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Influences of Nonmyristoylated α Subunit of G_{i2} on Adenylate Cyclase Signaling Pathway in COS-7 Cells†

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= Abstract = **To investigate the functional significance of myristoylation of inhibitory GTP binding protein α subunit, the rat cDNA encoding $G_{i2}\alpha$ was mutated at the glycine-2 modified normally by myristoylation. The glycine was replaced with alanine or deleted to construct G2A $G_{i2}\alpha$ and (Δ2-9)$G_{i2}\alpha$ mutants, respectively. The mutant and wild type $G_{i2}\alpha$ cDNAs were expressed transiently in COS-7 cells, and the intracellular localization of the proteins were analyzed by SDS-PAGE and immunoblot. Wild type $G_{i2}\alpha$ protein was localized mainly on the particulate fraction, but the nonmyristoylated G2A- and (Δ2-9)$G_{i2}\alpha$ proteins were localized to the cytosol. These results confirmed that myristoylation of $G_{i2}\alpha$ protein is required for its membrane binding as well as for $G_{i1}\alpha$ and $Go\alpha$. The basal level of cAMP in the COS cells transfected with $G_{i2}\alpha$ cDNAs ranged from 5.8 to 31 pmol/mg-proteins. When cells were treated with 10 μM isoproterenol, the cAMP level increased by 8- to 20 fold from the basal state, but the levels in COS cells expressing nonmyristoylated mutants were lower than that of control. Forskolin-stimulated accumulation of cAMP was increased in all the cells, and it also decreased in cells expressing nonmyristoylated $G_{i2}\alpha$ by 15% to 45% from the control. There was no significant alteration in the immunoreactivity of $G_s\alpha$ quantitated from the immunoblot. These results suggested that the nonmyristoylated $G_{i2}\alpha$ may decrease the adenylate cyclase activity. It might be possible that there is some crosstalk between the expression of $G_{i2}\alpha$ and that of adenylate cyclase.**

Key Words: *G-proteins, $G_{i2}\alpha$, Myristoylation, Adenylate cyclase, cAMP*

INTRODUCTION

Guanine nucleotide binding proteins (G-proteins) transduce the extracellular signals detected by a specific cell surface receptor into intracellular responses, and are composed of three subunits, α, β, γ (Gilman 1987). G proteins are activated when GTP binds to them, and inactivated when the bound GTP is hydrolyzed by G protein's intrinsic GTPase. The heterotrimeric G-proteins are involved in receptor-effector coupling of peptide hormones, neurotransmitters, prostaglandins, and sensory systems such as vision, olfaction and gustation. At least 16 genes encoding the α subunit have been cloned from various mammalian sources (Spiegel 1992), and 4 beta subunit cDNAs and 4 distinct gamma cDNAs have also been cloned(Simon *et al.* 1991). Since the α subunit of G proteins has the intrinsic GTPase activity, the sites for ADP-ribosylation by cholera toxin

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or pertussis toxin, and are involved in the interaction with receptors and effectors, the α subunit seems to perform most of the G protein functions. For the α subunit to receive signals it needs to bind to the membrane where the surface receptors detect extracellular signals. However, the amino acid sequences of the α subunit of G proteins (Gα) do not have hydrophobic transmembrane domains, and thus the Gαs must have some other way to attach to the plasma membrane even after activation and dissociation from $\beta\gamma$ complex which is more hydrophobic mainly due to isoprenylation of γ subunit(Maltese and Robishaw 1990). Pertussis toxin-sensitive α subunits i.e. inhibitory G proteins (Gis) and Go are known to be modified by myristic acid, which is a fatty acid (fourteen-carbon chain with no double bonds, C14:0) linked to the amino-terminal glycine. Such fatty acylation with myristic acid was found to be essential for $G_{i1}\alpha$ and $G_o\alpha$ for their membrane binding(Jones and Reed 1988; Mumby *et al.* 1990). Non-myristoylated Gi and Goα subunits showed reduced affinity for $\beta\gamma$, but the other physiological role of myristoylation is not clear.

In an attempt to examine the physiological function of fatty acylation, we constructed mutant forms of $G_{i2}\alpha$ lacking in myristoylation. The mutant proteins were expressed in COS cells and their intracellular localization and effects on the cyclic AMP signaling pathway were analyzed in this experiment.

MATERIALS AND METHODS

Construction of mutant $G_{i2}\alpha$ cDNAs

The cDNA for rat $G_{i2}\alpha$ (Jones and Reed 1988) was kindly provided by Dr. R. R. Reed (Johns Hopkins University, Baltimore, MD, U. S. A). Two $G_{i2}\alpha$ mutants which lacked the site for myristoylation were designed: one was mutated to replace glycine-2 with alanine (G2A $G_{i2}\alpha$) and the other had deletion from glycine-2 to aspartate-9 [$(\Delta 2\text{-}9)G_{i2}\alpha$]. The wild type $G_{i2}\alpha$ DNA was cloned into pAlter-1 vector (Promega Co., Madison, WI, U.S.A.), and the mutants were constructed according to the Promega's protocol with mutagenic oligonucleotides synthesized by the Inter-University Center for Natural Science Research Facility (Seoul National university, Seoul, Korea). In brief, a single stranded wild type $G_{i2}\alpha$ DNA in pAlter-1 vector was prepared and annealed with each mutagenic oligonucleotide, together with a second mutagenic oligonucleotide which restores ampicillin resistence to the mutant strand. The mutant strand was synthesized, ligated, and then transformed into repair minus strain of *E. coli* (BMH 71-18 mut S). A second round of transformation into JM109 was carried out to segregate mutant and wild type plasmid. Mutant plasmids were screened and confirmed by sequence analysis using the dideoxynucleotide chain termination method (Sanger *et al.* 1977). The changes of sequences in the $G_{i2}\alpha$ mutants and the respective mutagenic oligonucleotides used are illustrated in Fig. 1.

Expression of various forms of $G_{i2}\alpha$ in COS cells

The wild type and mutant $G_{i2}\alpha$ DNAs were cloned into an eukaryotic expression vector, pCD-PS (Bonner *et al.* 1988), and expressed transiently in monkey kidney cells, COS-7, by transfection using a DEAE-dextran method (Cullen 1987). The cells were harvested after 48 hours, homogenized, and fractionated by centrifugation at 100,000g for one hour twice.

SDS-PAGE, immunoblot and quantitation of immunoreactivity

Protein samples were separated on 12.5% SDS polyacrylamide gels, and then transferred onto nitrocellulose paper. The wild type and mutant $G_{i2}\alpha$ proteins were detected with a peptide specific antibody, AS7, which was generated against the carboxy terminal decapeptide of $G_{i2}\alpha$. The $G_{i2}\alpha$ was visualized by treating the blot with peroxidase labeled secondary antibody as described (Juhnn *et al.* 1992).

For quantitation of Gsα, the blot was reacted with RM antibody raised against the carboxy terminal decapeptide of Gsα, and the blot was visualized on a X-ray film by enhanced

Wild type Gi2α

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                                   1   2   3   4   5   6   7   8   9   10  11  12  13  14  15
                                   Met Gly Cys Thr Val Ser Ala Glu Asp Lys Ala Ala Ala Glu Arg
GGG CCG GCG GAC GGC AGG ATG GGC TGC ACC GTG AGC GCC GAG GAC AAG GCG GCA GCC GAG CGC
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G2A Gi2α

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                                       Ala
GGG CCG GCG GAC GGC AGG ATG GCC TGC ACC GTG AGC GCC GAG
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(Δ2-9) Gi2α

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GGG CCG GCG GAC GGC AGG ATG..........................................AAG GCG GCA GCC GAG CGC
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Fig 1. The DNA sequences near the amino terminus of wild type and mutant forms of rat $G_{i2}\alpha$. The mutant sequences shown in this figure are identical to the sequences of the respective mutagenic oligonucleotides. The sequences corresponding to glycine residue modified normally by myristoylation are displayed in outline.

chemiluminescent (ECL) detection kit (Amersham Co.) after incubation with the secondary antibody. The amount of Gsα was quantitated by measuring the band density with a image analyzer. Three different amounts of COS cell membranes were included in each blot as the reference standards.

Measurement of intracellular cAMP concentration

COS-7 cells were cultured in a 24-well plate, and transfected with $G_{i2}\alpha$ cDNAs. After 48 hours, the cells were treated with either 10 μM isoproterenol or 100 μM forskolin, and then the cAMP was extracted with 2.5 M perchloric acid. After the acid extract was neutralized, cAMP was quantified using a cAMP [^{3}H] assay system from Amersham Co. (Buckinghamshire, England) by following the manufacturer's protocol.

RESULTS

Expression of wild type and mutant $G_{i2}\alpha$s in COS cells

To evaluate the expression and membrane targeting of $G_{i2}\alpha$ mutants, we transfected COS cells with wild type and mutant cDNAs. We fractionated the transfected COS cells into particulate and soluble fractions and measured immunoreactivity in each fraction after SDS-PAGE and immunoblot. Expressed wild type $G_{i2}\alpha$ as well as the endogenous form was localized to the particulate fraction, which migrated as a 41 kD protein (Fig. 2). In contrast, both the non myristoylated mutant, G2A- and (Δ2-9)$G_{i2}\alpha$, were localized to soluble fraction as the non-myristoylated forms of $G_{i1}\alpha$ (Jones *et al.* 1990) and Goa (Mumby *et al.* 1990). However, the amount of expressed (Δ2-9)$G_{i2}\alpha$ was not as much as those of wild type and G2A $G_{i2}\alpha$.

Changes in cAMP signaling pathways of COS cells transfected by various forms of $G_{i2}\alpha$

In order to study the effect of $G_{i2}\alpha$ expression on cAMP signaling pathways, COS cells in a 24-well plate were transfected and treated with 10 μM isopropanol or 100 μM forskolin, and then cAMP content of the cell was determined.

As shown in Fig. 3, without any stimulation the basal level of cAMP was 31, 17, 6.0, and 5.9 pmol/mg-protein, respectively for control, wild type, G2A- , and (Δ2-9)$G_{i2}\alpha$ transfected COS cells. They exhibited a decreasing tendency in COS cells transfected with wild type or non-myristoylated mutant forms.

When the adenylate cyclase pathway was activated with isoproterenol which activated

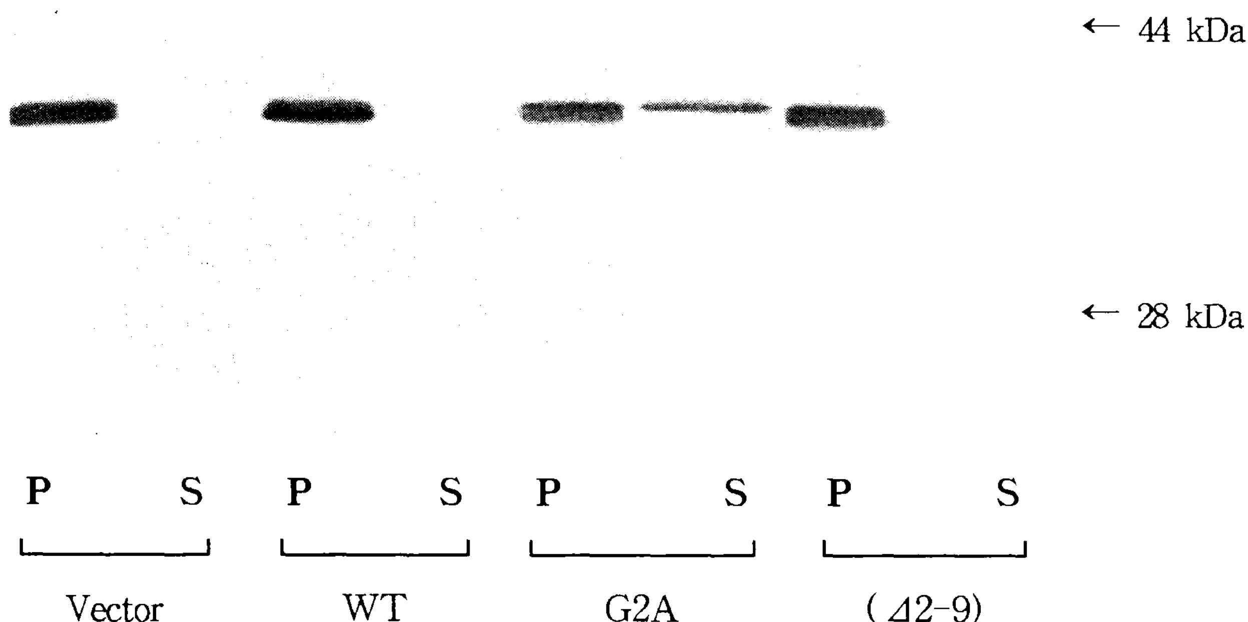

Fig. 2. Expression of wild type and mutant forms of $G_{i2}\alpha$ in COS cells. COS cells were transfected with wild type (WT), G2A- and $(\Delta 2\text{-}9)G_{i2}\alpha$ cDNA and the cells were harvested and fractionated into pellet (P) and supernatant (S) by centrifuging at 100,000 *g* for one hour. Each fraction, 50 μg proteins, was analyzed by 12.5% SDS-PAGE and western blot.

stimulatory G-proteins, the cAMP concentration in COS cells increased by about 8- to 30-fold its respective basal state. The cAMP concentration was 246, 232, 180 131 pmol/mg-protein in the order of control, wild type, G2A- , and $(\Delta 2\text{-}9)G_{i2}\alpha$ transfected COS cells. The cAMP level in the cells transfected with wild type $G_{i2}\alpha$ was comparable to that of cells without transfection. But the cAMP levels in COS cells transfected with a nonmyristoylated mutant decreased significantly, and about a 25% decrease was observed in cells transfected with G2A mutant and a 45% decrease in cells with $(\Delta 2\text{-}9)G_{i2}\alpha$.

To find the basis for the decreased cAMP response in COS cells transfected with mutant forms of $G_{i2}\alpha$, cAMP concentration was measured in the transfected cells after treatment with forskolin. Because forskolin stimulates adenylate cyclase without activation of G-proteins, the forskolin-stimulated cAMP response can reflect total activity of the enzyme (Seamon and Daly 1986). The concentrations of cAMP in the cells treated with forskolin were 229, 246, 195, 140 pmol/mg-protein. These cAMP levels were slightly higher than those of isoproterenol-treated cells, and this result indicated that there was some portion of adenylate cyclase which was not activated by isoproterenol. The cells transfected with wild type $G_{i2}\alpha$ exhibited comparable cAMP response to the control, but the cells transfected with G2A mutant and $(\Delta 2\text{-}9)G_{i2}\alpha$ displayed about a 15% and a 40% decrease, respectively (Fig. 3). These findings suggested that the decrease in cAMP responses in the cells transfected with nonmyristoylated mutant forms of $G_{i2}\alpha$ resulted from the decrease of adenylate cyclase activity.

The amount of $Gs\alpha$ in COS cells transfected with various forms of $G_{i2}\alpha$

The amount of $Gs\alpha$ was measured by ECL and densitometry, and the assay was reproducible when tested with standard samples. In all assay, three standard samples were analyzed together to ensure the linearity of the assay. The amount was expressed as the percentage to the immunoreactivity in mock transfected

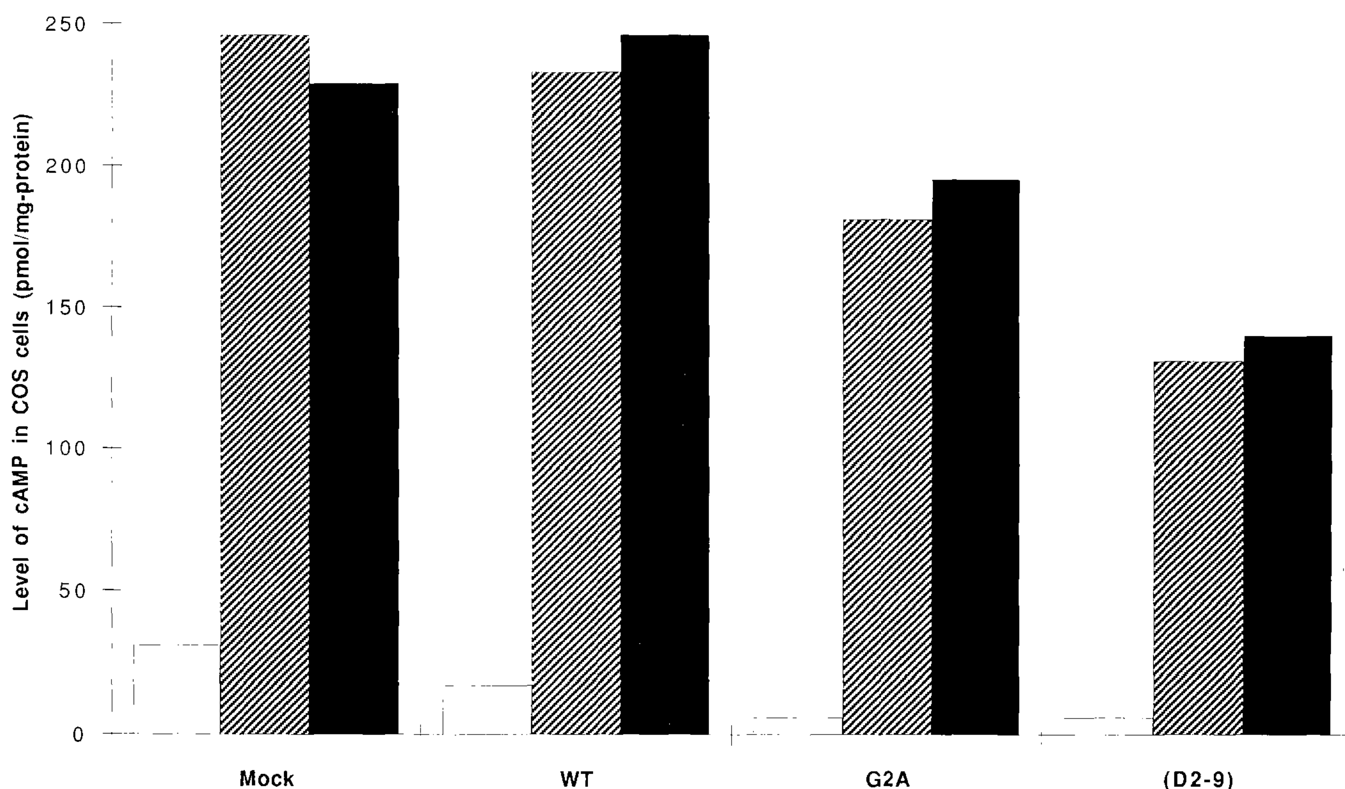

Fig. 3. Effects of transfected $G_{i2}\alpha$ on the cAMP level in COS cells. Intracellular cAMP level was measured in the COS cells transfected with wild type (WT), G2A- and $(\Delta 2\text{-}9)G_{i2}\alpha$ cDNA without any treatment (empty cloumn) or after treatment with 10 μM isoproterenol (hatched column) and 100 μM forskolin (filled column).

COS cell membrane. As shown in Table 1, there was no significant change in the immunoreactivity of $Gs\alpha$ without regard to the type of the transfected $G_{i2}\alpha$.

DISCUSSION

Heterotrimeric G-proteins transduce signals coming from an activated ligand-receptor complex into intracellular responses by regulating the activity of effector molecules such as adenylate cyclase, phospholipase and ion channels. It seems to be essential for the G proteins to possess membrane binding capacity to receive signals from receptors, because almost all the receptors coupled to G-proteins are integral membrane proteins with seven transmembrane helical domains (Birnbaumer *et al.* 1990). Ras protein and v-src protein are known to require membrane binding capacity to exert their transformation activity (Cross *et al.* 1984; Willumsen *et al.* 1984).

The α subunits of G-proteins are tightly bound to the cytosolic face of the plasma membrane, but the amino acid sequences of α subunit deduced from their nucleotide sequences contain no hydrophobic membrane spanning domains to account for their tight attachment. Posttranslational modification of proteins with lipids is one way to increase the hydrophobicity of the molecules and thus to promote membrane association. Fatty acylation with myristate was found in the α subunits of Gi and Go (Buss *et al.* 1987), and failure of membrane attachment was observed with mutant forms of $G_{i1}\alpha$ (Jones *et al.* 1990) and $G_o\alpha$ (Mumby *et al.* 1990) which were incapable of undergoing N-myristoylation. The nonmyristoylated G-proteins were also suggested to be decreased in their affinity for $\beta\gamma$ complex (Denker *et al.* 1992; Jones *et al.* 1990) and to be essential for signal transduction and regulation of effector enzymes in the cell (Gallego *et al.* 1992).

In this experiment, the wild type and

Table 1. The relative amount of Gsα in the COS cells transfected with various forms of $G_{i2}\alpha$ cDNAs

Transfected $G_{i2}\alpha$	Relative amount of Gsα (average ± SD)
Mock	100 ± 15
Wild type	96 ± 18
G2A $G_{i2}\alpha$	106 ± 10
(Δ2-9)$G_{i2}\alpha$	98 ± 11

The amount of Gsα was measured from the immunoblot by ECL and densitometry, and the amount was expressed as the percentage to the immunoreactivity in mock transfected COS cell membrane. The values are the averages of duplicate assays of triplicate transfections.

nonmyristoylated mutant forms of $G_{i2}\alpha$ were expressed in COS cells. The COS cells transfected with wild type and G2A mutant expressed increased amounts of the corresponding proteins evaluated by immunoblot analysis, but the expression of (Δ2-9)$G_{i2}\alpha$ was not as much as the former. Low level of expression might result from low activity of transcription, instability of mRNA, low activity of translation, and rapid degradation after protein biosynthesis. However, when considering the large amount of the expressed wild type and G2A mutant which had the same sequences except the mutation, the expression of (Δ2-9) $G_{i2}\alpha$ in low amount is more likely to result from rapid degradation of the protein after its synthesis because of structural instability from the deletion. Both of the nonmyristoylated mutants were localized to the soluble fraction as the comparable mutants of $G_{i1}\alpha$ and $G_o\alpha$ (Jones *et al.* 1990; Mumby *et al.* 1990), confirming that myristoylation is essential for membrane attachment for pertussis toxin-sensitive Gα proteins composed of $G_{i1}\alpha$, $G_{i2}\alpha$, G_{i3}, and $G_o\alpha$.

The cellular responses of receptor activation coupled to adenylate cyclase through $G_{i2}\alpha$ is dependent upon the type of the activated receptor, relative concentration of Gs and Gi proteins, and the distribution of adenylate cyclase subtypes in the cell(Choi *et al.* 1993). In COS cells, overexpression of wild type $G_{i2}\alpha$ did not decrease significantly the cAMP accumulation, and this result might suggest that the adenylate cyclase subtypes in COS cells cause adaptation in cAMP signaling pathway through mechanisms possibly such as inhibition by $\beta\gamma$ subunits.

Nonmyristoylated $G_{i1}\alpha$ was reported to fail to activate adenylate cyclase overexpressed in Sf cell membranes (Taussig *et al.* 1993). In our experiment, overexpression of analogous mutants of $G_{i2}\alpha$ decreased the activity of the adenylate cyclase signaling pathway when stimulated by either isoproterenol or forskolin, and such a decrease in cAMP response was not observed in COS cells overexpressing wild type protein. Since there was no significant change in the amount of Gsα, these results suggest that overexpression of $G_{i2}\alpha$ could be signaled, through unknown pathways, to the activity of adenylate cyclase. In case of (Δ2-9) $G_{i2}\alpha$, such a decrease of cAMP accumulation occurred even though the mutant protein was detected in a small amount, which might occur if there is some crosstalks between the transcription of $G_{i2}\alpha$ and that of adenylate cyclase. More reliable interpretation requires quantification of adenylate cyclase and G-proteins at the level of mRNA and protein. For a better understanding of the adenylate cyclase signaling pathway in COS cell, it is also required to analyze the distribution of adenylate cyclase subtypes in the cell.

In the present experiment, nonmyristoylated mutant $G_{i2}\alpha$ was observed to decrease the activity of adenylate cyclase without changing the amount of Gsα in COS cells. Investigation of the mechanisms for decrease in adenylate cyclase activity in COS cells expressing nonmyristoylated $G_{i2}\alpha$ mutants could disclose one part of the crosstalks between the components of signaling pathways.

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