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# Palmitoylation and its effect on the GTPase-activating activity and conformation of RGS2

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## Abstract

Regulator of G protein signaling (RGS) proteins act as negative regulators of G protein coupled signaling by accelerating the GTPase activity of the G proteins α subunits. Reversible palmitoylation, a common post-translational modification for various components of the G protein-coupled signaling pathway, plays an important role in the modulation of protein activity. RGS2 appears to act selectively to increase the GTPase activity of Gqα when single turnover assays are preformed in solution. However, less attention has been paid to the effects of palmitoylation of RGS2 on its conformation and GTPase-activating activity. Studies of palmitoylation on a series of RGS2 mutants in which alanine was substituted for cysteine revealed cysteine 106, 116 and 199 to be multiple putative palmitoylation sites in RGS2, the efficiency of palmitate incorporation being about 60% at each individual palmitoylation site. Palmitoylation of RGS2 inhibited the GTPase-activating activity toward a GTPase-deficient R183C mutant of Gqα in vitro, but mutation of cysteine 116 eliminated the inhibition of palmitoylation on GTPase-activating activity of RGS2. The effect of palmitoylation on conformation of RGS2 was examined by monitoring spectra of the intrinsic fluorescence and Circular Dichroism. The results suggested that GTPase-activating activity change of RGS2 might be related to conformational change of RGS2 upon palmitoylation. Taken together, these results provided clear and strong experimental evidence for palmitoylation sites in RGS2 as well as for effect of palmitoylation on the GTPase-activating activity and conformation of RGS2.
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*Keywords:* RGS2; Mutation; Palmitoylation; Conformation; GAP activity

## 1. Introduction

G protein coupled signaling is one of the most important pathway for transmembrane cellular signaling. This signal transmembrane transduction system is composed of G protein coupled receptor (GPCR), heterotrimeric G protein (Gαβγ) and effector. Activated receptors trigger the Gα subunit to exchange GTP for GDP resulting in the dissociation of the Gα subunit from the Gβγ thereby activating downstream effectors. The GTP on Gα subunit is hydrolyzed by the intrinsic GTPase-activity of Gα, and GDP-Gα reassociates with Gβγ subunits to reform the ground state of the complex, being once again capable of

*Abbreviations:* dR2, depalmitoylated RGS2; pR2, palmitoylated RGS2; p/d, palmitoylated or deplmitoylated; GAP, GTPase-activating protein

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interacting with the activated receptor (Gilman, 1987). A group of proteins called regulator of G protein signaling (RGS) that act as a key component of G protein signaling transduction system has recently been discovered. RGS negatively regulates G protein-linked signaling by accelerating the rate of GTP hydrolysis by the Gα subunit (Ross & Wilkie, 2000). To date, more than 30 subtypes of the RGS proteins have been identified. These proteins share a conserved structure of about 120 amino acids, designed as the "RGS domain" which is responsible for the interaction with Gα and necessary for GAP (GTPase-activating protein) activity (De Vries, Mousli, Wurmser, & Farquhar, 1995; Druey, Blumer, Kang, & Kehrl, 2002; Hollinger & Hepler, 2002).

Palmitoylation, as one of several post-translational protein modifications that covalent attach protein with lipid moieties, is a reversible and often dynamic modification. Reportedly occurring for most components of the G protein coupled signaling pathway, palmitoylation is speculated to be an important means of regulation for signal transduction, and has aroused extensive interest (Qanbar and Bouvier, 2003). However, the mechanism of reversible palmitoylation of protein remains to be elucidated (Linder & Deschenes, 2003).

RGS2 like other members of the RGS protein family possesses a RGS domain of 120-amino acid residues and was first identified in a screen for activation genes in human lymphocytes. It was originally named G0S8 but renamed RGS2 after the establishment of the RGS nomenclature (Siderovski, Heximer, & Forsdyke, 1994). Among the well-studied RGS proteins, RGS2 is unique because in vitro it interacts specifically with Gqα, but not with any other Gα subunits. Moreover, RGS2 binds to both Gsα and some adenylyl cyclase isoforms to attenuate cAMP production independent of GAP activity in vivo (Heximer, Watson, Linder, Blumer, & Hepler, 1997; Roy et al., 2006). In many cell types, especially in brain neurons, RGS2 expression is highly regulated. Knockout of RGS2 gene in mice impairs anti-viral immunity, increases anxiety levels and alters synaptic development in hippocampus CA1 neurons, but the mechanism behind the modulation of these biological functions by RGS2 remains unclear (Ingi et al., 1998; Oliveira-Dos-Santos et al., 2000).

Several studies have suggested that palmitoylation of RGS4, RGS7, RGS10 and RGS16 has a pivotal role in protein localization, GAP activity and interaction with Gα (Hiol et al., 2003; Osterhout et al., 2003; Takida, Fischer, & Wedegaertner, 2005; Tu, Popov, Slaughter, & Ross, 1999; Tu, Wang, & Ross, 1997). It is likely that most, if not all, functions of RGS in G protein coupled signaling pathways are regulated by palmitoylation, however, more direct evidence is needed to confirm this (De Vries, Elenko, Hubler, Jones, & Farquhar, 1996; Takida et al., 2005). Neither palmitoylation of RGS2 nor any effects of palmitoylation on RGS2 functions have yet been reported. Through studies of palmitoylation of RGS2 and its cysteine mutants, we have confirmed palmitoylation and palmitoylation sites of RGS2, and present direct experimental evidences for palmitoylation sites and their effects on GAP activity and conformation of RGS2.

## 2. Materials and methods

### 2.1. Materials

Immunobilized $Ni^{2+}$-NTA affinity chromatography column was purchased from Novagen, the BCA™ protein assay kit was from Pierce. Polyclonal antiserum against Gα (Sc-393) and RGS2 (Sc-7678) were from Santa Cruz Biotechnology Inc. [γ-$^{32}$P]-GTP and [$^{14}$C]-palmitoyl-Coenzyme A (Pal-CoA) were from Perkin-Elmer. Pfu DNA polymerase, Dpn I restriction endonuclease were the products of New England Biolabs. Dithiothreitol (DTT), imidazole, sodium cholate, GDP, activated charcoal (c5510) were the products of Sigma. GF/A glass fibre filter was from Whatman. Hepes was from Boehringer Mannheim. Top10 and JM109 (DE3) chemically competent cells were from TIANGEN (Beijing). Sephadex G-50 was from Amersham Pharmacia.

### 2.2. Purification of RGS2

Histidine-tagged RGS2 protein was prepared according to the method of Heximer et al. (1997) with some modifications. *Escherichia coli*. (*E. coli*) (JM109 (DE3)) was transformed with pET19-RGS2 plasmid (containing a full-length RGS2 cDNA) and grown in LB Medium (10% tryptone, 5% yeast extract and 10% NaCl supplemented with 100 μg/ml ampicillin, pH 7.2). After five hours induction with isopropyl-beta-D-thiogalactopyranoside (IPTG), the cells were harvested and lysed by sonication, followed by centrifugation at 15 000 × *g*, 4 °C for 1 h. The supernatant was applied to a $Ni^{2+}$-NTA affinity chromatography column and eluted with 250 mM imidazole. The eluate was collected and dialyzed against 200-fold volume buffer (20 mM Tris, pH 7.8, 0.5 M NaCl and 1 mM DTT) for 2–4 h at 4 °C. After another three times of the dialysis, the fraction was assayed by SDS-PAGE and Western blotting. The protein concentration was determined using the BCA protein assay kit with a known protein (bovine serum

albumin, BSA) as a standard, and the protein solution were thereafter stored at −8 °C.

### 2.3. Site-directed mutagenesis of RGS2

Site-directed mutagenesis of cysteine residues which are potential sites for palmitoylation of RGS2 was performed according to the protocol of Quickchange mutagenesis kit (Stratagene) with mutagenic oligonucleotide primers designed for the cysteine to alanine substitutions C13A, C106A, C116A, C169A and C199A, respectively. Mutant strands were synthesized with Pfu and the amplification products were digested with Dpn I, followed by transformation of Top 10 competent cells. Positive clones were verified by sequencing. The double-cysteine mutants (C106A/C116A, C116A/C199A and C106A/C199A) and the triple-cysteine mutants (C106A/C116A/C199A) were constructed sequentially in the same manner. In addition, three residues of the fluorescent amino acid tryptophan were mutated to alanine individually. After veracity of all the DNA constructs was verified by sequencing, the various mutants were expressed and purified in accordance with the purification procedure for intact RGS2.

### 2.4. Preparations of Gqα and Goα

Recombinant R183C Gqα was prepared and purified as described by Biddlecome, Berstein, and Ross (1996). Non-myristoylated Goα was synthesized in and purified from *E. coli* as described by Zhang, Yang, and Huang (2003). All recombinant proteins were analyzed by SDS-PAGE and Western blotting and stored at −80 °C.

### 2.5. Autopalmitoylation of RGS2 proteins

Autopalmitoylation of RGS2 and its mutants were performed as described previously for RGS4 (Tu et al., 1999) with some modification. The palmitoylation reaction was conducted in 50 mM Hepes, pH8.0, 0.005%Lubrol PX and 100 μM β-mercaptoethanol. RGS2 was added to a final concentration of 5 μM, and triggered the reaction by adding 120 μM radioactive ($^{14}$C-) or non-radioactive palmitoyl-CoA. The reaction mixture was incubated at 30 °C for up to 6 h and the proteins were then pelleted by addition of 10% TCA. Residual free pamitoyl-CoA was removed by collecting the pellet on a GF/A glass fibre filter and followed by washing with 6 × 1 ml washing buffer (50% ethanol and 3% TCA). The filter was counted by liquid scintillation spectrometry (1450 MicroBeta Liquid Scintillation & Luminescence Counter, Perkin-Elmer) and the efficiency of palmitoylation was calculated as described by Yang, Zhang, and Huang (2001). Fluorography was performed as described by Esther, Luis, Carlos, Maite, and Fernando (1998) with modifications. Palmitoylated RGS2 and its mutants were viewed by SDS-PAGE, the gel was dried at 80 °C for 2 h (Model 583 Gel Dryer, Bio-RAD) and exposed to the phosphor imaging screens for 24–48 h. Positive bands of the proteins were visualized utilizing a Typhoon Molecular Dynamics (TRIO$^+$ Amersham Bioscience) for scanning of the phosphor screen.

### 2.6. Measurement of GAP activity

The R183C Gqα has markedly reduced intrinsic GTP hydrolysis activity, but retains its responsiveness to RGS protein. The method used for measuring the GAP activity of RGS2 is a modification of that previously described by Ingi et al. (1998). Briefly, Gqα R183C was loaded with [γ-$^{32}$P]-GTP (5–10 μM) in a solution containing 50 mM Hepes, pH 7.4, 5.5 mM CHAPS, 1 mM DTT, 0.9 mM $MgSO_4$, 0.1 mg/ml BSA, 30 mM $(NH_4)_2SO_4$ and 4% glycerol, followed by removal of free [γ-$^{32}$P]-GTP by chromatography on Sephadex 50. The flow-through was kept on ice until needed. The hydrolysis reaction was initiated by adding RGS2 or its mutant forms and 100 μl aliquots were removed at the indicated times and immediately mixed with 900 μl of 10% (w/v) activated charcoal in 50 mM $NaH_2PO_4$. After centrifugation, 600 μl aliquots of the supernatant were counted by liquid scintillation spectrometry.

### 2.7. Measurement of conformational change in RGS2

#### 2.7.1. Circular Dichroism spectroscopy (CD)

RGS2 proteins were diluted in 20 mM sodium phosphate, pH 7.0 to a final concentration of 20 μM and the spectra were recorded from 250 to 190 nm at room temperature.

#### 2.7.2. Intrinsic fluorescence spectra of RGS2

The final concentrations of palmitoylated (pR2) and depalmitoylated (dR2) RGS2 protein and the various palmitoylated/depalmitoylated (p/d) mutants were 5 μM. Intrinsic fluorescence spectra of the RGS2 proteins were recorded from 300 to 390 nm at 30 °C with a slit 0.5 mm on the Hitachi F-4500 fluorescence spectrophotometer. The excitation wavelength was 295 nm.

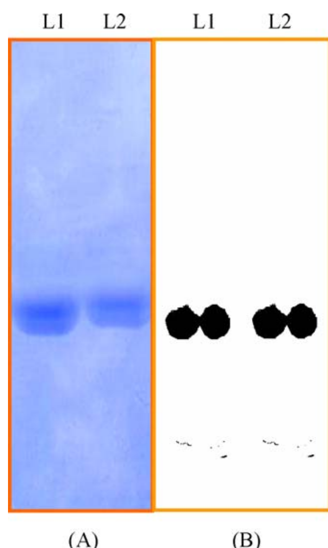


Fig. 1. Purification of RGS2 and its mutants on a Ni-NTA column. (A) Purified intact RGS2 (L1) and C199A (L2) RGS2 were subjected to SDS-PAGE and stained with Coomassie blue. (B) Western blotting of intact RGS2 (L1) and C199A (L2) RGS2.

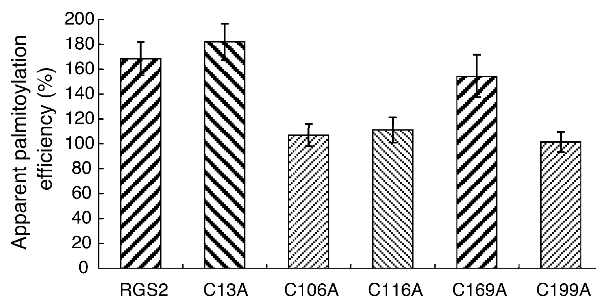


Fig. 2. Apparent palmitoylation efficiency of RGS2 and various single-cysteine mutants. Purified RGS2 or its mutants were incubated with $^{14}$C-palmitate CoA for up to 6 h at 30 °C, palmitoylation efficiency was calculated according to the results of liquid scintillation and expressed as mol of [C$^{14}$]-palmitate incorporation/mol of protein × 100%. Apparent palmitoylation efficiencies of C13A and C169A were similar to intact RGS2, but values for C106A, C116A and C199A decreased to 100–111%.

## 3. Results

### 3.1. Purification of RGS2 and its mutants

Recombinant RGS2 and its mutants were expressed in *E. coli* and purified by affinity chromatography. The purified proteins were visualized as a single band by SDS-PAGE and Western blotting. The profile of the intact RGS2 and the C199A RGS2 are shown in Fig. 1. The results of other mutants were similar to that of C199A RGS2 (data not shown). RGS2 and all the mutants were soluble and expressed in equivalent levels, indicating that they folded relatively normally. These results suggested that all the purified proteins would be suitable for further study of RGS palmitoylation.

### 3.2. Identification of palmitoylation and palmitoylation sites of RGS2

Palmitoylation usually occurs at cysteine residues. Five cysteine residues located at 13, 106, 116, 169 and 199 are found in the amino acid sequence of RGS2, and the C116 residue has been shown to be a highly conserved amino acid in the RGS family.

Palmitoylation of RGS2 and its mutants were performed as described in Section 2. As shown in Fig. 2, the apparent palmitoylation efficiency of intact RGS2 was 168.5 ± 13%, and mutation of the C106, C116 and C199 residues to alanine decreased the palmitoylation efficiency of RGS2 by 60–70%. However, the palmitoylation efficiency of C13A and C169A RGS2 mutants were similar to that of intact RGS2.

Lability to treatment with nucleophilic agents, thiol reagents and borohydride is a distinguishing feature of thioester linkages to cysteine on proteins (Qanbar and Bouvier, 2003). When palmitoylated RGS2 incubated with 50 mM DTT for 1h at 30 °C, all the incorporated $^{14}$C-palmitate would be released (data not shown). These results suggested that in vitro the palmitate was linked to RGS2 via a thioester bond.

The proceeding results demonstrated not only that RGS2 could be palmitoylated, but also that the cysteines 106, 116 and 199 are probable sites of palmitoylation. To study further the specific sites and degree of palmitoylation, we constructed a series of double-cysteine (C106A/C116A, C116A/C199A and C106A/C199A) and triple-cysteine (C106A/C116A/C199A) residue mutants, and measured the palmitoylation efficiency of these mutants. As shown in Fig. 3, double cysteine residue mutations of RGS2 decreased the palmitoylation efficiency to 70 ± 10% of intact RGS2. For the triple-cysteine mutant incorporation of palmitate was essentially eliminated which was similar to the result for the control protein (non-myristoylated Goα). These results strongly indicated that palmitoylation of RGS2 occurs at cysteine residues106, 116 and 199.

Fluorography is another approach commonly used to demonstrate acylation of proteins by radioactive acyl labeling studies. RGS2 and its mutants were analyzed by SDS-PAGE and fluorography after incubation with $^{14}$C-palmitate as described in Section 2. Commassie blue-stained gel showed that the amounts of protein in each reaction were identical (Fig. 4A). From analyses of fluorography intensity using ImageQuant Software (Amer-

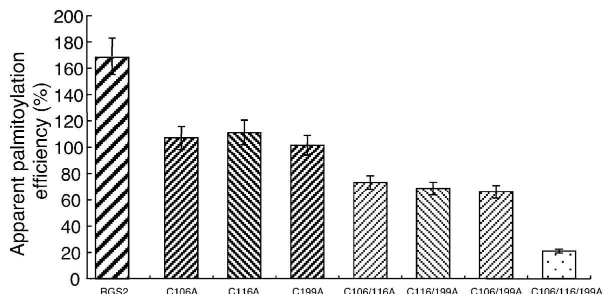


Fig. 3. Apparent palmitoylation efficiency of RGS2 and various plamitoylaton site-directed mutants. Palmitoylation efficiency of intact RGS2, single-cysteine mutants, double-cysteine mutants and triple cysteine mutant were calculated as described in Section 2. The value was168.5 ± 13% for intact RGS2, 100–111% for single palmitoylation site-directed mutants, 65–75% for double sites-directed mutants and 20% for triple sites-directed mutant which was close to that of the negative control.

sham Pharmacia), it was evident that the protein bands corresponding to C13A RGS2 and C169A RGS2 were similar to those of intact RGS2 but stronger than C106A, C116A and C199A RGS2 (Fig. 4B). Furthermore, the fluorography intensity of the bands corresponding to the double-cysteine mutants (Fig. 4C) were weaker than those of the single-cysteine mutants (C106A, C116A and C199A) and was nearly invisible for the triple-cysteine mutant (Fig. 4C). These results further demonstrated RGS2 can be modified in vitro with palmitate at cysteine 106, 116 and 199 residues. The fidelity of palmitoylation under our experimental condition was further indicated by the presences of an additional fluorography band corresponding to RGS4 (Fig. 4B), which can be palmitoylated (Tu et al., 1999), and an invisible band to nonmyristoylated Goα (Fig. 4B), which is known not to be palmitoylated (Duncan & Gilman, 1996).

### 3.3. Palmitoylation significantly attenuates RGS2-promoted GTPase activity of Gqα in solution assays

Because RGS2 is known to selectively accelerate the rate of GTP hydrolysis of Gqα, the effect of palmitoylation on the GAP activity of RGS2 was determined using Gqα as a substrate in single turnover GTPase assays (Ingi et al., 1998). We began with preparation of R183C Gqα, which significantly reduced $k_{cat}$ of GTP hydrolysis but retains the sensitivity to RGS proteins, followed by addition of $(NH_4)_2SO_4$ into the incubation mixture containing [γ-$^{32}$P]-GTP to facilitate the nucleotide exchange of R183C Gqα (from which GDP dissociates very slowly, Chidiac, Markin, & Ross, 1999). The GTP hydrolysis of [γ-$^{32}$P]-GTP-R183C Gqα was initiated in the presence or absence of RGS2 and its mutants, and the GAP activity was measured by the increase in the initial rate of GTP hydrolysis. Fig. 5A shows that dR2 (depalmitoylated RGS2) significantly accelerated the rate of GTP hydrolysis by R183C Gqα, and so did several of the depalmitoylated single-cysteine mutants (Fig. 5B). Conversely, palmitoylation of RGS2 decreased its capacity to activate the GTP hydrolysis to about 70% of the level shown by intact untreated RGS2. Further examination of the GAP activity of a series of the single palmitoyla-

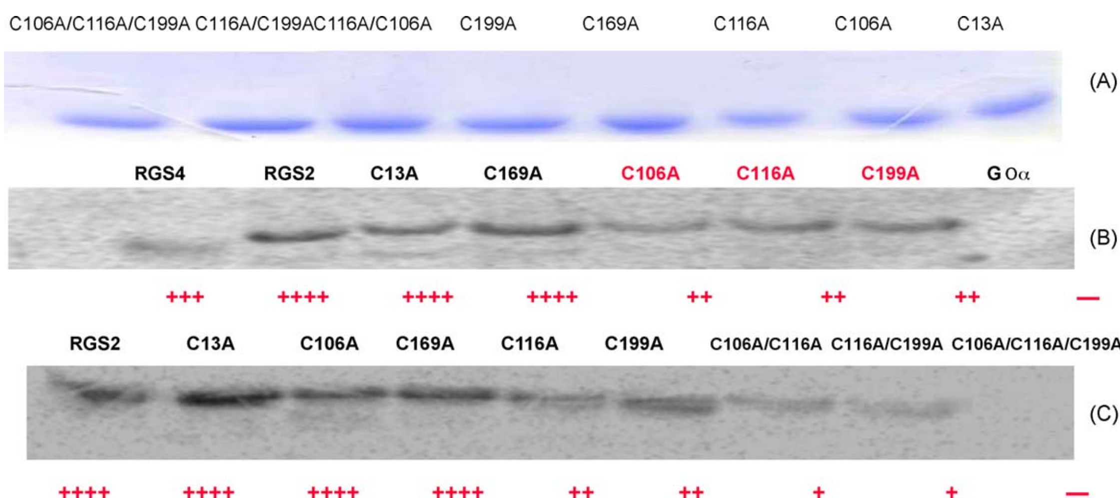


Fig. 4. Fluorographic analysis of RGS2 and its mutants. Palmitoylation was performed as described in Section 2. Palmitoylated samples were separated by SDS-PAGE, and $^{14}$C incorporation was detected by fluorography. (A) Coomassie blue staining of the SDS-PAGE of various protein samples used for fluorography. (B) The fluorography of intact RGS2, several single-cysteine mutants, RGS4 (Positive control) and non-myristoylated Goα (Negative control). (C) The fluorography of RGS2 and the palmitoylated site(s)-directed mutants.

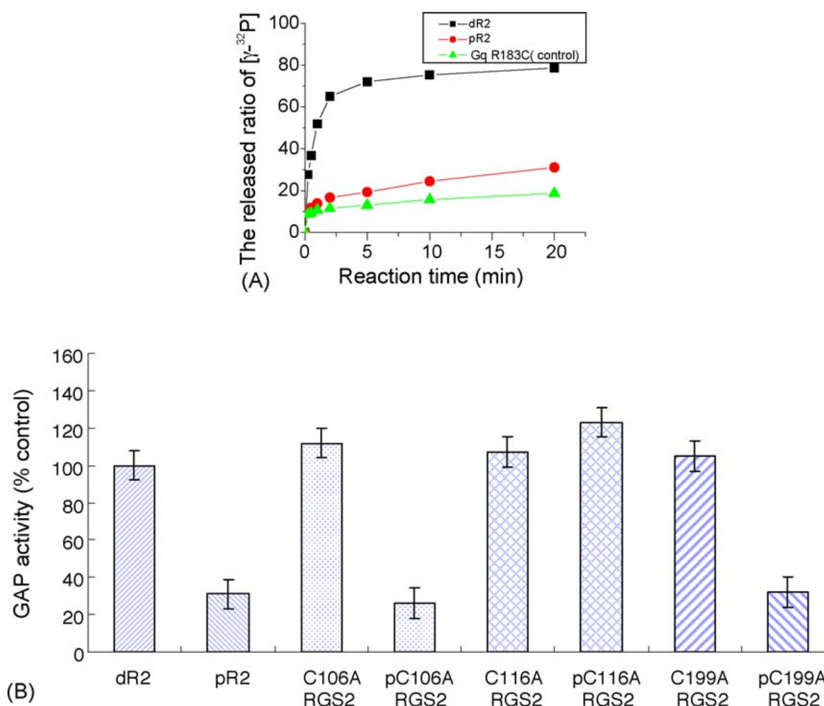


Fig. 5. GAP activity of palmitoylated and depalmitoylated RGS2 and its mutants on Gqα. (A) 200 nM of dR2 (■) and pR2 (●) were incubated with [$\gamma$-$^{32}$P]-GTP-R183C Gqα at room temperature with water control (▲). A 100 μl aliquots were removed at the indicated times and released $\gamma$-$^{32}$P was counted by liquid scintillation. (B) GAP activity of various d/p (depalmitoylated/palmitoylated) mutants of RGS2 was shown as percentage of the activity of intact dR2 (using as a control, its activity taken as 100%). Palmitoylation of intact RGS2 (pR2), C106A (pC106A RGS2, palmitoylated at C116 and C199) and C199A (pC199A RGS2, palmitoylated at C106 and C116) inhibited their GAP activity. But palmitoylation of C116A (pC116A RGS2, palmitoylated at C106 and C199) potentiated its GAP activity.

tion site-directed mutants of RGS2 revealed that for the C116A RGS2 mutant modified with palmitate at C106 and C199 there was no longer inhibitory effect of palmitoylation (Fig. 5B). The GAP activities of C106A RGS2 palmitoylated at C116 and C199, and C199A RGS2 palmitoylated at C106 and C116, were similar to that of palmitoylated intact RGS2. These results suggested that the effects of plamitoylation on the GAP activity of RGS2 depend on the sites of modification and may owe to the different structural domains in which the palmitoylation sites lies. It is also possible that the inhibitory effects of palmitoylation may stem mainly from the modification at the conserved C116 residue in the RGS box.

### 3.4. Conformational change of RGS2 and its mutants upon palmitoylation

#### 3.4.1. Circular Dichroism spectroscopy

The functional activity of a protein is associated with its conformational changes. To explore the correlation between the changes in GAP activity and RGS2 conformation upon palmitoylation, we first analyzed the secondary structural changes of pR2/dR2 (palmitoylated/depalmitoylated RGS2) by CD measurement at first. As shown in Fig. 6, pR2/dR2 formed typical α-helix coils as monitored by CD spectroscopy (double peaks at 208 and 222 nm), but the proportion of α-helices, β-sheets and β-turns were significantly different between dR2 and pR2 (Table 1). This might mean the secondary structure of RGS2 changed when palmitoylated.

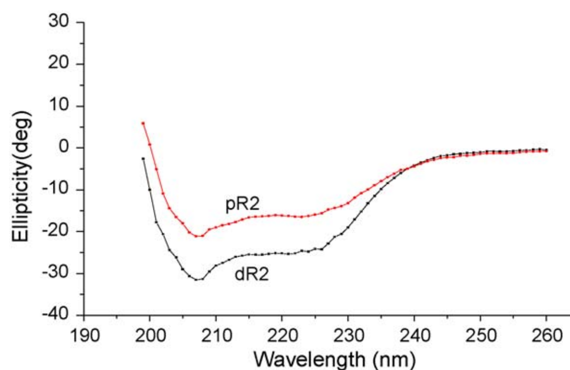


Fig. 6. CD spectra of pR2/dR2. CD spectra were recorded on JASCO J-720 Spectropolarimeter at room temperature.

Table 1
The secondary structure of RGS2 and pRGS2

| Parameters | Samples | |
|---|---|---|
| | RGS2 | pRGS2 |
| α-Helix | 30.6 ± 2.3 | 23.4 ± 1.9 |
| β-Sheet | 20.3 ± 1.7 | 29.4 ± 2.5 |
| β-Turn | 17.5 ± 2.1 | 13.7 ± 1.6 |

*3.4.2. Intrinsic fluorescence measurement*

*3.4.2.1. Palmitoylation induced intrinsic fluorescence change is mainly due to the tryptophan 113 residue.* Changes in intrinsic fluorescence also can be used to monitor conformational changes in a protein, the intrinsic fluorescence is routinely excited at 290 nm and mainly attributed to tryptophan residues. Fig. 7A shows that the intrinsic fluorescence intensity of RGS2 decreased significantly after palmitoylation (pR2), and also that there was a 3 nm blue shift of the emission maxima between pR2 and dR2.

Of three single tryptophan-directed mutants (W41A, W80A and W113A) two mutants (W41A and W80A; data not shown) gave fluorescence intensities similar to intact RGS2 (Fig. 7A), whereas the pattern of the third mutant (W113A RGS2; Fig. 7B) changed substantially compared with intact RGS2 (Fig. 7A). These results suggest that the intrinsic fluorescence changes of RGS2 upon palmitoylation might mainly result from the W113 residue.

*3.4.2.2. Changes in intrinsic fluorescence of pR2/dR2 stem mainly from palmitoylation at the C106 and C116 residues.* To further explore the correlation between intrinsic fluorescent changes of pR2/dR2 and palmitoylation sites, the intrinsic fluorescence of the three single palmitoylation site-directed mutants were measured. As shown in Fig. 8, the fluorescence spectra of p/d C199A RGS2 (Fig. 8A) were similar to those of intact pR2/dR2 (shown in Fig. 7A), but the fluorescence spectra of p/d C106A RGS2 (Fig. 8B) and p/d C116A RGS2 (Fig. 8C) were significantly different. These results suggested that most of the observed palmitoylation-dependent alteration in conformation of RGS2 stems from palmitoylation at the C106 and C116 residues.

## 4. Discussion

### *4.1. Direct experimental evidence for palmitoylation and palmitoylation sites of RGS2*

As one of several lipid modifications of protein, Palmitoylation has been shown to play an important role in the regulation of signal transduction in eukaryotic cells (Oliveira-Dos-Santos et al., 2000). Dynamic palmitoylation has been demonstrated for multiple signaling proteins (such as G proteins, RGS proteins, GPCRs, etc.) and implicated in the initiation and duration of signaling (Chen & Manning, 2001; Mumby, 1997; Riddle, Schwartzman, Bond, & Insel, 2005; Ross, 1995). Palmitate is usually linked to cysteine residues via a thioester linkage. Palmitoylation motifs are poorly characterized till date. In the case of signaling proteins, palmitate is attached either close to termini of proteins, such as the N-terminal in RGS16 and myristoylated Goa (Joyoti, 2004) and the C-terminal in isoprenylated H-Ras and N-Ras (Hancock, Magee, Childs, & Marshall, 1989), or to internal parts of proteins, such as in RGS4 and RGS16 (Osterhout et al., 2003; Tu et al., 1999). However, in the absence of cellular factors, palmitoyl-CoA is capable of spontaneously S-acylating cysteinyl thiols of short peptides as well as folded protein containing palmitoylation sites (Joyoti, 2004).

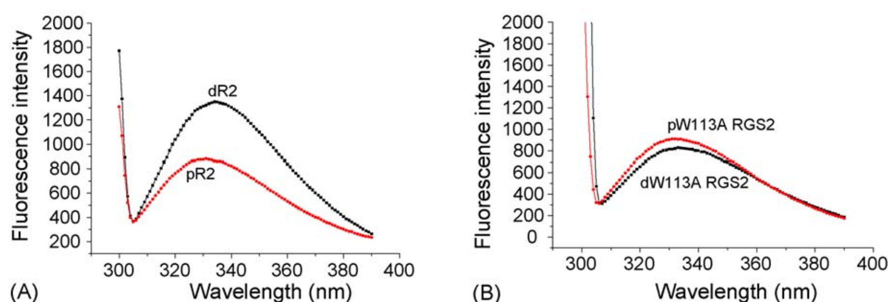


Fig. 7. Intrinsic fluorescence changes of RGS2 and its tryptophan site-directed mutants after palmitoylation. Fluorescent emission spectra were recorded with a Hitachi 4500 Spectrofluorometer. The excitation wavelength was 295 nm, the emission spectra were monitored from 300 to 390 nm. (A) Palmitoylation decreased significantly the fluorescence intensity of intact RGS2, and the fluorescence spectra of W41A and W80A RGS2 (data not shown) were similar to that of A. (B) The fluorescence spectra were similar between palmitoylated and depalmitoylated W113A RGS2, but they were different from that in A.

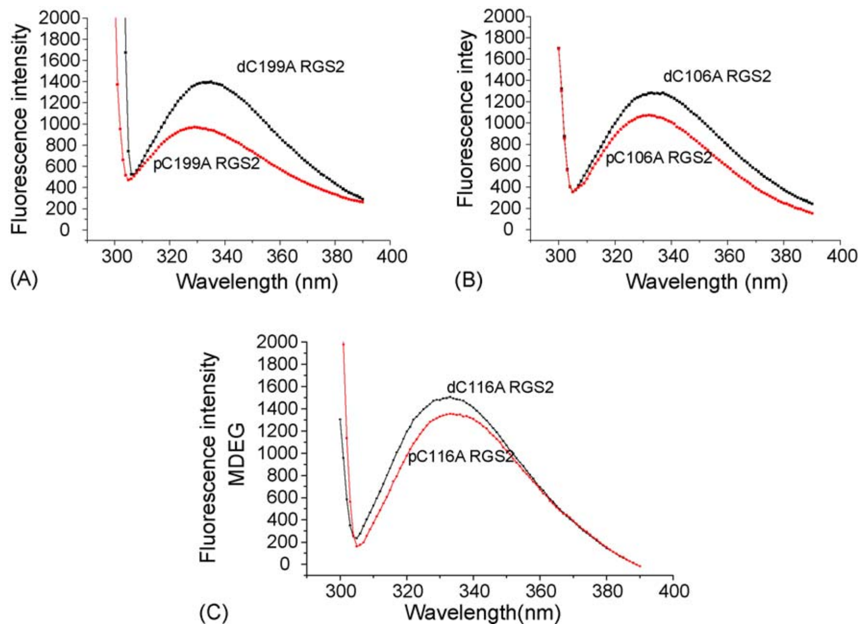


Fig. 8. Intrinsic fluorescence changes of single palmitoylation site-directed mutants of RGS2 after palmitoylation. Intrinsic fluorescence spectra were recorded from 300 to 390 nm at excitation 295 nm for p/d C106ARGS2, p/d C116ARGS2 and p/d C199ARGS2 as described in Section 2.

RGS2 as one of the important member of the RGS family, fall into the R4 subfamily together with RGS4, RGS5 and RGS16. Palmitoylation reportedly occurs for multiple RGS proteins (RGS4, RGS7, RGS10 and RGS16), and produces a variety of effects including alterations in subcellular localization, GAP activity and protein conformation (Hiol et al., 2003; Osterhout et al., 2003; Takida et al., 2005; Tu et al., 1999). It is likely that most RGS proteins are regulated by palmitoylation. Sequence homology analyses have showed that the amino acid residues of RGS2 corresponding to the palmitoylated N-terminal cysteine residues of RGS4 and RGS16 were glutamine and lysine (Ishii & Kurachi, 2003). In contrast, some cysteine residue positions in RGS2 are occupied by different amino acids in other RGS proteins. Therefore, studies of palmitoylation of RGS2 and its effects will be necessary to understand not only the functional activity of RGS2, but also the mechanism by which RGS proteins modulate G protein signaling. Here, the experimental results of palmitoylation of a series of single, double and triple site-directed cysteine mutants indicated the existence of distinct and specific palmitoylation sites in RGS2. Among these putatively palmitoylated cysteine residues, C106 lies on helix 4 in proximity close to the loop 3/4 and C116 reside in the center of α helix4, which form the part of the pocket that binds Gα switch I corresponding to RGS4 counterparts (Ser85 and C116). C199 is located close to the C-terminal of RGS2 (Tesmer, Berman, Gilman, & Sprang, 1997). C116 is highly conserved among RGS proteins (C95 of RGS4 and C98 of RGS16) whereas C106 and C199 are RGS2-specific residues. Based on the crystal structure of the RGS4-Giα1 complex, in RGS2, C106 and N184 would form parts of the floor and lip of the pocket, and a palmitate group on cysteine 106 might interact with the side chains of N184 to reduce the pocket size, which in turn might influence the ability to bind Gα subunits. Also, palmitate groups on cysteine 106 and 116 could increase the hydrophobicity of the domain and re-orient the adjacent contact residues (particularly F105, C106, E108 and N109). As a result, palmitoylation may alter the interactions occurring at the edge of RGS–Gα interface, including hydrophobic interactions, and impair its ability to bind Gα subunits (Posner, Mukhopadhyay, Tesmer, Gilman, & Ross, 1999; Srinivasa, Watson, Overton, & Blumer, 1998). In addition, palmitate attachment on cysteine 199 might increase the hydrophobicity of RGS2 and contribute to its membrane association and subcellular trafficking. Given the different locations of the palmitoylated cysteine residues, palmitoyl modifications might participate in modulation of multiple aspects of RGS2 function and activity (Heximer et al., 1999; Hiol et al., 2003; Osterhout et al., 2003). In this paper, we have provided direct and strong experimental evidence for palmitoylation and palmitoylation sites of RGS2 that has previously not been reported.

### *4.2. Palmitoylation influences the conformation and GAP activity of RGS2*

RGS proteins act as central participants in receptor signaling and cell physiology through interaction with various signaling proteins. Although RGS proteins use a variety of mechanisms to regulate signaling, their GAP activity is a primary characteristic. Studies of the effects of palmitoylation on the GAP activity and function of RGS2 will be crucial in revealing the modulatory role of RGS2 in the G protein coupled signaling pathway. Our results suggested that pR2 and dR2 have significantly different of GAP activity levels for Gqα. Palmitoylation of C106A and C199A RGS2 substantially decreased the GAP activity, whereas palmitoylated C116A RGS2 had even stronger GAP activity than dR2. Our results suggest that acylation at different sites of RGS2 may have different effects on the GAP activity, either elevating the GAP activity by palmitoylation at C106 and C199 (pC116A RGS2) or impairing the GAP activity by palmitoylation at C116 (pC106A and pC199A RGS2). Our findings are similar to those studies by Tu et al. who reported that palmitoylation inhibits the GAP activity of RGS4 and RGS10 in a solution-based assay (Tu et al., 1999).

It is well-known that conformational changes can be key events in the regulation of protein activity. Analyses of the CD spectra showed that the secondary structures of pR2 and dR2 were significantly different (Table 1). This difference was also further confirmed by the intrinsic fluorescence measurement (Fig. 7). As Fig. 7B shows, the tryptophan 113 residue is mainly responsible for this change in fluorescence. In addition, measurements of intrinsic fluorescence of various cysteine residues mutants of RGS2 showed the changes of fluorescence spectra between pR2 and dR2 results mainly from palmitoylation at C106 (which lies on α-helix 4 in close proximity to loop 3/4) and C116 (which reside in the center of α-helix 4) (Tesmer et al., 1997). Dual palmitoylation of helix 4 might increase its hydrophobicity, further altering the interaction on the RGS2-Gqα interface (α-helix 4 forms a part of the pocket that binds Gα; Posner et al., 1999; Srinivasa et al., 1998) which in turn influences the GAP activity of RGS2 for Gqα.

In summary, through studies of palmitoylation of RGS2 and its mutants, direct and strong experimental evidence for palmitoylation and palmitoylation sites of RGS2 have been presented in this study. Furthermore, the results of effects of palmitoylation on the GAP activity and RGS2 conformation imply an important role of palmitoylation in the interaction between RGS2 and Gqα.

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