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# 5 Biochemical Characterization of Phototransduction RGS9-1–GAP Complex

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## 5.1 INTRODUCTION

A major player in the regulation of timing and sensitivity of photoresponses is the GTPase accelerating protein, RGS9-1 (1–4). RGS9-1 binds to the  $\alpha$  subunits of the rod and cone G proteins,  $G_{\alpha 1}$  and  $G_{\alpha 2}$ , when they are in their activated GTP-bound conformations, and speeds up the rates at which they hydrolyze GTP, thereupon returning to the inactive GDP-bound conformations. This conceptually simple function is complex in its biochemical details and regulation. In addition to RGS9-1, two other subunits,  $G_{\beta 5L}$  (5–9) and R9AP (RGS9-1 anchor protein) (10–12), are required for the function and stability of the GTPase accelerating protein (GAP) complex, and the inhibitory subunit of the photoreceptor effector enzyme, PDE6 $\gamma$ , dramatically enhances the affinity of this complex for  $G_{\alpha}$ -GTP. The activity of the complex and the time resolution of vision depend on its concentration in the cells (13), and the concentrations are very different in rods and cones (1,14). The  $G_{\beta 5}$  gene is subject to alternative splicing, and although only one splice variant,  $G_{\beta 5L}$ , is found in rods (15), both  $G_{\beta 5L}$  and  $G_{\beta 5S}$  are found associated with RGS9-1 in cones (14). RGS9-1 is also subject to  $Ca^{2+}$ -regulated phosphorylation by protein kinase C (16,17), and may be further regulated by phosphoinositides. Levels of this complex have been shown to be essential for the timely recovery of photoresponse by loss-of-function studies in mice (18–21) and humans (22), and those proteins also control the rate-limiting step in vision confirmed by a recent discovery that overexpression of the whole complex speeds up the response (13). This chapter will focus on biochemical techniques used to characterize the molecular mechanisms of RGS9-1–GAP complex in phototransduction kinetics and their regulation.

## 5.2 EXPRESSION AND PURIFICATION OF RECOMBINANT COMPONENTS, FRAGMENTS, AND MUTANTS OF THE RGS9-1 COMPLEX

Characterization of the RGS9-1– $G_{\beta 5}$ –R9AP complex requires expression and purification of recombinant proteins, with either wild-type or reengineered sequences. Three major heterologous expression systems have been previously tested, and each has its own advantages. The choice of expression system depends on the requirement of these proteins for different applications: *in vitro* biochemical assay, generation of antibodies, or functional studies of domains and specific sites. There are some special considerations that must be taken into account when working with these proteins. One is that any RGS9-1 construct containing the GGL domain must be coexpressed with  $G_{\beta 5}$ , if soluble and functional protein is to be obtained, and, conversely, no success has been achieved in expression of functional  $G_{\beta 5}$  without coexpression of a GGL-domain-containing construct. R9AP is a transmembrane protein, in contrast to the RGS9-1– $G_{\beta 5}$  complex, so conditions for its expression and purification are necessarily different. Although R9AP is required for stability of both RGS9-1 and the long isoform of  $G_{\beta 5}$  *in vivo* in photoreceptors, *in vitro* expression systems do not require coexpression. All three proteins have been successfully expressed in both insect cells and mammalian tissue culture cells, and they appear to form functional complexes. The RGS9-1– $G_{\beta 5}$  complex can be expressed and purified without R9AP,

and then recombined either with full-length R9AP reconstituted into lipid vesicles, or with the cytoplasmic domain of R9AP, which binds tightly to RGS9-1 without the need for lipid or detergent.

### 5.2.1 EXPRESSION OF RGS9-1 FRAGMENTS IN *E. COLI*

The following procedure describes the expression and purification of either full-length RGS9-1 or fragments for antibody generation and functional assays (2,23,24). Both His<sub>6</sub> tags (pET vectors, Novagen) and glutathione-S-transferase (GST, pGEX vectors, GE) tags have been used successfully. Neither full-length RGS9-1 nor the RGS domain-containing fragments (i.e., containing residues 291–418) of RGS9-1 are initially soluble when overexpressed in *E. coli*. They are found primarily in inclusion bodies, so simply lysing the cells and washing the inclusion bodies gives a good first step of purification. Subsequently the proteins can be extracted under denaturing conditions. His<sub>6</sub>-tagged and GST-tagged proteins both renature well into active form upon dialysis to remove denaturants. The His-tagged protein is purified by metal ion affinity chromatography under denaturing conditions, and then renatured by step dialysis, whereas the GST-tagged protein is renatured by dialysis prior to affinity purification using glutathione beads. In contrast, full-length RGS9-1 does not fold properly without G<sub>β5</sub>, so for this protein, *E. coli* expression is useful only to produce denatured protein for antibody production or for use as a standard in gel-based (Coomassie-blue staining or Western blotting) quantification procedures.

#### PROTOCOL I: BACTERIAL EXPRESSION AND PURIFICATION OF TAGGED RGS9-1 FRAGMENTS

1. Cells (BL21 (DE3) pLysS) are freshly transformed with plasmid using standard procedures, and incubated overnight at 37°C on LB-ampicillin/chloramphenicol plates. The next day multiple colonies are collected and used to inoculate the LB medium. A typical volume is 1 L. pLysS strains, expressing T7 lysozyme, are useful for efficient disruption of cell walls. However, when a Microfluidizer® (Microfluidizer Corp.) is used to break open the cells, BL21 (DE3) without pLysS can be used, and no chloramphenicol is needed.
2. For vectors with the T7-lac promoter (e.g., pET-14B-derived expression plasmids), 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) is used to induce expression when the cells have reached an O.D.<sub>600</sub> of 0.6, and growth is allowed to continue at 37°C if the intent is to extract protein from inclusion bodies, or at 25°C or even lower temperatures if the intent is to optimize the amount of initially soluble protein. For vectors with the T7 promoter (e.g., pGEX-2TK), 0.1 mM IPTG is used to induce expression. In general, it is a good idea to remove samples for SDS-PAGE assessment of protein expression at different time points.
3. Cells are harvested by 4000 × g centrifugation (20 min, 4°C), and pellets are sonicated and washed twice with lysis buffer: 50 mM sodium phosphate, pH 7.4, 300 mM NaCl, 1 mM dithiothreitol (DTT). The supernatants from these washes can then be used for affinity purification on immobilized

metal or glutathione beads, following the manufacturer's instructions. However, for most RGS9-1 fragments, a much higher yield of soluble protein is obtained by extracting and renaturing proteins from inclusion bodies.

4. The inclusion bodies (pellets from previous washes) are solubilized in 35 mL/L culture of guanidinium HCl buffer: 6 M guanidinium HCl, 100 mM sodium phosphate, 10 mM Tris, pH 8.0. The samples are rotated at room temperature for 2 h.
5. For His-tagged proteins, the solubilized protein is then applied to nickel nitrilotriacetic ( $\text{Ni}^{2+}$ -NTA) beads (Qiagen) and purified under denaturing conditions according to the manufacturer's instructions. The purified protein is then diluted to a concentration of 0.1 mg/mL with urea buffer: 8 M urea, 100 mM sodium phosphate, 10 mM Tris, pH 8.0. The protein is then dialyzed overnight at 4°C against a 10,000-fold larger volume of renaturing buffer: 50 mM sodium phosphate, pH 7.4, 300 mM NaCl, 10% glycerol, 0.1% 2-mercaptoethanol. Insoluble protein is removed by centrifugation at  $20,000 \times g$  (20 min 4°C), and the protein in the supernatant is concentrated by centrifugal ultrafiltration (Centricon, Millipore). Most RGS9-1 fragments show a tendency to aggregate at concentrations above 10  $\mu\text{M}$ , so there is not much point in trying to exceed this concentration.
6. For GST-tagged proteins, the crude solubilized inclusion bodies are renatured as described earlier to renature the GST moiety, prior to their being loaded onto glutathione beads and purified according to the manufacturer's instructions.
7. Full-length RGS9-1 does not renature, but instead forms precipitates when denaturants are dialyzed away. The denatured protein can be stored at -20°C after adding glycerol to 40% (v/v) and used as a standard for quantifying recombinant proteins expressed by other means using Coomassie staining on gels or quantitative immunoblotting.

## 5.2.2 R9AP EXPRESSION AND PURIFICATION IN *E. COLI*

R9AP is a transmembrane protein, and thus the full-length form, containing its single C-terminal transmembrane helix, is expressed in insoluble form in bacteria. Fortunately, however, it is one of the few eukaryotic membrane proteins that can be solubilized from bacterial membrane pellets using nondenaturing detergents, purified, and reconstituted in functional form into lipid vesicles. Moreover, the cytoplasmic domain of R9AP can be expressed as a soluble fragment in bacteria. In both cases, the protein has been expressed in pET14b and purified using N-terminal His tags. Immobilized metal-ion affinity chromatography is not sufficient to obtain pure protein, so an additional step of ion exchange chromatography is used.

### PROTOCOL 2: EXPRESSION OF R9AP AND ITS SOLUBLE FRAGMENTS IN *E. COLI* AND PURIFICATION BY AFFINITY AND ION EXCHANGE CHROMATOGRAPHY

1. Transformation of BL21 (DE3) pLysS cells is carried out as described earlier for RGS9-1 fragments. Cells are induced when  $\text{O.D.}_{600} = 0.6$ – $0.8$  with 0.3 mM IPTG, and allowed to grow for 4–5 h at 30°C.

2. Cells are harvested by centrifugation at  $4000 \times g$  for 20 min at  $4^{\circ}\text{C}$ , and the pellets sonicated with lysis buffer (25 mM Tris, pH 8.0, 300 mM NaCl, 20 mM imidazole, 2 mM DTT, and  $\sim 20$  mg/L PMSF, phenylmethylsulfonyl fluoride).
3. His-tagged bovine R9AP fragments lacking the C-terminal transmembrane helix (His-R9AP- $\Delta\text{C}$ , amino acids 1–212 or His-R9AP- $\Delta\text{C}2$ , amino acids 1–191) are largely soluble because they do not contain the C-terminal transmembrane helix. In these cases, cell debris and insoluble materials are removed by centrifugation at  $24,000 \times g$  for 30 min, and the soluble fragments in the supernatant are purified under native conditions with immobilized metal ion beads following the manufacturer's protocol, and eluted with 200 mM imidazole in the lysis buffer. The purest fractions are combined and dialyzed against dialysis buffer (10 mM Tris-HCl, 1 mM DTT, pH 8.0). The protein is not exceptionally pure at this point, so it is applied onto a strong anion exchange column (e.g., POROS HQ HPLC anion exchange column, Applied Biosystems), washed with 20 mM NaCl in the dialysis buffer, and eluted with a linear gradient from 20 to 200 mM NaCl in the same buffer (at a flow rate of 2–3 mL/min for the POROS HPLC column). About 10 mg of protein can be obtained from 1 L of culture.
4. His-tagged mouse full-length R9AP is insoluble and is found in pellets after centrifugation of the cell lysates as described earlier. The proteins are extracted with 4% sodium cholate in lysis buffer for 0.5–1 h at  $4^{\circ}\text{C}$  with gentle agitation, followed by centrifugation; this procedure is repeated 3–4 times, yielding  $>70\%$  of total His-mR9AP extracted in a soluble form. The pooled detergent-solubilized R9AP is purified using  $\text{Ni}^{2+}$ -NTA beads in 4% sodium cholate in the lysis buffer according to the manufacturer's protocol. Routinely, at least 5 mg R9AP with 90–95% purity can be obtained from 2 L *E. coli* cultures. Reconstitution procedures are described in section 5.3.

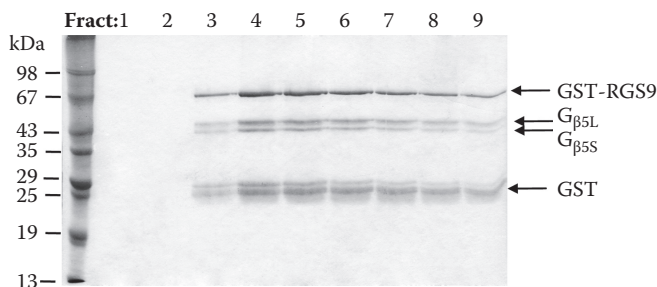
### 5.2.3 EXPRESSION OF THE RGS9-1 COMPLEX WITH $G_{\beta 5}$ IN INSECT CELLS USING BACULOVIRUS

The most useful expression system so far for production of functional full-length RGS9-1 and its partner  $G_{\beta 5}$  is baculovirus-directed expression in insect cells. Attempts to express each separately have produced low yields of most insoluble proteins, for which no activity has been detected; however, coexpression using simultaneous infection with two viruses produces useful amounts of soluble, active protein complex. It is generally necessary to test different ratios of RGS9-1 virus to  $G_{\beta 5}$  virus to determine the one that produces the highest yield of the complex; this ratio will vary over time if the titers of the virus stocks decline at different rates, so it is advisable to retiter the virus from time to time. The following procedure (protocol 3) uses adherent cells growing in plates. Alternatively, cells can be grown in suspension as described (25).

There are two known splice variants of  $G_{\beta 5}$ ,  $G_{\beta 5L}$ , and  $G_{\beta 5S}$ . In insect cells, viruses encoding the long-variant  $G_{\beta 5L}$  produce a mixture of  $G_{\beta 5L}$  and  $G_{\beta 5S}$ , with the cells apparently using the first methionine residue of  $G_{\beta 5S}$  as an alternative translation start site.

### PROTOCOL 3: INSECT CELL EXPRESSION AND PURIFICATION OF RGS9-1/G<sub>β5</sub>

1. If it is necessary to produce new virus, recombinant baculoviruses are isolated after cotransfection of the linearized BaculoGold viral DNA (PharMingen) and the recombinant transfer vector pVL1392 (PharMingen) with the proper insert into Sf9 cells following the manufacturer's protocol.
2. The cells are grown as monolayers in 150 mm culture dishes in Insect-Xpress medium (Bio Whittaker) supplemented with 8% fetal bovine serum and 10 μg/mL gentamycin. They are coinfecting with two types of recombinant viruses containing RGS9-1 or G<sub>β5L</sub> (the MOI [multiplicity of infection] for both viruses is approximately 1:1) at 80% confluency and the cells are harvested 48 h later.
3. Cells are removed from the plates, collected by centrifugation, and the cell pellet is resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM DTT, and 1% Nonidet P-40) with freshly added protease inhibitors (0.03 mg/mL leupeptin, 0.017 mg/mL pepstatin A, 0.005 mg/mL aprotinin, 0.03 mg/mL lima bean trypsin inhibitor, and ~20 mg/L solid PMSF) and mixed by rocking for 1 h at 4°C. The cell suspensions are sonicated on ice, centrifuged at 20,000 × g for 30 min at 4°C, and the supernatants are collected.
4. For His-tagged proteins, the supernatants are supplemented with 20 mM imidazole and loaded onto a Ni<sup>2+</sup>-NTA agarose column, washed with lysis buffer, and then with GAPN-H buffer (10 mM HEPES pH 7.4, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM DTT and ~20 mg/liter PMSF) containing 20 mM imidazole, and then eluted with 250 mM imidazole in GAPN-H buffer.
5. For GST-tagged proteins, the supernatants are loaded to a glutathione-sepharose 4B column, washed with lysis buffer followed by GAPN-H buffer (10 mM HEPES pH 7.0, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM DTT, and ~20 mg/L PMSF, and eluted with 40 mM glutathione in GAPN-H buffer.
6. Untagged G<sub>β5</sub> expressed in the cells consistently copurify with RGS9-1 after affinity chromatography (figure 5.1).



**FIGURE 5.1** Coomassie-blue-stained SDS-PAGE gel of glutathione elution fractions from GST-RGS9-1-G<sub>β5</sub> expressed in insect cells. After the column had been thoroughly washed with buffer, as described in protocol 3, glutathione was added to the buffer, and fractions collected, beginning with Fraction 1.

### 5.3 RECONSTITUTION OF FULL-LENGTH R9AP INTO LIPID VESICLES

R9AP appears to have several roles in regulating RGS9-1 function. These include ensuring the stability of the RGS9-1–G<sub>βSL</sub> complex (20), controlling its localization to the outer segments (26), anchoring it to the disk membrane (10,11) where it interacts with G<sub>αt</sub>-GTP and PDE6, and enhancing the catalytic activity of RGS9-1 (27). To study its membrane-dependent functions using purified proteins, it is necessary to reconstitute it into lipid vesicles. This is conveniently carried out with protein purified from *E. coli* in nondenaturing detergents, as described earlier, and well-defined phospholipid mixtures. This procedure can be carried out with or without the simultaneous presence of detergent-purified rhodopsin, and remarkably homogeneous vesicles as determined by cryoelectron microscopy are obtained (11).

#### 5.3.1 RECONSTITUTION OF PURIFIED R9AP INTO VESICLES CONTAINING PHOSPHOLIPIDS ONLY, OR PHOSPHOLIPIDS AND RHODOPSIN

##### PROTOCOL 4: RHODOPSIN PURIFICATION

1. Buffers used are lysis buffer (300 mM NaCl, 25 mM Tris, pH 8.0), GAPN-H buffer (100 mM NaCl, 2 mM MgCl<sub>2</sub>, 10 mM HEPES pH 7.4); ConA buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.0, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>), high-salt buffer (1 M NH<sub>4</sub>Cl, 10 mM HEPES, pH 7.4, 2 mM MgCl<sub>2</sub>). DTT, to 1 mM, and solid PMSF, to 20 mg/L, were added to each buffer just before use.
2. Purification of rhodopsin uses a modified version of a published procedure (28), with all procedures carried out either under dim red light or using near-infrared illumination and infrared image-converting goggles. Sepharose beads containing immobilized concanavalin A (Con A) are stabilized before use to prevent bleeding off of ConA during elution by treatment with 0.05% glutaraldehyde in 250 mM NaHCO<sub>3</sub> and prepared as described (28). Rod outer segments are prepared using a standard discontinuous sucrose gradient procedure (29), and extracted twice with high salt buffer at a concentration of 15 μM rhodopsin or lower. They are then washed twice at the same concentration with ConA buffer, and the pellets are solubilized in ConA buffer containing 4% (w/v) sodium cholate to the same concentration. The supernatant is loaded onto the column, which is washed first with 10 column volumes of Con A buffer containing 4% sodium cholate, and then with 3 column volumes of the same buffer supplemented with 300 mM α-methyl mannoside. The eluant is concentrated by ultrafiltration to obtain a final rhodopsin concentration (determined by absorbance at 500 nm) of 2–3 mg/mL.

##### PROTOCOL 5: RECONSTITUTION OF R9AP WITH OR WITHOUT RHODOPSIN IN LIPID VESICLES

1. A phospholipid solution is prepared by first mixing chloroform solutions of lipids from Avanti Polar Lipids or Molecular Probes at a mass ratio of

phosphatidylcholine:phosphatidyl-ethanolamine:phosphatidylserine:rhodamine-labeled phosphatidylethanolamine = 50:35:1:0.43, drying it under a stream of argon, and then dissolving it in sufficient lysis buffer containing 4% sodium cholate to achieve a final lipid concentration of 20 mg/mL. The mixture is sonicated as necessary under argon and on ice to achieve a homogeneous solution. Then, cholate solutions of either rhodopsin, or His-tagged R9AP, or both are added to the lipid solution to achieve a lipid-to-protein mass ratio between 20:1 and 40:1.

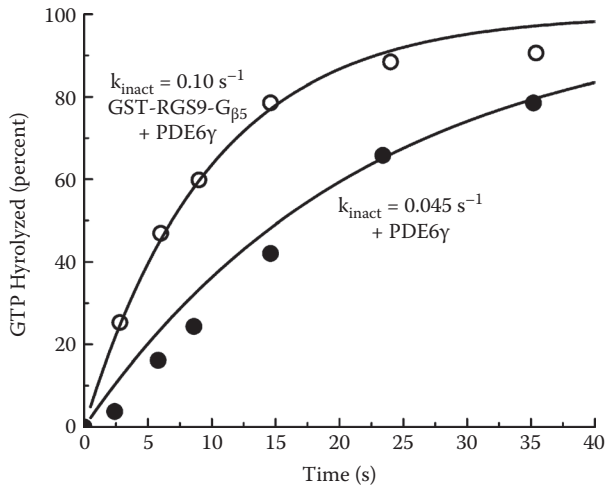
2. The same procedure without added proteins produces protein-free vesicles to use in control experiments.
3. Typical yields of protein incorporated into vesicles are 42 molecules of rhodopsin per vesicle, and 12 molecules of R9AP, determined by 500 nm absorbance for rhodopsin, or by densitometry of Coomassie-stained gels and comparison to a standard for R9AP.
4. The amount of accessible (i.e., cytoplasmic domain facing outward) rhodopsin and R9AP can be determined by titration with either transducin,  $G_{\alpha\beta\gamma}$ -GDP, for rhodopsin, or with RGS9-1- $G_{\beta5}$ , for R9AP, in centrifugation-based binding assays.
5. The unilamellar character of the vesicles and their size distribution can be readily assessed by cryoelectron microscopy.

#### 5.4 SINGLE-TURNOVER GAP ASSAY FOR PURIFIED RECOMBINANT OR RETINAL PROTEINS

To detect effects of different factors on GAP activity, two kinetic approaches have been commonly used (described in detail in Reference 30). The multiple-turnover method detects the steady-state GTP hydrolysis rate during cycles of GTPase activation and inactivation, whereas the single-turnover approach described here has been employed to avoid any possible interference caused by the G protein recycling. A limitation of the latter approach is that it must be carried out at substrate (i.e.,  $G_{\alpha}$ -GTP) concentrations well below saturating, and thus cannot be used by itself to determine values of the Michaelis-Menten constant,  $K_m$ . Rather, it yields a single-exponential rate constant,  $k_{\text{inact}}$ , which when divided by the RGS9-1 concentration yields  $k_{\text{cat}}/K_m$ , the catalytic efficiency. This value when extrapolated to *in vivo* conditions allows a comparison with the time constant for photoresponse recovery (figure 5.2).

##### PROTOCOL 6: PREPARATION OF UREA-WASHED ROS MEMBRANES

1. Rod outer segments are prepared in the dark from fresh or frozen bovine retinas (obtained from a local abattoir, or from Schenk Packing, Seattle, Washington, or Wanda Lawson Packing, Lincoln, Nebraska) using a standard sucrose gradient procedure (29,31).
2. Each wash is carried out using the following procedure in the dark: The membrane pellet is resuspended with a 22½ gauge needle and 16 mL of wash buffer (listed in the following text). Four aliquots of 4 mL each are removed and diluted to 55 mL in a Potter-Elvehjem homogenizer (with Tef-



**FIGURE 5.2** GAP assay. Enhancement of GTP hydrolysis in rod outer segment membranes by added recombinant RGS9-1-G $\beta_5$ . The release of [ $^{32}$ P]Pi from [ $\gamma$ - $^{32}$ P]GTP was monitored by scintillation counting of the supernatant from a charcoal suspension in samples quenched by acid at the indicated times after addition of GTP plus the indicated proteins at time zero, as described in protocol 7.

ion pestle) and homogenized with 10 slow strokes, care being taken not to add air bubbles to the suspension. The homogenized membranes are poured into Ti-45 centrifuge tubes (55 mL per tube), and centrifuged for 30 min at 42,000 rpm, 4°C, in a Type 45-Ti rotor (Beckman). All washes are carried out at a final concentration of 15  $\mu$ M rhodopsin or below. All buffers are supplemented with 1 mM DTT and solid PMSF just before use.

3. Wash once with 1  $\times$  GAPN-Tris buffer (10 mM Tris-HCl, pH7.4, 100 mM NaCl, 2 mM MgCl<sub>2</sub>), twice with Low Salt Buffer (5 mM Tris-HCl, pH 7.4, 0.5 mM MgCl<sub>2</sub>), twice with High Salt Buffer (5 mM Tris-HCl, pH 7.4, 0.5 mM MgCl<sub>2</sub>, 1 M NaCl), twice with Urea Wash Buffer (5 mM Tris-HCl, pH 7.4, 0.5 mM MgCl<sub>2</sub>, 4 M Urea—urea deionized with mixed-bed ion exchange resin). The final pellet is washed once in the final assay buffer, e.g., GAPN buffer (protocol 3) for the following GAP assay (protocol 7), and after resuspension in this buffer at the desired concentration is divided into 100–200  $\mu$ L aliquots wrapped in foil and stored at –80°C.

AU: OK?

#### PROTOCOL 7: SINGLE-TURNOVER GAP ASSAY

1. Urea-stripped ROS membranes are mixed with holo-transducin, G $_{\alpha\beta\gamma}$  purified from bovine retinas (see Reference 32 for purification procedure) at concentrations of 15  $\mu$ M rhodopsin and 1  $\mu$ M Gt in GAPN buffer (protocol 3).
2. Expose the sample to room light immediately before the assay and continuously vortex the reaction mechanically during the assay.
3. Initiate the assay by adding 50 nM [ $\gamma$ - $^{32}$ P]GTP (GTP  $\ll$  G $_{\alpha}$ ) premixed with any acceleration factors to be assayed (e.g., recombinant RGS9 or PDE $\gamma$ ,

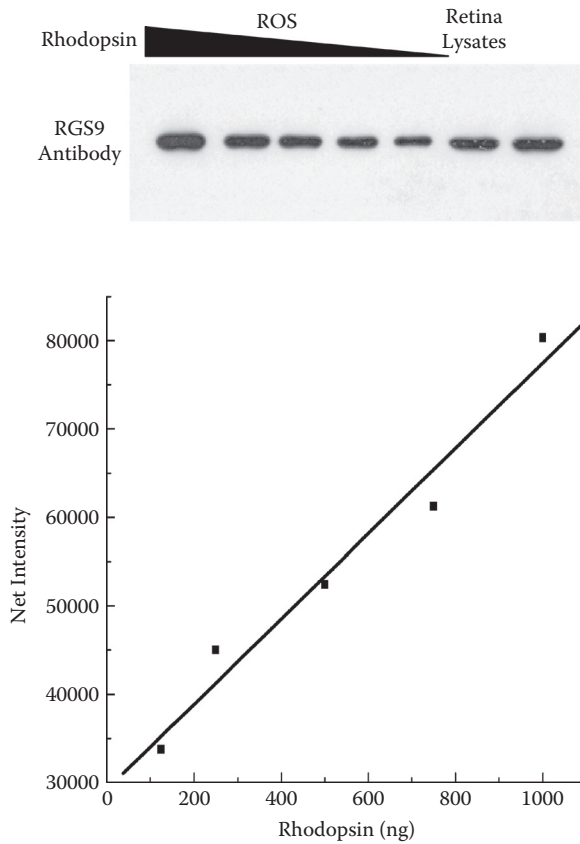
alone or together) with the tape recorder starting running and marking the first verbal note ( $t = 0$ ), then stop the reaction at various times up to 2 min by adding trichloroacetic acid (TCA) to 10% (w/v) and recording another verbal note upon quenching. For early time points, it is useful to have a pipette preloaded with TCA, and to have a second person either hold the sample on the vortexer or handle the second pipette.

4. Incubate the stopped reaction with 5% activated charcoal in phosphate buffer to determine released [ $^{32}$ P]Pi by charcoal binding and scintillation counting. GTP remains bound to the charcoal, while the released [ $^{32}$ P]Pi is collected in the supernatant.
5. The following control reactions are needed: To determine the background of [ $^{32}$ P]Pi released not due to hydrolysis by  $G_{\text{out}}$ , GTP $\gamma$ S in excess of total  $G_{\text{out}}$  is added prior to addition of GTP. To determine the total amount of releasable [ $^{32}$ P]Pi, one sample is incubated with 10-fold higher transducin for 10 min. For assaying effects of accelerating factors, one set of control samples should not have these factors.
6. Determine the GTP hydrolysis rate using single exponential curve fitting to: [ $^{32}$ P]Pi( $t$ ) - [ $^{32}$ P]Pi( $t$ , GTP $\gamma$ S) = ([ $^{32}$ P]Pi(10 min,  $10 \times Gt$ ) - [ $^{32}$ P]Pi( $t$ ,  $10 \times Gt$ , GTP $\gamma$ S))(1 -  $\exp[-k_{\text{inact}}t]$ ). The time values are determined accurately by using a stopwatch while playing back the tape recording of the assay.

## 5.5 QUANTIFICATION OF ROS PROTEINS INVOLVED IN GTPASE REGULATION BY QUANTITATIVE IMMUNOBLOTTING

A key method to study endogenous ROS proteins is to quantify protein concentrations in purified ROS or total retina using quantitative immunoblotting. The major ROS marker rhodopsin with known concentration in ROS is a commonly used standard. The method described in the following text uses chemiluminescence and x-ray film to detect horseradish peroxidase-labeled secondary antibodies. This method is very sensitive and convenient, but is well known to have a limited dynamic range for a given set of conditions, and to have a nonlinear response throughout much of its dynamic range. In addition to the nonlinearity inherent in the chemiluminescence detection method, quantitative immunoblotting is also subject to uncertainties due to unevenness of transfer from SDS-PAGE gel to nitrocellulose membrane, and possible nonlinearities in antibody binding as a function of immobilized antigen. At the upper end of the dynamic range of this method, it is easy to have too much antigen in a band, so that the peroxidase substrate is rapidly depleted, or excessive immobilized protein blocks antibody binding. In this case, there is actually a decrease in signal with increasing antigen, often seen as a negative image on x-ray film. For all these reasons, it is absolutely critical that every quantitative immunoblot be analyzed in replicates on the same blot as a series of samples for a standard curve prepared with varying known amounts of the antigen. In addition, multiple exposure times should be tested to increase the chances of the sample intensity falling within the linear range of the standard curve. The best way to determine the amount of protein in

the standard sample is to use absorbance spectrophotometry of purified protein and the molar extinction coefficient. For most proteins, the molar extinction coefficient at 280 nm can be accurately calculated from the sequence and the protein's absorbance measured in 6 N guanidinium hydrochloride using the method of Gill and Von Hippel (33). For rhodopsin the known molar extinction coefficient at 500 nm of  $42,700 \text{ M}^{-1}\text{-cm}^{-1}$  can be used. Once the molar ratio of a given antigen to rhodopsin in a sample of purified ROS is determined using these methods, that sample can then be used in place of purified protein to establish a standard curve, if the purified protein is present in limited supply. Note that in most cases samples derived from rod outer segment or other retinal membranes should not be boiled before use in SDS-PAGE, as such treatment tends to cause formation of large protein aggregates that do not enter the gel (figure 5.3).



**FIGURE 5.3** Quantitative immunoblots of RGS9-1. Top panel: x-ray film showing chemiluminescence signal from immunoblots using RGS9-1-specific antibody. Varying amounts of purified bovine rod outer segments were used to generate a standard curve (lanes 1–5) for comparison to a duplicate sample of retinal lysate (lanes 6 and 7). Lower panel: plot of the integrated intensity on the film as a function of amount of rhodopsin, determined by 500 nm absorbance.

**PROTOCOL 8: ISOLATION OF OSMOTICALLY INTACT RETINAL ROD OUTER SEGMENTS USING ISO-OSMOTIC DENSITY GRADIENTS**

The following procedure is modified from Reference 34 for a small-scale ROS preparation:

1. A 40% (w/v) iodixanol working solution is prepared by diluting 8 mL of OptiPrep™ (60% solution of iodixanol in water, density = 1.32 g/mL; Axis-Shield) to 12 mL Ringer's buffer (10 mM HEPES, pH7.4, 130 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl<sub>2</sub>, 1.2 mM CaCl<sub>2</sub>, 0.02 mM EDTA), and is diluted to a series of 4 mL iodixanol solution (4.8, 6, 10, 14, and 18%) with Ringer's buffer.
2. A gradient is formed by layering 300  $\mu$ L of the iodixanol solutions from 6 to 18% in polyallomer centrifuge tubes (11  $\times$  34 mm, 2.2 mL; Beckman) using a syringe attached with a 26½ gauge needle. A continuous gradient is formed by allowing gently rotating the tubes to a horizontal position and then allowing them to stand 45–60 min at room temperature, or by allowing iodixanol to diffuse at 4°C until the sharp lines between layers are no longer visible. For a reproducible gradient, the same procedure should be followed each time.
3. All the following procedures are performed under dim red light or using near-infrared illumination and infrared image-converting goggles. Four mouse retinas are placed in 120  $\mu$ L of 4.8% iodixanol solution, vortexed for 1 min, and then centrifuged at 200  $\times$  g for 1 min. The supernatant containing the ROS is carefully removed without disturbing the pellet. The pellet is resuspended in 120  $\mu$ L of 4.8% iodixanol solution, vortexed, and centrifuged again. This procedure of vortexing and centrifugation is repeated once.
4. The collected supernatants (approximately 350  $\mu$ L) are combined and placed on top of a 6–18% continuous gradient of iodixanol solution and centrifuged at 26,500  $\times$  g for 1 h in a TLS 55 swing bucket rotor (Beckman) using the slowest acceleration and deceleration modes.
5. The band containing ROS (about two-thirds of the way from the top of the gradient) is collected as a second band. Rhodopsin concentration is determined by measuring the absorbance at 500 nm.

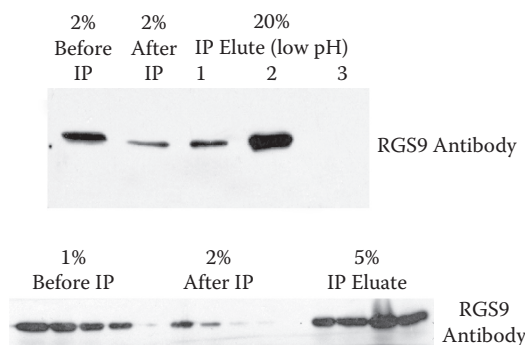
**PROTOCOL 9: QUANTITATIVE IMMUNOBLOTTING  
PROTOCOL FOR ROS PROTEINS**

1. To prepare mouse retina lysates, six whole retinas are collected in a Microfuge polyallomer tube (9.5  $\times$  38 mm, 1.5 mL; Beckman), sonicated in 500  $\mu$ L SDS-PAGE sample application buffer (5% SDS (w/v), 15% sucrose (w/v), 50 mM Na<sub>2</sub>CO<sub>3</sub>, small amounts of bromphenol blue, and 50 mM DTT). Insoluble debris is removed by centrifugation at 100,000  $\times$  g for 30 min at room temperature in a TLA 100.3 rotor (Beckman).
2. RGS9-1 quantification serves as an example in quantification of ROS proteins in retina lysates. Whole-retina lysates are applied 5  $\mu$ L per lane to a 12% polyacrylamide bis-tris gel. The bovine ROS containing rhodopsin

- 125, 250, 500, 750, and 1000 ng are loaded in equal volume on the same gel; these amounts of proteins could be a starting point to establish a standard curve for other ROS proteins with an unknown quantity but a determined molar ratio to rhodopsin.
3. The gel is electrophoresed at 90 V until the dye front passes the stacking gel and continued at 120 V for approximately 1 h. The proteins are wet-electroblotted to supported nitrocellulose membranes (NitroPure, Osmotics, Inc.) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v), 0.1% SDS, pH 8.3) for 90 min at 350 mA at 4°C.
  4. Blots are blocked with 5% (w/v) nonfat dry milk in TBST buffer (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and then incubated overnight at 4°C with polyclonal anti-RGS9-1 antibody R4432at a dilution of 1:1000 in 0.5% nonfat dry milk/TBST buffer. After being washed thrice for 10 min each with TBST buffer, blots are incubated 40 min at room temperature with horseradish peroxidase-conjugated anti-rabbit antibody (Promega) at a dilution of 1:10,000.
  5. Signals on the blot are developed on an x-ray film using Supersignal West Pico Chemiluminescent Substrate (Pierce), and exposure time is varied from 5 min to 1 s, depending on the signal intensity.
  6. The x-ray film is scanned, and protein band densities are measured using densitometric analysis software such as UN-SCAN-IT or ImageJ.
  7. A standard curve is generated by plotting band intensities on the blot against the known rhodopsin amount and fitted with a linear function. If the band intensities of the sample series fall within the linear range of this standard curve, the amount of RGS9-1 in retina lysates or ROS is predicted by the linear function or interpolating on the curve and the documented molar ratio of RGS9-1 to rhodopsin.

## 5.6 IMMUNOPRECIPITATION OF THE GAP COMPLEX

Immunoprecipitation is one of the most useful techniques to detect protein interactions in cells to understand the functional regulation of the proteins. Immunodepletion experiments were originally used to demonstrate that the PDE6 $\gamma$ -sensitive GAP activity in extracts of rod outer segment membranes was primarily due to RGS9-1 (1). Through RGS9-1 immunoprecipitation followed by mass spectrometry, a novel 25 kDa protein was identified and later characterized to be the membrane anchor and GAP activator (10). Immunoprecipitation was also essential in identifying the carboxy-terminal domain of RGS9-1 as a major site of Ca<sup>2+</sup>-dependent phosphorylation by protein kinase C (16,17). Protocol 11 is for RGS9-1 immunoprecipitation. G <sub>$\beta$ SL</sub> and R9AP immunoprecipitation procedures are similar except the elution using peptides for making G <sub>$\beta$ SL</sub> antibody or SDS-PAGE sample buffer for R9AP. Because the RGS9-1 GAP complex is tightly membrane associated because of anchoring by the transmembrane protein R9AP, detergent must be used to extract the proteins in soluble form before binding to immobilized antibodies. A persistent problem in immunoprecipitation is nonspecific binding by proteins that are not associated directly with the antigen of interest. In general, better results are obtained when the antibodies



**FIGURE 5.4** Immunoprecipitation of RGS9-1 from retinal extract. Detergent-solubilized bovine ROS proteins were immunoprecipitated as described in protocol 10, separated by SDS-PAGE, and immunoblotted with RGS9-1 antibody. (Above) In one immunoprecipitation trial, RGS9-1 was eluted by thrice the column volume of 0.1 M glycine, pH 2.5. (Below) In four trials, different amounts of starting ROS (containing 250, 125, 75, and 50  $\mu$ g rhodopsin from left to right in lane 1–4) were used and compared in “after IP” flowthrough and “IP eluate” with the same order to optimize immunoprecipitation conditions.

used are more specific, higher affinity, and purer. The cleanest results are obtained when elution from the immunoaffinity matrix is carried out by competition with a peptide containing the epitope recognized by the antibody. This is generally feasible for antibodies raised against and affinity-purified with peptides, or with monoclonal antibodies with known epitopes. It is not generally feasible with polyclonal antibodies raised against proteins of 5 kDa size or larger. In this case, elution can be carried out by high pH, low pH, high concentrations of monovalent or divalent salts, etc. The most complete elution, but also the one giving the highest level of nonspecifically bound proteins, is to elute with the strongly denaturing detergent, SDS. Another common problem with immunoprecipitation is bleeding of antibodies off the affinity matrix; these often interfere with immunoblotting procedures that are frequently carried out on immunoprecipitated proteins. One partial solution is to cross-link the antibodies to the matrix more securely using glutaraldehyde. This procedure usually has the drawback of decreasing somewhat the capacity of the matrix for antigen. Another approach is simply to wash the affinity matrix very extensively with the same solutions used for washing and elution (e.g., low pH solution) while testing the eluent for antibodies until no more are detected. This procedure also decreases the antigen-binding capacity somewhat. In general, whenever the immobilized antibody is prepared, it is essential to have a control matrix with preimmune antibody (preferably from the same animal) identically prepared (unless the specific antibody was prepared by an antigen-affinity procedure) and coupled at the same density to the matrix (figure 5.4).

#### PROTOCOL 10: PREPARATION OF IMMOBILIZED ANTIBODY MATRIX

The procedure begins with purification of the antibody. At a minimum, protein A or protein G can be used to purify the IgG fraction from polyclonal sera. The

following procedure is used for the RGS9-1 rabbit polyclonal R4432 raised against a recombinant fragment including amino acid residues 223–484.

1. The pH of the crude serum is adjusted to 8.0 by adding 1/10 volume of 1.0 M Tris (pH 8.0), and the serum is loaded to a protein A Sepharose column (Amersham Biosciences) following the manufacturer's recommendation for capacity of the column.
2. The column is washed with ten column volumes of first 100 mM Tris (pH 8.0), then with 10 mM Tris (pH 8.0), and the antibodies eluted stepwise with 0.1 M glycine, pH 3.0 into tubes with 1/10 volume of 1.0 M Tris (pH 8.0) to neutralize the eluate. Check the protein concentrations in the elution fractions by absorbance at 280 nm or by dye-based protein assays (35), and continue eluting until the concentration of eluting protein falls below the detection limit; usually 2–3 column volumes of low pH solution is sufficient to elute the antibody. The antibody purity is checked by SDS-PAGE. Antibody samples should be treated with both DTT and 2-mercaptoethanol and boiled in SDS before application to the gel to ensure complete denaturation and reduction of disulfides.
3. Purified IgG is coupled to CNBr-activated Sepharose 4B-CL (Amersham/GE) or Affi-gel 10 (Bio-Rad) at a ratio of 5–10 mg IgG to 1 mL beads following the manufacturer's protocol. The IgG concentration is assayed before and after the reaction to determine the yield of coupled antibody. Note that this number cannot be used to determine the antigen-binding capacity of the beads, as some antigen-binding activity is usually lost in the coupling reaction. To determine the capacity, test small aliquots of the beads with varying amount of antigen (either purified or in tissue extracts), and use immunoblots to determine the amount of beads necessary to remove all of a known amount of antigen or all of the antigen in a tissue extract from solution. The amount of beads used in the following procedures described should exceed by at least twofold the minimum amount needed to bind all the antigen in the sample (the amount of antigen in the sample can be determined ahead of time by quantitative immunoblotting, protocol 9).

#### **PROTOCOL II: RGS9-I IMMUNOPRECIPITATION**

1. ROS membranes or retinas are solubilized in detergent. For the RGS9-1–GAP complex, a solution of 1% Nonidet P-40 in GAPN-H buffer (see protocol 3) works well. For 40  $\mu$ L IgG-coupled beads, 300  $\mu$ L ROS containing 10–15  $\mu$ M rhodopsin are homogenized by passing through an 18 (for bovine ROS) or 23 (for mouse ROS) gauge needle in the previous buffer, or 8 to 10 retinas are sonicated in 300  $\mu$ L buffer for 5  $\times$  30 s on ice; insoluble material is removed by centrifugation at 100,000  $\times$  g for 20 min at 4°C in TLA 100.3 rotor (Beckman).
2. The best way to promote binding of the antigen to the immobilized antibody is to pour a slurry of the antibody beads into a column of narrow diameter, and run the detergent extract slowly through this column several

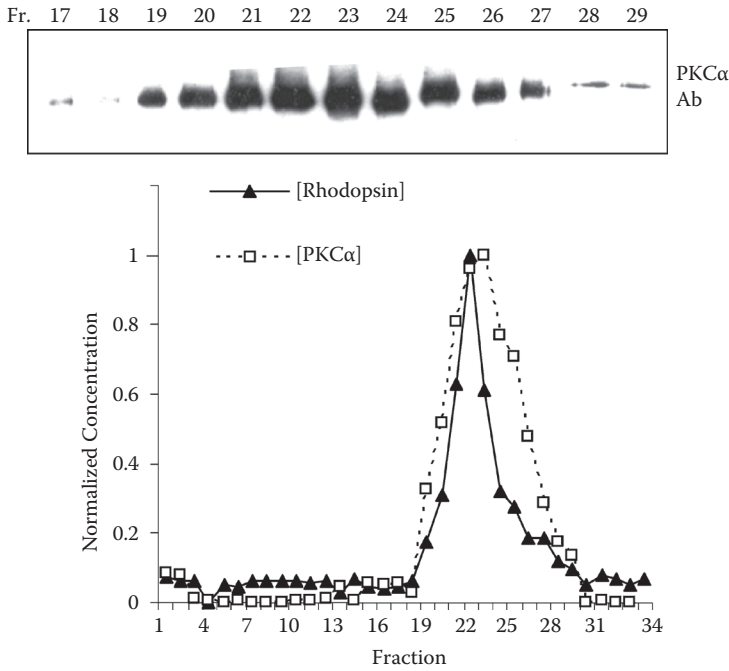
- times. Small samples are saved of each flowthrough fraction, as well as of the starting material to assess the course of antigen–antibody binding.
3. Alternatively, when the amount of sample and of beads is too small to make pouring a column practical, small batchwise reactions can be carried out. Solubilized ROS or retina lysate is mixed with IgG-coupled beads for 2.5 h at 4°C on a shaker. Care must be taken to make sure the shaking is sufficient to circulate the beads efficiently through the solution. The beads are separated from the supernatant by brief centrifugation.
  4. The columns (or beads in microfuge tubes) are washed with ten times the column volume of the solubilization buffer (three washes using the centrifuge for the batch procedure).
  5. The antigen can be eluted with peptide for antipeptide antibody (e.g., 300  $\mu$ L 1 mg/mL CT215 peptide is applied to the column and incubated for >1 h followed by elution, and this step is repeated multiple times for G $_{\beta 5}$  peptide elution) or with 0.1 M glycine at pH 3.0. Eluted antigen is often of too low a concentration to be assayed by UV absorbance or by dye-binding assays, so it is usually monitored by running immunoblots on all washes and elution fractions. Usually, thrice the column volume is sufficient for efficient elution. Two to three separate washes are used for the batch procedure.
  6. For analysis of coimmunoprecipitating proteins by mass spectrometry, the samples are generally loaded onto an SDS-PAGE gel and detected by Coomassie blue staining. For detection or quantification of small amounts of proteins, qualitative or quantitative (protocol 9) immunoblotting is usually used.

## 5.7 LOCALIZATION OF PROTEINS IN ROD OUTER SEGMENTS BY SUBCELLULAR FRACTIONATION AND IMMUNOFLOURESCENCE

Proteins must be localized to rod or cone outer segments to play a role in phototransduction. Two useful techniques for determination and confirmation of protein localization are gradient purification of rod outer segments with each fraction quantitatively assayed for the protein of interest, and immunolocalization using fluorescent secondary antibodies and confocal microscopy.

### 5.7.1 GRADIENT COPURIFICATION: A GENERAL WAY TO DETERMINE WHETHER A PROTEIN IS LOCALIZED TO ROS OR A CONTAMINANT FROM OTHER PARTS OF RETINA

The presence of a protein in a purified sample of rod outer segments is not sufficient to allow the conclusion to be drawn that it is a resident outer segment protein. The reason is that all purification procedures yield material that is only partially pure and always contains contamination at some level. However, it is highly unusual for a contaminating protein or organelle to display precisely the same profile across fractions of a density gradient following centrifugation as rod outer segments, especially across two different gradients, for example, one iso-osmotic gradient such



**FIGURE 5.5** Fractionation of rod outer segments, and assaying for protein comigration. ROSs were purified from bovine retinas by a discontinuous sucrose gradient, and fractions were analyzed for rhodopsin content absorbance at 500 nm and for PKC $\alpha$  by immunoblots.

as OptiPrep, and one sucrose gradient, which induces substantial volume loss in most organelles because of high osmolarity, but much less so for rod outer segments (figure 5.5).

**PROTOCOL 12: SUCROSE GRADIENT FRACTIONATION**

1. ROS membranes are prepared from wild-type mouse retinas in the dark by sucrose density gradient (16,29,31) or Optiprep® (iso-osmotic) density gradient (see protocol 8).
2. The best method for fractionating the gradient is by using an automatic gradient puller such as the Auto-Densi-Flow from Labconco. This instrument uses conductance to detect the liquid surface and automatically inserts the entry hole of a collection tube just below the surface. The flow into the tube (which is usually connected to a slow peristaltic pump or a slow gravity-flow system) is horizontal, so there is virtually no mixing of vertical fractions. Fractions of 200  $\mu$ L are collected for subsequent assays, and can be stored at  $-80^{\circ}$ C until use.
3. The concentration of the major ROS marker protein rhodopsin in each fraction is determined by measuring the absorbance at 500 nm before and after light bleaching in 1.5% LDAO (*N,N*-dimethyldodecylamine *N*-oxide) detergent and 10 mM hydroxylamine. If very low amounts are used (e.g., from

a single mouse), it may be necessary to quantify rhodopsin by quantitative immunoblotting, as described in protocol 9.

4. Proteins in these fractions are resolved by SDS-PAGE and analyzed by immunoblotting (protocol 9) to compare the purification profile of RGS9-1,  $G_{\beta\text{SL}}$ , and R9AP or other components with that of rhodopsin. The profile of a resident ROS protein should closely follow that of rhodopsin, especially with regard to the peak position, whereas the profiles of contaminants usually only partially overlap rhodopsin's but do not have coincident profiles.

### 5.7.2 PROTEIN LOCALIZATION BY IMMUNOFLUORESCENCE (12,14,19)

Those experiments are essential for visualization of protein subcellular localization, but very easily produce false results owing to antibody cross-reactivity. It is useful to compare the results using different antibody preparations or using knockout mice as negative controls if possible. Useful RGS9-1 antibodies include rabbit and goat antisera raised against a C-terminal fragment of RGS9-1 (aa 226–484) (2) and a mouse monoclonal antibody that recognizes an epitope including a small part of the RGS domain and adjacent portions of the C-terminal domain (1).

AU: Which experiments?

#### PROTOCOL 13: IMMUNOFLUORESCENCE STAINING OF RETINAL SECTIONS

1. Mice are humanely euthanized (e.g., by  $\text{CO}_2$  inhalation), and their eyes rapidly removed and placed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.2, GIBCO) for a 1 h fixation at  $4^\circ\text{C}$ . Some antigens require longer fixation times.
2. The eyes are cryoprotected by soaking in 30% sucrose in PBS at  $4^\circ\text{C}$  until tissue sinks, typically, for 6 h overnight.
3. The eyes are embedded in OCT (Tissue-Tek Compound) and rapidly frozen on dry ice or in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until use.
4. For cryosectioning, the embedded eyes are warmed to  $-20^\circ\text{C}$ , cut into sections of 12–40  $\mu\text{m}$  thickness using a cryomicrotome and placed on warm Superfrost Plus slides (Fisher). They are stored at  $-80^\circ\text{C}$  until use.
5. Prior to staining, sections are thawed at  $-20^\circ\text{C}$  for 1 h and  $4^\circ\text{C}$  for 1 h, then air dried for 30 min at room temperature.
6. Sections are dehydrated in methanol/acetone (1:1 v/v) at room temperature for 10 min.
7. Slides are washed in 0.1% Triton X-100 in PBST (PBS with 0.1% Tween 20). PBS alone may also be used; results should be compared for each antigen) at room temperature for  $2 \times 10$  min.
8. Sections are blocked with 10% sheep serum (Sigma) in PBST for 1 h at room temperature.
9. Slides are incubated with anti-RGS9 antibodies, anti-R9AP antiserum, or anti- $G_{\beta\text{SL}}$  antibody at various dilutions (1:100–1:500) in PBST (or PBS—see note in preceding paragraph) containing 10% sheep serum for 2–3 h or overnight at a chamber humidified with PBS. Wash slides with PBST for  $3 \times 5$  min.

AU: Corresponding opening parenthesis missing.

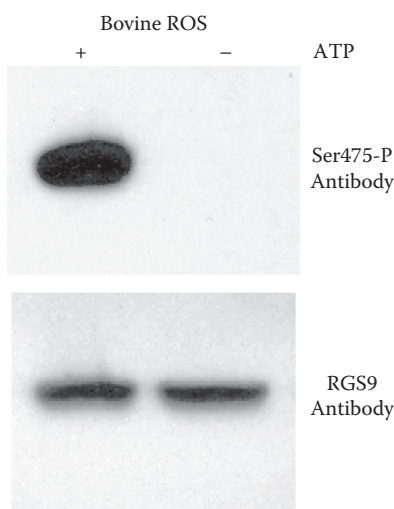
10. Dye-conjugated secondary antibody is added to the slides for 1 h at the dilution recommended by the manufacturer, typically, 1:25 to 1:100 (again, it is useful when optimizing the protocol for specific antigens, antibodies, and tissues; it is best to compare different dilutions) in PBST. Then, slides are washed with PBST for  $3 \times 5$  min.
11. The slides are mounted with a drop of Vectashield (Vector Laboratories) mounting medium and coverslipped for microscopy. Usually, the edges of the cover slip are sealed with colorless nail polish.
12. It is often useful to counterstain with a nuclear marker, such as propidium iodide, or with cell-specific markers such as peanut lectin for cone sheaths, or rhodopsin antibodies for rod outer segments.

## 5.8 PHOSPHORYLATION OF RGS9-1

Phosphorylation is a common mechanism for regulating protein function, including RGS proteins. RGS9-1 has been reported to be phosphorylated *in vitro* by either protein kinase C (PKC) (16,17,36) or protein kinase A (PKA) (37) and is phosphorylated at PKC site Ser<sup>475</sup> *in vivo* by a kinase whose activity is inhibited by light. *In vitro* phosphorylation is useful for preliminary detection of potential phosphorylation reactions and identification of sites, whereas *in vivo* studies using phosphorylation-specific antibodies provide insight into physiological relevance of those sites identified *in vitro*. Candidate kinases can be readily tested in the *in vitro* assays by addition of inhibitors or activators of known kinases. For these experiments, it is essential to have control substrates for the kinases in questions to ensure that the effective concentrations of these substances are sufficient to activate or inhibit the endogenous kinase. For example, amphipathic inhibitors may partition into membranes, reducing their effective concentrations in solution, and some activators, such as cyclic nucleotides or diacyl-glycerol, may be metabolized by the cell homogenates.

### PROTOCOL 14: ANALYSIS *IN VITRO* OF PHOSPHORYLATION OF RGS9-1 BY ENDOGENOUS KINASES IN ROD OUTER SEGMENTS

1. Bovine or mouse ROS are prepared as described earlier, typically as stocks with rhodopsin concentrations of 15–150  $\mu\text{M}$ . The following procedures are all carried out in complete darkness using infrared image converters, or in dim red light.
2. Purified ROS are homogenized as described in protocol 10 in GAPN-H buffer containing phosphatase inhibitors (and, if desired, any inhibitors or activators of specific kinases) at a dilution of 1:5, centrifuged at  $8400 \times g$  for 15 min, and then the pellet resuspended in the GAPN-H buffer to a final rhodopsin concentration of 6–60  $\mu\text{M}$ .
3.  $\text{NH}_2\text{OH}$  is added to the ROS at a final concentration of 10 mM to minimize rhodopsin phosphorylation, and ATP to 2–5 mM (with  $[\gamma\text{-}^{32}\text{P}]$  at a specific activity of 40–100 Ci/mol if detection will be by radioactivity). The mixture is incubated at 30°C for different times up to 20 min, and the reactions stopped by washing away the free ATP buffer (by centrifuging and resus-



**FIGURE 5.6** Detection of RGS9-1 phosphorylation by a phosphorylation-specific antibody. Purified bovine ROS were incubated with or without 2 mM ATP for 15 min as described in protocol 14. Proteins in ROS were analyzed by SDS-PAGE and immunoblotting with anti-Ser<sup>475</sup>-phosphate monoclonal antibody and anti-RGS9-1 antibody.

pending the pellet thrice) with phosphatase-inhibitor buffer (5 mM Tris-HCl, 2 mM EDTA, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 15 mM fenvalerate, 100 nM okadaic acid, 1 mM DTT). The pellets are immediately solubilized with SDS-PAGE sample buffer for detection by phospho-specific antibody, or first subjected to immunoprecipitation for detection by radioactivity.

4. ROS are solubilized in NP-40 detergent, and RGS9-1 immunoprecipitated as described earlier and subject to SDS-PAGE, followed by autoradiography or phosphoimager analysis to detect phosphorylation. The immunoprecipitation step is critical, as RGS9-1 comigrates with tubulin in SDS-PAGE, and tubulin is a kinase substrate.
5. Alternatively, SDS-PAGE and immunoblotting with anti-Ser<sup>475</sup>-phosphate monoclonal antibody (at a dilution ration of 1:500) are used to detect the specific Ser<sup>475</sup> phosphorylation (see section 5.5 for immunoblotting protocol) (figure 5.6).

#### **PROTOCOL 15: ANALYSIS IN VIVO OF PHOSPHORYLATION OF RGS9-1, AND REGULATION BY LIGHT**

1. Four to six wild-type mice are maintained in a dark room for a period of 16 h, followed by euthanasia and removal of retinas under dim red light or in complete darkness with the help of infrared goggles; control mice are kept for the same time in light of a specified intensity.
2. Retinas are immediately homogenized in the dark in a 1.5 mL tube using GAPN-H buffer with 1% Nonidet P-40 detergent, plus 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 15 μm

fenvalerate, 100 nM okadaic acid to inhibit phosphatase activities. First, a plastic pestle (Kontes) is used, and then the homogenates are sonicated on ice.

3. RGS9-1 is immunoprecipitated using rabbit polyclonal antibodies (e.g., R4432) as described earlier (protocol 10) and RGS9-1 phosphorylation is analyzed by immunoblotting with anti-Ser<sup>475</sup>-phosphate-specific antibodies following SDS-PAGE. Quantitative immunoblotting is carried out as described in section 5.5. It is difficult to obtain a standard for absolute quantification of phosphorylated RGS9-1, so usually only relative amounts (e.g., light versus dark) can be determined.

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