Closely related G protein coupled receptors use multiple and distinct domains on G protein α subunits for selective coupling

Janna E. Slessareva∗†, Hongzheng Ma∗‡, Karyn M. Depree∗, Lori A. Flood∗, Hyunsu Bae§, Theresa M. Cabrera-Vera§, Heidi E. Hamm§& and Stephen G. Graber∗¶

∗Department of Biochemistry and Molecular Pharmacology, West Virginia University School of Medicine, Morgantown, West Virginia 26506

§Institute for Neuroscience, Northwestern University, Chicago, Illinois 60611

†Current address: Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

‡Current address: Department of Medicine, Duke University Medical Center, Durham, NC 27710

&Current address: Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Running Title: Multiple and distinct domains of Ga couple related receptors

∗Send Correspondence to:

Stephen Graber
PO Box 9142 HSN
West Virginia University
Morgantown, WV 26506-9223

Phone: (304) 293-2305
FAX: (304) 293-6846
Email: sgraber@hsc.wvu.edu
Multiple and distinct domains of Gα couple related receptors
SUMMARY

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 Gi1α, Gtα and Gqα, 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 Gi1α and Gtα sequences, the 5-HT1A and 5-HT1B serotonin and M2 muscarinic receptors can couple with Gi1 but not Gt. 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 Gi1α are involved in 5-HT and M2 receptor interactions. Chimeric Gi1α/Gqα subunits verify the critical role of the Ga C-terminus in receptor coupling, however, the individual receptors differ in the C-terminal amino acids required for coupling. Furthermore, the EC50 for interactions with Gi1 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.
INTRODUCTION

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). 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-HT1B receptor coupling to Gi1 (11).
Later it was demonstrated that Gln304 and Glu308 in the α4 helix of Gi1α 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.

Gi1α and Gtα are closely related Gα subunits, which belong to the Gi/o class of G protein α subunits, share 68% homology, and have nearly identical overall structures. Although the 5-HT$_{1B}$ receptor discriminates between Gi1 and Gt (11;19), the fact that their C termini are identical render Gi1α/Gtα chimeras useless for exploring the role of this domain in receptor coupling. However, the extreme C terminus of Gqα differs from that of Gi1α by four amino acids, while their α5-helixes differ by an additional nine amino acids. Thus Gi1α/Gqα chimeras are ideal for studying the role of this domain in coupling. Since several different GPCRs$^1$ 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. demonstrated that 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors distinguish themselves by the affinity with which they interact with G proteins (20). Therefore we also wanted to test the generality of this mechanism with different receptors. Here we compare the coupling behavior of four Gi/o-coupled receptors (5-HT$_{1A}$ and 5-HT$_{1B}$ serotonin, A1 adenosine and M2 muscarinic) by reconstituting them with G protein heterotrimerers containing native or chimeric Gα subunits composed of Gai1, Gαt and Gαq. Our data demonstrate that selective coupling between Gi1 and the members of Gi/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**- [3H]-Oxotremorine-M Acetate ([3H]-OXO-M) (85.8 Ci/mmol), [3H]-Hydroxytryptamine Binoxalate ([3H]-5-HT) (25.5 Ci/mmol) and [3H]-Chloro-N6-cyclopentyladenosine ([3H]-CCPA) (30 Ci/mmol) were from New England Nuclear Life Science Products, Inc. (Boston, MA). Atropine Sulfate, 5-Hydroxytryptamine (5-HT) and R-phenylisopropyl adenosine (R-PIA) were from Sigma-Aldrich Corporation (St. Louis, MO). Adenosine deaminase was from Roche Molecular Biochemicals (Indianapolis, IN). The BCA Protein Assay reagents were from Pierce (Rockford, IL). All other chemicals were from Sigma-Aldrich Corporation (St. Louis, MO) or EMD Biosciences (formerly Calbiochem-Novabiochem Corporation; San Diego, CA).

**Expression and purification of proteins**- The expression and purification of the Gαi1 and Gβγ subunits was as previously described (21;22). The chimeric Gαi1/Gαt subunits were constructed, expressed in *E. coli* and purified as described (19). The Gi1/Q3C, Gi1/Q5C and Gi1/Q11C chimeras (which have the 3, 5 or 11 C-terminal residues of Gi1α replaced with those from Gqα) were made from pHis6Gαi1 using the silent BamHI site introduced at amino acid position 212 (19). The pHis6Gαi1 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 pHis6Gαi1. To construct Gi1/Q35C (which has the 35 C-terminal residues of Gi1α replaced with those from Gqα), the C-terminal portion of a Gqα 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, AlF₄⁻-dependent conformational change (measured as an
increase in intrinsic tryptophan fluorescence) or binding to the cGMP phosphodiesterase \(\gamma\) 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 15x 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 DTT, 0.1 mM PMSF, 20 \(\mu\)g/ml of benzamidine and 2 \(\mu\)g/ml of each of aprotinin, leupeptin and pepstatin A) and burst by nitrogen cavitation (600 psi, 20 minutes). Cavitated cells were centrifuged at 4 °C for 10 minutes at 500 x 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 x g. The supernatant was discarded and the pellets were resuspended and pooled in 35 ml of HE buffer (5 mM NaHEPES, 1 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, aliquotted, 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 \(\mu\)l. Typically, 1-2 \(\mu\)l of G protein subunits were added to 40 \(\mu\)l of membrane suspension, the mixture was incubated at 25 °C for 15 minutes 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 MgCl₂, 0.5 mM EDTA, pH 7.5. Binding buffer for A1 adenosine receptor was 10 mM HEPES, 5 mM MgCl₂, 1 mM EDTA, pH 7.4. Radioligand binding in the affinity shift assay was determined in the presence of the [³H]-OXO-M for M2 muscarinic receptor, [³H]-5-HT for 5-HT serotonin receptors and [³H]-CCPA for A1 adenosine receptor. Adenosine deaminase was added to the [³H]-CCPA solution at 12 μg/ml in binding buffer. Non-specific 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 hr for M2 receptor, 1.5 hrs for 5-HT receptors, 2 hrs 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 ice cold 50 mM Tris-Cl, 5 mM MgCl₂, 0.5 mM EDTA, 0.01% NaAzide, pH 7.5 at 4 °C, placed in 4.5 ml 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. [³H]-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 Gi1 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 Gi1 protein heterotrimers varied significantly among the individual receptors affinity shift activity was normalized to Gi1 activity and expressed as % affinity shift activity, which is (Chimera Reconstituted Binding - Control Binding/Gi1 Reconstituted Binding - Control Binding) x100.

**Analysis of the 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 3 or more times. Data represent the mean ± SEM from multiple experiments. One-way ANOVA 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 Gi1α are molecular determinants of 5-HT\textsubscript{1B} receptor coupling (11) and that two amino acids in the α4 helix of Gi1α (Gln304 and Glu308) are especially important for 5-HT\textsubscript{1B} receptor coupling (18). The goal of the present study was to examine the generality of these findings among closely related members of the Gi/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 Gi/o-coupled receptors: 5-HT\textsubscript{1A} and 5-HT\textsubscript{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\textsubscript{50} values for reconstitution of high affinity agonist binding were determined. The data indicate that A1, 5-HT\textsubscript{1A}, 5-HT\textsubscript{1B} and M2 receptors have different EC\textsubscript{50} values for Gi1 (Figure 1). A1 receptors have the highest apparent affinity (0.4 nM) and M2 receptors have the lowest apparent affinity (47 nM) for the Gi1 heterotrimer. 5-HT receptors have intermediate EC\textsubscript{50} values of 3.7 nM and 16.2 nM for the 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B} receptors respectively. Titration experiments similar to those shown in Figure 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\textsubscript{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** - Figure 2 depicts the secondary structures of the Gi1α/Gtα chimeras used in this study. All of these chimeras have been previously described and were used to study Gi1α domains involved in 5-HT₁B receptor coupling (11;18;19). Figure 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 Gtα and has the same functional properties as Gtα (19). Chi6 is primarily Gtα in character as it includes N-terminal amino acids 1-215 and C-terminal amino acids 295-350 of Gtα with the amino acids corresponding to 216-294 from Gi1α to maintain solubility. In this region there are just 26 amino acids that differ between Chi6 and Gtα. As shown in Figure 3, Chi6 was inactive with 5-HT₁A, 5-HT₁B 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 Figure 3 demonstrate Chi6 was 74% active with the A1 adenosine receptor, indicating that A1 adenosine receptor doesn't discriminate well between Gt and Gi1 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 Gi1, 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-HT₁A, 5-HT₁B and M2 muscarinic receptors to couple with Chi6 allowed us to use additional chimeras containing less Gtα sequence to more precisely identify the domains required for coupling.
We first examined whether the N-terminal or C-terminal portion of Gi1α was critical for receptor coupling. Chi21 has N-terminal amino acids 1-215 of Gtα with the rest of the molecule Gi1α sequence (Figure 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 Gi1α and Gtα (Figure 3). The activity of Chi21 with 5-HT1A, 5-HT1B and M2 receptors was significantly (p<0.001) reduced (44%, 57% and 42% respectively, Figure 3) demonstrating that amino acids 1-219 of Gi1α contain an important determinant of Gi coupling with these receptors. Chi2 has the C-terminal amino acids 295-350 of Gtα with the rest of the chimera Gi1α sequence (Figure 2). Figure 3 demonstrates that amino acids 299-354 of Gi1α contain residues critical for 5-HT1A, 5-HT1B 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 Gi1α and Gtα sequences. To further evaluate the role of amino acids 299-354 of Gi1α in 5-HT and M2 receptor coupling we tested two additional chimeras, Chi3 and Chi13 (Figure 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 Gi1α replaced with the corresponding amino acids of Gtα. As shown in Figure 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-HT1A, 5-HT1B and M2 receptor coupling, but not for A1 adenosine receptor coupling. In contrast, Chi13, with six amino acids variant from Gi1α, was active with all four receptors indicating that the 35 C-terminal amino acids of Gi1α and Gtα are functionally interchangeable 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\textsubscript{1A} (128%) and 5-HT\textsubscript{1B} (124.5%) receptors suggest subtle differences in the coupling mechanism of these receptors. The role of the extreme C-terminus of Gi1\textalpha cannot be evaluated with these chimeras because the eight C-terminal amino acids of Gi1\textalpha and Gt\textalpha are identical.

**Role of the \(\alpha 4\)-helix and \(\alpha 4/\beta 6\)-loop of Gi1\textalpha in receptor coupling**—In order to investigate \(\alpha 4-\alpha 4/\beta 6\) region of Gi1\textalpha in more detail we used several additional chimeras to subdivide this region (Figure 4). Chi22 has the \(\alpha 4\) helix of Gi1\textalpha replaced with that from Gt\textalpha while Chi25 has the \(\alpha 4/\beta 6\) loop of Gi1\textalpha replaced with that from Gt\textalpha. Chi23 has the \(\alpha 4/\beta 6\) loop of Gi1\textalpha replaced with that from Gt\textalpha and also switches the Glu in Gi1\textalpha at the end of the \(\alpha 4\) helix for the Leu found in Gt\textalpha. Chi24 has the central part of the \(\alpha 4/\beta 6\) loop with two variant amino acids switched between Gi1\textalpha and Gt\textalpha. These chimeras were fully active with the A1 adenosine receptor (data not shown), supporting our conclusion that the A1 receptor does not use the \(\alpha 4-\alpha 4/\beta 6\) region to distinguish between Gt and Gi1 (see Figure 3). Figure 5 shows the affinity shift activity of these chimeras with 5-HT\textsubscript{1A}, 5-HT\textsubscript{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 \(\alpha 4\) helix of Gi1\textalpha (Figure 5). For the 5-HT\textsubscript{1B} receptor, the activity of Chi22 was significantly higher than the activity of Chi3 (p<0.01), indicating that the \(\alpha 4/\beta 6\) loop may also play a role in 5-HT\textsubscript{1B} receptor coupling. This conclusion is supported by the Chi25 activity with the 5-HT\textsubscript{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 Gi1 activity) and was 121% as active with the 5-HT\textsubscript{1A} receptor (which was significantly (p<0.001) higher than Gi1). Taken together the data suggest the \(\alpha 4/\beta 6\) loop is utilized differently by these receptors. Chi24 was
fully active with all three receptors (Figure 5) which suggests that the reduced activity of Chi25 with the 5-HT$_{1B}$ receptor is due to the replacement of Asp309 by Glu at the beginning of the $\alpha_4/\beta_6$ loop (Figure 4). Figure 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 Glu308 from Gi1$\alpha$ for Leu from Gt$\alpha$) indicating that Glu308 is important for coupling to 5-HT$_{1A}$, 5-HT$_{1B}$ and M2 receptors. Taken together, the data indicate that the $\alpha_4$ helix (Glu308 in particular) is important for all three receptors, and that the $\alpha_4/\beta_6$ loop (probably Asp309) is also important for 5-HT$_{1B}$ receptors.

*Defining individual amino acids in the $\alpha_4-\alpha_4/\beta_6$ region of Gi1$\alpha$*—To prove the role of Glu308 in receptor coupling and also to study the role of other amino acids in the $\alpha_4-\alpha_4/\beta_6$ region of Gi1$\alpha$ we used chimeras in which amino acids Ala301, Gln304, Cys305, Glu308, Lys312 and Thr316 of Gi1$\alpha$ were replaced individually or in combinations with the corresponding amino acids of Gt$\alpha$. 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 Gi1$\alpha$ were replaced individually or in combinations with the corresponding amino acids of Gt$\alpha$ would be expected to exhibit reduced affinity shift activities if these amino acids were important for coupling. Replacement of Ala301 with Asn did not reduce activity (Gi1A301N, Figure 6) demonstrating that Ala301 is not important for coupling any of the receptors tested. When Gln304 was changed to Lys (Gi1Q304K, Figure 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 (Figure 6). The activity of Gi1C305V shows that Cys305 is important for M2
muscarinic receptors (67% activity, p<0.001) but not important for either 5-HT receptor (Figure 6). Glu308 is an important amino acid for all three receptors as the Gi1E308L mutant displays 62%, 73% and 61% of activity with 5-HT\textsubscript{1A}, 5-HT\textsubscript{1B} and M2 receptors respectively (p<0.001) (Figure 6). Lys312 and Thr316 are not important for coupling these receptors and the increased activity of Gi1K312M and Gi1T316V with the 5-HT\textsubscript{1A} receptor (p<0.001) is consistent with the increased activity of Chi25 with this receptor.

Data obtained with three double mutants (Gi1Q304K/C305V, Gi1Q304K/E308L, Gi1C305V/E308L) and a triple mutant (Gi1Q304K/C305V/E308V) support the conclusions drawn from the point mutants (Figure 6). The activity of the Gi1Q304K/E308L mutant was lower than the activity of either Gi1Q304K or Gi1E308L for all receptors supporting the importance of both Gln304 and Glu308 in receptor coupling. The role of Cys305 in M2 receptor coupling is supported by the observation that the activity of Gi1Q304K/C305V mutant was significantly lower than the activity of the Gi1Q304K mutant (p<0.05). Furthermore, the activity of the triple mutant (Gi1Q304K/C305V/E308V) was the lowest of all with the M2 receptor, supporting the idea that Gln304, Cys305 and Glu308 are all important for M2 receptor coupling. On the other hand, the conclusion that Cys305 is not important for coupling the 5-HT\textsubscript{1A} and 5-HT\textsubscript{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 Gi1\textalpha replaced those from Gt\textalpha in Chi22 were used to confirm the role of the amino acids identified in the loss of function assay. The data in Figure 7 demonstrate that substituting back Ala301 does not lead to gain of function
with any of the receptors tested, supporting the conclusion that Ala301 of Gi1α is not important for receptor coupling. Substituting back Gln304 (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 Gln304 was mutated to Lys in Gi1α. Similarly, substituting back Cys305 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 Glu308 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 Gln304 and Glu308 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 Gi1 (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 Gln304 and Glu308 of Gi1α are important for 5-HT$_{1A}$, 5-HT$_{1B}$ and M2 receptor coupling, and that Cys305 of Gi1α is important for M2 receptor coupling in addition to Gln304 and Glu308.

**Role of C terminus of Gi1α in receptor coupling**—Alignment of the C-terminal sequences of Gi1α and Gtα indicates that their extreme eight C-terminal amino acids are identical (Figure 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 Gi1α in 5-HT$_{1A}$, 5-HT$_{1B}$, A1 and M2 receptor coupling using Gi1α/Gqα C-terminal chimeras in which 3, 5, 11 or 35 C-terminal residues of Gi1α were replaced with those from Gqα. These chimeras
are designated Q3C, Q5C, Q11C and Q35C respectively. As shown in the sequence alignments in Figure 8, the extreme C-terminus of Gqα differs from that of Gi1α in just four amino acids. Loss of function experiments may demonstrate partial or complete loss of activity. As shown in Figure 9, replacement of just two of these amino acids with those from Gqα 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-HT1B 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-HT1A and M2 receptors suggest these amino acids are important, but not critical, for coupling. Substitution of the five C-terminal amino acids of Gi1α with those from Gqα eliminates coupling with the A1 adenosine receptor while substitution of 11 C-terminal amino acids are required for complete loss of 5-HT1A receptor coupling (Figure 9). These data indicate that the 5-HT1A, 5-HT1B, A1 adenosine and M2 muscarinic receptors differ in their utilization of the C-terminal amino acids of Gi1α 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 Gi1-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 Gi1α and Gtα sequences. In contrast, the 5-HT and M2 receptors couple with Gi1 but fail to couple with Gt. This selectivity allowed us to use Gi1α/Gtα chimeras to define domains on Gi1α 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 Asp309 at the beginning of α4/β6 loop for optimal coupling. The corresponding amino acid in Gtα is Glu305, 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 Gi1α the amino acids that are involved in receptor coupling differ slightly among the receptors. While all three receptors utilize Gln304 and Glu308, the M2 receptor also uses Cys305 and the 5-HT$_{1B}$ receptor may use Asp309. 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. demonstrated the role of Arg310 and Asp311 in interaction of Gtα with rhodopsin (12). Blahos et al. demonstrated that α4-α4/β6-β6-α5 region of Gα16 is important but not critical for interaction with metabotropic glutamate receptor 8 (13). In contrast, the work of Grishina and Berlot shows that α4/β6 loop of Gαs is not important for interactions with β2 adrenergic receptors (15). Using gain of function experiments, Ho and Wong demonstrated that incorporation of α4/β6 loop of Gαz into a Gαt backbone was not sufficient for δ-opioid receptor coupling (23). 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 Gi1α important for 5-HT and M2 receptor coupling is the N terminus, as affinity shift activity with Chi21 was lower than with Gi1 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 Gi1α and Gtα with 15 variant amino acids compared with just 9 variants from amino acids 31 to 219. Another significant difference between Gi1α and Gtα is that the αN helix of Gtα is 4 amino acids shorter than the αN helix of Gi1α. 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. Cys351 (position −4), Gly352 (position −3) and Phe354 (position −1) in Gi family members have been shown to be important for mediating
selectivity of receptor coupling (reviewed in) (2). Gain of function studies with Gq/i chimeras (5;24) indicate that five C terminal amino acids of Giα are sufficient for coupling to A1 and M2 receptors while three C terminal amino acids of Giα 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 αL type C-cap (26) with C terminal glycine (Gly348 in Gtα, Gly352 in Gi1α) 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 required to gain coupling may be explained by the fact that this αL C-cap, which is disrupted in Gi1/Q3C chimera, is required for A1 receptor coupling. This is probably also true for the 5-HT1B receptor. Our M2 receptor data indicate that although this αL C-cap structure is important, it is not critical for receptor coupling. For the 5-HT1A receptor three C terminal amino acids of Gi1α are important while amino acids at the positions −4 and −5 (Asp350 and Cys351) are not important since the activities of Gi1/Q3C and Gi1/Q5C are the same. Gi/Q5C and Gi/Q11C are different in three amino acids, which are probably involved in 5-HT1A receptor coupling. Some additional amino acids involved in 5-HT1A receptor coupling are located in the α5 helix (see Figure 8) as evident from the activity of Gi1/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.

Figure 10 depicts a structure of the Gαi1β1γ2 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 Gtα C-terminal decapeptide (27) have been docked to the crystal structure. The domains of Gi1α 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 Gi1α that eliminated coupling upon replacement with the corresponding regions from Gtα or Gqα have been colored red and yellow in Figure 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-HT1B receptors are sensitive to a very small (just two amino acids) change in the extreme C-terminus, while the M2 muscarinic and 5-HT1A receptors use a larger portion of the C-terminus to distinguish among the Gα subunits. Furthermore, slightly different residues within the α4-helix are used by the 5-HT1A, 5-HT1B, and M2 muscarinic receptors while this region is not used by A1 adenosine receptors. Amino acids Glu304, Cys305, Glu308 and Asp309 are surface exposed and so are available for receptor coupling. Molecular modeling indicates that Gi1Q304K, Gi1E308L and Gi1304/308 mutations alter the surface potential (18), while the Gi1D309E mutation alters steric interactions because Glu is one CH2 group larger then Asp (water-accessible surfaces of native Gi1 and Gi1D309E 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_{1A} and M2 muscarinic receptors (colored green in Figure 10), but not the A1 adenosine receptor. In summary, we have demonstrated that four closely related Gi/o coupled receptors distinguish themselves by the affinity with which they interact with Gi1 and by their use of multiple and distinct domains of Gi1α for selective coupling.

Acknowledgements— We thank Dr. Brenda Temple of the Structural BioInformatics Core Facility, University of North Carolina at Chapel Hill, Chapel Hill, NC, for assistance in preparing Figure 10.
REFERENCES


FOOTNOTES

1The abbreviations used are: GPCRs, G protein coupled receptors; GTPγS, guanosine 5'-3-O-(thio)triphosphate; OXO-M, Oxotremorine-M; 5-HT, Hydroxytryptamine; CCPA, chloro-N6-cyclopentyladenosine, R-PIA, R-phenylisopropyl adenosine.
FIGURE LEGENDS

FIGURE 1. Concentration dependence of Gi1 in affinity shift assays for individual Gi1-coupled receptors. Sf9 cell membranes expressing the indicated Gi1-coupled receptors were reconstituted with increasing concentrations of Gi1 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 Gi1 was 4.1 ± 0.51, n=17, for the 5-HT1A receptor; 3.8 ± 0.19, n=22, for the 5-HT1B 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 endpoint. Shown are the data from representative experiments. EC50 data are the mean ± SEM from 3 or more independent experiments.

FIGURE 2. Secondary structure of Ga subunits. Numbers above the chimeric structures indicate the junction points of Ga t and Ga i1 sequences and refer to the amino acid positions in Ga t. Numbers for the wild type forms of Ga t and Ga i1 represent their total amino acid residues. The bottom diagram depicts the secondary structural domains common to Ga subunits.

FIGURE 3. Functional coupling of receptors to the indicated Gi1/Gt chimeras. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Ga and βγ subunits. Data represent the % affinity shift activities as mean ± SEM from 3 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.
FIGURE 4. Primary sequence alignment of the α4-α4/β6 loop region of Gαi1 and Gαt. The boxes indicate the regions of Gαi1 that were substituted with the corresponding sequences from Gαt to generate the indicated Gαi1/Gαt chimeras.

FIGURE 5. Functional coupling of receptors to the indicated Gi1/Gt chimeras. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the % affinity shift activities as mean ± SEM from 3 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.

FIGURE 6. Functional coupling of receptors to the indicated Gi1α point mutants. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the % affinity shift activities as mean ± SEM from 3 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.

FIGURE 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 % affinity shift activities as mean ± SEM from 3 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.
FIGURE 8. Sequence alignment of 35 C terminal amino acids of Gtα, Gi1α and Gqα. The sequences of Gtα and Gqα are compared to Gi1α sequence. Depicted in bold are amino acids of Gtα and Gqα that are different from corresponding amino acids of Gi1α.

FIGURE 9. Functional coupling of receptors to the indicated Gi1/Gq chimeras. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the % affinity shift activities as mean ± SEM from 3 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.

FIGURE 10. Receptor recognition surfaces on Gi1α. 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 Gi1α are not present in the crystal structure of the trimer and are represented here by the NMR structure of the Gtα C-terminal peptide (27) that has been docked to the crystal structure. The α subunit is shown in blue and the βγ subunit in gray. The four panels represent the G protein surfaces required for functional coupling with the indicated receptors. The regions of Gi1α colored red and yellow (red only for the C-termini of the A1 and 5-HT1B receptors) eliminated receptor coupling upon replacement with the corresponding regions from Gtα or Gqα 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.
FIGURE 1
FIGURE 2
FIGURE 3
FIGURE 4
FIGURE 5
Figure 6: Affinity Shift Activity, % of Gi1

- **A301N**
- **Q304K**
- **C305V**
- **E308L**
- **K312M**
- **T316V**
- **304/305**
- **304/308**
- **305/308**
- **304/305/308**

**Gα Subunits**

**5-HT1A**

**5-HT1B**

**M2**
FIGURE 7
FIGURE 9
FIGURE 10
Closely related G protein coupled receptors use multiple and distinct domains on G protein α subunits for selective coupling

Janna E. Slessareva, Hongzheng Ma, Karyn M. Depree, Lori A. Flood, Hyunsu Bae, Theresa M. Cabrera-Vera, Heidi E. Hamm and Stephen G. Graber

J. Biol. Chem. published online October 2, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304417200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2003/10/02/jbc.M304417200.citation.full.html#ref-list-1