Ca\textsuperscript{2+}-dependent Conformational Changes in Guanylyl Cyclase-activating Protein 2 (GCAP-2) Revealed by Site-specific Phosphorylation and Partial Proteolysis*

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Guanylyl cyclase-activating proteins (GCAPs) are calcium sensor proteins of the EF-hand superfamily that inhibit retinal photoreceptor membrane guanylyl cyclase (retGC) in the dark when they bind Ca\textsuperscript{2+} but activate retGC when Ca\textsuperscript{2+} dissociates from GCAPs in response to light stimulus. We addressed the difference in exposure of GCAP-2 structure to protein kinase and a protease as indicators of conformational change caused by binding and release of Ca\textsuperscript{2+}. We have found that unlike its homolog, GCAP-1, the C terminus of GCAP-2 undergoes phosphorylation by cyclic nucleotide-dependent protein kinases (CNDPK) present in the retinal extract and rapid dephosphorylation by the protein phosphatase PP2C present in the retina. Inactivation of the CNDPK phosphorylation site in GCAP-2 by substitutions S201G or S201D, as well as phosphorylation or thiophosphorylation of Ser\textsuperscript{201}, had little effect on the ability of GCAP-2 to regulate retGC in reconstituted membranes in vitro. At the same time, Ca\textsuperscript{2+} strongly inhibited phosphorylation of the wild-type GCAP-2 by retinal CNDPK but did not affect phosphorylation of a constitutively active Ca\textsuperscript{2+}-insensitive GCAP-2 mutant. Partial digestion of purified GCAP-2 with Glu-C protease revealed at least two sites that become exposed or constrained in a Ca\textsuperscript{2+}-sensitive manner. The C\textsuperscript{2+}-dependent conformational changes in GCAP-2 affect the areas around Glu\textsuperscript{62} residue in the entering helix of EF-hand 2, the areas proximal to the exiting helix of EF-hand 3, and Glu\textsuperscript{136}–Glu\textsuperscript{138} between EF-hand 3 and EF-hand 4. These changes also cause the release of the C-terminal Ser\textsuperscript{201} from the constraint caused by the Ca\textsuperscript{2+}-bound conformation.

Photoisomerized rhodopsin triggers hydrolysis of cGMP through the activation of the G-protein transducin, which stimulates cGMP phosphodiesterase and thus causes cGMP-gated channels to close (see Refs. 1–3 for review). Rods and cones rapidly recover to their resting potential after excitation induced by a brief non-saturating exposure to light. They also adapt to a constant background illumination by decreasing the amplitude and accelerating the kinetics of their electrical responses to a standard test flash (see Refs. 1–3 for review). Reopening of the cGMP-gated channels during recovery and light adaptation requires acceleration of cGMP synthesis by guanylyl cyclase, a process controlled by the level of intracellular free Ca\textsuperscript{2+}. In the dark, Ca\textsuperscript{2+} extruded from the photoreceptor outer segment by a Na\textsuperscript{+}/K\textsuperscript{+} exchanger re-enters the outer segment through the open cGMP-gated channels so that the free intracellular outer segment Ca\textsuperscript{2+} concentrations reach 250 nM in mammals and 350–450 nM in lower vertebrates (4–6). In the light, cGMP is hydrolyzed, and these channels close while the exchanger continues to extrude Ca\textsuperscript{2+} ions from the photoreceptor outer segment; therefore, Ca\textsuperscript{2+} concentrations decrease nearly 10-fold (4–7). Ca\textsuperscript{2+}-binding proteins GCAP-1 and GCAP-2 activate retGC1 and retGC2 (and their homologs found in photoreceptors of different vertebrate species) in a Ca\textsuperscript{2+}-sensitive manner (see Refs. 8–11 for review). GCAPs are a separate subfamily within the broader family of neuronal calcium sensor proteins that includes many recoverin-like proteins. The recoverin-like neuronal calcium sensor proteins have four EF-hand-type Ca\textsuperscript{2+}-binding domains and an N-terminal Gly residue acylated with fatty acid residues. Only three of the EF-hand structures in GCAPs are true Ca\textsuperscript{2+}-binding domains in which Ca\textsuperscript{2+} sensitivity is adjusted to the range within which the intracellular free Ca\textsuperscript{2+} varies between light and dark by intracellular free Mg\textsuperscript{2+} (12). The N-terminal EF-hand-related motif EF-1 cannot bind Ca\textsuperscript{2+} but strongly contributes to the GCAP interaction with retGC (13, 14).

The partial solution structure of the Ca\textsuperscript{2+}-bound GCAP-2 established by Ames et al. (15) resembles those of recoverin and neurocalcin and consists of two globular halves connected by a flexible “hinge” region. The NMR structure was established only for the Ca\textsuperscript{2+}-loaded form of GCAP-2 and does not include the C-terminal portion of the protein; therefore, the exact conformational changes that occur when dissociation of the Ca\textsuperscript{2+} ions causes GCAP-2 to transition into the activator conformation are not immediately apparent.

In our present study, we used the accessibility for the CNDPK phosphorylation of a Ser\textsuperscript{201} residue proximal to the C terminus of GCAP-2 and limited proteolysis by protease V8 to assess the conformational changes in GCAP-2 related to the binding and release of Ca\textsuperscript{2+}. Our data indicate that the C terminus of GCAP-2 undergoes a conformational Ca\textsuperscript{2+}-dependent switch that releases the C terminus from a constraint

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† The abbreviations used are: GCAP, guanylyl cyclase-activating protein; retGC, retinal photoreceptor membrane guanylyl cyclase; EF-1, -2, -3, and -4, the first, second, third, and fourth EF-hand domains, respectively, in GCAP primary structure; CNDPK, cyclic nucleotide-dependent protein kinases; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; ATP\textsuperscript{S}, adenosine 5’-(O-thiotriphosphate); MOPS, 4-morpholinosopropanesulfonic acid; PP, protein phosphatase; WT, wild type; 8-Br-cGMP, 8-bromo-cyclic GMP.

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created by the Ca\textsuperscript{2+}-loaded structure of GCAP-2 and changes the exposure of the regions between EF-hands 2 and 3 and between EF-3 and -4.

**EXPERIMENTAL PROCEDURES**

Cyclic Nucleotide-dependent Protein Kinases—Purified cGMP-dependent kinase II (PKG II) and cAMP-dependent protein kinase (PKA) were kindly provided by Dr. Michael Uhler (University of Michigan). Purified cGMP-dependent protein kinase isoform I (PKG I) was from Calbiochem/EMD (San Diego, CA). Recombinant myristoylated bovine and mouse GCAP-2, GCAP-1, and neurocalcin were expressed from pET11d vector (Novagen/EMD) in a BLR(DE3)pLysS Escherichia coli strain harboring yeast N-myristoyltransferase and purified as described previously in full detail (12, 16, 17).

Site-directed Mutagenesis—All mutations were incorporated into bovine GCAP-2 cDNA by PCR using the splicing by overlap extension technique and Pfu DNA polymerase (Stratagene) as described previously (18, 19). All mutant cDNAs where then sequenced using Cy5-modified dideoxynucleoside triphosphates, a ThermoSequenase kit, and

**FIG. 1.** Purified CNDPK phosphorylates Ser\textsuperscript{201} in GCAP-2. A, Coomassie stain of proteins in phosphorylation mixtures separated by 15% SDS-PAGE. The strong upper band corresponds to purified bovine serum albumin (BSA) added to the phosphorylation reaction as the internal negative control. B, radioautograph of \(^{32}\)P phosphorylation products (duplicate from A). The molecular mass (MW) markers (Bio-Rad, broad range) shown in lane a are 200, 115, 97, 67, 43, 31, 21, 14, and 6 kDa. The position of GCAPs in the gel is indicated by the asterisk. The minor band, \(<1\) kDa above the main GCAP band, in every case corresponds to non-myristoylated GCAP. The 50-kDa reaction mixture contained purified PKG I (1,000 units, Calbiochem), 40 \(\mu\)g of bovine serum albumin, and 20 \(\mu\)g of purified recombinant WT GCAP-2 (b and c), partially purified C1\textDelta{}GCAP-2 mutant (d), or purified recombinant GCAP-1 (e). c–e, 150 \(\mu\)M cGMP was added; b, no cGMP added. After incubation for 30 min at 37 °C, each reaction mixture was diluted with Laemmli buffer, and 20 \(\mu\)l were loaded on SDS-PAGE and analyzed as described under “Experimental Procedures.” C, lack of PKG I\textsubscript{a}-dependent phosphorylation in GCAP-2 with Ser\textsuperscript{201} substituted by Asp (a) or Gly (b), non-substituted Ser\textsuperscript{201} GCAP-2 as a control. D, radioautograph of WT GCAP-2 phosphorylated in vitro by 1 \(\mu\)l each of PKG II (a), PKA (b), and PKG Ia (c). E, radioautograph of recombinant GCAP-2 phosphorylated by soluble retinal CNDPK in retinal extract as a function of Mg\textsuperscript{2+} concentration. a–e, 1.5 \(\mu\)g of WT GCAP-2; f–i, 1 \(\mu\)g of C1\textDelta{}GCAP-2. The reaction contained 10 \(\mu\)l of retinal S\textsubscript{100} fraction and 100 \(\mu\)M 8-Br-cGMP. F, GCAP-2 phosphorylation by S\textsubscript{100} soluble retinal fraction requires cyclic nucleotide. A radioautograph of 1 \(\mu\)g of WT GCAP-2 phosphorylated in the presence of 10 \(\mu\)l of S\textsubscript{100} fraction in the presence (a) or in the absence (b) of 100 \(\mu\)M 8-Br-cGMP is shown. c, no exogenous GCAP-2 was added. d, purified \[^{32}\]PGCAP-2 labeled \textit{in vitro} by PKG Ia, as in A, used as a protein marker. G and H, PKG I\textsubscript{a}-dependent phosphorylation does not affect electrophoretic mobility of bovine or mouse GCAP-2. G, bovine GCAP-2 (2 \(\mu\)g) that contains 50% phosphorylated myristoylated GCAP-2 migrates as a single band (e). The extent of GCAP-2 phosphorylation by PKG I was determined from the specific activity of the \[^{32}\]P label incorporated into \(0.75 \times 10^{-3}\)M GCAP-2 from 0.75 mM ATP containing 10 \(\mu\)Ci of \([\gamma-^{32}\]P\]ATP (300 Ci/mmol). H, left panel (Coomassie Blue stain) shows protein molecular mass markers (a), 14 \(\mu\)g of non-phosphorylated recombinant myristoylated mouse GCAP-2 (mouse GCAP-2 migrates faster than bovine GCAP-2) (b), and \[^{32}\]P-phosphorylated GCAP-2 (c). The position of the main protein band is marked with an arrow covered with radioactive ink. Right panel, radioautograph of the same gel.

Recombinant myristoylated bovine and mouse GCAP-2, GCAP-1, and neurocalcin were expressed from pET11d vector (Novagen/EMD) in a BLR(DE3)pLysS Escherichia coli strain harboring yeast N-myristoyltransferase and purified as described previously in full detail (12, 16, 17).

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an SEQ 4 × 4 automated sequencer (Amersham Biosciences). The CΔ1GCAP-2 mutant lacked the last nine amino acid residues. The Ca²⁺-insensitive GCAP-2, EF23/4, contains single substitutions in all three EF-hands that prevented high-affinity Ca²⁺ binding and was expressed and purified as described previously in detail (18, 19).

Photoreceptor outer segment membranes were isolated under infrared illumination from frozen dark-adapted bovine retinas (Lawson and Lawson) using sucrose density centrifugation and were washed in low salt buffer to remove endogenous GCAPs as described previously (12, 16).

Soluble Retinal Extract (S₁₀₀)—Fresh bovine eyes obtained at a local slaughterhouse were dark-adapted for 2 h on ice, dissected under infrared light, and homogenized in 0.5 ml of buffer A (5 mM sodium phosphate buffer, pH 7.2, 50 mM NaCl) per retina on ice by 10 strokes in a Dounce homogenizer. The homogenate was centrifuged at 20,000 g at 4 °C, and the supernatant was collected and centrifuged in 200-µl aliquots in a Beckman A100 rotor for 30 min at 80,000 rpm. The supernatant fraction, S₁₀₀, was collected and used immediately or divided into aliquots, frozen in dry ice, and stored at −70 °C. Typical total protein concentration in the S₁₀₀ fraction was near 40 mg/ml each, leupeptin and aprotinin (10 µg/ml each), 0.3 µM ATP, 4 µM cGMP, 1 mM GTP, 1 mM Ci of [8-3H]cGMP, 1 µM of [8-3H]-labeled GCAP-2. The assay mixture contained 20 µl of Tris/HCl, pH 7.5, 10 mM 2-mercaptoethanol, either 500 µM MgCl₂ or 100 µM EDTA, 46 µCi of purified GCAP-2, and 0.18 Ci of [32P]-32P-labeled GTP, 0.1 µCi of [8-3H]cGMP, 1 µl of washed bovine outer segment membranes (3.7 mg/ml rhodopsin), and 60 µM GCAP-2. The reaction was incubated at 23 °C for 2 h and then incubated overnight at 4 °C. After incubation, the reaction mixture was dialyzed against 10 mM Tris/HCl, pH 7.5.

Protein Phosphatase Assay—Wild-type GCAP-2 was 32P-labeled in the presence of PKI as described above, except instead of quenching the reaction by adding SDS sample buffer, the unincorporated label was removed by gel filtration on Sephadex G-50. The phosphate reaction typically contained 1 µg of [32P]GCAP-2 (~1 µCi/µg), 1 µl of S₁₀₀ protein fraction in 5 mM MgCl₂, 20 mM Tris, 7.5, and 6 mM dithiothreitol in a total volume of 20 µl. The reaction was stopped by adding 2 volumes of a Laemmli sample SDS buffer containing 4 mM EGTA and was boiled for 3 min followed by SDS-PAGE and radioautography as described above.

Immunoblot—Proteins were transferred from SDS-polyacrylamide gel onto Immobilon P membrane (Millipore) for 20 h at 60 V, stained for GCAP-2 using polyclonal rabbit antibody ΔNP24 (20), and developed using alkaline phosphatase-conjugated goat anti-rabbit IgG (ICN/Cappel).

Glu-C Proteolysis—The reaction contained 20 µl of 10 mM Tris/HCl, pH 7.5, 10 mM 2-mercaptoethanol, either 500 µM MgCl₂ or 100 µM EDTA, 46 µCi of purified GCAP-2, and 0.18 µg of Staphylococcus aureus protease V8 (Sigma). The components were mixed at room temperature and incubated for 7 min at 37 °C, and the reaction was stopped by heating to 100 °C for 5 min. 10-µl aliquots from each reaction were then loaded on a capillary C4 column. Peptides eluted with a gradient of acetonitrile with 0.1% trifluoroacetic acid were directly infused into a Sciex API III electrospray mass spectrometer and scanned for positive ions with m/z values between 600 and 1200. Mass spectrometry data were processed using Sciex MacProMass and MacSpec software.

RetGC Assay—All experiments were conducted under infrared light as described (12). The assay mixture (25 µl) contained 30 mM MOPS/KOH, pH 7.3, 60 mM KCl, 5 mM NaCl, 1 mM dithiothreitol, 2 mM Ca²⁺/EGTA buffer, 1 mM free Mg²⁺, zaprinast and diprydiamole (25 µM each), leupeptin and aprotinin (10 µg/ml each), 0.3 µM ATP, 4 µM cGMP, 1 mM GTP, 1 µCi of [γ-32P]GTP, 0.1 µCi of [8-3H]cGMP, 1 µl of washed bovine outer segment membranes (3.7 mg/ml rhodopsin), and purified recombinant GCAP-2 or its mutants as indicated in the figure legends. The products of the reaction were analyzed by TLC using alkaline phosphatase-conjugated goat anti-rabbit IgG (ICN/Cappel). The thiolphosphorylation reaction contained 50 mM Tris/HCl, pH 7.6, 2 mM EGTA, 5 mM MgCl₂, 0.5 mM 8-Br-cGMP, 10 µg/ml leupeptin, 20,000 units of PKG Ia, 5.0 mM ATP/β-S, and 60 µM GCAP-2. The reaction was incubated at 23 °C for 2 h and then incubated overnight at 4 °C. After incubation, the reaction mixture was dialyzed against 10 mM Tris/HCl, pH 7.5. The thiolphosphorylation reaction contained 50 mM Tris/HCl, pH 7.6, 2 mM EGTA, 5 mM MgCl₂, 0.5 mM 8-Br-cGMP, 10 µg/ml leupeptin, 20,000 units of PKG Ia, 5.0 mM ATP/β-S, and 60 µM GCAP-2. The reaction was incubated at 23 °C for 2 h and then incubated overnight at 4 °C. After incubation, the reaction mixture was dialyzed against 10 mM Tris/HCl, pH 7.5.

FIG. 3. Activation of retGC in vitro in outer segment membrane by GCAP-2 and its mutants. RetGC activity stimulation by WT GCAP-2 is shown at 10 nM free Ca²⁺ (●, ○), S201G GCAP-2 (●, ▲), and 201G GCAP-2 (○, ■) in the absence (●, ▲, ○, solid line) and in the presence (Δ, ○, ◯, dashed lines) of 0.5 mM ATP. Inset, Coomassie-stained gel after SDS-PAGE of purified S201G GCAP-2 (1.8 µg) (α), S201D (2 µg) (β), and WT GCAP-2 (2.4 µg) (γ).

RESULTS AND DISCUSSION

According to the NMR spectroscopy study by Ames et al. (15), the overall folding of Ca²⁺-loaded GCAP-2 resembles recoverin,
Ca\(^{2+}\)-dependent Conformational Changes in GCAP-2

FIG. 4. Phosphorylation of GCAP-2 and retGC regulation. A, Ca\(^{2+}\) sensitivity of retGC activation in washed outer segment membranes by 5 \(\mu\)M thiophosphorylated (●) or non-thiophosphorylated (○) GCAP-2. Inset, radioautograph of \([^{32}\text{P}]\)GCAP-2. Control (a) or thiophosphorylated (b) GCAP-2 was \([^{32}\text{P}]\)labeled using \([\gamma^{32}\text{P}]\)ATP. B, activation of retGC by thiophosphorylated (●) or non-thiophosphorylated (○) GCAP-2 in the presence of 2 mM EGTA. C and D, phosphorylation by PKG does not interfere with Ca\(^{2+}\)-sensitive dimerization of GCAP-2. Purified bovine myristoylated GCAP-2 was phosphorylated using \([\gamma^{32}\text{P}]\)ATP and purified PKG in the presence of EGTA as described under “Experimental Procedures.” After phosphorylation, free \([\gamma^{32}\text{P}]\)ATP was removed by repeated dilution/concentration using a Millipore Ultrafree-4 cartridge, and the \([^{32}\text{P}]\)GCAP-2 was subjected to gel permeation chromatography on an Amersham Superdex 200 HR 10/30 column connected to a fast protein liquid chromatography system. Samples contained either 2 mM CaCl\(_2\) (C) or 2 mM EGTA (D). The elution buffer contained 10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 60 mM KCl, 1 mM MgCl\(_2\), 1 mM dithiothreitol, and either 500 \(\mu\)M CaCl\(_2\) (C) or 500 \(\mu\)M EGTA (D).

C-terminal Ser\(^{201}\) in GCAP-2 Undergoes Phosphorylation by CNDPK

The RRKSAMF sequence at the C terminus of the GCAP-2 molecule (20) is a consensus recognition site for both cGMP- and cAMP-dependent kinases, so three different purified CNDPKs can phosphorylate the C-terminal Ser in GCAP-2 \textit{in vitro} (Fig. 1, A–D). Purified PKG I phosphorylates GCAP-2 with low efficiency in the absence of added cyclic nucleotide, and the addition of cGMP enhances the phosphorylation (Fig. 1B, lanes b and c). GCAP-1 lacks such a sequence (26), and we did not observe phosphorylation of GCAP-1 by CNDPK under the same conditions (Fig. 1, A and B, lane e). Unlike wild-type GCAP-2, a chimera mutant, in which the C-terminal region downstream of EF-4 was substituted with the corresponding fragment from the related recoverin-like protein neurocalcin (19), did not undergo phosphorylation under these conditions (not shown). Deletion (mutant CΔ1) of the last nine amino acid residues, including Ser\(^{201}\), from GCAP-2 (19) also completely abolished phosphorylation (Fig. 1, A and B, lane d). Apparently, the C terminus in GCAP-2 was the only part in GCAP-2 that was phosphorylated. To ensure that the CNDPK site Ser\(^{201}\) was the only site of phosphorylation in GCAP-2, we substituted Ser\(^{201}\) with either Gly or Asp and found that both mutations completely abolished \(^{32}\text{P}\) incorporation in GCAP-2 (Fig. 1C). In the presence of N-myristoyltransferase, GCAP-2 is expressed in \textit{E. coli} predominantly as a fatty acylated protein (16); however, a variable small fraction of GCAP-2 that remains non-acylated is often present in such preparations and appears in SDS-PAGE as a minor band just above the acylated GCAP-2 (16). When both myristoylated and non-myristoylated forms are present in preparation of GCAP-2, they incorporate the \(^{32}\text{P}\) label catalyzed by purified PKA and PKG (Fig. 1D). Hence, myristoylation is not required for GCAP-2 to become a substrate for CNDPK.

PKA, PKG II, and, at a lower level, PKG I have been reported in photoreceptors (27, 28). Consistent with that report, we have found that the retinal extract contains soluble GCAP-2 kinase activity stimulated by cGMP or its slow hydrolyzable analog, 8-Br-cGMP (Fig. 1, D and F). To minimize potential interference from phosphorylation of the endogenous GCAP-2, we extracted soluble retinal proteins (\(S_{100}\) fraction) at normal ionic strength when GCAP-2 remained mostly associated with the membrane fraction (16). Although the \(S_{100}\) fraction contains an ~33-kDa endogenous protein (presumably phosducin) that is highly phosphorylated by the CNDPK (27, 29), this band migrates substantially higher than GCAP-2 and therefore does not interfere with the analysis. Using 8-Br-cGMP produces more stable and reproducible results because the \(S_{100}\) fraction, even isolated from the dark-adapted retina, contains substantial traces of cGMP phosphodiesterase activity (not shown). In
the presence of cyclic nucleotide, purified recombinant GCAP-2 becomes phosphorylated by the S100 fraction. The Ser^{201} in GCAP-2 was the only site for the protein kinase activity present in the S100 fraction, and it was phosphorylated only by CNDPK. Removing the CNDPK site from GCAP-2 either by deletion or a by the point mutation S201D (Fig. 1, C and E) completely suppresses phosphorylation of GCAP-2 by the retinal extract, as does the removal of CNDPK activator (Fig. 1F). Although in other proteins phosphorylation can alter their electrophoretic mobility, phosphorylation of Ser^{201} in GCAP-2 (either of bovine or mouse origin) does not affect its electrophoretic mobility in denaturing SDS gel (Fig. 1, G and H).

Dephosphorylation of GCAP-2—GCAP-2 can be phosphorylated by CNDPK present in the S100 fraction, but the efficiency of phosphorylation in that case is drastically lower (10-fold lower, data not shown) compared with purified CNDPK because the S100 fraction also contains a protein phosphatase (Fig. 2). Purified [32P]-labeled GCAP-2, phosphorylated by PKG Iα and added to the retinal S100 fraction, becomes rapidly dephosphorylated (Fig. 2, A and D). The loss of radioactivity was due to phosphatase activity, not protein degradation, because leupeptin and aprotinin did not prevent the loss of the [32P] label from prephosphorylated GCAP-2 (not shown), and the integrity of GCAP-2 was not compromised after incubation with S100 (Fig. 2B).

Inhibitors of protein phosphatases PP1, PP2A, and PP2B (such as Fenvalerate, microcystine, and okadaic acid) do not affect dephosphorylation of [32P]GCAP-2, even at concentrations high enough to completely inhibit these protein phosphatases (Fig. 2). Similarly, neither bromotetramisole (an alkaline phosphatase inhibitor) nor the removal of Ca^{2+} by EGTA inhibits dephosphorylation. Inhibition of the [32P]GCAP-2 dephosphorylation occurred only when Mg^{2+} was removed from the reaction mixture by EDTA. EDTA is the only inhibitor of the fourth type of protein phosphatase, the Mg^{2+}-dependent phosphatase PP2C, which is also present in the retina (30) (Fig. 2, A, C, and D). Phospho-GCAP-2 appears to be the first PP2C-specific substrate identified in photoreceptors. The only covalent modification in GCAP-2 isolated from bovine retinas was fatty acylation (16), and the presence of potent phosphatase PP2C in retinal extract (30) may account for the lack of phosphorylated GCAP-2 after its isolation from the retina. Therefore, it remains unclear to what extent GCAP-2 can be phosphorylated in vivo.

Phosphorylation of GCAP-2 and RetGC Regulation

ATP is known to potentiate the stimulation of retGC by GCAP-2 in vitro (31–34). The light-activated photoreceptor-specific phosphodiesterase PDE6 can hydrolyze both cGMP and cAMP (35–38). However, neither ATP nor PKA could potentially affect retGC activity or Ca^{2+} sensitivity via phosphorylation of GCAP-2. However, neither inactivation of the phosphorylation site in GCAP-2 (Fig. 3) nor inclusion of CNDPK and its activators directly into the retGC assay reaction (not shown) had an effect on retGC regulation. Only a small change (<15%) in maximal activity of retGC was observed when Ser^{201} was substituted with either Gly or negatively charged Asp residues. Yet the potentiating effect of ATP on retGC activity remained present in both GCAP-2 mutants (Fig. 3). To completely exclude the possibility that PP2C, which is present in outer segment membranes, interferes with the analysis, we used modification by a phosphatase group that can be transferred to proteins by protein kinases but cannot be removed by protein phosphatases. Prior to use in retGC assay, GCAP-2 was incubated with PKG Iα and ATPγS, after which 92% of GCAP-2 became unavailable for subsequent phosphorylation using [γ-32P]ATP (Fig. 4A). Neither the affinity for guanylyl cyclase nor the Ca^{2+} sensitivity of the cyclase regulation by GCAP-2 changed after the Ser^{201} thiophosphorylation of GCAP-2 (Fig. 4, A and B). Apparently, the effect of ATP on retGC activation is because of either ATP binding by retGC (33) or phosphorylation of retGC itself (37) but not because of phosphorylation of GCAP-2. Phosphorylation by PKG does not affect Ca^{2+}-sensitive dimerization of GCAP-2 either (25) (Fig. 4, C and D). Distribution of [32P]PGCAP-2 in the fractions eluted from the gel filtration column demonstrates that the amount of radioactivity follows the protein profiles. The phosphorylated GCAP-2 elutes predominantly as a monomer in the presence of Ca^{2+} but distributes almost equally between the monomer and the dimer in the presence of EGTA.

This result is consistent with our previous observation that small C-terminal deletions in GCAP-2 have only a minor effect on retGC activation (19) and argues against GCAP-2...
phosphorylation playing a part in retGC regulation. Yet we cannot exclude the possibility that phosphorylation contributes to processes other than GCAP-2/retGC interaction because the C-terminal CNDPK site is preserved in all vertebrate species (38).

Conformational Change in GCAP-2 as a Function of Ca\textsuperscript{2+}/H\textsubscript{11001} Binding

Our preliminary experiments indicated that phosphorylation of Ser\textsuperscript{201} in GCAP-2 by the retinal CNDPK was suppressed when concentrations of free Ca\textsuperscript{2+} in the reaction mixture (not shown). However, the efficiency of GCAP-2 phosphorylation by the crude retinal CNDPK is dramatically influenced by PP2C activity present in the retinal extract, which can potentially interfere with the interpretation of these data. Therefore, to avoid interference from the PP2C activity, we used \(^{35}\text{S}\)ATP. Protein kinases can efficiently utilize this ATP analog as a substrate, whereas the resulting thiophosphoprotein is not recognized by protein phosphatases (39). By using this approach we found that retinal CNDPK indeed phosphorylates GCAP-2 in a Ca\textsuperscript{2+}-sensitive manner (Fig. 5). GCAP-2 fully saturates with Ca\textsuperscript{2+} when free Ca\textsuperscript{2+} concentrations are above 1 μM, even in the presence of 5 mM MgCl\textsubscript{2} (12, 15). The saturating free Ca\textsuperscript{2+} concentrations suppress GCAP-2 thiophosphorylation by the soluble retinal CNDPK (Fig. 5A, lanes a and b). S201D GCAP-2 was used as a control for the CNDPK-specific site (Fig. 5A, lane c), and both thiophosphorylation and phosphorylation of GCAP-2 by purified CNDPK were also inhibited (Fig. 5, B and C). In different experiments, the relative efficiency of the retinal CNDPK-dependent phosphorylation for the Ca\textsuperscript{2+}-loaded GCAP-2 varied between 6 and 40% compared with the Ca\textsuperscript{2+}-free GCAP-2, and on average, phosphorylation of the Ca\textsuperscript{2+}-bound GCAP-2 was suppressed nearly 5-fold (Fig. 5D).

Was the effect of Ca\textsuperscript{2+} because of a change in the specificity of the CNDPK activity or the result of a conformational change in GCAP-2? To answer this question, we used the constitutively active GCAP-2 mutant EP2/3/4 (18). This mutant remains constitutively active even in the presence of Ca\textsuperscript{2+}, because inactivation of all three active EF-hands by substitutions in the first or the last amino acid residues of a 12-amino acid Ca\textsuperscript{2+}-binding loop of the EF-hands 2, 3, and 4 locks GCAP-2 in a Ca\textsuperscript{2+}-free conformation (18). If the effect of Ca\textsuperscript{2+} was because of changes in CNDPK activity and specificity rather than related confor-
Ca\(^{2+}\)-dependent Conformational Changes in GCAP-2

**FIG. 7. Putative conformational changes in GCAP-2 caused by binding and release of Ca\(^{2+}\).** A, the C terminus in GCAP-2 is flexible and unstructured (15), but it is loosely constrained in Ca\(^{2+}\)-bound conformation and remains largely inaccessible for CNDPK. Dissociation of Ca\(^{2+}\) from GCAP-2 results in a conformational change that promotes both the dimerization of GCAP-2 (25) and the release of its C terminus from the constraint created by protein structure. Other explanations are in the text under “Results and Discussion.” B and C, change in exposure of Glu\(^{62}\), Glu\(^{136}\), and Glu\(^{138}\) side chains are shown space-filled in two projections based on three-dimensional partial NMR structure of the Ca\(^{2+}\)-loaded GCAP-2 (Ames et al.) (15). The model was drawn using SPDB Viewer 3.7 software. Positions of Ca\(^{2+}\) ions within the Ca\(^{2+}\)-binding loops of EF-hand 2, 3, and 4 are shown as green spheres. C, Glu\(^{62}\), shown with space-filled atoms in the entering alpha helix of EF-hand 2, is likely to move away from the exiting helix of EF-hand 3 in the absence of Ca\(^{2+}\) and thus become exposed for proteolysis when GCAP-2 undergoes transition into the activator conformation.

...the Glu\(^{62}\) located in the entering alpha helix of EF-hand 2 is only exposed when GCAP-2 undergoes the transition into its Ca\(^{2+}\)-free (cyclase activator) conformation. The three possible scenarios through which Ca\(^{2+}\) may affect the exposure of the parts of GCAP-2 are discussed in the following paragraphs.

**Compartmentalization—**GCAP-2 could associate with the membrane and/or the target cyclase in such a manner that in the Ca\(^{2+}\)-bound form, the C terminus is protected from phosphorylation by proximity to the membrane. However, we are justified in ruling out this possibility because (a) the GCAP-2 phosphorylation and proteolysis experiments described in this paper were carried out using only soluble protein, and (b) Ca\(^{2+}\)-loaded GCAP-2 tends to dissociate from the membrane more efficiently than the Ca\(^{2+}\)-free form (16).

**Ca\(^{2+}\)-dependent Association of GCAP-2—**Ca\(^{2+}\)-dependent association of GCAP-2 with unknown soluble retinal protein(s) could hamper its interaction with CNDPK. This possibility cannot be completely ruled out in the case of a complex protein mixture such as the S\(_{100}\) fraction. Yet it remains highly unlikely, because we observed Ca\(^{2+}\)-sensitivity of GCAP-2 phosphorylation even by purified CNDPK in the absence of other proteins in the reaction (Fig. 5, B and C). Therefore, the change in availability of the C-terminal Ser\(^{201}\) as a substrate for CNDPK reflects its constraint in the Ca\(^{2+}\)-bound form and its release from constraint in Ca\(^{2+}\)-free GCAP-2 (Fig. 7A).

**Dimerization of GCAP-2—**The Ca\(^{2+}\)-free form of GCAP-2 can undergo dimerization, and the dimer dissociates upon its transition back into the Ca\(^{2+}\)-loaded retGC inhibitor form (13, 25). Obviously, the exposure of some regions of the main and side chains in Ca\(^{2+}\)-bound GCAP-2 can be affected by the GCAP-2 dimerization. In particular, the flexible portion of GCAP-2 structure between EF-hands 3 and 4 could become masked when it forms the Ca\(^{2+}\)-free dimer. However, this seems an unlikely explanation, because even in the Ca\(^{2+}\)-free form of GCAP-2, we observed a mixture of monomers and dimers at a nearly equal (or at least comparable) ratio. Should the Glu\(^{136}\)-Glu\(^{138}\) region become constrained only as a result of dimerization, we would still expect almost one-half of the protein to undergo cleavage at these sites. Yet unlike Ca\(^{2+}\)-bound GCAP-2, none of the corresponding peptides was detectable in the case of the Ca\(^{2+}\)-free GCAP-2 (Fig. 6, left panel). Therefore, it is more reasonable to suggest that the flexible loop between EF-hands 3 and 4, well exposed in Ca\(^{2+}\)-bound GCAP-2 structure (15), becomes constrained in both Ca\(^{2+}\)-free monomers and dimers of GCAP-2. Even though dissociation of Ca\(^{2+}\) promotes GCAP-2 dimerization (25), its C-terminal Ser\(^{201}\) becomes more accessible for the CNDPK (Figs. 5 and 7).

In the case of recoverin, binding of Ca\(^{2+}\)-results in a calcium-myristoyl switch, during which the fatty acylated N-terminal segment of the molecule undergoes a Ca\(^{2+}\)-dependent extrusion from a hydrophobic pocket (40). A similar process may also take place in the case of other recoverin-like proteins (24). Yet, both functional and structural analyses of GCAP-2 strongly indicate that Ca\(^{2+}\)-dependent conformational changes in GCAP-2 are different from recoverin and do not include the N-terminal Ca\(^{2+}\)-myristoyl switch (16, 41). Our data support the possibility that, instead, the C terminus in GCAP-2 undergoes a Ca\(^{2+}\)-sensitive conformational switch. Although the flexibility and exposure of the C terminus is limited by the constraint created in the protein structure by Ca\(^{2+}\)-binding to EF-hands, the constraint at the C-terminal Ser\(^{201}\) is released after dissociation of Ca\(^{2+}\) from GCAP-2 (Fig. 7). Hence, if a Ca\(^{2+}\)-sensitive switch exists in GCAP-2, it is located at the C terminus of GCAP-2, which is opposite to the N-terminal Ca\(^{2+}\)-myristoyl switch in recoverin that extrudes the N terminus of the protein when Ca\(^{2+}\) is bound (42). The NMR spectroscopy...
does not show the presence of stable long-range interactions at the C terminus (amino acids 189–204). This would indicate that the C terminus remains unstructured and flexible even in Ca\(^{2+}\)-bound conformation, yet it is not extruded far enough from the protein core to become accessible for CNBDP.

Interestingly, neither the C terminus nor the flexible unstructured loop between EF-hands 2 and 3 in GCAPs apparently plays an essential role in retGC activation (19), so their exposure or constraint reflects the overall conformational change in GCAP-2 rather than playing an important role in cyclase regulation. In contrast to that role, the region between EF-hands 2 and 3 in GCAPs apparently destabilizes the interaction between the entering helix of EF-2 and the entering helix of EF-3 (15). Because the Glu\(^{62}\) residue located in the entering helix of EF-2 becomes Ca\(^{2+}\)-free recoverin, this helix becomes highly stabilized by its interaction with the helices of EF-3 at the domain interface (48, 49). Similar Ca\(^{2+}\) interaction with the helices of EF-3 at the domain interface (48, 49) is essential for retGC activation (19), so their exposure or constraint reflects the overall conformational change in GCAP-2 rather than playing an important role in cyclase regulation.

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