Inactivation of the visual G protein transducin, during recovery from photoexcitation, is regulated by RGS9-1, a GTPase-accelerating protein of the ubiquitous RGS protein family. Incubation of dark-adapted bovine rod outer segments with [$\gamma$-32P]ATP led to RGS9-1 phosphorylation by an endogenous kinase in rod outer segment membranes, with an average stoichiometry of 0.2–0.45 mol of phosphates/mol of RGS9-1. Mass spectrometry revealed a single major site of phosphorylation, Ser$^{475}$. The kinase responsible catalyzed robust phosphorylation of recombinant RGS9-1 and not of an S475A mutant. A synthetic peptide corresponding to the region surrounding Ser$^{475}$ was also phosphorylated, and a similar peptide with the S475A substitution inhibited phosphorylation by an endogenous kinase in rod outer segments. 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, protein kinase G, rhodopsin kinase, CaM kinase II, casein kinase II, or cyclin-dependent kinase 5, in contrast to other protein kinases such as CaM-dependent kinase II, protein kinase C, protein kinase A, and protein kinase G. RGS9-1 phosphorylation by an endogenous kinase in rod outer segments is stimulated by light and by [Ca$^{2+}$]$_{i}$ to nanomolar levels with EGTA; however, it was not stimulated by the addition of phorbol esters, under conditions that significantly enhanced rhodopsin phosphorylation. A monoclonal antibody specific for the Ser$^{475}$-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 in immunoblots of dark-adapted mouse retina.

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$_{t}$)α-subunit. The activated G$_{t}$α, 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$_{t}$α 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$_{t}$α-GTP and those that dampen or terminate it. Therefore, rhodopsin deactivation by phosphorylation and GTPase acceleration on G$_{t}$α are two major mechanisms for regulation in the recovery stage of normal vision.

RGS9-1, the GTPase-accelerating protein (GAP) for G$_{t}$α, is an important regulator of phototransduction and a key mediator of the recovery to a dark state (4–6). It belongs to the ubiquitous RGS (regulators of G protein signaling) family of GAPs (for reviews, see Refs. 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 γ-like domain, which tethers RGS9-1 to its partner subunit, G$_{o}$βγ (15, 16), a dephosphorylated EGL-10/plekstrin 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, sub-
cellular 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-tyroine kinases such as Sre (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 Gbg-binding protein phoshducin (32), Gbg, and the inhibitor γ subunit of cGMP phosphodiesterase (26, 33), 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 Srt, RGS2, RGS3, RGS4, RGS7, and GAI, 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 show 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 [Val6,Ala7]Leu-Arg-6.5, containing 4% SDS (w/v), 20% glycerol (v/v), 2.5 mM reducing agent; buffer C (100 mM NaCl, 5 mM Tris-HCl, 100 mM NaCl, 2 mM MgCl2, 1 mM DTT, –20 mg/liter phenylmethylsulfonyl fluoride); buffer D (10 mM MOPS, 30 mM NaCl, 60 mM KCl, 2 mM MgCl2, 1 mM DTT, –20 mg/liter phenylmethylsulfonyl fluoride); buffer E (5 mM Tris-HCl, 2 mM EDTA, 0.2 mM NaVO4, 15 μM fenvalerate, 100 mM aspartic acid, 1 mM DTT); buffer F (50 mM sodium phosphate, 50 mM NaCl, 10 mM KF, 2 mM MgCl2); buffer G (100 mM NaCl, 5 mM Tris-HCl). For all these buffers, the pH was adjusted to 7.4–7.5. Other buffer standards and conditions were varied as indicated throughout.

**Expression and Purification of Recombinant Proteins—** His-tagged RGS9-1 full-length protein in complex with Gbg was expressed in Sf9 cells using a baculovirus vector and affinity-purified as described (14). Polymerase chain reaction mutagenesis was used to generate a construct that was identical to the His-tagged RGS9-1 except for mutation of Ser475 to Ala (S475A). 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 Gbg, because the protein is not expressed in a stable form in the absence of Gbg or the natural partner subunit (16) of RGS9-1, Gbg(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 24,000 g, and then resuspended in buffer C to a rhodopsin concentration of 100–150 μM 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 NH4OH (to minimize photoisomerized rhodopsin phosphorylation), unless otherwise stated. ATP was added to final concentrations of 2–5 mM, with [γ-32P]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 immunofluorescence isolation of RGS9-1 as described below. The amount of RGS9-1 in the ROS membranes was determined by solubilization of excised 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 Escherichia coli in guanidine HCl by affinity chromatography. The extinction coefficient for RGS9-1 of 259,000 cm−1·M−1 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, KDRRS/QPLKKLPPK, 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 (KLDKRS/QPKLKKLPKK), was used to assure reactivity with the phosphoprotein in mice. However, the bovine phosphoprotein, bound to the phosphorylation domain, 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 of IgG to 1 ml of beads. For immunoprecipitation, ROS membranes were washed after ATP incubation by repeated centrifugation at 54,000 g 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 P-40 detergent at 60 μM rhodopsin. The insoluble material was removed by centrifugation for 20 min at 84,000 g. Typically, 300 μl of solubilized ROS were incubated with 40 μl of IgG-coupled beads for 2.5 h 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 redissolved by boiling in the SDS-PAGE sample buffer. Efficiency of immunoprecipitation was measured by autoradiography following SDS-PAGE and immunoblot using monoclonal antibody D7.

**Analytical Procedures—** Mice were maintained in a dark room (dark-adapted) or in full room light (light-adapted) for a period of 16 h 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 P-40 detergent, plus 0.2 mM NaVO4, 15 μM fenvalerate, 100 mM aspartic 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—** Mice were maintained in a dark room (dark-adapted) or in full room light (light-adapted) for a period of 16 h 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 P-40 detergent, plus 0.2 mM NaVO4, 15 μM fenvalerate, 100 mM aspartic 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.
gels. The gels were stained with Coomassie Blue R-250 and destained, and the RGS9-1 band was 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 polyvulized, and the total protein content was determined by overnight extraction by 8M Tris-HCl, pH 7.8, containing 1% β-mercaptoethanol (v/v), 0.2% SDS (w/v) for 5 h. 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 (v/v) to a final concentration of 10% trichloroacetic acid to precoat the glass filter. 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 μg) in 400 μl of 12.5 mM 1,3-bis(trihydroxymethyl)-methylamino]propane, a pH buffer, pH 7.9, containing 2% DMSO without radiolabel, followed by immunoprecipitation. Immunoblots were fitted with a nebulization-assisted electrospray ionization source (PE/Sciex, Thornhill, Ontario). For tandem MS/MS, precursor ions were collected, vacuum-dried, and used for the next step. Reverse-phase HPLC was carried out; one was identical to the second gradient described above, and the other was performed using a C18 HPLC column (Vydac 201HS52; 2.1 mm × 250 mm) with binary solvent systems (solvent A: H2O/0.1% trifluoroacetic acid; solvent B: CH3CN/0.1% trifluoroacetic acid; solvent C: H2O/0.2% HFBA; solvent D: CH3CN/2.0% HFBA) and linear gradients. The first gradient was 100% A/50% B to 10% A/90% B in 30 min at 0.3 ml/min, and the second was from 100% A/50% B to 75% A/25% B in 50 min at 0.2 ml/min. The peptide was further purified by Gn+-immobilized metal affinity chromatography (47) using 0.2 ml of Chelex-Sepharose (Amer- sham Pharmacia Biotech). Finally, two additional rounds of HPLC were carried out; one was identical to the second gradient described above, and the last 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 mass spectrometry and tandem mass spectrometry (MS/MS) using a Sciex API III triple quadruple 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 in an extraction buffer C as described under “Phosphorylation of RGS9-1 in ROS”, using 5 mM ATP without radiolabel, followed by immunoprecipitation. Immunoprecipitated RGS9-1 was separated by SDS-PAGE and 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 (MALDI-TOF), before and after in-gel trypsin digestion, 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 Gαs subunits was mixed with ROS and [γ32P]ATP at 30°C for the time indicated. Final concentrations of proteins and reagents were 60 μM rhodopsin, 0.2 μM His-RGS9-1 or 0.2 μM His-RGS9-1-S475A, and 2.5 mM [γ32P]ATP (100 Ci/mmol). Reactions were then quenched by the 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-RGS9-1 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-cGMP; 50 μM), PKG (8-Br-cGMP; 50 μM), or PKC (PMA; 1, 5, or 10 μM plus 1 or 2 mM CaCl2, referred to subsequently as PMA/Ca) were added under our standard assay conditions. The ability of the added cyclic nucleotide analogues or endogenous cyclic nucleotide-dependent protein kinase activity was verified by testing their ability to stimulate phosphorylation of RGS homogenates of [Val50,Ala19]Kemptide using [γ32P]ATP, with detection by binding to phosphocellulose paper, followed by scintillation counting (51). The concentrations used are 100 times the reported K value for PKA activation (52) and 100 times the reported K value for PKG activation (53). Efficacy of PMA/Ca for PKC stimulation under our conditions was verified by the previously charac- terized phosphorylation of rhodopsin (54, 55), using [γ32P]ATP and autoradiography. 2) Known substrates for candidate kinases were added and tested for phosphorylation using [γ32P]ATP and either autoradiography of SDS-PAGE gels or phosphocellulose paper binding followed by sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE). PKA (PKA/PKG and CDK5 substrates) were then used to test the 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 veri- fication of the ability of added kinase to phosphorylate the substrate. The substrate used for PKA and PKG was [Val50,Ala19]Kemptide (Sigma) at 15 μM. A530 was a substrate peptide (Calbiochem) at 100 μM, and the substrate used for CDK5 was the γ subunit of ROS cGMP phosphodiesterase (PDE6), PDEγ (26, 33) at 400 nM. Peptide phosphorylation was assayed using [γ32P]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 [γ32P]ATP and autoradiography following SDS-PAGE. 3) Known inhibitors were added, and their effects on RGS9-1 phosphorylation were determined by measuring RGS9-1 phosphorylation in immunoblotting using Ser475-phosphate-specific monoclonal antibody A4. Inhibitors used were as follows: for PKA and PKG, H8-diisohydrochloride (60 μM); for PKC, PMA was used (1, 5, 10 μM); for PKG, G8 was used (56 μM); for rhodopsin kinase, sartiamycin (10 μM) (Ki = 9 nM); for PKC, bisindolylmaleimide I (GF 109203X, 3-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)-maleimide, HCl) (1 μM) (Ki = 0.65 μM); for PKC, bisindolylmaleimide I (GF 109203X, 3-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)-maleimide, HCl); for PKC, bisindolylmaleimide I (GF 109203X, 3-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)-maleimide, HCl) (1 μM) (Ki = 0.65 μM). Inhibitors were preincubated with ROS at room temperature for 15 min before the addition of [γ32P]ATP to start phosphorylation. The 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, CKD5) or added (PKA/PKG, CK II) enzyme. Inhibition by RGS9-1-derived Peptide—For the peptide inhibitor, ROS were mixed with ATP in the presence of increasing concentrations (200 nM–20 μM) of the peptide derived from the mouse ROS. 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 Ser475-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 tech- nique (43). Samples in the sucrose gradient were then fractionated into 1-ml aliquots using a gradient puller (Auto-Densi-Flow from Labconco) and stored at −80°C. To check the kinase activity in these fractions, 20 μl of each fraction was mixed with purified His-RGS9-1 (in complex with Gαs subunits) and ATP at 25°C for 10 min. Final concentrations of proteins and reagents were as follows: 60 μM rhodopsin (in the peak fraction), 80 μg His-RGS9-1, 5 mM ATP, and 80 μM Ca2+. Reactions were quenched by adding equal volumes of standard SDS-PAGE sample buffer. Phosphorylation of His-RGS9-1 was detected by immunoblotting using Ser475-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 [γ32P]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 [γ32P]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 recombinant RGS9-1 (Fig. 1A). Phosphopeptides. 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, identifi- ing 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 \([\gamma-32P]ATP\) labeling clearly reveals RGS9-1 as one of the major phosphorylated proteins in ROS after ATP incubation (Fig. 1A). The stoichiometry of RGS9-1 phosphorylation was determined as described under “Experimental Procedures” for 15 min in the dark. After washing to remove ATP, membranes (ROS) were extracted by detergent as described, yielding pellet and supernatant (Sup’r) fractions. ROS-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, ROS-1; lower arrows, rhodopsin. 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, ROS-1; lower arrows, rhodopsin. B, ROS were incubated with \([\gamma-32P]ATP\) for the times indicated. The stoichiometry of RGS9-1 phosphorylation was determined as described under “Experimental Procedures.” The curve drawn is a nonlinear least squares fit of the data to a single exponential function with a rate constant of 0.36 min\(^{-1}\).

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\(^{475}\) 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\(^{475}\)—To test further the requirement of Ser\(^{475}\) for RGS9-1 phosphorylation, phosphorylation of both His-tagged RGS9-1 full-length protein and His-tagged RGS9-1 mutant with Ser\(^{475}\) 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 that the Ser\(^{475}\) residue is required for phosphorylation. Only very weak signals could be detected in the mutant RGS9-1 protein, probably due to phosphorylation at one or more minor sites. Immunoblotting with the monoclonal Ser\(^{475}\)-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\(^{2+}\) was required for RGS9-1 phosphorylation, because RGS9-1 phosphorylation was completely abolished in the presence of EDTA or Ca\(^{2+}\) alone and could only be partially restored by Mn\(^{2+}\) (Fig. 4A). Chelation of Ca\(^{2+}\) by EGTA in the presence of excess Mg\(^{2+}\) reduced phosphorylation (Fig. 4C). Free [Ca\(^{2+}\)] calculated to be on the order of 10\(^{-7}\) M was sufficient to restore full activity (Fig. 4C). For these immunoblots, we used monoclonal antibody A4 that specifically recognizes Ser\(^{475}\)-phosphate. It was raised against a phosphopeptide derived from murine RGS9-1 (KLDRR85/PQLKKE) 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\(^{475}\) is located in a se-
using a C18 HPLC column in the presence of 0.1% trifluoroacetic acid (solid line with open circles). The 32P 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 under “Experimental Procedures.” B, tandem MS/MS of the RGS9-1 phosphopeptide purified by HPLC in the presence of 0.2% HFBA (the major 32P-radioactivity peak in Fig. 2A). MS/MS spectrum of MH+ precursor ion (m/z 583.5) of the phosphorylated S475QLR yielded ions of y1-y4 (all dephosphorylated) and b1-b4 (all dephosphorylated and dehydrated). The same site was identified from two independent phosphorylation experiments.

Fig. 3. Phosphorylation of recombinant RGS9-1. ROS membranes were incubated with purified His-tagged RGS9-1-G8 complex (rRGS9) and [γ-32P]ATP. Phosphorylation of His-RGS9-1 or His-RGS9-1-S475A was detected by autoradiography following SDS-PAGE (32P). The amount of RGS9-1 in each sample was verified by immunoblot using a polyclonal anti-RGS9-1 antibody (RGS9 antibody). Upper bands are recombinant proteins (rRGS9), and lower bands are endogenous RGS9-1 (RGS9).

Fig. 4. Cation requirements for RGS9-1 phosphorylation. A, purified bovine ROS membranes were homogenized at 15 μM 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 μM rhodopsin: EDTA, buffer G with 1 mM EDTA; Mg2+, buffer G with 2 mM MgCl2 and 0.1 mM EDTA; Mn2+, buffer G with 2 mM MnCl2 and 0.1 mM EDTA; Ca2+, buffer G with 2 mM CaCl2 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 (32P). 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 Ca2+ chelation. ROS, ROS without ATP incubation; ROS + ATP, ROS plus 5 mM ATP; ROS + Ca2+, ROS plus 5 mM ATP plus 500 nM CaCl2; ROS + 5 mM ATP + 500 nM CaCl2, Ca2+ ≤ 1 nM; ROS + 5 mM ATP + 4.0 mM EGTA, [Ca2+] = 150 nM; ROS + 5 mM ATP + 4.0 mM EGTA + 500 nM CaCl2, [Ca2+] = 142 nM; Ca (300 nM), ROS plus 5 mM ATP, 4.0 mM EGTA, 3.3 mM CaCl2, [Ca2+] = 285 nM. Mg2+ was present at 6 mM total concentration in all samples. The program WinMAXC written by Chris Patton (Stanford University) was used to calculate free Ca2+ concentrations.
Effects on phosphorylation of any protein detectable upon the 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. Since 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 responsible for RGS9-1 phosphorylation and are also 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 bisindolylmaleimide I raises the possibility of a previously uncharacterized PKC isoforme or PKC-like enzyme in ROS, the inhibitor we used can also inhibit kinases other than PKC, such as phosphorylase kinase, (IC\textsubscript{50} = 0.7 \mu M at 250 \mu M ATP (60)). The kinase responsible for RGS9-1 phosphorylation is clearly distinct from the PKC isoforme 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\textsuperscript{475} 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\textsuperscript{475}-containing peptide was found to be phosphorylated by ROS membranes (data not shown). Therefore, 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\textsuperscript{475}-phosphate-specific antibody A4 to detect the phosphorylation. There was no detectable phosphorylation of this recombinant protein on Ser\textsuperscript{475} prior to incubation with preparations containing the RGS9-1 kinase, as demonstrated by its lack of activity 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 probably plays a role in regulation of phototransduc-
ROS proteins under a range of different conditions. The significance of 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 of RGS-1/1600 mol of rhodopsin), it incorporates more phosphate than all but a few other ROS proteins under a range of different conditions. The 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.

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).

Fig. 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 ultracentrifugation was fractionated into 1-ml 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 under “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 unsealed retinal membranes; RGS9 antibody, immunoblot using anti-RGS9-1c antibody; S475-P antibody, immunoblot using monoclonal anti-Ser475-phosphate antibody A4. B, purified ROS membranes were homogenized by repeated passage through a 23-gauge needle at 15 μm rhodopsin in one of the following buffers: isotonic buffer (Iso.) (100 mM NaCl, 5 mM Tris, pH 7.4, 2 mM MgCl₂, 1 mM DTT); hypertonic buffer (Hyper.) (1 mM NH₄Cl, 5 mM Tris, pH 7.4, 1 mM DTT); hypertonic buffer (Hypero.) (5 mM Tris, pH 7.4, 0.5 mM MgCl₂, 1 mM DTT). Membranes were then collected by centrifugation at 84,000 × g 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 μM for ATP incubations. Endogenous RGS9-1 phosphorylation was detected by autoradiography (32P) 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 unsealed membranes.

Fig. 7. RGS9-1 phosphorylation in mouse retina. A, RGS9-1 was immunoprecipitated from dark-adapted (Dark) or light-adapted (light) mouse retina and immunoblotted with Ser475-phosphate-specific antibody (S475-P antibody). The amount of RGS9-1 in the samples was measured on the same blot using monoclonal anti-RGS9-1 antibody D7 (RGS9 antibody). B, RGS9-1 phosphorylation was compared between dark- and light-adapted animals by densitometry of films. The data points from immunoblots similar to A were averaged, and RGS9-1 phosphorylation = (average densities of RGS9-1 bands in Ser475-phosphate-specific antibody Western blots)/ (average densities of RGS9-1 bands in monoclonal anti-RGS9-1 antibody D7 Western blots).
able 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, immunoblotting of retina from dark-adapted animals reveals Ser<sup>477</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, although not fully established, the function of its unique C-terminal domain is beginning to emerge. Recent work (14) suggests that 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 disc membranes (65). 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 a molecular level, or how its phosphorylation affects its function. Assays of GAP activity before and after phosphorylation have been difficult to interpret, because A TP 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>477</sup> phosphorylation. 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 Fig. 7, the endogenous membrane-associated protein is a much better substrate for the membrane-associated kinase than is the soluble RGS9-1-G<sub>p<sub>11</sub></sub> 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<sub>βγ</sub>, the PDE inhibitory subunit of cGMP phosphodiesterase, and G<sub>α</sub> in addition to its interactions with the kinase and with ROS membranes. Moreover, sensitivity and kinetics of photoresponse vary with the 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 that 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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