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Carboxyl-Terminal Mutations of $G_{q\alpha}$ and $G_{s\alpha}$ That Alter the Fidelity of Receptor Activation

BRUCE R. CONKLIN, PAUL HERZMARK, SEIKO ISHIDA, TATYANA A. VOYNO-YASENETSKAYA, YAN SUN, ZVI FARFEL, and HENRY R. BOURNE

Departments of Medicine and Cellular and Molecular Pharmacology (B.R.C., P.H., S.I., T.A.V.-Y., Y.S., H.R.B.) and The Gladstone Institute of Cardiovascular Disease (B.R.C.), University of California, San Francisco, California 94143, and the Department of Medicine, Sheba Medical Center, Tel Aviv University, Tel Hashomer, 52621 Israel (Z.F.)

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SUMMARY

The carboxyl terminus of the G protein α subunit is a key determinant of the fidelity of receptor activation. We have previously shown that the $G_{q\alpha}$ subunit (α_q) can be made to respond to α_i-coupled receptors by replacing its carboxyl terminus with the corresponding α_{i2}, α_o, or α_z residues. We now extend these findings in three ways: 1) carboxyl-terminal mutations of α_q/α_i chimeras show that the critical amino acids are in the –3 and –4 positions, 2) exchange of carboxyl termini between α_q and α_s allows activation by receptors appropriate to the carboxyl-terminal residues, and 3) we identify receptors that either do or do not activate the expected carboxyl-terminal chimeras (α_q/α_i, α_q/α_s, α_s/α_q). Replacement of the five carboxyl-terminal amino acids of α_q with the α_s sequence permitted an α_s-coupled receptor (the V2 vasopressin receptor but not the β_2-adrenergic receptor) to stimulate phospholipase C. Replacement of the five carboxyl-terminal amino acids of α_s with residues of α_q permitted certain α_q-coupled receptors (bombesin and V1a vasopressin receptors but not the oxytocin receptor) to stimulate adenylyl cyclase. Thus, the relative importance of the G_α carboxyl terminus in permitting coupling to a new receptor depends on the receptor with which it is paired. These studies refine our understanding and provide new tools with which to study the fidelity of receptor/G_α activation.

Agonist-bound receptors activate heterotrimeric ($\alpha\beta\gamma$) G proteins by catalyzing replacement by GTP of GDP bound to the α subunit, a reaction that causes α-GTP to dissociate from the $\beta\gamma$ subunit dimer (1–4). Correct sorting of hormone signals by G proteins depends on the fidelity of receptor/G protein interactions. For example, different subsets of hormone receptors specifically activate G_q, G_s, or G_i, whose α subunits (α_q, α_s, and α_i) stimulate PLC, stimulate adenylyl cyclase, or inhibit adenylyl cyclase, respectively. Several lines of evidence point to the extreme carboxyl terminus of the G protein α subunit as one of the regions involved in receptor coupling (5). Mutations in this region (6–9), covalent modification by pertussis toxin-catalyzed ADP-ribosylation (10), and binding of specific antibodies (11–13) uncouple G proteins from receptors. Peptides mimicking the last 10 residues of α_t (the α subunit of transducin) and another sequence near its carboxyl terminus inhibit stimulation of G_t by photorhodopsin, indicating that the corresponding regions of α_t directly bind to this receptor (14). NMR studies with these peptides indicate that the last four residues form a type II β-turn (15).

We have previously shown that replacement of carboxyl-terminal amino acids of α_q by the corresponding residues of α_i creates α_q/α_i chimeras that can mediate stimulation of PLC by the D_2R and A_1R, which otherwise couple only with α_i (16). The smallest mutation shown to confer new receptor activation of α_q was one with simultaneous replacements at the –1, –3, and –4 positions. We have also shown that carboxyl-terminal substitutions in α_{13} allow stimulation by the D_2R (17). We now expand our previous findings with more detailed mutagenesis of α_q and reciprocal exchange of carboxyl termini between α_q and α_s. These findings refine and extend our understanding of the role of the G_α carboxyl terminus in determining the fidelity of receptor interaction.

Materials and Methods

Reagents. UK-14304 [5-bromo-6-(imidazolin-2-ylamino)-quinoxaline], (+)-PIA, quinpirole, isoproterenol, and [Arg^8]vasopressin were

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ABBREVIATIONS: PLC, phosphatidylinositol-specific phospholipase C; D_2R, D_2 dopamine receptor; A_1R, A_1 adenosine receptor; HEK, human embryonic kidney; CHO, Chinese hamster ovary K1; α_2AR, α_2-adrenergic receptor; β_2AR, β_2-adrenergic receptor; V2R, V2 vasopressin receptor; V1aR, V1a vasopressin receptor; PCR, polymerase chain reaction; PIA, N^6-phenylisopropyladenosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

purchased from Research Biochemicals (Natick, MA). Cell culture reagents and restriction enzymes were obtained from Life Technologies (Grand Island, NY) and New England Biolabs (Beverly, MA). All other reagents were from Sigma Chemical (St. Louis, MO) unless otherwise noted.

DNA constructs. Murine α_q (18) and rat α_s (19) cDNAs in pcDNA-1 (InVitrogen, San Diego, CA) were used as templates for PCR mutagenesis by including the entire added sequence in the 3′ PCR primer. The PCR product was then digested with *Eco*RI and *Nsi*I (for α_q) or *Bgl*II and *Xho*I (for α_s). The resulting fragments were then subcloned back into α_q/pcDNA-1 and α_s/pcDNA-1, respectively. Each construct had an internal hemagglutinin epitope tag (DVPDYA) that has no effect on receptor coupling (20, 21). The sequences of the entire subcloned fragments were confirmed by DNA sequencing. The sequences of the oligonucleotides used for mutagenesis were (underlining indicates change from wild-type sequence) CGLV, ggc cat gca tta gac cag gcc aca ctc ctt cag gtt cag ctg cag; CNLF, ggc cat gca tta gaa cag att gca ctc ctt cag gtt cag ctg cag gat; YGLV, ggc cat gca tta gac cag gcc gta ctc ctt cag gtt cag ctg cag; CNLV, ggc cat gca tta gac cag att aca ctc ctt cag gtt cag ctg cag; CALF, ggc cat gca tta gaa cag agc gca ctc ctt cag gtt cag ctg cag; CGLFG, ggc cat gca tta acc gaa cag acc gca ctc ctt cag gtt; CGL, ggc cat gca tta cag acc gca ctc ctt cag gtt cag ctg cag gat; qs5, ggc cat gca tta gag cag ctc gta ttg ctt cag gtt cag ctg cag gat; and sq5, ggc cat gca tta gac cag att gta ctc gcg aag gtg cat gcg ctg gat gat. All of these constructs will be made available to any other investigator without any obligation on the part of the recipient.

Cell culture and transfection conditions. HEK 293 cells (American Type Culture Collection, Rockville, MD: CRL-1537) were used for all $\alpha_{q/i}$ chimeras, and CHO cells were used for all other experiments. The cell type was changed because of improved transfection efficiency (20% in HEK 293 cells versus 60% in CHO cells recently obtained with adenoviral cotransfection in CHO cells (22). Similar results were obtained in HEK 293 cells and CHO cells for the α_s and the α_q constructs. In HEK 293 cells, G protein α subunits and G_i-coupled receptors were transiently expressed by incubating the cells (1×10^6/60-mm dish) for 2 hr with 250 μg/ml DEAE-dextran, 100 μM chloroquine, and the indicated cDNAs: 1 μg of α_2AR/pCMV4 (23), 1 μg of D_2R/pcDNA-1 (24), 1 μg of A_1R/CDM8 (25) (kindly provided by P. D. Garcia, University of California, San Francisco), 3 μg of qWT, or 3 μg of α_q/α_{i2} chimera. Cells were shocked for 2 min with phosphate-buffered saline containing 10% dimethylsulfoxide, washed once with phosphate-buffered saline, and maintained for 24 hr in a growth medium composed of minimum essential medium supplemented with 10% fetal bovine serum. In CHO cells, G protein α subunits and G_q-coupled receptors were transiently expressed by incubating the cells (5×10^6/100-mm dish) for 2 hr with adenoviral stock solution (1:3 dilution with serum-free medium), DEAE-dextran (80 μM), and the indicated cDNAs: 1 μg of V2R (26) in pcDNA-1, 1 μg of β_2AR (27) in pcDNA-1, 1 μg of bombesin receptor (28) in pcDNA-1, 1 μg of V1aR (29) in pcDNA-1, 1 μg human oxytocin receptor (30) in pcDNA-1, 1 μg of m1R-CDM8 (human m1 muscarinic receptor, kindly provided by W. Sadee, University of California, San Francisco), 3 μg of G_α wild-type, or 3 μg of G_α mutant construct. Cells were shocked for 2 min with phosphate-buffered saline containing 10% dimethylsulfoxide and then maintained for 24 hr in growth medium.

PLC and adenylyl cyclase assays. To measure PLC, cells were trypsinized from each 60-mm dish, split into 12 wells of a 24-well plate, and incubated with [^{3}H]inositol (2 μCi/well) 24 hr after transfection. After a 24-hr labeling period, the cells were washed once with 1 ml of assay medium (20 mM HEPES-buffered Dulbecco's modified Eagle's medium without bicarbonate) and incubated at 37° for 1 hr with 1 ml of assay medium containing 5 mM LiCl and receptor agonists, as indicated. [^{3}H]IPs were resolved on Dowex columns (31). The results are expressed as the cpm of [^{3}H]IP ($\times$ 1000) divided by the sum of the cpm in both the [^{3}H]IP and [^{3}H]inositol fractions. To measure adenylyl cyclase activity, cells were trypsinized from each 60-mm dish, divided among 15 wells of a 24-well plate, and incubated with [^{3}H]adenine (2 μCi/well) 24 hr after transfection. After a 24-hr labeling period, the cells were washed once with 1 ml of assay medium (20 mM HEPES-buffered Dulbecco's modified Eagle's medium without bicarbonate) and incubated at 37° for 1 hr with 1 ml of assay medium containing 100-mM 3-isobutyl-1-methylxanthine and receptor agonists, as indicated. [^{3}H]cAMP was resolved on Dowex and alumina columns (32). The results are expressed as the cpm of [^{3}H]cAMP ($\times$ 1000) divided by the sum of the cpm of both [^{3}H]cAMP and [^{3}H]ATP. Data represent the mean $\pm$ standard deviation of triplicate determinations in a single experiment, unless otherwise noted.

Protein expression. Equivalent numbers of cells (1×10^6) of each transfection were solubilized in sodium dodecyl sulfate sample buffer. A 50-μg aliquot of each sample was electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel. The proteins were transferred to nitrocellulose membrane (no. 00860; Schleicher & Schuell, Keene, NH) and probed with 12CA5 monoclonal antibody against the hemagglutinin epitope for 1 hr, washed three times for 10 min each with Tris-buffered saline/Tween 20, and then probed with a horseradish peroxidase-linked anti-mouse antibody (1 mg/ml in Tris-buffered saline/Tween 20; Amersham, Arlington Heights, IL). The blot was rewashed three times for 10 min each with Tris-buffered saline/Tween 20 and developed with an enhanced chemiluminescence substrate (Amersham) according to the manufacturer's instructions.

Results and Discussion

We have previously shown that the carboxyl terminus of a G_α subunit can determine the fidelity of its receptor interactions. We chose α_q and α_{i2} for our first experiments because G_q and G_i discriminate among different subsets of receptors that regulate distinct and easily measurable second messenger pathways. Initially, we tested a series of 11 mutant α_q subunits in which carboxyl-terminal residues were progressively replaced with corresponding residues from α_{i2} (16). Once we narrowed the site that allowed activation by a new receptor to the –1, –3, and –4 positions (Fig. 1A), we made point-mutations of these residues in an effort to determine the minimal sequence necessary for activation by a new receptor (Fig. 1, B and C).

In HEK 293 cells, we transiently coexpressed DNAs encoding each α_q construct with DNAs encoding a panel of three different receptors. Each of these receptors couples predominantly or exclusively to G_i. Two of these, the A_1R and D_2R, activate G_i in HEK 293 cells (33) and do not affect PLC even when α_q is overexpressed (*qWT*, Fig. 1A). The third receptor, the α_2AR, shares with a small but growing class of receptors (34–37) the ability to activate more than one G protein. The α_2AR preferentially activates G_i but can also activate G_q when α_q is overexpressed in HEK 293 cells (31) (*qWT*, Fig. 1A). Thus, α_2AR stimulation of PLC indicates that the $\alpha_{q/i}$ chimera is functionally expressed, whereas A_1R or D_2R stimulation of PLC indicates that the chimera has gained an ability to interact with receptors that couple exclusively to G_i.

Measurements of receptor-stimulated PLC activity in cells expressing α_q constructs first showed (16) that replacement of just three of the last four amino acids of α_q with the corresponding α_{i2} sequence suffices to alter the fidelity of receptor activation (*qi4*, Fig. 1). More extensive substitutions beyond the –4 position did not further increase PLC stimulation (16). Therefore, we focused on these three positions (–4, –3, and –1), changing each one individually or in pairs to the corresponding α_{i2} residues. The changes at the –4 and –3

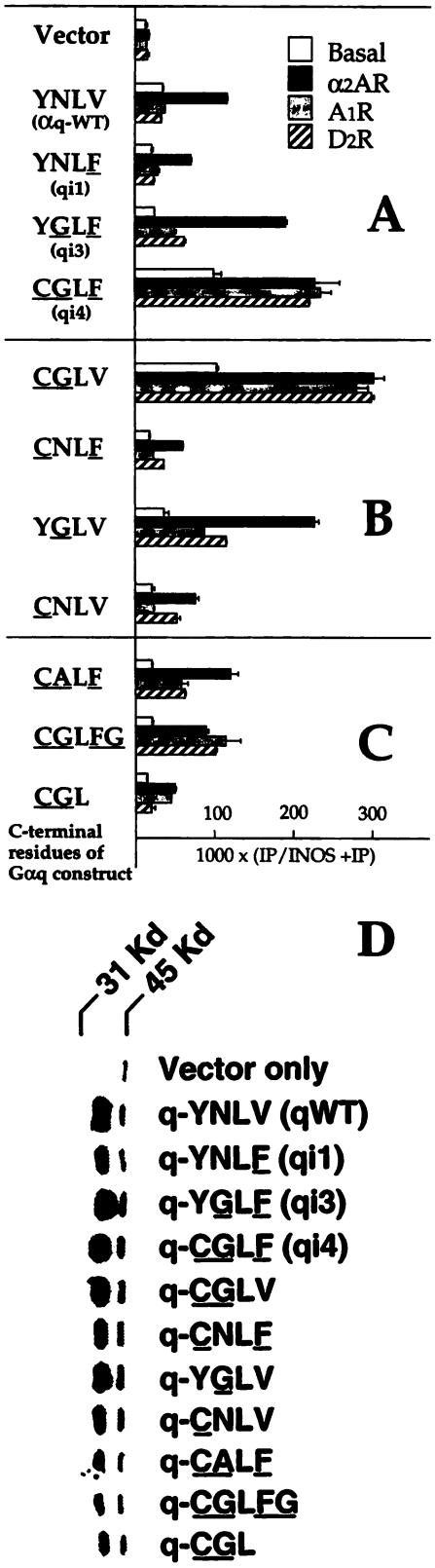

Fig. 1. Mutations of the carboxyl terminus of α_q that allow coupling to G_i-coupled receptors. A, Mutations made progressively from the carboxyl terminus as described previously (16). B, Alternate point-mutations that change residues at the –1, –3, and –4 positions from α_q to α_{i2} sequence. C, Constructs that alter CGLF (qi4) by substituting an alanine residue at position –3 or adding or subtracting a residue at the extreme carboxyl terminus. *Underlined*, change from the wild-type α_q sequence (YNLV). IP formation was measured after incubation with agonists to the indicated G_i-coupled receptors transfected as described in Materials and Methods in HEK 293 cells, plus pcDNA-1 (vector), qWT (YNLV), or individual α_q constructs. Agonists were 10 μM UK-14304 for α_2AR, 10 μM (+)-PIA for A_1R, and 10 μM quinpirole for D_2R. In similar studies with naturally occurring G_q-coupled receptors such as the bombesin or the oxytocin receptor, the inositol fraction is elevated to ~1200 units [1000 × IP/(inositol + IP)]. Data represent the mean ± standard deviation of triplicate determinations of a single experiment; three additional experiments showed similar results. D, Expression of α_q constructs in HEK 293 cells. Western analysis illustrates that the levels of expression of the constructs in A and B were similar and greater than those in C. Two additional blots yielded similar results.

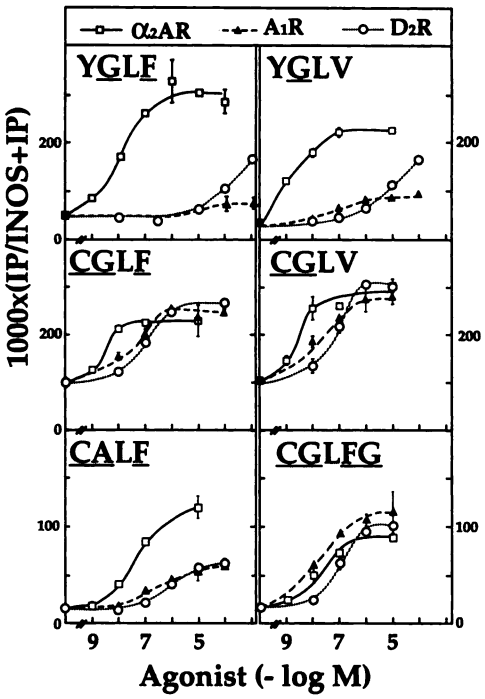

Fig. 2. Concentration-response curves for stimulation of PLC by three G_i-coupled receptors. HEK 293 cells were transfected with α_q mutants as indicated in the legend to Fig. 1 and treated with the various concentrations of the α_2AR agonist UK-14304, the A_1R agonist (+)-PIA, or the D_2R agonist quinpirole. Transfections and IP assays were performed as described in Materials and Methods. Data are expressed as the mean of duplicate determinations in a single experiment. For each construct, two additional experiments gave similar results.

positions allowed activation by G_i-coupled receptors, whereas changes in the extreme carboxyl-terminal residue had no discernible effect on the fidelity of receptor activation (Fig. 1B). In a third series of mutants (Fig. 1C), we used qi4 as a starting point to interfere with its function by replacing the –3 glycine with alanine, adding a glycine to the carboxyl terminus, or removing the carboxyl-terminal residue. All of the α_q mutants in Fig. 1, A and B, were expressed at comparable levels, whereas the α_q mutants in Fig. 1C were expressed at lower levels (see Fig. 1D). Concentration-response curves for six key α_q constructs (Fig. 2, with carboxyl-terminal sequences of YGLF, YGLV, CGLF, CGLV, CALF, and CGLFG) illustrate the relative importance of the –4 and –3 positions. Only by changing both residues simultaneously will the G_i-coupled receptors fully activate the resulting G proteins, as measured in this assay.

Interestingly, Garcia *et al.* (9) recently reported similar results with transducin, which has the identical four carbox-

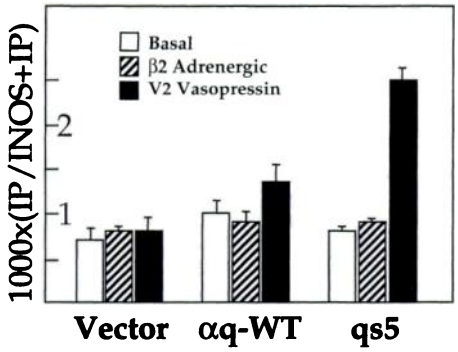

Fig. 3. PLC stimulation by two α_s-coupled receptors. CHO cells were transfected with DNA encoding the V2R and the β_2AR plus vector (pcDNA-1), α_q-WT, or qs5 as described in Materials and Methods. Agonists used were 1 μM [Arg^8]vasopressin for the V2R and 1 μM isoproterenol for the β_2-AR. Both α_q-WT and qs5 were expressed at similar levels as determined by Western blot analysis. Data represent the mean ± standard deviation of triplicate determinations of a single experiment; three additional experiments showed similar results.

yl-terminal residues (CGLF). In that study (9), alanine substitutions at the -2 and –3, but not at the –1 or –4, positions interfered with rhodopsin coupling. In our current study, a tyrosine at the –4 position interfered with coupling to G_i-coupled receptors (YGLF and CGLF, Fig. 1A). Alanine substitution was not attempted at this position. Like Garcia *et al.*, we found that an alanine substitution at the –3 position interferes with coupling to G_i-coupled receptors. We did not attempt a substitution at the –2 position because this residue is identical in all G protein α subunits and our purpose was to alter the fidelity of receptor activation.

We were surprised to find that exchanges of the extreme carboxyl-terminal residues had no apparent effect on the fidelity of receptor activation. In combination with the previously described qz5 and qo5 chimeras, which substitute the last five residues of α_z (YIGLC) and α_o (GCGLY), respectively (16), the current results indicate that coupling to α_2AR, A_1R, or D_2R was unaffected by changing the last residue to cysteine, tyrosine, or valine or by a glycine extension.

The decreased expression by the chimeras CALF, CGLFG, and CGL (Figs. 1C and 2) limits our ability to interpret their responses. The fact that CGLFG can still be activated by the D_2R and A_1R indicates that the fidelity of receptor activation remains altered despite the carboxyl-terminal extension. The decreased response of CALF adds to the notion that the –3 position is critical for receptor fidelity; however, the decreased expression precludes definitive conclusions based on this chimera alone.

Two constructs, CGLF (qi4) and CGLV, consistently elevated basal PLC activity. Our previous studies (16) showed that qi4, qi5, qi9, and qi11 elevated basal activity. Increased basal activity could be caused by decreased affinity for GDP, which would favor the GTP-bound state. Indeed, truncation of six residues from the carboxyl terminus of α_o (38) point-mutants of transducin (6) and a point-mutation 28 residues upstream from the end of α_s (39) were reported to decrease GDP binding affinity. In the absence of biochemically pure α_q/α_i chimeras, we could not determine the relative GDP affinity of these mutants. It is clear that the basal activity is not directly related to the fidelity of receptor activation; however, because several constructs with low basal activity are still activated by the A_1R and D_2R [CGLFG, Figs. 1C and 2; GCGLY in qo5 (16)].

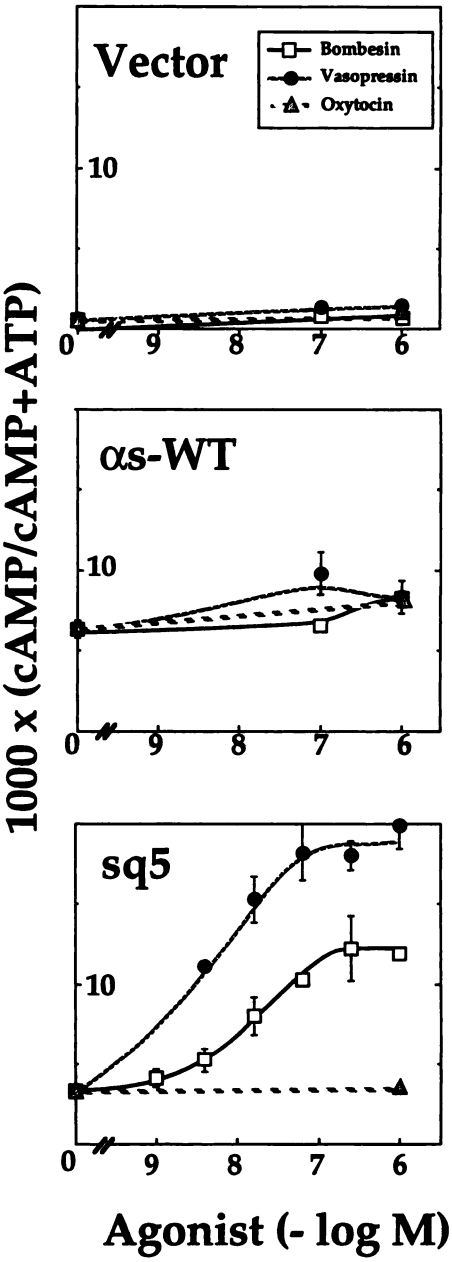

Fig. 4. Concentration-response curves for stimulation of cAMP accumulation by three G_q-coupled receptors in CHO cells. All cells were transfected with the indicated receptors as described in Materials and Methods plus pcDNA-1 (vector), α_s-WT, or sq5. Agonists were [Arg^8]vasopressin, oxytocin, and bombesin. Both α_s-WT and sq5 were expressed at similar levels as determined by Western blot analysis. In similar studies with naturally occurring G_s-coupled receptors such as the β_2AR or V2R, the cAMP fraction is elevated to ~50 units [1000 × cAMP/(cAMP + ATP)]. Data represent the mean ± standard deviation of triplicate determinations of a single experiment; three additional experiments showed similar results.

In our previous studies, all G_i-coupled receptors tested (D_2R, A_1R, and α_2AR) were able to activate α_q/α_i chimeras. This result is not invariable. The somatostatin receptor-1 was functionally expressed in four different experiments (data not shown), as indicated by its previously reported ability to inhibit adenylyl cyclase (40), but it had no effect on qi5.

TABLE 1

Activities of G_α carboxyl-terminal chimeras presented in the current study or previously reported

G_α construct	Expected to stimulate:	Receptors that worked	Receptors that did not work
qi5	PLC by α_i-coupled receptors	D_2 dopamine	Somatostatin 1
		A_1 adenosine	
		α_2-Adrenergic	
		m2 muscarinic	
qs5	PLC by α_s-coupled receptors	V_2 vasopressin	β_2-Adrenergic
sq5	Adenylyl cyclase by α_q-coupled receptors	V_{1a} vasopressin	Oxytocin
		Bombesin	
13/z	Sodium-proton pump by α_i-coupled receptors	D_2 dopamine	

Based on current study and Refs. 16, 17, and 41.

We next investigated whether our observations concerning the carboxyl termini of α_i and α_q were applicable to other G protein α subunits and different classes of receptors. We showed that the D_2R could be redirected to activate α_{13} by replacing five residues at the carboxyl terminus with α_z residues (17). To extend this analysis to other classes of receptors and G_α subunits, we have exchanged the carboxyl termini of α_q and α_s. Replacement of the five carboxyl-terminal amino acids of α_q (EYNLV) with those of α_s (QYELL) allowed the new construct (qs5) to respond to the α_s-coupled V2R but not to the β_2AR) (Fig. 3). Both the V2R and the β_2AR activated α_s and stimulated adenylyl cyclase 5–10-fold over basal activity under similar conditions, as described previously (26).

We were able to test the reciprocal chimera (sq5) because certain α_q-coupled receptors (bombesin receptor, oxytocin receptor, and V1R) cannot stimulate α_s or cAMP accumulation, even when α_s is overexpressed in the same cells (Fig. 4). Replacement of the last five residues of α_s (QYELL) with those of α_q (EYNLV) produced a new construct (sq5) that was activated by the bombesin receptor and the V1R. Interestingly, the oxytocin receptor did not activate sq5 (Fig. 4) even though all three receptors stimulate IP production by 3–5-fold over the basal level when transfected under identical conditions, as described previously (29, 30). Table 1 summarizes all data from the G_α carboxyl-terminal chimeras presented in this study or previously published (16, 17, 41).

Taken together, these findings indicate that the carboxyl terminus of G_α plays an important role in determining the fidelity of receptor activation. Detailed mutagenesis of the α_q carboxyl terminus shows that the –3 and –4 positions are critical for conferring new receptor activation, whereas the –1 position (the extreme carboxyl-terminal residue) is relatively unimportant. The carboxyl terminus cannot invariably confer susceptibility to stimulation by every receptor for a given G_α. This is in keeping with a wide variety of evidence pointing to other G_α regions that also help in the determination of the fidelity of receptor activation. The recently solved crystal structures of α_t (42) and α_i (43) provide valuable information for identifying the residues near the carboxyl terminus that are likely to contact the receptor. A study of α_{11}/α_{16} chimeras indicates that another region, near the termination of α helix 2, is also involved in determination of the fidelity of receptor activation (44). We predict that each receptor/G protein interaction will be found to involve common sets of protein/protein contacts. The relative importance of each of the contacts will vary, however, depending on the receptor/G protein combination involved. Eventual identification of these contacts will provide valuable insights into the mechanism by which G proteins sort hormonal signals.

Acknowledgments

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Send reprint requests to: Dr. Bruce R. Conklin, Gladstone Institute of Cardiovascular Disease, P.O. Box 419100, San Francisco, CA 94141-9100. E-mail: bruce_conklin.gicd@quickmail.ucsf.edu