

A Highly Conserved Glycine within Linker I and the Extreme C Terminus of G Protein α Subunits Interact Cooperatively in Switching G Protein-Coupled Receptor-to-Effector Specificity

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ABSTRACT

Numerous studies have attested to the importance of the extreme C terminus of G protein α subunits in determining their selectivity of receptor recognition. We have previously reported that a highly conserved glycine residue within linker I is important for constraining the fidelity of receptor recognition by G_{α_q} proteins. Herein, we explored whether both modules (linker I and extreme C terminus) interact cooperatively in switching G protein-coupled receptor (GPCR)-to-effector specificity and created as models mutant G_{α_q} proteins in which glycine was replaced with various amino acids and the C-terminal five G_{α_q} residues with the corresponding G_{α_i} or G_{α_s} sequence. Coupling properties of the mutated G_{α_q} proteins were determined after coexpression with a panel of 13 G_i - and G_s -selective receptors and compared with those of G_{α} proteins modified in

only one module. G_{α} proteins modified in both modules are significantly more efficacious in channeling non- G_q -selective receptors to G_q -mediated signaling events compared with those containing each module alone. Additive effects of both modules were observed even if individual modules lacked an effect on GPCR-to-effector specificity. Dually modified G_{α} proteins were also superior in conferring high-affinity agonist sites onto a coexpressed GPCR in the absence, but not in the presence, of guanine nucleotides. Together, our data suggest that receptor-G protein coupling selectivity involves cooperative interactions between the extreme C terminus and linker I of G_{α} proteins and that distinct determinants of selectivity exist for individual receptors.

Accurate signal transduction demands that seven transmembrane G protein-coupled receptors (GPCRs) selectively regulate a few of the many available and closely related G proteins expressed within a given cell. Conversely, G proteins must distinguish between selected groups of GPCRs, and each member of the four different subfamilies of G_{α} proteins ($G_{\alpha_{10}}$, G_{α_s} , G_{α_q} , and $G_{\alpha_{12/13}}$) is capable of interacting with multiple GPCRs. Understanding the structural requirements that determine the exquisite selectivity of receptor-G protein recognition will clarify how specificity is

maintained in living cells. Importantly, it may also suggest ways to manipulate signal transduction, pharmacologically or genetically, or simply enable development of assays that allow receptor activation to be linked to a signal transduction event of choice.

Progress has been made in defining the interface between GPCRs and G proteins, mainly aided by the availability of G protein crystal structures (for review, see Preininger and Hamm, 2004) as well as a wealth of information from site-directed mutagenesis studies (Conklin et al., 1993; Lee et al., 1995; Liu et al., 1995, 2002; Bae et al., 1997; Kostenis et al., 1997a; Blahos et al., 2001; Havlickova et al., 2003; for review, see Cabrera-Vera et al., 2003). On the receptor side, various

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ABBREVIATIONS: GPCR, G protein-coupled receptor; KOR, κ opioid receptor; GLP1, glucagon like peptide 1; GIP, gastric inhibitory peptide; NPY4, neuropeptide Y4; MCH-1R, melanin concentrating hormone-1 receptor; SSTR1, somatostatin type I receptor; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; BSA, bovine serum albumin; GTP γ S, guanosine 5'-O-(3-thio)triphosphate; PLC β , phospholipase C β ; AlF $_4^-$, fluoroaluminate; PTX, pertussis toxin; U50,488, (trans)-3,4-dichloro-N-methyl-N[1- β -pyrrolidinyl]-cyclohexyl]benzeneacetamide.

intracellular portions are involved in selective recognition of G proteins. These encompass the second (i2) and third (i3) intracellular loop as well as the receptor's C-terminal tail (Kobilka et al., 1988; Liu et al., 1995, 2004; Gomeza et al., 1996; Kostenis et al., 1997a; Orzel et al., 2000; Perroy et al., 2001; Kim et al., 2002). Within the rhodopsin family of GPCRs (also referred to as family A or class I receptors), the i3 loop plays a decisive role in selective recognition of G α proteins (Kobilka et al., 1988; Liu et al., 1995). Conversely, within family C or class III receptors, i3 loops are rather conserved, but i2 loops display remarkable sequence variability consistent with their decisive role in selective G α recognition (Gomeza et al., 1996; Havlickova et al., 2003). On the level of the G protein, both G α and the $\beta\gamma$ subunit complexes are likely to possess receptor contact sites (Conklin et al., 1993; Kisselev et al., 1995; Taylor et al., 1996). Within the G α subunit, several key regions governing GPCR-G protein coupling specificity have been identified so far; these include 1) the extreme C terminus (Conklin et al., 1993; Kostenis et al., 1997a; Liu et al., 2002; Havlickova et al., 2003), 2) the extreme N terminus (Kostenis et al., 1997b; Slessareva and Graber, 2003), 3) a region between $\alpha 4$ and $\alpha 5$ helices (Bae et al., 1997; Cai et al., 2001; Slessareva et al., 2003), and 4) a region within the loop linking the N-terminal α -helix to the $\beta 1$ strand of the Ras-like domain (Blahos et al., 2001) and the linker I region connecting the Ras-like with the helical domain (Heydorn et al., 2004). Whereas all of these domains have been evaluated independently for their specific interaction with GPCRs, relatively little is known about whether these regions interact in an additive or cooperative manner. We have previously reported that a glycine residue within linker I (glycine 66 in G α_q), which is highly conserved among G α subunits across all species, is a key residue for constraining the fidelity of G protein recognition by ligand-activated GPCRs (Heydorn et al., 2004). Molecular modeling studies suggested that glycine 66 is located in a key position within linker I and is the only amino acid that can be accommodated in this location without interfering with the proper arrangement of both helical and GTPase domains of G α . Perturbation of the structural arrangement of the helical and GTPase domains via mutagenesis of this key residue in linker I (glycine 66 within G α_q) increased the ease with which receptors otherwise selective for G α_i or G α_s gained the ability to activate G α_q . Previously, we have only assessed the coupling properties of G α_q in which glycine 66 was replaced by various other amino acids upon coexpression with G α_i , G α_s , and G α_q -selective receptors. The present study sought to clarify the contributions of glycine 66 in linker I and the sequence at the extreme C terminus of G α , a well established receptor recognition site, individually and in combination, and to study potential cooperative effects between these modules in determining receptor recognition and coupling efficiency. Our data reveal that glycine 66 plays an exquisite role in GPCR-G protein specificity both alone and as a facilitator for G α_q proteins modified at their extreme C termini to permit coupling to GPCRs otherwise linked to non G α_q -mediated signaling events.

Materials and Methods

DNA Construction

Construction of G α_q and G α_{qG66X} (X = D, N, V, K) in the pcDNA1.1 expression vector was reported previously (Heydorn et al., 2004). The C-terminally modified G α_q subunits G $\alpha_{q15/s5}$ were created by introducing, in a two-piece ligation, a polymerase chain reaction-based

175-base pair BglII-NsiI fragment containing the desired codon changes. To create G $\alpha_{qG66X15/s5}$, a 175-base pair BglII-NsiI fragment of G α_{qG66X} was replaced, in a two-piece ligation, with the corresponding BglII-NsiI fragment of G $\alpha_{q15/s5}$. All wild-type and mutant G α subunits contained an internal hemagglutinin epitope tag DVPDYA (Wedegaertner et al., 1993), replacing G α_{wt} residues 125 to 130 to monitor protein expression. Construction of expression plasmids encoding for the chemokine CCR5 and CXCR2 receptor, κ opioid receptor (KOR), adrenergic $\beta 2$ receptor, glucagon like peptide 1 (GLP1) receptor, prostaglandin D2 DP receptor, and the gastric inhibitory peptide (GIP) receptor were described previously (Heydorn et al., 2004). The Chemerin receptor ChemR23 was cloned from a bone marrow cDNA library and inserted into the pcDNA3.1(+) expression vector using BamHI and EcoRI. GPR7 was cloned from brain cDNA and inserted via EcoRI/XhoI into pcDNA3.1(+). The high-affinity nicotinic acid receptor HM74a was cloned from adipose tissue and inserted into pcDNA3.1(+) via HindIII/EcoRI sites. The human neuropeptide Y4 (NPY4), MCH-1, and somatostatin type I receptor (SSTR1) were cloned from brain cDNA and inserted via HindIII/EcoRI into the pcDNA3.1(+) expression vector.

Cell Culture and Transfections

COS-7 cells were maintained in Dulbecco's modified Eagle's medium 1885 supplemented with 10% fetal bovine serum and 10 μ g/ml gentamicin and kept at 37°C in a 10% CO₂-humidified atmosphere. The cells were plated at a density of 6 \times 10⁶ cells/175-cm² flask and were transfected using a calcium phosphate-DNA coprecipitation method, where 40 μ g of cDNA (equal amounts of receptor and G protein) was diluted in 480 μ l of CaCl₂ solution (10 mM Tris base, 1 mM EDTA, pH 8, and 250 mM CaCl₂) and dropwise added to 480 μ l of HBS [280 mM NaCl, 50 mM HEPES, and 1.5 mM sodium phosphate]. The precipitation was incubated for 45 min at room temperature and was added to the cells in 10 ml of fresh culture media supplemented with 0.1 mM chloroquine for 5 h at 37°C in a 10% CO₂-humidified atmosphere. One day after transfection, the cells were transferred to poly-D-lysine-treated 12-well culture plates at a density of 100,000 cells/well in growth media supplemented with 5 μ Ci of [³H]myo-inositol. HEK293 cells were maintained in minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM Glutamax-I, 1% nonessential amino acids, 1% sodium pyruvate, and 10 μ g/ml gentamicin. HEK293 cells were transfected with the FuGENE transfection reagent as per manufacturer's instructions. For all receptor G α protein coexpression experiments, a ratio of 1:1 was used for the respective cDNAs.

Inositol Phosphate Turnover Assays

Forty-eight hours after transfection, the cells were washed twice in HBSS buffer (including CaCl₂ and MgCl₂; catalog no. 14025-050; Invitrogen, Carlsbad, CA) and stimulated with the respective agonists in HBSS buffer supplemented with 5 mM LiCl for 45 min at 37°C. The reactions were terminated by the aspiration and addition of 10 mM ice-cold formic acid and incubated for 30 min on ice. The lysate was applied to AG 1-X8 anion exchange resin (Bio-Rad, Hercules, CA) and washed twice with buffer containing 60 mM sodium formate and 5 mM borax. The [³H]inositol-phosphate fraction was then eluted by adding 1 M ammonium formate and 100 mM formic acid solution and counted after addition of HiSafe3 scintillation fluid (PerkinElmer Life and Analytical Sciences, Boston, MA).

Calcium Mobilization Assay

Agonist-mediated intracellular Ca²⁺ release was measured in HEK293 cells coexpressing the human KOR and the respective set of G protein α subunits as indicated in Fig. 6. One day after transfection, cells were loaded for 60 min with the FLEX calcium assay kit (Molecular Devices, Sunnyvale, CA) and subsequently challenged with increasing concentrations of agonist ligand. Intracellular calcium mobilization was recorded in real time during 120 s in a FLEX

station (Molecular Devices): relative light units = maximum Ca^{2+} peak/cell number \times 1000.

ELISA

Determination of cell surface expression levels of the MCH-1 receptor was performed using an N-terminally HA-tagged MCH-1 receptor in an ELISA assay. Approximately 48 h after transfection, cells were fixed in 48-well tissue culture plates with 3.7% paraformaldehyde, washed three times with washing buffer (0.14 M NaCl, 25 mM Tris base, 2.7 mM KCl, and 0.1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 7.4), and then blocked with blocking buffer (3% dry milk, 1 mM CaCl_2 , and 50 mM Tris-HCl, pH 7.5). The primary monoclonal HA-11 antibody (Babco, Richmond, CA) was applied into the same buffer at a 1:300 dilution for 1 h at room temperature followed by three washes and a 1-h incubation with the secondary antibody (1:2500, goat anti-mouse coupled to alkaline phosphatase; Bio-Rad) in blocking buffer. The secondary antibody was detected and quantified after adding a colorimetric alkaline phosphatase substrate 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO). When adequate color change was reached, the reaction was terminated by addition of 0.5 M H_2SO_4 . Samples were then transferred to a 96-well plate and colorimetric readings were obtained at an optical density of 450 on a Tecan Sunrise absorbance reader (Tecan, Maennedorf, Switzerland). All experiments were performed in quadruplicate determinations.

Receptor Binding Studies

Whole Cell Binding. Twenty-four hours after transfection, COS-7 cells were seeded into 96-well plates at a density of 30,000 cells/well. Competition binding experiments on whole cells were then performed about 18 to 24 h later using 0.1 nM ^{125}I -MCH (74 TBq/mmol, 2000 Ci/mmol; Amersham Biosciences Inc., Piscataway, NJ) in a binding buffer consisting of 25 mM HEPES, pH 7.4, 10 mM MgCl_2 , 5 mM MnCl_2 , 10 mM NaCl, 0.1% bovine serum albumin (BSA), and 100 $\mu\text{g}/\text{ml}$ bacitracin. Total and nonspecific binding were determined in the absence and presence of 10 μM MCH. Binding reactions were routinely conducted for 3 h at room temperature and terminated by two washes (100 μl each) with ice-cold binding buffer and addition of 25 μl of 1 N NaOH. Radioactivity was determined by liquid scintillation counting in a TopCounter (Amersham Biosciences Inc.) after overnight incubation in Microscint 20. Determinations were made in duplicates.

Membrane Binding. Binding assays were carried out in a total volume of 200 μl , containing cell membranes (10 μg of protein), ^{125}I -MCH (0.1 nM), and varying concentrations of MCH or $\text{GTP}\gamma\text{S}$. Competitor drug dilutions, radioligand, and cell membranes were all prepared in assay buffer [25 mM HEPES, pH 7.4, 10 mM MgCl_2 , 5 mM CaCl_2 , 0.1% (w/v) bacitracin, and 0.1% (w/v) BSA (protease free)]. Each data point was performed in duplicate, and reactions were incubated at room temperature for 2 h to allow equilibration. Membrane-bound radioligand was separated from free radioligand by vacuum filtration and washing with assay buffer over glass fiber filters [pretreated with 0.1% (w/v) polyethyleneimine in assay buffer; grade GF/C, Brandel Inc., Gaithersburg, MD] using a Brandel M-24 harvester. Bound ^{125}I -MCH was measured using a Cobra II gamma-counter (Amersham Biosciences Inc.).

Membrane Preparation and Western Blotting

Membranes (standard P2 membrane preparations) from transiently transfected COS-7 cells were prepared essentially as described previously (Heydorn et al., 2004). Membrane proteins were quantified with the Bio-Rad protein assay kit using BSA as a standard. Samples (20–40 μg of membrane protein) were resolved by SDS-polyacrylamide gel electrophoresis (13%), transferred to nitrocellulose membranes, and probed with the 12CA5-peroxidase linked monoclonal antibody (1:1000 dilution in 10 mM Tris, pH 8, 150 mM NaCl, 10% SDS, and 0.1% Tween 20). Immunoreactive proteins were

visualized with the enhanced chemiluminescence kit from Amersham Biosciences Inc.

Receptor Ligands

^{125}I -Melanin-concentrating hormone (2000 Ci/mmol) was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK); Chemerin 9 was synthesized by UFPeptides (Ferrara, Italy); somatostatin 28 was obtained from Bachem (Bubendorf, Switzerland); neuropeptide W23 was obtained from Phoenix Pharmaceuticals, Inc. (Belmont, CA), regulated on activation normal T cell expressed and secreted (RANTES) and interleukin-8 were obtained from PreproTech (London, UK), prostaglandin D2 was from Cayman Chemical (Ann Arbor, MI), and human pancreatic polypeptide, GLP1, and GIP were obtained from Sigma-Aldrich (St. Louis, MO) as were all other chemicals that were of the highest grade possible.

Calculations

IC_{50} and EC_{50} values were determined by nonlinear regression using Prism 3.0 software (GraphPad Software Inc., San Diego, CA). Values of the dissociation and inhibition constants (K_d and K_i) were estimated from competition binding experiments using the equations $K_d = \text{IC}_{50} - L$ and $K_i = \text{IC}_{50}/(1 + L/K_d)$, where L is the concentration of radioactive ligand and K_d is its dissociation constant.

Results

Construction, Expression, and Functionality of Mutant $\text{G}\alpha_q$ Proteins. Replacement of the five C-terminal amino acids of $\text{G}\alpha_q$ with the corresponding $\text{G}\alpha_i$ sequence (the resulting chimera is referred to as $\text{G}\alpha_{qi5}$) has been shown to confer onto G_i -linked GPCRs the ability to stimulate the G_q pathway in assays measuring intracellular inositol phosphate generation or intracellular calcium mobilization (Conklin et al., 1993; Kostenis et al., 1997a; Milligan and Rees, 1999). Similarly, G_s -coupled receptors can be forced to stimulate $\text{PLC}\beta$ upon coexpression with $\text{G}\alpha_{qs5}$ (the C-terminal five amino acids of $\text{G}\alpha_q$ are replaced with the corresponding $\text{G}\alpha_s$ sequence) (Conklin et al., 1996). We have previously shown that mutation of a highly conserved glycine residue in linker I of the $\text{G}\alpha_q$ subunit to various other amino acids confers onto $\text{G}\alpha_q$ the ability to channel G_i - and G_s -selective receptors to the $\text{PLC}\beta$ pathway. Here, we created G protein α_q subunits that combine mutational replacement of the highly conserved glycine residue in linker I and harbor five amino acids of either $\text{G}\alpha_i$ or $\text{G}\alpha_s$ sequence at their extreme C termini. The mutants are referred to as $\text{G}\alpha_{qG66Xs5}$ and $\text{G}\alpha_{qG66Xi5}$ ($X = \text{D, N, V, K}$) and are schematically depicted in Fig. 1. The sequences of various wild-type $\text{G}\alpha$ subunits as well as chimeric $\text{G}\alpha_{qi5}$ and $\text{G}\alpha_{qs5}$ are included in the alignment for comparison. Initially, expression levels of the mutant $\text{G}\alpha_q$ subunits and functionality upon coexpression with two G_i - and two G_s -selective receptors were determined in transiently transfected COS-7 cells (Fig. 2). All $\text{G}\alpha_{qG66Xs5}$ mutants were coexpressed with the G_s -selective GLP1 and the adrenergic β_2 receptor, and the corresponding $\text{G}\alpha_{qG66Xi5}$ proteins with the G_i -selective NPY4 and the chemokine-like ChemR23 receptor, and ligand-stimulated inositol phosphate production was determined as a measure of coupling efficiency. Although replacing the five C-terminal amino acids of $\text{G}\alpha_q$ with $\text{G}\alpha_i$ or $\text{G}\alpha_s$ sequence, respectively, suffices to alter coupling specificity of the adrenergic β_2 , NPY4, and ChemR23 receptors, $\text{G}\alpha_{qs5}$ did not allow the GLP1 receptor to produce stimulation of the $\text{PLC}\beta$ pathway. Remarkably, however, all four GPCRs gained the ability to productively stim-

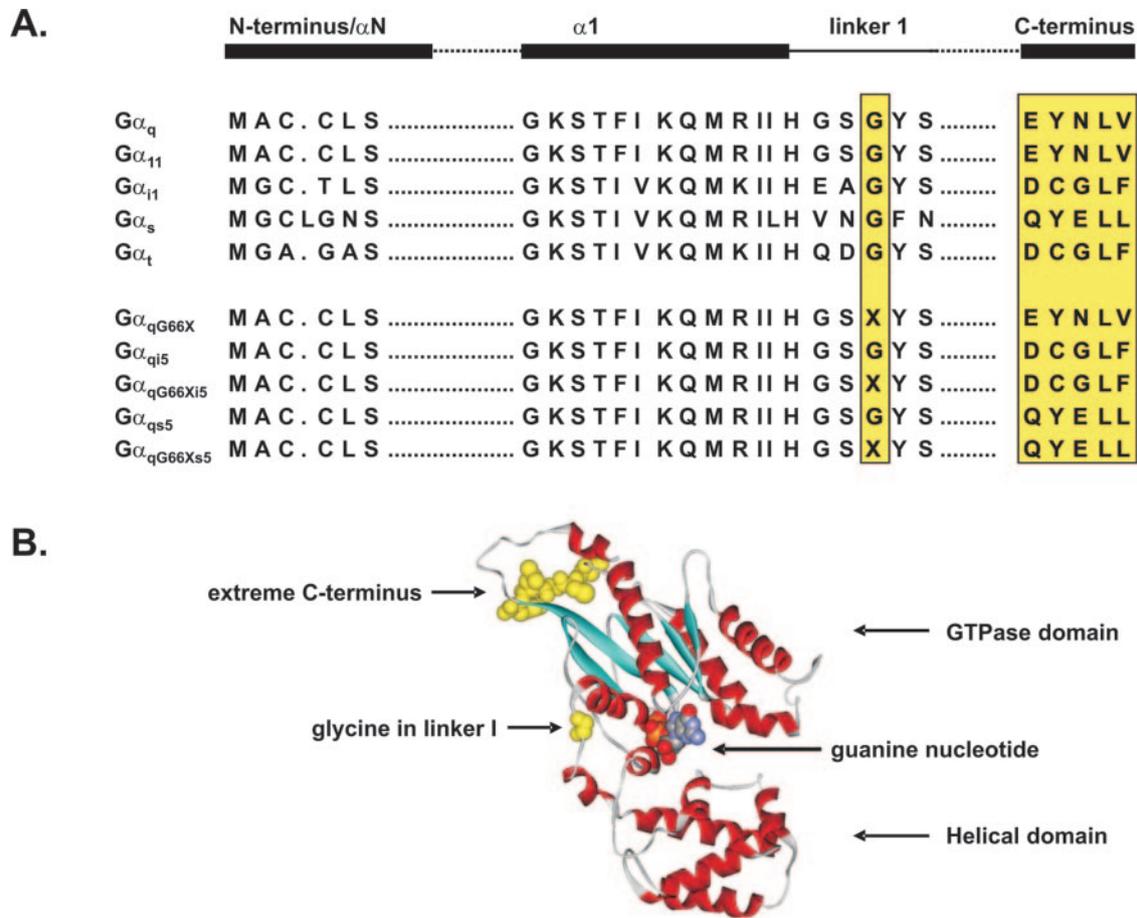


Fig. 1. A, alignment of the N- and C-terminal amino acid sequences of selected mutant and wild-type G protein α subunits. Gaps were introduced for optimum sequence alignment. G α_{qi5} and G α_{qs5} denote mutant G α_q constructs in which the C-terminal five amino acids are replaced with the corresponding G α_i (G α_{qi5}) or G α_s (G α_{qs5}) sequence, respectively. G α_{qG66X} denotes mutant α_q subunits in which the highly conserved glycine in linker I (boxed in yellow) is replaced with amino acids of different physicochemical properties (X = D, N, V, K). These single mutants have previously been shown to impart promiscuity onto G α_q in that they allow G $_i$ - and G $_s$ -linked receptors to activate the PLC β pathway (Heydorn et al., 2004). G $\alpha_{qG66Xi5/s5}$ are mutant G α subunits combining a C-terminal sequence switch and mutational replacement of glycine in the linker I region. The single-letter amino acid code is used. B, mapping of the highly conserved glycine residue and the extreme C terminus onto the crystal structure of a G α subunit. Shown is the crystal structure of the G α_i subunit in its GDP-Mg $^{2+}$ -bound form because it is assumed that this corresponds to the conformation of the G protein that interacts with the receptor (Coleman and Sprang, 1998). The guanine nucleotide GDP is buried between the helical and the GTPase-domain of the G α subunit. Both, the highly conserved glycine residue in the center of the linker I region and the extreme C terminus are highlighted in yellow. The structure was prepared in WebLab ViewerPro and the PDB identifier for the G α protein is 1BOF.

ulate inositol phosphate hydrolysis when coexpressed with the dually modified G α_q proteins (Fig. 2). Furthermore, measurement of PLC β activation showed that the gain in coupling efficiency of the dually modified G α proteins was independent of the nature of amino acid chosen to replace the highly conserved glycine residue in linker I. In parallel with the functional experiments, G α subunit expression was monitored by immunodetection using membrane preparations of the respective transient receptor-G protein cotransfections (Fig. 2). G α subunits were visualized with the monoclonal 12CA5-peroxidase linked antibody that recognizes an internal HA epitope tag (DVPDYA) that had been engineered into each G protein. SDS-polyacrylamide gel-electrophoresis and Western blot analysis indicated equal expression of wild-type and mutant G α_q proteins in the presence of cotransfected GPCRs. Thus, the possibility that the superior coupling efficiency of the GPCRs in the presence of the dually modified G α proteins was due to alterations in G α expression levels can be eliminated. Additional control experiments were performed to test whether the panel of dually modified G α pro-

teins was equally capable of adopting the active conformation as wild-type G α_q or the C-terminally modified G $\alpha_{qi5/s5}$ proteins. Toward this end, COS-7 cells transiently transfected with the various G α proteins were challenged with fluoroaluminate (AlF $_4^-$) and inositol phosphate production was determined as a measure of G α activity (not shown). AlF $_4^-$ typically activates G protein α subunits by binding to the GDP-bound form of G α . The resulting G α -GDP-AlF $_4^-$ complex assumes an active state conformation, which resembles the G α -GTP complex (Coleman et al., 1994). Both fluoroaluminate-activated and receptor-activated G α subunits are capable of stimulating intracellular effector molecules. All G α proteins were found to be equally capable to stimulate inositol phosphate hydrolysis (2.5–3-fold above basal levels), indicating that the mutations do not interfere with the ability to adopt an active conformation.

Functional Characterization of G $\alpha_{qG66Di5/s5}$ in Inositol Phosphate Hydrolysis Assays. Because we were interested to examine the generality of the observed cooperative effects between the linker I region and the extreme C termi-

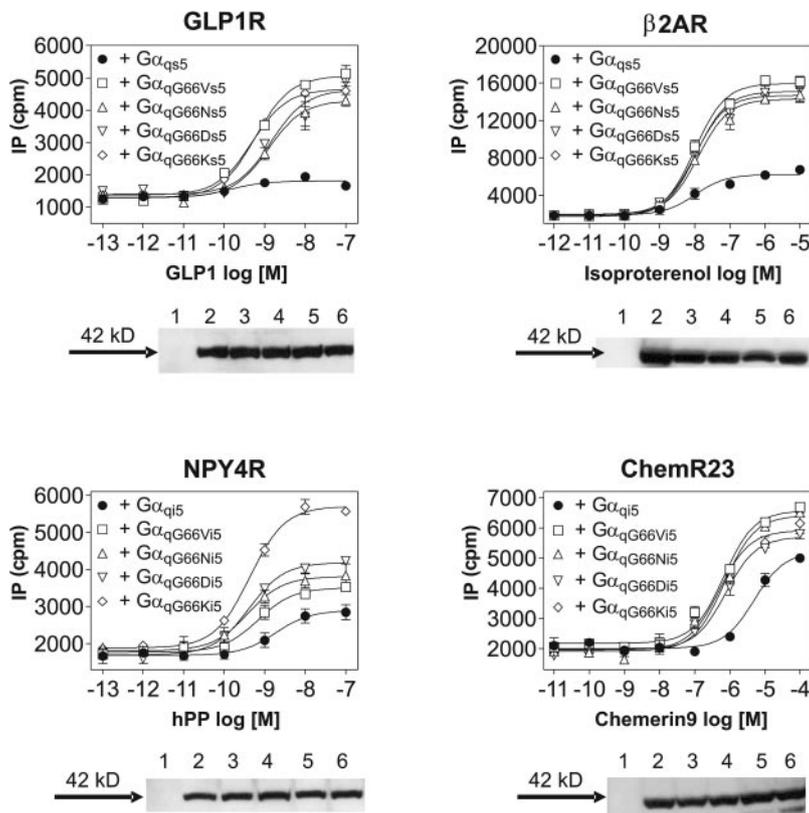


Fig. 2. Concentration-response curves for stimulation of PLC β by two G_i - and two G_s -selective receptors. COS-7 cells were cotransfected with expression plasmids coding for the G_s -selective GLP1 and adrenergic β 2 receptor or the G_i -selective NPY4 and ChemR23 receptor and the indicated set of G protein α subunits. About 48 h after transfection, cells were stimulated with increasing concentrations of the respective agonist ligands, and increases in intracellular inositol phosphates were determined as described under *Materials and Methods*. Data are expressed as means \pm S.D. of triplicate determinations of a single representative experiment; two additional experiments gave similar results. In parallel to each functional experiment, expression of the G α subunits was monitored by immunodetection. Forty-eight hours after transfection, membranes were prepared and analyzed (20–40 μ g each) by SDS-polyacrylamide gel electrophoresis (13%) and Western blotting using the 12CA5-peroxidase-linked monoclonal antibody. For the GLP1 and β 2 receptor-G protein cotransfections, lane 1 corresponds to mock-transfected cells; lane 2 to $G_{\alpha_{qs5}}$, lane 3 to $G_{\alpha_{qG66Vs5}}$, lane 4 to $G_{\alpha_{qG66Ns5}}$, lane 5 to $G_{\alpha_{qG66Ds5}}$, and lane 6 to $G_{\alpha_{qG66Ks5}}$. For the NPY4R and ChemR23 receptor cotransfections, lane 1 (mock-transfected cells), lane 2 ($G_{\alpha_{q15}}$), lane 3 ($G_{\alpha_{qG66Vs15}}$), lane 4 ($G_{\alpha_{qG66Ns15}}$), lane 5 ($G_{\alpha_{qG66Ds15}}$), and lane 6 ($G_{\alpha_{qG66Ks15}}$). Similar results were obtained in two additional experiments with different batches of membranes.

nus in altering GPCR-to-effector specificity, we then chose to compare coupling properties of a selected dually modified G_{α} protein ($G_{\alpha_{qG66Di5}}$ was chosen as a model) with G_{α} proteins containing the individual mutations ($G_{\alpha_{qG66D}}$ and $G_{\alpha_{q15}}$) upon coexpression of a broad range of G_i -linked receptors. To this end, the panel of G_{α} proteins was transiently coexpressed in COS-7 cells with each of the ChemR23, chemokine CCR5, and CXCR2, KOR, GPR7, MCH-1 receptor, HM74a, SSTR1, and neuropeptide Y NPY4 receptors, and agonist-induced mobilization of inositol phosphates was determined (Fig. 3). Of these nine G_i/o -coupled receptors, eight did not affect PLC even when wild-type G_{α_q} was overexpressed. In contrast, the MCH-1 receptor, which preferentially activates $G_{\alpha_{i/o}}$ proteins, is also capable of activating G_{α_q} (Saito et al., 1999) and therefore, gave rise to inositol phosphate production in the absence of any cotransfected G protein. Coexpression of the ChemR23, CCR5, CXCR2, and MCH-1 receptors with $G_{\alpha_{qG66D}}$ resulted in a significantly increased inositol phosphate response (compared with wild-type G_{α_q}). By contrast, no inositol phosphate generation was observed for the KOR, GPR7, HM74a, SSTR1, and NPY4 receptors. However, all these GPCRs produced significant increases in inositol phosphate levels after cotransfection with $G_{\alpha_{q15}}$. Remarkably, coexpression of all nine GPCRs with $G_{\alpha_{qG66Di5}}$ induced robust inositol phosphate increases that clearly exceeded those observed upon coexpression with the individual C-terminal or the G66D mutants. It should be noted that basal inositol phosphate levels in cells transfected with G_{α} protein alone were not significantly different from receptor-G protein-cotransfected cells (not shown). Inositol phosphate production was also determined in COS-7 or HEK293 cells cotransfected with the G_s -linked GLP1, GIP, prostaglandin D2 DP, and adrenergic β 2 receptor and vector DNA as a control

or the various mutant G_{α_q} and $G_{\alpha_{q/s}}$ chimeric constructs (Fig. 4). In agreement with the results obtained with G_i -selective receptors, combination of the G66D mutation with the C-terminal $G_{\alpha_{q\Delta}}$ G_{α_s} sequence switch gave rise to a mutant G_{α} superior to the individual domain modified G_{α} proteins with respect to stimulation of PLC β . Furthermore, it seems as if distinct determinants of selectivity exist for receptors from different coupling classes. Although G_i -selective receptors interacted more productively with C-terminally compared with linker-modified G_{α} proteins, G_s -selective receptors displayed the opposite phenotype and interacted more productively with the linker-modified G_{α} proteins. Collectively, the data provided evidence that both the linker I region and the extreme C terminus of G_{α} determine receptor-G protein coupling specificity and that both regions act in concert in a cooperative manner to achieve maximal PLC β stimulation by non- G_q -selective GPCRs.

Functional Characterization of $G_{\alpha_{qG66Di5}}$ in Calcium Mobilization Assays. We next tested whether the functional superiority of $G_{\alpha_{qG66Di5}}$ over the other mutant G_{α} proteins was also visible in calcium assays where more signal amplification exists and used the KOR as a model. Figure 5A shows concentration-response curves of HEK293 cells cotransfected with the KOR and the set of G_{α} proteins and challenged with the KOR-selective agonist U50,488. As was the case in inositol phosphate assays (Fig. 3), $G_{\alpha_{qG66Di5}}$ was clearly superior to $G_{\alpha_{q15}}$ in channelling the G_i -selective KOR to intracellular calcium mobilization, whereas no detectable calcium mobilization was visible in the presence of the other G_{α} proteins. Importantly, mock- or G_{α} subunit transfected cells did not respond to U50,488, whereas they responded equally to 10 nM ATP that stimulates an endogenously expressed purinoceptor (not shown). We were interested in

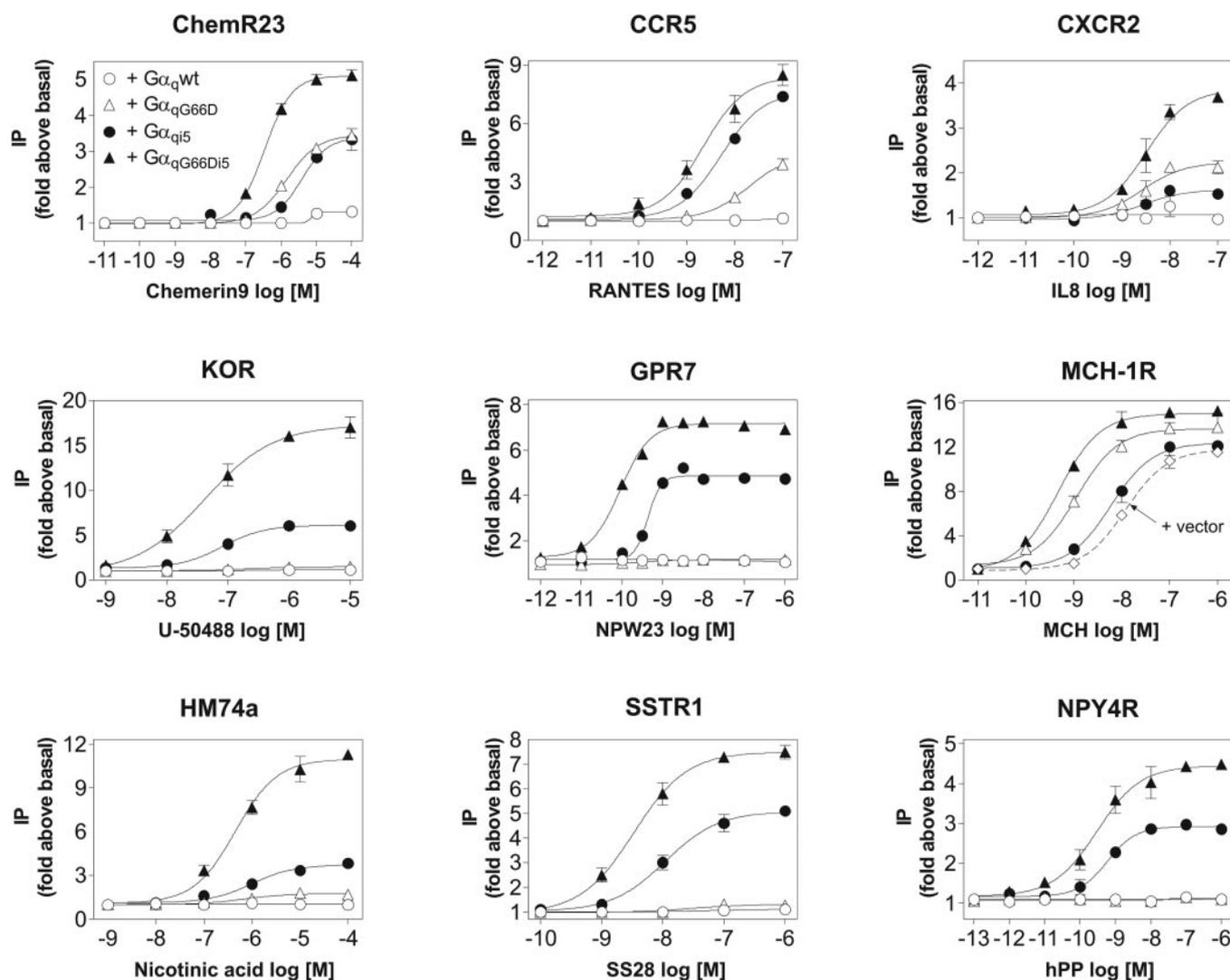


Fig. 3. Functional interaction of selected Gi-coupled receptors with wild-type $G\alpha_q$, $G\alpha_{qG66D}$, $G\alpha_{q15}$, and $G\alpha_{qG66Di5}$. COS-7 cells coexpressing pcDNA1.1 vector DNA or the indicated $G\alpha$ subunits and different G_i -linked receptors were incubated for 45 min (37°C) in the absence or presence of increasing concentrations of the appropriate agonist ligands. The resulting increases in intracellular inositol phosphate levels were determined as described under *Materials and Methods*. Data are means \pm S.E. of three to five experiments each performed in duplicate.

calcium mobilization assays for a second reason and tested the hypothesis that functional superiority of $G\alpha_{qG66Di5}$ may be due to the fact that it confers onto G_i -selective GPCRs the ability to maintain the active conformation for a longer period. The inability of a GPCR to convert from a high- to a low-affinity agonist state is detectable when monitoring real time calcium dynamics. Typically, GPCRs induce rapid increases of intracellular calcium that decrease rapidly. In contrast, GPCRs that are “locked” in the high-affinity conformation generate calcium peaks that increase quickly but decrease very slowly (Okuno et al., 2003). Real-time calcium peaks are depicted in Fig. 5, B and C, for the KOR coexpressed with either $G\alpha_{q15}$ (B) or $G\alpha_{qG66Di5}$ (C), respectively, and show that they only differ in magnitude but not in their kinetic profiles. These data suggest that the functional superiority of $G\alpha_{qG66Di5}$ over $G\alpha_{q15}$ is not due to interference with the ability of the GPCR to convert from the high- to the low-affinity conformation and thus its signal termination.

Comparison of Wild-Type and Mutant $G\alpha_q$ Proteins in Their Temporal Response to Receptor Stimulation. To investigate whether the series of $G\alpha_q$ proteins differ in

their mechanism of interaction with the ligand-activated GPCRs, we coexpressed the G_s -selective GLP1 receptor with wild-type $G\alpha_q$ or the different mutant $G\alpha_q$ proteins and measured inositol phosphate production in response to 100 nM GLP1 over time (Fig. 6). In agreement with the observed coupling profile of the GLP1 receptor (Fig. 4), no detectable inositol phosphate accumulation was measured at any time in the presence of coexpressed wild-type $G\alpha_q$. Conversely, linker as well as C-terminally modified subunits gave rise to significant increases in inositol phosphates over time. Temporal resolution of inositol phosphate production clearly showed that the various subunits differ only in the quantity of second messenger production but not in their quality of interaction with the ligand-stimulated GLP1 receptor.

Induction of High-Affinity Agonist Sites onto a GPCR upon Coexpression of Different $G\alpha$ Proteins. G proteins are known to affect the structure and the ligand binding properties of GPCRs. Likewise, agonists demonstrate high affinity for receptor states due to the promotion of G protein coupling. It is well accepted that high-affinity agonist binding depends on the formation of a ternary com-

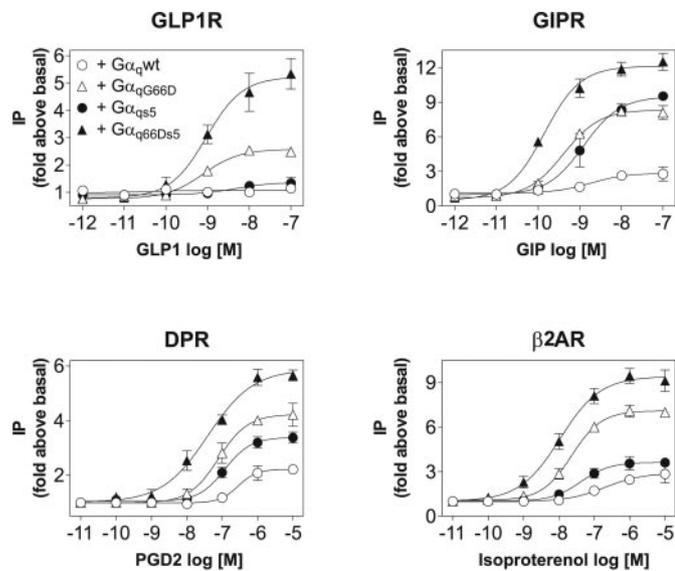


Fig. 4. Functional interaction of selected Gs-coupled receptors with wild-type $G\alpha_q$, $G\alpha_{qG66D}$, $G\alpha_{qS5}$, and $G\alpha_{qG66D\Delta 5}$. COS-7 cells coexpressing the GLP1, GIP, prostaglandin D2 DP, and adrenergic β_2 receptor and pcDNA1.1 vector DNA or the indicated $G\alpha$ subunits were incubated for 45 min (37°C) in the absence or presence of increasing concentrations of the appropriate agonist ligands. The resulting increases in intracellular inositol phosphate levels were determined as described under *Materials and Methods*. Data are means \pm S.D. of two to five independent experiments each performed in duplicate.

plex between agonist, receptor and G protein (Hepler and Gilman, 1992; Samama et al., 1993). We therefore hypothesized that the different $G\alpha$ proteins described in this study might be distinguished by their ability to confer high-affinity agonist sites onto a given GPCR and used the MCH-1 receptor as a model. Figure 7A depicts that high-affinity agonist binding of the MCH-1 receptor is indeed increased in the presence of various $G\alpha$ proteins in the following rank order: $G\alpha_{qG66D\Delta 5} > G\alpha_{qi5} = G\alpha_{qG66D} >$ wild-type $G\alpha_q$ (note that wild-type $G\alpha_q$ exerts a “dominant negative” effect on ^{125}I -MCH binding, most likely via competing with endogenous $G\alpha_i$, whereas cotransfection of the MCH-1 receptor with $G\alpha_i$ leads to a further increase in high-affinity agonist sites (Fig. 7A, inset). We would have preferred to perform these binding experiments after treatment with pertussis toxin (PTX) to prevent the receptor from interacting with the $G\alpha_i$ proteins endogenous to the membranes; however the five C-terminal residues of $G\alpha_i$ are sufficient to confer PTX sensitivity onto the chimeric $G\alpha_{qi}$ proteins and therefore precluded use of PTX in this experimental setup. To eliminate that the increase in MCH agonist binding sites reflected a nonspecific increase of the GPCR at the cell surface as opposed to a higher percentage of receptors residing in the high-affinity conformation, the total number of receptors was monitored with an ELISA using an N-terminally HA-tagged MCH-1 receptor. Results of the ELISA (Fig. 7B) clearly proved that, when coexpressed with the MCH-1 receptor, the various $G\alpha$ proteins selectively influence the number of receptors in the high-affinity state as opposed to exerting nonspecific effects on receptor expression. In agreement with functional data on inositol phosphate production (cf. Fig. 3), $G\alpha_{qG66D\Delta 5}$ was superior to both $G\alpha_{qi5}$ and $G\alpha_{qG66D}$ in conferring high-affinity sites onto the MCH-1 receptor. Collectively, the binding and intracellular calcium elevation data suggest that the differ-

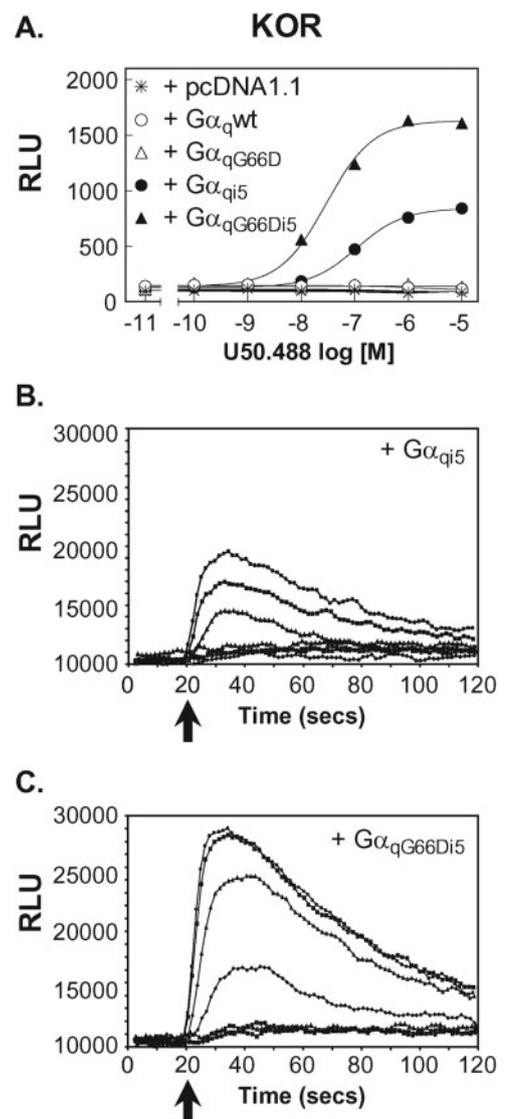


Fig. 5. Agonist-dependent mobilization of intracellular calcium in cells cotransfected with the κ opioid receptor and the indicated $G\alpha$ subunits. HEK293 cells were transiently cotransfected with the KOR and the indicated $G\alpha$ subunits. Agonist-mediated changes in intracellular calcium were measured by the FLEX-station as described under *Materials and Methods*. A, transformation of real-time calcium peaks into dose response curves. B, real-time calcium peaks for the KOR/ $G\alpha_{qi5}$ cotransfection. C, real-time calcium peaks for the KOR/ $G\alpha_{qG66D\Delta 5}$ cotransfection. The arrow in B and C indicates the addition of agonist. Shown is one of three representative experiments, each performed in triplicate determinations.

ent $G\alpha$ proteins can be distinguished by their ability to confer high-affinity signaling competent binding sites to a given receptor rather than interfere with its ability to convert from the high- to the low-affinity conformation and thus signal termination after ligand activation.

Effects of $GTP\gamma S$ on High-Affinity Agonist Binding. We next examined the effects of $GTP\gamma S$, a hydrolysis-resistant GTP analog, on ^{125}I -MCH binding to membrane preparations from COS-7 cells transiently cotransfected with the MCH-1 receptor and the two selected $G\alpha$ proteins $G\alpha_{qi5}$ and $G\alpha_{qG66D\Delta 5}$. Excess $GTP\gamma S$ is known to lower the affinity of receptor agonists through disassembly of ternary complexes, and this approach can be applied to estimate the affinity of a GPCR for a given G protein. Figure 7C shows that ^{125}I -MCH

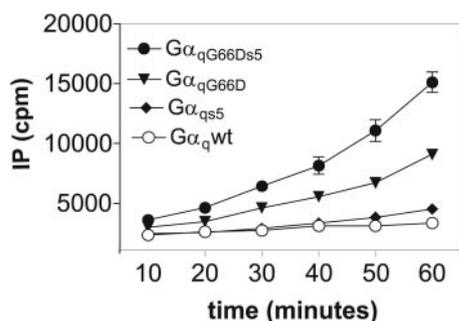


Fig. 6. Time-dependent generation of [^3H]inositol phosphates in COS-7 cells cotransfected with the GLP1 receptor and the indicated $G\alpha$ subunits. COS-7 cells coexpressing the GLP1 receptor and the indicated $G\alpha$ subunits were incubated for different times (37°C) in the absence or presence of 100 nM GLP1. The resulting increases in intracellular inositol phosphate levels were determined as described under *Materials and Methods*. Data are means \pm S.D. of three independent experiments each performed in duplicate.

binding to the MCH-1 receptor was decreased by increasing concentrations of GTP γ S in the $G\alpha_{q15}$ and $G\alpha_{qG66Di5}$ cotransfections. The fraction of ^{125}I -MCH displaced with high affinity by GTP γ S was substantially greater after cotransfection of the MCH-1 receptor with $G\alpha_{qG66Di5}$ ($85.8 \pm 2.3\%$, mean \pm S.E.M., $n = 3$) than with $G\alpha_{q15}$ ($44.6 \pm 3.6\%$). Surprisingly, though, we could not detect a substantial difference in potency of GTP γ S to reduce ^{125}I -MCH binding to the MCH-1 receptor cotransfected with either $G\alpha$ protein. Together, these results suggest that the two $G\alpha$ proteins differ in their ability to confer high-affinity binding sites to the MCH-1 receptor and may form ternary complexes with different stability that would reflect different affinities of the receptor for the individual $G\alpha$ proteins.

Discussion

Interactions between GPCRs and their cognate G proteins are known to involve several different domains on both the receptor and the G protein heterotrimer. Within $G\alpha$, the best characterized GPCR contact site is the extreme C terminus where residues at positions -3 and -4 are particularly important for specific receptor recognition (Conklin et al., 1993, 1996; Kostenis et al., 1997c; Bahia et al., 1998; Blahos et al., 1998; Liu et al., 2002). We have recently demonstrated the importance of the linker I region of $G\alpha_q$ proteins in constraining the fidelity of receptor recognition. A highly conserved glycine residue in linker I (glycine 66) regulates coupling selectivity indirectly by playing a role in the specificity of nucleotide exchange within $G\alpha_q$ induced by ligand-activated GPCRs (Heydorn et al., 2004). Here, we analyzed 1) the relationship between the linker I region and the extreme C terminus of $G\alpha$ in determining selective GPCR coupling and 2) whether different GPCRs use different $G\alpha$ domains to achieve specific coupling. To this end, we generated $G\alpha$ proteins carrying a glycine-to-aspartate mutation within linker I and five amino acids of either $G\alpha_i$ or $G\alpha_s$ sequence at their extreme C termini ($G\alpha_{qG66Di5}$ and $G\alpha_{qG66Ds5}$). Coupling properties of the mutant $G\alpha$ proteins were compared with $G\alpha$ proteins individually modified within linker I ($G\alpha_{qG66D}$) or the C terminus ($G\alpha_{q15}$ and $G\alpha_{qs5}$) upon coexpression with G_i - or G_s -selective receptors. Our coexpression studies confirmed the importance of the extreme C terminus of $G\alpha$ in selective GPCR recognition because 12 of 13 tested non- G_q -linked

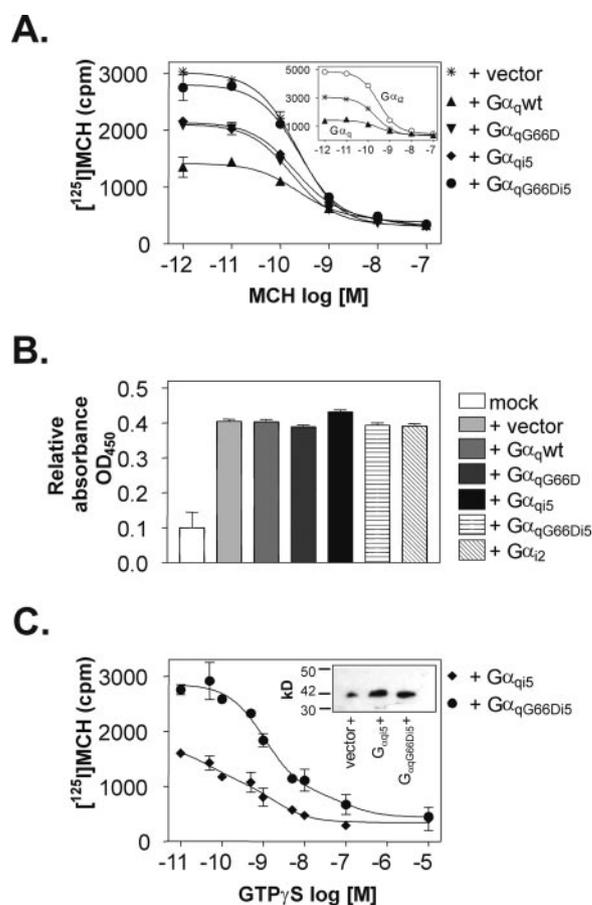


Fig. 7. A, induction of high-affinity agonist binding to MCH-1 receptors in whole cells by cotransfection of $G\alpha$ subunits. COS-7 cells were cotransfected with the MCH-1 receptor and pcDNA1.1 vector DNA or the indicated $G\alpha$ subunits. After 48 h, whole cell binding experiments were performed for 3 h at room temperature as described under *Materials and Methods*. Receptor-bound radioactivity was measured by liquid scintillation counting. Results are means \pm S.D. of one representative experiment; three additional experiments gave similar results. B, comparison of the surface expression level of the MCH-1 receptor cotransfected with the indicated $G\alpha$ proteins in an ELISA assay. Cotransfection of the MCH-1 receptor with the various $G\alpha$ proteins does not alter its surface expression level as verified by ELISA using an HA-antibody. Mock represents the signal obtained with mock-transfected cells using both the HA and the secondary antibody. Values are means \pm S.D. of triplicate determinations of a representative experiment, two additional experiments gave similar results. C, disruption of high-affinity agonist binding by GTP γ S. COS-7 cells were cotransfected with the MCH-1 receptor and $G\alpha_{q15}$ (diamonds) or $G\alpha_{qG66Di5}$ (circles). Forty-eight hours after transfection, cells were harvested and membranes were prepared. Membrane protein (10 μg) was incubated in binding buffer (for composition, see *Materials and Methods*) with 0.1 nM ^{125}I -MCH and the indicated concentrations of GTP γ S for 2 h at room temperature. Radioactivity bound to the membranes was measured by liquid scintillation counting. Results are means \pm S.D. of one of three representative experiments. Inset, verification of $G\alpha$ subunit expression in the membrane preparations used for determination of high affinity agonist binding. $G\alpha$ proteins were detected with a primary anti C-terminal antiserum and a secondary horseradish peroxidase-linked anti-rabbit IgG (1:5000 dilution; Amersham Biosciences Inc.). The band in the vector lane represents $G\alpha_i$ endogenous to the membrane.

receptors gained the ability to generate inositol phosphates when coexpressed with $G\alpha_{q15}$ or $G\alpha_{qs5}$, respectively. Although almost all GPCRs use the last five amino acids for specific $G\alpha$ activation, eight GPCRs (ChemR23, CCR5, CXCR2, MCH-1-R, GLP1R, GIPR, DPR, and β 2AR) stimulated robust inositol phosphate production upon coexpression of $G\alpha_{qG66D}$ and thus bypassed the requirement of the ex-

treme C terminus for specific $G\alpha$ activation. Remarkably, all GPCRs coexpressed with either $G\alpha_{qG66D15}$ or $G\alpha_{qG66D55}$ and stimulated with the appropriate agonist ligands gave rise to inositol phosphate increases exceeding those observed upon coexpression with the $G\alpha$ proteins modified in only one of these two domains, even if they were unable to signal when coexpressed with $G\alpha_{qG66D}$ (KOR, GPR7, HM74a, SSTR1, and NPY4R) or $G\alpha_{q55}$ (GLP1R). Thus, we demonstrated that 1) the relative importance of $G\alpha$ domains for specific GPCR coupling varies for different receptors and that 2) participation of certain $G\alpha$ domains may first be uncovered in the context of additional modifications. Our data are in agreement with a study from Slessareva et al. (2003) who have shown that four G_i -coupled receptors use slightly different domains on $G\alpha$ subunits to achieve selective coupling. It will be interesting to determine whether linker I of $G\alpha$ proteins interacts in a similarly cooperative manner with the $\alpha 4$ -helix- $\alpha 4/\beta 6$ loop, another region shown to be critical for specific GPCR-G protein recognition (Bae et al., 1997).

To differentiate between whether increased inositol phosphate or calcium levels generated upon coexpression of GPCRs with $G\alpha_{qG66D15}$ compared with $G\alpha_{qG66D}$ or $G\alpha_{q15}$ was due to different abilities of the $G\alpha$ proteins to induce/stabilize high-affinity agonist binding sites as opposed to inhibit conversion of the GPCR from the high- to the low-affinity state after ligand activation, agonist radioligand binding studies were performed as well as experiments monitoring real-time calcium dynamics. According to the ternary complex model, high-affinity agonist binding requires a complex between agonist, receptor and G protein (Hepler and Gilman, 1992; Samama et al., 1993). We therefore tested whether the $G\alpha$ proteins differed in their ability to induce a high-affinity agonist state of the MCH-1 receptor. We found that $G\alpha_{qG66D15}$ was superior to each of $G\alpha_{q15}$, $G\alpha_{qG66D}$, and wild type $G\alpha_q$ in increasing the fraction of high-affinity agonist binding sites of this receptor (Fig. 7A). Because $G\alpha_{qG66D15}$ allowed detection of a larger fraction of MCH-1 receptors in the high-affinity state compared with $G\alpha_{q15}$ or $G\alpha_{qG66D}$, it is tempting to speculate that the extent of functional inositol phosphate production correlates with the number of high-affinity receptor sites induced by the respective $G\alpha$ proteins. To test whether the $G\alpha$ proteins also interfered with the ability of the receptor to terminate intracellular signaling upon ligand activation, they were cotransfected with the KOR as a selected example, and calcium dynamics was monitored as a measure of $G\alpha$ activation. Calcium dynamics are particularly suitable to detect an altered conversion of receptors from the high- to the low-affinity state (Okuno et al., 2003). Typically, ligand-activated GPCRs mobilize intracellular calcium displaying peaks that rapidly increase and decrease within about 120 s. Receptors deficient in shutdown of intracellular signaling due to the inability to convert to the low-affinity state after activation, display calcium peaks that increase rapidly but decrease very slowly. We noted that the calcium peaks observed upon cotransfection of the KOR with $G\alpha_{qG66D15}$ or $G\alpha_{q15}$ differed only in magnitude but not in their kinetic profile, suggesting that increased functional signals of the KOR in inositol phosphate and calcium assays is not due to prolonged intracellular signaling of the receptors. This is most likely the case for the other GPCRs examined in this study because, consistent with this notion is the inability of the receptors to signal in a ligand-independent fashion in the

presence of the respective $G\alpha$ proteins. Receptors deficient in shutdown of an intracellular signal because they are trapped in the active conformation by a G protein could in fact be expected to display intrinsic signaling, as has been shown for the adrenergic $\beta 2$ receptor, which signals constitutively in the presence but not in the absence of a $G\alpha 16/z$ chimera (Mody et al., 2000). However, none of the 13 GPCRs tested in our study showed significant increases in second messenger production in the absence of an activating ligand. To gain more insight into the molecular mechanism underlying the induction of high-affinity agonist sites and to confer robust functional signaling characteristics onto non-Gq linked GPCRs, we sought to determine the affinity of receptors for two selected $G\alpha$ proteins $G\alpha_{q15}$ and $G\alpha_{qG66D15}$ using the MCH-1 receptor as a selected example. We found that GTP γ S was able to disassemble ternary complexes in a concentration-dependent manner (Waldhoer et al., 1998; Welsby et al., 2002; Alves et al., 2004) as evidenced by a decrease in the binding of a concentration of ^{125}I -MCH that is expected to occupy only high-affinity sites. Although the fraction of ^{125}I -MCH binding that was abolished by the G protein uncoupling agent GTP γ S with high affinity was greater when the receptor was coexpressed with $G\alpha_{qG66D15}$ than with $G\alpha_{q15}$, the absolute potency of GTP γ S for the high-affinity site was not substantially different. A similar lack of correlation between receptor-G protein affinity and GTP γ S potency was recently reported by Alves et al., (2004) who investigated interaction of the human δ opioid receptor with the different G protein subtypes $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$. Thus, the superiority in functional and binding assays of $G\alpha_{qG66D15}$ over $G\alpha_{q15}$ is reflected by its ability to induce significantly more high-affinity agonist sites; at present, however, it cannot be stated unequivocally that this arises as a consequence of receptor G protein affinity. Alternative methods, such as plasmon-waveguide resonance spectroscopy will be required to clarify the question of receptor-G protein affinity.

It should also be of interest to determine whether the activation by GPCRs of the engineered $G\alpha$ proteins differentially affects GDP release of $G\alpha$ and hence their ability to reside in the very short-lived empty pocket conformation. In fact, the G66D mutation in the linker I region is ideally placed to generate a $G\alpha$ protein with a slightly "opened" nucleotide binding pocket, a condition that may well facilitate receptor-mediated nucleotide exchange. Since the free energy required for GDP release upon receptor stimulation is related to the stability of the ternary complex (Roberts and Waelbroeck, 2004), association and dissociation kinetics of agonist tracers may help to explore this issue in more detail. Such studies are currently underway in our laboratory.

Together, our data indicate that the extreme C terminus that is part of the receptor G protein interface and the linker I region outside the receptor G protein interface of $G\alpha$ subunits interact cooperatively in switching GPCR-to-effector specificity of GPCRs. The observed cooperativity is reminiscent of the cooperativity between different intracellular regions of the GPCRs in controlling their coupling to G proteins and underscores the hypothesis that specificity of GPCR-G protein interaction results from a multiplicity of contact zones between both proteins and cooperative interactions among various $G\alpha$ domains within and outside the GPCR-G protein interface. We expect that the novel $G\alpha$ proteins presented here will prove valuable for linking an even wider

range of receptors than those examined here to efficient stimulation of the G α_q -PLC β -inositol phosphate-calcium pathway and may aid in the deorphanization process of orphan GPCRs as well as allow G α_i - and G α_s -selective GPCRs to be screened alongside G α_q -selective receptors in assays recording inositol phosphate or calcium mobilization.

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