Phosphorylation of RGS9-1 by an Endogenous Protein Kinase in Rod Outer Segments*

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Received for publication, December 21, 2000, and in revised form, April 2, 2001
Published, JBC Papers in Press, April 5, 2001, DOI 10.1074/jbc.M011539200

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 [γ-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, Ser475. The kinase responsible catalyzed robust phosphorylation of recombinant RGS9-1 and not of an 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, protein kinase C, PKG, cGMP-dependent protein kinase; PKC, protein kinase C; PFK, cGMP-dependent protein kinase; PMA, phorbol esterase; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; 8-Br-cAMP, 8-bromo-cyclic AMP; 8-Br-cGMP, 8-bromo-cyclic GMP.

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

RGS9-1, the GTPase-accelerating protein (GAP) for Gtα, 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 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); PDE, phosphodiesterase; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; 8-Br-cAMP, 8-bromo-cyclic AMP; 8-Br-cGMP, 8-bromo-cyclic GMP.
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-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 Gβγ-binding protein phosducin (32), Gγ (30), and the inhibitory β subunit of GMP 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 Set2, RGS2, RGS3, RGS4, RGS7, and GAI P, 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 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

We describe here experiments suggesting that one of these may be phosphorylated by other protein kinases, play an important role as well.

39). Therefore, it is likely that the function of RGS9-1 may also be

Antibodies and Immunoprecipitation—Rabbit anti-RGS9-1 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/QQLKELLPPK, 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 (KLDRLSQLKEEPFPK), was used to assay reactivity with the phosphoprotein in mice. However, the bovine phosphoprotein, both nonphosphorylated and phosphorylated, was 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 5,000 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 resolved by boiling in the SDS-PAGE sample buffer. Efficiency of immunoprecipitation was measured by autoradiography following SDS-PAGE and immunoblot using monoclonal antibody D7.
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 pulverized, and the resulting supernatant was collected by shaking Triton X-100, 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 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 μg) in 400 μl of 12.5 μl 1.3-his(tris(hydroxymethyl)-methylamino)propane, a pH buffer, pH 7.9, containing 2 μl of 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 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 μg) in 400 μl of 12.5 μl 1.3-his(tris(hydroxymethyl)-methylamino)propane, a pH buffer, pH 7.9, containing 2 mM urea, 0.125% mercaptoethanol (v/v), 1 mM CaCl₂. The suspension was incubated at room temperature for 7 h with occasional vortexing. Insoluble material was separated by centrifugation and treated again with trypsin until 1234 was undetectable in the pellet. Phosphopeptides were isolated by chromatography with detection by scintillation counting. After each elution, a single major 1234-containing peak was collected, vacuum-dried, and used for the next step. Reverse-phase HPLC was carried out on a C18 HPLC cartridge (20 μm; 0.2 ml/ min) with binary solvent systems (solvent A: H₂O/0.1% trifluoroacetic acid; solvent B: CH₃CN/0.1% trifluoroacetic acid; solvent C: H₂O/0.2% HFBFA; solvent D: CH₃CN/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 was from 100% A/0% B to 75% A/25% B in 50 min at 0.2 ml/min. The peptide was further purified by a 3 times the reported Kᵣ value for PKA activation (52) and 100 times the reported Kᵣ value for PKG activation (53). Efficacy of PKA/Ca for PKC stimulation under our conditions was verified by the previously characterized phosphorylation of rhodopsin (54, 55), using [γ-32P]ATP and autoradiography. 2) Known substrates for candidate kinases were added and tested for phosphorylation using [γ-32P]ATP and either autoradiography of SDS-PAGE gels or phosphocellulose paper binding assay. Known substrates for 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 verification of the ability of added kinase to phosphorylate the substrate. The substrate used for PKA and PKG was [Valᵦ,Alaᵦ]Kemptide (Sigma) at 5 μM, rhodopsin kinase (Calbiochem) at 100 μM, and the substrate used for CKD5 was the γ subunit of ROS cGMP phosphodiesterase (PDE6), PDEγ (26, 33) at 400 nM. Peptide phosphorylation was assayed using [γ-32P]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 [γ-32P]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-dihydrochloride (60 μM) (Kᵣ for PKA = 1.2 μM, Kᵣ for PKG = 0.45 μM (56)); for rhodopsin kinase, sangivamycin (10 μM) (Kᵣ = 5 μM (57)); for CK II, A3-HCl (250 μM) (Kᵣ = 5.1 μM (59)); for PKC, bisindolylmaleimide I-HCl (GF 109203X, 3-(1-(3-dimethylamino)propyl)-indol-3-yl)-3-(indol-3-yl)-maleimide, HCl; Calbiochem) (500 nM) (Kᵣ = 10 μM (60, 61)); for CaM-dependent kinase II (CaMK II), CaM-binding domain (2.5 μM) (Kᵣ = 52 nm (62)). Inhibitors were preincubated with ROS at room temperature for 15 min before the addition of [γ-32P]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, CDK5) or added (CK II) enzyme. Inhibition by RGS9-1-derived Peptide—For the peptide inhibitor, ROS was preincubated with ATP in the presence of increasing concentrations of RGS9-1 from the mouse RGS9-1 with the phosphorylation site mutated to Ala. KLDQRQAKKELPPK (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 technique (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 Gₛ), and ATP at 25 °C for 10 min. Final concentrations of proteins and reagents were as follows: 60 μM rhodopsin (in the peak fraction), 5 μM His-RGS9-1, 5 mM MgCl₂, 10 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 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 [γ-32P]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 [γ-32P]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 endogenous RGS9-1 (Fig. 1A). The phosphoprotein (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...
A single exponential function with a rate constant of 0.36 min per unit of time. The curve drawn is a nonlinear least squares fit of the data to this function.

Phosphorylation was determined as described under “Experimental Procedures.”

When phosphorylated RGS9-1 was digested with trypsin and the phosphorylated peptide was then extensively purified by two rounds of reverse-phase HPLC, Ga<sup>3+</sup>-immobilized metal affinity chromatography, 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<sub>1</sub><sup>+</sup>), corresponding to a monophosphorylated peptide at Ser<sup>475</sup> with a sequence of S<sup>475</sup>QLR (calculated average mass for MH<sub>1</sub><sup>+</sup> = 583.56). The precursor ion with the loss of HPO<sub>4</sub><sup>−</sup> was also observed at m/z 503.5 (calculated mass 503.58). The precursor ion underwent elimination of phosphate (MH<sub>1</sub><sup>+</sup>−H<sub>3</sub>PO<sub>4</sub>, y4) and elimination of phosphate and water (MH<sub>1</sub><sup>+</sup>−H<sub>2</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 that the Ser<sup>475</sup> 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. Immunoblots 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 (KLDBRS5/PQLKKELPP) 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 se-
using a C18 HPLC column in the presence of 0.1% trifluoroacetic acid (solid line) 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 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 MH1+ 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. 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 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 MH1+ 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-GTP, complex (rRGS9) and 2-[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 50 μM ATP plus 500 μM CaCl2; [Ca2+] = 500 μM; EDTA, ROS plus 5 μM ATP plus 4.0 μM EDTA, [Ca2+] = 1 μM; Ca (150 nM), ROS plus 5 μM ATP, 4.0 μM EDTA, 2.9 mM CaCl2, [Ca2+] = 142 nM; Ca (300 nM), ROS plus 5 μM ATP, 4.0 μM EDTA, 3.3 mM CaCl2, [Ca2+] = 285 μM. 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 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 additional kinases whose presence is less certain. Although the inhibition by the PKC inhibitor bisindolylmaleimide 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, (IC50 0.7 μM at 250 μM 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 Ser475 mutated to Ala (KLDRAQLKKELPP) 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 Ser475-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 Ser475-phosphate-specific antibody A4 to detect the phosphorylation. There was no detectable phosphorylation of this recombinant protein on Ser475 prior to incubation with ROS membranes. To determine whether the RGS9-1 kinase is Ser475 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 unshredded 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-

**Fig. 5.** Effects of kinase activators and inhibitors on RGS9-1 phosphorylation. A, ROS membranes were incubated with 5 mM γ-32P]ATP (40 Ci/mol) at 60 μM 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 A and B are indicated by arrows. B, phosphorylation of RGS9-1 in the presence of PKA (5 units/μl), PKC (1 unit/μl), and CaMKII (2 units/μl) was performed as in A, with detection by γ-P in the upper panel and immunoblotting using Ser475-phosphate-specific antibody A4 (S475-P antibody) in the lower panel. C, ROS were preincubated at room temperature for 15 min in buffer C with one of the following kinase inhibitors at the concentrations listed under “Experimental Procedures”; CK II, A3-HCl, PKC, bisindolylmaleimide I-HCl, CaMK II, CaM-binding domain; RK, sangivamycin; CDK5, ros covitine, PKA/PKG, H8 dihydrochelroide. 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 in C. D, RGS9-1 was incubated with 5 mM ATP in the presence of the peptide inhibitor KLDRAQLKKELPPK. Increasing concentrations of the peptide (peptide) were used: 0, 0.5, 2.0, and 8.0 mM. RGS9-1 phosphorylation was determined as in C. E, ROS were incubated with ROS (60 μM rhodopsin) and 5 mM γ-32P]ATP in buffer C for 15 min. Rhodopsin phosphorylation was monitored by autoradiograms following SDS-PAGE. F, recombinant PDE-γ (400 mM) was phosphorylated with or without CDK5/p35 inhibitor (Rosec.; 20 μM) in the presence of ROS (60 μM rhodopsin) and 5 mM γ-32P]ATP in buffer C. PDE-γ (γ-P) phosphorylation was measured by autoradiograms following SDS-PAGE. G, [Val1,Ala2]{\textsuperscript{5}}Kemptide (Kemptide (Kemp; 100 μM) was phosphorylated by ROS kinases (ROS; 60 μM rhodopsin) in the presence of 0.2 mM γ-32P]ATP, 8-Br-cAMP (cAMP, 50 μM), or 8-Br-cGMP (cGMP, 50 μM), with or without PAK/PKG inhibitor (H8; 60 μM). Kemptide phosphorylation was measured by phosphocellulose binding and scintillation counting. H, ROS (60 μM rhodopsin) were incubated with 0.2 mM γ-32P]ATP in the presence of CK II peptide substrate (Sub.; 100 μM), CK II inhibitor (A3; 250 mM), or recombinant CK II (5 units/μl). Phosphorylation of CK II substrate was determined as in G.
The lack of detectable endogenous RGS9-1 phosphorylation in the fractions containing the minor peak of broken ROS (fractions 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 (Fig. 6A, boxed area) when the levels of phosphorylation are compared with 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).

**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 RGS9-1/1600 mol of 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. Immunoblots of ROS purified and washed in the absence of added ATP reveal no detectable phosphorylation by endogenous kinase. Immunoblots of ROS purified and washed in the absence of added ATP reveal no detectable phosphorylation by endogenous kinase.
able Ser^{475} 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^{475} 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^{2+}] from the range of hundreds of nanomolar to 10 nM or less (64).

The Ser^{475} 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 475. Although there is a serine residue at a similar position (Ser^{474}) in RGS9-2, it is in a completely different sequence context. Thus, the Ser^{475} 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 rendered ambiguous by the difficulty in obtaining stoichiometric inactivation of RGS9-1 GAP activity by a mechanism that is difficult to interpret, because ATP treatment of ROS leads to changes of GAP activity before and after phosphorylation have been found to regulate the function of RGS9-1, although not fully established, the function of its localization, GAP activity, or interactions with other regulatory proteins in RGS9-1, essential role in RGS9–2 binding to rod disc membranes (65).

Acknowledgment—We thank Jing Huang for helping in the preparation of the monoclonal anti-phosphorylated peptide antibody A4.

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J. Biol. Chem. 2001, 276:22287-22295.
doi: 10.1074/jbc.M011539200 originally published online April 5, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011539200

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