Differential Ca2+ Sensor Guanylate Cyclase Activating Protein Modes of Photoreceptor Rod Outer Segment Membrane Guanylate Cyclase Signaling

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Differential Ca^{2+}-Sensor GCAPs Modes of Photoreceptor ROS-GC1 Signaling

Teresa Duda[*], **Alexandre Pertzev**, and **Rameshwar K. Sharma**[*]
Research Divisions of Biochemistry and Molecular Biology, The Unit of Regulatory and Molecular Biology, Salus University, 8360 Old York Road, Elkins Park, PA 19027

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 nM to near 1000 nM range. Modulated by Ca^{2+} sensors GCAP1 and GCAP2, lowering of $[Ca^{2+}]_i$ from 200 to 20 nM progressively turns it "ON". Similarly, does the modulation by the Ca^{2+} sensor S100B, raising $[Ca^{2+}]_i$ 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* fluorescent spectroscopy and *in vitro* reconstitution experiments the present 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 the GCAP2 upstream. And, importantly, (3) the isolated catalytic domain, translating the GCAPs-modulated Ca^{2+} signal into the generation of cyclic GMP, *in vivo*, exists as a homodimer, the two subunits existing in the antiparallel conformation. Furthermore, the findings demonstrate that the N-terminally placed signaling helix domain (SHD) 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.

Keywords

ROS-GC1; Ca^{2+}; GCAPs; phototransduction; membrane guanylate cyclase

Introduction

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: Fig. 1 of reference 3). A steady influx of Na^+ and Ca^{2+} passes through the open channels and keeps the photoreceptor depolarized. Photons

Correspondence: R.K. Sharma, rsharma@salus.edu, phone: 215-780-3124, T. Duda tduda@salus.edu, phone: 215-780-3112.

"Supporting Information Available": 1) Western blot showing that GCAP1 and GCAP2 expressed alone in COS cells are soluble proteins (Figure S1); 2) 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.

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 fall in $[Ca^{2+}]_i$ in mammalian rods from 250 nM to about 20 nM [5]. Guanylate cyclase activating proteins (GCAPs: 1 and 2) sense the fall 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 current 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 (Fig. 12 of reference 7). The presence and functional linkage of ROS-GC1 with Ca^{2+}-sensor, S100B, has 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 $K_{1/2}$ 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 for 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], the present study solidifies and advances this concept by demonstrating *in vivo* that the two GCAP-modulated pathways of ROS-GC1 are dynamically different. Furthermore, the study for the first time discloses a new model of the membrane guanylate cyclase signaling; this model, applicable to the GCAP-modulated Ca^{2+} signaling of ROS-GC1, demonstrates that at the structural and the dynamic level the mode of GCAP1 signaling of the photoreceptor ROS-GC1 is opposite to that of GCAP2.

Experimental Procedures

Mutagenesis

ROS-GC1 mutants were constructed using 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 identically as the 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 coding for the core catalytic domain aa M^{816}-Y^{965} was amplified by PCR. A linker sequence (R-S-I-A-T) and a Bam*H1* recognition site were added at the 5’-end of the amplified cDNA and a Bgl*II* recognition site, at the 3’-end. The amplified product was digested with Bam*H1* and Bgl*II* and subcloned into the Bgl*II* site of the YFP(1-158)/pcDNA1/Amp vector. To construct the CCD-C-YFP(159-238) vector the same as for the N-YFP(1-158)-CCD construct ROS-GC1 cDNA fragment was amplified except that the R-S-I-A-T linker sequence was added to the 3’-end. The Bam*H1* and Bgl*II* recognition sites were added at the 5’- and 3’- ends, respectively. The amplified product was digested with Bam*H1* and Bgl*II* and subcloned into the Bam*H1* 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) were constructed. The constructs were verified by sequencing.

Expression in COS cells

COS-7 cells were transfected with appropriate expression constructs using calcium-phosphate coprecipitation technique[15]. Sixty hours after transfection, the cells were harvested and their membranes 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 radioimmunoassay[16]. All experiments were done 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. 72 hr after transfection 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 TBS/0.5% Triton X-100 (TTBS) for 1hr at room temperature, washed with TTBS, incubated with ROS-GC1 antibody (diluted 50:1) in blocking solution overnight at 4°C, washed with TTBS for and then incubated with DyLight 488-conjugated donkey anti-rabbit antibody (200:1) for 1 hr, 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 software.

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, 1 and 2, are bound to ROS-GC1 and they delicately control its Ca^{2+}-modulated activity. The mechanisms of their controls are ill defined. To understand these in the isolated fashions, the heterologous *in vivo* expression system of COS cells was used 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. Advantage was taken 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] (Fig. 1). The GCAP2 domain borders the C-terminal side of the catalytic domain and GCAP1 is juxtaposed to the transmembrane domain (Fig. 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) (Fig. 1) in the GCAPs signaling of ROS-GC1 was investigated.

COS cells were co-transfected 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 co-transfected with these individual GCAPs and the wild type (wt)ROS-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 (Fig. 2A); (2) when expressed alone both GCAPs are soluble proteins (Fig. 2B and

2F): their expression is observed throughout the entire cell including the nucleus [that the GCAPs expressed alone exist as soluble proteins was verified by analyzing the cytosolic fraction of the transfected cells by Western blot using anti GCAP1 and anti GCAP2 antibodies (Supplemental figure 1)]; (3) when co-expressed with wtROS-GC1, both GCAPs migrate to the membrane and target their specific sites on ROS-GC1 which act as the anchors (Figures 2C and 2E for GCAP1 and 2G and 2I for GCAP2). The anchoring of GCAPs by ROS-GC1 is clearly visible in figures 2G, 2H and 2I. The intensity of GCAP2 fluorescence follows the pattern of ROS-GC1 expression (indicated by arrows). However, when co-expressed with JMD-KHD$^-$ mutant of ROS-GC1, the two GCAPs behave differently. The fluorescence of GFP-GCAP1 is scattered all over the cell (Fig. 3B). Its pattern is identical to that when GFP-GCAP1 is expressed alone (compare figures 3B with 2B), and differs completely from the pattern of JMD-KHD$^-$ mutant immunofluorescence (Fig. 3A). Although some yellow color after merging images 3A and 3B 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 co-expressed with JMD-KHD$^-$ mutant does not migrate to the membrane indicates that it lost its anchoring site therefore, remains as the soluble protein in the cell. In contrast, the fluorescence of YFP-GCAP2 is observed in the cellular membranes (Fig. 3E), it follows the red immunofluorescence of the JMD-KHD- mutant (Fig. 3D) and merges with it (Fig. 3F) indicating that 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 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 GCAP2 case, JMD-KHD structural domains of ROS-GC1 have no signaling role[9, 18]. The GCAP2 target site in ROS-GC1 resides outside the JMD-KHD domains; at the opposite end of the intracellular domain (ICD) it borders the catalytic domain (Fig. 1). It is therefore concluded that the trajectory of the GCAP1 and GCAP2 pathways are opposite, they do not overlap and, thereby, 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 (Supplemental figure 2). The additiveness 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, COS cells were transfected with JMD-KHD$^-$mutant and recombinant ROS-GC1 served 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 the respective EC_{50} values of 0.7 and 0.8 μM (Fig. 4A for GCAP1 and 4B for GCAP2). However, the JMD-KHD$^-$ mutant responds differently to the two GCAPs. It is totally unresponsive to GCAP1 (Fig. 4A) but its response to GCAP2 is hardly different from the response of the wtROS-GC1 (Fig. 4B). Of the latter, identical kinetics of activation, are confirmed by the calculated values of Hill's coefficients, 2.4 ±0.2 for the 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. Since 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 GCAP2 signal starts beyond the JMD-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) (Fig. 1) in the GCAPs 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 the 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 as 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 5-heptad conserved structure, which was universally present between two signaling domains[19]. The characteristic of this structure was that it is helical, that it bridges the preceding KHD and the following catalytic domains, and in this manner functionally connects them. The authors named this domain SHD (Signaling Helix Domain)[19]. The L^{770}-P^{807} structure of the ROS-GC1 showed 57.1 % homology with the DD domain of ANF-RGC and about 50% homology 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 αβ turns and coiled coil conformation. For this reason, the present authors have named L^{770}-P^{807} domain of ROS-GC1 as the SHD domain; and according to its postulated function[19], it bridges the KHD and CCD domains (Fig. 1) and functions as a transmitter station for CCD activation.

To assess if the above bridging criteria are met for the SHD domain in the two GCAPs signaling of ROS-GC1, immunofluorescent 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 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 the 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, the COS cells were co-transfected with the ROS-GC1 mutant lacking the SHD (SHD^-) and GFP-GCAP1 or YFP-GCAP2 cDNAs. The results are presented in figure 5. In both cases when GCAP1 or GCAP2 is co-expressed with the SHD^- mutant, they are membrane-bound proteins co-present with the mutant (Fig. 5). The green fluorescence emitted by GCAP1 (Fig. 5A) merges with that of the red emitted by the mutant guanylate cyclase (Fig. 5B), resulting in the merged yellow fluorescence (Fig. 5C). Similarly, the yellow fluorescence emitted by GCAP2 (Fig. 5D) merges with that of the red emitted by ROS-GC mutant (Fig. 5E), resulting in the merged orange fluorescence (Fig. 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 the ROS-GC in the membrane. Note: in absence of the ROS-GC1 they are soluble proteins.

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

To solve 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+} (Fig. 6). In contrast to the wt-ROS-GC1, the mutant was totally unresponsive to GCAP1 at all its concentrations tested (0-6 μM) (Fig. 6A). However, opposite was the case with the GCAP2 signaling of the mutant ROS-GC1. Like, the wtROS-GC1, the mutant responded almost identically to the GCAP2 signaling in a dose-dependent

fashion (Fig. 6B). The EC_{50} values of the GCAP2 for both the wt- and the mutant guanylate cyclases were identical, about 0.8 μM, V_{max} was achieved at 2 μM and the saturation activity of the mutant was 80% to that of the wild-type ROS-GC1. Furthermore, the calculated Hill coefficients of both the wtROS-GC and the mutant were almost identical, respectively, 2.4±0.2 and 2.1±0.2, demonstrating their identical kinetic profiles. It is therefore concluded that for the catalytic activation of ROS-GC1 the SHD domain 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 SHD domain role in maintaining the above secondary structure and the catalytic activity of CCD was ruled out[21]. Incorporating this information into the protein-based homology domain modeling the 3D-structure of the CCD domain showed that conformation of the ROS-GC1 CCD homodimer is antiparallel[21]. To validate this conformational state *in vivo*, the bimolecular fluorescence complementation (BiFC) technique was used.

Principle of the 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 within proximity, 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); aa 1-158] in 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); aa 159-238] in the pcDNA1/Amp vector was fused to the C-terminus of the CCD. This resulted in the CCD-C-YFP. It is noteworthy that the constructs were designed that the fluorescence will be emitted only if the CCD domain dimerizes in the antiparallel orientation.

These two constructs were co-expressed in COS cells and analyzed for the fluorescence under confocal microscope. The results are presented in figure 7.

The selected cells emit yellow fluorescence (red arrows). The fluorescence is spread throughout the entire cells, a marked feature 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 co-expressed in COS cells indicating that the CCD does not form parallel homodimers or that their formation is minimal, below the detection level. It is therefore concluded that *in vivo* the isolated CCD of ROS-GC1 forms spontaneously a homodimer in which the two monomers are in antiparallel orientation (Fig. 1).

Discussion

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 20 nM to near 1000 nM range of free [Ca^{2+}] in rods and cones. It does so through its remarkable structural design, reversing its operation from “GCAP mode” to the “S100B mode” with transition of free [Ca^{2+}] range between 100 nM to 20 nM to between 100 to 1000 nM (Fig. 1 of reference 3). The present study is a part of the overall goal to decode the molecular principles by which [Ca^{2+}] exhibits such a 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 domain exists as a homodimer where 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 of GCAP2 via the subdomain Y^{965}-N^{981} of CTE[9] (Fig. 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 the present study nothing was known about their mechanisms of signal transduction. Also, there was a critical gap in the understanding: that being soluble proteins, how they can signal the membrane bound ROS-GC?

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

If the 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 and its functional property. Are these assumptions correct?

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

These results guide to the conclusion that the two GCAPs signal ROS-GC1 activation through different modes and the differences reside in the spatial characteristic 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: the GCAP1 downstream from M^{445}-L^{456} and L^{503}-I^{522} to P^{808}-K^{104} CCD site, and the GCAP2 upstream from Y^{965}-N^{981} to P^{808}-K^{104} (Fig. 1).

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

In keeping with the assumption, SHD deletion disrupts the GCAP1 signaling of ROS-GC1 (Fig. 6A). Importantly, the deletion does not affect the GCAP1 binding modes to ROS-GC1: GCAP1 remains bound to ROS-GC1 (Fig. 5). In the case of GCAP2, its signaling of ROS-GC1 stays intact: both its binding to ROS-GC1 (Fig. 5) and its activation of the cyclase (Fig. 6B). These results further attest to the conclusion that the two GCAPs modes of ROS-GC1 signaling are different, so 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 the GCAP2 and runs opposite to that of GCAP1, upstream

from Y^{965}-N^{981} site in CTE to CCD for its translation into the accelerated production of cyclic GMP (Fig. 1).

Final part of the present investigation deals with CCD, the common translation site for the GCAP1 and GCAP2 signals (Fig. 1). The current model, based on biochemical analysis, is that CCD has intrinsic basal catalytic activity; and in its isolated form it exists as a homodimeric[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]. The present study using BIFC technique shows that the above conclusions are correct. In living cells, CCD is present in dimeric form; the two subunits are fused in the antiparallel orientations.

In summary, the present study has demonstrated that operation of the two Ca^{2+}-modulated GCAP signaling pathways are different and it has defined these differences in molecular terms. GCAP1 pathway is driven by $[Ca^{2+}]_i$ levels which are one order of magnitude higher than the GCAP2 pathway[11]. It is meant to detect dim light while the GCAP2 pathway is meant to detect bright light. The immediate question awaiting answer is: Are the same principles applicable to the other Ca^{2+}-modulated phototransduction-linked guanylate cyclase ROS-GC2? It is anticipated that application of these findings will have a direct impact in better explaining the physiology of phototransduction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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

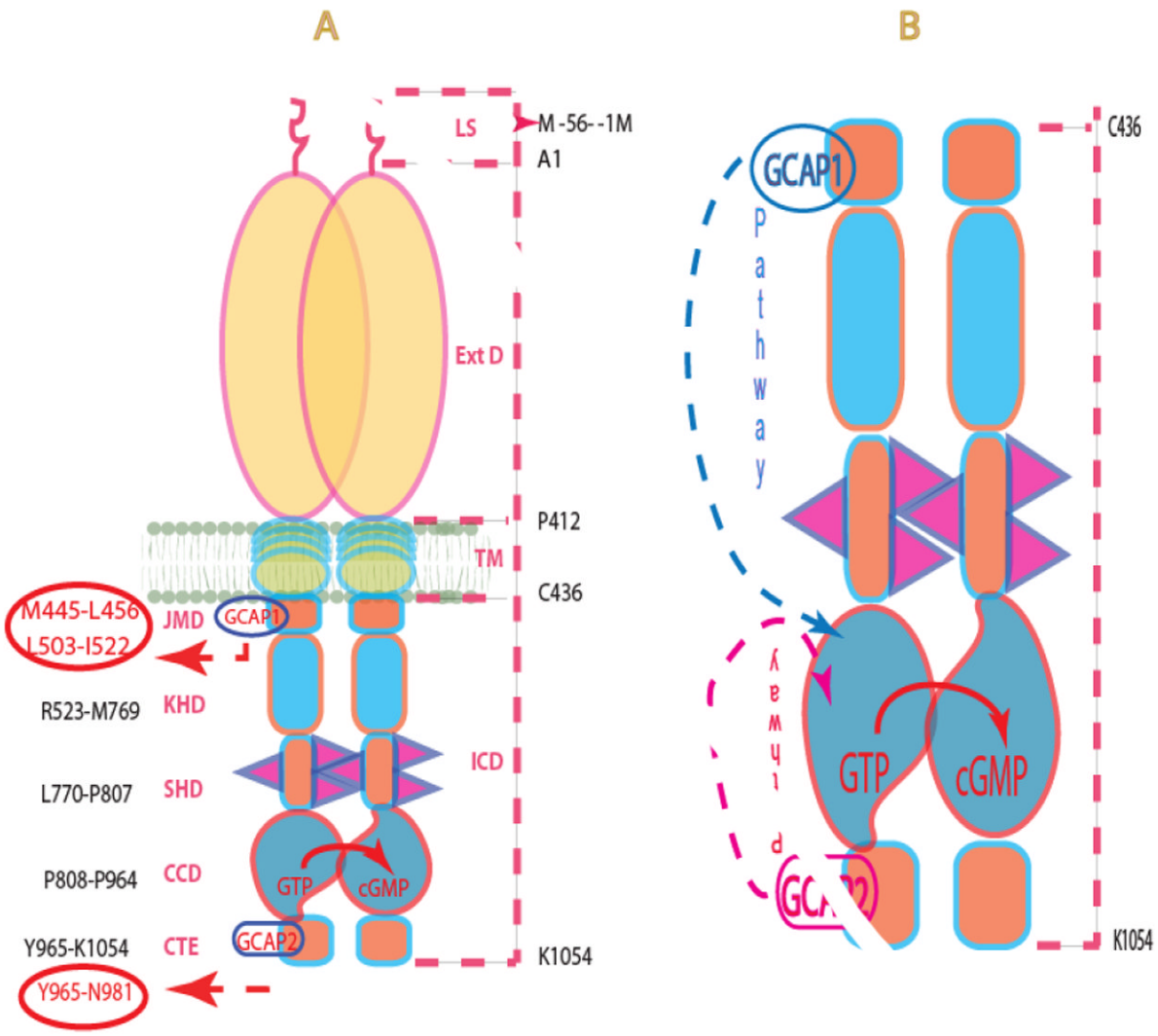

Figure 1. (A) Schematic representation of the structural topography of ROS-GC1

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 ICD, 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 in blue dashed arrow is downstream. From its origin in the JMD, it passes through the structural domains of KHD and SHD in its course to CCD. In contrast, the trajectory of the GCAP2 pathway (shown in pink dashed arrow) is upstream. From its origin in the CTE, it directly flows to 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.

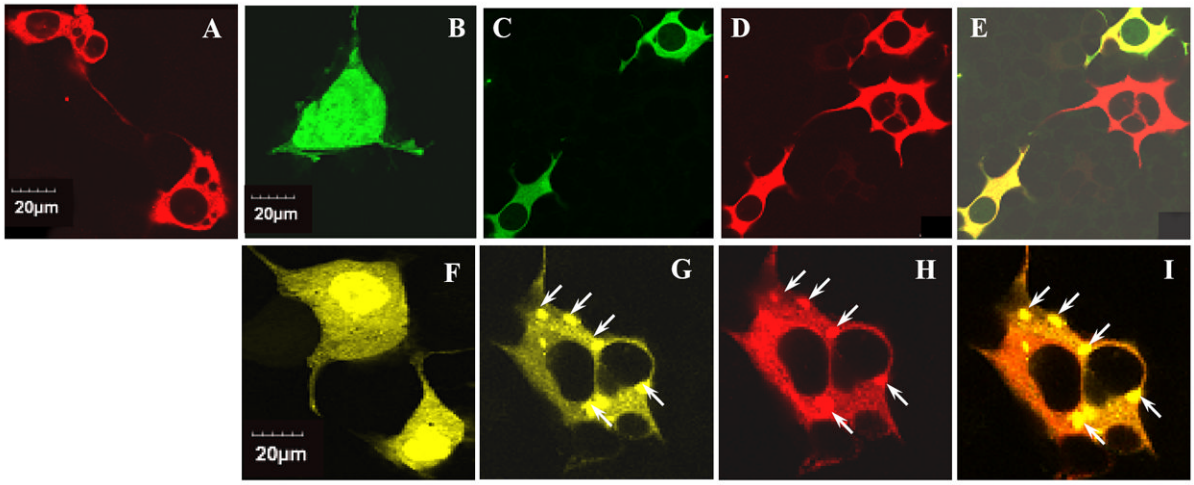

Figure 2. GCAP1 and GCAP2 bind ROS-GC1 in COS cells

COS cells were transfected with GFP-GCAP1, YFP-GCAP2 or/and ROS-GC1 cDNAs. 72 hr after transfection the cells were viewed directly (laser excitation 531 nm for ZsYFP1 and 475 nm for AcGFP) or fixed and incubated with ROS-GC1 antibody followed by the incubation with secondary antibody conjugated with DyLight 649 (excitation at 652 nm). The cells were viewed using 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-GCAP1co-expressed with ROS-GC1 **(D)** Immunofluorescence of ROS-GC1 co-expressed with GFP-GCAP1. **(E)** merge of (C) and (D). **(F)** Fluorescence of YFP-GCAP2 expressed alone. **(G)** Fluorescence of YFP-GCAP2 co-expressed with ROS-GC1. **(H)** Immunofluorescence of ROS-GC1 co-expressed with YFP-GCAP2. **(I)** merge of (G) and (H). In figures G, H, and I the arrows indicate sites where the intensity of GCAP2 fluorescence follows quantitatively the pattern of ROS-GC1 expression.

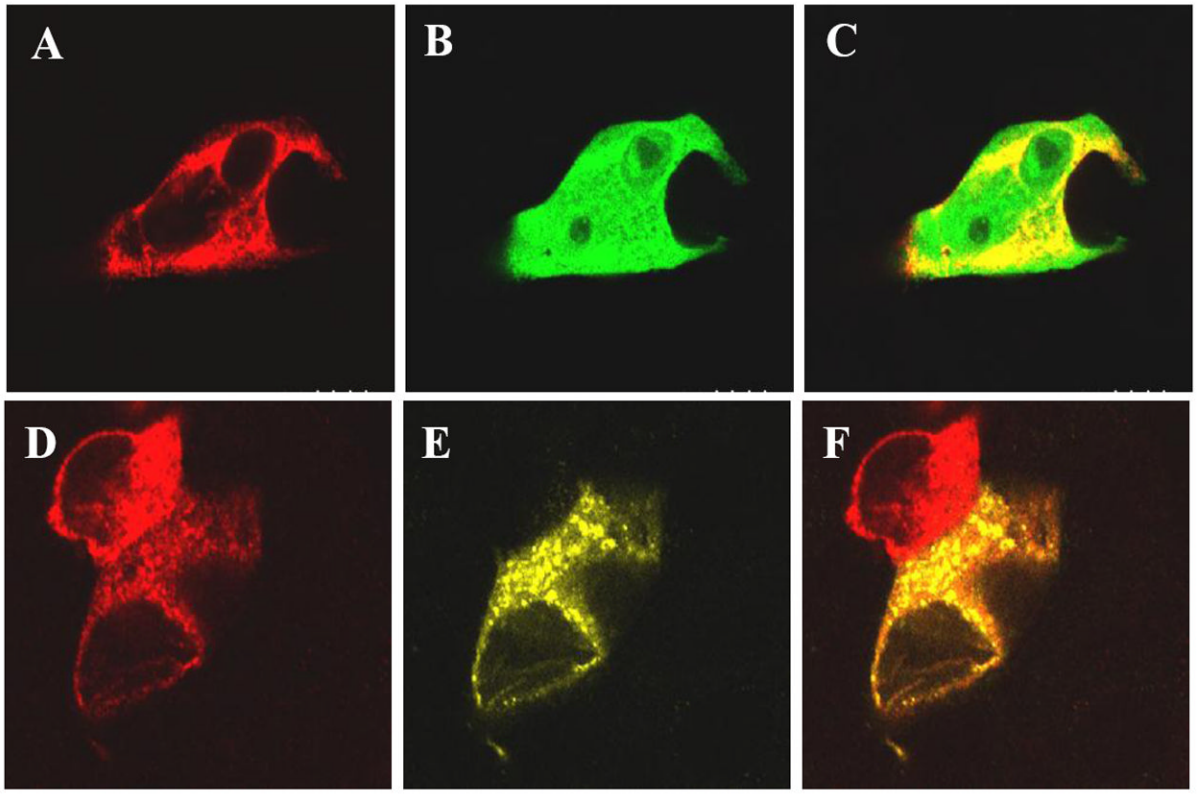

Figure 3. The kinase homology domain of ROS-GC1 is obligatory for GCAP1 but not GCAP2 binding to ROS-GC1

COS cells were co-transfected with the ROS-GC1-JMD-KHD$^-$ mutant and GFP-GCAP1 or YFP-GCAP2 cDNAs. 72 hr after transfection the cells were viewed as described in the figure 2 legend or fixed and incubated with the ROS-GC1 antibody and the secondary antibody conjugated with DyLight 649. **(A)** Immunofluorescence of the JMD-KHD$^-$ mutant co-expressed with GFP-GCAP1. **(B)** Fluorescence of GFP-GCAP1 co-expressed with the JMD-KHD$^-$ mutant. **(C)** merge of (A) and (B). **(D)** Immunofluorescence of the JMD-KHD$^-$ mutant co-expressed with YFP-GCAP2. **(E)** Fluorescence of YFP-GCAP2 co-expressed with the JMD-KHD$^-$ mutant. **(F)** merge of (D) and (E).

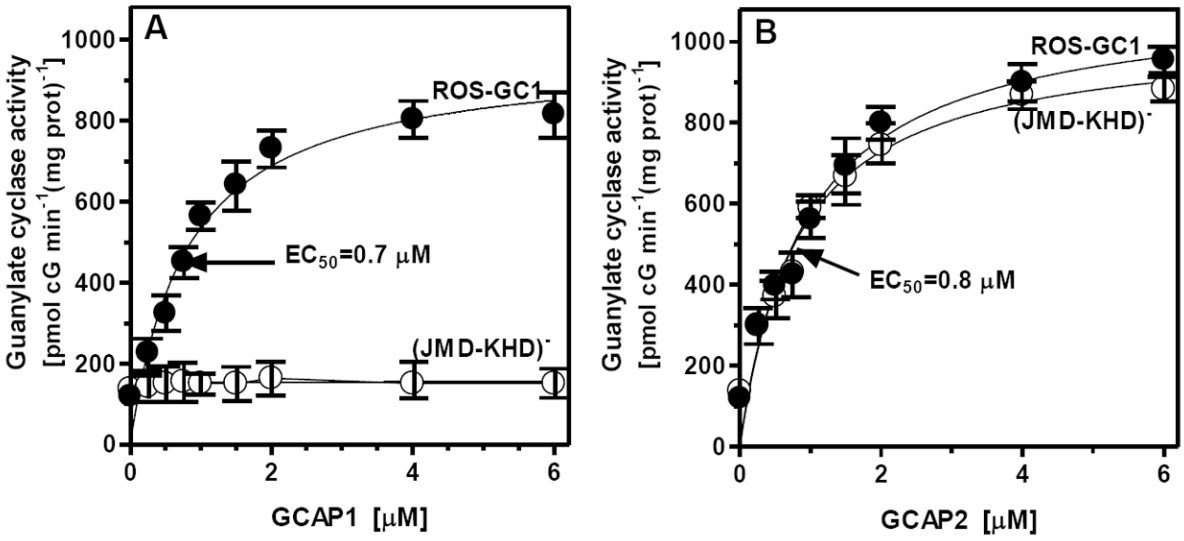

Figure 4. Role of the JMD-KHD in GCAP signaling of ROS-GC1 activity

WtROS-GC1 or its JMD-KHD$^-$ mutant were 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 done in triplicate and repeated three times. The results presented (mean±SD) are from these experiments. The EC_{50} values were determined graphically.

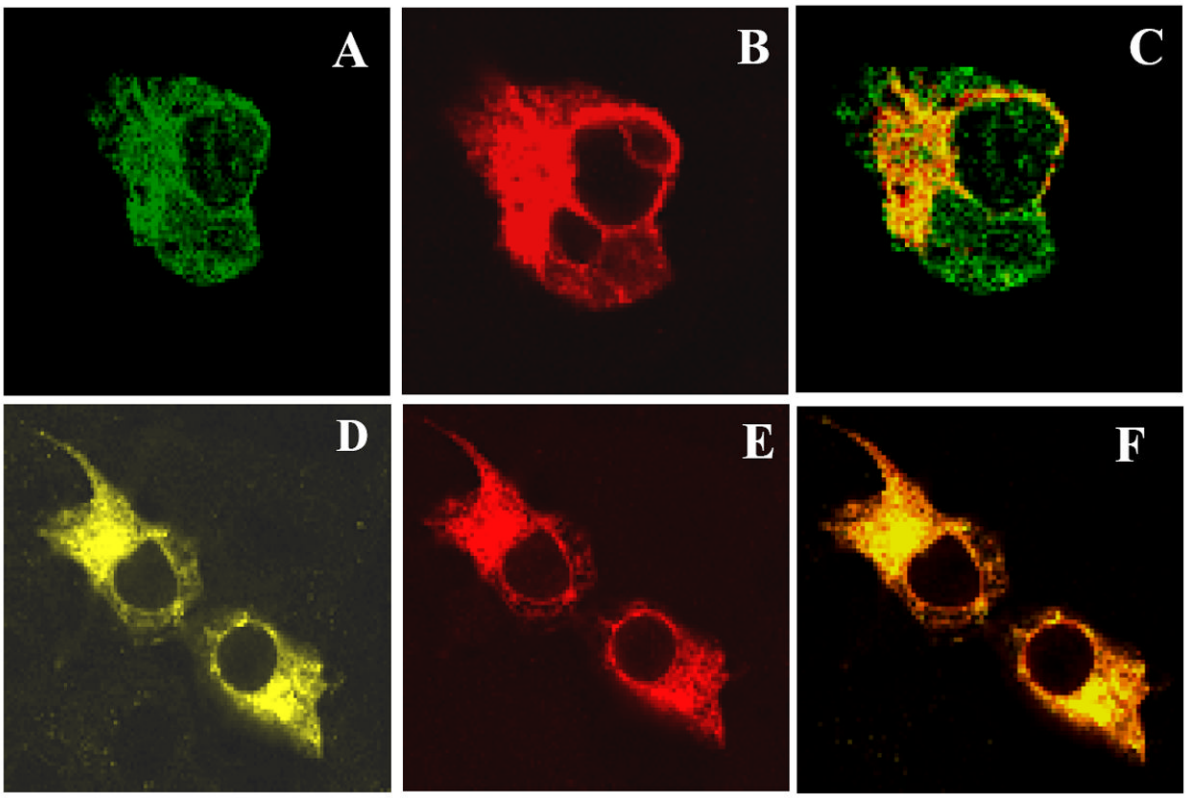

Figure 5. The signaling helix domain (SHD) of ROS-GC1 is not required for GCAP binding to ROS-GC1

COS cells were co-transfected with the ROS-GC1-SHD$^-$ mutant and GFP-GCAP1 or YFP-GCAP2 cDNAs. 72 hr after transfection the cells were viewed as described in the figure 2 legend or fixed and incubated with the ROS-GC1 antibody and the secondary antibody conjugated with DyLight 649. **(A)** Immunofluorescence of the SHD$^-$ mutant co-expressed with GFP-GCAP1. **(B)** Fluorescence of GFP-GCAP1 co-expressed with the SHD$^-$ mutant. **(C)** merge of (A) and (B). **(D)** Immunofluorescence of the SHD$^-$ mutant co-expressed with YFP-GCAP2. **(E)** Fluorescence of YFP-GCAP2 co-expressed with the SHD$^-$ mutant. **(F)** merge of (D) and (E).

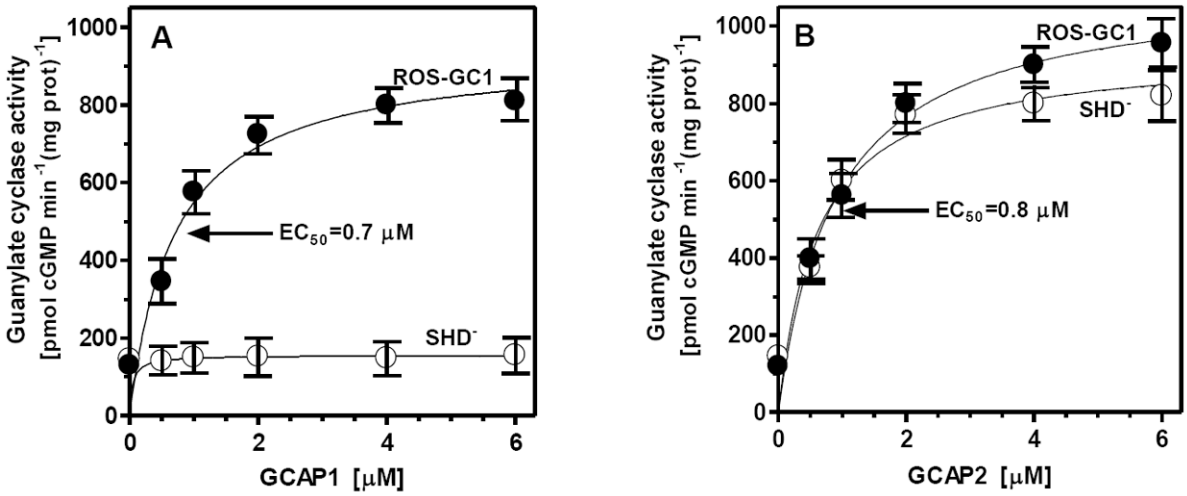

Figure 6. Role of the SHD in GCAP signaling of ROS-GC1 activity

WtROS-GC1 or its SHD$^-$ mutant were 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 done in triplicate and repeated three times. The results presented (mean±SD) are from these experiments. The EC_{50} values were determined graphically.

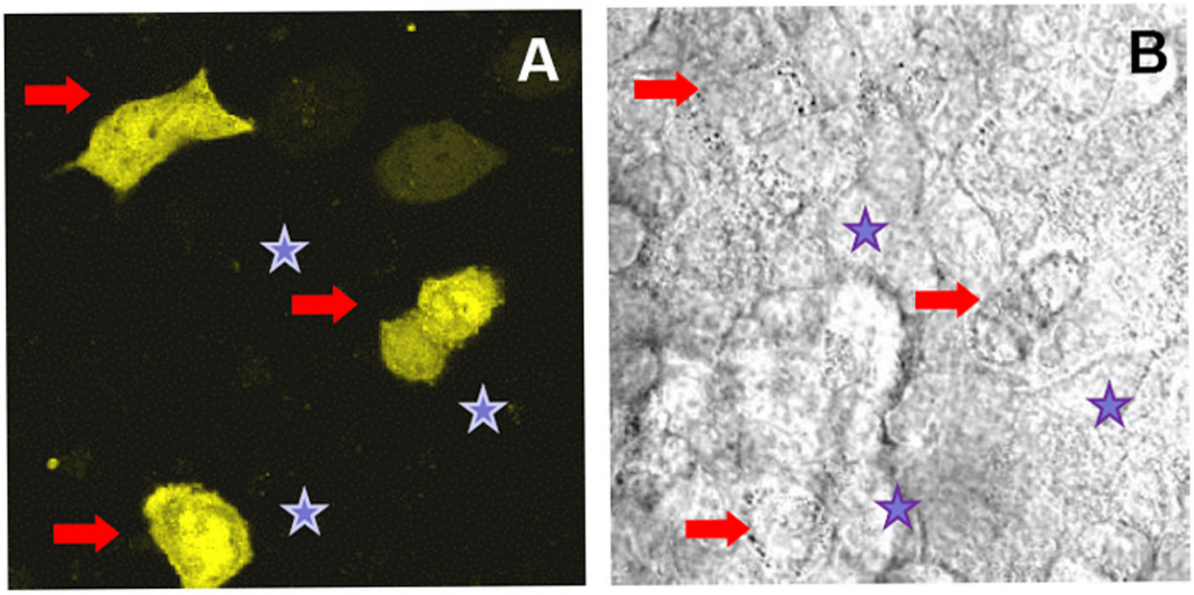

Figure 7. Isolated catalytic domain of ROS-GC1 forms spontaneously antiparallel homodimer

YFP(1-158)-CAT and CAT-YFP(159-238)STOP constructs were prepared as described in Experimental Procedures. The constructs were co-expressed in COS cells. 72 hr after transfection the cells were viewed directly under 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 co-transfected cells which fluoresce due to the presence of antiparallel homodimer. The purple stars point to some of the untransfected cells or cells transfected with only one construct which do not fluoresce.