Identification of a stretch of six divergent amino acids on the $\alpha 5$ helix of $G\alpha_{16}$ as a major determinant of the promiscuity and efficiency of receptor coupling

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A broad repertoire of G-protein-coupled receptors shows effective coupling with the haematopoietic G_{16} protein. In the present study, individual residues along the C-terminal $\alpha 5$ helix of $G\alpha_{16}$ were examined for their contributions in defining receptor-coupling specificity. Residues that are relatively conserved within, but diverse between, the subfamilies of cloned $G\alpha$ subunits were mutated into the corresponding $G\alpha_z$ residues. Six G_i -linked receptors with different coupling efficiencies to $G\alpha_{16}$ were examined for their ability to utilize the various $G\alpha_{16}$ mutants to mediate agonist-induced inositol phosphate accumulation and Ca^{2+} mobilization. Co-operative enhancements of receptor coupling were observed with chimaeras harbouring multiple mutations at Glu³⁵⁰, Lys³⁵⁷ and Leu³⁶⁴ of $G\alpha_{16}$. Mutation of Leu³⁶⁴ into isoleucine appeared

to be more efficient in enhancing receptor recognition compared with mutations at the other two sites. Mutation of a stretch of six consecutive residues (362–367) lying towards the end of the $\alpha 5$ helix was found to broaden significantly the receptor-coupling profile of $G\alpha_{16}$, and the effect was mediated partly through interactions with the $\beta 2$ – $\beta 3$ loop. These results suggested that a stretch of six distinctive residues at the $\alpha 5$ helix of $G\alpha_{16}$ is particularly important, whereas other discrete residues spreading along the $\alpha 5$ helix function co-operatively for determining the specificity of receptor recognition.

Key words: G_{16} , G-protein, phospholipase C, receptor coupling, site-directed mutagenesis.

INTRODUCTION

Heterotrimeric G-proteins transmit extracellular signals from heptahelical GPCRs (G-protein-coupled receptors) to trigger intracellular signalling events. The fidelity and specificity of the interactions between GPCRs and G-proteins determine the scope and characteristics of the ultimate cellular response. The α subunit of G-protein, $G\alpha$, is a guanine nucleotide-binding protein controlling the activation process of the G-protein and serves as the major contact for GPCRs. Using chimaera construction techniques, a number of $G\alpha$ subunits, including $G\alpha_q$ [1–4], $G\alpha_s$ [5], $G\alpha_{i1}$ [6], $G\alpha_{i2}$ [7], $G\alpha_z$ [8] and $G\alpha_{16}$ [9], have been studied previously for their receptor-coupling domains. Each $G\alpha$ subunit has a distinct coupling profile to particular types of GPCRs and, in the extreme case, $G\alpha_{i1}$ interacts almost exclusively with rhodopsin. The receptor-coupling domains of these $G\alpha$ subunits have significant overlaps, mostly confined to a number of secondary-structural elements such as the N- and C-termini and various loops on the GTPase (GTP hydrolase) domain [1–5,8]. The C-terminus of $G\alpha$ is probably the most important receptor-interacting region. Mutations of the last five residues are often sufficient to change the receptor-coupling specificity [1,2]. Although it has not been rigorously proven, such switches in specificity may be due to the incorporation of a better interacting surface for particular categories of receptors. This notion is supported by the results obtained with a variety of $G\alpha$ chimaeras that exhibit preferences on particular categories of GPCRs according to the choice of parental $G\alpha$ [1,2].

$G\alpha_{16}$ is a member of the $G\alpha_q$ subfamily, which stimulates phospholipase C β , but its broad receptor-coupling characteristics are

unique among all known $G\alpha$ subunits [10]. This peculiarity of $G\alpha_{16}$ has been investigated by manipulating its amino acid sequence in the receptor-coupling domains [11–13]. Swapping the last 25 or 44 amino acids of $G\alpha_{16}$ with those of $G\alpha_z$, $G\alpha_{i2}$ and $G\alpha_o$ allows the chimaeric $G\alpha$ subunits to couple with more G_i -linked receptors [9,14,15]. The same strategy has also been applied for redirecting the coupling specificity of $G\alpha_{16}$ towards G_s -coupled receptors [16]. The 25 C-terminal residues of $G\alpha_{16}$ correspond to the complete $\alpha 5$ helix, and the last 44 residues cover additional secondary structures encompassing the C-terminal portion of the $\alpha 4/\beta 6$ loop and the complete $\beta 6$ strand. Both the $\alpha 4/\beta 6$ loop and the $\alpha 5$ helix constitute exposed surfaces of $G\alpha_{16}$ and they probably contribute to the receptor-coupling specificity of $G\alpha_{16}$.

Expression of two $G\alpha_{16/z}$ chimaeras, 16z25 and 16z44, in monkey kidney fibroblast COS-7 cells enhances the basal levels of IP (inositol phosphate) production without receptor activation, suggesting that they are constitutively active [9]. This result implies that modifications of the C-terminal $\alpha 5$ helix can perturb the conformation of $G\alpha_{16}$ and shift its equilibrium to the GTP-bound conformation. Similar observations have been reported in earlier studies of $G\alpha_s/G\alpha_i$ chimaeras, where constitutive activation of adenylate cyclase was observed when the C-terminus of $G\alpha_s$ was replaced with the corresponding region of $G\alpha_{i2}$ [17,18]. Some studies have reported that residues at the C-terminus of $G\alpha$ could enhance the GDP dissociation rate, the rate-determining step in the GTPase cycle of $G\alpha$ [19–21]. Furthermore, these constitutively active chimaeras retained their ability to interact productively with GPCRs. It is not clear whether perturbation of basal activity results in enhanced promiscuity of the chimaeras.

Abbreviations used: D₂R, dopamine D₂ receptor; DMEM, Dulbecco's modified essential medium; DOR, KOR, MOR, δ -, κ - and μ -opioid receptors; GPCR, G-protein-coupled receptor; IP, inositol phosphate; SST1, type 1 somatostatin receptor; XMR, *Xenopus* melatonin receptor.

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The present study aims to decipher the specific residues on the $\alpha 5$ helix that are responsible for receptor specificity and the constitutive activity of $G\alpha_{16}$. We report that discrete residues lying on the exposed surface of the $\alpha 5$ helix of $G\alpha_{16}$ may serve as critical determinants for receptor specificity. Their contributions were co-operative, since the mutants containing combinations of specific mutations coupled better with a variety of GPCRs. Interestingly, mutations in a stretch of six consecutive residues in the middle of the $\alpha 5$ helix significantly affected both the receptor coupling and constitutive activity of $G\alpha_{16}$.

EXPERIMENTAL

Materials

The plasmids containing various $G\alpha$ subunits and GPCRs were obtained or constructed as described previously [9,14]. Monkey kidney fibroblast COS-7 cells were obtained from A.T.C.C. (CRL-1651). Various receptor agonists were obtained from Tocris Cookson (Bristol, U.K.) and Sigma–Research Biochemicals International (St. Louis, MO, U.S.A.). [^3H]myo-Inositol was purchased from PerkinElmer Life Sciences (Boston, MA, U.S.A.). Plasmid purification columns were obtained from Qiagen (Hilden, Germany). Restriction endonucleases were from Roche Molecular Biochemicals. PCR Supermix High Fidelity premix, customized primers, LIPOFECTAMINETM 2000 and cell-culture reagents were obtained from Invitrogen (Carlsbad, CA, U.S.A.). $G\alpha_{16}$ -specific antiserum against the epitope CLTEDEKAAARVDQE (residues 13–27 of $G\alpha_{16}$) was made by Gramsch Laboratories (Schwabhausen, Germany). Black-walled clear-bottomed 96-well plates (no. 3603) were from Corning Costar (Acton, MA, U.S.A.). Fluo-4 AM Ca^{2+} -sensitive fluorophore was purchased from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals were from Sigma–Aldrich (St. Louis, MO, U.S.A.).

Construction of $G\alpha_{16}$ mutants

PCR was used for introducing mutations to $G\alpha_{16}$ using a pair of mutation primers for each single-point mutation. For convenience of construction and sequencing, a silent G $\rightarrow$ T mutation was introduced to the nucleotide position 966 of the open reading frame of $G\alpha_{16}$ cDNA, thereby generating a *PinAI* restriction site (primers used were 16PinAI/S for ACCGGTGCCTGGACG and 16PinAI/AS for CACGCAACCGGTGTACA; mutated nucleotides are indicated by lower-case letters). The full-length PCR product was generated with flanking primers annealed to the T7 and SP6 promoter regions of pcDNAI and was sequenced using ABI PRISM 310 genetic analyser (Foster City, CA, U.S.A.). The modified $G\alpha_{16}$ cDNA was subcloned in the *XbaI* site of the expression vector pcDNAI (Invitrogen) and served as the template for creating all the mutants studied here. Introduction of various mutations was achieved using the following mutation primers: QS/S, GACActtGAACATCCGC; QS/AS, GTTCgaaGTGTCTGTGG; KD/S, TCTTCgAtGACGTGCGGG; KD/AS, ACGTcaTcGAAGACCTTG; LI/S, TCGGTGaTCGCCCCGCTA; LI/AS, GGCGatCACCGAGTCC; SV6/S, atcatagaaataacCTGGACGA-GA; SV6/AS, attctgtatgatgacGTCCCGCACG; LI4/S, GTGatacagaacaatCTGGACGAGATCAAC; LI4/AS, CAGattgtctgtatCACCGAGTCCCGCAC; RN2/S, ctgcgAACAAATCTGGACGAGATCAAC; RN2/AS, GTCCAGATTGTTcgcgagCACCGAGTC; SV/S, GTGCGGGACgtcGTGCTCGCCCCGCTAC; SV/AS, GGCGAGCACgacGTCCCGCACGTCCTT. The flanking primers used were 16PinAI/S and SP6. A 233 bp DNA fragment bearing the mutation(s) was digested with *XbaI* and then subcloned into the *EcoRV* and *XbaI* sites of pcDNAI for sequencing. Finally, an *HindIII*–*PinAI* fragment from the template was subcloned back

to the mutated fragments to form the full-length open reading frames of the mutants. The full-length product of ST7 was separately constructed using ST7/S (acctcaaggagctcaccttcCGGATCGTGGACGTC) and ST7/AS (aaggtgagctcttgaaggtGAAGCA-GTACTCGTTG) as mutation primers, and T7 and SP6 as flanking primers. ST7 was then subcloned back into pcDNAI as the template for making ST7SV6.

Transfection of COS-7 cells and IP accumulation assay

COS-7 cells were cultured with DMEM (Dulbecco's modified essential medium), supplemented with 10 % (v/v) foetal bovine serum, 50 units/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin at 37 °C in humidified air with 5 % CO_2 . They were co-transfected with various recombinant DNA constructs using the DEAE-dextran/chloroquine method as described previously [15]. Transfected cells were labelled with 750 μl of 2.5 $\mu\text{Ci}/\text{ml}$ [^3H]myo-inositol in inositol-deficient DMEM containing 5 % foetal bovine serum. Labelled cells were preincubated with 20 mM Hepes-buffered DMEM containing 20 mM LiCl (assay medium) for 10 min and then treated with proper receptor agonists in assay medium for 60 min for accumulation of IP. The reactions were terminated by adding ice-cold 20 mM formic acid. Separation of labelled IP from other inositol species was achieved by ion-exchange chromatography as described previously [22]. The IP levels were interpreted as the ratio of the counts/min of [^3H]IP fractions to those of the total labelled inositol fractions and are expressed as $[\text{IP}/(\text{IP} + \text{Total}) \times 1000]$. The absolute values for IP accumulation varied between experiments, but variability within a given experiment was in general $<10\%$. Results are expressed as the means $\pm$ S.E.M. for at least three individual experiments. One-way ANOVA with Tukey–Kramer's multiple-comparison tests was performed using GraphPad Prism 3 to verify the significance between selected treatment groups within the experiments.

Immunodetection of $G\alpha_{16}$ mutants

Western-blot analysis was performed as described previously [9]. $G\alpha_{16}$ -specific antiserum against residues 13–27 of $G\alpha_{16}$ was used (1:2000 dilution) for the detection of $G\alpha_{16}$ and the mutants.

Intracellular Ca^{2+} -mobilization assay using the fluorimetric image plate reader FLIPR[®] I

COS-7 cells were seeded into black-walled clear-bottomed 96-well plates at a cell density of $2 \times 10^4/\text{well}$ and were transfected with various DNAs using LIPOFECTAMINETM 2000 according to the manufacturer's instructions. Each plasmid DNA (20 ng) was transfected into the cells and left for 48 h until assay. Each well containing cells was labelled with 200 μl of 1 μM Fluo-4 AM in 20 mM Hepes-buffered Hanks balanced salt solution containing 2.5 mM probenecid (anion exchanger inhibitor, freshly prepared) for 1 h at 37 °C. Mobilization of intracellular Ca^{2+} triggered by agonist administrations was monitored using FLIPR[®] I obtained from Molecular Devices (Sunnyvale, CA, U.S.A.). Agonists (50 μl) in appropriate concentrations were injected into each well at the speed of 50 $\mu\text{l}/\text{s}$ at the beginning of the 11th sampling time. The power of the laser and the exposure time were set at 0.4 W and 0.4 s respectively, with proper adjustments to maintain the basal fluorescence signals at 8000–12 000 units. Measurement was made for 70 s at 1 s intervals and then for 60 s with 3 s intervals. The peak values (max–min) were obtained from the averaged data of triplicates and plotted against the concentrations of agonists. Dose-dependent response curves were fitted by the non-linear regression function of GraphPad Prism 3 and the corresponding EC_{50} and V_{max} values were estimated accordingly.

Figure 1 Amino acid sequence alignments

(A) Alignment of the C-termini of $G\alpha$ subunits. The 30 C-terminal amino acid sequences corresponding to a part of the $\beta 6$ - $\alpha 5$ loop, the complete $\alpha 5$ helix and the C-terminal tail of the $G\alpha$ subunits were extracted from the complete alignment of all known $G\alpha$ subunits using the CLUSTAL X program (numbers flanking the sequences indicate the positions of the residues). Identical residues are represented by asterisks, while those marked with double and single dots are highly and marginally conserved respectively. **(B)** Alignment of chimera sequences: alignment of the same region as in **(A)** for $G\alpha_{16}$, $G\alpha_z$ and the various mutants. Residues of $G\alpha_z$ and various mutants that are identical with $G\alpha_{16}$ are indicated by dots.

Sequence alignment and molecular modelling

Complete amino acid sequence alignment of $G\alpha$ subunits was generated using CLUSTAL X [23]. Molecular models were created by SWISS-MODEL web server [24] using the co-ordinates of the crystal structures of $G\alpha_{i1}$ [25] and $G\alpha_{t1}$ [26] in their corresponding heterotrimers, retrieved from Protein Data Bank (maintained by Research Collaboratory for Structural Bioinformatics, Department of Chemistry and Chemical Biology and the Center for Molecular Biophysics and Biophysical Chemistry, Rutgers University, Piscataway, NJ, U.S.A.) [27] as the modelling templates. The models were visualized and modified with Swiss-PDBViewer Deep View version 3.7 [24]. The extreme C-terminal tail of $G\alpha_{i16}$ missing in the generated model was restored by incorporating the averaged co-ordinates of the NMR structures of a peptide corresponding to the rhodopsin-bound 11 C-terminal residues of transducin $G\alpha_{i1}$ [28] and mutating the corresponding residues into those of $G\alpha_{i16}$. Energy minimization was then performed for the modified model with the GROMOS96 implementation of Swiss-PDBViewer. The final images were rendered by POV-Ray 3.5 (<http://www.povray.org/>).

RESULTS

Based on the alignment of the amino acid sequences of the $\alpha 5$ helices of various $G\alpha$ subunits (Figure 1A), three particular amino acids were initially selected for homologous mutagenesis. They are either unique for $G\alpha_{16}$ (Gln³⁵⁰ and Lys³⁵⁷) or different from the amino acids of $G\alpha$: subfamily members (Leu³⁶⁴). These residues

Figure 2 Molecular modelling of G α_{16}

(A) The $\alpha 5$ helix on the GTPase domain. The ribbon structure of a putative $G\alpha_{16}$ model was generated as described in the Experimental section and a part of the GTPase domain is shown. The three residues mutated in various constructs are labelled and highlighted as space-filled structures using Corey–Pauling–Koltun colour scheme on the red-coloured $\alpha 5$ helix. (B) A helical wheel diagram of the $\alpha 5$ helix is displayed in view from the C-terminal end, with the residues coloured according to their side-chain properties. Blue, positively charged; grey, aliphatic; dark grey, aromatic; orange, slightly negatively charged; red, strongly negatively charged. The residues mutated in the present study are green-coloured. The left and right halves are the buried and exposed sides respectively of the $\alpha 5$ helix, and the secondary structures interacting at approximate positions are indicated. $L_{\beta 2-\beta 3}$ refers to the $\beta 2$ – $\beta 3$ loop. (C) Interaction of the $\alpha 5$ helix and the $\beta 2$ – $\beta 3$ loop: another view of the same $G\alpha_{16}$ model as (A), with the SV6 (bright red) and ST7 regions (pale green; refer to Figure 7) highlighted on the $\alpha 5$ helix (deep red) and the $\beta 2$ – $\beta 3$ loop (yellow) respectively. Residues corresponding to these two regions are displayed as sticks and coloured according to their accessibilities in the ascending order with blue, cyan, green and red (the most accessible).

were mutated to the corresponding residues of $G\alpha_z$ and named as QS, KD and LI (Figure 1B). As shown in the hypothetical model of $G\alpha_{16}$ (Figure 2A), the three selected residues are positioned along the same side of the $\alpha 5$ helix. Gln³⁵⁰ is located at the N-terminal end of the $\alpha 5$ helix, much closer to the cleft between the GTPase and helical domains, whereas Lys³⁵⁷ and Leu³⁶⁴ are in the middle and C-terminal ends respectively. Double and triple mutants

Figure 3 Expression of $G\alpha_{16}$ mutants

COS-7 cells transfected with plasmid DNA encoding $G\alpha_{16}$ and the various mutants were harvested, and membrane proteins were extracted for Western-blot analysis. Vector-transfected cells served as the negative control. Recombinant proteins were detected using a custom-made anti- $G\alpha_{16}$ serum against the region of residues 13–27 of $G\alpha_{16}$. Images are visualized with chemiluminescence reagents and recorded on an X-ray film. Two separate sets of transfected cells yielded similar results.

were subsequently generated by combining the selected mutation sites and the sequences of the mutants are given in Figure 1.

The expression levels of all the mutants generated for the present study were examined first by Western-blot analysis using a specific antibody raised against the N-terminal region of $G\alpha_{16}$ (Figure 3). All of the recombinant proteins were expressed to similar levels in COS-7 cells, suggesting that any functional variability of the mutants is probably not due to uneven expression levels. We have demonstrated previously that the densities of transiently expressed GPCRs (usually in the range of several hundred fmol/mg of protein) are not significantly affected by the co-expression of various $G\alpha$ subunits [29,30].

The functional coupling of the three mutants with selected GPCRs was examined first. The GPCRs were selected based on our previous studies on $G\alpha_{16}$ and 16z chimaeras, wherein the receptors have different capabilities of coupling with $G\alpha_{16}$ but all of them showed enhanced coupling with 16z44 [9,14]; the 16z44 chimaera served as a reference in the various functional assays of the present study (Figures 4–7). In terms of their efficiency in coupling with $G\alpha_{16}$, DOR (δ -opioid receptor) and KOR (κ -opioid receptor) are relatively strong [31]. The D_2 R (dopamine D_2 receptor) and SSTR1 (type 1 somatostatin receptor) modestly coupled to $G\alpha_{16}$ [9,14], whereas MOR (μ -opioid receptor) and XMR (*Xenopus* melatonin receptor) poorly coupled to $G\alpha_{16}$ [32,33].

Individual GPCRs were co-expressed with one of the 16z chimaeras in COS-7 cells and the receptor-driven accumulation of IP was monitored in the absence or presence of appropriate agonists. The responses mediated by various chimaeras were compared with those of $G\alpha_{16}$ and 16z44, and the results are shown in Figure 4. QS and KD showed no significant enhancement of receptor coupling compared with $G\alpha_{16}$. LI coupled with DOR much better than $G\alpha_{16}$ and was comparable with the response mediated by 16z44. However, LI was no better than $G\alpha_{16}$ in all other cases. The double mutant QSKD also failed to couple better with the tested GPCRs. Instead, double mutants containing the LI mutation (QSLI and KDLI) exhibited significant enhancement in their coupling with DOR, KOR and SSTR1, but not in their coupling with D_2 R, MOR or XMR (Figure 4). The coupling of DOR with QSLI or KDLI resembled that of the LI mutant, suggesting that Leu³⁶⁴ of $G\alpha_{16}$ was a critical determinant for the recognition of DOR among the three selected residues. For KOR and SSTR1, the differences in the responses between LI and either QSLI or KDLI suggested that particular GPCRs required multiple proper interactions with the $\alpha 5$ helix to activate $G\alpha_{16}$. This idea was further confirmed for QSKDLI, which contained all three point mutations. The DOR-mediated IP production in QSKDLI-expressing cells was even greater than the responses of

Figure 4 Effects of the QS, KD and LI mutations and their combinations on receptor coupling of $G\alpha_{16}$

Individual GPCRs were co-expressed with $G\alpha_{16}$, 16z44 or one of the $G\alpha_{16}$ mutants in COS-7 cells. Transfectants were labelled with [³H]myo-inositol and assayed for agonist-induced IP accumulation. Agonist treatment was performed with the proper ligands (300 nM [D-Pen^{2,5}]enkephalin for DOR, 300 nM (±)-U-50488 for KOR, 1 μ M dopamine for D_2 R, 100 nM somatostatin for SSTR1, 300 nM [D-Ala², NMe-Phe⁴, Gly-oI⁵]enkephalin for MOR and 100 nM 2-iodomelatonin for XMR) to induce receptor-mediated IP formation. *Significantly higher than the $G\alpha_{16}$ -mediated responses; **significantly higher than the 16z44-mediated responses; Tukey–Kramer's test, $P < 0.05$, $n \geq 3$.

16z44. Significant enhancement of coupling was also observed for D_2 R. Modest improvements in the coupling of QSKDLI with MOR or XMR were observed. Taken together, the three individual mutations on the $\alpha 5$ helix of $G\alpha_{16}$ appeared to enhance its coupling with GPCR in a co-operative manner. In particular, Leu³⁶⁴ seemed to be a more important site compared with Gln³⁵⁰ and Lys³⁵⁷ in determining the coupling of $G\alpha_{16}$ with GPCRs.

Leu³⁶⁴ lies within a stretch of six amino acids (362–367) on the $\alpha 5$ helix. These six residues are particularly diverse among various $G\alpha$ subunits (Figure 1). Based on the distinctive phenotypes of LI mutants, it would be interesting to study their roles in determining

Figure 5 Mutation of a stretch of six non-conserved residues in the $\alpha 5$ helix enhanced the receptor-coupling capability of $G\alpha_{16}$

Experimental details are the same as described in the legend to Figure 4. *Significantly higher than the $G\alpha_{16}$ -mediated responses; **significantly higher than the 16z44-mediated responses; Tukey–Kramer's test, $P < 0.05$, $n \geq 3$.

the receptor-coupling characteristics of $G\alpha_{16}$. Another series of 16z chimaeras was then constructed. Swapping the six residues of $G\alpha_{16}$ with those of $G\alpha_z$ generated SV6. Surprisingly, as shown in Figure 5, SV6 behaved in a manner very similar to 16z44. Receptor-mediated IP productions for five of the six GPCRs tested in the present study were significantly enhanced. Both 16z44 and SV6 increased the basal activity of PLC to a similar extent ($P < 0.001$ for both 16wt versus 16z44 and SV6, one-way ANOVA with Tukey–Kramer's test; $n = 3-8$). The phenotype of SV6 indicated that the six mutated residues contributed to most, if not all, of the properties of 16z44. The lack of coupling between SV6 and XMR suggested that additional structural elements were required for some types of GPCRs. Next, chimaeras QSSV6, KDSV6 and QSKDSV6 were constructed as derivatives of SV6 by incorporating the mutations in QS and KD (Figure 1), analogous to the combined mutants in the first series. The responses mediated by these three chimaeras varied between different GPCRs. For DOR, further increases (approx. 2-fold) in both the basal activities and agonist-stimulated responses were enhanced for all three chimaeras when compared with SV6 and 16z44. For KOR, incorporation of either QS or KD mutation, but not both, also increased the receptor-mediated responses without increasing the basal activities. For SSTR1, consistently small increases in IP production, although statistically insignificant, were seen with QSSV6, KDSV6 and QSKDSV6 when compared with SV6. There was no significant increase in the D_2R -mediated responses for the combined mutants with SV6. QSKDSV6 also coupled with MOR and XMR more efficiently compared with SV6, even though the fold stimulation was actually lower.

The SV6 region was further dissected to define the relative importance of the six residues in facilitating better receptor-coupling efficiency. Three more mutants were constructed. LI4 has residues

364–367 of $G\alpha_{16}$ mutated to those of $G\alpha_z$, whereas RN2 has only two residues (366–367) mutated (Figure 1). These two mutants performed even better compared with SV6 for DOR, and performed more or less the same as SV6 and 16z44 for KOR and D_2R , which highlighted the critical roles of residues 366–367 in controlling the receptor-coupling event (Figure 5). Although the coupling of these two mutants with SSTR1 and MOR was less efficient when compared with 16z44 and SV6, they still outperformed $G\alpha_{16}$. In contrast, mutation of the single residue Ser³⁶² into valine (the SV mutant) has weaker effects on receptor coupling (Figure 5). These results indicated that the C-terminal side of the SV6 region was more critical in controlling the receptor-coupling event of $G\alpha_{16}$.

Among the two series of chimaeras, those which coupled better with various GPCRs were usually associated with higher spontaneous basal activity. In the first series, both the basal activity and the agonist-stimulated IP production mediated by QSKDLI were significantly enhanced to levels similar to those of 16z44. In the second series, all chimaeras containing the SV6 mutation also have much higher basal activities (Figure 5). However, it appeared that the spontaneous activities of the mutants were diminished as the number of mutated residues within the SV6 region was decreased. The basal responses of the cells expressing LI4, RN2 and SV were lower compared with those of 16z44 and SV6 in most of the cases ($P > 0.05$ for 16wt versus LI4, RN2 or SV, 16z44 versus LI4, not significant; $P < 0.05$ for 16z44 versus RN2 and SV, SV6 versus LI4; $P < 0.01$ for SV6 versus RN2 and SV; one-way ANOVA with Tukey–Kramer's test; $n = 3-8$). The decrease in spontaneous activities apparently also affected the efficiencies of receptor coupling. Overall, mutations of the C-terminal residues could increase the basal activity of $G\alpha_{16}$; this may in turn, at least partially, potentiate its coupling with those weakly-coupled GPCRs.

To examine further the characteristics of the receptor-coupling properties of the 'best couplers' among the two series of mutants (16z44, SV6, QSKDLI and QSKDSV6), agonist dose–response studies were performed by measuring the receptor-driven mobilization of intracellular Ca^{2+} using fluorimetric assays. Expressions of various G_i -coupled receptors alone in COS-7 cells could not support agonist-induced intracellular Ca^{2+} mobilization unless $G\alpha_{16}$ or 16z chimaeras were co-expressed [14]. In cells co-expressing $G\alpha_{16}$ with either D_2R or MOR, addition of increasing doses of dopamine or DAMGO [Tyr-D-Ala-Gly-(Me)Phe-Gly-ol] resulted in dose-dependent increases in the peak fluorescence. Co-expression of D_2R with 16z44, SV6, QSKDLI or QSKDSV6 also supported dopamine-induced Ca^{2+} mobilization. Although the maximal response elicited by dopamine in 16z44-expressing cells was similar to that of $G\alpha_{16}$ -expressing cells, the EC_{50} value for the former was lower by more than 100-fold (Figure 6 and Table 1). In terms of EC_{50} values, 16z44 remained the best, but SV6, QSKDLI and QSKDSV6 produced higher maximal responses compared with either $G\alpha_{16}$ or 16z44. Similar trends of the results were obtained for MOR, although less significant compared with D_2R . Since MOR coupled poorly with $G\alpha_{16}$ in the IP accumulation assay (Figures 4 and 5), the observed coupling between MOR and $G\alpha_{16}$ in the fluorimetric assay suggested that this assay was much more sensitive in detecting weak signals. The two mutants containing the SV6 mutations performed better than QSKDLI for both the GPCRs examined, which further supported the importance of the SV6 region in determining receptor-coupling efficiency.

As shown in the helical wheel diagram in Figure 2(B), the $\alpha 5$ helix of $G\alpha$ is surrounded by a series of secondary structures of the GTPase domain, including the $\alpha 1$ helix and all six β strands. In particular, the SV6 region is in contact with the $\beta 2$ – $\beta 3$ loop, wherein the amino acid sequences are fairly conserved within but

Figure 6 Dose-response curves of D₂R- or MOR-driven Ca²⁺ mobilization via selected G α_{16} mutants

Cells expressing D₂R or MOR with one of the indicated G α subunits were labelled with Fluo-4 AM, and receptor-driven intracellular Ca²⁺ mobilization was monitored after treatment with increasing doses of proper agonists by using FLIPR® I as described in the Experimental section. The results shown are representative of three independent experiments performed in triplicate. Data were imported into GraphPad Prism 3 and non-linear regression was performed to obtain the best-fit curves and estimate the corresponding EC₅₀ and V_{max} values, which are presented in Table 1.

diverged between G α subfamilies. This allowed us to consider that this loop may play a specific role in conveying conformational changes from the $\alpha 5$ helix to the $\beta 2$ and $\beta 3$ strands. A chimaera

named ST7 was thus made by replacing the seven residues forming the $\beta 2$ – $\beta 3$ loop (residues 198–204) in G α_{16} with those of G α_z to examine their impact on receptor coupling (Figure 7, top panel). Interestingly, the ST7 mutant did not perform any better than G α_{16} for all six GPCRs tested (Figure 7, bottom panel). The ST7SV6 mutant was then created by combining the ST7 and SV6 mutations together. In this case, interactions between these two regions, if any, would be essentially restored as in G α_z . As shown in Figure 7, ST7SV6 coupled with five out of six GPCRs tested in a slightly, but significantly, better manner when compared with G α_{16} , but the coupling was much reduced when compared with SV6 except for DOR. These results indicated that the extraordinarily effective receptor-coupling property of SV6 (and probably 16z44) may be partly due to alteration in the interactions with the $\beta 2$ – $\beta 3$ loop.

DISCUSSION

For the first series of mutants, we compared the effects of mutating three distinct residues lying on the exposed side of the $\alpha 5$ helix. The QS mutation replaced the long, terminal amide group with a relatively short hydroxy group, and its effect on enhancing receptor coupling of G α_{16} was relatively minor. Similarly, only minor changes in rhodopsin-mediated nucleotide exchange and GTP-induced activation were observed for a Q326A (Gln³²⁶ → Ala) mutant of G α_{11} [19,34]. However, rates of both spontaneous and receptor-mediated activations of a G α_{11} Q326P mutant were significantly decreased [21]. Since this glutamine residue is situated at the junction of the $\alpha 5$ helix and the $\beta 6$ strand, these findings suggested that its primary role was to provide a flexible linkage between the two secondary structures for transmitting the receptor signal into the GTP-binding pocket. The KD mutation has the side chain shortened and the charge reversed. Similar to the alanine mutation at the same position of G α_{11} [19,34], the effect on the receptor-mediated activation caused by mutating Lys³⁵⁷ of G α_{16} was negligible. A possible role for Lys³⁵⁷ of G α_{16} was to maintain the integrity of the α helix structure, since the cognate G α_{11} D333P mutant exhibited a significant increase in spontaneous activation, but impaired the receptor-mediated activation [21]. Surprisingly, LI mutation significantly increased the coupling of G α_{16} with DOR. Isoleucine is more hydrophobic than leucine in various hydrophobicity scales [35]. Synthetic peptides derived from the last 11 residues of G α_{11} bound to metarhodopsin II with much

Table 1 EC₅₀ values and relative maximal Ca²⁺-mobilization responses of the coupling of D₂R and MOR with G α_{16} and various mutants

The EC₅₀ values and maximal responses were estimated from the non-linear regression plots in Figure 6 using GraphPad Prism 3.03 for Windows. Results are the means $\pm$ S.E.M. for five individual experiments performed in triplicate. Percentage responses mediated by the corresponding mutants were calculated with reference to the G α_{16} -mediated maximal response. One-way ANOVA with Tukey–Kramer's test was performed using GraphPad Prism to obtain the *P* values against the responses of either G α_{16} or 16z44. N.S., not significant (*P* > 0.05).

G α	Average EC ₅₀ (nM) $\pm$ S.E.M. (<i>n</i> = 5)	<i>P</i> versus 16wt	<i>P</i> versus 16z44	Average % response $\pm$ S.E.M. (<i>n</i> = 5)	<i>P</i> versus 16wt	<i>P</i> versus 16z44
D₂R						
16wt	1471 $\pm$ 899	–	< 0.001	100 $\pm$ 30	–	N.S.
16z44	1.27 $\pm$ 0.63	< 0.001	–	117 $\pm$ 15	N.S.	–
SV6	14.76 $\pm$ 3.90	< 0.001	< 0.01	228 $\pm$ 12	< 0.01	< 0.05
QSKDLI	26.07 $\pm$ 7.45	< 0.001	< 0.001	185 $\pm$ 13	N.S.	N.S.
QSKDSV6	9.67 $\pm$ 2.04	< 0.001	< 0.01	178 $\pm$ 10	N.S.	N.S.
MOR						
16wt	72.46 $\pm$ 19.14	–	< 0.001	100 $\pm$ 17	–	N.S.
16z44	4.77 $\pm$ 1.66	< 0.001	–	91 $\pm$ 9	N.S.	–
SV6	13.69 $\pm$ 4.51	< 0.01	N.S.	140 $\pm$ 7	N.S.	< 0.05
QSKDLI	29.59 $\pm$ 14.28	N.S.	< 0.05	130 $\pm$ 8	N.S.	N.S.
QSKDSV6	15.90 $\pm$ 3.45	< 0.05	N.S.	123 $\pm$ 7	N.S.	N.S.

Figure 7 Interactions between the $\alpha 5$ helix and the $\beta 2$ – $\beta 3$ loop contribute to the enhanced receptor-coupling property

Top panel: schematic representations of the mutants with mutations at the $\beta 2$ – $\beta 3$ loop (ST7) and the $\alpha 5$ helix (SV6). Middle panel: the sequence alignment of the corresponding regions in $G\alpha_{16}$ and $G\alpha_z$ with the representative member of each $G\alpha$ subfamily. Bottom panel: experimental details are essentially the same as described in the legend to Figure 4. *Significantly higher than the $G\alpha_{16}$ -mediated responses; Tukey–Kramer's test, $P < 0.05$, $n \geq 3$.

higher affinities when compared with the original sequence if the first two residues (corresponding to Leu³⁶⁴ and Ala³⁶⁵ of $G\alpha_{16}$) were changed into more hydrophobic residues [36–38]. Presumably, the acquaintance of higher hydrophobicity to these residues substantially stabilized the reverse capping structure of the last seven residues [28] and thus favoured its interaction with receptors. This idea is also in line with the notion that the loss of hydrophobicity from isoleucine to alanine at this position caused defective receptor-mediated activation [19]. Despite the supporting evidence from $G\alpha_{11}$, subtle change in hydrophobicity may not be sufficient to promote an enhanced coupling of $G\alpha_{16}$ with all GPCRs. Increasing the hydrophobicity of Leu³⁶⁴ of $G\alpha_{16}$ may only partially enhance its coupling with particular GPCRs.

The co-existence of LI mutation with either QS or KD significantly enhanced the coupling of $G\alpha_{16}$ with KOR, SSTR1 and

DOR. It unmasked the significance of QS and KD for specific GPCRs to recognize $G\alpha_{16}$, and further supported the idea that different GPCRs may have their own structural requirements for $G\alpha_{16}$ interactions. This point was further illustrated by the phenotypes of the triple mutant QSKDLI (Figure 4), the only mutant in the first series capable of enhancing the coupling with MOR (and XMR, to a lesser extent). Studies of chimaeric GPCRs suggested that there are at least two separate G-protein-interacting domains on every GPCR, including a 'selectivity domain' that governs the preferences to specific G-proteins and an 'activation domain' for activating the bound G-protein irrespective of the selectivity [39]. It can be envisaged that the selectivity domains on MOR and XMR may be highly selective to $G\alpha_z$ -specific residues for effective coupling. It should be noted that the coupling of QSKDLI with MOR or XMR was substantially weaker when compared with 16z44. One of the major receptor-binding sites is believed to be the $\alpha 4/\beta 6$ loop [8], but this loop in $G\alpha_{16}$ is very different in length and sequence from other $G\alpha$ subunits. 16z44 has the entire $\alpha 5$ helix and a part of the $\alpha 4/\beta 6$ loop replaced with the $G\alpha_z$ sequence, which may restore a more compact loop structure of $G\alpha_z$ and thus confer higher receptor-coupling efficiency.

The SV6 mutants have provided additional insights on receptor–G-protein coupling not addressed previously. A relevant previous study suggested that differences in the G-protein-coupling selectivity between bovine (G_i -coupled) and scallop (G_o -coupled) rhodopsins depend on the identities of residues –6 to –11 of the $G\alpha$ subunits [40]. For SV6 mutants, Ser³⁶² and Val³⁶³ were mutated into the more hydrophobic residues valine and isoleucine respectively. The enhancement of receptor coupling in SV6 mutants might thus be the result of an increase in overall hydrophobicity at and before the –11 position. The decreased receptor-coupling efficiency of LI4 mutant compared with SV6 further illustrated this point. By combining SV6 with QS and/or KD, the resultant chimaeras were the most efficient couplers for the receptors tested (Figure 5). Particularly, QSKDSV6 outperformed 16z44 and SV6 with most of the GPCRs tested. These results further indicated that the identities of the residues throughout the whole $\alpha 5$ helix are critical for determining the receptor-coupling specificity of $G\alpha_{16}$.

Expressions of the 'best couplers', including QSKDLI, SV6 and its derivatives, significantly increased the basal IP levels, suggesting that these mutants, similar to 16z44, were spontaneously active. Studies on $G\alpha_{11}$ have already shown that the GDP/GTP exchange rate is promoted by C-terminal mutations [21,34]. Our previous study on 16z44 indicated that such a behaviour cannot be reversed by inverse agonists [9]. The same might apply to SV6 and its related mutants. The spontaneous activation of QSKDSV6 did not mask its robust coupling with various GPCRs (except perhaps XMR). Our findings were consistent with previous studies on $G\alpha_{12}/G\alpha_s$ chimaeras [17,18] and $G\alpha_{11}$ mutants [19–21], implying that modifications of the C-terminal region perturbed the activation state of the $G\alpha$ subunits. Moreover, our results can be taken as evidence for such potentiation of basal activity in the $G\alpha_q$ subfamily.

The mutants LI4, RN2 and SV also showed coupling efficiencies better than $G\alpha_{16}$ and, in some cases, better than 16z44. Unlike SV6, LI4 and RN2 mutants had much decreased spontaneous basal activities. The first two residues Ser³⁶² and Val³⁶³ make major contacts with the β strand network ($\beta 4$, $\beta 5$ and $\beta 6$; Figure 2C), whereas the rest may make contacts with the $\alpha 4/\beta 6$ loop or the extreme C-terminal turn depending on the activation state of $G\alpha$. Since the phenotypes of LI4 and RN2 were similar, it suggested that the two residues Arg³⁶⁶ and Tyr³⁶⁷ are more critical in determining the receptor-coupling efficiencies. We have preliminary evidence indicating that the interactions between

Arg³⁶⁶ and Tyr³⁶⁷ with cognate residues at the extreme C-terminus (Asp³⁶⁹ and Glu³⁷⁰) are essential for successful receptor coupling, and the misplacement of 'uncoupled pairs' of amino acids might prevent G α_{16} from coupling with GPCR. A previous study using conformational-constrained C-terminal peptides derived from G α_{11} sequences has revealed that the close interactions between these two pairs of amino acids, which pre-organized the terminal helical turn of the $\alpha 5$ helix, are important for efficient receptor binding [36]. The slight enhancement of receptor coupling caused by S362V mutation supported the idea that increased hydrophobicity of the inner side of the $\alpha 5$ helix at this position may also facilitate the receptor-mediated activation of G α .

The $\beta 2/\beta 3$ loop interacts with the SV6 region (Figures 2B and 2C) and conveys the receptor signals to the guanine nucleotide-binding pocket at the other ends of the two β strands. Alternatively, it may directly interact with GPCR and contribute to the coupling specificity and efficiency, as it extends to the plasma membrane-facing surface of G α . The ST7 mutant with the $\beta 2/\beta 3$ loop of G α_{16} mutated to that of G α_z did not promote receptor coupling (Figure 7), suggesting that it might not interact with GPCR directly. Combining ST7 with SV6 mutations significantly decreased the enhanced receptor-coupling efficiencies, implying that the superior receptor-coupling efficiencies of SV6 were caused by the re-formation of intramolecular interactions, at least partly with the $\beta 2/\beta 3$ loop. For the ST7SV6 mutant, the normal intramolecular interactions as in G α_z were presumably resumed and thus reversed the phenotypes of SV6. It has been postulated that the network of β strands in the GTPase domain, particularly $\beta 1$ – $\beta 3$, makes contacts with $\beta 6$ and $\alpha 5$ helices to convey the receptor signals to the guanine nucleotide-binding pocket [19]. The phenotypes of the ST7 mutants are surprisingly the first evidence showing the importance of the coupling between the $\beta 2/\beta 3$ loop and the $\alpha 5$ helix for promoting the receptor-mediated activation of G-protein.

G α_{16} permits investigations of receptor–G-protein coupling in a much broader context. The contributions of individual secondary structural elements have been examined against a wide variety of GPCRs with different coupling efficiencies, thereby allowing a more comprehensive comparison of their relative importance in receptor-coupling events. The present study has provided prominent functional evidence to illustrate the mechanism of receptor-mediated activation of a G-protein with reference to multiple GPCRs. The GPCR–G α_{16} may be considered as a generalized model for studying the changes in receptor–G-protein interactions.

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