

Dependence of RGS9–1 Membrane Attachment on Its C-terminal Tail*

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RGS9–1 is a GTPase-accelerating protein (GAP) required for rapid recovery of the light response in vertebrate rod and cone photoreceptors. Similar to its phototransduction partners transducin (G_t) and cGMP phosphodiesterase, it is a peripheral protein of the disc membranes, but it binds membranes much more tightly. It lacks the lipid modifications found on G_t and cGMP phosphodiesterase, and the mechanism for membrane attachment is unknown. We have used limited proteolysis to generate a fragment of RGS9–1 that is readily removed from membranes under moderate salt conditions. Immunoblots reveal that this soluble fragment lacks a 3-kDa fragment from the C-terminal domain, the only domain within RGS9–1 that differs in sequence from the brain-specific isoform RGS9–2. Recombinant fragments of RGS9–1 with or without the partner subunit $G\beta_{5L}$ were constructed with or without the C-terminal domain. Those lacking the C-terminal domain bound to photoreceptor membranes much less tightly than those containing it. Removal by urea of $G\beta_{5L}$ from endogenous or recombinant RGS9–1 bound to rod outer segment membranes left RGS9–1 tightly membrane-bound, and recombinant RGS9–1 was urea-soluble in the absence of membranes. Thus the C-terminal domain of RGS9–1 is critical for membrane binding, whereas $G\beta_{5L}$ does not play an important role in membrane attachment.

Phototransduction in the vertebrate retina occurs on the surfaces of disc membranes in rod and cone outer segments. The key interactions occur between proteins that are tightly membrane-associated: the heptahelical receptor, rhodopsin (R^*),¹ the heterotrimeric G protein, transducin (G_t), and the

effector, cGMP phosphodiesterase (PDE). Part of the explanation for the impressive rapidity of light responses may lie in the efficiency of protein encounters mediated by diffusion largely restricted to a two-dimensional search (1, 2). A recently identified participant in this pathway, clearly required for proper kinetics of the recovery phase of the light response (3), is the GTPase-accelerating protein for G_t , consisting of a complex between the photoreceptor-specific RGS protein RGS9–1 (4) and the photoreceptor-specific G protein β subunit, $G\beta_{5L}$ (5). With the help of the inhibitory subunit of PDE ($PDE\gamma$), this complex accelerates $G_t\alpha$ GTPase from its basal rate of one turnover per tens of seconds to a rate compatible with the subsecond recovery of the mammalian light response (6). Before the molecular identity of RGS9–1 was determined, biochemical experiments demonstrated the presence of its GTPase-accelerating protein activity in rod outer segment (ROS) membranes (7, 8). The detergent concentrations necessary to remove this activity suggested that it might be a transmembrane protein (8, 9). Molecular cloning allowed the production of antibodies, which revealed that while RGS9–1 is indeed tightly bound to membranes, it can be removed by treatment with sodium carbonate, pH 12 (9), as expected for a peripheral membrane protein.

The sequence of RGS9–1 has provided few clues as to the mechanism by which it is tethered to membranes. There are no recognizable signals for N-terminal acylation or C-terminal isoprenylation. There are multiple cysteine residues, which might be sites for palmitoylation as proposed for other RGS proteins, such as GAIP (10), RGS4 (11), and RGS16 (12), but hydrolytic treatment with hydroxylamine under conditions that cleave thioester-linked palmitates failed to release RGS9–1 from the membranes (9). There are many basic residues in RGS9–1, which has a predicted pI of 9.53, so electrostatics may play a role in binding to negatively charged membrane surfaces. However, high ionic strength removes only a little RGS9–1 from membranes (9), suggesting that general electrostatic attraction alone is unlikely to account for the very tight membrane binding.

To elucidate the mechanism by which the RGS9–1- $G\beta_{5L}$ complex binds the membrane, we have begun by asking what part of this protein is important for membrane binding. Our results rule out an important role for the entire $G\beta_{5L}$ polypeptide in membrane tethering but indicate clearly that the C-terminal tail of RGS9–1 plays an essential role in tethering RGS9–1 and $G\beta_{5L}$ to the membrane. This role of the C-terminal domain may help to explain the need for photoreceptor-specific RNA processing, which leads to one isoform, RGS9–1, in the retina, and a distinct isoform, RGS9–2, to be expressed in the brain (13–15). The amino acid sequences of these proteins differ only in their C termini.

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¹ The abbreviations used are: R^* , photoexcited rhodopsin; PDE, cGMP phosphodiesterase (PDE6); ROS, rod outer segment; lwROS, low salt-washed ROS; hwROS, high salt-washed ROS; uwROS, urea-washed ROS; OG, *n*-octyl- β -D-glucopyranoside; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; GST, glutathione *S*-transferase.

EXPERIMENTAL PROCEDURES

Buffers—Compositions were as follows. GAPN buffer: 10 mM Tris, pH 7.4, 100 mM NaCl, 2 mM MgCl₂; low salt buffer: 5 mM Tris, pH 7.4, 0.5 mM MgCl₂; high salt buffer 1: 5 mM Tris, pH 7.4, 1 M NaCl, 0.5 mM MgCl₂; high salt buffer 2: 10 mM Tris, pH 7.4, 1 M NH₄Cl; urea buffer: 5 mM Tris, pH 7.4, 1 or 4 M urea as indicated. 1 mM DTT and solid phenylmethylsulfonyl fluoride were added to the above buffers before use. Digestion buffer: 10 mM MOPS, pH 7.4, 200 mM NaCl, 2 mM MgCl₂, 1 mM DTT.

Preparation and Washing of Rod Outer Segments—Rod outer segments from frozen bovine retinas were prepared in dim red light by a standard sucrose gradient method as described (16). Membrane washing was performed in dim red light using ice-cold buffers. ROS were first diluted 3-fold with GAPN buffer and sedimented at 80,000 × *g* for 30 min at 4 °C. ROS membranes were subsequently washed twice by GAPN buffer. Low salt-washed ROS (lwROS) membranes were prepared by washing ROS membranes twice more with low salt buffer. High salt-washed ROS (hwROS) membranes were made by washing lwROS membranes once further with high salt buffer 1 in the dark. Urea-washed ROS (uwROS) membranes were made by washing hwROS membranes once further with urea buffer. All washing was at a concentration of 75 μM rhodopsin.

Limited Proteolysis—Bleached ROS membranes, which had been stripped by low salt and low salt/GTP (100 μM GTP) to remove most of the PDE and transducin, were incubated with protease V8 in digestion buffer at final concentrations of 30 μM R* and 0.01 mg/ml protease V8 (Sigma). The proteolysis was stopped by 0.20 mg/ml 1-chloro-3-tosyl-amido-7-amino-2-heptanone (Invitrogen). For detecting the total amount of RGS9 left, Na₂CO₃ (0.1 M final) was added to the quenched reaction mixture, and then the membranes were homogenized by extrusion through a 23-gauge needle and centrifuged at 80,000 × *g* for 30 min. For sequential washing, the quenched membranes were first spun down from the moderate salt digestion buffer and then sequentially resuspended by homogenization and centrifuged, first in high salt buffer 2 and then in 100 mM Na₂CO₃. All supernatants were precipitated by 10% trichloroacetic acid and resolved by SDS-PAGE.

Membrane Binding Assays—ROS membranes or synthetic lipid vesicles were mixed, by vortexing, with recombinant proteins using the buffers and volumes indicated in the figure legends, incubated on ice for 30 min, and sedimented at 80,000 × *g* for 30 min at 4 °C. The unbound recombinant proteins left in the supernatants (75% of the total volume for Coomassie staining or 2.5% of the total volume for immunoblotting) were either precipitated by 10% trichloroacetic acid and resolved by SDS-PAGE for Coomassie Blue staining or directly resolved by SDS-PAGE for immunoblot analysis. For vesicles, phosphate assays (17) were used to verify >90% efficiency of sedimentation.

Expression and Purification of Recombinant Proteins—GST-RGS9-1d (g9D, residues 291–418; lowercase g is used to denote the GST tag) and GST-RGS9-1dc (g9DC, residues 291–484) were expressed in bacteria using pGEX-2TK vector and purified as described previously (18). GST and GST-RGS9-1c (g9C, residues 413–484) were expressed in bacteria using pGEX-2TK vector and purified as soluble proteins using glutathione affinity chromatography and glutathione-Sepharose 4 Fast Flow resin, following the manufacturer's standard protocol (Amersham Biosciences, Inc.). The proteins g9NGDC (residues 1–484)-Gβ_{5S} and g9NGD (residues 1–431)-Gβ_{5S} were expressed in Sf9 cells and purified as described previously (20). Briefly, monolayer Sf9 cells were infected with recombinant baculoviruses encoding g9NGDC (or g9NGD) and Gβ_{5S} at 80% confluency and harvested 48 h later. Then GST-tagged g9NGDC-Gβ_{5S} and g9NGD-Gβ_{5S} complexes were purified from the cell lysates following the standard protocol (Amersham Biosciences, Inc.). The concentrations of g9d, g9dc, GST, and g9c were determined by Bradford assays on the highly purified protein using bovine serum albumin as standard. The concentrations of partially purified g9NGDC-Gβ₅ and g9NGD-Gβ₅ were determined by densitometry of the Coomassie Blue-stained bands following their resolution on SDS-PAGE together with bovine serum albumin standards.

Antibodies—A polyclonal antibody raised against RGS9-1 residues 291–418 (*i.e.* the RGS domain and beginning of the C-terminal domain, referred to as anti-RGS9-1c in Ref. 3), polyclonal anti-RGS9-1 C-terminal peptide (residue 462–475) antibody CT-0, polyclonal anti-RGS9N antibody (residue 1–219), and monoclonal anti-RGS9-1 antibody (D7) were generated as described (3, 7, 13, 15, 20). Polyclonal anti-Gβ₅ antibody was a generous gift from the laboratory of M. I. Simon, California Institute of Technology.

Western Blot Analysis—After SDS-PAGE, proteins were electrotransferred to supported nitrocellulose. Membranes were blocked with

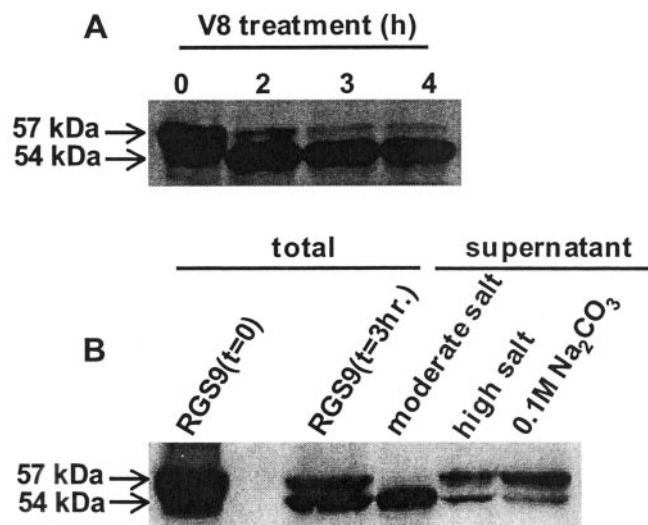


FIG. 1. Generation of soluble fragments of RGS9-1 by proteolysis of ROS membranes with protease V8. ROS membranes were digested with protease V8 for the indicated times and then analyzed by SDS-PAGE and immunoblotting using a monoclonal antibody that recognizes all fragments of RGS9 containing the RGS domain (A). ROS membranes were treated by protease V8 for 3 h and sequentially washed by digestion buffer (*moderate salt*), high salt buffer 2 (*high salt*), and 0.1 M Na₂CO₃ (pH 12) solutions (B). The supernatants were analyzed as in panel A except that detection was with polyclonal antiserum raised against an RGS9-1 fragment containing residues 227–484.

5% nonfat dry milk/Tris-buffered saline solution for 1 h at room temperature, incubated at 4 °C overnight with antibodies (1:1,000 dilution of polyclonal anti-RGS9 residues 291–418 serum and polyclonal anti-RGS9-1 C-terminal peptide antibody, 1:250 dilution of monoclonal anti-RGS9-1 antibody, 1:500 dilution of anti-RGS9N or 1:5,000 dilution of polyclonal anti-Gβ₅ antibody) followed by a 45-min incubation with 1:10,000 dilution of anti-rabbit or anti-mouse IgG peroxidase antibodies. Western blots were developed with the ECL kits (Amersham Biosciences, Inc.) and exposed to Eastman Kodak Co. Biomax MR films.

Extraction of Lipids from Rod Outer Segment—Bleached ROS (R* = 100 μM) in GAPN buffer were mixed with an equal volume of CHCl₃/MeOH (2:1) by vortexing for 1 min. The mixture was then centrifuged at 22,000 × *g* for 1 min, and the organic fraction containing most of the lipids was removed. The remaining aqueous layer was extracted twice more with an equal volume of CHCl₃/MeOH (2:1). All organic fractions were pooled and dried by a stream of argon. The extracted lipids were redissolved in CHCl₃ and used for further experiments.

Rhodopsin Purification and Reconstitution—Rhodopsin was purified from rod outer segments as described (19) using *n*-octyl-β-D-glucopyranoside (OG). For reconstitution, ROS lipids and DOPC were mixed at a 1:1 molar ratio in organic solvent, dried down under argon, and then resolubilized in 150 mM OG. Rhodopsin reconstitution was performed by mixing purified rhodopsin with the OG-solubilized lipid mixture (1 mol of rhodopsin/70 mol of lipids) and then dialyzing away OG in GAPN/1 mM DTT. As a control, DOPC/ROS lipid vesicles were made by dialyzing the OG-solubilized lipids against GAPN/1 mM DTT. The phospholipid concentration was determined by assay of phosphate (17) as described (20).

RESULTS

Limited Proteolysis of Endogenous RGS9-1—We used limited proteolysis to look for small fragments of RGS9-1 whose removal would release it from the membranes. When ROS membranes were treated for various times with trypsin, papain, and endoprotease Glu-C (protease V8) and the results were analyzed by SDS-PAGE and RGS9 immunoblots, we found that trypsin rapidly cleaved this highly basic protein into small fragments and that papain rapidly generated a 32-kDa fragment, representing only about one-half of the full-length protein (data not shown). Only protease V8 yielded fairly efficient production of a reasonably large fragment (Fig. 1A), one whose apparent mass (54 kDa) indicated that it resulted from the removal of an ~3-kDa peptide from the full-length 57-kDa

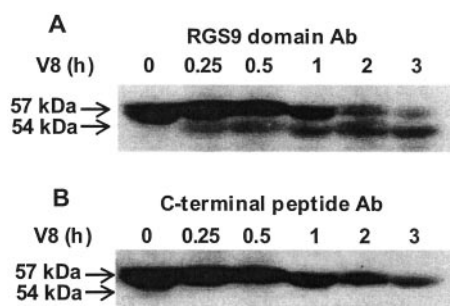


FIG. 2. **Failure of C-terminal-specific antibodies to recognize the soluble proteolysis product.** ROS membranes were treated by protease V8 for the indicated times and then analyzed as described in the Fig. 1 legend with detection by monoclonal antibodies (Ab) against RGS9 domain (A) or polyclonal antibodies (Ab) against a C-terminal peptide (residues 462–475) (B). Arrows indicate the positions of full-length RGS9–1 and the 54-kDa fragment.

RGS9–1. Interestingly, V8 cleavage was highly inefficient in the low salt buffer, suggesting that strengthening electrostatic interactions may protect the cleavage site.

Solubility of the 54-kDa Proteolytic Product—When we subjected V8-treated membranes to sequential washes with increasingly stringent conditions (Fig. 1B), we found that the 54-kDa fragment was freely soluble in the moderate salt buffer used for the protease digestion. After protease treatment, some full-length RGS9–1 protein remained and displayed its typical membrane binding behavior: virtually none was removed by moderate salt, a little was removed by high salt, and only sodium carbonate, pH 12, was able to remove most of it from the membranes. Thus the release of the 54-kDa fragment was due to the loss of a portion of the RGS9–1 needed for membrane binding and was not due to proteolysis of another ROS membrane protein needed for membrane binding of RGS9–1. When exposed to protease V8 for extended periods of time, the 54-kDa fragment is itself subject to cleavage (Fig. 2 and additional data not shown), indicating that there are other susceptible sites. However, because cleavage at these additional sites occurs much more slowly than generation of the soluble 54-kDa fragment, cleavage at these sites is not informative with regard to domains involved in membrane binding.

C-terminal Origin of the Membrane Binding Fragment—Examination of the amino acid sequence of RGS9–1 revealed potential cleavage sites for protease V8 in both the C-terminal and N-terminal domains. Cleavage at E-21 would release a 2.7-kDa peptide from the N terminus. Cleavage at E-450 or E-457 would release a 3.9- or 3.1-kDa peptide, respectively, from the C terminus. To determine whether this ~3-kDa peptide is chopped from the N terminus or C terminus of RGS9–1, we prepared C-terminal-specific antibodies by two different procedures. In one, we started with a polyclonal antiserum (3) that primarily recognized C-terminal epitopes as revealed by a much weaker signal obtained in immunoblots with a construct containing only the RGS domain as compared with one containing the RGS domain and the complete C terminus (data not shown). Antibodies recognizing epitopes outside residues 449–484 were depleted using an immobilized protein lacking these C-terminal residues but containing the rest of the protein, which was used for immunization. This preparation (CT-1) gave no detectable signal in immunoblots with constructs lacking the C-terminal residues 449–484 but strongly recognized polypeptides containing the C-terminal 36 amino acids (data not shown). A second set of polyclonal antibodies (CT-0) was prepared by immunizing and affinity purifying with a peptide corresponding to the C-terminal residues 462–475 of RGS9–1. When RGS9–1 was digested with protease V8 and blotted by monoclonal antibodies, whose epitope is near the end of RGS

domain, both full-length RGS9–1 and the ~54-kDa RGS-1 fragment were detected (Fig. 2A). However, the ~54-kDa RGS9–1 fragment was not detected by the C-terminal-specific antibodies CT-1 (data not shown) or CT-0 (Fig. 2B). If removal of an N-terminal fragment of 2.7 kDa or greater were responsible for release from the membranes, then the ~54-kDa fragment released would react with the C-terminal antibodies. If there were a concerted cleavage simultaneously at both the N and C termini, then the soluble fragment would migrate to a significantly lower position on the gel. Thus the 3-kDa peptide, which is essential for the tight membrane association of RGS9–1 and which is removed by protease V8 digestion, comes from the unique C terminus of RGS9–1.

Binding of Recombinant Proteins to ROS Membranes—An important role for the C terminus in membrane binding was also found with recombinant fragments of RGS9–1. Initial experiments were carried out with the RGS domain (residues 291–418) expressed in *Escherichia coli*. Because the relatively small amounts of bound recombinant protein are difficult to discern on the background of a greater than hundredfold excess of total ROS membrane proteins, we used depletion of recombinant RGS9–1 from the supernatant to detect binding. Recombinant GST-RGS9–1d (RGS domain only, g9D) bound ROS membranes only very weakly (Fig. 3A), whereas a construct containing the RGS domain plus the C terminus (g9DC) bound much more tightly (Fig. 3B). The RGS9–1 C terminus-specific antibodies blocked the membrane binding of g9DC (data not shown). Binding does not appear to be light-sensitive as the membrane binding of these two recombinant proteins was not significantly changed by using bleached ROS membranes (data not shown). GST alone does not show significant binding to ROS membranes (Fig. 3D). The C-terminal domain appears to mediate membrane binding only in the context of the RGS domain; a construct containing the C-terminal domain fused to GST did not interact with membranes significantly (Fig. 3C). Results with vesicles reconstituted from purified components indicated that the binding mediated by the C-terminal domain of RGS9–1 is not primarily due to interactions with either of the two most abundant constituents of the membranes, phospholipids or rhodopsin (Fig. 3, E and F). Vesicles containing the natural lipids of ROS membranes (diluted 1:1 with DOPC to facilitate bilayer formation (8)) bound g9DC (GST-RGS9–1dc) much more weakly than did ROS membranes (compare the 280 μ M lipid lanes for panels A and E). Vesicles containing the same lipids plus the natural molar ratio of purified rhodopsin bound the C-terminal-containing RGS domain construct possibly even more weakly than the protein-free vesicles (Fig. 3F).

Similar results were obtained with full-length recombinant RGS9–1 expressed as a complex with $G\beta_5$ using baculovirus (21). Fig. 4 shows that whereas the complex containing an intact C-terminal domain of RGS9–1 (g9NGDC- $G\beta_{5S}$, see Ref. 21 and Fig. 4A) bound tightly to ROS membranes, even in a high salt buffer, much less of a complex lacking the C-terminal domain (g9NGD- $G\beta_{5S}$, see Ref. 21 and Fig. 4B) bound to ROS membranes and what little bound was easily removed by high salt buffer.

Removal of $G\beta_{5L}$ from Membranes without Loss of RGS9–1—One domain that might be involved in membrane recognition is the $G\gamma$ -like domain, identified as the binding site for $G\beta_5$ in RGS11 (22) and responsible for binding of $G\beta_{5L}$ (5, 21) to RGS9–1. $G\beta_{5L}$ (23) and RGS9–1 (9) were both found to bind ROS membranes tightly, and when RGS9–1 was dissolved using a non-ionic detergent, $G\beta_{5L}$ was present in the extract, tightly bound to RGS9–1 (5). However, we have found conditions that can remove $G\beta_{5L}$ but leave RGS9–1 tightly bound to the membrane. Fig. 5, A and B, shows that treatment with 4 M

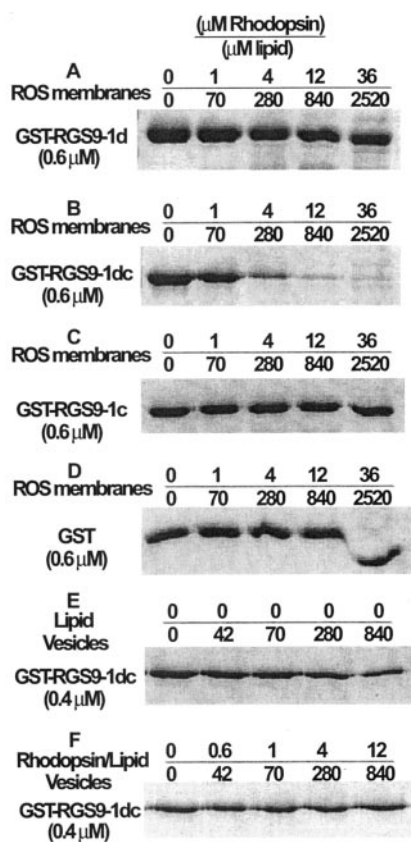


FIG. 3. Membrane binding of GST-tagged recombinant RGS9 fragments with or without the C-terminal domain of RGS9. *A–D*, binding to ROS membranes. Concentrations are expressed as concentrations of rhodopsin (*upper lines*) and the 70-fold-higher phospholipid concentrations (*lower lines*). *A*, binding of GST-RGS9-1d (g9D, residues 291–418) to ROS membranes. *B*, binding of GST-RGS9-1dc (g9DC, residues 291–484) to ROS membranes. *C*, binding of GST-RGS9-1c (g9C, residues 413–484) to ROS membranes. *D*, binding of GST to ROS membranes. *E* and *F*, binding of GST-RGS9-1dc to lipid vesicles. Concentrations are expressed as for *panels A–D*. *E*, vesicles with 1:1 ratio of ROS lipid/DOPC (mol/mol). *F*, vesicles of identical lipid composition to those described in *panel E*, containing purified rhodopsin. ROS membranes or synthetic lipid vesicles were mixed with recombinant proteins in GAPN buffer at a volume of 200 μl by vortexing, incubated on ice for 30 min, and sedimented at 80,000 $\times g$ for 30 min. The unbound recombinant proteins left in the supernatants (75% of the total volume) were precipitated by 10% trichloroacetic acid and resolved by SDS-PAGE for Coomassie Blue staining.

urea removes nearly all the $G\beta_{5L}$ from the membranes, whereas nearly all the RGS9-1 stays in the membrane fraction. Fig. 5C shows that recombinant RGS9-1 does not precipitate when exposed to 4 M urea in the absence of membranes but does bind to ROS membranes in the presence of 4 M urea, whereas recombinant $G\beta_5$ does not. Thus $G\beta_{5L}$ is not important for membrane binding, and the RGS9-1- $G\beta_{5L}$ interaction is more easily disrupted than that between RGS9-1 and the membrane.

DISCUSSION

Members of the RGS protein family in general contain multiple structural domains in addition to their conserved RGS domains. The functions of these, and their relationships to GTPase acceleration by the RGS domains, are just beginning to be discovered, but it seems likely that they will hold many of the answers to the puzzling question of how superficially promiscuous RGS domains accomplish specific tasks in particular signaling pathways. In the case of RGS9-1, the clear identification of its highly specific role in vision facilitates the process of understanding how domain properties fit with physiological

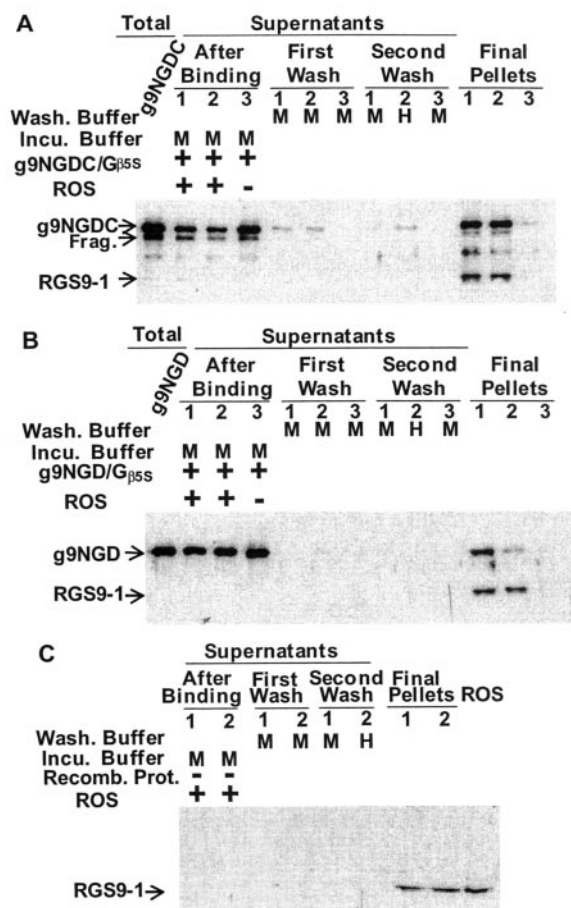
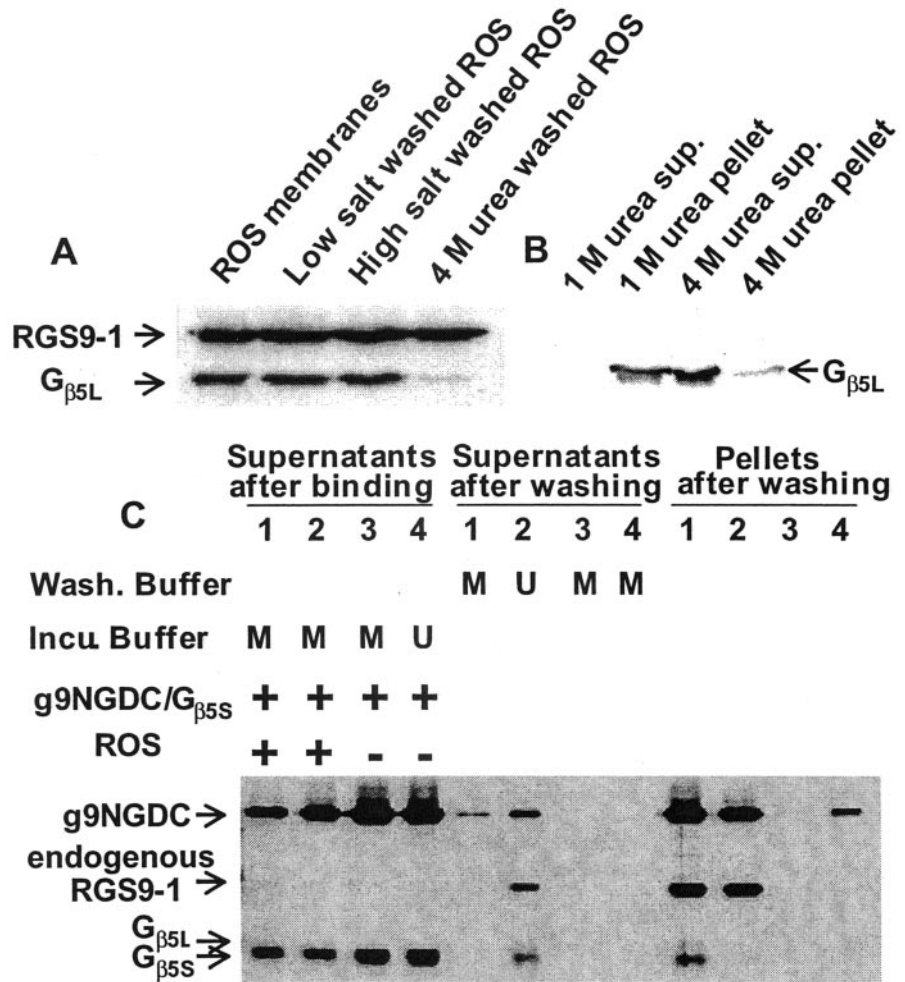


FIG. 4. ROS membrane binding of recombinant GST-tagged RGS9- $G\beta_{5S}$ complexes with or without the C-terminal domain of RGS9. *A*, binding of g9NGDC- $G\beta_{5S}$ (residues 1–484 of RGS9-1) to ROS membranes. *Frag.* denotes a contaminating proteolytic fragment of g9NGDC. *B*, binding of g9NGD- $G\beta_{5S}$ (residues 1–431 of RGS9-1). *C*, controls with ROS membranes and no recombinant proteins. Recombinant g9NGDC- $G\beta_{5S}$ or g9NGD- $G\beta_{5S}$ (50 nM) was mixed with or without ROS membranes (3 μM R^{*}) in indicated buffers at a volume of 100 μl by vortexing, incubated on ice for 30 min, and sedimented at 80,000 $\times g$ for 30 min. The supernatants were designated as *after binding*. The membranes were further washed twice by resuspension and sedimentation with 200 μl of buffers as indicated each time. The supernatants from these two washing steps were designated as *first wash* and *second wash*. *M* represents moderate salt buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM MgCl_2 , 1 mM DTT); *H* represents high salt buffer 1 (5 mM Tris, pH 7.4, 1 M NaCl, 0.5 mM MgCl_2 , 1 mM DTT). Equal portions of supernatants and final pellets (2.5% of the total in each case) were analyzed by SDS-PAGE and immunoblotting using polyclonal antiserum raised against an RGS9-1 fragment containing residues 1–219. Recombinant g9NGDC and g9NGD migrate more slowly on SDS-PAGE than the endogenous RGS9-1 in ROS membranes due to the GST tag on their N termini.

functions. Thus the role of the $G\gamma$ -like domain (22) can be clearly identified with its binding of the photoreceptor-specific $G\beta$ protein $G\beta_{5L}$ (5, 21) because the expression of each subunit in this complex is restricted to photoreceptors (4, 23). Recent results from mice lacking the *rgs9* gene (3) and from co-expression studies in cultured cells (21) indicate that each is critical for intracellular stability of the other. The present study rules out an important role for the $G\gamma$ -like domain and its bound $G\beta_{5L}$ subunit in membrane tethering.

In contrast, the C-terminal domain of RGS9-1 can now be reliably assigned an important role in attaching the RGS9-1- $G\beta_{5L}$ complex to rod outer segment membranes. Analysis of the potential C-terminal peptides removed by protease V8, residues 451–484 (pI 9.52/ M_r 3880.44) or residues 458–484 (pI

FIG. 5. Selective removal of $G_{\beta 5L}$ from ROS membranes without loss of RGS9. ROS membranes were washed with low salt buffer, high salt buffer 1, or urea buffer containing 4 M urea at a concentration of 75 μ M rhodopsin as described under "Experimental Procedures" and then analyzed by a mixture of polyclonal antibodies raised against an RGS9-1 fragment containing residues 227-484 and polyclonal antibodies against $G_{\beta 5L}$ (A). ROS membranes were washed as in panel A with urea buffer containing either 1 or 4 M urea (B). Supernatants (*sup.*) and pellets (2.5% of the total sample in each case) were analyzed by SDS-PAGE and immunoblotting using the $G_{\beta 5}$ polyclonal antibodies. Recombinant g9NGDC- $G_{\beta 5S}$ (50 nM) was mixed with or without ROS membranes (3 μ M R*) in indicated buffers at a volume of 100 μ l by vortexing, incubated on ice for 30 min, and sedimented at $80,000 \times g$ for 30 min (C). The membranes were further washed by resuspension and sedimentation with 200 μ l of buffers as indicated for each. *M* represents moderate salt buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT); *U* represents urea buffer (5 mM Tris, pH 7.4, 4 M urea, 1 mM DTT). Equal portions (2.5% of the total in each case) of supernatants and final pellets were analyzed by SDS-PAGE and immunoblotting as described in panel A.



10.42/ M_r 3622.20), reveals no striking differences in terms of hydrophobicity or charge distribution as compared with the full sequence of RGS9-1 (pI 9.53/ M_r 56680.15). Because the C-terminal membrane binding domain is unique to RGS9-1 and differs even in the striatal isoform RGS9-2 (13-15), it seems likely that the interactions it mediates are specific to ROS. Recent studies have revealed that the C-terminal domain is important for regulation of RGS9-1 GTPase-accelerating protein activity by the effector subunit PDE γ (21) and is the site for a highly specific phosphorylation reaction regulated by light and intracellular calcium (24).

It has been proposed that RGS-GAIP (10) and RGSZ1 (25), which are also membrane-bound in cells, are tethered via palmitoylation of the N-terminal cysteines found in clusters. Endogenous RGSZ1 in brain binds membranes so tightly that it cannot be solubilized by either 1% cholate or high ionic strength. RGS3, which is cytosolic in transfected mammalian cells, was translocated to plasma membranes through its N-terminal 380 amino acids after agonist stimulation (26). More than 90% of the endogenous RGS4 in NG108 cells was found to be soluble (27), and in mammalian cells transfected with RGS4 and RGS16, the proteins were largely soluble (12, 28). However, in yeast cells transformed with RGS4 (11) and RGS16 (28), they are clearly membrane-bound. Palmitoylation of Cys-2 and Cys-12 of either RGS4 or RGS16 was found not to be critical for their membrane association in either yeast or mammalian cells. An amphipathic helix preceding the RGS domain appears to be important for their membrane interactions (28). Reconstitution of RGS4 in lipid vesicles revealed a strong dependence of function on the mode of membrane association

(29). Membrane association of RGS7 has been reported to be regulated by interactions with polycystin (30). RGS5 requires its N-terminal domain for membrane association but not for activity (31), and the N-terminal domain of RGS2 is necessary and sufficient for its membrane localizing, which is enhanced by activated G_q (32). Thus the mechanisms for membrane localization and attachment for RGS proteins may be as diverse as their domain structures and the membranes on which they function.

RGS9 contains a DEP domain (4, 33), which in the protein Disheveled (Dsh) has been reported to be important for membrane localization as well as for important elements of its signaling function (34, 35). Several considerations argue against the DEP domain being important for membrane binding of RGS9-1. First, in *Drosophila*, endogenous Dsh is observed to be cytoplasmic and only shows a cytoplasmic vesicular pattern in late stage embryos (36). In a heterologous expression system, deletion and mutagenesis studies indicate a requirement of a functional DEP domain for plasma membrane localization of Dsh, but this membrane relocalization is dependent on activation of the receptor Frizzled (Fz), suggesting that it is mediated by protein-protein interactions. Moreover, binding of Dsh to the membrane surfaces of cytoplasmic vesicles does not depend on the DEP domain but rather on the DIX domain (34), suggesting that the latter is responsible for general membrane interactions. Secondly, localization of RGS7, which has a DEP domain very similar to that of RGS9-1, has been studied in retina (37) and in brain (38, 39), and it does not appear to have the tight membrane binding properties of RGS9-1 in ROS unless it is palmitoylated (40). Finally, the

results reported here show that a fragment of RGS9-1 containing the DEP domain but not the C-terminal tail is readily released from membranes in moderate salt. Thus although it may well be that the DEP domain of RGS9-1 interacts with other membrane-associated proteins in ROS and thus contributes somewhat to membrane binding, it clearly does not play the sort of decisive role played by the C-terminal tail.

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