# The Kinase Homology Domain of Retinal Guanylyl Cyclases 1 and 2 Specifies the Affinity and Cooperativity of Interaction with Guanylyl Cyclase Activating Protein-2†

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ABSTRACT: RetGC-1 and RetGC-2 are photoreceptor membrane guanylyl cyclases that are regulated by the $Ca^{2+}$-binding protein, GCAP-2. We used a protease protection assay to localize regions of the intracellular domains of RetGCs important for the interaction with GCAP-2 and found that GCAP-2 reduces the access of trypsin to a site in the kinase homology domain (KHD) of RetGC-1. The protective effect of GCAP-2 is independent of $Ca^{2+}$. We also found that RetGC-2 and GCAP-2 interact cooperatively with high affinity, but RetGC-1 and GCAP-2 interact noncooperatively with low affinity. By analyzing RetGC-1/RetGC-2 chimeras we demonstrated that the affinity and cooperativity of the interaction with GCAP-2 is dictated by the structure of the KHD. These findings suggest that GCAP-2 interacts constituitively with the KHDs of RetGC-1 and RetGC-2 and that cGMP synthesis is controlled by $Ca^{2+}$-dependent conformational changes in the RetGC/GCAP complex.

The guanylyl cyclase (GC)[1] activity of vertebrate photoreceptors is represented by at least two membrane GCs, RetGC-1 and RetGC-2. These isozymes were first cloned from a human retinal cDNA library and subsequently from bovine and rat libraries (*1*−*5*). Expression of RetGC-2 has been detected only in photoreceptors, and RetGC-1 has been found in both photoreceptors and pineal (*1*, *2*, *4*, *6*). Electron microscopy studies of primate retina have localized RetGC-1 primarily to the disks of rod outer segments and to the invaginated membranes of cones (*7*). More recently, a similar subcellular localization was established in rat retina where both RetGC-1 and RetGC-2 were found in the rod outer segment disks (*8*). The activities of recombinant RetGC-1 and RetGC-2, like the endogenous GC activity of bovine photoreceptor outer segments (OS), are activated by the photoreceptor proteins, GCAP-2 and GCAP-1, at low concentrations (nM) of free $Ca^{2+}$ (*2*, *6*, *9*, *10*). $Ca^{2+}$ inhibits RetGC activation by GCAP-1 and GCAP-2 with $IC_{50}$s between 100 and 300 nM (*2*, *5*, *6*, *11*−*13*). The localization of RetGC-1 and RetGC-2 and their regulation by $Ca^{2+}$-sensor proteins suggest that they function in the recovery phase of photoexcitation and light adaptation.

Both RetGC-1 and RetGC-2 are members of the membrane GC family that includes the natriuretic peptide receptor-GCs (NPR-A/GC-A and NPR-B/GC-B), the heat-stable enterotoxin receptor-GC (StaR/GC-C) and the sea urchin sperm GCs (*1*, *2*, *14*). Each member of the membrane GC family is a type I transmembrane protein that has an extracellular domain (ECD) linked by a single transmembrane domain (TMD) to an intracellular domain. Within the intracellular domain the membrane-proximal region is homologous to protein kinases (KHD). Adjacent to the KHD is a dimerization domain (DD) which contains a 41 amino acid stretch predicted to form an amphipathic α-helix. This amphipathic α-helix is necessary and sufficient for dimerization of the NPR-A intracellular domain (*15*). On the carboxyl terminal side of the DD lies the cyclase catalytic domain (CAT) extending to the end of the protein for NPR-A and NPR-B. There is an additional hydrophilic domain of unknown function on the carboxyl terminus of RetGC-1, RetGC-2, and StaR (CT).

NPR-A, NPR-B, StaR, and the sperm GCs are all activated by peptide ligands that bind to their extracellular domains (*14*, *16*). In contrast, RetGC-1 is activated through its intracellular domain. Deletion mutants of RetGC-1 lacking the ECD or both the ECD and TMD are activated by GCAP-1 or GCAP-2 in a manner indistinguishable from full-length RetGC-1 (*11*, *13*). The reconstitution of $Ca^{2+}$-sensitive GC activity using ECD/TMD truncated RetGC-1 membranes and purified recombinant GCAP-2 strongly suggested that GC activation occurs through binding of GCAPs to the intracellular face of RetGCs (*11*). Additional support for a direct binding model of GCAPs to RetGCs

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[1] Abbreviations: RetGC, retinal guanylyl cyclase; GC, guanylyl cyclase; OS, photoreceptor outer segments; GCAP, guanylyl cyclase activating protein; NPR-A and NPR-B, natriuretic peptide receptors; StaR, heat-stable enterotoxin receptor; ECD, extracellular domain; TMD, transmembrane domain; KHD, kinase homology domain; DD, dimerization domain; CAT, cyclase catalytic domain; CT, carboxyl terminus.

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comes from a cross-linking experiment that yielded a large protein complex immunoreactive for both RetGC-1 and GCAP-1 (*13*). Deletion of the KHD of RetGC-1 interferes with its ability to be stimulated by GCAP-1 suggesting that the KHD is a potential interaction site for the GCAPs (*13*). These observations suggested that GCAPs regulate RetGC activity by binding to the KHD of the RetGCs. However, it was recently reported that deletion of most of the RetGC-1 KHD is not required for activation by GCAP-2 (*17*).

To further clarify the molecular mechanism by which GCAPs regulate RetGC activity we used two strategies to examine the interaction between GCAP-2 and the RetGCs. First, we used proteolysis protection assays to demonstrate a $Ca^{2+}$-independent interaction between GCAP-2 and RetGC-1 in OS. Second, we found that GCAP-2 interacts with RetGC-1 and RetGC-2 with different affinity and cooperativity. Exploiting these observations, we generated RetGC-1/RetGC-2 chimeras and found that the KHD determines the affinity and cooperativity of the interaction with GCAP-2. The results of both studies suggest that the GCAP-2 binding site lies at least partially within the KHD of RetGCs.

## MATERIALS AND METHODS

*Photoreceptor Outer Segment Preparations*. Outer segments (OS) were isolated from frozen dark-adapted bovine retinas (J. A. Lawson Co., Lincoln, NE) using sucrose flotation. OS membranes were washed three times by homogenization at 0.5 mg rhodopsin/mL in 5 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM ATP, 10 mM 2-mercaptoethanol, and 5% (v/v) glycerol in the dark. Washed membranes were resuspended in 10 mM Tris-HCl, 5 mM 2-mercaptoethanol, frozen on dry ice, and stored in foil wrapped tubes at −70 °C. The washed OS contained less than 5% of the endogenous $Ca^{2+}$-sensitive GC activity. However, $Ca^{2+}$-sensitive GC activity could be restored by reconstitution of washed OS with purified recombinant GCAP-2. The rhodopsin concentration of OS preparations was measured by absorbance at 500 nm in 1.5% lauryldimethylamine oxide.

*Recombinant GCAP-2, Recoverin, and Neurocalcin*. Expression and purification of myristoylated bovine GCAP-2 in *Escherichia coli* has been described previously (*18*). Purified myristoylated bovine neurocalcin and bovine recoverin expressed in *E. coli* were provided by Dr. E. Faurobert and prepared as described previously (*19*). Recoverin preparations were additionally purified over a Superdex 200 (Pharmacia) gel filtration column in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM $CaCl_2$, and 10 mM 2-mercaptoethanol to further remove contaminant *E. coli* proteins which cross-reacted with some of our GC antibodies. Protein concentrations were determined with the BioRad Protein assay, BCA assay (Pierce), and by amino acid analysis. Both protein assays yielded protein concentrations equivalent to that determined by amino acid analysis.

*RetGC-1 Antibodies*. Amino acids $Met^{443}$-$Ser^{746}$ of human RetGC-1 were expressed as a carboxyl-terminal fusion to glutathione-S transferase in BL21 *E. coli* using the pGEX 2T vector (Pharmacia). The insoluble fusion protein was isolated by preparative SDS−PAGE, electroelution, and acetone precipitation and then resuspended in PBS for inoculation into rabbits. The polyclonal antibodies produced were designated as "KHD". KHD recognizes both bovine RetGC-1 and recombinant human RetGC-1. $Met^{747}$-$Ser^{1052}$ of human RetGC-1 encompassing the DD, CAT, and CT were expressed in BL21 *E. coli* using the pET 11d vector (Novagen). The insoluble recombinant protein was purified and inoculated as described above. The antibodies produced were designated as "CAT" and recognized both bovine RetGC-1 and recombinant human RetGC-1. Immunoblot analyses with both the KHD and CAT immune sera reveal a 115 kDa band from OS or from recombinant HEK 293 cell membranes containing RetGC-1, but not from control HEK 293 cell membranes. Pre-immune sera did not recognize the 115 kDa protein. The CAT and KHD antibodies were affinity-purified by incubating the antiserum diluted 1:10 in TTBS (Tris-buffered saline, 0.05% Tween-20) with strips of Immobilon P membranes containing 100−200 $\mu$g of the corresponding gel-purified antigens. After extensive washing with TTBS, specific antibodies were eluted from the Immobilon P strips with 20 mM Glycine (pH 2.5) and 0.05% Tween 20, and the eluates were neutralized with 1 M $Na_2HPO_4$. To obtain antibodies against the carboxyl terminus of RetGC-1, amino acids $Asn^{1011}$-$Ser^{1052}$ were fused to glutathione-S transferase (pGEX 2T) and expressed in BL21 *E. coli*. The soluble fusion was purified by affinity chromatography using glutathione Sepharose 4B (Pharmacia), then coupled to CNBr activated Sepharose 4B. The coupled fusion protein was used to purify a subset of antibodies from the CAT antiserum specific for the carboxyl terminus of RetGC-1. The affinity-purified antibodies were designated as "CT". The carboxyl terminus specificity was demonstrated by the ability of gst-$Asn^{1011}$-$Ser^{1052}$ to compete away the RetGC-1 signal obtained from these antibodies on immunoblots. Neither the CAT or KHD antibody recognize RetGC-2 from OS based on the mobility of RetGC-1 and RetGC-2 immunoreactive signals resolved by 6% SDS−PAGE (*2*). Starting from the first amino acid in the mature processed protein, the numbering of amino acids for the aligned sequences of human and bovine RetGC-1 are the same; however, bovine RetGC-1 has two additional amino acids at the carboxyl terminus.

*Other Antibodies.* The monoclonal antibody RGC-6G7 is directed against the ECD of bovine RetGC-1 and was a gift from the laboratory of Dr. Robert Molday. The epitope within the ECD of RetGC-1 recognized by this antibody has not been defined.

*Immunoblot Analysis*. Samples to be analyzed with antibodies were electrophoresed on SDS−PAGE gels and then transferred to Immobilon P membrane (Millipore). Immunoblots to be directly compared for signal intensity (OD) were transferred in the cold room overnight at 40 V in the same cassette of a large BioRad transblot cell to ensure even transfer of protein on each membrane. Otherwise, transfers were carried out in a BioRad mini-PROTEAN II apparatus for 1.5 h at 100 V. SDS (0.02%) was included in all transfer buffer to ensure complete transfer of large proteins. Membranes were blocked in TTBS containing 10% nonfat dry milk, incubated with primary antibody, washed with TTBS, and incubated with horseradish peroxidase-linked secondary antibody (Amersham). Immunoreactive bands were revealed using chemiluminescence reagents (DuPont) and exposed with Reflection autoradiography film (DuPont). Optical density of immunoblot signals was obtained with a

model GS-670 BioRad imaging densitometer. Relative comparison between blots was made only if the blots were transferred in the same cell and developed on the same film.

*Proteolysis Protection Assay*. Washed OS at a final concentration of 1.3 mg rhodopsin/mL were incubated in the absence or presence of increasing amounts of TPCK-treated trypsin (Sigma) in 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 0.5 mM ATP, and either 1 mM EGTA or 1 mM $CaCl_2$ in a final volume of 30 $\mu$L. The assays were carried out in the dark for 10 min at 30 °C. The samples contained GCAP-2, or as a control, neurocalcin or recoverin at the concentrations indicated in the figure legends. The control proteins, recoverin and neurocalcin, were chosen because they are in the same protein family as GCAP-2, but, they do not affect RetGC. Neither recoverin nor neurocalcin (30 $\mu$M each) activates RetGC-1 or interferes with activation of RetGC-1 by 1.7 $\mu$M GCAP-2 (not shown). Reactions were stopped by heating for 4 min at 95 °C. Time course proteolysis experiments were conducted similarly except that all samples contained the same concentration of trypsin and were incubated for the indicated time. The samples were analyzed by SDS−PAGE and immunoblotting. Membranes were stained with Ponceau S to confirm even transfer of proteins. Some of the bands observed in Figure 1 are contaminant *E. coli* proteins common to the neurocalcin preparation and the RetGC-1 fragments used to generate the antibodies, both of which were expressed in *E. coli*. The contaminant proteins are not significantly proteolyzed during the assay and did not interfere with the analysis.

*Construction of Human RetGC Chimeras*. The construction of pRC CMV RetGC-1 and pRC CMV RetGC-2 has been described previously (*2, 11*). Two RetGC-1/RetGC-2 chimeras were constructed by inserting a silent *Cla*I restriction site into the coding region of RetGC-1 and RetGC-2 at points where the amino acids were identical. Initially, a silent *Cla*I site was created at $Ile^{766}$-$Asp^{767}$ of RetGC-1 and RetGC-2 using the Quick Change Site Directed mutagenesis kit (Stratagene). The *Cla*I constructs were then further modified by creation of an *Sgr*AI site at $Arg^{437}$-$Arg^{439}$ of RetGC-1 and $Arg^{441}$-$Arg^{443}$ of RetGC-2. Creation of the *Sgr*AI site in RetGC-1 required changing $His^{488}$ to Arg; however, the point mutation had no measurable effect on the properties of the recombinant protein. The chimeras RetGC-121 and RetGC-212 (refer to Figure 5A for schematics) were constructed by exchanging the *Cla*I/*Sgr*AI digest fragments from pRC CMV RetGC-1 and pRC CMV RetGC-2. Formation of chimeras was demonstrated by DNA sequencing of the constructs. At the protein level, the domain defined as the KHD (running from adjacent to the TMD to adjacent to the DD) was exchanged.

*Expression in HEK 293 Cells and Isolation of Recombinant Membranes*. Transient transfection of human embryonic kidney (HEK) 293 or COS 7 cells with pRC CMV RetGC expression constructs was carried out using the calcium phosphate precipitation method as described previously (*11*). Cells were isolated 60 h post transfection. Cells were washed with PBS, removed from dishes by agitating in PBS, 0.5 mM EDTA (HEK 293 cells), or scraping (COS-7 cells) and pelleted by centrifugation. A 100 mM dish of cells was resuspended in 1.5 mL of hypotonic lysis buffer [10 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 1 mM ATP], and the cells were allowed to swell for 10 min on ice. Cells were lysed by passing 8 times through a 26 gauge needle. Lysates were clarified by centrifugation for 10 min at 2500 rpm in an SS-34 rotor. Clarified supernatants were brought to 200 mM NaCl and centrifuged for 20 min at 90000 rpm in a Beckman tabletop ultracentrifuge using a TLA 100.1 rotor to isolate recombinant membranes. The membranes were resuspended in 10 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, frozen on dry ice, and stored as aliquots at −70 °C.

*Guanylyl Cyclase Assay*. GC assay of recombinant membranes was carried out for 30 min at 30 °C as described previously (*9, 11*) except that a final concentration of 0.5 mM ATP was present in all assays.

## RESULTS

*GCAP-2 Protects RetGC-1 from Proteolysis*. To examine how GCAP-2 interacts with RetGC-1, we analyzed the ability of GCAP-2 to protect RetGC-1 from proteolysis. Washed OS were reconstituted either with recombinant GCAP-2 or with recombinant neurocalcin as a control. Neurocalcin and recoverin are $Ca^{2+}$-binding proteins that have no effect on the GC activity of OS. The mixtures were then treated with increasing concentrations of trypsin. The resulting pattern of RetGC-1 proteolysis was analyzed by immunoblot analysis using polyclonal antibodies generated against intracellular fragments of RetGC-1 or using a monoclonal antibody against the extracellular domain. The regions of RetGC recognized by the antibodies are summarized in Figure 1B. The pattern of proteolysis revealed by each antibody is shown in Figure 1A. Immunoblots on the left reveal proteolysis of RetGC-1 in the presence of the control protein, neurocalcin, and on the right, GCAP-2.

By comparing the pattern of proteolytic fragments recognized by each antibody, we identified the approximate location of the trypsin cleavage sites as shown schematically in Figure 1B. The uppermost immunoblots probed with the CAT antibody show that trypsin produces a 62 kDa fragment that includes most of the intracellular region of RetGC-1. This assignment is based on the size of the fragment and its immunoreactivity with antibodies against the 42 carboxyl terminal amino acids of RetGC-1 and against the KHD (Figure 1A,B). Trypsin also produces a 54 kDa band, detected strongly by the ECD antibody and more weakly by the KHD antibody. This fragment is not proteolyzed further even at the highest trypsin concentration used in the experiment (Figure 1A). Together, these observations suggest that the initial trypsin cleavage site is in the KHD close to the TMD. Cleavage at this site splits RetGC-1 into 62 kDa and 54 kDa fragments as diagrammed in Figure 1B. It should be noted that formations of the 62 and 54 kDa fragments do not appear simultaneously on the immunoblots. There are two reasons for this. First, the antibodies used to detect these fragments appear to have different sensitivities. This explains why we detect the 62 kDa fragment with the CT, CAT, and KHD antibodies before we detect the 54 kDa fragment with the ECD antibody. Second, some of the 62 kDa fragment is being further proteolyzed even as it is formed, resulting in a maximum signal intensity for the 62 kDa fragment which precedes that of the 54 kDa fragment. Densitometry analysis of full-length RetGC-1 at each trypsin concentration shows that GCAP-2 does not notably protect the first cleavage site (Figure 1C).

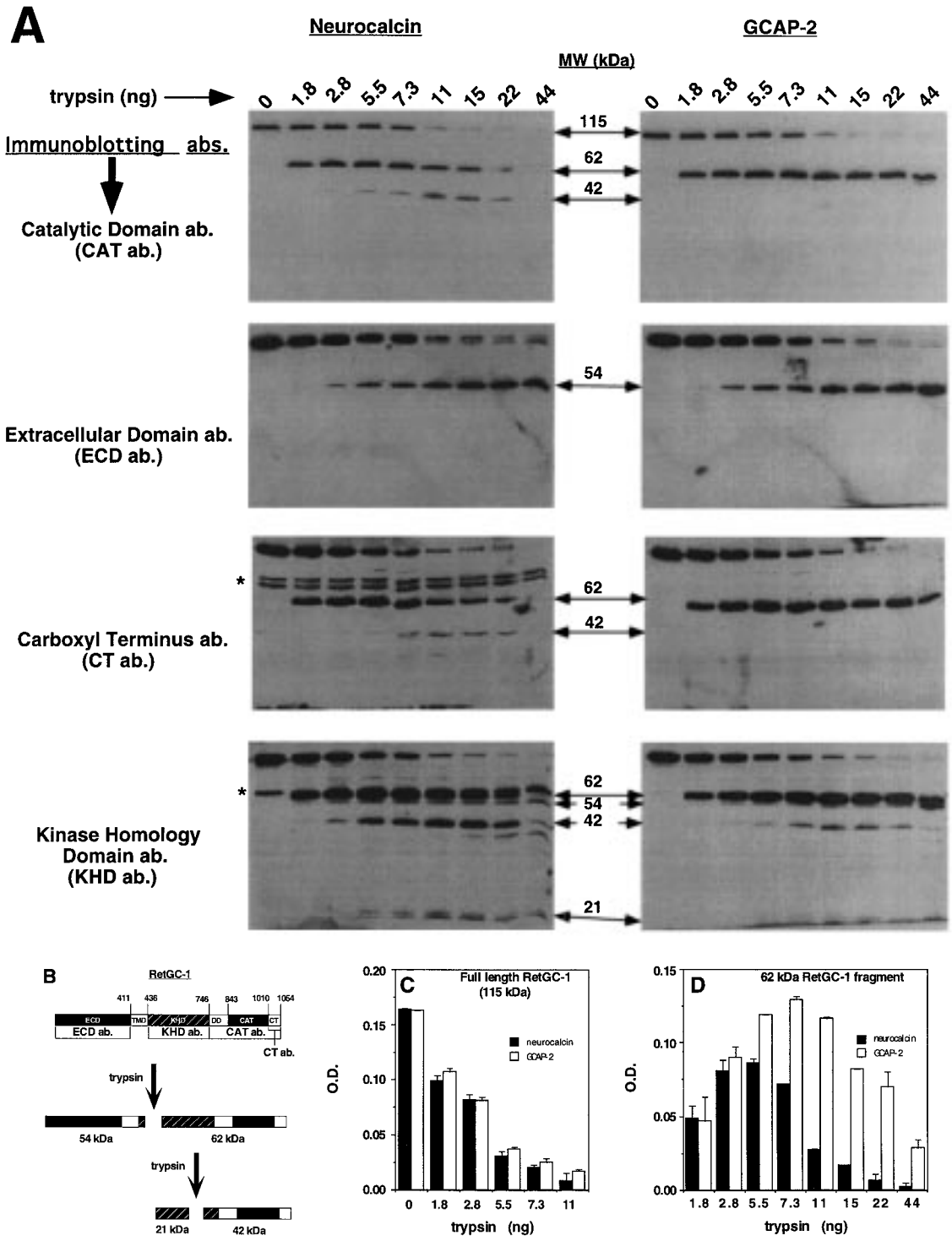


FIGURE 1: GCAP-2 protects RetGC-1 from proteolysis by trypsin. Duplicate samples of washed OS membranes (40 $\mu$g rhodopsin/sample) were treated with increasing concentrations of trypsin for 10 min at 30 °C in the dark. Proteolysis was carried out in the presence of 3 $\mu$M neurocalcin or GCAP-2. (A) The samples were separated by SDS−PAGE and analyzed by immunoblot using the RetGC-1 domain-specific antibodies indicated on the left margin. The antibodies are described in detail in Materials and Methods. Arrows and numbers between each pair of immunoblots denote the MW of proteolysis fragments estimated from samples resolved on a 10 cm gel and analyzed by immunoblot. The asterisk denotes immunoreactive contaminates in the neurocalcin preparation (see Materials and Methods). Two additional experiments using different preparations of washed OS or GCAP-2 yielded equivalent results. (B) A schematic representation of the domain structure of bovine RetGC-1 and qualitative representation of the RetGC-1 fragments formed by the trypsin digest. Brackets enclose potential epitopes for the KHD, CAT, and CT antibodies. The epitope within the RetGC-1 ECD recognized by the monoclonal antibody has not been defined. Numbers labeling the domain boundaries correspond to the last amino acid of that domain as defined previously for human RetGC-1 (15). (C, D) Optical density (OD) measurements of bands corresponding to (C) full-length 115 kDa RetGC-1 and (D) the 62 kDa fragment in the presence of neurocalcin (black bars) or GCAP-2 (white bars) generated from this experiment. OD and ranges were determined by densitometry using immunoblots from both replicates probed with the CAT antibody.

The 62 kDa fragment is specifically protected by GCAP-2 from further proteolysis (Figure 1A,D). In the absence of GCAP-2, it is rapidly cleaved into 42 kDa and 21 kDa fragments detected as single bands or doublets depending

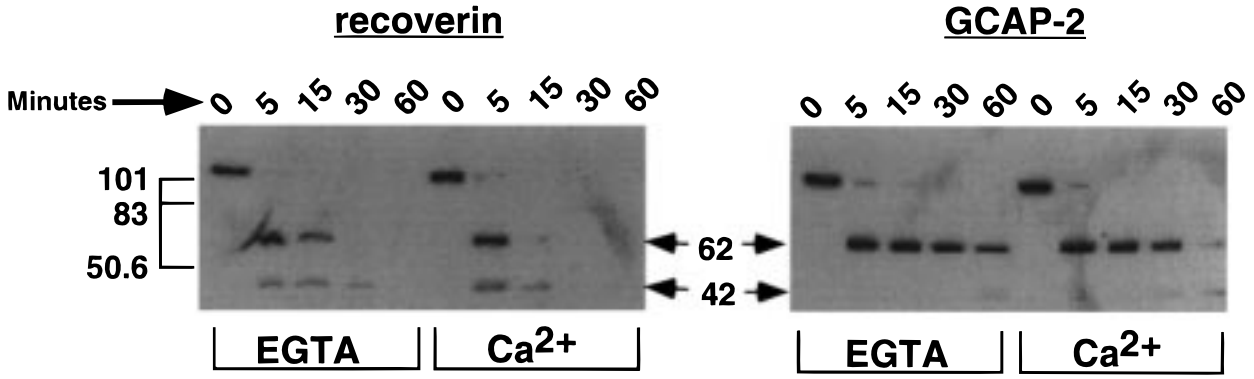


FIGURE 2: RetGC-1 is protected from proteolysis in a $Ca^{2+}$-independent manner by GCAP-2. Washed OS (30 $\mu$g/sample) were reconstituted with 5 $\mu$M GCAP-2 or 5 $\mu$M recoverin and treated with 20 ng trypsin for the indicated times at 30 °C in the dark. Reactions were carried out in 1 mM EGTA or 1 mM $CaCl_2$ as indicated. The samples were analyzed by immunoblot with affinity-purified CAT antibody (shown here) and the KHD antibody (not shown).

on the trypsin concentration. The sum of the MWs and the immunoreactivity of the 42 kDa and 21 kDa fragments support this conclusion (Figure 1A). The 42 kDa fragment is recognized by the CT, CAT, and KHD antibodies, whereas the 21 kDa fragment is recognized only by the KHD antibody (Figure 1A). The absence of an effect of GCAP-2 on the sizes of the tryptic fragments together with the effect of GCAP-2 on the kinetics of proteolysis indicates that GCAP-2 hinders access of trypsin to a specific site in RetGC-1 near the carboxyl boundary of the KHD (Figure 1B).

*Proteolysis in $Ca^{2+}$ vs EGTA.* Previous biochemical data has shown that $Ca^{2+}$-bound GCAP-2 blocks activation of OS GC by a dominant positive EF-hand knockout of GCAP-2 (*18*). Furthermore, $Ca^{2+}$-loaded GCAP-1 or GCAP-2 decrease the basal activity of OS GCs (*18, 20*). These results suggest that the RetGC/GCAP interaction may be $Ca^{2+}$-independent. To directly examine whether the interaction of GCAP-2 with RetGC-1 is $Ca^{2+}$-sensitive, we evaluated the ability of GCAP-2 to protect RetGC-1 from proteolysis in the presence or absence of $Ca^{2+}$. We found that GCAP-2, but not recoverin, protects the 62 kDa fragment of RetGC-1 from proteolysis in both high and low $Ca^{2+}$ (Figure 2). There is a small $Ca^{2+}$-dependent component to the trypsin sensitivity of RetGC-1, but it is independent of GCAP-2. These results are consistent with RetGC-1 and GCAP-2 interacting at free $Ca^{2+}$ concentrations that both activate and inhibit cyclase activity.

*Differences in Regulation of RetGC-1 and RetGC-2 by GCAP-2.* Previously we reported that the half-saturation ($K_{1/2}$) for activation of human RetGC-1 by GCAP-2 was approximately 40 times higher than that measured for activation of OS GCs (*11*). The disparate apparent affinities led us to compare the sensitivities of recombinant human RetGC-1 and RetGC-2 to GCAP-2. Figure 3A shows that stimulation of both RetGC-1 and RetGC-2 by GCAP-2 is inhibited by $Ca^{2+}$ with nearly identical $IC_{50}$s for $Ca^{2+}$ of 130 $\pm$ 2 nM and 106 $\pm$ 1.7 nM, respectively. Close inspection of the $Ca^{2+}$ titration data reveals significant differences between the two GCs. RetGC-2 has a higher activity in the absence of GCAP-2 (basal activity) than RetGC-1 when compared to their normalized maximal GCAP-2 stimulated activities. The higher basal activity of RetGC-2 is also substantially inhibited by GCAP-2 at concentrations of $Ca^{2+}$ > 500 nM, whereas the basal activity of RetGC-1 is not (Figure 3A). Inhibition of GC activity at high $Ca^{2+}$ does not occur in the absence of GCAP-2, demonstrating that GCAP-2 causes both activation and inhibition. The overall ranges of both RetGC-1 and RetGC-2 activity are similar when both activation above and inhibition below their basal activities are considered.

Another difference between RetGC-1 and RetGC-2 is that RetGC-1 has a 20−25-fold lower apparent affinity ($K_{1/2}$ = 2.4 $\pm$ 0.12 $\mu$M) than RetGC-2 ($K_{1/2}$ = 112 $\pm$ 3 nM) for GCAP-2 (Figure 3B). Hill coefficients ($n$) of 1.7 $\pm$ 0.07 for RetGC-2 and 1.1 $\pm$ 0.04 for RetGC-1 were calculated from the GCAP-2 titrations by fitting the data with the Hill equation. The higher cooperativity of the RetGC-2/GCAP-2 interaction is evident as a steeper slope in Figure 3B.

$K_{1/2}$ and $n$ values were constant over a range (30-fold) of cyclase expression levels tested using HEK 293 cell membranes. These values were also independent of the concentration of recombinant membranes (not shown). This demonstrated that the $K_{1/2}$ and $n$ values are not affected by the RetGC/lipid ratio and that GCAP-2 was always present in a large molar excess over active RetGC in our assay system. $K_{1/2}$ and $n$ values were the same whether RetGCs were expressed in HEK 293 or in COS-7 cells (not shown).

We also examined inhibition of RetGC basal activity under high $Ca^{2+}$ conditions as a function of GCAP-2 concentration. The $K_{1/2}$ (67 $\pm$ 3 nM) and $n$ (2.0 $\pm$ 0.16) for inhibition of RetGC-2 basal activity by $Ca^{2+}$-loaded GCAP-2 are similar to the corresponding values for activation by $Ca^{2+}$-free GCAP-2 (Figure 3C). RetGC-1 activity is slightly stimulated by a specific concentration window of $Ca^{2+}$-loaded GCAP-2 (Figure 3C). The small stimulation was variable in magnitude and was sometimes followed by inhibition of RetGC-1 basal activity (~30%) at the highest concentrations of GCAP-2. The significance of this variability remains unclear. No stimulation was observed when GCAP-2 was replaced with recoverin under high $Ca^{2+}$ conditions (not shown).

The affinity and cooperativity with which bovine GCAP-2 regulates human RetGC-2 and GC activity in bovine OS membranes are nearly identical. However, regulation of human RetGC-1 by GCAP-2 is different. To determine if these differences reflected species-specific interactions we subsequently measured activation of bovine RetGC-1 and bovine RetGC-2 by bovine GCAP-2. We found $K_{1/2}$ and $n$ values to be 1.18 $\pm$ 0.07 $\mu$M and 0.99 $\pm$ 0.05 for bovine RetGC-1 and 124 $\pm$ 2 nM and 1.95 $\pm$ 0.2 for bovine RetGC-2 (Figure 4). These values suggest that recombinant

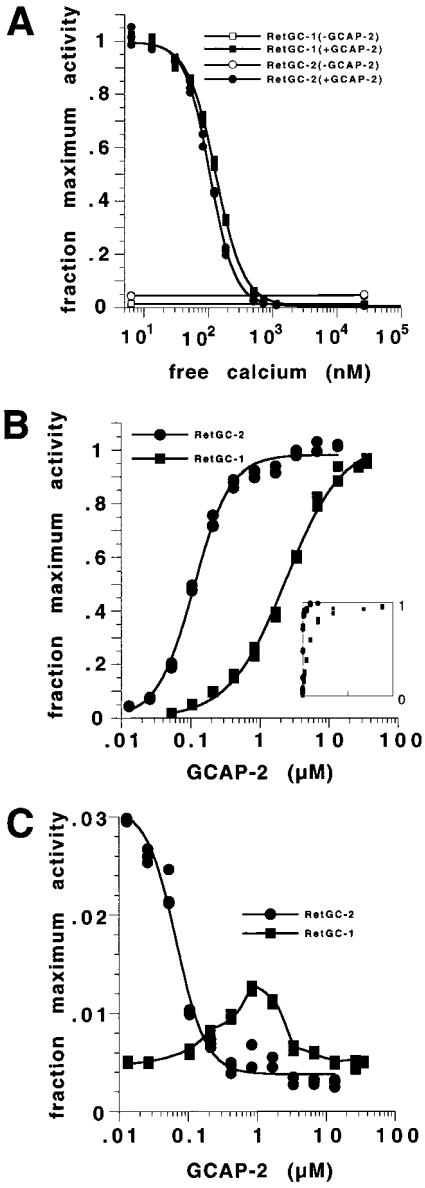


FIGURE 3: Comparative regulation of RetGC-1 and RetGC-2 activity by GCAP-2. (A) The sensitivity of GC activity to increasing $Ca^{2+}$ levels in the presence of 13 $\mu$M (RetGC-1) or 1.7 $\mu$M (RetGC-2) GCAP-2. The $IC_{50}$; Hill coefficient ($n$) for $Ca^{2+}$ are 130 nM; 1.94 for RetGC-1 and 106 nM; 2.20 for RetGC-2. $IC_{50}$, $n$, and apparent $v_0$ were determined by fitting the data to the equation: $v = v_0 - (V_{sat/min}\,[Ca^{2+}]^n/(IC_{50}{}^n + [Ca^{2+}]^n))$. The data shown has been normalized to GCAP-2 stimulated activity in the absence of $Ca^{2+}$ ($v_0$) to facilitate comparison of RetGC-1 and RetGC-2 $Ca^{2+}$-sensitivities. (B) GCAP-2 saturation curves for activation of RetGC-1 and RetGC-2 at 7 nM $Ca^{2+}$. Inset shows the same data plotted against a linear $x$-axis. The $K_{1/2}$; $n$ of GCAP-2 for RetGC-1 and RetGC-2 were 2.4 $\mu$M; 1.1 and 112 nM; 1.7, respectively. $K_{1/2}$, $n$, and $V_{sat/max}$ were obtained by fitting the data to the equation: $v = v_0 + (V_{sat/max}[GCAP\text{-}2]^n/(K_{1/2}{}^n + [GCAP\text{-}2]^n))$ where $v_0$ is the activity in the absence of GCAP-2. The data shown are normalized to maximal activation ($V_{sat/max}$). (C) GCAP-2 saturation curves for the inhibition of RetGC-1 and RetGC-2 by GCAP-2 at 27 $\mu$M $Ca^{2+}$. $K_{1/2}$ and $n$ values of 68 nM and 2.0, respectively, were obtained only for RetGC-2 by fitting the data to the equation $v = v_0 - (V_{sat/min}[GCAP\text{-}2]^n/(K_{1/2}{}^n + [GCAP\text{-}2]^n))$. Data were normalized to the maximum activity under the assay conditions ($v_0$) which was set equal to the fraction of maximal activation obtained by GCAP-2 in 7 nM $Ca^{2+}$ as in (B). The results shown are representative of at least two independent experiments, each with triplicate data points.

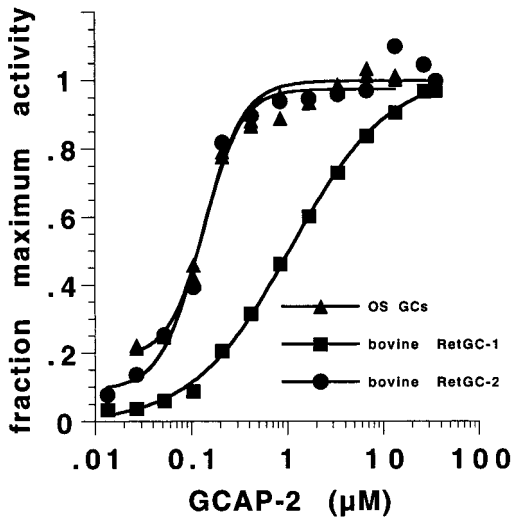


FIGURE 4: Activation of OS GCs and recombinant bovine RetGC-1 and RetGC-2 by GCAP-2. GCAP-2 saturation curves for activation of washed bovine OS or bovine RetGC-1 and bovine RetGC-2 at 7 nM $Ca^{2+}$. The $K_{1/2}$; $n$ of GCAP-2 for bovine OS, bovine RetGC-1, and bovine RetGC-2 were 130 nM and 2.2, 1.18 $\mu$M and 0.99, or 124 nM and 1.95, respectively. Data were normalized and plotted as described in previous figure legends. An additional experiment yielded equivalent results.

human and bovine RetGCs function essentially the same with respect to GCAP-2. However, the absence of a prevalent low-affinity target of GCAP-2 in OS representative of the RetGC-1/GCAP-2 complex is perplexing. One possible explanation is that RetGC-2 is responsible for most of the GC activity in bovine OS. Because rod photoreceptors predominate in the bovine retina, this might be expected if RetGC-2 represents the rod form of photoreceptor GCs.

*RetGC-1/RetGC-2 Chimeras*. The apparent affinity of RetGC-1 for GCAP-2 is weak, but regulation of RetGC-2 by GCAP-2 is cooperative and 20−25-fold stronger (Figure 3B,C). To identify determinants in the primary structure of RetGC-1 and RetGC-2 responsible for these differences we generated chimeras, RetGC-121 and RetGC-212, in which the KHDs of RetGC-1 and RetGC-2 are exchanged (Figure 5A). The characteristics of activation of these chimeras by GCAP-2 under low $Ca^{2+}$ conditions ($n = 2.7 \pm 0.16$; $K_{1/2} = 88 \pm 2$ nM for RetGC-121 and $n = 1.1 \pm 0.03$; $K_{1/2} = 2.3 \pm 0.06$ $\mu$M for RetGC-212) resemble those of the RetGC parent from which the KHD originated (Figure 5B). The inhibition characteristics of RetGC-121 ($n = 1.8 \pm 0.15$; $K_{1/2}$=56 $\pm$ 2 nM) or the modest concentration-dependent stimulation of RetGC-212 by GCAP-2 at high $Ca^{2+}$ also segregate with the corresponding KHDs (Figure 5C). These observations, together with the proteolysis protection results described above, highlight the importance of the KHD in defining the binding affinity of RetGC/GCAP-2 interactions and/or the favorability of allosteric conformational changes induced by GCAP-2. These results do not exclude additional involvement of other intracellular regions of RetGCs in binding or conformational changes that may be quantitatively similar for RetGC-1 and RetGC-2.

We attempted to more precisely define structures within the KHD which confer affinities of RetGC-1 and RetGC-2 for GCAP-2 by producing two additional chimeras. A silent *Pac*I restriction site that spans $Leu^{599}$-$Lys^{601}$ of RetGC-1 and RetGC-2 was created. Then, using *Cla*I and *Pac*I restriction

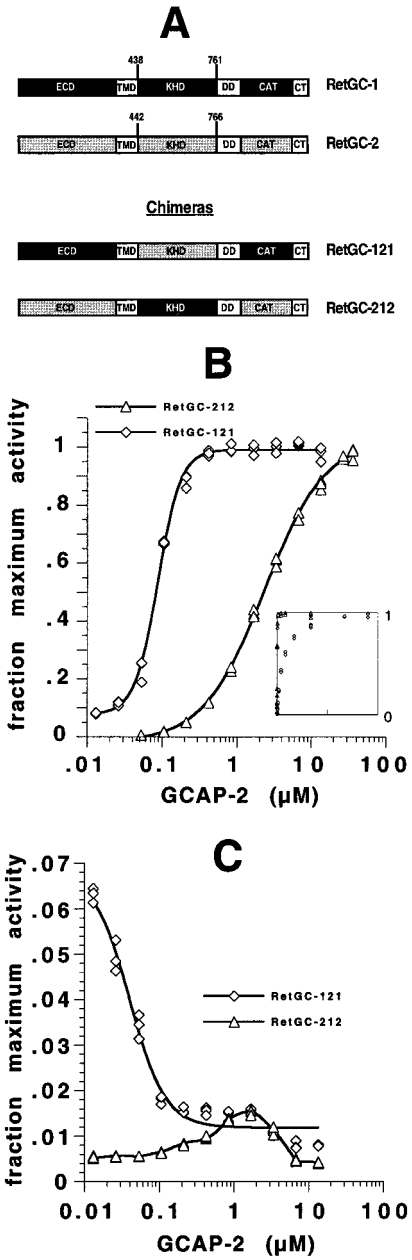


FIGURE 5: The affinity and cooperativity of the RetGC/GCAP-2 interaction is linked to the KHD of RetGC. (A) Schematic representation of the chimeras constructed from human RetGC-1 and RetGC-2 using silent restriction sites as described in Materials and Methods. The first distinct amino acids within the region exchanged are numbered. (B) GCAP-2 saturation curves for activation of RetGC-121 and RetGC-212 at 7 nM $Ca^{2+}$. Inset shows the same data plotted against a linear *x*-axis. The $K_{1/2}$; *n* of GCAP-2 for RetGC-121 and RetGC-212 were 88 nM; 2.7 and 2.3 $\mu$M; 1.1, respectively. $K_{1/2}$, *n*, and $V_{sat/max}$ were obtained by fitting the data to the equation: $v = v_0 + (V_{sat/max}[GCAP\text{-}2]^n/(K_{1/2}^n + [GCAP\text{-}2]^n))$ where $v_0$ is the activity in the absence of GCAP-2. The data shown are normalized to maximal activation ($V_{sat/max}$). (C) GCAP-2 saturation curves for the inhibition of RetGC-121 and RetGC-212 by GCAP-2 at 27 $\mu$M $Ca^{2+}$. $K_{1/2}$ and *n* values of 56 nM and 1.8 were obtained only for RetGC-121 by fitting the data to the equation $v = v_0 - (V_{sat/min}[GCAP\text{-}2]^n/K_{1/2}^n + [GCAP\text{-}2]^n)$. Data were normalized to the maximum activity under the assay conditions ($v_0$) which was set equal to the fraction of maximal activation obtained by GCAP-2 at 7 nM $Ca^{2+}$ as in (B). The results shown are representative of at least two independent experiments, each with triplicate data points.

sites, we swapped the carboxyl terminal portion of the KHDs of RetGC-1 and RetGC-2. Both chimeras expressed well in HEK 293 cells but had diminished basal cyclase activity and no sensitivity to GCAP-2 (not shown). Folding or stability problems may be responsible since it was shown previously that RetGC KHDs are easily denatured (*21*).

## DISCUSSION

The GC activity of RetGCs is regulated by GCAP-2 in a $Ca^{2+}$-sensitive manner. In this study we used two complementary approaches to characterize the interaction of GCAP-2 with RetGCs.

In the first approach we showed that a site within the KHD of RetGC-1 is protected by GCAP-2 from cleavage by trypsin. GCAP-2 affects the kinetics of proteolysis, but the same proteolytic fragments of RetGC-1 are generated in both the absence and presence of GCAP-2. The most direct interpretation of this finding is that the binding site for GCAP-2 includes, or is near to, the protected site in the KHD. Alternatively, GCAP-2 may bind elsewhere, but it indirectly protects a site in the KHD either by inducing a conformational change in the RetGC KHD or by stabilizing its structure.

GCAP-2 protects a trypsin cleavage site within the RetGC-1 KHD from proteolysis independently of $Ca^{2+}$. This suggests that protection of the site by GCAP-2 reflects a constant interaction which is independent of $Ca^{2+}$-sensitive interactions that control GC activity.

GCAP-2 regulates both RetGC-1 and RetGC-2 in vitro by what appear to be similar interactions. However, the apparent affinity and cooperativity of these interactions are different. Evidence suggests that RetGCs are dimers (*15*, *22*, *23*). The Hill coefficients that fit our GCAP-2 titrations suggest that activation of the RetGC-1 dimer occurs upon binding a single GCAP-2. Activation of RetGC-2 is cooperative with a Hill coefficient near 2. This suggests that RetGC-2 exists almost exclusively in two forms, one free of GCAP-2 and the other associated with two GCAP-2s. Another difference between the two RetGCs is that $Ca^{2+}$-bound GCAP-2 has a simple inhibitory effect on RetGC-2, whereas its effect on RetGC-1 is complex (see Figure 3C). The narrow range of $Ca^{2+}$-GCAP-2 concentrations that activate RetGC-1 may be explained by assuming that a single $Ca^{2+}$-GCAP-2 bound asymmetrically to a RetGC-1 dimer acts as a partial agonist, whereas two $Ca^{2+}$-GCAP-2 produces the state of lowest activity. Alternatively, heterogeneity of recombinant RetGC-1 due to incomplete processing or post-translational modification might also be responsible for the activation and experimental variability in the extent of activation by $Ca^{2+}$-GCAP-2.

By swapping the KHDs of RetGC-1 and RetGC-2 to produce the chimeras RetGC-121 and RetGC-212, we have shown that all properties unique to each protein segregate with the respective KHDs. Therefore, the KHD determines whether the RetGC-GCAP-2 interaction is cooperative and it encodes the affinity difference between RetGC-1 and RetGC-2 for GCAP-2.

Both the proteolysis protection and chimera results highlight the importance of the KHD in the RetGC/GCAP-2 interaction. Whether or not GCAP-2 actually binds to the KHD remains to be shown directly. Our findings do not exclude a GCAP-2 interaction with the highly conserved CAT domain. However, it is unlikely that GCAP-2 interacts

with the carboxyl terminus (CT) or dimerization domain (DD). In separate experiments we investigated the role of the CT domain in the RetGC-1/GCAP-2 interaction by deleting the 37 carboxyl terminal amino acids of RetGC-1. This deletion did not affect the $K_{1/2}$ for activation by GCAP-2 (not shown). We also made soluble gst-fusions from the CT and DD of RetGC-1 and found that neither interferes with activation or inhibition of GC activity when added into the assay system. It should be noted that while our manuscript was under revision, Goraczniak et al. reported a similar study using RetGC-1/RetGC-2 chimeras to examine determinants of the RetGC/GCAP-2 interaction (*24*). Surprisingly, those investigators found the apparent affinity of RetGCs for GCAP-2 segregates with the carboxy-terminal amino acids of the cyclase. These results appear to differ from our finding that the affinity for GCAP-2 segregates with the KHD. But a direct comparison of these two sets of results reveals that the splice junction used by Goraczniak et al. in their chimeric cyclases is 36 amino acids more into the KHD than the splice junction in our chimeras. These results suggest that the 36 amino acid stretch ($I^{730}$-$I^{765}$ of human RetGC-1 and $L^{735}$-$I^{770}$ of human RetGC-2) between the splice sites used by us and Goraczniak et al. determines the affinity of RetGC for GCAP-2. Efforts are under way to examine this stretch of amino acids in more detail and to determine whether the influence of the KHD on the RetGC/GCAP-2 interaction reflects changes in binding affinity, conformation, or both.

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