Accelerated Publications

Calcium Modulation of Bovine Photoreceptor Guanylate Cyclase[†]

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ABSTRACT: Bovine photoreceptor guanylate cyclase (ROS-GC) consists of a single transmembrane polypeptide chain with extracellular and intracellular domains. In contrast to non-photoreceptor guanylate cyclases (GCs) which are activated by hormone peptides, ROS-GC is modulated in low Ca^{2+} by calmodulin-like Ca^{2+}-binding proteins termed GCAPs (guanylate cyclase-activating proteins). In this communication we show that, like the native system, ROS-GC expressed in COS cells is activated 4−6-fold by recombinant GCAP1 at 10 nM Ca^{2+} and that the reconstituted system is inhibited at physiological levels of Ca^{2+} (1 μM). A mutant ROS-GC in which the extracellular domain was deleted was stimulated by GCAP1 indistinguishable from native ROS-GC indicating that this domain is not involved in Ca^{2+} modulation. Deletion of the intracellular kinase-like domain diminished the stimulation by GCAP1, indicating that this domain is at least in part involved in Ca^{2+} modulation. Replacement of the catalytic domain in a non-photoreceptor GC by the catalytic domain of ROS-GC yielded a chimeric GC that was sensitive to ANF/ATP and to a lesser extent to GCAP1. The results establish that GCAP1 acts at an intracellular domain, suggesting a mechanism of photoreceptor GC stimulation fundamentally distinct from hormone peptide stimulation of other cyclase receptors.

Cyclic GMP and Ca^{2+} are intracellular messengers that mediate a series of key events in vertebrate phototransduction, light adaptation, and recovery of the dark state (Pugh, & Lamb, 1993; Lagnado, & Baylor, 1992, 1994). Hydrolysis of cyclic GMP, initiated by bleaching of rhodopsin, causes closure of cyclic GMP-gated cation channels and hyperpolarization of the photoreceptor plasma membrane (Fesenko et al., 1985; Stryer, 1991). As a consequence, cytoplasmic Ca^{2+} concentrations drop, which in turn accelerates cyclic GMP synthesis by a photoreceptor guanylate cyclase (GC).[1] The Ca^{2+} sensitivity of GC is mediated by a Ca^{2+}-binding protein termed guanylate cyclase-activating protein (GCAP) (Gorczyca et al., 1994). Two types of GCAPs (GCAP1 and GCAP2) and several types of GCs have

been identified in the retina. The GCAPs (molecular mass ∼23 kDa) are members of the calmodulin superfamily of Ca^{2+}-binding proteins and are predicted to have three functional EF hand Ca^{2+}-binding sites. GCAP1 mRNA was shown to be expressed in photoreceptors (Palczewski et al., 1994; Subbaraya et al., 1994), and the gene product was shown by immunocytochemistry to be present in the outer segment where phototransduction occurs (Gorczyca et al., 1995). The related but distinct GCAP2 (p24) appears also to be expressed in photoreceptors (Dizhoor et al., 1994, 1995), but its precise subcellular distribution has not been determined. Both GCAPs modulate photoreceptor GC(s) in a Ca^{2+}-dependent manner (Gorczyca et al., 1995; Dizhoor et al., 1995).

The GC family consists of soluble (two subunits) and particulate (one subunit) isozymes (Garbers, 1992; Sharma et al., 1994; Garbers, & Lowe, 1994). Depending on their sensitivity to hormone peptides, the membrane form of GC is divided into several subtypes. Of the natriuretic receptor form, at least two types have been cloned from the retina (Kutty et al., 1992; Duda et al., 1992, 1993; Ahmad & Barnstable, 1993) and were shown to be expressed in non-photoreceptor cells. Two distinct types of photoreceptor GCs which are insensitive to natriuretic peptides have been cloned from human retina libraries, retGC1 (Shyjan et al., 1992), and retGC2 (Lowe et al., 1995). The corresponding mRNAs from rat retina, GC-E and GC-F (Yang et al., 1995), were recently identified. A bovine rod outer segment (ROS) GC of the GC1 type has been recently biochemically isolated

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[1] Abbreviations: ROS, rod outer segments; GC, guanylate cyclase; ANF, atrial natriuretic factor; GCAP, guanylate cyclase activating protein; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of 0.1% SDS.

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and cloned (Goraczniak et al., 1994), and its identity as photoreceptor GC has been unambiguously established by comparison with four ROS-GC tryptic peptide fragments (Margulis et al., 1993). The biochemical identity of ROS-GC is very close to the revised sequence of retGC1 (Genbank accession no. M92432, January 1995), establishing that retGC1 is the human counterpart of ROS-GC.

The membrane GCs are single polypeptide chains, divided into roughly equal extracellular and intracellular portions by a transmembrane section. The extracellular portion (ext) of hormone-sensitive GCs contains the signal receptor domain. Sequence analysis predicts two major intracellular portions, one showing sequence similarity to tyrosine kinases (kinase-like domain, kin), and the other containing the catalytic domain (cat). In photoreceptor GCs, the functions of the extracellular and kinase-like domains are unknown. In the present study, we reconstitute the Ca^{2+} modulation of ROS-GC by GCAP1 in a recombinant system. We further show by expression of mutant GCs that GCAP1 acts at an intracellular domain(s) to modulate cyclase activity.

MATERIALS AND METHODS

Cross-Linking Experiments. Native ROS (containing GCAP1 and 30 μM rhodopsin) or washed ROS (depleted of GCAP1) (Gorczyca et al., 1994), suspended in a final volume of 64 μL of 50 mM HEPES, pH 7.8, 60 mM KCl, 20 mM NaCl, 10 mM $MgCl_2$, were cross-linked in the presence of 2 mM DTSSP (3,3′-dithiobis[sulfosuccinimidylpropionate]) (Pierce, U.S.A.). In pilot experiments, reaction conditions of 0 °C, 10 min, 2 mM cross-linker were identified as optimal for cross-linking GCAP1 and GC. The reactions were carried out in the presence of 50 nM or 1 μM free Ca^{2+} and stopped by the addition of 0.5 M glycine in 10 mM Tris-HCl, pH 7.4 (Clerc et al., 1992). ROS membranes were collected by centrifugation, washed with HEPES buffer, and resuspended in SDS sample buffer (without β-mercaptoethanol), and an aliquot ($^1/_5$ of the total) was subjected to electrophoresis in the presence of 0.1% SDS using a 5%−15% acrylamide gradient. After electrophoresis, proteins were visualized by staining with Coomassie Brilliant Blue R250 or by immunostaining after electrotransfer to immobilon (Millipore). The antibodies were a polyclonal monospecific anti-GCAP1 antibody (UW-14) (Gorczyca et al., 1995) and a polyclonal anti-ROS-GC antibody (Yoshida et al., 1995).

Isolation of Recombinant GCAP1. Full-length bovine GCAP1 cDNA (Palczewski et al., 1994) was cloned into pVL941 to yield pVLgcap. The construct was cotransfected with BaculoGold helper virus into Hi5 cells (Pharmingen), and the expressed protein purified by immunoaffinity chromatography as described previously (Gorczyca et al., 1995). rGCAP1 was further purified by HPLC on a CN W-Porex column (5 μm, 300 Å, 250 × 2 mm, Phenomenex) equilibrated with 10 mM BTP, pH 7.5. rGCAP1 was eluted isocratically at a flow rate of 1.5 mL/min in the same buffer, and the protein was detected at 230 nm. Native GCAP1 from ROS extracts was purified in an identical manner.

Construction of GC Deletion and Hybrid Mutants. For generation of ext⁻, two *Hpa*I restriction sites were created by oligonucleotide-directed mutagenesis (Kunkel et al., 1987) at nucleotide positions 241 and 1446 (Goraczniak et al., 1994) in ROS-GC cDNA subcloned into pAlter1 vector

Table 1: Specific Activities of ROS-GC, Deletion Mutants, and Hybrid ANF-RGC/ROS-GC[a]

Mutant	[A]	Domain Structure
ROS-GC ext⁻	105	
ROS-GC kin⁻	145	
ROS-GC ext⁻/kin⁻	67	
ROS-GC	110	
Hybrid	60	
ANF-RGC	59	
pcDNA3	<1	

[a] Rows 4 and 6 show unmodified ROS-GC and ANF-RGC (shaded). Unshaded rows show mutant GCs, the bottom row indicates specific activity of cells infected with vector only. The middle column shows the specific activities, [A] (nmol of cyclic GMP/mg of protein/min), of the expressed GC or mutant constructs as measured in crude COS membrane fractions. The column on the right graphically depicts domains of ROS-GC, ANF-RGC and the mutants. The domains are labeled lp, leader sequence; ext, extracellular domain; tm, transmembrane section; kin, area with tyrosine kinase sequence similarity; cat, catalytic site. In ext⁻, kin⁻, and ext⁻/kin⁻, the deleted portions are indicated by horizontal lines. Domains of ANF-RGC are shown in capital letters.

(Promega Biotech mutagenesis kit). The 1.2 kb *Hpa*I fragment was excised, and the remaining part was religated, resulting in deletion of the extracellular domain amino acid residues 64−465. For kin⁻, two *Hpa*I sites were generated at positions 1557 and 2409, the 852 bp fragment was excised with *Hpa*I, and the remainder was religated, resulting in deletion of amino acids 503−786. For ext⁻/kin⁻, two *Bgl*II restriction sites were generated at positions 1557 and 2408 in the ext⁻ mutant. Excision of the 851 bp fragment and religation of the remainder cyclase clone resulted in deletion of amino acids 503−786. To construct the hybrid mutant consisting of ANF-RGC (amino acids 1−745) and ROS-GC (amino acids 787−1100), an *Hpa*I restriction site was introduced into ANF-RGC cDNA at position 2638, and another *Hpa*I site was introduced into ROS-GC at position 2409. The mutant RGC cDNA was subcloned into pBluescript, and the mutant ROS-GC into pcDNA3. The 2.4 kb *Kpn*I/*Hpa*I fragment of ANF-RGC was replaced by the corresponding fragment of ROS-GC cDNA. All constructs were sequenced to confirm their identities and correct orientations. The mutant cDNAs were finally cloned into the *Kpn*I/*Xba*I sites of of the pcDNA3 vector for COS cell expression. A schematic representation of these mutants is given in Table 1.

Expression of ROS GC and Mutants. COS 7 cells (simian virus 40 [SV40] transformed African Green Monkey kidney cells), maintained in Dulbecco's modified Eagle's medium with penicillin/streptomycin and supplemented with 10% fetal bovine serum (Sigma) were transfected with the appropriate expression constructs using the calcium phosphate precipitation technique. At 60 h post-transfection, cells were washed twice with 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ buffer, scraped into 1 mL of the same buffer,
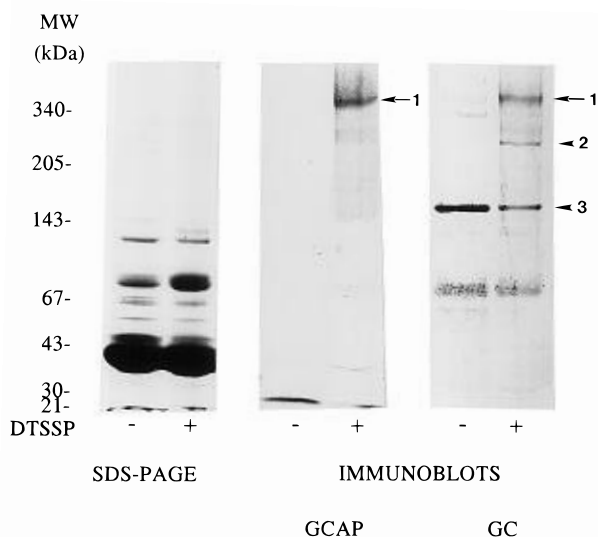
FIGURE 1: Cross-linking of GCAP1 and ROS-GC in bovine ROS membranes. Native ROS (30 μM rhodopsin) were cross-linked as described in Materials and Methods and analyzed by non-reducing SDS−PAGE. Proteins were visualized either by staining with Coomassie Brilliant Blue or with polyclonal antibodies specific to GCAP1 or ROS-GC after Western blotting. Arrow 1 indicates positions of the cross-linked, presumably multimeric GCAP1/ROS-GC complex. Arrow 2 points at a faster moving cross-linked polypeptide, presumably a GC dimer. Arrow 3 identifies monomeric GC. The mobilities of known protein standards are indicated on the left. "+" and "−" denote membranes which were and were not cross-linked, respectively.

homogenized, centrifuged for 15 min at 5000g, and washed three times with the same buffer. The pellet represented the crude membranes.

GC Assay. The crude membranes were assayed for GC activity similarly as described previously (Paul et al., 1987). Briefly, membranes were preincubated on ice with or without GCAP1 in the assay system containing 10 mM theophilline, 15 mM phosphocreatine, 20 μg of creatine kinase, 50 mM Tris-HCl (pH 7.5), 0.4 mM EGTA and CaCl$_2$ adjusted to the appropriate free Ca^{2+} concentrations calculated using Maxchelator version 6.5 (Bers et al., 1994). Total assay volume was 50 μL. The reaction was initiated by the addition of the substrate solution containing 4 mM MgCl$_2$ and 1 mM GTP. Incubation (37 °C, 10 min) was terminated by the addition of 450 μL of 50 mM sodium acetate buffer (pH 6.2) followed by heating in a boiling water bath for 3 min. The amount of cyclic GMP formed was determined by a radioimmunoassay (Nambi et al., 1982).

RESULTS AND DISCUSSION

ROS-GC/GCAP1 Cross-Linking. Ca^{2+}-dependent modulation of the integral membrane protein ROS-GC by the peripherally bound GCAP1 requires interaction of the two components on the ROS membrane surface. To study this interaction in the presence and absence of Ca^{2+}, limited cross-linking experiments with ROS membranes containing both the ROS-GC (120 kDa) and GCAP1 (23 kDa) were performed. The homobifunctional cross-linker DTSSP was allowed to react for 10 min. A high molecular mass product (400−500 kDa) was formed and was found to be antigenic to both anti-GCAP1 and anti ROS-GC antibodies (Figure 1, arrow 1). The cross-linking was independent of the concentration of free Ca^{2+} (50 nM and 1 μM). No product was detected with either antibody when extensively washed ROS

membranes containing ROS-GC, but devoid of GCAP1, were used (not shown). Consistent with recently reported physiological experiments (Koutalos et al., 1995), the results suggest that GCAP1 is in close contact with ROS-GC at high and low Ca^{2+}.

Reconstitution of GC Ca^{2+} Modulation by Recombinant Components. Under physiological conditions, native ROS-GC accelerates cyclic GMP synthesis when free Ca^{2+} is lowered to 50 nM or below as a response to photobleaching. To determine whether ROS-GC and GCAP1 are the exclusive components responsible for this feedback mechanism, both proteins were cloned and expressed in heterologous systems. Bovine GCAP1 was expressed in insect cells as described previously (Gorczyca et al., 1995), and purified by reverse phase HPLC. Purified rGCAP1 displayed the characteristic change in mobility during SDS−PAGE in the presence of EDTA, identical to purified native GCAP1 (not shown). Wild-type bovine ROS-GC was expressed in COS cells.

To reconstitute the Ca^{2+} modulated system, COS cell membranes containing rROS-GC were recombined with purified rGCAP1 at various Ca^{2+} concentrations. At low Ca^{2+}, rGCAP1 stimulated ROS-GC in a dose dependent manner, with half-maximal activation occurring at 0.8 μM, and saturating at about 2.5 μM GCAP1 (Figure 2A). The GC stimulation decreased as free Ca^{2+} increased with a half-maximal inhibition at ∼100 nM Ca^{2+} (Figure 2B). The cyclase activity was completely inhibited at 1 μM and higher concentrations of free Ca^{2+}. Similar results were obtained using the human ROS-GC homolog, retGC1 (Shyjan et al., 1992), expressed in human embryonic 293 cell lines (results not shown). In contrast, ANF-RGC was not stimulated by all tested rGCAP1 concentrations (Figure 2A,B). These findings show that ROS-GC is a Ca^{2+}-modulated cyclase, that the modulation is mediated through GCAP1, and that no other components are required to reconstitute the Ca^{2+} modulation of ROS-GC.

ROS-GC Domains and Construction of Mutants. According to cDNA cloning, unprocessed ROS-GC is predicted to consist of 1110 amino acids (Goraczniak et al., 1994). Sequencing of an N-terminal peptide of ROS-GC (Margulis et al., 1993) established that the prepeptide has a 56-residue leader sequence. The N-terminal 411 amino acids of mature ROS-GC (A[57] to E[468]) are presumed to be extracellular, and no function has been assigned to this relatively divergent domain (∼50% sequence similarity in retina GCs). To identify potential domains of interaction with GCAP1, four ROS-GC mutants were constructed (Table 1). In ext$^-$, the extracellular domain (from residue L[64] to G[465]) was deleted. In kin$^-$, most of the kinase-like domain (from S[503] to I[786]), and in ext$^-$/kin$^-$, both the extracellular and the kinase-like domains were deleted. Among retina GCs, the kin domain shows very high sequence similarity, but its function in ROS-GC is unknown. In all deletion mutants, the leader peptide and the first eight amino acids of the mature protein were retained in the construct to ensure proper translocation of the mutant GC. In the hybrid mutant, the catalytic cyclase domain of natriuretic receptor GC was substituted by the corresponding domain of ROS-GC (C-terminal 320 residues, from Q[787] to the C-terminus, K[1100], Table 1).

Expression in COS Cells. All mutants were transiently expressed in COS cells. The expression levels were determined by measurement of basal GC activity of the wild-
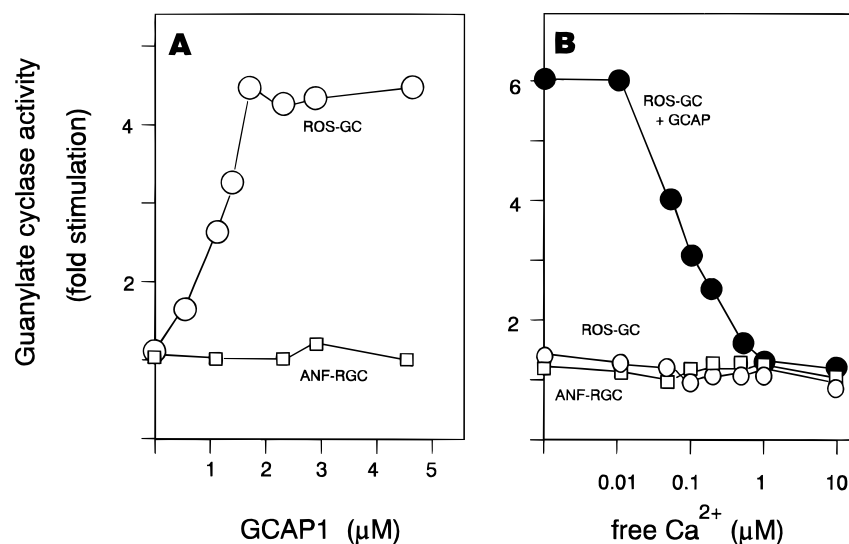
FIGURE 2: Effect of recombinant GCAP1 and Ca2+ on recombinant ROS-GC activity. COS cell membranes were prepared as described in Materials and Methods, and assayed for GC activity using Mg^{2+} as a cofactor in the presence of 10 nM free Ca^{2+} and GCAP1 as indicated. Each experiment was done in triplicate, and repeated three times. The results shown are from one representative experiment. (A) ROS-GC (O) and ANF-RGC (□) activity expressed in COS cells. (B) GC activity of deletion mutants [ROS-GC + GCAP1 (●); ROS-GC without GCAP1 (O); ANF-RGC, (□)]. Error bars are within the size of the symbols.
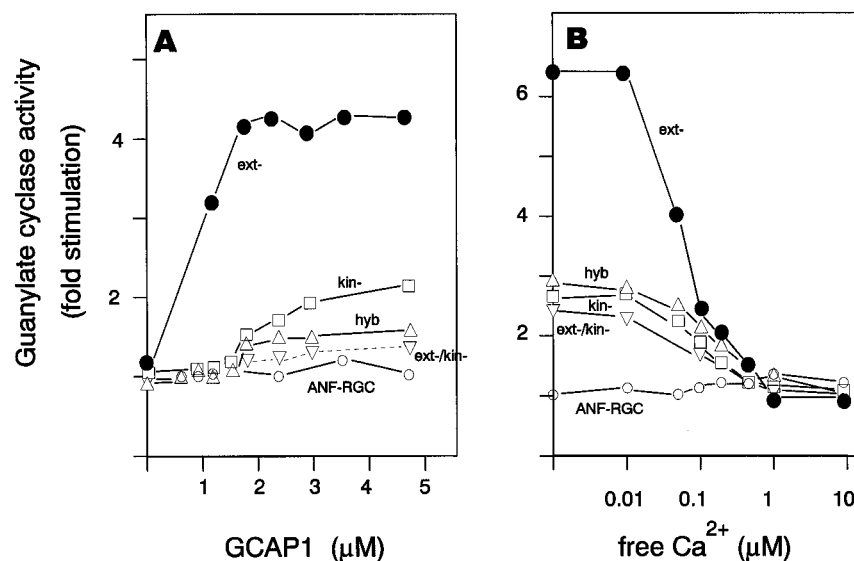

FIGURE 3: Stimulation of cyclase activity in ROS-GC mutants. (A) Effect of added rGCAP1 on mutant ROS-GCs at constant Ca^{2+} (10 nM); (●) ext⁻ mutant; (□) kin⁻; (▲) hybrid GC; (▼) ext⁻/kin⁻; (O) ANF-RGC. (B) Effect of free Ca^{2+} on rROS-GC and ROS-GC mutant cyclase activity at constant rGCAP1 (3 μg). The symbols and assay conditions are as in Figure 3.

type cloned ROS-GC and the mutant GC. The results show that the basal cyclase activities of the wild-type recombinant ROS-GC, deletion mutants and of the hybrid GC ranged between 50−150 pmol of cyclic GMP formed/min/mg of protein (Table 1). The basal activity is 100-fold higher than control cells transfected with vector only. As expected, the expressed hybrid GC, which contains the EXT and KIN domains of ANF-RGC and the cat domain of ROS-GC, could be fully stimulated by ANF/ATP (not shown). The biological activity of the hybrid cyclase and its sensitivity to ANF indicate that the mutant GC has been translocated and folded properly and confirms that the active site (cat) of the enzyme is contained in the C-terminal 320 amino acid residues. The nearly identical basal activities of the expressed mutant and native GCs (Table 1) suggest that they are expressed to approximately the same levels in the COS cell system.

Intracellular Location of Domains Involved in Ca²⁺ Modulation. To locate the domain(s) involved in GCAP1

modulation of ROS-GC, the membrane fractions of cells expressing ext⁻, kin⁻, ext⁻/kin⁻, or hybrid mutants were assayed in the presence of rGCAP1 and Ca^{2+}. At low Ca^{2+} concentrations (10 nM), rGCAP1 stimulated the cyclase activity of ext⁻ in a dose-dependent fashion virtually identical to the stimulation of wild-type rROS-GC (Figure 3A). At 1.5 μM rGCAP1, the stimulation reached a plateau, which was sustained up to the tested 4 μM of rGCAP1. The maximal GCAP1-stimulated cyclase activity (at 2.5 μM) decreased in a dose-dependent fashion as the free Ca^{2+} increased (Figure 3B). Both stimulatory and inhibitory patterns were virtually identical to those observed for wild-type rROS-GC (Figure 2).

In contrast, analysis of the kin⁻ and ext⁻/kin⁻ mutants showed only little (1.5-fold or less) stimulation at the GCAP1 concentration (1.7 μM) that maximally stimulated wild-type ROS-GC or ext⁻ (Figures 2A and 3A). At the highest GCAP1 concentrations tested (4 μM), the stimulation of kin⁻

was only about 2-fold. With the hybrid and ext⁻/kin⁻ mutants (Figure 3A), the cyclase activity at the highest GCAP1 concentration tested was only about half as much as in kin⁻. The diminished cyclase stimulations of the mutant GCs observed with the high GCAP1 concentrations were also inhibited by the free Ca^{2+} concentrations (Figure 3B). These results show that the domain modulated by GCAP1 and Ca^{2+} must be present in the intracellular region of ROS-GC. The low GCAP1 stimulation of the kin⁻ and ext⁻/kin⁻ mutants suggest that the kinase-like domain of ROS-GC is important for Ca^{2+} modulation, and if replaced by the KIN domain of ANF-RGC, the ectopic domain is unable to substitute fully for the endogenous domain.

Conclusion. The conceptual framework of ROS-GC modulation by a photoreceptor specific Ca^{2+}-binding protein was originally postulated by Stryer and associates (Koch & Stryer, 1988; Dizhoor et al., 1991). The individual components, ROS-GC and GCAP1, have now been identified, cloned, and expressed *in vitro*. On the basis of the present findings, it appears that GCAP1 modulates ROS-GC through interaction (docking) at an intracellular domain while all known membrane guanylate cyclases of the ANF-type are modulated extracellularly. At low free Ca^{2+}, GCAP1 activates ROS-GC through a yet unknown mechanism, and at high free Ca^{2+}, the activation is disabled. The activation/inactivation mechanism is reversible. The cross-linking experiments (Figure 1) suggest that at high free Ca^{2+}, GCAP1 remains in close contact with the target enzyme.

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