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Identification of a Novel Site within G Protein α Subunits Important for Specificity of Receptor-G Protein Interaction

Arne Heydorn, Richard J. Ward, Rasmus Jorgensen, Mette M. Rosenkilde, Thomas M. Frimurer, Graeme Milligan and Evi Kostenis

Laboratory for Molecular Pharmacology, the Panum Institute, University of Copenhagen, Copenhagen, Denmark (A.H., M.M.R.); 7TM Pharma A/S, Hørsholm, Denmark (R.J., T.M.F., E.K.); Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, Scotland, United Kingdom (R.J.W., G.M.)

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ABSTRACT

Several domains of G protein α subunits are implicated in the control of receptor-G protein coupling specificity. Among these are the extreme N-and C-termini, the α4/β6-loops, and the loop linking the N-terminal α-helix to the β1-strand of the ras-like domain. In this study, we illustrate that single-point mutations of a highly conserved glycine residue within the linker I region of the $G\alpha_q$ subunit confers upon the mutant $G\alpha_q$ the ability to be activated by $G\alpha_i$- and $G\alpha_s$-coupled receptors, as evidenced by guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding and inositol phosphate turnover assays. The mutations did not affect expression of $G\alpha_q$ proteins nor their ability to stimulate phospholipase Cβ. It is noteworthy that both mutant and wild-type $G\alpha_q$ proteins are indistinguishable in their ability to reconstitute a functional Gq-PLCβ-calcium signaling pathway when cotransfected with the $G\alpha_q$-coupled neurokinin 1 or muscarinic M3 receptor into mouse embryonic fibroblasts derived from $G\alpha_{q/11}$ knockout mice. On a three-dimensional model of the receptor-G protein complex, the highly conserved linker I region connecting the helical and the GTPase domain of the Gα protein is inaccessible to the intracellular surface of the receptors. Our data indicate that receptor-G protein coupling specificity is not exclusively governed by direct receptor-G protein interaction and that it even bypasses the requirement of the extreme C terminus of Gα, a well accepted receptor recognition domain, suggesting a novel allosteric mechanism for G protein-coupled receptor-G protein selectivity.

Heterotrimeric G proteins consisting of α, β, and γ subunits relay signals from activated seven transmembrane G protein-coupled receptors (GPCRs) to downstream effector proteins. Stimuli as diverse as light, odorants, nucleotides, neurotransmitters, and hormones exert their effects via the GPCR-G protein signal transduction cascade. Heterotrimeric G proteins are currently classified according to the nature of the α-subunit and are grouped into four families ($G\alpha_s$, $G\alpha_q$, $G\alpha_{i/o}$, and $G\alpha_{12/13}$) based on structural and functional similarity (Simon et al., 1991). A given GPCR typically interacts only with a limited subset of the G protein α-subunits, and the specific interaction of GPCRs with their cognate G proteins is essential for coordinated cellular signaling.

Extensive investigation of receptor peptides and chimeras has shown that the third intracellular loop sequence determines Gα coupling selectivity more often than does the second intracellular loop (Kobilka et al., 1988; Wess et al., 1989; Gomeza et al., 1996; Havlickova et al., 2003). Likewise, the first intracellular loop (Strader et al., 1994, 1995; Ho et al., 2002) or the receptor's C terminal tail (Perroy et al., 2001; Lai et al., 2002) rarely determine coupling specificity. On the level of the G protein α subunit, several different regions have been implicated in recognition of GPCRs and thus determine the specificity of receptor-G protein coupling: the extreme C terminus (Conklin et al., 1993; Kostenis et al., 1997a; Blahos et al., 1998; Havlickova et al., 2003; Slessareva et al., 2003), the extreme N terminus (Kostenis et al., 1997b; Slessareva and Graber, 2003), a region between the α4- and α5-helices (α4–β6 loop) (Mazzoni and Hamm, 1996; Bae et al., 1997; Onrust et al., 1997; Slessareva et al., 2003), and a region within the loop linking the N-terminal α-helix to the β1-strand of the ras-like domain (Blahos et al., 2001). Current models of receptor-G protein interaction based on available crystal structures of G protein α subunits predict these

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ABBREVIATIONS: GPCR, G protein-coupled receptor; IP_3, inositol 1,4,5-trisphosphate; GLP, glucagon-like peptide; GIP, gastric inhibitory peptide; IL, interleukin; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; S1P, Sphingosine 1-phosphate; UK14304; HA, hemagglutinin; CHO, Chinese hamster ovary; HEK, human embryonic kidney; IP, inositol phosphate; PBS, phosphate-buffered saline; PLC, phospholipase C; PTX, pertussis toxin; MEF, mouse embryonic fibroblast; KO, knockout.

four regions to be in direct contact with the GPCR and to determine the specificity of receptor-G protein coupling (Bourne, 1997; Blahos et al., 2001). No region within the G protein α subunit is yet known to influence the fidelity of GPCR-G protein interaction from a distance. X-ray crystallographic studies have revealed that Gα subunits consist of a GTPase domain and a helical domain that are connected by two highly conserved linker regions (Noel et al., 1993; Coleman et al., 1994). According to X-ray structures, the guanine nucleotide-binding site lies in a cleft between these two domains and about 30 Å from the cytoplasmic surface of the plasma membrane. To the best of our knowledge, mutational alterations of the linker I region have not been performed, and this region has not been implicated in functions other than keeping both Gα domains in the proper distance from each other. The linker I region lies near two of the three "switch regions" in the Gα structure that undergo conformational rearrangement during nucleotide exchange. We therefore speculated that interference with the conformational freedom of the linker I region may influence the specificity of receptor-mediated nucleotide exchange. We report herein the identification of a highly conserved glycine (glycine 66 in $G\alpha_q$) as a "hot spot" for constraining the fidelity of receptor-$G\alpha_q$ interaction. Mutational replacement of glycine by amino acids with different physicochemical properties (acidic, basic, uncharged side chains) and hence reduced conformational flexibility confers upon $G\alpha_q$ the ability to link various non–Gq-coupled receptors to the phospholipase Cβ-IP_3-signaling pathway. This is the first report to describe fidelity of receptor-G protein interaction governed by residues other than those at the direct receptor G protein interface and suggests a novel mechanism for receptor-G protein selectivity.

Materials and Methods

Materials. Glucagon-like peptide 1 (GLP1), gastric inhibitory peptide (GIP), and interleukin-8 (IL-8) were from Bachem (Torrance, CA); RANTES (regulated on activation normal T cell expressed and secreted) was from PeproTech (London, UK); (−)-quinpirole and carbachol were from RBI-Sigma (Natick, MA). [^{35}S]GTPγS was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). *myo*-[^{3}H]inositol with PT6-271 stabilizer was purchased from Amersham Biosciences (Uppsala, Sweden). Chemerin9 was synthesized by UFpeptides (Ferrara, Italy), and PGD2 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Sphingosine 1-phosphate (S1P), UK14304, substance P, isoproterenol, acetyl-β-methylcholine chloride (methacholine), pertussis toxin, and the remaining chemicals were purchased from Sigma (St. Louis, MO).

DNA Materials and Construction. Murine wild-type $G\alpha_q$ was cloned from Mouse brain by reverse transcription-polymerase chain reaction and inserted as a BamHI-NsiI fragment into the pcDNA1.1 expression plasmid. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to generate $G\alpha_{qG66D/N/V/K}$ using $G\alpha_q$ as a template. $G\alpha_{qQ209L}$ was obtained from Guthrie cDNA Resource Center (http://www.cdna.org). $G\alpha_{qG66D/Q209L}$ was created with the QuikChange kit using $G\alpha_{qG66D}$ as a template. All wild-type and mutant $G\alpha_q$ proteins contained an internal hemagglutinin (HA) epitope tag, DVPDYA, replacing $G\alpha_q$wt residues 125 to 130. The sequences of the Gα constructs were confirmed by automated sequencing. Sequences of primers used are available upon request. The expression plasmids for the human chemokine CXCR1, CXCR2, and CCR5 receptors were kindly provided by Timothy N. C. Wells (Serono Pharmaceutical Research Institute, Geneva, Switzerland); human dopamine D2 receptor was kindly provided by Hubert H. M. Van Tol (University of Toronto, Toronto, ON, Canada); human M2 and M3 muscarinic acetylcholine receptors were kindly provided by Tom I. Bonner (National Institutes of Health, Bethesda, MD); and human β_2 adrenergic receptor was kindly provided by Brian Kobilka (Stanford University, Stanford, CA). The human GIP receptor was cloned from a stomach cDNA library and inserted as a BamHI-EcoRI fragment into pcDNA3.1(+). The human GLP1 receptor was cloned from a pancreas cDNA library and inserted via EcoRI sites into pcDNA3.1(+). The adrenergic α_{2A} receptor was cloned from a human cDNA library into pSI. The intronless coding sequences of the human $S1P_1$ (edg1) and $S1P_5$ (edg8) receptors were amplified from human genomic DNA and inserted as an XbaI-HindIII fragment into pcDNA3.1(−) ($S1P_1$ receptor) and as a HindIII-EcoRI fragment into pcDNA3.1(+) ($S1P_5$ receptor). ChemR23 and the prostaglandin D2 DP receptor were cloned from a bone marrow cDNA library and inserted as a BamHI-EcoRI (ChemR23) or HindIII-BamHI (DP receptor) fragment into pcDNA3.1(+).

Cell Culture and Transfections. COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 180 units/ml penicillin and 45 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. COS-7 cells were transiently transfected with the indicated receptor and G protein constructs at a 1:1 ratio using the FuGENE 6 transfection reagent (Roche Applied Science, Mannheim, Germany) according to the supplied protocol.

CHO-K1 cells were grown in basal Iscove medium (Biochrom KG, Berlin, Germany) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 μg/ml gentamicin, 180 units/ml penicillin, and 45 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. CHO-K1 cells were transiently transfected with the indicated receptor and G protein constructs at a 1:1 ratio using LipofectAMINE (Invitrogen, Carlsbad, CA) according to the supplied protocol.

HEK293 cells were grown in Dulbecco's modified Eagle's medium with GlutaMAX and 4500 mg/l glucose supplemented with 10% (v/v) fetal calf serum, 180 units/ml penicillin, and 45 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. HEK293 cells were transiently transfected with the indicated receptor and G protein constructs at a 1:1 ratio using LipofectAMINE 2000 (Invitrogen) according to the supplied protocol.

An embryonic fibroblast cell line (EF88) derived from a combined $G\alpha_q/G\alpha_{11}$ double knockout mouse was kindly provided by Prof. Stefan Offermanns (University of Heidelberg, Heidelberg, Germany) and used in IP turnover assays, single-cell Ca^{2+} imaging, and Western blots (Offermanns et al., 1998). EF88 cells were grown in Dulbecco's modified Eagle's medium with GlutaMAX and 4500 mg/l glucose supplemented with 10% (v/v) fetal calf serum and 1% (v/v) nonessential amino acids (Invitrogen), 180 units/ml penicillin, and 45 μg/ml streptomycin. For transfections of EF88 cells with NK1 for IP turnover assays (Fig. 5A, inset), cells were transiently transfected with the NK1 receptor and G protein constructs or empty vector at a 1:1 ratio using LipofectAMINE 2000 (Invitrogen) according to the supplied protocol. EF88 cells used for single-cell calcium imaging assays and immunoblot analysis (Fig. 5, B and C) were transfected using the Nucleofection system (Amaxa Biosystems, Cologne, Germany), according to the general protocol for Nucleofection of adherent cells. Cells were washed with 1× PBS, trypsinized, and then counted. The appropriate volume of cells (enough for 3×10^6 cells per well) was centrifuged, and the media were completely removed. The cells were then resuspended in 100 μl of Nucleofection solution V for each well. The cell suspension (100 μl) was added to 5 μg of DNA and then transferred to a Nucleofection cuvette. Program T-20 of the Nucleofector was used, and then 500 μl of prewarmed media were added to the cuvette. The contents of the cuvette were transferred to one well of a six-well dish containing 1.5 ml of media. This was repeated for each transfection. The cells were incubated overnight at 37°C, and the media were changed the following morning.

Inositol Phosphate Assays (IP Accumulation). One day after transfection, cells (1×10^5 cells/well) were incubated for 24 h with 2 μCi of *myo*-[^{3}H]inositol in 0.3 ml of growth medium per well. Cells

were then washed twice in 20 mM HEPES, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, and 0.05% (w/v) bovine serum albumin. Cells were then stimulated in 0.5 ml of the same buffer supplemented with 10 mM LiCl at 37°C for 45 min with the appropriate agonist ligands or aluminum fluoride (final concentrations, 10 mM NaF and 300 μM AlF_3). Cells were extracted by addition of 1 ml of 10 mM formic acid to each well followed by incubation on ice for 60 min. The generated [^{3}H]inositol phosphates were purified on AG 1-X8 anion exchange resin from Bio-Rad (Hercules, CA) (Berridge et al., 1983). Determinations were made in duplicates.

Membrane Preparation and Western Blotting. COS-7 cells were transfected in 100-mm dishes with 4 μg of G protein plasmid DNA and 4 μg of pCDNA3.1 vector DNA as a control or 4 μg of CCR5 or GIP receptor using the FuGENE 6 transfection reagent (Roche) according to the supplied protocol. For 8 μg of plasmid DNA, 12 μl of Fugene was used. 72 h after transfection, cells were washed in ice-cold PBS and harvested in a total volume of 2 ml of PBS containing 1 mM phenylmethylsulfonyl fluoride. Cells were pelleted and either stored at −80°C or immediately processed to membranes. Cell pellets were resuspended in an ice-cold buffer containing 50 mM Tris, pH 8, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (buffer A), and ruptured with 20 strokes of a hand-held glass homogenizer followed by passage (10 times) through a 27-gauge needle. Nuclei and cell debris were pelleted by centrifugation (1000*g*, 5 min, 4°C), and the resulting supernatant was fractionated (100,000*g*, 30 min, 4°C) into a membrane pellet and supernatant. Pellets were then resuspended in buffer A. 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 an anti–HA-peroxidase–linked monoclonal antibody (12CA5; Roche) diluted 1:1000 in 10 mM Tris, pH 8, 150 mM NaCl, 10% SDS, and 0.1% Tween 20. Immunoreactive proteins were visualized with the ECL kit from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Standard P2 membrane preparations from EF88 cells and Western blotting were performed essentially as described previously (Liu et al., 2002).

[^{35}S]GTPγS Binding Assays. [^{35}S]GTPγS binding assays were done essentially as described previously (Carrillo et al., 2002). HEK293 cells were transfected with either the wild-type $S1P_1$ receptor, the GIP receptor, or vector DNA and cotransfected with either $G\alpha_q$wt, $G\alpha_{qG66D}$, or a pertussis toxin-insensitive variant (C351I) of $G\alpha_{i1}$ (Bahia et al., 1998). Twenty-four hours later, cells were treated with pertussis toxin (25 ng/ml) for 24 h, harvested, and membrane fractions were prepared. Membrane protein (10 μg) was incubated in GTPγS binding buffer with 50 nCi of [^{35}S]GTPγS and 1 μM GDP with or without 20 μM S1P or 2 μM GIP. Parallel assays containing 100 μM nonradioactive GTPγS were used to define nonspecific binding. Samples were incubated for 15 min at 30°C and then immunoprecipitated with the anti-Gq/G11 antiserum CQ (Mitchell et al., 1991) or the anti Gi1/Gi2 antiserum SG (Green et al., 1990). Samples were then counted. Data are means ± S.E.M. of three independent experiments, each performed in triplicate.

Results

Construction, Expression, and Functionality of Wild-Type and Mutant Gα_q Subunits. We tested the hypothesis of whether conformational flexibility of the linker I region of Gα proteins is a determinant of receptor-G protein coupling specificity using the $G\alpha_q$ subunit as a model system. We initially generated various mutant α_q subunits in which a glycine residue at position 66 within linker I, highly conserved in all Gα subunits, was replaced by amino acids with different side chains and hence presumably less conformational freedom. The mutant $G\alpha_q$ proteins are referred to as $G\alpha_{qG66D}$, $G\alpha_{qG66V}$, $G\alpha_{qG66N}$, and $G\alpha_{qG66K}$. Glycine was chosen for its high conservation in all Gα subunits irrespective of their species origin and thus presumably its common role in Gα subunit structure and functionality. All mutant and wild-type α_q subunits contained an internal HA epitope tag replacing α_q residues 125 to 130. Previous studies have shown that the presence of the epitope tag does not affect the receptor and effector coupling properties of $G\alpha_q$ wild type (Conklin et al., 1993). Immunoblots with an anti-HA monoclonal antibody (peroxidase-linked 12CA5) showed that all mutant $G\alpha_q$ subunits were expressed at levels comparable with $G\alpha_q$ wild type, independent of the presence or absence of a cotransfected G protein-coupled receptor (Fig. 1A).

To determine whether the mutant $G\alpha_q$ proteins were capable of assuming the active conformation, fluoroaluminate (AlF_4^-) was used to activate the respective α-subunits in transiently transfected COS-7 cells. AlF_4^- typically activates G protein α subunits by binding to the GDP-bound form of Gα. The resulting Gα-GDP-AlF_4^- 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. We tested the effects of 300 μM AlF_4^- on Gα subunit-mediated IP accumulation and found that both wild-type and mutant $G\alpha_q$ proteins were capable of stimulating IP turnover to about 2.2- to 2.8-fold above basal levels, indicating that the introduced mutations did not interfere with the ability of the Gα proteins to assume an active conformation.

Interaction of Mutant Gα_q Subunits with Prototypical Gi- and Gs-Coupled Receptors. To study the effects of the various mutations on the fidelity of receptor-G protein interactions, all mutant $G\alpha_q$ subunits were initially coexpressed in COS-7 cells with the Gs-coupled GLP1 receptor, GIP receptor, prostaglandin D2 DP receptor, or the Gi-coupled chemokine CCR5 or ChemR23 receptor (Fig. 1, B–F). Transfected cells were incubated with increasing concentrations of the respective agonists, and the ability of each receptor to activate the various $G\alpha_q$ constructs was determined by measuring IP turnover. Coexpression of the receptors with either vector DNA (pcDNA1) or $G\alpha_q$ wild type, followed by ligand stimulation, resulted in no measurable (GLP1, CCR5) or relatively small (GIP, DP, ChemR23) increases in PLCβ activity (Fig. 1, B–F). It was remarkable that all five Gi- and Gs-coupled receptors gained the ability to productively couple to all mutant α_q subunits, resulting in a 3- to 10-fold stimulation of IP accumulation above basal levels (Fig. 1, B–F). Conformational freedom of the linker I imparted by the glycine residue is apparently crucial for maintaining the fidelity of $G\alpha_q$ recognition by ligand-activated GPCRs.

Role of G66D Mutation in Gi- and Gs-Coupled Receptor-Mediated Activation of Phospholipase Cβ. To test the importance of the linker I region, in particular the highly conserved glycine residue for receptor coupling selectivity, we used the $G\alpha_{qG66D}$ mutant as a model system for investigating coupling promiscuity upon cotransfection with eight additional non–Gq-coupled receptors in functional assays monitoring IP accumulation (Fig. 2). To this end, CHO or COS-7 cells were transfected with cDNAs encoding the D2 dopamine receptor, muscarinic M2 receptor, $S1P_1$ and $S1P_5$ receptors, chemokine CXCR1 and CXCR2 receptors, and α_{2A}-

and β_2-adrenergic receptors. Each receptor was cotransfected with $G\alpha_q$ wild type, $G\alpha_{qG66D}$, or vector DNA as a control. Cells transfected with only vector DNA or Gα subunit did not respond to any concentrations of the agonists tested in this study (data not shown). Likewise, cotransfection of the various non–Gq-linked receptors with $G\alpha_q$ wild type did not stimulate IP accumulation ($S1P_5$, CXCR2) or only marginally stimulated IP accumulation (D2, M2, $S1P_1$, CXCR1, α_{2A}, β_2). The only Gi-coupled receptor that robustly stimulated IP accumulation upon coexpression with $G\alpha_q$wt was the α_{2A} receptor. The α_{2A} receptor is known to preferentially interact with $G\alpha_i$ but gains the ability to stimulate adenylylcyclase or phospholipase C when the respective $G\alpha_s$ and $G\alpha_q$ proteins are overexpressed (Chabre et al., 1994). It was interesting that all tested receptors responded with a robust increase in IP turnover upon cotransfection with $G\alpha_{qG66D}$. Thus, the G66D mutant has clearly lost the ability to discriminate between receptors from different coupling classes.

Receptor Coupling Properties of $G\alpha_{qG66D}$ to the Gi-Coupled CCR5 Receptor in the Presence of Pertussis Toxin. To make sure that the IP turnover observed upon coexpression of Gi-coupled receptors with $G\alpha_{qG66D}$ was caused by receptor-mediated activation of $G\alpha_{qG66D}$ rather than increased βγ-mediated PLCβ stimulation via an activated $G\alpha_i$ protein, we measured CCR5 receptor-mediated IP turnover in the presence or absence of PTX (Fig. 3). CCR5 did not mediate IP turnover when expressed alone or in combination with $G\alpha_q$ wild type but robustly stimulated IP turnover when coexpressed with $G\alpha_{qG66D}$. It was noteworthy that IP turnover mediated by CCR5 coexpressed with $G\alpha_{qG66D}$ was virtually the same in the presence or absence of PTX. This strongly suggests that the IP turnover generated by the Gi-coupled CCR5 receptor is caused by receptor-mediated activation of the $G\alpha_{qG66D}$ protein.

Role of the G66D Mutation in G Protein Activation by Gi- and Gs-Coupled Receptors as Assessed by Incorporation of [^{35}S]GTPγS into $G\alpha_q$ Proteins. Replacement of GTP for GDP on the α subunit of a trimeric G protein represents the first step for signal transduction mediated via G protein-coupled receptors and is—as opposed to classic second messenger assays—a direct way of determining the effectiveness of GPCR-G protein interaction. As measurement of GTPγS binding on $G\alpha_q$ proteins is difficult to assess in standard membrane [^{35}S]GTPγS assays, we combined the GTPγS activation step with $G\alpha_q$-selective immunoprecipitation to enrich for the $G\alpha_q$ species and thus increase the signal to background ratio (Milligan, 1993). Figure 4 shows agonist-induced stimulation of [^{35}S]GTPγS binding followed by immunoprecipitation with a $G\alpha_q$-selective antiserum in membranes prepared from COS-7 cells transiently transfected with the G_i-coupled $S1P_1$ or the G_s-coupled GIP receptor and the respective $G\alpha_q$ proteins. It was evident that sphingosine-1-phosphate (20 μM) and gastric inhibitory peptide (2 μM) stimulate significant ($p < 0.05$) [^{35}S]GTPγS incorporation

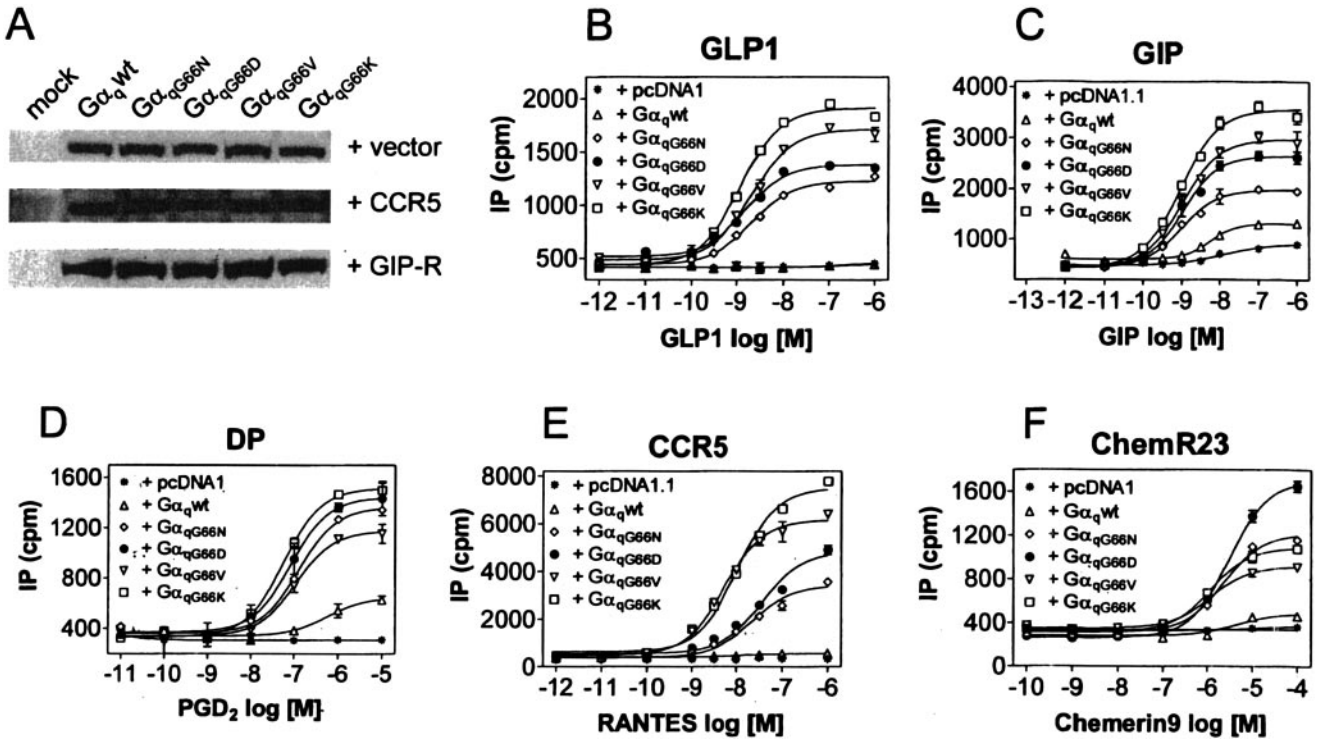

Fig. 1. Expression and functional analysis of wild-type and mutated $G\alpha_q$ subunits carrying single-point mutations of a highly conserved glycine residue (G66) within the linker I region. A, membranes were prepared from COS-7 cells transiently transfected with expression plasmids coding for the various wild-type and mutant $G\alpha_q$ proteins alone or in combination with the chemokine CCR5 or the GIP receptor, respectively, and Gα proteins were separated by SDS-polyacrylamide gel electrophoresis. After blotting onto nitrocellulose membranes, G protein α subunits were visualized with the anti–HA-peroxidase–linked monoclonal antibody as described under *Materials and Methods*. Immunoreactive Gα subunits run at approximately 42 kDa. B–F, COS-7 cells were cotransfected with expression plasmids coding for the Gi-coupled chemokine CCR5, ChemR23 receptor, or the Gs-coupled GIP, GLP1, or DP prostaglandin D2 receptor and the indicated G proteins or pCDNA1.1 vector DNA as a control. Transfected cells were incubated for 45 min at 37°C in the presence of various concentrations of the respective agonists, and IP turnover was determined as described under *Materials and Methods*. Basal IP turnover levels (no ligand added) were similar for vector and Gα subunit transfected cells, and the presence of receptors did not cause any alteration in basal second messenger production (not shown). Data show one of three representative experiments, each carried out in duplicate.

only when their receptors are coexpressed with $G\alpha_{qG66D}$, not with vector or $G\alpha_q$ wild-type DNA or a $G\alpha_i$ subunit rendered unresponsive to PTX. This result provides additional evidence that receptors otherwise not or at best poorly coupled to $G\alpha_q$ gain the ability to directly stimulate Gq and thus the Gq-PLCβ-IP_3 effector pathway when coexpressed with the $G\alpha_{qG66D}$ mutant.

Role of G66D Mutation in Gq-Coupled Receptor-Mediated Activation of Phospholipase Cβ. To test whether the G66D mutant also affects Gq-coupled receptor signaling, we used COS-7 cells transiently transfected with the neurokinin NK1 receptor and measured ligand-stimulated IP accumulation (Fig. 5A). Although these cells express $G\alpha_q$ endogenously, IP accumulation in response to stimulation with the NK1 agonist substance P (SP) could be further enhanced upon cotransfection with $G\alpha_q$wt. It was notable that coexpression of $G\alpha_{qG66D}$ lead to a leftward shift of the agonist dose response curves comparable in magnitude to the shift observed with $G\alpha_q$ wild type. Because the analysis of coupling properties of Gq-linked receptors with mutant $G\alpha_q$ subunits is hampered by the fact that Gq-coupled receptors generate robust IP accumulation in almost all cell lines, we used mouse embryonic fibroblasts (MEFs) derived from a $G\alpha_q/G\alpha_{11}$ double-knockout (KO) mouse to confirm the coexpression data obtained in COS-7 cells. Figure 5A, inset, depicts IP accumulation in $G\alpha_{q/11}$ KO MEFs transfected with the NK1 receptor alone or the wild-type and mutant $G\alpha_q$ protein. Substance P clearly does not stimulate IP accumulation in cells transfected with the NK1 receptor alone but causes robust IP accumulation when the NK1 receptor is coexpressed with either $G\alpha_q$wt or $G\alpha_{qG66D}$. Equal effectiveness of $G\alpha_q$wt and $G\alpha_{qG66D}$ was also observed when they were coexpressed in $G\alpha_{q/11}$ KO MEFs with the Gq-selective muscarinic M3 receptor and analyzed in a single-cell calcium imaging assay (Fig. 5B). It should be noted that $G\alpha_q$wt and $G\alpha_{qG66D}$ were expressed equally well in the $G\alpha_{q/11}$ KO MEFs (Fig. 5C). The data obtained in COS-7 and $G\alpha_{q/11}$ knockout cells show collectively that the G66D mutant is indistinguishable from $G\alpha_q$wt in recognizing receptors from the Gq class and hence provide strong support for the notion that

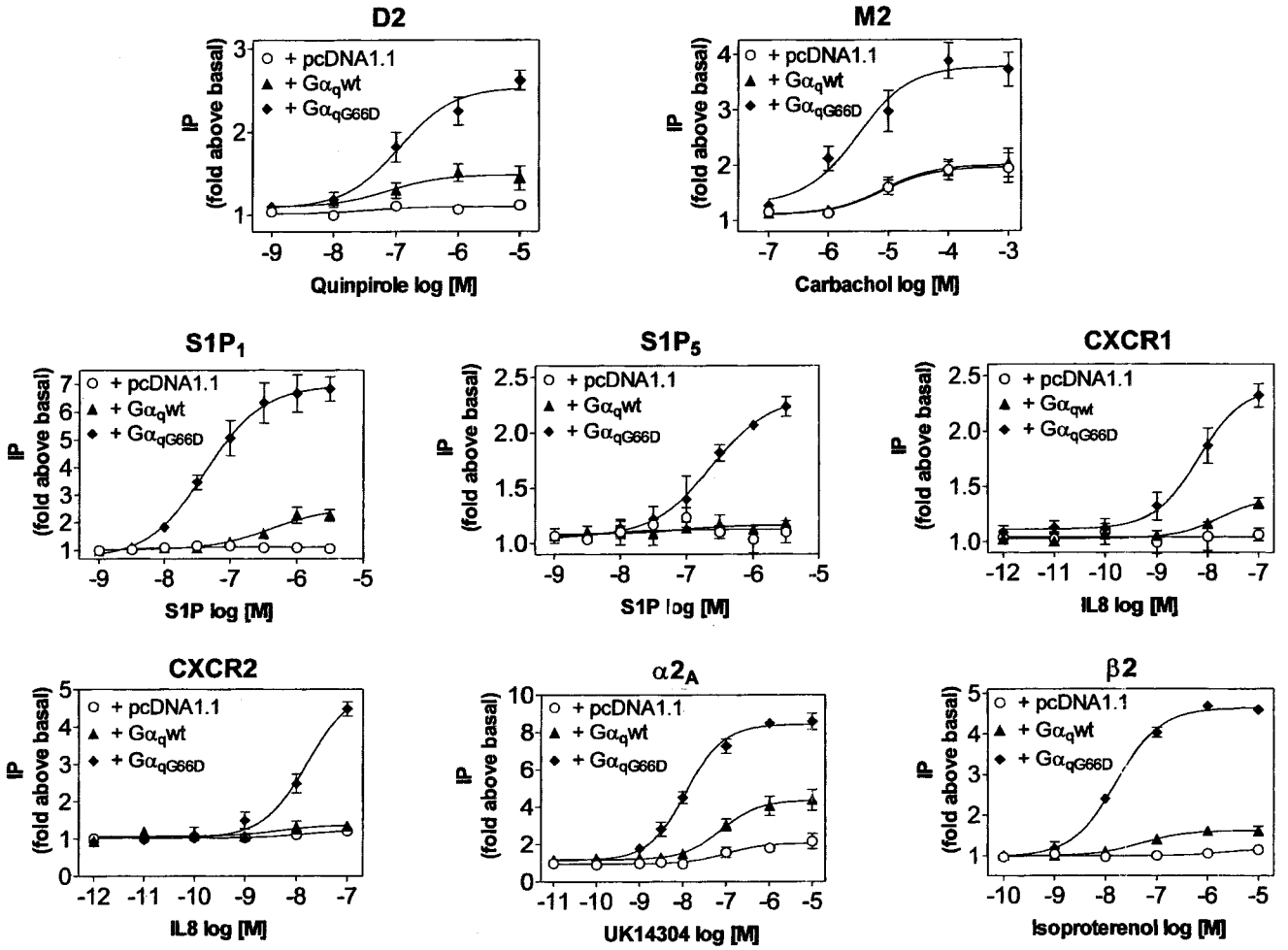

Fig. 2. Functional interaction of selected non–Gq-coupled receptors with $G\alpha_q$wt and $G\alpha_{qG66D}$. COS-7 or CHO cells were cotransfected with expression plasmids coding for the D2 dopamine receptor, muscarinic M2 receptor, $S1P_1$ or $S1P_5$ receptors, chemokine CXCR1 and CXCR2 receptor, α_{2A}-adrenergic receptor, β_2-adrenergic receptor, and the indicated G proteins or pCDNA1.1 vector DNA as a control. CHO cells were used for functional expression of the sphingosine-1-phosphate receptors and the β_2-adrenergic receptors because mock-transfected cells do not accumulate IP in response to the agonists S1P and isoproterenol. Transfected cells were incubated for 45 min at 37°C in the presence of various concentrations of the appropriate agonist ligands. Basal IP turnover levels (no ligand added) were similar for vector- and Gα-subunit–transfected cells, and the presence of receptors caused no alteration in basal second messenger production (not shown). The resulting increases in intracellular IP turnover levels (-fold stimulation above basal) were determined as described under *Materials and Methods*. Data are given as means ± S.E. of three to five independent experiments, each carried out in triplicate.

$G\alpha_{qG66D}$ represents a truly universal G protein capable of linking receptors from all major coupling classes to intracellular IP accumulation. In addition, the data strongly support the notion that $G\alpha_{qG66D}$ is a truly promiscuous as opposed to a Gq-hypersensitizing mutant.

$G\alpha_{qG66D}$ Retains the Ability to Stimulate PLCβ but Does Not Display Intrinsic PLCβ Stimulation. Next, we tested whether the $G\alpha_{qG66D}$ mutant protein has retained the ability to stimulate PLCβ by using a constitutively active mutant of G66D. Mutation at codon 209 of $G\alpha_q$ is known to confer constitutive activity onto $G\alpha_q$ by impairing the intrinsic GTPase activity, and we therefore generated a mutant protein harboring the G66D mutation in combination with the Q209L mutation (the mutant protein is referred to as $G\alpha_{qG66D/Q209L}$). Expression of $G\alpha_{qG66D/Q209L}$ in COS-7 cells also led to increased basal IP accumulation, comparable in extent with the IP accumulation induced by the GTPase-deficient $G\alpha_{qQ209L}$ (Fig. 6). The intrinsic IP accumulation caused by $G\alpha_{qG66D/Q209L}$ suggests that IP accumulation generated upon coexpression of $G\alpha_{qG66D}$ with various non–Gq-coupled receptors is indeed mediated via stimulation of PLCβ isoforms. It should also be highlighted that the G66D mutant alone did not augment basal IP accumulation levels nor did it cause an apparent alteration in basal IP accumulation when coexpressed with the various receptors used in this study.

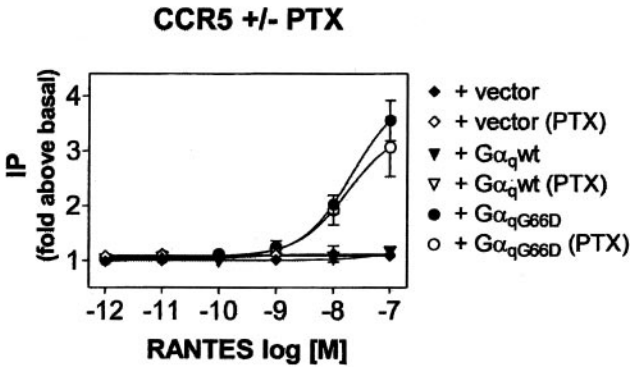

Fig. 3. Effect of PTX on IP accumulation in cells cotransfected with $G\alpha_q$wt or $G\alpha_{qG66D}$ and the $G_{i/o}$-coupled CCR5 receptor. COS-7 cells were cotransfected with expression plasmids coding for the wild-type CCR5 receptor and vector DNA (pcDNA1.1), $G\alpha_q$wt, or $G\alpha_{qG66D}$. Transfected cells were incubated for 45 min at 37°C in the presence of various concentrations of the agonist RANTES and in the absence or presence of PTX (100 ng/ml). Increases in intracellular IP turnover levels were determined as described under *Materials and Methods*. Data are given as the means ± S.E. of three independent experiments, each carried out in triplicate.

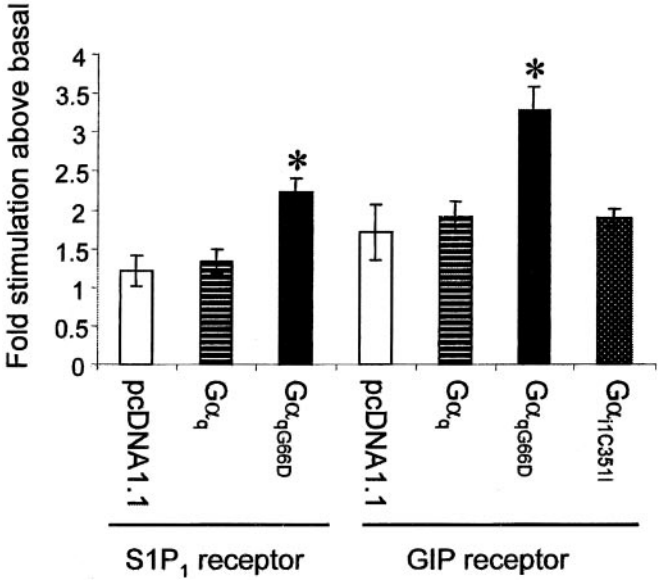

Fig. 4. Agonist-induced [^{35}S]GTPγS binding to the $S1P_1$ and GIP receptor in the absence and presence of the indicated Gα proteins. HEK293 cells were transfected with either the wild-type $S1P_1$ receptor or the GIP receptor and cotransfected with either vector DNA, $G\alpha_q$wt, $G\alpha_{qG66D}$, or a pertussis toxin-insensitive variant (C351I) of $G\alpha_{i1}$ (Bahia et al., 1998). [^{35}S]GTPγS binding assays were done as described under *Materials and Methods*. Because binding of [^{35}S]GTPγS to endogenously expressed $G\alpha_i$ subtypes is much greater than to receptor-activated $G\alpha_q$, even in the absence of agonist stimulation, at the termination of incubation, samples were immunoprecipitated with antipeptide antisera directed against the C-terminal decapeptides of $G\alpha_{q/11}$ or $G\alpha_{i1/2}$, thereby extending the utility of [^{35}S]GTPγS binding assays (Milligan, 2003). Statistically significant increases in [^{35}S]GTPγS binding caused by the agonists S1P and GIP are indicated as *, $p < 0.05$.

Molecular Modeling of Linker I and the Adjacent α-Helices of $G\alpha_q$ Proteins. To gain more insight into the molecular mechanism underlying the observed promiscuity of the $G\alpha_q$ proteins, a molecular model of the linker I region and its adjacent αA and α1 helices was generated (Fig. 7). The model was based on the coordinates 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 highly conserved glycine 66 is located centrally within the short linker sequence and is visualized in ball shape (Fig. 7B) and highlighted in bold (Fig. 7A). Glycine 64 terminates the α1 helix of the helical domain and engages in a hydrogen bond with the backbone carbonyl moiety of arginine 60 (Fig. 7). It is interesting that the backbone nitrogen of the highly conserved glycine 66 can form a hydrogen bond to the same carbonyl of arginine 60. It is important to note that hydrogen bonds can also be formed between arginine 60 and tyrosine 67, as well as between serine 68 and aspartate 71, indicating that both α1- and αA-helices are stabilized by an intra- and interhelical hydrogen bond network. Glycine 66 is the central residue within linker I and represents the only amino acid that would be tolerated at this position without interfering with the local packing of α1-and αA-helix, respectively, or the "conformational freedom" of the linker because it contains only a hydrogen atom as a sidechain. The unique role of glycine 66 for Gα protein structure and function also becomes evident when the high-resolution crystal structure of $G\alpha_i$ is viewed as a Ramachandran plot (not shown). The φ,ψ dihedral angles for glycine 66 amount to 93 and −118, respectively, indicating that no amino acid other than glycine allows adoption of the required peptide backbone conformation.

Discussion

Several regions within the Gα protein sequence are known to be involved in the selectivity of its activation by cell surface GPCRs. By far the best characterized region is the extreme C terminus of the Gα protein comprising the last nine amino acids (Conklin et al., 1993; Kostenis et al., 1997a; Blahos et al., 1998; Sasamura et al., 2000), and it has been shown that mutational alteration of the C-terminal sequence comprising as few as one amino acid may be sufficient to change the coupling specificity of a given Gα subunit (Kostenis et al., 1997a). Other regions determining coupling selectivity of Gα

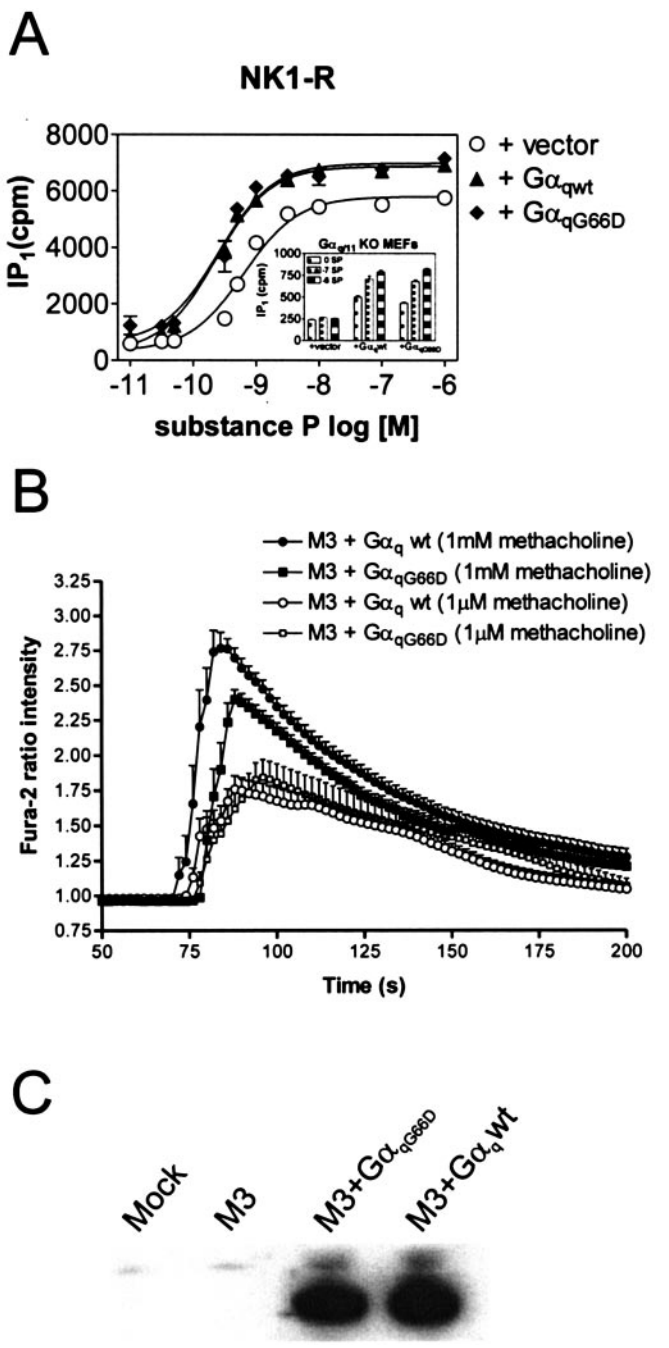

Fig. 5. A, $G\alpha_q$wt and $G\alpha_{qG66D}$ are activated equally by the $G\alpha_q/G\alpha_{11}$-coupled neurokinin NK1 receptor in COS-7 cells and $G\alpha_q/G\alpha_{11}$ knockout cells. COS-7 cells were cotransfected with expression plasmids coding for the neurokinin NK1 receptor and the indicated G proteins or pcDNA1.1 vector DNA as a control. Transfected cells were incubated for 45 min at 37°C in the presence of various concentrations of substance P (SP). The resulting increases in intracellular IP turnover levels were determined as described under *Materials and Methods*. Data show one of three independent experiments, each carried out in triplicate. Inset, SP-induced IP accumulation in $G\alpha_{q/11}$ knockout mouse embryonic fibroblasts (EF88) transfected with the NK1 receptor alone or in combination with either $G\alpha_q$wt or $G\alpha_{qG66D}$. Transfected cells were challenged with SP (100 and 1000 nM), and IP accumulation was determined as described above. Data shown are means ± S.E. of three independent experiments, each performed in duplicate. B, $G\alpha_q$ and $G\alpha_{qG66D}$ are activated equally by the $G\alpha_q$-coupled muscarinic M3 receptor in $G\alpha_q/G\alpha_{11}$ knockout cells. Single-cell Ca^{2+} imaging (Liu et al., 2002) was performed on EF88 cells transfected to coexpress the M3 muscarinic acetylcholine receptor along with either wild-type $G\alpha_q$ or $G\alpha_{qG66D}$ and stimulated with a saturating (1 mM) or subsaturating (1 μM) concentrations of acetyl-β-methylcholine chloride (methacholine). Data are means ± S.E.M. derived from analysis of 15 individual cells for each condition. No elevation of $[Ca^{2+}]$ was recorded unless the receptor and one of the forms of $G\alpha_q$ were cotransfected (data not shown). C, equivalent expression of $G\alpha_q$ and $G\alpha_{qG66D}$ in $G\alpha_q/G\alpha_{11}$ knockout cells. EF88 cells were mock transfected or transfected to express the M3 muscarinic acetylcholine receptor along with either $G\alpha_{qG66D}$ or $G\alpha_q$wt. After cell harvest, P2 membrane preparations were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with antiserum CQ, which identifies the C-terminal decapeptide common to $G\alpha_q$ and $G\alpha_{11}$. Immunoreactive $G\alpha$ subunits run at approximately 42 kDa.

proteins comprise the N terminus (Kostenis et al., 1997b; Slessareva and Graber, 2003; Slessareva et al., 2003), the region between the α4- and α5-helices (α4–β6 loop) (Bae et al., 1997; Onrust et al., 1997), and a region within the loop linking the N-terminal α-helix to the β1-strand of the ras-like domain (Blahos et al., 2001) (all receptor-Gα protein contact sites are colored magenta in Fig. 8). Evidence from mutagenesis and X-ray crystallographic studies as well as models of receptor-G protein complexes based on these studies suggest that all regions highlighted in magenta in Fig. 8 are in close contact with the intracellular surface of the receptor protein. No region within Gα is yet known to control specificity of receptor-G protein interaction from a region other than the postulated direct receptor-G protein interface.

G-protein α-subunits consist of two domains: a ras-like domain (also referred to as GTPase domain), structurally homologous to monomeric G proteins and so called because of its resemblance to the oncoprotein $p21^{ras}$, and a more divergent domain, unique to heterotrimeric G-proteins, referred to as the helical domain (Fig. 8). G-protein activation requires the exchange of bound GDP for GTP, and because the guanine nucleotide is buried in a deep cleft between the domains, it has been postulated that activation may involve a conformational change that will allow the opening of this cleft (Rondard et al., 2001). The movement of the GTPase domain away from the helical domain is crucial for the opening process, and the linker regions are thought to act as hinges because they are relatively close together toward the phosphate end of the nucleotide (Noel et al., 1993). Whereas the linker II region constitutes one of the three switch regions known to undergo severe conformational alteration upon Gα activation, nothing is known about linker I except that it acts as a tether between the helical and ras-like domain (Coleman et al., 1994). There are, however, naturally occurring G protein variants that differ in the composition of the otherwise highly conserved linker I region; the predominant splice variants of $G\alpha_s$ ($G\alpha_s$ short and $G\alpha_s$ long) differ in the amino acid

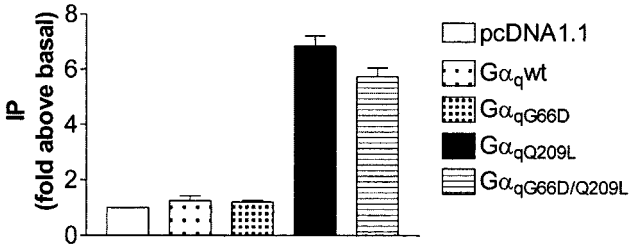

Fig. 6. Functional characterization of $G\alpha_q$wt, $G\alpha_{qG66D}$, $G\alpha_{qQ209L}$, and $G\alpha_{qG66D/Q209L}$ proteins. Basal IP accumulation (intrinsic stimulation of PLCβ) was determined in COS-7 cells transiently transfected with vector DNA and the various wild-type and mutant $G\alpha_q$ proteins as described in detail under *Materials and Methods*. Data represent mean values ± S.E.M. of three independent experiments, each performed in duplicate.

sequence of linker I in that linker I of $G\alpha_s$-long contains a 15-amino acid insert at position 72 (Bray et al., 1986). Both splice variants have been shown to act differentially with G protein-coupled receptors (Unson et al., 2000) and to exhibit differences in the kinetics of receptor-mediated nucleotide exchange or altered nucleotide affinity (Seifert et al., 1998). Whether the linker I region plays a role in specificity of receptor-G protein interaction has not been tested so far. In this study, we have shown that the linker I region does indeed control coupling to GPCRs. Upon mutation of a highly conserved glycine in linker I to amino acids with uncharged, positively charged, or negatively charged side chains, we generated mutant Gα proteins that lost the ability to discriminate between functional GPCR classes and link Gi-, Gs-, and Gq-coupled receptors to the $G\alpha_q$-PLCβ pathway. To exclude the possibility that greater coupling efficiency of receptors in the presence of the various $G\alpha_q$ mutants is attributable to a higher level of transient expression, we performed Western blot analysis of cells transfected with the wild-type and mutant $G\alpha_q$ proteins in the absence or presence of two selected receptors (chemokine CCR5 and GIP receptor). Although different levels of expression were unlikely given that the chimeras were constructed in the same expression vector and that they differ in only one amino acid in the linker I region, it was a necessary control experiment to rule out an effect of G protein expression level on the measured functional responses. Our data show that both wild-type $G\alpha_q$ and all mutant $G\alpha_q$ proteins are expressed at equivalent levels; thus, the increased coupling efficiencies observed with all mutant $G\alpha_q$ subunits are not caused by simple expression artifacts.

It is interesting to note that replacement of glycine with any other amino acid chosen in this study gave rise to a $G\alpha_q$ protein that lost its ability to discriminate between different functional GPCR classes (Fig. 1). As can be inferred from a receptor-G protein model (Fig. 8), the mutation is highly unlikely to be located at a direct receptor-G protein contact site. Whereas the mutation is about 30 Å away from the intracellular side of the plasma membrane (comparable in distance to the site where GDP is bound), the longest intracellular loop of any given receptor may at best reach only halfway to the GDP binding site (Bourne, 1997; Hamm, 1998). These data provide strong support for the notion that specificity of GPCR signaling can be controlled from a "distance" in addition to the well characterized receptor-G protein interface. Glycine residues are known to play pivotal

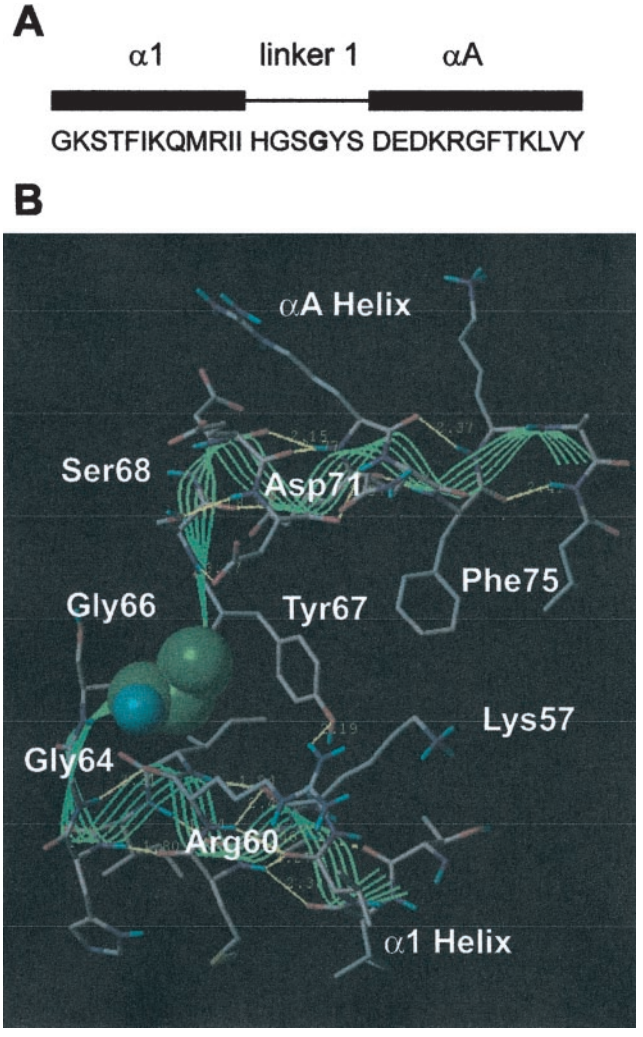

Fig. 7. Molecular model of the linker I region and the adjacent αA and α1 helices. The model was generated using the coordinates of $G\alpha_i$ in its GDP-Mg^{2+} bound form (coordinate code 1BOF; obtained from the Protein Data Bank of Brookhaven National Laboratory; http://www.pdb.bnl.gov/). The highly conserved glycine residue is shown as a ball model in the center of the linker I region. Yellow lines represent the hydrogen bond network within and between the αA- and α1-helices, respectively.

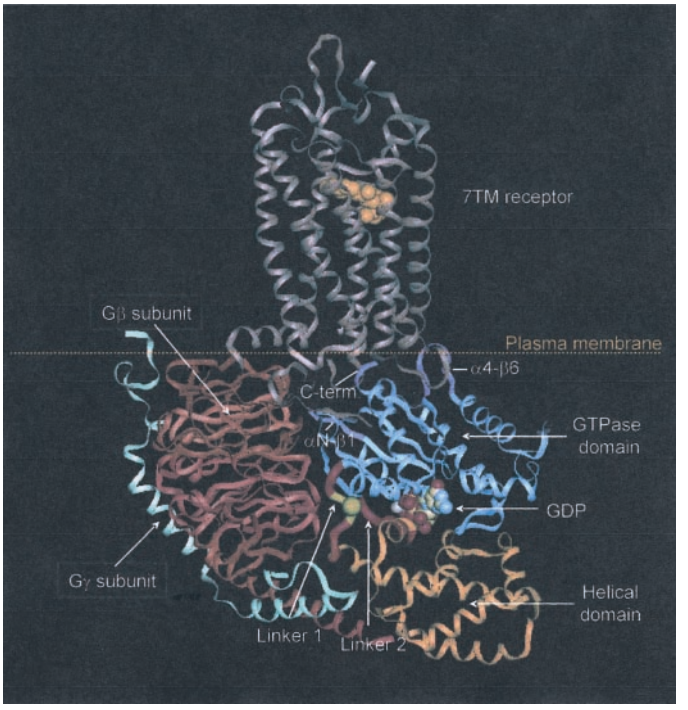

Fig. 8. Mapping of residues important for receptor-G protein coupling specificity onto a receptor-G protein model. Positioning of a heterotrimeric G protein ($G\alpha_t$-β-γ) relative to a G protein-coupled receptor is based on a model proposed by Bourne (1997). Regions known to determine specificity of receptor-G protein interaction are highlighted in magenta: the extreme C terminus, the α4-β6 loop, and the small loop connecting the N-terminal α-helix with the β1-strand of the ras-like domain. The guanine nucleotide GDP is buried between the helical (yellow) and the GTPase (blue) domains of the Gα-subunit. The two linker regions (red) connect the helical and GTPase domains of Gα. The colored green circle within linker I indicates the location of the G66D mutation imparting promiscuity onto $G\alpha_q$. The β-subunit is colored orange, and the γ-subunit is white. The C terminus of Gγ, which contains a lipid anchor, is facing the plasma membrane. The PDB identifiers for the receptor, Gα, and βγ are 1F88, 1BOF, and 1TBG, respectively.

structural roles in proteins in that they allow for a large range of dihedral angles at the Cα atoms and hence a higher degree of conformational flexibility compared with any other amino acid. It is attractive to speculate, therefore, that promiscuity observed with the various Gα_q mutants is caused by the lack of conformational freedom within the mutated linker sequence. Indeed, upon modeling of the various mutations into the structure of Gα_q (data not shown), only glycine residues can be accommodated in the central linker position without interfering with the local packing of the α1- and αA-helices, respectively. Thus, replacement of glycine with any other amino acid is not compatible with maintaining the tight intra- and interhelix hydrogen bond network of the respective region (Fig. 7), suggesting that its integrity is key for maintaining the fidelity of Gα activation by GPCRs.

It is interesting to note that many of the classic Gi- or Gs-coupled receptors used within this study exhibited at least a small tendency to activate Gq. Therefore, it was important to distinguish whether the Gα_{qG66D} protein was truly promiscuous as opposed to simply endowed with the ability to release $\beta\gamma$ subunits more readily upon receptor activation. We tested this possibility of enhanced $\beta\gamma$ release by using classic Gq-coupled receptors (Fig. 5) as model systems. We made the important observation that Gα_{qG66D} does not display higher sensitivity compared with G$\alpha_q$wt to genuine Gq-coupled receptors, thus clearly showing that the Gα_{qG66D} protein is not simply hypersensitizing Gq toward receptors in general but rather is making it promiscuous. Thus, only Gi- and Gs- but not Gq-coupled receptors give rise to more efficient PLCβ stimulation, strongly suggesting that Gα-receptor but not Gα-effector interaction is the basis for the IP_3 gain-of-function phenotype of the various Gi- and Gs-linked receptors tested in this study.

The ability of the GTPase-deficient versions of G$\alpha_q$wt and Gα_{qG66D} (Fig. 6) to intrinsically stimulate PLCβ with equal effectiveness helped not only to rule out the possibility that Gα-PLCβ interaction was differentially affected by both Gα proteins but also to underscore that Gα_{qG66D} retains its ability to recognize the Gq effector molecule PLCβ. These data provide strong support for the notion that the IP turnover generated upon coexpression of various non–Gq-linked receptors with Gα_{qG66D} is caused by activation of the well known Gq effector protein PLCβ. Although it was very unlikely that Gα_{qG66D} lost its ability to stimulate PLCβ, it was a necessary control experiment, in that the mutation in linker I may have had an indirect impact on effector recognition. Effector recognition domains within Gα_q have previously been mapped to the α4-helix and α4/β6 loops as well as several residues around the switch III region (Arkinstall et al., 1995; Medina et al., 1996).

It is interesting to note that our mutant Gα protein not only channels family A receptors (CCR5, D2, M2, α_{2A}, CXCR1, CXCR2, $S1P_1$, $S1P_5$, β_2) but also family B receptors (GIP, GLP1) to the PLCβ pathway. Family B receptors, such as those for secretin, glucagon, GIP, and GLP1, generally share little sequence similarity with any other G protein-coupled receptors. Therefore, identification and comparison of molecular determinants of receptor-G protein coupling specificity within the different receptor families may help unravel general rules involved in this protein/protein interaction and allow comparison of coupling mechanisms of both receptor families. In conclusion, we have identified a new region within the Gα subunit that acts as a regulator of receptor-G protein coupling specificity, most probably acting distantly from the proposed cytoplasmic receptor-G protein interface (Fig. 8). This region has never been previously reported to be involved in the control of receptor-G protein coupling. Although the precise molecular mechanism for inducing a specificity switch of Gα remains elusive, Gα_{qG66D} constitutes a new tool for linking Gi- and Gs-coupled receptors to the PLCβ pathway and adds to the repertoire of promiscuous G proteins. It will be interesting to determine whether introduction of this mutation into other chimeric or promiscuous G proteins such as G$\alpha_{15/16}$ increases their promiscuity and allows an even wider range of receptors to signal to the PLCβ pathway. It is intriguing to note that the glycine residue is highly conserved in all Gα subunits, even in sequences from distantly related organisms, suggesting that Gα subunit functionality is similarly affected in other Gα proteins by the corresponding mutation.

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Address correspondence to: Evi Kostenis, 7TM Pharma A/S, Fremtidsvej 3, 2970 Hørsholm, Denmark. E-mail: ek@7tm.com