Reconstitution Reveals Additional Roles for N- and C-Terminal Domains of Gα in Muscarinic Receptor Coupling†

Janna E. Slessareva and Stephen G. Graber*

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

Received January 23, 2003; Revised Manuscript Received April 14, 2003

ABSTRACT: The molecular basis for selectivity of M1 and M2 muscarinic receptor coupling to heterotrimeric G proteins has been studied using receptors expressed in Sf9 cell membranes and reconstituted with purified chimeric Gα subunits containing different regions of Gi1α and Gqα. The abilities of G protein heterotrimers containing chimeric α subunits to stabilize the high-affinity state of the receptors for agonist and to undergo receptor stimulated guanine nucleotide exchange was compared with G protein heterotrimers containing either native Gi1α or Gqα. The data confirm the importance of the proper context of the C-terminus of Gα by demonstrating that the C-terminus of Gi1α, when placed in the context of Gqα, prevents coupling to muscarinic M1 receptors, while the C-terminus of Gqα, when placed in the context of Gi1α, prevents coupling to muscarinic M2 receptors. However, C-terminal amino acids of Gqα placed in the context of Gi1α were not sufficient to allow M1 receptor coupling, nor were C-terminal amino acids of Gi1α placed in the context of Gqα sufficient for M2 receptor coupling. The unique six amino acid N-terminal extension of Gqα when added to the N-terminus of Gi1α neither prevented M2 receptor coupling nor permitted M1 receptor coupling. A Gi1α-based chimera containing both N- and C-terminal regions of Gqα gained the ability to productively couple M1 receptors suggesting that the proper context of both N- and C-termini is required for muscarinic receptor coupling.

Heptahelical receptors transfer a variety of extracellular signals to intracellular effectors by coupling to heterotrimeric guanine nucleotide-binding proteins (G proteins) (*1*). The mechanisms responsible for selectivity in G protein-mediated signaling pathways remain poorly understood (*2*). Direct contacts with receptors have been demonstrated for both α and $\beta\gamma$ subunits (*3*−*9*). Although recent evidence suggests both that β and γ interact selectively with receptors (*10*−*13*), the α subunits appear to play a more prominent role in establishing the selectivity of G protein−receptor coupling. Using different receptor systems and various methodologies, the C-terminus and α5 helix (*14*−*17*), N-terminus and αN helix (*18*−*21*), α4 helix and α4/β6 loop (*20*), α2 helix and α2/β4 loop (*22*), and α3/β5 loop (*23*) domains of Gα subunits have been shown to be involved in receptor coupling.

Muscarinic receptors are typical class I heptahelical receptors. When expressed in Sf9 cells, they demonstrate structural integrity and display the expected ligand binding properties (*24*, *25*). The family of human muscarinic receptors includes five molecularly distinct members. M1, M3, and M5 are Gq/11-coupled receptors that stimulate phospholipid breakdown through activation of phospholipase Cβ (PLCβ),[1] while M2 and M4 are Gi/o-coupled receptors that mediate the inhibition of adenylyl cyclase and activation of K^+ channels (*26*, *27*). Muscarinic receptors also regulate additional signaling processes, including stimulation of MAP kinase pathways and regulation of other ion channels (*27*, *28*).

The C-terminus of Gα subunits is the most studied determinant of receptor coupling selectivity to date. Numerous studies, including transfection experiments (*15*), synthetic peptide competition experiments (*14*), ADP-ribosylation of Gα by Pertussis toxin (PTX) (*29*), and direct reconstitution studies (*17*), indicate that the extreme C-terminus of Gα interacts with receptors. With respect to selectivity, Conklin, Bourne, and Wess demonstrated that substitution of the last three to five C-terminal residues of Gqα with the corresponding sequences of Giα, Goα, or Gzα allowed PLCβ stimulation by A1 adenosine, D2 dopamine, α2 adrenergic, and M2 muscarinic receptors, which are normally coupled to Gi/o family members (*15*, *30*). Furthermore, site-directed mutagenesis revealed receptor contact sites for the C-terminus of Giα and Gqα on M2 and M3 muscarinic receptors respectively (*30*−*32*). The N-terminus of Gα has also been shown to be a determinant of G protein-receptor coupling selectivity (*18*−*21*). Importantly, Kostenis et al. suggested a role for the unique N-terminal extension of Gqα in constraining receptor coupling selectivity by showing that deletion of the six N-terminal amino acids of Gqα (MTLESI)

† This work was supported by NSF Grants MCB9870839 and MCB0110839.

* Corresponding author. Address: P.O. Box 9142 HSN, West Virginia University, Morgantown, WV 26506-9223. Phone: (304) 293-2305.FAX: (304) 293-6846. E-mail: sgraber@hsc.wvu.edu.

[1] Abbreviations: PLCβ, phospholipase Cβ; PTX, pertussis toxin; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; oxo-M, oxotremorine-M; NMS, *N*-methyl scopolamine.

10.1021/bi034133j CCC: $25.00 © 2003 American Chemical Society
Published on Web 05/31/2003

led to promiscuous coupling of this Gq mutant with several Gi/o- and Gs-coupled receptors (*18*).

Our study directly examines the interactions of M1 and M2 Muscarinic receptors with Gq and Gi1 proteins in an Sf9 cell membrane-based reconstitution system in which the coupling behavior of receptors of interest can be studied by reconstitution with exogenously supplied, purified G protein subunits (*33*). This system allows the analysis of both the ability of G proteins to stabilize the high-affinity state of receptor for agonist and the ability of the receptors to catalyze guanine nucleotide exchange on Gα. Our results show that the C-terminus of Gα, although critical, is not sufficient for M1 or M2 receptor coupling. Furthermore, the six amino acid N-terminal extension of Gqα neither allows M1 receptor coupling nor constrains M2 receptor coupling. In summary, the data suggest that the appropriate context of both N- and C-termini is required for selective receptor coupling.

MATERIALS AND METHODS

Materials. [^{3}H]-oxotremorine-M (85.8 Ci/mmol) and [^{35}S]-GTPγS (1250 Ci/mmol) were from New England Nuclear Life Science Products, Inc. (Boston, MA). Molecular biology enzymes were from New England Biolabs (Beverly, MA). Wild-type *baculovirus* DNA and lipofectin were from Invitrogen (Carlsbad, CA). Anti-G$\alpha i_{1/2}$ C-terminal antibodies were from Calbiochem-Novabiochem Corporation (San Diego, CA). Anti-Gαi1 internal antibodies and anti-Gαq/11 C-terminal antibodies were from Santa Cruz Biotechnology. Pertussis Toxin was from Calbiochem-Novabiochem Corporation (San Diego, CA). The ProtoBlot Western blot reagents were from Promega (Madison, WI). The BCA protein assay reagents were from Pierce (Rockford, IL). All other chemicals were from Sigma-Aldrich Corporation (St. Louis, MO) or Calbiochem-Novabiochem Corporation (San Diego, CA).

Construction of Gα Chimeric Genes. H_6pQE-60-Gi1Q3C and H_6pQE-60-Gi1Q35C plasmids were the kind gift of Dr. Heidi Hamm (Vanderbilt University, Nashville, TN) and are based on H_6pQE-60-Gi1, originally described by Lee et al. (*34*). The pcDNA1-Gqi5C plasmid was the kind gift of Dr. Bruce Conklin (Gladstone Institute of Cardiovascular Disease, University of California, San Francisco). To construct pVLSG-Gi1Q6N, the nucleotides coding for the six N-terminal amino acids of mouse Gαq (MTLESI) were inserted into the BamH1/Nco1 sites of pVLSG-Gi1 (*35*) using synthetic oligonucleotides to create a duplex linker. H_6pQE-60-Gi1Q3C was used to construct both Gi1Q6N3C and Gi1Q6N35C. H_6pQE-60-Gi1Q3C was digested with EcoR1 and Nco1 and recircularized using synthetic oligonucleotides to create a duplex linker encoding the six N-terminal amino acids of mouse Gαq (MTLESI), resulting in the removal of the His_6 tag and creation of the intermediate pQE-60−Gi1Q6N3C. A duplex linker was used to subclone the NcoI/HindIII fragment of pQE-60-Gi1Q6N3C into NcoI/EcoRI sites of pVLKD. The pVLKD vector was constructed from the commercially available baculovirus expression vector pVL1393 by removing the BamH1 site and adding an Nco1 site with a duplex linker between the BamH1 and XbaI sites of the pVL1393 poly-linker. The BamH1/HindIII fragment of H_6pQE-60-Gi1Q35C was subcloned into the BamH1/EcoRI sites of pVLKD-Gi1Q6N3C using a duplex linker. This resulted in the replacement of the BamH1/HindIII fragment of pVLKD-Gi1Q6N3C with the corresponding fragment from H_6pQE-60-Gi1Q35C, creating pVLKD-Gi1Q6N35C. To create pVL1393-Gqi5C, the BamHI/NsiI fragment coding for Gqi5C was cut from pcDNA1 and subcloned into the BamHI/PstI sites of pVL1393. All constructs were verified by sequencing and restriction analysis. PVLSG-Gi1Q6N, pVLKD-Gi1Q6N35C, and pVL1393-Gqi5C transfer vectors were used to create recombinant baculoviruses as described (*36*).

Expression and Purification of G Protein α and βγ Subunits. The chimeric Gi1Q35C α subunit was constructed, purified after expression in *Escherichia coli*, and kindly provided by Dr. Heidi Hamm of Vanderbilt University. While bacterially expressed subunits lack the N-terminal acylation of native and baculovirus expressed subunits and contain a HIS_6 tag at the extreme N-terminus, their EC_{50} values for receptor interactions are indistinguishable from those of native or baculovirus expressed subunits (*20*). Gq was purified as detergent extracted $G\alpha_q\beta_1\gamma_{2HIS}$ heterotrimer from Sf9 cells infected with baculoviruses expressing Gqα, β_1 and γ_{2HIS}, as described by Kozasa and Gilman (*37*), using sequential chromatography on DEAE anion exchange, Ni−NTA Superflow resin and QHR15 anion exchange columns. Free $\beta_1\gamma_{2HIS}$ subunits were eluted at 100−125 mM NaCl, and $G\alpha q\beta_1\gamma_{2HIS}$ heterotrimers were eluted at 200−220 mM NaCl from the QHR15 column. Gqi5 was purified in the same manner as Gq. The HA-epitope tag present between residues 125 and 130 of Gαqi5C has been shown not to affect functionality (*15*). Gαq and Gαqi5C were purified from heterotrimers by elution from Ni−NTA Superflow resin with 10 mM NaF and 50 μM $AlCl_3$, followed by final purification on a QHR15 column. Gi1α was expressed in Sf9 insect cells and purified as described (*38*). Gi1Q6N and Gi1Q6N35C were expressed and purified from Sf9 cells using identical procedures as for Gi1α. All G protein α subunits except Gi1Q35C and Gqi5 contained only native protein sequences. Protein concentrations were determined with the BCA protein assay using BSA as a standard.

Intrinsic Tryptophan Fluorescence Assay. The ability of the Gα subunits to bind GDP and adopt the active conformation in the presence of AlF^-_4 was verified by monitoring intrinsic tryptophan fluorescence (*39*) with a QM-2000−4 spectrofluorimeter (PTI fluorescence system hardware and Felix software) at room temperature in a buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, and 1μM GDP. The AlF^{4-}-dependent conformational changes of activated Gα subunits were monitored by intrinsic tryptophan fluorescence changes with excitation at 280 nm and emission at 340 nm. The relative increase in fluorescence of 400 nM Gα subunits was determined from absorbance readings before and after addition of 10 mM NaF and 20 μM $AlCl_3$ in a total volume of 100 μL. The increase in fluorescence was calculated as $\Delta F\% = (F - F_o)/F_o \times 100\%$. The relative change in intrinsic fluorescence for individual Gα subunits was consistent with the literature (*39*) and as follows: Gi1 48.3 ± 3.2 ($n = 11$), q6N 45 ± 12.2 ($n = 8$), q35C 56.7 ± 5.2 ($n = 3$), q6N35C 54.5 ± 15.6 ($n = 4$), qi5C 47.7 ± 8.4 ($n = 3$), Gq 50 ± 22.9 ($n = 4$).

Preparation of Sf9 Cell Membranes Containing Recombinant Receptors. Baculoviruses expressing M1 and M2 muscarinic receptors were the kind gift of Dr. Elliott Ross

(University of Texas Southwestern Medical Center, Dallas, Texas). Sf9 cell membranes containing recombinant muscarinic receptors were produced as described (*38*). In short, Sf9 cells were infected at a density of 3.0×10^6 cells/mL with a recombinant baculovirus expressing M1 or M2 muscarinic receptors at a 2-fold multiplicity of infection. Infected cells were harvested 60−72 h post-infection for M1 receptors and 48−56 h post-infection for M2 receptors. To prepare membranes, cell pellets were thawed in 15× their wet weight of ice-cold SGHB1 buffer (10 mM Tris/HCl, 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, pH 8.0) with protease inhibitors (2 mg/mL aprotinin, 20 mg/mL benzamidine, 2 mg/mL leupeptin, 2 mg/mL pepstatin and 0.1 mM PMSF) and burst by nitrogen cavitation (20 min at 600 PSI). Cavitated cells were centrifuged at 4 °C for 10 min at 500 × *g*. The supernatant was centrifuged at 4 °C for 30 min at 28 000 × *g*. The pellets were resuspended in 35 mL of HE buffer (5 mM Hepes, 1 mM EDTA, pH 7.5) with protease inhibitors. The membranes were washed twice, resuspended in HE buffer at 1−3 mg protein/ml, snap frozen in liquid nitrogen, and stored at −70 °C. M1 receptor coupling required removal of endogenous proteins with 6M urea essentially as described by Northup (*40*). For urea stripping, membranes were thawed, resuspended at about 0.15 mg/mL in binding buffer (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA) with 120 μM oxotremorine-M and 6 μM GTPγS, and incubated for 30 min at 25 °C. Pellets obtained by centrifugation at 288 000 × *g* for 30 min at 4 °C were resuspended at approximately 0.6 mg/mL in stripping buffer (HE + 6M Urea) and extracted on ice for 30 min. The urea extract was centrifuged at 142 000 × *g* for 30 min at 4 °C and the pellets were washed twice in HE buffer. Urea stripped membranes were resuspended in HE buffer at a concentration of about 2 mg/mL and snap frozen in liquid nitrogen. Receptor number in membrane preparations was determined using the muscarinic receptor antagonist [^{3}H]-*N*-methyl Scopolamine (NMS) at concentrations from 0.03 to 8 nM of free [^{3}H]-NMS under the same conditions as used in the affinity shift assay (see below). Membrane protein concentrations were determined with the BCA protein assay using BSA as a standard.

Reconstitution of Receptors with Exogenous G Proteins. Frozen membranes were thawed, pelleted in a refrigerated microcentrifuge at 10 000 × *g* for 10 min, and resuspended at 5−10 mg/mL in reconstitution buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 500 nM GDP, 0.08% CHAPS). G protein α and $\beta\gamma$ subunits were mixed at a ratio of 1:1.3 and diluted in reconstitution buffer to keep the reconstitution volume the same for each sample. Typically, 0.8 pmol of receptor was reconstituted with 80 pmol of G protein heterotrimers (based on Gα). The mixture was incubated at 25 °C for 20 min and held on ice briefly until the start of the binding assay.

Affinity Shift Assay. Just prior to the binding assay the reconstitution mixture was diluted 10−12-fold with binding buffer (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA). Agonist binding was determined in the presence of 5 nM [^{3}H]-oxotremorine-M. Nonspecific binding was determined in the presence of 10 μM atropine sulfate. Binding to equilibrium was for 1 h at 25 °C in a temperature controlled shaker and was terminated by filtration over Whatman GF/C filters using a Brandel Cell Harvester. The filters were rinsed three times with 4 mL of ice-cold wash buffer (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.01% NaAzide), placed in 4.5 mL CytoScint (ICN Pharmaceuticals, Costa Mesa, CA) and counted to constant error in a scintillation counter. The final concentrations of receptors and G protein heterotrimers in the binding assay were 1.5−1.7 and 150−500 nM, respectively, in a final volume of 150 μL. Specific binding in the absence of exogenous G-proteins averaged 50 DPM for M1 receptors and 326 DPM for M2 receptors. Because muscarinic receptors exhibit ratios as high as 30 000-fold for the high- and low-affinity states for agonists such as oxotremorine M (oxo-M) and acetylcholine (*41*, *42*), specific binding for M1 receptors reconstituted with Gq averaged 1614 DPM, while that for M2 receptors reconstituted with Gi1 averaged 7682 DPM. The actual concentrations of agonist varied slightly among experiments which resulted in different magnitudes of absolute binding. To compare the results among experiments, it was necessary to express the data as affinity shift activities. Affinity shift is defined as the fold-enhancement above buffer controls of high-affinity agonist binding in membranes expressing recombinant receptors reconstituted with saturating concentrations of G protein heterotrimers. Subunits that cannot stabilize the high-affinity state of receptors for agonist (as well as buffer controls) have-affinity shift activities of 1, while active subunits have affinity shift activities significantly greater than 1. The concentrations of G proteins required to saturate the affinity shift activities of all receptor G-protein combinations used in this study were determined in control experiments (Figure 3).

Gα Saturation Binding Experiments. To determine the concentration of Gα needed to saturate the affinity shift, increasing amounts of G protein heterotrimers were added to the receptors in the reconstitution, and agonist binding was detected in the binding assay as described above. Typically, 7 different G protein concentrations in triplicates in the range 10−500 nM were used. EC_{50} values were generated from nonlinear regression analysis of data fit to a one-site interaction model between receptor and G protein. These data are presented in Figure 3.

ADP-Ribosylation of Gα by Pertussis Toxin. Pertussis Toxin (1.1 μg/μL) was activated by the addition of an equal volume of activation buffer (5 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.08% CHAPS, 40 mM DTT, 2μM GDP) and incubation for 40 min at 32 °C. G protein heterotrimers (3 μg α plus 3.6 μg $\beta\gamma$) were ribosylated for 1 h at 32 °C in a total volume of 20 μL containing 10 μL of activated PTX and 400 μM NAD^+. Ribosylation reactions were reconstituted with membranes under the same conditions described above. At the end of the reconstitution incubation, the mixture was diluted 9-fold with binding assay buffer and used in the affinity shift assay.

Receptor-Catalyzed [^{35}S]-GTPγS Binding Assay. A GTPγS binding assay was used to analyze receptor-catalyzed GDP/GTP exchange on G protein α subunits in response to agonist. Typically 1.3 pmol of receptors was reconstituted with 80 pmol of G protein heterotrimers (1.3-fold excess of α over $\beta\gamma$) for 20 min at 30 °C in reconstitution buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 40 μM GDP, 0.08% CHAPS). After reconstitution the mixture was diluted 10-fold with GTPγS Binding buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 25 mM $MgCl_2$, 1 mM

DTT, 0.1 mg/mL BSA). Reactions were initiated by the addition of 25 μL of membrane mixture to 25 μL of GTPγS with or without oxotremorine-M. Final assay conditions were 50 nM Gα, 0.8 nM receptor, 2 μM GDP, and 100 nM GTPγS as a mixture of 1 nM [^{35}S]-GTPγS (~125,000 cpm/well) and cold GTPγS, with or without 2 μM oxotremorine-M in a final volume of 50 μL. Use of MultiScreen 96 well plates (Millipore) allowed collection of samples at 30 s intervals for 4−6 min for M2 receptors or 3 min intervals for 18 min for M1 receptors. Filters were rinsed three times with 100 μL of ice-cold wash buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM $MgCl_2$). Radioactivity retained on the filters was counted to constant error in a liquid scintillation counter. Greater than 1500 cpm was observed at the shortest time points, and ligand depletion was less than 15% at the longest time-points. Data were analyzed by linear regression, and agonist-driven GTPγS binding was reported as the ratio of the slopes in the presence and absence of agonist provided the slopes were significantly different ($p < 0.05$). If the slopes did not differ significantly, a value of 1.0 was used for the slope ratio. GTPγS binding in the absence of agonist (moles of GTPγS per mole of receptor) are reported in the legend to Figure 5.

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 2−5 times as indicated. Rates of GTPγS binding were determined from linear regression of eight individual determinations at 30 s or 3 min intervals within each experiment, and experiments were repeated 3−5 times as indicated. Data represent the mean ± SEM from multiple experiments unless otherwise indicated.

RESULTS AND DISCUSSION

Molecular mechanisms of selectivity in G protein−receptor coupling are still not completely understood (*2*). Intact organisms employ various mechanisms including regulation of receptor and G protein expression levels, compartmentalization of signaling complexes, and the activity of accessory proteins, to regulate the selectivity of signaling along G protein-mediated pathways. However, at the level of the G protein−receptor interface, the primary recognition is governed by the amino acid sequences of both G protein and receptor. G protein α subunits are considered the major determinant of coupling selectivit,y and several domains of Gα have been shown to be important for receptor coupling. In this study we investigated the roles of the N- and C-termini of Gqα and Gi1α in directing the selectivity of M1 and M2 muscarinic receptor coupling in a reconstituted system. To do so we tested a series of chimeric Gα subunits composed from different portions of Gqα and Gi1α for their ability to stabilize the high-affinity agonist binding state of receptors and for their ability to undergo agonist stimulated guanine nucleotide exchange.

Figure 1A shows the secondary structure of the chimeric Gα subunits used in this study. The q6N chimera has been constructed by addition of the unique six amino acid N-terminal extension of Gqα (MTLESI) to the N-terminus of Gi1α. Q35C has been constructed by the replacement of 35 C-terminal amino acids of Gi1α with the corresponding amino acids of Gqα. There are 14 amino acids that are different between Gi1α and Gqα in this region. Q6N35C has been constructed by addition of the six N-terminal amino acids of Gqα (MTLESI) to the q35C construct. The qi5C chimera (*15*) has five C-terminal amino acids of Gqα (EYNLV) replaced with the corresponding amino acids of Gi1α (DCGLF). All constructs used for protein expression were verified by DNA sequencing. All proteins except q35C were purified after expression in Sf9 cells. Q35C was purified after expression in bacteria and is the only Gα subunit in this study with a HIS_6 tag at the extreme N-terminus. Previous work, with bacterially expressed subunits containing a HIS_6 tag at the extreme N-terminus, demonstrated that their EC_{50} values for receptor interactions are indistinguishable from those of native or baculovirus expressed subunits (*20*). Furthermore, all of the α subunits used in this study bound GDP and adopted the active conformation in the presence of AlF^-_4 as shown by an enhancement of intrinsic tryptophan fluorescence (see Materials and Methods) comparable to that reported for other G protein preparations (*39*). To verify that the purified proteins had the expected domain switching between Gα subunits, chimeric proteins were separated on 12% SDS-polyacrylamide gel and immunoblotted with antibodies selective for unique sequences within the Gα subunits. Immunoblotts of purified chimeric Gα subunits are shown in Figure 1B. As expected, Gi1, q6N, q6N35C, and q35C, but not qi5C or Gq, were recognized by an antibody against internal Gαi1 sequence. Although the antibody directed against Gαi1/2 C-terminal sequence recognized Gi1 and q6N, it did not recognize q6N35C and q35C verifying that the C-terminal domains of q6N35C and q35C are different from the C-terminal domain of Gi1. In addition, the antibody directed against Gαi1/2 C-terminal sequence recognized the qi5C chimera demonstrating that C-terminal amino acids of Gi1α are present in qi5C. Q6N35C and q35C were also recognized by the antibody against Gαq/11 C-terminal sequences, as was Gqα, further supporting the domain switching. The antibody against Gqα internal sequence recognized Gq only. This antibody did not recognize qi5C because an internal HA tag in the qi5C construct (amino acid positions 125−130) disrupts the recognition epitope of the antibody against Gqα internal sequence (amino acids 115−133). The presence of the qi5C chimera was proven by immunoblotting with anti-HA tag antibodies (Figure 1B). Therefore, immunoblotting with different antibodies verified the domain switching between wild type α subunits in the α subunit chimeras used in this study. Figure 1C shows Coomassie Blue stained Gα subunits resolved on a 12% SDS-polyacrylamide gel. All of the α subunits used in this study were estimated to be at least 80% pure. The presence of doublets and triplets on the gel is likely to be due to the presence of α subunits in different states of lipid modification or guanine nucleotide binding. This interpretation is supported by the fact that these bands are recognized by the antibodies specific to the protein of interest (Figure 1B).

To study the role of N- and C-termini of Gqα and Gi1α in M1 and M2 muscarinic receptor coupling, G protein heterotrimers containing chimeric α subunits were reconstituted with Sf9 cell membranes containing expressed M1 or M2 muscarinic receptors, and their ability to form a high-affinity ternary complex with the expressed receptors was

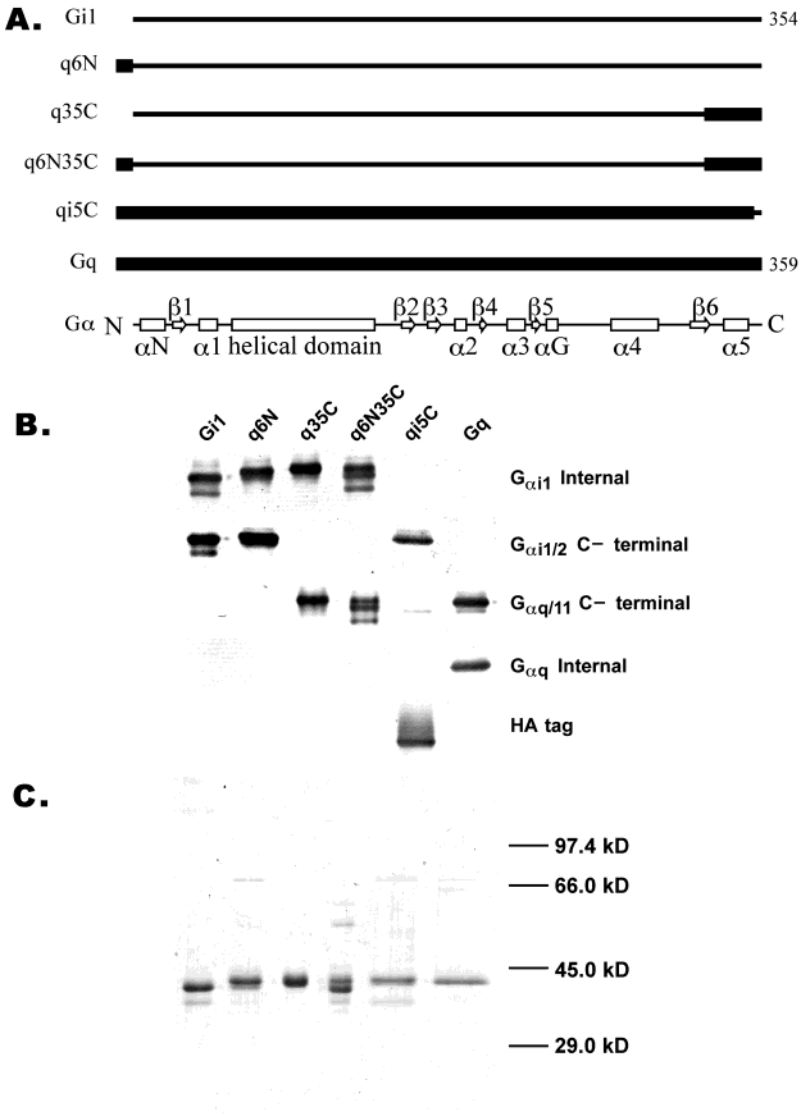

FIGURE 1: A. Secondary structure of chimeric Gα subunits. Numbers on the right of the wild-type forms of Gαi1 and Gαq indicate their total amino acid residues. Numbers within the designations for the chimeras indicate the number of amino acids switched between Gαi1 and Gαq. The diagram on the bottom depicts the secondary structural domains common to Gα subunits. B. Immunoblot analysis of purified chimeric α subunits. Proteins were purified as described in Materials and Methods, electrophoresed on SDS-polyacrylamide gels and immunoblotted with the antibodies indicated at the right of each panel. Each lane contained 75 ng of the indicated purified α subunit. C. Coomassie stain of purified Gα subunits. 500 ng of purified α subunits were resolved on a 12% SDS-polyacrylamide gel and visualized with coomassie blue stain. The order of proteins on coomassie gel is the same as that indicated above the immunoblot. Lane 1 is Gi1, lane 2 is q6N, lane 3 is q35C, lane 4 is q6N35C, lane 5 is qi5C, and lane 6 is Gq.

determined by affinity shift assay. Figure 2 shows the affinity shift activities of wild type and chimeric α subunits with M1 and M2 muscarinic receptors which were determined with saturating concentrations of G proteins. The affinity shift assay has been completely described for several receptors expressed in Sf9 cells (*33*, *43*) and is based on earlier work with native receptors (*44*, *45*). In short, the affinity shift assay detects the formation of the high-affinity state of the receptor as an enhanced level of agonist binding upon ternary complex formation. Control experiments, shown in Figure 3, in which G proteins were titrated at fixed agonist concentration (5 nM [^{3}H]-oxo-M), were used to determine the concentration of G protein required to saturate affinity shift activity. The EC_{50} values for reconstitution of high-affinity agonist binding for individual Gα subunits shown in the inset tables of Figure 3 are not significantly different, which is in agreement with earlier work suggesting that differences in G protein−receptor coupling manifest themselves as differences in agonist affinities rather than differences in EC_{50} values (*20*). The Gα subunits for which inset data are lacking displayed either weak saturable activity (as shown in experiments where doubling G protein concentrations did not increase affinity shift activity) or no activity even at high concentrations (300−500 nM) of G proteins (data not shown). By saturating affinity shift activities, we eliminated the possibility that the differences in the affinity shifts shown in Figure 2 were caused by differences in specific activities of the Gα subunit preparations.

As expected, in the affinity shift assay M1 receptors coupled with Gq and M2 receptors coupled with Gi1 (Figure 2). We used both loss of function and gain of function approaches to investigate the role of the C-terminus of Gqα in M1 muscarinic receptor coupling. As shown in Figure 2, the qi5C chimera has very little ability to couple M1 receptors compared with native Gqα, verifying the important role of the Gqα C-terminus. Because Conklin, Bourne, and

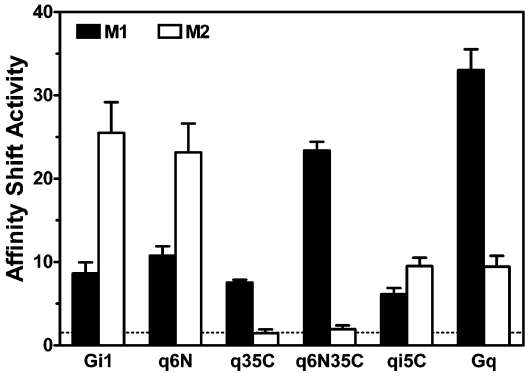

FIGURE 2: Affinity shift activity of Gα subunits with M1 and M2 muscarinic receptors. Affinity shift activities represent the fold enhancement above buffer controls of high-affinity [^{3}H]-oxo-M binding in membranes expressing the indicated muscarinic receptor (1−2 nM) reconstituted with a saturating excess (100−200 nM) of G protein heterotrimers containing the indicated Gα subunits. Data are the mean ± SEM from 3−5 experiments where the concentration of oxo-M was ~5 nM. The dotted line represents an affinity shift activity of 1, the level of activity observed in the absence of exogenous G proteins or in the presence of inactive G protein heterotrimers.

Wess have demonstrated in transfection studies that the five C-terminal amino acids of Gi1α, when placed in the Gqα context, were sufficient to couple M2 muscarinic receptors (*15*, *30*), we used a gain of function approach as a criteria to test if the C-terminus of Gqα placed in the Gi1α context was sufficient to couple M1 muscarinic receptors. Interestingly, even 35 C-terminal amino acids of Gqα in the Gi1α context were not sufficient to couple M1 receptors (Figure 2). We have also tested chimeras with three, five, and 11 C-terminal amino acids of Gqα in place of those from Gi1α and found they were not sufficient for M1 receptor coupling (data not shown). We conclude, therefore, that the C-terminus of Gqα, although required, is not sufficient for proper Gq−M1 receptor coupling and that other domains of Gqα must be present. Intriguingly, Natochin et al. reported that eleven C-terminal residues of Gtα in the Gsα context were sufficient for rhodopsin coupling in a reconstituted system (*17*). Such differences seem to indicate that molecular determinants of G protein-receptor coupling selectivity differ among individual receptors and their cognate G proteins.

To investigate the role of the unique six amino acid N-terminal extension of Gqα (MTLESI) in M1 receptor coupling, we added these amino acids of Gqα to the N-terminus of Gi1α. However, this chimera (q6N) also did not gain the ability to couple M1 receptors (Figure 2, compared with Gi1 and Gq). To investigate the possibility that both N- and C-termini of Gqα are required for proper M1 receptor coupling, we created the double chimera, q6N35C, which has the six N-terminal amino acids and 35 C-terminal amino acids of Gqα in the Gi1α context. Interestingly, q6N35C had a significantly enhanced ability ($p < 0.01-0.001$, Tukey's multiple comparison test) to shift the agonist affinity of M1 muscarinic receptors, indicating that the appropriate context of N- and C-termini is required for proper receptor coupling (Figure 2). However, the activity of q6N35C chimera in the affinity shift assay was significantly lower ($p < 0.05$, Tukey's multiple comparison test) than the activity of Gq (Figure 2). At least two explanations can be suggested for this difference in activity. First, other domains of Gqα or, possibly, a longer N-terminal portion of Gqα, are needed for full M1 receptor coupling. Second, since Cys9 and Cys10 of Gqα have been shown to be important but not critical for receptor coupling (*21*), the lack of Cys10 in the q6N35C chimera may explain the lower activity. Kostenis and others have suggested that the unique six amino acid N-terminal extension of Gqα prevents Gi/o-coupled receptors (including M2 muscarinic) and Gs-coupled receptors from coupling to Gq (*18*). We tested if these six N-terminal amino acids of Gqα could restrict M2 receptor coupling in the affinity shift assay. As shown in Figure 2, the six N-terminal amino acids of Gqα added to the N-terminus of Gi1α did not prevent M2 receptor coupling in a reconstitution system.

The important role of the C-terminus of Gi1α in receptor coupling was verified by the loss of function of the q35C chimera in the affinity shift assay with M2 receptors (Figure 2). In similar experiments, q5C and q11C chimeras also failed to couple with M2 receptors (data not shown). The q6N35C chimera also had no coupling activity with the M2 receptor (Figure 2). As discussed below, the low-level affinity shift activity of both Gq and qi5C in coupling the M2 receptor is due to a small amount of contaminating Sf9 cell Gi/o-like

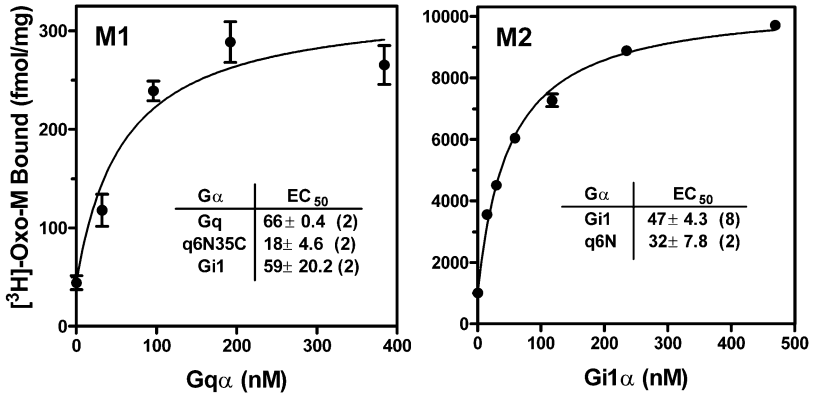

FIGURE 3: Concentration dependence of Gα subunits in agonist binding to M1 and M2 muscarinic receptors. Sf9 cell membranes expressing either M1 (left panel) or M2 (right panel) muscarinic receptors were reconstituted with increasing concentrations of G protein heterotrimers containing the indicated Gα subunit. Receptor concentrations were 0.95 nM for M1 and 0.82 nM for M2 in a final volume of 150 μL. High-affinity agonist binding was determined in the presence of 5 nM [^{3}H]-OXO-M. Data were fit to a single-site interaction between receptor and G protein and are the mean ± SD of triplicate determinations from a representative experiment. The inset tables show the EC_{50} values (mean ± SEM) from the indicated number of experiments for individual Gα subunits. For each receptor, the individual EC_{50} values did not differ significantly from one another (One-way ANOVA or t-test).

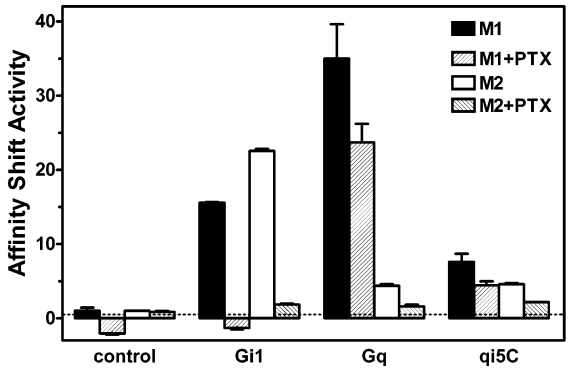

FIGURE 4: Affinity shift activity of Gα subunits following ADP-ribosylation by Pertussis Toxin. Affinity shift activities represent the fold enhancement above buffer controls of high-affinity [^{3}H]-oxo-M binding in membranes expressing the indicated muscarinic receptor (1−2 nM) reconstituted with a saturating excess (100−200 nM) of G protein heterotrimers containing the indicated Gα subunits in a final volume of 150 μL with or without ADP-ribosylation by Pertussis Toxin. Data are the mean ± SD of triplicate determinations from a representative experiment repeated twice with similar results. Gα subunits without pertussis toxin treatment contained all of the reagents (except Pertussis Toxin) and underwent the same treatment as those treated with toxin. The dotted line represents an affinity shift activity of 1, the level of activity observed in the absence of exogenous G proteins or in the presence of inactive G protein heterotrimers.

protein in these preparations (see Figure 4). Therefore, the data indicate that the M2 receptor has an absolute requirement for the proper context of the C-terminus of the coupling G protein. Interestingly, gain of function experiments indicated that the five C-terminal amino acids of Gi1α placed in the context of Gqα were not sufficient for optimal M2 receptor coupling as measured by the affinity shift assay (qi5C chimera, Figure 2). Because qi5C only minimally couples M2 receptors, it is tempting to speculate that cooperation between appropriate N- and C-termini are also critical for full Gi1-M2 muscarinic receptor coupling. Previous reconstitution studies demonstrated a role for a poorly defined region within the first 210 amino acids of Gi1α that is important for 5-HT_{1B} receptor coupling (*20*). However, since only the six amino acid N-terminal extension that is unique to Gqα subunits was tested in this study, such speculation may be premature, and the structural determinants of coupling may differ significantly between M1 and M2 receptors. The failure of qi5C to couple appreciably in our reconstitution assays is in contrast with the results of Liu, Conklin, and others, who demonstrated in transfection assays that the qi5C construct conferred coupling to several Gi/o-coupled receptors, including the M2 receptor (*30*). While the precise reasons for such different observations in transfection versus reconstitution assays are unknown, several factors are likely to contribute. In transfection assays, proteins of interest are overexpressed in a limited percentage of cells, and coupling is inferred from effector activities which are substantially amplified at several points in the signal transduction cascade. On the other hand, all cellular components are present and the integration and assembly of membrane structures occurs in a cellular context. Reconstitution experiments directly examine the G protein−receptor interface and allow controlled stoichiometries of components; however, they require the G protein to associate with the receptor after the receptor has been integrated into cellular membranes and may not allow the participation of all components responsible for receptor−G protein coupling. Thus, reconstitution experiments are likely to more precisely identify the G protein domains directly involved in the receptor−G protein interface, while transfection experiments are likely to identify the domains that permit coupling in a cellular context without revealing the precise mechanism or required stoichiometries. Ultimately both contribute to our understanding of coupling although neither may completely reflect the actual situation in living tissues.

In addition to the expected Gq−M1 and Gi1−M2 receptor coupling, we observed a low level of Gi1−M1 and Gq−M2 receptor coupling in the affinity shift assay (Figure 2). The basis for this coupling was explored with the use of pertussis toxin (PTX). PTX is known to uncouple Gi/o proteins from their cognate receptors by ADP-ribosylating a C-terminal cysteine in Gi/oα subunits (*29*). We ADP-ribosylated G protein heterotrimers using PTX and reconstituted them with M1 or M2 muscarinic receptors. The results shown in Figure 4 indicate that PTX treatment completely uncoupled Gi1 from M2 receptors as expected. Surprisingly, PTX treatment also completely uncoupled Gq from M2 receptors, but not Gq from M1 receptors. As Gqα is not a substrate for ADP-ribosylation by PTX, we attribute the Gq−M2 receptor coupling to the presence of small amounts of endogenous Sf9 Gi/o family proteins in our Gq preparation, which was undetectable by immunoblotting (Figure 1A). A similar contamination of Gq purified after expression in Sf9 cells by endogenous Gi/o proteins was reported by Kozasa (*37*). This contaminating activity is not present in the q35C and q6N35C preparations (see Figure 2), as they are purified from the soluble fraction of expressing cells which contains few, if any, endogenous G proteins, while Gq and qi5C are purified from a detergent extract of the particulate fraction from expressing cells which would be expected to contain endogenous G proteins (see Materials and Methods). In addition, PTX treatment prevented Gi1−M1 receptor coupling (Figure 4). Therefore, our data indicate that M1 muscarinic receptors weakly couple with Gi1, while M2 muscarinic receptors probably do not couple with Gq. Our data agree well with the data of Burford et al., who demonstrated that M1 receptors, especially when expressed at high levels, can couple to both pertussis toxin sensitive and insensitive G proteins, while M2 receptors couple only with pertussis toxin sensitive G proteins (*46*). DeLapp et al. also demonstrated that M1 receptors were able to couple to inhibitory G proteins while M2 receptors coupled to Gq/11 only weakly (*47*).

To compare the ability of Gα subunits to be activated by receptors, muscarinic receptor-catalyzed GDP/GTP exchange was measured as agonist-stimulated GTPγS binding. Figure 5 shows the fold differences in slopes between agonist-driven and basal nucleotide exchange on various Gα subunits. The inset graph shows moles of GTPγS bound in membranes expressing M2 muscarinic receptors with or without reconstitution with Gi1 in the presence or absence of agonist. All chimeras were analyzed in the same fashion and the rates of GTPγS binding were determined from the slopes of the lines. Data for M2 receptor-catalyzed GTPγS binding demonstrated that q6N exchanged guanine nucleotides at a rate comparable to Gi1, while the rest of the Gα subunits were inactive (Figure 5). With the M1 receptor, both Gq and q6N35C exchanged guanine nucleotides upon agonist stimulation,

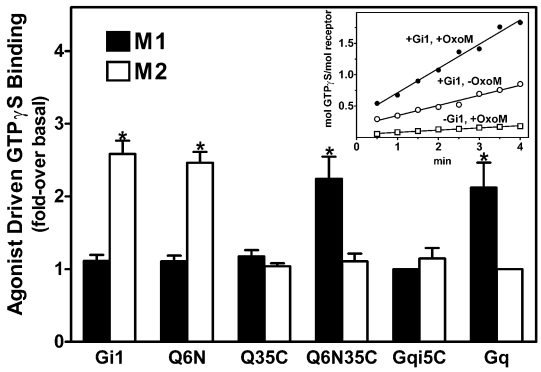

FIGURE 5: Muscarinic receptor-catalyzed GDP/GTP exchange on Gα subunits. GDP/GTP exchange was measured as GTPγS binding in membranes expressing the indicated muscarinic receptor reconstituted with G protein heterotrimers containing the indicated Gα subunits. Bars represent the fold enhancement by agonist above basal nucleotide exchange in the absence of agonist on Gα subunits and are the mean ± SEM from 3−8 experiments. Final conditions in the binding assay were 50 nM Gα, 0.8 nM receptor, 2 μM GDP, 100 nM GTPγS (~125 000 cpm/well), with or without 2 μM oxotremorine-M in a final volume of 50 μL. The asterisks above the bars indicate values significantly different than 1.0 ($p < 0.05$, one-sample t-test), and the absence of error bars indicates that there was no significant difference between basal and agonist driven rates in any experiment. The inset graph depicts moles of GTPγS bound per mole of receptor in membranes expressing M2 muscarinic receptors with or without reconstitution with Gi1 in the presence and absence of 2 μM oxo-M as indicated. The lines are least squares regression lines and the slopes represent the rate of GTPγS binding. Rates of GTPγS binding to all Gα subunits were determined as shown in the inset graph and the mean basal rates of GTPγS binding (mol GTPγS/mol receptor/min) were as follows: Gi1, 0.23; Q6N, 0.25; Q35C, 0.10; Q6N35C, 0.12; Gqi5C, 0.04; Gq, 0.08. The basal rate for Gi1 was significantly greater than all other subunits except Q6N ($p < 0.05$, Tukey's multiple comparison test).

while the rest of the Gα subunits were inactive (Figure 5). These data agree well with the affinity shift data (Figure 2) and support our main conclusion that both the N- and C-termini of Gqα are required for effective M1 receptor coupling.

Although the affinity shift data indicate that the M1 receptor couples weakly with Gi1 (Figure 2) and that this coupling is abolished by PTX treatment (Figure 4), we did not see agonist stimulated M1 receptor-mediated exchange on Gi1. Thus, while the high-affinity agonist binding state of G protein-coupled receptors is thought to be a ternary complex containing nucleotide free G protein and GDP release is accepted as the rate-limiting step in nucleotide exchange (*1*), our data indicate that it is possible to stabilize the high-affinity state of the receptor without stimulating guanine nucleotide exchange. Such discrepancies have been noted in a previous study (*38*) and are likely to be related to the fact that the affinity shift assay measures an equilibrium condition among agonist, receptor, and G protein in the absence of guanine nucleotides after 1 hour or more of incubation, while the agonist driven GTPγS binding assay measures the kinetics of GTPγS binding in the first few minutes after mixing these components.

The N-terminus of Gα subunits, together with amino acids from switch I and switch II, are binding sites for Gβγ subunits in the G protein heterotrimer (*48*). Mutations at the N-terminus of Gα may affect its interactions with βγ, which acts as a guanine nucleotide dissociation inhibitor for Gα. Kostenis et al. have suggested that the six N-terminal amino acids of Gqα (MTLESI) form a tightly folded protein subdomain that prevents Gi/o- and Gs-coupled receptors from accessing Gqα, thereby regulating coupling selectivity (*49*). Our results demonstrate that the addition of the six N-terminal amino acids of Gqα to the N-terminus of Gi1α does not restrict M2 receptor coupling. Therefore, we can say that the q6N chimera interacted with βγ properly. Our data suggest that N- and C-termini of Gqα cooperate to couple M1 receptors. While the crystal structure of Gq is not available, and the crystal structure of the Gi1 heterotrimer (with GDP bound) lacks four amino acids from the N-terminus and six amino acids are from the C-terminus (*48*), it appears that the Gα C-terminus in the heterotrimer is accessible and not buried within a compact microdomain. The N-terminus and αN helix are turned away from the body of Gα to interact with βγ. The structure of the Gi1 heterotrimer indicates that Val34 and Arg32 are the N-terminal amino acids of Gi1α that come close to Leu348, the most C-terminal Gα residue in the structure. However, because the structural orientation of the extreme N- and C-termini in Gq is not known and also because the crystal structure of a nucleotide-free G protein heterotrimer is not available, the structural basis for cooperativity between the extreme N- and C-termini of Gqα is not yet understood. One possibility is that the N- and C-termini of Gα might interact to form a single receptor binding site. Alternatively, there might be two separate sites on the receptor for the N- and C-termini which have to be recognized for the receptor to activate G proteins. Muradov and Artemyev suggested that coupling between N- and C-terminal domains of Gtα is responsible for low basal GDP/GTP exchange in Gtα (*50*). Since Gqα is also known to have low basal GDP/GTP exchange, this concept may in principle apply to Gqα.

Taken together, our data indicate that the context of the C-terminus of Gα, although critical, is not sufficient for M1 or M2 muscarinic receptor coupling. In addition, the unique six amino acid N-terminal extension of Gqα is neither sufficient to allow M1 muscarinic receptor coupling, nor sufficient to prevent M2 muscarinic receptor coupling. Rather, the appropriate context of both N- and C-termini of Gα is required for functional coupling to M1, and possibly, M2, muscarinic receptors. Q6N35C represents an easily purified, relatively stable, and soluble Gα subunit with the ability to functionally couple with M1 muscarinic receptors. As a reagent, it therefore offers significant advantages to native Gqα, which is difficult to purify, requires detergents for solubility, and is easily inactivated by denaturation.

ACKNOWLEDGMENT

We thank Lori Flood and Karyn Depree for excellent technical assistance and Chris Van Dyke for help in preparing the figures. We also thank Bruce Conklin for the qi5C cDNA, Heidi Hamm for the q35C protein, and Elliott Ross for the M1 and M2 muscarinic receptor baculoviruses.

REFERENCES

1. Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615−649.
2. Wess, J. (1998) *Pharmacol. Ther. 80*, 231−264.
3. Conklin, B. R., and Bourne, H. R. (1993) *Cell 73*, 631−641.
4. Phillips, W. J., and Cerione, R. A. (1992) *J. Biol. Chem. 267*, 17032−17039.

5. Heithier, H., Frohlich, M., Dees, C., Baumann, M., Haring, M., Gierschik, P., Schiltz, E., Vaz, W. L., Hekman, M., and Helmreich, E. J. (1992) *Eur. J. Biochem. 204*, 1169−1181.
6. Taylor, J. M., Jacob-Mosier, G. G., Lawton, R. G., VanDort, M., and Neubig, R. R. (1996) *J. Biol. Chem. 271*, 3336−3339.
7. Kisselev, O. G., Ermolaeva, M. V., and Gautam, N. (1994) *J. Biol. Chem. 269*, 21399−21402.
8. Azpiazu, I., Cruzblanca, H., Li, P., Linder, M., Zhuo, M., and Gautam, N. (1999) *J. Biol. Chem. 274*, 35305−35308.
9. Ernst, O. P., Meyer, C. K., Marin, E. P., Henklein, P., Fu, W. Y., Sakmar, T. P., and Hofmann, K. P. (2000) *J. Biol. Chem. 275*, 1937−1943.
10. Kisselev, O., Ermolaeva, M., and Gautam, N. (1995) *J. Biol. Chem. 270*, 25356−25358.
11. Yasuda, H., Lindorfer, M. A., Woodfork, K. A., Fletcher, J. E., and Garrison, J. C. (1996) *J. Biol. Chem. 271*, 18588−18595.
12. Richardson, M., and Robishaw, J. D. (1999) *J. Biol. Chem. 274*, 13525−13533.
13. Hou, Y., Azpiazu, I., Smrcka, A., and Gautam, N. (2000) *J. Biol. Chem. 275*, 38961−38964.
14. Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Koenig, B., and Hofmann, K. P. (1988) *Science 241*, 832−835.
15. Conklin, B. R., Farfel, Z., Lustig, K. D., Julius, D., and Bourne, H. R. (1993) *Nature 363*, 274−276.
16. Gilchrist, A., Mazzoni, M. R., Dineen, B., Dice, A., Linden, J., Proctor, W. R., Lupica, C. R., Dunwiddie, T. V., and Hamm, H. E. (1998) *J. Biol. Chem. 273*, 14912−14919.
17. Natochin, M., Muradov, K. G., McEntaffer, R. L., and Artemyev, N. O. (2000) *J. Biol. Chem. 275*, 2669−2675.
18. Kostenis, E., Degtyarev, M. Y., Conklin, B. R., and Wess, J. (1997) *J. Biol. Chem. 272*, 19107−19110.
19. Swift, S., Sheridan, P. J., Covic, L., and Kuliopulos, A. (2000) *J. Biol. Chem. 275*, 2627−2635.
20. Bae, H., Anderson, K., Flood, L. A., Skiba, N. P., Hamm, H. E., and Graber, S. G. (1997) *J. Biol. Chem. 272*, 32071−32077.
21. Hepler, J. R., Biddlecome, G. H., Kleuss, C., Camp, L. A., Hofmann, S. L., Ross, E. M., and Gilman, A. G. (1996) *J. Biol. Chem. 271*, 496−504.
22. Lee, C. H., Katz, A., and Simon, M. I. (1995) *Mol. Pharmacol. 47*, 218−223.
23. Grishina, G., and Berlot, C. H. (2000) *Mol. Pharmacol. 57*, 1081−1092.
24. Dong, G. Z., Kameyama, K., Rinken, A., and Haga, T. (1995) *J. Pharmacol. Exp. Ther. 274*, 378−384.
25. Weill, C., Autelitano, F., Guenet, C., Heitz, F., Goeldner, M., and Ilien, B. (1997) *Eur. J. Pharmacol. 333*, 269−278.
26. Wess, J. (1996) *Crit. Rev. Neurobiol. 10*, 69−99.
27. Hosey, M. M., Pals-Rylaarsdam, R., Lee, K. B., Roseberry, A. G., Benovic, J. L., Gurevich, V. V., and Bunemann, M. (1999) *Life Sci. 64*, 363−368.
28. Gutkind, J. S. (1998) *J. Biol. Chem. 273*, 1839−1842.
29. West, R. E., Jr., Moss, J., Vaughan, M., Liu, T., and Liu, T. Y. (1985) *J. Biol. Chem. 260*, 14428−14430.
30. Liu, J., Conklin, B. R., Blin, N., Yun, J., and Wess, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11642−11646.
31. Kostenis, E., Conklin, B. R., and Wess, J. (1997) *Biochemistry 36*, 1487−1495.
32. Kostenis, E., Gomeza, J., Lerche, C., and Wess, J. (1997) *J. Biol. Chem. 272*, 23675−23681.
33. Clawges, H. M., Depree, K. M., Parker, E. M., and Graber, S. G. (1997) *Biochemistry 36*, 12930−12938.
34. Lee, E., Linder, M. E., and Gilman, A. G. (1994) *Methods Enzymol. 237*, 146−164.
35. Graber, S. G., Figler, R. A., and Garrison, J. C. (1992) *J. Biol. Chem. 267*, 1271−1278.
36. Graber, S. G., Figler, R. A., and Garrison, J. C. (1994) *Methods Enzymol. 237*, 212−226.
37. Kozasa, T., and Gilman, A. G. (1995) *J. Biol. Chem. 270*, 1734−1741.
38. Bae, H., Cabrera-Vera, T. M., Depree, K. M., Graber, S. G., and Hamm, H. E. (1999) *J. Biol. Chem. 274*, 14963−14971.
39. Skiba, N. P., Bae, H., and Hamm, H. E. (1996) *J. Biol. Chem. 271*, 413−424.
40. Hartman, J. I., and Northup, J. K. (1996) *J. Biol. Chem. 271*, 22591−22597.
41. Berrie, C. P., Birdsall, N. J., Hulme, E. C., Keen, M., and Stockton, J. M. (1984) *Br. J. Pharmacol. 82*, 853−861.
42. Florio, V. A., and Sternweis, P. C. (1989) *J. Biol. Chem. 264*, 3909−3915.
43. Figler, R. A., Graber, S. G., Lindorfer, M. A., Yasuda, H., Linden, J., and Garrison, J. C. (1996) *Mol. Pharmacol. 50*, 1587−1595.
44. Asano, T., Ui, M., and Ogasawara, N. (1985) *J. Biol. Chem. 260*, 12653−12658.
45. Pobiner, B. F., Northup, J. K., Bauer, P. H., Fraser, E. D., and Garrison, J. C. (1991) *Mol. Pharmacol. 40*, 156−167.
46. Burford, N. T., Tobin, A. B., and Nahorski, S. R. (1995) *J. Pharmacol. Exp. Ther. 274*, 134−142.
47. DeLapp, N. W., McKinzie, J. H., Sawyer, B. D., Vandergriff, A., Falcone, J., McClure, D., and Felder, C. C. (1999) *J. Pharmacol. Exp. Ther. 289*, 946−955.
48. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell 83*, 1047−1058.
49. Kostenis, E., Zeng, F. Y., and Wess, J. (1998) *J. Biol. Chem. 273*, 17886−17892.
50. Muradov, K. G., and Artemyev, N. O. (2000) *Biochemistry 39*, 3937−3942.

BI034133J