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Differential Activation of Rod Outer Segment Membrane Guanylate Cyclases, ROS-GC1 and ROS-GC2, by CD-GCAP and Identification of the Signaling Domain

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The ROS-GC is one of the two subfamilies of membrane guanylate cyclases. It distinguishes itself from the other surface receptor subfamily in that its members are not regulated by extracellular peptides; instead, they are modulated by intracellular Ca^{2+} signals. There are two members of the subfamily, ROS-GC1 and ROS-GC2. An intriguing feature of ROS-GC1 is that it has two Ca^{2+} switches. One switch inhibits the enzyme at micromolar concentrations of Ca^{2+}, and the other stimulates. The inhibitory switch is linked to phototransduction, and it is likely that the stimulatory switch is linked to retinal synaptic activity. Ca^{2+} acts indirectly via Ca^{2+}-binding proteins, GCAPs and CD-GCAP. GCAPs modulate the inhibitory switching component of the cyclase and CD-GCAP turns on the activation signaling switch. The activating switch of ROS-GC2 has not so far been scrutinized. The present study shows that CD-GCAP is linked to the activation signaling switch of ROS-GC2, but the linkage is about 10-fold weaker than that of the ROS-GC1. Thus, CD-GCAP is a specific ROS-GC1 activator. Furthermore, through a series of expression studies on the mutants involving deletion, building of hybrids, and reconstruction of a heterologous cyclase, the study confirms that the CD-GCAP regulated switch resides within the amino acid segment 736-1053 of the cyclase.

During the course of studies aimed at defining the characteristics of rod outer segment membrane guanylate cyclase (ROS-GC) linked to phototransduction, identity of the first member of ROS-GC, ROS-GC1 (1), was established by direct cloning of the native ROS-GC residing in the bovine rod outer segments (2), and by the reconstitution studies in a heterologous system (3). Consistent with its role in phototransduction (4 - 6), the cloned cyclase mimicked the regulation of the native ROS-GC, i.e., 100 nM or lower of free Ca^{2+} concentration stimulated, and the higher (1 μM or more) Ca^{2+} concentrations inhibited the cyclase. The reconstitution studies also established three other features of the native recombinant (r) ROS-GC1. First, the cyclase does not belong to the surface receptor guanylate cyclase family, because it is not stimulated by any of the peptide hormones (1). Second, unlike the latter cyclases, ROS-GC1 is modulated by the intracellular Ca^{2+} signal via Ca^{2+}-binding proteins, termed GCAPs (3, 7 - 9); the cyclase domain modulated by the GCAP resides within the segment covering amino acids (aa) 447-730 (3). Third, the most intriguing feature is that the cyclase is not only inhibited by the submicromolar concentrations of Ca^{2+}, as in phototransduction, but also stimulated by the micromolar (1 μM and above) concentrations of intracellular Ca^{2+} (10, 11). Like the inhibition, the stimulation also occurs indirectly, but this time not via GCAP but via CD-GCAP (10); and the regulated domain, aa 731-1054, is different from the one involved in phototransduction (12). Thus, ROS-GC1 has two Ca^{2+} switches, one linked to the inhibitory and the other to the stimulatory mode of Ca^{2+}.

Reconstitution studies with the second member of ROS-GC, ROS-GC2 (13 - 15), have established that this cyclase is also modulated in a manner consistent with its linkage to phototransduction, and the modulation is via GCAP2 (13, 15). The present study shows that, besides phototransduction, ROS-GC2 is also linked to the stimulatory mode of Ca^{2+} via CD-GCAP and CD-GCAP is a selective activator of ROS-GC1. Thus, there is a CD-GCAP concentration-dependent differentiation of the stimulatory switching-components of the two ROS-GCs.

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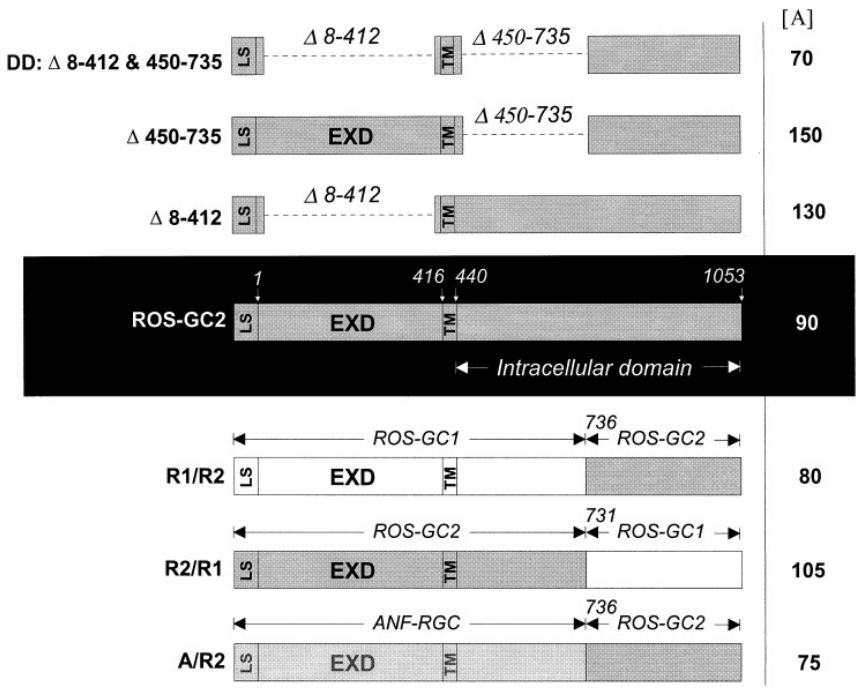

FIG. 1. Schematic representation of ROS-GC2, deletion and hybrid mutants. The predicted domains of ROS-GC2 are denoted by: LS - leader sequence; EXD, extracellular domain; TM, transmembrane domain. For the deletion mutants the dotted lines and the numbers above them correspond to the deleted fragments; for the hybrid mutants, the vertical lines depict the divide between portions of different cyclases. In all mutants, LS was retained to ensure proper translation and functional expression of the protein in the membrane portion of the cell. *Right:* specific activities [A] of the cyclases expressed in COS cells (pmoles cyclic GMP formed/min/mg protein).

MATERIALS AND METHODS

CD-GCAP was either purified from the bovine retina (10) or was purchased commercially as S100B ($\beta\beta$) protein from Sigma. The protein (1 mg) was reconstituted in 50 mM Tris-HCl, pH 7.0/50 mM NaCl (12) and adjusted to a final concentration of 1 mg/ml.

Construction of ROS-GC2 deletion and hybrid mutants. Three ROS-GC2 deletion mutants: Δ8-412 [deleted aa 8-412], Δ450-735 (deleted aa 450-735), double deletion mutant -- DD-- (deleted aa 8-412 and 450-735) were constructed. To generate Δ8-412 and Δ450 - 735 mutants, two *Hpa*1 restriction sites were introduced into ROS-GC2 cDNA by mutagenesis. For the former mutant, the sites were at nucleotide positions 496 and 1711, and for the latter, they were at 1825 and 2680. The desired fragments were excised and the remaining parts were religated. For the DD mutant, two *Bgl*II sites at nucleotide positions 1825 and 2680 were introduced into the Δ8-412 mutant cDNA, the 855 bp fragment was excised and the remaining part religated. All constructs were sequenced to confirm their identities. These mutants are schematically represented in Fig. 1. The mutated cDNAs were cloned into *Kpn*1/*Xba*1 sites of the pcDNA3 vector for expression in COS cells.

Three hybrids of ROS-GC2 were constructed. They were: R1/R2, consisting of aa 1-730 of ROS-GC1 (R1) and aa 736-1053 of ROS-GC2 (R2); R2/R1, consisting of aa 1-735 of ROS-GC2 and aa 731-1054 of ROS-GC1 [note: residues 730 and 731 of ROS-GC1 correspond to the residues 735 and 736 of ROS-GC2 (15)]; and A/R2, consisting of aa 1-745 of ANF-RGC (A) and aa 736-1053 of ROS-GC2 (R2). To construct these mutants an *Hpa*1 restriction site was introduced into ROS-GC1, ROS-GC2 and ANF-RGC cDNAs at nucleotide positions 2409, 2680 and 2638, respectively. In addition, in ROS-GC2 cDNA, the *Kpn*1 restriction site at nucleotide positions 3220-3225 was deleted by a T^{3222}→C mutation, which does not alter Gly^{915} in the encoded protein. The mutated cDNAs were subcloned into *Kpn*1/*Xba*1 sites of the pcDNA3 expression vector, digested with *Kpn*1/*Hpa*1 or *Hpa*1/*Xba*1 and the appropriate fragments were religated. Identities of the generated hybrids were verified by sequencing. These mutants are graphically represented in Fig. 1.

Expression of ROS-GC1, ROS-GC2 and the mutants. ROS-GC1, ROS-GC2 and the mutants were expressed in COS cells according to the previously described protocols (12, 16).

Guanylate cyclase assay. The crude membranes were assayed for guanylate cyclase activity (17). The incubation protocol involving membranes, CD-GCAP and Ca^{2+} was precisely as that described earlier (12). Total assay volume was 25 μl. The amount of cyclic GMP formed was quantified by radioimmunoassay (18).

RESULTS AND DISCUSSION

The purification and characterization of CD-GCAP and its stimulation of both the native and cloned rROS-GC1, suggested a new mode of Ca^{2+} signaling (10). It indicated that ROS-GC1 is not only inhibited by the submicromolar levels of free Ca^{2+} (3, 7, 19), as in phototransduction (4 - 6), but also stimulated by the micromolar concentrations of Ca^{2+} (10). The study noted that the stimulatory feature of the cyclase was unrelated to phototransduction since, in ROS, Ca^{2+} concentration never reaches the micromolar range. Because the immunocytochemical evidence had shown the presence of ROS-GC1 outside ROS, in the synaptic layers (20, 21), and because the depolarization of neurons causes inter-

nal free Ca^{2+} concentrations to rise to the range of 10 to 100 μM (22), the study predicted that the high Ca^{2+}-stimulatory mode of ROS-GC1 is consistent with a possible linkage to the synaptic activity (10). Subsequent biochemical, molecular cloning and expression studies showed that CD-GCAP is identical to S100B, a member of the S100 proteins family (2, 12, 23). Thus, it is now established that S100B ($\beta\beta$), when bound to Ca^{2+}, is a positive regulator of ROS-GC1 and there is a potential for linkage of this regulation with the retinal synaptic activity. Additional studies with ROS-GC1 deletion-mutants have revealed that the low and the high Ca^{2+}-dependent cyclase responses occur through different ROS-GC1 domains (12). Thus, one portion of the cyclase acts as a low Ca^{2+} switch and the other as a high Ca^{2+} switch.

Like ROS-GC1, ROS-GC2 is localized outside and inside ROS (13), it is possible that this cyclase also, through high Ca^{2+} is linked to synaptic activity. To determine this possibility, Ca^{2+}-dependent ROS-GC2 regulation was assessed by reconstitution experiments in a heterologous system of COS cells. To directly compare ROS-GC2 results with those of ROS-GC1 activation by CD-GCAP, the experiments with ROS-GC1 were done side by side under identical conditions.

The membranes from the cells expressing ROS-GC2 (or ROS-GC1) were incubated with a series of incremental concentrations of purified CD-GCAP at a fixed (1mM) Ca^{2+} concentration. CD-GCAP stimulated both ROS-GC1 and ROS-GC2 in a dose-dependent fashion, but their stimulatory patterns were different (Fig. 2A). In ROS-GC1 expressing membranes, the half-maximal effective concentration (EC_{50}) of CD-GCAP was reached at about 1 μM, in the case of ROS-GC2 it was about 8 μM (Fig. 2A). The CD-GCAP concentration causing maximal ROS-GC1 activation was about 5 μM, it was about 15 μM for ROS-GC2 (Fig. 2A), and even at this high concentration of CD-GCAP, the maximal activation of ROS-GC2 was almost half of that of ROS-GC1. The stimulatory effect of CD-GCAP on both ROS-GCs was calcium-dependent and increased as free Ca^{2+} increased. In the absence of CD-GCAP, the cyclases did not respond to changes in concentration of Ca^{2+} (data not shown).

These results show that both ROS-GCs are regulated by high Ca^{2+} levels through CD-GCAP, but CD-GCAP is 8-fold more potent and is twice as effective for ROS-GC1 activation as it is for ROS-GC2. Thus, CD-GCAP is a selective Ca^{2+} regulator of ROS-GC1.

A previous study has shown that the CD-GCAP regulated ROS-GC1 domain resides at its C-terminal portion, aa 731-1054 (12). The corresponding ROS-GC2 segment is 736-1053. To determine if this ROS-GC2 segment represents the CD-GCAP-regulated domain, and if the specificity of CD-GCAP for the cyclases is associated with its isolated fragments, three strategies were used: (1) by deletion-mutation, identify the ROS-GC2-regulated domain; (2) exchange the domain with the corresponding ROS-GC1 domain and determine its functionality in the new cyclase; and in the final analysis (3) construct a heterologous guanylate cyclase, which is transformed from being less specific to being more specific for CD-GCAP regulation. Accordingly, the mutants schematically represented in figure 1 were constructed, appropriately expressed in the membranes of COS cells, and studied for their responsiveness to CD-GCAP. The protocols for these studies were identical to those described earlier for the wild-type ROS-GC1, where the Ca^{2+} concentration was fixed at 1 mM.

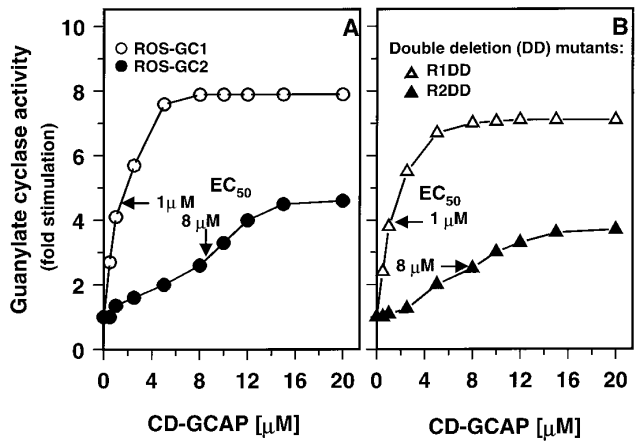

FIG. 2. Effect of CD-GCAP on the cyclase activity of ROS-GC1, ROS-GC2 (A) and their double deletion (DD) mutants (B). COS cells were transfected with ROS-GC1, ROS-GC2 or their DD mutants (R1DD, R2DD) cDNAs and the membranes were prepared as described in "Materials and Methods". These were assayed for guanylate cyclase activity in the presence of 1 mM Ca^{2+} and incremental concentrations of CD-GCAP. The molecular mass of 21,000 Da was used to calculate the concentrations of CD-GCAP. Each experiment was done in triplicate and repeated at least three times with separate membranes preparations. The depicted curves are from the normalized data of these experiments. Error bars are within the size of the symbols. The ROS-GC1 DD was constructed as in reference 3.

The expression studies with three ROS-GC2 deletion mutants--Δ8-412 (deleted segment: aa 8-412; Δ450-735 [deleted segment: aa 450-735] and double deletion mutant (DD) [two deleted segments: aa 8-412 and, aa 450-735]--showed almost identical profiles in response to CD-GCAP (Fig. 2B), which, in turn, were virtually identical to their parent ROS-GCs (Fig. 2A). For brevity, the results with only one, DD mutant are presented. This is because the mutant represents the core cyclase regulated domain.

Figure 2B shows that CD-GCAP stimulated ROS-GC1 DD mutant like the parent ROS-GC1 and ROS-GC2 DD mutant like the parent ROS-GC2: The EC_{50} of CD-GCAP for the ROS-GC1 mutant was about 1 μM and for the ROS-GC2 mutant, it was about 8 μM. CD-GCAP was twice as effective in ROS-GC1 activation than ROS-GC2 activation. These results demonstrate

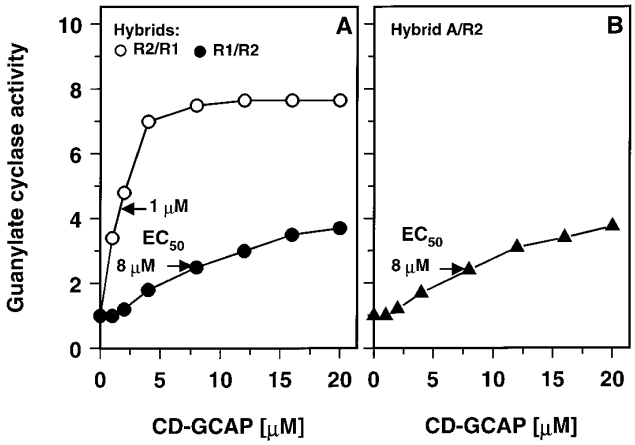

FIG. 3. Effect of CD-GCAP on the cyclase activity of ROS-GC hybrid mutants. Membranes of COS cells expressing hybrids: R1/R2, R2/R1 and A/R2 were prepared as described in "Materials and Methods". These were assayed for the guanylate cyclase activity in the presence of 1 mM Ca^{2+} and incremental concentrations of CD-GCAP. Experimental conditions were identical to those in figure 2. Experiments were repeated three times with different membrane preparations. The depicted curves are from the normalized data of these experiments. Error bars are within the size of the symbols.

that the cyclase domains--aa 8-412 and 450-735--have no role in the CD-GCAP regulation of ROS-GC2, and similarly, as shown earlier (12), they have no role in the ROS-GC1 regulation. Thus, the CD-GCAP-regulated domain must lie within 736-1053 residues of ROS-GC2, a domain located identically to the ROS-GC1-regulated domain (12). This conclusion is supported through the Hybrid R1/R2 and Hybrid R2/R1 expression studies as described below.

An earlier study showed that CD-GCAP-regulated domain resides within the aa 731-1054 segment of ROS-GC1 (12). The present study shows that CD-GCAP-regulated domain of ROS-GC2 resides within its aa 736-1053 segment. To determine if the exchange of these segments will bring into the cyclase CD-GCAP-dependent specificity, hybrids: R1/R2 and R2/R1 were constructed (Fig. 1). In R1/R2, aa 731-1054 segment of ROS-GC1 was replaced by the corresponding segment of ROS-GC2, aa 736-1053; and in Hybrid R2/R1, the C-terminal region of ROS-GC2 (aa 736-1053) was replaced by ROS-GC1 region, aa 731-1054.

The cyclase activity of both hybrids was stimulated by CD-GCAP in a dose- dependent fashion, but stimulatory patterns of the hybrids were different (Fig. 3A): R1/R2 responded almost like ROS-GC2 with EC_{50} of $\sim 8\mu M$, and R2/R1 responded like ROS-GC1 with EC_{50} of $\sim 1\ \mu M$. The results clearly demonstrated specificity of CD-GCAP for ROS-GC1 and showed that the cyclase segments regulated by CD-GCAP are identically located in their parent ROS-GCs, a conclusion further supported by the third analysis.

ANF-RGC does not belong to the ROS-GC subfamily of membrane guanylate cyclases. In contrast to ROS-GC, ANF-RGC is not intracellularly regulated by Ca^{2+} (3). The previous study has established that the appropriate introduction of aa 731-1054 segment of ROS-GC1 in ANF-RGC converts the remodeled ANF-RGC into functional ROS-GC1, i.e., the ANF-RGC hybrid now appropriately responds to CD-GCAP (12). To determine if the appropriate introduction of aa 736-1053 segment of ROS-GC2 in ANF-RGC will also convert the remodeled ANF-RGC (A/R2) into functional ROS-GC2, the ANF-RGC segment (aa 746-1030) was replaced by the R2 segment (aa 736-1053) of ROS-GC2. The construct was expressed in mammalian cells and the GCAP2-mediated regulation by Ca^{2+} was assessed as described earlier.

The stimulatory pattern of A/R2 was nearly identical to that of the parent ROS-GC2 (Fig. 3B): The EC_{50} values of CD-GCAP for A/R2 and ROS-GC2 were 8 μM (Compare: Fig. 2A with Fig. 3B), and the maximal cyclase activation in both A/R2 and the parent cyclase was achieved at about 15 μM of CD-GCAP, and the maximal activation of A/R2 was about half to that of the wild-type ROS-GC1.

The results establish that CD-GCAP is a selective Ca^{2+} regulator of ROS-GC1 and that the ROS-GC2-regulated domain resides within the segment covering aa 736-1053.

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