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The Mutation in the *N*-terminal Domain of RGS4 Disrupts PA-conferred Inhibitory Effect on GAP Activity

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Regulator of *G* protein signaling (RGS) proteins are GTPase-activating proteins (GAP) for *G* protein α-subunits and are thought to be responsible for rapid deactivation of *G* protein mediated signaling pathway. In this present study, we demonstrate that PA is the most efficient candidate to inhibit GAP activity of RGS4. The functional significance of *N*-terminus of RGS4 in respose to PA-granted inhibition on GAP activity has been studied with the site mutation in the *N*-terminus of RGS4. These site-directed mutations in the *N*-terminal domain do not severely disrupt its association with liposomes of PA. However, RGS4L23E diminishes the inhibition of GAP activity by PA compared with the wild type RGS4, whereas RGSR22E abrogates the inhibitory effect by PA on GAP activity. The correspondent conformational discrepancy in the RGS domain of these mutants in the presence of PA vesicles was detected from fluorescence experiments. It is suggested that the functional pertinence between the *N*-terminus and RGS domain may be important to modulate PA-conferred inhibitory effect on its GAP activity.

KEY WORDS: RGS4; *N*-terminal mutation; PA-conferred inhibition; GAP; RGS domain; conformational change.

ABBREVIATIONS: RGS, regulator of G-protein signaling; GAP, GTPase-activating proteins; *G* protein, guanine nucleotide-binding protein; $G_{i\alpha 1}$, myristoylated $G_{i\alpha 1}$; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; SUVs, small unilamellar vesicles; Trp. tryptophan.

INTRODUCTION

A variety of cellular signals are transmitted through G-proteins mediated signaling pathway. Regulation of G-protein intrinsic GTPase activity represents a critical mechanism for establishing proper signal strength and duration (Neer *et al*., 1995). Recently a novel protein family of RGS named after Regulator of G-protein Signaling has been discovered to serve as GTPase-activating proteins (GAP) to stimulate GTP hydroysis by G-proteins (Berman *et al*., 1996; Watson *et al*., 1996; Hunt *et al*., 1996; Zheng *et al*., 2001). RGS proteins from yeast to mammals share a highly

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conserved RGS domain that retains full GAP activity toward G_α subunits *in vitro* as full-length RGS proteins (Popov *et al.*, 1997).

The ubiquity and stimulation on GTP hydrolysis of RGS proteins virtually demand their regulation (reviewed by Ross *et al.*, 2000). The GAP activities of RGS proteins are modulated by phosphorylation of RGS proteins themselves and of their G_α substrates (Wang *et al.*, 1998; Ogier-Denis *et al.*, 2000; Cunningham *et al.*, 2001). In addition to the regulation role of phosphorylation in signaling proteins, palmitoylation is increasingly recognized as an important modification of eukaryotic signaling proteins (Dunphy *et al.*, 1998). Recent studies demonstrate that GAP activities of RGS proteins can also be regulated by the reversible palmitoylation of both RGS proteins and their counterparts G_α (Tu *et al.*, 1997, 1999).

Since RGS proteins must act at the inner surface of the plasma membranes to modulate membrane-bound *G* proteins signaling pathway (Srinivasa *et al.*, 1998a; Zeng *et al.*, 1998; Chen *et al.*, 1999), it is reasonable to hypothesize that certain phospholipids in the cellular membrane may influence the interaction between RGS proteins and their targets G_α. We found that PA shows much greater potency to inhibit GAP activity of RGS4 than other phospholipids tested. It is reported that the amphiphathic structure of RGS4 in the *N*-terminus plays a critical role in its association with membranes (Bernstein *et al.*, 2000), so the corresponding residues in the *N*-terminus of RGS4 were mutated to further test their response to the inhibitory effect by PA. We report herein that RGS4L23E attenuates its response to PA-conferred inhibition, whereas, RG4R22EE abrogates the inhibitory effect by PA on GAP activity. These mutants in the *N*-terminus do not have significant effects on the binding of RGS4 to PA vesicles. To elucidate the mechanism of the disruption of PA-granted inhibitory effect on GAP activities, the conformation of RGS4 and these mutants in the presence of PA vesicles was compared. It is revealed that more compact conformation in the RGS domain was associated with the wild type RGS4 in the presence of PA vesicles and that remarkable discrepancy in the conformation within the RGS domain occurs when such residues as Arg^{22}(R22) or Leu^{23}(L23) in the *N*-terminus were substituted. By virtue of the structure of RGS4-$G_{i\alpha 1}$ complex obtained from the crystallographic studies (Tesmer *et al.*, 1997), it can be deduced that such difference in the conformation occurred at the interface between RGS4 and $G_{i\alpha 1}$, which is triggered by the site mutation in the *N*-terminus, are probably correlated with the differential disruption of PA-granted inhibitory effect on GAP activities.

MATERIALS AND METHODS

Materials

The cDNA constructs used for expression of RGS4 and $G_{i\alpha 1}$ have been described in (Tu *et al.*, 1999). RGS4 and its mutants, $G_{i\alpha 1}$ were purified from *E. coli* (Tu *et al.*, 1997; Mumby *et al.*, 1994). The specific activity of $G_{i\alpha 1}$ protein was determined by [^{35}S]GTPγS binding assay (Northup *et al.*, 1982). PC (bovine brain), PG (bovine brain), PS (bovine brain), and PA (egg yolk) were from Avanti Polar Lipids Inc.

Imidazole and Tween 20 were purchased from Sigma. Guanosine-5′-O-(3-thiotriphosphate) (GTPγS) and GTP were purchased from Boehringer-Mannheim. Acrylamide (ultra purity) was from Gibco. NI^{2+}-nitrilotriacetic acid (NTA)-agarose column wa purchased from Qiagen Company. [γ-^{32}P]GTP (30 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were obtained from Perkin Elmer Life Sciences. An antibody to RGS4 C-terminus and secondary antibody were products of Santa Cruz Biotechnology. P-nitrophenyl phosphate (pNPP) was from Amersco. The 96-well microtiter plates were obtained from Nunc. All other chemicals were of analytical grade available in China.

Site-directed Mutagenesis of RGS4

Point mutations of RGS4 cDNA were performed by polymerase chain reaction using the Quick change mutagenesis kit (Stratagene) and specific mutagenic primers as follows (the mutated codons are in bold):

RGS4R22E: primer 1, GGATATGAAACAT**GAG**CTGGGATTTCTG,
primer 2, CAGAAATCCCAG**CTC**ATGTTTCATATCC;
RGS4R22E: primer 1, GATATGAAACATGG**GAG**GGATTTCTGCTG,
primer 2, CAGCAGAAATCC**CTC**CCGATGTTTCATATC.

The RGS4 coding regions of all constructs were sequenced to verify that only the desired mutations had been introduced.

Preparation of SUVs

Desired phospholipids in chloroform solution were dried under a stream of nitrogen. Residual solvent were removed under high vacuum for 3–4 hr. The phospholipid films were then suspended in a solution of 25 mM Hepes, pH 7.4, 1 mM DTT (Buffer A) and vortexed thoroughly for hydration. Small unilamellar vesicles (SUVs) were prepared by using a probe sonicator for approximately 2–3 min intervals to optical clarity. Then the supension was centrifuged to remove large vesicles and metal debris from the titanium tip of the probe. SUVs in the supernatant were used the same day as prepared.

Binding of RGS4 or its Mutants to PA Vesicles Assayed by ELISA

All procedures were performed similar to Ghoh *et al.* (Ghosh *et al.*, 1996). In brief, phospholipid solutions (10 μg per well) diluted in methanol were allowed to bind overnight at 4°C. The following ELISA steps were performed at room temperature. The wells were blocked with 3% bovine serum albumin (BSA) in PBS (pH 7.5) for 2 hr. RGS4 and its mutants diluted in PBS containing 0.3% BSA were added to the well, and incubated for 1 hr. Bound RGS4 and its mutants were detected by employing anti-RGS4 C-terminus goat polyclonal antibody (1/500) and a donkey anti-goat IgG-alkaline phosphatase conjugate (1/8000). After excess secondary antibody had been washed off, a 10 mg/ml solution of pNPP in buffer (10 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) was added to the wells for color

development. The reactions were terminated by the addition of 33 mM EDTA (final concentration). The resulting absorbance was quantitated at 405 nm on an ELISA plate reader (Bio-Rad). The magnitude of the absorbance was directly related to the amount of RGS4 and its mutants bound, and used to determine the apparent dissociation constant (Kd).

RGS4 GAP Assays

Single turnover [γ-^{32}P]GTPase assays at low temperature (4°C) were performed as described previously (Tu *et al.*, 1997). To study the influence of anionic phospholipids on RGS4 and its mutants GAP activity, 2 μM RGS4 or its mutants were preincubated with 200 μM anionic phospholipid or buffer alone as a control in buffer (25 mM Hepes, pH 7.5, 10 mM EDTA, 50 mM NaCl, 1 mM Dtt) at 30°C for 30 min, and then paced on ice for 10 min before initiation of the reaction. The final concentrations of RGS4 and phospholipids in assay were 0.08 and 8 μM respectively. In this assay. GAP activity is defined as the increase in the first-order hydrolysis rate constant (k_{hydrol}) (Tu *et al.*, 1997).

Fluorescence Spectroscopy

Fluorescence measurements were performed with a Hitachi F4500 fluorescence spectrophotometer using a 1 cm quartz fluorescence cuvette. The temperature was maintained at 30°C. RGS4 or its mutants were incubated with PA vesicles at 30°C for 30 min before measurement. Excitation wavelength was set at 280 nm. Tryptophan emission spectra were recorded from 300 to 400 nm. Background intensity in samples without protein was subtracted, and the data of emission fluorescence intensity were determined from the corrected spectra.

For fluorescence quenching experiment, aliquots of 6 M acrylamide stock solution freshly prepared were added to the protein solution in the absence of or presence of SUVs to achieve the desired acrylamide concentration. After each addition of the quencher, the mixture was stirred and equilibrated for at least 5 min before the emission intensity at 340 nm was recorded. The concentrations of RGS4 and its mutants were 1.4 μM, and the molar ratios of PA vesicles to proteins were 100:1. Excitation was set at 295 nm instead of 280 nm to reduce the absorbance by acrylamide and the inner filter effects of acrylamide itself were corrected (Kroon *et al.*, 1990). The data were corrected for dilution and background contribution and fitted by the Stern–Volmer equation (Eftink *et al.*, 1981): $F_0/F = 1 + K_{SV}[Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, $[Q]$, is the concentration of quencher, K_{SV} is the Stern–Volmer quenching constant (M^{-1}).

RESULT AND DISCUSSION

The Mutation in the *N*-terminus of RGS4 Disrupts the Inhibition of GAP Activity by PA

Heterotrimeric *G* proteins must be associated with the plasma membrane to transduce extracellular signals across the plasma membrane to intracellular effector

molecules, thereby eliciting cellular response. In addition, it has been well established that plasma membrane-association is required for RGS4 to regulate the G-protein signaling pathway *in vivo* (Srinivasam *et al.*, 1998a; Zeng *et al.*, 1998), so it is conceivable that certain phospholipids in the cellular membrane may influence the interaction between RGS proteins and their targets G_α. It can be seen from Table 1 that RGS4-accelerated GTP hydrlysis by G_α was markedly decreased in the presence of anionic phospholipids, while neutral phospholipids PC was completely inactive at the same molar ratio of phospholipid to protein 100:1. Among the anionic phospholipids tested, 8 μM phosphatidic acid (PA) inhibited about 80% GAP activity of RGS4, which indicates that PA has the greatest ability to inhibit GAP activity of RGS4. As a control, the intrinsic GPAase activity of $G_{i\alpha 1}$ was unaffected by the anionic phospholipids (data not shown). It has been demonstrated that phospholipid metabolism is often altered in respone to a wide variety of hormones, growth factors, and other extracellular agonists. Membrane PA levels increases rapidly upon stimulation of cells with these stimuli (reviewed in English *et al.*, 1996). As shown in Table 1, RGS4 GAP activity is mostly inhibited by PA. This suggests that PA may participate in the regulation of interaction between RGS4 and G_α.

It has been reported that *N*-terminal amphiphatic domain of RGS4 is responsible for its association with membranes (Bernstein *et al.*, 2000). So it is rational to assume that the mutation in the *N*-terminal domain of RGS4 may influence its association with membranes, and thus may alter its response to PA-granted inhibitory effect on GAP activity. Based on this assumption, we introduced respectively mutation to the basic and hydrophobic residues in the *N*-terminus of RGS4 and examined their sensitivities to the inhibitory effects by PA. As shown in Fig. 1, these site-directed mutants in the *N*-terminus behaved to such diferent degrees in response to the inhibitory effects by PA in that RGS4L23E attenuated the inhibition by PA on GAP activity as compared with the wild type RGS4, whereas RGS4R22E failed to respond to the inhibitory effect by PA on GAP activity at the same molar ratio

Table 1. Effects of Anionic Phospholipids on GAP Activity of RGS4

	K_{hydrol} (min^{-1})
Control	0.2 ± 0.04
RGS4	1.51 ± 0.05
RGS4/PC	1.48 ± 0.07
RGS4/PS	0.9 ± 0.10
RGS4/PG	1.0 ± 0.05
RGS4/PA	0.34 ± 0.04

RGS4 was preincubated with different phospholipids before initiation of assay. The final RGS4 concentration in assay was 0.08 μM and the molar ratios of phospholipids to RGS4 were all about 100:1. Control represents the first-order hydrolysis rate constant (K_{hydrol}) determined in the absence of RGS4. These values are averages of three experiments.

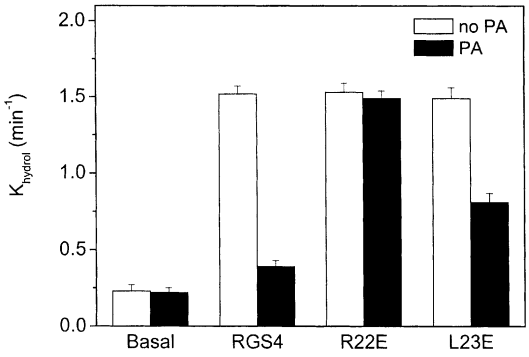

Fig. 1. The change in GAP activities of RGS4 and its mutants in the presence of PA vesicles. 2 μM RGS4 or its mutants were preincubated with 200 μM PA ir buffer as a control for 30 min at 30°C before initiation of the reaction. The final concentrations of RGS4 and PA or buffer as a control for 30 min at 30°C before initiation of the reaction. The final concentrations of RGS4 and PA in assay were 0.08 and 8 μM respectively, PA to proteins molar ratio wa 100:1. Basal activity was assayed in the absence of RGS4. GAP activity was assayed as described under "Materials and Methods."

of PA to proteins. It should be noted that all the mutants including RGS4R22E and RGS4L23E were as effective GAPs in solution as the wild type RGS4 (Fig. 1).

Membrane Binding Charistic of RGS4 and Its Mutant

To evaluate quantitatively the binding competence of these mutants to PA vesicles, we utilized the antibody to RGS4 to recognize the membrane-bound proteins. Fitting these data obtained to the one site binding equation revealed an apparent dissociation constants (K_d) that can reflect the affinity of proteins for PA membranes. Although the relatively bound ratio of RGS4R22E and RGS4L23E was about 85% of that in the wild type RGS4 according to the fitting data, RGS4R22E exhibited PA binding affinity almost identical to the wild type RGS4 with apparent K_d around 23 nM; as compared with RGS4 and RGS4R22E, RGS4L23E yielded a slightly higher K_d, i.e., 32.0 ± 2.9 nM (Fig. 2). These data indicate that the mutants in the *N*-terminus do not completely disrupt RGS4 binding to PA vesicles and have an obvious affinity with PA membranes although there is some slight affinity difference between them.

It is worthy of being noted that while the Arg^{22}(R22) mutation in the *N*-terminus has almost the same apparent K_d (approximately 23 nM) for PA as the wild type RGS4 (Fig. 2), however, RGS4R22E abrogates the inhibitory effect by PA on GAP activity as compared with the wild type RGS4 (Fig. 1). This finding renders unlikely the possibility that the binding competence is exclusively responsible for such notably distinctive response of these mutants to PA-granted inhibitory effect

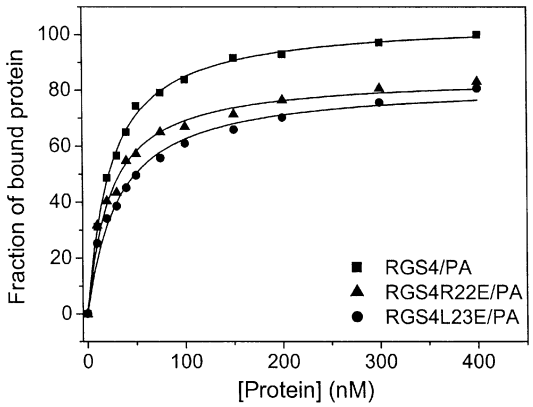

Fig. 2. Binding isotherms of RGS4 or its mutants to PA as determined by ELISA. Each well of an ELISA plate was coated with 10 μg PA and subequently blocked by incubation with BSA. Thereafter, the wells were incubated with the increasing amounts of RGS4 or its mutants as indicated in the figures. The bound proteins were analyzed by the antibody to the *C*-terminus of RGS4.

on GAP activity (Fig. 1); however, other factors may contribute to the difference in GAP activity upon association with PA vesicles.

It is demonstrated that the RGS domain is responsible for GAP activity of RGS4 toward G_α subunits (Popov *et al.*, 1997). The interaction at this interface between RGS4 and $G_{i\alpha 1}$ is very crucial for RGS4 GAP activity obtained from the crystallographic studies (Tesmer *et al.*, 1997; Moy *et al.*, 2000). It has been reported that disturbance at this interface have a disadvantageous effect on GAP activities of RGS proteins (Srinivasa *et al.*, 1998b; Natochin *et al.*, 1998; Posner *et al.*, 1999). From the crystal structure, it can be noticed that one of RGS4 tryptophan (Trp), Trp^{92} is localized just in the vincity of the interface between RGS4 and $G_{i\alpha 1}$ (Tesmer *et al.*, 1997). Therefore, it is interesting to further explore whether the conformational change in the RGS domain triggered by the mutation in the *N*-terminus may lead to such notable difference in response to inhibitory effect by P on GAP activity.

Conformational Alterations of RGS4 and Its Mutants upon Interaction with PA Vesicles Intrinsic Fluorescence Measurements

The sensitivity of intrinic fluorescence to the environment of the Trp residue is well established and has been widely used in studies of peptide or protein-phopholipid bilayer interaction (Lakowicz *et al.*, 1983). It can be seen from Fig. 3 that the emission spectrum of RGS4 in solution showed a peak at 332 ± 1 nm (Fig. 3A in solid line). Upon interaction with PA vesicles, RGS4 yielded a noticeable red shift in emission maximum (from 332 to 338 nm) and substantial enhancement in the

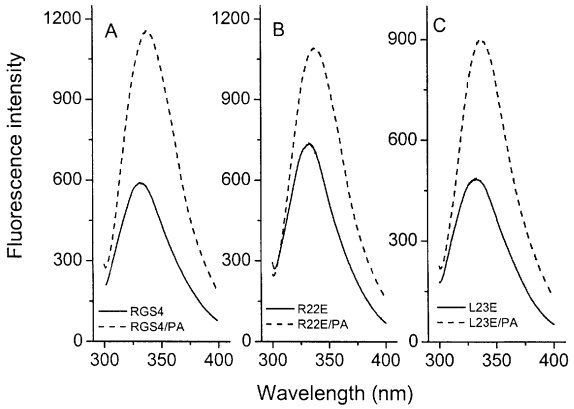

Fig. 3. The change in the fluorescence emission spectra of RGS4 and its mutants upon interaction with PA vesicles. RGS4 and its mutants were 0.54 μM, and PA to proteins molar ratio was 100:1. Excitation wavelength was set at 280 nm. The emission spectra were shown as background subtracted. The emission and excitation slit width were set both at 10 nm.

fluorescence intensity (Fig. 3A in dash line), which suggests that a significant conformational change does occur when RGS4 is associated with PA vesicles. We have observed that RGS4 failed to bind to neutral phospholipid PC membranes as noticed by Linder and his colleagues (Bernstein *et al.*, 2000) and that there was neither increase in fluorescence intensity nor the red shift in the maximal emission wavelength when RG4 wa incubated with PC vesicles (data not shown), which indicates that the global conformation in RGS4 is not altered in the presence of PC vesicles. This further confirms the idea that such a conformational change in the presence of PA vesicles is caused by RGS4 binding to membranes and thus the incompetence of PC to inhibit GAP activity of RGS4 as shown in Table 1 is also readily explained.

When the mutation was introduced to the *N*-terminus of RGS4, RGS4R22E, and RGS4L23E both yielded the similar emission spectra in solution as the wild type RGS4 with identical maximal emission wavelength at about 332 nm except some differences in the fluorescence intensities (Fig. 3B and C in solid line). In the presence of PA vesicles, analogous to the wild type RGS4, both mutants underwent red shifts in the emission maxima from 332 to 338 nm, which suggests that the remarkable conformational change occurred is due to membrane binding of these mutants RGS4R22E and RGS4L23E as revealed in Fig. 2. However, there were differences in altered amplitude of fluorescence intensities, implying that although microenvironments of Trp in the wild type RGS4 and the mutants RG4R22E and RGS4L23E were all altered in the presence of PA vesicles, the conformational changes in proteins were not of the same extents (Fig. 3). There are two Trp residues in RGS4 that includes Trp^{59} and Trp^{92}. It is logical to ask which one of two Trp residues in RGS4 contributes to such conformational changes of proteins observed in the presence of PA vesicles. To address this question, we generated one mutant RGS4W59A to possess solely Trp^{92} in the RGS domain but not RGS4W92A

because it has been reported that RGS4W92A fails to acclerate GTP hydrolysis of G_α and thus cannot reflect what it happens in the wild type RGS4 upon association with PA vesicles (Srinivasa *et al.*, 1998b), while RGS4W59A mutant retains full GAP activity as the wild type RGS4 (data not shown). The emission spectrum of RGS4W59A protein, which contains only Trp^{92} in the RGS domain, also red-shifted from 332 to 338 nm and increased its fluorescence intensity similar to the wild type RGS4 upon interaction with PA vesicles (data not shown), which suggests that the conformational change observed in RGS4 and its mutants in the presence of PA vesicles was indeed mainly donated by the Trp^{92} residue in the RGS domain.

Fluorescence Quenching Studies

According to the published three-dimenional structure of RGS4 and the RGS4-$G_{i\alpha 1}$ complex, Trp^{92} is in the RGS domain that is in charge of GAP activity, proximate to the interface between RGS4 and $G_{i\alpha 1}$, and exposed to the hydrophilic environment, while the Trp^{59} residue is completely buried in the structure (Tesmer *et al.*, 1997; Moy *et al.*, 2000). Acrylamide is a neutral polar and efficient quencher of tryptophan fluorescence that is very sensitive to the exposure of Trp in proteins and has been extensively used to probe the conformational change in the hydrophilic region of proteins (Eftink *et al.*, 1981; Lackowicz *et al.*, 1983; Kroon *et al.*, 1990). To further monitor the conformational change at the interaction interface between RGS4 and $G_{i\alpha 1}$, based on the crystal structure described above, acrylamide was applied to quench fluorescence emitted from Trp^{92} located in the RGS domain. We have observed that PC vesicles have no effect on the accessibility of Trp^{92} to acrylamide (data not shown), which suggests that the change in acrylamide quenching could be thought to be a real conformational change in proteins and exclude the possibility that phospholipids simply shield the Trp residue in proteins from accessing the quencher acrylamide. As shown in Fig. 4A, the degree of quenching

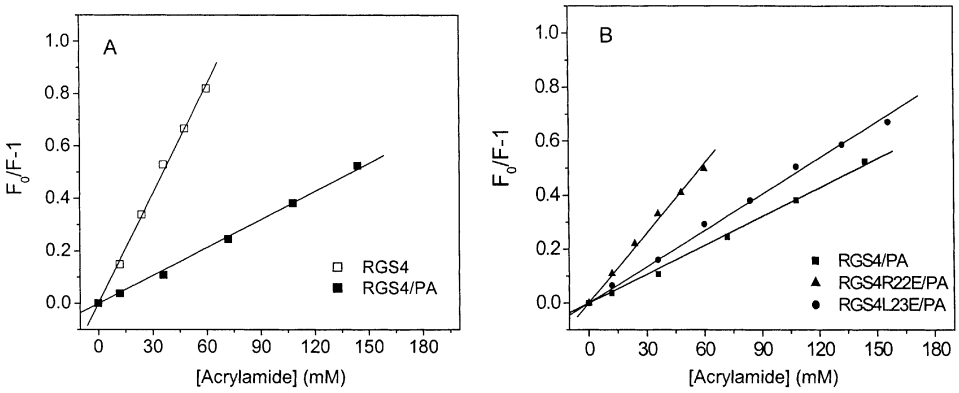

Fig. 4. Quenching of the intrinsic fluorescence intensities of RGS4 and its mutants by hydrophilic quencher acrylamide. The concentrations of RGS4 and its mutants were 1.4 μM, PA to proteins molar ratio was 100:1. Excitation wavelength, 295 nm; emission wavelength, 340 nm. F_0 and F represent the fluorescence intensities of proteins in the absence and presence of the quencher respectively.

for RGS4 in the presence of PA vesicles was sharply decreased by about 75% (Fig. 4A), suggesting that the conformation in the RGS domain bcomes more compact as compared with that in solution and thus the accessibility of Trp^{92} to acrylamide is considerably decreased. Therefore, it can be inferred that such remarkably altered microenvironment around the Trp^{92} residue induced by PA may have a significant influence on the interaction of neighboring residues involved in RGS4 with its counterpart $G_{i\alpha 1}$ as shown in the RGS4-$G_{i\alpha 1}$ complex crystal (Tesmer *et al.*, 1997) and thus inhibits RGS4 GAP activity at the same molar ratio of phospholipids to protein (100:1) (Fig. 1).

When the site-mutations were introduced in the *N*-terminus of RGS4, these mutants had the similar accessibility to acrylamide in solution as the wild type RGS4 (data not shown). However, as opposed to the sharp decrease of accessibility to acrylamide (about 75%) observed in the wild type RGS4 in the presence of PA vesicles, the acrylamide-induced quenching of RGS4R22E fluorescence was slightly dcreased by approximately 15% at the same concentration of PA vesicles. These data suggested that RGS4R22E mutant could not poise the more compact conformation around Trp^{92} residue seen in the wild type RGS4 in the presence of PA vesicles (Fig. 4A), so RGS4R22E failed to respond to the inhibitory effect by PA on GAP activity (Fig. 1). As summarized in Table 2, the quenching constants (Ksv) are significantly different with changes in the order: RGS4 < RGS4L23E < RGS4R22E when these proteins bind to PA vesicles. RGS4L23E displayed intermediate quenching to acrylamide in the presence of PA (Fig. 4B and Table 2) and thus reduced the inhibition by PA on GAP activity compared with the wild type RGS4 (Fig. 1). The results obtained support the idea that the more compact conformation in the RGS domain, the greater was the inhibitory effect by PA on GAP activity.

It has been demonstrated that RGS4 binding to membranes is dependent on its *N*-terminus (Bernstein *et al.*, 2000). Upon binding to vesicles made of anionic phopholipids through its *N*-terminus, the conformation in the RGS domain was altered significantly as characterized by the fluorescence measurements (Figs. 3 and 4), thus leading to the inhibition of GAP activity of RGS4. Our work presented here revealed the functional connection of the *N*-terminal domain and the RGS domain of RGS4. Furthermore, it has been found that Arg^{22}(R22) in the *N*-terminus was critical for the PA-conferred inhibitory effect on GAP activity, which is undertaken by the RGS domain. Analysis of conservation of the residues in the *N*-terminus revealed that Arg^{22}(R22), Leu^{23}(L23) are highly conserved in the RGS4 class family, indicating that these conserved residues are important for the functional specificity of this class family. Moreover, a model for the *N*-terminal amphipathic α-helix has shown that Arg^{22} lies on the polar and nonpolar interface of α-helix, whose side

Table 2. Comparisons of Quenching by Acrylamide for RGS4 and its Mutants in the Presence of PA

	RGS4/PA	R22E/PA	L23E/PA
K_{SV} (M^{-1})	3.55	8.61	4.43

Data in Fig. 4 were fitted the Stern–Volmer equation to obtain the values of K_{SV}.

chain extends outside the α-helix just like an antena and could associate wth the head groups of anionic phospholipids of membranes, whereas Leu^{23} along with other hydrophic residues are thought to yield hydrophobic interactions with the phospholipid acyl chains in the inner surface of membranes (Chen *et al*., 1999), so RGS4 association with membranes recognized by these residues such as Arg^{22} or Leu^{23} in the *N*-terminus may influence its orientation in membranes, and thus could induce more compact conformation in the RGS domain by PA through domain–domain communications. In related with such a conformational change, the GAP activity of RGS4 is notably inhibited by PA.

To date, RGS4 mutational work focused on the RGS domain to elucidate the functional importance of this domain, but yet it has alo been admitted that flanking sequences outside the RGS domain are indispensable for its divergent function (reviewed by DeVries *et al*., 1999). It has been shown that the RGS4 *N*-terminal domain is required for the efficient interaction with G_q (Zeng *et al*., 1998). Our data strengthen the argument of allosteric interaction between the *N*-terminus and the RGS domain of RGS4 and highlight a putative role of RGS4 *N*-terminus in modulation of PA-conferred inhibitory effet on its GAP activity.

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