

# **Phosphorylation of RGS9-1 by an endogenous protein kinase in Rod Outer Segments\***

Guang Hu¶, Geeng-Fu Jang§, Christopher W. Cowan¶, Theodore G. Wensel¶, and Krzysztof Palczewski§#\*\*

¶Verna and MARRS McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX 77030, Departments of §Ophthalmology, #Chemistry, and

\*\*Pharmacology, University of Washington, Seattle, WA 98195.

Key words: phototransduction, RGS9-1, transducin, phosphorylation.

Running title: Phosphorylation of RGS9-1.

Correspondence to:

**Theodore G. Wensel**, Ph.D., Verna and MARRS McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030; Phone: 713-798-6994; Fax: 713-796-9438; E-mail: [twensel@bcm.tmc.edu](mailto:twensel@bcm.tmc.edu).

\*This research was supported by United States Public Health Service Research Grants EY08061, EY11900, training Grant EY07001, the Welch Foundation, a grant from Research to Prevent Blindness (RPB) for the University of Washington, Department of Ophthalmology and a grant from the E.K. Bishop Foundation. We thank Jing Huang for helping in the preparation of the monoclonal anti-phosphorylated peptide antibody A4. KP is a RPB Senior Investigator.

<sup>1</sup>The abbreviations used are: CaM, calmodulin; CaMK II, CaM-dependent kinase II; CK II, casein kinase II; GAP, GTPase-accelerating protein(s); HFBA, heptafluorobutyric acid; MS, mass spectrometry; PKA, cAMP-dependent protein kinase; PKC: protein kinase C; PKG, cGMP-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; ROS, rod outer segment(s).

**SUMMARY**

Inactivation of the visual G protein transducin, during recovery from photoexcitation, is regulated by RGS9-1, a GTPase accelerating protein (GAP) of the ubiquitous RGS protein family. Incubation of dark-adapted bovine rod outer segments (ROS) with [ $\gamma$ - $^{32}$ P]-ATP led to RGS9-1 phosphorylation by an endogenous kinase in ROS membranes, with an average stoichiometry of 0.2 to 0.45 mol phosphates per mol RGS9-1. Mass spectrometry revealed a single major site of phosphorylation, Ser<sup>475</sup>. The kinase responsible catalyzed robust phosphorylation of recombinant RGS9-1, and not of a Ser<sup>475</sup>Ala mutant. A synthetic peptide corresponding to the region surrounding Ser<sup>475</sup> was also phosphorylated, and a similar peptide with the Ser<sup>475</sup>Ala substitution inhibited RGS9-1 phosphorylation. The RGS9-1 kinase is a peripheral membrane protein that co-purifies with rhodopsin in sucrose gradients, and can be extracted in buffers of high ionic strength. It is not inhibited or activated significantly by a panel of inhibitors or activators of protein kinase A (PKA), protein kinase G (PKG), rhodopsin kinase, CaM kinase II, casein kinase II (CK II) or cyclin-dependent kinase 5, at concentrations 50 or more times higher than their reported IC<sub>50</sub> or K<sub>i</sub> values. It was inhibited by the protein kinase C (PKC) inhibitor bis-indolylmaleimide I, and by lowering Ca<sup>2+</sup> to nanomolar levels with EGTA; however, it was not stimulated by addition of phorbol ester, under conditions which significantly enhanced rhodopsin phosphorylation. A monoclonal antibody specific for the Ser<sup>475</sup>-phosphorylated form of RGS9-1 recognized RGS9-1 in immunoblots of dark-adapted mouse retina. Retinas from light-adapted mice had much lower levels of RGS9-1 phosphorylation. Thus RGS9-1 is phosphorylated on Ser<sup>475</sup> *in vivo*, and the phosphorylation level is regulated by light and by [Ca<sup>2+</sup>], suggesting importance of the modification in light adaptation

## Introduction

Phototransduction in vertebrate rod cells is a prototypical G protein signal transduction pathway (1,2). In the activation phase, the receptor rhodopsin captures a photon and activates the rod's heterotrimeric G protein transducin ( $G_t$ )  $\alpha$ -subunit. The activated  $G_{t\alpha}$ , in its GTP-bound form, then activates its downstream effector cGMP phosphodiesterase (PDE), which in turn hydrolyzes cGMP and lowers the cellular cGMP level to close the cGMP gated cation channels. In the recovery phase, rhodopsin is deactivated by mechanisms involving phosphorylation and arrestin binding (3), and  $G_{t\alpha}$  is deactivated by hydrolysis of its bound GTP. The intensity and duration of the G-protein coupled signaling is determined by the balance between reactions that amplify or sustain the amount of activated  $G_{t\alpha}$ -GTP and those that dampen or terminate it. Therefore, rhodopsin deactivation by phosphorylation and GTPase acceleration on  $G_{t\alpha}$  are two major mechanisms for regulation in the recovery stage of normal vision.

RGS9-1, the GTPase Accelerating Protein (GAP) for  $G_{t\alpha}$ , is an important regulator of phototransduction and a key mediator of the recovery to a dark state (4-6). It belongs to the ubiquitous Regulators of G protein Signaling (RGS) family of GAPs (for reviews, see (7-9)) and shares with them a conserved catalytic core or RGS domain that is responsible for the GAP activity (10). Like most other RGS proteins (11-13), RGS9-1 also contains multiple additional functional domains, which have been speculated to be involved in GTPase regulation (14). These include a G protein  $\gamma$ -like (GGL) domain, which tethers RGS9-1 to its partner subunit,  $G_{\beta 5L}$  (15,16), a DEP (Dishevelled/EGL-10/Pleckstrin) domain (17,18), and a C-terminal domain unique to RGS9-1 (4,19,20). Deletion of the RGS9 gene in mice results in profound slowing of photoresponse recovery and transducin GTP hydrolysis (21), and adding exogenous RGS9

catalytic core to excised patches from ROS dramatically attenuates the phototransduction cascade (22). It has been proposed that RGS9-1-regulated GTP hydrolysis is the rate-limiting step in phototransduction (23,24), but this hypothesis has yet to be tested in a definitive way. Thus, modulation of GTPase accelerating activity provides a plausible mechanism for attenuating, sensitizing, speeding up, or slowing down light responses in order to accommodate changes in background light or other conditions.

Phosphorylation events are often employed to regulate signal transduction because they can change the activities, subcellular localization, protein-protein interactions, or stability of transduction components. In ROS, where the reactions of phototransduction take place, there are many protein kinases and many phosphoproteins. Kinases including rhodopsin kinase (25), CDK5 (26), PKC (27), PKA (28), CK II (29) and protein tyrosine kinases such as src (30) have all been reported to be present in photoreceptors. Phosphorylation by endogenous kinases in ROS has been reported for phototransduction components including rhodopsin (whose phosphorylation is known to be essential for normal recovery kinetics (31)), the  $G_{\beta\gamma}$ -binding protein phosducin (32),  $G_{\alpha}$  (30), the inhibitory  $\gamma$  subunit of cGMP phosphodiesterase (26,33) has also been reported, but the roles of most of these phosphorylation reactions in regulation of phototransduction are not currently well understood. Recently, phosphorylation of other RGS proteins, such as Sst2, RGS2, RGS3, RGS4, RGS7 and GAIP, has been reported to modulate their functions (34-39). Therefore, it is likely that the function of RGS9-1 may also be subject to regulation by mechanisms involving phosphorylation.

ATP has long been known to have profound effects on the kinetics of the recovery phase of the light response (40,41), but the mechanisms of its actions have not been fully determined either. Clearly, one of the main roles of ATP in recovery is to serve as a substrate for rhodopsin

kinase (3,42), but it seems likely that other ATP-dependent reactions, including those catalyzed by other protein kinases, play an important role as well. We describe here experiments suggesting that one of these may be phosphorylation of RGS9-1, and that this reaction is catalyzed by a protein kinase not identified previously in ROS.

## EXPERIMENTAL PROCEDURES

*Reagents-* Buffer reagents, nucleotides and analogues, PKA catalytic subunit from bovine heart, and PKA substrate (Val<sup>6</sup>, Ala<sup>7</sup>)-kemptide were purchased from Sigma. Protein kinase inhibitors (A3-HCl, H8-dihydrochloride, Bis-indolylmaleimide I-HCl (3-[1-(3-Dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)-maleimide, HCl, also known as GF 109203X), CaM-binding domain, Sangivamycin, Roscovatine), recombinant CK II, and CK II substrate were purchased from Calbiochem.

*Buffers-* Standard buffers were: Buffer A: 10 mM HEPES, 100 mM NaCl; Buffer B: 5 mM HEPES, 0.5 mM MgCl<sub>2</sub>; Buffer C: 10 mM Tris-HCl, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, ~20 mg/L PMSF; Buffer D: 10 mM MOPS, 30 mM NaCl, 60 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM DTT, ~20 mg/L PMSF; Buffer E: 5 mM Tris-HCl, 2 mM EDTA, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 15 μM Fenvalerate, 100 nM okadaic acid, 1 mM DTT. Buffer F: 50 mM sodium phosphate, 50 mM NaCl, 10 mM KF, 2 mM MgCl<sub>2</sub>. Buffer G: 100 mM NaCl, 5 mM Tris. For all these buffers, the pH was adjusted to 7.4-7.5. Other buffer components and conditions were varied as indicated in the text and figure legends.

*Expression and purification of recombinant proteins-* His<sub>6</sub>-tagged RGS9-1 full-length protein in complex with G<sub>β5S</sub> was expressed in Sf9 cells using a baculovirus vector and affinity purified as described (14). PCR mutagenesis was used to generate a construct that was identical to the His-tagged RGS9-1 except for mutation of Ser<sup>475</sup> to Ala (Ser<sup>475</sup>Ala). The expression and

purification of the mutant protein was essentially the same as that of the full-length His-RGS9-1. Full-length recombinant RGS9-1 had to be prepared and used as a complex with G $\beta_{5S}$  because the protein is not expressed in stable form in the absence of G $\beta_{5S}$  or the natural partner subunit (16) of RGS9-1, G $\beta_{5L}$  (14). Truncated recombinant constructs containing only the RGS and C-terminal domains of RGS9-1 were not effective substrates for endogenous ROS kinases (data not shown).

*Phosphorylation of RGS9-1 in ROS-* Bovine ROS were prepared in dim red light by sucrose gradient centrifugation (43). After ROS membranes were collected from the sucrose gradient, they were homogenized by passage through an 18 gauge needle in buffer C at a dilution ratio of 1:5, pelleted by centrifugation at x 24,000g, then resuspended in buffer C to a rhodopsin concentration of 100-150  $\mu$ M and stored at -80°C. Phosphorylation experiments were carried out in the dark. Kinase assays were performed by incubating ROS with ATP at 30°C in Buffer C in the presence of 1 mM DTT and 10 mM NH $_2$ OH (to minimize photoisomerized rhodopsin phosphorylation), unless otherwise stated. ATP was added to final concentrations of 2 mM to 5 mM, with [ $\gamma$ - $^{32}$ P]-ATP specific activity ranging from 40 to 100 Ci/mol. Phosphorylation was detected by autoradiography of immunoprecipitated RGS9-1 (see below) following SDS-PAGE. The quantity of phosphate incorporation into RGS9-1 was determined by scintillation counting of the RGS9-1 bands excised from SDS-PAGE gels following immuno-affinity isolation of RGS9-1 as described below. The amount of RGS9-1 in the sample was determined by densitometry of the Coomassie Blue-stained gel using known amounts of bovine serum albumin as standards. Differences in dye binding by the standards and RGS9-1 were accounted for by UV absorbance spectrophotometry of recombinant His tagged RGS9-1, which was purified from *E. coli* in guanidine-HCl by affinity chromatography. The extinction coefficient for RGS9-1 of

93,910 M<sup>-1</sup>-cm<sup>-1</sup> calculated from its amino acid sequence (44) was used to determine the amount of protein present, which was found to be 0.98 +/- 0.01 of that determined by dye binding using standards.

*Antibodies and immunoprecipitation-* Rabbit anti-RGS9-1c polyclonal antibody and monoclonal anti-RGS9-1 antibody D7 were generated as described previously (4,5,20). Mouse anti-phosphorylated RGS9-1 monoclonal antibodies (A4) were raised against a peptide from the C-terminus of mouse phosphorylated RGS9-1, KLDRRS(P)QLKKELPPK, where the (P) refers to phosphorylation of the preceding serine residue (Quality Controlled Biochemicals, Inc./Hopkinton, MA), coupled to a carrier protein, KLH (Sigma) as described previously (45). The mouse sequence, which differs slightly from the bovine sequence (KLDRRSQLRKEPPP), was used to assure reactivity with the phosphoprotein in mice. However the bovine phosphoprotein, but not the unphosphorylated form, was also strongly recognized by the antibody. For use in immunoprecipitation, IgG was affinity purified by protein A beads from rabbit anti-RGS9-1c antisera. Purified IgG was then covalently attached to CNBr-activated Sepharose 4B-CL from Amersham following the manufacturer's instructions at a ratio of 10 mg IgG to 1 ml beads. For immunoprecipitation, ROS membranes were washed after ATP incubation by repeated centrifugation at 84,000g for 15 min, homogenized three times with Buffer E at 15 μM rhodopsin 0-4°C, and then solubilized for 30 min on ice in Buffer C with 1% Nonidet-P40 detergent at 60 μM rhodopsin. The insoluble material was removed by centrifugation for 20 min at 84,000g . Typically, 300 μl solubilized ROS were incubated with 40 μl IgG coupled beads for 2.5 hr at 4°C upon mixing on a shaker. The beads were separated from the supernatant by a brief centrifugation and washed three times with the solubilization buffer. Bound proteins were eluted from the beads by 0.1 M glycine at pH 3.0, concentrated by

trichloroacetic acid precipitation, and re-dissolved by boiling in the SDS-PAGE sample buffer. Efficiency of immunoprecipitation was measured by autoradiography following SDS-PAGE and immunoblot using monoclonal antibody D7.

*Immunoblotting-* Immunoblotting was carried out using a standard protocol (46) on proteins separated by SDS-PAGE. For electrophoretic transfer, the buffer used was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, and the membranes were supported nitrocellulose (NitroPure, Osmonics, Inc.). After 60 min at 350 mA (room temperature), membranes were blocked with with 5% nonfat dry milk/TBS solution for 1 h, followed by incubation with primary antibody for 2 hr. Monoclonal antibody D7 and A4 were used at a dilution of 1:500, and polyclonal anti-RGS9-1c serum was used at a dilution of 1:1000. The secondary antibodies used were horseradish peroxidase-conjugated (Promega) anti-mouse (for monoclonals) or anti-rabbit (for polyclonal), with detection by chemiluminescence, using the ECL® system (Amersham). For autoradiography, X-ray films were exposed to dried SDS-PAGE gels, typically for 2-3 days, before being developed.

*Detection of in vivo phosphorylation.* Mice were maintained in a dark room (dark adapted) or in full room light (light adapted) for a period of 16 hr prior to euthanasia and removal of retinas under dim red light. The retinas were homogenized by pestle (Kontes) in 1.5 ml microtubes using buffer C with 1% Nonidet-P40 detergent, plus 0.2 mM  $\text{Na}_3\text{VO}_4$ , 15  $\mu\text{M}$  Fenvalerate, 100 nM okadaic acid to inhibit phosphatase activities. Immunoprecipitation of RGS9-1 was carried out using rabbit polyclonal antibodies, as described above, and the immunoprecipitated protein was analyzed by SDS-PAGE and immunoblotting with the mouse monoclonal antibody A4 specific for the phosphopeptide.

*Identification of the phosphorylation site-* ROS were incubated at 37°C for 40 min at a concentration of 69  $\mu$ M rhodopsin with 0.2 mM [ $\gamma$ -<sup>32</sup>P]-ATP (0.56 Ci/mmol) in Buffer F. After the phosphorylation reaction, all separation procedures prior to HPLC were carried out at 0-4°C. The phosphorylated membranes were collected by centrifugation (86,000g for 10 min), and washed several times with buffer A and then twice with buffer B before being dissolved in 125 mM Tris-HCl, pH 6.5, containing 4% SDS (w/v), 20% glycerol (v/v), 2.5 mM reducing agent TCEP (*tris* (2-carboxyethyl) phosphine hydrochloride), 2.5 mM MgCl<sub>2</sub>. Phosphorylated proteins were separated by 12% (w/v) preparative SDS-PAGE gels. The gels were stained with Coomassie blue R-250, destained, and the RGS9-1 band identified by its mobility as calibrated by immunoblotting with monoclonal antibody D7 on an identical gel run in parallel. After the gels were washed with water and then pH 7.8 sodium bicarbonate solution, the RGS9-1 band was excised and pulverized, and the protein was extracted by shaking with 10 mM Tris-HCl, pH 7.8, containing 1%  $\beta$ -mercaptoethanol (v/v), 0.2% SDS (w/v) for 5 hr. The gel was extracted again with extraction buffer and water, and the combined extracts were vacuum dried. The dried extract was redissolved in water and subjected to centrifugation to remove insoluble material. The resulting supernatant was mixed with 100% trichloroacetic acid (w/v) to a final concentration of 10% trichloroacetic acid to precipitate proteins. The supernatant was subjected to another round of precipitation by 15% trichloroacetic acid. The pellets were pooled, and washed sequentially with acetone, acetone/methanol (1/1), and water. The pellet was digested with trypsin (10-20  $\mu$ g) in 400  $\mu$ l 12.5 mM BTP (1,3-bis[tris(hydroxymethyl)-methylamino]propane), a pH buffer), pH 7.9, containing 2 M urea, 0.125% mercaptoethanol (v/v), 1 mM CaCl<sub>2</sub>. The suspension was incubated at room temperature for 7 hr with occasional vortexing. Insoluble material was separated by centrifugation and treated again with trypsin until

$^{32}\text{P}$  was undetectable in the pellet. Phosphopeptides were isolated by chromatography with detection by scintillation counting. After each elution, a single major  $^{32}\text{P}$ -containing peak was collected, vacuum-dried, and used for the next step. Reverse-phase HPLC (RP-HPLC) was performed using a C18 HPLC column (Vydac 201HS52; 2.1 x 250 mm) with binary solvent systems (solvent A:  $\text{H}_2\text{O}$ / 0.1% trifluoroacetic acid; solvent B:  $\text{CH}_3\text{CN}$ / 0.1% trifluoroacetic acid; solvent C:  $\text{H}_2\text{O}$ / 0.2% HFBA; solvent D:  $\text{CH}_3\text{CN}$ / 0.2% HFBA) and linear gradients. The first gradient was 100%A/0% B to 10%A/90% B in 30 min at 0.3 ml/min, and the second from 100%A/0% B to 75%A/25% B in 50 min at 0.2 ml/min. The peptide was further purified by  $\text{Ga}^{3+}$ -immobilized metal affinity chromatography (IMAC) (47) using 0.2 mL of Chelex-Sepharose (Pharmacia). Finally, two additional rounds of HPLC were carried out: one identical to the second gradient described above, and the last wash was also identical to the second except that solvents C and D were used. The major peak was dried down and subjected to analysis by electrospray (ES/MS) and tandem MS/MS using a Sciex API III triple quadrupole mass spectrometer fitted with a nebulization-assisted electrospray ionization source (PE/Sciex, Thornhill, Ontario). For tandem MS/MS, precursor ions were selected with the first of three quadrupoles (Q1) for collision-induced dissociation with argon in the second quadrupole (Q2), and product ions were scanned by the third quadrupole (Q3) (48).

To verify that the same site is phosphorylated under our standard assay conditions, phosphorylation reactions were carried out using Buffer C as described above under “*Phosphorylation of RGS9-1 in ROS*”, using 5 mM ATP without radiolabel, followed by immunoprecipitation. Immunoprecipitated RGS9-1 was separated by SDS-PAGE, subjected to in-gel trypsin digestion, and the phosphorylation sites in the tryptic peptides were identified by mass spectrometry. The method used was a combination of matrix-assisted laser

desorption/ionization time-of-flight mass spectrometry, before and after phosphatase treatment, and on-line capillary liquid chromatography electrospray tandem ion trap mass spectrometry described previously (49). Two-dimensional phosphopeptide mapping was carried out as described (50).

*Phosphorylation of recombinant proteins-* Purified His-tagged RGS9-1 in complex with G $\beta_{5s}$  was mixed with ROS and [ $\gamma$ - $^{32}$ P]-ATP at 30 °C for the time indicated. Final concentrations of proteins and reagents were 60  $\mu$ M rhodopsin, 0.2  $\mu$ M His-RGS9-1 or 0.2  $\mu$ M His-RGS9-1-Ser $^{475}$ Ala, and 2.5 mM [ $\gamma$ - $^{32}$ P]-ATP (100 Ci/mol). Reactions were then quenched by addition of equal volumes of SDS-PAGE sample buffer. Phosphorylation of His-RGS9-1 was determined by autoradiography following SDS-PAGE, and the amounts of His-RGS9-1 in the reactions were determined by immunoblotting using rabbit anti-RGS9-1c polyclonal antibody.

*Tests of candidate protein kinases-* Three different approaches were used to test the possible involvement of known protein kinases in RGS9-1 phosphorylation: 1) Activators of PKA (8-Br-cAMP, 50  $\mu$ M), PKG (8-Br-cGMP, 50  $\mu$ M) or PKC (PMA: 1, 5, 10  $\mu$ M plus 1 or 2 mM CaCl $_2$ , referred to subsequently as PMA/Ca) were added under our standard assay conditions. The ability of the added cyclic nucleotide analogues to activate endogenous cyclic nucleotide-dependent protein kinase activity was verified by testing their ability to stimulate phosphorylation in ROS homogenates of (Val $^6$ , Ala $^7$ ) kemptide using [ $\gamma$ - $^{32}$ P]-ATP, with detection by binding to phosphocellulose paper, followed by scintillation counting (51). The concentrations used are 1000 times the reported K $_a$  value for PKA activation (52) and 100 times the reported K $_a$  value for PKG activation (53). Efficacy of PMA/Ca for PKC stimulation under our conditions was verified by the previously characterized phosphorylation of rhodopsin (54,55), using [ $\gamma$ - $^{32}$ P]-ATP and autoradiography. 2) Known substrates for candidate kinases were

added and tested for phosphorylation using [ $\gamma$ - $^{32}$ P]-ATP and either autoradiography of SDS-PAGE gels or phosphocellulose paper binding and scintillation counting. Those which were phosphorylated (PKA/PKG and CDK5 substrates) were then used to test efficacy of activators (see above) and inhibitors (see below). Lack of phosphorylation (CK II substrate) was taken as evidence for absence of the kinase after verification of the ability of added kinase to phosphorylate the substrate. The substrate used for PKA and PKG was (Val<sup>6</sup>, Ala<sup>7</sup>)-kemptide (Sigma) at 100  $\mu$ M, the substrate for CK II was a substrate peptide (Calbiochem) at 100  $\mu$ M, and the substrate used for CDK5 was the  $\gamma$  subunit of ROS cGMP phosphodiesterase (PDE6), PDE $\gamma$  (26,33) at 400 nM. Peptide phosphorylation was assayed using [ $\gamma$ - $^{32}$ P]-ATP under the same conditions as for RGS9-1 phosphorylation, with detection by binding to phosphocellulose paper, followed by scintillation counting (51). Phosphorylation of proteins was also detected using [ $\gamma$ - $^{32}$ P]-ATP and autoradiography following SDS PAGE. 3) Known inhibitors were added, and their effects on RGS9-1 phosphorylation determined by measuring RGS9-1 phosphorylation in immunoblotting using Ser<sup>475</sup>-phosphate specific monoclonal antibody A4. Inhibitors used were: For PKA and PKG, H8-dihydrochloride 60 $\mu$ M, ( $K_i$  for PKA = 1.2 $\mu$ M,  $K_i$  for PKG = 0.48 $\mu$ M, (56)); for rhodopsin kinase, sangivamycin, 10  $\mu$ M ( $K_i$  = 180 nM, (57)); for CDK5/p35, roscovitine, 20  $\mu$ M ( $IC_{50}$  = 0.2  $\mu$ M, (58)); for CK II, A3-HCL, 250  $\mu$ M ( $K_i$  = 5.1  $\mu$ M, (59)); for PKC, bisindolylmaleimide I-HCl (GF 109203X, 3-[1-(3-Dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)-maleimide, HCl, Calbiochem), 500 nM ( $K_i$  = 10 nM, (60,61)); for CaMK II (CaM-dependent kinase II), CaM-binding domain 2.5  $\mu$ M ( $K_i$  = 52 nM, (62)). Inhibitors were pre-incubated with ROS at room temperature for 15 min before addition of [ $\gamma$ - $^{32}$ P]-ATP to start phosphorylation. Efficacy of the inhibitors under our conditions was verified by determining their effects on phosphorylation of endogenous (PKC and rhodopsin kinase) or added

(PKA/PKG, CK II, CDK5) substrates by endogenous (PKA/PKG, CDK5) or added (CK II) enzyme.

*Inhibition by RGS9-1-derived peptide-* For the peptide inhibitor, ROS were mixed with ATP in the presence of increasing concentrations of a synthetic peptide derived from the mouse RGS9-1 C-terminus with the phosphorylation site mutated to Ala: KLDRRAQLKKELPPK (Quality Controlled Biochemicals, Inc). Reactions were then quenched by SDS-PAGE sample buffer, and phosphorylation was detected by Ser<sup>475</sup>-phosphate specific monoclonal antibody A4.

*Kinase activity in fractionated retinal membranes-* Retinal membranes from frozen bovine retinas were prepared in dim red light and separated by sucrose gradient centrifugation using a standard technique (43). Samples in the sucrose gradient were then fractionated into 1ml aliquots using a gradient puller (Auto-Densi-Flow from Labconco) and stored at  $-80^{\circ}\text{C}$ . To check the kinase activity in these fractions, 20  $\mu\text{l}$  of each fraction was mixed with purified His-RGS9-1 (in complex with  $\text{G}_{\beta 5}$ ) and ATP at  $25^{\circ}\text{C}$  for 10 min. Final concentrations of proteins and reagents were: 60  $\mu\text{M}$  rhodopsin (in the peak fraction), 200 nM His-RGS9-1, 5 mM ATP. Reactions were quenched by adding equal volumes of standard SDS-PAGE sample buffer. Phosphorylation of His-RGS9-1 was detected by immunoblotting using a Ser<sup>475</sup>-phosphate specific monoclonal antibody A4, and the amount of endogenous RGS9-1 and recombinant proteins in each sample was determined by the anti-RGS9-1c polyclonal antibody.

## RESULTS

*RGS9-1 is phosphorylated by an endogenous kinase on the ROS membranes-* When purified bovine ROS membranes were incubated with  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ , radioactivity was detected in a protein migrating to the same position as that of RGS9-1. To determine if this phosphoprotein

is indeed RGS9-1, we incubated ROS with [ $\gamma$ - $^{32}$ P]-ATP, washed away free ATP, and immunoprecipitated RGS9-1 from detergent solubilized ROS. Two strongly labeled radioactive bands were detected in the total ROS proteins, migrating to the positions corresponding to rhodopsin and RGS9-1 ( Fig. 1A), respectively. At the position of RGS9-1, the radioactivity came from a detergent soluble and a detergent insoluble species, implying the presence of two co-migrating phosphoproteins. Both the autoradiogram and the immunoblot showed that the detergent soluble phosphoprotein was quantitatively precipitated by anti-RGS9-1 antibody, identifying it as phosphorylated RGS9-1. The detergent insoluble phosphoprotein was later identified by mass spectrometry analysis to be tubulin (29,63) (data not shown). Comparison of the radioactive signals from RGS9-1 and other ROS proteins after [ $\gamma$ - $^{32}$ P]-ATP labeling clearly reveals RGS9-1 as one of the major targets for phosphorylation in ROS under our phosphorylation conditions.

To test whether RGS9-1 phosphorylation level is high enough to be a potential regulatory mechanism, we studied the stoichiometry by immunoprecipitating phosphorylated RGS9-1. The highest phosphorylation level we achieved was 0.45 mole phosphate per mole RGS9-1 after 20 min of ATP incubation (Fig. 1B). However, the stoichiometry varied among different batches of ROS preparations, ranging from 0.23 to 0.45 (from 3 independent experiments, data not shown), with the differences possibly due to loss of kinase activity during the ROS preparation. This explanation is supported by the observation that RGS9-1 kinase is slightly soluble in the isotonic buffers that were used for ROS preparation (see below). Likely, the phosphorylation stoichiometry is determined by the balance of phosphatase and kinase activity, and phosphatase activity may also vary somewhat in different preparations. As discussed below, RGS9-1 is not phosphorylated in our ROS preparations prior to ATP treatment.

*RGS9-1 is specifically phosphorylated at Ser<sup>475</sup> near the carboxyl terminus-* When phosphorylated RGS9-1 was digested with trypsin, and the phosphorylated peptide was then extensively purified by two rounds of RP-HPLC, Ga<sup>3+</sup>-IMAC, and two additional rounds of HPLC with different solvent systems (Fig. 2A), in each separation procedure, a single major phosphopeptide peak was eluted and collected. Electrospray ionization MS revealed (Fig. 2B) that this phosphopeptide has an m/z 583.5 (the precursor ion, MH<sup>+</sup><sub>1</sub>), corresponding to a monophosphorylated peptide at Ser<sup>475</sup> with a sequence of S<sup>475</sup>QLR (calculated average mass for MH<sup>+</sup><sub>1</sub> = 583.56). The precursor ion with the loss of HPO<sub>3</sub> was also observed at m/z 503.5 (calculated mass 503.58). The precursor ion underwent elimination of phosphate (MH<sup>+</sup><sub>1</sub>-H<sub>3</sub>PO<sub>4</sub>, y4) and elimination of phosphate and water (MH<sup>+</sup><sub>1</sub>-H<sub>3</sub>PO<sub>4</sub>-H<sub>2</sub>O, b4). Further decompositions of y4 and b4 yielded ions y1-y3 and b1-b3, respectively. The observed fragmentation pattern is in agreement with the sequence SQLR where the Ser residue is phosphorylated. The same peptide was identified in two different preparations. These results demonstrate clearly that the single major phosphorylation site in RGS9-1 is Ser<sup>475</sup>.

In a completely different trial to identify the phosphorylation site under our standard reaction conditions, RGS9-1 was phosphorylated in Buffer C, immunoprecipitated, resolved by SDS-PAGE, and subjected to in-gel trypsin digestion for MS analysis of the phosphorylation site using an established procedure (49). Again, Ser<sup>475</sup> was revealed as the only phosphorylation site (data not shown). Under these conditions both endogenous RGS9-1, and recombinant phosphorylated RGS9-1 (see below) each yielded a single major radiolabeled spot upon two-dimensional phosphopeptide mapping (data not shown).

*Recombinant RGS9-1 can be phosphorylated by the endogenous kinase and its phosphorylation requires Ser<sup>475</sup>.*- To test further the requirement of Ser<sup>475</sup> for RGS9-1

phosphorylation, phosphorylation of both His-tagged RGS9-1 full-length protein and His-tagged RGS9-1 mutant with Ser<sup>475</sup> mutated to Ala by the endogenous kinase was examined. Only full-length RGS9-1 with the wild-type sequence was phosphorylated significantly by ROS kinases (Fig. 3), suggesting the Ser<sup>475</sup> residue is required for phosphorylation. Only very weak signals could be detected in the mutant RGS9-1 protein, likely due to phosphorylation at one or more minor sites. Immunoblotting with the monoclonal Ser<sup>475</sup>-phosphate specific antibody A4 confirmed that only the wild-type, and not the mutant protein could be detected after phosphorylation (data not shown).

*Divalent cation dependence of RGS9-1 phosphorylation-* We tested the requirement of RGS9-1 phosphorylation for metal ions by performing kinase assays in the presence of different cations. ROS membranes were first washed with EDTA to remove contaminating metal ions, and then resuspended in buffers containing the desired cations for phosphorylation. As expected for most phosphotransfer reactions, we found that Mg<sup>2+</sup> was required for RGS9-1 phosphorylation, because RGS9-1 phosphorylation was completely abolished in the presence of EDTA or Ca<sup>2+</sup> alone, and could only be partially restored by Mn<sup>2+</sup> (Fig. 4A). Chelation of Ca<sup>2+</sup> by EGTA in the presence of excess Mg<sup>2+</sup> reduced phosphorylation (Fig. 4C).. Free [Ca<sup>2+</sup>] calculated to be on the order of 10<sup>-7</sup> M was sufficient to restore full activity (Fig. 4C). For these immunoblots we used monoclonal antibody A4 that specifically recognizes Ser<sup>475</sup>-phosphate. It was raised against a phosphopeptide derived from murine RGS9-1 (KLD~~RRS~~(P)QLKKELPP), and was found to react with RGS9-1 only in ATP treated ROS (Fig. 4B).

*Effects of kinase activators and inhibitors--* In order to determine if the kinase responsible for RGS9-1 phosphorylation is one of the well-characterized protein kinases, we first tried to stimulate RGS9-1 phosphorylation by adding kinase activators for PKA, PKC and PKG, since

the Ser<sup>475</sup> is located in a sequence similar to the consensus sequence for PKA and PKG phosphorylation, and both PKA and PKC activities in purified ROS have been reported (27,28). We found that RGS9-1 phosphorylation levels did not change significantly in the presence of 8-Br-cAMP, 8-Br-cGMP or PMA/Ca<sup>2+</sup>, although PMA/Ca<sup>2+</sup> did greatly enhance rhodopsin phosphorylation (Fig. 5A, 5B). Both 8-Br-cAMP and 8-Br-cGMP enhanced the phosphorylation of added kemptide substrate in the presence of ROS (Fig. 5G). We then tested the effects of several kinase inhibitors, including inhibitors for PKA, PKC, PKG, CK II, CaMK II, rhodopsin kinase and CDK5/p35. No significant inhibition of RGS9-1 phosphorylation was observed in any of these experiments (< 10% inhibition at concentrations of 50 times of their reported K<sub>i</sub> values as detailed under Experimental Procedures; Fig. 5C), except for the PKC inhibitor bis-indolylmaleimide I. Rhodopsin phosphorylation was strongly inhibited by inhibitors of both rhodopsin kinase and PKC (Fig. 5E), verifying their efficacies under our conditions. The PKA/PKG inhibitor H8 greatly reduced kemptide phosphorylation in ROS stimulated by 8-Br-cGMP 8-Br-cAMP (Fig. 5G) verifying its potency under our conditions. PDE $\gamma$  was phosphorylated by an endogenous kinase, most likely CDK5/p35 (33), and the phosphorylation was inhibited 67% by added roscovitine, verifying its efficacy as well (Fig. 5F). No phosphorylation of the CK II substrate in ROS membranes was observed, and there were no effects on phosphorylation of any protein detectable upon addition of the CK II inhibitor A3-HCl. However, this inhibitor did reduce phosphorylation of the CK II substrate peptide by added CK II in the presence of ROS. As no activity of CK II or CaMK II was detected using either substrate (CK II) or inhibitors (CK II and CaMK II), it seems likely that they are not only not responsible for RGS9-1 phosphorylation, but also are absent in our membrane preparation. Thus we can rule out, to varying degrees of certainty, the involvement in RGS9-1 phosphorylation of

all protein kinases known to be present in ROS, as well as of additional kinases whose presence is less certain. Although the inhibition by the PKC inhibitor bis-indolylmaleimide I raises the possibility of a previously uncharacterized PKC isozyme or PKC-like enzyme in ROS, the inhibitor we used can also inhibit kinases other than PKC, such as phosphorylase kinase, ( $IC_{50}=0.7 \mu\text{M}$  at  $250 \mu\text{M}$  ATP, (60)). The kinase responsible for RGS9-1 phosphorylation is clearly distinct from the PKC isozyme responsible for PMA-induced phosphorylation of rhodopsin (54,55).

We next tested a peptide derived from the mouse RGS9-1 C-terminus containing the phosphorylation site with Ser<sup>475</sup> mutated to Ala (KLDRRAQLKKELPP) for its effect on RGS9-1 phosphorylation. We found that the peptide containing the mutated phosphorylation site did inhibit RGS9-1 phosphorylation in a concentration dependent manner, probably by competing for the kinase (Fig.5D). Indeed, the Ser<sup>475</sup>-containing peptide was found to be phosphorylated by ROS membranes (data not shown). Thus the RGS9-1 kinase has a sequence specificity not previously reported for any known protein kinase.

*RGS9-1 kinase co-purifies with rhodopsin and RGS9-1 in fractionated retinal membranes and is tightly membrane associated-* To determine whether the RGS9-1 kinase is an endogenous component of ROS or a contaminant from elsewhere in the retina, we checked the presence of RGS9-1 kinase activity in fractions from homogenized retina separated by sucrose gradient centrifugation. In these experiments we used recombinant His-tagged RGS9-1 protein as substrate and the monoclonal Ser<sup>475</sup>-phosphate specific antibody A4 to detect the phosphorylation. There was no detectable phosphorylation of this recombinant protein on Ser<sup>475</sup> prior to incubation with preparations containing the RGS9-1 kinase, as demonstrated by its lack of reactivity with the A4 antibody. The major kinase activity peaks correlated very well with the

peaks of rhodopsin and RGS9-1 in those fractions corresponding to ROS, broken ROS, and unsheared retinal membranes (Fig. 6A), implying that within the retina the kinase is predominantly localized to ROS, and likely plays a role in regulation of phototransduction. The lack of detectable endogenous RGS9-1 phosphorylation in the fractions containing the minor peak of broken ROS (fraction 25-27) may result from competition by recombinant protein. The minor kinase activity peak in the fractions corresponding to soluble proteins (fractions 1-3) can be attributed to the slight solubility of RGS9-1 kinase in isotonic buffer (see below). Phosphorylation of endogenous RGS9-1 can be seen to be more efficient than phosphorylation of recombinant proteins (the boxed area in Fig. 6A) when the levels of phosphorylation are compared to the amounts of substrates that were present, indicating that the kinase may be localized in close vicinity to or even form a complex with RGS9-1. We were only able to achieve phosphate incorporation stoichiometries of less than 10% for the recombinant protein.

To characterize the kinase further, we tested whether the kinase displays similar membrane-binding properties to those of RGS9-1, a tightly bound peripheral membrane protein, by comparing the kinase activity remaining on ROS membranes before and after buffer extractions. All assays were adjusted to the same final ionic strength. We found that low salt (5 mM ionic strength) and moderate salt (100 mM ionic strength) extractions removed only about 20% of the kinase activity, but the hypertonic extractions removed at least 60-70% (Fig. 6B). Assays of kinase activity in the high salt extracts using recombinant proteins (data not shown) revealed that while high salt does inhibit kinase activity somewhat, there is substantial kinase activity in the extracts, confirming that high ionic strength extracts the kinase into the supernatant rather than permanently inactivating it in the membranes. These results indicate that the kinase itself is tightly membrane bound and that its membrane-binding has a large

electrostatic component. These membrane-binding properties are very similar to those of RGS9-1 (5).

*RGS9-1 phosphorylation in mouse retina-* To find out if RGS9-1 phosphorylation actually occurs *in vivo*, we isolated endogenous RGS9-1 from freshly dissected mouse retina and used the Ser<sup>475</sup>-phosphate specific monoclonal antibody to detect its phosphorylation by immunoblotting. The immunoblot (Fig. 7A) demonstrated that RGS9-1 was phosphorylated by endogenous kinase in the retina. A further comparison on the phosphorylation level between light and dark adapted animals revealed that light reduced signal due to RGS9-1 phosphorylation about 80% (Fig. 7B). As revealed by phospho- and site-specific monoclonal antibodies, these results demonstrate clearly that RGS9-1 is phosphorylated on Ser<sup>475</sup> *in vivo*, and that this phosphorylation is regulated by light, the signal transduced by the pathway whose recovery is regulated by RGS9-1.

## DISCUSSION

The very robust phosphorylation of RGS9-1 can be seen qualitatively simply by the fact that despite not being a particularly abundant protein (~1 mol RGS-1 per 1600 mol rhodopsin), it incorporates more phosphate than all but a few other ROS proteins under a range of different conditions. The significant stoichiometry of phosphorylation that we observe suggests that under some conditions a substantial fraction of RGS9-1, and perhaps even all of it, may be subject to phosphorylation by the endogenous kinase. Immunoblots of ROS purified and washed in the absence of added ATP reveal no detectable Ser<sup>475</sup> phosphorylation, suggesting that RGS9-1 phosphorylation is highly dynamic and may be subject to regulation at the levels of both addition and removal of phosphate.

In contrast to results obtained with purified ROS, immunoblots of retina from dark adapted animals reveal Ser<sup>475</sup> phosphorylation of RGS9-1 *in vivo*. The dramatic decrease in phosphorylation levels observed in animals exposed to light underscores the physiological regulation of RGS9-1 phosphorylation. Inhibition of phosphorylation by lowering of calcium is consistent with the direction of light regulation, because light lowers intracellular [Ca<sup>2+</sup>] from the range of hundreds of nanomolar to 10 nM or less (64).

The Ser<sup>475</sup> residue phosphorylated by the ROS kinase is within the RGS9-1 C-terminal domain, which is not conserved in other RGS proteins. Interestingly, there is another isoform of RGS9 named RGS9-2 in striatum, which results from alternative RNA processing (19,20). Its amino acid sequence differs from that of RGS9-1 only in the C-terminal domain beginning at residue 467. Although there is a serine residue at a similar position (Ser<sup>474</sup>) in RGS9-2, it is in a completely different sequence context. Thus, the Ser<sup>475</sup> phosphorylation is unique for RGS9-1, and must be highly specific for photoreceptors, because of the exclusive expression of RGS9-1 in these cells.

Phosphorylation of several other RGS proteins has been reported, including Sst2, RGS2, RGS3, RGS4, RGS7 and GAIP (34-39). Phosphorylation was found to regulate the function of these RGS proteins by affecting their stability, subcellular localization, GAP activity or interactions with other regulatory molecules. For example, phosphorylation of RGS2 by PKC inhibits its GAP activity, and phosphorylation of RGS3 and RGS4 translocates them from cytosol to cell membrane. In the case of RGS9-1, though not fully established, the function of its unique C-terminal domain is beginning to emerge. Recent work (14) suggests it contributes to the tight regulation of GAP activity of RGS9-1, and there is also evidence suggesting an essential role in RGS9-1 binding to rod disk membranes (Wei He, Ph.D. Dissertation, Baylor College of

Medicine, 2001). Therefore, it will be interesting to determine whether phosphorylation in the C-terminal tail has any direct effects on RGS9-1 GAP activity and membrane association.

From the studies we have conducted so far it is not clear how the phosphorylation state of RGS9-1 is regulated on molecular level, or how its phosphorylation affects its function. Assays of GAP activity before and after phosphorylation have been difficult to interpret because ATP treatment of ROS leads to inactivation of RGS9-1 GAP activity by a mechanism that seems to be distinct from, and to precede, Ser<sup>475</sup> phosphorylation (C. Cowan and G. Hu, unpublished observations). Likewise, assays of recombinant RGS9-1 have so far been rendered ambiguous by the difficulty in obtaining stoichiometric phosphorylation of the recombinant protein. As can be seen in Figure 7, the endogenous membrane-anchored protein is a much better substrate for the membrane-associated kinase than is the soluble RGS9-1/G $\beta_{5s}$  complex, and no Ser<sup>475</sup> phosphorylation of the latter can be detected on the complex as purified from insect cells.

Nevertheless, it would be very surprising if RGS9-1 phosphorylation did not regulate its function in some way. The high specificity of the reaction, and the localization of the kinase to ROS membranes imply a role in phototransduction. RGS9-1 interacts with a number of other proteins in ROS, including G $\beta_{5L}$ , the PDE $\gamma$  inhibitory subunit of cGMP phosphodiesterase, and G $\tau\alpha$  in addition to its interactions with the kinase and with ROS membranes. Moreover, sensitivity and kinetics of photoresponses vary with intensity of excitation light and background light. Because of its pivotal position in the inactivation phase of the light response, modulation of RGS9-1 GAP activity through phosphorylation is a plausible mechanism for bringing about some of these changes. Even if GAP activity turns out not to be regulated by phosphorylation, RGS9-1 phosphorylation has revealed the presence of a membrane-associated protein kinase which appears to be distinct from those previously identified in ROS. This kinase is clearly

active in ROS *in vivo*, and its activity is regulated by light. Identification and characterization of this kinase at the molecular level should provide insight into its functional role, and will be facilitated by the procedures we describe here for extracting it in soluble form and for assaying it in the absence of ROS membranes.

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**FIGURE LEGENDS**

**Figure 1. Phosphorylation of RGS9-1 by endogenous kinase.** **A.** ROS membranes (60  $\mu$ M rhodopsin) were incubated with 5 mM [ $\gamma$ - $^{32}$ P]-ATP as described in the text for 15 min in the dark. After washing to remove ATP, membranes (*ROS*) were extracted by detergent as described in the text, yielding *Pellet* and supernatant (*Sup't*) fractions. RGS9-1 was immunoprecipitated, again yielding pellet (*IP*) and supernatant (*After IP*) fractions. Identical SDS-PAGE gels loaded with equal proportions of each fraction were visualized by dye staining (*Coomassie*), autoradiography (*Auto-Rad*) or immunoblotting with monoclonal RGS9-1 antibody D7 (*Western*). *Upper arrows*, RGS9-1; *lower arrows*, rhodopsin. **B.** ROS were incubated with [ $\gamma$ - $^{32}$ P]-ATP for the times indicated. The stoichiometry of RGS9-1 phosphorylation was determined as described in the text. The curve drawn is a non-linear least squares fit of the data to a single exponential function with a rate constant of 0.36 min $^{-1}$ .

**Figure 2. Isolation of tryptic phosphopeptide by reverse phase-HPLC and identification of the phosphorylation site by mass spectrometry.** **A.** HPLC elution profiles of the RGS9-1 phosphopeptide(s) in the presence of 0.1% trifluoroacetic acid or 0.2% HFBA. The proteolysis and preliminary separations of peptides were as described under Experimental Procedures. The RGS9-1 phosphopeptide was purified using a C18 HPLC column in the presence of 0.1% trifluoroacetic acid (*solid line with open circles*). The  $^{32}$ P- radioactivity-containing fractions were pooled, dried down, and further purified on the same column in the presence of 0.2% HFBA (*dashed line with solid circles*) as described in Experimental Procedures. **B.** Tandem MS/MS of the RGS9-1 phosphopeptide purified by HPLC in the presence of 0.2% HFBA (the major  $^{32}$ P-

radioactivity peak in Fig. 2A). MS/MS spectrum of  $MH_1^+$  precursor ion ( $m/z$  583.5) of the phosphorylated  $S^{475}QLR$  yielded ions of y1-y4 (all dephosphorylated) and b1-b4 (all dephosphorylated and dehydrated). The same site was identified from two independent phosphorylation experiments.

**Figure 3. Phosphorylation of recombinant RGS9-1.** ROS membranes were incubated with purified His-tagged RGS9-1/ $G_{\beta 5s}$  complex (*rRGS9*) and  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ . Phosphorylation of His-RGS9-1 or His-RGS9-1-Ser<sup>475</sup>Ala was detected by autoradiography following SDS-PAGE (<sup>32</sup>P). The amount of RGS9-1 in each sample was verified by immunoblot using a polyclonal anti-RGS9-1c antibody (*RGS9 antibody*). Upper bands are recombinant proteins (*rRGS9*) and lower bands are endogenous RGS9-1 (*RGS9*).

**Figure 4. Cation requirements for RGS9-1 phosphorylation.** **A.** Purified bovine ROS membranes were homogenized at 15  $\mu\text{M}$  rhodopsin in buffer G plus 1 mM EDTA and centrifuged twice to remove contaminating metal ions. ROS were then homogenized in the following buffers once and resuspended in corresponding buffers to a final concentration of 60  $\mu\text{M}$  rhodopsin: *EDTA*: buffer G with 1 mM EDTA; *Mg<sup>2+</sup>*: buffer G with 2 mM  $\text{MgCl}_2$  and 0.1 mM EDTA; *Mn<sup>2+</sup>*: buffer G with 2 mM  $\text{MnCl}_2$  and 0.1 mM EDTA; *Ca<sup>2+</sup>*: buffer G with 2 mM  $\text{CaCl}_2$  and 0.1 mM EDTA. RGS9-1 was phosphorylated in these buffers as described for those in buffer C, and phosphate incorporation was detected by autoradiography in immunoprecipitated RGS9-1 after SDS-PAGE (<sup>32</sup>P). Equivalent loading of immunoprecipitated RGS9-1 was verified by immunoblot using monoclonal antibody D7 (*RGS9 antibody*). *Control*: Phosphorylation of RGS9-1 in ROS membranes without any washes. **B.** Specificity of

monoclonal antibody A4. Proteins in ROS membranes were analyzed by SDS-PAGE and immunoblotting with mAb A4 after incubation with (+ *ATP*) or without (- *ATP*) ATP. Similar results were obtained with recombinant RGS9-1 isolated from insect cells (data not shown). **C.** Inhibition by  $\text{Ca}^{2+}$  chelation. *ROS*, ROS without ATP incubation; *ROS+ATP*, ROS + 5 mM ATP + 500 nM  $\text{CaCl}_2$ ,  $[\text{Ca}^{2+}] \geq 500$  nM; *EGTA*, ROS + 5mM ATP + 4.0 mM EGTA,  $[\text{Ca}^{2+}] \leq 1$  nM; *Ca (150 nM)*, ROS + 5mM ATP, 4.0 mM EGTA, 2.9 mM  $\text{CaCl}_2$ ,  $[\text{Ca}^{2+}] = 142$  nM; *Ca (300 nM)*, ROS + 5mM ATP, 4.0 mM EGTA, 3.3 mM  $\text{CaCl}_2$ ,  $[\text{Ca}^{2+}] = 285$  nM.  $\text{Mg}^{2+}$  was present at 6 mM total concentration in all samples. The program WinMAXC written by Chris Patton at Stanford University was used to calculate free  $\text{Ca}^{2+}$  concentrations.

**Figure 5. Effects of kinase activators and inhibitors on RGS9-1 phosphorylation.** **A.** ROS membranes were incubated with 5 mM  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$  (40 Ci/mol) at 60  $\mu\text{M}$  rhodopsin in buffer C in the presence of 8-Br-cAMP (*cA*) or 8-Br-cGMP (*cG*). The reactions were allowed to proceed at 30 °C for 15 min and then quenched by SDS-PAGE sample buffer. RGS9-1 phosphorylation was detected by autoradiography following SDS-PAGE. The positions of RGS9-1 in this and the next panel are indicated by arrows. **B.** Phosphorylation of RGS9-1 in the presence of phorbol 12-myristate 13-acetate (*PMA*: 0, 1, 5, 10  $\mu\text{M}$ ) and  $\text{CaCl}_2$  (0 or 2 mM, upper panel, 0 or 1 mM, lower panel) was performed as in A, with detection by  $^{32}\text{P}$  in the upper panel, and immunoblotting using Ser<sup>475</sup>-phosphate specific antibody A4 (*S475-P antibody*) in the lower panel. **C.** ROS were pre-incubated at room temperature for 15 min in buffer C with one of the following kinase inhibitors at the concentrations listed in *Experimental Procedures*: CK II, A3-HCl; *PKC*, Bisindolylmaleimide I-HCl; CaMK II, CaM-binding domain; *RK*, Sangivamycin; *CDK5*, Roscovitine; *PKA/PKG*: H8 dihydrochloride. ATP was then added to a final

concentration of 0.2 mM, and the reactions were allowed to proceed for 15 min. before adding SDS-PAGE sample buffer to quench. RGS9-1 phosphorylation was determined as in *C. D.* RGS9-1 was incubated with 5 mM ATP in the presence of the peptide inhibitor: KLD RRAQLKKELPPK. Increasing concentrations of the peptide (*peptide*) were used: 0, 0.5 mM, 2.0 mM and 8.0 mM. RGS9-1 phosphorylation was determined as in *C. E.* ROS were incubated with rhodopsin kinase inhibitor (*San*, 2 $\mu$ M) or PKC inhibitor (*Bis*, 100nM) and 5 mM [ $\gamma$ -<sup>32</sup>P]-ATP at 60  $\mu$ M rhodopsin in buffer C for 15 min. Rhodopsin phosphorylation was monitored by autoradiograms (<sup>32</sup>P) and coomassie (*Coom.*) staining following SDS-PAGE. *F.* Recombinant PDE- $\gamma$  (400 nM) was phosphorylated with or without CDK5/p35 inhibitor (*Rosc.*, 20  $\mu$ M) in the presence of ROS (*ROS*, 60  $\mu$ M rhodopsin) and 5 mM [ $\gamma$ -<sup>32</sup>P]-ATP in buffer C. PDE- $\gamma$  (*P- $\gamma$* ) phosphorylation was measured by autoradiograms (<sup>32</sup>P) following SDS-PAGE. *G.*, (Val<sup>6</sup>, Ala<sup>7</sup>)-kemptide (*Kemp.*, 100  $\mu$ M) was phosphorylated by ROS kinases (*ROS*, 60  $\mu$ M rhodopsin) in the presence of 0.2 mM [ $\gamma$ -<sup>32</sup>P]-ATP, 8-Br-cAMP (*camp*, 50  $\mu$ M), or 8-Br-cGMP (*cGMP*, 50  $\mu$ M), with or without PKA/PKG inhibitor (*H8*, 60  $\mu$ M). Kemptide phosphorylation was measured by phosphocellulose binding and scintillation counting. *H.* ROS (*ROS*, 60  $\mu$ M rhodopsin) were incubated with 0.2 mM [ $\gamma$ -<sup>32</sup>P]-ATP in the presence of CK II peptide substrate (*Sub.*, 100  $\mu$ M), CK II inhibitor (*A3*, 250  $\mu$ M), or recombinant CK II (5 U/ $\mu$ l) Phosphorylation of CK II substrate was determined as in *G.*

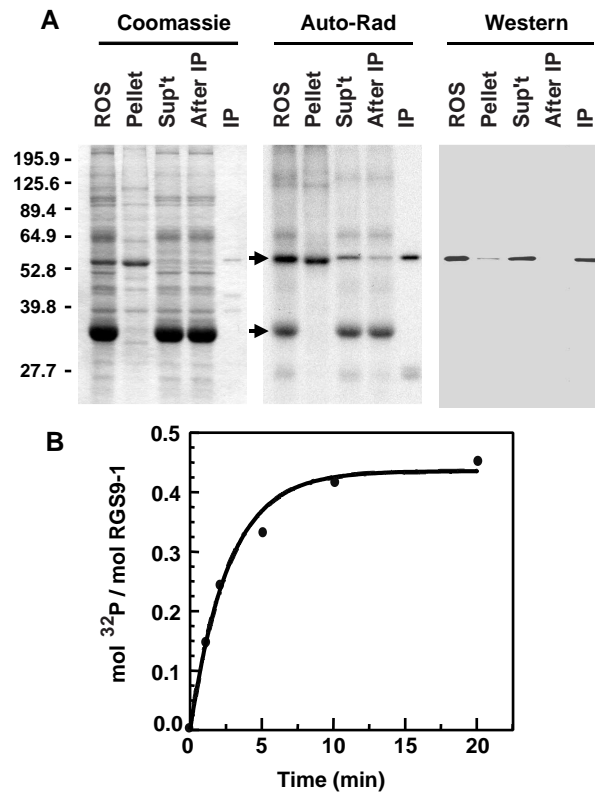
**Figure 6. Co-purification of RGS9-1 kinase activity with rhodopsin and RGS9-1 in fractionated retinal membranes, and tight membrane-binding of RGS9-1 kinase. A.** ROS were purified by a standard discontinuous sucrose density gradient technique (43). The sucrose gradient after ultra-centrifugation was fractionated into 1ml fractions. Rhodopsin concentrations

in each of the fractions were determined by absorbance at 500 nm, and were normalized to the highest concentration value. RGS9-1 kinase activity in each of the fractions was detected using His-tagged RGS9-1 as substrate, as described in *Experimental Procedures*. The boxed area shows an enlarged view of the fractions corresponding to the rhodopsin peak. *ROS*: fraction containing peak of ROS; *Broken ROS*: fraction containing peak of broken ROS membranes; *Pellet*: fraction containing unshered retinal membranes; *RGS9 antibody*: immunoblot using anti-RGS9-1c antibody; *S475-P antibody*: immunoblot using monoclonal anti-Ser<sup>475</sup>-phosphate antibody A4. **B.** Purified ROS membranes were homogenized by repeated passage through a 23 gauge needle at 15  $\mu$ M rhodopsin in one of the following buffers: Isotonic buffer (*Iso.*): 100 mM NaCl, 5 mM Tris, pH 7.4, 2 mM MgCl<sub>2</sub>, 1 mM DTT; Hypertonic buffer (*Hyper.*): 1 M NH<sub>4</sub>Cl, 5 mM Tris, pH 7.4, 1 mM DTT; Hypotonic buffer (*Hypo.*): 5 mM Tris, pH 7.4, 0.5 mM MgCl<sub>2</sub>, 1 mM DTT. Membranes were then collected by centrifugation at 84,000g for 20 min. The washing step was repeated three times for each sample, and the washed ROS were finally resuspended in buffer C to a rhodopsin concentration of 60  $\mu$ M for ATP incubations. Endogenous RGS9-1 phosphorylation was detected by autoradiography (<sup>32</sup>P) following immunoprecipitation from detergent extracts and SDS-PAGE, and the amount of immunoprecipitated RGS9-1 was verified by immunoblot using the monoclonal antibody D7 (*RGS9 antibody*). *ROS*: RGS9-1 phosphorylation on unwashed membranes.

**Figure 7. RGS9-1 phosphorylation in mouse retina.** **A.** RGS9-1 was immunoprecipitated from dark adapted (*Dark*) or light adapted (*light*) mouse retinas and immunoblotted with Ser<sup>475</sup>-phosphate specific antibody (*S475-P antibody*). The amount of RGS9-1 in the samples was measured on the same blot using anti-RGC9-1c polyclonal antibody (*RGS9 antibody*). **B.** RGS9-

1 phosphorylation was compared between dark and light adapted animals by densitometry of films. Three data points from immunoblots similar to A were averaged, and RGS9-1 phosphorylation = (average densities of RGS9-1 bands in Ser<sup>475</sup>-phosphate specific antibody western blots) / (average densities of RGS9-1 bands in anti-RGC9-1c polyclonal antibody western blots).

**Fig. 1**



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Figure 2

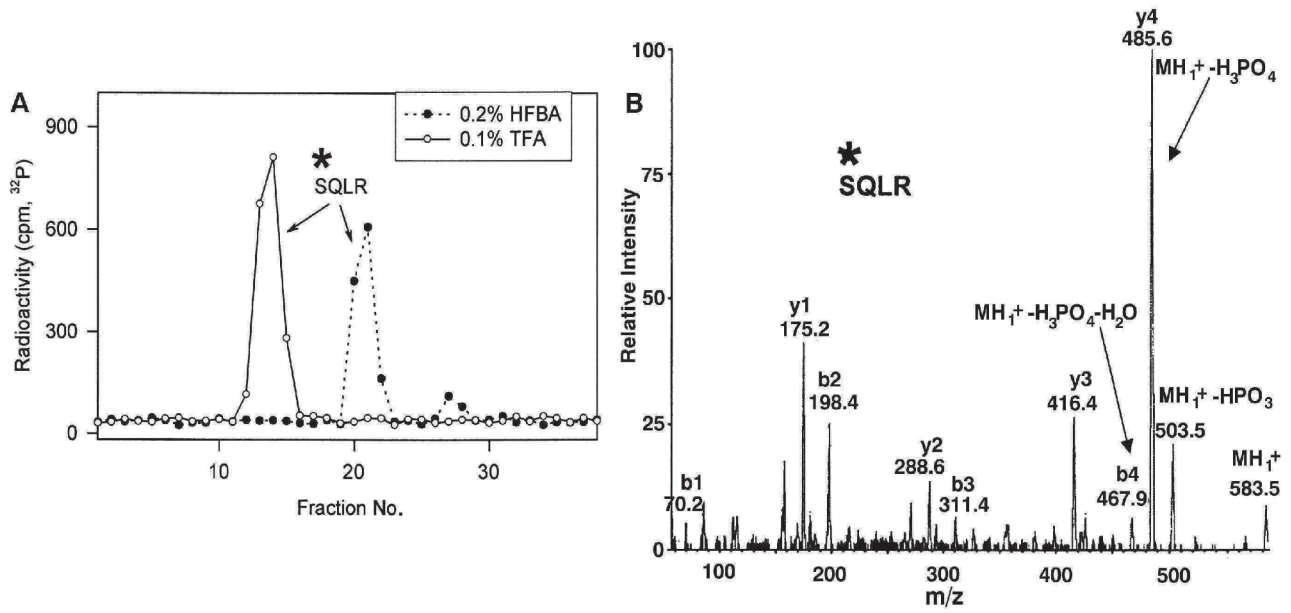
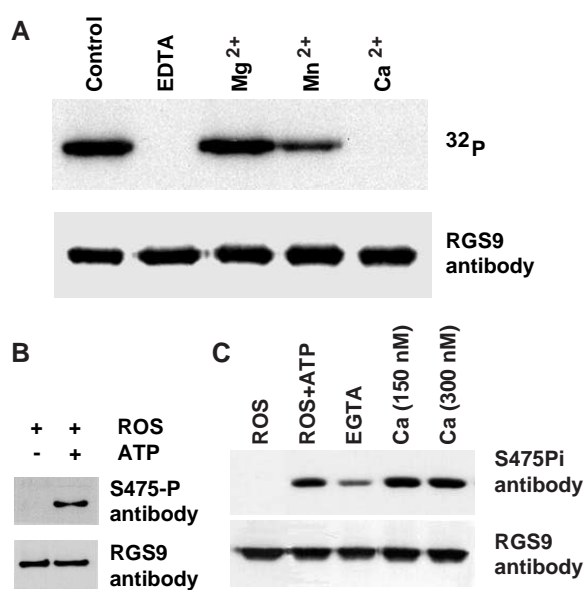




Fig. 4



**Fig. 5**

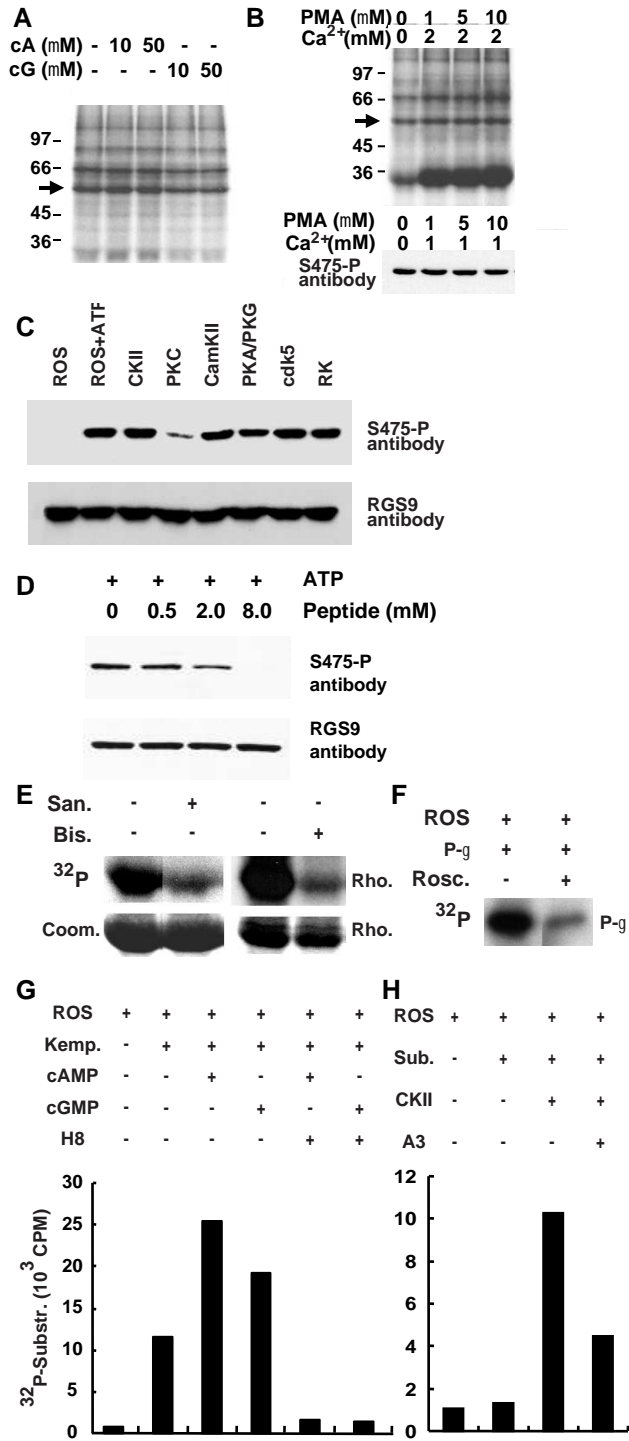
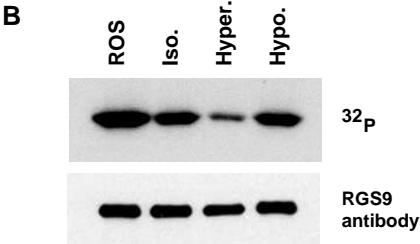
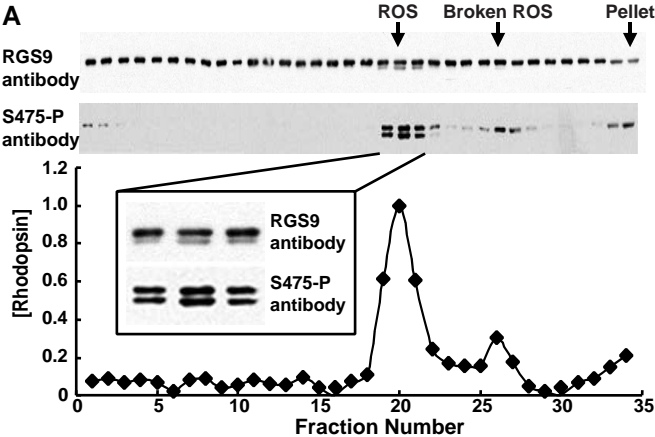


Fig. 6



**Fig. 7**

