A Second Calcium Regulator of Rod Outer Segment Membrane Guanylate Cyclase, ROS-GC1: Neurocalcin†

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ABSTRACT: ROS-GC represents a membrane guanylate cyclase subfamily whose distinctive feature is that it transduces diverse intracellularly generated Ca^{2+} signals into the production of the second messenger cyclic GMP. An intriguing feature of the first subfamily member, ROS-GC1, is that it is both stimulated and inhibited by these signals. The inhibitory signals are processed by the cyclase activating proteins, GCAPs. The only known stimulatory signal is by the Ca^{2+}-dependent guanylate cyclase activating protein, CD-GCAP. There are two GCAPs, 1 and 2, which link the cyclase with phototransduction, and one CD-GCAP, which is predicted to link ROS-GC1 with its retinal synaptic activity. Individual switches for these GCAPs and CD-GCAP have been respectively defined as CRM1, CRM3, and CRM2. This report defines the identity of a new ROS-GC1 regulator: neurocalcin. A surprising feature of the regulator is that it structurally is a GCAP but functionally behaves as a CD-GCAP. Recombinant neurocalcin stimulates ROS-GC1 in a dose-dependent fashion; the stimulation is Ca^{2+}-dependent with an EC_{50} of 20 μM; and the modulated domain resides at the C-terminal segment, between amino acids 731 and 1054. Previously, the residence of CRM2 has also been defined in this segment of the cyclase. However, the present study shows that the neurocalcin-regulated domain is distinct from CRM2. This is now designated as CRM4. Thus, the signal transduction mechanisms of neurocalcin and CD-GCAP are different, occurring through different modules of ROS-GC1. Neurocalcin signaling of ROS-GC1 is highly specific. It does not influence the activity of its second subfamily member, ROS-GC2, and of the other retinal guanylate cyclase, atrial natriuretic factor-receptor guanylate cyclase. In conclusion, the findings extend the concept of ROS-GC1's sensing diverse Ca^{2+} signals, reveal the identity of its unexpected new Ca^{2+} regulator, and show that the regulator acts through its specific cyclase domain. This represents an additional transduction mechanism of Ca^{2+} signaling via ROS-GC1.

The capture of a photon by a vertebrate rod and a cone generates an electrical signal. The biochemical process involved in the generation of this signal is termed phototransduction (reviewed in ref *1*). Two critical molecules of phototransduction are Ca^{2+} and cyclic GMP.[1] They are interlocked. The captured photon causes a decline in the level of cyclic GMP, closure of the cyclic GMP-gated cation channel, and hyperpolarization of the photoreceptor plasma membrane. This results in the fall of intracellular Ca^{2+} from ~500 nM in the dark-adapted photoreceptor cell to below 100 nM (*2*−*5*) and in the signaling of the activation of a rod outer segment (ROS) membrane guanylate cyclase (ROS-GC). Increased synthesis of cyclic GMP along with the inactivation of the phototransduction cascade leads to the reopening of the cyclic GMP-gated channels and, thus, restoration of the dark current state of the photoreceptors.

Two isoforms of ROS-GC, ROS-GC1 and ROS-GC2, have been cloned (*6*−*10*). Immunostaining and in situ hybridization studies indicate the presence of both forms in photoreceptor outer segments (*6*, *7*, *11*−*13*). The physiological significance of the heterogeneity in ROS-GCs and its relationship with rod/cone expression pattern are not known. However, the emerging evidence strongly supports ROS-GC1 linkage with ROS-specific phototransduction: (1) to date, only the existence of ROS-GC1 has been demonstrated biochemically in ROS, the site of phototransduction (14); and (2) only ROS-GC1 mutations specific to a rod-specific abnormality, Leber's congenital amaurosis (LCA1) (*15*, *16*),

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[1] Abbreviations: ANF, natriuretic peptide hormone type A; ANF-RGC, atrial natriuretic factor-receptor guanylate cyclase; CRM, calcium regulatory module; CD-GCAP, calcium-dependent guanylate cyclase activating protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GCAP, guanylate cyclase activating protein; GMP, guanosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; LCA, Leber's congenital amaurosis; (r), recombinant; ROS-GC, rod outer segment membrane guanylate cyclase.

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mimic the disease phenotype in precise molecular terms (*17*). Another modulatory feature of ROS-GC1, unique to itself, has started to develop. ROS-GC1 responds to three Ca^{2+} signals. One signal is modulated by GCAP1, the second is modulated by GCAP2, and the third is regulated by CD-GCAP (*18*−*25*, *27*−*30*). Each signal is transduced by a ROS-GC1 signal-specific domain. These domains have been termed CRM1, CRM3, and CRM2, respectively (model in Figure 6 of ref *27*). In contrast, ROS-GC2 responds only to the Ca^{2+} signal mediated by GCAP2 (*26*). Compared to ROS-GC1, ROS-GC2 is about 10-fold less responsive to the CD-GCAP signal (*31*). There is an intriguing aspect to the GCAPs and CD-GCAP regulation of ROS-GC1. Ca^{2+} inhibits GCAP's ability to activate the cyclase, but it stimulates CD-GCAP's. Ca^{2+} linkage of the ROS-GC1 with phototransduction is through GCAPs (*18*, *32*). Presumably, Ca^{2+} linkage of the ROS-GC1 with the retinal synaptic activity is through CD-GCAP (*28*). The terms modulation and regulation are used to indicate the Ca^{2+}-dependent inhibitory and stimulatory features of ROS-GC1. Thus, GCAPs are modulators and CD-GCAP is the regulator.

GCAPs and CD-GCAP are members of the two respective, unrelated subfamilies of Ca^{2+}-binding proteins, recoverin and S100. Both subfamilies, in turn, are a part of the calmodulin superfamily (*33*). CD-GCAP is identical in its structure and function to S100b (*34*). Besides GCAPs, some other members of the recoverin family are S-modulin, visnin, vilip, and neurocalcin (*35*, *36*). These family members share 34−45% sequence identity, a conserved myristoylation signal at the amino terminus, two pairs of classical EF-hand Ca^{2+}-binding loop sequences—EF1, EF2, EF3, EF4—and 20−25 kDa subunit mass. In contrast, S100 protein family members share only minimal (∼15%) sequence identity with recoverin family members. Among themselves, they share 30−50% sequence identity and are considerably smaller in their subunit masses, about 10 kDa (*37*).

The present study discloses the identity of a second Ca^{2+}-dependent regulator of ROS-GC1, neurocalcin. Neurocalcin stimulates ROS-GC1 in a Ca^{2+}-dependent fashion. It, however, does not affect ROS-GC2, the other member of the ROS-GC subfamily. Also, it does not influence the activity of ANF-RGC. ANF-RGC is the receptor for natriuretic peptide hormone type A (ANF) (*38*−*41*). Thus, neurocalcin is specific for ROS-GC1. This property of neurocalcin is remarkable, because it is opposite to its expected structure; neurocalcin belongs to the GCAP family, yet it behaves like CD-GCAP, a S100 family member. Furthermore, the study shows that the neurocalcin-regulated ROS-GC1 domain is distinct from that of the CD-GCAP-regulated domain. These findings extend the concept that ROS-GC1 senses diverse Ca^{2+} signals, reveal the identity of its unexpected new Ca^{2+} regulator, and show that the regulator acts through its specific cyclase domain. This represents an additional transduction mechanism of Ca^{2+} signaling via ROS-GC1.

MATERIALS AND METHODS

Expression and Purification of Neurocalcin. The coding sequence of the bovine neurocalcin δ cDNA (*42*) was amplified via polymerase chain reaction, inserted into the *Stu*I and *Eco*RI sites of pMAL-C expression vector and expressed in *Escherichia coli* DH5α strain. Neurocalcin was overexpressed as a fusion product with maltose-binding protein and purified (*43*). The purity was about 95% on the basis of Coomassie staining after sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis.

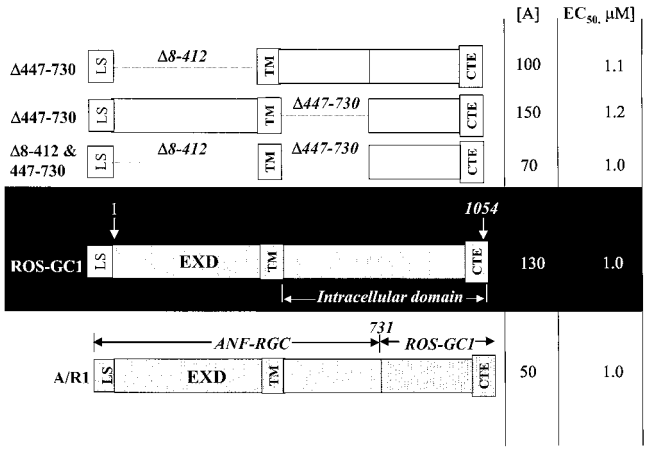

FIGURE 1: Left panel: Schematic representation of ROS-GC1, deletion, and hybrid mutants. The predicted domains are denoted as follows: LS, leader sequence; EXD, extracellular domain; TM, transmembrane domain. Numbers indicated correspond to the mature protein (*8*). Right panel: Basal cyclase activities of ROS-GC1, the mutants, and the respective EC_{50} values of activation by neurocalcin.

CD-GCAP was purchased commercially as S100b ($\beta\beta$) from Sigma. The protein (1 mg) was reconstituted in 50 mM Tris-HCl, pH 7.0/50 mM NaCl and adjusted to a final concentration of 1 mg/mL.

Construction of ROS-GC1 Mutants. Four ROS-GC1 mutants were constructed. Three were deletion, Δ8−412 [deleted amino acids (aa) 8−412], Δ447−730 (deleted aa 447−730), double deletion --DD-- (deleted aa 8−412 and 447−730), and one was the hybrid, A/R1. In A/R1, the 2.4 kb *Kpn*I/*Hpa*I fragment of ANF-RGC cDNA was replaced by the corresponding fragment of ROS-GC1 cDNA. Detailed construction of these mutants is described in ref *18*. The mutant recombinants were individually subcloned into pcDNA3 expression vector (Invitrogen). These mutants with their respective expression levels and EC_{50} values of activation by neurocalcin are represented in Figure 1.

Expression Studies. COS7 cells (simian virus 40-transformed African green monkey kidney cells), maintained in Dulbecco's modified Eagle's medium with penicillin, streptomycin, and 10% fetal bovine serum, were transfected with the expression constructs by the calcium phosphate coprecipitation technique (*44*). Transfection with vector alone served as a control. Sixty hours after transfection, cells were washed twice with 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$ buffer, scraped into 2 mL of cold buffer, homogenized, centrifuged for 15 min at 5000*g*, and washed several times with the same buffer. The pellet represented the crude membranes.

Guanylate Cyclase Assay. The crude membranes were assayed for guanylate cyclase activity as described previously (*40*). Briefly, membranes were preincubated on an ice bath with, or without, neurocalcin. The assay system consisted of 10 mM theophylline, 15 mM phosphocreatine, 20 μg of creatine kinase, and 50 mM Tris-HCl (pH 7.5). This was adjusted to appropriate free Ca^{2+} concentrations with preca-

librated Ca^{2+}/ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) solutions (Molecular Probes). The total assay volume was 25 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 225 μL of 50 mM sodium acetate buffer (pH 6.25) followed by heating in a boiling water bath for 3 min. The amount of cyclic GMP was determined by radioimmunoassay (*45*).

RESULTS AND DISCUSSION

Reconstitution of Neurocalcin-Dependent Ca^{2+} Regulation of ROS-GC1. Reconstitution studies on the cloned ROS-GC1 have shown that the cyclase mimics the physiological conditions of phototransduction; i.e., the cyclase potentiates the synthesis of cyclic GMP when the free Ca^{2+} intracellular level is lowered to 100 nM or below (*18*). Ca^{2+} modulates ROS-GC1 activity via GCAP1 and GCAP2, through their cyclase-specific domains, respectively termed CRM1 and CRM3 (*27*). Neurocalcin is a member of the GCAP family of Ca^{2+}-binding proteins (*43*). The bovine neurocalcin shares 34% homology with bovine GCAP1 and 37% with bovine GCAP2. Between themselves, the bovine forms of GCAP1 and GCAP2 share 34% homology. To determine if neurocalcin is able to duplicate the stimulatory effect of GCAPs, COS cell membranes expressing recombinant (r) ROS-GC1 were mixed with purified (r)neurocalcin at different Ca^{2+} concentrations. There was no cyclase stimulation at nanomolar concentrations of Ca^{2+} (Figure 2B), indicating that neurocalcin does not duplicate GCAP's effects, and thus has no role in phototransduction-linked ROS-GC1 activity. This conclusion is in harmony with the information that neurocalcin does not reside in photoreceptors (*46*), which is the site of phototransdution.

Immunocytochemical studies show the presence of neurocalcin in the retinal amacrine and ganglion cells (*46*) and also of ROS-GC1 (*12*). To determine whether neurocalcin influences ROS-GC1 activity, COS cell membranes expressing (r)ROS-GC1 were incubated with a series of incremental concentrations of purified (r)neurocalcin at a fixed high (1 mM) Ca^{2+} concentration. Neurocalcin stimulated ROS-GC1 activity in a concentration-dependent manner; half-maximal activation of the cyclase occurred at $\sim$1 μM and saturation at $\sim$3 μM (Figure 2A). The stimulatory effect of neurocalcin was Ca^{2+}-dependent with its EC_{50} of $\sim$20 μM (Figure 2B). In the absence of neurocalcin, the cyclase did not respond to any concentration of Ca^{2+} (Figure 2B). Thus, Ca^{2+} regulation of ROS-GC1 occurs via neurocalcin. Under similar conditions, neurocalcin did not stimulate ROS-GC2 (Figure 2A) and it did not influence the activity of ANF-RGC (Figure 2A). ANF-RGC is expressed in the retina (*47*, *48*). Thus, neurocalcin is specific for ROS-GC1 regulation, the regulation is Ca^{2+}-dependent, and it is unrelated to phototransduction. The copresence of neurocalcin and ROS-GC1 in the amacrine and ganglion cells suggests that the regulation may be linked to the synaptic activities of these cells. A similar interpretation regarding the role of CD-GCAP's regulation of ROS-GC1-linked retinal synaptic activity has been made earlier (*28*).

Localization of the Neurocalcin-Regulated ROS-GC1 Domain. To localize the neurocalcin-regulated domain,

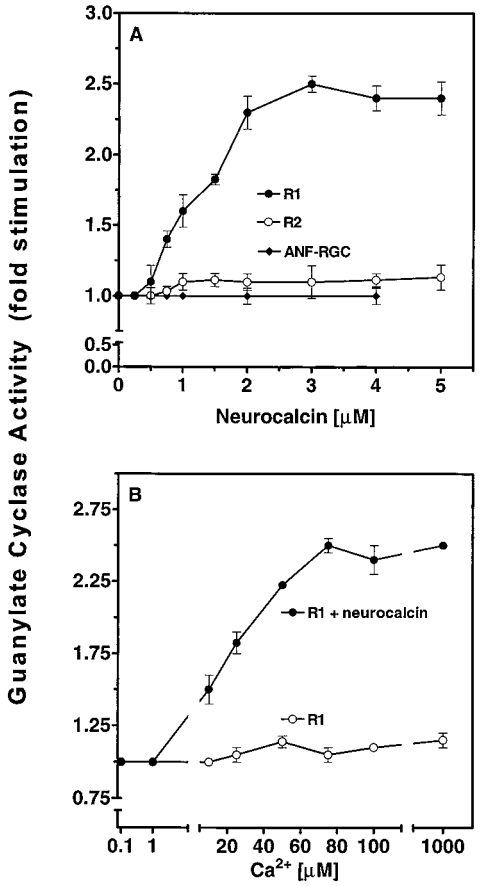

FIGURE 2: Effect of neurocalcin on the cyclase activities of ROS-GC1 (R1), ROS-GC2 (R2), and ANF-RGC and of free Ca^{2+} on the cyclase activity of ROS-GC1. COS7 cells were transfected with ROS-GC1, ROS-GC2, or ANF-RGC cDNA, and the membranes were prepared as described in Materials and Methods. These were assayed for guanylate cyclase activities in the presence of 1 mM free Ca^{2+} and incremental concentrations of neurocalcin (A). The effect of free Ca^{2+} concentration on ROS-GC1 activity was determined in the presence or absence of 4 μM neurocalcin and the indicated free Ca^{2+} concentrations (B). Experiments were done in triplicate and repeated three times with separate membrane preparations. The results from these experiments are depicted as the mean $\pm$ SD.

expression studies with the ROS-GC1 deletion mutants, Δ8−412, Δ447−730 and Δ8−412, Δ447−730 [double deletion (DD)], and hybrid A/R1 were conducted. These mutants are graphically represented in Figure 1. They were transiently expressed in COS cells, and their membrane fractions were appropriately treated and studied for their cyclase activities. At a fixed Ca^{2+} concentration of 1 mM, neurocalcin stimulated all ROS-GC1 mutants in a dose-dependent fashion with comparable EC_{50} values of about 1 μM (Figure 3A,B), which again were comparable to that observed for the wild-type ROS-GC1 (Figure 2A). The enzyme saturation of the wild-type and the mutant cyclases also occurred at comparable concentrations of neurocalcin, between 3 and 4 μM. The maximal cyclase stimulation of Δ447−730 and DD mutants were comparable, but they were about 20% lower than the wild type (Figure 3A). Because similar lowering of the Δ447−730 and DD maximal cyclase stimulation in response to GCAP2 and CD-GCAP has been observed previously (*27*, *31*), it is concluded that the neurocalcin-regulated domain of ROS-GC1 does not reside in the aa segment 8−730. Hence, it must reside at the C-terminus,

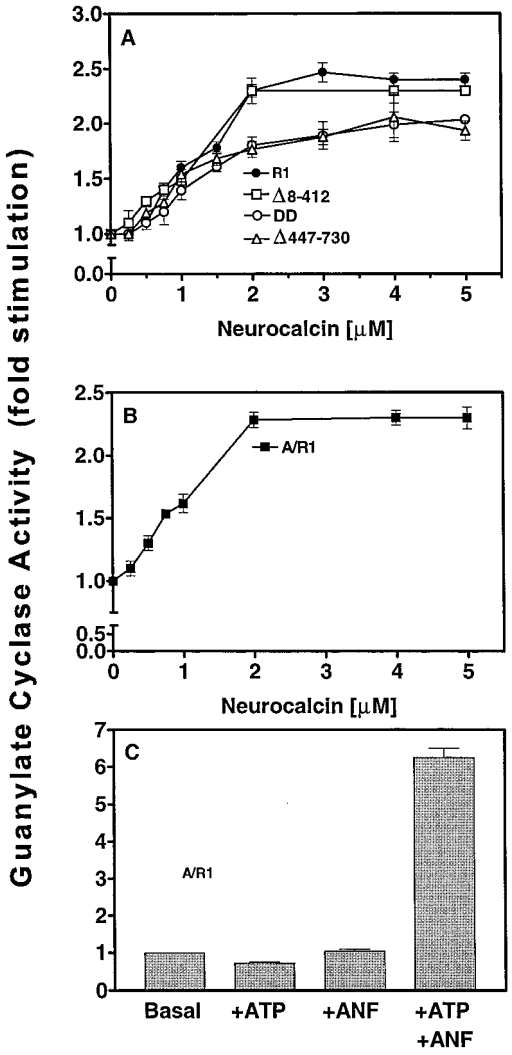

FIGURE 3: Effect of neurocalcin on the cyclase activity of ROS-GC1 deletion mutants (A) and on the A/R1 hybrid (B) and effect of ANF and/or ATP on the A/R1 hybrid (C). COS7 cells were transfected with the appropriate expression construct, and the membranes were prepared as described in Materials and Methods. Membranes were assayed for guanylate cyclase activity in the presence of 1 mM Ca^{2+} and increasing concentrations of neurocalcin (A and B) or in the presence of 0.1 μM ANF and/or 0.4 mM ATP (C). Experiments were done in triplicate and repeated three times with separate membrane preparations. The results from these experiments are depicted as the mean ± SD.

between aa 731−1054. That this is, indeed, the case is shown by the results obtained with A/R1 (Figure 3B). Here, the ROS-GC1 ectopic domain—aa 731−1054—mimics the neurocalcin-dependent activity. The hybrid is now the sensor of both types of signals: one generated intracellularly by Ca^{2+}/neurocalcin and the other generated extracellularly by ANF (Figure 3C). In contrast, the respective parent cyclases, ANF-RGC and ROS-GC1, show no functional cross-reactivity (*8*, *30*).

Previous studies have established CD-GCAP to be a Ca^{2+}-dependent regulator of ROS-GC1 (*28*−*31*, *34*). Like neurocalcin, CD-GCAP has no role in phototransduction-related ROS-GC1 activity, and like neurocalcin, CD-GCAP is implicated in ROS-GC1-related retinal synaptic activity (*28*). There are other parallels between CD-GCAP's and neurocalcin's mode of ROS-GC1 regulation: (1) both agents stimulate ROS-GC1 almost identically (CD-GCAP's values

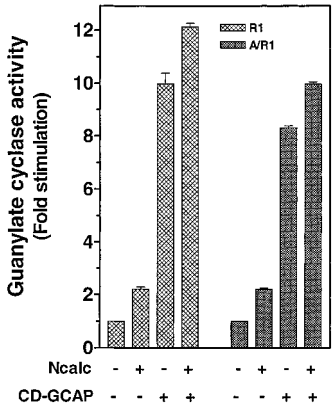

FIGURE 4: Additiveness of the neurocalcin (Ncalc) and CD-GCAP effects on the cyclase activities of ROS-GC1 (R1) and the A/R1 hybrid. Membranes of COS7 cells expressing ROS-GC1 or the A/R1 hybrid mutant were assayed for guanylate cyclase activity in the presence of 4 μM neurocalcin and 2 μM CD-GCAP at 1 mM Ca^{2+} concentration. The experiment was done in triplicate and repeated two times. The results were analyzed through the *t* test ($p < 0.001$). The results from these experiments are depicted as the mean ± SD.

discussed here are provided in refs *30* and *31*), EC_{50} of ~1 μM and V_{max} about 3 μM; (2) a similar Ca^{2+}-dependent fashion compares EC_{50} of 17−40 μM for CD-GCAP vs 20 μM for neurocalcin; (3) in a highly specific manner, neurocalcin has no effect on ROS-GC2, and CD-GCAP is 10-fold more selective for ROS-GC1 than for ROS-GC2 (*31*), and both have no effect on ANF-RGC; and finally (4) the modulated cyclase domain in both cases resides at the C-terminal region of ROS-GC1, between aa 731−1054. Despite these shared features, there is one notable difference between the two regulators. The achievable cyclase activity at the saturable concentrations of the two regulators is vastly different: it is about 3-fold more with CD-GCAP than with neurocalcin. To determine whether neurocalcin and CD-GCAP use common or distinct ROS-GC1 domains in activating its catalytic domain, ROS-GC1 was exposed to the combined saturating concentrations of the two activators (4 μM neurocalcin and 2 μM CD-GCAP) at a fixed free Ca^{2+} concentration (1 mM). The resulting stimulation of the cyclase was approximately the sum of those caused by neurocalcin and CD-GCAP alone (Figure 4). The difference between the cyclase stimulation in the presence of CD-GCAP alone and together with neurocalcin is statistically significant ($p < 0.001$). Similar results were obtained with the hybrid, where the sole segment of ROS-GC1 was aa 731−1054 (Figure 4; $p < 0.001$). Hence, the neurocalcin- and CD-GCAP-regulated ROS-GC1 domains are distinct, and the results further support the conclusion that the neurocalcin-regulated domain resides in the segment covering aa 731−1054. Previous studies have shown that this segment also covers the residence of CD-GCAP. Thus, the aa 731−1054 segment of ROS-GC1 contains two distinct domains, one belonging to neurocalcin and the other to CD-GCAP. The CD-GCAP domain has been termed CRM2 (*27*), and the neurocalcin domain now is being termed CRM4.

The physiological implications of these findings are unclear. They predict, however, that under identical conditions ROS-GC1 will sense both Ca^{2+} signals carried by CD-GCAP and neurocalcin, but the amplitude of response will be far higher with CD-GCAP as compared to the neurocalcin-

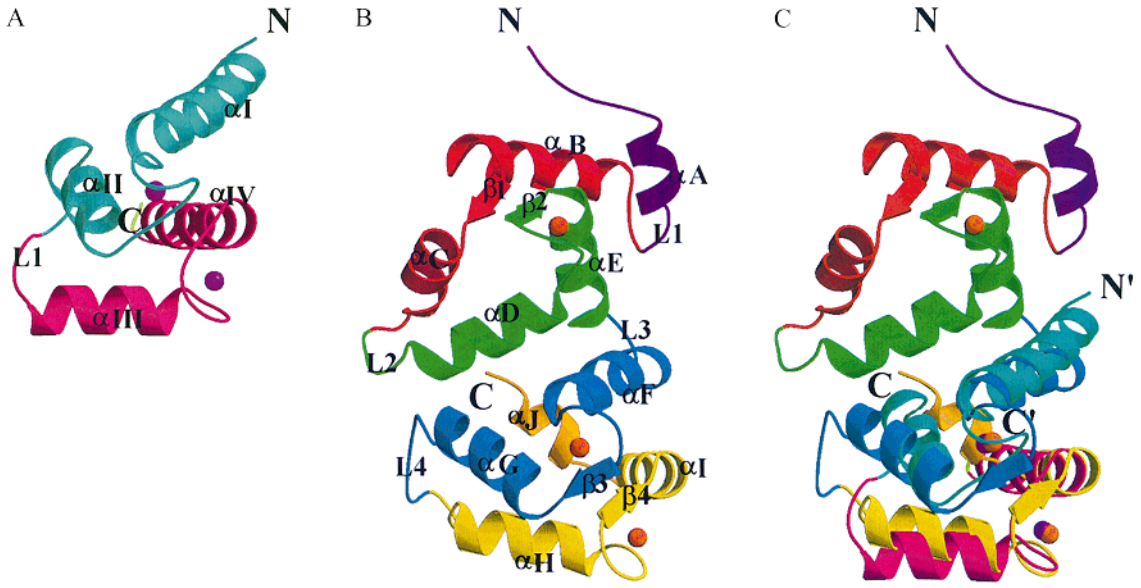

FIGURE 5: (A) Bovine Ca^{2+}-S100b monomer crystal structure. The bound Ca^{2+} ions are represented as cyan spheres. The two EF-hand Ca^{2+}-binding domains are color coded: EF1 is in light blue and EF2 is in cyan. The helices are labeled I−IV, and the linker region connecting the two pairs of EF hands is labeled L1. The C-terminal region is colored in green. (B) Bovine Ca^{2+}-neurocalcin monomer structure. The four Ca^{2+}-binding EF-hand domains are color coded: EF1 is in red, EF2 is in green, EF3 is in blue, and EF4 is in yellow. The N- and C-terminal regions are represented in magenta and gold, respectively. The Ca^{2+} ions that are bound at EF2-, EF3-, and EF4-hand domains are represented as orange spheres. The helices are labeled A−J, and the linker regions connecting EF hands are labeled L1−L4. (C) Best-fit superposition of main chain atoms of the bovine Ca^{2+}-S100b monomer on the C-terminal half of the Ca^{2+}-neurocalcin monomer. The EF1 and EF2 hands of S100b superpose on the EF3 and EF4 hands of neurocalcin.

mediated Ca^{2+} signals. The depolarization of neurons causes internal free Ca^{2+} concentrations to rise to the range of 10−100 μM (*49*). Given the paired presence of CD-GCAP/ROS-GC1 or neurocalcin/ROS-GC1, the present study provides a conceptual framework for two alternative mechanisms by which ROS-GC1 may be linked with the neuronal synapse of a variety of retinal cells.

The finding that neurocalcin stimulates ROS-GC1 is intriguing and was unexpected. Structurally, neurocalcin is a GCAP, yet functionally it behaves in altogether an opposite fashion, like a CD-GCAP. Why? With the recent availability of the crystal structures of neurocalcin (*50*) and CD-GCAP (S100b) (*51*), an attempt was made to address this enigmatic issue.

Comparison of Neurocalcin and CD-GCAP. To provide structural insights that result in neurocalcin's behavior like CD-GCAP, the configurational arrangements of the two proteins, based on their Ca^{2+}-bound crystallographic models (*50*, *51*), were compared. The monomeric subunit of Ca^{2+}-CD-GCAP is a relatively compact and globular structure. It consists of four helices, I−IV. These participate in two EF-hand domains, the helix−loop−helix structural motif for Ca^{2+} binding, and are linked by a flexible linker region L1 (Figure 5A). The four helices (I−IV) in the monomeric Ca^{2+}-CD-GCAP structure exhibit some differences to those observed with most Ca^{2+}-binding proteins, which contain two isolated EF-hand domains. Inspection of the CD-GCAP monomer reveals a splayed helix relationship. This is similar to that of Ca^{2+}-bound neurocalcin, involving helices B and C and helices D and E, or helices F and G and helices H and I (Figure 5B). The comparison reveals three significant structural relationships between neurocalcin and CD-GCAP (Figure 5C): (1) the CD-GCAP monomer superposes comfortably on either the N- or C-terminal pair of EF hands of neurocalcin with root-mean-square deviations for main chain atoms being 3.6 and 4.0 Å, respectively; (2) the four helix-packing arrangements, involving either an N-terminal pair or a C-terminal pair of EF hands of neurocalcin, are very similar to those of CD-GCAP (Figure 5C shows the superposition of the CD-GCAP structure over the C-terminal pair of EF hands of neurocalcin); and (3) related to their crystal packing arrangements, the two CD-GCAP monomers pack in an antiparallel fashion about its C-terminal helix, and the neurocalcin monomers pack around the N-terminal helix of the EF3 hand.

In addition to its Ca^{2+}-binding EF hands, two regions in CD-GCAP have been suggested to play an important role in target recognition. One is the linker region L1: aa Leu32−Leu42 (Figure 5A). The region connects the two EF hands in CD-GCAP and supposedly interacts with ROS-GC1 and activates the cyclase (*52*). The corresponding linker region in neurocalcin is aa Met130−Ser142 from loop L4 (Figure 5B). This is two amino acids longer than the CD-GCAP region, but in both proteins the region is rich in hydrophobic and charged residues.

The second CD-GCAP region is the C-terminus. This is comprised of helix IV and C-terminal residues, covering aa residues 81−91 (Figure 5A). This region, upon transition of the protein from the apo to the holo form, undergoes a significant conformational change. This results in the exposure of hydrophobic residues—Met74, Ala75, Met79, and Ala83—to the surface. It is predicted that the change enables the residues to interact with the target molecule, ROS-GC1, and the cyclase activation. A similar hydrophobic patch comprised of the residues Leu182, Leu183, Ile184, and Leu185 is observed in neurocalcin. By analogy with CD-GCAP, the patch is the effector region of ROS-GC1 activation. Consistent with this theoretical prediction, the effector role of these residues has been experimentally supported for ROS-GC activation (*52*) and for their interac-

tion with CapZ (*53*). Thus, the conformational identity of these residues between neurocalcin and CD-GCAP may explain their functional identity toward ROS-GC1.

An attempt has also been made to rationalize the opposite behaviors of GCAPs and neurocalcin toward ROS-GC1. In this matter it is important to keep in mind the structural similarity between bovine GCAPs, 1 and 2 (36%), vs neurocalcin, 34% compared to GCAP1 and 37% compared to GCAP2; i.e., these three proteins are significantly homologous. Recent mutagenesis studies with GCAP2 show that its three Ca^{2+}-binding EF hands have important implications on their ability to regulate ROS-GCs stimulation (*54*). Crystal structure comparison of neurocalcin and recoverin, the proteins of the same family, shows a highly conserved topology (*50*). However, recoverin has a disabled EF4 hand and is unable to activate ROS-GCs (*55*). This supports the importance of active EF hands in ROS-GC1 activation. A closer study between neurocalcin and other members of this subfamily of proteins reveals two regions of significant sequence divergence: the linker region, connecting EF3 and EF4 hands, and the C-terminal region. As noted earlier, these regions of the GCAPs (and neurocalcin) correlate with the linker and C-terminal region of CD-GCAP. Because the experimental findings (*52*) support the role of these regions in the protein targeting mechanism, it is tempting to suggest that the residues in neurocalcin and GCAPs interact with ROS-GC1. This raises an important biological question: Do these diverse regions undertake a specific conformation at varying Ca^{2+} concentrations, thus giving each protein a distinct function?

That this may, indeed, be the case is supported by the recent mutagenesis studies with GCAPs. A point mutation Y to C in the EF3 hand of GCAPs results in varying effects on the activation of ROS-GC1 by GCAP1 and GCAP2 (*56*). This is consistent with the authors' suggestion that these mutations have a profound effect on the surrounding regions of the GCAPs and that the regions may play an important role in guanylate cyclase activation. This hypothesis is further supported by the Ca^{2+}-dependent studies on GCAP-2 and recoverin (*57*). In this case the linker region between EF3 and EF4 hands is susceptible to V8 proteolysis only in the Ca^{2+}-bound form of GCAP-2 but has no effect on recoverin.

These observations suggest that (1) all three EF hands of the GCAP class of proteins contribute to the overall regulation of ROS-GC1 activity; (2) inactivation of any EF hand in these proteins has a profound effect in the cyclase stimulation, the example being recoverin, which is unable to stimulate ROS-GC1; and (3) the two divergent regions play an important part in recognizing the target protein, ROS-GC1.

In conclusion, this study has made several significant contributions in clarifying the understanding of the ROS-GC-coupled Ca^{2+}-signal transduction field: (1) it has broadened the scope of ROS-GC1 regulation of neuronal signaling by the identification of its additional regulator, neurocalcin; (2) neurocalcin regulation is contrary to its expected structure, based on its crystallographic structure; an explanation for the anomaly has been provided; and most significantly (3) by identifying an additional ROS-GC1 regulator, the study has verified the most puzzling biochemical feature of the enzyme, which is that ROS-GC1 senses both the negative and positive signals of Ca^{2+}. The negative signals are linked to phototransduction and the positive presumably to the neuronal signaling. This provides a variety of ways by which Ca^{2+} via ROS-G1 is able to influence presumed neuronal signaling. In a final note, neurocalcin is distributed in a wide range of neuronal-specific tissues, viz., brain, spinal cord, and retina. A question of enormous biological interest is, do those other tissues, like retina, also contain a ROS-GC? If they do, this will represent a general neuronal signaling pathway. In this connection, in a recent study, the presence of ROS-GC/CD-GCAP-like molecules has been demonstrated in the pineal gland (*58*).

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