Stimulation and conformational change of $G_o\alpha$ induced by GAP-43

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Abstract GAP-43 and G_o are peripheral membrane proteins enriched in neuronal growth cone. GAP-43 was highly purified from bovine cerebral cortex and myristoylated $G_o\alpha$ was highly purified from *Escherichia coli* cotransformed with pQE60 ($G_o\alpha$) and pBB131 (NMT). GAP-43 stimulated GTPγS binding to $G_o\alpha$ and the stimulation effect was dependent on concentration of GAP-43. Protein-protein binding experiments using CaM-Sepharose affinity media revealed that $G_o\alpha$ · GDP bound GAP-43 directly to form intermolecular complex. This interaction induced conformational change of $G_o\alpha$. In the presence of GAP-43, fluorescence spectrum of $G_o\alpha$ · GDP blue shifted 4 nm; fluorescence intensity increased 35.3% and apparent quenching constant (Ksv) increased from $(1.1 \pm 0.22) \times 10^5$ to $(4.1 \pm 0.43) \times 10^5$ (M^{-1}). However, no obvious changes of fluorescence spectra of $G_o\alpha$ · GTPγS were observed in the absence or presence of GAP-43. Our results indicated that GAP-43 induced conformational change of $G_o\alpha$ · GDP so as to accelerate GDP release and subsequent GTPγS binding, which activates G proteins to trigger signal transduction and amplification. These results provided insights into understanding the function of G proteins in coupling between receptors and effectors and the key role of GDP/GTP exchange mode in GTPase cycle.

Keywords: GAP-43, G_o, GDP/GTP exchange, conformation.

GAP-43, a major component of the neuronal growth cone is closely correlated with neural development and regeneration[1—5]. G_o is the predominant noncytoskeletal protein in the growth cone membrane[6]. It is a kind of heterotrimeric GTP-binding proteins, which transduce signals across the plasma membrane by coupling between receptors and effectors[7]. Stimulation or inhibition of G proteins altered neurite outgrowth[3]. Mastoparan, which activates heterotrimeric G proteins of the G_o and G_i subtypes, induces growth cone collapse in embryonic dorsal root ganglion cells[8]. The correlation of cellular function and location between G_o and GAP-43 is attractive. A large number of experiments carried out in the early period of the 1990s demonstrated that like G protein-coupled receptors, GAP-43 enhances GDP release from G_o and increases the initial rate of GTPγS binding without altering the intrinsic k_{cat} for the GTPase[9]. *In vivo*, GAP-43 augmented G protein-coupled receptor transduction in *Xenopus laevis* oocytes[10]. These results implied that GAP-43 might exert its cell biological roles through G protein-coupled signal transduction pathways as an intracellular regulator, but its molecular mechanism of action has remained unclear. An interesting question is whether GAP-43 binds $G_o\alpha$ · GDP to enhance GDP release or

binds $G_o\alpha$ • GTP to accelerate GDP/GTP exchange. To further elucidate the molecular mechanism of interaction between G_o and GAP-43, we prepared $G_o\alpha$ and GAP-43 with high purity and investigated the stimulation and conformational change of $G_o\alpha$ induced by GAP-43.

1 Materials and methods

1.1 Materials

Bovine brains were obtained from slaughterhouse in Dachang County, Hebei Province, China. Tris, DTT, PMSF, $(NH_4)_2SO_4$, NBT, BCIP, goat anti-rabbit IgG linked to alkaline phosphatase were purchased from Promega. GTPγS, Hepes were purchased from Boehringer Mannheim. CaM-Sepharose affinity column was purchased from Amersham Pharmacia Biotech Inc. ^{35}S-GTPγS and ^{3}H-Palmitate were purchased from Dupont NEN. ^{3}H-Palmitoyl CoA was synthesized from ^{3}H-Palmitate and coenzyme A. Bio-beads were purchased from BioRad. Nitrocellulose membrane was obtained from Gelman. Rabbit anti-mouse polyclonal antibody specific to $G_o\alpha$ was purchased from Santa Cruz Biotechnology. Plasmid pQE60 ($G_o\alpha$) was a generous gift of Prof. Susanne Mumby (University of Texas Southwestern Medical Center, USA); plasmid pBB131 (NMT) was a generous gift of Prof. Gordon (University of Washington, USA). HB (hypocrellin B) was provided by associate Prof. Yue Jiachang in our laboratory. All other chemicals were of reagent grade.

1.2 Methods

1.2.1 Expression and purification of myristoylated $G_o\alpha$. Myristoylated $G_o\alpha$ was prepared by the method of Mumby[11] with a few modifications. *Escherichia coli* strain JM109 was cotransformed with pQE60 ($G_o\alpha$) and pBB131 (NMT) and grown in T_7 enriched medium (2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, and 50 mmol/L KH_2PO_4, pH 7.2) supplemented with 50 μg/mL ampicillin and 50 μg/mL kanamycin. IPTG were added to a final concentration of 60 μmol/L to induce synthesis of NMT and $G_o\alpha$. The cells were harvested and lysed by lysozyme. The lysate was centrifuged at 30000 *g* for 1 h. The supernatant was applied to DEAE-Sephacel column and eluted with 300 mmol/L NaCl. The DEAE eluate was brought to 1.2 mol/L in $(NH_4)_2SO_4$ and 25 μmol/L in GDP and applied to Phenyl-Sepharose column and eluted with a gradient of $(NH_4)_2SO_4$ (1.2—0 mol/L). In this step, myristoylated $G_o\alpha$ and nonmyristoylated $G_o\alpha$ were separated. The pooled myristoylated $G_o\alpha$ was dialyzed against buffer Q (50 mmol/L Tris-HCl, pH 8.0, 0.02 mmol/L EDTA, 1 mmol/L DTT) overnight to remove $(NH_4)_2SO_4$. The protein sample was subsequently applied to Q-Sepharose column and eluted with a gradient of NaCl (0—300 mmol/L) in buffer Q. The protein fractions were assayed by SDS-PAGE and the fractions of highly purified $G_o\alpha$ were pooled and stored in liquid nitrogen.

1.2.2 Purification of GAP-43. GAP-43 was purified according to the methods of Oestreicher[12] and Andreasen[13] with few modifications. All procedures were carried out at 4℃. Fresh bovine cerebra were dissected to remove myelinated tissue, blood clots, and any remaining meninges.

The dissected tissue (~100 g/brain) was homogenized in buffer I (0.32 mol/L sucrose, 14 mmol/L β-mercaptoethanol, 1 mmol/L PMSF) for 30 s. The homogenate was centrifuged at 900 *g* for 10 min and the supernatant subsequently for 20 min at 10000 *g*. The pellet was subjected to hypo-osmotic shock for 15 min in 500 mL double distilled water supplied with 1 mmol/L PMSF and 14 mmol/L β-mercaptoethanol. The preparation was further disrupted by five strokes and centrifuged at 48000 *g* for 20 min. The pellet was suspended in buffer II (5 mmol/L magnesium acetate, pH 8.1, 14 mmol/L β-mercaptoethanol); the pH of the suspension was adjusted to 11.5 with 2 mol/L NaOH to a final volume of 10 mL/g wet weight. The mixture was stirred at 4℃ for 30 min and centrifuged at 48000 *g* for 20 min. The supernatant was acidified to pH 5.5 with 1 mol/L sodium acetate-acetic acid buffer at pH 5.0. After stirring at 4℃ for 30 min, the mixture was centrifuged at 48000 *g* for 20 min. The supernatant was treated with ammonium sulfate of 57% saturation. After centrifugation at 48000 *g* for 20 min, the supernatant was then re-saturated to 82%. The precipitation was collected by centrifugation at 48000 *g* for 20 min and suspended in 10 mL buffer III (50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L DTT). The suspension was dialyzed against 1 L buffer III for 7 h. After centrifugation at 25000 *g* for 15 min, the supernatant was brought to 5 mmol/L in EGTA and 0.1% in Lubrol PX and applied to CaM-Sepharose column equilibrated in buffer III containing 5 mmol/L EGTA and 0.1% Lubrol PX. The column was washed with equilibration buffer until the absorbance at 280 nm reached zero and proteins were eluted with buffer III containing 3 mmol/L $CaCl_2$ and 0.1% Lubrol PX. Fractions were assayed by SDS-PAGE and those highly purified were pooled. After removal of Lubrol PX by the following method, proteins were concentrated and stored in liquid nitrogen.

1.2.3 Remove of Lubrol PX by Bio-beads absorption. Bio-beads can absorb Lubrol PX effectively after activation by methanol[14]. The purified GAP-43 proteins were concentrated and divided into 1 mL per tube. 100 mg (wet weight) Bio-beads were added to every tube and the mixture was rotated at room temperature for 30 min. Then 50 mg (wet weight) Bio-beads were supplied and the mixture was rotated for another 30 min. After precipitation of Bio-beads, the supernatants containing GAP-43 proteins were pooled and concentrated and stored in liquid nitrogen. With this method, almost all the Lubrol PX in protein samples could be removed.

1.2.4 ^{35}S-GTPγS binding assay. ^{35}S-GTPγS binding assays were conducted as described previously[15]. $G_o\alpha$ and GAP-43 were diluted into buffer A (20 mmol/L Hepes, pH 8.0, 1 mmol/L EDTA, 1 mmol/L DTT) to the determined concentration. 30 μL of diluted samples was pre-incubated at 30℃ for 5 min and 30 μL of GTPγS binding buffer B (100 mmol/L Hepes, pH 8.0, 15 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L DTT, 4 μmol/L GTPγS and ^{35}S-GTPγS (100000 cpm/tube)) was added. After incubation for 10 min at 30℃, the reaction was stopped with ice-cold buffer C (20 mmol/L Tris-HCl, pH 8.0, 5 mmol/L $MgCl_2$). Then samples were filtered over millipore filter and membrane-bound radioactivity was determined. For non-specific binding, 2 μL of

10 mmol/L GTPγS was added to an assay tube.

1.2.5 Nonenzymatic palmitoylation of myristoylated $G_o\alpha$. Nonenzymatic palmitoylation of myristoylated $G_o\alpha$ was generally conducted according to the methods of Duncan[16] and Yang[17].

1.2.6 The direct interaction between GAP-43 and $G_o\alpha$. CaM-Sepharose beads were equilibrated in buffer D (50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L $MgCl_2$, 6 mmol/L EDTA, 0.1% Lubrol PX, 1 mmol/L DTT). 50 μL of CaM-Sepharose emulsion was mixed with 48 μL of 21 μmol/L GAP-43 and 2 μL of 50 μmol/L $G_o\alpha$. The mixture was incubated for 1 h at 4℃ with vortex every 10 min. CaM-Sepharose beads were pelleted by centrifugation and the supernatant was aspirated out. Buffer D was added to wash beads for three times. Then 100 μL of buffer E (50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 0.1% Lubrol PX, 9 mmol/L $CaCl_2$, 1 mmol/L DTT) was added and the mixture was incubated at 4ºC for 30 min. After centrifugation, 50 μL of supernatant was subjected to 12% SDS-PAGE and Western blotting assay using anti-$G_o\alpha$ antibody. In control experiments, GAP-43 was displaced with BSA.

1.2.7 Western blotting assay of $G_o\alpha$. Blots were processed as described[18]. The proteins were electrophoretically transferred to nitrocellulose membrane in a Bio-Rad Mini Trans-Blot Cells apparatus with constant voltage of 100 V for 2 h. Membranes were blocked with 3% BSA overnight. After being washed for three times, the membranes were incubated with the rabbit anti-mouse polyclonal antibody apecific to $G_o\alpha$ (1:100) at 37℃ for 2 h and goat anti-rabbit Ig G linked to alkaline phosphatase (1:7000) at 37ºC for 2 h, respectively. NBT and BCIP were added to detect protein bands.

1.2.8 Fluorescence measurement and HB quenching. The intrinsic fluorescence emission spectrum of $G_o\alpha$ with or without GAP-43 was measured on a Hitachi F4010 spectrofluorometer. $G_o\alpha$ • GDP or $G_o\alpha$ • GTPγS and GAP-43 were pre-incubated at 30℃ for 20 min prior to the measurement. The data were calibrated to eliminate the influence of background fluorescence and dilution of samples. As for HB quenching, the fluorescence intensity was titrated with 1.89 mmol/L HB in concentration from 0 to 7.6 μmol/L. The data were fitted to modified Stern-Volmer equation as $F_o/\Delta F = A+B*1/[Q]$, where F_o and ΔF represent fluorescence intensity in the absence of the quencher and decrease of fluorescence intensity in the presence of the quencher respectively, and [Q] is the concentration of the quencher. Ksv (A/B) is the apparent quenching constant.

2 Results

2.1 Purification of myr-$G_o\alpha$ and GAP-43

E. coli cells expressing $G_o\alpha$ were harvested, lysed and centrifuged. The supernatant was applied to DEAE-Sephacel, Phenyl-Sepharose, Q-Sepharose in series to get highly purified $G_o\alpha$. SDS-PAGE showed only one band with purity greater than 95% (fig. 1(a), lane G) and the band

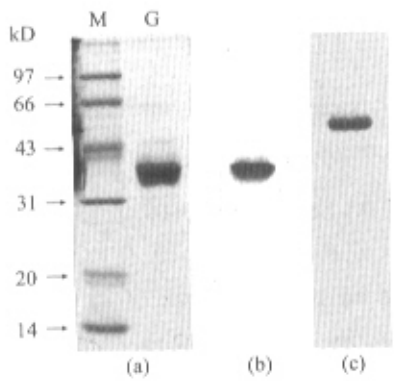

Fig. 1. SDS-PAGE of the purified recombinant $G_o\alpha$ and GAP-43. (a) 12% polyacrylamide gel: Lane M, molecular weight standards; lane G, recombinant myr-$G_o\alpha$; (b) Western blotting analysis of purified $G_o\alpha$; (c) 11% SDS-PAGE of purified GAP-43.

could be recognized by anti-$G_o\alpha$ antibody (fig. 1(b)). The purified $G_o\alpha$ was successfully palmitoylated at the same site as *in vivo* (data not shown) and the palmitoylation ratio was greater than 99%. Since only myristoylated $G_o\alpha$ can be palmitoylated and nonmyristoylated $G_o\alpha$ fails to incorporate palmitate[16], our results demonstrated the $G_o\alpha$ was myristoylated. The highly purified $G_o\alpha$ facilitated the further study on the interaction between $G_o\alpha$ and GAP-43.

GAP-43 was prepared by alkali extraction from bovine cerebral cortex. The protein having an apparent molecular mass of 50 kD was highly purified with purity greater than 95% (fig. 1(c)), but its actual molecular mass is 24 kD deduced from the sequence analysis (226 amino acids). The anomalous migration of GAP-43 may be due to its acidic nature, which results in poor binding to SDS and slow migration during electrophoresis[12].

2.2 The influence of Lubrol PX at different concentrations on GTPγS binding assay

Since GAP-43 will not bind to CaM unless some detergent or lipid is included in the buffer[13], 0.1% Lubrol PX must be added during CaM-Sepharose affinity chromatography to purify GAP-43. Lubrol PX is a nonionic detergent with very low CMC value, which is difficult to pass through the ultrafiltration membrane to separate from protein when concentrated, so the concentration of Lubrol PX is very high in the concentrated samples. The GAP-43 without removal of Lubrol PX could not stimulate GTPγS binding to $G_o\alpha$, but it inhibited GTPγS binding activity of $G_o\alpha$. As described[6] previously, GTPγS binding activity of $G_o\alpha$ seems sensitive to the detergent used in the reaction buffer. To explore the influence of Lubrol PX on GTPγS binding assay, we measured ^{35}S-GTPγS binding to $G_o\alpha$ in the presence of various concentrations of Lubrol PX. In the presence of 0.05% Lubrol PX, the GTPγS binding activity of $G_o\alpha$ was twice the control, while in the presence of 0.1% Lubrol PX, the GTPγS binding activity of $G_o\alpha$ was comparable to the control. With the increase of concentration of Lubrol PX, the GTPγS binding activity of $G_o\alpha$ decreased dramatically (fig. 2). Our results indicated that Lubrol PX interfered with the GTPγS binding assay significantly. In order to measure the stimulation of $G_o\alpha$ by GAP-43 successfully, it is necessary to remove Lubrol PX in purified GAP-43 samples. Bio-beads are a kind of hydrophobic absorbent, which can remove detergent of low CMC value effectively[14]. After Bio-beads absorption, almost all the Lubrol PX was removed from the assay buffer.

2.3 Stimulation of GTPγS binding to $G_o\alpha$ by GAP-43

To examine the stimulation of GTPγS binding to $G_o\alpha$ by GAP-43, we found that Lubrol PX concentration inhibiting $G_o\alpha$ activity also inhibited GAP-43 activation. Fig. 3 shows both of the GTPγS binding activity of $G_o\alpha$ and stimulation extent of $G_o\alpha$ by GAP-43 decreased when the concentration of Lubrol PX was greater than 0.05%. The GTPγS binding assay is usually conducted in 0.1% Lubrol PX as reported in references[6,9], which is disadvantageous to examine the stimulation of $G_o\alpha$ by GAP-43.

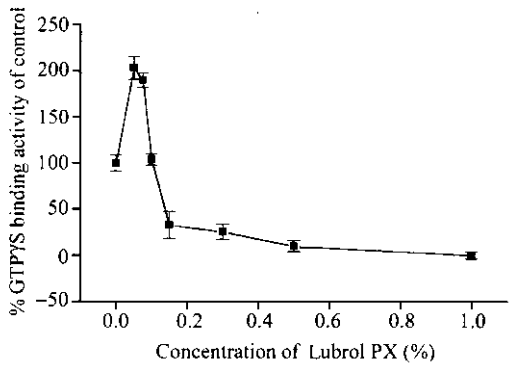

Fig. 2. The influence of Lubrol PX at different concentrations on GTPγS binding assay. ^{35}S-GTPγS binding to 20 nmol/L $G_o\alpha$ was measured in the presence of Lubrol PX at the indicated concentrations (0%, 0.05%, 0.075%, 0.1%, 0.15%, 0.3% 0.5% and 1%). The GTPγS binding activity of $G_o\alpha$ in the absence of Lubrol PX was set as a control. The results are the average of three independent experiments and the standard errors are shown.

When Lubrol PX was removed from the assay buffer, GAP-43 can stimulate GTPγS binding to $G_o\alpha$ effectively (fig. 4). The stimulation effect increased with the increase of concentration of Lubrol PX. It should be noticed that the same stimulation level as those reported[6,9] could be obtained when we used only one-tenth of GAP-43 proteins of that reported.

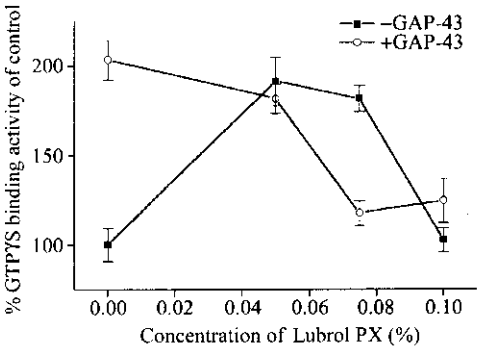

Fig. 3. The influence of Lubrol PX at different concentrations on the stimulation of $G_o\alpha$ by GAP-43. ^{35}S-GTPγS binding to 20 nmol/L $G_o\alpha$ in the presence of 0.2 μmol/L GAP-43 and Lubrol PX at the indicated concentrations (0%, 0.05%, 0.075% and 0.1%) was measured. The GTPγS binding activity of $G_o\alpha$ in the absence of GAP-43 and Lubrol PX was set as a control. The results are the average of three independent experiments and the standard errors are shown.

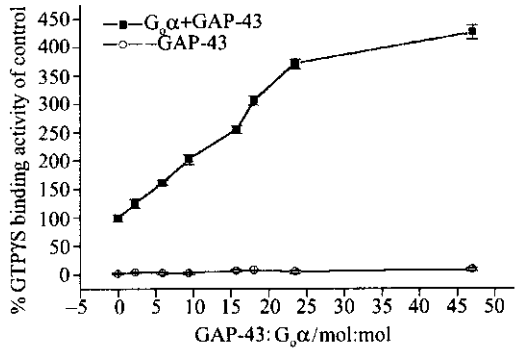

Fig. 4. The stimulation of $G_o\alpha$ by GAP-43 at different concentrations. ^{35}S-GTPγS binding to $G_o\alpha$ in the presence of GAP-43 at the indicated concentrations was measured. The GTPγS binding activity of $G_o\alpha$ in the absence of GAP-43 was set as a control. The results are the average of three independent experiments and the standard errors are shown.

2.4 The direct interaction between GAP-43 and $G_o\alpha$

Although it is clear that GAP-43 stimulates GTPγS binding to $G_o\alpha$, the direct interaction between GAP-43 and $G_o\alpha$ to form intermolecular complex lacks direct evidence. To clarify this

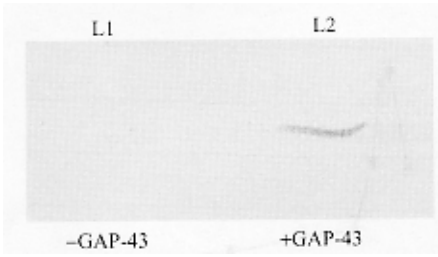

Fig. 5. Western blotting assay of direct interaction between GAP-43 and $G_o\alpha$. Proteins were subjected to 12% SDS-PAGE and subsequent Western blotting assay using rabbit anti-mouse polyclonal antibody specific to $G_o\alpha$. Lane L1, Proteins eluted from CaM-Sepharose in the absence of GAP-43; lane L2, proteins eluted from CaM-Sepharose in the presence of GAP-43. The procedures were performed as described in sec. 1.2.6. Similar results were obtained in three independent experiments.

issue, protein-protein binding experiments were carried out using CaM-Sepharose affinity media. Fig. 5 shows that in the absence of GAP-43, $G_o\alpha$ could not bind to CaM-Sepharose (lane L1) but in the presence of GAP-43, $G_o\alpha$ could be bound with CaM-Sepharose and was eluted from it with Ca^{2+}, which could be detected by anti-$G_o\alpha$ antibody (lane L2).

2.5 Conformational change of $G_o\alpha$ induced by GAP-43

GAP-43 contains a single phenylalanine without tryptophan and tyrosine[19], so its intrinsic fluorescence intensity is very weak when excited at 295 nm. We can measure the tryptophan fluorescence spectra to examine the interaction between GAP-43 and $G_o\alpha$ and conformational change of $G_o\alpha$ induced by GAP-43[20]. Fig. 6(a) shows when the concentration ratio of GAP-43 to $G_o\alpha$ was ten to one, the fluorescence intensity of GAP-43 itself was very weak (curve 3), which could be taken as background; the emission maximum of $G_o\alpha$ • GDP itself was at 338.8 nm when scanned from 300 nm to 400 nm (curve 2); in the presence of GAP-43, the fluorescence intensity of the complex of $G_o\alpha$ • GDP·GAP-43 increased 35.3% significantly and the spectral maximum was also blue shifted to 334.8 nm (curve 1). As for $G_o\alpha$ • GTPγS, no obvious changes of fluorescence spectra were observed in the absence or presence of GAP-43 (fig. 6(b)).

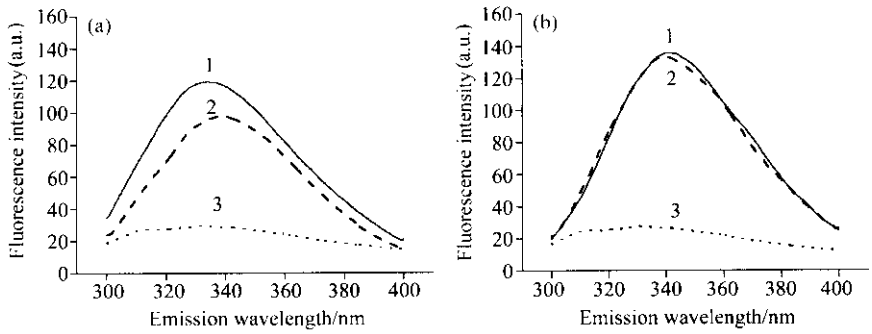

Fig. 6. Tryptophan emission spectrum of $G_o\alpha$. 1 μmol/L $G_o\alpha$ and/or 10 μmol/L GAP-43 were added. (a) Fluorescence spectrum of $G_o\alpha$·GDP in the absence or presence of GAP-43; (b) fluorescence spectrum of $G_o\alpha$·GTPγS in the absence or presence of GAP-43. $G_o\alpha$·GTPγS was prepared by adding 10 μmol/L GTPγS to $G_o\alpha$·GDP and pre-incubaed at 30℃ for 20 min. Similar results were obtained in three independent experiments.

To further explore conformational change of $G_o\alpha$, the intrinsic fluorescence of $G_o\alpha$ • GDP or $G_o\alpha$ • GTPγS in the absence or presence of GAP-43 was quenched with HB. As shown in fig. 7, the quenching data of HB were fitted to modified Stern-Volmer equation and Ksv calculated from equation is shown in table 1. It can be seen from the results that in the presence of GAP-43, the

quenching efficiency of HB of $G_o\alpha$ • GDP increased significantly, but that of HB for $G_o\alpha$ • GTPγS changed little.

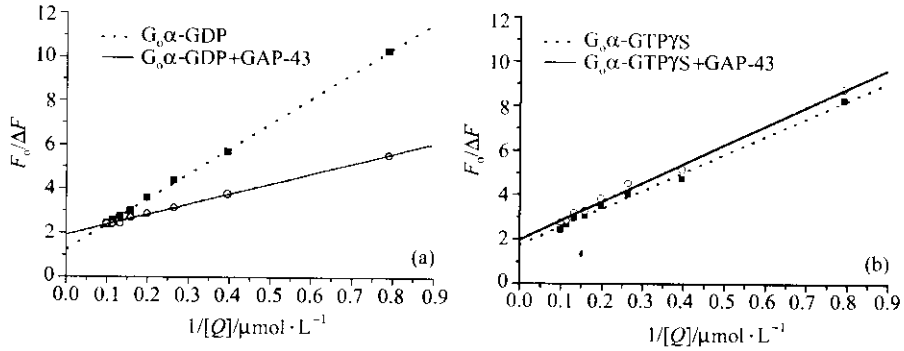

Fig. 7. Modified Stern-Volmer curve of HB quenching of intrinsic fluorescence of $G_o\alpha$. 1 μmol/L $G_o\alpha$ and/or 10 μmol/L GAP-43 were added. (a) Modified Stern-Volmer curve of HB quenching of intrinsic fluorescence of $G_o\alpha$·GDP in the absence or presence of GAP-43; (b) modified Stern-Volmer curve of HB quenching of intrinsic fluorescence of $G_o\alpha$·GTPγS in the absence or presence of GAP-43. $G_o\alpha$·GTPγS was prepared as described above. The intrinsic fluorescence of $G_o\alpha$ was quenched with HB at the indicated concentrations and a linear plot of $F_o/\Delta F$ vs. [Q] was obtained. This result is representation of five independent experiments.

Table 1 Apparent quenching constant of HB quenching of intrinsic fluorescence of $G_o\alpha$

	Ksv/M^{-1}	
Samples	$G_o\alpha$ • GDP	$G_o\alpha$ • GTPγS
–GAP-43	$(1.1 \pm 0.22)\times10^5$	$(2.1 \pm 0.26)\times10^5$
+GAP-43	$(4.1 \pm 0.43)\times10^5$	$(2.3 \pm 0.17)\times10^5$

The data of fig. 7 were fitted to modified Stern-Volmer equation and Ksv was calculated as described in sec. 1.2.8.

3 Discussion

G_o is the major heterotrimeric GTP-binding protein in neuronal growth cone membrane. Activated G protein-coupled receptor enhances GDP release and GTP binding to transduce extracellular signals into cells. The elucidation of regulation of GDP/GTP exchange in GTPase cycle may be helpful to understanding the diversity and specificity of G protein-coupled signal transduction pathways. GAP-43 is a neuron-specific protein enriched on the cytosolic face of the plasma membrane of growth cones. GAP-43 can stimulate G_o like G protein-coupled receptors[9], which may imply GAP-43 is an intracellular regulator of G proteins and exert its function through G protein-coupled pathways, but the molecular mechanism by which GAP-43 affects G_o remains unclear.

In this paper, we examined the interaction between $G_o\alpha$ and GAP-43 using highly purified proteins. Our results revealed that GAP-43 stimulated GTPγS binding to $G_o\alpha$ effectively (fig. 4) and the stimulation was only viable to $G_o\alpha$ • GDP, but not to $G_o\alpha$ • GTPγS. These investigations suggested that GAP-43 affected the rate of GDP/GTP exchange of $G_o\alpha$ • GDP by inducing conformational change from inactivated state to activated state. Once GTP is bound, G_o becomes activation to fulfill its primary role as a regulator of effectors.

Although the stimulation of $G_o\alpha$ by GAP-43 has been reported previously, the direct interac-

tion between GAP-43 and $G_o\alpha$ to form intermolecular complex remains an open question. We examined protein-protein binding between GAP-43 and $G_o\alpha$ using CaM-Sepharose affinity media, which exploits the special binding property of GAP-43 for CaM that GAP-43 has higher affinity for CaM in the absence of bound Ca^{2+} and is dissociated from CaM by Ca^{2+} [13]. It can be seen clearly from fig. 5 that $G_o\alpha$ • GDP bound CaM-Sepharose only in the presence of GAP-43, but not in the absence of GAP-43. This provided powerful evidence for the direct interaction between GAP-43 and $G_o\alpha$ • GDP.

From the results above, it is interesting to further explore the mechanism by which GAP-43 affects $G_o\alpha$. Activity of the protein or enzyme is closely correlated with its proper conformation. Thus we examined conformational change of $G_o\alpha$ induced by GAP-43 by measurement of fluorescence change and HB quenching. In the presence of GAP-43, the spectral maximum of $G_o\alpha$ • GDP blue shifted; its fluorescence intensity increased (fig. 6(a)) and its apparent quenching constant also changed significantly (table 1), but no obvious changes of $G_o\alpha$ • GTPγS were observed (fig. 6(b) and table 1). This demonstrated clearly that GAP-43 induced conformational change of $G_o\alpha$ • GDP so as to enhance GDP/GTP exchange. It is known that $G_o\alpha$ contains two Trp residues, which are Trp132 and Trp212. Trp132 is in the helical domain of $G_o\alpha$ and Trp212 is in the Switch II domain. Trp132 is more buried, while Trp212 is relatively exposed. Thus the intrinsic fluorescence is the major contribution of Trp212 and the change of fluorescence spectrum is mainly corresponding to the change of microenvironment that Trp212 is involved in ref. [21]. According to the published three-dimensional structure of Gα[22], upon activation, conformation of three Switch domains changes obviously, in which Trp212 becomes more embedded into hydrophobic environment. Combining the results described above with three-dimensional structure of Gα, we can speculate that conformational change of $G_o\alpha$ • GDP induced by GAP-43 is related to more exposure of Trp212 to a relatively hydrophobic environment, which is a more active state than $G_o\alpha$ • GDP itself.

In summary, protein-protein interaction experiments using highly purified GAP-43 and $G_o\alpha$ revealed that GAP-43 bound $G_o\alpha$ • GDP directly and induced conformational change of $G_o\alpha$ to stimulate its GTPγS binding. Our studies may be helpful to further understanding the mechanism by which GAP-43 stimulates $G_o\alpha$ and the sense of GDP/GTP exchange in GTPase cycle.

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