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Closely Related G-protein-coupled Receptors Use Multiple and Distinct Domains on G-protein α-Subunits for Selective Coupling*

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The molecular basis of selectivity in G-protein receptor coupling has been explored by comparing the abilities of G-protein heterotrimers containing chimeric Gα subunits, comprised of various regions of $G_{i1}\alpha$, $G_t\alpha$, and $G_q\alpha$, to stabilize the high affinity agonist binding state of serotonin, adenosine, and muscarinic receptors. The data indicate that multiple and distinct determinants of selectivity exist for individual receptors. While the A1 adenosine receptor does not distinguish between $G_{i1}\alpha$ and $G_t\alpha$ sequences, the 5-HT_{1A} and 5-HT_{1B} serotonin and M2 muscarinic receptors can couple with G_{i1} but not G_t. It is possible to distinguish domains that eliminate coupling and are defined as "critical," from those that impair coupling and are defined as "important." Domains within the N terminus, α4-helix, and α4-helix-α4/β6-loop of $G_{i1}\alpha$ are involved in 5-HT and M2 receptor interactions. Chimeric $G_{i1}\alpha/G_q\alpha$ subunits verify the critical role of the Gα C terminus in receptor coupling, however, the individual receptors differ in the C-terminal amino acids required for coupling. Furthermore, the EC_{50} for interactions with G_{i1} differ among the individual receptors. These results suggest that coupling selectivity ultimately involves subtle and cooperative interactions among various domains on both the G-protein and the associated receptor as well as the G-protein concentration.

A large number of diverse seven transmembrane-spanning cell surface receptors mediate signaling to a variety of intracellular effectors by coupling to the heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins)[1] (1). The mechanisms responsible for selectivity in G-protein-mediated signaling pathways are not fully understood (2, 3). Although it is known that at the molecular level the selectivity in G-protein receptor coupling is determined by amino acid sequences of both receptor and G-protein, the individual amino acids involved in this selective recognition have not been completely identified. Different receptor systems and different methodologies indicate that the Gα subunit C terminus and α5-helix (4–7), N terminus, and αN-helix (4, 8–10), α4-helix, and α4/β6-loop (11–13), α2-helix, and α2/β4-loop (14), α3/β5-loop (15), αN/β1-loop (13) and amino acids 110–119 from the α-helical domain (16) are involved in receptor-coupling selectivity. Some of these domains contact the receptor directly, while others regulate receptor-coupling selectivity indirectly by playing a role in nucleotide exchange. Despite the fact that many of the receptor-interacting domains have been identified, the relationship between receptor subtypes and Gα domains involved in receptor coupling has not been clearly established. Thus, it is difficult to predict which Gα domains will be utilized by a specific receptor. Here we propose that individual receptors recognize specific patterns formed by amino acids of Gα thus making G-protein interface look different for different receptors. The C terminus of Gα is a well accepted receptor recognition domain, which contacts receptors directly (17). Although individual C-terminal amino acids important for receptor coupling have been identified in several Gα subunits, the specific Gα amino acids participating in receptor recognition may differ among receptors. The α4-helix-α4/β6-loop domain, first described as an effector domain, has been shown to be important for 5-HT_{1B} receptor coupling to G_{i1} (11). Later it was demonstrated that Gln-304 and Glu-308 in the α4-helix of $G_{i1}\alpha$ are important for 5-HT_{1B} receptor coupling (18). However the generality of the role for the α4-helix-α4/β6-loop domain in receptor coupling selectivity has not been determined.

$G_{i1}\alpha$ and $G_t\alpha$ are closely related Gα subunits, which belong to the $G_{i/o}$ class of G-protein α-subunits, share 68% homology, and have nearly identical overall structures. Although the 5-HT_{1B} receptor discriminates between G_{i1} and G_t (11, 19), the fact that their C termini are identical render $G_{i1}\alpha/G_t\alpha$ chimeras useless for exploring the role of this domain in receptor coupling. However, the extreme C terminus of $G_q\alpha$ differs from that of $G_{i1}\alpha$ by four amino acids, while their α5-helices differ by an additional nine amino acids. Thus $G_{i1}\alpha/G_q\alpha$ chimeras are ideal for studying the role of this domain in coupling. Since several different GPCRs can couple to the same G-protein, we wanted to test the hypothesis that individual receptors utilize slightly different domains on Gα subunits to achieve coupling. G-protein receptor coupling selectivity may also be regulated at the level of G-protein concentration. In fact, Clawges *et al.* (20) demonstrated that 5-HT_{1A} and 5-HT_{1B} receptors distinguish themselves by the affinity with which they interact with G-proteins. Therefore we also wanted to test the generality of this mechanism with different receptors. Here we compare the coupling behavior of four $G_{i/o}$-coupled receptors (5-HT_{1A} and

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[1] The abbreviations used are: G-proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; GPCRs, G-protein-coupled receptors; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; OXO-M, oxotremorine-M; 5-HT, hydroxytryptamine; CCPA, chloro-N^6-cyclopentyladenosine, R-PIA, R-phenylisopropyl adenosine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

5-HT_{1B} serotonin, A1 adenosine and M2 muscarinic) by reconstituting them with G-protein heterotrimers containing native or chimeric Gα subunits composed of $G\alpha_{i1}$, $G\alpha_t$, and $G\alpha_q$. Our data demonstrate that selective coupling between G_{i1} and the members of $G_{i/o}$-coupled receptor family is directed by multiple and distinct Gα domains and is regulated at the level of G-protein concentration.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Oxotremorine-M acetate ([^{3}H]OXO-M) (85.8 Ci/mmol), [^{3}H]hydroxytryptamine binoxalate (5-[^{3}H]HT) (25.5 Ci/mmol), and chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA) (30 Ci/mmol) were from PerkinElmer Life Science Products, Inc. (Boston, MA). Atropine sulfate, 5-hydroxytryptamine (5-HT) and *R*-phenylisopropyl adenosine (R-PIA) were from Sigma-Aldrich Corporation. Adenosine deaminase was from Roche Applied Science (Indianapolis, IN). The BCA protein Assay reagents were from Pierce. All other chemicals were from Sigma-Aldrich Corporation or EMD Biosciences (formerly Calbiochem-Novabiochem Corporation; San Diego, CA).

Expression and Purification of Proteins—The expression and purification of the $G\alpha_{i1}$ and Gβγ subunits was as previously described (21, 22). The chimeric $G\alpha_{i1}/G\alpha_t$ subunits were constructed, expressed in *Escherichia coli* and purified as described (19). The G_{i1}/Q3C, G_{i1}/Q5C, and G_{i1}/Q11C chimeras (which have the 3, 5, or 11 C-terminal residues of $G_{i1}\alpha$ replaced with those from $G_q\alpha$) were made from $pHis_6G\alpha_{i1}$ using the silent BamHI site introduced at amino acid position 212 (19). The $pHis_6G\alpha_{i1}$ cDNA was amplified by PCR reaction with primer oligonucleotides containing the desired mutations. The PCR products were digested with BamHI and HindIII, and the BamHI-HindIII fragment was used to replace the corresponding fragment from $pHis_6G\alpha_{i1}$. To construct G_{i1}/Q35C (which has the 35 C-terminal residues of $G_{i1}\alpha$ replaced with those from $G_q\alpha$), the C-terminal portion of a $G_q\alpha$ cDNA was amplified by PCR reaction, followed by digestion with BglII and HindIII. The digested PCR fragment was inserted into the BglII and HindIII sites of the Chi13 plasmid (11). Functional characterization of all bacterial subunits included GTPγS binding, AlF_4^--dependent conformational change (measured as an increase in intrinsic tryptophan fluorescence) or binding to the cGMP phosphodiesterase γ-subunit (11, 18, 19).

Preparation of Sf9 Membranes Containing Expressed Receptors—Sf9 cells were infected with a recombinant baculovirus expressing the desired receptor, cultured, and harvested as previously described (22). To prepare membranes, harvested cells were thawed in 15× their wet weight of ice-cold homogenization buffer (10 mM Tris-Cl, pH 8.0 at 4 °C, 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml of benzamidine, and 2 μg/ml of each of aprotinin, leupeptin, and pepstatin A) and burst by nitrogen cavitation (600 psi, 20 min). Cavitated cells were centrifuged at 4 °C for 10 min at 500 × *g* to remove the unbroken nuclei and cell debris. The supernatant from the low speed spin was centrifuged at 4 °C for 30 min at 28,000 × *g*. The supernatant was discarded, and the pellets were resuspended and pooled in 35 ml of HE buffer (5 mM NaHEPES, l mM EDTA, pH 7.5) containing the same protease inhibitors as used in the homogenization buffer. Adenosine receptor HE buffer included 100 mM NaCl in addition to the above components. The membranes were washed twice in HE, resuspended in the same buffer at a concentration of 1–3 mg 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, 12,000 rpm), and resuspended at about 10 mg/ml in a reconstitution buffer consisting of 5 mM NaHEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 500 nM GDP, 0.04% CHAPS (0.08% CHAPS for M2 receptor), pH 7.5. 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 40 μl of membrane suspension, the mixture was incubated at 25 °C for 15 min and held on ice until the start of the binding assay.

Radioligand Binding—Just prior to the start of the binding assay the reconstitution mixture was diluted 10–12-fold with binding assay buffer appropriate to the receptor of interest such that the desired amount of membranes (5–25 μg/assay tube) were contained in 10–50 μl. Binding buffer for 5-HT and M2 receptors was 50 mM Tris, 5 mM $MgCl_2$, 0.5 mM EDTA, pH 7.5. Binding buffer for A1 adenosine receptor was 10 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4. Radioligand binding in the affinity shift assay was determined in the presence of the [^{3}H]OXO-M for M2 muscarinic receptor, 5-[^{3}H]HT for 5-HT serotonin receptors and [^{3}H]CCPA for A1 adenosine receptor. Adenosine deaminase was added to the [^{3}H]CCPA solution at 12 μg/ml in binding buffer. Nonspecific binding was determined by addition of 1000-fold excess of unlabeled ligand, 5-HT for 5-HT receptors, atropine sulfate for M2 receptor and R-PIA for A1 receptor. Incubations were for times sufficient to achieve equilibrium in a temperature controlled shaker (1 h for M2 receptor, 1.5 h for 5-HT receptors, 2 h for A1 receptor) and were terminated by filtration over Whatman GF/C filters using a Brandel Cell Harvester. The filters were rinsed thrice with 4 ml of ice-cold 50 mM Tris-Cl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.01% sodium azide, pH 7.5 at 4 °C, placed in 4.5 ml of CytoScint (ICN Pharmaceuticals, Costa Mesa, CA) and counted to constant error in a scintillation counter. For reconstitution of high affinity agonist binding in affinity shift assays, a single concentration of radioligand near the high affinity K_D of the receptor of interest was used in a final volume of 150 μl. [^{3}H]-5-HT radioligand purity was monitored by HPLC or TLC using an appropriate mobile phase. Radioligands were repurified or replaced when the radiochemical purity fell below 85%.

Affinity Shift Activity Assay—The Sf9 cell membranes expressing individual receptors were reconstituted with saturating amounts of native or chimeric G_{i1} protein heterotrimers (≥25 nM or 40–400-fold molar excess over receptors) to achieve the maximal specific binding during the binding assays. Because the magnitude of the affinity shifts observed with native G_{i1} protein heterotrimers varied significantly among the individual receptors affinity shift activity was normalized to G_{i1} activity and expressed as percent affinity shift activity, which is (Chimera Reconstituted Binding − Control Binding/Gil Reconstituted Binding − Control Binding) × 100.

Analysis of Data—Data analysis was done using the GraphPad Prism software package (GraphPad Software, San Diego, CA). For affinity shift assays, triplicate determinations were used within each experiment, and experiments were repeated three or more times. Data represent the mean ± S.E. from multiple experiments. One-way analysis of variance with Tukey's multiple comparison post-test was used to compare the activities of chimeras.

RESULTS

Previously we have shown that amino acids 299–318 and 1–219 of $G_{i1}\alpha$ are molecular determinants of 5-HT_{1B} receptor coupling (11) and that two amino acids in the α4-helix of Gi1α (Gln-304 and Glu-308) are especially important for 5-HT_{1B} receptor coupling (18). The goal of the present study was to examine the generality of these findings among closely related members of the $G_{i/o}$-coupled receptor family. Our general strategy involves reconstitution of purified G-proteins containing chimeric α-subunits with receptors expressed in Sf9 insect cell membranes and comparison of the abilities of these chimeric G-proteins to stabilize the high affinity agonist binding state of the receptors in an affinity shift activity assay. In the present study we compared the coupling behavior of four different $G_{i/o}$-coupled receptors: 5-HT_{1A} and 5-HT_{1B} serotonin receptors, M2 muscarinic receptors, and A1 adenosine receptors.

Affinities of Individual Receptors for G-proteins—First we determined the concentration of G-proteins in the binding assay that produced the maximum affinity shift for each receptor. Increasing amounts of G-protein heterotrimers were reconstituted with individual receptors and EC_{50} values for reconstitution of high affinity agonist binding were determined. The data indicate that A1, 5-HT_{1A}, 5-HT_{1B}, and M2 receptors have different EC_{50} values for G_{i1} (Fig. 1). A1 receptors have the highest apparent affinity (0.4 nM) and M2 receptors have the lowest apparent affinity (47 nM) for the G_{i1} heterotrimer. 5-HT receptors have intermediate EC_{50} values of 3.7 and 16.2 nM for the 5-HT_{1A} and 5-HT_{1B} receptors, respectively. Titration experiments similar to those shown in Fig. 1 were used to determine the concentration of chimeric G-proteins needed to saturate affinity shift activities with individual receptors. In agreement with earlier studies (11, 18, 20), the EC_{50} values of the active G-proteins were not significantly different for individual receptors, and even high concentrations (>600 nM) of inactive chimeras did not have affinity shift activity (data not shown). All affinity shift activities were determined with saturating concentrations of G-proteins.

Affinity Shift Activity of Chimeric Gα Subunits—Fig. 2 depicts the secondary structures of the $G_{i1}\alpha/G_t\alpha$ chimeras used in this study. All of these chimeras have been previously described and were used to study $G_{i1}\alpha$ domains involved in 5-HT_{1B} receptor coupling (11, 18, 19). Fig. 3, in which 100% activity corresponds to the affinity shift activity of Gi1, shows the percent affinity shift activity of Chi2, Chi3, Chi6, Chi13, and Chi21. Chi6 was constructed as a soluble analog of $G_t\alpha$ and has the same functional properties as $G_t\alpha$ (19). Chi6 is primarily Gtα in character as it includes N-terminal amino acids 1–215 and C-terminal amino acids 295–350 of $G_t\alpha$ with the amino acids corresponding to 216–294 from $G_{i1}\alpha$ to maintain solubility. In this region there are just 26 amino acids that differ between Chi6 and $G_t\alpha$. As shown in Fig. 3, Chi6 was inactive with 5-HT_{1A}, 5-HT_{1B}, and M2 muscarinic receptors. Earlier experiments with native transducin demonstrated it also failed to couple with the 5-HT receptors (20). In contrast, the data in Fig. 3 demonstrate Chi6 was 74% active with the A1 adenosine receptor, indicating that A1 adenosine receptor does not discriminate well between G_t and G_{i1} sequences. Similarly, native transducin was 80% active with the A1 adenosine receptor, which is not significantly different from Chi6 (data not shown). Although the activity of Chi6 (and native transducin) with the A1 adenosine receptor was significantly lower ($p < 0.001$) than the activity of G_{i1}, the magnitude of the difference was too small to be of use in identifying the precise domains responsible for the reduced activity. However, the inability of the 5-HT_{1A}, 5-HT_{1B}, and M2 muscarinic receptors to couple with Chi6 allowed us to use additional chimeras containing less $G_t\alpha$ sequence to more precisely identify the domains required for coupling.

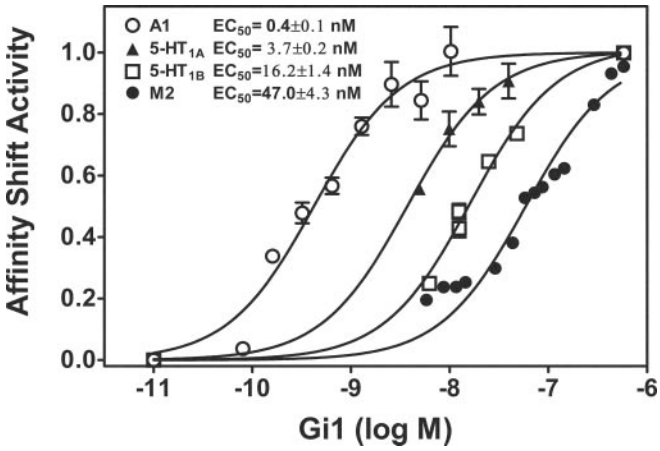

FIG. 1. **Concentration dependence of G_{i1} in affinity shift assays for individual G_{i1}-coupled receptors.** Sf9 cell membranes expressing the indicated G_{i1}-coupled receptors were reconstituted with increasing concentrations of G_{i1} heterotrimer. The affinity shift activities for each receptor were normalized and fit to a single site interaction between receptor and G-protein. The magnitude of the affinity shift activity (-fold enhancement of agonist binding above non-reconstituted controls) with saturating amounts of G_{i1} was 4.1 ± 0.51, $n = 17$, for the 5-HT_{1A} receptor; 3.8 ± 0.19, $n = 22$, for the 5-HT_{1B} receptor, 4.4 ± 0.37, $n = 17$, for the A1 receptor; and 12.2 ± 1.04, $n = 35$, for the M2 receptor. Saturation was achieved for each receptor, however for visual purposes the curves have been extended to a common end point. Shown are the data from representative experiments. EC_{50} data are the mean ± S.E. from three or more independent experiments.

We first examined whether the N-terminal or C-terminal portion of $G_{i1}\alpha$ was critical for receptor coupling. Chi21 has N-terminal amino acids 1–215 of $G_t\alpha$ with the rest of the molecule $G_{i1}\alpha$ sequence (Fig. 2). Chi21 was fully active with the A1 adenosine receptor, indicating that the A1 receptor does not distinguish between N-terminal amino acid sequences of $G_{i1}\alpha$ and $G_t\alpha$ (Fig. 3). The activity of Chi21 with 5-HT_{1A}, 5-HT_{1B}, and M2 receptors was significantly ($p < 0.001$) reduced (44, 57, and 42% respectively, Fig. 3) demonstrating that amino acids 1–219 of $G_{i1}\alpha$ contain an important determinant of G_i coupling with these receptors. Chi2 has the C-terminal amino acids 295–350 of $G_t\alpha$ with the rest of the chimera Gi1α sequence (Fig. 2). Fig. 3 demonstrates that amino acids 299–354 of $G_{i1}\alpha$ contain residues critical for 5-HT_{1A}, 5-HT_{1B}, and M2 receptor coupling because the affinity shift activity of Chi2 with these receptors (2, 9, and 23% respectively) was not significantly different from Chi6 activity. In contrast, Chi2 was fully active with A1 adenosine receptors supporting our conclusion that A1 adenosine receptor does not distinguish well between $G_{i1}\alpha$ and $G_t\alpha$ sequences. To further evaluate the role of amino acids 299–354 of $G_{i1}\alpha$ in 5-HT and M2 receptor coupling we tested two additional chimeras, Chi3 and Chi13 (Fig. 2). Chi3 has amino acids 299–319 of Gi1α replaced with the corresponding amino acids of Gtα (amino acids 295–315) while Chi13 has the 35 C-terminal amino acids of $G_{i1}\alpha$ replaced with the corresponding amino acids of $G_t\alpha$. As shown in Fig. 3, the affinity shift activities of Chi3 show that amino acids 299–319 of Gi1α (α4-helix and α4/β6-loop) are critical for 5-HT_{1A}, 5-HT_{1B}, and M2 receptor coupling, but not for A1 adenosine receptor coupling. In contrast, Chi13, with six amino acids variant from $G_{i1}\alpha$, was active with all four receptors indicating that the 35 C-terminal amino acids of $G_{i1}\alpha$ and $G_t\alpha$ are functionally inter-

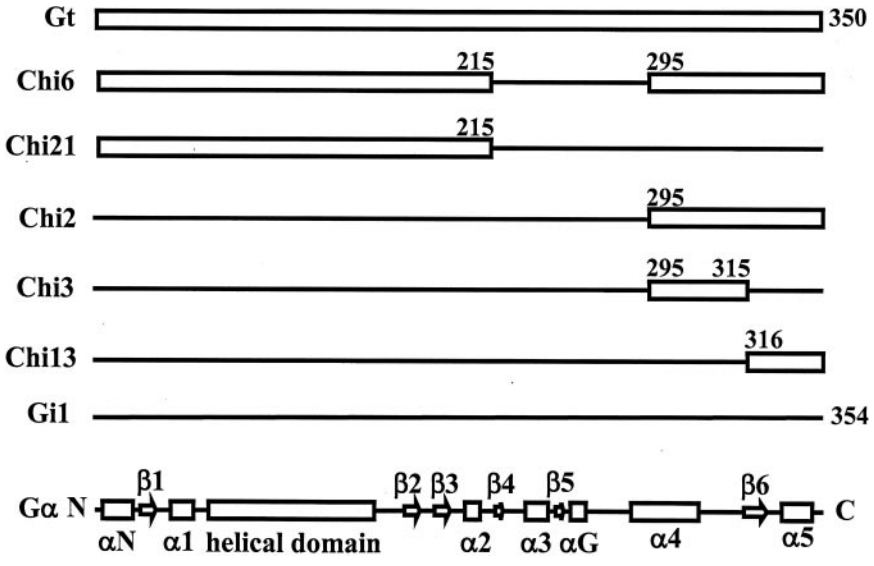

FIG. 2. **Secondary structure of Gα subunits.** Numbers above the chimeric structures indicate the junction points of $G\alpha_t$ and $G\alpha_{i1}$ sequences and refer to the amino acid positions in $G\alpha_t$. Numbers for the wild-type forms of $G\alpha_t$ and $G\alpha_{i1}$ represent their total amino acid residues. The *bottom diagram* depicts the secondary structural domains common to Gα subunits.

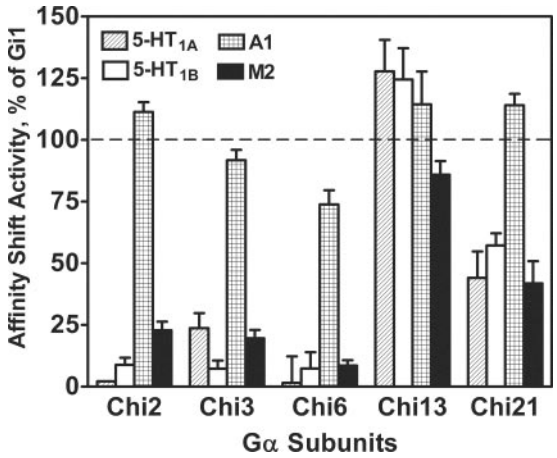

FIG. 3. **Functional coupling of receptors to the indicated G_{i1}/G_t chimeras.** Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the percent affinity shift activities as mean ± S.E. from three or more independent experiments for each receptor. Exogenous G-proteins were present in 40–200-fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

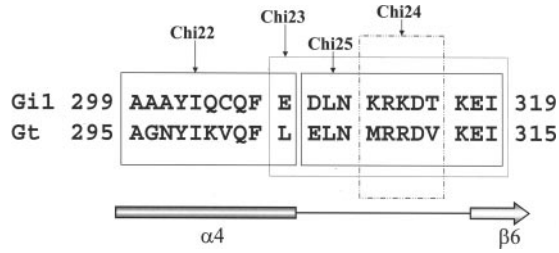

FIG. 4. **Primary sequence alignment of the α4-α4/β6-loop region of $G\alpha_{i1}$ and $G\alpha_t$.** The *boxes* indicate the regions of $G\alpha_{i1}$ that were substituted with the corresponding sequences from $G\alpha_t$ to generate the indicated $G\alpha_{i1}/G\alpha_t$ chimeras.

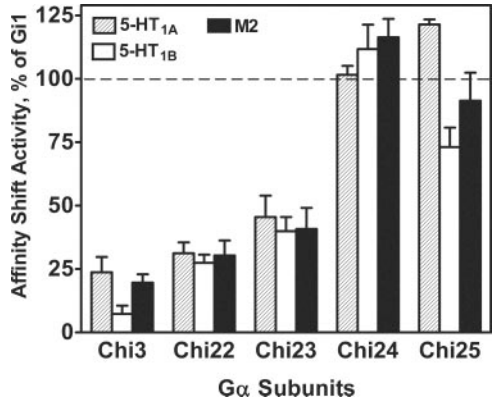

FIG. 5. **Functional coupling of receptors to the indicated G_{i1}/G_t chimeras.** Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the percent affinity shift activities as mean ± S.E. from three or more independent experiments for each receptor. Exogenous G-proteins were present in 40–200-fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

changeable in coupling these receptors. Nevertheless, the significantly ($p < 0.01$) reduced activity of Chi13 (85.9%) with the M2 receptor and the significantly ($p < 0.01$) increased activity with both 5-HT_{1A} (128%) and 5-HT_{1B} (124.5%) receptors suggest subtle differences in the coupling mechanism of these receptors. The role of the extreme C terminus of $G_{i1}\alpha$ cannot be evaluated with these chimeras because the eight C-terminal amino acids of $G_{i1}\alpha$ and $G_t\alpha$ are identical.

Role of the α4-Helix and α4/β6-Loop of $G_{i1}\alpha$ in Receptor Coupling—In order to investigate the α4-α4/β6 region of $G_{i1}\alpha$ in more detail we used several additional chimeras to subdivide this region (Fig. 4). Chi22 has the α4-helix of $G_{i1}\alpha$ replaced with that from $G_t\alpha$ while Chi25 has the α4/β6-loop of $G_{i1}\alpha$ replaced with that from $G_t\alpha$. Chi23 has the α4/β6-loop of $G_{i1}\alpha$ replaced with that from $G_t\alpha$ and also switches the Glu in $G_{i1}\alpha$ at the end of the α4-helix for the Leu found in Gtα. Chi24 has the central part of the α4/β6-loop with two variant amino acids switched between $G_{i1}\alpha$ and $G_t\alpha$. These chimeras were fully active with the A1 adenosine receptor (data not shown), supporting our conclusion that the A1 receptor does not use the α4-α4/β6 region to distinguish between G_t and G_{i1} (see Fig. 3). Fig. 5 shows the affinity shift activity of these chimeras with 5-HT_{1A}, 5-HT_{1B}, and M2 receptors. Chi22 had low affinity shift activity with all three receptors indicating that a critical determinant of coupling selectivity for these receptors is located in the α4-helix of Gi1α (Fig. 5). For the 5-HT_{1B} receptor, the activity of Chi22 was significantly higher than the activity of Chi3 ($p < 0.01$), indicating that the α4/β6-loop may also play a role in 5-HT_{1B} receptor coupling. This conclusion is supported by the Chi25 activity with the 5-HT_{1B} receptor (73%), which was significantly ($p < 0.001$) lower than the activity of Gi1 (100%). However, Chi25 was 91% as active with M2 muscarinic receptor) which was not significantly different ($p > 0.05$) from G_{i1} activity) and was 121% as active with the 5-HT_{1A} receptor (which was significantly ($p < 0.001$) higher than G_{i1}). Taken together the data suggest the α4/β6-loop is utilized differently by these receptors. Chi24 was fully active with all three receptors (Fig. 5), which suggests that the reduced activity of Chi25 with the 5-HT_{1B} receptor is due to the replacement of Asp-309 by Glu at the beginning of the α4/β6-loop (Fig. 4). Fig. 5 also shows the affinity shift activity of Chi23 was significantly reduced ($p < 0.001$) compared with the activity of both Gi1 and Chi25 for all three receptors. Chi23 differs from Chi25 by just one amino acid (replacement of Glu-308 from $G_{i1}\alpha$ for Leu from $G_t\alpha$) indicating that Glu-308 is important for coupling to 5-HT_{1A}, 5-HT_{1B}, and M2 receptors. Taken together, the data indicate that the α4-helix (Glu-308 in particular) is important for all three receptors, and that the α4/β6-loop (probably Asp-309) is also important for 5-HT_{1B} receptors.

Defining Individual Amino Acids in the α4-α4/β6 Region of $G_{i1}\alpha$—To prove the role of Glu-308 in receptor coupling and also to study the role of other amino acids in the α4-α4/β6 region of $G_{i1}\alpha$ we used chimeras in which amino acids Ala-301, Gln-304, Cys-305, Glu-308, Lys-312, and Thr-316 of $G_{i1}\alpha$ were replaced individually or in combinations with the corresponding amino acids of $G_t\alpha$. All of the mutants used here have been previously described (18). First we studied the role of these amino acids with a loss of function assay. Mutants in which amino acids of $G_{i1}\alpha$ were replaced individually or in combinations with the corresponding amino acids of $G_t\alpha$ would be expected to exhibit reduced affinity shift activities if these amino acids were important for coupling. Replacement of Ala-301 with Asn did not reduce activity (G_{i1}A301N, Fig. 6) demonstrating that Ala-301 is not important for coupling any of the receptors tested. When Gln-304 was changed to Lys (G_{i1}Q304K, Fig. 6) activity with 5-HT_{1A} and M2 receptors was significantly ($p < 0.001$) reduced, but as reported previously (18), this single amino acid replacement did not significantly reduce affinity shift activity with 5-HT_{1B} receptors (Fig. 6). The activity of Gi1C305V shows that Cys-305 is important for M2 muscarinic receptors (67% activity, $p < 0.001$) but not important for either 5-HT receptor (Fig. 6). Glu-308 is an important amino acid for all three receptors as the G_{i1}E308L mutant displays 62, 73, and 61% of activity with 5-HT_{1A}, 5-HT_{1B}, and

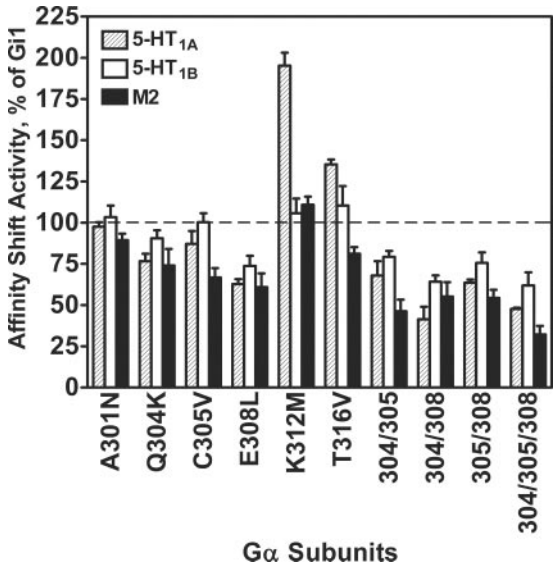

FIG. 6. **Functional coupling of receptors to the indicated $G_{i1}\alpha$ point mutants.** Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the percent affinity shift activities as mean ± S.E. from three or more independent experiments for each receptor. Exogenous G-proteins were present in 40–200-fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

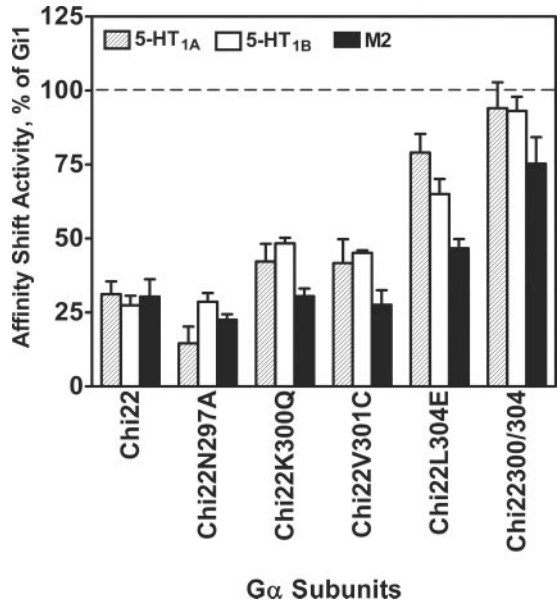

FIG. 7. **Functional coupling of receptors to the indicated Chi22 point mutants.** Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the percent affinity shift activities as mean ± S.E. from three or more independent experiments for each receptor. Exogenous G-proteins were present in 40–200-fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

M2 receptors, respectively ($p < 0.001$) (Fig. 6). Lys-312 and Thr-316 are not important for coupling these receptors and the increased activity of G_{i1}K312M and G_{i1}T316V with the 5-HT_{1A} receptor ($p < 0.001$) is consistent with the increased activity of Chi25 with this receptor.

Data obtained with three double mutants (G_{i1}Q304K/C305V, G_{i1}Q304K/E308L, G_{i1}C305V/E308L) and a triple mutant (G_{i1}Q304K/C305V/E308V) support the conclusions drawn from the point mutants (Fig. 6). The activity of the G_{i1}Q304K/E308L mutant was lower than the activity of either G_{i1}Q304K or G_{i1}E308L for all receptors supporting the importance of both Gln-304 and Glu-308 in receptor coupling. The role of Cys-305 in M2 receptor coupling is supported by the observation that the activity of G_{i1}Q304K/C305V mutant was significantly lower than the activity of the G_{i1}Q304K mutant ($p < 0.05$). Furthermore, the activity of the triple mutant (G_{i1}Q304K/C305V/E308V) was the lowest of all with the M2 receptor, supporting the idea that Gln-304, Cys-305, and Glu-308 are all important for M2 receptor coupling. On the other hand, the conclusion that Cys-305 is not important for coupling the 5-HT_{1A} and 5-HT_{1B} receptors is supported by the observations that the 304/305 and 305/308 double mutants have similar activities with these receptors as the Q304K and E308L single mutants and the 304/305/308 triple mutant is similar in activity to the 304/308 double mutant with these receptors.

Gain of function assays, in which amino acids from $G_{i1}\alpha$ replaced those from $G_t\alpha$ in Chi22 were used to confirm the role of the amino acids identified in the loss of function assay. The data in Fig. 7 demonstrate that substituting back Ala-301 does not lead to gain of function with any of the receptors tested, supporting the conclusion that Ala-301 of $G_{i1}\alpha$ is not important for receptor coupling. Substituting back Gln-304 (Chi22K300Q) resulted in significant ($p < 0.001$) gain of activity with 5-HT_{1B} receptors, which is in contrast to the absence of a loss of activity with 5-HT_{1B} receptors when Gln-304 was mutated to Lys in $G_{i1}\alpha$. Similarly, substituting back Cys-305 in the Chi22V301C mutant resulted in significant ($p < 0.05$) gain of activity with 5-HT_{1B} receptors but had no effect with M2 receptors. The precise reasons for these anomalies are unknown but may be related to the actual role of these amino acids in the context of their neighbors. Substituting back Glu-308 alone (Chi22L304E) resulted in a gain of affinity shift activity of 48% with 5-HT_{1A} receptors ($p < 0.001$), 38% with 5-HT_{1B} receptor ($p < 0.001$) but only 17% ($p > 0.05$) with M2 receptors. However, when both Gln-304 and Glu-308 were substituted back into Chi22 sequence (Chi22K300Q/L304E), a full gain of activity was observed with 5-HT_{1A} and 5-HT_{1B} receptors, as Chi22K300Q/L304E activity was not significantly different from activity of G_{i1} (100%). The gain of function with M2 receptors was significant (45% gain of activity, $p < 0.001$), though still less than the activity of Gi1. Taken together, the data indicate that Gln-304 and Glu-308 of $G_{i1}\alpha$ are important for 5-HT_{1A}, 5-HT_{1B}, and M2 receptor coupling, and that Cys-305 of $G_{i1}\alpha$ is important for M2 receptor coupling in addition to Gln-304 and Glu-308.

Role of C Terminus of $G_{i1}\alpha$ in Receptor Coupling—Alignment of the C-terminal sequences of $G_{i1}\alpha$ and $G_t\alpha$ indicates that their extreme eight C-terminal amino acids are identical (Fig. 8). Because numerous studies have indicated the C terminus of Gα plays a significant role in receptor coupling, we decided to investigate the role of C terminus of $G_{i1}\alpha$ in 5-HT_{1A}, 5-HT_{1B}, A1, and M2 receptor coupling using $G_{i1}\alpha/G_q\alpha$ C-terminal chimeras in which 3, 5, 11, or 35 C-terminal residues of $G_{i1}\alpha$ were replaced with those from $G_q\alpha$. These chimeras are designated Q3C, Q5C, Q11C, and Q35C, respectively. As shown in the sequence alignments in Fig. 8, the extreme C terminus of $G_q\alpha$ differs from that of $G_{i1}\alpha$ in just four amino acids. Loss of function experiments may demonstrate partial or complete loss of activity. As shown in Fig. 9, replacement of just two of these amino acids with those from $G_q\alpha$ in the Q3C mutant significantly lowers the affinity shift activity with all four receptors. The nearly complete loss of affinity shift activity (0.3 and 11.2%, respectively) with 5-HT_{1B} serotonin and A1 adenosine receptors suggests that these amino acids are critical for coupling, while the more modest decrease in activity (65 and 68% activity, respectively) with the 5-HT_{1A} and M2 receptors sug-

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Gtα 316 YSHMTCATDTQNVKFVFDAVTDIIIKENLKDCGLF 350
Gi1α 320 YTHFTCATDTKNVQFVFDAVTDVIIKNNLKDCGLF 354
Gqα 331 YSHFTCATDTENIRFVFAAVKDTILQLNLKEYNLV 359
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β6 α5 C

FIG. 8. **Sequence alignment of 35 C-terminal amino acids of $G_t\alpha$, $G_{i1}\alpha$ and $G_q\alpha$.** The sequences of $G_t\alpha$ and $G_q\alpha$ are compared with $G_{i1}\alpha$ sequence. Depicted in *bold* are amino acids of $G_t\alpha$ and $G_q\alpha$ that are different from corresponding amino acids of $G_{i1}\alpha$.

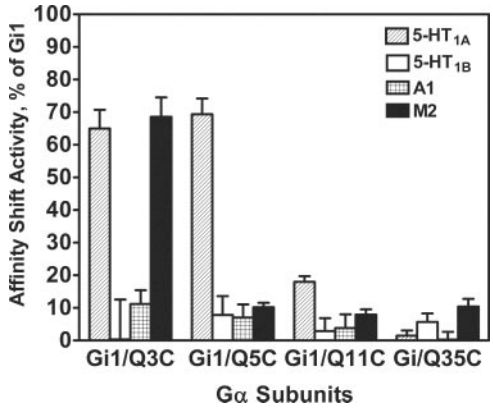

FIG. 9. **Functional coupling of receptors to the indicated G_{i1}/G_q chimeras.** Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the percent affinity shift activities as mean ± S.E. from three or more independent experiments for each receptor. Exogenous G-proteins were present in 40–400-fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

gest these amino acids are important, but not critical, for coupling. Substitution of the five C-terminal amino acids of $G_{i1}\alpha$ with those from $G_q\alpha$ eliminates coupling with the A1 adenosine receptor while substitution of 11 C-terminal amino acids are required for complete loss of 5-HT_{1A} receptor coupling (Fig. 9). These data indicate that the 5-HT_{1A}, 5-HT_{1B}, A1 adenosine, and M2 muscarinic receptors differ in their utilization of the C-terminal amino acids of $G_{i1}\alpha$ for coupling.

DISCUSSION

G-protein receptor coupling can be regulated by a variety of mechanisms (2, 3). At the G-protein-receptor interface, the selectivity of coupling is regulated by the amino acid sequences of both receptor and G-protein. By comparing the coupling mechanism of four closely related receptors to the same G-proteins, we found that receptors use multiple and distinct domains on Gα to achieve selective coupling. Coupling selectivity is also regulated by the G-protein concentration as demonstrated by the significant differences among the EC_{50} values for G_{i1} receptor interactions. This suggests that in living cells the expression levels of specific G-protein subunits may regulate receptor-coupling preferences.

At the level of Gα domains, the major difference we found is that the A1 adenosine receptor does not discriminate well between $G_{i1}\alpha$ and $G_t\alpha$ sequences. In contrast, the 5-HT and M2 receptors couple with G_{i1} but fail to couple with G_t. This selectivity allowed us to use $G_{i1}\alpha/G_t\alpha$ chimeras to define domains on $G_{i1}\alpha$ important for coupling with these receptors. Our findings indicate that amino acids especially important for receptor coupling are located in the α4-helix. In addition, the 5-HT_{1B} receptor may require Asp-309 at the beginning of α4/β6-loop for optimal coupling. The corresponding amino acid in $G_t\alpha$ is Glu-305, and while both are negatively charged, glutamate is one -CH_2 group bigger than aspartate. Thus replacement of aspartate with glutamate may decrease 5-HT_{1B} receptor coupling because of the change in the size of the receptor interacting surface on Gα. In addition, we demonstrated that within the α4-helix-α4/β6-loop region of $G_{i1}\alpha$ the amino acids that are involved in receptor coupling differ slightly among the receptors. While all three receptors utilize Gln-304 and Glu-308, the M2 receptor also uses Cys-305 and the 5-HT_{1B} receptor may use Asp-309. Interestingly, interaction of the 5-HT_{1A} receptor with the K312M mutant actually leads to an increased affinity shift. This increase in affinity shift activity may represent tighter coupling of the receptor with the chimera. Other investigators have also demonstrated the importance of this region of Gα in receptor coupling. Natochin *et al.* (12) demonstrated the role of Arg-310 and Asp-311 in interaction of $G_t\alpha$ with rhodopsin. Blahos *et al.* (13) demonstrated that α4-α4/β6-β6-α5 region of $G\alpha_{16}$ is important but not critical for interaction with metabotropic glutamate receptor 8. In contrast, the work of Grishina and Berlot (15) shows that the α4/β6-loop of $G\alpha_s$ is not important for interactions with β2 adrenergic receptors. Using gain of function experiments, Ho and Wong (23) demonstrated that incorporation of α4/β6-loop of Gαz into a Gαt backbone was not sufficient for δ-opioid receptor coupling. Taken together, these results support the idea that even if different receptors recognize the same general domain on Gα subunits, the specific amino acids involved in receptor interactions may be different.

Another region of $G_{i1}\alpha$ important for 5-HT and M2 receptor coupling is the N terminus, as affinity shift activity with Chi21 was lower than with G_{i1} for these receptors. According to the literature, the amino acids that bind to the receptor map to approximately positions 1–30 of the α-subunits (4). This region, which includes the N terminus and the αN-helix, contains the most differences between $G_{i1}\alpha$ and $G_t\alpha$ with 15 variant amino acids compared with just 9 variants from amino acids 31 to 219. Another significant difference between $G_{i1}\alpha$ and $G_t\alpha$ is that the αN-helix of $G_t\alpha$ is 4 amino acids shorter than the αN-helix of $G_{i1}\alpha$. Thus it is possible that amino acids 1–30 are important but not critical for 5-HT and M2 receptor coupling.

Although the C terminus of Gα subunits is postulated to directly contact the receptor and mediate receptor coupling selectivity, our data show that the specific amino acids involved in this recognition differ among the receptors studied. Cys-351 (position-4), Gly-352 (position-3), and Phe-354 (position-1) in G_i family members have been shown to be important for mediating selectivity of receptor coupling (reviewed in Ref. 2). Gain of function studies with $G_{q/i}$ chimeras (5, 24) indicate that five C-terminal amino acids of $G_i\alpha$ are sufficient for coupling to A1 and M2 receptors while three C-terminal amino acids of $G_i\alpha$ are not enough for A1 receptor coupling (5). Although so far it has not been possible to successfully solve the structure of the Gα C terminus in the context of the whole molecule (the C terminus is disordered in the crystal), the structure of the C-terminal undecapeptide of Gtα bound to activated rhodopsin has been resolved by NMR spectroscopy (25). In this C- terminal decapeptide, the first eight residues form an α-helix, which is terminated by an α_L type C-cap (26) with C-terminal glycine (Gly-348 in $G_t\alpha$, Gly-352 in $G_{i1}\alpha$) in the center of the reverse turn (27). Thus the observation (5) that for the A1 receptor three C-terminal amino acids of Gi1α are critical in the loss of function experiments but five C-terminal amino acids are re-

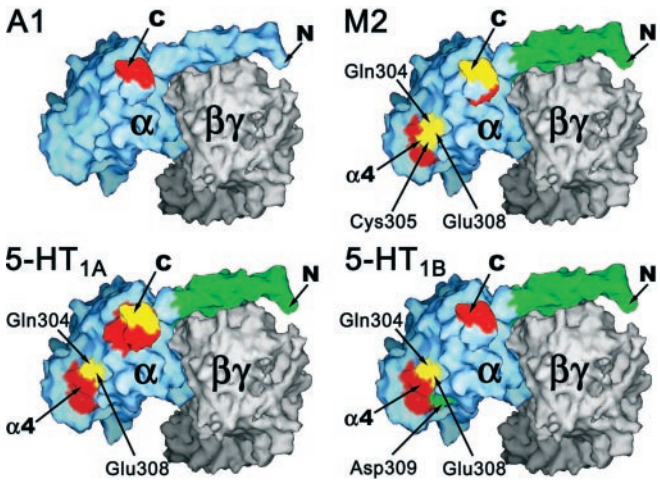

FIG. 10. **Receptor recognition surfaces on $G_{i1}\alpha$.** The images of the molecular surfaces were generated using SPOCK (29) with coordinates from the crystal structure of the heterotrimer solved by Wall *et al.* (28). The six C-terminal residues of $G_{i1}\alpha$ are not present in the crystal structure of the trimer and are represented here by the NMR structure of the $G_t\alpha$ C-terminal peptide (27) that has been docked to the crystal structure. The α-subunit is shown in *blue* and the $\beta\gamma$-subunit in *gray*. The four panels represent the G-protein surfaces required for functional coupling with the indicated receptors. The regions of $G_{i1}\alpha$ colored *red* and *yellow* (red only for the C termini of the A1 and 5-HT_{1B} receptors) eliminated receptor coupling upon replacement with the corresponding regions from $G_t\alpha$ or $G_q\alpha$ and are termed critical. Within these critical regions the residues colored *yellow* reduced coupling when tested alone and are termed important. Regions colored *green* also reduced coupling upon replacement and are termed important but were not found to be part of a larger region that eliminated coupling.

quired to gain coupling may be explained by the fact that this α_L C-cap, which is disrupted in G_{i1}/Q3C chimera, is required for A1 receptor coupling. This is probably also true for the 5-HT_{1B} receptor. Our M2 receptor data indicate that although this α_L C-cap structure is important, it is not critical for receptor coupling. For the 5-HT_{1A} receptor three C-terminal amino acids of $G_{i1}\alpha$ are important while amino acids at the positions −4 and −5 (Asp-350 and Cys-351) are not important since the activities of G_{i1}/Q3C and G_{i1}/Q5C are the same. G_i/Q5C and G_i/Q11C are different in three amino acids, which are probably involved in 5-HT_{1A} receptor coupling. Some additional amino acids involved in 5-HT_{1A} receptor coupling are located in the α5-helix (see Fig. 8) as evident from the activity of G_{i1}/Q35C chimera. Taken together, our results support the idea that different receptors may recognize a specific pattern of amino acids, which form receptor recognition surfaces.

Fig. 10 depicts a structure of the $G\alpha_{i1}\beta_1\gamma_2$ G-protein heterotrimer. Six amino acids from the C terminus and four amino acids from the N terminus are missing from the crystal structure of the heterotrimer solved by Wall *et al.* (28) and so the C-terminal residues from the NMR structure of the $G_t\alpha$ C-terminal decapeptide (27) have been docked to the crystal structure. The domains of $G_{i1}\alpha$ discussed herein are surface exposed and located on the G-protein surface that is presumed to face the receptor. They are therefore available for receptor coupling. However, while some amino acids may be involved in coupling by making direct contact with receptors, others may be involved indirectly by playing a role in guanine nucleotide exchange, and it is not possible to distinguish between these possibilities based on our functional coupling assays. Regions of $G_{i1}\alpha$ that eliminated coupling upon replacement with the corresponding regions from $G_t\alpha$ or $G_q\alpha$ have been colored *red* and *yellow* in Fig. 10, with the yellow portions defining residues whose replacement merely reduced coupling. The *green* regions also merely reduced coupling but were not found to be part of a larger region that eliminated coupling. Clearly the regions responsible for coupling the individual receptors are subtly different. The adenosine A1 and 5-HT_{1B} receptors are sensitive to a very small (just two amino acids) change in the extreme C terminus, while the M2 muscarinic and 5-HT_{1A} receptors use a larger portion of the C terminus to distinguish among the $G\alpha$ subunits. Furthermore, slightly different residues within the α4-helix are used by the 5-HT_{1A}, 5-HT_{1B}, and M2 muscarinic receptors while this region is not used by A1 adenosine receptors. Amino acids Glu-304, Cys-305, Glu-308, and Asp-309 are surface-exposed and so are available for receptor coupling. Molecular modeling indicates that G_{i1}Q304K, G_{i1}E308L, and G_{i1}304/308 mutations alter the surface potential (18), while the G_{i1}D309E mutation alters steric interactions because Glu is one CH2 group larger then Asp (water-accessible surfaces of native G_{i1} and G_{i1}D309E were constructed and superimposed in Insight II; not shown). Therefore, structural considerations are consistent with a role for these residues in receptor coupling. Similarly, the N terminus is used by the 5-HT_{1A}, 5-HT_{1B}, and M2 muscarinic receptors (colored *green* in Fig. 10), but not the A1 adenosine receptor. In summary, we have demonstrated that four closely related $G_{i/o}$-coupled receptors distinguish themselves by the affinity with which they interact with G_{i1} and by their use of multiple and distinct domains of $G_{i1}\alpha$ for selective coupling.

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