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Inhibition of Retinal Guanylyl Cyclase by the RGS9-1 N-Terminus

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Cyclic GMP plays a key role in retinal phototransduction and its photoreceptor concentration is precisely controlled by the cooperative action of cGMP phosphodiesterase (PDE) and retinal guanylyl cyclase (retGC). However, studies of the relationship between these two systems have focused only on a Ca^{2+}-mediated, indirect connection. Using a retinal "regulator of G-protein signaling" (RGS9-1) and its fragments, we show that the N-terminus of RGS9-1 inhibits retGC activity. We also indicate that the GGL domain and/or the RGS domain function as an internal suppressor against the N-terminus, suggesting that proteins bound to these domains regulate the inhibitory activity of the N-terminus. Direct interaction of retGC with RGS9-1 and its N-terminus is also proved by immunoprecipitation and an overlay technique. Since RGS9-1 also controls the lifetime of transducin-activated PDE through regulating GTPase activity of transducin, this study strongly suggests that RGS9-1 mediates the direct interaction between PDE and retGC systems, and that this ingenious mechanism plays an important role in tuning of cGMP concentration in photoreceptors. © 2001 Academic Press

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In vertebrate retinal rod outer segments (ROS), activation by light of cGMP phosphodiesterase (PDE) is essential for visual excitation (1, 2). Under dark conditions, PDE is composed of P$\alpha\beta$ (the catalytic subunits) and two Pγs (the regulatory subunits) and is represented as P$\alpha\beta\gamma\gamma$. After illumination, rhodopsin changes its conformation and its meta II form stimulates GTP/GDP exchange on Tα (the α subunit of transducin). Then, the GTP/Tα formed is dissociated from T$\beta\gamma$ (the $\beta\gamma$ subunits of transducin), interacts with P$\alpha\beta\gamma\gamma$ and releases the inhibitory constraint of Pγ from P$\alpha\beta$. PDE is thereby activated. The resulting decrease of cytoplasmic cGMP concentration in ROS leads to the closure of cGMP-gated channels and the hyperpolarization of plasma membranes. During the PDE activation, retinal guanylyl cyclase (retGC), the enzyme for the synthesis of cGMP from GTP in ROS, remains inactive. Its basal activity appears to be too low to account for the increase of cGMP concentration in ROS, although the interaction between PDE and retGC systems has not been thoroughly investigated.

Restoration of the dark membrane potential requires recovery to the dark level of cytoplasmic cGMP concentration in ROS (1, 2). The recovery of the dark level of cGMP concentration needs two processes: deactivation of the PDE cascade and an increase of cytoplasmic cGMP concentration. In the deactivation of the PDE cascade, two mechanisms have been mainly studied: phosphorylation of illuminated rhodopsin and deactivation of GTP/Tα-activated PDE. For the deactivation of PDE, GTP-hydrolysis by Tα is essential in the prevailing model, although there have been several questions in this mechanism including regulation of the lifetime of GTP/Tα-activated PDE (3), the requirement of GTP hydrolysis (4–9) and the role (10) and rate (11) of GTP hydrolysis. For the rate of GTP hydrolysis, the intrinsic GTPase activity of Tα measured *in vitro* (11) is much slower than the rate of termination of phototransduction. Subsequently, a protein to stimulate the GTPase activity of Tα was isolated from ROS (12). The protein is a member of a gene family called "*r*egulators of *G* protein *s*ignaling" (RGS) (13) and is termed RGS9 (RGS9-1). Later a nonretinal form of RGS9, RGS9-2 (14, 15), was also found. However, "RGS9" represents RGS9-1 in this study because this study deals with only RGS9-1.

RGS proteins are characterized by a homologous ~120-amino acid region, referred to as the RGS do-

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main (Fig. 1), that is responsible for association with the α subunit of G protein and stimulation of its GTPase activity (13). However, outside of this RGS domain, the more than 30 members of the family are structurally diverse. Depending these outside domains, RGS proteins can be subclassified into small and large proteins. The large subfamily of RGS proteins, including RGS9, contains additional sequences that may mediate subcellular targeting and assemble signaling complexes. For example, RGS9 contains a G protein γ-like (GGL) domain that is responsible for the specific interaction with the neuro-specific G protein β subunit, Gβ5 (16, 17). RGS9 complexed with Gβ5L (the long splice variant of Gβ5) acts as a GTPase accelerator for Tα in concert with Pγ *in vitro* (18, 19). RGS9 also contains a DEP (Disheveled/EGL-10/pleckstrin homology) domain in its N-terminus (13) and a C-terminal tail (12). Very recent studies suggest that all domains of RGS9, except the N-terminus, contribute to the GTPase-accelerating activity and Pγ coupling (18, 20). However, a target protein(s) for the N-terminus and its function remain unknown.

For the increase of cytoplasmic cGMP concentration in photoreceptors, RetGC has been studied as a main enzyme (1, 2). After activation of PDE and subsequent reduction of cytoplasmic cGMP concentration, the closing of cGMP-gated channels block Na^{2+} and Ca^{2+} influx, and allow a Na^{2+}/Ca^{2+}, K^+ exchanger to decrease cytoplasmic Ca^{2+} concentration (1, 2). Then, retGC is activated (21). The Ca^{2+}-sensitive stimulation of retGC is mediated by at least two calmodulin-like Ca^{2+}-binding proteins termed by GCAPs (*g*uanylyl *c*yclase *a*ctivating *p*roteins), 1 and 2 (22, 23). It has been suggested that retGC dimerization is involved in the activation of retGC by GCAPs in the presence of low Ca^{2+} concentration (24). Thus, in the prevailing model of phototransduction, PDE and retGC systems are indirectly connected by the Ca^{2+}-mediated mechanism. However, several studies (25, 26), although preliminary, have suggested that PDE and retGC are also directly connected by proteins involved in these two systems. One of these studies has shown that RGS9 interacts with and slightly inhibits purified retGC (25). In this study, we show that the N-terminus of RGS9, the domain whose function is unclear in very recent studies (18, 20), inhibits retGC activity even in the presence of GCAPs. Moreover, the GGL domain and/or the RGS domain appear to function as an internal repressor against the N-terminus. Thus, this study implies that proteins bound to the GGL domain and/or the RGS domain, such as Gβ5L and/or GTP/Tα, control the inhibition of retGC by the RGS9 N-terminus. This novel relationship between PDE and retGC systems seems to reflect the complexity of mechanisms required for the precise control of cGMP concentration in photoreceptors.

MATERIALS AND METHODS

Materials. Dark-adapted frozen bovine retinas were purchased from Dr. Yee-Kin Ho (University of Illinois, Department of Biochemistry, Chicago, IL). SP-Sepharose Fast Flow was obtained from Pharmacia Biotech, Inc. Other materials were purchased from various sources as described (24). GCAP2 was a gift from Dr. Alexander Dizhoor (Wayne State University, Kresge Eye Institute, Detroit, MI). Antibodies against retGC and RGS9 were reported previously (25).

Preparation of ROS membranes and retGC. Bovine ROS, its homogenate (ROS homogenate) and GCAP-free ROS membranes were prepared as described (24). RetGC was purified from bovine ROS (27) and its purity was greater than 95%. The purified retGC was stored in liquid nitrogen until use.

Cloning, expression, solubilization, and purification of RGS9 and its fragments. Recombinant RGS9 and its fragments (Fig. 1) were expressed as His-tagged proteins. Total RNA isolated from bovine retinas was converted to cDNA using Superscript II RT (Gibco BRL). The resulting cDNA was amplified by PCR using oligonucleotide pairs (Table 1) containing unique sites for restriction endonucleases *Nde*I or *BamH*I. These sites were used for cloning amplified cDNA fragments into the prokaryotic vector pET-19b (Novogene). The vector was used for protein expression in *E. coli* BRL(DE3)LysS (Novogene). The attached His-tag sequence is MGHHHHHHHHHHSSGHIDDDDKH (molecular weight 2784 Da). Bacteria culture was grown at 37°C. At an A_{600} ~0.5, protein expression was induced by the addition of 1 mM (final) isopropyl β-D-thiogalactopyranoside, and cells were incubated for another 3–4 h at room temperature. Then, cells were spun down (1700*g*, 20 min, 4°C). The pellet was suspended in 1/10 volume of Buffer A (50 mM Tris/HCl (pH 7.5), 2 mM EDTA, 0.1 mM PMSF, 50 mM NaCl, and 1 mM DTT) and sonicated at 0°C. Then, insoluble materials were spun down (40,000*g*, 20 min, 4°C). The pellet was further washed with 20 ml of cold Buffer A (3 times). The washed pellet was suspended in 20 ml of Buffer B (20 mM Tris/HCl (pH 7.5), 0.1 mM PMSF, 1 mM DTT) containing 6 M urea. After incubation for 1 h at room temperature with rotation, the solution was centrifuged (45,000*g*, 30 min, 4°C). The clear supernatant was dialyzed against Buffer B containing 75% glycerol, as described (28). Proteins in the 75% glycerol solution was further dialyzed against Buffer B containing 20% glycerol and stored at −70°C. Purity of recombinant proteins was 90–95%. If an additional purification step was required, recombinant proteins were applied to a Ni-agarose column in the presence of 6 M urea. Protein fractions were dialyzed against Buffer B containing 75% glycerol and then against Buffer B containing 20% glycerol. We also used a SP-Sepharose column to purify proteins (Fig. 3). Details of the SP-Sepharose column chromatography are in the figure legend. We note that RGS9 and its fragments expressed in *E. coli* formed inclusion bodies, and after solubilization with chaotropic reagents, simple dialysis of these proteins against a chaotropic reagent-free buffer resulted in unwanted reprecipitation. Dialysis against a buffer containing high concentration glycerol and subsequent dialysis against a buffer containing low concentration of glycerol (28) were appropriate to obtain soluble proteins from these inclusion bodies. These proteins also gradually formed precipitates after several weeks at 4°C. Such aggregated preparations were not used for further study.

Interaction of RGS9 and its fragments with retGC. (a) Immunoprecipitation of retGC by a RGS9 antibody in a ROS homogenate. ROS (500 μg) was homogenated with 0.5 ml of Buffer C (50 mM Tris/HCl (pH 7.5), 1 mM DTT, 0.1 mM PMSF, 5 μM pepstatin A, 5 μM leupeptin, 100 mM KCl, 5% *n*-dodecyl-β-maltoside, and 1 M KCl) for 2 h at 0°C. After centrifugation (100,000*g*, 30 min, 4°C), 5 μl of an anti-RGS9 antiserum was added to the supernatant and incubated for 1 h at 0°C. Then, protein-A Sepharose (25 μl) was added and the mixture was incubated for 1 h at 0°C. The beads were washed with Buffer D (50 mM Tris/HCl (pH 7.5), 1 mM DTT, 0.1 mM PMSF, 5 μM pepstatin A, 5 μM leupeptin, 100 mM KCl, and 0.1% *n*-dodecyl-β-

TABLE 1

PCR Primers for cDNA Amplification

Fragment	Sequence	Primers
RGS9	Met1-Lys484	5′-CCCAGGAGCCCAT**ATG**ACCATCCGACACCAAGGCC-3′ 5′-AGTGGATCC**TTA**TTTGGGAGGCGGCTCTTTTCTGAGTTGG-3′
RGS9N	Met1-Val190	5′-CCCAGGAGCCCAT**ATG**ACCATCCGACACCAAGGCC-3′ 5′-GGAGGGATCCT**TTA**CACCAGCCAGTACGCC-3′
RGS9N-DelN50	(Met)Gly52-Val190	5′-TCCCTCACCAT**ATG**ACGGGAAGTGACGTCCTCC-3′ 5′-GGAGGGATCCT**TTA**CACCAGCCAGTACGCC-3′
RGS9-DelC58	Met1-Lys132	5′-CCCAGGAGCCCAT**ATG**ACCATCCGACACCAAGGCC-3′ 5′-TCTAGGATCCC**CTA**CTTCTTGATATTTCGCTTGGCC-3′
RGS9NR	Met1-Ser427	5′-CCCAGGAGCCCATATGACCATCCGACACCAAGGCC-3′ 5′-AATGGGATCCTTTAGCTTTTCCTCCGTAGTTCCC-3′
RGS9R	(Met)Pro288-Ser427	5′-ACTGGTGCAT**ATG**CCAACCAAGATGCGGGTGGAG-3′ 5′-AATGGGATCCT**TTA**GCTTTTCCTCGTAGTTCCC-3′
RGS9RC	(Met)Pro288-Lys484	5′-ACTGGTGCAT**ATG**CCAACCAAGATGCGGGTGGAG-3′ 5′-AGTGGATCC**TTA**TTTGGGAGGCGGCTCTTTTCTGAGTTGG-3′

Note. The following pairs of primers were used in PCR to obtain cDNA fragments. Bold letters in these oligonucleotide sequences indicate start and stop codons.

maltoside). The bound proteins were eluted with a SDS-sample buffer and analyzed by SDS–PAGE. The proteins were transferred to a PVDF membrane and retGC coprecipitated with RGS9 was detected with an anti-retGC-1 antibody and chemiluminescent autoradiography using ULTRA Super Signal substrate. (b) Overlay of purified retGC on RGS9 and its fragments. RetGC overlay on RGS9 and its fragments was performed as described previously (25). The amounts of RGS9 and its fragments applied to SDS–PAGE were 2 μg per lane. The concentration of purified retGC was 0.5 μg/ml.

Analytical methods. RetGC activity of ROS homogenate (5 μg) and GCAPs-free membranes (5 μg) was measured in the presence of 1 mM EGTA, as described (24). RetGC activity of GCAPs-free membranes were assayed in the presence of 1 μM GCAP2. Under our assay conditions, retGC activity, with negligible hydrolysis of cGMP, was high enough to show inhibitory activities of RGS9 and its fragments. We note that some retGC activities were measured in the presence of glycerol and/or urea (Fig. 3). However, these compounds used under conditions described did not affect retGC activity. Dimerization of retGC was detected as described (24). SDS–PAGE was carried out by the method previously described (24) using a 10% polyacrylamide gel. Protein concentration in samples was assayed with bovine serum albumin as standard (29).

RESULTS AND DISCUSSION

RGS9 and its fragments used in this study. The sequence of the bovine RGS9 open reading frame predicts a 484 amino acid protein of 56.7 kDa calculated molecular mass (12). In addition to the RGS domain (~125 amino acid residues), the protein contains the N-terminal domain (~190 amino acid residues), the GGL domain (Lys216-Leu280) and the C-terminus (Fig. 1). The N-terminal domain contains a DEP domain (Gly33-Thr111). We have expressed the following proteins; RGS9, RGS9N, RGS9N-DelN50, RGS9N-DelC58, RGS9NR, RGS9R, and RGS9RC (Fig. 1). In the N-terminal fragments, RGS9N and RGS9N-DelC58 contain intact DEP domain, but in RGS9N-DelN50 19 amino acid residues are missing from the N-terminus of the DEP domain. Both RGS9 and RGS9NR contained also the GGL domain.

Effects of RGS9 and its fragments on GCAPs-activated retGC activity. It has been shown that RGS9 isolated from bovine ROS slightly, but constantly, inhibits the activity of purified retGC (25), suggesting that RGS9 inhibits basal activity of retGC. In this study, we measured effects of RGS9 and its fragments on retGC activity stimulated by GCAPs with low Ca^{2+} concentration. We found that the retGC activity in a ROS homogenate was inhibited by RGS9, RGS9N, and RGS9NR (Fig. 2A). RGS9 and RGS9N also inhibited retGC activity stimulated by recombinant GCAP2 (Fig. 2C). Since RGS9, RGS9N, and RGS9NR contain only the N-terminus as a common domain, these observations strongly suggest that the N-terminus of RGS9 functions as the inhibitor of retGC. We also found that both RGS9N-DelN50 and RGS9N-DelC58 did not show any inhibitory activity on GCAP-activated retGC in the ROS homogenate under the conditions that RGS9N clearly inhibited retGC activity (Fig. 2B). This observation suggests that amino acid residues located in Thr2-Thr51 and Gly133-Val190 are involved in the inhibition.

In addition, we found that the RGS domain (RGS9R), the fundamental domain in RGS proteins, showed no inhibitory activity on GCAP-activated retGC (Fig. 2A, C). The interesting point is that the inhibitory effect of the N-terminus (RGS9N) on retGC in a ROS homogenate was clearly stronger than that of the entire protein (RGS9) and the protein with the C-terminus deleted (RGS9NR) (Fig. 2A). The inhibitory activity of RGS9N was also stronger than that of RGS9 in recombinant GCAP2-activated retGC (Fig. 2C). The order of inhibition is RGS9N $\gg$ RGS9 $>$ RGS9NR. These observations strongly suggest that addition of the GGL domain and/or the RGS domain to the N-terminus weakens the N-terminal inhibitory activity on retGC. These results suggest that the GGL domain and/or the RGS

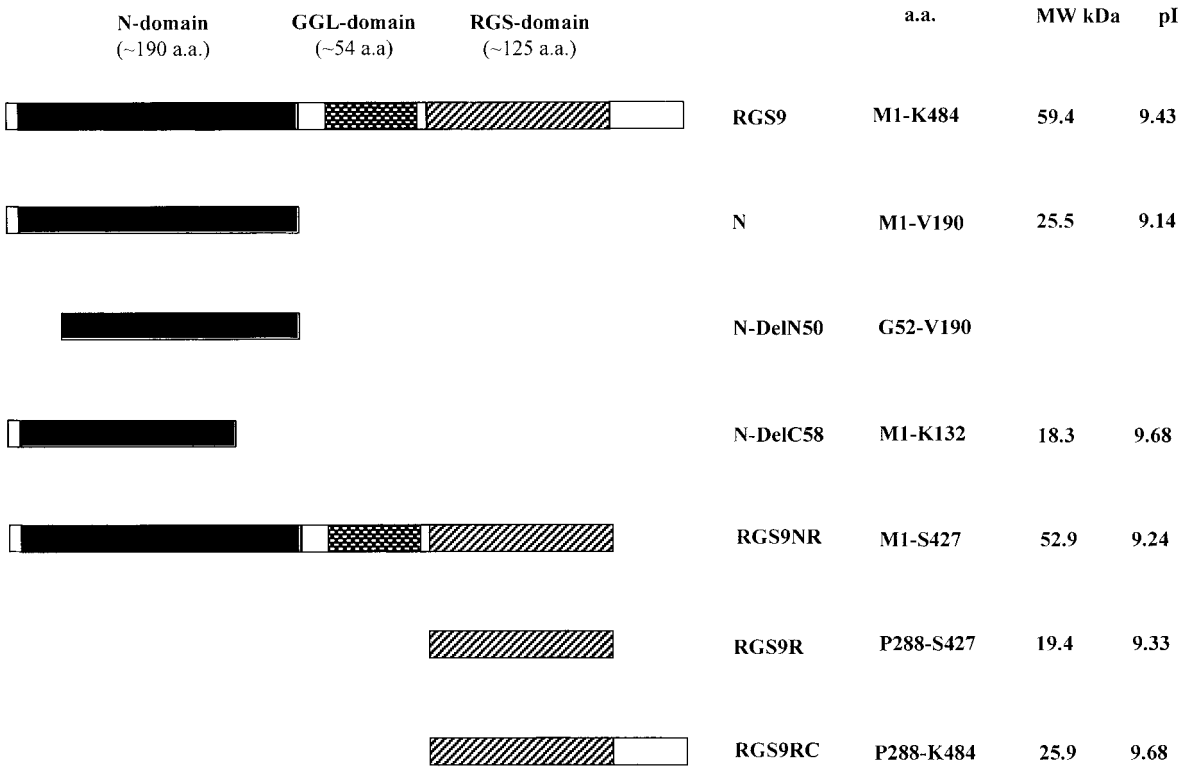

FIG. 1. Structure of RGS9 and its fragments used in this study. Recombinant RGS9 and its fragments were expressed in *E. coli* as His-tagged proteins, solubilized, and purified as described. Molecular weights and pI values were estimated from their sequences with His-tag. Without His-tag, their molecular weights (kDa) and pI values are as follows: RGS9, 56.7 and 9.54; RGS9N, 22.7 and 9.46; RGS9N-DelN50, 17 and 8.9; RGS9N-DelC58, 15.5 and 9.78; RGS9NR, 50.1 and 9.39; RGS9R, 16.6 and 9.65; RGS9RC, 23.2 and 9.9.

domain function as an internal suppressor against the N-terminus, and that proteins bound to these domains control the suppressor activity of these domains.

In this study we observed that GCAP-activated retGC in membranes is inhibited by RGS9 and its fragments containing the N-terminus. However, a previous study showed that RGS9 inhibits the basal activity of purified retGC (25). Thus, it is expected that the retGC inhibition by RGS9 and its fragments is not due to the blocking of active conformational change of retGC by GCAPs, such as retGC dimerization (24). Indeed, the inhibitory effect of high Ca^{2+} concentration on GCAP-activated retGC was detected even in the presence of RGS9 and RGS9N (data not shown). Moreover, we saw no evidence that RGS9 and RGS9N inhibited that GCAP-stimulated retGC dimerization under conditions where the stimulation of retGC dimerization by GCAP2 was clearly observed (data not shown). The inhibition of retGC by RGS9 may result from simple blocking of access of GTP to the catalytic cavity of retGC.

Inhibition of retGC activity by RGS9 and its fragments after column chromatography. In order to rule out the possibility that an unidentified component(s) contaminated in the preparations of RGS9 and its fragments inhibits retGC activity, we measured effects of these proteins on GCAP-activated retGC after isolation of these proteins by SP-Sepharose column chromatography (Fig. 3). These protein preparations were applied to and eluted from the same column under the same conditions because all proteins have alkaline pIs (Fig. 1). We found that fractions containing RGS9 (Fig. 3A), RGS9N (Fig. 3B), and RGS9NR (Fig. 3C) clearly inhibited retGC activity in a concentration-dependent manner. However, RGS9R (the peak fraction is #26) showed no inhibitory activity on retGC (data not shown). These observations strongly suggest that RGS9 proteins containing its N-terminus, but not a contaminant(s) in these preparations, inhibit GCAP-activated retGC activity. Comparison of protein contents in fractions with their retGC inhibitory activities (Fig. 3) indicates that the inhibitory activity of RGS9N is much higher than that of RGS9 and RGS9NR. These results support our suggestions that the N-terminus of RGS9 has inhibitory activity on retGC, and that the GGL domain and/or the RGS domain function as an internal repressor against the N-terminal inhibitory activity.

We note that the RGS9 preparation showed two inhibitory peaks (fraction #22 to 26, and fraction #28 to 32) in the column chromatography (Fig. 3A). Two inhibitory peaks were detected in only the RGS9 prepa-

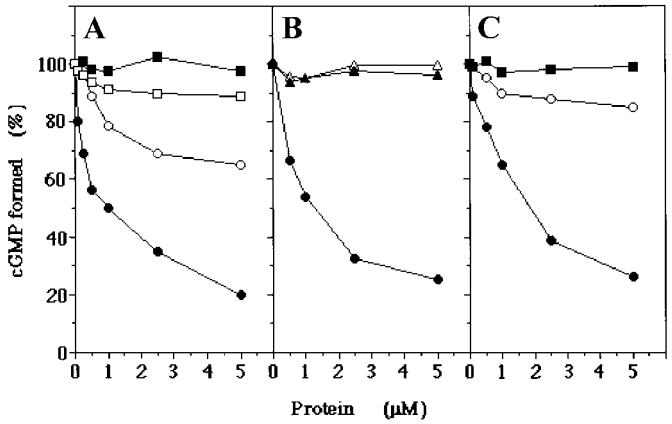

FIG. 2. Effects of RGS9 and its fragments on retGC activity. Inhibitory effects of RGS9 and its fragments on retGC activity were measured using bovine ROS homogenate (A and B) and GCAP-free membranes (C). When the ROS homogenate was used, 1 mM EGTA was added to activate endogenous GCAPs. In the case of GCAP-free membranes, 1 μM GCAP2 was added to the reaction mixture. Under these conditions, 100% activities of ROS homogenate and GCAP-free membranes were 8.0 and 10.0 nmol cGMP formed/min/mg, respectively. (○) RGS9; (●) RGS9N; (□) RGS9NR; (■) RGS9R; (△) RGS9N-DelN50; and (▲) RGS9N-DelC58.

ration. SDS–PAGE showed that the former peak fractions did not contain RGS9, but the latter peak fractions contained RGS9, as described above. In the former peak fractions, several proteins were detected by Coomassie blue staining (data not shown). However, the inhibitory protein could not be identified because either amounts of the peptide were small or the peptide might be resistant to staining with Coomassie Blue. We note that this inhibitory compound did not derive from buffer components because same elution fractions (fraction #22 to 26) of RGS9N (Fig. 3B) and RGS9R (data not shown) did not show the inhibitory activity against retGC. We also note that a non-transformed cell extract of *E. coli* used for the expression of RGS9 and its fragments did not show any inhibitory activity on retGC (data not shown), indicating that the inhibitory compound is specifically expressed. We speculate that RGS9 may be susceptible to an *E. coli* protease(s) during bacterial expression, and that a digested product (a peptide) may have a high inhibitory activity against retGC, as the RGS9 N-terminus (RGS9N) has a higher inhibitory activity against retGC than intact RGS9 (Figs. 2A and 2C). It should be noted that the higher inhibitory activity of RGS9 than that of RGS9NR (Fig. 2A) might be due to the unidentified compound in the RGS9 preparation to some extent.

Evidence for the direct interaction of RGS9 and its fragments with retGC. In order to establish the direct interaction between RGS9 and retGC, we first analyzed the interaction between retGC and RGS9 using immunoprecipitation (Fig. 4A). We found that retGC in a ROS homogenate was precipitated by an RGS9-specific antibody. This observation strongly suggests that retGC in the ROS homogenate, if not all, forms a complex with RGS9 and the complex is precipitated by the RGS9 antibody. We note that the antibody used is specific to RGS9 and cannot recognize retGC (25). We also note that the amino acid sequence recognized by the antibody is located between Thr2 and Leu17 in RGS9. However, binding of the antibody to the site appears not to disturb the interaction of RGS9 with retGC although the N-terminus (Met1-Val190) is responsible for the retGC inhibition (Fig. 2). It is possible that RGS9 amino acid residues located after Arg18 are

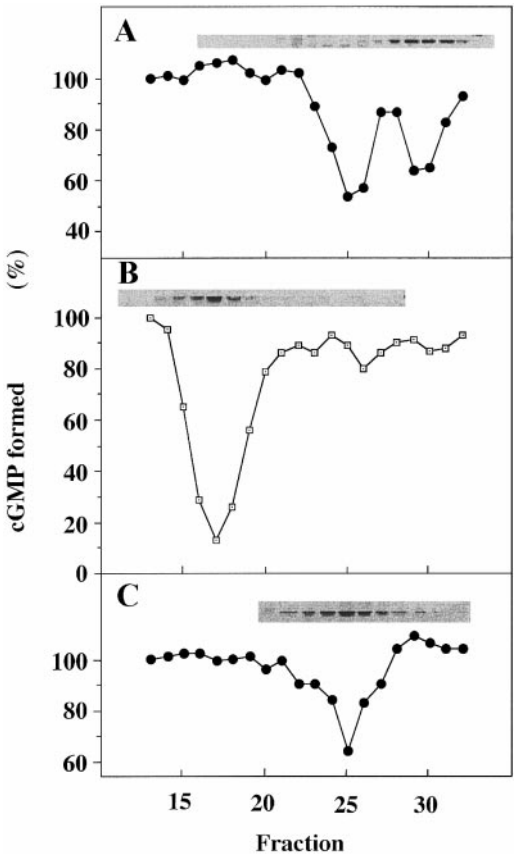

FIG. 3. Inhibition of retGC activity by RGS9 and its fragments after SP-Sepharose column chromatography. After expression and solubilization, RGS9 and its fragments (1 mg) were applied to a SP-Sepharose column (2 × 10 cm) which had been equilibrated with buffer B containing 6 M urea. Then, the column was washed with the same buffer, and proteins were eluted with a NaCl gradient (0–0.5 M) in 100 ml buffer B containing 6 M urea and 20% glycerol (flow rate 20 ml/h, fraction vol 2 ml). RGS9 and its fragments were identified by their molecular weights after analysis of all fractions (20 μl) by SDS–PAGE. Effect of each fraction (10 μl) on retGC activity was measured using a ROS homogenate. The reaction mixture contained 300 mM urea and 20% glycerol (final); however, we found that neither urea (up to 1 M) nor 20% glycerol affected retGC activity under our conditions. We note that during enzyme assay, and precipitation was not detected in the reaction mixtures. Under these conditions, 100% activity of ROS homogenate was 8.0 nmol cGMP formed/min/mg. Inserts are profiles of RGS9 and its fragments in peak fractions. (A) RGS9; (B) RGS9N; (C) RGS9NR.

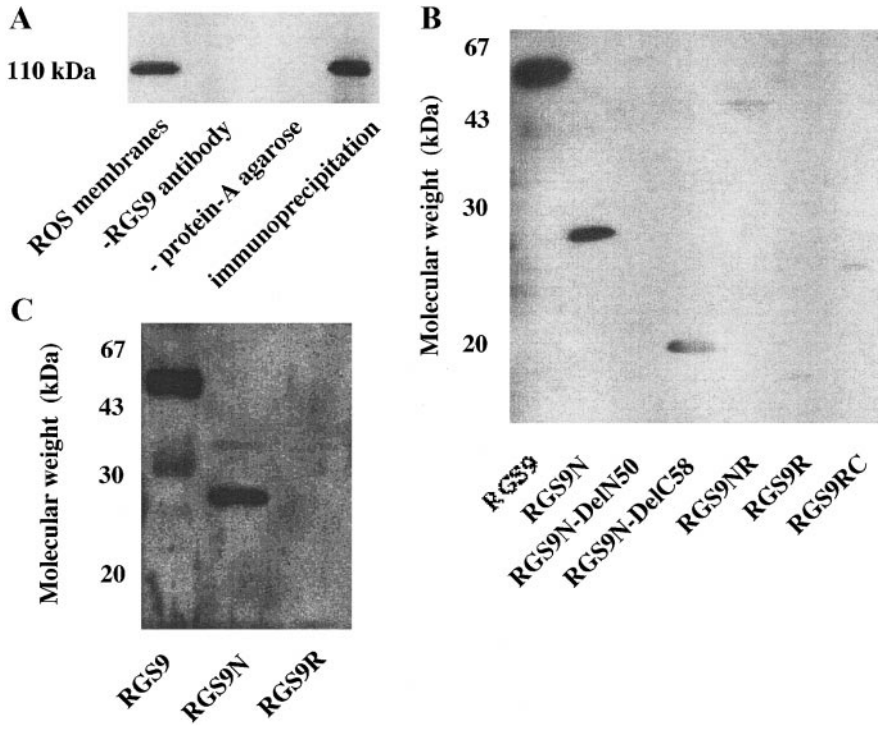

FIG. 4. Direct interaction of retGC with RGS9 and its fragments. (A) Immunoprecipitation of retGC-RGS9 complex. Bovine ROS membranes (500 μg) were solubilized using 5% *n*-dodecyl-β-D-maltoside as described. After centrifugation, rabbit anti-RGS9 antiserum was added and incubated. The mixture was further incubated with protein-A agarose. After washing the beads, proteins bound the beads were eluted and separated by SDS–PAGE. These proteins were transferred to a PVDF membrane and retGC precipitated with RGS9 was detected using an anti-retGC antibody and chemiluminescent autoradiography. As a control for the protein, ROS membranes (50 μg) were applied to the gel without any procedures. As controls for the procedure, same procedures were carried out without RGS9 antibody or protein-A agarose. (B and C) RetGC overlay. After isolation of RGS9 and its fragments (~2 μg) by SDS–PAGE, these proteins were blotted to a PVDF membrane. After denaturation and renaturation of these proteins on the membrane, the membrane was incubated with purified retGC (0.5 μg)/ml. RetGC bound to RGS9 and its fragments were identified using a retGC-specific antibody and chemiluminescence as described. (C) In addition to a ~60 kDa protein, a retGC-binding protein, ~32 kDa, was occasionally detected in the RGS9 preparation, but not in RGS9N and RGS9R preparations.

involved in its interaction with retGC. Since RGS9N-DelN50 (Gly52-Val190) did not show any inhibitory activity on retGC (Fig. 2B), this observation indicates that amino acid residues located in Arg18-Thr51 are involved in the retGC inhibition. The possibility that the DEP domain (Gly33-Thr111) is involved in the inhibition of retGC (also in the interaction with retGC) is great.

In order to analyze the direct interaction of RGS9 fragments with retGC, we utilized an overlay technique. After purification of RGS9 and its fragments by SDS–PAGE, these proteins were blotted to a PVDF membrane. Then, following denaturation and renaturation of these proteins, purified retGC was overlaid and retGC bound to these proteins was detected using an antibody specific to retGC. This overlay technique has essentially advantages and disadvantages. As an advantage, interaction of a specific protein with various proteins can be analyzed simultaneously even without antibodies specific to these proteins. In addition, both purification of various proteins and interaction of these proteins with a specific protein can be simultaneously performed in the procedure. Thus, this method is suitable to analyze interaction of retGC (specific protein) with various RGS9 fragments. However, the level of denaturation and renaturation may be different in each fragment and the effect of the fragment interaction with a PVDF membrane on their retGC inhibitory activities may not be ignored. Under these conditions, we found that retGC bound clearly to RGS9 and RGS9N (Figs. 4B and 4C). These observations are consistent with the main conclusion obtained in Figs. 2 and 3; the N-terminus of RGS9 has an inhibitory activity on retGC stimulated by GCAPs. Although faint bands of retGC were also detected on some of fragments (Fig. 4B), these bands were deemed inconsequential since detection of these bands was variable and the intensity of these bands, if observed, was much less than that of RGS9 and RGS9N.

However, two points, as follows, are in conflict with results of Figs. 2 and 3. Reasons for these conflicts are unknown; however, we speculate that these conflicts are resulted from essential disadvantages of this overlay method as described above. (i) Binding of retGC to

RGS9 is stronger than that to RGS9N although the inhibitory activity of RGS9N is higher than that of RGS9. We emphasize that this overlay method includes denaturation and renaturation of proteins on a PVDF membrane. It is possible that levels of denaturation and renaturation of these proteins were different. For example, renaturation of RGS9 on a PVDF membrane might be more efficient than that of RGS9N. Domains deleted for the preparation of RGS9N might be important for the stability of RGS9 (19). It is also possible that under our conditions, binding of RGS9 to a PVDF membrane releases the inhibitory effect of the GGL domain and/or the RGS domain on the N-terminus and the N-terminus strongly interacts with retGC. (ii) The binding of retGC to RGS9NR was not detected although RGS9NR slightly inhibited retGC activity. We expect that the interaction between retGC and RGS9NR was too weak to be detected by this overlay technique. It is possible that the real conformation of RGS9NR may not be completely recovered by the renaturation process.

We also note that in addition to a ~60 kDa protein, an additional retGC-binding protein, ~32 kDa, was occasionally detected in a RGS9 preparation, but not in RGS9N and RGS9R preparations (Fig. 4C). The 60 kDa band was RGS9. We speculate that the 32 kDa protein may be an inhibitory compound detected in fraction #22 to 26 in SP-Sepharose column chromatography (Fig. 3A). We also note that the C-terminus itself may not have the ability to inhibit retGC or the inhibitory activity, if present, may be very small, because retGC did not bind to RGS9R and RGS9RC (Fig. 4B). However, this result does not rule out the possibility that the C-terminus affects the N-terminus inhibition of retGC because the inhibitory ability of RGS9 is higher than that of RGS9NR (Fig. 2A).

Conclusion. This study has demonstrated that the RGS9 N-terminus is mainly responsible for the inhibition of retGC, and that the inhibition is due to the direct interaction of retGC and the N-terminus. At present, we do not have any evidence showing that these two proteins form a complex under specific conditions *in vivo.* However, previous studies have suggested that both RGS9 (25) and retGC (27) are found in similar Triton X-100-insoluble fractions. These properties may indicate the possibility that these two proteins are located in a similar membranous domain. Another important point is that the GGL domain and/or the RGS domain appear to function as an internal inhibitory domain against the N-terminus. We do not know now how the inhibitory effect of the GGL domain and/or the RGS domain on the N-terminus is released. However, recent studies (16–20) show that RGS9 interacts with GTP/Tα, Gβ5L, and Pγ. It is possible that the RGS9 interaction with these proteins may release the inhibitory effect of these domains because these proteins bind to these domains. If so, our observations imply that during PDE activation by GTP/Tα, GTP/Tα also suppresses retGC activity. This mechanism may be crucial for photoreceptor adaptation. Although more experiments (e.g., analysis of the effects of GTP/Tα, Gβ5L, and Pγ on the regulation of retGC by RGS9) are needed to establish the role of RGS9 in the regulation of retGC activity, this study strongly suggests that RGS9 is directly involved in the interaction between PDE and retGC systems, and that in addition to Ca^{2+}-mediated mechanism, this RGS9-mediated mechanism plays an important role in regulating the cGMP concentration in ROS.

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