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High expression levels in cones of RGS9, the predominant GTPase accelerating protein of rods

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ABSTRACT **RGS9 is a member of the RGS family of GTPase accelerating proteins (GAPs) for heterotrimeric G proteins. We have explored its contribution to GTPase acceleration in mammalian rod and cone photoreceptors. When RGS9 was specifically removed from detergent extracts of rod outer segments by immunodepletion, the extracts lost nearly all of their GAP activity stimulatable by the inhibitory subunit of cGMP phosphodiesterase. Immunolocalization using monoclonal antibodies and confocal microscopy revealed that RGS9 is present in cones at significantly higher levels than in rods. Thus, RGS9 is the predominant source of GAP activity in rod outer segments, and RGS9 concentration emerges as a potentially important determinant of the faster response kinetics and lower sensitivity of mammalian cones, as compared with rods.**

Recovery of the G protein cascade of vision to the dark state on a sub-second time scale is facilitated by a GTPase accelerating protein (GAP) (1) whose activity is potentiated by phosphodiesterase-γ (PDEγ), an inhibitory subunit of the effector enzyme cGMP phosphodiesterase (2, 3). RGS9, a member of the RGS (regulators of G protein signaling) family (4) of GTPase accelerating proteins for Gα subunits (5), has been identified as a photoreceptor-specific GAP for the visual G protein transducin (G_t), whose activity is potentiated by PDEγ (6). Immunoblots revealed that RGS9 is enriched in rod outer segments (ROS), and *in situ* hybridization experiments revealed that RGS9 mRNA in the retina is restricted to the photoreceptor layer. The experiments reported here address two questions not resolved by previous work: what fraction of PDEγ-sensitive GAP activity in rod outer segments can be reliably attributed to RGS9, and what role, if any, does GTPase acceleration by RGS9 have in the much faster recovery kinetics of cones?

The first question arises because numerous RGS proteins and mRNAs encoding them have been identified in mammalian retina (6–8), and because other outer segment proteins have been proposed to act as transducin GAPs (2, 9, 10). The second question, concerning RGS9 in cones, is of great interest because cones employ a very similar transduction machinery to that of rods but have greatly accelerated response kinetics and lowered sensitivity compared with rods. Mammalian cones (but not those of amphibians) display signal amplification characteristics similar to those of rods (11), and so the interesting possibility arises that both sensitivity and temporal resolution are governed in mammalian cones by inactivation kinetics. Inactivation kinetics in rods and cones are limited by a "dominant time constant" for each cell type (12, 13) whose biochemical basis has recently been suggested to be GTP hydrolysis by transducin (14), an idea supported by the observation that this rate-limiting recovery time constant is unaffected by preventing [Ca^{2+}] changes that regulate the other likely candidate, decay of photoactivated rhodopsin, R* (13).

We have addressed these questions in the bovine retina using antibodies raised against recombinant RGS9. First, we examined the solubility properties of RGS9 and found that conditions allowing extraction of PDEγ-sensitive GAP activity in soluble form also efficiently extract RGS9. Next, we used polyclonal antibodies (6) to deplete RGS9 from detergent extracts of rod outer segments and found this treatment also removes most of the PDEγ-sensitive GAP activity from those extracts. Immunofluorescence techniques employed previously to localize other transduction components within the retina (15, 16) have allowed us to identify RGS9 as a major component of cones and have provided support for the idea that accelerated recovery in cones may be, at least in part, due to higher concentrations of RGS9 than those found in rods.

MATERIALS AND METHODS

Preparation and Washing of Rod Outer Segments. Rod outer segments were purified in dim red light from fresh bovine retina as described (17) or from frozen bovine retinas using a standard sucrose gradient technique (18). Membrane washing procedures (Fig. 1) were carried out in dim red light using ROS that had been washed with GAPN buffer [10 mM Tris·HCl, pH 7.4/100 mM NaCl/2 mM $MgCl_2$/solid phenylmethylsulfonyl fluoride (PMSF)], then homogenized and stored in GAPN buffer at −80°C. The following buffers were used for sequential washing experiments: hypertonic wash buffer, 5 mM Tris·HCl, pH 7.4, 1 M NH_4Cl, 1 mM dithiothreitol (DTT), and solid PMSF; hypotonic wash buffer, 5 mM Tris·HCl, pH 7.4, 3 mM EDTA, 1 mM DTT, and solid PMSF; urea buffer, 4 M urea, 5 mM Tris·HCl, pH 7.4, 1 mM DTT, and solid PMSF. ROS buffer contained 10 mM MOPS, pH 7.4, 30 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and solid PMSF. Sodium carbonate buffer contained 100 mM Na_2CO_3, with the pH adjusted to 12.0 with NaOH. For each sequential washing step, ROS membranes were diluted, typically to 11 μM rhodopsin, in the indicated buffer, homogenized extensively on ice using a glass/Teflon homogenizer, and centrifuged in a Ti-45 rotor (Beckman) at 44,000 rpm for 20 min (hypertonic buffer, GAPN buffer), 35 and 50 min (hypotonic buffer), or 25 min (urea buffer). The order (and number) of washing steps was hypertonic buffer (two), hypotonic buffer (two), urea buffer (one), GAPN (one). At each step of the wash proce-

Abbreviations: GAP, GTPase accelerating proteins; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; Mops, 4-morpholinepropanesulfonic acid; ROS, rod outer segments; OG, *n*-octyl glucoside; GTP[γS], guanosine 5′-[γ-thio]triphosphate; PDE, phosphodiesterase.

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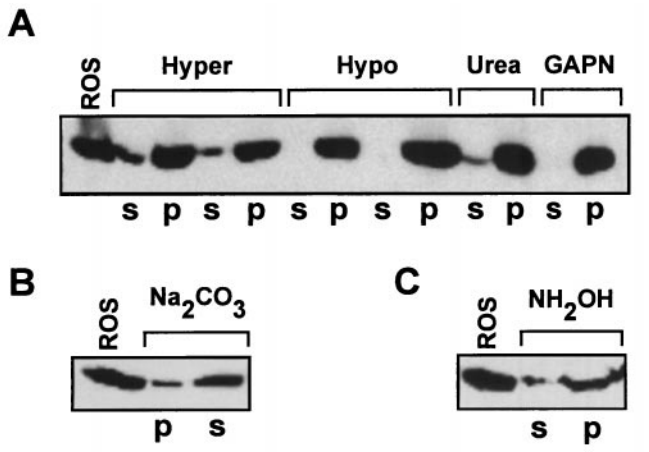

FIG. 1. Peripheral association of RGS9 with rod outer segment membranes. RGS9 immunoblots are shown for ROS membranes subjected to various washing procedures, as described in the text. Lanes were loaded with equivalent fractions of pellets (p) or supernatants (s). (*A*) Sequential washes with hypertonic buffer (Hyper); hypotonic buffer (Hypo); 4 M urea (Urea); GAPN moderate ionic strength buffer (GAPN). (*B*) Treatment with 100 mM sodium carbonate, pH 12.0. (*C*) Treatment with 1 M hydroxylamine.

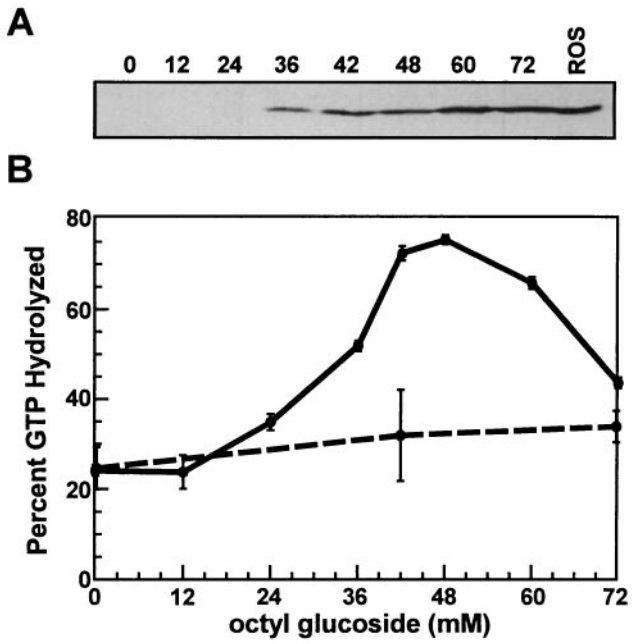

FIG. 2. Solubilization of RGS9 and ROS GAP activity by octyl glucoside. (*A*) Immunoblot of supernatants from ROS membranes washed with the indicated concentrations (mM) of octyl glucoside. (*B*) GTP hydrolysis acceleration induced by supernatants of ROS membranes washed with indicated concentrations of octyl glucoside. GTP hydrolysis of transducin was measured under single-turnover conditions in the presence of 920 nM PDEγ.

dure, an aliquot of homogenized membrane suspension or supernatant was removed, and 40 μl used for electrophoresis and immunoblotting. For sodium carbonate extraction, ROS membranes were washed with ROS buffer and resuspended to 1.5 μM rhodopsin, in 100 mM Na_2CO_3, pH 12.0, homogenized by repeated extrusion through a 25-gauge needle, incubated on ice for 20 min, and centrifuged at 70,000 $\times$ *g* for 20 min at 4°C. For hydroxylamine washing, ROS were homogenized using a 23-gauge needle, diluted to 10 μM rhodopsin in 1 M NH_2OH (pH 7.5), incubated at room temperature for 4 h with constant mixing, then rehomogenized and centrifuged at 80,000 $\times$ *g* for 20 min at 4°C. The supernatant was carefully removed, and 77 μl was precipitated with 10% trichloroacetic acid.

Detergent Solubilization. ROS were diluted to 60 μM rhodopsin in varying concentrations of *n*-octyl glucoside (OG) in GAPN buffer, homogenized by repeated extrusion through a 23-gauge needle, and incubated on ice for 20 min. Samples were centrifuged at 73,000 $\times$ *g* for 30 min at 4°C. Supernatants were assayed for PDEγ-enhanced GAP activity in single-turnover GTPase assays using His-PDEγ (6) and for RGS9 protein by immunoblots. Each 100-μl GAP assay sample included 50 μl of solubilized ROS supernatant (corresponding to a final concentration of 30 μM rhodopsin if fully solubilized) and 920 nM PDEγ. Immunoblots were prepared using 12.5 μl (Fig. 2) or 25 μl of each supernatant. Different preparations of ROS and buffers were tested in the solubilization procedures and gave essentially identical results.

Antibodies. Recombinant RGS9 fragments (with His_6 tags) were prepared as described (6). Polyclonal rabbit antisera specific for RGS9 were described previously (6). For monoclonal antibody (mAb) production, full-length recombinant RGS9 was dialyzed against 70 mM sodium phosphate buffer, pH 7.5, and injected with Ribi adjuvant (Ribi Immunochem) into BALB/c mice. Monoclonal antibodies were produced according to standard procedures (19). Monoclonal antibodies were purified from culture supernatant using a protein A-Sepharose CL-4B column (Pharmacia).

Electrophoresis and Immunoblots. Proteins in the samples from washing and solubilization experiments were precipitated with 10% trichloroacetic acid prior to electrophoresis. A Hoefer mini-gel system was used for SDS/PAGE (20) and for electrotransfer of proteins to Immobilon (mAb) or to supported nitrocellulose (polyclonal Ab). For Immobilon, membranes were first blocked with 3% wt/vol gelatin in 20 mM Tris·HCl buffer, pH 7.5, containing 500 mM NaCl and then incubated for 1 h with primary antibody. Alkaline phosphatase-conjugated secondary antibody was diluted 1:5000 and used in a 1-h incubation before development with the ECL system (Amersham). For nitrocellulose, membranes were incubated with 5% nonfat dry milk/TBS solution for 1 h at room temperature. Primary RGS9 antibodies (6) were used at a dilution of 1:1,000 (His-RGS9c anti-serum A23A7) or 1:10,000 (His-RGS9c anti-serum, A13C7) in 0.5% nonfat dry milk/TBS and incubated overnight. Blots were washed with TBS containing 0.1% Tween-20 (TBS-T) and incubated with a 1:10,000 dilution of anti-rabbit IgG-peroxidase secondary antibodies (Amersham) for 45 min in 0.5% nonfat dry milk/TBS. Blots were washed again with TBS-T and then developed using the ECL system.

Immunocytochemistry. Bovine eyes obtained from a local slaughterhouse were held briefly on ice prior to dissection. The anterior segment and vitreous humor were removed from each eye, and the eye cups were immersed for 6 h in chilled 4% formaldehyde in 0.086 M sodium phosphate buffer, pH 7.3. Pieces of retina 5 mm^2 were excised, washed in phosphate buffer, and embedded in 5% agarose in phosphate-buffered saline (21). Sections of retina (100 μm thick) were cut with a VT1000E vibrating microtome (Leica). Antibody labeling was assessed by indirect immunofluorescence. Sections were first incubated in normal goat serum diluted 1:50 in ICC buffer (PBS containing 0.5% BSA, 0.2% Triton X-100, and 0.05% sodium azide, pH 7.3) for 1 h to reduce nonspecific labeling. Mouse monoclonal RGS9 antibody (A_{280} = 0.69) diluted 1:25 in ICC buffer was added to retinal sections for incubation overnight in a humidified chamber. Negative controls for antibody labeling were produced by omitting primary antibody from the incubation buffer or by preadsorbing RGS9 antibody with purified full-length RGS9 coupled to CNBr-activated Sepharose (0.5 ml, 1 mg/ml RGS9). After repeated washing in buffer, sections were incubated for 4 h in Cy 3-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, Inc.) diluted

1:200 in ICC buffer. Sections were washed repeatedly with ICC buffer, mounted in 5% *n*-propyl gallate in glycerol, coverslipped, and examined on a Bio-Rad MRC 600 laser scanning confocal microscope. Scan head parameters influencing image intensity and resolution (pinhole aperture, PMT gain and black level, attenuation of laser by neutral density filters) were standardized for all samples. Single plane and Z series images were collected and stored as unprocessed files. Images for publication were processed with ADOBE PHOTOSHOP 3.0.

RGS9 Immunodepletion from Detergent-Solubilized ROS. Rabbit IgG from pre-immune serum or immune serum was purified by protein A-Sepharose CL-4B (Pharmacia) using standard techniques (22). Purified RGS9 immune IgG (0.75 mg) was incubated with either Ni^{2+}-NTA agarose (100-μl beads) or Ni^{2+}-NTA agarose (100-μl beads) pre-incubated with 1.5 mg of recombinant full-length RGS9 (to deplete RGS9-specific antibodies), for 2 h at 4°C with constant mixing, followed by sedimentation of the agarose beads at low speed. IgG remaining in the supernatant was transferred to 50 μl of protein A-Sepharose CL-4B beads and incubated for 1 h at 4°C with constant mixing. The protein A beads were then pelleted by low speed centrifugation, and the supernatant was removed. The beads were then washed three times with GAPN buffer containing 40 mM OG. ROS (70 or 55 μM rhodopsin) were solubilized in GAPN buffer containing 40 mM OG under dim-red light, as described above, and centrifuged at 73,000 × *g* for 30 min at 4°C. The supernatant was incubated overnight at 4°C with protein A-Sepharose CL-4B with bound pre-immune IgG, RGS9 immune IgG, or RGS9 immune IgG depleted of RGS9-specific antibodies. The beads were then pelleted by low-speed centrifugation, and the supernatants were assayed for GAP activity and PDEγ-enhanced GAP activity in single-turnover GTPase assays as well as for RGS9 protein by immunoblotting. Blots were stained with 0.1% Ponceau S and imaged prior to blocking to check for nonspecific protein loss from the immunodepletion procedures.

In similar experiments, purified RGS9 immune IgG was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's recommended protocol, using 5 mg of purified IgG with 1 ml of resin. Rabbit whole IgG-agarose (Sigma) was used as a control resin. 500 μl of solubilized ROS (70 μM rhodopsin) was incubated with 50 μl of RGS9 IgG-Sepharose or rabbit IgG-agarose overnight at 4°C. The resin was pelleted by low-speed centrifugation, and supernatants were assayed for ROS GAP activity in single-turnover GTPase assays, in the presence or absence of 1.17 μM PDEγ.

Single-Turnover GTPase Assays. Single-turnover GTPase assays were performed to test detergent-solubilized or RGS9-immunodepleted ROS extracts for GAP activity, with or without added PDEγ, essentially as described (1, 3, 6). Specifically, urea-washed ROS membranes containing 15 μM rhodopsin were reconstituted with 1 μM transducin in GAPN buffer and 20 μM AMP-PNP. GTP hydrolysis was measured in a final assay volume of 100 μl and performed at 23°C. All concentrations in the text refer to the final assay concentrations in 100 μl. The assays were initiated by addition of 100 nM [γ-^{32}P]GTP, followed by simultaneous addition of 50 μl of test sample (GAPN buffer with 40 mM OG, 2 μM GTP[γS] in the same buffer, or OG-solubilized ROS) and 5 μl of PDEγ in GAPN buffer, or buffer alone. Assays were stopped by acid quench at 10 sec, and the amount of GTP hydrolyzed was determined as described (6).

RESULTS

Membrane Binding of RGS9 in ROS. We analyzed the membrane binding of endogenous RGS9 protein by subjecting purified ROS membranes to buffers of various ionic strengths. The moderate ionic strength buffer (ROS buffer) used during preparation of ROS did not remove RGS9 from ROS (Fig. 1, ref. 6). Consistent with this observation, further washes in GAPN buffer did not remove any detectable RGS9 from ROS membranes (Fig. 1*A*). Following repeated homogenization in hypertonic buffer, very little RGS9 was removed from the membranes, but a detectable amount was present in the supernatant (Fig. 1*A*). No detectable RGS9 was removed by hypotonic buffer (Fig. 1*A*), which removes the peripheral membrane proteins transducin and PDE. It was previously reported (1) that ROS GAP was inactivated by treating ROS membranes with 4 M urea; however, no activity was recovered in the supernatant upon removal of urea. We find here that 4 M urea does not remove significant amounts of RGS9 from the membranes, indicating that the effect of 4 M urea is to inactivate RGS9, without removing most of it from the membranes.

As a more stringent treatment to remove peripherally bound membrane proteins, we used an alkaline (pH 12.0) sodium carbonate treatment and found that RGS9 protein was largely removed (Fig. 1*B*), confirming that it is a peripheral membrane protein in ROS. This result is consistent with the lack of any predicted transmembrane segments within the primary structure of RGS9 (6). Other RGS proteins, GAIP (23) and SSt2 (24), have also been found to be tightly membrane-bound, and in the case of GAIP, thioester-linked palmitoylation has been suggested as the mechanism for membrane association. We investigated the possibility that palmitoylation of RGS9 might anchor the protein to ROS membranes by treating ROS membranes with hydroxylamine, to cleave thioester linkages, and found that this treatment did not remove a significant amount of RGS9 from the membranes (Fig. 1*C*). Thus, thioester-linked lipids cannot account for membrane binding of RGS9. The amino acid sequence of RGS9 does not contain canonical isoprenylation or N-terminal myristoylation sequences, so these modifications are unlikely. The fact that a small fraction of RGS9 is extracted at high ionic strength (1 M), and almost all RGS9 is removed by sodium carbonate, indicates that electrostatic interactions play an important role, as might be expected for a basic protein (predicted pI = 9.5) binding to negatively charged ROS membranes.

Solubilization of RGS9 from Rod Outer Segments. Previous work (25) revealed that the ROS GAP could be solubilized in active form by the nonionic detergent, OG, and retained its susceptibility to enhancement by PDEγ at 40 mM OG. When we compared the solubilization of PDEγ-sensitive ROS GAP activity with that of RGS9 protein (Fig. 2), we observed a strong correlation, with extraction of RGS9 and GAP activity becoming detectable only above ≈36 mM OG. Maximal GAP activity was observed at 48 mM OG, whereas higher OG concentrations (60–72 mM) were inhibitory. These results are consistent with RGS9 constituting the major PDEγ-sensitive GAP in these membranes and reveal conditions for testing this idea by immunodepletion of detergent-solubilized RGS9.

Immunodepletion of RGS9 from Rod Outer Segments. The polyclonal antibodies used in this study react primarily with epitopes outside the RGS domain and do not inhibit GAP activity significantly (C.W.C., unpublished observations). However, we were able to use them to test RGS9 contribution to the endogenous GAP activity in ROS by physically removing RGS9 from detergent extracts of ROS membranes. When solubilized ROS membranes were treated with immobilized antibodies from pre-immune serum (Fig. 3*A*, control 2), there was no detectable loss of RGS9 and very little loss of GAP activity, as compared with untreated extract (Fig. 3*A*, control 1). IgG from RGS9-specific serum depleted of antibodies recognizing recombinant RGS9 (Fig. 3*A*, control 3) removed some GAP activity but also removed some of the endogenous RGS9, suggesting that most, but not all, of the RGS9-binding antibodies had been removed. Immobilized IgG containing a full complement of RGS9-specific antibodies (Fig. 3*A*, de-

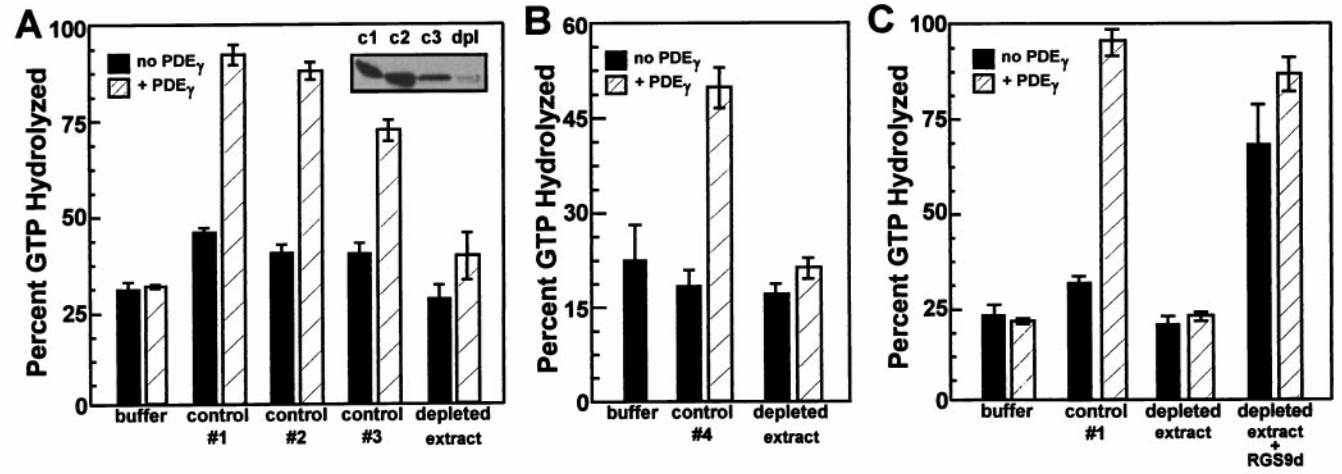

FIG. 3. Removal of ROS GAP activity by immunodepletion of RGS9. (*A*) RGS9 antibodies were immobilized on protein A-Sepharose and incubated with OG-solubilized ROS as described in the text. After removal by centrifugation, the ROS supernatants were analyzed for ROS GAP activity, PDEγ-enhanced ROS GAP activity, and RGS9 protein remaining in the supernatant (immunoblot, *inset*). Control samples are: 1, solubilized ROS extract; 2, solubilized extract after incubation with immobilized pre-immune rabbit antibodies; and 3, solubilized extract after incubation with RGS9 antibodies, which had been pre-incubated with recombinant RGS9 bound to Ni^{2+}-NTA agarose prior to immobilization on protein A-Sepharose. Depleted extract, solubilized ROS extract after incubation with RGS9 antibodies, which had been pre-incubated with Ni^{2+}-NTA agarose only prior to immobilization on protein A-agarose. For assays containing PDEγ (hatched boxes), a final concentration of 1.84 μM was used. (*B*) Immunodepletion by directly coupled antibodies. Either CNBr-activated Sepharose 4B with covalently coupled purified RGS9 antibodies or rabbit IgG-agarose was incubated with OG-solubilized ROS (control 4), and the supernatants were assayed for PDEγ-enhanced ROS GAP activity. PDEγ (*hatched boxes*) was present at 1.17 μM. (*C*) Extract was immunodepleted as in *A*, without preincubation of IgG with Ni^{2+}-NTA agarose, and recombinant RGS9d (6) was added (10 μM) with or without PDEγ (0.3 μM). Control #1 is as in *A*.

pleted extract) removed nearly all of the RGS9, and with it nearly all of the PDEγ-stimulated GAP activity. These results indicate that the greatest part of endogenous GAP activity in these extracts is due to RGS9. To verify these results, we used a different immunodepletion procedure with the purified antibodies directly coupled to CNBr-activated Sepharose (Fig. 3*B*) and obtained essentially the same result: IgG specific for RGS9, but not normal rabbit IgG, removed nearly all of the endogenous GAP activity. To verify that the immunodepletion procedure did not introduce an inhibitor of GAP activity, we added back recombinant RGS9d (6) and found that it was sufficient to restore GAP activity to the depleted extracts (Fig. 3*C*).

Recognition of RGS9 in Photoreceptors by Monoclonal Antibody. Homogenized fractions of bovine retina enriched in photoreceptor outer segments were analyzed by SDS/PAGE and immunoblotting with monoclonal antibody. The antibody labels a single band approximately 55 kDa (Fig. 4*A*). Full-length and truncated forms of bacterially expressed RGS9 are also labeled with this antibody, but a fragment containing only the RGS domain (amino acid residues 291–418) labels much

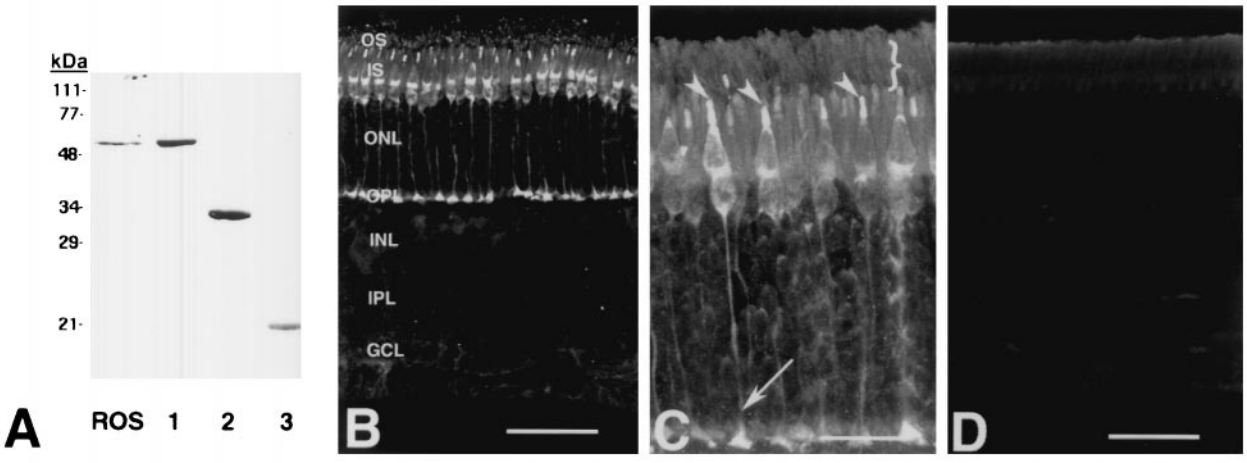

FIG. 4. Immunolocalization of RGS9 in bovine retina by monoclonal antibody. (*A*) RGS9 immunoblot showing labeling of RGS9 from bovine ROS and full-length and truncated forms of bacterially expressed RGS9. ROS, bovine ROS (20 μg rhodopsin). Lane 1, His-RGS9 (delta30N, 0.6 μg) corresponding to aa 31–484. Lane 2, His-RGS9c (0.6 μg) corresponding to aa 226–484. Lane 3, His-RGS9d (0.6 μg) corresponding to aa 291–418. (*B–D*) Confocal immunolocalization. RGS9 immunolabeling (*B* and *C*) is present in rod and cone photoreceptors. These sensory neurons extend from the outer segment layer (OS) to the outer plexiform layer (OPL) where their axons terminate. Antibody labeling is more prominent in the cones, whose cell soma are adjacent to the IS layer, in the outer most tier of the outer nuclear layer (ONL). Antibody labeling was not observed in the inner nuclear layer (INL), inner plexiform layer (IPL), or ganglion cell layer (GCL). At higher magnification (*C*), the subcellular distribution of RGS9 in rods and cones is demonstrated. Cone OS are heavily labeled (arrowheads), whereas rod OS ({) are less intensely labeled. Rod and cone IS are also RGS9 immunopositive, although labeling is more pronounced in the cones. Cone photoreceptors terminate in the OPL where their axons form synaptic terminals. Cone axons and synaptic terminals (arrow) are positive for RGS9. (*D*) RGS9 immunolabeling is abolished by preadsorbing the antibody with purified RGS9 protein coupled to CNBr-activated Sepharose. Scale bars are 50 μm in *B* and *D* and 20 μm in *C*.

more weakly. Thus, the epitope recognized primarily involves residues outside the RGS core domain.

Photoreceptor-Specific Immunolabeling with RGS9 Antibody. Bovine retina contains large numbers of rod and cone photoreceptors, and immunolabeling demonstrates that both classes of sensory neurons contain RGS9 (Fig. 4 *B* and *C*), whereas no labeling is apparent elsewhere in the retina (Fig. 4*B*). Although both rod and cone photoreceptors are immunolabeled, cones are labeled more intensely than rods. In cones, the highest levels of immunolabeling are in the outer segment, the perinuclear cytoplasm, and the axon and synaptic terminal (Fig. 4*C*). In rods, low intensity RGS9 immunolabeling is visible in the inner and outer segments but not in the perinuclear cytoplasm or synaptic terminals of these cells (Fig. 4*C*). RGS9 immunolabeling of rods and cones is abolished by preadsorbing the primary antibody with bacterially expressed, purified RGS9 prior to incubation (Fig. 4*D*). Sections from which the primary antibody was omitted showed no fluorescence signal (data not shown). To ensure that the differences in rod and cone staining were not artifacts of fixation techniques or epitope masking effects, we employed a different protocol using retinal flat mounts, fixed with methanol and H_2O_2, and immunoperoxidase staining and again observed the same striking pattern of rod and cone staining, with a much stronger signal in the cones (data not shown).

DISCUSSION

RGS9 Membrane Association. Like the other components of the transduction cascade, RGS9 is tightly bound to disc membranes. Its peripheral association with the membranes is a property it shares with transducin and with the cGMP phosphodiesterase (PDE), but the mechanism for this association is quite different. Transducin and PDE are both acidic proteins whose association with membranes is likely conferred largely by covalently attached lipids (26–32). Their electrostatic interactions with the membranes are largely repulsive, as revealed by their efficient extraction at low ionic strengths (33, 34). In contrast, there is currently no evidence for lipidation of RGS9, and electrostatic attraction seems to be important; however, other interactions not yet identified may be involved as well. It is likely that membrane association of RGS9 enhances the efficiency with which it interacts with $G_{t\alpha}$-GTP and perhaps with other disc membrane proteins as well.

RGS9 as the Major Source of PDEγ-Sensitive GAP Activity in ROS. RNA species encoding a number of different RGS proteins have been identified in the retina (6–8). Furthermore, proteins other than RGS proteins, such as phospholipase Cβ (35) and PDE subunits (2, 9, 10) have been reported to act as GAPs for Gα subunits, so it was not clear previously what portion of ROS GAP activity was attributable to RGS9. The results reported here, although not ruling out a supplemental role for other proteins, indicate that the PDEγ-enhanced GAP activity described previously in bovine ROS (1, 3, 36) is primarily RGS9-dependent. Therefore, regulation of $G_{t\alpha}$ GTPase in the light response, and its role in determining recovery kinetics, are likely to revolve around the properties and regulation of RGS9-catalyzed GTPase acceleration.

RGS9 as a Major Component of Cones. One of the most striking conclusions to be drawn from the results presented here is that RGS9 concentrations are significantly higher in cones than in rods. A previous estimate of one molecule of RGS9 per 7 holo–PDE complexes or per ≈85 transducins in rod outer segments (6) suggests that the abundance of RGS9 in cones is probably similar to that of PDE, and possibly even higher. This observation is particularly interesting in light of the important role that enhanced recovery kinetics seem to play in two features of cone photo-responses that distinguish them from those in rods: they are much faster and much less sensitive (37–40). A consideration of the amplification constants of photo-responses in mammalian cones and rods (11) indicates that they are too similar to account for the observed differences in peak sensitivity. Moreover, photoreceptor recovery kinetics are in general much slower than activation, so they are expected to be rate-limiting for establishing the temporal resolution of cones. Thus, faster recovery in mammalian cones may account for both their lower peak sensitivity and for their finer discrimination of rapid changes in illumination as compared with rods.

Several components in the cone recovery phase of phototransduction have already been identified to be faster than those in rods. First, the activated form of the cone pigment decays much more rapidly than photoactivated rhodopsin (41). Second, the rate of calcium clearance by an exchanger from rods is five to eight times slower than in cones (42), and cGMP-gated channels from cone photoreceptors conduct more Ca^{2+} than those from rod photoreceptors (43). Thus, calcium changes in cones can occur more rapidly than in rods. Calcium has a profound effect on the rate of cGMP production by guanylate cyclase(s) through its regulation by Ca^{2+}-binding guanylate cyclase-activating proteins, GCAPs (reviewed in ref. 44), so more rapid changes in calcium facilitate rapid recovery. Based on immunolocalization studies, it seems that cones express more guanylate cyclase 1 and GCAPs than do rods (15, 45), suggesting more rapid changes in cGMP production through rapid activation and inactivation of guanylate cyclase. Third, regeneration of cone visual pigment is approximately five times faster than regeneration of rhodopsin (reviewed in ref. 46). Based on the results presented here, faster GTP hydrolysis, resulting from higher concentrations of RGS9 in cones versus rods, represents yet another biochemical mechanism by which this faster recovery might be achieved.

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