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Palmitoylation regulates GDP/GTP exchange of G protein by affecting the GTP-binding activity of Goα

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Abstract

The effect of palmitoylation on the GTP-binding activity and conformation of Goα protein in hydrophobic and hydrophilic environments was studied. The binding assay was performed with an isotope labeled analog of GTP, GTP-γ-^{35}S, and its fluorescent analog, BODIPY FL-GTPγS was used to detect conformational change in the GTP-binding domain of Goα. Investigation of the GTP-γ-^{35}S binding activity of Goα shows that in a hydrophobic environment, mimicked by the presence of detergent, the apparent dissociation constant for palmitoylated Goα ($K_D = 25.5 \times 10^{-9} \pm 1.7 \times 10^{-9}$ M) increased threefold compared with that of non-palmitoylated Goα ($K_D = 9.9 \times 10^{-9} \pm 0.8 \times 10^{-9}$ M), while in an aqueous environment without detergent there is no significant difference between palmitoylated ($K_D = 50.1 \times 10^{-9} \pm 5.2 \times 10^{-9}$ M) and non-palmitoylated ($K_D = 65.5 \times 10^{-9} \pm 7.6 \times 10^{-9}$ M) Go(. This indicates that in a membrane environment palmitoylation may weaken the GTPγS binding ability of Go(. Fluorescent quenching studies using BODIPY FL-GTPγS as a probe showed that the conformation of the GTP-binding domain of Go(tends to become more compact after palmitoylation. These results imply that palmitoylation may regulate the GTP/GDP exchange of Goα by influencing the GTP-binding activity of Goα and facilitating the on–off switch function of the G protein in G protein-coupled signal transduction.

Keywords: G protein; Palmitoylation; GTP-binding activity; Conformation change; Signal transduction

1. Introduction

G proteins are located on the cell membrane in the form of heterotrimers and perform a pivotal role in signal transmembrane transduction (Gilman, 1987; Neer, 1995). G protein heterotrimers consist of α, β and γ subunits. The function of G proteins in signal transduction depends on the guanine nucleotide (GTP or

Abbreviations: GTPγS, guanosine-5′-O-(3-thiotriphosphate); HB, hypocrellin B; GPCR, G-protein-coupled receptor

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GDP) bound state and GTP/GDP exchange triggered by activated receptors. G protein-coupled signal transduction is under the regulation of various factors, such as the regulator of G-protein signaling (RGS) protein, phosphorylation and lipid modification.

Lipidation, such as myristoylation, palmitoylation and prenylation, is an important covalent modification of G proteins. Palmitoylation represents the covalent linkage between the palmitoyl group (C16:0) and the cystine residue of the protein. The palmitoylation of the Goα subunit usually occurs at a cystine residue located at the 3rd position from the N-terminus of the protein. It was recently reported that the N-terminal glycine of Gsα can also be palmitoylated (Kleuss & Krause, 2003). Both palmitoylation and de-palmitoylation are thought to be enzyme-catalyzed processes. Unlike myristoylation and prenylation, palmitoylation is a reversible covalent modification, which places Goα in a dynamic palmitoylation-depalmitoylation cycle (Wedegaertner, Wilson, & Bourne, 1995). It has been reported that the palmitoylation-depalmitoylation cycle is prompted by the activation of the G protein by (G protein-coupled receptors (Stanislaus, Ponder, Ji & Conn, 1998; Wedegaertner & Bourne, 1994). Therefore, palmitoylation is speculated to be an important means of regulation for G protein-coupled signal transduction. To date there exist different viewpoints concerning the regulatory role of palmitoylation in G protein-coupled signaling. Wedegaertner et al. reported that palmitoylation may affect the distribution of Gα between the membrane and the cytoplasm (Wedegaertner & Bourne, 1994; Wedegaertner, Chu, Wilson, Levis & Bourne, 1993). Iiri and co-workers proposed that palmitoylation may regulate the interaction between the Gα subunit, β/γ subunits and RGS (Iiri, Backlund, Jones, Wedegaertner, & Bourne, 1996; Tu, Wang, & Ross, 1997). Song and co-workers showed that palmitoylation may cause Gα locate on different microdomains in the membrane (Mumby, 1997; Song, Sargiacomo, Galbiati, Parenti, & Lisanti, 1997). Studies on Goα and other members of the Gi family show that the loss of potential of the α subunit to be palmitoylated can cause mislocalization on the plasma membrane (Huang, Duncan, Gilman, & Mumby, 1999; Morales, Fishburn, Wilson, & Bourne, 1998). Additionally, palmitoylation may facilitate location of the G protein into specialized membrane domains including caveolae and lipid rafts, which are rich in sphingolipids and cholesterol (Moffett, Brown, & Linder, 2000; Song et al., 1997). In spite of the viewpoints and supporting evidence mentioned above, the detailed mechanism of how the dynamic change of the palmitoylation state of Gα regulates its function remains unclear. Furthermore, the membrane-anchorage theory has been challenged by reports that the palmitoylation-disabled mutant Gαz can still properly locate in the membrane and in its non-palmitoylated state, it can still bind with the membrane through its myristoylation modification and interaction with β/γ subunits (Fishburn, Herzmark, Morales, & Bourne, 1999; Fishburn, Pollitt, & Bourne, 2000). These conflicting experimental results indicate that it is difficult to demonstrate the regulating function of palmitoylation for G proteins merely on the basis of the interaction between Gα subunits and the plasma membrane. Given that the critical switch function of the G protein in signal transduction is performed by its guanine nucleotide binding ability and GTP/GDP exchange, the aim of this study is to understand the effect of palmitoylation on the GTP-binding activity and conformation of Goα and to discuss palmitoylation as a regulation mechanism of Goα GTP/GDP exchange.

2. Materials and methods

2.1. Materials

Guanosine 5′-3-O-(thio)triphosphate (GTPγS) was purchased from Roche Molecular Biochemicals (Mannheim, Germany); Phenyl Sepharose High Performance, DEAE Sephacel and Q Sepharose High Performance were from Amersham Pharmacia (Uppsala, Sweden); palmitoyl-coenzyme A and acrylamide were from Sigma (St. Louis, USA); [^{3}H]-palmitoyl-coenzyme A was synthesized from palmitate and coenzyme A; [^{35}S]-GTPγS and [^{3}H]-palmitate were from Perkin–Elmer (Boston, USA); BODIPY FL-GTPγS was purchased from Molecular Probes (Eugene, USA); the expression plasmid pQE60-Goα was a gift from Professor Susanne Mumby (University of Texas, Southwestern Medical Center); the *N*-myristoyltransferase expression plasmid pBB131-NMT was a gift from Professor Gordon (University of Washington); Hypocrellin B (HB) was generously provided by Professor Jiachang Yue (Institute of Biophysics, Chinese Academy of Science,

Beijing). All other chemicals were of analytical grade.

2.2. Preparation of myristoylated Goα

Myristoylated Goα was prepared by the method of Zhang and Huang (2003) with a few modifications. *Escherichia coli* strain JM109 was cotransformed with pQE60-Goα and pBB131-NMT and grown in T_7 enriched medium (2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol and 50 mM KH_2PO_4, pH 7.2, supplemented with 50 μg/ml kanamycin and 50 μg/ml ampicillin). Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 60 μM when the $O.D._{600}$ reached 0.4–0.6 and the cells were grown at 30 °C overnight. The cells were harvested and lysed by freezing–thawing with liquid nitrogen and digested with lysozyme. The lysate was centrifuged at 30,000 × *g* for 1 h. The supernatant was applied to a DEAE Sephacel column and eluted with 300 mM NaCl. The DEAE elute was adjusted to 1.2 M $(NH_4)_2SO_4$ and 25 μM GDP, and then applied to Phenyl Sepharose and Q Sepharose columns for further purification. The GTPγS-binding activity of the purified Goα was determined and the purity evaluated by SDS–PAGE stained with Coomassie blue R-250.

2.3. In vitro palmitoylation of Goα

Goα was palmitoylated in vitro according to the method of Duncan & Gilman, 1996) with modifications. The palmitoylation reaction was conducted in buffer A (20 mM Hepes, pH 8.0, 1 mM EDTA, 2 mM $MgCl_2$, 0.015% Lubrol PX), Goα was added to a final concentration of 3 μM, then palmitoyl-CoA was added in a final concentration of 30 μM to trigger the reaction. The reaction mixture was incubated at 20 °C for 1 h. The efficiency of palmitoylation was measured as described by Yang and Huang (2001).

2.4. Assay for apparent dissociation constant (K_D) of Goα with GTPγS

The GTPγS binding activities of Goα in different concentrations of [^{35}S] GTPγS were determined and the data obtained were used to calculate the K_D value. The [^{35}S] GTPγS binding assay was performed according to Northup's method (Northup, Smigel, Sternwise & Gilman, 1983).

2.5. Fluorescent quenching assay

Fluorescence measurements were performed on a Hitachi 4010 spectrofluorometer at 20 °C with λ_{ex} of 470 nm and λ_{em} of 510 nm. The fluorescent assay using BODIPY FL-GTPγS as a probe was conducted according to McEwen's method with modifications (McEwen, Gee, Kang & Neubig, 2001McEwen, Gee, Kang & Neubig, 2001). Specifically, fluorescence measurements were carried out in a binding buffer (50 mM Hepes, pH 8.0, 1 mM EDTA, 20 mM $MgCl_2$). Non-palmitoylated or palmitoylated Goα as described above was diluted to 500 nM for use, and BODIPY FL-GTPγS was added to a final concentration of 50 nM. After stabilization of the fluorescence resulting from the association of Goα with BODIPY FL-GTPγS, the fluorescence quencher was added in aliquots of certain concentrations and the change in fluorescence was measured.

2.6. Data analysis

To analyze the binding activity data, the receptor–ligand bimolecule association equation in its original form (without simplification) was used (Receptor–ligand interactions, 1992):

$$[\text{bound ligand}] = \frac{(L_{\text{total}} + R_{\text{total}} + K_D) - [(L_{\text{total}} + R_{\text{total}} + K_D)^2 - 4 \times R_{\text{total}} \times L_{\text{total}}]^{0.5}}{2}$$

where L_{total} and R_{total} refer to the concentrations of GTPγS and the maximal bound Goα present in the assay, respectively. It should be noted that we used the original form of the bimolecule association equation for data analysis. In this case, the logarithm curve ([bound ligand] versus [total ligand]) is not meaningful, as this type of plot is only appropriate when the simplified form of the bimolecule association applies.

The classical Stern–Volmer equation was used in the analysis of the quenching data (Eftink & Ghiron, 1981):

$$F_0/F = 1 + K_{sv} \times Q$$

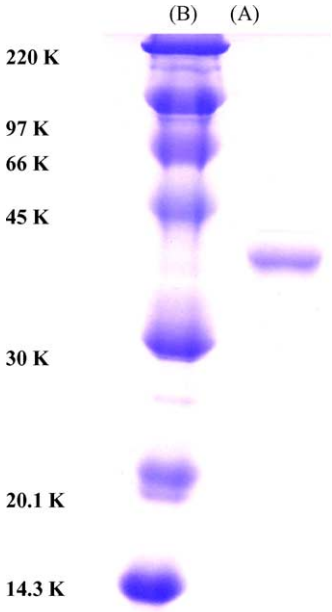

Fig. 1. Purification of the Goα expressed in *E. coli*. Two micrograms of the purified Goα was subjected to SDS–PAGE (13.5%) and loaded onto lane (A). Lane (B) is molecular weight standards. The gel was stained with Coomassie blue R-250.

where Q refers to concentration of the quencher, while F_0 and F are fluorescence intensity in the absence and presence of the quencher, respectively.

3. Results

3.1. Expression and purification of myristoylated Goα

Myristoylated Goa was expressed efficiently in *E. coli* JM109 and the purity of the Goα (shown in Fig. 1) was above 85%. The [^{35}S]-GTPγS binding assay indicated that the binding activity of the purified Goα was about 20 nmol/mg (the maximal binding activity in theory is 24.4 nmol/mg), which would mean above 80% of the purified Goα remained higher binding activity.

3.2. In vitro palmitoylation of Goα

An in vitro [^{3}H]-Palmitoyl-CoA binding assay showed a palmitoylation efficiency above 70%. The concentration of the Goα in the palmitoylation reaction was found to affect the efficiency and repeated experiments indicated that the palmitoylation efficiency decreased to 10% when the concentration of the Goα was below 1 μM (data not shown).

3.3. Effects of palmitoylation on the binding activity of Goα with GTPγS

Assay of the [^{35}S]-GTPγS binding activity of palmitoylated and non-palmitoylated Goα was performed by measuring the apparent dissociation constant (K_D) of Goα with [^{35}S]-GTP(S in the presence or absence of 0.05% Lubrol PX, which was used to mimic a hydrophobic membranes environment. The results indicated that the apparent dissociation constant (K_D) of Go α with GTP(S under hydrophobic conditions increased from $9.9 \times 10^{-9} \pm 1.1 \times 10^{-9}$ M for non-palmitoylated Goα to $25.5 \times 10^{-9} \pm 2.5 \times 10^{-9}$ M for palmitoylated Goα. In a control assay system without detergent there was no significant difference in the K_D values ($P > 0.05$ in t-test, $n = 3$) between the non-palmitoylated Goα ($65.5 \times 10^{-9} \pm 7.0 \times 10^{-9}$ M) and palmitoylated Goα ($50.1 \times 10^{-9} \pm 14.2 \times 10^{-9}$ M). Each K_D value shown above is the average result of three to five experiments. Table 1 shows a group of representative results of the [^{35}S] GTP(S binding assay of non-palmitoylated and palmitoylated Goα from three parallel experiments and a clear difference between the effects of palmitoylation on GTP(S binding activity in a hydrophobic and in a hydrophilic reaction system can be observed.

3.4. Fluorescent change of BODIPY FL-GTPγS in the presence or absence of Goα

BODIPY FL-GTPγS contains a fluorescent group, the BODIPY fluorophore, and a GTP analog group, GTP(S, and is used as a specific probe for a guanine nucleotide binding (McEwen et al., 2001). In the absence of a binding protein, the fluorescence of the probe is very weak due to quenching of the guanine base on BODIPY. The fluorescence of BODIPY FL-GTPγS increases substantially upon the addition of Goα, which results from the specific association of GTPγS with Goα and the movement of the guanine base away from the BODIPY group (McEwen et al., 2001). As shown in Fig. 2, when the concentration of Goα exceeds that of the probes by fivefold, the maximal fluorescence

Table 1
Data obtained in the GTPγS-binding assay for apparent dissociation constant (K_D) of Goα

Total [GTPγS] (nM)	1.25	5	12.5	25	50	100	150	250	1000	5000
BoundGTPγS (nM)										
1	1.09	4.30	10.84	21.91	46.86	91.91	120.02	178.15	190.76	185.21
2	1.09	4.49	10.55	22.86	41.84	67.53	74.50	94.10	105.10	111.65
3	0.84	3.33	7.81	13.90	27.99	52.71	73.40	76.09	113.98	100.22
4	0.99	3.59	8.11	16.40	26.80	36.30	45.41	49.00	63.80	67.80

The GTPγS binding assay was performed as described in Section 2. Assays for groups 1 and 2 in the Table were performed in the binding buffer (50 mM Hepes, pH 8.0, 1 mM EDTA, 20 mM $MgCl_2$, 200 nM Goα) with 0.05% Lubrol PX, while assays for groups 3 and 4 were performed in the binding buffer without Lubrol PX. Goα used in groups 1 and 3 was in non-palmitoylated form, while Goα used in group 2 and 4 was palmitoylated. The data set shown in any group is a representative result from three parallel experiments.

change is three to fivefold higher than the baseline. After the addition of Goα, it takes about 10 minutes for the fluorescence to reach a maximum, after which it remains stable in subsequent quenching experiments (for further about 10 min). The results in Fig. 3 showed that the fluorescence increase when BODIPY FL-GTPγS was incubated with either non-palmitoylated (Fig. 3A) or palmitoylated Goα (Fig. 3B), although in the latter case it rose more slowly and reached a lower maximal value (Fig. 3B). The addition of Lubrol PX up to 0.1% did not interfere with the fluorescent profile of the probe (data not shown). This indicates that the BODIPY FL-GTPγS is a suitable probe to study conformational changes between palmitoylated and non-palmitoylated Goα.

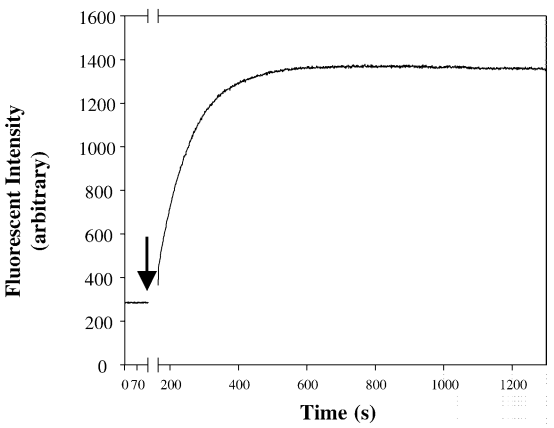

Fig. 2. Fluorescent profile of BODIPY FL-GTPγS. The fluorescence of BODIPY FL-GTPγS was measured as described in Section 2. At the time indicated by the arrow, Goα was added to give a final concentration of 500 nM and the change in fluorescence was monitored.

3.5. Fluorescence quenching of BODIPY FL-GTPγS bound to Goα

Fluorescence quenching is commonly used to investigate the conformational characteristics of a protein. Fig. 4A and B show the fluorescence quenching of BODIPY FL-GTP(S bound to Goα by using acrylamide as a hydrophilic quencher. The results show that in the presence of 0.05% Lubrol PX, the fluorescence of BODIPY FL-GTPγS in the presence of non-palmitoylated Goα was significantly quenched (Fig. 4A), while the fluorescence for the palmitoylated

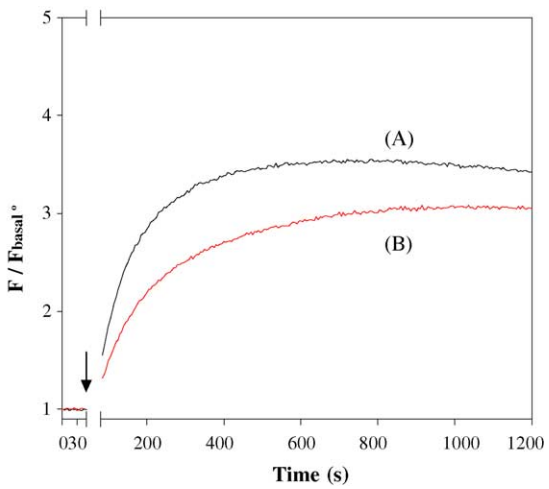

Fig. 3. Association of BODIPY FL-GTPγS with non-palmitoylated and palmitoylated Goα. At the time indicated by the arrow, non-palmitoylated Goα (A) and palmitoylated Goα (B) were added to the same final concentration of 500 nM and the change in fluorescence was monitored. To compensate for different fluorescent baselines (F_{basal}), changes in fluorescence (F) are shown in the form of the ratio of F/F_{basal}.

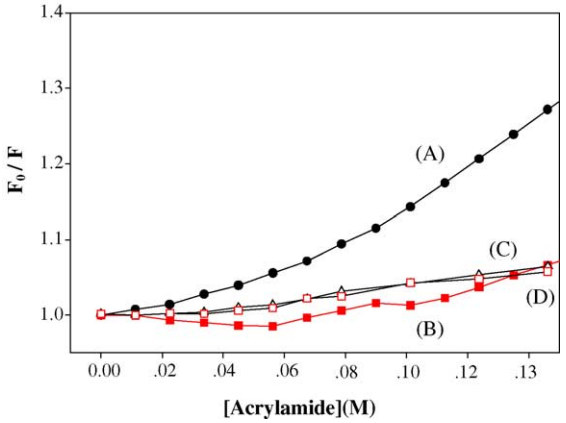

Fig. 4. Fluorescence quenching with acrylamide of BODIPY FL-GTPγS bound to Goα. Solid circles (●) indicate non-palmitoylated Goα (A) in a hydrophobic assay system (with 0.05% Lubrol PX), while solid squares (■) indicate palmitoylated Goα (B) in the same assay system. Open triangles (Δ) indicate non-palmitoylated Goα (C) in a hydrophilic assay system (without the detergent), while open squares (▭) indicate palmitoylated Goα (D) in the same system. The quencher was added after the fluorescence level of BODIPY FL-GTPγS bound to Goα became stable. The curves shown (and in Fig. 5) are representative results from three parallel experiments.

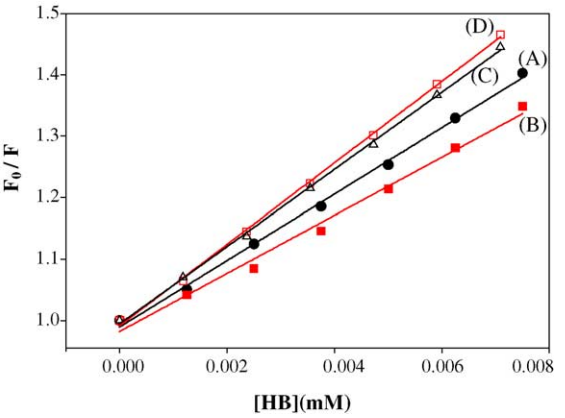

Fig. 5. Fluorescence quenching with HB of BODIPY FL-GTPγS bound to Goα. Solid circles (●) indicate non-palmitoylated Goα (A) in a hydrophobic assay system (with 0.05% Lubrol PX), while solid squares (■) indicate palmitoylated Goα (B) in the same system. Open triangles (Δ) indicate non-palmitoylated Goα (C) in a hydrophilic assay system (without detergent), while open squares (▭) indicate palmitoylated Goα (D) in the same system. The quencher was added after the fluorescence level of BODIPY FL-GTPγS bound to Goα became stable.

Goα remained unchanged during the corresponding experiment (Fig. 4B). A similar difference in the fluorescence quenching was observed when using a hydrophobic quencher, Hypocrellin B. As shown in Fig. 5A and B, although the fluorescence of BODIPY FL-GTPγS in the presence of either non-palmitoylated (Fig. 5A) or palmitoylated Goα (Fig. 5B) was quenched by HB, the quenching effect of HB decreased substantially in the case of palmitoylated Goα. The quenching data were analyzed using the classical Stern–Volmer equation (Eftink & Ghiron, 1981). The results show clearly that in a hydrophobic environment the K_{SV} value of non-palmitoylated Goα ($K_{SV} = 58.47 \pm 4.37$) was increased compared with that of palmitoylated Goα ($K_{SV} = 46.04 \pm 1.16$). In a control system without Lubrol PX, neither acrylamide (Fig. 4C and D) nor HB (Fig. 5C and D) caused a significant difference between the fluorescence quenching of BODIPY FL-GTPγS for non-palmitoylated and palmitoylated Goα. Data analysis of HB quenching showed similar K_{SV} values for non-palmitoylated ($K_{SV} = 62.9 \pm 0.89$) and palmitoylated ($K_{SV} = 66.3 \pm 0.83$) Goα (Fig. 5C and D, respectively).

4. Discussion

The guanine nucleotide binding state (GDP or GTP bound) of Gα plays a key role in turning on and off G protein-coupled signal transduction. GTP bound Gα subunits direct the signals to effectors, and GDP bound Gα subunits tend to bind with βγ subunits and return to their inactive state. Although the effect of G protein palmitoylation on Gα-membrane interaction has been studied by several groups, the literature concerning its influence on guanine nucleotide binding activity of Gα is limited. β/γ subunits function as dominant regulators when they bind to the α subunit. When associated with β/γ, the Gα subunit is stabilized in the GDP-binding form, and thus palmitoylation plays little role in GTP-binding activity of Gα in the presence of β/γ. However, if palmitoylation does have a regulatory function on affinity of Gα for GTP, this function may work only after dissociation of Gα from β/γ. Our interest is to understand the effect of palmitoylation on the GDP/GTP exchange of Gα by modulating GTP-binding activity of Gα. This necessitated the use of purified Gα. On the other hand, because G proteins are peripheral membrane proteins and perform their functions in the membrane, a hydrophobic environment is likely to be

important for their natural conformation and function. Given that the Gα shows poor binding to liposomes, an assay system containing detergent molecules was used in the present experiments to mimic a membrane environment (Micheal Garavito & Shelagh, 2001). The results from the experiments performed by using purified Goα in the presence or absence of lubrol PX clearly showed that palmitoylation influences the GTP-binding activity of Goα. The K_D of the palmitoyated Goα decreased by at least threefold compared with that of the non-palmitoylated Goα. If we take the palmitoylation efficiency of 70% into account, a threefold increase in K_D after palmitoylation indicates that the affinity of the palmitoylated Gα for GTP decreases by at least threefold. Although a threefold change is not very large, it is similar to the changes in the affinity observed for other components of the Go protein-mediated signal pathway. For example, Alves et al. reported that the K_D of Goα with opioid receptor shifts from 20 nM (with liganded receptor) to 10 nM (with unliganded receptor) (Isabel, Zdzislaw, Eva, Henry, Gordon, & Victor, 2003).

There are many reports that activation of Gα by G protein-coupled receptors is accompanied by an increase in the turnover of palmitate on the α subunits (Degtyarev, Spiegel, & Jones, 1993; Mumby, Kleuss & Gilman, 1994), which is thought to be a result of increased susceptibility of activated α subunits to palmitoylthioesterase (Mumby, 1997). Combining these reports with our results, it suggests that the depalmitoylation of Gα triggered by activation of GPCRs facilitates the association of Gα with GTP, and thus it becomes the activated (Gα-GTP) form and further interacts with effectors, and enhances signal transduction. Further support for this viewpoint comes from the co-localization of palmitoyl-acyltransferase (PAT) activity and the effectors of G proteins. In spite of the failure to purify a PAT specific for Gα to date, there is evidence that the Gα-specific PAT activity is enriched in so-called "lipid rafts" and caveolae (Wedegaertner et al., 1993). These are rich in sphingolipids and cholesterol and also have been shown to be rich in effectors (Seno, Kishimoto, Abe, Higuchi, Mieda, Owada, Yoshiyama, Liu, & Hayashi, 2001; Rybin, Xu, Lisanti, & Steinberg, 2000). This co-localization implies a re-palmitoylation mechanism for Gα, which would mean the active Gα (GTP-binding state) could be re-palmitoylated when interacting with effectors and its affinity for GTP thus reduced by its palmitoylation. The re-palmitoylated Gα would then recover its affinity for β/γ subunits and become inactivated (the Gα-GDP form) due to the decrease in binding activity with GTP. Thus, through its effect on guanine nucleotide binding activity, palmitoylation could play an important role in enhancement and attenuation of signal pathways.

Assay of intrinsic fluorescence of proteins is an important means to investigate the conformational changes and mainly reflects the environment of tryptophan residues. When a protein has more than one tryptophan residue, it can be difficult to interpret the data in terms of the regions involved in structural change. To investigate the structural basis of the effect of palmitoylation on the binding activity of Goα, a specific fluorescent probe for Gα, BODIPY FL-GTPγS, was used (McEwen et al., 2001). Because of the specific association of the probe with the GTP-binding domain of Gα, the fluorescent characterization of the probe reflects conformational changes in the binding domain of Gα during palmitoylation. As shown in Figs. 4 and 5, in a hydrophobic system the fluorescence quenching with either hydrophobic (HB) or hydrophilic (acrylamide) quenchers decreased upon palmitoylation of Goα. The results indicate that the GTP-binding domain of palmitoylated Goα was more compact compared to non-palmitoylated Goα, and hence is less accessible to the quenching agents. This conformational change is related to the low affinity of the palmitoylated Goα for GTPγS as measured by the binding assay mentioned above. This is the first report on the relationship between GTP-binding activity changes of palmitoylated Goα and the accompanying conformational changes. In summary, we present evidence for a regulatory role of palmitoylation in GDP/GTP exchange of Gα due to its influence on the GTP-binding activity. Further studies on the mechanism of the regulation of the GTP-binding activity of different G proteins by palmitoylation should shed light on the role of palmitoylation in the G protein-coupled signal transduction pathway.

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