EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 30, No 4, 235-239, December 1998

Retainment of membrane binding capacity of non-palmitoylated Gsα mutants expressed in COS-1 cells

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Accepted 16 October 1998

Abbreviations: G protein, guanine nucleotide-binding regulatory protein; Gi, adenylyl cyclase inhibitory G protein; Gs, adenylyl cyclase stimulatory G protein; Gsα, α subunit of Gs; Gα, α subunit of G protein ; Gβγ, βγ subunit of G protein

Abstract

Heterotrimeric guanine nucleotide binding regulatory proteins (G proteins) transduce extracellular signals into intracellular signals by coupling receptors and effectors. Because most of the G protein-coupled receptors are integral proteins, the G proteins need to have a membrane binding capacity to receive signals from the receptors. The α subunit of G protein binds tightly to the cytoplasmic face of the plasma membrane without any membrane spanning domain. Fatty acylation of Gα with myristic acid or palmitic acid, in addition to the βγ subunits, plays an important role in anchoring the Gα subunit. The reversible and dynamic palmitoylation of the alpha subunit of stimulatory G protein (Gsα) has been suggested as essential for its membrane attachment. However, in our previous experiments, Gsα deleted in the amino terminus containing palmitoylation site, retained its binding capacity when expressed in COS cells. Thus, to evaluate the role of palmitoylation in Gsα membrane binding, we constructed and expressed non-palmitoylated mutants of Gsα and analyzed their subcellular distributions in COS-1 cells. We found that non-palmitoylated mutants of Gsα, C3S- and G2A/C3S Gsα, retained their membrane binding capacities in COS-1 cells, demonstrating that palmitoylation is not essential for membrane binding of Gsα in COS-1 cells. We also found that the palmitoylation did not change significantly the distribution of Gsα in Triton X-114 partition. These results suggest that the palmitoylation of Gsα may produce different effects on membrane binding depending on cell types.

Keywords: G proteins, membrane binding, palmitoylation, site-directed mutagenesis, transfection, immunoblot, Triton X-114

Introduction

Heterotrimeric guanine nucleotide binding regulatory proteins (G proteins) transduce extracellular signals such as hormones, neurotransmitters and photons into intracellular signals by receptor-effector coupling. When the G protein binds to the ligand-activated receptor, it replaces the bound GDP with GTP. The GTP-bound activated G protein regulates effectors such as enzymes and ion channels (Gilman, 1987; Kaziro *et al.*, 1991; Neer, 1995). Because most of the receptors coupled to G proteins are integral proteins that have seven hydro-phobic transmembrane domains, the G proteins need to have a membrane binding capacity to receive signals from the membrane receptors. The α subunit of G protein is known to bind tightly to the cytoplasmic face of the plasma membrane without any membrane spanning hydrophobic domain. The βγ subunit complex has a high affinity for the membrane because of isoprenylation of the gamma subunit, and plays an important role in anchoring the α subunit of G proteins (Sternweis, 1986). However, fatty acylation with myristic acid and palmitoylation was found to be important for membrane binding by α subunits of the G protein family (Jones *et al.*, 1990, Mumby, 1997). The reversible and dynamic palmitoylation had been claimed to be critical in membrane binding by the alpha subunit of the stimulatory G protein (Gsα) (Wedegaertner *et al.*, 1993; Wedegaertner and Bourne, 1994), but it was also argued that this domain of Gsα was not essential for membrane binding from the finding that Gsα mutants deleted in this domain retained membrane binding capacity (Degtyarev *et al.*, 1993; Juhnn, 1993). Thus it is necessary to make clear the importance of this modification in the binding of Gsα.

The present study was performed to evaluate the role of palmitoylation in membrane binding of Gsα. We constructed Gsα mutants by replacing the glycine-2, the cognate site of myristoylation in Giα with alanine, and cysteine-3, the site of palmitoylation with serine, and then analyzed the intracellular distribution of the mutant proteins expressed in COS-1 cells by quantitative

western blotting.

Materials and Methods

Construction of mutant Gsα cDNAs

The cDNA coding for rat Gsα-1, the 47 kDa Gsα containing 394 residues, was kindly provided by Dr. R. R. Reed (Johns Hopkins University), and was cloned into the pCD-PS vector, a eukaryotic expression vector that contains simian virus 40-derived DNA sequences(Bray *et al.*, 1986). The glycine-2 residue at the amino terminus was replaced with alanine in the construct of G2A Gsα, and the cysteine-3 was replaced with serine in C3S Gsα construct, respectively. The combination of the two mutations was made in the G2A/C3S Gsα construct. The mutants were constructed using the pAlter mutagenesis system according to the manufacturer's protocol (Promega Co., Madison, WI, U.S.A.). In brief, a single stranded wild type Gsα DNA in pAlter-1 vector was prepared and annealed with a mutagenic oligonucleotide, together with the second mutagenic oligonucleotide that restored ampicillin resistance to the mutant strand. The mutant strand was synthesized, ligated, and then transformed into an repair minus strain of *E. coli* (BMH 71-18 mutS). A second transformation into JM109 was carried out to segregate the mutant and wild type plasmid. Mutant plasmids were screened and confirmed by sequence analysis using the dideoxynucleotide chain termination method.

Expression of mutant forms of Gsα in COS-I cells and preparation of the subcellular fractions

Transformed monkey kidney cells, COS-1, were transfected with the wild type and mutant forms of Gsα cDNA by the DEAE-dextran method (Aruffo, 1991), and cells transfected with reagents without DNA or the vector DNA without insert, were used as controls.

The transfected COS-1 cells were harvested after 48 hours. The cells were spun down and resuspended in 10 volumes of an homogenization buffer composed of 20 mM sodium phosphate, pH 7.4, 1 mM phenylmethyl sulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM dithiothreitol. The cells were homogenized by passage through a 25 gauge needle 15 times. The cell lysate was centrifuged at 100,000 *g* for 60 min in a table top ultracentrifuge (Beckman Instrument Inc. CA, U.S.A.). The resulting supernatant was transferred to a fresh tube, the pellet was suspended in the original volume of buffer, and then both fractions were centrifuged again. The washed pellet was suspended in 10 volumes of the homogenization buffer and designated as the particulate (P) fraction, and the cleared supernatant was designated as the soluble (S) fraction (Juhnn *et al.*, 1992). The transfected COS-1 cells were also treated with 10 μM isoproterenol for 10 min before harvest and fractionation.

The protein content of the preparation was determined by the Lowry method with bovine serum albumin as the reference standard (Lowry *et al.*, 1951).

Labeling Gsα with [^{3}H]palmitate

COS-1 cells transfected with various forms of Gsα were serum starved for 14 h, incubated in DMEM with 5% fetal bovine serum for 30 min, and then incubated further in the same medium containing 100 μCi/ml [^{3}H]palmitate (49 Ci/mmol, DuPont NEN) for 2 h at 37˚C (Kostenis *et al.*, 1997). The cells were washed with phosphate buffered saline and harvested using a cell scraper in the homo-genization buffer. After cells were homogenized, sixty micrograms of the proteins were separated on 12.5% SDS-PAGE. The gel was dried for 1 h, and then exposed to the exposure cassette of an image analyzer (BAS 1000, Fuji, Japan) for 3 days.

Fractionation of COS-1 cells with Triton X-114

COS-1 cells were harvested and resuspended in the homogenization buffer containing 2% triton X-114. The suspension was incubated for 60 min on a rotator at 4˚C, and spun down at 10,000 *g* for 10 min at 4˚C. After transfer of the resulting supernatant to a fresh microfuge tube, the solution was incubated for 1 minute in a water bath at 30˚C, and then spun down at 10,000 *g* for 1 min at room temperature. The upper aqueous phase was transferred to a fresh microfuge tube. Each fraction was treated with 2% Triton X-114 repeatedly. The volumes of both the aqueous and detergent phases were finally adjusted to 500 μl with the homogenization buffer or the buffer containing 2% Triton X-114. The distribution of Gsα in both the phases was analyzed by SDS-PAGE and immunoblot.

SDS-polyacrylamide gel electrophoresis and immunoblot

The subcellular distribution of Gsα was analyzed by electrophoresis on a 12.5% sodium dodecyl sulfate polyacrylamide gel. Proteins on the gel were electrotransferred onto a piece of nitrocellulose paper, and the blot was blocked in 5% non-fat milk. The blot was incubated with RM antibody that was generated against carboxyl decapeptide of Gsα (Simonds *et al.*, 1989), and then with peroxidase-labeled goat anti-rabbit antibody (Pierce, IL, U.S.A.). The Gsα was visualized by exposing an X-ray film to the blot after incubation in the substrate mixture from an enhanced chemiluminescence kit (Amersham International plc, England). The density of visualized bands corresponding to the wild type and mutant Gsα was quantified with an image analyzer (BioRad, Model GS-700, USA), and the density was compared with those of standard samples applied on the same gel.

Results

Palmitoylation of the mutant Gsα expressed in COS-1 cells

The endogenous forms of Gsα exhibited two groups of bands on western blots when the blot was visualized with an antibody against Gsα, RM (Juhnn *et al.*, 1992). The larger slow moving immunoreactivity increased in the cells transfected with wild type or mutant forms of Gsα cDNA demonstrating respective overexpression in the COS-1 cells (Figure 1a). Increase of [^{3}H]palmitate incorporation into Gsα was observed in the cells transfected with the wild type and G2A mutant Gsα, while such an increase was not demonstrated in cells transfected with the C3S- and G2A/C3S mutant Gsα (Figure 1b). These results confirmed that the cysteine at position 3 of the Gsα amino terminus was the site for palmitoylation, and that palmitoylation of the cysteine did not need the glycine residue next to the cysteine (Linder *et al.*, 1993).

Subcellular distribution of the mutant Gsα expressed in COS-1 cells

When COS-1 cells were fractionated, most of the immunoreactivity was localized in the particulate fraction, and less than 10% of the Gsα was detected in the cytosol fraction. The relative distribution of Gsα in COS-1 cells did not change by transfection with the pCD-PS vector (data not shown). When wild type and mutant Gsαs were overexpressed, the corresponding slowly migrating band became denser in both particulate and soluble fractions than that of the controls. However, the relative distribution to the soluble fraction of the wild type and mutant forms of Gsα did not increase significantly from the control in the COS-1 cells (Figure 2a, b).

To analyze the effect of activation of Gsα proteins on their subcellular distribution, cells were treated with isoproterenol. Treatment with isoproterenol increased the Gsα in the soluble fraction of the COS-1 cells transfected with Gsα. About 20% of overexpressed wild type and C3S Gsα were found in the soluble fraction, and about 30% of G2A- and G2A/C3S Gsα were in the soluble fraction. However, there were no significant differences in the Gsα in the soluble fractions between plamitoylated and non-palmitoylated Gsα after treatment with isoproterenol(Figure 3a, b).

Fractionation of the mutant Gsα expressed in

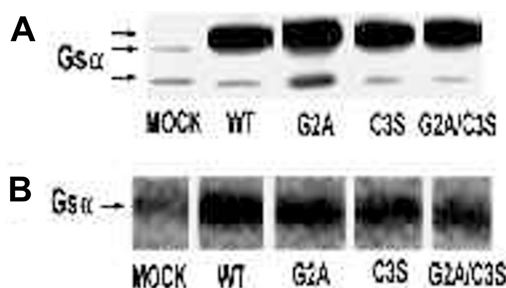

Figure 1. Expression of wild type and mutant forms of Gsα in COS-1 cells. The COS-1 cells were transfected with the reagents only (MOCK), wild type Gsα (WT), Gly-2-Ala Gsα (G2A), Cys-3-Ser Gsα (C3S), and Gly-2-Ala/Cys-3-Ser Gsα (G2A/C3S) by the DEAE-dextran method. **A** About one-third of the homogenate prepared from the cells cultured in a 100 mm dish was separated by 12.5 % SDS-PAGE and transferred onto nitrocellulose paper. The blot was incubated with RM antibody directed against Gsα, and then with peroxidase labeled goat anti-rabbit IgG antibody. The Gsα was visualized by incubating the blot with the substrate mixture, and then exposing it on X-ray film. Arrows indicate Gsα bands. **B** The transfected COS-1 cells were labeled with [^{3}H]palmitate, and then 60 μg of the cell lysate were separated by 12.5 % SDS-PAGE, the gel was dried for 1 hour, and then exposed on the exposure cassette of an image analyzer for 3 days.

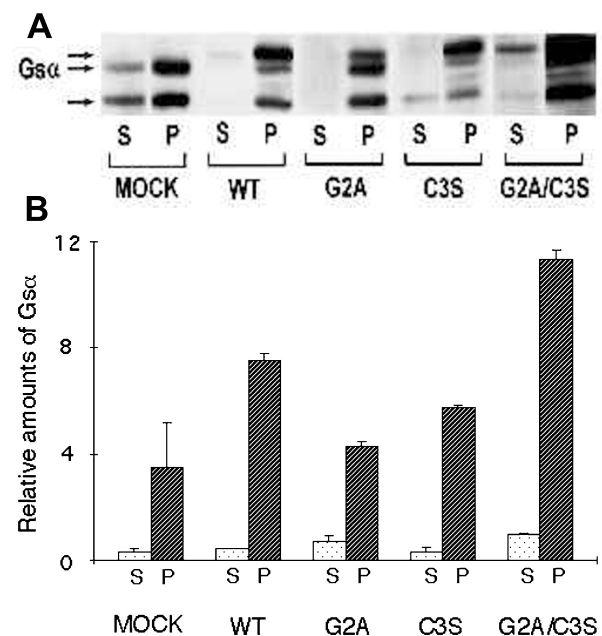

Figure 2. Subcellular localization of wild type and mutant forms of Gsα in COS-1 cells. The COS-1 cells were transfected with the reagents only (MOCK), wild type Gsα (WT), Gly-2-Cys Gsα (G2A), Cys-3-Ser Gsα (C3S), and Gly-2-Cys/Cys-3-Ser Gsα (G2A/C3S) by the DEAE-dextran method. The particulate fraction (P) and the soluble fraction (S) were prepared from COS-1 cells by centrifuging the cell lysate at 100,000 *g* for 60 minutes followed by washing with an homogenization buffer. Each fraction was analyzed by SDS-PAGE and immunoblot using an anti-Gsα antibody. Also see legend for Figure 1. **A** Representative immunoblot of subcellular fractions from COS-1 cells transfected with various forms of Gsα. **B** Subcellular distribution of various forms of Gsα analyzed by densitometry of immunoblots. Dotted bars represent the soluble fraction and hatched bar the particulate fraction. The results are average values of two separate experiments. Transfection was performed in duplicate or triplicate for each experiment.

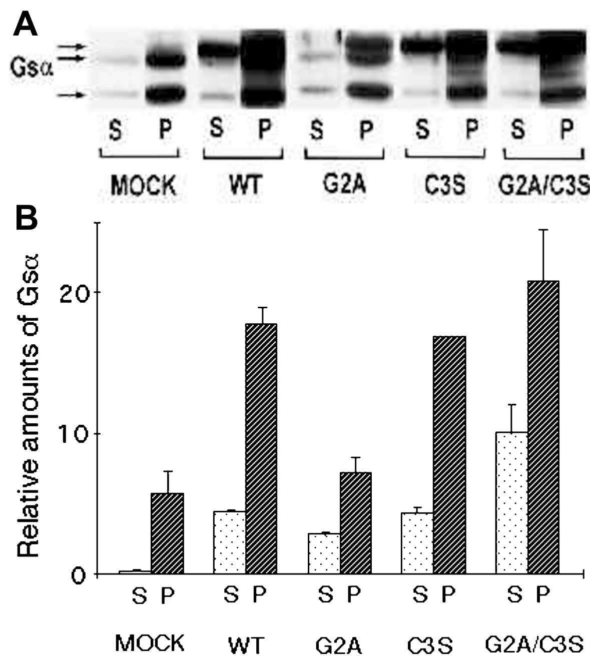

Figure 3. Effects of isoproterenol treatment on the subcellular localization of wild type and mutant forms of Gsα in COS-1 cells.

A Representative immunoblot of subcellular fractions from COS-1 cell after treatment with isoproterenol. The transfected COS-1 cells were treated with 10 μM isoproterenol for 10 minutes before harvest. **B** Subcellular distribution of various forms of Gsα after treatment with isoproterenol analyzed by densitometry of immunoblots. Dotted bars represent the soluble fraction and hatched bar the particulate fraction. See the legends for Figure 1 and 2.

COS-1 cells with Triton X-114

When COS-1 cells were fractionated with Triton X-114, most of the endogenous Gsα was extracted in the aqueous phase. Major portions of the overexpressed wild type Gsα and more than half of C3S Gsα also partitioned in the aqueous phase, in spite of occasional shift of both the endogenous and transfected Gsα in the detergent phase. (Figure 4).

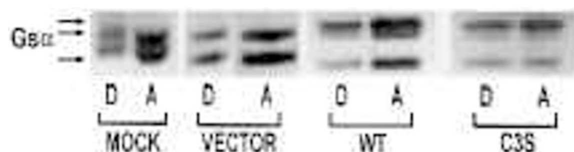

Figure 4. Partition to aqueous phase of Triton X-114 extract of endogenous and G2A/C3S Gsα from COS-1 cells. COS-1 cells transfected with vector, wild type Gsα , and G2A/C3S Gsα were extracted with 2% Triton X-114, and the resulting detergent(D) and aqueous(A) fractions were analyzed by SDS-PAGE and immunoblot. Also see legend for Figure 1.

Discussion

In this experiment, we confirmed that most Gsα retained membrane binding capacity without modification by palmitoylation in COS-1 cells. These results are consistent with our previous findings that no single region of Gsα, including the amino terminal region, was solely responsible for membrane binding in COS-1 cells (Juhnn *et al.*, 1992, 1993). However, our results do not support the model of activation coupled depalmitoylation with the release of Gsα to cytosol (Wedegaertner *et al.*, 1993; Wedegaertner and Bourne, 1994). They proposed that regulated palmitoylation cycles provide a potential general mechanism for controlling cellular localization and activity of many proteins including Gsα.

The reason for these differences is not clear, but it might be due to the different cell lines used in the study. Wedegaertner *et al*. (1993) used HEK293 cells and S49 murine lymphoma cells and reported most of non-palmitoylated Gsα distributed in the cytosol. In contrast, we used COS-1 cells and found that the non-palmitoylated Gsα stayed in the particulate fraction, in agreement with the results of Degtyarev *et al.* (1993) who reported similar findings using COS-7 cells. Thus, it is likely that the role of palmitoylation in the membrane binding of Gsα may differ depending upon cell types. This means that palmitoylation can regulate membrane attachment dynamically in some cells, but in other cells palmitoylation alone is not enough for the dynamic regulation of membrane attachment of Gsα. It is also noteworthy that some investigators have not observed the activation-induced release of G proteins (Mumby, 1997), thus necessitating more study before the activation-plamitoylation hypothesis is accepted as a general regulatory mechanism for G proteins.

Because hydrophobicity is one of the major contributing factors that regulate the membrane binding of a molecule in cells, we also analyzed the effects of Gsα palmitoylation on its membrane binding affinity by fractionation with Triton X-114 to complement the subcellular fractionation study using centrifugal separation. Because Triton X-114 extract remains single phase at 4˚C but separates into aqueous and detergent phase at 30˚C, extraction with the detergent is very useful to fractionate cellular components based on hydrophobicity of molecules (Pryde, 1986). However, our results showed that both wild type and the non-palmitoylated form of Gsα partitioned in the aqueous phase of Triton X-114 fractionate, demonstrating that palmitoylation on Gsα was not sufficient solely to determine the partition. This partition pattern of Gsα is different from the report of Kleuss and Gilman (1997), who described partition of purified liver Gsα into the detergent phase and non-myristoylated Gsα in the aqueous phase. This difference might result from the difference of Gsα preparation. Because they used purified Gsα, the partition of Gsα in their system could differ from partition in the

whole cell extract that was analyzed in the present experiment. The results suggest that palmitoylation of Gsα does not increase its hydrophobicity enough to make it partition from the aqueous phase to the detergent phase in the Triton X-114 extract of COS-1 cells. This could be associated with the finding that non-palmitoylated Gsα retained its membrane binding capacity, and these two results may imply that palmitoylation alone is not enough to determine the cellular localization of Gsα in a COS-1 cell.

In this experiment, we found that Gsα retained its membrane binding capacity in COS-1 cells without modification by palmitoylation. Thus, it is suggest that the palmitoylation of Gsα may produce different effects on membrane binding depending upon the cell type. Since the binding of G proteins to the membrane seems to be regulated by multiple factors such as βγ subunit complex, fatty acylation, and interactions with other proteins (Wedegaertner *et al.*, 1995), these factors must also be considered when evaluating the effect of Gsα palmitoyl-ation on membrane binding.

Acknowledgment

This research was supported by a grant from the Basic Medical Research Fund of the Ministry of Education, 1994.

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