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A highly conserved glycine within linker I and the extreme C-terminus of G protein α subunits interact cooperatively in switching GPCR-to-effector specificity.

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Running Title Page

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[1]**Abbreviations:** GPCR: G protein-coupled receptor, IP, inositol phosphate, PLCβ

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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 $G\alpha_q$ proteins (Heydorn *et al.*, 2004). Herein, we explored whether both modules (linker I and extreme C-terminus) interact cooperatively in switching GPCR-to-effector specificity and created as models mutant $G\alpha_q$ proteins in which glycine was replaced with various amino acids and the C-terminal five $G\alpha_q$ residues with the corresponding $G\alpha_i$ or $G\alpha_s$ sequence. Coupling properties of the mutated $G\alpha_q$ proteins were determined following coexpression with a panel of thirteen Gi-and Gs-selective receptors and compared with those of $G\alpha$ proteins modified in only one module. $G\alpha$ proteins modified in both modules are significantly more efficacious in channelling non-Gq selective receptors to Gq-mediated signalling events as 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 $G\alpha$ 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 $G\alpha$ proteins and that distinct determinants of selectivity exist for individual receptors.

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INTRODUCTION

Accurate signal transduction demands that seven transmembrane G protein-coupled receptors (7TM GPCRs, hereafter referred to as 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 Gα proteins ($G\alpha_{i/o}$, $G\alpha_{s}$, $G\alpha_{q}$, $G\alpha_{12/13}$) 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; Liu *et al.*, 1995; Lee *et al.*, 1995; Kostenis *et al.*, 1997a; Bae *et al.*, 1997; Blahos *et al.*, 2001; Liu *et al.*, 2002; Havlickova *et al.*, 2003) for review see (Cabrera-Vera *et al.*, 2003)). On the receptor side, various 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; Gomeza *et al.*, 1996; Kostenis *et al.*, 1997a; Orcel *et al.*, 2000; Perroy *et al.*, 2001; Kim *et al.*, 2002; Liu *et al.*, 2004). 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 Gα 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 Gα recognition (Gomeza *et al.*, 1996; Havlickova *et al.*, 2003). On the level of the G protein both Gα and the βγ subunit complexes are likely to possess receptor contact sites (Conklin *et al.*, 1993; Kisselev *et al.*, 1995; Taylor *et al.*, 1996). Within the Gα subunit several key regions governing GPCR-G protein coupling specificity have been identified so far: These include (i) the extreme C-terminus (Conklin *et al.*, 1993; Kostenis *et al.*, 1997a; Liu *et al.*, 2002; Havlickova *et al.*, 2003), (ii) the extreme N-terminus (Kostenis *et al.*, 1997b; Slessareva and Graber, 2003), (iii) a region between α4 and α5 helices (Bae *et al.*, 1997; Cai *et al.*, 2001; Slessareva *et al.*, 2003), (iv) a region within the loop linking the N-terminal α-helix to the β1 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 fashion. We have previously reported that a glycine residue within linker I (glycine 66 in $G\alpha_q$), which is highly conserved among Gα 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 modelling 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 Gα. Perturbation of the structural arrangement of the helical and GTPase domains via mutagenesis of this key residue in linker I (glycine 66 within $G\alpha_q$) increased the ease with which receptors otherwise selective for $G\alpha_i$ or $G\alpha_s$ gained the ability to activate $G\alpha_q$. Previously we have only assessed the coupling properties of $G\alpha_q$ in which glycine 66 was replaced by various other amino acids upon coexpression with Gi-,

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Gs-, and Gq-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 Gα – 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 $G\alpha_q$ proteins modified at their extreme C-termini to permit coupling to GPCRs otherwise linked to non Gq-mediated signalling events.

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MATERIALS AND METHODS

DNA Construction

Construction of $G\alpha_q$ and $G\alpha_{qG66X}$ (X= D,N,V,K) in the pcDNA1.1 expression vector was reported previously (Heydorn *et al.*, 2004). The C-terminally modified $G\alpha_q$ subunits $G\alpha_{qi5/s5}$ were created by introducing, in a two piece ligation, a PCR-based 175 bp *Bgl*II-*Nsi*I fragment containing the desired codon changes. To create $G\alpha_{qG66Xi5/s5}$ a 175-bp *Bgl*II-*Nsi*I fragment of $G\alpha_{qG66X}$ was replaced, in a two piece ligation, with the corresponding *Bgl*II-*Nsi*I fragment of $G\alpha_{qi5/s5}$. All wild type and mutant $G\alpha$ subunits contained an internal hemagglutinin epitope tag DVPDYA (Wedegaertner *et al.*, 1993) replacing $G\alpha_q$wt residues 125-130 to monitor protein expression. Construction of expression plasmids encoding for the chemokine CCR5 and CXCR2 receptor, kappa opioid receptor (KOR), adrenergic β2 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 *BamH*I and *EcoR*I. GPR7 was cloned from brain cDNA and inserted via *EcoR*I/*Xho*I into pcDNA3.1(+). The high affinity nicotinic acid receptor HM74a was cloned from adipose tissue and inserted into pcDNA3.1(+) via *Hind*III/*EcoR*I sites. The human NPY4, MCH-1, and somatostatin type I receptor (SSTR1) were cloned from brain cDNA and inserted via *Hind*III/*EcoR*I into the pcDNA3.1(+) expression vector.

Cell Culture and Transfections

COS-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) 1885 supplemented with 10% fetal bovine serum and 10 μg/ml Gentamicin, and kept at

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37°C in a 10% CO_2 humidified atmosphere. The cells were plated at a density of $6x10^6$ cells per 175 cm^2 flask and were transfected using a calcium phosphate-DNA co-precipitation method, where 40 μg cDNA (equal amounts of receptor and G protein) was diluted in 480 μl $CaCl_2$ solution (10 mM Tris base, 1 mM EDTA (pH 8), 250 mM $CaCl_2$) and drop-wise added to 480 μl HBS (280 mM NaCl, 50 mM Hepes and 1.5 mM sodium phosphate (sec.)). The precipitation incubated for 45 minutes at room temperature, and was added to the cells in 10 ml fresh culture-media supplemented with 0.1 mM chloroquine for 5 hours at 37°C in a 10% CO_2 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 c/w in growth media supplemented with 5 μCi of ^{3}H-*myo*-inositol. HEK293 cells were maintained in Minimum Essential medium (MEM) supplemented with 10% (v/v) heat inactivated fetal calf serum (HIFCS), 2 mM Glutamax™-I, 1% non essential amino acids (NEAA), 1% sodium pyruvate, and 10 μg/ml Gentamicin. HEK293 cells were transfected with the Fugene transfection reagent as per manufacturer's instructions. For all receptor Gα protein coexpression experiments, a ratio of 1:1 was used for the respective cDNAs.

Inositol phosphate turnover assays

48 hours after transfection the cells were washed twice in HBSS buffer (including $CaCl_2$ and $MgCl_2$, GIBCO cat. 14025-050) and stimulated with the respective agonists in HBSS buffer supplemented with 5 mM LiCl for 45 minutes at 37°C. The reactions were terminated by aspiration and addition of 10 mM ice-cold formic acid, and incubated for 30 minutes 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 [^{3}H]-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, Boston, MA).

Calcium mobilization assay

Agonist-mediated intracellular Ca^{2+}-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 Corp.) and subsequently challenged with increasing concentrations of agonist ligand. Intracellular calcium mobilization was recorded in real time during 120 seconds in a FLEX station (Molecular Devices Corp). RLU (= relative light units) = maximum Ca^{2+}-peak/cell number x 1000.

ELISA

Determination of cell surface expression levels of the MCH1 receptor was performed using an N-terminally HA-tagged MCH1 receptor in an ELISA assay. Approximately 48 hours 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, 0.1 mM $CaCl_2.2H_2O$, pH 7.4), and then blocked with blocking buffer (3% dry-milk, 1 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5). The primary monoclonal HA-11 antibody (BabCo, Covance, CA) was applied into the same buffer at a 1:300 dilution for 1 hour at room temperature followed by three washes and a 1 hour 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 TMB (3, 3', 5, 5'-Tetramethylbenzidine (Sigma, 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 OD450 on a Tecan Sunrise absorbance reader (Tecan Corp., Maennedorf, Switzerland). All experiments were performed in quadruplicate determinations.

Receptor binding studies

Whole cell binding – 24 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 18-24 hours later using 0.1 nM [^{125}I]MCH (74TBq/mmol, 2000 Ci/mmol; Amersham Biosciences) 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% BSA, and 100 μg/ml Bacitracin. Total and nonspecific binding were determined in the absence and presence of 10 μM MCH. Binding reactions were routinely conducted for 3 hours at room temperature and terminated by 2 washes (100 μl each) with ice cold binding buffer and addition of 25 μl NaOH (0.1 N). Radioactivity was determined by liquid scintillation counting in a TOPCOUNTER (Packard) following over night 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 protein), [^{125}I]MCH (0.1 nM) and varying concentrations of MCH or GTPγS. 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 hours to allow equilibration. Membrane-bound radioligand was separated from free radioligand by vacuum filtration and washing with assay buffer

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over glass fibre filters (pre-treated with 0.1 % (W/V) polyethleneimine in assay buffer; Grade GF/C, Brandel Inc.) using a Brandel M-24 harvester. Bound [^{125}I]MCH was measured using a Packard Cobra II γ-counter.

Membrane preparation and Western blotting

Membranes (standard P2 membrane preparations) from transiently transfected COS-7 cells were prepared essentially as described (Heydorn *et al.*, 2004). Membrane proteins were quantified with the Bio-Rad protein assay kit using bovine serum albumin (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 TBS-T (10 mM Tris pH 8, 150 mM NaCl, 10% SDS, 0,1% Tween 20)). Immunoreactive proteins were visualized with the enhanced chemiluminescence (ECL) kit from Amersham.

Receptor Ligands

[^{125}I]-Melanin Concentrating Hormone (2000 Ci/mmol) was from Amersham Biosciences UK Ltd. Chemerin 9 was synthesized by UFPeptides (Ferrara, Italy); Somatostatin 28 (SS28) was from Bachem, neuropeptide W23 (NPW23) was from Phoenix peptides, Rantes and interleukin8 (IL8) were from Preprotech, prostaglandin D2 (PGD2) was from Cayman lipids, human pancreatic polypeptide (hPP), glucagon-like peptide 1 (GLP1), and gastric inhibitory peptide (GIP) were from Sigma as were all other chemicals which were of the highest grade possible.

Calculations - IC_{50} and EC_{50} values were determined by nonlinear regression using the Prism 3.0 software (GraphPad Software, San Diego). Values of the dissociation

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and inhibition constants (K_d and K_i) were estimated from competition binding experiments using the equations $K_d = IC_{50} - L$ and $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioactive ligand and K_d is its dissociation constant.

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RESULTS

Construction, expression and functionality of mutant Gα_q proteins

Replacement of the five C-terminal amino acids of Gα_q with the corresponding Gα_i sequence (the resulting chimera is referred to as Gα_{qi5}) has been shown to confer onto Gi-linked GPCRs the ability to stimulate the Gq-pathway in assays measuring intracellular inositol phosphate (IP) generation or intracellular calcium mobilization (Conklin *et al.*, 1993; Kostenis *et al.*, 1997a; Milligan and Rees, 1999). Similarly, Gs-coupled receptors can be forced to stimulate PLCβ upon coexpression with Gα_{qs5} (the C-terminal five amino acids of Gα_q are replaced with the corresponding Gα_s sequence) (Conklin *et al.*, 1996). We have previously shown that mutation of a highly conserved glycine residue in linker I of the Gα_q subunit to various other amino acids confers onto Gα_q the ability to channel Gi- and Gs-selective receptors to the PLCβ 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 Gα_i or Gα_s sequence at their extreme C-termini. The mutants are referred to as G$\alpha_{qG66Xi5}$ and G$\alpha_{qG66Xs5}$ (X = D, N, V, K) and are schematically depicted in Fig. 1. The sequences of various wild type Gα subunits as well as chimeric Gα_{qi5} and Gα_{qs5} are included in the alignment for comparison. Initially, expression levels of the mutant Gα_q subunits and functionality upon coexpression with two Gi- and two Gs-selective receptors were determined in transiently transfected COS-7 cells (Fig. 2). All G$\alpha_{qG66Xs5}$ mutants were coexpressed with the Gs-selective glucagon-like peptide 1 (GLP1) and the adrenergic β2 receptor, and the corresponding G$\alpha_{qG66Xi5}$ proteins with the Gi-selective neuropeptide Y4 (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 $G\alpha_q$ with $G\alpha_i$ or $G\alpha_s$ sequence, respectively, suffices to alter coupling specificity of the adrenergic β2, NPY4 and ChemR23 receptors, $G\alpha_{qs5}$ did not allow the GLP1 receptor to produce stimulation of the PLCβ pathway. Remarkably, however, all four GPCRs gained the ability to productively stimulate inositol phosphate hydrolysis when coexpressed with the dually modified $G\alpha_q$ proteins (Fig. 2). Furthermore, measurement of PLCβ activation showed that the gain in coupling efficiency of the dually modified Gα 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, Gα subunit expression was monitored by immunodetection using membrane preparations of the respective transient receptor-G protein cotransfections (Fig. 2). Gα 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 $G\alpha_q$ 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 Gα proteins was due to alterations in Gα expression levels can be eliminated. Additional control experiments were performed to test whether the panel of dually modified Gα proteins was equally capable of adopting the active conformation as wild type $G\alpha_q$ or the C-terminally modified $G\alpha_{qi5/s5}$ proteins. Towards this end COS7 cells transiently transfected with the various Gα proteins were challenged with fluoroaluminate ($AlF4^-$) and inositol phosphate production was determined as a measure of Gα activity (not shown). $AlF4^-$ typically activates G protein α subunits by binding to the GDP-bound form of Gα. The resulting Gα-GDP-$AlF4^-$ complex assumes an active state conformation, which resembles the

Gα-GTP complex (Coleman *et al.*, 1994). Both fluoroaluminate-activated and receptor-activated Gα subunits are capable of stimulating intracellular effector molecules. All Gα 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. As we were interested to examine the generality of the observed cooperative effects between the linker I region and the extreme C-terminus in altering GPCR-to-effector specificity we then chose to compare coupling properties of a selected dually modified Gα protein ($G\alpha_{qG66Di5}$ was chosen as a model) with Gα proteins containing the individual mutations ($G\alpha_{qG66D}$, $G\alpha_{qi5}$) upon coexpression of a broad range of Gi-linked receptors. To this end the panel of Gα proteins was transiently coexpressed in COS-7 cells with each of the ChemR23, chemokine CCR5 and CXCR2, kappa opioid receptor (KOR), GPR7, melanin concentrating hormone receptor-1 (MCH-1R), HM74a, somatostatin receptor type I (SSTR1), and neuropeptide Y NPY4 receptors and agonist-induced mobilization of inositol phosphates was determined (Fig. 3). Out of these nine Gi/o-coupled receptors, eight did not affect PLC even when wild type $G\alpha_q$ was overexpressed. In contrast, the MCH-1 receptor, which preferentially activates $G\alpha_{i/o}$ proteins is also capable of activating $G\alpha_q$ (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 (as compared to wild type $G\alpha_q$). 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 following cotransfection with $G\alpha_{qi5}$. 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 $G\alpha$ 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 Gs-linked glucagon like peptide 1 (GLP1), gastric inhibitory peptide (GIP), prostaglandin D2 DP, and adrenergic β2 receptor and vector DNA as a control or the various mutant $G\alpha_q$ and $G\alpha_{q/s}$ chimeric constructs (Fig. 4). In agreement with the results obtained with Gi-selective receptors, combination of the G66D mutation with the C-terminal $G\alpha_q \rightarrow G\alpha_s$ sequence switch gave rise to a mutant $G\alpha$ superior to the individual domain modified $G\alpha$ proteins with respect to stimulation of PLCβ. Furthermore, it appears as if distinct determinants of selectivity exist for receptors from different coupling classes. While Gi-selective receptors interacted more productively with C-terminally as compared with linker-modified $G\alpha$ proteins, Gs-selective receptors displayed the opposite phenotype and interacted more productively with the linker-modified $G\alpha$ proteins. Collectively, the data provided evidence that both the linker I region and the extreme C-terminus of $G\alpha$ determine receptor- G protein coupling specificity and that both regions act in concert in a cooperative manner to achieve maximal PLCβ stimulation by non-Gq-selective GPCRs.

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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 $G\alpha$ proteins was also visible in calcium assays where more signal amplification exists and used the KOR as a model. Fig. 5A shows concentration response curves of HEK293 cells cotransfected with the KOR and the set of $G\alpha$ 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 $G\alpha_{qi5}$ in channelling the Gi-selective KOR to intracellular calcium mobilization while no detectable calcium mobilization was visible in the presence of the other $G\alpha$ proteins. Importantly, mock- or $G\alpha$ 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 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 Gi-selective GPCRs the ability to maintain the active conformation for a longer period of time. The inability of a GPCR to convert from a high to a low agonist affinity 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. 5B/C for the KOR coexpressed with either $G\alpha_{qi5}$ (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 $G\alpha_{qi5}$ 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.

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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 Gs-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 complex 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. Fig. 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

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rank order: $G\alpha_{qG66Di5}$ > $G\alpha_{qi5}$ = $G\alpha_{qG66D}$ > wild type $G\alpha_q$ (note: wild type $G\alpha_q$ exerts a 'dominant negative' effect on [^{125}I]MCH binding, most likely via competing with endogeneous $G\alpha_i$, while cotransfection of the MCH-1 receptor with $G\alpha_i$ leads to a further increase in high affinity agonist sites (inset Fig. 7A). We would have preferred to perform these binding experiments following treatment with pertussis toxin (PTX) to prevent the receptor from interacting with the $G\alpha_i$ proteins endogeneous 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 non-specific 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 MCH1-R, the various $G\alpha$ proteins selectively influence the number of receptors in the high-affinity state as opposed to exerting non-specific effects on receptor expression. In agreement with functional data on inositol phosphate production (cf. Fig. 3), $G\alpha_{qG66Di5}$ 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 different $G\alpha$ proteins can be distinguished by their ability to confer high affinity signalling 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.

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Effects of GTPγS on high affinity agonist binding.

We next examined the effects of GTPγS, a hydrolysis resistant GTP analogue, on [^{125}I]MCH binding to membrane preparations from COS-7 cells transiently cotransfected with the MCH-1 receptor and the two selected Gα proteins $G\alpha_{qi5}$ and $G\alpha_{qG66Di5}$. Excess GTPγS 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. Fig. 7C shows that [^{125}I]MCH binding to the MCH-1 receptor was decreased by increasing concentrations of GTPγS in the $G\alpha_{qi5}$ and $G\alpha_{qG66Di5}$ cotransfections. The fraction of [^{125}I]MCH displaced with high affinity by GTPγS was substantially greater following cotransfection of the MCH-1 receptor with $G\alpha_{qG66Di5}$ (85.8 +/- 2.3%, mean +/- SEM, n= 3) than with $G\alpha_{qi5}$ (44.6 +/- 3.6%). Surprisingly, though, we could not detect a substantial difference in potency of GTPγS to reduce [^{125}I]MCH binding to the MCH1 receptor cotransfected with either Gα protein. Together, these results suggest that the two Gα proteins differ in their ability to confer high affinity binding sites to the MCH-1 receptor and may form ternary complexes with different stability which would reflect different affinities of the receptor for the individual Gα proteins.

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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 Gα, 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; Conklin *et al.*, 1996; Kostenis *et al.*, 1997c; Blahos *et al.*, 1998; Bahia *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 (i) the relationship between the linker I region and the extreme C-terminus of Gα in determining selective GPCR coupling, and (ii) whether different GPCRs utilize different Gα domains to achieve specific coupling. To this end we generated Gα proteins carrying a glycine to aspartate mutation within linker I and 5 amino acids of either Gαi or Gαs sequence at their extreme C-termini ($G\alpha_{qG66Di5}$, $G\alpha_{qG66Ds5}$). Coupling properties of the mutant Gα proteins were compared with Gα proteins individually modified within linker I ($G\alpha_{qG66D}$) or the C-terminus ($G\alpha_{qi5}$, $G\alpha_{qs5}$) upon coexpression with Gi- or Gs-selective receptors. Our coexpression studies confirmed the importance of the extreme C-terminus of Gα in selective GPCR recognition as twelve out of thirteen tested non Gq-linked receptors gained the ability to generate inositol phosphates when coexpressed with $G\alpha_{qi5}$ or $G\alpha_{qs5}$, respectively. While almost all GPCRs utilize the last five amino acids for specific Gα activation, eight GPCRs (ChemR23, CCR5, CXCR2, MCH1-R, GLP1R, GIPR, DPR, and the β2AR) stimulated robust inositol phosphate production upon coexpression of $G\alpha_{qG66D}$

and thus bypassed the requirement of the extreme C-terminus for specific Gα activation. Remarkably, all GPCRs coexpressed with either $G\alpha_{qG66Di5}$ or $G\alpha_{qG66Ds5}$ and stimulated with the appropriate agonist ligands gave rise to inositol phosphate increases exceeding those observed upon coexpression with the Gα 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, NPY4R) or Gαqs5 (GLP1R). Thus, we demonstrated that (i) the relative importance of Gα domains for specific GPCR coupling varies for different receptors and that (ii) participation of certain Gα 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 Gi-coupled receptors utilize slightly different domains on Gα subunits to achieve selective coupling. It will be interesting to determine whether linker I of Gα proteins interacts in a similarly cooperative manner with the α4-helix-α4/β6 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 $G\alpha_{qG66Di5}$ when compared with $G\alpha_{qG66D}$ or $G\alpha_{qi5}$ was due to different abilities of the Gα 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 Gα proteins differed in their ability to induce a high affinity agonist state of the MCH-1 receptor. We found

that $G\alpha_{qG66Di5}$ was superior to each of $G\alpha_{qi5}$, $G\alpha_{qG66D}$, and wild type $G\alpha_{q}$ in increasing the fraction of high affinity agonist binding sites of this receptor (Fig. 7A). As $G\alpha_{qG66Di5}$ allowed detection of a larger fraction of MCH-1 receptors in the high affinity state as compared to $G\alpha_{qi5}$ 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 Gα proteins. To test whether the Gα proteins also interfered with the ability of the receptor to terminate intracellular signalling upon ligand activation, they were cotransfected with the KOR as a selected example and calcium dynamics were monitored as a measure of Gα 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 seconds. Receptors deficient in shutdown of intracellular signalling 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 $G\alpha_{qG66Di5}$ or $G\alpha_{qi5}$ 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 signalling 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 Gα proteins. Receptors deficient in shutdown of an intracellular signal because there are trapped in the active conformation by a G protein could in fact be expected to display intrinsic signalling, as has been shown for the adrenergic β2 receptor which signals constitutively in the presence but not in the absence of a

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Gα16/z chimera (Mody et al., 2000). However, none of the thirteen 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 signalling characteristics onto non-Gq linked GPCRs, we sought to determine the affinity of receptors for two selected Gα proteins $G\alpha_{qi5}$ and $G\alpha_{qG66Di5}$ using the MCH-1 receptor as a selected example. We found that GTPγS 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 GTPγS with high affinity was greater when the receptor was co-expressed with $G\alpha_{qG66Di5}$ than with $G\alpha_{qi5}$ the absolute potency of GTPγS for the high affinity site was not substantially different. A similar lack of correlation between receptor-G protein affinity and GTPγS potency was recently reported by Alves et al., (2004) who investigated interaction of the human δ opioid receptor (DOR) 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 $G\alpha_{qG66Di5}$ over $G\alpha_{qi5}$ 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 (PWR) 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 Gα proteins differentially affects GDP release of Gα and hence their ability to reside in the very shortlived empty pocket conformation. In fact the G66D

mutation in the linker I region is ideally placed to generate a Gα 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.

Taken together, our data indicate that the extreme C-terminus which is part of the receptor G protein interface and the linker I region outside the receptor G protein interface of Gα 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 Gα domains within and outside the GPCR-G protein interface. We expect that the novel Gα proteins presented here will prove valuable for linking an even wider range of receptors than those examined here to efficient stimulation of the $G\alpha_q$-PLCβ- inositol phosphate-calcium pathway and may aid in the deorphanization process of orphan GPCRs as well as allow Gi-and Gs-selective GPCRs to be screened alongside Gq-selective receptors in assays recording inositol phosphate or calcium mobilization.

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FIGURE LEGENDS

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. $G\alpha_{qi5}$ and $G\alpha_{qs5}$ denote mutant $G\alpha_q$ constructs in which the C-terminal five amino acids are replaced with the corresponding $G\alpha_i$ ($G\alpha_{qi5}$) or $G\alpha_s$ ($G\alpha_{qs5}$) sequence, respectively. $G\alpha_{qG66X}$ 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 $G\alpha_q$ in that they allow Gi- and Gs-linked receptors to activate the PLCβ pathway (Heydorn *et al.*, 2004). $G\alpha_{qG66Xi5/s5}$ are mutant Gα 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 Gα subunit.** Shown is the crystal structure of the $G\alpha_i$ 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 Gα 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 Gα protein is 1BOF.

Fig. 2. Concentration response curves for stimulation of PLCβ by two Gi- and two Gs-selective receptors. COS-7 cells were cotransfected with expression plasmids coding for the Gs-selective glucagon like peptide 1 (GLP1) and adrenergic

β2 receptor or the Gi-selective neuropeptide Y4 (NPY4) and ChemR23 receptor and the indicated set of G protein α subunits. About 48 hours 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 & Methods". Data are expressed as means + 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 Gα subunits was monitored by immunodetection. 48 hours after transfection membranes were prepared and analyzed (20-40 µg each) by SDS-polyacrylamide gel electrophoresis (13%) and Western blotting using the 12CA5-peroxidase linked monoclonal antibody. For the GLP1 and β2 receptor-G protein cotransfections, lane 1 corresponds to mock transfected cells, lane 2 ($G\alpha_{qs5}$), lane 3 ($G\alpha_{qG66Vs5}$), lane 4 ($G\alpha_{qG66Ns5}$), lane 5 ($G\alpha_{qG66Ds5}$), and lane 6 ($G\alpha_{qG66Ks5}$). For the NPY4R and ChemR23 receptor cotransfections, lane 1 (mock transfected cells), lane 2 ($G\alpha_{qi5}$), lane 3 ($G\alpha_{qG66Vi5}$), lane 4 ($G\alpha_{qGssNi5}$), lane 5 ($G\alpha_{qG66Ds5}$), and lane 6 ($G\alpha_{qG66Ks5}$). Similar results were obtained in two additional experiments with different batches of membranes.

Fig. 3. Functional interaction of selected Gi-coupled receptors with wild type $G\alpha_q$, $G\alpha_{qG66D}$, $G\alpha_{qi5}$, and $G\alpha_{qG66Di5}$. COS-7 cells coexpressing pcDNA1.1 vector DNA or the indicated Gα subunits and different Gi-linked receptors were incubated for 45 minutes (37°C) in the absence or presence of increasing concentrations of the appropriate agonist ligands. The resulting increases in intracellular inositol phosphates levels were determined as described under "Materials & Methods". Data are means ± SE of three to five experiments each performed in duplicate.

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Fig. 4. Functional interaction of selected Gs-coupled receptors with wild type $G\alpha_q$, $G\alpha_{qG66D}$, $G\alpha_{qs5}$, and $G\alpha_{qG66Ds5}$. COS-7 cells coexpressing the GLP1, gastric inhibitory peptide (GIP), prostaglandin D2 DP, and adrenergic β2 receptor and pcDNA1.1 vector DNA or the indicated Gα subunits were incubated for 45 minutes (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 "Experimental Procedures". Data are means ± SD of 2-5 independent experiments each performed in duplicate.

Fig. 5. Agonist-dependent mobilization of intracellular calcium in cells cotransfected with the kappa opioid receptor and the indicated Gα subunits. HEK293 cells were transiently cotransfected with the KOR and the indicated Gα subunits. Agonist-mediated changes in intracellular calcium were measured by the FLEX-station as described under "Materials & 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/$G\alpha_{qG66Di5}$ cotransfection. The arrow in panel B and C indicates the addition of agonist. Shown is one out of three representative experiments, each performed in triplicate determinations.

Fig. 6. Time-dependent generation of [^{3}H] inositol phosphates in COS-7 cells cotransfected with the GLP1 receptor and the indicated Gα subunits. COS-7 cells coexpressing the GLP1 receptor and the indicated Gα subunits were incubated for different time intervals (37°C) in the absence or presence of 100 nM GLP1. The resulting increases in intracellular inositol phosphate levels were determined as

described under “Experimental Procedures”. Data are means ± SD of three independent experiments each performed in duplicate.

Fig. 7. A, Induction of high affinity agonist binding to MCH-1 receptors in whole cells by cotransfection of Gα subunits. COS-7 cells were cotransfected with the MCH-1 receptor and pcDNA1.1 vector DNA or the indicated Gα subunits. After 48 hours whole cell binding experiments were performed for 3 hours at room temperature as described under “Materials & Methods”. Receptor-bound radioactivity was measured by liquid scintillation counting. Results are means ± SD 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 Gα proteins in an ELISA assay.** Cotransfection of the MCH-1 receptor with the various Gα 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 ± SD of triplicate determinations of a representative experiment, two additional experiments gave similar results. **C, Disruption of high affinity agonist binding by GTPγS.** COS-7 cells were cotransfected with the MCH-1 receptor and $G\alpha_{qi5}$ (diamonds) or $G\alpha_{qG66Di5}$ (circles). 48 hours after transfection cells were harvested and membranes were prepared. 10 μg of membrane protein was incubated in binding buffer (for composition see “Materials & Methods”) with 0.1 nM [^{125}I]MCH and the indicated concentrations of GTPγS for 2 hours at room temperature. Radioactivity bound to the membranes was measured by liquid scintillation counting. Results are means + SD of one out of three representative experiments. Inset: Verification of Gα subunit expression in the membrane

preparations used for determination of high affinity agonist binding. Gα proteins were detected with a primary anti C-terminal antiserum and a secondary horseradish peroxidase linked anti-rabbit IgG (1:5000 dilution; Amersham Biosciences). The band in the vector lane represents Gαi endogeneous to the membrane.

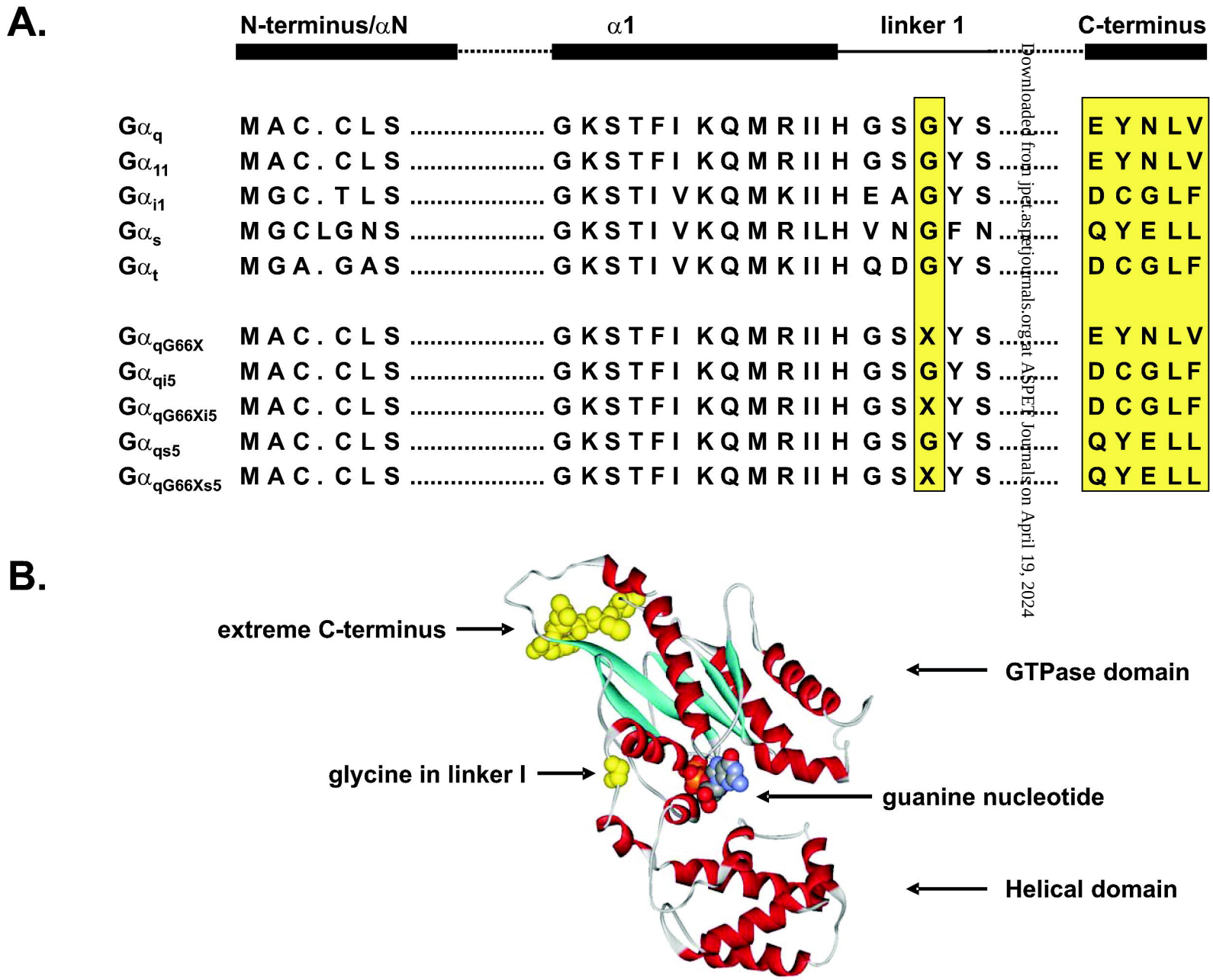

Fig. 1

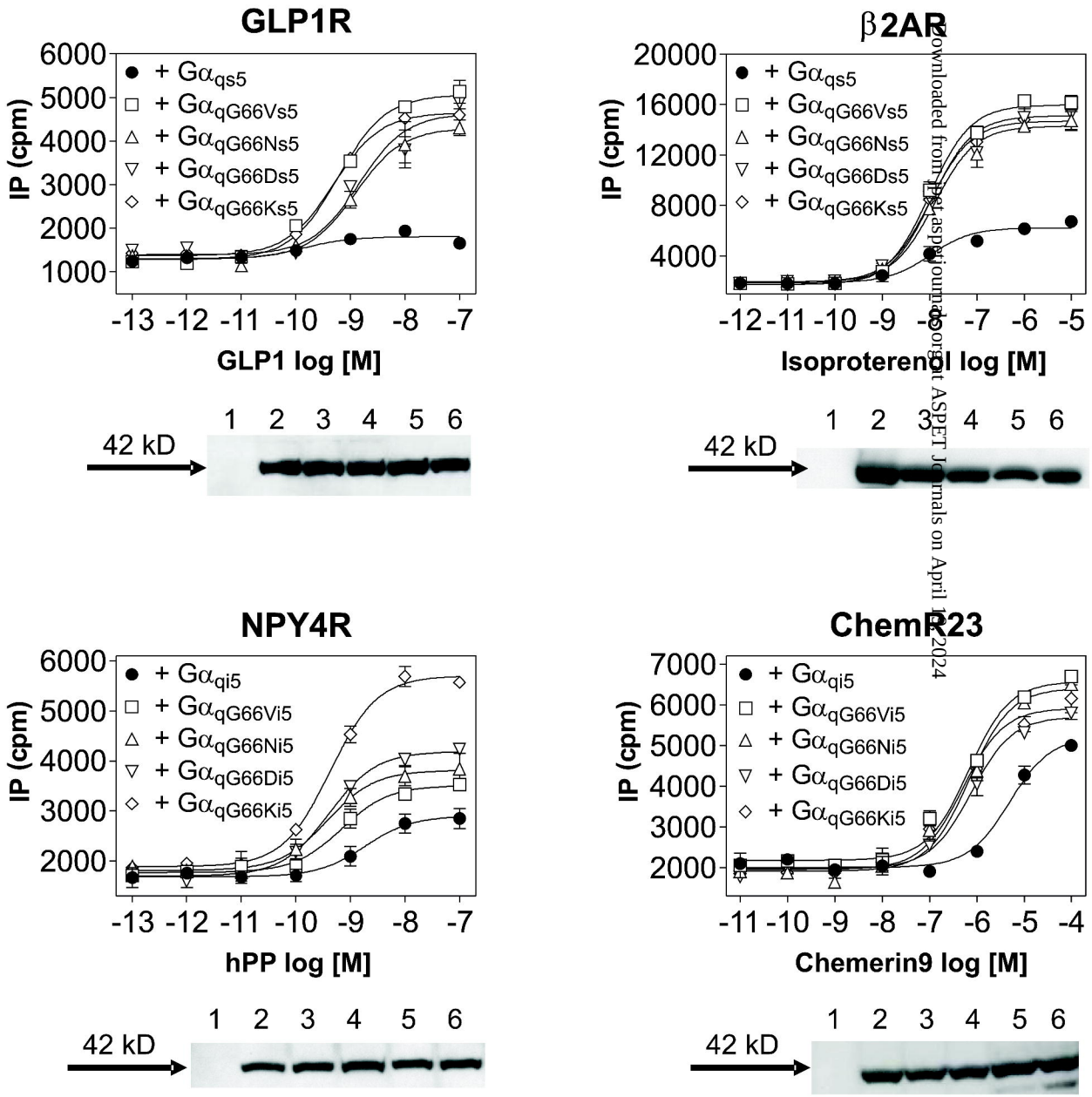

Fig.2

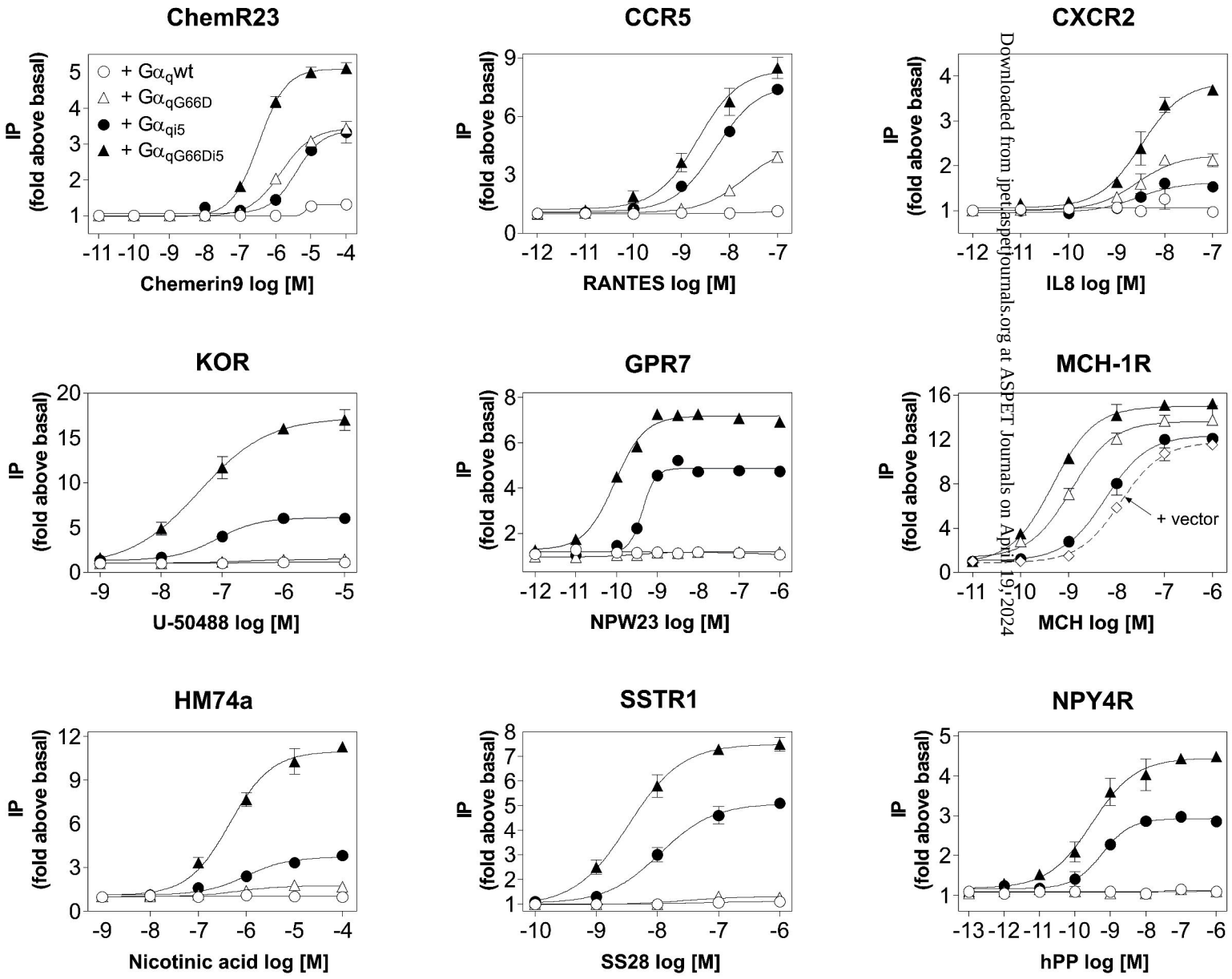

Fig. 3

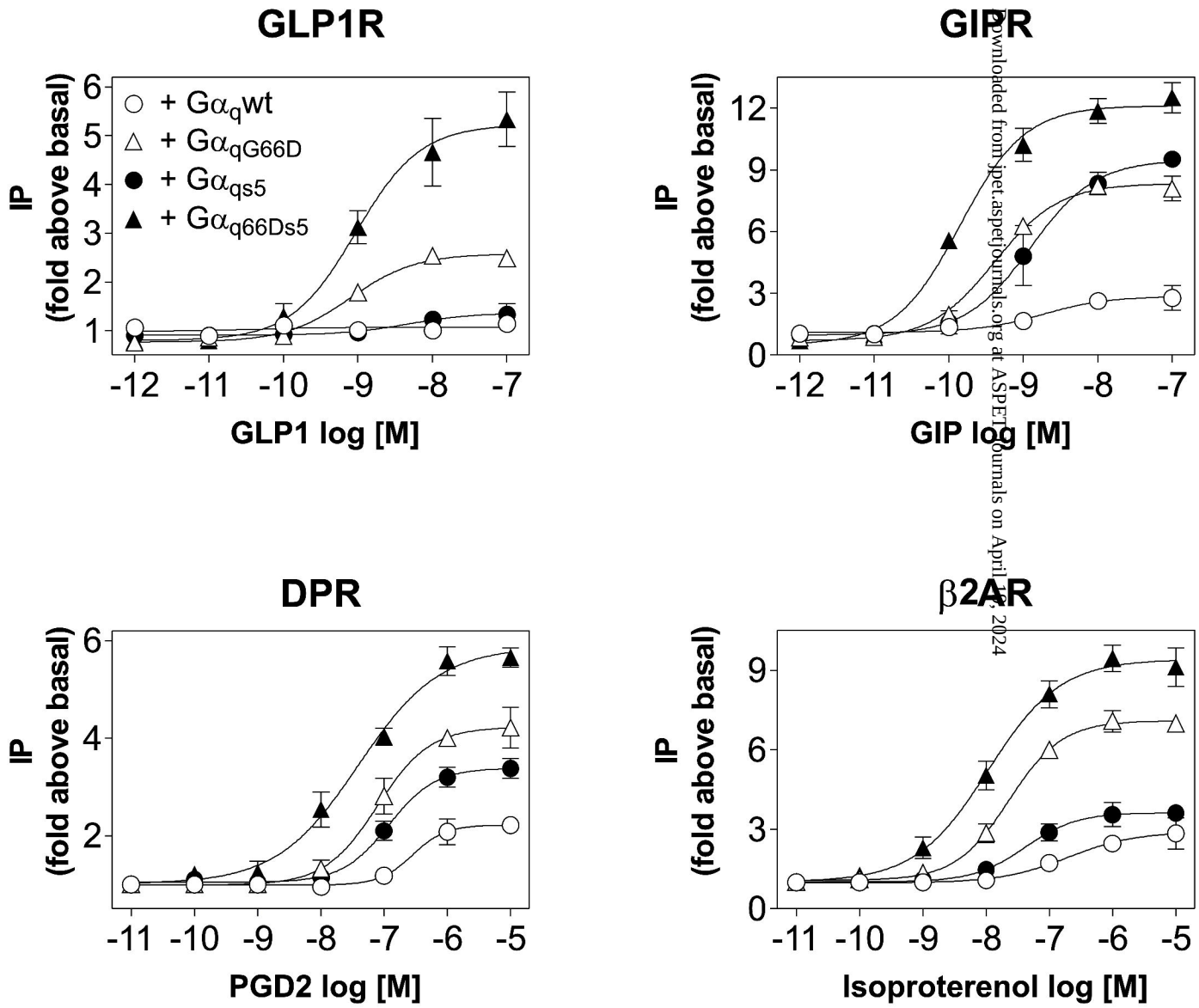

Fig.4

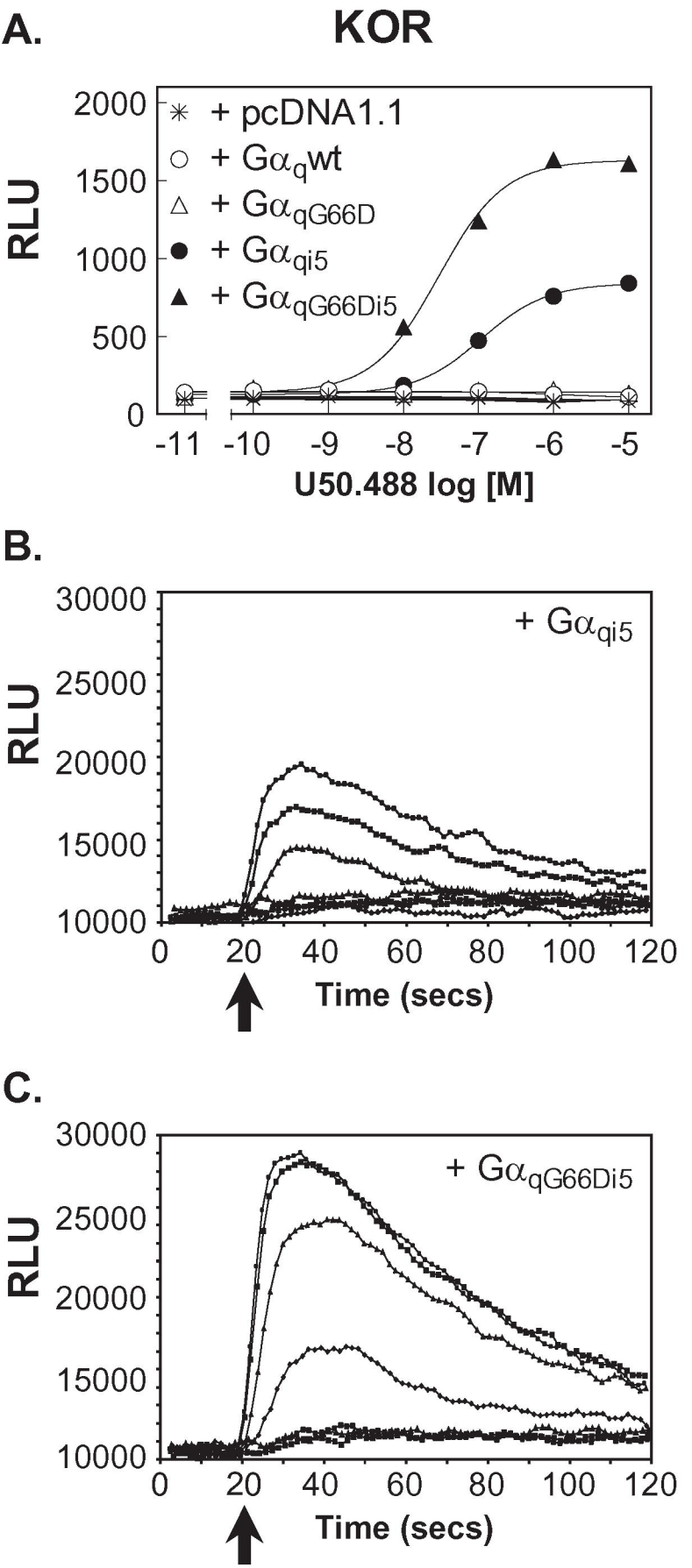
A.
KOR
RLU
2000
1500
1000
500
0
+ pcDNA1.1
+ Gαqwt
+ GαqG66D
+ Gαqi5
+ GαqG66Di5
-11
-10
-9
-8
-7
-6
-5
U50.488 log [M]
B.
RLU
30000
25000
20000
15000
10000
+ Gαqi5
0
20
40
60
80
100
120
Time (secs)
C.
RLU
30000
25000
20000
15000
10000
+ GαqG66Di5
0
20
40
60
80
100
120
Time (secs)

Fig. 5

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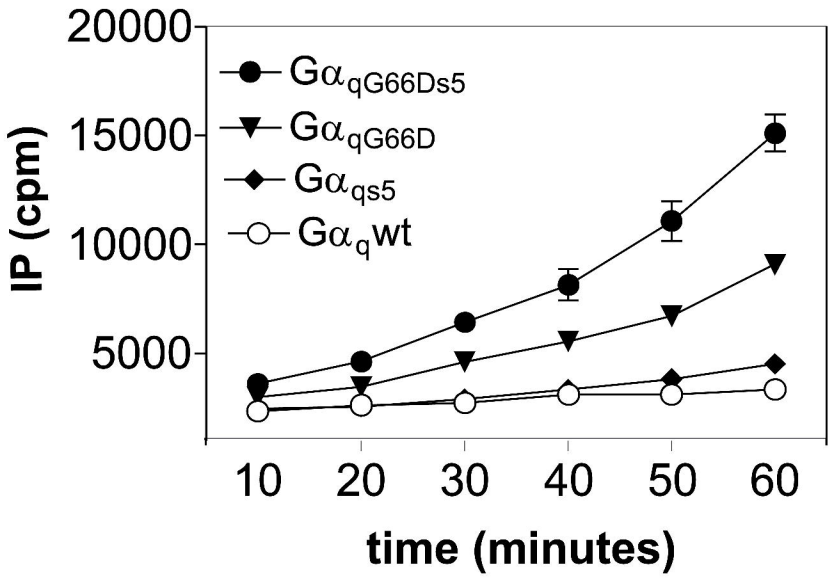

Fig. 6

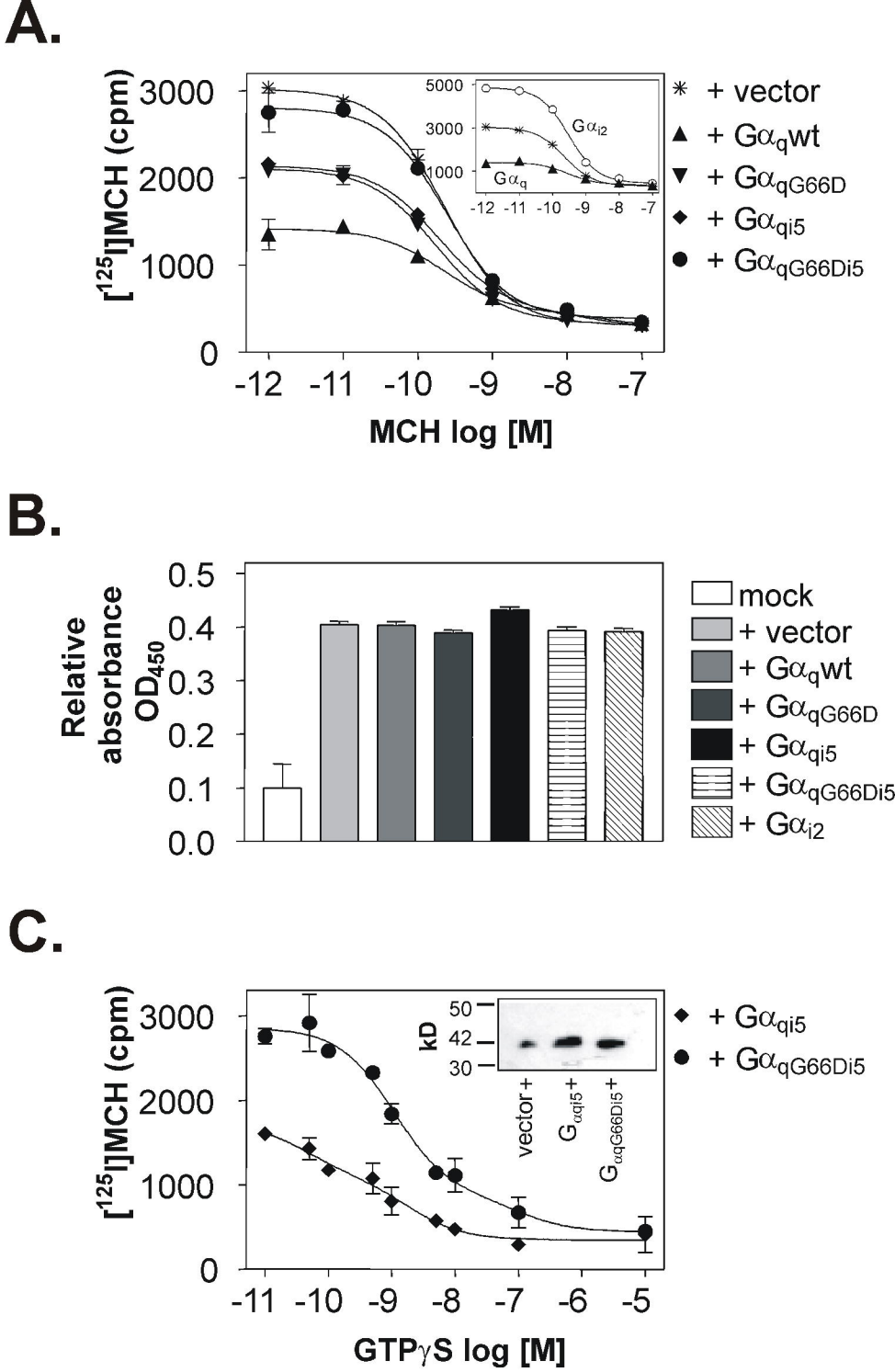
A.
[125I]MCH (cpm)
3000
2000
1000
0
-12 -11 -10 -9 -8 -7
MCH log [M]
5000
3000
1000
Gαi2
Gαq
-12 -11 -10 -9 -8 -7
+ vector
+ Gαqwt
+ GαqG66D
+ Gαqi5
+ GαqG66Di5
B.
Relative absorbance OD450
0.5
0.4
0.3
0.2
0.1
0.0
mock
+ vector
+ Gαqwt
+ GαqG66D
+ Gαqi5
+ GαqG66Di5
+ Gαi2
C.
[125I]MCH (cpm)
3000
2000
1000
0
-11 -10 -9 -8 -7 -6 -5
GTPγS log [M]
kD
50
42
30
vector +
Gαqi5 +
GαqG66Di5 +
+ Gαqi5
+ GαqG66Di5

Fig. 7