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

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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 G α_{i1} or G α_t . G α_{i1} can interact with the 5-HT_{1B} receptor and stabilize a high affinity agonist binding state of this receptor, but G α_t cannot. A series of G α_i /G α_{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 G α_i /G α_{i1} chimeras to 5-HT_{1B} receptors using high affinity agonist binding and receptor-stimulated guanosine 5'-3-O-(thio)triphosphate (GTP γ S) binding. In the presence of $\beta\gamma$ subunits, amino acid residues 299–318 of G α_{i1} increase agonist binding to the 5-HT_{1B} receptor and receptor stimulation of GTP γ S binding. Moreover, G α_{i1} containing only G α_t amino acid sequences from this region does not show any coupling ability to 5-HT_{1B} receptors. Our studies suggest that the $\alpha 4$ helix and $\alpha 4$ - $\beta 6$ loop region of G α_s are an important region for specific recognition between receptors and G α_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 α_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_{i6} 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 (GTP γ S binding).

EXPERIMENTAL PROCEDURES

Materials—GTP, GDP, GTP γ S, 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. [³⁵S]GTP γ S and [³H]5-HT were purchased from NEN Life Science Products.

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¹ The abbreviations used are: 5-HT, 5-hydroxytryptamine; GTP γ S, 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 $G_{\alpha_i}/G_{\alpha_{11}}$ Chimeric Genes—We used *Escherichia coli* expression vectors pHis₆G α_{11} and pHis₆G α_i , which contain G α_{11} or G α_i 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 G α_{11} or G α_i cDNAs using PCR amplification with corresponding oligonucleotide primers followed by replacement of G α_{11} cDNA fragments with corresponding G α_i cDNA fragments or vice versa. Insertion of a *Nae*I site in the G α_{11} cDNA corresponding to amino acid residues 298–299 of G α_{11} , resulted in the replacement of Ala²⁹⁹ 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 $G_{\alpha_i}/G_{\alpha_{11}}$ Chimeras—The chimeric G α 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 MgCl₂, 50 μ M 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 mM NaCl, and 160 mM imidazole) before being loaded onto 5 ml of Ni²⁺-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 μ M 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 (G α_v) and $\beta\gamma$ subunits used for the GTP γ S 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 MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, 20 μ g/ml of benzamidine, and 2 μ g/ml each of aprotinin, leupeptin, and pepstatin A) and burst by N₂ 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 MgCl₂, 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 μ l. Typically, 1–2 μ l of G protein subunits were added to 15–40 μ l 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 MgCl₂, 0.5 mM EDTA) such that the desired amount of membranes were contained in 50 μ l.

[³H]5-Hydroxytryptamine Binding Assay—[³H]5-HT binding to

20–50 μ g of membrane protein was determined in binding assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 0.5 mM EDTA) in the presence of the desired components. Nonspecific binding was determined by addition of 10 μ M 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 MgCl₂, 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 [³H]5-HT ranged from 0.07 to 250 nM in a final volume of 500 μ l. For reconstitution of high affinity agonist binding in affinity shift assays, a single concentration (0.4–1.2 nM) of [³H]5-HT was used in a final volume of 150 μ l. 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 G α 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 MgCl₂). The AlF₄⁻-dependent conformational changes of activated G α 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 G α subunits was determined from absorbance readings before and after addition of 10 mM NaF and 20 μ M AlCl₃.

5-HT Receptor-stimulated GTP γ S 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 μ g of protein, 3.4 fmol of receptor/ μ g) were reconstituted with the indicated G α subunits and retinal $\beta\gamma$ subunits on ice in 70 μ l of reaction buffer (25 mM Hepes, pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol) for 30 min. Following reconstitution, the suspended membranes were diluted by the addition of 200 μ l of reaction buffer containing 150 nM GDP and 60 nM GTP γ S. The reaction was initiated by transferring 30 μ l of reaction buffer containing carrier-free [³⁵S]GTP γ S (~7 \times 10⁶ 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 μ g of protein/reaction, 3.4 fmol of receptor/ μ g), 40 nM G α , and 40 nM retinal $\beta\gamma$. For agonist activation, 1 μ M 5-HT was included. Samples (20 μ l) 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 μ l of ice-cold wash buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 25 mM MgCl₂). The filters were presoaked by washing 2 times with 200 μ l 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 G $\alpha_i/G_{\alpha_{11}}$ chimeric proteins produced in *E. coli* to map the regions of the G α_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 G α_{11} 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 AlF₄⁻-dependent increase in tryptophan

TABLE I

Affinity shift activity and AlF₄⁻-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 [³H]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*.

Chimera	Structure	AlF ₄ ⁻ Δ ^a %	Affinity Shift Activity
Group 1	Gα _t -GDP	70	1.13 ± 0.04 (13) *
	Chi 6	40-45	1.06 ± 0.14 (3)
	Chi 15	40-45	1.03 ± 0.13 (3)
	Chi 3	40-45	0.99 ± 0.08 (3)
	Chi 2	40-45	1.25 ± 0.06 (3)
Group 2	Chi 21	40-45	2.12 ± 0.13 (3)
	Chi 14	40-45	2.44 ± 0.08 (5)
Group 3	Chi 13	40-45	3.45 ± 0.21 (3)
	Gα _{ii}	40-45	2.96 ± 0.16 (5) **
Gα			

^a Percent increase of tryptophan fluorescence in the presence of 10 mM NaF and 20 μM AlCl₃ (see "Experimental Procedures" for detail).

fluorescence (20). This assay is based on the ability of AlF₄⁻, which mimics the γ-phosphate of GTP, to induce the active conformation resulting in an increase in intrinsic fluorescence of Trp²⁰⁷ in Gα_t (Trp²¹¹ in Gα_{i1}). Tryptophan fluorescence of all chimeras and Gα_{i1} used in this study increases 40–45% upon the addition of AlF₄⁻ (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 [³H]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. [³H]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 G_α subunits with an excess of βγ subunits and used in a [³H]5-HT binding assay as described under "Experimental Procedures." The concentration of [³H]5-HT used was 0.5 nM, and the G_α 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.

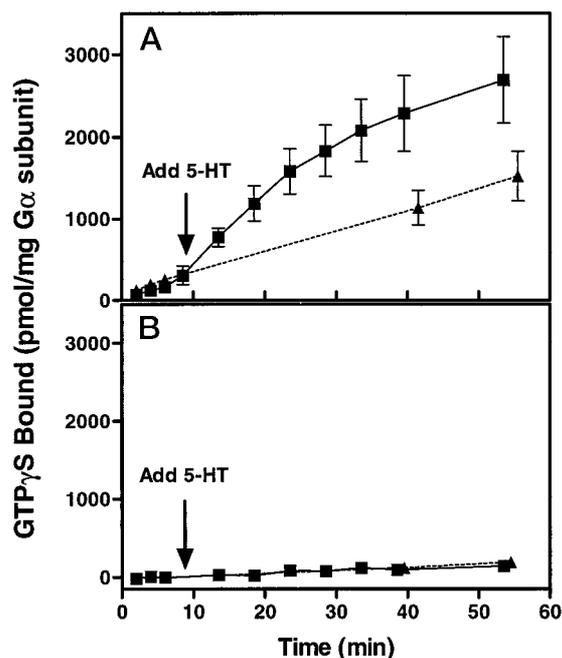
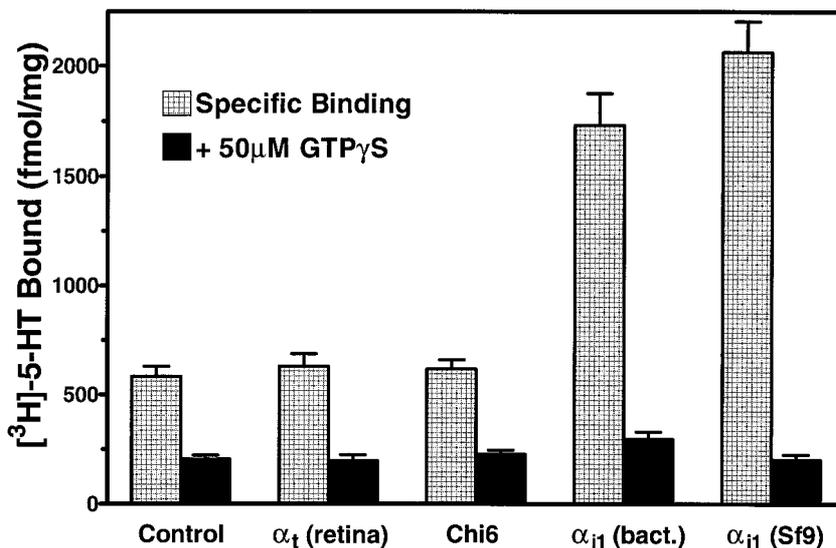


FIG. 2. 5-HT_{1B} receptor catalyzed GTP/GDP exchange on G_t versus G₁₁. Membranes expressing 5-HT_{1B} receptor were reconstituted with either G_{α₁₁} (panel A) or Chi 6 (panel B) and βγ subunits. Squares indicate GTPγS binding in the presence of 1 μM 5-HT (added at the 8.5 min point), and triangles indicate the binding in the absence of agonist. Final concentrations used were: 40 nM G_αβγ subunits, 1 nM 5-HT_{1B} receptors (6 μg of protein/filter, 3.4 fmol of receptor/μg), 40 nM [³⁵S]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_{α_{i/o}} subunit, whereas the amount of the G_{α_{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

G_{α_t}/G_{α₁₁} 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 [³H]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_{α_{i/o}} subunits would have affinity shift activities between 3 and 4. Fig. 3 summarizes the affinity shift activities of the G_{α_t}/G_{α₁₁} 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 [³H]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 G_α subunits with minimal (native G_{α_t} or Chi6) and maximal (G_{α₁₁} 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 G_{α₁₁} 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 G_{α₁₁} (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 G_{α₁₁}. Examination of the amino acid sequences of G_{α₁₁} and G_{α_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 G_{α_t} with the corresponding residues of G_{α₁₁} (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 G_{α_t} sequence in the context of G_{α₁₁}. 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-

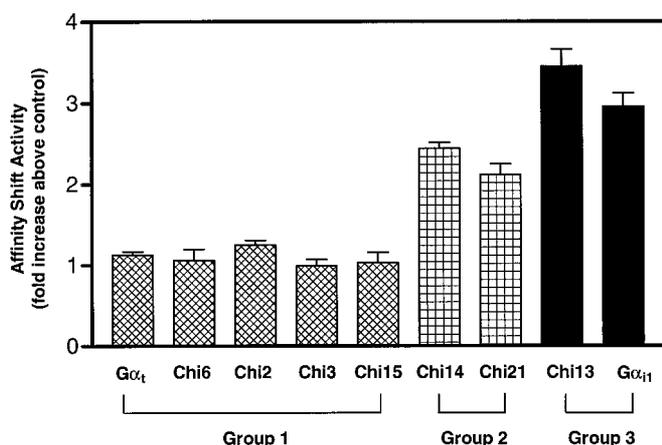


FIG. 3. Affinity shift activity of various G α_t /G α_{i1} chimeras with 5-HT_{1B} receptors. Affinity shift activities refer to the -fold enhancement above buffer controls of high affinity [³H]5-HT binding to 5-HT_{1B} receptors reconstituted with G protein heterotrimer containing the indicated α subunits (see "Results" for further explanation). Data represent the mean \pm 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 μ M during reconstitution. The concentration of [³H]5-HT was between 0.6 and 1.2 nM in all experiments. G α_t represents the activity of native transducin, and G α_{i1} represents the activity of α_{i1} expressed in *E. coli*.

terminal regions ($\alpha 5$, and C-terminal tail) of G α 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 G α_{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 G α_{i1} and G α_t in this region. To understand why high affinity agonist binding with Chi14 and Chi21 is less than with G α_{i1} (Fig. 3), Chi13 was constructed containing the key residues 299–318 of G α_{i1} , as well as the N-terminal half of G α_{i1} . Chi13 enhanced high affinity agonist binding to the same extent as G α_{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 G α_{i1} , but they are dependent on the presence of the C-terminal determinants (amino acids 299–318 of G α_{i1}). The nature of this role of the G α 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 G α_{i1} resulted in different agonist affinities rather than different affinities between receptor and G protein, we compared the concentration dependence of the G α subunits in an affinity shift assay. The data in Fig. 4 show that, while the EC₅₀ (3.8 \pm 1.2 versus 6.4 \pm 1.7 nM for Chi14 and G α_{i1} , respectively) for formation of high affinity agonist binding sites by Chi14 and G α_{i1} are not significantly different, the \sim 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 α_{i0} subunits have been seen with both 5-HT_{1A} and 5-HT_{1B} receptors (25).

Table I summarizes the affinity shift activity of G α_t , G α_{i1} , and chimeric G α subunits on 5-HT_{1B} receptors in the presence of $\beta\gamma$ subunits. We can categorize three groups of G α_t /G α_{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

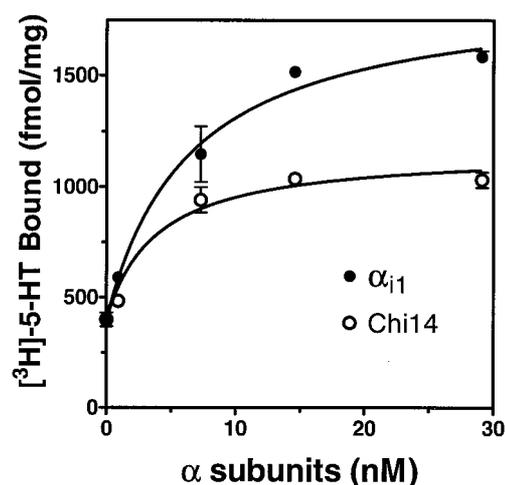


FIG. 4. Concentration dependence of α_{i1} and Chi14 in an affinity shift assay with 5-HT_{1B} receptors. Sf9 cell membranes (17.5 μ g of protein/filter, 4.8 fmol of receptor/ μ g) expressing 5-HT_{1B} receptors were reconstituted with the indicated concentrations of G α_{i1} or Chi14 with an excess of $\beta\gamma$ subunits. The highest concentration of α subunits represents an estimated 75-fold molar excess over expressed receptors. The concentration of [³H]5-HT used was 1.2 nM. Data points represent specific binding expressed as the mean \pm 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.

chimeras in the first group contain the 295–314 amino acid sequence from G α_t ($\alpha 4$ helix and $\alpha 4/\beta 6$ loop). The second group, including Chi14 and Chi21, have an intermediate effect on high affinity agonist binding compared with G α_{i1} . These two chimeras contain amino acid residues 299–318 of G α_{i1} sequence and the N-terminal 215 amino acid residues from G α_t . Amazingly, replacing only the 295–314 region of Chi6 with the homologous region from G α_{i1} (Chi14) recovers \sim 82% of the affinity shift activity of G α_{i1} . The third group, which includes Chi13 and G α_{i1} , exhibits the maximum affinity shift activity. Thus, the N-terminal domain of G α_{i1} may act synergistically with the 299–318 region of G α_{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 G α_{i1} N terminus without the 299–318 region of G α_{i1} (Chi2 and Chi3).

Agonist-Stimulated GTP/GDP Exchange of G α_t /G α_{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 \pm 0.71 to 23.99 \pm 2.73 pmol/mg-min) although only half as well as G α_{i1} (46.46 \pm 4.37 pmol/mg-min). Chi13 becomes activated as well as G α_{i1} by the receptor; however, Chi2 does not show any significant agonist-stimulated guanine nucleotide exchange, suggesting that the N-terminal domain of G α_{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 G α_{i1} on receptor stimulated G protein activation. Thus, the regions of the G α_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 G α_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 G α subunits. We provide strong evidence that the α 4 helix and α 4- β 6 loop are critical for specific 5-HT_{1B} receptor-G α_{i1} interaction and are required for G protein activation by the receptor. Amino acid sequence alignment shows that G α_{i1} , G α_{i2} , and G α_{i3} are highly conserved in the α 4 helix and α 4- β 6 loop region while G α_t has 9 residues which differ from G α_{i1} (Fig. 6). In addition, the amino acid sequence of this region is diverse among the different families of G α subunits, supporting the idea that the α 4 helix and α 4- β 6 loop of G α is another binding domain necessary for selective interaction with appropriate heptahelical receptors. This domain has previously been implicated in receptor interaction. Mazzone *et al.* reported that Arg³¹⁰ of G α_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 G α_t or heterotrimeric G α_t (30), suggesting that the α 4- β 6 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 α 4- β 6 loop region of G α_t (Arg³⁰⁹, Asp³¹¹, Val³¹², and Lys³¹³) are each defective in their ability to interact with light-activated rhodopsin (12).

Interestingly, the α 4 helix and α 4- β 6 loop of the G α subunit was originally identified as an effector coupling site. Synthetic peptides from amino acids 293–314 of G α_t activate cGMP-

phosphodiesterase (PDE) (31), and PDE γ 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 G α subunits. In support of this, Artyemyev (34) recently found that cGMP phosphodiesterase γ subunit, an effector of G α_t , can prevent the interaction between G α_t and light-activated rhodopsin.

This study also implicates another region of G α_{i1} important for 5-HT_{1B} receptor interaction. The N-terminal 210 amino acids of G α_{i1} imparted increased high affinity agonist binding to the 5-HT_{1B} receptor. Some part of the N-terminal half of G α_{i1} (not further specified) acts synergistically with the α 4 helix and α 4- β 6 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 G α_{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 G α 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" G α subunit is different from the previously crystallized GDP- and GTP γ S-bound forms of G α subunits (6, 8). Thus, any defect in receptor-catalyzed GDP release from the G α 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 G α subunit, GTP/GDP exchange should be a direct reflection of GDP release. Therefore, we speculate that the α 4 helix and α 4- β 6 loop of G α is necessary for GDP release following binding to an activated receptor, and subsequently results in the empty state of G α 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 α_i -coupled receptors to activate G α_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 α_{i1} , the amino acid sequences of the C-terminal 35 amino acids of G α_{i1} are so highly similar to G α_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 G α_t to G α_{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 G α_{i1} has a secondary role in 5-HT_{1B} receptor coupling. In the case of

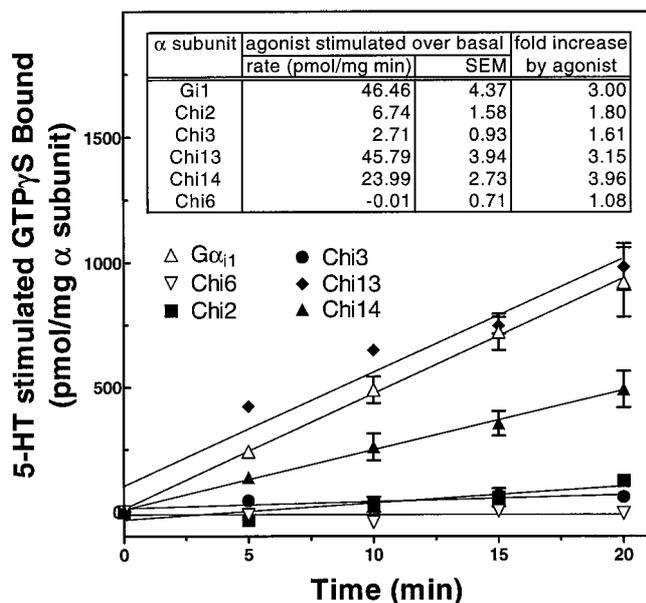


FIG. 5. 5-HT_{1B} receptor catalyzed GTP/GDP exchange on various G α /G α_{i1} chimeras. Membranes expressing 5-HT_{1B} receptors were reconstituted with the indicated chimeras and $\beta\gamma$ subunits. Curves depict the difference between the rates of GTP γ S binding in the presence and absence of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

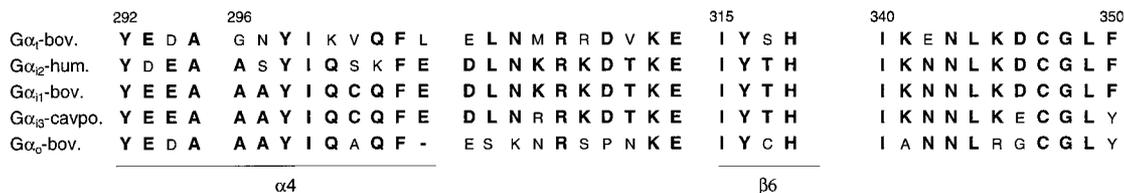


FIG. 6. Alignment of α 4- α 4/ β 6 region and the C terminus of G α family. Numbers above the sequences refer to amino acid positions in the context of G α_t . Boldface letters represent identical amino acid residues among G α_{i1} subunits. bov., bovine; hum., human; cavpo., guinea pig.

G α_{16} and the C5a receptor, a chimeric protein approach revealed that yet another region, including the $\alpha 2$ helix and the $\alpha 2/\beta 4$ 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, $\beta 6$, 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 $\alpha 4$ - $\alpha 4/\beta 6$ loop and the $\alpha 5$ -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 ($\alpha 4$ - $\alpha 4/\beta 6$ loop and $\alpha 5$ -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 G α subunits.

In this study, we clearly show that proper fit between the 5-HT_{1B} receptor and the $\alpha 4$ - $\alpha 4/\beta 6$ 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 $\alpha 4$ - $\alpha 4/\beta 6$ loop region of G α 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 G α important for selective coupling with other receptors.

In summary, we have identified two distinct regions in G α_{i1} important in specific G protein 5-HT_{1B} receptor interaction: 1) 299–318 G α_{i1} ($\alpha 4$ and $\alpha 4$ - $\beta 6$ loop), and 2) 1–219 G α_{i1} . The region of 299–318 G α_{i1} is necessary for both high affinity agonist binding and agonist-stimulated G protein activation. The other region, 1–219 G α_{i1} has a minor and conditional effect on the apparent affinity state of the receptor. Data on the regions of G α_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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