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A Functional Kinase Homology Domain Is Essential for the Activity of Photoreceptor Guanylate Cyclase 1*[S]

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Phototransduction is carried out by a signaling pathway that links photoactivation of visual pigments in retinal photoreceptor cells to a change in their membrane potential. Upon photoactivation, the second messenger of phototransduction, cyclic GMP, is rapidly degraded and must be replenished during the recovery phase of phototransduction by photoreceptor guanylate cyclases (GCs) GC1 (or GC-E) and GC2 (or GC-F) to maintain vision. Here, we present data that address the role of the GC kinase homology (KH) domain in cyclic GMP production by GC1, the major cyclase in photoreceptors. First, experiments were done to test which GC1 residues undergo phosphorylation and whether such phosphorylation affects cyclase activity. Using mass spectrometry, we showed that GC1 residues Ser-530, Ser-532, Ser-533, and Ser-538, located within the KH domain, undergo light- and signal transduction-independent phosphorylation *in vivo*. Mutations in the putative Mg^{2+} binding site of the KH domain abolished phosphorylation, indicating that GC1 undergoes autophosphorylation. The dramatically reduced GC activity of these mutants suggests that a functional KH domain is essential for cyclic GMP production. However, evidence is presented that autophosphorylation does not regulate GC1 activity, in contrast to phosphorylation of other members of this cyclase family.

In photoreceptor outer segments, photoreceptor guanylate cyclases GC1 and GC2 (also known as GC-E and GC-F)[3] produce cGMP, the second messenger of phototransduction (1–4) (reviewed in Refs. 5 and 6). GC1 is critical for human vision because mutations in its gene result in Leber congenital amaurosis, a cause of early onset blindness (7). Photoreceptor GCs belong to a family of membrane-bound GCs composed of an extracellular (EC), transmembrane (TM), kinase homology (KH), dimerization (DIM), and catalytic (CAT) domain (Fig. 1*A*). How these domains cooperate to achieve precisely regulated cGMP synthesis was proposed for a homolog of GC1, the natriuretic peptide receptor A (NPR-A). According to this model based on extensive data (reviewed in Ref. 8), NPR-A exists as a constitutive homodimer. In the peptide-unliganded state, the KH domains inhibit the CAT domains, a conclusion drawn from results demonstrating that the KH domain deletion mutant is constitutively active (9, 10). Binding of a single peptide ligand between the two EC domains results in their relative reorientation, relieving the inhibitory effect of the KH domains (11, 12). Consequently, the repositioned CAT domains form two active sites per dimer with both monomers contributing critical residues to each active site (13, 14). The mechanism by which KH domains mediate communication between the EC and the CAT domains is not fully understood; however, the contributions of both phosphorylation and direct binding of ATP are evident. The KH domain of NPR-A undergoes phosphorylation on four Ser and two Thr residues within a stretch of 15 residues near its intracellular N terminus. Importantly, phosphorylation of these sites is obligatory for peptide ligand-dependent activation, but it does not affect the activity of the unliganded receptor (15). No protein kinase responsible for this activity has been identified. Surprisingly, ATP, besides being used for this phosphorylation, also directly binds to this domain, enhancing the peptide ligand-dependent cyclase activity (16, 17). Whether ATP is absolutely required for such ligand-induced activation or just potentiates it is still controversial (17–19).

The degree of sequence similarity between GC1 and NPR-A differs for various domains. The extracellular domains have a low sequence identity of only 15%, whereas the intracellular portions of the receptors are more similar, with the KH, DIM, and CAT domains, respectively, sharing 32, 49, and 53% sequence identity, as calculated for mouse enzymes. Because GC1 and NPR-A are predicted to have a similar domain organization and their intracellular portions share considerable sequence identity, it seems reasonable to expect that both enzymes are regulated similarly. However, no evidence of extracellular ligand binding to the EC domain of GC1 has yet been demonstrated. Such ligand regulation is presumably

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[3] The abbreviations used are: GC, guanylate cyclase; CAT, catalytic; DIM, dimerization; EC, extracellular; EGFP, enhanced green fluorescent protein; GCAP, guanylate cyclase-activating protein; HEK, human embryonic kidney; KH, kinase homology; LRAT, lecithin:retinol acyltransferase; NPR, natriuretic peptide receptor; PP, protein phosphatase; PP2Ac, catalytic subunit of PP2A; RIPA, radioimmune precipitation assay; ROS, rod outer segment(s); WT, wild-type.

replaced by guanylate cyclase-activating proteins (GCAPs). GCAPs are small (~23 kDa), soluble, N-terminally myristoylated proteins (20–22). GCAPs activate photoreceptor GCs when intracellular Ca^{2+} levels are low (supplemental Fig. S1*A*) by interacting with their intracellular domains (23–25). It is possible that extracellular ligands, in the case of NPRs, and intracellular GCAPs, in the case of photoreceptor GCs, both activate the catalytic domains of their target enzymes by inducing structural changes. However, currently no structural or biophysical data exist that address this point. GC1 and NPR-A are both activated by ATP binding to their KH domains (17, 26). Furthermore, this ATP binding effect is at least in part direct, without involving phosphorylation, as both enzymes can be activated by nonhydrolyzable ATP analogs (26, 27). ATP activates GC1 up to 2-fold (supplemental Fig. S1*B*), whereas activation of NPR-A by ATP is more substantial but requires peptide ligand binding as well. In the absence of the peptide ligand, stimulation of NPR-A by ATP was reported to be between 0 and 20% (16, 18, 28).

Both NPR-A and GC1 are phosphoproteins (15, 26). In contrast to NPR-A, phosphorylation sites in GC1 have not been identified. Curiously, the KH domain of GC1 might undergo autophosphorylation, a surprising finding considering that the invariable "catalytic" Asp residue required for efficient kinase activity is absent in this enzyme (26). The function of this phosphorylation is also unknown. In NPR-A both phosphorylation and binding of peptide ligand to the EC domain clearly constitute a common regulatory mechanism, with phosphorylation alone having no effect but being required for peptide ligand activation (reviewed in Ref. 8). Hence, because GC1 presumably does not have a peptide ligand, the role of its phosphorylation is unclear. Studies demonstrating that NPRs, which are homologs of GC1, are regulated by phosphorylation (15) and that photoreceptor GCs are phosphoproteins (15, 26) (Fig. 1*B*) suggest that phosphorylation of GC1 constitutes an important but unappreciated regulatory mechanism, which prompted us to study its function. Furthermore, technological advances, mainly in the field of mass spectrometry (29), and specific staining of phosphoproteins (30, 31) allowed us to examine phosphorylation of GC1 more precisely. Here we report the identification of phospho-Ser residues in GC1 purified from bovine and mouse rod outer segments (ROS). We demonstrate that mutations in the putative Mg^{2+} binding site within the KH domain located in the primary sequence far away from the phosphorylation site are crucial for GC1 and protein kinase activities, suggesting regulation of cGMP production and autophosphorylation by the KH domain. In contrast to findings in other cyclases, however, our results suggest that autophosphorylation does not regulate the guanylate cyclase activity of GC1. Finally, our data from studies of wild-type (WT) and knock-out mice provide evidence that phosphorylation of photoreceptor GCs is independent of GCAPs and either direct or indirect activation by light.

MATERIALS AND METHODS

Animals—C57BL/6 mice were purchased either from Taconic or The Jackson Laboratory; BALB/c mice were acquired from The Jackson Laboratory. *Gnat1*$^{-/-}$ mice, characterized previously (32), were bred with C57BL/6 mice for two generations and then inbred to obtain knock-out homozygotes. The generation of *Lrat*$^{-/-}$ mice (33) and *Gcap1/2*$^{-/-}$ mice was reported previously (34, 35). All mice were maintained on a normal diet in a 12-h light/12-h dark cyclic environment. Experimental procedures involving animals were approved by the Case Western Reserve University Animal Care Committee and conformed to the recommendations of the American Veterinary Medical Association Panel on Euthanasia and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation of Mouse ROS and ROS Membranes—A protocol for rapid isolation of mouse ROS from 8–20 retinas was developed to minimize changes in protein phosphorylation during this procedure. All buffers used were ice-cold. Following cervical dislocation, the retina was removed from the eye through an incision in the cornea and immediately immersed in 0.5 ml of phosphatase/kinase stop buffer (7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, pH 7.2, 50 mM NaCl, 50 mM NaF, and 10 mM EDTA) in a microtube. Retinas then were fragmented by pipetting five times through a 1-ml tip, and ROS were detached from cell bodies by vigorous shaking for 1 min. Cells and larger photoreceptor fragments were pelleted by a 1-min centrifugation at 300 × *g* at 4 °C, and the ROS-containing supernatant was transferred into a fresh microtube. To maximize the yield, the pellet was resuspended in 0.5 ml of phosphatase/kinase stop buffer and subjected to one more round of pipetting, shaking, and low speed centrifugation. Finally, ROS were pelleted by centrifugation at 16,000 × *g* for 2 min at 4 °C, frozen in liquid nitrogen, and stored at −80 °C.

To remove soluble proteins, ROS pellets were resuspended in 0.5–1 ml of wash buffer (7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, pH 7.2, 10 mM NaF, and 10 mM EDTA), sonicated for 10 s with a Sonifier Model 150D set at a power level of 3, and centrifuged at 16,000 × *g* for 30 min at 4 °C. The collected ROS membrane pellet was frozen in liquid nitrogen stored at −80 °C. In experiments comparing phosphorylation level of GC1 in dark-adapted and light-exposed mice, the wash buffer was supplemented with 1 μM microcystin-LR (a specific inhibitor of protein phosphatase 1 (PP1) and PP2A).

Purification of GC from ROS Membranes by Immunoprecipitation—ROS membranes obtained from 20 retinas were resuspended in 0.8 ml of PB-RIPA buffer (7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, pH 7.2, 50 mM NaCl, 50 mM NaF, 10 mM EDTA, 1% ANAPOE-NID-P40, 0.5% sodium deoxycholate, and 0.1% SDS) and incubated with shaking at 37 °C for 30 min to solubilize membrane proteins. Next, the sample was centrifuged at 100,000 × *g* for 1 h at 25 °C to remove insoluble debris, and the supernatant was transferred to a fresh microtube. To immunoprecipitate GC1/GC2, 20 μg of anti-GC polyclonal antibody UW28 (purified by protein A-Sepharose) was added followed by a 1-h incubation with mixing at room temperature.

The protein A-Sepharose resin (50 μl) was washed three times with 150 μl of PB-RIPA buffer and resuspended in 50 μl of PB-RIPA buffer to obtain a 1:1 slurry. Between washes, the resin was pelleted by 2-min centrifugations at 3,000 × *g*. Twenty-five μl of protein A-Sepharose slurry was added to the sam-

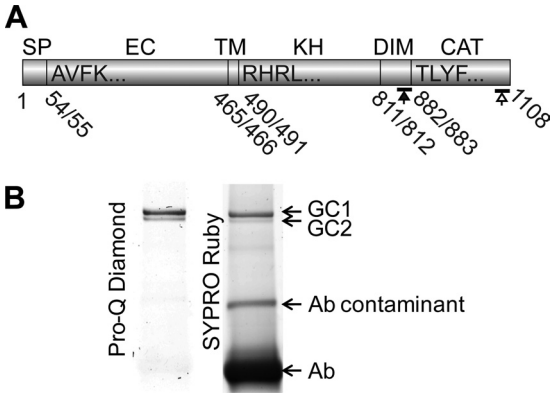

FIGURE 1. **Structural features and phosphorylation of photoreceptors GCs.** *A*, linear diagram of GC1 domain organization. The GC1 protein sequence begins with the signal peptide (*SP*) followed consecutively by domains referred to as EC, transmembrane (*TM*), KH, DIM, and CAT. Domain lengths are drawn to scale. The mature N terminus is at Ala-55. *Numbers* denote predicted domain boundaries based on information obtained from the UniProtKB data base (version 80) with small modifications, *i.e.* the DIM domain is included, and the CAT domain is extended to the end of the polypeptide. Epitopes for polyclonal UW28 and monoclonal IS4 antibodies are indicated by *black* and *white arrows*, respectively. *B*, phosphorylation of photoreceptor GCs. GC1 and GC2 were purified by immunoprecipitation with UW28 polyclonal antibodies from mouse ROS, resolved by SDS-PAGE, and stained sequentially with Pro-Q Diamond and SYPRO Ruby. Staining with Pro-Q Diamond indicates that both photoreceptor GCs are phosphoproteins, whereas failure to stain antibody (*Ab*) with this dye demonstrates its selectivity.

ple followed by a 40-min incubation with vortexing at 25 °C to prevent resin sedimentation. Then, the resin was pelleted by centrifugation at 3,000 × *g* for 3 min, and the supernatant was discarded. To remove nonspecifically bound proteins, the resin was resuspended in 0.5 ml of PB-RIPA buffer, gently vortexed for 2 min, and than pelleted by centrifugation at 3000 × *g* for 3 min. This wash cycle was performed three times. After removal of the last supernatant, GC was released from the resin by the addition of 30 μl of gel loading buffer (90 mM Tris, pH 6.85, 3% SDS, 18% (w/v) glycerol, and 7.5% (v/v) 2-mercaptoethanol) and a 5-min incubation at 100 °C. The sample was centrifuged at 16,000 × *g* for 1 min to pellet the resin, and the supernatant was loaded (15 μl/lane) onto an 8% Laemmli minigel and resolved at a current of 80 V for 20 min followed by 120 V for 100 min. GC was then visualized by Coomassie Blue staining. A typical high purity immunoprecipitate is shown in Fig. 1*B*.

Mass Spectrometry—All solutions were freshly prepared, and all tubes were washed with methanol. GC was purified from 80 mouse retinas by immunoprecipitation and resolved on SDS-PAGE as described above. Coomassie Blue-stained bands corresponding to GC were excised from eight gel lanes, chopped into 1-mm^2 cubes, and transferred into a microtube. To remove the Coomassie stain, the gel was incubated sequentially with 200 μl water for 15 min, 50 μl of 50% acetonitrile for 15 min, 50 μl of 100% acetonitrile for 15 min, and 50 μl of 100 mM NH_4HCO_3 for 5 min. Next, 50 μl of 100% acetonitrile was added to obtain final concentrations of 50% acetonitrile and 50 mM NH_4HCO_3. After 15 min of incubation, the solution was replaced with 100% acetonitrile to dehydrate the gel, and the sample was incubated for 3 min. Following aspiration of acetonitrile, the sample was dried by SpeedVac. To reduce and alkylate GC, 50 μl of 20 mM dithiothreitol in 100 mM NH_4HCO_3 was added, and the sample was incubated at room temperature for 30 min. Next, the dithiothreitol solution was replaced with 50 μl of 100 mM iodoacetamide in 100 mM NH_4HCO_3, and the sample was alkylated for 30 min in the dark. Following aspiration of the iodoacetamide solution, the sample was sequentially treated with 200 μl of 100% acetonitrile for 5 min, 200 μl of 100 mM NH_4HCO_3 for 5 min, and 200 μl of 100% acetonitrile for 5 min and, after removal of the acetonitrile, dried by SpeedVac. GC was usually digested with 0.4 μg of trypsin in 60 μl of 50 mM NH_4HCO_3, pH 8.0, overnight at 37 °C. In some experiments, GC was digested with both Lys-C and Asp-N endoproteinases. The sample was initially incubated with 0.4 μg of Lys-C endoproteinase in 60 μl of 50 mM NH_4HCO_3 or 50 mM Tris overnight at 37 °C followed by protease inactivation by boiling for 3 min and rapid cooling on ice. Next, 0.4 μg of Asp-N endoproteinase in 60 μl of 50 mM NH_4HCO_3 or 50 mM Tris, pH 8.0, was added, and the digestion was carried out overnight at 37 °C.

To recover peptides, the supernatant was transferred to a new microtube, and 30 μl of extraction solution composed of 50% acetonitrile and 5% formic acid was added to the gel fragments followed by a 10-min incubation with intermittent mixing at room temperature. The sample then was centrifuged at 16,000 × *g* for 30 min, and the supernatant containing GC peptides was collected. This extraction was repeated three more times with 5-min incubations at room temperature. In some experiments, one of the incubations was carried out in a water bath sonicator. All supernatants were combined and concentrated to 5–10 μl by SpeedVac. MonoTip TiO_2 pipette tips were employed to enrich phosphopeptides. These tips are filled with silica monolith coated with TiO_2 that selectively traps phosphopeptides. The sample in 50% acetonitrile in 0.1% formic acid (v/v) was loaded onto the TiO_2 resin, processed according to the supplier's protocol, eluted with 50 μl of 5% ammonia in water, concentrated to near dryness, resuspended in 30 μl of 0.1% formic acid, and analyzed by mass spectrometry (36).

Purification of GC from HEK-293 Cells by Immunoprecipitation—HEK-293-GC cells were seeded at 1 × 10^7 cells/75 cm^2 bottle and grown for 24 h at 37 °C. Next, cells were washed once with 5 ml of phosphate-buffered saline (5.6 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.1, and 154 mM NaCl) and once with 5 ml of phosphatase/kinase stop buffer (7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, pH 7.2, 50 mM NaCl, 50 mM NaF, and 10 mM EDTA) with 1 μM leupeptin, collected by scraping in 5 ml of phosphatase/kinase stop buffer, and pelleted by centrifugation at 300 × *g* for 5 min. After aspiration of the supernatant, cell pellets were frozen and stored at −80 °C. To purify GC, cell pellets were processed similarly to ROS pellets as described under "Isolation of ROS and ROS Membranes" and "Purification of GC from ROS Membranes by Immunoprecipitation," with the following two modifications to address the higher protease activity in cells as compared with ROS. Leupeptin (protease inhibitor) was added to PB-RIPA buffer to a 1 μM final concentration, and the solubilization temperature was decreased to 25 °C.

Phosphoprotein Staining, Visualization, and Quantification—Following SDS-PAGE, phosphoproteins were stained with Pro-Q Diamond phosphoprotein gel stain according to the manufacturer's manual. The gel was imaged with either a

Molecular Imager FX with a 532-nm laser and 555-nm long pass filter or a Typhoon 9410 scanner with a 532-nm laser and a 580 ± 30-nm filter. For visualization of all proteins, the gel was then stained with SYPRO Ruby protein gel stain following instructions in the manufacturer's manual and imaged with a Molecular Imager FX with a 532-nm laser and 640 ± 35-nm filter or a Typhoon 9410 with a 532-nm laser and 610 ± 30-nm filter. Protein quantification was performed with Quantity One or ImageQuant TL software.

Cloning and Mutagenesis of GC1—The full-length murine GC1 cDNA clone was obtained from mouse photoreceptor cDNA by PCR amplification with primers 5′-CTGCCAGGGGAGACCGAAG-3′ and 5′-GGACAGAAGCCTTGGGCCTTA-3′ followed by ligation into a pGEM-T-Easy vector to generate plasmid pGTE-mGC1. To construct the GC1 retroviral expression vector (pMiLG3), EcoRI and NotI sites were introduced at the ends of the GC1 coding sequence by PCR with pGTE-mGC1 as the template and with primers 5′-CCCCGGAATTCCCGAAGAAGGCAATGAGC-3′ and 5′-ATACTCGCGGCCGCAGTAGAGCTTCACTTCCCAG-3′. The GC1 coding sequence was then cloned into the pMXs-IG3 retroviral vector between the EcoRI and NotI sites. The insert was sequenced and confirmed to be identical to the GC1 reference sequence from Ensembl Genome Browser (ID: ENSMUST00000021259) except for a silent, single nucleotide polymorphism, 1386A > G (relative to the initiation ATG). Mutations within the KH domain were introduced by PCR amplification of the entire plasmid with the Phusion high fidelity polymerase. The fragment encompassing the GC1-IRES-EGFP cassette (where IRES is the internal ribosome entry site) was sequenced to ensure that only the desired mutations were introduced.

Generation of Stable Cell Lines Expressing GC and Its Mutants—HEK-293 cells and Phoenix Ampho cells were cultured in growth media consisting of Dulbecco's Modified Eagle's medium with 4 mM L-glutamine, 4.5 g/liter glucose, 110 mg/liter sodium pyruvate, 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum. To generate stable cell lines, the WT or mutant GC1 transgene was delivered to HEK-293 cells by a retrovirus produced with Phoenix Ampho cells. To generate this retrovirus, 1.6×10^6 Phoenix Ampho-cells in 6 ml of growth medium were seeded into a 6-cm dish. Twenty-four h later and 5 min prior to transfection, the medium was replaced with fresh, warm growth medium with 25 μM chloroquine. Transfections were performed by a calcium phosphate method in a manner similar to that described previously (37). Fifteen μg of DNA in 938 μl of water was combined with 62 μl of 2 M $CaCl_2$ in a 2-ml microtube. One ml of room temperature buffer (50 mM HEPES, pH 7.05, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, and 1.5 mM Na_2HPO_4 (molecular weight 141.96)) was then added, and the solution was immediately mixed several times by rapid inversion of the tube. After a 1-min incubation, this solution was added dropwise to the Phoenix Ampho cells. The medium was replaced with fresh growth medium at 10 h and then again at 24 h after transfection. To harvest the virus (48 h post-transfection), the medium was transferred to 15-ml conical tubes, spun at 450 × *g* for 5 min to remove detached cells, aliquoted (0.5 ml) into cryovials, frozen in liquid nitrogen, and stored at −80 °C. The target HEK-293 cells were seeded at 6×10^5 cells/6-cm dish in 4 ml of growth medium 24 h prior to transduction. To transduce the target cells, 3 ml of growth medium collected from target cells was combined with 0.5 ml of viral supernatant and 3.5 μl of Polybrene (final concentration 5 μg/ml). This was added back to the target cells after removal of residual medium. Transduction was carried out for 24 h at 32 °C (to extend retroviral half-life) in 5% CO_2 with intermittent swirling. Twenty-four h after transduction, the medium was replaced with fresh growth medium. After another 24–48 h, cells were split 1:5, and this transduction protocol was repeated to increase the expression level. After recovery, cells were separated with a cell sorter to ensure similar EGFP fluorescence intensity profiles that corresponded to similar levels of mRNA encoding WT and mutant GCs.

GC Activity Assay—The GC activity was measured in HEK-293 cells stably expressing GC1. Cells were seeded at 6×10^6 cells/60-cm^2 dish and grown for 24 h at 37 °C. Cells were washed twice with 4 ml of protein buffer (50 mM HEPES, pH 7.4, 90 mM KCl, and 10 mM NaCl), collected by scraping in 4 ml of the same buffer, and pelleted by centrifugation at 300 × *g* for 5 min. After removal of the supernatant, cell pellets were frozen in liquid nitrogen and stored at −80 °C. Just before assaying, cell pellets were resuspended in 250 μl of the protein buffer with 2 μM leupeptin and sonicated for 5 s with a Sonifier Model 150D set at a power level of 3.

For experiments that required washed cell membranes, cell pellets were resuspended in 1 ml of water with 2 μM leupeptin and sonicated for 5 s with a Sonifier Model 150D set to power level 3, and membranes were collected by a 20-min centrifugation at 16,000 × *g*. After the supernatant was removed, washed membranes were resuspended in 150 μl of protein buffer with 2 μM leupeptin and used directly, without freezing, in the assay. The GC activity assays were carried out as described previously (23, 27) with slight modifications. Briefly, each assay sample (50 μl) consisted of the following: 10 μl of assay buffer concentrate (150 mM HEPES, pH 7.4, 270 mM KCl, 30 mM NaCl, 50 mM $MgCl_2$, and 2.5 mM EGTA), 10 μl of 5 mM 3-isobutyl-1-methylxanthine in water, 10 μl of membranes resuspended in protein buffer (50 mM HEPES, pH 7.4, 90 mM KCl, and 10 mM NaCl), 10 μl of GCAP1 (~0.5 mg/ml) in protein buffer, 5 μl of $CaCl_2$ in water (at the desired concentration as described below), and 5 μl of nucleotide premix (10 mM GTP, pH 7.4, and 100 μCi/ml [α-^{33}P]GTP, stored previously at −80 °C) added last to initiate the reaction. If GCAP1 was added, samples were mixed and preincubated for 5 min to allow for GC-GCAP1 binding prior to addition of the nucleotide premix. For GCAP-free samples, protein buffer was added instead. $CaCl_2$ was added to reach final total concentrations of either 1.24 or 4.40 mM to obtain either 45 nM (low) or 1 μM (high) free Ca^{2+} concentrations, resulting from EGTA-Ca^{2+} buffering as calculated with WEBMAXC STANDARD (version 5/21/2007). Reactions were carried out for 10 min at 30 °C. To stop the reactions, samples were placed on ice, and 15 μl of 0.4 M HCl was added followed by mixing and a 4-min centrifugation at 16,000 × *g* to pellet the membranes. Forty μl of the supernatant was transferred to 150 mg of alumina suspended in 500 μl of alumina buffer (200 mM Tris, pH 7.4, and 50 mM EDTA) and vortexed

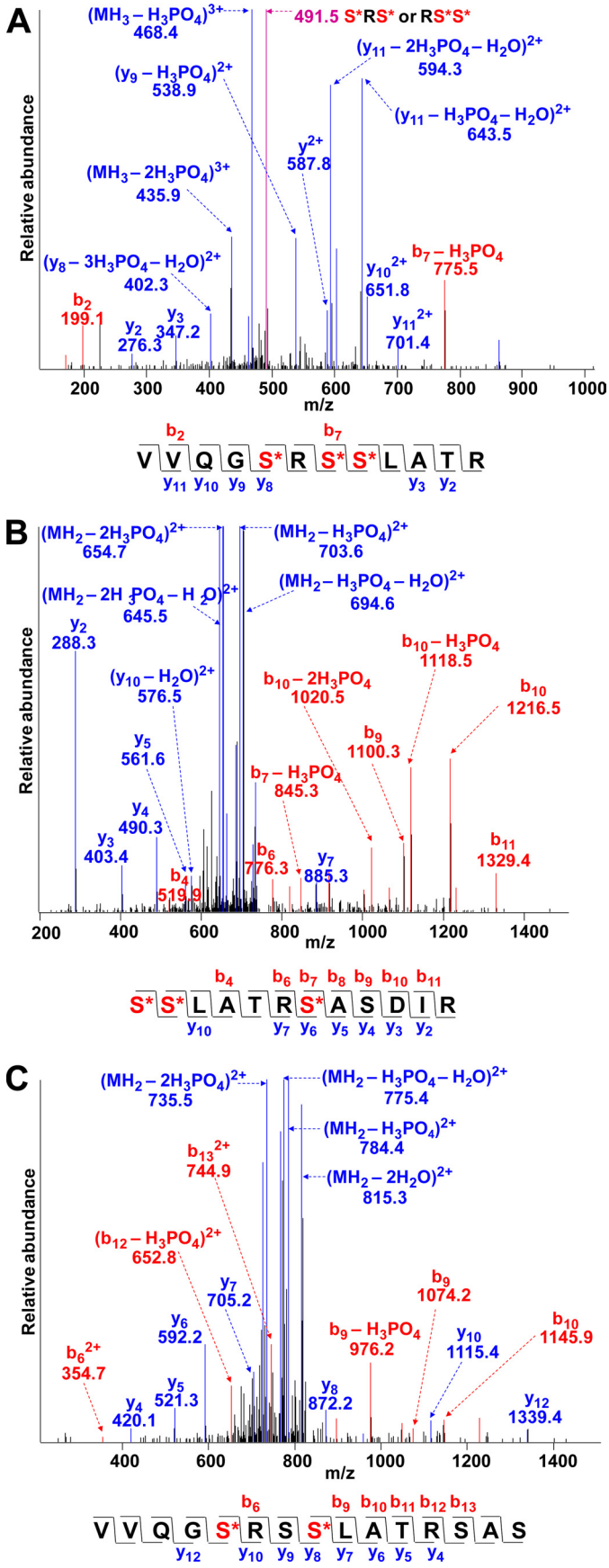

FIGURE 2. **Phosphorylation sites in GC1.** *A* and *B*, tandem mass spectra of two overlapping phosphopeptides, VVQGpS^{530}RpS^{532}pS^{533}LATR (*m/z* 501.07^{3+}) and pS^{532}pS^{533}LATRpS^{538}ASDIR (*m/z* 752.74^{2+}), generated by a partial trypsin digestion of GC1 purified by immunoprecipitation from ROS of dark-adapted mice. *C*, tandem mass spectrum of phosphopeptide VVQGpS^{530}SRpS^{533}LATRSAS (*m/z* 833.37^{2+}) generated by a Lys-C/Asp-N digestion of mouse GC1 purified by immunoprecipitation from HEK-GC1 cells. For clarity, only selected species are labeled in the spectrum. Sequences of the observed phosphopeptides are presented below the spectra,with *b* and *y* indicating the N- and C-terminal fragment ions, respectively.

for 8 min. Under these conditions, alumina binds GTP selectively compared with cGMP. Next, samples were centrifuged at 16,000 × *g* for 4 min, 300 μl of supernatant was transferred to scintillation vials filled with 3 ml of Opti-Fluor scintillation liquid and, after a thorough mixing, radioactivity was measured with an LS-6500 scintillation counter.

RESULTS

Identification of the Phosphorylated Residues in Mouse GC1—Initially, to determine which region of GC1 undergoes phosphorylation, we performed mass spectrometry analysis of GC1 purified from bovine ROS and found a doubly phosphorylated VAQGpSRTpSLAAR peptide from the N-terminal region of the KH domain (not shown). Because of the availability of genetic mouse models, we further characterized phosphorylation of GC1 purified from mouse retinas. We digested GC1 with trypsin and, after phosphopeptide enrichment, analyzed the peptides by mass spectrometry. We detected two overlapping GC1 phosphopeptides derived from the N terminus of the KH domain, and their tandem mass spectra (MS/MS) revealed four phosphorylated residues: Ser-530, Ser-532, Ser-533, and Ser-538 (Fig. 2, *A* and *B*). Because we planned to use GC1 stably expressed in HEK-293 cells to further investigate the effect of phosphorylation on its enzymatic activity, we also identified phosphorylated residues within the same region in recombinant GC1 (Fig. 2*C*). The phospho-residues identified herein for GC1 and previously for NPRs are summarized in supplemental Fig. S2.

Activity of Heterologously Expressed GC1 Is Not Regulated by Phosphorylation—Next, we stably expressed GC1 mutant variants in which identified Ser residues were replaced by either Ala residues (to eliminate potential phosphorylation) or Asp residues (to mimic potential phosphorylation sites by means of a negative charge) (Fig. 3). In addition to cDNA encoding the respective GC1 protein variants, all transgene constructs contained cDNA encoding EGFP preceded by an internal ribosome entry site, allowing translation of both genes from a single bicistronic mRNA transcript. This arrangement allowed us to generate cell lines with equivalent mRNA levels for WT and mutant GC1 by selecting cells with similar EGFP fluorescence and precisely counting the cells with a cell sorter. The cellular localization of WT and mutant GCs was analyzed by immunocytochemistry and confocal imaging (supplemental Fig. S3, *A* and *B*). These analyses revealed that all variants of GC were distributed in a similar pattern in the HEK-293 cells. We also confirmed by immunoblotting that all GC1 variants were expressed in similar amounts (supplemental Fig. S3*C*). To test whether phosphorylation levels had been altered, we purified different GC1 variants from cell lines by immunoprecipitation. After SDS-PAGE, the proteins were stained with Pro-Q Diamond (Fig. 4*A*,

NAME	SEQUENCE
PHOSPHORYLATION SITE MUTANTS	
WT	^{529}GSRSSLATRSA
AAAA	^{529}GARAALATRAA
DDDD	^{529}GDRDDLATRDA
ASAS	^{529}GARSALATRSA
SDSD	^{529}GSRDSLATRDA
SDSS	^{529}GSRDSLATRSA
Mg^{2+} BINDING SITE MUTANTS	
N674A	^{669}RLKSRACVVDG
D687A	^{682}VLKVTAHGHGR

FIGURE 3. **Mutations in the KH domain of GC1 analyzed in this study.** Mutations in the phosphorylation and Mg^{2+} binding sites are shown. The name of each mutant is followed by an amino acid sequence of an altered fragment of the KH domain. In the case of the WT, Ser residues found phosphorylated *in vivo* are in *bold*, and those in HEK-293 cells are in *red*. The *red* color also indicates a negative charge contributed by either phospho-Ser or Asp residues, and the *green* color represents lack of charge due to the presence of unphosphorylated Ser or Ala residues.

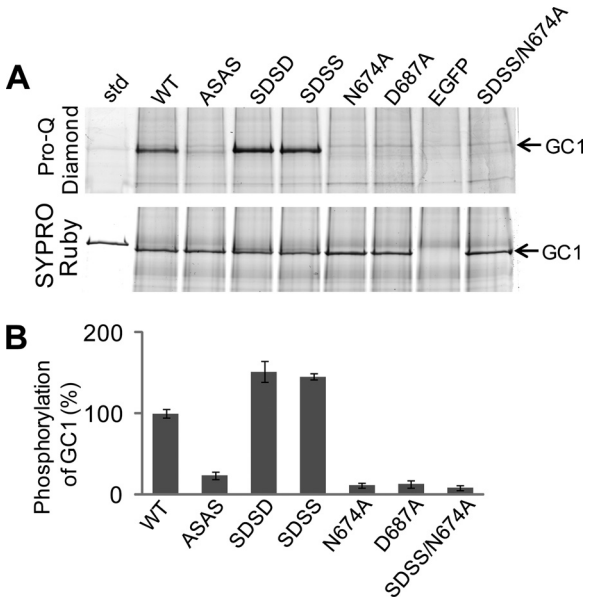

FIGURE 4. **Phosphorylation of GC1 mutants stably expressed in HEK-293 cells.** Mutants of GC1 were purified by immunoprecipitation, resolved by SDS-PAGE, and stained with Pro-Q Diamond phosphoprotein stain (*A, upper*) and SYPRO Ruby protein stain (*A, lower*). The data presented are representative of two experiments with samples analyzed in triplicate. Std denotes protein molecular weight standard. *B*, a chart comparing relative phosphorylation levels calculated from the ratios of Pro-Q Diamond to SYPRO Ruby signals. The *vertical lines* represent S.D. These results demonstrate that the phosphorylation of GC1 was altered by mutations. Furthermore, lack of phosphorylation in the mutants defective in Mg^{2+} binding (N674A and D687A) indicates that binding of Mg^{2+} to the KH domain is essential for phosphorylation, thereby suggesting that this domain might be capable of autophosphorylation in WT GC1.

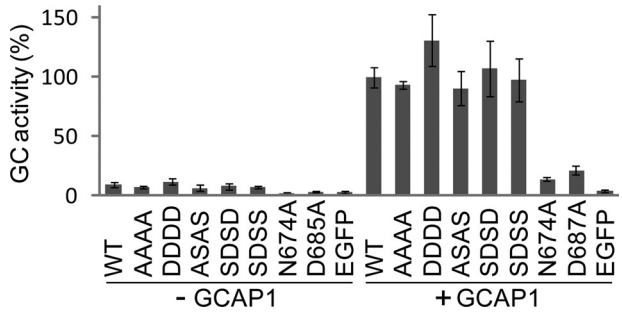

FIGURE 5. **Effect of phosphorylation and Mg^{2+} binding on GC1 activity.** Enzymatic activities of WT and mutant GC1 stably overexpressed in HEK-293 cells were assayed in cell sonicates in the presence of 45 nM (low) free Ca^{2+} and in the absence or presence of 4 μM GCAP1. Because expression levels of various mutants differed slightly from each other, activities were normalized to GC1 protein levels quantified by immunoblotting (not shown) with fluorescent secondary antibodies as described under "Materials and Methods." The data presented in the chart are representative of two experiments with samples analyzed in triplicate. The *vertical lines* represent S.D. Data obtained for cells labeled EGFP (*right*), which do not express GC1, show that the background GC activity in HEK-293 cells was negligible. GC activity recorded in all phosphorylation site mutants was similar, indicating that the GC enzymatic activity of heterologously expressed GC1 is independent of phosphorylation. Surprisingly, mutants defective in binding of Mg^{2+} to the KH domain had a very low activity, suggesting a critical role for Mg^{2+} in GC1 activation.

upper) followed by SYPRO Ruby (Fig. 4*A*, *lower*) to visualize the phosphorylated GC1 and total GC1, respectively. Quantification of these data is shown in Fig. 4*B*. These analyses demonstrated that the phosphorylation level was low (23 ± 4%) in the ASAS mutant compared with WT GC1. Residual phosphorylation of this mutant indicates that residues other than Ser-530 and Ser-533 are phosphorylated in HEK-293 cells at low levels. In SDSD and SDSS mutants the overall phosphorylation level was increased (152 ± 13 and 145 ± 3%) suggesting enhanced phosphorylation or lower dephosphorylation rates. In the AAAA mutant phosphorylation was undetectable (not shown), and in the DDDD mutant, it was either undetectable or near the detection limit (not shown).

To determine whether phosphorylation affects GC1 activity, we measured both the basal and GCAP1-stimulated activities of all GC1 variants. To account for small differences in the expression level of different variants, we normalized the activity data to the respective expression levels quantified by immunoblotting with fluorescent detection (not shown). The accuracy of this quantification was ensured by preparing an appropriate standard curve for a serially diluted sample (not shown). Our results show that GC1 activities were low in GCAP1-free samples and elevated in GCAP1-stimulated samples (Fig. 5). Importantly, the activities were similar within groups, indicating that in our experimental system neither basal nor GCAP1-stimulated GC1 activity was affected by phosphorylation. We also tested whether phosphorylation effects regulation of GC1 by Ca^{2+} or ATP by measuring the concentration dependence of cyclase activity for WT and two of the mutants, AAAA and DDDD (supplemental Fig. S4). The assays were performed in the presence of GCAP1. These results demonstrated that phosphorylation does not affect the sensitivity of GC1 to Ca^{2+} or ATP.

GC1 Is Substrate for the Catalytic Subunit of PP2A (PP2Ac)—To examine whether the GC1 activity can be altered by dephosphorylation, we treated fragmented ROS with PP2Ac. As expected from mutagenesis study, the cyclase activity assays did not show any significant difference between phosphatase-treated and control samples (supplemental Fig. S5*A*). To demonstrate that the activity measured originated from GCAP1-stimulated GCs and not from contaminating enzymes, we added purified GCAP1 to the samples. As expected, the addition of GCAP1 resulted in higher cyclase activity. To test the efficiency of dephosphorylation, photoreceptor GCs were immunoprecipitated from the assay samples, resolved by SDS-

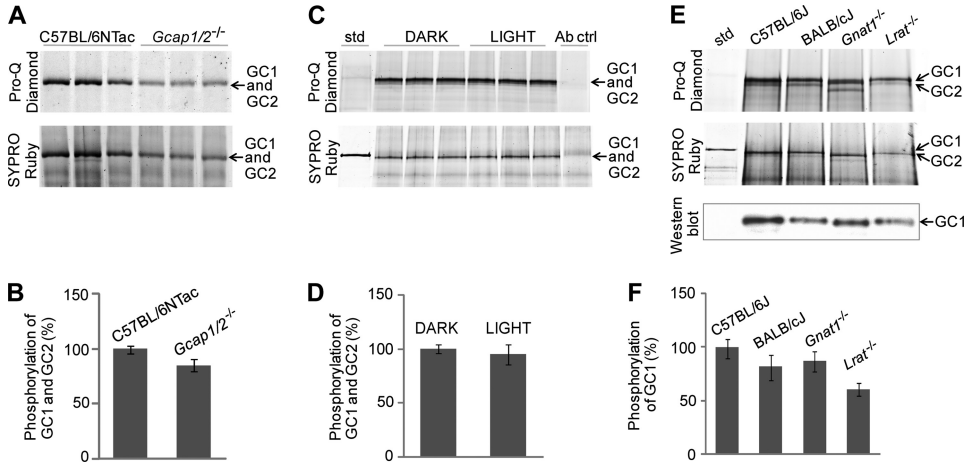

FIGURE 6. **Phosphorylation of GCs in mice with genetically altered phototransduction and under different light conditions.** SDS-polyacrylamide gels showing GCs purified by immunoprecipitation from ROS of WT (C57BL/6NTac) and *Gcap1/2*$^{-/-}$ mice (*A*), dark-adapted or light-exposed WT (C57BL/6J) mice (*C*), and WT (C57BL/6J or BALB/cJ), *Gnat1*$^{-/-}$, and *Lrat*$^{-/-}$ mice (*E*). Each *lane* contains GC purified from eight retinas. The data presented are representative of two experiments with samples analyzed in triplicate. The gels were stained with Pro-Q Diamond (*top panels* of *A*, *C*, and *E*) followed by SYPRO Ruby (*bottom panels* of *A* and *C* and *middle panel* of *E*). In *A* and *C*, both GCs migrated as one band, and thus the phosphorylation level was quantified for both proteins combined (*B* and *D*). In *E*, because of improvements in our technique, GC1 was resolved from GC2, and quantification was performed selectively for GC1 (*F*). The phosphorylation level of GC2 was not quantified because the abundance of GC2 was insufficient for accuracy. *Vertical lines* represent S.D. An immunoblot probed with IS4 anti-GC1 antibody is also presented (*E*, *bottom*). These results demonstrate that the phosphorylation levels of GCs are largely independent of light or severe perturbations in the phototransduction cascade and recovery. Quantification indicates that the phosphorylation of GC1 in *Lrat*$^{-/-}$ mice is slightly lower than in WT. However, the photoreceptor degeneration was pronounced in these mice resulting in lower amount of purified GC1, which most likely affected the accuracy of this quantification.

PAGE, and stained with Pro-Q Diamond to visualize phosphoproteins (supplemental Fig. S5*B*). Significant dephosphorylation of GC1, which occurred upon phosphatase treatment, was observed. This result suggested that GC1 is a good substrate for PP2Ac. Immunoblotting demonstrated that all samples contained equivalent amounts of GC1 (supplemental Fig. S5*C*). Thus, the activity of native GC1 was not affected by phosphorylation within ROS.

GC Is an Autophosphorylating Kinase—The ability of photoreceptor GCs to undergo autophosphorylation has been suggested previously (26). However, the possibility of contamination of the preparation of photoreceptor GCs with a kinase was not unambiguously excluded, and thus these authors suggested that mutagenesis of the KH domain should be performed to "irrefutably demonstrate that the kinase is inherent to the GC" (26). Continuing these studies, and keeping in mind that Mg^{2+} is indispensable for the activity of nearly all kinases (38), we generated GC1 mutants with altered putative Mg^{2+} binding site and analyzed their phosphorylation. Lack of phosphorylation in this case would suggest that the modification of the Mg^{2+} binding site abolished the intrinsic, autophosphorylating activity of the KH domain. On the basis of sequence alignments with various kinases and homology modeling (supplemental Fig. S6), we predicted that residues Asn-674 and Asp-687 of the GC1 KH domain were Mg^{2+} ligands. First, we replaced these residues with Ala residues, then stably expressed the mutant enzymes, and finally analyzed their expression and phosphorylation levels exactly as described above for the Ser residue mutants. Both immunocytochemistry (supplemental Fig. S3) and SYPRO Ruby staining (Fig. 4*A*, *lower*) confirmed that these mutants have cellular localizations and levels of expression similar to that of WT GC1. The Pro-Q Diamond and SYPRO Ruby staining of immunoprecipitated GCs as resolved by SDS-PAGE showed that phosphorylation was abolished in mutants with altered putative Mg^{2+} binding site (Fig. 4), providing new evidence for intrinsic kinase activity in the KH domain.

Proper Conformation of the KH Domain Is Essential for GC Activity—The cyclase activity assays revealed that heterologously expressed mutants with altered putative Mg^{2+} binding site (Asn-674 and Asp-687) had greatly diminished activity (Fig. 5, *right*). These results suggested that proper conformation of the KH domain, possibly stabilized by Mg^{2+}/nucleotide, is necessary for activation of GC1. To assess whether the KH domains of these mutants retain the ability to bind ATP, indicative of an unaltered overall fold, we performed an 8-N_3-[α-^{32}P]ATP photolabeling experiments. Our results showed that the extent of labeling was similar in all samples and was diminished to a similar extent by 4 mM ATP or 4 mM GTP, suggesting that all of the GC1 variants tested bound ATP with similar efficiency (supplemental Fig. S7). Our inability to completely compete out labeling by the addition of 4 mM ATP and 4 mM GTP is in agreement with the low binding affinity of ATP (supplemental Fig. S1*B*). The observation that both GTP and ATP decreased labeling to similar levels indicates that the nucleotide specificity of KH is low and/or the CAT domain is also labeled to some extent. In either case, labeling of the KH domain is expected to predominate, given that we used 8 μM 8-N_3-[α-^{32}P]ATP, and ATP at this concentration stimulates GC1 activity (supplemental Fig. S1*B*) presumably by binding to the KH domain. The inhibition of cyclase activity by binding of ATP to the CAT domain is significant only at much higher (millimolar) concentrations. We were also able to label WT GC1 efficiently in the absence of Mg^{2+}, further confirming that Mg^{2+} is not required for the binding of ATP to the KH domain.

Phosphorylation of GC Is Independent of GCAP1—Because GCAPs regulate the activity of photoreceptor GCs, we investigated whether this mode of regulation involves changes in phosphorylation. Accordingly, phosphorylation levels of photoreceptor GCs were compared between WT mice and mice that do not express GCAPs (*Gcap1/2*$^{-/-}$). Photoreceptor GCs were purified from both strains of mice by immunoprecipitation, resolved by SDS-PAGE, and treated with phosphoprotein-specific stain (Pro-Q Diamond) to quantify phosphorylation levels (Fig. 6*A*, *top*). To account for possible differences in the levels of GC expression between mouse strains, gels were also treated with SYPRO Ruby protein stain (Fig. 6*A*, *bottom*), and phosphoprotein amounts were compared with total protein

amounts (ratiometric analysis). Quantification of this data (Fig. 6*B*) showed that photoreceptor GCs are phosphorylated at similar levels in WT and *Gcap1/2*$^{-/-}$ mice, indicating that GCAPs are not required for phosphorylation of GCs. In these experiments our technique did not permit separation of GC1 from GC2, and thus both enzymes were analyzed together.

Phosphorylation of GC Is Independent of Light—As light is the primary regulator of processes occurring within the retina and because the α-subunit of transducin, which relays the signal from photoactivated rhodopsin to the downstream effectors (39), was recently suggested to directly interact with GC1 (40), we hypothesized that phosphorylation of photoreceptor GCs might be light-dependent. To test this hypothesis we compared the phosphorylation levels of GCs from dark-adapted and light-exposed mice. During the course of this experiment, we first dark-adapted 24 mice and then exposed half of them to ambient light for 5–15 min. We then isolated retinas from dark-adapted mice in a darkroom and retinas from light-exposed mice under ambient light. To compare GC phosphorylation between the two groups, we purified GCs from retinas by immunoprecipitation and resolved them by SDS-PAGE, and after sequential staining with fluorescent Pro-Q Diamond (a phosphoprotein-specific stain) and SYPRO Ruby (which stains for all proteins) we quantified the level of phosphorylation of GCs relative to the amount of GC protein expressed (Fig. 6, *C* and *D*). This analysis did not show any effect of light on GC phosphorylation.

To further confirm that our results were not biased by experimental design, we analyzed the phosphorylation levels of GCs in both WT mice and mouse strains with genetically altered phototransduction, namely G protein transducin knock-out mice (*Gnat1*$^{-/-}$) and LRAT knock-out mice (*Lrat*$^{-/-}$). In *Gnat1*$^{-/-}$ mice the phototransduction cascade is disrupted, mimicking the condition of permanent darkness (32), whereas *Lrat*$^{-/-}$ mice lack visual chromophore, and thus unliganded opsin constitutively activates transducin, thereby mimicking the condition of constant illumination (33, 41–43). Both knock-out strains were on the C57BL/6 background. However, because *Gnat1*$^{-/-}$ mice were backcrossed to C57BL/6 for only two generations and still had some of the mixed genetic makeup characteristic of transgenic mice, with a large contribution from BALB/c, we included BALB/c mice as an additional control. To compare the GC phosphorylation levels, we isolated photoreceptor GCs and quantified the phosphorylation levels of GC1 as in the previous "light dependence" experiment. The results demonstrate that GC1 phosphorylation levels were similar in all mouse strains (Fig. 6, *E* and *F*), suggesting that GC1 phosphorylation is not regulated by light *in vivo* and that transducin is not required for the phosphorylation. Although the amount of GC2 was too low for us to accurately quantify its phosphorylation level, phosphorylation of this cyclase was most likely unaffected as well. Interestingly, both photoreceptor GCs isolated from *Gnat1*$^{-/-}$ mice migrated faster in SDS-PAGE. This mobility shift was unlikely to have been caused by a difference in phosphorylation state, because substantial dephosphorylation failed to alter the migration of GC1 in a different experiment (supplemental Fig. S5*B*).

DISCUSSION

New Evidence for Autophosphorylation of GC1—Aparicio and Applebury (26) demonstrated that membrane GC purified from photoreceptors incorporates radiolabeled phosphate from ATP, suggesting that its KH domain has kinase activity. However, the KH domain of this protein lacks the Asp residue considered to be invariably conserved in kinases and indispensable for efficient phosphotransfer activity (44, 45). Interestingly, cAMP-dependent protein kinase retains residual kinase activity, despite substitution of Asp for Ala in the catalytic loop (46). To confirm that the findings of Apparicio and Applebury (26) were not affected by a contaminating kinase, we attempted to reproduce their results with methods established in our laboratory that took advantage of the fact that nearly all kinases require Mg^{2+} for activity (38, 45). On the basis of homology modeling of the GC1 KH domain, we predicted that residues Asn-674 and Asp-687 coordinate Mg^{2+}. Subsequently, we showed that substitution of either of these residues with Ala eliminates phosphorylation of the KH domain, providing new evidence for its intrinsic kinase activity. That mutagenesis altered the KH domain structure to prevent binding of an upstream kinase is unlikely because (i) the results for two different mutants were similar; (ii) mutant GCs were expressed at similar levels compared with the WT enzyme, indicative of their stability; and (iii) proper folding of mutant KH domains was suggested by the 8-N_3-[α-^{32}P]ATP photolabeling experiment demonstrating that nucleotide binding was unaffected.

A Functional KH Domain Is Important for GC1 Activity—Surprisingly, GCAP1-stimulated cyclase activity was severely diminished in mutants with alterations in the putative Mg^{2+} binding site; namely, N674A and D687A had 14 ± 2 and 21 ± 4% of the WT level. Moreover, this decreased activity did not seem to arise from lack of autophosphorylation, because our mutagenesis and dephosphorylation experiments demonstrated that cyclase activity was unaffected by the autophosphorylation level. To better understand how binding of Mg^{2+} might affect the KH domain, we examined the Mg^{2+} binding site in a homology model, which showed that Mg^{2+} ions along with nucleotide bridge the two lobes of the kinase together via a hydrogen bond network. This implies that altering the Mg^{2+} binding residues disrupts the proper interaction between these lobes, which in turn stabilizes an inactive conformation of the CAT domain. Furthermore, Mg^{2+} ions form multiple direct and water-mediated hydrogen bonds with the phosphate moiety of the nucleotide, which strongly suggests that the nucleotide is also required for binding of Mg^{2+} to the KH domain and thus is important for cyclase activity. So we propose that ATP and Mg^{2+} do not regulate GC1 in response to light but instead constitute a structural element of the KH domain. This conclusion is supported by the observation that as low as 0.1 mM ATP maximally stimulates GC1, whereas the concentration of ATP in ROS is thought to be much higher, irrespective of its light-induced fluctuations (47). This indicates that ATP remains bound to GC1 regardless of the lighting conditions. Moreover, the concentration of Mg^{2+} is reported to be steady within ROS (48), precluding the idea that Mg^{2+} has a regulatory function.

Each Family Member of Membrane-bound GCs Is Distinctly Regulated by Phosphorylation—We have demonstrated that GC1 isolated from mouse ROS is phosphorylated on four Ser residues (Ser-530, Ser-532, Ser-533, and Ser-538) located at the N terminus of the KH domain. We altered the phosphorylation of GC1 through mutagenesis and tested the GCAP1-dependent activity of mutant enzymes expressed in HEK-293 cells. The design of these mutants was guided by the results of mass spectrometry analyses, which revealed phosphorylation of Ser-530 and Ser-533 in heterologously expressed GC1. Consequently, we generated mutants with greatly reduced phosphorylation by replacing Ser-530 and Ser-533 with Ala residues as well as mutants mimicking fully phosphorylated GC1 by replacing Ser-532 and Ser-538 with Asp residues (49, 50). Additionally, mutants were generated with all four Ser residues of interest changed to Ala or Asp residues. All of these mutants were stably expressed in HEK-293 cells and their GC activities measured. The results demonstrate that basal and ATP- and GCAP1-stimulated activities all were unaffected by the phosphorylation state of GC1. However, because there was no proof in this instance that Asp residues could maintain the functionality of phospho-Ser residues, the findings obtained with these mutants should be interpreted with caution; an additional factor(s) mediating the effect of phosphorylation could have been missing. To address both of these issues, we dephosphorylated native GC1 directly in fragmented ROS by treating them with PP2Ac, naturally present in the native cells (51, 52). The results demonstrated that dephosphorylation by PP2Ac did not affect cyclase activity. Interestingly, these findings separate GC1 from NPRs, in which activity is strongly dependent on their phosphorylation status.

What Is the Role of GC1 Phosphorylation?—From previous studies of NPRs, it appears that the KH domain exerts an inhibitory effect on the catalytic domain. Upon ligand binding, dephosphorylation and desensitization occurs. Binding of the extracellular hormone peptide sends a signal that is transmitted through the KH domain occupied by the nucleotide to the catalytic site (53). Interestingly, NPRs that are homologs of GC1 are also known to undergo phosphorylation in the same region of the KH domain (8), but the responsible kinase has not been identified. Like GC1, other membrane-bound GCs could undergo autophosphorylation. In NPRs and GC1, the first phospho-Ser is located 35 and 40 residues from the end of the predicted transmembrane helix and is flanked by Gly and Arg. These striking similarities in phosphorylation suggest a common regulatory mechanism, probably with distinct and specific elements for each cyclase. We suggest that the presence of nucleotide and Mg^{2+} in the nucleotide binding pocket of the GC1 KH domain stabilizes the conformation required for activation of GC1 by GCAP1. Whereas phosphorylation is required for peptide ligand-dependent activation of NPRs (8), GC1 activity is unaffected, and equivalent peptide ligands for photoreceptor GCs have not been reported. GC1 isolated from mouse ROS is phosphorylated on four Ser residues (Ser-530, Ser-532, Ser-533, and Ser-538) located at the N terminus of the KH domain. Likely in many ATP-binding enzymes, particularly in protein kinases, a flexible region becomes autophosphorylated. Moreover, instead of being activated by an extracellular ligand, phosphorylated GC1 could represent a partially active conformation that is constitutively preactivated and poised to have its activity further stimulated 10-fold by Ca^{2+}-sensing GCAPs (23) interacting with the intracellular portion of the cyclase (54). Although known activators of photoreceptor GCs and NPRs are unrelated, the KH domains of both types of enzymes would relay the signal from the activator to the CAT domain (8, 54). Thus, we hypothesize that KH domains might be a common off-switch for both types of enzymes, rendering NPRs insensitive to peptide ligands and maintaining photoreceptor GCs at basal activity but still poised for further GCAP activation. The basal activity is essential to sustain phototransduction. It would also be of interest to determine the tertiary structure of this domain to gain insights into the mechanism of catalysis.

In summary, we have demonstrated that GC1 possesses protein kinase activity in addition to its cGMP cyclase activity. Our results suggest that phosphorylation does not regulate either the basal or the GCAP1-stimulated activity of GC1 and is not linked to activation of phototransduction. The Ser residues that undergo phosphorylation are highly conserved in various species (supplemental Fig. S8) and among various types of GCs (supplemental Fig. S2), suggesting an evolutionary conservation among GCs. Phosphorylation of NPRs dramatically regulates peptide-stimulated activity. Finally, it is possible that besides phosphorylating itself, GC1 also phosphorylates other substrates in ROS. Whether members of membrane-bound GCs are protein kinases has not been established; and perhaps this study will prompt a re-evaluation of NPR phosphorylation.

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