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The Amino Terminus of $G\alpha_z$ is Required for Receptor Recognition, Whereas its $\alpha4/\beta6$ Loop Is Essential for Inhibition of Adenylyl Cyclase

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ABSTRACT

G_z couples to most of the known G_i-linked receptors and its α subunit ($G\alpha_z$) inhibits adenylyl cyclases as efficiently as $G\alpha_i$ subtypes. A series of chimeric $G\alpha$ subunits with different portions of $G\alpha_z$ and $G\alpha_{t1}$ (a regulator of cGMP phosphodiesterase) were constructed to study the essential structural elements of $G\alpha_z$ that determine receptor coupling and effector interaction. The receptor-mediated functions of the chimeras were assessed in two aspects: 1) stimulation of type 2 adenylyl cyclase through the release of $\beta\gamma$ subunits from the chimeras, and 2) inhibition of isoproterenol-stimulated adenylyl cyclase by the chimeric $G\alpha$ subunits. The results suggested that the presence of both termini of $G\alpha_z$ were critical for coupling to δ-opioid receptor, with the N-terminal region being more important. Moreover, a stretch of amino acids (295–319) corresponding to the $\alpha4/\beta6$ loop was identified as one of the adenylyl cyclase inhibitory domains of $G\alpha_z$.

The signaling properties of α subunit of G_z protein ($G\alpha_z$) are very similar to the three $G\alpha_i$ subtypes. All G protein-coupled receptors that have been shown to interact with G_z are known couplers of G_i proteins (Fields and Casey, 1997; Ho and Wong, 1998). Although the overall amino acid identity of $G\alpha_z$ and $G\alpha_{i2}$ is about 60%, they inhibit adenylyl cyclase (AC) in a similar fashion (Wong et al., 1992; Kozasa and Gilman, 1995). The sequence identities are even higher when only their C-terminal halves are compared, where the putative receptor- and effector-interacting domains were located. However, subtle differences are found between the amino acid sequences of $G\alpha_{i2}$ and $G\alpha_z$, which suggest that they may use different structural elements to achieve similar functions. Incorporation of the last five residues of either $G\alpha_{i2}$ or $G\alpha_z$, which shared very low homology to each other, to the corresponding region of $G\alpha_q$ broadened its receptor coupling profile to both G_q- and G_i-linked receptors (Conklin et al., 1993). However, the replacement of the last 36 amino acids of $G\alpha_z$ with those of $G\alpha_{t1}$ did not block the coupling of the resultant $G\alpha_z/G\alpha_{t1}$ to G_i-linked receptors (Tsu et al., 1997). Indeed, a number of studies suggested that the amino terminus of $G\alpha$ subunit might be crucial for receptor coupling (Hamm et al., 1988; Kostenis et al., 1997). Furthermore, a stretch of amino acids, 220 to 240 of $G\alpha_{16}$, has been shown to be essential for receptor coupling (Lee et al., 1995). It prompted us to identify the essential determinants of $G\alpha_z$ for specifying its receptor coupling property.

The effector interacting domains of various $G\alpha$ subunits are generally localized at the C-terminal half of the amino acid sequence (Berlot and Bourne, 1992; Medina et al., 1996; Grishina and Berlot, 1997). Although $G\alpha_s$ and $G\alpha_{i2}$ regulate AC in opposite fashions, they have evolved similar and different stretches of amino acids for effector interactions (Berlot and Bourne, 1992; Grishina and Berlot, 1997). The AC inhibiting residues of $G\alpha_{i2}$ were localized at the Switch II region (similar to $G\alpha_s$) and $\alpha4/\beta6$ loop (unlike $G\alpha_s$) by alanine mutagenesis (Grishina and Berlot, 1997). It is unclear if regions similar to those found in $G\alpha_{i2}$ are responsible for effector interaction in $G\alpha_z$. Interestingly, mutations of the acylation-modified N-terminal residues altered the constitutive inhibitory action of $G\alpha_z$ to AC (Wilson and Bourne, 1995). Moreover, alanine substitution of the protein kinase C-phosphorylation sites of mutationally active $G\alpha_z$ attenuated its inhibitory effect on AC (Ho and Wong, 1997). It is still possible that the N-terminus of $G\alpha_z$ is also involved in effector regulation.

We attempted to localize the receptor interacting as well as AC inhibiting domains of $G\alpha_z$ by constructing chimeric $G\alpha$ subunits using $G\alpha_z$ and $G\alpha_{t1}$. $G\alpha_{t1}$ is approximately 60% identical with $G\alpha_z$; hence, their tertiary structures should have considerable resemblance. Within the $G\alpha_i$-subfamily,

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ABBREVIATIONS: AC, adenylyl cyclase; HEK, human embryonic kidney; DOR, δ-opioid receptor; AC2, type 2 adenylyl cyclase; PTX, pertussis toxin; DPDPE, [*d*-Pen^{2,5}]enkephalin; PCR, polymerase chain reaction; MEM, Eagle's minimal essential medium.

$G\alpha_{t1}$ is primarily coupled to rhodopsin and regulates cGMP phosphodiesterase. Introduction of $G\alpha_{t1}$ portions into a $G\alpha_z$ backbone would be expected to have minimal interference on the protein folding and AC inhibition. Desired recombinant proteins were transiently expressed in human embryonic kidney (HEK) 293 cells and the functions of the chimeras were assessed in two aspects. To study the receptor coupling events, the abilities of chimeric $G\alpha$ subunits to couple to δ-opioid receptor (DOR) were monitored by the $\beta\gamma$-mediated stimulation of type 2 adenylyl cyclase (AC2). For studying the effector interaction, the abilities of the chimeras to inhibit isoproterenol-stimulated cAMP accumulation upon the activation of DOR were monitored. Another series of chimeras were constructed with a point mutation that rendered GTPase-deficient phenotypes (Freissmuth and Gilman, 1989; Graziano and Gilman, 1989). They were applied to investigate the constitutively inhibitory effects of the chimeras on isoproterenol-stimulated AC activity.

Experimental Procedures

Materials. AC2 and DOR cDNA were gifts from Randall Reed (Johns Hopkins School of Medicine, Baltimore, MD) and Christopher Evans (University of California, Los Angeles, CA), respectively. Pertussis toxin (PTX) was purchased from List Biological Laboratories, Inc. (Campbell, CA). HEK 293 cells were obtained from the American Type Culture Collection (Rockville, MD). [*d*-Pen^{2,5}]enkephalin (DPDPE) was from Research Biochemicals Inc. (Natick, MA). Antisera against $G\alpha_z$ and $G\alpha_{t1}$ were from Gramsch Laboratories (Schwabhausen, Germany) and Transduction Laboratories (Lexington, KY), respectively. [^{3}H]Adenine was purchased from Amersham (Buckinghamshire, UK). Plasmid purification columns were obtained from Qiagen (Hilden, Germany). *Taq* DNA polymerase, customized primers, restriction endonucleases, and cell culture reagents were obtained from Life Technologies Inc. (Grand Island, NY). All other chemicals were purchased from Sigma (St. Louis, MO).

Construction of Chimeric α Subunits. A complete list of chimeric and mutational $G\alpha$ subunits is shown in Table 1. The chimeras zt40 and zt43 were constructed by polymerase chain reaction (PCR) using a pair of chimeric primers (Table 2). The full-length PCR product was sequenced using Sequenase Version 2.0 DNA sequencing kit from Amersham. Other chimeras were constructed by making use of convenient unique restriction sites and exchanging the corresponding restriction fragments of $G\alpha_{t1}$, zt40, or zt43 with $G\alpha_z$ (Table 1). For example, the junctional restriction sites of tz36, tz60, and tz143 are *Bgl*II, *Afl*III, and *Bam*HI, respectively. The insertion mutant +4t and PTX-resistant $G\alpha_{t1}$CG mutant were made by PCR using $G\alpha_{t1}$ as the template, and +4t/CG is generated by combining +4t and $G\alpha_{t1}$CG together through restriction cutting. −4z Is derived from $G\alpha_z$ with the residues 11 to 14 deleted. For si143 and sz143, the portion of $G\alpha_s$ was obtained by partial digestion with *Bam*HI and then ligation with the C-terminal portion of either $G\alpha_{i2}$ or $G\alpha_z$. GTPase-deficient mutants of various chimeras were made by replacing $G\alpha_{t1}$ and $G\alpha_z$ with $G\alpha_{t1}$RC (Ho et al., 1999) and $G\alpha_z$QL (Wong et al. 1992), respectively in the construction procedures. All the cDNA constructs were subcloned in the mammalian expression vector pcDNAI at *Eco*RI site.

Transfection of HEK 293 Cells and cAMP Accumulation Assay. HEK 293 cells were cultured with Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (v/v), 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in humidified air with 5% CO_2. They were cotransfected with various recombinant DNA constructs using DEAE-dextran/chloroquine method as described previously (Wong, 1994). Transfected cells were labeled with 1 μCi/ml of [^{3}H]adenine in MEM with 1% fetal calf serum and treated with 100 ng/ml PTX as appropriate. Labeled cells were treated with proper receptor agonists in 20 mM HEPES-buffered MEM with 1 mM 1-methyl-3-isobutylxanthine for 30 min and the reactions were terminated by adding ice-cold 5% trichloroacetic acid with 1 mM ATP. Separation of labeled cAMP from other nucleotides was achieved by sequential ion exchange chromatography as described previously (Salomon, 1991). The cAMP levels were interpreted as the ratios of the counts per minute of [^{3}H]cAMP fractions to those of [^{3}H]ATP fractions and expressed as [cAMP/(cAMP + Total) × 1000]. Absolute values for cAMP accumulation varied between experiments, but variability within a given set of transfection was in general <10%. Data shown in the figures were the mean ± S.E.M. of three to five individual experiments performed in triplicate. ANOVA and paired *t* test with 95% confidence was used to analyze the significance between different treatment groups.

Western Blotting Analysis. Crude membrane proteins from HEK 293 cells transfected with various chimeric $G\alpha$ subunits were extracted as described previously (Ho and Wong, 1997). Each protein sample (50 μg) was resolved in 10% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membrane. Antiserum 3A-170

TABLE 1

List of mutational and chimeric $G\alpha$ subunits

Except for the last three constructs, the nomenclatures of the chimeras are according to the parental $G\alpha$ subunits (templates) and the numbers of amino acids of $G\alpha_z$ present in the chimeras. All chimeras are constructed either by PCR using the primers listed in Table 2 or by restriction digestion (RD) of templates and subsequent ligation. The restriction sites shown are the junctional sites of the ligated fragments.

Construct	Template	Details	Construction Method
zt40	$G\alpha_{t1}$, $G\alpha_z$	$G\alpha_z$ (1–40) + $G\alpha_{t1}$ (37–355)	PCR
zt43	$G\alpha_{t1}$, $G\alpha_z$	$G\alpha_z$ (1–43) + $G\alpha_{t1}$ (40–355)	PCR
tz36	$G\alpha_{t1}$, $G\alpha_z$	$G\alpha_{t1}$ (1–315) + $G\alpha_z$ (320–355)	RD, *Bgl*II
tz60	$G\alpha_{t1}$, $G\alpha_z$	$G\alpha_{t1}$ (1–291) + $G\alpha_z$ (296–355)	RD, *Afl*III
tz143	$G\alpha_{t1}$, $G\alpha_z$	$G\alpha_{t1}$ (1–207) + $G\alpha_z$ (213–355)	RD, *BamH*I
si143	$G\alpha_s$, $G\alpha_{i2}$	$G\alpha_s$ (1–235) + $G\alpha_{i2}$ (213–355)	RD, *BamH*I
sz143	$G\alpha_s$, $G\alpha_z$	$G\alpha_s$ (1–235) + $G\alpha_z$ (213–355)	RD, *BamH*I
zt212	$G\alpha_{t1}$, $G\alpha_z$	$G\alpha_z$ (1–212) + $G\alpha_{t1}$ (208–350)	RD, *BamH*I
zt295	$G\alpha_{t1}$, $G\alpha_z$	$G\alpha_z$ (1–295) + $G\alpha_{t1}$ (290–350)	RD, *Afl*III
zt319	$G\alpha_{t1}$, $G\alpha_z$	$G\alpha_z$ (1–319) + $G\alpha_{t1}$ (315–350)	RD, *Bgl*II
ztz40/36	zt40, $G\alpha_z$	$G\alpha_z$ (1–40) + $G\alpha_{t1}$ (37–315) + $G\alpha_z$ (320–355)	RD, *Afl*III
ztz40/60	zt40, $G\alpha_z$	$G\alpha_z$ (1–40) + $G\alpha_{t1}$ (37–291) + $G\alpha_z$ (296–355)	RD, *Afl*III
ztz40/143	zt40, $G\alpha_z$	$G\alpha_z$ (1–40) + $G\alpha_{t1}$ (37–207) + $G\alpha_z$ (213–355)	RD, *BamH*I
ztz43/36	zt43, $G\alpha_z$	$G\alpha_z$ (1–43) + $G\alpha_{t1}$ (40–315) + $G\alpha_z$ (320–355)	RD, *Bgl*II
ztz43/60	zt43, $G\alpha_z$	$G\alpha_z$ (1–43) + $G\alpha_{t1}$ (40–291) + $G\alpha_z$ (296–355)	RD, *Afl*III
ztz43/143	zt43, $G\alpha_z$	$G\alpha_z$ (1–43) + $G\alpha_{t1}$ (40–207) + $G\alpha_z$ (213–355)	RD, *BamH*I
−4z	$G\alpha_z$	$G\alpha_z$ (1–10) + $G\alpha_z$ (15–355)	PCR
$G\alpha_{t1}$CG	$G\alpha_{t1}$	C347 → G	PCR
+4t/CG	$G\alpha_{t1}$CG	$G\alpha_{t1}$ (1–10) + $G\alpha_z$ (11–14) + $G\alpha_{t1}$ (11–355), C347 → G	PCR

(Gramsch Laboratories, Schwabhausen, Germany) against the carboxyl terminus of $G\alpha_z$ was used for the detection of $G\alpha_z$, tz, and ztz chimeras, whereas $G\alpha_{t1}$, zt40, and zt43 were identified with anti-$G\alpha_{t1}$ antiserum (Transduction Laboratories).

Results

The Role of the C-Terminus of $G\alpha_z$ in Coupling with DOR. The abilities of chimeric $G\alpha$ subunits to couple to DOR were monitored by the $\beta\gamma$-mediated stimulation of AC2. This reporter system is advantageous because it could be generally applied to assess the coupling between different categories of receptor-G protein pairs without considering the functions of the $G\alpha$ subunits. Moreover, AC2 is relatively insensitive to $G\alpha_i$-mediated inhibition (Taussig et al., 1994). It eliminates the possibility that $G\alpha_z$ and $\beta\gamma$ complex act on AC2 in an antagonistic fashion. The AC2 system is especially useful for checking the receptor coupling efficiencies of particular chimeric $G\alpha$ subunits that have lost the integrity of effector interacting domains. A schematic representation of the first series of chimeric $G\alpha$ subunits was shown in Fig. 1A. We attempted to examine the importance of the C-terminal tail of $G\alpha_z$ on receptor coupling using this series of chimeras. In the vector control, HEK 293 cells expressing $G\alpha_s$QL, DOR, and AC2 were treated with or without PTX. Addition of 100 nM DPDPE stimulated the cAMP production to about two times the basal level in the absence of PTX treatment (Fig. 1B). The DPDPE-induced enhancement was abolished in cells treated with PTX, indicating that the coupling between DOR and the endogenous G_i proteins was obstructed. Coexpression of recombinant $G\alpha_z$ provided a larger pool of G protein trimers capable of coupling to DOR; hence, in the absence of PTX treatment, a 150% enhancement of the cAMP production was observed compared with the corresponding response in vector control. PTX treatment partially reduced the cAMP level of $G\alpha_z$-expressing cells, which suggested that G_z could functionally replace the role of endogenous G_i and mediated the DPDPE-induced cAMP production in a PTX-resistant manner. In the case of $G\alpha_{t1}$, the response in the absence of PTX treatment was significantly lower than that of vector control and was close to the basal value. Suppression of $\beta\gamma$-mediated AC2 activity was caused by the strong $\beta\gamma$-scavenging property of $G\alpha_{t1}$. Moreover, there was no increase of cAMP level in cells treated with PTX, which inactivated endogenous G_i and the recombinant G_{t1}. These results are consistent with those reported earlier (Tsu et al., 1997).

One of our recent studies showed that the $G\alpha_z$/$G\alpha_{t1}$ and $G\alpha_z$/$G\alpha_{i2}$ chimeras containing the last 36 amino acids of $G\alpha_{t1}$ and $G\alpha_{i2}$, respectively, can couple to DOR efficiently (Tsu et al., 1997). To determine whether the N-terminus is required for receptor coupling, a reversed chimera tz36, an $G\alpha_{t1}$ subunit with the C-terminal 36 residues of $G\alpha_z$ (Fig. 1A, the third construct), was constructed and examined for its ability to couple to DOR. In this assay system, tz36 did not release $\beta\gamma$ subunits for stimulating AC2 upon DOR activation (Fig. 1B). Subsequent substitution of larger C-terminal portions of $G\alpha_{t1}$ with $G\alpha_z$ sequence up to 143 amino acids (tz60 and tz143) also did not rescue the coupling to DOR. Two other chimeras sz143 and si143 (Fig. 1A, the sixth and seventh constructs) were constructed by replacing the C-terminal tail of $G\alpha_s$ with that of $G\alpha_z$ or $G\alpha_{i2}$ portions. These two chimeras resembled tz143 and did not release $\beta\gamma$ complex for AC2 stimulation upon DOR activation. These results further suggested neither the C-terminal 143 amino acids of $G\alpha_{i2}$ nor $G\alpha_z$ could rescue the receptor coupling and the loss of function of tz143, sz143, and si143 may not be related to the choice of parental $G\alpha$ subunits for chimera construction. Obviously, other essential receptor interacting regions were located on the N-terminal half of $G\alpha_z$ sequence.

The last three chimeras of this series were constructed as mirror images of tz36, tz60, and tz143. The chimeras were denoted as zt319, zt295, and zt212, respectively (Fig. 1A, the eighth to tenth constructs; numbers refer to the last amino acid of $G\alpha_z$). Replacement of the C-terminal tail of $G\alpha_z$ with that of $G\alpha_{t1}$ may alter its coupling to DOR. However, all three zt chimeras seemed to couple to DOR and showed substantial increases of cAMP accumulation in the absence of PTX treatment (enhanced by 60, 50, and 65% for zt319, zt295, and zt212, respectively; see Fig. 1B). These results further reinforced the idea that the C-terminal 143 amino acids of $G\alpha_z$ are sufficient for receptor recognition and some critical structural elements are located on the N-terminal half of $G\alpha_z$. Because these zt chimeras acquired the C-terminal tail of $G\alpha_{t1}$, they should be sensitive to PTX-mediated inactivation. Accordingly, there was no increment of cAMP levels in all three cases when the cells were pretreated with PTX (Fig. 1B).

TABLE 2
List of primers

Primer	Sequence
zt40/AS	ccggattcaccggcGCCCAGCAGGAGAA[a]
zt40/S	TTCTCCTGCTGGGCgccggtgaatccgg
zt43/AS	gtactcttcccggaGTTGCTGGTGCCCA
zt43/S	TGGGCACCAGCAACtccgggaagagtac
−4z/AS	TCTCCTCGACCGTTTTTCCTCTGA
−4z/S	TCAGAGGAAAAACGGTCGAGGAGA
tCG/AS	ccgaattcagaagagcccgccgtctttgaggtt[b]
+4t/AS	ctcccttgagtgCCGCGCTGCCTCcttctcctcagc
+4t/S	gctgaggagaagGAGGCAGCGCGGcactcaagggag

[a] Nucleotides corresponding to $G\alpha_z$ and $G\alpha_{t1}$ sequences are shown in upper and lower cases, respectively.
[b] Mismatch nucleotides are underlined in the case of tCG/AS.

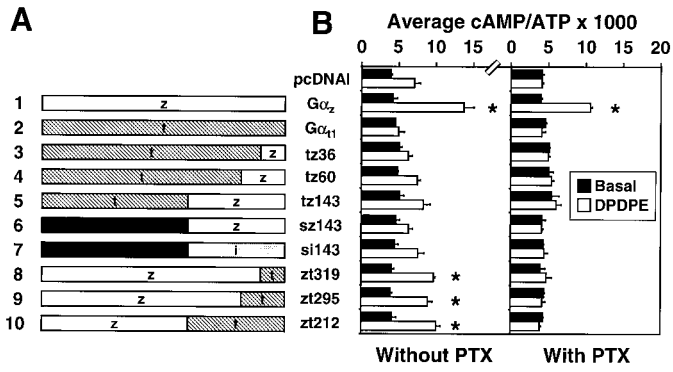

Fig. 1. The first series of chimeric $G\alpha$ subunits and receptor-mediated regulation of AC2. The parental ($G\alpha_z$ and $G\alpha_{t1}$) and chimeric $G\alpha$ subunits are diagrammatically shown in A. B, mutationally active $G\alpha_s$QL (0.025 μg/ml), AC2 (0.25 μg/ml), DOR (0.25 μg/ml), and 0.25 μg/ml of one of the $G\alpha$ subunits in A were coexpressed in HEK 293 cells. Transfected cells were treated with or without 100 ng/ml PTX as indicated. cAMP levels were measured in the absence or presence of 100 nM DPDPE. Asterisks indicate the DPDPE-induced cAMP levels are significantly higher than those of vector control.

The N-Terminus of α_z Is Essential for Receptor Coupling. Recent studies highlighted the importance of the N-terminus of Gα subunit for receptor specificity (Kostenis et al., 1997). A second series of chimeras were thus constructed to verify the role of the N-terminus of $G\alpha_z$ on DOR coupling. The chimera zt40 was constructed so that the N-terminal helix became $G\alpha_z$-like (Fig. 2A, the third construct). In the absence of PTX, DPDPE-induced enhancement of cAMP accumulation in zt40-transfected cells resembled that of $G\alpha_z$-transfected cells. However, zt40 is PTX-sensitive because it acquired the C-terminus of $G\alpha_{t1}$ and the DPDPE-induced cAMP production was abolished in the presence of PTX (Fig. 2B). This result suggested that the N-terminal region of $G\alpha_z$ was an essential determinant for receptor coupling. The chimera ztz40/36 was then constructed based on zt40 and tz36 (Fig. 2A, the fourth construct) to check whether the inclusion of the $G\alpha_z$-specific C-terminus would confer PTX resistance to the ztz40/36 chimera. The phenotype of ztz40/36 was more or less the same as $G\alpha_z$, except that the percentage response over basal value was slightly lower than that of $G\alpha_z$ (Fig. 2B). It suggested that the C-terminus of $G\alpha_z$ only conferred PTX-resistance but did not further enhance the receptor coupling efficiency. Two other ztz chimeras, ztz40/60 and ztz40/143 (Fig. 2A, sixth and seventh constructs), were also able to couple to DOR and stimulate AC2 to slightly greater degree than ztz40/36 (Fig. 2B).

$G\alpha_z$ has distinct biochemical properties in that the loading and hydrolysis of GTP by $G\alpha_z$ are much slower than other Gα subunits (Casey et al., 1990). The main reason is correlated to the variance of sequence identity in the G-1 GTP-binding region (amino acids 41–43). It is unknown whether the triplet variations affect the receptor coupling efficacies of the chimeras. zt43 and the corresponding series of ztz chimeras (ztz43/36, ztz43/60, and ztz43/143) were made so that the G-1 region resembled the $G\alpha_z$ sequence (Fig. 2A, the seven to tenth constructs). However, the profile of responses was similar to the zt40 series. All three ztz chimeras with N-terminal 43 residues of $G\alpha_z$ but not zt43 coupled to DOR and stimulated AC2 in the presence of PTX (Fig. 2B). The results eliminated the assumption that the variation of the G-1 GTP-binding region of $G\alpha_z$ affected the receptor interaction and subsequent release of βγ subunits.

The Amino Acids 11 to 14 of α_z Are Essential for Receptor Coupling. The N-terminus of $G\alpha_{t1}$ is considerably divergent from those of $G\alpha_{i2}$ and $G\alpha_z$ in two aspects. First, the N-terminus of $G\alpha_z$ is more homologous to $G\alpha_{i2}$ (47.1%) than to $G\alpha_{t1}$ (33.3%). Second, the length of the theoretical N-terminal helix of $G\alpha_{t1}$ is shorter than $G\alpha_{i2}$ or $G\alpha_z$ by four amino acids (Fig. 3A). Progressive deletion of the six N-terminal residues of $G\alpha_q$ broadened its receptor coupling specificity (Kostenis et al., 1997). It is conceivable that the length of the N-terminal region of the chimeras may affect receptor activation. In the case of zt40, its ability to couple to DOR may be attributable to the elongation of its N-terminal region. To test this possibility, an insertion mutant of $G\alpha_{t1}$ was constructed by inserting the four residues of $G\alpha_z$ between residues 10 and 11 of $G\alpha_{t1}$. The cysteine residue at −4 position of the C-terminus of $G\alpha_{t1}$ was mutated into glycine and the $G\alpha_{t1}$ mutant (termed +4t/CG) became resistant to PTX-mediated inactivation. This mutation allowed us to examine the coupling of +4t/CG with DOR without the interference caused by endogenous G_i proteins. In the presence of PTX, application of DPDPE can induce a modest increase of cAMP level in +4t/CG-transfected cells, which is significantly higher than that of $G\alpha_{t1}$CG- or vector-transfected cells (Fig. 3B). It indicated that +4t/CG can indeed couple to DOR in a PTX-insensitive manner although the response is relatively weak (~40% increase as compared with the basal). To further confirm the role of these four amino acids, a deletion mutant of $G\alpha_z$ was constructed with the amino acids 11 to 14 removed (termed −4z) and the mutant was tested for its ability to recognize DOR. In AC2 assays, −4z-transfected

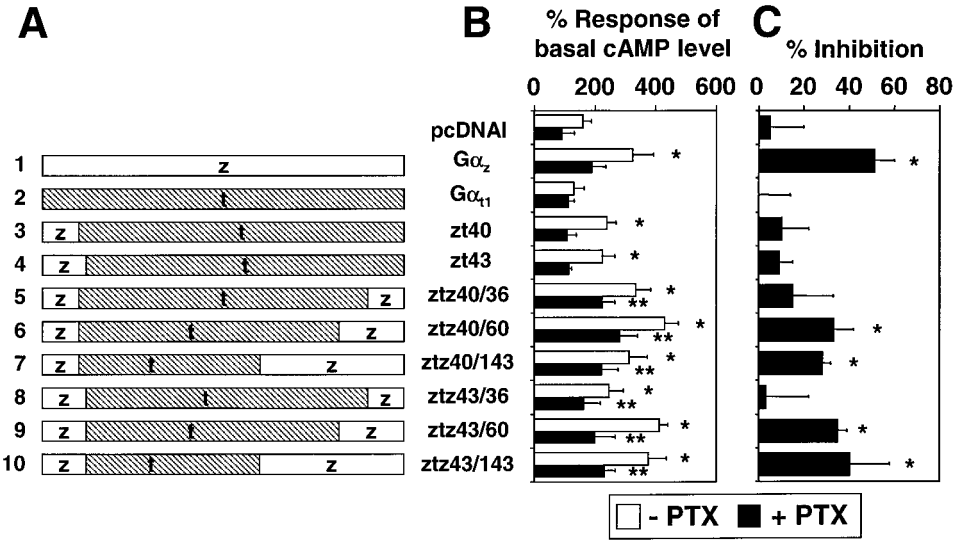

Fig. 2. The second series of chimeric Gα subunits and receptor-mediated regulation of ACs. The parental ($G\alpha_z$ and $G\alpha_{t1}$) and chimeric Gα subunits are diagrammatically shown in A. B, HEK 293 cells were transfected, labeled, and treated as indicated in the legend to Fig. 1B. Basal cAMP levels ranged from 4.11 ± 0.33 to 6.72 ± 0.18. Single and double asterisks indicate that the basal and DPDPE-induced cAMP levels, respectively, are significantly higher than those of vector control. C, HEK 293 cells were cotransfected with β_2AR (0.15 μg/ml), DOR (0.025 μg/ml), and 0.25 μg/ml of one of the Gα subunits in A. Transfected cells were all treated with 100 ng/ml PTX. cAMP production was triggered by treating the cells with 10 μM isoproterenol alone or in the presence of 100 nM DPDPE. DPDPE-induced inhibition was expressed as percentage inhibition of the isoproterenol-stimulated cAMP levels that ranged from 10.55 ± 1.78 to 14.08 ± 1.24. Asterisks indicate the DPDPE-induced inhibition of cAMP production is significantly greater than that of vector control.

cells showed only slight reduction of DPDPE-induced cAMP accumulation compared with $G\alpha_z$ (Fig. 3B). The reduction of response was also observed when the cells were pretreated with PTX. It suggested that residues 11 to 14 of $G\alpha_z$ form one of the minor determinants for DOR coupling. The reduction of DOR-induced inhibitory response in −4z-transfected cells is probably independent to its expression level (Fig. 4) and effector interaction. A constitutively active mutant of −4z, −4z/QL, inhibited isoproterenol-induced cAMP production in the cells cotransfected with β_2-adrenoceptor to a similar extent as $G\alpha_z$QL (Fig. 3C). Overall, these results strongly suggested that the N-terminal residues 11 to 14 of $G\alpha_z$ are involved in receptor recognition.

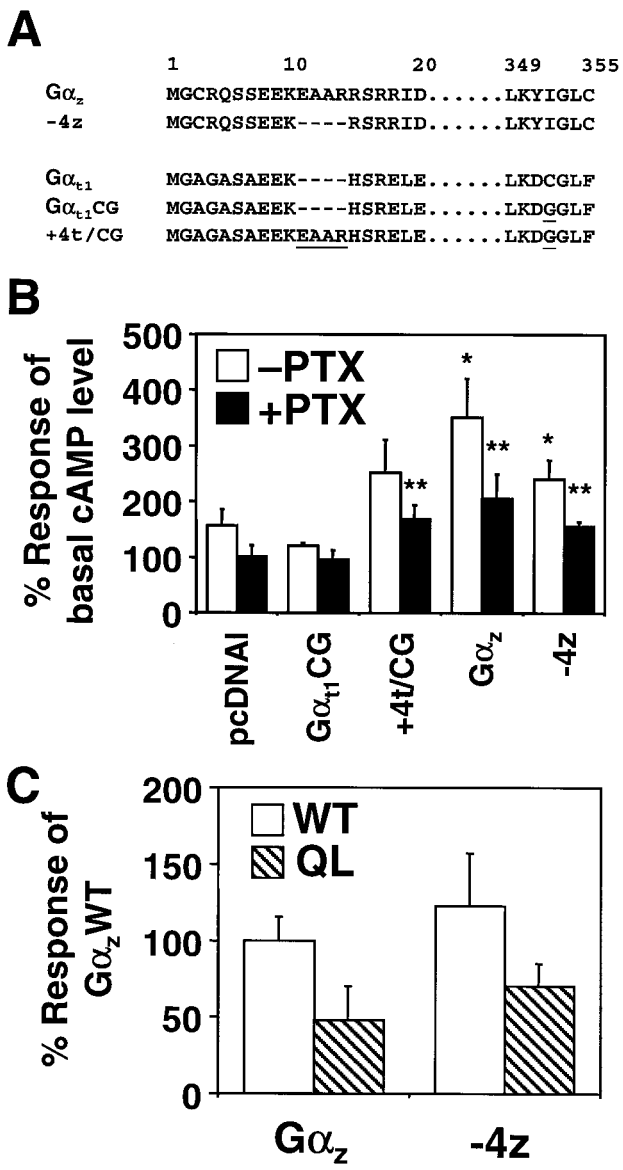

Fig. 3. Amino acids 11 to 14 of $G\alpha_z$ are essential for DOR coupling. A, the sequences of the two termini of $G\alpha_z$, $G\alpha_{t1}$, and three mutants were aligned. Deletion of the residues 11 to 14 of $G\alpha_z$ yielded the mutant −4z. A cysteine-to-glycine mutation was introduced to the −4 position of $G\alpha_{t1}$ to form $G\alpha_{t1}$CG. +4t/CG was made by inserting the residues 11 to 14 of $G\alpha_z$ between residues 10 and 11 of $G\alpha_{t1}$CG. Underlined residues are the mutated and inserted residues. B, HEK 293 cells were transfected, labeled, and treated as indicated in the legend to Fig. 1B. Basal cAMP levels ranged from 4.51 ± 0.73 to 5.45 ± 0.44. Single and double asterisks indicate that the basal and DPDPE-induced cAMP levels, respectively, are significantly higher than those of vector control. C, HEK 293 cells were cotransfected with 0.15 μg/ml of β_2AR and 0.25 μg/ml of one of the $G\alpha$ subunits. cAMP production was triggered by treating the cells with 10 μM isoproterenol. The cAMP levels were expressed as percentage responses of $G\alpha_z$. WT, wild type; QL, active mutant.

Protein Expression of Chimeric $G\alpha$ Subunits. Membrane proteins of the cells transfected with various chimeras studied here were separated by denaturing gel electrophoresis and the chimeras were detected by specific antisera. The expression levels of zt40 and zt43 were similar to tz36, but lower than $G\alpha_{t1}$ in HEK 293 cells as detected by an anti-$G\alpha_{t1}$ antiserum (Fig. 4A). Both $G\alpha_{t1}$CG and +4t/CG were expressed strongly in HEK 293 cells. Detection with an antiserum against the last 15 residues of $G\alpha_z$ facilitated direct comparison of the expression levels of various chimeras (Fig. 4B). All chimeric α subunits were expressed to similar levels in HEK 293 cells as $G\alpha_z$. The results indicated that most of the chimeras studied here are expressed to similar levels.

Receptor-Mediated Inhibition of AC by Chimeric α Subunits. Some of the chimeras contained increasing lengths of C-terminal tails of $G\alpha_z$, and could serve as useful tools for localizing the AC-inhibiting regions on $G\alpha_z$. Thus, we tested these chimeras for their ability to inhibit AC. HEK 293 cells transfected with β_2-adrenoceptor, DOR, and a parental or chimeric $G\alpha$ subunit were pretreated with PTX and then stimulated with 10 μM isoproterenol to elevate the intracellular cAMP level. Coadministration of DPDPE to the cells expressing $G\alpha_z$ showed about 30 to 40% reduction of isoproterenol-induced cAMP level, which was not observed in the cases of vector control or $G\alpha_{t1}$-transfected cells (Fig. 2C). Both zt40 and zt43-transfected cells lacked DPDPE-induced inhibition of AC activity. The N-terminal $G\alpha_z$-specific sequence of these two chimeras did not contain sufficient structural elements for AC inhibition. Next, we examined the six

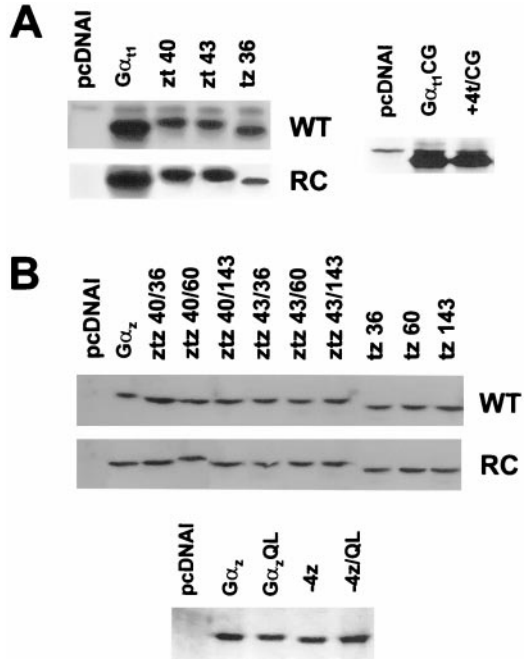

Fig. 4. Western blotting analysis of chimeric $G\alpha$ subunits. 0.25 μg/ml of each chimeric $G\alpha$ subunit cDNA was transfected into HEK 293 cells and membrane proteins were extracted for immunodetection. Antisera specific against $G\alpha_{t1}$ and $G\alpha_z$ were applied for blots in A and B, respectively. Two individual experiments using different batches of protein samples showed similar results.

ztz chimeras, because it has been shown that the N-terminal region of $G\alpha_z$ was important for the coupling to DOR. ztz40/36 and ztz43/36 showed no significant inhibitory effect on the cAMP levels (Fig. 2C). Chimeras containing 60 or more C-terminal amino acids of $G\alpha_z$ inhibited AC significantly. Such results indicated that one of the essential AC inhibitory domains must be located within amino acids 296 to 319 of $G\alpha_z$.

Inhibition of AC by Constitutively Active Chimeric Gα Subunits. Introduction of a point mutation at the GTP-binding regions of Gα subunit created receptor-independent constitutively active mutants (Freissmuth and Gilman, 1989; Graziano and Gilman, 1989). This approach has been successfully applied for studying the effector interacting domains of various Gα subunits even when the receptor interacting domains were disrupted in the chimeras (Medina et al., 1996; Grishina and Berlot, 1997). For the constructs used in this study, Arg-174 of the $G\alpha_{t1}$ domain and Gln-205 of the $G\alpha_z$ portion were mutated to cysteine and leucine, respectively, and these chimeras were used to examine the receptor-independent constitutive inhibition of AC activity. Results are summarized in Fig. 5. As a positive control, the $G\alpha_z$ mutant $G\alpha_z$QL (Wong et al., 1992) inhibited isoproterenol-stimulated cAMP level by 40 to 50% compared with the vector control, whereas $G\alpha_{t1}$ exhibited no observable inhibition on the AC activity. Among the three tz chimeras, tz60(RC) and tz143(RC) inhibited AC significantly, albeit to a lesser extent than that of $G\alpha_z$QL. Among the three zt chimeras, only zt319(QL) inhibited AC efficiently. Of the six ztz chimeras, only four—ztz40/60(RC), ztz40/143(RC), ztz43/60(RC), and ztz43/143(RC)—were able to inhibit AC significantly. The degrees of inhibition for all these chimeras were comparable with each other but slightly weaker than $G\alpha_z$QL. The protein expression levels of all these constitutively active chimeras were similar to each other and also comparable with those of their wild-type counterparts (Fig. 4). Collectively, the results confirmed the essential role of amino acids 295 to 319 of $G\alpha_z$ for AC inhibition because all chimeras that possess the ability to inhibit AC contained this stretch of residues from $G\alpha_z$.

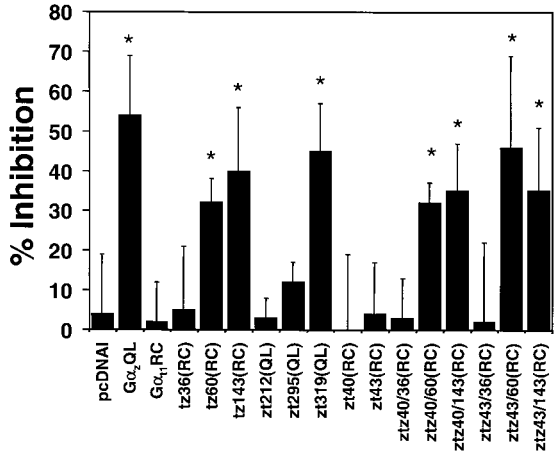

Fig. 5. Constitutive inhibition of AC by mutationally active chimeric Gα subunits. HEK 293 cells were cotransfected with 0.15 μg/ml of β_2AR and 0.25 μg/ml of one of the Gα subunits. cAMP production was triggered by treating the cells with 10 μM isoproterenol. The cAMP levels of the chimera-transfected cells were expressed as percentage inhibition of the mean value of the isoproterenol-stimulated cAMP levels of the vector control. Asterisks indicate the cAMP levels are significantly greater than the vector control.

Discussion

G_z is not simply a PTX-resistant substitute of the three G_i subtypes but it may be involved in a number of novel signaling events. The specific functional interactions of $G\alpha_z$ with newly discovered proteins such as RGSZ1 (Glick et al., 1998), Rap1GAP (Meng et al., 1999), and GRIN1 (Chen et al., 1999) suggested that $G\alpha_z$ has its distinct roles in signal transduction in addition to the regulation of ACs. Localization of the functional domains of $G\alpha_z$ could provide valuable information for a better understanding of the roles of $G\alpha_z$ in different molecular events. The present study investigated the receptor and AC-interacting domains of $G\alpha_z$. Two major findings could be concluded. First, the N-terminal sequence of $G\alpha_z$ seemed to be a critical determinant for the coupling to DOR. Second, one of the AC inhibiting domains of $G\alpha_z$ is located at amino acids 296–319.

A series of chimera studies indicated that the last five amino acids of Gα subunits are essential for determining the receptor specificity (Conklin et al., 1993, 1996). The last five amino acids of $G\alpha_{t1}$ are identical with those of $G\alpha_{i2}$ (Fig. 6), yet $G\alpha_{t1}$ primarily couples to rhodopsin. Both an evolutionary trace analysis (Lichtarge et al., 1996) and an extensive mutagenesis study (Onrust et al., 1997) suggested that residues spanning the whole α5 helix of the GTPase domain of Gα subunit interacts with receptor. Moreover, a recent study indicated that the intramolecular interaction between the GTPase and helical domains (β4/α3 and αG/α4 loops) of Gα subunit is important for receptor-mediated activation (Marsh et al., 1998). The series of tz chimeras retaining up to 143 C-terminal $G\alpha_z$ residues essentially covered all the regions identified in the previous studies for receptor-mediated activation. The lack of DOR coupling with tz chimeras clearly indicated that the N-terminal half of Gα subunit also contain essential elements for receptor coupling.

Alignment of the amino acid sequences of $G\alpha_{i2}$, $G\alpha_z$, and $G\alpha_{t1}$ yielded useful information (Fig. 6). The most interesting point is that the N-terminal helix of $G\alpha_{t1}$ is 4 amino acids shorter than $G\alpha_{i2}$ and $G\alpha_z$, but the charge distribution of the aligned residues are reasonably similar. A fair prediction would be the length of the N-terminal helix determined the receptor coupling efficiency. The phenotypes of the mutants +4t/CG and −4z (Fig. 3) supported the importance of N-terminus of Gα subunit in receptor coupling. Recent studies on the receptor coupling of $G\alpha_q$ also support this notion. Deletion of the N-terminal six amino acids of $G\alpha_q$ allowed the mutant to be activated by G_i-linked receptors (Kostenis et al., 1997). Alignment of the N-terminal sequences of various Gα subunits showed that the first six residues were unique for $G\alpha_q$. Indeed, the sequence identities and lengths of the N-terminal helices of different Gα subunits are very divergent and may be related to the peculiarities of the functions of each Gα subunit. For example, the N-terminal residues of $G\alpha_q$ are involved in membrane attachment (in addition to the palmitate attached) and phospholipase C activation (Hepler et al., 1996). In one of our previous studies (Ho and Wong, 1997), mutation of the two protein kinase C-phosphorylation sites at the N-terminus of $G\alpha_z$ abolished its constitutive inhibitory effect on AC. Further studies on the roles of N-

terminal residues of other Gα subunits should provide more insights on their specific functions.

The crystal structures of trimeric G proteins (Wall et al., 1995; Lambright et al., 1996) provided clear evidence that the N-terminus of Gα subunit is one of the major βγ-interacting sites. An early study of the interaction between $G\alpha_o$ and βγ subunits suggested that the reduction of the length of the N-terminus of $G\alpha_o$ by four residues (positions 7–10) diminishes its binding to βγ subunits (Denker et al., 1992). Preservation of the integrity of the N-terminal helix seems to be necessary for binding βγ subunits properly. In the present study, changes in the ability of the chimeras to associate with the βγ subunits might affect their coupling efficiencies to DOR. Moreover, it has been suggested that the N-terminal helix of Gα subunit is sandwiched by Gβ subunit and receptor (Wall et al., 1995; Lichtarge et al., 1996), and it may play a role in transmitting the conformational changes of ligand-bound receptor to the subsequent intra-and intermolecular conformational changes of G protein trimer. Additional studies are required to address these possibilities.

One of the well-characterized representatives in the $G\alpha_i$-subfamily in effector interaction is $G\alpha_{i2}$, which is probably the closest member to $G\alpha_z$. Berlot and coworkers have identified a region of 79 amino acids of $G\alpha_{i2}$, which was sufficient to convey the AC inhibition (Medina et al., 1996). The residues of $G\alpha_{i2}$ for effector regulation were resolved eventually using alanine mutagenesis by the same laboratory (Grishina and Berlot, 1997) and they are localized on two structural elements of $G\alpha_{i2}$, the Switch II region and the α4/β6 loop. Our results coincided with the findings in $G\alpha_{i2}$, the amino acids 291 to 314 of $G\alpha_z$ actually corresponded to α4/β6 loop on the GTPase domain of $G\alpha_z$. The same loop structure (but not the same residues at the corresponding positions) in $G\alpha_s$, $G\alpha_{i2}$, and $G\alpha_{t1}$ has also been shown to be an effector interacting region (Berlot and Bourne, 1992; Spickofsky et al., 1994; Grishina and Berlot, 1997; Natochin et al., 1999). It is interesting that different categories of G protein-regulated effector enzymes have evolved to interact with the similar structural elements of various Gα subunits. The α4/β6 loop may be more than just a structural element bearing a single function. Using $G\alpha_{t1}/G\alpha_{i1}$ chimeras, Bae et al. (1997) showed that the area bounded by the α4 helix and the α4/β6 loop of $G\alpha_{i1}$

A. Receptor interacting domains

N-terminus:

```
Gαi2  (1-35)    MGCTVSAEDKAAAERSKMIDKNLREDGEKAAREVK
Gαz   (1-35)    MGCRQSSEEKEAARRSRRIDRHLRSESQRQRREIK
Gαt1  (1-31)    MGAGASAEEK----HSRELEKKLKEDAEKDARTVK
                **.  *.*.*     *. ... *. . ..  * .*
```

C-terminus:

```
Gαi2  (325-355)   TCATDTKNVQFVFDAVTDVIIKNNLKDCGLF
Gαz   (325-355)   TCATDTSNIQFVFDAVTDVIIQNNLKYIGLC
Gαt1  (320-350)   TCATDTQNVKFVFDAVTDIIIKENLKDCGLF
                  ****** *..********.**. ***  **
```

B. Effector interacting domains

Switch II:

```
Gαi2  (200-220)    FDVGGQRSERKKWIHCFEGVT
Gαz   (200-220)    VDVGGQRSERKKWIHCFEGVT
Gαt1  (195-215)    FDVGGQRSERKKWIHCFEGVT
                    ********************
```

α4/β6 Loop:

```
Gαi2  (296-319)    KYDEAASYIQSKFEDLNKRKDTKE
Gαz   (296-319)    TYEEAAVYIQRQFEDLNRNKETKE
Gαt1  (291-314)    TYEDAGNYIKVQFLELNMRRDVKE
                    *..*  **. .* .**  .. **
```

Fig. 6. Alignment of the amino- and carboxyl-terminal regions of $G\alpha_{i2}$, $G\alpha_{t1}$, and $G\alpha_z$. Portions of the amino acid sequences of $G\alpha_{i2}$, $G\alpha_{t1}$, and $G\alpha_z$ were extracted from their complete alignment using CLUSTAL X program (numbers in brackets indicate the positions of the residues; Jeanmougin et al., 1998). The strictly and homologously conserved amino acids are marked with asterisks and dots, respectively, at the bottom of the aligned sequences. A, *inverted* and *underlined* residues are essential and nonessential for receptor coupling, respectively (Conklin et al., 1993; Beck et al., 1997; Ho and Wong, 1997; Onrust et al., 1997). B, *inverted* and *underlined* residues are important and unrelated to effector interactions, respectively (Faurobert et al., 1993; Spickofsky et al., 1994; Grishina and Berlot, 1997).

is important for the coupling of 5-HT_{1B} receptor. Subsequent studies by the same group (Bae et al., 1999) demonstrated that two α4 helical residues in $G\alpha_{i1}$ (Gln-304 and Glu-308) are critical determinants of receptor-G protein coupling. In the present study, the incorporation of the α4/β6 loop of $G\alpha_z$ in the $G\alpha_{t1}$ backbone (such as tz60 and tz143) did not allow coupling to DOR. Although the α4 helix of $G\alpha_z$ might be involved in receptor recognition, it alone was insufficient to support coupling to DOR.

We did not investigate further the importance of the Switch II region of $G\alpha_z$ on AC inhibition because $G\alpha_{t1}$ and $G\alpha_z$ have sequences identical with $G\alpha_{i2}$ at that region (Fig. 6) and so the corresponding region of $G\alpha_{t1}$ could provide essentially the same effector interacting residues. Inhibition of AC by $G\alpha_z$ was not related to the N-terminal sequence of $G\alpha_z$ because the constitutively active chimera tz60(RC) already exerted the inhibitory action (Fig. 4). The contribution of the variation of the G-1 GTP-binding region of $G\alpha_z$ to the regulation of AC was only minimal (Fig. 2C). It implied that the slow GTP hydrolysis rate of $G\alpha_z$ might not be related to the abilities of the chimeras to inhibit AC.

In conclusion, this report provides clear evidence that the N-terminal helix of $G\alpha_z$ (and possibly that of $G\alpha_i$) is crucial for receptor-mediated activation. The length of the N-terminal helix is also critical for determining the efficiency of receptor coupling. The AC-inhibiting domains of $G\alpha_z$ seem to be very similar to those of $G\alpha_{i2}$, including the α4/β6 loop and Switch II region. Our results strongly suggested that $G\alpha_z$ inhibited AC in a fashion similar to other inhibitory $G\alpha$ subunits.

Acknowledgments

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