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Differential Ca^{2+} Sensor Guanylate Cyclase Activating Protein Modes of Photoreceptor Rod Outer Segment Membrane Guanylate Cyclase Signaling

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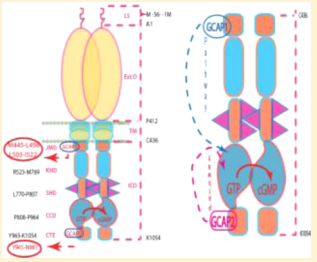
Supporting Information

ABSTRACT: Photoreceptor ROS-GC1 (rod outer segment membrane guanylate cyclase) is a vital component of phototransduction. It is a bimodal Ca^{2+} signal transduction switch, operating between 20 and ~1000 nM. Modulated by Ca^{2+} sensors guanylate cyclase activating proteins 1 and 2 (GCAP1 and GCAP2, respectively), decreasing $[Ca^{2+}]_i$ from 200 to 20 nM progressively turns it "on", as does the modulation by the Ca^{2+} sensor S100B, increasing $[Ca^{2+}]_i$ from 100 to 1000 nM. The GCAP mode plays a vital role in phototransduction in both rods and cones and the S100B mode in the transmission of neural signals to cone ON-bipolar cells. Through a programmed domain deletion, expression, in vivo fluorescence spectroscopy, and in vitro reconstitution experiments, this study demonstrates that the biochemical mechanisms modulated by two GCAPs in Ca^{2+} signaling of ROS-GC1 activity are totally different. (1) They involve different structural domains of ROS-GC1. (2) Their signal migratory pathways are opposite: GCAP1 downstream and GCAP2 upstream. (3) Importantly, the isolated catalytic domain, translating the GCAP-modulated Ca^{2+} signal into the generation of cyclic GMP, in vivo, exists as a homodimer, the two subunits existing in an antiparallel conformation. Furthermore, the findings demonstrate that the N-terminally placed signaling helix domain is not required for the catalytic domain's dimeric state. The upstream GCAP2-modulated pathway is the first of its kind to be observed for any member of the membrane guanylate cyclase family. It defines a new model of Ca^{2+} signal transduction.



Visual perception involves the transformation of patterns of light and darkness received by the retinal receptors into images of shape defined with depth and color intensity in the visual cortex of the brain. It is a multistage process. The first one, termed phototransduction, occurs in the outer segments of the rods and cones (ROS), where captured photons are transduced by a biochemical cascade into electric signals.[1−3] The second messenger of the photon signal is cyclic GMP, and its source is ROS-GC membrane guanylate cyclase.[4] In darkness, a pair of ROS-GCs, 1 and 2, through their basal synthesis of cyclic GMP keep a fraction of cyclic nucleotide-gated (CNG) ion channels in the plasma membrane of the rods and cones open (phototransduction model, in Figure 1 of ref 3). A steady influx of Na^+ and Ca^{2+} passes through the open channels and keeps the photoreceptor depolarized. Photons trigger the hydrolysis of cyclic GMP, closing the channels and blocking the entry of cations, thus hyperpolarizing the photoreceptor. Because extrusion of Ca^{2+} by the $Na^+/Ca^{2+},K^+$ exchanger persists, there is a light-induced decrease in $[Ca^{2+}]_i$ in mammalian rods from 250 to ~20 nM.[5] Guanylate cyclase activating proteins (GCAP1 and GCAP2, respectively) sense the decrease in Ca^{2+} and stimulate ROS-GC to synthesize cyclic GMP at a faster rate, limiting the effect of the phototransduction cascade and helping the photoreceptor to return to its resting state.[2,6]

In a recent study, another Ca^{2+}-modulated operational mode of ROS-GC1 has been observed, making ROS-GC1 a bimodal Ca^{2+} signal transduction switch in the photoreceptor cells (Figure 12 of ref 7). The presence and functional linkage of ROS-GC1 with Ca^{2+} sensor S100B have been mapped in the cone outer segments of the mouse retina.[7] Intriguingly, this pathway is not present in the mouse rods. The pathway modulates the transmission of neural signals to cone ON-bipolar cells. The $[Ca^{2+}]_i$ with a $K_{1/2}$ of 500 nM turns it "on". ROS-GC1 interacts with S100B; S100B undergoes conformational change, binds at the two sites, Gly^{962}−Asn^{981} and Ile^{1030}−Gln^{1041}, and stimulates ROS-GC1.[8] The binding site for GCAP2 overlaps with the S100B site,[9] but the sites for GCAP1, Met^{445}−Leu^{456} and Leu^{503}−Ile^{522}, are distinct,[10] raising the intriguing possibility of simultaneous binding of S100B and GCAP1 to the same ROS-GC1 molecule.

Together with the prior biochemical information that the two GCAPs selectively modulate the Ca^{2+} signaling of the photoreceptor ROS-GC1 and may thereby participate in defining the shades of light,[9−13] this study solidifies and advances this concept by demonstrating in vivo that the two GCAP-modulated pathways of ROS-GC1 are dynamically different. Furthermore, this study for the first time discloses a new model of membrane guanylate cyclase signaling; this model, which can be applied to the GCAP-modulated Ca^{2+} signaling of ROS-GC1, demonstrates that at the structural and dynamic level the mode of GCAP1 signaling of the photoreceptor ROS-GC1 is opposite to that of GCAP2.

Received: May 2, 2012
Revised: May 25, 2012
Published: May 29, 2012

dx.doi.org/10.1021/bi300572w | *Biochemistry* 2012, 51, 4650−4657

EXPERIMENTAL PROCEDURES

Mutagenesis. ROS-GC1 mutants were constructed using the Quick-change mutagenesis kit (Stratagene) and appropriate mutagenic primers. The mutations were verified by sequencing.

Recombinant FP-Tagged GCAP1 and GCAP2. The construction of YFP-GCAP2 was described previously.[14] GFP-GCAP1 was constructed in a manner identical to that used for YFP-GCAP2 except that the GCAP1 cDNA was inserted into the pAc-GFP-Hyg vector (Clontech).

Construction of the N-YFP(1−158)-CCD and CCD-C-YFP(159−238) Vectors. To construct the N-YFP(1−158)-CCD vector, the ROS-GC1 cDNA fragment encoding the core catalytic domain of amino acids M^{816}−Y^{965} was amplified by polymerase chain reaction. A linker sequence (R-S-I-A-T) and a BamHI recognition site were added at the 5′-end of the amplified cDNA, and a BglII recognition site was added at the 3′-end. The amplified product was digested with BamHI and BglII and subcloned into the BglII site of the YFP(1−158)/pcDNA1/Amp vector. To construct the CCD-C-YFP(159−238) vector, via the same method that was used for the N-YFP(1−158)-CCD construct, the ROS-GC1 cDNA fragment was amplified except that the R-S-I-A-T linker sequence was added to the 3′-end. The BamHI and BglII recognition sites were added at the 5′- and 3′-ends, respectively. The amplified product was digested with BamHI and BglII and subcloned into the BamHI site of the YFP(159−238)STOP/pcDNA1/Amp vector. Following the same protocol, the N-YFP(1−158)-CCD and C-YFP(159−238)-CCD vectors (N-YFP-CCD and C-YFP-CCD, respectively) were constructed. The constructs were verified by sequencing.

Expression in COS Cells. COS-7 cells were transfected with appropriate expression constructs using the calcium phosphate coprecipitation technique.[15] Sixty hours after being transfected, the cells were harvested, and their membranes were prepared.

Guanylate Cyclase Activity Assay. Membranes of COS cells expressing ROS-GC1 or its mutants were assayed for guanylate cyclase activity as described previously.[14] The amount of cyclic GMP formed was quantified by a radio-immunoassay.[16] All experiments were conducted in triplicate.

Immunocytochemistry. GFP-GCAP1 and YFP-GCAP2 were expressed individually or together with ROS-GC1 or its mutants in COS cells grown in coverslip chambers. Seventy-two hours after being transfected, the cells were viewed directly or fixed in 4% paraformaldehyde in Tris-buffered saline (TBS) for 15 min at room temperature. The fixed cells were washed with TBS, blocked in 10% normal donkey serum in a TBS/0.5% Triton X-100 (TTBS) mixture for 1 h at room temperature, washed with TTBS, incubated with ROS-GC1 antibody (diluted 50:1) in blocking solution overnight at 4 °C, washed with TTBS, incubated with DyLight 488-conjugated donkey anti-rabbit antibody (200:1) for 1 h, and washed with TTBS. Images were acquired using an inverted Olympus IX81 microscope/FV1000 spectral laser confocal system and analyzed using Olympus FluoView FV10-ASW software. Digital images were processed using Adobe Photoshop.

Expression of GCAP1 and GCAP2 was as described previously.[14]

RESULTS

In Vivo Mechanisms of GCAP1 and GCAP2 Signal Transduction Are Different and Autonomous. In their native states in the outer segments of rods and cones, the two GCAPs, GCAP1 and GCAP2, are bound to ROS-GC1 and delicately control its Ca^{2+}-modulated activity. The mechanisms of their controls are ill defined. To understand these in an isolated fashion, we used the heterologous in vivo expression system of COS cells, and the system was analyzed by confocal immunofluorescence microscopy. The benefit of this isolated cell system is that the given property of each GCAP and ROS-GC1 can be analyzed individually and then in the reconstituted state in each other's presence. We took advantage of the previous information that the target sites of two GCAPs reside at the opposite ends of the intracellular domain of ROS-GC1[9,10] (Figure 1). The GCAP2 domain borders the C-terminal side of the catalytic domain, and GCAP1 is juxtaposed with the transmembrane domain (Figure 1A). In these orientations, the two GCAP pathways will involve different structural domains of ROS-GC1. To assess this hypothesis, first, the structural role of the juxtamembrane and the kinase homology domains (JMD-KHD) (Figure 1) in the GCAP signaling of ROS-GC1 was investigated.

COS cells were cotransfected with the cDNAs of a ROS-GC1 mutant lacking the JMD and KHD (JMD-KHD$^-$) and GCAP1 or GCAP2 tagged with fluorescent protein [green fluorescent protein (GFP)-GCAP1 and yellow fluorescent protein (YFP)-GCAP2]. The controls consisted of the cells transfected with ROS-GC1, GFP-GCAP1 or YFP-GCAP2 alone, and of COS cells cotransfected with these individual GCAPs and wild-type ROS-GC1 (wtROS-GC1). The results are presented in Figures 2 and 3. They demonstrate that in accordance with the previous observations.[14,17] (1) ROS-GC1 is exclusively the membrane protein (Figure 2A). (2) When expressed alone, both GCAPs are soluble proteins (Figure 2B,F): their expression is observed throughout the entire cell, including the nucleus [the fact that the GCAPs expressed alone exist as soluble proteins was verified by analyzing the cytosolic fraction of the transfected cells by Western blotting using anti-GCAP1 and anti-GCAP2 antibodies (Figure 1 of the Supporting Information)]. (3) When coexpressed with wtROS-GC1, both GCAPs migrate to the membrane and target their specific sites on ROS-GC1 that act as the anchors (panels C and E of Figure 2 for GCAP1 and panels G and I of Figure 2 for GCAP2). The anchoring of GCAPs by ROS-GC1 is clearly visible in panels G−I of Figure 2. The intensity of GCAP2 fluorescence follows the pattern of ROS-GC1 expression (indicated by arrows). However, when coexpressed with the JMD-KHD$^-$ mutant of ROS-GC1, the two GCAPs behave differently. The fluorescence of GFP-GCAP1 is scattered all over the cell (Figure 3B). Its pattern is identical to that seen when GFP-GCAP1 is expressed alone (compare Figures 2B and 3B) and differs completely from the pattern of JMD-KHD$^-$ mutant immunofluorescence (Figure 3A). Although some yellow color after merging panels A and B of Figure 3 is visible, it is attributed to the deficiency of the technique rather then to physical interaction as observed in Figure 2E. The fact that GCAP1 coexpressed with the JMD-KHD$^-$ mutant does not migrate to the membrane indicates that it lost its anchoring site and, therefore, remains the soluble protein in the cell. In contrast, the fluorescence of YFP-GCAP2 is observed in the cellular membranes (Figure 3E); it follows the red immunofluorescence of the JMD-KHD$^-$ mutant (Figure 3D) and merges with it (Figure 3F), indicating that the GCAP2 anchoring site on ROS-GC1 is still present in the JMD-KHD$^-$ mutant. These results demonstrate that under in vivo conditions the GCAP1 signal requires the JMD-KHD

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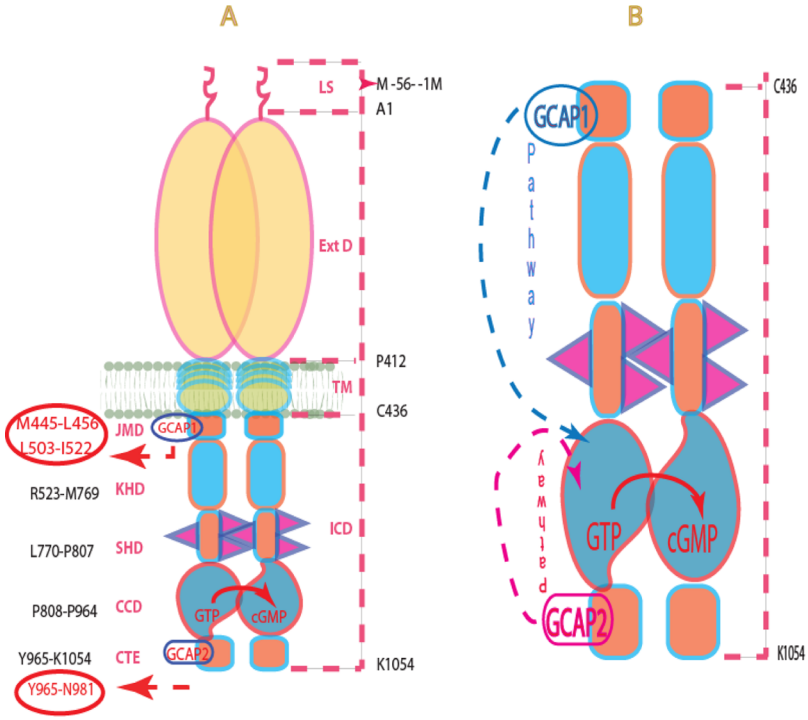

Figure 1. (A) Schematic representation of the structural topography of ROS-GC1. ROS-GC1 is a single transmembrane-spanning homodimer protein. The dashed lines on the right show the defined boundaries of its segments: LS, leader sequence; ExtD, extracellular domain; TM, transmembrane domain; ICD, intracellular domain. All its functional domains are housed in the ICD, and their designated names and the amino acid residues constituting their boundaries are indicated at the left: JMD, juxtamembrane domain housing the indicated GCAP1-targeted domain; KHD, kinase homology domain; SHD, signaling helix domain; CCD, core catalytic domain; CTE, C-terminal extension housing the GCAP2-targeted domain. It is noteworthy that the sites targeted by the two GCAPs (encircled) are at the opposite ends of CCD. (B) The two GCAP pathways run in opposite directions. The trajectory of the GCAP1 pathway shown with the blue dashed arrow is downstream. From its origin in the JMD, it passes through the structural KHD and SHD in its course to the CCD. In contrast, the trajectory of the GCAP2 pathway (shown with a pink dashed arrow) is upstream. From its origin in the CTE, it directly flows to the CCD. The CCD exists as an antiparallel homodimer. Both GCAP signals are translated at the CCD into the production of cyclic GMP, which serves the second messenger of the light signals.

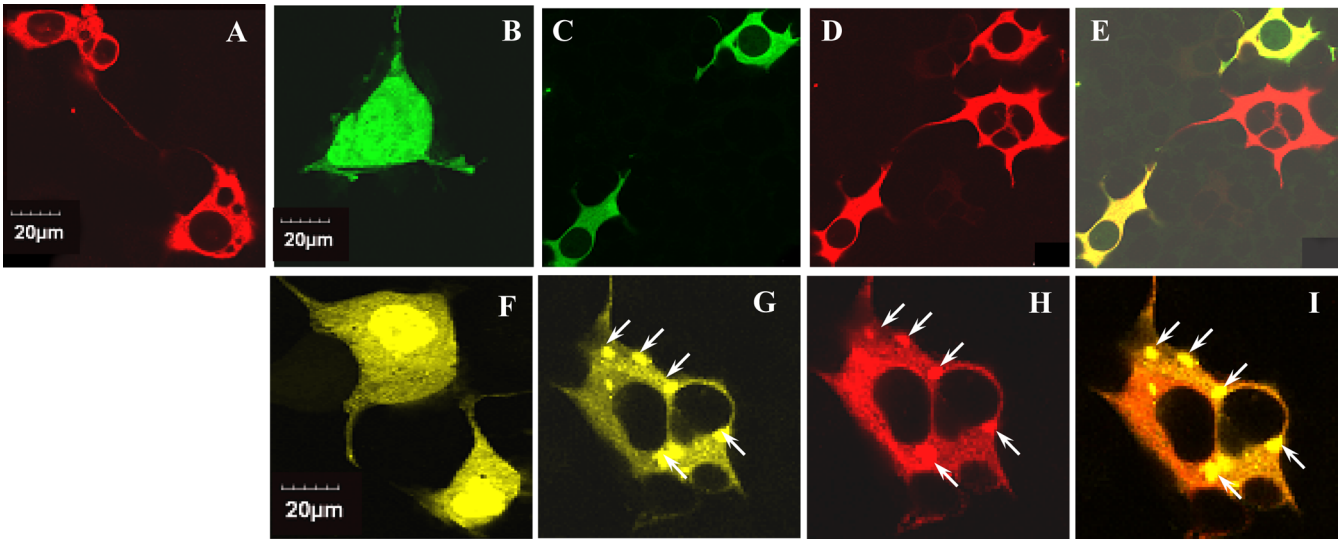

Figure 2. GCAP1 and GCAP2 bind ROS-GC1 in COS cells. COS cells were transfected with GFP-GCAP1, YFP-GCAP2, and/or ROS-GC1 cDNAs. Seventy-two hours after being transfected, the cells were viewed directly (laser excitation at 531 nm for ZsYFP1 and 475 nm for AcGFP) or fixed and incubated with ROS-GC1 antibody followed by incubation with a secondary antibody conjugated with DyLight 649 (excitation at 652 nm). The cells were viewed using an inverted Olympus IX81 microscope/FV1000 spectral laser confocal system: (A) immunofluorescence of ROS-GC1 expressed alone, (B) fluorescence of GFP-GCAP1 expressed alone, (C) fluorescence of GFP-GCAP1 coexpressed with ROS-GC1, (D) immunofluorescence of ROS-GC1 coexpressed with GFP-GCAP1, (E) panels C and D merged, (F) fluorescence of YFP-GCAP2 expressed alone, (G) fluorescence of YFP-GCAP2 coexpressed with ROS-GC1, (H) immunofluorescence of ROS-GC1 coexpressed with YFP-GCAP2, and (I) of panels G and H merged. In panels G–I, the arrows indicate sites where the intensity of the GCAP2 fluorescence follows quantitatively the pattern of ROS-GC1 expression.

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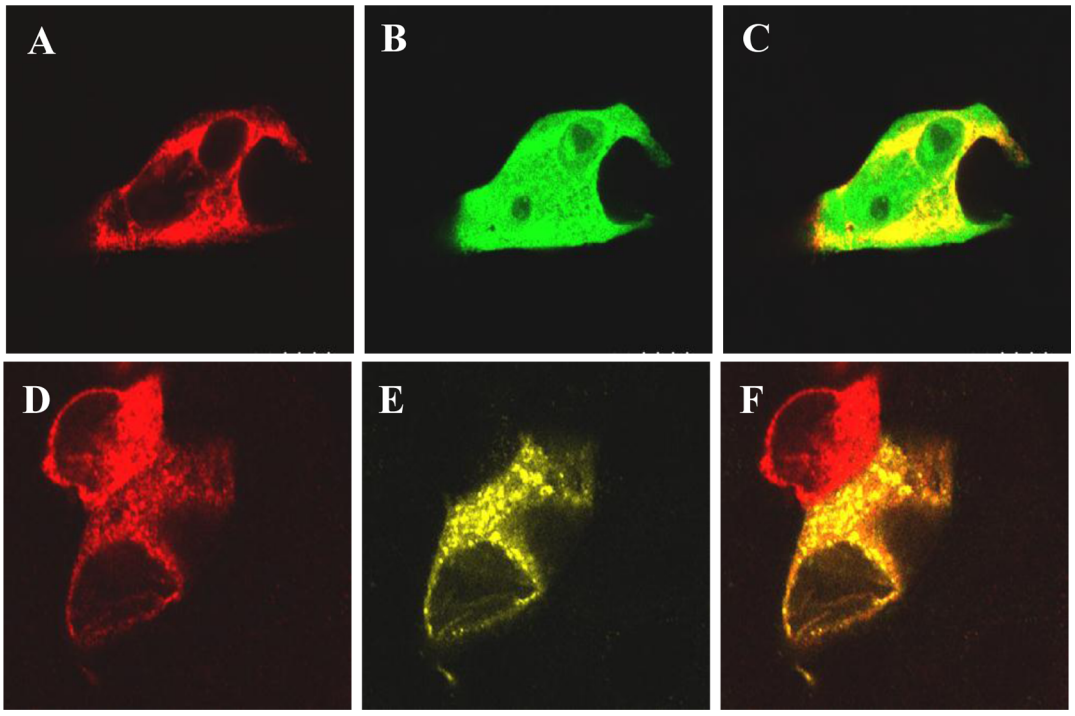

Figure 3. Kinase homology domain of ROS-GC1 that is obligatory for binding of GCAP1 but not GCAP2 to ROS-GC1. COS cells were cotransfected with the ROS-GC1-JMD-KHD$^-$ mutant and GFP-GCAP1 or YFP-GCAP2 cDNA. Seventy-two hours after being transfected, the cells were viewed as described in the legend of Figure 2 or fixed and incubated with the ROS-GC1 antibody and the secondary antibody conjugated with DyLight 649: (A) immunofluorescence of the JMD-KHD$^-$ mutant coexpressed with GFP-GCAP1, (B) fluorescence of GFP-GCAP1 coexpressed with the JMD-KHD$^-$ mutant, (C) panels A and B merged, (D) immunofluorescence of the JMD-KHD$^-$ mutant coexpressed with YFP-GCAP2, (E) fluorescence of YFP-GCAP2 coexpressed with the JMD-KHD$^-$ mutant, and (F) panels D and E merged.

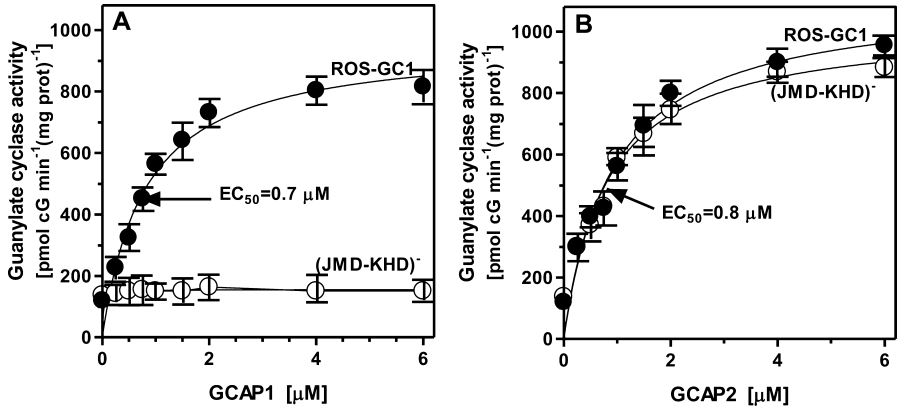

Figure 4. Role of the JMD and KHD in GCAP signaling of ROS-GC1 activity. wtROS-GC1 or its JMD-KHD$^-$ mutant was individually expressed in COS cells. Their membranes were prepared as described in Experimental Procedures and assayed for guanylate cyclase activity in the presence of 1 mM EGTA and the indicated concentrations of GCAP1 (A) or GCAP2 (B). The experiment was conducted in triplicate and repeated three times. The results presented (mean ± standard deviation) are from these experiments. The EC$_{50}$ values were determined graphically.

structural domain of ROS-GC1 but GCAP2 does not. The findings are in accord with the previous biochemical conclusion that the GCAP1 signal transduction site resides in the JMD.[10]

In the case of GCAP2, the structural JMD and KHD of ROS-GC1 have no signaling role.[9,18] The GCAP2 target site in ROS-GC1 resides outside the JMD and KHD; at the opposite end of the intracellular domain (ICD), it borders the catalytic domain (Figure 1). We therefore conclude that the trajectories of the GCAP1 and GCAP2 pathways are opposite and do not overlap; therefore, the signal transduction mechanisms of the two GCAPs are different and autonomous. This conclusion was verified biochemically by analyzing the combined effect of the two GCAPs on ROS-GC1 activity. In the presence of both GCAP1 and GCAP2, the stimulated ROS-GC1 activity was approximately equal to the sum of GCAP1- and GCAP2-dependent ROS-GC1 activities (Figure S2 of the Supporting Information). The additivity of GCAP1 and GCAP2 effects strongly supports the conclusion that each GCAP signals ROS-GC1 activation autonomously.

To bring these findings to the catalytic level, we transfected COS cells with the JMD-KHD$^-$ mutant and used recombinant ROS-GC1 as a control. The results are presented in Figure 4. In accordance with its well-established feature, $[Ca^{2+}]_i$-free GCAP1 and GCAP2 stimulate ROS-GC1 in a dose-dependent fashion with EC$_{50}$ values of 0.7 and 0.8 μM, respectively (Figure 4A for

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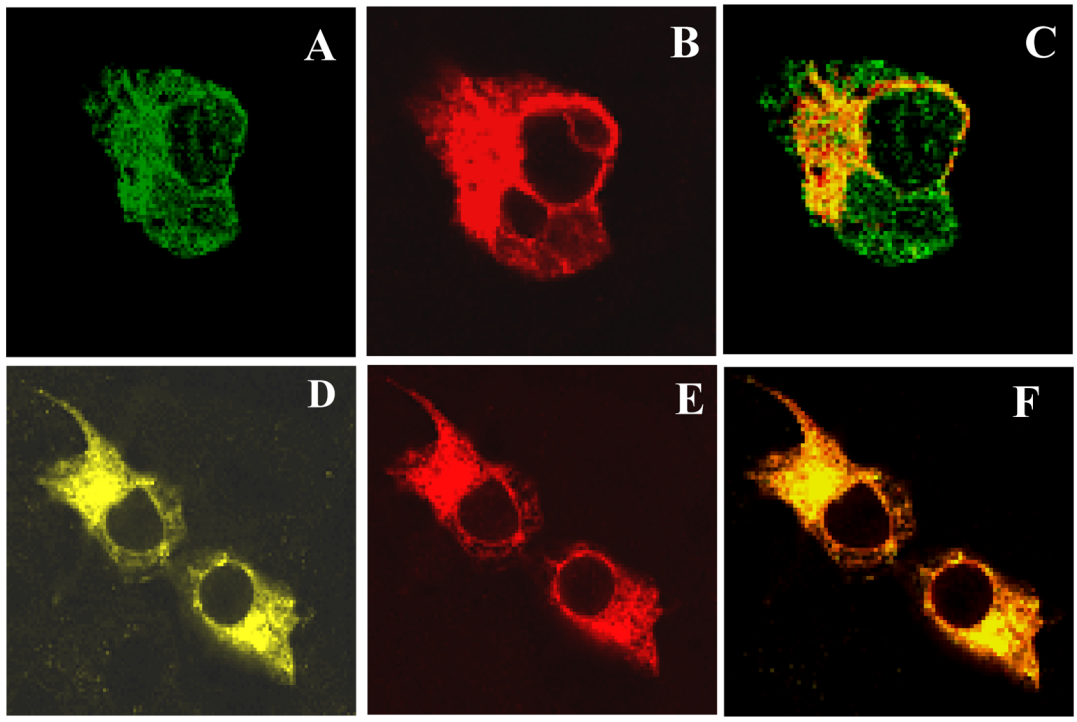

Figure 5. Signaling helix domain (SHD) of ROS-GC1 that is not required for binding of GCAP to ROS-GC1. COS cells were cotransfected with the ROS-GC1-SHD$^-$ mutant and GFP-GCAP1 or YFP-GCAP2 cDNA. Seventy-two hours after being transfected, the cells were viewed as described in the legend of Figure 2 or fixed and incubated with the ROS-GC1 antibody and the secondary antibody conjugated with DyLight 649: (A) immunofluorescence of the SHD$^-$ mutant coexpressed with GFP-GCAP1, (B) fluorescence of GFP-GCAP1 coexpressed with the SHD$^-$ mutant, (C) panels A and B merged, (D) immunofluorescence of the SHD$^-$ mutant coexpressed with YFP-GCAP2, (E) fluorescence of YFP-GCAP2 coexpressed with the SHD$^-$ mutant, and (F) panels D and E merged.

GCAP1 and Figure 4B for GCAP2). However, the JMD-KHD$^-$ mutant responds differently to the two GCAPs. It is totally unresponsive to GCAP1 (Figure 4A), but its response to GCAP2 is hardly different from the response of wtROS-GC1 (Figure 4B). Of the latter, identical kinetics of activation are confirmed by the calculated values of the Hill coefficient, 2.4 ± 0.2 for wtROS-GC and 2.1 ± 0.2 for the mutant. These results demonstrate that in accordance with the previously established binding and transduction sites,[10] GCAP1 signaling of ROS-GC1 begins at the JMD of ROS-GC1. Because it is absent in the mutant, the signal cannot be initiated and transmitted to the catalytic domain for its translation into the accelerated production of cyclic GMP. However, because the GCAP2 signal starts beyond the JMD and KHD,[9] this domain has no role in transmitting this signal to the catalytic domain of ROS-GC1 for its translation into the production of cyclic GMP.

In the Second Part of the Hypothesis, the Structural Role of the Signaling Helix Domain (SHD) (Figure 1) in the GCAP Signaling of ROS-GC1 Was Investigated. While marking the precise boundaries of the KHD and core catalytic domain (CCD), wedged between them a domain comprising residues L^{770}–P^{807} was found in ROS-GC1.[19] A similar domain in the ANF-RGC membrane guanylate cyclase was previously biochemically defined and termed the dimerization domain (DD), meaning its role is to keep the two catalytic subunits in the dimeric form, and it was proposed that the dimeric form is obligatory for the catalytic activity of the guanylate cyclase.[20] A later study indicated, however, that the L^{770}–P^{807} domain in ROS-GC1 is not required for the dimeric state and for the catalytic core CCD activity.[21] In addition, a systematic sequence analysis of the global signaling proteins revealed a general five-heptad conserved structure, which was universally present between two signaling domains.[19] The characteristics of this structure were that it is helical and that it bridges the preceding KHD and the following catalytic domains and in this manner functionally connects them. The authors named this domain the SHD (signaling helix domain).[19] The L^{770}–P^{807} structure of ROS-GC1 was 57.1% homologous with the DD of ANF-RGC and ∼50% homologous with the predicted SHD helical structural element.[19] Structural analysis indicated that it is composed of five heptads, its pI is 4.60, and its secondary structure conforms to the $\alpha\beta$ turns and coiled coil conformation. For this reason, we have named the L^{770}–P^{807} domain of ROS-GC1 as the SHD, and according to its postulated function,[19] it bridges the KHD and CCD (Figure 1) and functions as a transmitter station for CCD activation.

To assess if the bridging criteria described above are met for the SHD in the two GCAPs signaling of ROS-GC1, immunofluorescence studies were conducted. The expectation was that because the SHD does not harbor the binding site for any of the GCAPs, both GCAPs should bind ROS-GC1, yet because the trajectory of the GCAP1 pathway is downstream and requires its passage through SHD, the signaling of CCD would be disrupted if SHD is absent and is required for GCAP1 signaling activity. The reverse would be the case with the GCAP2 signaling of CCD: the GCAP2 pathway does not pass through SHD, and it should stay intact. However, the events involved in binding of both GCAPs to their ROS-GC1 domains should remain intact.

To assess this hypothesis, we cotransfected the COS cells with the ROS-GC1 mutant lacking the SHD (SHD$^-$) and GFP-GCAP1 or YFP-GCAP2 cDNA. The results are presented in Figure 5. In both cases, when GCAP1 or GCAP2 is coexpressed with the SHD$^-$ mutant, they are membrane-bound proteins

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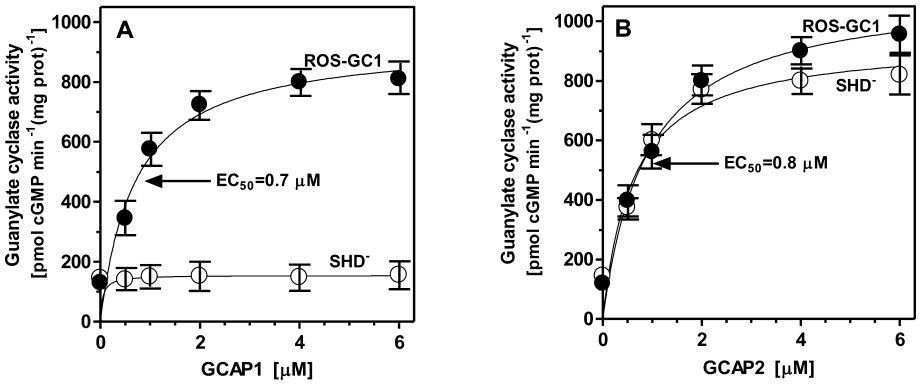

Figure 6. Role of the SHD in GCAP signaling of ROS-GC1 activity. wtROS-GC1 or its SHD$^-$ mutant was individually expressed in COS cells. Their membranes were prepared as described in Experimental Procedures and assayed for guanylate cyclase activity in the presence of 1 mM EGTA and the indicated concentrations of GCAP1 (A) or GCAP2 (B). The experiment was conducted in triplicate and repeated three times. The results presented (mean ± standard deviation) are from these experiments. The EC_{50} values were determined graphically.

copresent with the mutant (Figure 5). The green fluorescence emitted by GCAP1 (Figure 5A) merges with the red fluorescence emitted by the mutant guanylate cyclase (Figure 5B), resulting in the merged yellow fluorescence (Figure 5C). Similarly, the yellow fluorescence emitted by GCAP2 (Figure 5D) merges with the red fluorescence emitted by ROS-GC mutant (Figure 5E), resulting in the merged orange fluorescence (Figure 5F).

These results demonstrate that the SHD has no influence on the events involved in initiating the signals for both GCAPs: in the presence of ROS-GC1, both GCAPs are bound to ROS-GC in the membrane. Note that in the absence of ROS-GC1 they are soluble proteins.

Does SHD then have a role in transmission of the GCAPs signals to the CCD?

To answer this question, the membranes of the SHD$^-$ mutant expressed in COS cells were analyzed for their responses to the increasing concentrations of GCAP1 and GCAP2 in the absence of Ca^{2+} (Figure 6). In contrast to wt-ROS-GC1, the mutant was totally unresponsive to GCAP1 at all the concentrations tested (0–6 μM) (Figure 6A). However, the opposite was the case with the GCAP2 signaling of the mutant ROS-GC1. Like wtROS-GC1, the mutant responded almost identically to the GCAP2 signaling in a dose-dependent fashion (Figure 6B). The EC_{50} values of GCAP2 for both the wild-type and mutant guanylate cyclases were identical, ~0.8 μM; the V_{max} was 2 μM, and the saturation activity of the mutant was 80% of that of wild-type ROS-GC1. Furthermore, the calculated Hill coefficients of both wtROS-GC and the mutant were almost identical, 2.4 ± 0.2 and 2.1 ± 0.2, respectively, demonstrating their identical kinetic profiles. We therefore conclude that for the catalytic activation of ROS-GC1 the SHD is, indeed, the signal-transmitting center of GCAP1 but not of GCAP2.

The CCD in Vivo Exists in the Dimeric Antiparallel Conformation. With the information that the isolated form of CCD exists in the dimeric form and that this form has intrinsic catalytic activity, the role of the SHD in maintaining the secondary structure described above and the catalytic activity of CCD was ruled out.[21] Incorporating this information into the protein-based homology domain modeling the three-dimensional (3D) structure of the CCD showed that conformation of the ROS-GC1 CCD homodimer is antiparallel.[21] To validate this conformational state in vivo, we used the bimolecular fluorescence complementation (BiFC) technique.

The principle of BiFC is that the two proteins that are expected to interact with each other are fused to complementary fragments of a fluorescent reporter protein and expressed in live cells. Interaction of these proteins brings the fluorescent fragments into the proximity of each other, allowing emission of a fluorescent signal.[22,23] The complementary fragments of the fluorescent protein do not fluoresce by themselves.

The N-terminal half of the yellow fluorescent protein [(N-YFP), amino acids 1–158] in the pcDNA1/Amp vector was fused to the N-terminus of the ROS-GC1 CCD, M^{816}–Y^{965}. This resulted in the N-YFP-CCD construct. The C-terminal half of the YFP [(C-YFP), amino acids 159–238] in the pcDNA1/Amp vector was fused to the C-terminus of the CCD. This resulted in CCD-C-YFP. It is noteworthy that the constructs were designed so that the fluorescence will be emitted only if the CCD dimerizes in the antiparallel orientation.

These two constructs were coexpressed in COS cells and analyzed for the fluorescence under a confocal microscope. The results are presented in Figure 7.

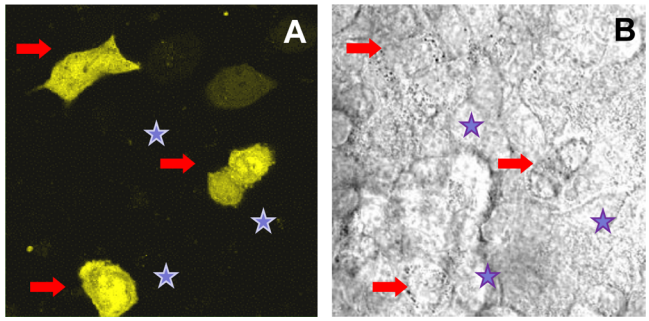

Figure 7. Isolated catalytic domain of ROS-GC1 forms spontaneously the antiparallel homodimer. YFP(1–158)-CCD and CCD-YFP(159–238)STOP constructs were prepared as described in Experimental Procedures. The constructs were coexpressed in COS cells. Seventy-two hours after being transfected, the cells were viewed directly under a confocal microscope (excitation at 531 nm and emission at 540 nm). (A) Fluorescence of the transfected cells. (B) Differential interference contrast image of the cells. The red arrows point to the cotransfected cells that fluoresce due to the presence of the antiparallel homodimer. The purple stars denote some of the untransfected cells or cells transfected with only one construct that do not fluoresce.

The selected cells emit yellow fluorescence (red arrows). The fluorescence is spread throughout the cells, a marked feature

dx.doi.org/10.1021/bi300572w | *Biochemistry* 2012, 51, 4650–4657

reflecting the characteristic of a soluble protein when present in a heterologous cell.[14,17] Importantly, the untransfected cells or the cells transfected with only one construct do not emit any fluorescence (marked with purple stars). No comparable fluorescence was observed when N-YFP-CCD and C-YFP-CCD constructs were coexpressed in COS cells, indicating that the CCD does not form parallel homodimers or that their formation is minimal, below the detection level. We therefore conclude that in vivo the isolated CCD of ROS-GC1 forms spontaneously a homodimer in which the two monomers are in an antiparallel orientation (Figure 1).

DISCUSSION

The photoreceptor ROS-GC transduction system is a central Ca^{2+}-modulated component of the phototransduction machinery. Its striking feature is that its operation is stringently controlled by the concentration range of free Ca^{2+} from 20 to ~1000 nM in rods and cones. It does so through its remarkable structural design, reversing its operation from the "GCAP mode" to the "S100B mode" with the transition of the free Ca^{2+} concentration from values between 100 and 20 nM to values between 100 and 1000 nM (Figure 1 of ref 3). This study is a part of the overall goal of decoding the molecular principles by which Ca^{2+} concentration exhibits such stringent control over the ROS-GC transduction machinery.

It focuses on two GCAPs and demonstrates that at the molecular level GCAPs in vivo signal ROS-GC1 activation through different modes. Dissection of these modes in structural terms discloses that the GCAP2 signal transduction pathway is unique, never observed before for any member of the membrane guanylate cyclase family. Finally, the study shows that in vivo the CCD exists as a homodimer in which two of its subunits are bound in an antiparallel configuration. These topics are elaborated below.

GCAP1 signal transduction occurs through M^{445}–L^{456} and L^{503}–I^{522} subdomains of JMD[10] and GCAP2 signal transduction via the Y^{965}–N^{981} subdomain of CTE[9] (Figure 1). These subdomains are at the opposite ends of CCD, and they are the sites where the signal of the respective GCAPs originates. Prior to this study, nothing was known about their mechanisms of signal transduction. Also, there was a critical gap in the understanding: how can soluble proteins signal the membrane-bound ROS-GC?

This study solves this puzzle. In living cells, both GCAPs by themselves are, indeed, soluble proteins (Figure 2B,F). Only when they are present with the ROS-GC do they become membrane-bound, and importantly, they are bound to their specific sites on ROS-GC (Figure 3B,E). This suggests that ROS-GC is the one that bestows upon them the property of being membrane-bound; the anchoring sites are M^{445}–L^{456} and L^{503}–I^{522} for GCAP1 and Y^{965}–N^{981} for GCAP2.

If residues M^{445}–L^{456} and L^{503}–I^{522} define the GCAP1 anchor in ROS-GC1, structural disruption of this anchor should dislodge GCAP1 and also its functional characteristic of ROS-GC1 activation. The disruption, however, should not affect the GCAP2 anchor or its functional property. Are these assumptions correct?

The answer is yes. Deletion of the JMD and KHD results in the dissociation of ROS-GC1 from GCAP1. GCAP1 no longer binds to the dismantled structure of ROS-GC1 (Figure 3B). It becomes a soluble protein and loses its catalytic property of ROS-GC activation (Figure 4A). In contrast, GCAP2 remains ROS-GC1-bound (Figure 3E) and retains its property of being the ROS-GC1 activator (Figure 4B).

These results guide us to the conclusion that the two GCAPs signal ROS-GC1 activation through different modes and the differences reside in the spatial characteristics of ROS-GC1. Additionally, because orientations of the two domains of their signal origins are different, the results suggest that their signal migration pathways are also different: GCAP1 downstream from M^{445}–L^{456} and L^{503}–I^{522} to the P^{808}–K^{104} CCD site and GCAP2 upstream from Y^{965}–N^{981} to P^{808}–K^{104} (Figure 1).

To validate this assumption, we deleted the SHD of the ROS-GC. The choice for selection of this domain was based on three factors. (1) The SHD is downstream from the JMD, the site of the GCAP1 signal. (2) It immediately follows the JMD and KHD and, thus, represents the direction of the trajectory of the GCAP1 pathway. (3) Being on the opposite end of the pathway, the SHD will have no influence on the GCAP2 signaling of ROS-GC1.

In a manner consistent with the assumption, deletion of the SHD disrupts the GCAP1 signaling of ROS-GC1 (Figure 6A). Importantly, the deletion does not affect the modes of binding of GCAP1 to ROS-GC1: GCAP1 remains bound to ROS-GC1 (Figure 5). In the case of GCAP2, its signaling of ROS-GC1 stays intact: both its binding to ROS-GC1 (Figure 5) and its activation of the cyclase (Figure 6B). These results further support the conclusion that the two GCAP modes of ROS-GC1 signaling are different, as are their structural requirements, and strikingly, they reveal an intriguing signaling pathway, never observed before for the membrane guanylate cyclase family. This pathway is unique to GCAP2 and runs opposite to that of GCAP1, upstream from the Y^{965}–N^{981} site in CTE to the CCD for its translation into the accelerated production of cyclic GMP (Figure 1).

The final part of this investigation deals with the CCD, the common translation site for the GCAP1 and GCAP2 signals (Figure 1). The current model, based on biochemical analysis, is one in which CCD has intrinsic basal catalytic activity and in its isolated form exists as a homodimer.[21] Through molecular modeling, this secondary structure has been advanced to its 3D form, which reveals that its two subunits are in the antiparallel conformation.[21,24] This study using the BiFC technique shows that these conclusions are correct. In living cells, the CCD is present in dimeric form; the two subunits are fused in the antiparallel orientations.

In summary, this study demonstrates that the modes of operation of the two Ca^{2+}-modulated GCAP signaling pathways are different and defines these differences in molecular terms. The GCAP1 pathway is driven by $[Ca^{2+}]_i$ levels that are 1 order of magnitude higher than that of the GCAP2 pathway.[11] It is meant to detect dim light, while the GCAP2 pathway is meant to detect bright light. The following question awaits an answer. Are the same principles applicable to the other Ca^{2+}-modulated phototransduction-linked guanylate cyclase, ROS-GC2? We anticipate that application of these findings will have a direct impact in better explaining the physiology of phototransduction.

ASSOCIATED CONTENT

Supporting Information

Western blot showing that GCAP1 and GCAP2 expressed alone in COS cells are soluble proteins (Figure S1) and additive effects of GCAP1 and GCAP2 on ROS-GC1 activity (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

The study was supported by National Heart, Lung and Blood Institute Grants HL084584 and S82701 and by the Pennsylvania Lions Sight Conservation and Eye Research Foundation.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thankfully acknowledge Dr. C. Berlot (Weis Center for Research, Geisinger Clinic, Danville, PA) for her generosity in providing the BiFC vectors [YFP(1−158)/pcDNA1/Amp, YFP(159−238)/pcDNA1/Amp, YFP(1−158)STOP/pcDNA1/Amp, and YFP(159−238)STOP/pcDNA1/Amp] and the detailed protocols for preparing the expression constructs.

ABBREVIATIONS

CCD, core catalytic domain; GCAP, guanylate cyclase activating protein; GFP, green fluorescent protein; JMD, juxtamembrane domain; KHD, kinase homology domain; ROS-GC, rod outer segment guanylate cyclase; SHD, signaling helix domain; YFP, yellow fluorescent protein.

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