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Palmitoylation modification of $G\alpha_o$ depresses its susceptibility to GAP-43 activation

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ABSTRACT

Interaction between GAP-43 (growth associated protein-43) and $G\alpha_o$ (alpha subunit of Go protein) influences the signal transduction pathways leading to differentiation of neural cells. GAP-43 is known to increase guanine nucleotide exchange by $G\alpha_o$, which is a major component of neuronal growth cone membranes. However, it is not clear whether GAP-43 stimulation is related to the $G\alpha_o$ palmitoylation or the conversion of $G\alpha_o$ from oligmers to monomers, which was shown to be a necessary regulatory factor in GDP/GTP exchange of $G\alpha_o$. Here we expressed and purified GAP-43, GST-GAP-43 and $G\alpha_o$ proteins, detected their stimulatory effect on [^{35}S]-GTPγS binding of $G\alpha_o$. It was found that the EC_{50} of both GAP-43 and GST-GAP-43 activation were tenfold lower in case of depalmitoylated $G\alpha_o$ than palmitoylated $G\alpha_o$. Non-denaturing gel electrophoresis and *p*-PDM cross-linking analysis revealed that addition of GST-GAP-43 induced disassociation of depalmitoylated $G\alpha_o$ from oligomers to monomers, but did not influence the oligomeric state of palmitoylated $G\alpha_o$, which suggests that palmitoylation is a key regulatory factor in GAP-43 stimulation on $G\alpha_o$. These results indicated the interaction of GAP-43 and $G\alpha_o$ could accelerate conversion of depalmitoylated $G\alpha_o$ but not palmitoylated $G\alpha_o$ from oligomers to monomers, so as to increase the GTPγS binding activity of $G\alpha_o$. Results here provide new evidence about how signaling protein palmitoylation is involved in the G-protein-coupled signal transduction cascade, and give a useful clue on the participation of GAP-43 in G-protein cycle by its preferential activation of depalmitoylated $G\alpha_o$.

1. Introduction

Growth associated protein-43 (GAP-43) is concentrated in the neuronal growth cone and is regarded as an important nerve ending "signal" protein (Mosevitsky, 2005). It is expressed at high level during neuronal development and regeneration (Skene, 1989; Strittmatter, 1992; Strittmatter et al., 1995) and is linked to axonal elongation and synaptic plasticity (Strittmatter, 1992; Strittmatter et al., 1992).

At the molecular level, GAP-43 may function by stimulating heterotrimeric GTP-binding proteins (such as Go protein) which are also enriched in the growth cone membrane (Strittmatter et al., 1990) and transduce extracellular signals that determine the extent and direction of axonal growth (Kater and Mills, 1991; Strittmatter et al., 1991a). Agents that alter G-protein activity have dramatic effects on neurite outgrowth in tissue culture (Skene, 1989; Doherty et al., 1991). Studies using *Xenopus laevis* oocytes have shown that GAP-43 alters the response of Go protein-coupled signaling to extracellular stimuli (Strittmatter et al., 1993), suggesting that GAP-43 participates in the regulation of Go protein-coupled signaling cascade. However, the specific molecular mechanisms underlying this regulation remain unclear.

Both GAP-43 and $G\alpha_o$ undergo post-translational palmitoylation modification, that is, a 16 carbon saturated fatty acid is linked to a cysteine residue via a thioester bond. Compared to other lipid modifications of proteins such as myristoylation and isoprenylation, palmitic acid turns over with a much shorter half-life than that of the proteins (Staufenbiel and Lazarides, 1986; Skene and Virag, 1989). The rapid turnover of palmitic acid with extracellular stimuli such as the binding of agonist and GPCR (G-protein-coupled receptor) as well as the variation in palmitoylation levels (Huang, 1989; Wedegaertner and Bourne, 1994) strongly suggest that addition or removal of palmitate regulates the functional activity of proteins. Palmitoylation of GAP-43 occurs on the 3rd and 4th cysteine residues from its N-terminal (Skene and Virag, 1989) and was shown to suppresses the stimulation of GAP-43 on $G\alpha_o$ *in vitro* (Sudo et al., 1992). $G\alpha_o$ is palmitoylated at the N-terminus 3rd

Abbreviations: GAP-43, growth associated protein-43; $G\alpha_o$, α subunit of heterotrimeric Go protein; GTPγS, guanosine-5′-O-(3-thiotriphosphate); *p*-PDM, N,N′-1,4-phenylenedimaleimide; IPTG, isopropyl-β-D-thiogalactoside.

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cysteine residue, and depalmitoylation of Gα occurs extensively during the G-protein-coupled signal transduction triggered by activated receptors (Wedegaertner and Bourne, 1994; Stanislaus et al., 1998; Barclay et al., 2005). Although there is evidence that palmitoylation influences Gα membrane association and cellular location (Wedegaertner and Bourne, 1994; Wedegaertner et al., 1993), little is known about how depalmitoylation modulates Gα activity and its interactions with other proteins. Strittmatter et al. (1993) demonstrated that intracellular GAP-43 not only stimulates the Go protein-coupled signal cascade directly, but also increases the receptor activation effect on this system in a cooperative manner. This suggests that the stimulatory effect of intracellular GAP-43 on $G\alpha_o$ is closely related to the activation of G-protein-coupled transduction cascades following receptor activation. Therefore, interesting questions to address are whether the depalmitoylation of $G\alpha_o$ associated with receptor activation directly influences the interaction between GAP-43 and $G\alpha_o$, if so, how does this happen?

According to the "disaggregation-coupling" model by Rodbell (Rodbell, 1997), $G\alpha_o$ exists as oligomers in its basal state (GDP bound) and disaggregates into monomers in its active state (GTP bound). Further results from our laboratory have shown that depalmitoylation weakens the $G\alpha_o$ oligomerization and consequently facilitates its GTP-binding (Yang et al., 2008). These data suggest that the degree of $G\alpha_o$ oligomerization associated with the palmitoylation/depalmitoylation cycle might be a crucial regulatory factor on GTP-binding. Therefore, we examined the relationship between GAP-43 stimulation and the palmitoylation modification of $G\alpha_o$ protein as well as corresponding changes of $G\alpha_o$ oligomerization states.

2. Materials and methods

2.1. Materials

Fresh bovine brain was obtained from Nanying village, Hebei province; CAM-Sepharose, Glutathione Sepharose™ 4B, Phenyl Sepharose High Performance and Q Sepharose High Performance were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA); Tris, HEPES, DTT, PMSF, $(NH_4)_2SO_4$, NBT, BCIP and IPTG from Promega (Madison, WI, USA); Bio-beads from Bio-Rad (Hercules, CA, USA); nitrocellulose membrane from Millipore (Billerica, MA, USA); the expression plasmid pGEX-4T-1-GAP-43 was provided by Dr. Xiaoli Duan (the Fourth Military Medical College, Xian, China), the expression plasmid pQE60-$G\alpha_o$ 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 Jeffrey Gordon (Washington University School of Medicine, St. Louis, MO); rabbit anti-mouse polyclonal antibody specific to GAP-43 (Chemicon, Temecula, CA, USA), rabbit anti-mouse polyclonal antibody specific to $G\alpha_o$ and goat anti-rabbit IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology, Santa Cruz, CA, USA); palmitoyl-Coenzyme A and acrylamide (Sigma, St. Louis, MO, USA); GTPγS (Roche Molecular Biochemicals, Basel, Switzerland); [^{35}S]-GTPγS and [^{14}C]-palmitoyl-Coenzyme A (PerkinElmer, Waltham, MA, USA); *p*-PDM (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Preparation of natural GAP-43 protein

GAP-43 was prepared from fresh bovine brain according to the "improved alkali extraction method" (Zhang and Huang, 2003). The purity of GAP-43 was evaluated by 12% SDS-PAGE stained with Coomassie blue R-250 and the protein concentration was determined using the BCA™ Protein Assay Kit (Pierce). The same identification was applied on GST-GAP-43 and $G\alpha_o$.

2.3. Preparation of GST-GAP-43 fusion protein

The *E. coli* strain JM109 transformed with pGEX-4T-1-GAP-43 was grown in LB medium supplemented with 50 μg/ml ampicillin. When the $O.D._{600}$ reached to ~0.6, 0.5 mM IPTG was added, the cells were then grown for 6 h and harvested by low speed centrifugation. The precipitated cells were re-suspended in 50 ml PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and lysed by ultrasonic shock before being centrifuged at 15,000 × *g* for 30 min. The supernatant was applied to a Glutathione Sepharose™ 4B affinity chromatography column equilibrated by PBS buffer as above. After washing the column with 100 ml PBS, the protein was eluted by PBS supplemented with 50 mM reduced glutathione.

2.4. Preparation of myristoylated $G\alpha_o$

The *E. coli* strain JM109 co-transformed with pQE60-$G\alpha_o$ and pBB131-NMT was grown in T_7 enriched medium with 50 μg/ml kanamycin and 50 μg/ml ampicillin. When the $O.D._{600}$ reached to ~0.6, 60 μM IPTG was added and the cells were then grown at 30 °C overnight. The cells were harvested and lysed by freezing–thawing with liquid nitrogen and digested with 0.2 mg/ml lysozyme before the lysate was centrifuged at 30,000 × *g* for 1 h. The supernatant 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 highly purified $G\alpha_o$ fractions were pooled and stored at −70 °C.

2.5. Western blot analysis

Western blots of all the samples were performed according to «Protein methods» (Bollag et al., 1996). After the electrophoresis, the proteins were transferred onto nitrocellulose membrane with a Mini Trans-Blot Cell (Bio-Rad) at constant voltage of 100 V for 1–2 h. After blocked with 3% (w/v) BSA overnight, the membranes were incubated with the rabbit anti-mouse polyclonal antibody specific to $G\alpha_o$ (1:100) or with the rabbit anti-mouse polyclonal antibody specific to GAP-43 (1:100) at 37 °C for 4 h and then incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:2000) at 37 °C for 4 h. NBT and BCIP were added to detect the protein bands.

2.6. In vitro palmitoylation of $G\alpha_o$ protein

$G\alpha_o$ expressed in *E. coli* JM109 is depalmitoylated (non-palmitoylated) because of a lack of palmitoyl acyl transferase (PAT) in prokaryotic cells. Purified $G\alpha_o$ was palmitoylated *in vitro* according to Duncan and Gilman (1996). The palmitoylation reaction was set up with 20 μM $G\alpha_o$ in palmitoylation reaction buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 2 mM $MgCl_2$, 0.02% (v/v) Lubrol PX), and 200 μM palmitoyl-coenzyme A was added to trigger the reaction. The reaction mixture was incubated at 22 °C for 1 h to obtain palmitoylated $G\alpha_o$. Non-palmitoylated $G\alpha_o$ was treated likewise, but without addition of palmitoyl-coenzyme A. Efficiency of the palmitoylation was measured as previously described, palmitoylation efficiency = $[[^{14}C] - \text{palmitate}]_{bound}/[G\alpha_o]_{total}$. $[G\alpha_o]_{total}$ in the assay system is known, and $[[^{14}C]\text{-palmitoyl}]_{bound}$ could be determined by Liquid scintillation counter (Yang et al., 2008).

2.7. [^{35}S]-GTPγS binding assay

The [^{35}S]-GTPγS binding assays were conducted according to Sudo's method with few modifications (Sudo et al., 1992). $G\alpha_o$ and GAP-43 (or GST-GAP-43) were diluted into buffer A (20 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT). The assay system contained either

no or 400 nM palmitoylated $G\alpha_o$ or depalmitoylated $G\alpha_o$, while the concentration of GAP-43 or GST-GAP-43 changed from 16 nM to 128 μM. Twenty μl of the diluted samples were mixed with 20 μl of [^{35}S]-GTPγS binding buffer B (50 mM HEPES, pH 8.0, 15 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 4 μM [^{35}S]-GTPγS (~50,000 cpm/(μmol l))). After incubation at 30 °C for 30 min, the reaction was stopped by adding 1 ml ice-cold buffer C (20 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$). Then samples were filtered through a millipore filter and membrane-bound radioactivity was measured on a 1450 MicroBeta Liquid Scintillation and Luminescence Counter (PerkinElmer™ Life Sciences). For the test of non-specific binding, 2 μl of 10 mM cold GTPγS was added to an assay tube and treated/counted likewise.

2.8. Non-denaturing gradient gel electrophoresis

Palmitoylated or depalmitoylated $G\alpha_o$ was diluted to 2 μM with buffer A (20 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT), and GST-GAP-43 was added to a final concentration of 10 μM when included. The $G\alpha_o$ samples with or without GST-GAP-43 were incubated at 30 °C for 30 min. Twenty μl of each sample was mixed with 5 μl sample buffer (300 mM Tris–HCl, pH 8.0, 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue) and subjected to 3–20% non-denaturing gradient gel electrophoresis with a constant voltage of 200 V for 5 h. Western blot analyses were carried out as in Section 2.5.

2.9. p-PDM cross-linking

p-PDM cross-linking of the depalmitoylated $G\alpha_o$ in different guanine nucleotide binding states were examined as follows. The purified $G\alpha_o$ (depalmitoylated $G\alpha_o$) was in a GDP bound state ($G\alpha_o$·GDP) because 25 μM GDP was added during the purification (Section 2.4). $G\alpha_o$·GDP was diluted to 16 μM in exchange buffer D (20 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, 7.5 mM $MgCl_2$, 500 μM GTPγS) and incubated at 30 °C for 30 min to obtain $G\alpha_o$·GTPγS. $G\alpha_o$·GDP and $G\alpha_o$·GTPγS were then dialysed against buffer E (20 mM HEPES, pH 8.0, 1 mM EDTA, 7.5 mM $MgCl_2$) to remove DTT. $G\alpha_o$·GDP or $G\alpha_o$·GTPγS (2 μM) was mixed with varying concentrations (0–20 μM) of GST-GAP-43 and incubated at 30 °C for 30 min. The *p*-PDM cross-linking reaction was then performed according to the method of Coulter and Rodbell (1992). Twenty μl of each sample was mixed with 5 μl SDS-PAGE sample buffer (300 mM Tris–HCl, pH 6.8, 30% (v/v) glycerol, 1% SDS, 0.5 M DTT, 0.05% (w/v) bromophenol blue) and subjected to 3–20% gradient SDS-PAGE with a constant voltage of 200 V for 5 h. Western blot analysis on $G\alpha_o$ was performed as in Section 2.5.

3. Results

3.1. Purification of GAP-43 and GST-GAP-43

As determined by SDS-PAGE, the apparent molecular mass of GAP-43 extracted from bovine cerebral cortex was 45 kDa and the purity was above 95%. Western blot analysis further confirmed that this band corresponds to GAP-43 (Fig. 1A).

The recombinant GST-GAP-43 protein was abundantly expressed in *E. coli* JM109 and was purified by Glutathione Sepharose™ 4B affinity chromatography. SDS-PAGE affirmed its apparent molecular mass to be 66 kDa (Fig. 1B), which is consistent with the estimated molecular mass of GST-GAP-43. Immunoblot analysis with a polyclonal antibody specific to GAP-43 further confirmed that the purified protein was a GAP-43 fusion protein (Fig. 1B).

3.2. Expression and purification of $G\alpha_o$ with high activity

Myristoylated $G\alpha_o$ expressed in *E. coli* strain JM109 was separated from non-myristoylated $G\alpha_o$ by its stronger affinity with Phenyl Sepharose and was applied to a Q-Sepharose column for further purification. Purified myristoylated $G\alpha_o$ was identified by 12% SDS-PAGE (purity > 90%) and western blot analysis using an antibody specific to $G\alpha_o$ (Fig. 1C).

3.3. Both GAP-43 and GST-GAP-43 could stimulate [^{35}S]-GTPγS binding of $G\alpha_o$

Both GAP-43 and GST-GAP-43 increased [^{35}S]-GTPγS binding of $G\alpha_o$ in a concentration-dependent manner that achieved saturation. The maximal stimulatory effect on GTPγS binding activity of Gαo reached three times that of its basal activity (Table 1). GAP-43 and GST-GAP-43 alone did not show any [^{35}S]-GTPγS binding activity (data not shown).

3.4. In vitro palmitoylation of $G\alpha_o$

We have reported that in vitro non-enzymatic palmitoylation modification of $G\alpha_o$ happened at the N-terminal 3rd Cysteine

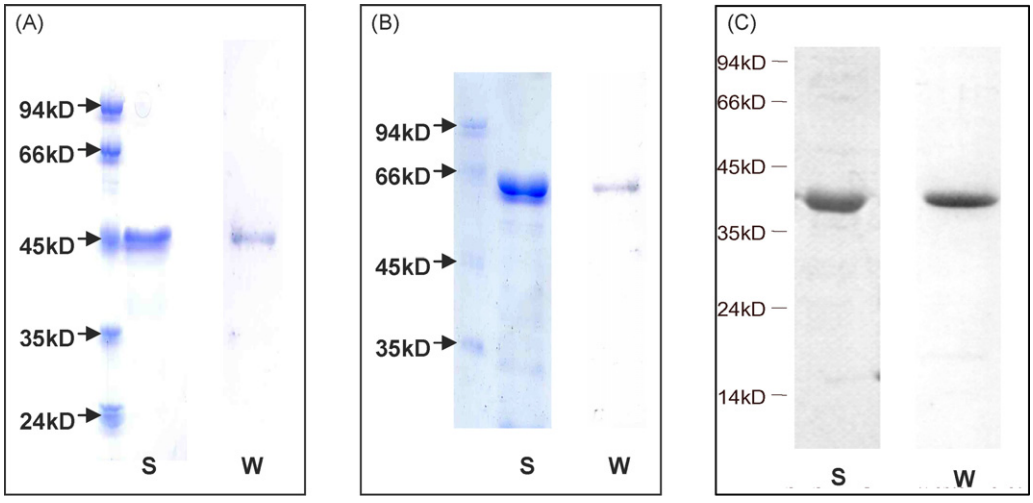

Fig. 1. SDS-PAGE (S) and western blot (W) analysis of several purified proteins. (A) GAP-43, (B) GST-GAP-43 and (C) $G\alpha_o$. About 2 μg of each protein was subjected to 12% SDS-PAGE. Mobility of molecular size standards is indicated separately on the left.

Table 1
Effect of GAP-43/GST-GAP-43 on [^{35}S]-GTP$_\gamma$S binding activity of Gα_o.

	Gα_o protein	E_b (nmol/mg)	E_m (nmol/mg)	Increase (%)	EC_{50}
GAP-43	dGα_o	6.2 ± 0.3	18.8 ± 1.03	305 ± 15.1	3.98 ± 0.52
	pGα_o	2.9 ± 0.16	8.6 ± 0.24	296 ± 12.5	35.98 ± 3.16
GST-GAP-43	dGα_o	5.9 ± 0.39	16.8 ± 0.85	285 ± 16.7	3.4 ± 0.6
	pGα_o	2.8 ± 0.21	8.2 ± 0.40	294 ± 18.2	38.5 ± 4.2

E_b, the basal GTPγS binding activity of Gα_o; E_m, the maximal GTPγS binding activity of Gα_o with stimulation of GAP-43/GST-GAP-43; EC_{50}, the required molar ratio of GAP-43/GST-GAP-43: Gα_o when half of the maximum stimulation was reached from the concentration response curves in Fig. 2. The data represent means ± S.E. of three independent experiments. dGα_o, depalmitoylated Gα_o; pGα_o, palmitoylated Gα_o.

residue and the palmitate incorporated was thioesterified (Yang and Huang, 2001).

In vitro [^{14}C]-Palmitoyl-CoA binding assay showed that palmitoylation reaction could hardly carry through when Gα_o concentration in the reaction system was lower than 1 μM, while the palmitoylation efficiency of Gα_o maintain at 80–90% when Gα_o concentration changed between 3–20 μM, but Gα_o tended to aggregate when its concentration was higher than 30 μM (data not shown). So in this study, we applied 20 μM Gα_o in the system for palmitoylation modification. The [^{35}S]-GTPγS binding assay showed that Gα_o retained about 30% of its GTPγS binding activity after the palmitoylation modification (Table 1).

3.5. Different stimulatory effects of GAP-43 on depalmitoylated and palmitoylated Gα_o

Fig. 2A shows the concentration response curves of GAP-43 stimulation on palmitoylated and depalmitoylated Gα_o. GAP-43 stimulated the GTPγS binding activity of Gα_o with or without palmitate up to threefold that of the background (Table 1), but depalmitoylated Gα_o was obviously more easily activated by GAP-43 than palmitoylated Gα_o, as the EC_{50} of GAP-43 stimulation on depalmitoylated Gα_o (GAP-43:Gα_o = 3.9, mol:mol) was only one tenth of that on palmitoylated Gα_o (GAP-43:Gα_o = 35.98, mol:mol) (Fig. 2A and Table 1).

3.6. Different stimulatory effects of GST-GAP-43 on depalmitoylated and palmitoylated Gα_o

Fig. 2B shows the concentration response curves of GST-GAP-43 stimulation on depalmitoylated and palmitoylated Gα_o. Similar to GAP-43, GST-GAP-43 increased GTPγS binding activity of depalmitoylated and palmitoylated Gα_o up to three times that of the background binding (Table 1). However, at the concentration of GST-GAP-43 required to stimulate Gα_o to half maximum stimulation, the molar ratio of GST-GAP-43 to Gα_o was 3.40 for depalmitoylated Gα_o compared with 38.50 for palmitoylated Gα_o (Table 1 and Fig. 2B), suggesting that GST-GAP-43 stimulates the GTPγS binding of depalmitoylated Gα_o more strongly than that of palmitoylated Gα_o.

3.7. GST-GAP-43 influences oligomerization of depalmitoylated Gα_o but not of palmitoylated Gα_o

It has been shown (Yang et al., 2008) that both depalmitoylated Gα_o and palmitoylated Gα_o in their basal states could form oligomers in non-denaturing gel electrophoresis. To detect the influence of GST-GAP-43 on the oligomerigation of Gα_o, the same amount of palmitoylated or depalmitoylated Gα_o were incubated with 10 uM of GST-GAP-43 and then resolved by non-denaturing gel electrophoresis and western blot analysis (Fig. 3A). Consist with the different effect of GAP-43 on Gα_o activity with or without palmitate, after GST-GAP-43 treatment, the oligomerization of palmitoylated Gα_o including dimers, trimers, tetramers and pentamers remained unchanged (Fig. 3A, L3, L4), on the contrary, no oligomeric or monomeric band of depalmitoylated Gα_o was detected on the membrane (Fig. 3A, L1, L2). The same amount of Gα_o samples with/without the GST-GAP-43 treatment as above were also applied to SDS-PAGE. As shown in Fig. 3B, no quantity change was found for depalmitoylated Gα_o after incubated with GST-GAP-43, which means no aggregates was formed in the process of GST-GAP-43 treatment and the same quantity of Gα_o before and after GST-GAP-43 treatment were loaded onto the SDS-PAGE as well as the non-denaturing gel electrophoresis.

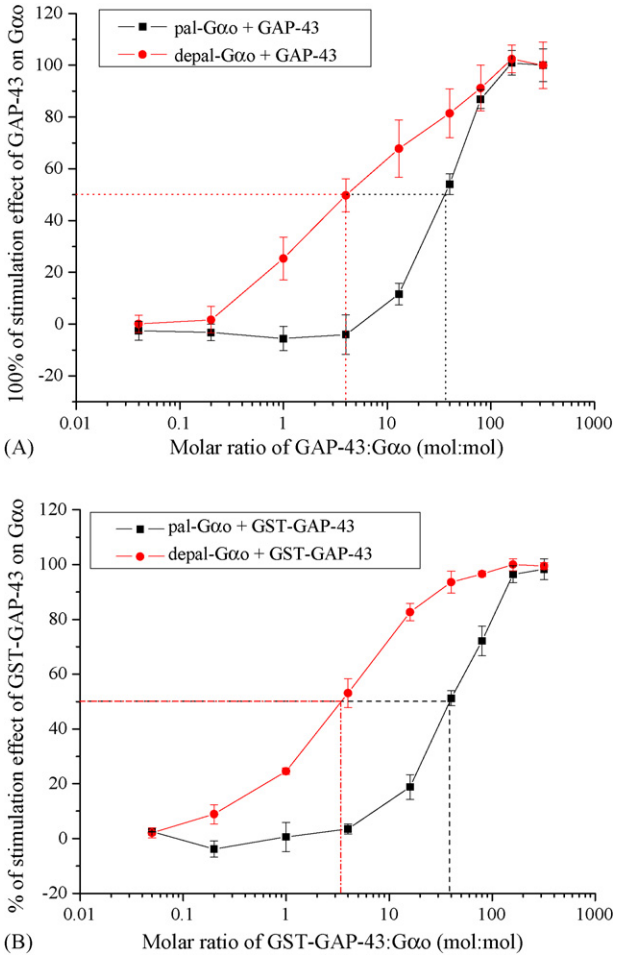

Fig. 2. Concentration response curves of GAP-43(A) or GST-GAP-43(B) stimulation on [^{35}S]-GTPrS binding activity of palmitoylated(■) and depalmitoylated(●) Gα_o. All the assays were performed at 30 °C for 30 min using 400 nM Gα_o and increasing amounts of GAP-43 or GST-GAP-43 (from 16 nM to 128 μM). Maximum stimulation of GAP-43 (or GST-GAP-43) on the GTPγS binding of depalmitoylated and palmitoylated Gα_o were set as 100%, respectively. The dashed lines in the figures indicated the molar ratio of GAP-43 (or GST-GAP-43) to Gα_o when 50% of the maximum stimulation in each condition were reached. The data represent means ± S.E. of three independent experiments.

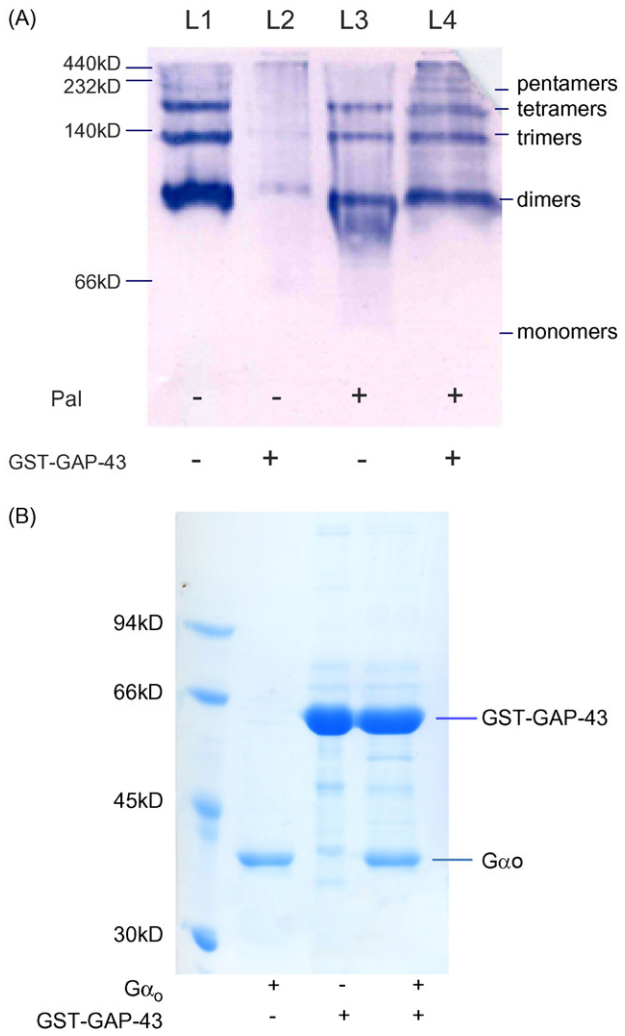

Fig. 3. Oligomerization of palmitoylated and depalmitoylated $G\alpha_o$ in the presence of GST-GAP-43. (A) The concentration of $G\alpha_o$ was 2 μM, for the samples with GST-GAP-43(+), $G\alpha_o$ was pre-incubated with 10 μM GST-GAP-43 at 30 °C for 20 min prior to non-denaturing gel electrophoresis. Western blotting analysis was performed with anti-$G\alpha_o$ antibody. (B) 2 μM $G\alpha_o$ with/without 10 μM GST-GAP-43 was pre-incubated at 30 °C for 20 min prior to SDS-PAGE, 10 μM GST-GAP-43 was loaded as a control.

The previous result from our group (Zhang and Huang, 2003) has demonstrated a direct interaction between GAP-43 and depalmitoylated $G\alpha_o$ by protein–protein binding experiments using CaM-Sepharose affinity media. This indicated a possible mechanism that the interactions between GST-GAP-43 and $G\alpha_o$ might prevent $G\alpha_o$ from transferring onto the nitrocellulose membrane. But we cannot evaluate how could GST-GAP-43 influence $G\alpha_o$ oligomerization by using non-denaturing gel electrophoresis, in case some kinds of protein complex of these two proteins formed in the non-denaturing condition.

3.8. GST-GAP-43 depressed oligomerization of depalmitoylated $G\alpha_o$ to form monomer by p-PDM cross-linking assay

As mentioned above, non-denaturing gel electrophoresis has some limitation in identifying the disaggregation of depalmitoylated $G\alpha_o$ in the presence of GST-GAP-43, we therefore applied a *p*-PDM cross-linking approach, which could eliminate the possible influence from a $G\alpha_o$ and GST-GAP-43 interaction.

p-PDM (N,N′-1,4-phenylenedimaleimide) is a homobifunctional cross-linking reagent reacting with sulfhydryl groups. In the presence of *p*-PDM, oligomeric $G\alpha_o$ should form cross-linking products that could be resolved by SDS-PAGE. As shown in Fig. 4, $G\alpha_o$·GDP and $G\alpha_o$·GTP without *p*-PDM (Fig. 4, L0 and L0′) existed as monomer by SDS-PAGE. After *p*-PDM treatment, $G\alpha_o$·GDP formed several cross-linking products (Fig. 4, L1) larger than the monomer, but almost no cross-linking product was found for $G\alpha_o$·GTP (Fig. 4, L1′). Interestingly, with an increasing quantity of GST-GAP-43, the amount of $G\alpha_o$·GDP cross-linking products decreased and the $G\alpha_o$·GDP monomer gradually increased (Fig. 4, L2, L3, L4). These results confirmed the observation that GST-GAP-43 influenced the oligomerization of depalmitoylated $G\alpha_o$ by depressing the amount of oligomers (Fig. 3, L2), and further indicated that GST-GAP-43 could promote depalmitoylated $G\alpha_o$ turning from an oligomeric to a monomeric state. On the other hand, addition of GST-GAP-43 did not influence the organization structure of $G\alpha_o$·GTP (Fig. 4, L2′, L3′, L4′)

4. Discussion

Palmitoylation of Gα is a readily reversible covalent modification which is considered an important means to regulate G-protein-coupled signal transduction process. During G-protein-coupled receptor activation, the depalmitoylation process of Gα protein is evidently accelerated (Wedegaertner and Bourne, 1994; Barclay et al., 2005). The precise functional regulation of the palmitoylated/depalmitoylated cycle on $G\alpha_o$, especially under physiological conditions, is still not well understood. GAP-43 is a candidate intracellular activator of $G\alpha_o$ that was shown several years ago to activate $G\alpha_o$ *in vitro* (Strittmatter et al., 1990). Currently, there is little research focus on the relationship between the GAP-43 and $G\alpha_o$ interaction and the palmitoylation modification of $G\alpha_o$. In the present study, we have attempted to explore the impact of $G\alpha_o$ palmitoylation on its stimulation induced by GAP-43, as well as the underlying structural basis of this stimulation.

In the nerve growth cone, GAP-43 and $G\alpha_o$ have the similar distribution patterns (Strittmatter et al., 1991b), while $G\alpha_o$ here includes depalmitoylated and palmitoylated species. In this study, prokaryotic expression and purification of $G\alpha_o$ and *in vitro* palmitoylation modification on this protein were performed to obtain palmitoylated and depalmitoylated $G\alpha_o$, respectively. The activation effect of GAP-43 on $G\alpha_o$ was tested by using a [^{35}S]-GTPγS binding assay, in which the molar ratio of GAP-43 to $G\alpha_o$ ranged from 0.04 to 320. GAP-43 stimulated both depalmitoylated and palmitoylated $G\alpha_o$ in a saturating manner (Fig. 2A), while the [^{35}S]-GTPγS binding efficiency of these two proteins stimulated by GAP-43 were significantly different (Fig. 2A). The EC_{50} of GAP-43 stimulation on depalmitoylated $G\alpha_o$ (GAP-43:$G\alpha_o$ = 3.98, mol:mol) was only one tenth of that on palmitoylated $G\alpha_o$(GAP-43:$G\alpha_o$ = 35.98, mol:mol), which suggests that the palmitoylation modification could depress the susceptibility of $G\alpha_o$ to GAP-43 stimulation. Further analysis of the stimulatory effect of GST-GAP-43 fusion protein on $G\alpha_o$ activity confirmed this finding (Fig. 2B). These results suggest that depalmitoylated $G\alpha_o$ might be the main target and the most sensitive "substrate" of GAP-43.

Studies on the Go protein-coupled signal transduction in *Xenopus laevis* oocytes (Strittmatter et al., 1993) revealed that activation of the M_2 acetylcholine receptor by carbachol (agonist) and microinjection of GAP-43 into cells can both improve GTP binding activity of $G\alpha_o$. However, when these two factors act simultaneously, the increased GTP-binding activity of $G\alpha_o$ is more than the sum of the activity increases induced by these two factors separately. It is known that in association with the receptor activation, the proportion of depalmitoylated Gα protein inside cells increases dramatically (Wedegaertner and Bourne, 1994; Stanislaus et al., 1998). According to the present results, depalmitoylated $G\alpha_o$, as the more sensitive "substrate" of GAP-43, would be more easily

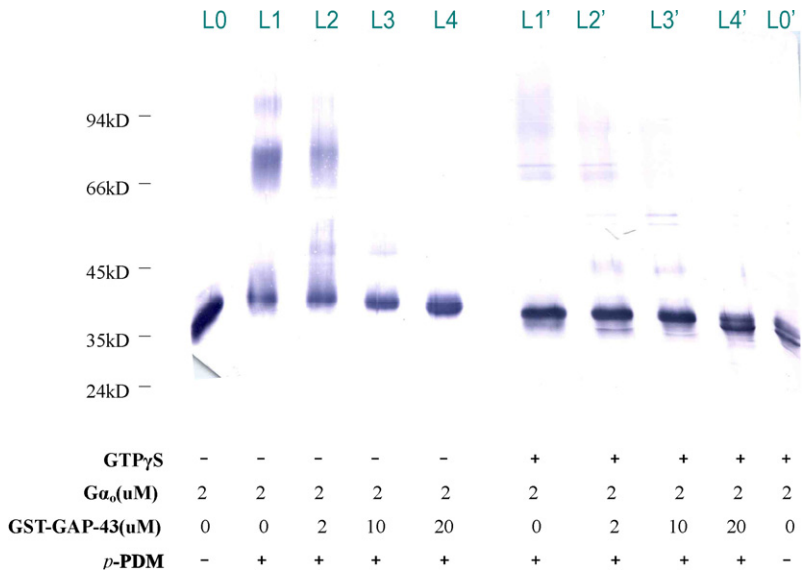

Fig. 4. Influence of GST-GAP-43 on *p*-PDM cross-linking of the depalmitoylated $G\alpha_o$. L0 and L0′, $G\alpha_o$ without *p*-PDM as a monomer control; L1- 4, 2 μM $G\alpha_o$·GDP (GTPγS(−)) with different concentration of GST-GAP-43 subjected to cross-linking; L1′–4′, 2 μM $G\alpha_o$·GTPγS (GTPγS(+)) with different concentrations of GST-GAP-43 subjected to cross-linking. All the samples were subjected to 3–20% SDS-PAGE followed by western blotting analysis using anti-$G\alpha_o$ antibody.

activated. That is, when an extracellular signal initiates G-protein-coupled signal pathway and the depalmitoylation of $G\alpha_o$ occurs, the intracellular activator GAP-43 should exert a stronger stimulation on $G\alpha_o$. So, the effects of extra- and intra-cellular activation factors should be cooperative, not just additive. The results obtained in this study, that GAP-43 stimulated depalmitoylated $G\alpha_o$ much easier than palmitoylated $G\alpha_o$, provide support at the molecular level for previously reported findings at the cellular level (Strittmatter et al., 1993), and suggest a molecular mechanism for the cooperative action between GAP-43 and the G-protein-coupled signal pathway.

GAP-43 can also be palmitoylated and its palmitoylation decreases its activation of $G\alpha_o$ *in vitro* (Sudo et al., 1992). On the other hand, GAP-43 can also be palmitoylated and its palmitoylation decreases its activation of $G\alpha_o$ *in vitro*. Palmitoylation of GAP-43 could initiate its binding to the membrane but did not serve as a permanent membrane anchor (Denny, 2006; Liang et al., 2002). At steady-state, most of the GAP-43 in COS-1 or PC12 cells was found to be depalmitoylated (Liang et al., 2002). Depalmitoylation of Gα occurs extensively during the G-protein-coupled signal transduction triggered by activated receptors (Wedegaertner and Bourne, 1994; Stanislaus et al., 1998; Barclay et al., 2005). Our previous work (Yang et al., 2008) further showed that depalmitoylation depresses $G\alpha_o$ oligomerization, so as to facilitate GDP/GTP exchange of $G\alpha_o$, indicating that the depalmitoylation process induced by receptor activation might be an important step in the activation of the GPCR signal pathway. Until now, there had been no evidence indicating the relationship of GAP-43 depalmitoylation and any extracellular/intracellular signal. So we could hardly figure out whether there exist a coordinated regulation fashion of palmitoylation of these two proteins *in vivo*. The results here from purified GAP-43 and $G\alpha_o$ suggested that depalmitoylation of $G\alpha_o$ facilitates GAP-43 activation on $G\alpha_o$, through which GAP-43 may participate in the G-protein activation cycle. This would imply studies on palmitoylation/depalmitoylation of $G\alpha_o$ has obvious significance for exploring the molecular mechanism regulating G-protein-coupled signal transduction pathways. Certainly, it would be a fascinating problem to explore the interaction between them as well as the underlying mechanisms and the physiological significance *in vivo*.

Previous studies on the oligomerization behavior of $G\alpha_o$ *in vivo* (Nakamura and Rodbell, 1991; Jahangeer and Rodbell, 1993) and *in vitro* (Yang et al., 2008; Zhang et al., 2003) provided much evidence that $G\alpha_o$ exists as oligomers in its basal state and is disaggregated into monomers by activation. An oligomerization–disassociation cycle has been proposed in the Gα activation process which has been elucidated in the "disaggregation-coupling" model by Martin Rodbell (Jahangeer and Rodbell, 1993). In view of the relationship between the activity and organization structure of $G\alpha_o$, knowledge concerning the influence of GAP-43 on $G\alpha_o$ oligomerization might be essential to elucidate the mechanism of GAP-43 stimulation on $G\alpha_o$. In this study, we took two potent approaches to characterize the oligomerization of $G\alpha_o$ with or without palmitate in the presence of GST-GAP-43. Non-denaturing gel electrophoresis analysis showed that GST-GAP-43 depressed the amount of the depalmitoylated $G\alpha_o$ oligomers (Fig. 3a, L2) but had no effect on palmitoylated $G\alpha_o$ (Fig. 3a, L4). Investigation by *p*-PDM cross-linking (Fig. 4) confirmed this result and further indicated that GST-GAP-43 promoted the conversion of depalmitoylated $G\alpha_o$ from its oligomeric to monomeric state. The oligomeric form of $G\alpha_o$ corresponds to its basal state, while the monomeric form corresponds to its active state (Rodbell, 1997; Zhang et al., 2003). Data documented here show that GST-GAP-43 could induce the oligomeric depalmitoylated $G\alpha_o$ but not palmitoylated $G\alpha_o$, to a more active monomeric state, which should help illuminate the structural basis for the stronger stimulation of GST-GAP-43 on depalmitoylated $G\alpha_o$ than on palmitoylated $G\alpha_o$.

In summary, this article provides direct evidence that depalmitoylation of $G\alpha_o$ protein remarkably enhances its susceptibility to stimulation from natural GAP-43 or GST-GAP-43 fusion protein. The discrepant influence of GST-GAP-43 on the disaggregation of $G\alpha_o$ oligomers in the depalmitoylated or palmitoylated state may shed light on the structural basis for this regulation. These results therefore suggest that palmitoylation/depalmitoylation of $G\alpha_o$ helps regulate the stimulation of its activity by GAP-43.

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