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Effects of overexpression of stimulatory G proteins on the expression of the other G proteins in COS-1 cells

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Abbreviations: G protein, guanine nucleotide-binding regulatory protein; G_i, adenylyl cyclase inhibitory G protein; G_s, adenylyl cyclase stimulatory G protein; $G_s\alpha$, α subunit of G_s; $G\beta$, β subunit of G protein

Abstract

To investigate the interaction of stimulatory GTP binding protein (G_s) pathways with others, we overexpressed wild type alpha subunit of G_s ($G_s\alpha$), constitutively activated R201E $G_s\alpha$, and dominant negative G226A $G_s\alpha$ in COS-1 cells by transfection with DEAE-dextran, respectively. The expression of various G proteins in the transfected cells was analyzed after 72 h by quantitative Western blots, and cAMP production by stimulation with isoproterenol and forskolin was quantitated using cAMP binding proteins. The expression of $G_s\alpha$ increased about 5-fold in the transfected cells, with concomitant increase in the small forms. However, there was no significant alteration the in the level of the alpha subunit of inhibitory G protein (G_i) and G_q, and the beta subunits of G proteins. The cAMP level without stimulation increased in the cells transfected with $G_s\alpha$ regardless to the type of mutation. Treatment with either isoproterenol or forskolin resulted in comparable increase of the cAMP level in all the transfected cells, though the ratio to its respective basal level was smaller in the $G_s\alpha$-transfected cells. From this experiment, we found that the expression of the other G proteins and the signaling pathway producing cAMP did not change significantly by transiently expressing wild type, constitutively activated type, and dominant negative type of $G_s\alpha$. Analysis of the effects of long-term expression of $G_s\alpha$ would contribute to better understanding on how the $G_s\alpha$ signaling system interacts with other signaling pathways and how it adapts to the changed environments.

Keywords: adenosine cyclic monophosphate, guanine nucleotide regulatory proteins, recombinant proteins, protein engineering, Western blotting

Introduction

Heterotrimeric signal transducing G proteins transduce extracellular signals into intracellular cellular response by coupling receptors and effectors (Gilman, 1987; Neer, 1995). The activated receptors activate G proteins by stimulating the replacement of bound GDP with GTP and then the G proteins are inactivated by hydrolysis of the bound GTP by its intrinsic GTPase activity (Bourne *et al.*, 1990). The various signals making G proteins active include peptide hormones, neurotransmitters, cytokines and photons (Watson and Arkinstall, 1994). The activated G proteins regulate intracellular effectors such as ion channels and enzymes including adenylate cyclase and phospholipase C that produce intracellular second messengers (Bourne *et al.*, 1991). The G protein signaling system usually results in immediate responses such as activation of glycogen phosphorylase to break-down glycogen, but it also induces long term effects such as regulation of cell proliferation and differentiation (Chen and Iyengar, 1995).

Cells receive numerous signals from surroundings continuously, and multiple signals may stimulate cells at the same time. Thus cells need to integrate various signals before making cellular responses to these stimuli, and the interactions between the various signaling pathways are necessary for this integration. Understanding the mechanisms of such interactions or cross-talks among signaling pathways is very important to understand how the extracellular signals evoke intracellular responses and how they are regulated in cells (Barritt, 1992).

G protein signaling pathways cross-react with other pathways including mitogen activated protein kinases (Dikic *et al.*, 1996), and they also cross-react with other G protein pathways (Liu and Simon, 1996; Wayman *et al.*, 1994). The stimulatory G protein (G_s) activates adenylate cyclase to increase intracellular cAMP, and it distributes ubiquitously in the tissues composing a housekeeping signaling pathway. The G_s-cAMP system is known to interact calcium signaling pathway and to inhibit Raf activation (Chen and Iyengar, 1995), but it is not clear how the alteration of G_s signaling pathway influence other signaling pathways including the expression of other G proteins.

Thus to investigate the interactions of stimulatory G protein signaling pathways with others, we analyzed the effects of overexpression of stimulatory G protein on the expression of other G protein and cAMP production in these cells.

Materials and Methods

Cell culture

African green monkey kidney cell line, COS-1, was obtained from Korean Cell Line Bank of SNU College of Medicine. COS-1 cells were grown in Dulbecco's modified minimal essential media (DMEM) containing 10% fetal bovine serum and incubated in a CO_2 incubator at 37˚C.

Construction of mutant $G_s\alpha$

The cDNAs encoding the alpha subunit of stimulatory G protein ($G_{s\alpha}$) from rat olfactory epithelium (Jones and Reed, 1988) were kindly provided by Dr. R. R. Reed (Johns Hopkins University), and it was subcloned into pCD-PS (Juhnn *et al.*, 1992). Constitutively activated mutant of $G_{s\alpha}$ (R201E $G_{s\alpha}$) was constructed by replacing arginine at 201 with glutamine (Freissmuth and Gilman, 1989), and dominant negative mutant, G226A $G_{s\alpha}$, was formed by replacing glycine at 226 with alanine (Miller *et al.*, 1988).

Expression of $G_s\alpha$ in COS cells and preparation of the cell homogenate

Transformed monkey kidney cells, COS-1, were transfected with the cDNA of wild type and mutated $G_{s\alpha}$ by the DEAE-dextran method (Aruffo, 1991), and reagents without DNA or the vector DNA without insert was used as controls.

The transfected COS-1 cells were harvested after 72 h with a cell scraper, and the cells were resuspended in 10 volumes of a homogenization buffer composed of 20 mM phosphate buffer, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM dithiothreitol. The cells were homogenized by passing 15 times through a 25 gauge needle on ice. Protein content was determined according to the Lowry method (1951).

SDS-PAGE and Western blot analysis

Six to 40 μg of the homogenate protein was separated on a 12.5% SDS-polyacrylamide gel and was transferred to nitrocellulose paper as described previously (Shin *et al.*, 1995). The blot was blocked with 5% non-fat milk in phosphate buffered saline (PBS) for 1 h, and then incubated at room temperature with an antibody against each G protein (1 to 5 μg/ml) overnight.

The antibody against each G protein was generated by immunizing rabbits with specific peptides of the G proteins and purified by affinity chromatography on a column of Affi-Gel 15 gel to which corresponding antigenic peptides had been coupled according to manufacturer's manual (Bio-Rad, CA, U.S.A.). The RM antibody was raised against the carboxyl terminal decapeptides of $G_{s\alpha}$, and it cross-reacts with $G_{olf\alpha}$ that differs in only one amino acid residue from the $G_{s\alpha}$ peptides. The AS antibody was generated against carboxyl decapeptides of the peptides of transducin, and it reacts not only with the α subunit of transducin but also with the $G_i1\alpha$ and $G_i2\alpha$ that differs in a single amino acid residue from the α subunit of transducin. The QL antibody was generated against the carboxyl decapeptides of the α subunit of G_q, and it detects both $G_{q\alpha}$ and $G_i1\alpha$. GO was directed against carboxyl decapeptides of the α subunit of G_o. The SW antibody was generated against carboxyl decapeptides of beta subunit and it reacts with $G_{\beta1}$, $G_{\beta2}$, $G_{\beta3}$, and $G_{\beta4}$.

The nitrocellulose paper was then washed with 0.1% Tween 20 in PBS and incubated with peroxidase-labeled goat anti-rabbit IgG antibody preparation (1:5,000 dilution, Pierce, IL, U.S.A.) for 2 h at room temperature. The blot was washed with PBS and then incubated with enhanced chemiluminescence (ECL) substrate mixture (Amersham, England) for 1 min, and then exposed to an X-ray film (AGFA Curix RPI) for 1-5 min to obtain an image.

The density of the bands corresponding to each G protein subunit in the autoradiogram was quantified using a densitometer (Model GS-700, Bio-Rad), and the relative amount was calculated by comparing the density with the standard curve prepared with the brain cholate extract analyzed simultaneously in the same blot. The amount of each G protein was expressed as the equivalents to that of rat brain extract used as the reference standard, which was prepared by extracting rat whole brains with 1% cholate.

Measurement of cAMP formation in the cells

The COS-1 cells were transfected with vector and each $G_{s\alpha}$ cDNA and incubated for 24 h. The cells were plated into 24-well plate, and were allowed to grow for 48 h. The cells were washed twice with PBS, and then treated with 500 μl DMEM medium containing 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) and 10 μM isoproterenol or 50 μM forskolin for 30 min at room temperature. The control cells were treated with the medium with 3-isobutyl-1-methylxanthine only. After washing the cells with PBS, cAMP was extracted with 500 μl of 0.42 M perchloric acid by incubating at 4˚C for 30 min. The cell extract was neutralized with 4.2 M KOH, and its cAMP was quantitated by cAMP assay system (Amersham) that uses cAMP binding proteins. The cAMP content was normalized by the protein content of the COS-1 cells extract.

The Kruskal-Wallis test and the Mann-Whitney U test were performed to compare the expression of G proteins and cAMP formation in the transfected cells.

Results

Expression of G proteins in COS-1 cells transfected with $G_s\alpha$

The COS-1 cells expressed enough $G_{s\alpha}$, $G_{i\alpha}$, $G_{q\alpha}$, and $G\beta$ for measuring by western blot, but the expression of $G_{o\alpha}$ was not high enough to measure it reliably. Thus the three alpha subunits and beta subunits were analyzed in this experiment. The amount of G proteins was expressed as the equivalent to rat brain extract.

The COS-1 cells express two bands of $G_{s\alpha}$, 45 kDa and 42-kDa, and the amount of 42-kDa forms was approximately 1.5-fold larger than the 45-kDa form. The cells transfected with wild type and mutant forms of $G_{s\alpha}$ cDNA expressed large amount of the 45 kDa form of $G_{s\alpha}$ up to 6-fold of endogenous $G_{s\alpha}$ (Figure 1, Table 1). The extent of $G_{s\alpha}$ overexpression was not different significantly among the cells transfected with wild type or mutant types $G_{s\alpha}$. The expression of the small forms of $G_{s\alpha}$ also exhibited a little increase, and it did not seem to result from overflow of overexpressed large form of $G_{s\alpha}$.

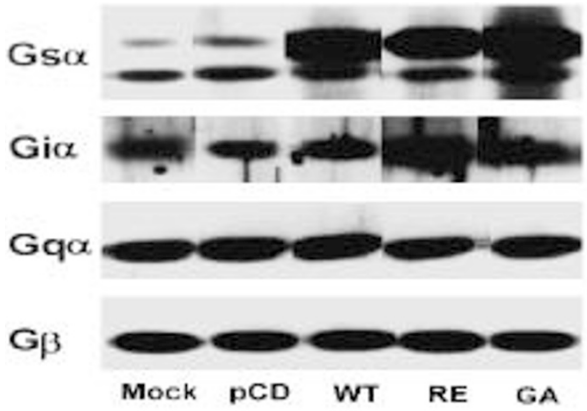

Figure 1. Representative immunoblot analysis of various G proteins in COS-1 cells expressing various types of $G_s\alpha$. The COS cells were transfected with reagents only (Mock), vector only (pCD), wild type $G_s\alpha$ (WT), constitutively activated R201E $G_s\alpha$ (RE), and dominant negative G226A $G_s\alpha$ (GA) by the DEAE-dextran method. Six to 40 μg of protein was separated by 12.5 % SDS-PAGE and transferred onto nitrocellulose paper. The blot was incubated with a specific antibody directed against each G protein, and then with peroxidase labeled goat anti-rabbit IgG antibody. The blot was incubated with enhanced chemiluminescence (ECL) substrate mixture for 1 minute, and then exposed to an X-ray film to obtain an image.

The expression of the alpha subunit of inhibitory G protein ($G_{i\alpha}$) showed decreasing tendency in the cells transfected with wild type and constitutively activated forms of $G_{s\alpha}$ without statistical significance. There was no change in the expression of $G_{i\alpha}$ in the cells transfected with dominant negative mutant, G226A $G_{s\alpha}$, either. There was no statistically significant change in the expression of the $G_{q\ \alpha}$ subunit in COS-1 cells transfected with vector, wild type or mutant types of $G_{s\alpha}$.

The expression beta subunits exhibited slight decrease in the COS-1 cells transfected with wild type and constitutively activated mutant R201E $G_{s\alpha}$ without statistical significance.

Formation of cAMP in COS-1 cells transfected with various forms of $G_s\alpha$

Formation of cAMP was analyzed in the cells transfected with various forms of $G_{s\alpha}$ and controls after stimulation with isoproterenol and forskolin. Without stimulation, the basal level of cAMP increased about 2- to 3-fold in all the transfected COS-1 cells, but the cells transfected with wild type and mutant type $G_{s\alpha}$ exhibited increase in the cAMP production in comparison with vector transfected control (Figure 2). The increase of basal level of cAMP has no correlation with the type of $G_{s\alpha}$ expressed in the COS-1 cells.

When the COS-1 cells were stimulated with isoproterenol, a beta adrenergic agonist, the cAMP level increased by 4.5- to 8-fold in the controls and about 2.5-fold in $G_{s\alpha}$-transfected cells. Though the ratio of basal to stimulated level of cAMP decreased significantly in the $G_{s\alpha}$ transfected cells, the stimulated level of cAMP was comparable in the control and transfected cells in spite of some

Table 1. Expression of G proteins in COS-1 cells transfected with various forms of $G_s\alpha$. Western blot was performed as described in the legend of Figure 1, and the expression of G proteins was quantitated by analyzing the blot with a densitometer. The amount of G proteins is expressed as the equivalent per mg-protein of cell homogenate to rat brain extract that was used as reference standard. The values are the average ±standard error, and n means the number of transfected samples analyzed.

	$G_s\alpha$			$G_i\alpha$	$G_q\alpha$	$G\beta$
	Long form	Short form	Total			
Mock (n=5)	1.21 ± 0.16	1.88 ± 0.16	3.09 ± 0.29	0.45 ± 0.13	0.75 ± 0.11	0.51 ± 0.06
Vector (n=4)	1.75 ± 0.12	2.26 ± 0.17	4.01 ± 0.29	0.35 ± 0.05	0.85 ± 0.07	0.41 ± 0.04
WT $G_s\alpha$ (n=7)	7.66 ± 1.14	3.17 ± 0.43	10.83 ± 1.78	0.36 ± 0.09	0.89 ± 0.12	0.36 ± 0.06
RE $G_s\alpha$ (n=6)	7.29 ± 1.69	3.17 ± 0.45	10.46 ± 2.07	0.38 ± 0.17	0.82 ± 0.19	0.37 ± 0.05
GA $G_s\alpha$ (n=6)	6.72 ± 1.17	2.86 ± 0.54	9.58 ± 1.70	0.46 ± 0.13	0.68 ± 0.19	0.41 ± 0.07

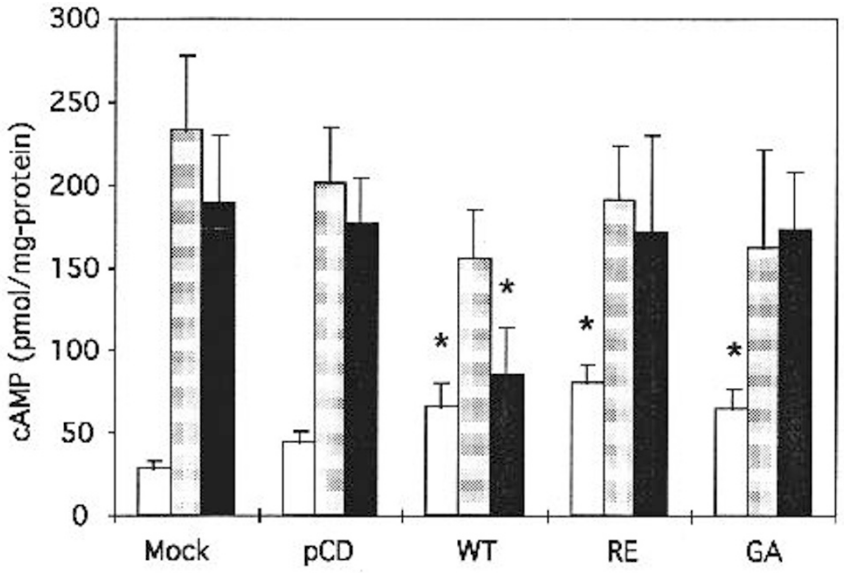

Figure 2. The cAMP response in COS-1 cells transiently expressing wild type and mutant forms of $G_s\alpha$. Twenty-four after transfection, the COS-1 cells were plated into 24-well plate, and were allowed to grow 48 hours. The cells were then treated with 10 mM isoproterenol (dotted bar), 50 mM forskolin (filled bar) or 0.1 mM IBMX only (empty bar) for 30 minutes at room temperature. The cAMP was extracted with perchloric acid and then quantitated by cAMP assay system using cAMP binding proteins. The protein content was measured by Lowry method, and the cAMP content was normalized by the protein content. The Kruskal-Wallis test was performed and the Mann-Whitney U test was used to compare the expression of G proteins and cAMP formation in the transfected cells. The asterisk (*) indicates a statistically significant difference ($P < 0.05$) of the mean from that of vector transfected cells.

decreasing tendency in the cAMP formation in the transfected cell.

The cells stimulated by forskolin, an activator of adenylate cyclase, produced equivalent amount of cAMP to that of stimulated by isoproterenol, and its formation decreased in the COS-1 cells transfected with wild type $G_{s\alpha}$. The cAMP formation stimulated by forskolin was not different among the cells transfected with R201E $G_{s\alpha}$ or G226A $G_{s\alpha}$.

These results suggest that the signaling pathway from beta adrenergic receptor to adenylate cyclase remains largely unchanged by transient expression of wild type, constitutively activated type or dominant negative type of $G_{s\alpha}$.

Discussion

Our experiment shows that transient overexpression of various forms of $G_{s\alpha}$ in COS-1 cells does not change G protein signaling pathways significantly. The expression of other G proteins and the cAMP production stimulated by isoproterenol and forskolin remains unchanged.

The expression of other G protein remains unchanged in COS-1 cells with regard to $G_{s\alpha}$ forms transfected. Because the constitutively activated mutants of $G_{s\alpha}$ were found in the tumors of pituitary and thyroid tumor and they exhibited increase in adenylate cyclase activity (Lyons *et al.*, 1990), expression of 201E $G_{s\alpha}$ was anticipated to increase cAMP production and to increase expression of inhibitory G protein $G_{i\alpha}$ by compensatory mechanisms (Kinane *et al.*, 1993). However, none of such changes was observed in this experiment. We obtained similar results with the cells transfected dominant negative G226A $G_{s\alpha}$. Transient expression of G_i2 did not change in the beta subunit expression in COS cells and human embryonic kidney cell line 293 (Simonds *et al.*, 1989; Wong *et al.*, 1991). However, increase in the beta subunit expression was observed in the membrane of NIH 3T3 cells and Chinese hamster ovary (CHO) cells stably expressing G_i2 (Osawa *et al.*, 1990; Hermouet *et al.*, 1991). Our results may reflect the same phenomena observed with G_i2 overexpression, that is, changes in the expression of G protein are observed only in the cells expressing the G proteins stably. Thus we need further analysis of the expression of G proteins in the cells stably expressing various forms of $G_{s\alpha}$ and we also need to analyze time course of changes in G protein expression to understand how the cell signaling pathways adapt to their new environments.

Since there was no significant change in the expression of other G proteins, we analyzed the cAMP response of the cells expressing various forms of $G_{s\alpha}$. The basal cAMP level of COS-1 cells transfected with the constitutively activated R201E $G_{s\alpha}$ was expected to increase higher than the cells transfected with wild type and dominant negative mutant of $G_{s\alpha}$, but the extent of the increase was comparable without regard to the forms of $G_{s\alpha}$ transfected. This results might come from the fact that only a fraction of cells expresses the protein by transient transfection, and thus the endogenous $G_{s\alpha}$ might have blurred the cAMP response by the transfected $G_{s\alpha}$.

When the transfected cells were stimulated with isoproterenol, the cells produced comparable amount of cAMP regardless of the $G_{s\alpha}$ types transfected. The cAMP response to isoproterenol was expected to blunted in the COS-1 cells transfected with dominant negative mutant G227A $G_{s\alpha}$, but no significant difference was observed. This means that the signaling pathway from beat-adrenergic receptor to adenylate cyclase has not

been changed by transient expression of various forms of $G_{s\alpha}$.

The cAMP production decreased in the cells transfected with wild type $G_{s\alpha}$ followed by treatment with forskolin compared to the control, but such decreased response was not observed in the cells transfected with other forms of $G_{s\alpha}$, either activated or negative mutant. Thus the significance of this change remains to be cleared in the further study.

From this experiment, we found that the signaling pathway producing cAMP does not change significantly by transiently expressing wild type, constitutively activated type, and dominant negative type of $G_{s\alpha}$. Analysis of the effects of long-term expression of $G_{s\alpha}$ would contribute to better understanding on how the $G_{s\alpha}$ signaling system interacts with other signaling pathways and how it adapts to the changed environments.

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