In vitro palmitoylation of native bovine brain G_oa

YANG Shengyu (杨胜于) & HUANG Youguo (黄有国)

National Laboratory of Biomacromolecule, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

Correspondence should be addressed to Huang Youguo (email: huang@sun5.ibp.ac.cn)

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Abstract The native $G_o\alpha$ was purified from bovine brain cortex and palmitoylated *in vitro*. The *in vitro* palmitoylation site was the same as that *in vivo*. The internal palmitoylation of purified native $G_o\alpha$ was found to be largely maintained. The apparent palmitoylation ratio was significantly increased after the $G_o\alpha$ was treated with DTT. The GTPγS binding characteristic of $G_o\alpha$ was not influenced by palmitoylation, however, the affinity for LUVs was increased dramatically. The *in vitro* palmitoylation model of $G_o\alpha$ provides a better basis for studying the functional role of G protein palmitoylation in signal transduction.

Keywords: native G_oa , *in vitro* palmitoylation, specificity, GTPγS binding activity, signal transduction.

Heterotrimeric GTP-binding proteins (G proteins) function as a coupling protein between receptor and effector, so that signals activating receptors can be transduced to effectors and thus initiate series of physiological and biochemical events[1]. Effective signal transduction of G protein depends on its association with the cytoplasmic side of plasma membrane[2,3] and lipid modification may influence its distribution on membranes. Three kinds of covalent lipid modification, i.e. prenylation, myristoylation and palmitoylation have been found, however, only palmitoylation is reversible and is regulated by numerous factors[2,4—6]. The functional roles of G protein palmitoylation have aroused extensive interest. Palmitoylated oligopeptide exhibits higher affinity for large unilamilar liposomes (LUVs)[7], indicating that plamitoylation may regulate the G protein-mediated signal transduction pathway by regulating the distribution of Gα between plasma membrane and cytosole. *In vitro* research showed that palmitoylation influenced the interactions of Gα with GAP (GTPase activating protein)[8] and $\beta\gamma$ subunits. In addition, palmitoylation may regulate the distribution of G protein between the membrane microdomains[2]. It was reported recently that the palmitoylation state of $G_{12}\alpha$ was related to the transformation of normal cell to cancer cell[9], which suggested that G protein palmitoylation was not only an important theoretical issue for signal transduction, but also important for clinical activities.

Research on the palmitoylation regulation of G protein signal transduction is difficult because thioester modification is liable to hydrolysis and DTT or β-mercatpoethanol reduction, and because there are no effective methods to separate palmitoylated protein from depalmitoylated protein[2]. Although several mechanisms have been proposed for the palmitoylation regulation of

G protein signal transduction, the funtional roles of G protein palmitoylation are difficult to progress. With Palmitoyl CoA as palmitate donor, several recombinant Gα have been nonenzymatically palmitoylated *in vitro*[8,10], which sheds new light on the *in vitro* research. The *in vitro* palmitoylation of native Gα has not been reported yet. We have purified $G_o\alpha$ from bovine brain cortex membrane[11] and succeeded in *in vitro* palmitoylation of native $G_o\alpha$. Some characteristics of palmitoylated $G_o\alpha$ were also studied. *In vitro* palmitoylation of native $G_o\alpha$ lays the foundation for studies on the role of Gα palmitoylation in signal transduction.

1 Material and methods

1.1 Material

Bovine brain was purchased from Dachang county, Hebei Province. Sodium cholate, Triton X-100, GDP, Palmitate, CoA and Acyl CoA ligase were purchased from Sigma. ^{3}H-Palmitate and ^{35}S-GTPγS were purchased from Du Pont NEN. Tris and DTT were from Promega. DEAE-Sephacel was the product of Amersham Pharmacia. GF/A glass fibre filter was from Whatman. Heptylamine Sepharose was synthesized according to ref. [15]. GTPγS and Hepes were the products of Boehringer Mannheim. Ultroscan laser light density meter was from LKB.

1.2 Methods

1.2.1 Purification of native $G_o\alpha$. Bovine brain $G_o\alpha$ was purified according to Sternweis[12] with some modification[11]. 290 g bovine brain cortex membrane was suspended with 700 mL cold TED (20 mmol/L Tris-Cl pH 8.0, 1 mmol/L EDTA, 1 mmol/L DTT). Sodium cholate was added to a final concentration of 1% and the mixture was incubated on ice for 40 min. After centrifugation at 100 000 *g* for 60 min, the supernatant was loaded onto a 200 mL DEAE-Sephacel column pre-equilibrated with three volumes of TED/1% sodium cholate. A single GTPγS binding activity peak was eluted with NaCl linear gradient (0 –225 mmol/L NaCl, TED, 1% sodium cholate). Fractions with high GTPγS binding activity were diluted with three volumes of TED containing 100 mmol/L NaCl and 1 mmol/L GDP and then loaded onto a 50 mL Heptylamine-Sepharose column pre-equilibrated with TED containing 100 mmol/ L NaCl, 0.25% sodium cholate and 1 mmol/L GDP. The column was then washed with Buffer A (TED, 300 mmol/L NaCl, 0.25 % sodium cholate, 1 mmol/ L GDP, 35 μmol/L $AlCl_3$, 10 mmol/L $MgCl_2$, 10 mmol/L NaF). The G proteins were eluted with a linear gradient from Buffer A to Buffer B (TED, 300 mmol/L NaCl, 0.25% sodium cholate, 1 mmol/ L GDP, 35 μmol/L $AlCl_3$, 10 mmol/L $MgCl_2$, 10 mmol/L NaF). The purity of each fraction was checked with 12% SDS-PAGE and stained with silver. The pure $G_o\alpha$ was concentrated with Amicon PM10 and flash-frozen with liquid nitrogen.

1.2.2 Synthesis, purification and purity determination of ^{3}H-Palmitoyl CoA. ^{3}H-Palmitoyl CoA was synthesized and purified according to Duncan[10]. 0.5 mCi ^{3}H-Palmitate was dried under nitrogen and redissolved with 50 μL ethanol. 450 μL of 0.05% Triton X-100, 1 mmol/L CoA, 5

mmol/L ATP, 5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH 7.7) and 1unit of Acyl CoA ligase were added. After incubation at 30℃ for 60 min, 5 mL chloroform:methanol (1∶1) was added and mixed. After centrifugation at 2 000 *g* for 10 min, the supernatant was removed and added to 2.5 mL chloroform and 1.25 mL H_2O and then mixed vigorously. After centrifugation at 2 000 *g* for 10 min, the water phase was divided into 100 μL aliquots, dried under vaccum and stored at −70 ℃. The radio-purity of ^{3}H-palmitoyl CoA was analyzed by thin-layer chromatography on silica gel G plate with isobutanol∶H_2O∶acetic acid (50∶30∶20) as the mobile phase, followed by fluorography.

1.2.3 *In vitro* palmitoylation of native $G_o\alpha$. *In vitro* palmitoylation was performed according to Duncan[10]. The buffer of purified $G_o\alpha$ was exchanged to Buffer C (20 mmol/L Hepes, pH 8.0, 1 mmol/L EDTA, 2 mmol/L $MgCl_2$) and then incubated with an equal volume of Buffer C containing 40 μmol/L ^{3}H-Palmitoyl CoA (~2 000 cpm/pmol) and 15 mmol/L CHAPS at 30℃ for 180 min. To determine palmitoylation ratio, 1 μL of palmitoylated $G_o\alpha$ was added to 30 μL stop solution (1% SDS, 2 mg/L BSA). The proteins were pelleted by the addition of 100 μL 10% TCA. The pellet was collected on a GF/A glass fibre filter and washed with 5×200 μL washing buffer (50% ethanol, 3% TCA). The filter was dried, placed on 4 mL scintillation fluid and counted by liquid scintillation spectrometry.

Fig. 1. G proteins purified with two-step chromatography. 1, SDS-PAGE of $G_o\alpha$ purified with two-step chromatography (silver-stained); 2, SDS-PAGE of partially purified G_i.

2 Results and discussion

2.1 Purification of native $G_o\alpha$

We have reported the purification of $G_o\alpha$, G_i and βγ subunits of G proteins by DEAE-Sephacel, Ultragel AcA34, Heptylamine Sepharose and BioScale Q_2 chromatography. To prepare $G_o\alpha$ sample for *in vitro* palmitoylation, we have made some modifications. The Ultrogel AcA gel filtration chromatography was omitted, and appropriate cocktail of AMF (35 μmol/L $AlCl_3$, 10 mmol/L $MgCl_2$/10 mmol/L NaF) was added to the Heptylamine Sepharose hydrophobic chromatography. After DEAE-Sephacel anion exchange chromatography and Heptylamine Sepharose hydrophobic chromatography, $G_o\alpha$ could be purified to homogeneous. In this purification strategy, the purification time was shortened greatly, which is more desirable for the preservation of protein activity. The two-step purification is suitable for the rapid preparation of small amounts of pure $G_o\alpha$.

2.2 Purity analysis of ^{3}H-Palmitoyl CoA

Preparation of ^{3}H-Palmitoyl CoA is critical for the $G_o\alpha$ palmitoylation, and the separation of ^{3}H-Palmitate from ^{3}H-Palmitoyl CoA will directly influence the quantitative analysis of palmitoylation ratio. Duncan et al. have analyzed the purity of ^{3}H-Palmitoyl CoA with Whatman C_{18} reverse phase thin layer chromatography[10]. We found that silica gel G with isobutanol : H_2O : acetic acid (50 : 30 : 20) as mobile phase could also be a convenient method for the same purpose. In fig. 2(a), lane 1 is the thin layer chromatography of synthesized ^{3}H- Palmitoyl CoA before the purification; lane 2 is the ^{3}H-Palmitoyl CoA after the purification. Fig. 2(b) is the calculated purity of ^{3}H-Palmitoyl CoA according to the integration of fig. 2(a) with the Ultroscan laser light density meter. Fig. 2 shows that the ^{3}H-Palmitoyl CoA has been purified to greater than 98%. For the determination of ^{3}H-Palmitoyl CoA purity, silica gel G plate will be a convenient and cheaper substitute for Whatman C_{18} reverse phase plate. The high purity preparation of ^{3}H-Palmitoyl CoA provides a basis for the quantitative analysis of $G_o\alpha$ palmitoylation.

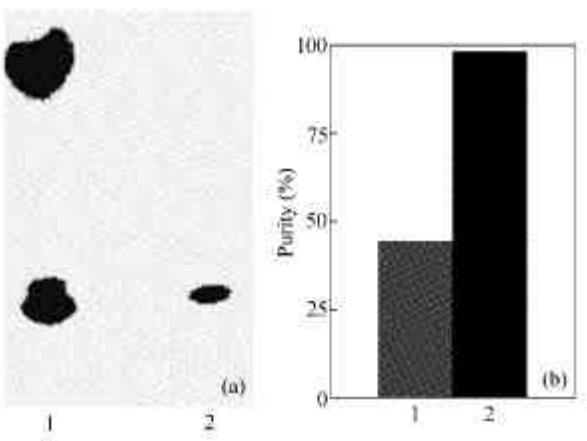

Fig. 2. Purification of ^{3}H-Palmitoyl CoA and purity analysis. ^{3}H-Palmitoyl CoA was synthesized under the catalysis of acyl CoA synthase from ^{3}H-Palmitate and CoA. ^{3}H-Palmitoyl CoA was highly purified after the chloroform extraction of ^{3}H-Palmitate. (a) Fluoroautography of ^{3}H-Palmitoyl CoA before and after the purification: lane 1, ^{3}H-Palmitoyl CoA before the purification; lane 2, ^{3}H-Palmitoyl CoA after the purification; (b) ^{3}H-Palmitoyl CoA calculated according to the integration of light density meter.

2.3 *In vitro* palmitoylation of $G_o\alpha$

2.3.1 Specificity of *in vitro* palmitoylation. All α subunits of heterotrimetric G protein except $G_t\alpha$ are palmitoylated on the N-terminal Cys residues[2]. Although $G\alpha$ subunits contain several Cys residues, their *in vivo* palmitoylation site(s) is(are) specific. The palmitoylation sites of $G_q\alpha$ family are Cys9 and Cys10, while the site of other $G\alpha$, including $G_o\alpha$, is Cys3[10,13]. Hydroxylamine and DTT treatment and trypsin restrictive digestion demonstrated that the *in vitro* palmitoylation of $G_o\alpha$ was also specific. $G\alpha$ has two kinds of fatty acid modification, i.e. amide-linked myristoylation and thioester-linked palmitoylation, among which palmitoylation can be removed by hydroxylamine, β-mercaptoehanol and DTT while myristoylation cannot[7,10,13]. As shown in fig. 3(D) and (H), the incorporated ^{3}H-Palmitate could be removed by hydroxylamine and DTT, which meant that the palmitated was linked by thioester. Trypsin digestion can remove the N-terminal of activated $G_o\alpha$ and thus produce a stable 38 ku product[12,14]. The removal of labeled ^{3}H-Palmitate by trypsin indicated that the palmitoylation site was within the N-terminal of

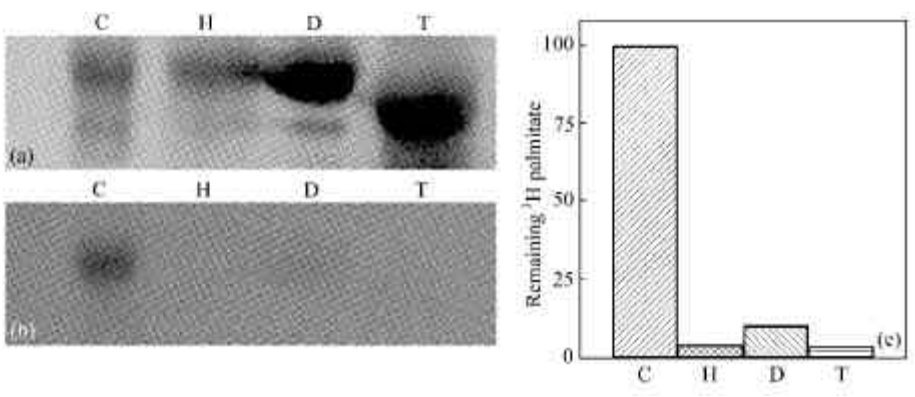

Fig. 3. The *in vitro* palmitoylation site of native $G_o\alpha$ was specific. Palmitoylated $G_o\alpha$ was incubated with buffer (C), l mol/L hydroxylamine (H), or 29 mmol/L DTT (D) at 30℃ for 90 min or was digested with 0.1 mg/mL trypsin (T) for 10 min at 30℃. The reaction was terminated by the addition of SDS-PAGE loading buffer without reducing agent and the subsequent boiling. (a) The commasie blue staining of the SDS-PAGE gel; (b) the fluoroautography of (a); (c) the remaining ^{3}H-Palmitate after various treatments calculated according to the integration of (a) and (b).

$G_o\alpha$. Since the only cystein of $G_o\alpha$ N-terminal is Cys3, this cystein residue should be the palmitoylation site. That is to say that the *in vitro* palmitoylation site of native $G_o\alpha$ is the same as the site *in vivo.*

2.3.2 Apparent palmitoylation ratio was greatly improved after DTT treatment. DTT was routinely added during the purification of G proteins. Since palmitation is liable to DTT reduction and purification usually takes several days, it is taken for granted that the internal palmitoylation of the G protein has been largely removed after purification[5]. The characteristics of purified G protein were considered to be the characteristics of depalmitoylated G protein. We found that the maximum apparent palmitoylation ratio of native $G_o\alpha$ was only 43%, however, after depalmitoylation with 20 mmol/L DTT for 90 min the apparent ratio would be increased to 85% (table 1). The increment of apparent palmitoylation ratio after DTT treatment, which indicated that the remaining endogenous palmitoylation of purified $G_o\alpha$ cannot be neglected. To study the function of depalmitoylated $G_o\alpha$, DTT or thioesterase depalmitoylation of native G protein is absolutely necessary.

Table 1 Apparent palmitoylation ratio improved after DTT treatment

DTT treatment	Apparent palmitoylation ratio
Depalmitoylated $G_o\alpha$	43
Non-depalmitoylated $G_o\alpha$	85

2.4 The influence of palmitoylation on the functions of $G_o\alpha$

Because it is difficult to determine the palmitoylation ratio of purified G proteins, site-directed mutation of the palmitoylation site(s) and subsequent comparison of the functions between the wild type and the mutant G protein in an eukoryotic system have been widely

used[16–19]. conclusions from different laboratories are controversial and mutation of the palmitoylation site itself may influence G protein function[20]. Furthermore, various unknown factors of the eukaryotic system make the explanation of the intracellular data difficult, *in vitro* researchs on the functional roles of $G\alpha$ palmitoylation are therefore necessary.

GTPase cycle is the central step of the coupling function of G proteins and non-hydrolyzable GTP analogue binding activity is an important characteristic of G protein. We have measured the GTPγS binding activity of palmitoylated $G_o\alpha$ and depalmitoylated $G_o\alpha$, however the data showed that palmitoylation has no significant influence upon the GTPγS binding activity of $G_o\alpha$. The GTPγS binding activity of palmitoylated $G_o\alpha$ was 2.95 nmol/mg, while that of depalmitoylated $G_o\alpha$ was 3.0 nmol/mg. Our further studies on the time course and dissociation constant of $G_o\alpha$-GTPγS interaction showed that there was not much difference between palmitoylated $G_o\alpha$ and depalmitoylated $G_o\alpha$. However, preliminary experiments addressing the interaction between LUVs and palmitoylated/depalmitoylated $G_o\alpha$ suggested that palmitoylation may function as a regulator of the $G\alpha$-membrane interaction *in vivo* (data not shown). Under the physiological conditions, palmitoylation may regulate the GTPase cycle by regulating the interaction between $G\alpha$ and GAP[7].

In sum, we have purified the native $G_o\alpha$ from bovine brain cortex and accomplished its *in vitro* palmitoylation. The *in vitro* palmitoylation was specific. The GTPγS binding activity of $G_o\alpha$ was not significantly influenced, however the affinity for membrane may be increased.

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