C-terminal helix $\alpha_5$ of Ga$\alpha$ form the contact surface with the activated receptor and that a rigid body rotation/translation of helix $\alpha_5$ induced by the activated receptor is necessary for GDP release (85), it appears that helix $\alpha_5$ of Ga$\alpha$ plays a key role in the interaction of Ga$\alpha$ with the activated receptor. It is known that the extreme C-terminal helix $\alpha_5$ undergoes a conformational change upon the binding of the activated receptor (i.e., disordered when unbound to the receptor but highly ordered when bound to the receptor) (85, 92). It appears that the conformational changes in helix $\alpha_5$ govern the degree of the interaction of Ga$\alpha$ with the receptor, as shown in the RMSD values of Ga$\alpha$ in close association with the tilt angle change in helix $\alpha_5$ of Ga$\alpha$ (Fig. 2B). The results of the present simulation also show that the conformation of helix $\alpha_5$ of Ga$\alpha$ changes dynamically in response to conformational changes in IC2 and IC3 of the CB1 receptor (Fig. 3D).

The position of the G$\alpha_i$CT peptide in the metarhodopsin II-G$\alpha_i$CT complex (63) is tilted by ~30° away from TM6, compared with the position of the corresponding helix $\alpha_5$ of Ga$\alpha$ in the nucleotide-free active state of G$\alpha_i$ ($\beta$$_{2}$AR-G$\alpha_i$) (12). Based on these observations, Rasmussen et al. (12) has proposed that the initially bound C-terminal helix $\alpha_5$ of Ga$\alpha$ of the $\beta$$_{2}$AR-G$\alpha_i$ complex requires a rotation of Ga$\alpha$ ~90° along the axis parallel to the membrane normal. As a result, the N terminus of Ga$\alpha$ is aligned with the G$\alpha_i$CT peptide (i.e., inactive state) becomes overlapped with the N terminus of Ga$\alpha$ in the $\beta$$_{2}$AR-G$\alpha_i$ complex (12) (i.e., active state). Superposition of the present model of the inactive state G$\alpha_i$ (CB1-Gi) on the active state of G$\alpha_i$ ($\beta$$_{2}$AR-G$\alpha_i$) (12) reveals that IC2 and TM6/IC3 of $\beta$$_{2}$AR move away from the middle and the C-terminal end of helix $\alpha_5$, respectively, compared with the CB1 receptor (Fig. 3E). On the other hand, both helix $\alpha_5$ and helix $\alpha_6$ of Ga$\alpha$Ras are in the same orientation but with slightly different tilt angles in these complexes (Fig. 3E). Thus, it appears that in order for the CB1-Gi complex to achieve the active state of Gi (i.e., GDP release) a rotation along the axis perpendicular to the membrane side is required, as shown in Fig. 3E. Such rotation, somewhat different from the proposed rotation around the axis parallel to the membrane normal (12), would lead to GDP release as a result of the disruption of (i) loop $\beta_i$-$\alpha_i$ (P-loop), a contact region for the purine ring moiety of GDP, attached to helix $\alpha_n$ of Ga$\alpha$Ras, and (ii) loop $\beta_i$-$\alpha_i$ (TCAT motif), a contact region for the $\beta_i$-phosphate moiety of GDP, attached to helix $\alpha_c$ of Ga$\alpha$Ras. A similar mechanism for receptor-mediated GDP release has recently been proposed (79).

Homology models of the CB1 receptor using the known x-ray structures of GPCRs as templates have been justified by sequence alignment with these GPCRs according to the highly conserved (>90%) TM residues (82). In this study, under our working hypothesis that the conformational change in Ga$\alpha$ is small during G protein activation, compared with that in GaAH as seen in the x-ray structure of the $\beta$$_{2}$AR-G$\alpha_i$ complex (12), we started with a model of the CB1-Gi complex, where G$\alpha_i$ represents the GDP-bound inactive state, using the x-ray structure of the $\beta$$_{2}$AR-G$\alpha_i$ complex, where G$\alpha_i$ represents the nucleotide-free active state (12), the only available x-ray structure of the GPCR-G$\alpha_i$ complex, as the docking template and determined the specific orientation of the two proteins (i.e., the CB1 receptor and G$\alpha_i$) through an extensive MD simulation. To keep the CB1 receptor and G$\alpha_i$ in a reasonably close distance for favorable complex formation and at the same time to minimize a biased complex conformation, we applied a minimum set of interresidual distances between the CB1 receptor and the G protein only at the receptor-Ga$\alpha_i$ C-terminal interface while allowing the rest of the intermolecular interactions to be freely adjusted during the simulation. At the end of the simulation, most of the applied interresidual distances of the receptor-Ga$\alpha_i$ C-terminal interface were lost. In fact, the resulting CB1-Gi complex appears to resemble the metarhodopsin II-G$\alpha_i$CT complex (63) (Fig. 3C), which is the inactive GDP-bound G protein at an early stage of complex formation, rather than the $\beta$$_{2}$AR-G$\alpha_i$ complex, which is at the activated “empty complex” stage. This result suggests that although we started with a structure similar to the known $\beta$$_{2}$AR-G$\alpha_i$ complex (12), we ended with a structure unique to the CB1-Gi complex. As discussed above, the position of the extreme C-terminal helix $\alpha_5$ of Ga$\alpha$ in the CB1-Gi complex is distinct from the corresponding helix of G$\alpha_i$ (Fig. 3E), whereas there is little change in the Ga$\alpha$Ras structure of Ga$\alpha$ in these structures, as indicated by small RMSDs (<2.0 Å). Because the overall orientation of the G protein relative to the
Key CB1 Contacts with the C-terminal Helix $\alpha_2$ of Go$_i$
Key CB1 Contacts with the C-terminal Helix $\alpha_5$ of G$\alpha_5$

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Key CB1 Contacts with the C-terminal Helix $\alpha_5$ of $\alpha_i$