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Hyunsu Bae

Kristin Anderson

Lori A. Flood

Nikolai P. Skiba

Heidi E. Hamm

See next page for additional authors

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Molecular Determinants of Selectivity in 5-Hydroxytryptamine$_{1B}$ Receptor-G Protein Interactions*

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Hyunsu Bae‡§, Kristin Anderson¶, Lori A. Flood¶, Nikolai P. Skiba§, Heidi E. Hamm§, and Stephen G. Graber¶‖

From the §Institute for Neuroscience, Northwestern University, Chicago, Illinois 60611, the ‡Department of Physiology & Biophysics, College of Medicine, University of Illinois, Chicago, Illinois 60612, and the ¶Department of Pharmacology & Toxicology, West Virginia University, Morgantown, West Virginia 26506

The recognition between G protein and cognate receptor plays a key role in specific cellular responses to environmental stimuli. Here we explore specificity in receptor-G protein coupling by taking advantage of the ability of the 5-hydroxytryptamine$_{1B}$ (5-HT$_{1B}$) receptor to discriminate between G protein heterotrimers containing Gα_{i1} or Gα_t. G$_{i1}$ can interact with the 5-HT$_{1B}$ receptor and stabilize a high affinity agonist binding state of this receptor, but G$_t$ cannot. A series of Gα_t/Gα_{i1} chimeric proteins have been generated in *Escherichia coli*, and their functional integrity has been reported previously (Skiba, N. P., Bae, H., and Hamm, H. E. (1996) *J. Biol. Chem.* 271, 413–424). We have tested the functional coupling abilities of the Gα_t/Gα_{i1} chimeras to 5-HT$_{1B}$ receptors using high affinity agonist binding and receptor-stimulated guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) binding. In the presence of $\beta\gamma$ subunits, amino acid residues 299–318 of Gα_{i1} increase agonist binding to the 5-HT$_{1B}$ receptor and receptor stimulation of GTPγS binding. Moreover, Gα_{i1} containing only Gα_t amino acid sequences from this region does not show any coupling ability to 5-HT$_{1B}$ receptors. Our studies suggest that the α4 helix and α4-β6 loop region of Gαs are an important region for specific recognition between receptors and G$_i$ family members.

The heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) mediate signaling from a large number of diverse heptahelical cell surface receptors to a variety of intracellular effectors. These pathways control numerous essential functions in all tissues and are ubiquitous throughout eukaryotes (1–3). A large body of work investigating the mechanisms underlying receptor-G protein interactions now exists. The early view that signaling selectivity would manifest itself on the basis of specific protein interactions allowing a receptor to couple with a unique G protein to modulate a single effector is no longer tenable with the accumulating evidence of a network of interactions that converge and diverge at multiple levels. Even in the earliest receptor-G protein reconstitution studies using phospholipid vesicles, it was clear that, while there were large differences in the efficiencies of coupling among the major families of G proteins, receptors were capable of activating multiple G proteins from distinct families (4, 5).

Elucidation of the crystal structures of α subunits in both active (6, 7) and inactive conformations (8), an isolated $\beta\gamma$ subunit (9) and the $\alpha\beta\gamma$ heterotrimer (10, 11), has begun to define a mechanistic basis for data from mutagenesis, chimera, and peptide studies defining functional domains on G protein subunits (12–17). A variety of studies have implicated the C terminus of α subunits in mediating receptor-G protein selectivity (13–15). Synthetic peptides from the C terminus of α_t (amino acids 340–350) have been shown to stabilize the active conformation of metarhodopsin II (17) while alanine scanning mutagenesis of the same region has identified four specific residues crucial for α_t activation by rhodopsin (12). Similarly, two C-terminal peptides from α_s (354–372 and 384–394), but not the corresponding peptides from α_{i2}, could evoke high affinity agonist binding to β-adrenergic receptors and block their ability to activate α_s (16). Substitution of three to five C-terminal amino acids of α_q with the corresponding residues from α_i allowed receptors that normally signal exclusively through α_i subunits to activate the chimeric α subunits and stimulate the G$_q$ effector, phospholipase C-β (PLC-β) (13). However, a similar chimeric approach revealed that sequences in addition to the C terminus were required for specificity of activation of α_{16} subunits by the C5a receptor (18). Other studies have also implicated a role for N-terminal sequences in receptor-G protein coupling (17, 19). Thus it appears that the molecular determinants of receptor-G protein coupling vary somewhat among specific families of receptors and G proteins. Studies presented below have revealed a previously unappreciated region involved in receptor-G protein coupling that mediates the discrimination between α_i and α_t subunits by 5-hydroxytryptamine$_{1B}$ (5-HT$_{1B}$)[1] receptors. Furthermore, these studies implicate a secondary role for an N-terminal α subunit region, but not a C-terminal region, in stabilizing high affinity agonist binding to 5-HT$_{1B}$ receptors as well as G protein activation (GTPγS binding).

EXPERIMENTAL PROCEDURES

Materials—GTP, GDP, GTPγS, AMP-PNP, deoxyribonucleotides, and imidazole were purchased from Boehringer Mannheim. Restriction enzymes, DNA modifying enzymes, and *Taq* DNA polymerase were from Boehringer Mannheim and Pharmacia Biotech Inc. 5-HT was a product of Sigma. [^{35}S]GTPγS and [^{3}H]5-HT were purchased from NEN Life Science Products.

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‖ To whom correspondence should be addressed: 3152 Health Sciences North, P.O. Box 9223, Morgantown, WV 26506-9223. Tel.: 304-293-2305; Fax: 304-293-6854; E-mail: sgraber@wvu.edu.

[1] The abbreviations used are: 5-HT, 5-hydroxytryptamine; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; AMP-PNP, adenosine 5′-(β,γ imino)triphosphate; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; β-ME, β-mercaptoethanol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

Construction of Gα_t/Gα_{i1} Chimeric Genes—We used *Escherichia coli* expression vectors pHis$_6$Gα_{i1} and pHis$_6$Gα_t, which contain Gα_{i1} or Gα_t cDNAs, respectively, preceded by a nucleotide sequence encoding a hexahistidine tag under the control of a T7 promoter (20). Chimeric genes were constructed by insertion of unique restriction enzyme sites into Gα_{i1} or Gα_t cDNAs using PCR amplification with corresponding oligonucleotide primers followed by replacement of Gα_{i1} cDNA fragments with corresponding Gα_t cDNA fragments or vice versa. Insertion of a *Nae*I site in the Gα_{i1} cDNA corresponding to amino acid residues 298–299 of Gα_{i1}, resulted in the replacement of Ala^{299} with Gly in Chi13 and Chi14. All other chimeras have only native amino acid residues from the specific gene product as indicated.

Expression and Purification of Gα_t/Gα_{i1} Chimeras—The chimeric Gα subunits were expressed in *E. coli* BL21(DE3) cells and purified as described previously (20). Briefly, cell pellets were resuspended in Buffer A (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 50 μM GDP, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM β-mercaptoethanol (β-ME)) and were disrupted by ultrasonication. The crude cell lysate was centrifuged at 100,000 $\times$ *g* for 60 min, and the supernatant was adjusted to 500 mM NaCl and 20 mM imidazole by addition of 8 $\times$ binding buffer (160 mM Tris-HCl, pH 8.0, 4 M NaCl, and 160 mM imidazole) before being loaded onto 5 ml of Ni^{2+}-nitrilotriacetic acid-agarose resin column (His-Bond, Novagen) prepared according to the manufacturer protocol. The column was washed with 10 volumes of 1 $\times$ binding buffer followed by elution of the protein using Buffer I-100 (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 100 mM imidazole). The protein sample was dialyzed overnight against Buffer A in the presence of 20% glycerol, PMSF, and β-ME and then further purified by high performance liquid chromatography using anion exchange resin (Waters Protein-Pak QHR-15, Waters Chromatography) packed in an AP-1 column (Waters Chromatography). The samples were adjusted to 25 μM GDP, 2 mM β-ME, 0.1 mM PMSF, and 40% glycerol and then stored at −80 °C. The purity of proteins was verified by SDS-polyacrylamide gel electrophoresis. The protein concentrations were determined using the Coomassie Blue method (21) with bovine serum albumin (Pierce) as the standard.

Expression and Purification of G Proteins and 5-HT$_{1B}$ Receptors—The expression in Sf9 cells and purification of the recombinant G protein α and $\beta\gamma$ subunits were performed as described previously (22, 23) except that the final chromatography step was performed on 15 micron Waters Protein-Pak QHR (Waters Chromatography). Dr. Eric Parker (Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT) provided the 5-HT$_{1B}$ receptor baculovirus. Membranes from Sf9 cells infected with these viruses typically contained 2–6 pmol of receptor/mg of membrane protein. The native retinal α (Gα_t) and $\beta\gamma$ subunits used for the GTPγS binding experiment were purified as described previously (24).

Preparation of Sf9 Membranes Containing Expressed Receptors—Sf9 cells were infected with recombinant baculovirus containing cDNA for the 5-HT$_{1B}$ receptor, cultured, and harvested as described previously (22). To prepare membranes, harvested cells were thawed in 15 times their wet weight of ice-cold homogenization buffer (10 mM Tris-HCl, pH 8.0, 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, 20 μg/ml of benzamidine, and 2 μg/ml each of aprotinin, leupeptin, and pepstatin A) and burst by N_2 cavitation (600 p.s.i., 20 min). Cavitated cells were centrifuged at 500 $\times$ *g* for 10 min at 4 °C to remove the unbroken nuclei and cell debris. The supernatant from the low speed spin was centrifuged at 28,000 $\times$ *g* for 30 min at 4 °C. The supernatant was discarded, and the pellets were resuspended and pooled in 35 ml of buffer (5 mM NaHEPES, pH 7.5, 1 mM EDTA, and the same protease inhibitors as used in the homogenization buffer). The membranes were washed twice, resuspended in the same buffer (1–3 mg of protein/ml), aliquoted, snap frozen in liquid nitrogen, and stored at −70 °C.

Reconstitution of Receptors with Exogenous G Proteins—Frozen membranes were thawed, pelleted in a refrigerated microcentrifuge (10 min), and resuspended at 8–10 mg/ml in a reconstitution buffer (5 mM NaHEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 500 nM GDP, 0.04% CHAPS). G protein subunits were diluted in the same buffer such that the desired amount of subunit was contained in 1–5 μl. Typically, 1–2 μl of G protein subunits were added to 15–40 μl of membrane suspension, and then the mixture was incubated at 25 °C for 15 min and held on ice until the start of the binding assay. Just prior to the start of the binding assay, the reconstitution mixture was diluted 10–12-fold with binding assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA) such that the desired amount of membranes were contained in 50 μl.

[^{3}H]5-Hydroxytryptamine Binding Assay—[^{3}H]5-HT binding to 20–50 μg of membrane protein was determined in binding assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA) in the presence of the desired components. Nonspecific binding was determined by addition of 10 μM 5-HT. Incubations were for 1.5 h in a room temperature shaker and were terminated by filtration over Whatman GF/C filters using a Brandel cell harvester. The filters were rinsed 3 times with 4 ml of ice-cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.01% NaAzide), placed in 4.5 ml of CytoScint (ICN Pharmaceuticals, Costa Mesa, CA), and analyzed for amount of retained radioactivity in a scintillation counter. To characterize levels of expressed receptors in membrane preparations, saturation binding isotherms were used. In these studies, the concentration of [^{3}H]5-HT ranged from 0.07 to 250 nM in a final volume of 500 μl. For reconstitution of high affinity agonist binding in affinity shift assays, a single concentration (0.4–1.2 nM) of [^{3}H]5-HT was used in a final volume of 150 μl. Radioligand purity was monitored by chromatography on a Zorbax ODS column (4.6 $\times$ 150 mm) using 1% triethylamine acetate (pH 4):methanol (95:5) as the mobile phase. Radioligand was repurified or replaced when the radiochemical purity fell below 85%.

Fluorescence Assay—To measure the folded state of chimeric Gα molecules, intrinsic fluorescence was measured with a Perkin-Elmer Corp. LS5B spectrofluorometer at room temperature in buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$). The AlF_4^--dependent conformational changes of activated Gα subunits were monitored by intrinsic tryptophan fluorescence changes with excitation at 280 nm and emission at 340 nm. The relative increase in fluorescence of 200 nM Gα subunits was determined from absorbance readings before and after addition of 10 mM NaF and 20 μM $AlCl_3$.

5-HT Receptor-stimulated GTPγS Binding Assay—Prior to the assay, the Sf9 cell membranes expressing 5-HT$_{1B}$ receptors were incubated with 1 mM AMP-PNP at 37 °C for 1 h. Membranes (90 μg of protein, 3.4 fmol of receptor/μg) were reconstituted with the indicated Gα subunits and retinal $\beta\gamma$ subunits on ice in 70 μl of reaction buffer (25 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol) for 30 min. Following reconstitution, the suspended membranes were diluted by the addition of 200 μl of reaction buffer containing 150 nM GDP and 60 nM GTPγS. The reaction was initiated by transferring 30 μl of reaction buffer containing carrier-free [^{35}S]GTPγS (~7 $\times$ 10^6 cpm) to the diluted membranes, and the reaction tube was incubated at 25 °C in a water bath. In these experiments, the following final concentrations were used: 1 nM 5-HT$_{1B}$ receptor (90 μg of protein/reaction, 3.4 fmol of receptor/μg), 40 nM Gα, and 40 nM retinal $\beta\gamma$. For agonist activation, 1 μM 5-HT was included. Samples (20 μl) were withdrawn at various times, and the reaction was terminated by passing through a Millipore Multiscreen-HA 96-well filtration plate followed immediately by 6 washes with 200 μl of ice-cold wash buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 25 mM $MgCl_2$). The filters were presoaked by washing 2 times with 200 μl of ice-cold washing buffer before adding samples. The filters were dried and punched out using a Millipore Multiscreen Puncher, and the amount of retained radioactivity was quantified in a liquid scintillation counter.

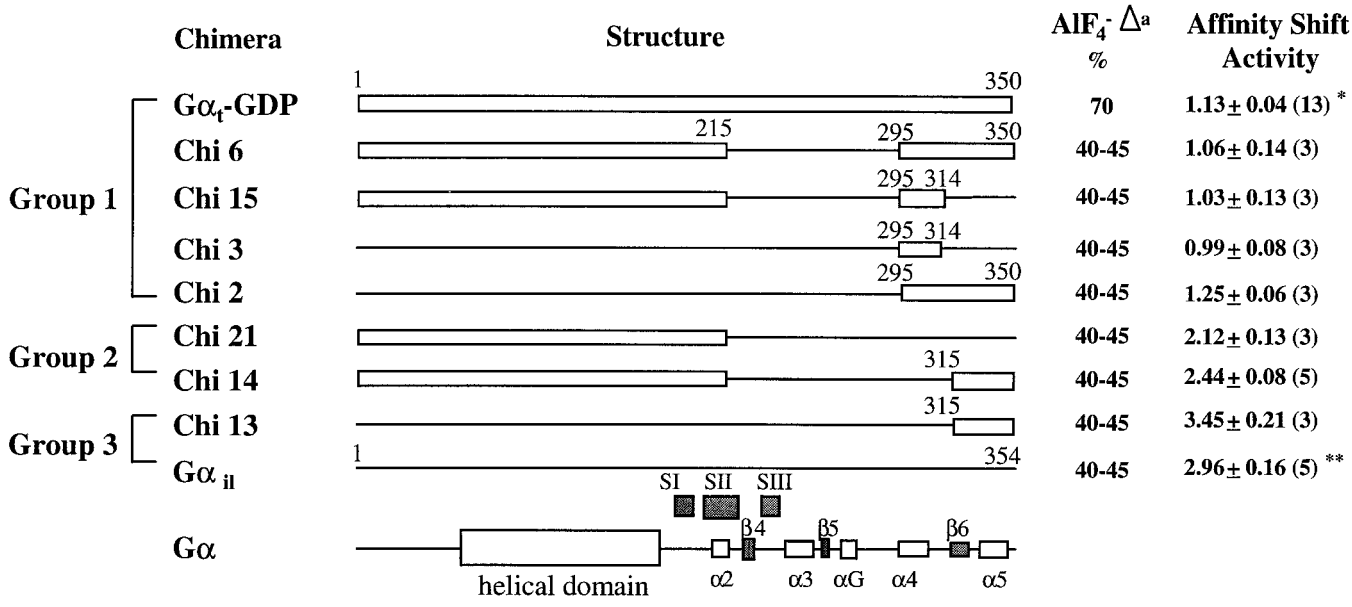
RESULTS

Selective Interaction of G$_i$ Family Members with 5-HT$_{1B}$ Receptors—Previous work has demonstrated the ability of 5-HT$_{1B}$ receptors to form a high affinity agonist binding state in the presence of G protein heterotrimers containing $\alpha_{i/o}$ but not α_t subunits (25). Moreover, the discrimination was shown to be entirely at the level of the α_t subunit as bovine brain or retinal $\beta\gamma$ subunits were equally effective in formation of the high affinity agonist binding state in the presence of $\alpha_{i/o}$ subunits (25). These observations made it possible to use recombinant Gα_t/Gα_{i1} chimeric proteins produced in *E. coli* to map the regions of the Gα_i subunit responsible for differential coupling with this receptor. These chimeric proteins are properly folded and functional by a variety of criteria: they bind GDP, undergo catalyzed GTP/GDP exchange in the presence of retinal $\beta\gamma$ subunits and light activated rhodopsin, and take on the active, GTP-bound conformation. The chimeric proteins and Gα_{i1} contain a hexahistidine tag at the N terminus to facilitate purification using metal ion affinity chromatography. The ability of chimeric proteins to bind GDP and undergo conformational change upon binding to GTP was tested by determining if the proteins undergo an AlF_4^--dependent increase in tryptophan

TABLE I

Affinity shift activity and Alf$_4^{23}$-dependent tryptophan fluorescence change of Gα subunits with secondary structure

Numbers above the structures symbolizing chimeras indicate the junction points of Gα$_t$ and Gα$_{i1}$ sequences and refer to amino acid positions in Gα$_t$. Affinity shift activities refer to the -fold enhancement above buffer controls of high affinity [^{3}H]5-HT binding in membranes expressing 5-HT$_{1B}$ receptors reconstituted with G protein heterotrimers containing the indicated Gα subunits (see text and Fig. 3 for additional explanation). The affinity shift activity data represent the mean ± S.E. from the indicated number of experiments. *, activity of native Gα$_t$; **, activity of Gα$_{i1}$ expressed in *E. coli*.



[a] Percent increase of tryptophan fluorescence in the presence of 10 mM NaF and 20 μM $AlCl_3$ (see "Experimental Procedures" for detail).

fluorescence (20). This assay is based on the ability of AlF_4^-, which mimics the γ-phosphate of GTP, to induce the active conformation resulting in an increase in intrinsic fluorescence of Trp^{207} in Gα$_t$ (Trp^{211} in Gα$_{i1}$). Tryptophan fluorescence of all chimeras and Gα$_{i1}$ used in this study increases 40–45% upon the addition of AlF_4^- (Table I), which is consistent with our previous results (20). The chimeric Gα$_t$/Gα$_{i1}$ proteins used are shown schematically in Table I.

Fig. 1 compares the abilities of the indicated heterotrimers to interact functionally with 5-HT$_{1B}$ receptors in an "affinity-shift" assay. This assay takes advantage of the fact that the majority of the receptors expressed in Sf9 cells are in a low affinity state for agonist and can be converted to a high affinity state for agonist by the addition of appropriate exogenous G protein heterotrimers (25). Agonist binding to reconstituted membranes using a single, low concentration of agonist (near the K_D for the high affinity state and well below the K_D for the low affinity state) readily detects the formation of the high affinity state as an enhanced level of binding. This assay has been completely described for several receptors expressed in Sf9 cells (25, 26) and is based on earlier work with native receptors (27, 28). In the absence of any added G protein, Sf9 cell membranes expressing 5-HT$_{1B}$ receptors exhibit a small amount of high affinity agonist binding, which is decreased by GTPγS. This suggests that endogenous G proteins present in Sf9 cells couple a small number of the expressed receptors. Fig. 1 shows that bacterially expressed, non-myristoylated α$_{i1}$ was able to support high affinity agonist binding although the apparent affinity for agonist is somewhat less than with acylated α$_{i1}$ expressed in Sf9 cells. Although the G proteins were present in saturating amounts (data not shown), ~14% less [^{3}H]5-HT was bound in the presence of Gα$_{i1}$ expressed in *E. coli* compared with Gα$_{i1}$ expressed in Sf9 cells. Fig. 1 also shows that GTPγS eliminates the high affinity agonist binding stabilized by either Gα$_{i1}$, consistent with the interpretation that the added G proteins are coupling the expressed receptor in the accepted fashion. Retinal G$_t$ has no effect on agonist binding. Importantly, as shown in Fig. 1, Chi6 is also unable to functionally couple with the 5-HT$_{1B}$ receptor. This chimera (Chi6) differs from native α$_t$ at just 26 positions between amino acids 215–295 of α$_t$ (see Table I) and closely resembles native α$_t$ in its interactions with cGMP phosphodiesterase-γ (the transducin effector) and rhodopsin (the visual receptor that activates transducin), as well as, in its intrinsic guanine nucleotide exchange properties (20). Additional experiments, in which Chi6 has been reconstituted at a concentration of 3 μM in a 310-fold molar excess over receptor, have given no indication that Chi6 can functionally couple with 5-HT$_{1B}$ receptors (data not shown).

The 5-HT$_{1B}$ receptor also distinguishes between G$_i$ and G$_t$ in terms of G protein activation. We used a GTPγS binding assay to quantitate agonist-dependent receptor-catalyzed GTP/GDP exchange. The data in Fig. 2 show that Gα$_{i1}$ undergoes GTP/GDP exchange by agonist-activated 5-HT$_{1B}$ receptor in the presence of retinal βγ. In contrast, Chi6, the Gα$_t$-like chimera, does not exchange GDP for GTP in the presence of agonist-activated receptors. The relatively high basal rate of GTP/GDP exchange on Gα$_{i1}$ seen in this assay is similar to previously reported data (20, 29). Based on the high affinity agonist binding and GTP/GDP exchange data, we decided to explore the molecular basis of this discrimination between Gα$_{i1}$ and Gα$_t$ by 5-HT$_{1B}$ receptors.

Affinity Shift Activity of Different Gα$_t$/Gα$_{i1}$ Chimeras with 5-HT$_{1B}$ Receptors—With respect to the ability of specific G proteins to induce the high affinity agonist binding state of heptahelical receptors, previous work with both 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors has shown that differences among various Gα$_{i/o}$ subunits are largely in the affinity of the receptor for agonist rather than in the affinity of the receptor for the G protein (25). Thus as increasing amounts of G proteins were added to Sf9 cell membranes containing expressed receptors, maximal levels of high affinity agonist binding (as measured in

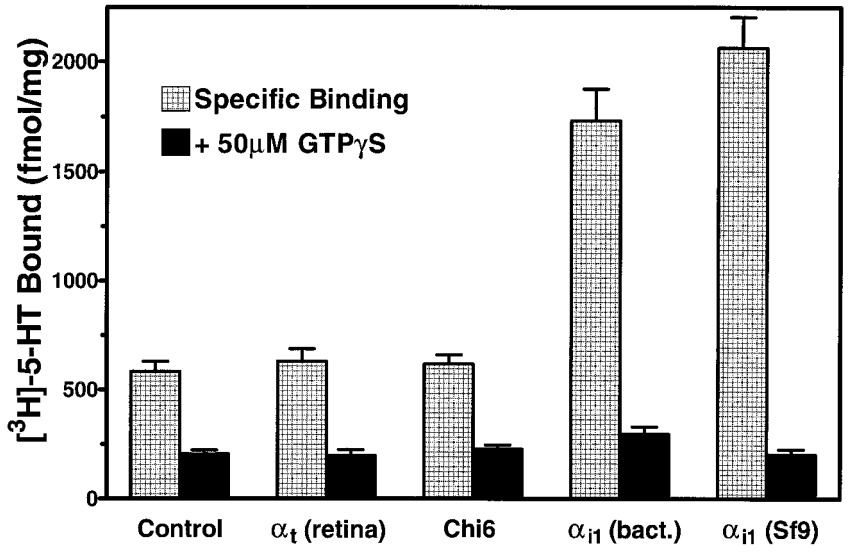


FIG. 1. **[^{3}H]5-HT binding in membranes from Sf9 cells reconstituted with G protein subunits.** Membranes (20 μg of protein/filter, 3.4 fmol of receptor/μg) from Sf9 cells expressing 5-HT$_{1B}$ receptors were reconstituted with buffer (*Control*) or the indicated Gα subunits with an excess of $\beta\gamma$ subunits and used in a [^{3}H]5-HT binding assay as described under "Experimental Procedures." The concentration of [^{3}H]5-HT used was 0.5 nM, and the Gα subunits were present in 100-fold molar excess (45 nM final concentration) over receptors. *Bars* represent specific binding as the mean ± S.D. of triplicate determinations from a representative experiment.

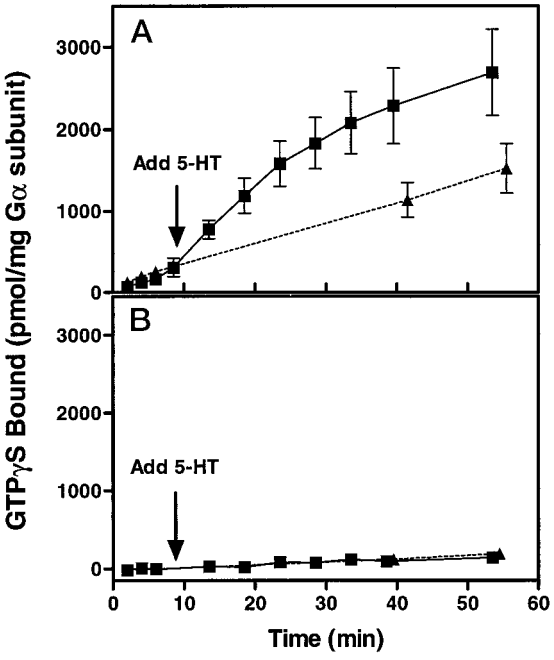


FIG. 2. **5-HT$_{1B}$ receptor catalyzed GTP/GDP exchange on G$_t$ *versus* G$_{i1}$.** Membranes expressing 5-HT$_{1B}$ receptor were reconstituted with either Gα_{i1} (*panel A*) or Chi 6 (*panel B*) and $\beta\gamma$ subunits. *Squares* indicate GTPγS binding in the presence of 1 μM 5-HT (added at the 8.5 min point), and *triangles* indicate the binding in the absence of agonist. Final concentrations used were: 40 nM G$\alpha\beta\gamma$ subunits, 1 nM 5-HT$_{1B}$ receptors (6 μg of protein/filter, 3.4 fmol of receptor/μg), 40 nM [^{35}S]GTPγS, and 100 nM GDP. Aliquots were withdrawn at the indicated times, filtered, and counted. Data shown are the mean ± S.E. of four independent experiments.

the presence of a single low concentration of agonist) were achieved. The maximal levels achieved differed depending on the identity of the G$\alpha_{i/o}$ subunit, whereas the amount of the G$\alpha_{i/o}$ subunits required to produce the maximal level of binding did not differ significantly for a given receptor (25). Typically, maximal levels of high affinity agonist binding have been achieved with a 5–20-fold molar excess of G protein over receptor (25, 26). In the present study, the actual concentration of agonist varied slightly in separate experiments, and small variations in agonist concentration at the low concentrations used in affinity shift assays result in significant differences in the absolute levels of binding observed. Therefore, it was necessary to define an affinity shift activity to compare the abilities of the Gα_t/Gα_{i1} chimeras to undergo functional interactions with 5-HT$_{1B}$ receptors. Affinity shift activity has been defined as the -fold enhancement above buffer controls of high affinity [^{3}H]5-HT binding in membranes expressing 5-HT$_{1B}$ receptors reconstituted with G protein heterotrimers in 40–100-fold molar excess over receptors. Subunits with no ability to produce a high affinity agonist binding state in 5-HT$_{1B}$ receptors would have an affinity shift activity of 1, whereas those subunits with similar abilities as fully myristoylated G$\alpha_{i/o}$ subunits would have affinity shift activities between 3 and 4. Fig. 3 summarizes the affinity shift activities of the Gα_t/Gα_{i1} chimeras depicted in Table I. These determinations have been made in 3–13 independent experiments using four separate membrane preparations where 5-HT$_{1B}$ receptors were expressed between 3–10 pmol/mg membrane protein and exogenous G protein concentrations ranged from 0.3–1.0 μM during reconstitution. The concentration of [^{3}H]5-HT used in the binding assays was 0.6–1.2 nM in all experiments. Within each experiment, the concentrations of G proteins and receptors were identical for all chimeras being tested, and each experiment included Gα subunits with minimal (native Gα_t or Chi6) and maximal (Gα_{i1} expressed in *E. coli* or Sf9 cells) affinity shift activities.

Based upon the observation that Chi6, the α_t-like chimera, cannot couple to the 5-HT$_{1B}$ receptor, we constructed Chi2 and Chi21 to determine whether the N-terminal or C-terminal region of Gα_{i1} underlies functional coupling. Fig. 3 shows that Chi2 still has no ability to induce high-affinity agonist binding, whereas Chi21 is nearly as effective as Gα_{i1} (2.12 ± 0.13-fold and 2.96 ± 0.16-fold increase, respectively). From this, we conclude that functional coupling is contributed by residues in the C-terminal 55 residues of Gα_{i1}. Examination of the amino acid sequences of Gα_{i1} and Gα_t show that they are highly similar in sequence from β-sheet 6 to the C terminus. Thus we constructed Chi14, which is identical to Chi6 except for the replacement of residues 295–314 of Gα_t with the corresponding residues of Gα_{i1} (residues 299–318). This chimera supports high affinity agonist binding to the same degree as Chi21. To examine how critical these residues are for receptor interaction, Chi3 was constructed, which changed just these residues back to the Gα_t sequence in the context of Gα_{i1}. Chi3, even when reconstituted at a concentration of 1.7 μM in a 175-fold molar excess over receptor, has no ability to stabilize high affinity agonist binding to 5-HT$_{1B}$ receptors (data not shown), proving that this region contains a key determinant of receptor coupling.

Although several lines of experiments suggest that the C-

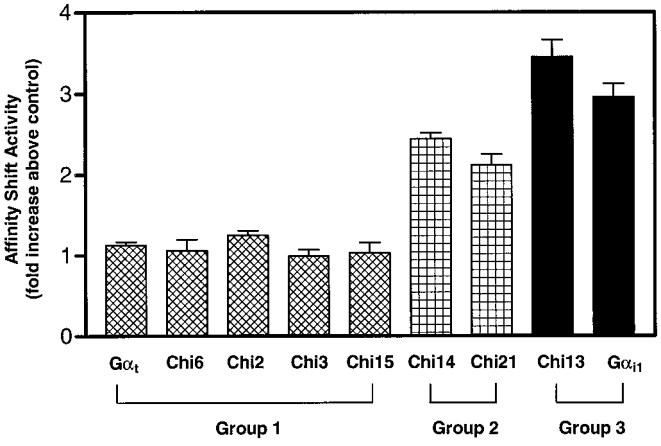


FIG. 3. **Affinity shift activity of various Gα_t/Gα_{i1} chimeras with 5-HT$_{1B}$ receptors.** Affinity shift activities refer to the -fold enhancement above buffer controls of high affinity [^{3}H]5-HT binding to 5-HT$_{1B}$ receptors reconstituted with G protein heterotrimers containing the indicated α subunits (see "Results" for further explanation). Data represent the mean ± S.E. from three to thirteen independent determinations using four separate membrane preparations where 5-HT$_{1B}$ receptors were expressed between 3–10 pmol/mg membrane protein, and exogenous G protein concentrations ranged from 0.3 to 1.0 μM during reconstitution. The concentration of [^{3}H]5-HT was between 0.6 and 1.2 nM in all experiments. Gα_t represents the activity of native transducin, and Gα_{i1} represents the activity of α_{i1} expressed in *E. coli*.

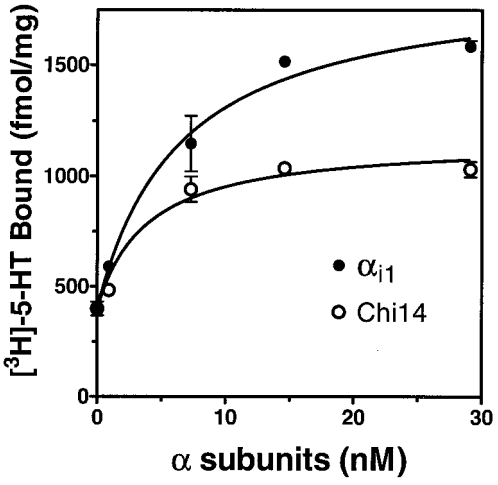


FIG. 4. **Concentration dependence of α_{i1} and Chi14 in an affinity shift assay with 5-HT$_{1B}$ receptors.** Sf9 cell membranes (17.5 μg of protein/filter, 4.8 fmol of receptor/μg) expressing 5-HT$_{1B}$ receptors were reconstituted with the indicated concentrations of Gα_{i1} or Chi14 with an excess of $\beta\gamma$ subunits. The highest concentration of α subunits represents an estimated 75-fold molar excess over expressed receptors. The concentration of [^{3}H]5-HT used was 1.2 nM. *Data points* represent specific binding expressed as the mean ± S.D. of triplicate determinations from a representative experiment. The *solid lines* indicate the best fit to the data of a single-site interaction between receptor and G protein governed by the law of mass action.

terminal regions (α5, and C-terminal tail) of Gα subunits are critical for receptor coupling and selective G protein-receptor interaction (12–17), examination of the role of these regions by comparing Chi14 with Chi21, which includes an additional C-terminal 35 amino acid residues of Gα_{i1} not present in Chi14, shows that Chi14 and Chi21 have similar affinity shift activity. This may be because there are few amino acid differences between Gα_{i1} and Gα_t in this region. To understand why high affinity agonist binding with Chi14 and Chi21 is less than with Gα_{i1} (Fig. 3), Chi13 was constructed containing the key residues 299–318 of Gα_{i1}, as well as the N-terminal half of Gα_{i1}. Chi13 enhanced high affinity agonist binding to the same extent as Gα_{i1} and to a significantly greater extent than either Chi14 or Chi21. Comparing the affinity shift activity of Chi14 and Chi13 allows the conclusion that important determinants of receptor interaction are also present in the N-terminal half of Gα_{i1}, but they are dependent on the presence of the C-terminal determinants (amino acids 299–318 of Gα_{i1}). The nature of this role of the Gα N-terminal region in receptor interaction is not based on the N-terminal acyl modifications since none of the bacterially expressed proteins are acylated. To demonstrate that receptor coupling with Chi14 and Gα_{i1} resulted in different agonist affinities rather than different affinities between receptor and G protein, we compared the concentration dependence of the Gα subunits in an affinity shift assay. The data in Fig. 4 show that, while the EC$_{50}$ (3.8 ± 1.2 *versus* 6.4 ± 1.7 nM for Chi14 and Gα_{i1}, respectively) for formation of high affinity agonist binding sites by Chi14 and Gα_{i1} are not significantly different, the ~38% difference in the amount of agonist binding is highly significant ($p < 0.0001$). Although smaller in magnitude, significant differences in levels of high affinity agonist binding among G$\alpha_{i/o}$ subunits have been seen with both 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors (25).

Table I summarizes the affinity shift activity of Gα_t, Gα_{i1}, and chimeric Gα subunits on 5-HT$_{1B}$ receptors in the presence of $\beta\gamma$ subunits. We can categorize three groups of Gα_t/Gα_{i1} chimeric proteins according to differences in their abilities to enhance high affinity agonist binding. The first group of chimeric proteins (Chi6, Chi2, Chi3, and Chi15) do not produce any significant increase in high affinity agonist binding. All chimeras in the first group contain the 295–314 amino acid sequence from Gα_t (α4 helix and α4/β6 loop). The second group, including Chi14 and Chi21, have an intermediate effect on high affinity agonist binding compared with Gα_{i1}. These two chimeras contain amino acid residues 299–318 of Gα_{i1} sequence and the N-terminal 215 amino acid residues from Gα_t. Amazingly, replacing only the 295–314 region of Chi6 with the homologous region from Gα_{i1} (Chi14) recovers ~82% of the affinity shift activity of Gα_{i1}. The third group, which includes Chi13 and Gα_{i1}, exhibits the maximum affinity shift activity. Thus, the N-terminal domain of Gα_{i1} may act synergistically with the 299–318 region of Gα_{i1} to enhance high affinity agonist binding to the 5-HT$_{1B}$ receptor. However, the N-terminal region is not sufficient for functional coupling with 5-HT$_{1B}$ receptors since no significant effects on high affinity agonist binding were observed with the chimeras containing the Gα_{i1} N terminus without the 299–318 region of Gα_{i1} (Chi2 and Chi3).

Agonist-Stimulated GTP/GDP Exchange of Gα_t/Gα_{i1} Chimeras—We next determined the relationship between high affinity agonist binding to the 5-HT$_{1B}$ receptor and G protein activation by the receptor. The data in Fig. 5 demonstrate that Chi14 undergoes significant 5-HT$_{1B}$ receptor-stimulated GTP/GDP exchange (from 0.01 ± 0.71 to 23.99 ± 2.73 pmol/mg·min) although only half as well as Gα_{i1} (46.46 ± 4.37 pmol/mg·min). Chi13 becomes activated as well as Gα_{i1} by the receptor; however, Chi2 does not show any significant agonist-stimulated guanine nucleotide exchange, suggesting that the N-terminal domain of Gα_{i1} has only a minor and conditional effect on the receptor-stimulated G protein activation. In addition, the absence of agonist-stimulated GTP/GDP exchange on Chi3 clearly proves the importance of amino acid residues 299–318 of Gα_{i1} on receptor stimulated G protein activation. Thus, the regions of the Gα_i subunit important for stabilizing the high affinity agonist binding state of the 5-HT$_{1B}$ receptor are also important for receptor catalyzed GTP/GDP exchange.

DISCUSSION

In this study, we found a new region of Gα_i important for specific interaction with 5-HT$_{1B}$ receptors using *in vitro* recon-

stitution of Sf9 cell membranes containing overexpressed 5-HT$_{1B}$ receptors with chimeric Gα subunits. We provide strong evidence that the α4 helix and α4-β6 loop are critical for specific 5-HT$_{1B}$ receptor-G$_{i1}$ interaction and are required for G protein activation by the receptor. Amino acid sequence alignment shows that Gα_{i1}, Gα_{i2}, and Gα_{i3} are highly conserved in the α4 helix and α4-β6 loop region while Gα_t has 9 residues which differ from Gα_{i1} (Fig. 6). In addition, the amino acid sequence of this region is diverse among the different families of Gα subunits, supporting the idea that the α4 helix and α4-β6 loop of Gα is another binding domain necessary for selective interaction with appropriate heptahelical receptors. This domain has previously been implicated in receptor interaction. Mazzoni *et al.* reported that Arg^{310} of Gα_t is completely protected from tryptic proteolysis in the presence of light-activated rhodopsin while it is a major site of tryptic cleavage of free Gα_t or heterotrimeric G$_t$ (30), suggesting that the α4-β6 loop may be a point of receptor contact. In addition, a recent site-directed mutagenesis study demonstrated that four single alanine substituted mutants in the α4-β6 loop region of Gα_t (Arg^{309}, Asp^{311}, Val^{312}, and Lys^{313}) are each defective in their ability to interact with light-activated rhodopsin (12).

Interestingly, the α4 helix and α4-β6 loop of the Gα subunit was originally identified as an effector coupling site. Synthetic peptides from amino acids 293–314 of Gα_t activate cGMP-phosphodiesterase (PDE) (31), and PDEγ can be cross-linked to this loop (32, 33). Our data thus suggest that the receptor binding domain is adjacent to or partially overlaps the effector interacting domain in Gα subunits. In support of this, Artemyev (34) recently found that cGMP phosphodiesterase γ subunit, an effector of Gα_t, can prevent the interaction between G$_t$ and light-activated rhodopsin.

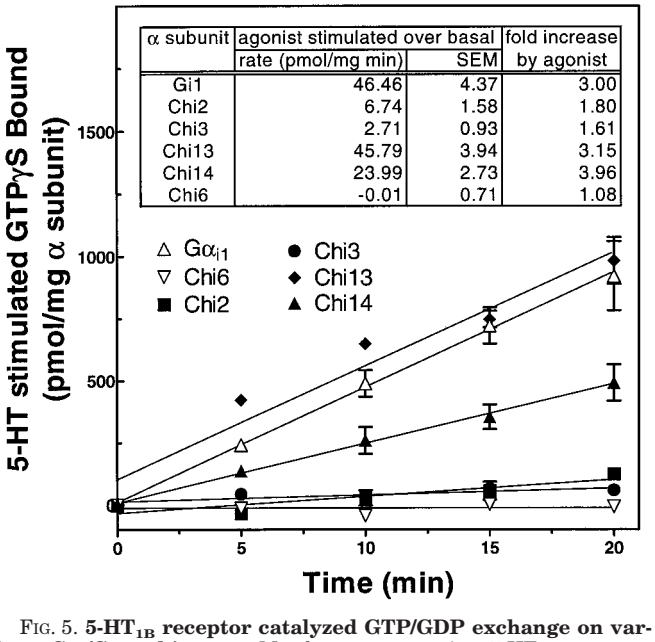


FIG. 5. **5-HT$_{1B}$ receptor catalyzed GTP/GDP exchange on various Gα_t/Gα_{i1} chimeras.** Membranes expressing 5-HT$_{1B}$ receptors were reconstituted with the indicated chimeras and $\beta\gamma$ subunits. *Curves* depict the difference between the rates of GTPγS binding in the presence and absence of 1 μM 5-HT. Final concentrations used were: 40 nM G protein subunits, 1 nM 5-HT$_{1B}$ (6 μg of protein/filter, 3.4 fmol of receptor/μg), 40 nM [^{35}S]GTPγS, and 100 nM GDP. Data shown are the mean ± S.E. of four independent experiments. The *inset* depicts the rate of agonist-stimulated GTPγS binding and -fold increase over basal based on this figure.

This study also implicates another region of Gα_{i1} important for 5-HT$_{1B}$ receptor interaction. The N-terminal 210 amino acids of Gα_{i1} imparted increased high affinity agonist binding to the 5-HT$_{1B}$ receptor. Some part of the N-terminal half of Gα_{i1} (not further specified) acts synergistically with the α4 helix and α4-β6 loop to enhance high affinity agonist binding to 5-HT$_{1B}$ receptors. However, the N-terminal region is not sufficient on its own for functional coupling with 5-HT$_{1B}$ receptors since no significant effect on high affinity agonist binding was observed with chimeras containing only the N-terminal half of Gα_{i1}.

It has been postulated that the GDP bound form of the G protein interacts with agonist-activated receptors and results in the release of GDP from the Gα subunit. This guanine nucleotide-free G protein is able to tightly bind agonist-bound receptors, and this complex can be released from the receptor by GDP or GTP (41, 42), suggesting that the conformation of "empty-state" Gα subunit is different from the previously crystallized GDP- and GTPγS-bound forms of Gα subunits (6, 8). Thus, any defect in receptor-catalyzed GDP release from the Gα subunit would result in a failure to form the ternary complex of agonist, receptor, and G protein. This hypothesis is supported by our finding that all chimeras which lacked the ability to undergo receptor-catalyzed GTP/GDP exchange also failed to induce high affinity agonist binding by the receptor (Table I and Fig. 3). As the rate-limiting step in G protein activation is release of the GDP from the Gα subunit, GTP/GDP exchange should be a direct reflection of GDP release. Therefore, we speculate that the α4 helix and α4-β6 loop of Gα is necessary for GDP release following binding to an activated receptor, and subsequently results in the empty state of Gα that can further stabilize the ligand bound receptor.

How does this finding relate to the well-established role of the G protein C terminus in receptor interactions? The C-terminal region clearly plays a central role in coupling G proteins to receptors (12–17). A body of evidence also demonstrates that it is important in class-specific selectivity of receptor-G protein interactions; changing the last few amino acids at the C terminus can recruit, for example, G$_i$-coupled receptors to activate G$_q$ (13). In the case of receptor selectivity within a class of G proteins, in this study the 5-HT$_{1B}$ receptor interaction with G$_{i1}$, the amino acid sequences of the C-terminal 35 amino acids of Gα_{i1} are so highly similar to Gα_t that it is unlikely that selectivity is imparted by this region. We show, in fact, that switching the C-terminal 35 amino acids from Gα_t to Gα_{i1} has no effect on coupling with the 5-HT$_{1B}$ receptor. Other regions of G proteins may also be important for receptor interaction. The N-terminal region has been implicated in receptor interaction (17, 19), and in the present study, the N-terminal half of Gα_{i1} has a secondary role in 5-HT$_{1B}$ receptor coupling. In the case of

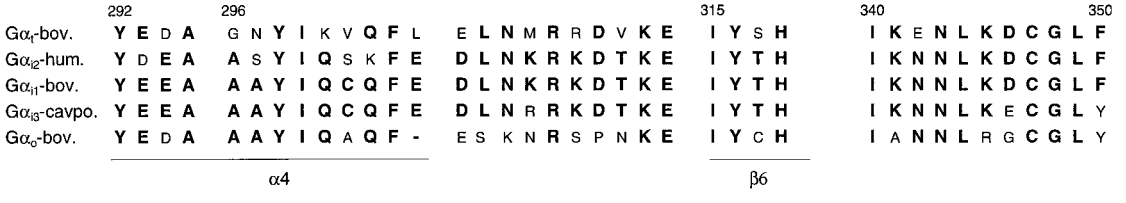


FIG. 6. **Alignment of α4-α4/β6 region and the C terminus of Gα_i family.** Numbers above the sequences refer to amino acid positions in the context of Gα_t. *Boldface letters* represent identical amino acid residues among Gα_{i1} subunits. *bov.*, bovine; *hum.*, human; *cavpo.*, guinea pig.

Gα_{16} and the C5a receptor, a chimeric protein approach revealed that yet another region, including the α2 helix and the α2/β4 loop, is involved in receptor-mediated G protein activation (18). In addition to the α subunit, the C-terminal region of the γ subunit of G proteins is also important for receptor coupling and specificity (35–38).

Thus multiple regions of G proteins make up interfaces for interactions with receptors. The region we have defined is positioned in the three-dimensional structure to potentially affect GDP binding affinities. This region is connected by a short β sheet, β6, to nucleotide binding residues TCAT that engage the guanine ring (6, 8). Mutations in this region dramatically decreased affinity for GDP (12, 39, 40). This conserved TCAT is in fact located between the α4-α4/β6 loop and the α5-C terminus, which were previously identified as receptor binding domains (12–17). Consistent with the structural and biochemical findings, the selective interaction of these regions (α4-α4/β6 loop and α5-C terminus) with an activated receptor may induce a conformational change in the TCAT motif and catalyze GDP release from the nucleotide binding pocket in the Gα subunits.

In this study, we clearly show that proper fit between the 5-HT$_{1B}$ receptor and the α4-α4/β6 loop region is required for GDP release from G$_{i1}$. It also appears that different receptors have different structural requirements for G protein coupling. Some receptors, such as rhodopsin, do not distinguish between G$_i$ family members. Other G$_i$ coupled receptors are able to distinguish to different degrees among G$_i$ family members (25, 43–47). The 5-HT$_{1B}$ receptor is an example of such selectivity as this receptor fails to couple with transducin or Chi6, the transducin-like chimera. The degree of contact between receptor and the α4-α4/β6 loop region of Gα subunits might vary depending on the three-dimensional surface of each receptor type. Considering the high degree of structural homology among G proteins, we speculate that the general mechanism of G protein-receptor coupling will be conserved among different classes of G proteins and receptors. Future studies will determine in more detail regions on Gα important for selective coupling with other receptors.

In summary, we have identified two distinct regions in Gα_{i1} important in specific G protein 5-HT$_{1B}$ receptor interaction: 1) 299–318 Gα_{i1} (α4 and α4-β6 loop), and 2) 1–219 Gα_{i1}. The region of 299–318 Gα_{i1} is necessary for both high affinity agonist binding and agonist-stimulated G protein activation. The other region, 1–219 Gα_{i1} has a minor and conditional effect on the apparent affinity state of the receptor. Data on the regions of Gα_i involved in the high affinity agonist binding and in receptor-mediated GTP/GDP exchange correlated well. These observations suggest a close relationship between the mechanism of G protein activation by the receptor and the ability of G proteins to stabilize the high affinity agonist binding state of receptors. An important goal is to define the nature of this interaction, the mechanisms by which receptors catalyze GDP release, and the roles of the various contact regions in determining selectivity of receptor G protein interaction.

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