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Specific Binding of RGS9-Gβ5L to Protein Anchor in Photoreceptor Membranes Greatly Enhances Its Catalytic Activity*

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The complex between the short splice variant of the ninth member of the RGS protein family and the long splice variant of type 5 G protein β subunit (RGS9-Gβ5L) plays a critical role in regulating the duration of the light response in vertebrate photoreceptors by activating the GTPase activity of the photoreceptor-specific G protein, transducin. RGS9-Gβ5L is tightly associated with the membranes of photoreceptor outer segments; however, the nature of this association remains unknown. Here we demonstrate that rod outer segment membranes contain a limited number of sites for high affinity RGS9-Gβ5L binding, which are highly sensitive to proteolysis. In membranes isolated from bovine rod outer segments, all of these sites are occupied by the endogenous RGS9-Gβ5L, which prevents the binding of exogenous recombinant RGS9-Gβ5L to these sites. However, treating membranes with urea or high pH buffers causes either removal or denaturation of the endogenous RGS9-Gβ5L, allowing for high affinity binding of recombinant RGS9-Gβ5L to these sites. This binding results in a striking ~70-fold increase in the RGS9-Gβ5L ability to activate transducin GTPase. The DEP (disheveled/EGL-10/pleckstrin) domain of RGS9 plays a crucial role in the RGS9-Gβ5L membrane attachment, as evident from the analysis of membrane-binding properties of deletion mutants lacking either N- or C-terminal parts of the RGS9 molecule. Our data indicate that specific association of RGS9-Gβ5L with photoreceptor disc membranes serves not only as a means of targeting it to an appropriate subcellular compartment but also serves as an important determinant of its catalytic activity.

Vertebrate photoreceptors produce rapid electrical responses to light and rapidly recover from excitation upon extinction of illumination (see Refs. 1–4 for recent reviews). Essential for the high speed of the photoresponse are the rates at which the G protein, transducin, is first activated by photoexcited rhodopsin and at which it is then inactivated by RGS9-Gβ5L.[1] The rates of activation and inactivation were both documented to be at the high end of the range observed in the characterized G protein-based signaling pathways (Refs. 5 and 18; reviewed in Ref. 4). The rapid rates of both processes may be explained, at least in part, by the membrane association of most of the signaling proteins participating in phototransduction (reviewed in Ref. 1). Membrane association may increase the probability that these proteins encounter each other in orientations optimized for their productive interactions.

The membrane association of most phototransduction proteins results from either their transmembrane position (*e.g.* rhodopsin) or their posttranslational modifications by fatty acids of isoprenoids (*e.g.* the subunits of transducin) (reviewed in Ref. 1). However, the nature of RGS9-Gβ5L membrane attachment remains unknown. Neither RGS9 nor Gβ5L has been shown to contain transmembrane domains or lipophilic posttranslational modifications. However, RGS9-Gβ5L is bound to photoreceptor membranes so tightly that it can be extracted only under denaturing conditions (7–9). Most recently, He and colleagues (9) reported that the C terminus of RGS9 may contribute to the tight membrane association of RGS9-Gβ5L and that Gβ5L is unlikely to be involved.

Here we report that photoreceptor membranes contain a limited number of specific sites responsible for the high affinity binding of RGS9-Gβ5L. These sites are susceptible to proteolysis, indicating that they are represented by a membrane-associated protein. Most importantly, we demonstrate that specific membrane association of RGS9-Gβ5L enhances its catalytic activity by ~70-fold. We further show that this binding requires the presence of the N-terminal DEP domain of RGS9 but not its C-terminal domain.

EXPERIMENTAL PROCEDURES

Purification of Photoreceptor Membranes—ROS were isolated from the frozen bovine retinas (obtained from T. A. & W. L. Lawson Co., Lincoln, NE) as described in Ref. 10. All manipulations were conducted under infrared illumination. Urea-treated ROS membranes (uROS) lacking the GAP activity of RGS9 were prepared using a two-step protocol (11). First, photoreceptor discs were prepared from ROS in the dark (12). Second, the residual activity of RGS9 was inactivated by treating discs with 6 M urea for 30 min on ice (13). Urea was then removed from the membrane preparation by five consecutive washes of the membranes with buffer A containing 250 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris·HCl (pH 8.0), and 1 mM dithiothreitol. Hypotonically washed membranes (ROS_{hyp}) were prepared from bleached ROS by washing them twice with a buffer containing 0.5 mM EDTA, 5 mM

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[1] The abbreviations used are: RGS, regulators of G protein signaling; Gβ5L, the long splice variant of type 5 G protein β subunit; ROS, rod outer segments; DEP, disheveled/EGL-10/pleckstrin homology domain; GGL, G protein γ subunit-like domain; Gβ5, type 5 G protein β subunit; PDE, type 6 cyclic nucleotide phosphodiesterase from rod outer segments; PDEγ, the inhibitory γ subunit of type 6 cyclic nucleotide phosphodiesterase from rod outer segments; uROS, urea-treated rod outer segment disc; ROS_{hyp}, rod outer segment membrane washed by a hypotonic buffer; HPLC, high performance liquid chromatography; GAP, GTPase-activating protein; GTPγS, guanosine 5′-*O*-(thiotriphosphate).

Tris·HCl (pH 8.0), and 1 mM dithiothreitol followed by two transducin extractions by a buffer containing 5 mM $MgCl_2$, 5 mM Tris·HCl (pH 8.0), and 1 mM dithiothreitol. Rhodopsin concentration in all membranes was determined spectrophotometrically from the difference in absorbance at 500 nm before and after rhodopsin bleaching, using the molar extinction coefficient of 40,000 (14). Bleached rhodopsin in the hypotonically washed membranes was regenerated by 11-*cis*-retinal (2 h at 37 °C by ~5-fold molar excess of 11-*cis*-retinal) prior to the determinations of rhodopsin concentration.

Protein Purification—Transducin was purified from bovine ROS as described in Ref. 15. The concentration of active transducin used in all calculations was determined based on the maximum amount of rhodopsin-catalyzed GTPγS binding performed as described in Ref. 16. The PDEγ-(63–87) peptide was synthesized by the solid-phase Merrifield method on an automated peptide synthesizer and purified to homogeneity by reverse-phase HPLC. Purity and chemical formula of the peptide were confirmed by mass spectrometry and analytical reversed-phase HPLC.

RGS9 and its truncated variants containing the GGL domain were co-expressed with Gβ5L in the Sf9/baculovirus system and purified as described previously (6). The N-terminally His_6-tagged DEP was expressed in *Escherichia coli* BL-21 (DE3). Its boundaries were defined as the sequence between residues 14 and 115 using the structure-based multiple sequence alignment (17). This region was amplified by PCR with primers containing upstream *Nde*I and downstream *Eco*RI sites. The fragment treated with corresponding endonucleases was cloned to pET-28b(+) vector. Resulting construct contained the coding region for the DEP domain placed after a His_6 tag coding sequence and separated by a thrombin cleavage site. Transformed cells were grown at room temperature until they reached $A_{600} = 0.8$. At this point the culture was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and allowed to grow for additional 3 h. Cells were disrupted by sonication and soluble protein was purified on nickel-nitrilotriacetic acid-agarose as described (6).

The purity of all recombinant proteins was at least 80%. Their concentrations were determined spectrophotometrically, based on the theoretically calculated values of the corresponding extinction coefficients at 280 nm. The N-terminal His_6 tag sequence of all recombinant RGS9 constructs provided a specific antibody recognition epitope in experiments addressing the binding of these proteins to photoreceptor membranes.

Membrane Binding Assays—All binding experiments were conducted on ice in buffer A. Recombinant RGS9-Gβ5L or one of its truncated variants was mixed with ROS membranes (final rhodopsin concentration in most experiments was 60 μM) in the volume of 100 μl, and the mixture was loaded on a 50-μl cushion containing 10% sucrose in the same buffer. The mixture was sedimented at 120,000 × *g* for 15 min in an air-driven ultracentrifuge (Airfuge, Beckman). The sucrose cushion significantly reduced contaminations of the sedimented ROS membranes by unbound proteins. It was essential to use polyethylene centrifuge tubes (product 343622, Beckman) in these experiments to prevent nonspecific binding of soluble proteins to the plastic surfaces of the tubes. Supernatants were collected, whereas the sucrose cushions were discarded. The pellet was washed once by buffer A containing 1 M NaCl to reduce nonspecific protein binding and once by buffer A, and then resuspended in 100 μl of buffer A. 20-μl aliquots of resuspended pellet and supernatant fractions were subjected to SDS-PAGE on 10% polyacrylamide gels and Western-blotted using the His probe (H3) mouse monoclonal antibodies (Santa Cruz Biotechnology) recognizing the His_6 tag epitopes of the recombinant proteins. The blots were developed with the Super Signal West Pico Western blot detection kit (Pierce). The use of H3 antibodies rather than antibodies derived against the polypeptides of RGS9-Gβ5L was essential in distinguishing between immunostaining of recombinant RGS9 and endogenous RGS9 present in ROS.

Proteolytic Digestions—Proteolytic digestion of the uROS membranes was performed in 300-μl aliquots containing 90 μM rhodopsin at 22 °C in the presence of either 10 ng of trypsin or 7 μg of Glu-C (V8) protease for 10 min (both proteases were obtained from Sigma). The reaction was stopped by an addition of concentrated mixture of protease inhibitors (Complete™ EDTA-free from Roche Diagnostics) at the final concentration ten times higher than recommended by the manufacturer. uROS were sedimented in an Airfuge for 10 min and washed twice with buffer A containing the same amount of protease inhibitors. Proteolytic digestion of recombinant RGS9-Gβ5L was performed by incubating 250 nM RGS9-Gβ5L with 7 μg of Glu-C protease in 100 μl of buffer A for 15 min at 22 °C. The reaction was stopped by adding protease inhibitors at the same concentration as above.

GTPase Measurements—Transducin GTPase activity was determined by using either a multiple-turnover ([GTP] > [transducin]) or single-turnover ([GTP] < [transducin]) technique as described previously in Refs. 18 and 19, respectively. GTPase assays were conducted at room temperature (22–24 °C) in buffer A. uROS lacking the endogenous GAP activity of RGS9 were used as a source of rhodopsin for assaying the GAP activity of recombinant RGS9 constructs. Rhodopsin in these membranes was activated by illuminating the membranes on ice immediately before the experiments. The reaction was started by the addition of 10 μl of [γ-^{32}P]GTP at desired concentration (~10^5 dpm/sample) to 20 μl of membranes with transducin and RGS9-Gβ5L at required concentrations. The reaction was stopped by the addition of 100 μl of 6% perchloric acid. $^{32}P_i$ formation was measured with activated charcoal (19). Because both native and recombinant RGS9-Gβ5L interact much more efficiently with the complex of transducin with its effector, PDEγ, than with free activated transducin (13, 18, 20, 21), we conducted all GTPase experiments in the presence of 50 μM C-terminal PDEγ peptide, PDEγ-(63–87). This peptide has been shown to completely replace the full-length PDEγ in this assay (18, 20). All data fitting and statistical analyses were performed with SigmaPlot software, version 6.

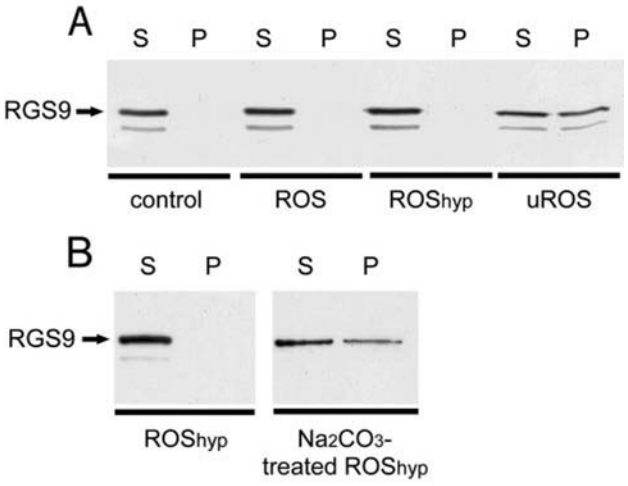

FIG. 1. **The binding of recombinant RGS9-Gβ5L to various preparations of photoreceptor membranes.** *A*, membrane binding assays of recombinant RGS9-Gβ5L (200 nM) were performed with three different preparations of ROS membranes (each containing 60 μM rhodopsin) as described under "Experimental Procedures." No membranes were added in the control experiment. *S* denotes the supernatant fractions; *P* denotes the fractions of washed pellets. Recombinant RGS9 was visualized with antibodies recognizing the N-terminal His_6 tag. The *arrow* indicates the position of the full-length RGS9. An additional band with a molecular mass ~3 kDa lower than the full-length RGS9 represents its proteolytic fragment lacking the distal part of the C terminus. The position of the truncation is evident from the fact that the His_6 tag used as the antibody recognition epitope was located at the N terminus of recombinant RGS9. The presence of such fragment in many preparations of recombinant RGS9-Gβ5L has been previously documented by others (9). *B*, the binding of RGS9-Gβ5L (100 nM) to ROS_{hyp} membranes (60 μM) treated by 100 mM Na_2CO_3 (pH 12.0). Each data set represents one of five similar experiments.

RESULTS

Photoreceptor Membranes Contain Specific Sites for RGS9-Gβ5L Binding—The ability of recombinant RGS9-Gβ5L to interact with photoreceptor membranes was studied in three ROS preparations described under "Experimental Procedures" (Fig. 1*A*). No evidence of high affinity binding was obtained with either whole ROS or hypotonically washed ROS_{hyp}. However, a significant fraction of RGS9-Gβ5L was found bound to urea-treated uROS membranes. ROS and ROS_{hyp} are different from uROS because they contain a functionally active endogenous RGS9-Gβ5L, whereas RGS9-Gβ5L in uROS is completely inactivated (9, 13). We suggest that treating photoreceptor membranes with urea caused the denaturation of endogenous RGS9-Gβ5L resulting in its dissociation from specific binding sites on the membranes. Consequently, these sites became

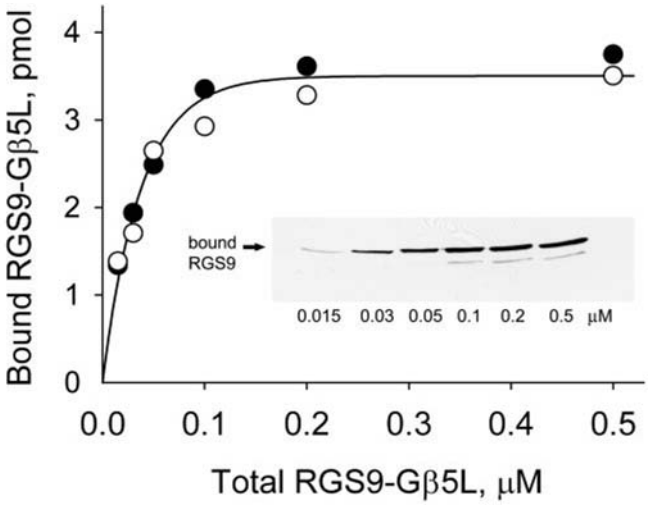

FIG. 2. **The concentration dependence of recombinant RGS9-Gβ5L association with uROS membranes.** Membrane binding assays were performed as described under "Experimental Procedures" with uROS containing 60 μM rhodopsin and various amounts of recombinant RGS9-Gβ5L. The amount of RGS9 in membrane-bound fractions was determined by densitometric analysis of the Western blots stained by the antibodies recognizing the N-terminal His_6 tag of RGS9 (*inset*). The absolute amounts of RGS9 in each band were determined by comparing the band densities with those of the RGS9-Gβ5L standards of known concentrations loaded on the same gel (data not shown). The data are taken from two identical experiments with *open* and *closed symbols* representing each individual experiment.

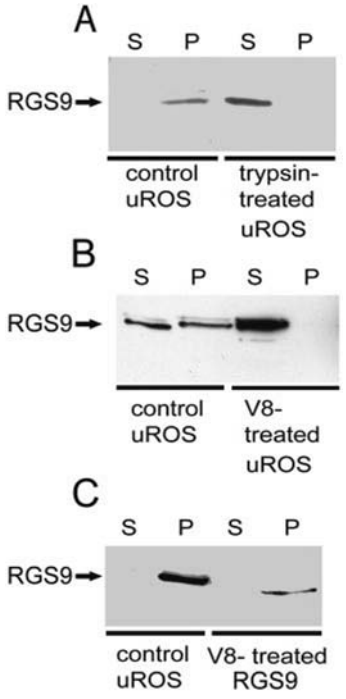

FIG. 3. **The effects of proteolytic treatments on RGS9-Gβ5L association with uROS membranes.** uROS membranes were treated with either trypsin (*A*) or protease V8 (*B*) as described under "Experimental Procedures." In control experiments, protease inhibitors were added to uROS prior to the additions of proteases. Aliquots of proteolyzed and control uROS were used in the binding assays with 15 nM (*A*) or 30 nM (*B*) RGS9-Gβ5L and 60 μM uROS. *C*, RGS9-Gβ5L was proteolyzed by protease V8 also as described under "Experimental Procedures" and used in the membrane binding assays at the 30 nM concentration. The binding of untreated RGS9-Gβ5L is shown as a control. The data in each panel are taken from one of three similar experiments. *S* denotes the supernatant fractions; *P* denotes the fractions of washed pellets.

available for the binding of exogenous recombinant RGS9-Gβ5L. This suggestion is further supported by the observation that membrane binding of recombinant RGS9-Gβ5L also took place after treating ROS with alkaline sodium carbonate buffer (Fig. 1*B*). This treatment has been previously shown to result in the extraction of endogenous RGS9-Gβ5L from photoreceptor membranes (8).

We then determined the amount of membrane-associated sites that are available for the binding of recombinant RGS9-Gβ5L in uROS (Fig. 2). We incubated various concentrations of RGS9-Gβ5L (ranging from 0.015 to 0.5 μM) with uROS membranes containing 60 μM rhodopsin and determined the amount of membrane-associated RGS9-Gβ5L after removing nonspecifically bound protein by two washes, as described under "Experimental Procedures." The binding of RGS9-Gβ5L saturated at the ~3.5 pmol level, which corresponds to 1 molecule of bound RGS9-Gβ5L/~1700 molecules of rhodopsin. This value is within the range of the published RGS9/rhodopsin ratio in bovine ROS (1:1640 ± 620 from Ref. 7). Therefore, the maximal amount of recombinant RGS9-Gβ5L that can bind to the specific sites in uROS membranes is approximately the same as the amount of native RGS9-Gβ5L originally present in these membranes.

Specific Sites for RGS9-Gβ5L Binding Contain a Protein Component—In the next set of experiments, we tested whether high affinity binding of RGS9-Gβ5L to photoreceptor membranes may involve a specific interaction with a protein component located in the photoreceptor membranes. The binding of recombinant RGS9-Gβ5L was analyzed after proteolytic treatments of uROS membranes by trypsin or endoproteinase Glu-C (also known as protease V8). We choose trypsin as the most commonly used nonspecific protease and V8 as the protease previously reported to remove endogenous RGS9-Gβ5L from ROS membranes (9). The treatment with either protease resulted in a complete loss of the uROS ability to bind RGS9-Gβ5L (Fig. 3, *A* and *B*), indicating that the binding sites indeed contain a protein component.

We then addressed whether the ability of RGS9-Gβ5L to specifically bind to photoreceptor membranes was also affected by its partial proteolysis by V8, as reported by He and colleagues (9). An experiment shown in Fig. 3*C* indicates that a short treatment of recombinant RGS9-Gβ5L by V8, using the protease concentration identical to that in Fig. 3*B*, resulted in a formation of a single major proteolytic fragment with the molecular mass of ~3 kDa lower than the mass of the full-length RGS9. This fragment could represent only the C-terminally truncated RGS9 because it was recognized by the antibodies against the N-terminal His_6 tag. This observation is in complete agreement with the data reported by He and colleagues (9). However, in contrast to their report, the ability of the C-terminally truncated RGS9-Gβ5L to bind to membranes was essentially the same as that of the full-length protein. These data indicate that protease V8 releases RGS9-Gβ5L from photoreceptor membranes by proteolyzing its membrane-associated partner rather than RGS9-Gβ5L itself.

Specific Membrane Binding of RGS9-Gβ5L Enhances Its Catalytic Activity—In the next set of experiments, we analyzed the effects of RGS9-Gβ5L association with photoreceptor membranes on its ability to activate transducin GTPase. We first utilized the single-turnover GTPase methodology that has been ubiquitously used in studying transducin GTPase regulation in the past (*cf.* Ref. 19). The data in Fig. 4 indicate that, in the presence of excess RGS9-Gβ5L, the rate of GTP hydrolysis was limited by the amount of uROS present in the assay. No significant effect was observed when the concentration of uROS was increased in control experiments in the absence of RGS9-Gβ5L. Because the increase in the amount of uROS provided

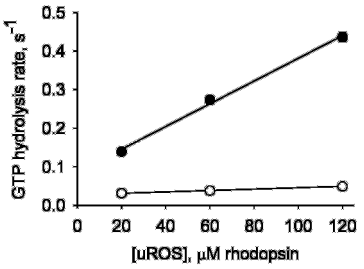

FIG. 4. **The effect of uROS concentration on the ability of RGS9-Gβ5L to activate transducin GTPase in single-turnover assays.** Single-turnover transducin GTPase assays were performed as described under "Experimental Procedures" at 2 μM transducin 50 μM PDEγ-(63–87) and 0.2 μM [γ-^{32}P]GTP with (●) or without (○) 1 μM recombinant RGS9-Gβ5L. The reaction was carried out for five different durations; the values of the GTP hydrolysis rates were determined from the exponential fits to the data and plotted as a function of uROS concentration in the assays. The data shown are taken from one of three independent experiments with *error bars* representing S.E. obtained from individual exponential fits (note that, in most cases, *error bars* are smaller than the size of the points).

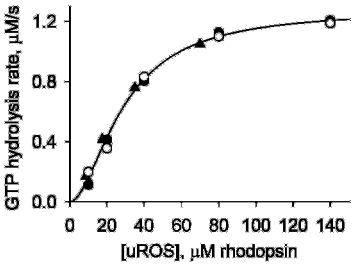

FIG. 5. **The effect of uROS concentration on the catalytic activity of RGS9-Gβ5L determined in multiple-turnover GTPase assays.** Multiple-turnover transducin GTPase assays were performed as described under "Experimental Procedures" at 20 μM transducin, 50 nM recombinant RGS9-Gβ5L, 50 μM PDEγ-(63–87), and 200 μM [γ-^{32}P]GTP. The reaction was carried out for 10 s during which no more than 30% GTP was hydrolyzed at any uROS concentration used. The basal GTPase activity of transducin measured at each uROS concentration without RGS9-Gβ5L (data not shown) was subtracted from the values measured with RGS9-Gβ5L, and the resulting values were plotted on the *graph*. The data are taken from three independent experiments with different *symbols* representing different experiments. The data were fitted with the Hill equation: $y = V_{max}\cdot[\text{uROS}]^n/(K_m{}^n + [\text{uROS}]^n)$, where the maximal rate of RGS9-Gβ5L-catalyzed GTP hydrolysis, V_{max} = 1.28 ± 0.04 (S.E.) μM GTP hydrolyzed/s, apparent Michaelis constant, K_m = 29 ± 2 μM rhodopsin, and the Hill coefficient, n = 1.8 ± 0.1.

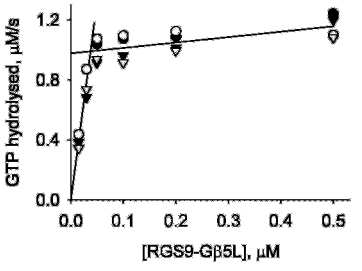

FIG. 6. **The dependence of recombinant RGS9-Gβ5L catalytic activity on its concentration.** Multiple-turnover transducin GTPase assays were performed at a fixed uROS concentration (60 μM rhodopsin) as described under "Experimental Procedures." All samples contained 20 μM transducin, 50 μM PDEγ-(63–87), and 200 μM [γ-^{32}P]GTP and various concentrations of RGS9-Gβ5L. The basal GTPase activity of transducin measured without RGS9-Gβ5L (data not shown) was subtracted from the values measured with RGS9-Gβ5L, and the resulting values were plotted on the *graph*. The data were fitted with *two straight lines* with slopes of 26 ± 2 s^{-1} for the initial steep rise and 0.36 ± 0.10 s^{-1} for the slow rise. The data are taken from four independent experiments with different *symbols* representing each individual experiment.

an increase in the number of specific sites for RGS9-Gβ5L binding, this observation supports the idea that, as RGS9 becomes membrane-bound, its activity increases.

To quantify the degree of RGS9-Gβ5L activity stimulation by uROS, we used an alternative kinetic approach: multiple-turnover transducin GTPase assay. In this approach, the maximal catalytic activity of RGS9-Gβ5L (expressed as the number of transducin molecules whose GTPase activity may be stimulated by one RGS9-Gβ5L molecule per time unit) is determined when small catalytic amounts of RGS9-Gβ5L are supplemented by saturating concentrations of transducin and GTP (6, 18). In the experiment shown in Fig. 5, maximal catalytic activity of RGS9-Gβ5L was determined at various amounts of uROS. The activity rose until saturation was achieved as the amount of uROS became sufficient to provide membrane binding sites for all RGS9-Gβ5L molecules present in the assay. Fitting the data with the Hill equation and dividing the obtained V_{max} value by the RGS9-Gβ5L concentration in the assay yielded the maximal catalytic activity of RGS9-Gβ5L equal to ~26 turnovers/s.

Further support of the hypothesis that membrane binding stimulates the catalytic activity of RGS9-Gβ5L comes from the data shown in Fig. 6. We measured the rates of multiple-turnover transducin GTPase at various RGS9-Gβ5L concentrations under experimental conditions essentially replicating those used to determine the concentration dependence of RGS9-Gβ5L association with uROS membranes in Fig. 2. The rate of GTP hydrolysis rose sharply before RGS9-Gβ5L concentration reached ~50 nM, after which its increase slowed by approximately 2 orders of magnitude. This concentration corresponds to practically the same amount of RGS9-Gβ5L as the maximal amount of RGS9-Gβ5L bound to uROS membranes in Fig. 2. Therefore, we conclude that RGS9-Gβ5L association with specific sites on the photoreceptor membranes leads to a dramatic stimulation of its ability to activate transducin GTPase. This conclusion is also consistent with the observation that removing the excess of nonbound RGS9-Gβ5L by membrane sedimentation did not reliably reduce the rate of transducin GTPase measured with these membranes in similar assays (we used 1 μM RGS9-Gβ5L in these experiments; data not shown). The degree of RGS9-Gβ5L activity stimulation caused by RGS9-Gβ5L membrane association was estimated from the ratio of the slopes of linear regression fits to the data before and after the slowdown point in Fig. 6. This ratio was equal to ~70, provided that the steep slope was 26 ± 2 (mol of GTP)·(mol of RGS9-Gβ5L)$^{-1}\cdot s^{-1}$ (S.E.) and the shallow slope was 0.36 ± 0.1 (mol of GTP)·(mol of RGS9-Gβ5L)$^{-1}\cdot s^{-1}$.

High Affinity Membrane Binding of RGS9-Gβ5L Requires the Presence of DEP Domain of RGS9 but Not Its C Terminus— The role of individual domains of RGS9 in the membrane binding of RGS9-Gβ5L was addressed in experiments with a series of deletion mutants lacking various structural elements of RGS9. We used the same recombinant constructs as in a previous study, where we examined the role of these domains in regulating RGS9-Gβ5L catalytic activity (6). We conducted this analysis only with recombinant constructs possessing the catalytic activity of RGS9 to avoid potential artifacts resulting from misfolding of protein fragments.

Schematic illustrations of RGS9 domain composition and protein constructs used in these experiments are drawn in Fig. 7*A* (see Refs. 4 and 22 for recent reviews on RGS9 domain structure). The N-terminal domain of RGS9 containing ~110 amino acids is called DEP because highly homologous domains are also present in disheveled, EGL-10, and pleckstrin. DEP is followed by an ~80-amino acid sequence with the lowest degree of homology to other members of the RGS protein family, which we term "interdomain." The next domain of ~80 residues is the

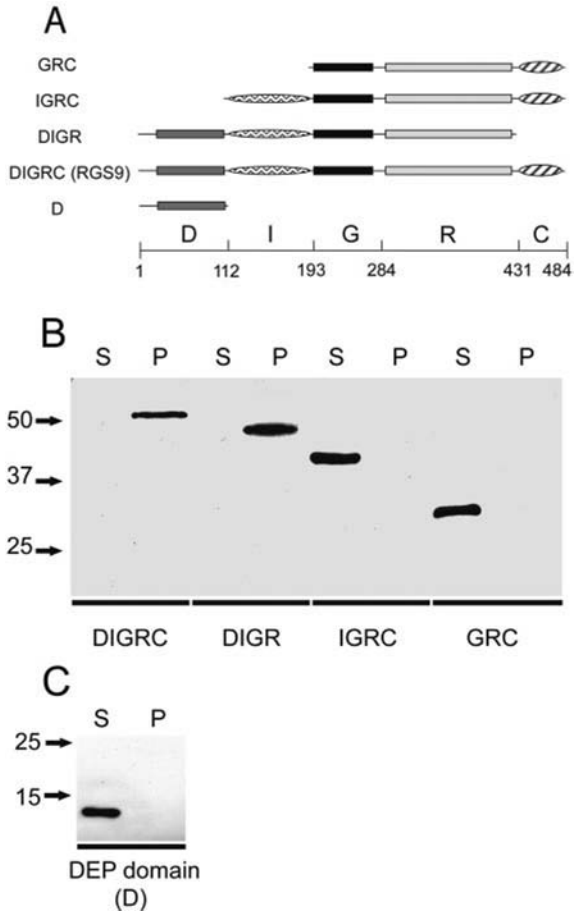

FIG. 7. **The DEP domain of RGS9 is necessary for the high affinity binding of RGS9-Gβ5L to photoreceptor membranes.** *A*, schematic structure of the RGS9 domain composition and recombinant constructs used in this study. Established structural domains (DEP, GGL, and RGS) are shown as *rectangles*, and other structures are shown as *ellipsoids*. The domains are abbreviated as follows: *D*, DEP; *I*, interdomain; *G*, GGL; *R*, RGS homology domain; *C*, C terminus. The *numbers* shown at the *bar* indicate the positions of the amino acids where constructs start and end. Each construct used in this study contained a N-terminal His_6 tag. *B*, membrane binding assays of recombinant RGS9-Gβ5L constructs containing truncated mutants of RGS9 (each at 15 nM) were performed with uROS membranes (containing 60 μM rhodopsin) as described under "Experimental Procedures." *C*, membrane binding assay with recombinant DEP domain (50 nM). Each data set represents one of six (*B*) or four (*C*) similar experiments.

G protein g subunit-like domain (GGL), which provides the binding site for Gβ5L. GGL is followed by the RGS homology domain of ~120 residues and finally by the C-terminal extension of ~50 residues that is often called C-terminal domain. Because it has been previously shown that the expression of all RGS9 constructs containing GGL domain in soluble form was possible only in the presence of Gβ5 (21), we co-expressed all GGL domain-containing RGS9 constructs with Gβ5L.

We conducted membrane-binding assays at the relatively low, 15 nM concentration of the RGS9-Gβ5L constructs so that the amount of binding sites within the uROS membranes was sufficient to bind all RGS9-Gβ5L present. The data of Fig. 7*B* indicate that the construct lacking the C-terminal domain of RGS9 binds to uROS as well as the full-length recombinant protein. To the contrary, no membrane association was observed with the constructs lacking either DEP alone or both DEP and the interdomain. The lack of membrane association for these constructs was also observed at the 10-fold higher construct concentrations used in similar assays (data not shown). These data clearly demonstrate that the DEP domain of RGS9 but not its C terminus is essential for RGS9-Gβ5L association with specific binding sites within the photoreceptor membranes. Interestingly, the DEP domain alone did not show any sign of membrane binding (Fig. 7*C*). This may be explained by the requirement of the other structural elements of RGS9-Gβ5L for membrane association, although a possibility of the DEP domain misfolding could not be ruled out as well.

DISCUSSION

Membrane Association of RGS9-Gβ5L Leads to a Dramatic Increase in Its Catalytic Activity—The central observation of this study is that specific binding of RGS9-Gβ5L to proteinaceous sites within the photoreceptor membranes enhances its ability to activate transducin GTPase by approximately 2 orders of magnitude. The absolute catalytic activity of recombinant RGS9-Gβ5L achieved upon its membrane association is ~26 turnovers/s, which is on the same order as the activity of native RGS9-Gβ5L ($98 \pm 40\ s^{-1}$ from Ref. 18). In combination with recent findings that recombinant and native RGS9-Gβ5L have the same affinity for transducin and the same degree of cooperation with PDEγ (6), this observation indicates that the catalytic properties of recombinant and native RGS9-Gβ5L are very similar. In fact, the difference between the catalytic activities of native and recombinant RGS9-Gβ5L may be even smaller than it appears. It may be explained by assuming that some of the recombinant protein used in this study was not functionally active but retained its ability to bind to the protein anchor in the uROS membranes, whereas the entire concentration of recombinant RGS9-Gβ5L was used in the calculations of catalytic activity. It may be also accounted for by some unavoidable inaccuracies in protein concentration determinations employed in either case.

Our data also clarify a discrepancy raised in previous investigations with recombinant RGS9-Gβ5L. It has been consistently noted that its absolute catalytic activity was significantly lower than the activity of native protein from rod outer segments (6, 21). For example, the maximal activity of only ~1.8 turnovers/s was reported for recombinant RGS9-Gβ5L studied in the multiple turnover GTPase assays (6). We now understand that the amount of recombinant RGS9-Gβ5L used in that and most other investigations by far exceeded the capacity of specific binding sites within the uROS membranes present in the assays. As a result, RGS9-Gβ5L was likely to be distributed between a small fraction of the membrane-bound protein with high catalytic activity and a large fraction of unbound protein whose activity was very low. Thus, the number of $1.8\ s^{-1}$ reflected an average activity of all RGS9-Gβ5L molecules present in those assays and not the activity of the most active fraction associated with the membranes.

The DEP Domain of RGS9 Is Crucial for RGS9-Gβ5L Membrane Attachment—RGS9 is one of the most intensively studied representative of the RGS protein subfamily whose members share similar domain composition and exist as complexes with Gβ5 (reviewed in Refs. 4, 22, and 23). Several recent publications have significantly advanced our understanding of structure-function relations within the catalytic domain of RGS9 (24, 25). Functional roles of the other structures of RGS9 and Gβ5L remain poorly understood, although two independent studies indicate that all of them are important for regulating the activity of the RGS catalytic domain (6, 21). These studies argue that noncatalytic domains of the RGS9-Gβ5L complex, including the DEP domain, contribute to its ability to interact preferentially with the transducin-PDEγ complex as opposed to activated transducin alone.

The data presented in Fig. 7 of this study indicate that another function of the DEP domain of RGS9 is to enable RGS9-Gβ5L to bind tightly to specific sites within the photo-

receptor membranes. The amount of these binding sites is limited, and in rod outer segments they are all occupied by endogenous RGS9-Gβ5L. The high affinity DEP-dependent membrane association of recombinant RGS9-Gβ5L becomes possible only after endogenous RGS9-Gβ5L is either extracted or denatured. The specificity of this association is supported by two considerations. First, this association is resistant to the same membrane treatments as the membrane association of native RGS9-Gβ5L. Second, the catalytic activity of membrane-bound recombinant RGS9-Gβ5L is on the same order as the activity of native protein, whereas the activity of nonbound RGS9-Gβ5L is much lower.

The properties of the high affinity RGS9-Gβ5L membrane association revealed in this study allow us to understand an apparent contradiction between our conclusion that the binding requires the N-terminal DEP domain and the recent report by He and colleagues (9), who argue for the crucial role of the C-terminal domain of RGS9 in binding. We noted that ROS membranes used by He and colleagues were prepared in a way that retained the intactness of endogenous RGS9-Gβ5L. Thus, their membranes were unlikely to contain high affinity sites available for the binding of recombinant RGS9-Gβ5L and its deletion mutants. Therefore, we conclude that recombinant RGS9-Gβ5L in that study was not bound to the same sites that we studied here, the sites to which the native RGS9-Gβ5L is normally bound. Furthermore, we did not observe wash-resistant RGS9-Gβ5L binding to non-urea-treated membranes, which He and colleagues described, although exact explanation for this difference is not clear.

Another argument by He and colleagues (9) in favor of the role of RGS9 C terminus in RGS9-Gβ5L membrane attachment was that essentially only C-terminally cleaved RGS9 was released from ROS membranes upon their proteolytic digestion by protease V8. Our data (Fig. 3) indicate that the effect of V8 protease on the RGS9-Gβ5L membrane attachment originates from the proteolysis of the protein component of membrane-associated binding sites rather than from the C-terminal proteolysis of RGS9. In this context, it is logical to assume that, although the C terminus of RGS9 is not important for binding the anchor protein, it is protected from proteolysis when RGS9-Gβ5L is membrane-bound. The proteolysis of the membrane anchor leading to the release of RGS9-Gβ5L from membranes to solution may significantly increase the accessibility of RGS9 C terminus to V8 protease.

In summary, our data indicate that the membrane attachment of RGS9-Gβ5L in photoreceptors serves not only as a means to contain it in the appropriate intracellular compartment, but also serves as a crucial determinant of its catalytic activity. The goal of the future experiments is to identify the membrane-associated anchoring protein for RGS9-Gβ5L and to elucidate the mechanism by which it regulates RGS9-Gβ5L activity.

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