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Incorporation of $G\alpha_z$-Specific Sequence at the Carboxyl Terminus Increases the Promiscuity of $G\alpha_{16}$ toward G_i-Coupled Receptors

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ABSTRACT

Although the promiscuous nature of G_{16} allows it to interact with numerous G protein-coupled receptors, several G_i-linked receptors are incapable of activating phospholipase C via G_{16}. A series of chimeras between $G\alpha_{16}$ and $G\alpha_z$ were constructed and assayed for their ability to mediate receptor-induced stimulation of phospholipase C. Two $G\alpha_{16/z}$ chimeras harboring 25 or 44 $G\alpha_z$-specific sequences at their C termini (named 16z25 and 16z44) were capable of responding to 14 different G_i-coupled receptors tested, including those that were either unable to associate with $G\alpha_{16}$ (melatonin Mel1c) or activate $G\alpha_{16}$ weakly (μ-opioid and type 1 somatostatin). Agonist-induced stimulation of phospholipase C was more efficiently mediated (higher maximal and lower EC_{50} value) by 16z44 than by $G\alpha_{16}$. Both 16z25 and 16z44 were also coupled to G_s- and G_q-linked receptors. Incorporation of $G\alpha_z$ sequence at the N terminus of $G\alpha_{16}$ did not further enhance the ability of the chimeras to interact with G_i-coupled receptors. Expression of the various chimeras was verified by immunodetection and functional analysis of their constitutively activated mutants. These results show that the incorporation of $\alpha 4/\beta 6$ and $\alpha 5$ regions of $G\alpha_z$ into a $G\alpha_{16}$ backbone can improve the recognition of G_i-coupled receptors. $G\alpha_{16/z}$ chimeras with expanded capability to interact with G_i-linked receptors may be used to link orphan receptors to the stimulation of phospholipase C.

The G proteins are responsible for the efficient transmission of signals from agonist-bound cell surface receptors to different intracellular effectors (Bourne, 1997). Currently, the total number of G protein-coupled receptors (GPCRs) far exceeds the number of known G proteins. Each member of the four different subfamilies of G proteins must therefore be capable of interacting with multiple GPCRs. Depending on their coupling specificity, most GPCRs are often referred as G_q, G_i, or G_s coupled, which reflects their primary signal transduction pathway. Some G proteins are more promiscuous than others by possessing the ability to interact with a large panel of GPCRs. The most notable examples of promiscuous G proteins are the human G_{16} and its murine homolog, G_{15}. Both G_{15} and G_{16} link a variety of G_q-, G_i-, and G_s-coupled receptors to the stimulation of phospholipase C (PLC; Offermanns and Simon, 1995; Lee et al., 1998). Because of their promiscuity, G_{15} and G_{16} are ideal candidates for linking orphan receptors (cloned receptors without a known ligand) to PLC and its downstream effectors. Hence, G_{16} has received considerable attention as a potential tool for drug discovery (Milligan et al., 1996).

Although G_{15} and G_{16} are more promiscuous than other G proteins, they cannot be considered as true universal adapters for GPCRs. For example, the CCR2a chemokine receptor (Kuang et al., 1996), the α_{1A}- and α_{1C}-adrenoceptors (Wu et al., 1992) are unable to recognize G_{16}. Indeed, of the 33 different GPCRs examined to date (Wu et al., 1992, 1993; Offermanns and Simon, 1995; Zhu and Birnbaumer, 1996; Kuang et al., 1996; Lee et al., 1998; Parmentier et al., 1998), at least six receptors are incapable of activating G_{16}. Most of the GPCRs that fail to activate G_{16} belong to the G_i-coupled receptor subfamily. Because approximately 18% of the total number of G_i-coupled receptors examined to date cannot activate G_{16}, the molecular structure of $G\alpha_{16}$ may not be optimal for association to GPCRs with a high preference for G_i proteins. This is perhaps unavoidable if $G\alpha_{16}$ were to possess the ability to recognize G_q- and G_s-coupled receptors as well. The G_i-coupled receptors constitute an exceedingly large GPCR subfamily that encompasses many newly discovered receptors such as those for chemokines. This poses a serious concern for the use of G_{16} as an adapter of orphan receptors in drug-screening protocols.

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ABBREVIATIONS: GPCR, G protein-coupled receptor; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; PLC, phospholipase C; PCR, polymerase chain reaction; PTX, pertussis toxin; IP, inositol phosphates; DMEM, Dulbecco's modified Eagle's medium.

The overall receptor contact regions on the α-subunit of several G proteins have been mapped (reviewed in Conklin and Bourne, 1993; Onrust et al., 1997), and they are in good agreement with the available crystal structure data (Wall et al., 1995; Lambright et al., 1996). Both the N and C termini are sites for receptor contact. Promiscuity for GPCRs can be increased by altering the amino acid sequence of either the N or C terminus. Modification of the last five residues of G_q (Conklin et al., 1993) or G_s (Liu et al., 1995; Conklin et al., 1996) expanded the number of GPCRs that can use the resultant chimeras to regulate the corresponding effectors beyond those of the parental constructs. Likewise, alterations at the N terminus of G_q resulted in additional coupling to several GPCRs that do not normally use G_q for signal transduction (Kostenis et al., 1998). These studies illustrate the possibility of changing the coupling specificity of G proteins by modifying their putative receptor contact sites on the α-subunit. Many G_i-coupled receptors share the ability to inhibit adenylyl cyclase via the pertussis toxin (PTX)-insensitive G_z (Chan et al., 1995, 1998; Lai et al., 1995; Shum et al., 1995; Tsu et al., 1995a,b; Yung et al., 1995). Thus, the incorporation of G_z-specific sequences into a G_{16} backbone might improve the ability of the resultant chimera to recognize G_i-coupled receptors. The present study describes the construction and characterization of a series of chimeras between the α-subunits of G_{16} and G_z ($G\alpha_{16}$ and $G\alpha_z$). Indeed, some of the resultant chimeras has increased promiscuity toward G_i-coupled receptors and are more efficient than G_{16} in their interactions with GPCRs.

Experimental Procedures

Materials. The $G\alpha_{16}$ cDNA was a kind donation from Dr. M. Simon (California Institute of Technology, Pasadena, CA), and all other cDNAs were constructed or obtained as previously described (Wong et al., 1992; Tsu and Wong, 1996; Lee et al., 1998). Simian kidney fibroblast COS-7 cells were obtained from the American Type Culture Collection (ATCC CRL-1651; Rockville, MD). Various receptor agonists were purchased from Research Biochemicals Inc. (Natick, MA), Sigma Chemical Co. (St. Louis, MO), or Tocris Cookson (Bristol, UK). Sequenase Version 2.0 DNA sequencing kit and ECL enhanced chemiluminescence detection kit were purchased from Amersham Pharmacia Biotech. *myo*-[^{3}H]Inositol was from DuPont-New England Nuclear (Boston, MA). Plasmid DNA purification columns were obtained from Qiagen (Hilden, Germany). $G\alpha_z$-specific antisera 3A-170 (C-terminal) and 3-18 (N-terminal) were obtained from Gramsch Laboratories (Schwabhausen, Germany) and Calbiochem (La Jolla, CA), respectively. *Taq* DNA polymerase, restriction endonucleases, custom mutation primers, and cell culture reagents were obtained from Life Technologies Inc. (Grand Island, NY), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction of Chimeras and Mutants. All of the chimeras were constructed by polymerase chain reactions (PCRs) using human $G\alpha_{16}$ and rat $G\alpha_z$ cDNAs (subcloned in the *Xba*I and *Eco*RI sites of pcDNAI, respectively) as templates with T7 and SP6 promoter sequences as outer flanking priming regions. A pair of chimeric primers covering both the nucleotide sequences of $G\alpha_{16}$ and $G\alpha_z$ were designed as appropriate for each chimeric construct. First, two overlapping fragments that corresponded to the portions of $G\alpha_{16}$ and $G\alpha_z$ were generated. The 5′ fragment was made with T7 primer and the reversed chimeric primer, whereas the 3′ fragment was made with the forward chimeric primer and a primer annealed to the SP6 promoter on the vector sequence (SP6 primer). The two PCR products were annealed together, and the full-length fragments were made using the T7 and SP6 primers. Specific primers used for the construction of the various chimeras were listed below, with the nucleotide sequences of $G\alpha_z$ underlined: 16z25/S, CAC TAC ACA TGT GCC ACA GAC ACC AGT AAC ATC; 16z25/AS, GAT GTT ACT GGT GTC TGT GGC ACA TGT GTA GTG; 16z44/S, GGC CCC GAG GGC AGC AAC CGA AAC AAG GAG; 16z44/AS, CTC CTT GTT TCG GTT GCT GCC CTC GGG GCC; 16z66/S, GCT ACC TAT TTC CCC GAG TAC AAG GGT CAG; 16z66/AS, CTG ACC CTT GTA CTC GGG GAA ATA GGT AGC; 30z16/S, GAG AGC CAG CGG CAG CGC GGG GAG CTG AAG; and 30z16/AS, CTT CAG CTC CCC GCG CTG CCG CTG GCT CTC. $MgCl_2$ (1.5 mM) was included in the PCR mixture, and the PCR products were amplified with thermal cycling at 94°C for 60 s, 50°C for 90 s, and 72°C for 90 s for 30 cycles using Robocycler 40 from Stratagene (La Jolla, CA). The 30z16z44 and 30z16z66 chimeras were constructed in a similar manner except one of the half-products was amplified by PCR using either the 16z44 or 16z66 chimera as the template. Full-length chimeric α-subunit cDNAs were subcloned into either pcDNA3 or pcDNA3.1Zeo(+) mammalian expression vectors (InVitrogen, San Diego, CA). DNA sequences of the mutants were checked by dideoxynucleotide sequencing method using Sequenase V2.0 kit and restriction mapping.

A GTPase-deficient mutant of $G\alpha_{16}$ ($G\alpha_{16}$QL) with Gln^{212} mutated into Leu was constructed essentially as described previously (Qian et al., 1994). Functional constitutive activity of $G\alpha_{16}$QL was confirmed by transiently expressing the construct in COS-7 cells and determining the basal PLC activity in the transfectants. A *Hin*dIII/*Xcm*I fragment from $G\alpha_{16}$QL was used to construct the GTPase-deficient mutants of 16z25, 16z44, and 16z66. The resultant mutant chimeric constructs were verified by restriction mapping.

Transfection of COS-7 Cells. Simian kidney fibroblast COS-7 cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (v/v), 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in humidified air with 5% CO_2. Then, 1×10^5 cells/well were seeded onto 12-well plates the day before transfection. DEAE-dextran-mediated transfection was performed as described previously (Wong, 1994). Briefly, appropriate amounts of various DNA samples purified by Qiagen column chromatography were mixed with growth medium containing 250 μg/ml DEAE-dextran and 100 μM chloroquine. Cells were incubated with the transfection cocktails for ~3.5 h and then shocked for 1 min at room temperature in PBS containing 10% dimethyl sulfoxide (v/v). After rinsing with PBS, the cells were returned to growth media for 24 h. Approximately 50% of the cell population will take up the cDNAs as indicated by cotransfecting a plasmid DNA encoding β-galactosidase as a reporter.

Inositol Phosphates (IP) Accumulation Assay. An aliquot (750 μl) of inositol-free DMEM containing 5% FCS and 2.5 μCi/ml *myo*-[^{3}H]inositol was added to each well of transfected COS-7 cells and incubated for 18 to 24 h. The labeling media were subsequently replaced by 1 ml of inositol phosphate assay medium (DMEM buffered with 20 mM HEPES, pH 7.5, containing 20 mM LiCl) for 10 min, and then 1 ml of IP assay medium containing the appropriate agonist was added to the cells for an additional 1 h at 37°C. Reactions were stopped by adding 750 μl of ice-cold 20 mM formic acid and stored at 4°C for 30 min. [^{3}H]IP were separated from other labeled inositol species by sequential ion-exchange chromatography as described previously (Tsu et al., 1995a).

Membrane Protein Preparation and Immunodetection of Recombinant Proteins. COS-7 cells were grown on 150-mm dishes to 70 to 80% confluence. Transfection was performed as on 12-well plates with proper adjustments to the volumes and amounts of the reagents used. After 48 h in normal growth conditions, cells were washed with Ca^{2+}/Mg^{2+}-free PBS and harvested with 5 ml of Ca^{2+}/Mg^{2+}-free PBS containing 10 mM EDTA. The following procedures were performed at 4°C. Cells were spun down briefly (200*g*, 5 min); resuspended in hypotonic lysis buffer (50 mM Tris · HCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, and 1 mM dithiothreitol, pH 7.4); and lysed by one

cycle of freeze-thawing followed by 10 passages through a 27-gauge needle. Nuclei were removed by brief spinning, and membranes were collected by spinning the supernatants at 15,000*g* for 15 min. Membrane pellets were finally resuspended in lysis buffer. Protein concentrations were determined using the Bio-Rad Protein Assay Kit. For immunodetection, 50 μg of each membrane protein sample was resolved on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes through electroblotting. Protein molecular weight markers were visualized by Coomassie blue staining. Several chimeras were detected by the $G\alpha_z$-specific antiserum from Calbiochem and with enhanced chemiluminescence (ECL kit; Amersham).

Data Analysis. [^{3}H]IP was estimated by determining the ratios of [^{3}H]IP to [^{3}H]inositol plus [^{3}H]IP as previously described (Tsu et al., 1995a). Absolute values for IP accumulation varied between experiments, but variability within a given experiment was less than 10% in general. Unless otherwise stated, data shown in the figures and tables represent the mean ± S.E. of three or more independent experiments performed in triplicate. Bonferroni's *t* test with 95% confidence limit was adopted to verify the significance between different treatment groups within the experiments.

Results

Design of C-Terminal Chimeras. Although multiple regions in the primary structure of $G\alpha_{16}$ are required for receptor coupling (Lee et al., 1995), the molecular determinants for the promiscuous property of $G\alpha_{16}$ has not been fully delineated. Numerous studies on other α-subunits have implicated the C-terminal tail of the α-chain as one of the major receptor contact regions (Sullivan et al., 1987; Conklin et al., 1993, 1996; Tsu et al., 1997). As a first step toward enhancing the ability of $G\alpha_{16}$ to recognize G_i-coupled receptors, we constructed a series of $G\alpha_{16/z}$ chimeras by incorporating different lengths of $G\alpha_z$ sequences at the C terminus of $G\alpha_{16}$. $G\alpha_z$ was selected as a donor for the construction of the chimeras because it recognizes practically all G_i-coupled receptors (Wong et al., 1992; Chan et al., 1995, 1998; Lai et al., 1995; Shum et al., 1995; Tsu et al., 1995a,b; Yung et al., 1995) but is insensitive to PTX. Signals transduced by chimeric $G\alpha_{16/z}$ can be easily discerned from G_i-mediated signals with the use of PTX. Based on the alignment of the C-terminal sequences of $G\alpha_{16}$ and $G\alpha_z$ (Fig. 1), we selected three junctional sites for the construction of $G\alpha_{16/z}$ chimeras.

Because the α5 helix is a known contact region for receptors (Lichtarge et al., 1996), we replaced the entire α5 helix of $G\alpha_{16}$ with that of $G\alpha_z$. Based on the crystal structures of $G\alpha_{t1}$ (Lambright et al., 1996) and $G\alpha_{i1}$ (Wall et al., 1995), the α5 helix of $G\alpha_{16}$ is predicted to be composed of the last 25 residues. The resultant chimera was therefore named 16z25; for 16z25 and subsequent chimeras, the number following the letter z indicates the number of $G\alpha_z$ residues present in the C terminus of the construct.

A unique structural feature of $G\alpha_{16}$ (and $G\alpha_{15}$) is an insertion of 11 residues (amino acids 326–336; based on the alignment of all mammalian Gα-subunits using the Clustal X program), which is absent in all other Gα-subunits, between the α4 helix and the β6 strand. To test whether this insertion is critical for the promiscuity of $G\alpha_{16}$, we constructed the

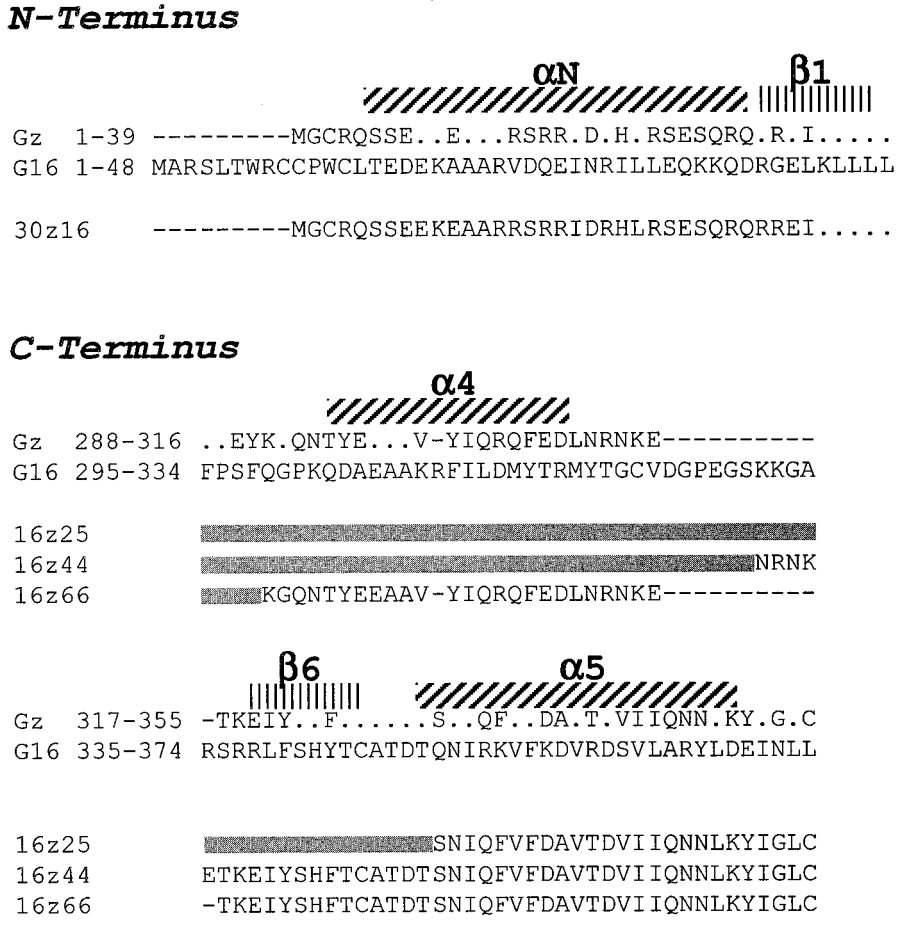

Fig. 1. Alignment of amino acid sequences of $G\alpha_{16}$, $G\alpha_z$, and their chimeras. The amino acid sequences of of the N and C termini $G\alpha_{16}$ and $G\alpha_z$ are aligned using the Clustal X program. Gaps introduced for better alignment of the sequences are hyphenated. Residues of $G\alpha_z$ that are identical to $G\alpha_{16}$ are shown as dots. Numbers shown represent the relative positions of the amino acids of $G\alpha_{16}$ and $G\alpha_z$. Shaded horizontal columns depict sequences of the chimeras that are identical to $G\alpha_{16}$. Putative secondary structures based on the $G\alpha_{t1}$ crystal structure are indicated by striped (α-helix) and solid (β-strand) horizontal columns above the $G\alpha_z$ sequence.

16z44 chimera. In this chimera, half of the $\alpha 4/\beta 6$ insertion was replaced by $G\alpha_z$ residues without shortening the insertion (Fig. 1). Last, we replaced the $\alpha 4/\beta 6/\alpha 5$ domains of $G\alpha_{16}$ with the cognate regions of $G\alpha_z$ by creating the 16z66 chimera with a junctional site between the αG and $\alpha 4$ helices (Fig. 1). The 16z66 chimera has approximately 20% of the C-terminal residues of $G\alpha_{16}$ substituted by those of $G\alpha_z$ and is shorter than $G\alpha_{16}$ by 12 amino acids. No epitope tag was engineered into the chimeras in case it disrupts receptor recognition.

Functional Coupling of $G\alpha_{16/z}$ Chimeras to δ-Opioid Receptor. We used a well established transient expression system to examine the ability of the $G\alpha_{16/z}$ chimeras to interact with G_i-coupled receptors. We have previously shown that coexpression of the δ-opioid receptor (a typical G_i-coupled receptor) and $G\alpha_{16}$ in COS-7 cells permits the δ-selective opioid agonist [D-Pen^2,D-Pen^5]enkephalin (DPDPE) to stimulate the formation of IP (Lee et al., 1998). Here, we adopted the same approach to study the $G\alpha_{16/z}$ chimeras. In accordance with our earlier report (Lee et al., 1998), 100 nM DPDPE stimulated IP formation by 3-fold in COS-7 cells coexpressing the $G\alpha_{16}$ and the δ-opioid receptor (Fig. 2). In contrast, agonist treatment failed to evoke formation of IP in COS-7 cells cotransfected with cDNAs encoding the δ-opioid receptor and $G\alpha_z$ (Fig. 2). In COS-7 cells coexpressing the δ-opioid receptor with either 16z25 or 16z44, 100 nM DPDPE stimulated the formation of IP by 3- to 4.5-fold (Fig. 2). Interestingly, the DPDPE response was significantly higher in cells transfected with the 16z44 cDNA than those expressing 16z25 or $G\alpha_{16}$ (Fig. 2 and Table 1).

Unlike 16z25 and 16z44, the 16z66 chimera failed to mediate the DPDPE response under identical experimental conditions (Fig. 2). Hence, we checked the expression of $G\alpha_{16/z}$ chimeras by Western blot analysis. Because most of the $G\alpha_{16/z}$ chimeras contained $G\alpha_z$ C-terminal sequences, we could not use commercially available $G\alpha_{16}$-specific C-terminal antiserum to verify the expression of these chimeras. Instead, we used a $G\alpha_z$-specific antiserum for the immunodetection of the 16z25, 16z44, and 16z66 chimeras. As shown in Fig. 3, all three chimeras were detected by the $G\alpha_z$-specific antiserum 3A-170 in membranes prepared from COS-7 cells transfected with the chimeras.

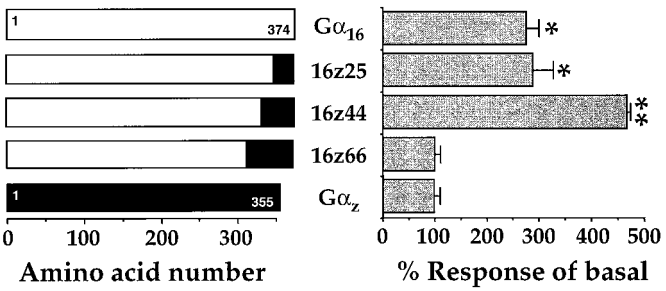

Fig. 2. Stimulation of PLC by the δ-opioid receptor via the 16z25 and 16z44 chimeras. $G\alpha_{16/z}$ chimeras were constructed as described in *Materials and Methods*. Left, schematic representation of the chimeras. Activation of PLC was determined by transfecting COS-7 cells with cDNAs (0.25 μg/ml each) encoding the δ-opioid receptor and one of the three chimeric G proteins: 16z25, 16z44, or 16z66. Additional transfections with the wild-type $G\alpha_{16}$ and $G\alpha_z$ were carried out as controls. Transfected cells were labeled with 2.5 μCi/ml *Myo*-[^{3}H]inositol 20 to 24 h before the assay. Formation of IP was measured in the absence and presence of 100 nM DPDPE. Results are expressed as percentage stimulation of IP production compared with basal activity. Basal values expressed as the ratio ($\times 10^3$) of IP to total inositols ranged from 10.5 ± 0.8 to 16.2 ± 1.8. *DPDPE-induced IP formation was significantly higher than basal levels. **DPDPE-stimulated IP accumulation was significantly higher than that observed in the $G\alpha_{16}$ transfected cells; Bonferroni's t test, $P < .05$.

Next, we tested the possibility that 16z66 has lost the ability to stimulate PLC by using a constitutively active mutant of 16z66. Mutation at codon 212 of $G\alpha_{16}$ has been shown to constitutively activate PLC in Swiss 3T3 cells (Qian et al., 1994). Expression of $G\alpha_{16}$QL (harboring the Q212L mutation) in COS-7 cells also led to increased basal accumulation of IP, whereas the expression of $G\alpha_{16}$ or $G\alpha_z$QL (Wong et al., 1992) did not affect the PLC activity (Fig. 4). The constitutively active mutants of the $G\alpha_{16/z}$ chimeras were similarly expressed in COS-7 cells. Except for 16z66QL, all $G\alpha_{16/z}$ chimeras harboring the Q212L mutation constitutively stimulated PLC activity by 3- to 6-fold above those of their corresponding wild-type chimeras (Fig. 4). The lack of constitutive activity of 16z66QL was not due to a deficiency in expression because 16z66QL was expressed to the same level as 16z25QL and 16z44QL (Fig. 3). These results support the notion that 16z66 cannot stimulate PLC.

Promiscuity of 16z25 and 16z44. The ability of 16z25 and 16z44 to interact productively with the δ-opioid receptor prompted us to further investigate into their capacity to functionally associate with other G_i- and G_s-coupled receptors. COS-7 cells were cotransfected with $G\alpha_{16}$, 16z25, or 16z44 and a receptor (0.25 μg/ml per construct) chosen from a panel of G_i- or G_s-coupled receptors that were available in our laboratory. The selected receptors include the adenosine A_1, α_2- and β_2-adrenergic, complement C5a, dopamine D_1 and D_2, formyl peptide, luteinizing hormone, opioid receptor-like (ORL_1), vasopressin V_2, somatostatin-1 and -2 (SSTR1 and SSTR2), three subtypes each of melatonin (1a, 1b, and 1c) and opioid (μ, δ, and κ) receptors (Table 1). Transfected cells were assayed for IP formation in the absence or presence of saturating concentrations of the appropriate agonists. All 14 G_i-coupled receptors examined were capable of activating 16z25 and elicit agonist-induced PLC activation (Table 1). The magnitude of PLC stimulation by the various receptors ranged from 1.5- to 4.5-fold. Activation of 16z25 by aminergic receptors (dopamine and melatonin receptors) resulted in up to 3.5-fold stimulation of PLC activity. The receptors for peptide ligands gave slightly higher responses in general. Similar results were obtained with the 16z44 chimera (Table 1). All of the G_i-coupled receptors tested were efficiently coupled to 16z44 and stimulated PLC. The 16z44-mediated PLC responses ranged from 1.7- to 5.5-fold stimulation. It should be noted that none of the G_i-coupled receptors, except SSTR2, was capable of stimulating IP production in the absence of 16z25 or 16z44 (unpublished data; Lee et al., 1998). Collectively, these results indicate that both 16z25 and 16z44 are capable of linking a large variety of G_i-coupled receptors to the stimulation of PLC. In terms of absolute amount of IP formation, 16z25 was generally less efficient than 16z44 in transducing signals from G_i-coupled receptors. When G_i-coupled receptors such as the formyl peptide, melatonin Mel1a, and ORL_1 receptors were tested against the 16z66 chimera, no functional coupling could be detected.

Compared with $G\alpha_{16}$, the 16z25 and 16z44 chimeras exhibited enhanced capability to interact with G_i-coupled receptors. Most notable of all was their ability to interact productively with the melatonin Mel1c receptor. The Mel1c was unable to activate $G\alpha_{16}$, whereas both Mel1a and Mel1b

TABLE 1

Coupling of $G\alpha_{16}$, 16z25, and 16z44 to Various G_i- or G_s-Coupled Receptors

COS-7 cells were cotransfected with cDNAs encoding 16z25 or 16z44 and the indicated receptors (0.25 μg/ml per construct). Transfected cells were labeled with 2.5 μCi/ml [^{3}H]inositol 20 to 24 h before assay. IP formations were determined in the absence (basal) or presence of specific agonists for the indicated receptors.

fMLP, *N*-formylmethionyl-leucyl-phenylalanine; hCG, human choriogonadotropin; LHR, luteinizing hormone receptor; PIA, (+)-N^6-(2-phenylisopropyl)-adenosine.

Receptor	Ligand	IP Formation								
		$G\alpha_{16}$			16z25 Chimera			16z44 Chimera		
		Basal	Agonist	% Res.	Basal	Agonist	% Res.	Basal	Agonist	% Res.
G_i-coupled										
Adenosine A_1	10 μM PIA	11.7 ± 0.9	90.3 ± 11.4*	766[a]	30.3 ± 1.4	59.7 ± 5.3*	197	49.9 ± 3.2	93.9 ± 7.8*	188
α_2-Adrenergic	1 μM UK14,304	14.7 ± 1.6	107.4 ± 9.6*	730	31.8 ± 2.5	116.1 ± 8.8*	365	42.6 ± 0.9	126.7 ± 1.5*	297
C5a	100 nM C5a	11.1 ± 1.2	67.5 ± 8.1*	608	18.4 ± 3.0	79.6 ± 6.5*	432	19.1 ± 1.1	87.4 ± 7.2*	457
Dopamine D_2	10 μM dopamine	11.6 ± 1.5	24.0 ± 2.3*	207[a]	12.3 ± 0.9	40.1 ± 0.9*	324	28.0 ± 3.4	84.5 ± 6.2*	302
fMLP	200 nM fMLP	12.1 ± 0.9	68.6 ± 5.4*	566	16.6 ± 2.5	58.4 ± 5.1*	352	21.4 ± 1.6	91.6 ± 7.2*	428
Melatonin Mel1a	1 μM 2-iodomelatonin	15.4 ± 1.2	45.6 ± 6.0*	296	53.4 ± 2.9	80.4 ± 4.0*	150	53.7 ± 5.8	91.9 ± 8.8*	171
Melatonin Mel1b	1 μM 2-iodomelatonin	16.2 ± 2.6	31.7 ± 2.3*	195	69.7 ± 3.8	108.3 ± 3.6*	155	63.6 ± 2.0	113.2 ± 7.3*	177
Melatonin Mel1c	1 μM 2-iodomelatonin	13.9 ± 1.4	16.1 ± 3.7	115	15.7 ± 1.3	42.5 ± 6.7*	270	28.2 ± 3.0	86.8 ± 7.0*	307
δ-Opioid	100 nM DPDPE	10.1 ± 1.5	42.2 ± 6.1*	417	23.2 ± 4.1	66.8 ± 7.1*	287	22.2 ± 1.4	121.8 ± 19.1*	548
κ-Opioid	100 nM U50,488	10.3 ± 2.6	33.7 ± 3.4*	327	23.5 ± 3.6	44.2 ± 3.6*	188	27.3 ± 3.8	70.3 ± 5.2*	257
μ-Opioid	100 nM DAGO	11.9 ± 1.5	16.0 ± 0.8*	134	32.9 ± 2.0	74.0 ± 3.7*	224	25.4 ± 4.6	68.5 ± 8.7*	269
ORL_1	100 nM nociceptin/OFQ	10.2 ± 1.3	64.1 ± 8.7*	628	18.2 ± 1.1	74.3 ± 6.7*	408	29.3 ± 0.5	111.5 ± 10.8*	380
SSTR1	100 nM somatostatin	12.6 ± 1.6	24.2 ± 2.6*	198[a]	17.5 ± 0.2	60.7 ± 7.4*	347	32.7 ± 1.8	121.8 ± 4.0*	372
SSTR2	100 nM somatostatin	16.5 ± 2.5	106.4 ± 10.8*	645[a]	13.3 ± 1.3	62.2 ± 5.5*	467	36.6 ± 0.4	169.1 ± 5.5*	462
G_s-coupled										
β_2-Adrenergic	10 μM isoproterenol	16.1 ± 1.5	127.0 ± 5.9*	784[a]	15.5 ± 2.1	55.9 ± 5.0*	360	30.6 ± 0.4	94.2 ± 5.8*	307
Dopamine D_1	10 μM dopamine	10.8 ± 1.7	98.3 ± 8.1*	904[a]	16.3 ± 3.4	34.9 ± 0.3*	214	32.7 ± 0.8	94.0 ± 2.2*	287
LHR	1 μg/ml hCG	10.2 ± 1.1	30.6 ± 1.2*	300[a]	13.7 ± 0.3	16.1 ± 0.8	117	19.3 ± 0.3	22.7 ± 1.9	117
Vasopressin V_2	100 nM vasopressin	11.8 ± 1.0	39.2 ± 1.5*	332	14.5 ± 1.6	42.2 ± 2.5*	291	18.6 ± 1.2	59.8 ± 4.9*	321

* Agonist treatments significantly increased IP formation over basal levels; Bonferroni *t* test, $P < .05$.

[a] Data were extracted from Lee et al., 1998, with permission from the publisher.

receptors stimulated PLC via $G\alpha_{16}$ (Table 1). Other examples of enhanced linkage to G_i-coupled receptors by the 16z25 and 16z44 chimeras included the μ-opioid receptor and SSTR1. Among the three opioid receptor subtypes, activation of $G\alpha_{16}$ by the μ-opioid receptor is relatively weak (Lee et al., 1998). In terms of both the absolute activity and the fold of stimulation, μ-opioid receptor-induced responses were more robust with the 16z25 and 16z44 chimeras (Table 1). As for SSTR1, 100 nM somatostatin stimulated IP formation by only 2-fold in COS-7 cells coexpressing $G\alpha_{16}$, whereas the same concentration of agonist elicited 3.5-fold of stimulation in cells coexpressing either 16z25 or 16z44 (Table 1).

The 16z44 chimera seemed to produce greater stimulations of PLC activity on receptor activation compared with either $G\alpha_{16}$ or 16z25 (Fig. 2 and Table 1). The magnitudes of the agonist-induced responses mediated via 16z44 were generally higher than those obtained with $G\alpha_{16}$ or 16z25. Hence, we examined the efficiency of coupling between 16z44 and several G_i-coupled receptors. The melatonin Mel1c, SSTR1, and δ-opioid receptors were chosen on the basis of their varying abilities to associate with $G\alpha_{16}$. Each receptor was coexpressed with either $G\alpha_{16}$ or the 16z44 chimera in COS-7 cells and assayed for IP accumulation in response to increasing concentrations of the corresponding agonist. Activation of the melatonin Mel1c receptor by 2-iodomelatonin did not stimulate PLC activity in cells coexpressing the receptor and $G\alpha_{16}$ (Fig. 5). In the presence of 16z44, however, 2-iodomelatonin dose-dependently stimulated the formation of IP with an EC_{50} value of ~0.4 nM (Fig. 5). Likewise, the SSTR1 was weakly coupled to $G\alpha_{16}$ (Fig. 5 and Lee et al., 1998), whereas it efficiently stimulated the PLC activity in the presence of 16z44 with an EC_{50} value of ~3 nM somatostatin (Fig. 5); the EC_{50} of somatostatin for $G\alpha_{16}$ cotransfected cells was ~20 nM. The δ-opioid receptor has been shown to activate $G\alpha_{16}$ in a dose-dependent manner (Lee et al., 1998). Replacement of $G\alpha_{16}$ by the 16z44 chimera resulted in a more efficient stimulation of PLC by DPDPE (Fig. 5). The 16z44-mediated DPDPE response gave a higher maximal stimulation (twice that of the $G\alpha_{16}$-mediated response) and a reduced EC_{50} value (10 versus 40 nM). Taken together, these studies showed that the 16z44 chimera exhibited enhanced linkage to G_i-coupled receptors compared with its parental $G\alpha_{16}$. Preliminary results suggest that such enhanced linkage can be extended to include the CCR1, CCR2b, and CCR5 chemokine receptors.

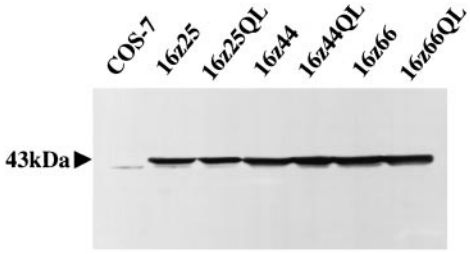

Fig. 3. Immunodetection of chimeric $G\alpha_{16/z}$ subunits. COS-7 cells were transiently transfected with cDNAs encoding the wild-type or constitutively active mutant of 16z25, 16z44, and 16z66. Plasma membranes were prepared 48 h post-transfection. Membrane proteins (50 μg) were separated on an SDS-12.5% polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membranes. Protein markers were localized by Ponceau S staining, and the chimeras were immunodetected with the 3A-170 antiserum against the C terminus of $G\alpha_z$. Two independent experiments with different batches of membrane proteins yielded similar results.

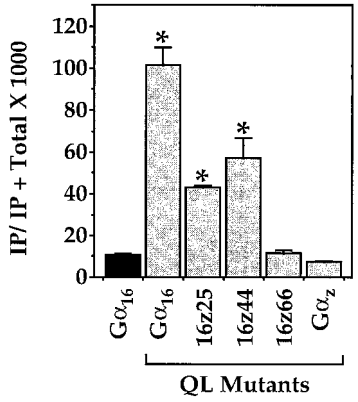

Fig. 4. Constitutively activated $G\alpha_{16/z}$ chimeras stimulate PLC except for 16z66QL. Wild-type $G\alpha_{16}$ and $G\alpha_{16/z}$ chimeras were constitutively activated by altering the amino acid at position 212 from Q (Gln) to L (Leu). The cDNAs (0.25 μg/ml) encoding the mutant chimeras were transiently expressed in COS-7 cells. $G\alpha_{16}$ and $G\alpha_z$QL were included as negative controls. Transfected cells were labeled with *Myo*-[^{3}H]inositol and assayed for IP accumulation in the absence of agonist as in the legend to Fig. 2. *Basal IP formation was significantly higher than that obtained with wild-type $G\alpha_{16}$; Bonferroni's t test, $P < .05$.

Coupling of 16z25 and 16z44 to G_s- *and* G_q-Linked Receptors. A distinguishing feature of $G\alpha_{16}$ is its ability to link a large number of GPCRs to the stimulation of PLC, including those receptors that normally utilize G_s for signal propagation. To confirm that the $G\alpha_{16/z}$ chimeras can also recognize G_s-coupled receptors, we assessed the ability of 16z25 and 16z44 to interact productively with four different

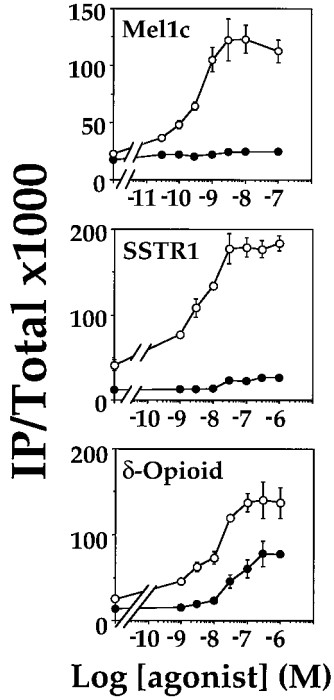

Fig. 5. Dose-dependent agonist stimulation of PLC by the Mel1c, SSTR1, and δ-opioid receptors via $G\alpha_{16}$ or 16z44. COS-7 cells were transiently cotransfected with the cDNAs encoding $G\alpha_{16}$ (●) or 16z44 (○) and one of the three selected receptors: Mel1c, SSTR1, or δ-opioid (at 0.25 μg/ml per construct). Transfected cells were then labeled with *Myo*-[^{3}H]inositol and assayed for IP formation in the absence or presence of various concentrations of 2-iodomelatonin (0.03–100 nM), somatostatin (1 nM to 1 μM), or DPDPE (1 nM to 1 μM). Data represent the mean ± S.D. of triplicate determinations of a single representative experiment; two additional experiments yielded similar results.

G_s-linked receptors. COS-7 cells were transiently cotransfected with either 16z25 or 16z44 and a G_s-coupled receptor (β_2-adrenergic, dopamine D_1, luteinizing hormone, or vasopressin V_2). Transfected cells were subsequently challenged with the appropriate agonists. In cells coexpressing the β_2-adrenergic, dopamine D_1, or vasopressin V_2 receptors with either 16z25 or 16z44, activation of the receptor led to increased production of IP (Table 1). Among these three receptors, only the vasopressin V_2 receptor has the ability to utilize endogenous G_q to weakly stimulate IP formation (66.5 ± 17.8% over basal, $n = 6$). The magnitudes of agonist-induced stimulations mediated via 16z25 or 16z44 were all ~3-fold and were generally lower than those observed with $G\alpha_{16}$ in previous reports (Offermanns and Simon, 1995; Lee et al., 1998). In contrast, activation of the luteinizing hormone receptor did not significantly stimulate IP formation in the presence of either 16z25 or 16z44 (Table 1).

The inability of the luteinizing hormone receptor to interact with 16z25 and 16z44 was unlikely to be due to lack of receptor expression because it was functionally associated to stimulation of adenylyl cyclase in the transfectants (data not shown). Moreover, under identical experimental conditions, coexpression of luteinizing hormone receptor and $G\alpha_{16}$ allowed the transfected cells to produce a 3-fold stimulation of PLC in response to 1 μg/ml human choriogonadotropin (Table 1). The lack of coupling of 16z25 and 16z44 to the prostanoid DP and luteinizing hormone receptors suggests that even though these chimeras exhibited enhanced ability to recognize G_i-coupled receptors, their linkage to G_s-coupled receptors seemed to be impaired. We have also examined the ability of 16z66 to interact with the G_s-coupled β_2-adrenergic receptor. The β_2-adrenergic receptor was coexpressed with one of the three chimeras in COS-7 cells. The 16z66 chimera was not able to stimulate IP formation in response to 10 μM isoproterenol, despite the fact that the same concentration of agonist potently stimulated cAMP accumulation.

Productive coupling of $G\alpha_{16}$ to G_q-linked receptors usually leads to a potentiation of agonist-induced stimulation of PLC when $G\alpha_{16}$ is coexpressed (Offermanns and Simon, 1995). We adopted the same approach to examine the ability of 16z25 and 16z44 to recognize G_q-coupled receptors and selected the bombesin, serotonin$_{1c}$, and muscarinic m1 receptors for the study. Each of the three G_q-coupled receptors was transiently expressed in COS-7 cells in the absence or presence of 16z44. Agonist-induced activation of these receptors significantly increased IP formation even in the absence of 16z44 (Table 2). Such stimulations were mediated through endogenous $G_{q/11}$ proteins. The magnitude of the agonist-induced responses became greater by 35 to 55% in cells coexpressing the 16z44 chimera (Table 2). Similar results were obtained for the 16z25 chimera (data not shown). These studies demonstrated that both 16z25 and 16z44 chimeras retained the ability to interact with G_q-coupled receptors.

Construction and Characterization of N-terminal $G\alpha_{z/16}$ Chimeras. Recent studies by Wess and coworkers (Kostenis et al., 1998) have focused on the extreme N-terminal region of $G\alpha_q$ as a determinant for the selectivity of receptor coupling. Compared with the α-subunits of the G_i subfamily, $G\alpha_q$ and $G\alpha_{11}$ are longer by six residues at their N termini. Progressive deletion or substitution with alanine of these "extra" residues produced $G\alpha_q$ mutants that can effectively interact with G_i-coupled receptors (Kostenis et al., 1998). Alignment of the $G\alpha_{16}$ and $G\alpha_z$ sequences revealed that their predicted N-terminal α-helices share little homology and that the $G\alpha_{16}$ N terminus is longer than that of $G\alpha_z$ by nine residues (Fig. 1). To assess whether the N terminus of $G\alpha_z$ is required for efficient coupling to G_i-linked receptors, we replaced the entire αN helix with that of the first 30 residues of $G\alpha_z$. The resultant 30z16 chimera is shorter than $G\alpha_{16}$ by nine residues (Fig. 1).

As with the $G\alpha_{16/z}$ chimeras, we tested the ability of the 30z16 chimera to interact with the δ-opioid receptor in COS-7 cells. The δ-agonist DPDPE doubled the IP formation in COS-7 cells coexpressing the δ-opioid receptor and 30z16 (Fig. 6). The 30z16-mediated stimulation was lower than that obtained with $G\alpha_{16}$. Replacement of the αN helix of $G\alpha_{16}$ by the cognate region of $G\alpha_z$ seemed to impair the ability of the chimera to interact with the δ-opioid receptor. Additional experiments showed that 30z16 was also capable of transducing stimulatory signals from the ORL_1 and β_2-adrenergic receptors to PLC (Fig. 7). However, GPCRs such as the melatonin Mel1c and μ-opioid receptors, which are weak or ineffective activators of $G\alpha_{16}$, could not stimulate PLC via 30z16 (data not shown). Although the 30z16 chimera can interact with both G_i- and G_s-coupled receptors, its promiscuous property was compromised.

Because the N and C termini of the Gα-subunit are in close proximity, the ability of $G\alpha_{16/z}$ chimeras to recognize G_i-coupled receptors may be enhanced by having $G\alpha_z$-specific sequences at both termini of the chimera. Two "z-16-z" chimeras were constructed. We constructed the 30z16z44 and 30z16z66 chimeras to examine whether the inclusion of a

TABLE 2

Coupling of 16z44 to G_q-Coupled Receptors

COS-7 cells were cotransfected with cDNAs encoding one of the three receptors indicated, with or without (control) 16z44 (0.25 μg/ml per construct). Transfected cells were labeled with [^{3}H]inositol (2.5 μCi/ml) 20 to 24 h before assay. IP formations were determined in the absence (basal) or presence of specific agonists for the indicated receptors. Agonists used were 100 nM bombesin, 1 μM serotonin, and 200 μM carbachol. Data represent the mean ± S.D. of triplicate determinations of a single representative experiment; two additional experiments yielded similar results.

Receptor	IP Formation				
	Control		16z44 chimera		Enhancement
	Basal	Agonist	Basal	Agonist	
					%
Bombesin	7.6 ± 0.2	148.9 ± 4.8*	41.1 ± 0.5	233.9 ± 11.1*	57**
5-HT_{1C}	26.0 ± 2.2	145.9 ± 8.5*	87.0 ± 0.9	208.6 ± 9.6*	43**
Muscarinic m1	20.3 ± 1.9	182.8 ± 1.1*	77.3 ± 4.9	245.0 ± 0.3*	34**

* Agonist treatments significantly increased IP formation over basal levels.

** Agonist-induced responses mediated via 16z44 were significantly greater than those mediated via endogenous $G_{q/11}$ proteins; Bonferroni's t test, $P < .05$.

$G\alpha_z$-specific αN helix can enhance or rescue the ability of 16z44 and 16z66 to recognize G_i-coupled receptors. The 30z16z44 chimera behaved like $G\alpha_{16}$, whereas 30z16z66 remained unable to respond to agonist-activated δ-opioid receptor (Fig. 6). One interesting observation is that the 30z16z44 chimera was no better than $G\alpha_{16}$ in coupling to the δ-opioid receptor but was markedly better than the 30z16 chimera. The magnitude of DPDPE-induced PLC stimulation was actually lower with 30z16z44 than when it was mediated via 16z44 (cf. Fig. 2). Further experiments showed that the 30z16z44 chimera can interact productively with the ORL_1 and β_2-adrenergic receptors (Fig. 7), as well as the μ-opioid, κ-opioid, and the three melatonin receptors (data not shown).

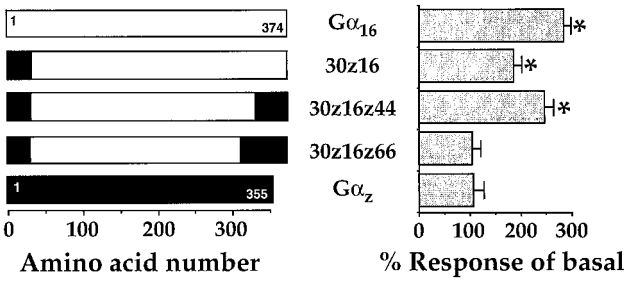

Fig. 6. Stimulation of PLC by the δ-opioid receptor via the 30z16 and 30z16z44 chimeras. $G\alpha_{z/16}$ chimeras were constructed as described in *Materials and Methods*. Left, schematic representation of the chimeras. COS-7 cells were cotransfected with cDNAs (0.25 μg/ml each) encoding the δ-opioid receptor and $G\alpha_{16}$, $G\alpha_z$, or one of three $G\alpha_{z/16}$ chimeras: 30z16, 30z16z44, or 30z16z66. Transfected cells were assayed as in the legend to Fig. 2. Results are expressed as percentage stimulation of IP production compared with basal activity. Basal values expressed as the ratio ($\times 10^3$) of IP to total inositols ranged from 11.3 ± 1.2 to 14.7 ± 1.1. *DPDPE-induced IP formation was significantly higher than basal levels; Bonferroni's t test, $P < .05$.

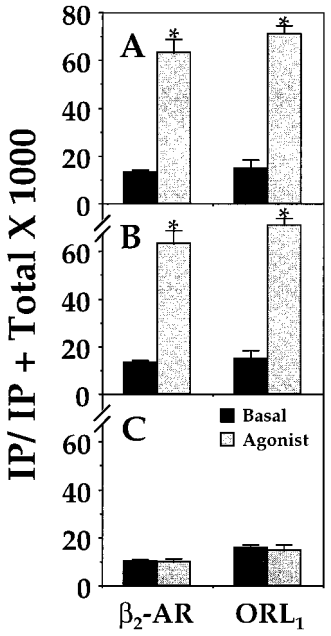

Fig. 7. Coupling of β_2-adrenergic (β_2-AR) and ORL_1 receptors to the 30z16 and 30z16z44 chimeras. COS-7 cells were cotransfected with cDNAs (0.25 μg/ml each) encoding the β_2-adrenergic or ORL1 receptor with one of three $G\alpha_{z/16}$ chimeras: 30z16 (A), 30z16z44 (B), or 30z16z66 (C). Transfected cells were assayed for IP formation in the absence or presence of agonist (1 μM isoproterenol or 100 nM nociceptin). *Agonist-induced IP formation was significantly higher than the corresponding basal values; Bonferroni's t test, $P < .05$.

The expression of 30z16, 30z16z44, and 30z16z66 was confirmed by immunoblot analysis using a $G\alpha_z$-specific N-terminal antiserum (Fig. 8). Thus, the inability of 30z16z66 to interact with GPCRs was not due to a lack of expression. Overall, replacement of the αN helix of $G\alpha_{16}$ by $G\alpha_z$-specific sequence did not seem to enhance the specificity of coupling to G_i-linked receptors.

Effects of Inverse Agonists on Receptors Coupled to 16z44. A notable feature of cells coexpressing a GPCR and 16z25 or 16z44 was their elevated basal IP production. The increased basal IP accumulation can be seen with the vast majority of the receptors tested (Table 1), and it resembles the constitutive activity of GPCRs. This interpretation is supported by the fact that no elevation of basal activities could be observed in COS-7 cells expressing 16z44 alone; coexpression with a G_i- or G_s-coupled receptor is required (Y. H. Wong, unpublished results). If the enhanced linkage of the chimeras to GPCRs promotes the formation of constitutively active receptors, it may offer an opportunity to identify

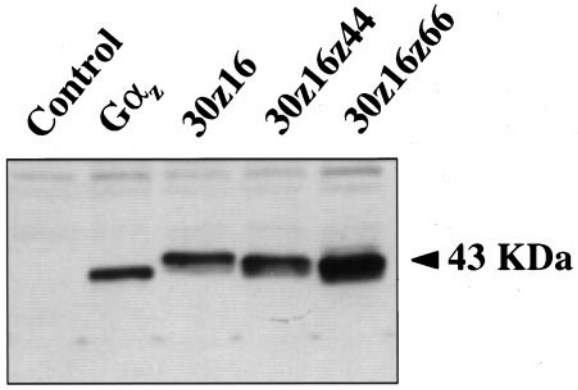

Fig. 8. Immunodetection of chimeric $G\alpha_{z/16}$ subunits. COS-7 cells were transiently transfected with cDNAs encoding the wild-type $G\alpha_z$, 30z16, 30z16z44, or 30z16z66 chimeras. Immunodetection of the various chimeras was performed as described in the legend to Fig. 3, except that a $G\alpha_z$ N-terminal specific antiserum 3-18 was used.

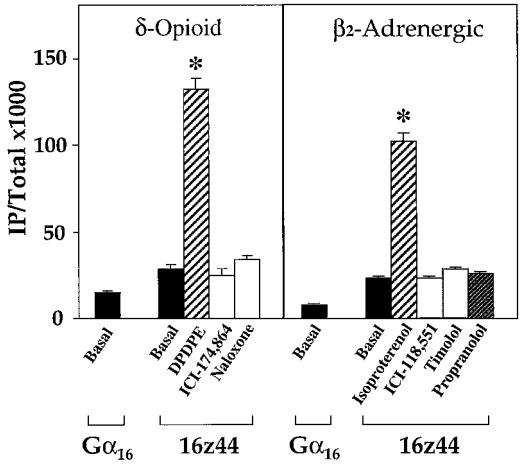

Fig. 9. Lack of inverse agonist effect on δ-opioid and β_2-adrenergic receptors coexpressed with 16z44. COS-7 cells were transfected with cDNAs encoding $G\alpha_{16}$ or 16z44 together with either the δ-opioid or the β_2-adrenergic receptor (0.25 μg/ml each). Transfected cells were assayed for IP accumulation in the absence (basal) or presence of the indicated ligands: 100 nM DPDPE, 10 μM ICI-174,864, 10 μM naloxone, 1 μM isoproterenol, 10 μM ICI-118,551, 10 μM timolol, or 10 μM propranolol. *Agonist-induced IP formation was significantly higher than the corresponding basal values; Bonferroni's t test, $P < .05$.

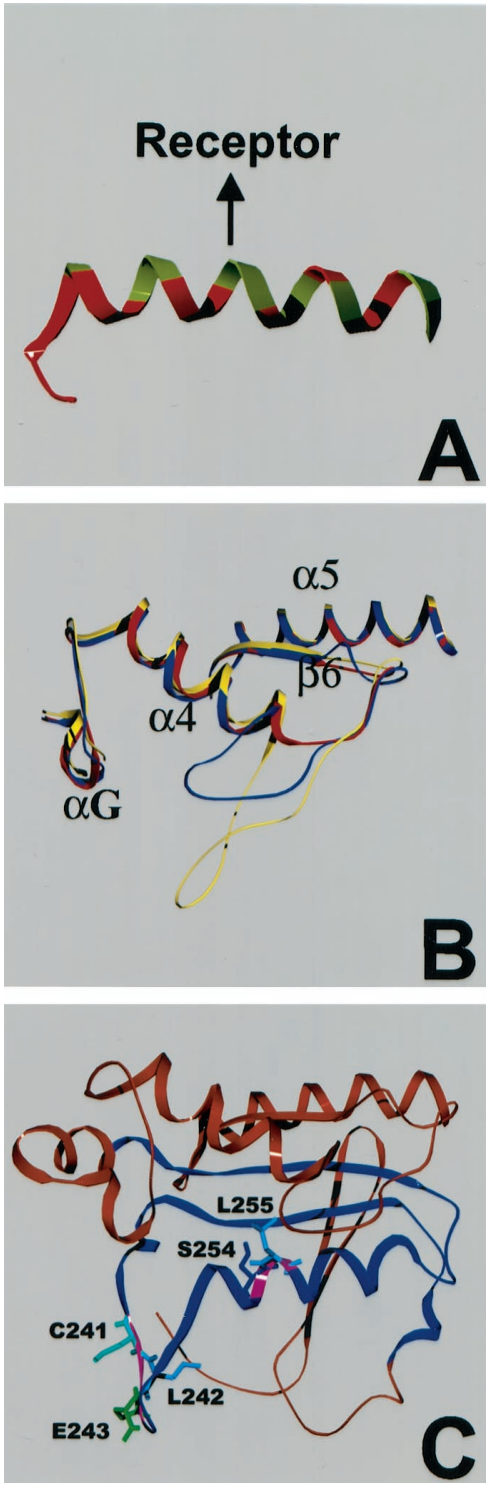

Fig. 10. Molecular models of $G\alpha_{16/z}$ chimeras. Coordinates of the hypothetical structural models of various Gα-subunits were generated by Swiss-Model modeling service available from World Wild Web (http://www.expasy.ch/swissmod/SWISS-MODEL.html) using $G\alpha_{t1}$ and $G\alpha_{i1}$ complexed with βγ-subunits as template structures (coordinate codes 1GOT and 1GP2; obtained from the Protein Data Bank of Brookhaven National Laboratory, http://www.pdb.bnl.gov/). Models were visualized by Swiss-PDB Viewer v3.1 (http://www.expasy.ch/spdbv/mainpage.htm) and rendered by POV-Ray for Windows v3.1 (http://www.povray.org/). A, the α5 helix of $G\alpha_{16}$ is shown with the putative receptor-facing side on top, as indicated. The last seven residues have not been mapped to the structure due to the lack of the corresponding residues in the template. Green patches correspond to the residues different from $G\alpha_z$ in their nature. B, the structures from αG to α5 of 16z25 (blue), 16z44 (yellow), and 16z66 (red) are displayed in an overlapping view. Receptor-facing side of α5 helix is inside the plane. The α4/β6 loops of 16z25 and 16z44 are longer than that of the template structures and thus are not in well defined structures. C, the GTPase domain of $G\alpha_{16}$ is in the same orientation as in B. The putative PLC-activating domain is marked in blue color according to the study of Medina et al. (1996), and the five residues important for PLC activation are shown in sticks (Venkatakrishnan and Exton, 1996).

inverse agonists that act at various GPCRs. To test this hypothesis, COS-7 cells were cotransfected with cDNAs encoding 16z44 and either the δ-opioid or β_2-adrenergic receptor, and the ability of known inverse agonists to suppress the elevated basal IP levels of the transfectants was examined. Compared with cells coexpressing the δ-opioid receptor and $G\alpha_{16}$, 16z44 transfectants exhibited increased basal IP production, but neither ICI-174,864 (an inverse agonist) nor naloxone (a neutral antagonist) affected the elevated basal levels (Fig. 9). The expression of δ-opioid receptors in the transfectants was confirmed by the DPDPE-induced stimulation of PLC (Fig. 9). Similar results were obtained with cells coexpressing the β_2-adrenergic receptor and 16z44. Two inverse agonists of β_2-adrenergic receptor, ICI-118,551 and timolol, were incapable of reducing the elevated basal level associated with the coexpression of 16z44 (Fig. 9). These results suggest that although 16z44 may provide enhanced linkage to GPCRs, it does not necessarily promote the formation of constitutively active GPCRs.

Discussion

As one of the largest protein families found in nature, it is estimated that several thousand different GPCRs may exist in the human genome. The recent discovery of more than 1000 genes encoding known and orphan GPCRs in the *Caenorhabditis elegans* genome (Bargmann, 1998) lends further credibility to this estimation. Human $G\alpha_{16}$ possesses the rare ability to recognize a wide spectrum of GPCRs, which can facilitate the characterization of orphan GPCRs. Numerous biochemical, structural, and molecular genetic studies have revealed that the docking site for receptors is composed of multiple regions on the Gα-subunit (reviewed by Bourne, 1997). The five regions of the Gα-subunit involved in receptor recognition are the α2 helix, the β6-α5 loop, the α5 helix, and the two extreme termini. By replacing one or more of these regions in $G\alpha_{16}$ with sequences from $G\alpha_z$, we have successfully created chimeric $G\alpha_{16/z}$ proteins that exhibit enhanced linkage to G_i-coupled receptors. The 16z44 chimera appeared to be particularly effective in this respect.

Compared with $G\alpha_{16}$, both 16z25 and 16z44 can additionally couple to the melatonin Mel1c receptor and produce higher magnitudes of PLC stimulation with some of the G_i-coupled receptors (e.g., μ-opioid and SSTR1). In the 16z25 chimera, the α5 helix of $G\alpha_{16}$ was replaced by that of $G\alpha_z$. Numerous studies have attested to the importance of the α5 helix in receptor coupling. Amino acids within the α5 helix of $G\alpha_s$ (Conklin et al., 1996; Sullivan et al., 1987), $G\alpha_i$ (Tsu et al., 1997), $G\alpha_{t1}$ (Martin et al., 1996), and $G\alpha_q$ (Conklin et al., 1993, 1996) have been shown to alter the specificity of receptor coupling. The α5 helix of $G\alpha_z$ is quite different from that of $G\alpha_{16}$ with less than 35% homology (Fig. 1). Alignment of the α5 helices of $G\alpha_{16}$ and $G\alpha_z$ shows that every third or fourth amino acid from position −1 are different between the

two Gα-subunits. Using the crystal structures of $G\alpha_{t1}$ and $G\alpha_{i1}$ as a basis, we generated a molecular model to highlight the structural differences in the α5 helices of $G\alpha_{16}$ and $G\alpha_z$ (Fig. 10A). When the $G\alpha_z$-specific residues are superimposed on the α5 helix of $G\alpha_{16}$, the receptor-facing plane are predominantly composed of $G\alpha_z$-specific amino acids.

Not only did 16z44 recognize more G_i-coupled receptors than $G\alpha_{16}$, the magnitudes of the stimulations were also higher. Comparison of the EC_{50} values obtained with 16z44 and $G\alpha_{16}$ transfectants reflected that 16z44 was more efficient in linking the G_i-coupled receptors to the activation of PLC. Another notable feature of 16z44 is its elevated basal activities. Cells coexpressing 16z44 generally exhibited basal PLC activities that were much higher than those coexpressing $G\alpha_{16}$ (Table 1). This elevation in basal IP accumulation resembles the constitutive activity of GPCRs, but we were unable to detect inverse agonist effects on the δ-opioid and β_2-adrenergic receptors. As a result of elevated basal activities and in terms of percentage stimulation, 16z44-mediated responses were not significantly better than those mediated by $G\alpha_{16}$. However, the absolute levels of IP accumulation transduced by 16z44 were often greater than those of $G\alpha_{16}$. Basal IP accumulation was also higher in cells coexpressing the 16z25 chimera, albeit to a lesser extent.

Structural differences between 16z25 and 16z44 are primarily located in the α4/β6 loop (residues 318–335 of $G\alpha_{16}$). The α4/β6 loop is one of several secondary structures forming the contact surface for receptors (part of the A1 cluster as described by Lichtarge et al., 1996). The α4 helix and α4/β6 loop region of $G\alpha_{i1}$ are important for specific recognition of receptors (Bae et al., 1997). The α4/β6 loop of 16z25 is identical with that of $G\alpha_{16}$ because there is no substitution of $G\alpha_z$-specific residues in this region of 16z25 (Fig. 1). Based on the crystal structure coordinates of trimeric G_{t1} (Lambright et al., 1996), the α4/β6 loop of 16z44 is predicted to be a more flexible structure with an energy level higher than those of 16z25 and $G\alpha_{16}$ (Fig. 10B). This increased flexibility may partially account for the enhanced potency of 16z44 to transduce signals from G_i-coupled receptors. The model generated for 16z66 (Fig. 10B) fitted well to the known structures of $G\alpha_{t1}$ and $G\alpha_{i1}$. Compared with $G\alpha_{16}$, 16z66 has a smaller α4/β6 loop and a tighter α4 helix. One would presume that the close resemblance of 16z66 to $G\alpha_{t1}$ and $G\alpha_{i1}$ implies enhanced capability of the chimera to interact with G_i-coupled receptors, yet our results were contrary to such a prediction.

Because 16z66 was expressed to the same extent as the other chimeras, its inability to mediate receptor-induced stimulation of PLC might be due to the disruption of effector recognition domains like the α4/β6 region. The α4 and the α4/β6 loops of $G\alpha_q$ are known to be involved in the activation of PLC (Arkinstall et al., 1995; Medina et al., 1996). When the putative PLC regulatory domains of $G\alpha_q$ are mapped onto a molecular model of $G\alpha_{16}$ (shown in blue, Fig. 10C), these regions were not substituted by $G\alpha_z$-specific residues in 16z66. Several residues around the switch III region of $G\alpha_q$ have also been shown to be critical for the regulation of PLC (Venkatakrishnan and Exton, 1996), and they are conserved in $G\alpha_{16}$: residues 241 to 243 and 254 to 255 (Fig. 10C). We used molecular modeling of $G\alpha_{16}$ to identify amino acids distal to or in the α4 helix that may interact with these PLC-activating residues. Residue Leu^{254} is predicted to interact with several amino acids in the α4 helix (Ile^{312}, Met^{315}, Tyr^{316}, and Thr^{317}) and the α4/β6 loop (Asp^{325}). Four of these five potential sites (except Ile^{312}) were actually replaced by $G\alpha_z$-specific residues in 16z66 (Fig. 1). Like mutating Leu^{254} itself, the disruption of intramolecular interactions may severely curtail the ability of 16z66 to stimulate PLC, resulting in a lack of constitutive activity of 16z66QL. Another possibility is that 16z66 cannot adopt the GTP-bound active form. Further experiments are needed to distinguish the molecular basis for the lack of function of 16z66.

Although we did not embark on an extensive study to determine the importance of N-terminal sequences of $G\alpha_z$ in receptor recognition, the present study shows that the αN helix of $G\alpha_z$ alone could not confer specificity for G_i-coupled receptors. This is in stark contrast to results obtained in similar studies where the N terminus of $G\alpha_z$ alone was sufficient to allow a $G\alpha_{z/t1}$ chimera to respond to the δ-opioid receptor (Tsu et al., 1997). Close proximity of the two termini in the crystal structures of $G\alpha_{t1}$ (Lambright et al., 1996) and $G\alpha_{i1}$ (Wall et al., 1995) supports the involvement of the N terminus in receptor recognition. Biochemical evidence are also available to substantiate this notion for the coupling of receptors to $G\alpha_o$ (Denker et al., 1995) and $G\alpha_{t1}$ (Dratz et al., 1993), two members of the G_i subfamily. Hence, an intact N terminus may be required for $G\alpha_{16}$ to efficiently associate with GPCRs. In this respect, our results are in accordance with those reported by Lee et al. (1995), where the N-terminal 209 residues of $G\alpha_{16}$ were found to be essential for activation by the G_i-coupled C5a receptor.

Despite their enhanced linkage to G_i-coupled receptors, neither 16z25 nor 16z44 can be considered as a universal adapter for GPCRs. Compared with $G\alpha_{16}$, the ability of 16z25 and 16z44 to recognize the luteinizing hormone receptor was actually diminished. Naturally, one would expect that when the specificity for G_i-coupled receptors increases in a Gα-subunit, its specificity for G_s-linked receptors will be inversely affected because the two sets of GPCRs are designed to produce opposite effects on adenylyl cyclase. Perhaps it is impractical to engineer a universal G protein adapter for GPCRs even though such a construct is highly desirable for the characterization of orphan receptors. Nevertheless, our study has successfully demonstrated the feasibility of improving the recognition of specific subsets of GPCRs by altering receptor contact regions on $G\alpha_{16}$. With the recent explosion of the number of orphan receptors being cloned, the chimeras described herein may become invaluable tools for their characterization. For example, the 16z44 chimera can be incorporated into a variety of cell-based assays for the rapid detection of receptor activation. Conceivably, the ability of $G\alpha_{16}$ to recognize G_s-coupled receptors can be similarly enhanced by incorporating $G\alpha_s$-specific regions on a $G\alpha_{16}$ backbone. Several chimeric $G\alpha_{16}$ with expanded capability of receptor recognition may collectively serve as a true "universal adapter" for orphan GPCRs.

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