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Mapping Sites in Guanylyl Cyclase Activating Protein-1 Required for Regulation of Photoreceptor Membrane Guanylyl Cyclases*

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Guanylyl cyclase activating protein (GCAP)-1 regulates photoreceptor membrane guanylyl cyclase, RetGC, in a Ca^{2+}-sensitive manner. It contains four Ca^{2+}-binding motifs, EF-hands, three of which are capable of binding Ca^{2+}. GCAP-1 activates RetGC in low Ca^{2+} and inhibits it in high Ca^{2+}. In this study we used deletion and substitution analysis to identify regions of GCAP-1 sequence that are specifically required for inhibition and activation. A COOH-terminal sequence within Met^{157} to Arg^{182} is required for activation but not for inhibition of RetGC. We localized one essential stretch to 5 residues from Arg^{178} to Arg^{182}. Another sequence essential for activation is within the N-terminal residues Trp^{21} to Thr^{27}. The region between EF-hands 1 and 3 of GCAP-1 also contains elements needed for activation of RetGC. Finally, we found that inhibition of RetGC requires the first 9 amino-terminal residues of GCAP-1, but none of the residues from Gln^{33} to the COOH-terminal Gly^{205} are specifically required for inhibition. The ability of GCAP-1 mutants to regulate RetGC was tested on total guanylyl cyclase activity present in rod outer segments. In addition, the key mutants were also shown to produce similar effects on recombinant bovine outer segment cyclases GC1 and GC2.

RetGC1 and RetGC2 are membrane guanylyl cyclases that catalyze the conversion of GTP into cyclic GMP (cGMP) in vertebrate rods and cones. RetGCs are implicated in restoring cGMP levels following activation of cGMP phosphodiesterase by light (1–5). The ability of RetGC to catalyze cGMP synthesis is sensitive to Ca^{2+} but only in the presence of guanylyl cyclase activator proteins (GCAPs)[1] (6–8).

GCAPs were identified and purified as Ca^{2+}-binding proteins that impart Ca^{2+} sensitivity to RetGCs *in vitro* (9, 10). Two isoforms, GCAP-1 and GCAP-2, have been found. Both stimulate RetGC activity in homogenates of rod outer segments (ROS) at low free Ca^{2+} concentrations (below 200 nM) and inhibit it at high free Ca^{2+} concentrations (11, 12). GCAP-1 and GCAP-2 share the following primary structural features: (i) four EF-hand motifs in the core of the protein, three of which bind Ca^{2+}, (ii) an acylated NH_2 terminus, and (iii) a molecular mass of roughly 24 kDa. However, there are significant differences between GCAP-1 and GCAP-2. They display little sequence conservation in their NH_2 and COOH termini. Also, a naturally occurring point mutation (13) affects the two proteins differently (14, 15).

The ability of GCAPs to regulate RetGC in a Ca^{2+}-sensitive manner is well established (7, 10, 11, 16). However, specific structures within GCAPs that are responsible for regulating RetGCs have not yet been clearly defined. Peptide competition experiments have suggested that three structures in GCAP-1 are involved in activation of RetGCs. The first is between residues Gly^2 and Glu^{28}. The second one is contiguous with the first; it runs from Glu^{28} to Glu^{57} (9), and the third one is the EF-hand 3 motif (17).

In order to more precisely define sites in GCAP-1 that interact with RetGC, we constructed deletion mutants of GCAP-1 and chimeras of GCAP-1 with recoverin, a closely related Ca^{2+}-binding protein that does not regulate RetGC. Chimeras were used in cases where deletions were not desirable. For instance, deletions from the NH_2 terminus are especially likely to complicate folding. Moreover, mere deletions would change the length of the peptide, thus introducing another variable into the experiments. Chimeras with recoverin, on the other hand, allowed us to conserve the total length of the constructs and improve the chances of proper folding.

These assumptions were borne out by the fact that nearly all constructs displayed one or more kinds of assayable activity: (i) ability to stimulate RetGC in low Ca^{2+}, (ii) ability to inhibit it in high Ca^{2+}, (iii) ability to block activation of RetGC by wt GCAP-1 in low Ca^{2+}, or (iv) ability to block activation by a Ca^{2+}-insensitive GCAP-1 mutant in high Ca^{2+}.

In the study described here we have addressed the following specific questions. Is regulation of RetGCs by GCAP-1 mediated by a single contiguous stretch of GCAP-1 sequence? Or, if multiple regions of the sequence contribute to the interaction and regulation, what are they and what are their roles in regulating RetGC?

EXPERIMENTAL PROCEDURES

DNA Constructs—All mutants were derived from bovine GCAP-1 and recoverin cDNA clones (18). All chimeras were generated by the polymerase chain reaction-based "splicing by overlap extension" method (19). Cloned Pfu polymerase (Stratagene) was used in all polymerase chain reactions. GCAP-1 truncations were generated by introducing a stop codon into the reverse polymerase chain reaction primers used to amplify the cDNA. It was found that the wt sequence of GCAP-1 does not provide for complete myristoylation of the protein in our expression system even at saturating myristate concentrations in the media and with overexpression of yeast NMT. The wt GCAP-1 sequence does not have a Ser in the sixth position. This residue is part of the myristoylation consensus (20). The GCAP-1 sequence was mutated to encode Ser in the sixth position. This substitution alone provided for complete myristoylation of GCAP-1 as confirmed by mass spectrometry. The properties of this D6S GCAP-1 in regard to activat-

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[1] The abbreviations used are: GCAP, guanylyl cyclase activating protein; ROS, rod outer segment; wt, wild type; BSA, bovine serum albumin.

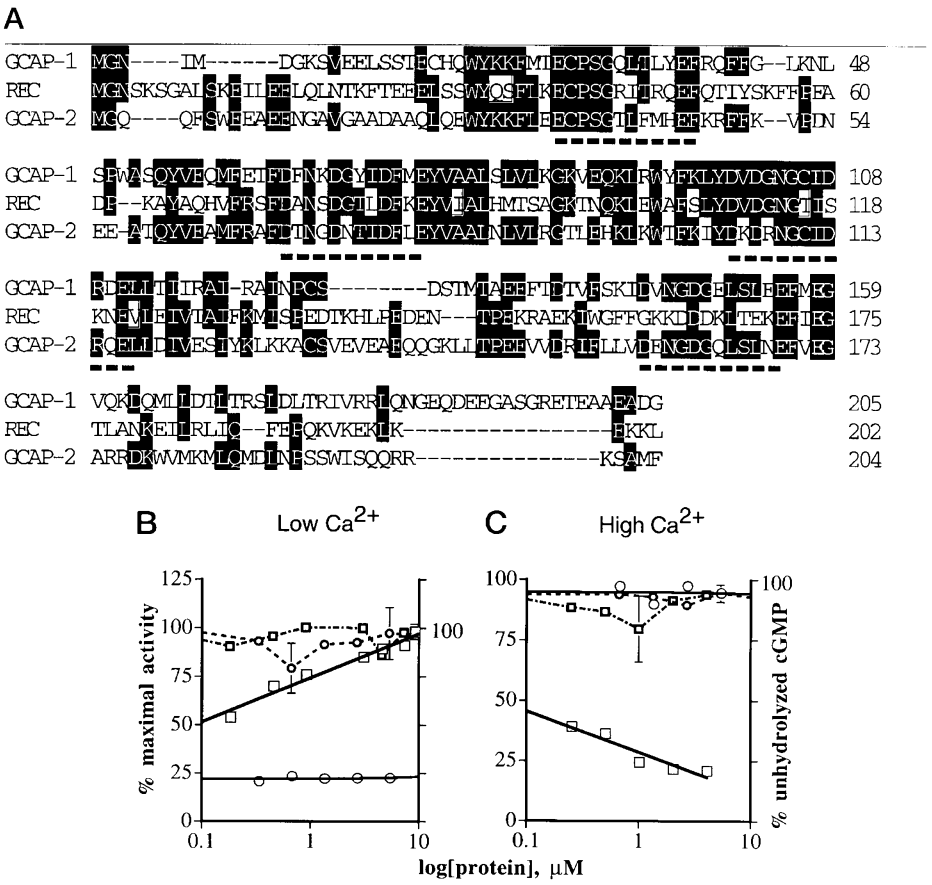

FIG. 1. **Effects of GCAP-1 and recoverin on RetGC activity and lack of PDE activation.** *A*, an alignment of bovine GCAP-1, recoverin, and GCAP-2. Identical residues are *boxed*. EF-hands are *underlined* in *dashed lines*. *B*, purified recombinant myristoylated GCAP-1 and recoverin were added to washed ROS membranes, and RetGC activity was assayed in 1 mM EGTA. □ denotes GCAP-1, ○ denotes recoverin. *Solid lines* represent guanylate cyclase activity, *broken* and *dashed lines* represent cGMP levels. The left *y* axis is plotted in percent of maximal GC activity, the *right y* axis in percent unhydrolyzed cGMP recovered from the assay. *C*, GC activity was assayed in the presence of >10 μM Ca^{2+}. The data shown are the average of duplicate data points in one experiment. They are representative of two or more independent experiments.

ing and inhibiting the cyclase were found to be indistinguishable from those of fully myristoylated wt GCAP-1. For the sake of brevity the D6S GCAP-1 is referred to as wt in the rest of the paper. A Ca^{2+}-insensitive GCAP-1 mutant was produced by the following substitutions in EF-hands 2, 3, and 4: E75Q, E111Q, D144N. The mutagenesis strategy is described in Ref. 11. The mutant protein activated RetGC in high Ca^{2+} in our assay system. Some constructs were confirmed by DNA sequencing. The masses of all of the expressed proteins were confirmed to be correct by electrospray mass spectrometry. All constructs were at least 90% myristoylated.

Expression of GCAP-1 Mutants—The cDNA constructs were ligated into pET11d or pET11a vectors (Novagen) using the *Nco*I or *Nde*I sites respectively at the 5′ and *Bam*HI site at the 3′ end. The expression plasmids were transformed into *Escherichia coli* (BL21 DE3pLysE) that harbored p88131 encoding yeast *N*-myristoyl transferase (NMT) and kanamycin resistance. Expression was carried out essentially as described in Ref. 11. 30 min prior to induction of expression with 1 mM isopropyl-1-thio-β-D-galactopyranoside bacterial media were supplemented with free myristic acid. After expression (2–5 h) cells were collected and sonicated on ice in lysis buffer: 40 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1 mM β-mercapthoethanol, 20 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride. The resulting lysate was centrifuged at 30,000 × *g*. Most of the expressed protein was recovered in the pellet. The pellet was washed twice with lysis buffer by resuspension and centrifugation at 30,000 × *g*. It was then dissolved in 6 M urea and dialyzed 3 times against 1000 volumes of lysis buffer without protease inhibitors. The renatured fraction typically contained 50% or more of the expressed protein. Construction of the ΔLQ truncation mutant required an introduction of non-endogenous TAA stop codon. The use of the endogenous stop codon in this truncation produced a read-through peptide of a higher than predicted molecular weight.

Expression of RetGC1 and RetGC2—Bovine GC1 and GC2 were expressed in the HEK293 cell line. The expression vector pCDNA3 (Invitrogen) containing corresponding cDNAs was a gift from Dr. R. Sharma (24). Cells at 80% confluency were transfected using calcium phosphate. 15 μg of vector DNA was used per 100-mm dish. Cells were harvested 48 h after transfection and lysed by passaging three times through a 26-gauge needle in hypotonic buffer. A 500 × *g* supernatant was collected and centrifuged at 400,000 × *g* for 10 min. The resulting pellet was resuspended in the buffer containing 10 mM Tris (pH 7.5) and 10 mM β-mercapthoethanol to the concentration of 4 μg/μl of total protein as measured by the Bradford assay.

Circular Dichroism—All experiments were performed on circular dichroism spectrometer 62A DS from AVIV™, Lakewood, NJ, in a 1-mm optical path cell. We used purified proteins at 20–30 μM in 10 mM phosphate buffer (pH 7.0) and 50 μM EDTA. Denaturation curves were obtained by monitoring ellipticity at 222 nm. Ellipticities were normalized according to the formula: $\Theta_{MRW} = \Theta_o M_r/lc$, where Θ_o is observed ellipticity in degrees, M_r is the average molecular weight of an amino acid in the protein, l is the optical path length in mm, and c is protein concentration in grams/liter.

GC Assays—The expressed proteins were assayed for their ability to regulate RetGCs in parallel with wtGCAP-1 and nonspecific protein (BSA or recoverin). The assays were carried out as described previously (10). In brief, rod outer segments were washed to remove endogenous GCAPs and were then assayed for GC activity under infrared illumination. Ca^{2+} concentrations were controlled by 1 mM EGTA or EGTA/Ca^{2+} buffers. The substrate was 5 mM cold GTP and 0.1 μCi of [α-^{32}P]GTP (Amersham). The reactions were carried out at 30 °C for 20 min, and the products were analyzed by TLC and scintillation counting. Typically, synthesized cGMP was labeled to 1,000–10,000 cpm. The background was typically 50 cpm. The amount of cGMP hydrolysis was controlled in every reaction by adding 25 mM cold cGMP and 20,000 dpm of ^{3}H-labeled cGMP. For recombinant cyclases each assay point contained membranes with 10 μg of total membrane protein.

RESULTS

Recoverin Does Not Regulate RetGC in Our Experimental System—In order to perform an interpretable analysis of chimeras we first established that recoverin is not a regulator of RetGC in our system. Bovine recoverin and GCAP-1 share roughly 30% amino acid sequence identity (Fig. 1*A*). Previous studies have shown that pure recoverin does not stimulate photoreceptor guanylyl cyclase (RetGC) (21). To confirm this result in our system and to determine whether or not recoverin inhibits RetGC we assayed GC activity in washed ROS membranes titrated with recombinant myristoylated recoverin (Fig. 1*B*). Recoverin did not stimulate RetGC even at concentrations

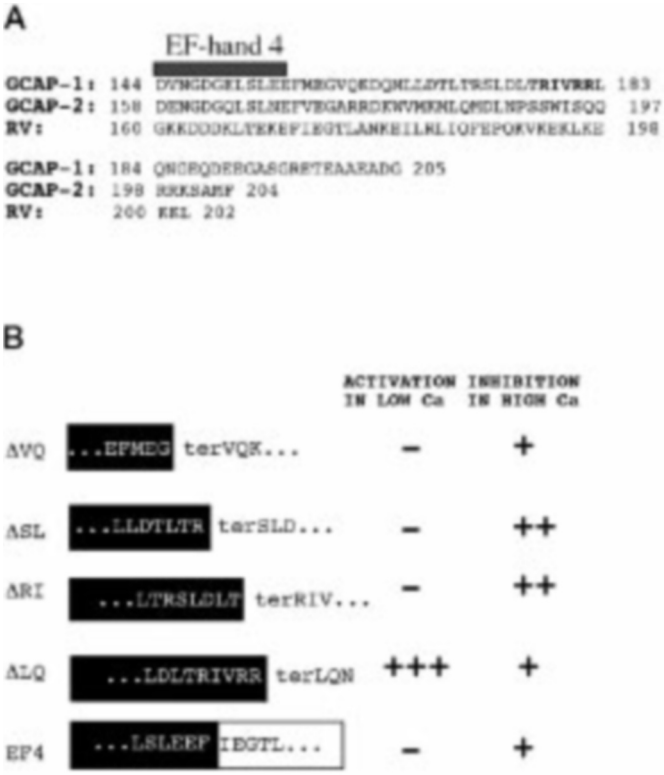

FIG. 2. **COOH-terminal constructs.** *A*, an alignment of the COOH-terminal sequences of GCAP-1, GCAP-2, and recoverin. *B*, a series of truncation mutants: ΔVQ, ΔSL, ΔRI, ΔLQ is represented here. EF4 is a chimera. *Closed* area denotes GCAP-1, and *open* area denotes recoverin. A minimum 2-fold difference from the negative control (BSA) was considered as a positive effect (+). Less than 2-fold difference was considered as no effect (−). ΔLQ is the shortest truncation mutant that can activate GC. The difference between ΔLQ and the next shortest truncation ΔRI is the sequence RIVRR.

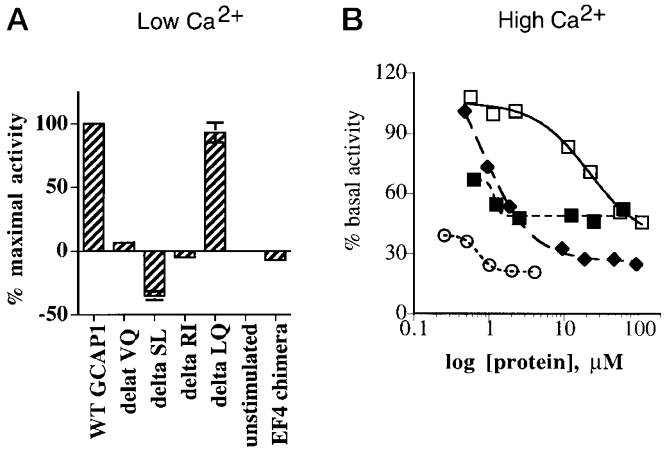

FIG. 3. *A*, effect of COOH-terminal mutants on RetGC in low Ca^{2+}. The mutant proteins shown in Fig. 2 were tested in a GC assay using washed ROS. All assays were performed in a 25-μl volume in the presence of 1 mM EGTA. The following concentrations were used: ΔVQ at 43 μM, ΔSL at 87 μM, ΔRI at 56 μM, ΔLQ at 52 μM, EF4 at 65 μM, GCAP-1 at 1 μM. The data shown are the average of duplicate data points in one experiment. They are representative of two or more independent experiments. Basal GC activity in the presence of a nonspecific protein (BSA) was taken to be 0 and % maximal activation was calculated using the formula: %(X) = ((activity X − basal activity)/(maximal stimulated activity − basal activity))*100%. A saturating amount of GCAP-1 was taken as 100% activation (3–5-fold stimulation depending on the ROS preparation). Suppression of the activity below the basal level is represented as a negative value. *B*, inhibition of GC in high Ca^{2+} by COOH-terminal mutants. □ denotes ΔVQ; ◆, ΔSL; ■, EF4; ○, GCAP-1. The mutant proteins were tested in a GC assay using washed ROS membranes. All assays were performed in a 25-μl volume in the presence of >10 μM free Ca^{2+}. The data shown are the average of duplicate data points in one experiment. They are representative of two or more independent experiments. Basal GC activity in the presence of a nonspecific protein (BSA) was taken as 100%. Saturation with wt GCAP-1 is reached at 2.5 μM.

up to 30 μM whereas GCAP-1 stimulated it 4-fold at 10 μM concentration. Similarly, recoverin did not inhibit RetGC in >10 μM Ca^{2+} while GCAP-1 did (Fig. 1*C*).

These results confirmed that recoverin indeed does not regulate RetGC in our assay. None of the structural elements in our chimeras derived from recoverin are in themselves sufficient to regulate RetGC. This suggested that we could indeed use chimeric proteins to identify GCAP-1-specific structural elements that are responsible for activating and inhibiting RetGC.

Lack of Phosphodiesterase Activation in the Assay System—It is a formal possibility that the changing levels of cGMP in our assay system result from variations in PDE activity present in ROS preparations. We monitored hydrolysis of cGMP in all our assays as described under "Experimental Procedures." As evident from the *dashed lines* on Fig. 1, *B* and *C*, the level of cGMP hydrolysis did not depend on increasing concentrations of GCAP-1 and recoverin in low as well as high Ca^{2+}. Similarly we observed no effect on cGMP hydrolysis by any of the mutants we produced (data not shown). We were able to conclude that the varying amounts of cGMP in our assay system result solely from varied guanylyl cyclase activity.

The Role of the COOH Terminus—For the purpose of this work we consider the COOH terminus of GCAP-1 as the residues from Phe^{156} at the end of EF-hand 4 to the very COOH-terminal Gly^{205}. To study the role of the COOH terminus in regulating RetGC we constructed several truncation mutants and chimeras with the COOH terminus of recoverin (Fig. 2, *A* and *B*). The truncation mutant that ended after Gly^{159} (ΔVQ) had only 7% of the stimulatory activity of wt GCAP-1 (low Ca^{2+} conditions) when its concentration in the assay was 25 μM (Fig. 3*A*). The stimulatory effect of GCAP-1 saturated below 10 μM (Fig. 1*B*).

The longer truncation mutants ΔSL (ends at Arg^{172}) and ΔRI (ends at Thr^{176}) also do not stimulate RetGC. In fact they suppress RetGC in low Ca^{2+} below the basal level (Fig. 3*A*).

The truncation mutant ΔLQ ends after Arg^{182}. In contrast to the shorter deletion mutants it stimulated RetGC to 93% of the wt GCAP-1 level when assayed at 50 μM. Essentially, only ΔLQ of all truncation mutants described here is capable of activating RetGC to significant levels.

The truncation mutants that failed to activate RetGC do inhibit RetGC in high Ca^{2+} (>10 μM) as shown in Fig. 3*B*. They also block activation by wt GCAP-1 in low Ca^{2+} in a competition experiment with a half-maximal effect reached at a molar excess of 35–100 (data not shown). Even the most extensive COOH-terminal truncation mutant, ΔVQ, and the EF4 chimera inhibited the cyclase in high Ca^{2+}. The EF4 chimera contains GCAP-1 sequence from the NH_2 terminus down to Phe^{156} following EF-hand 4 (Fig. 2). The rest of the chimera consists of Ile^{172} to Leu^{202} of recoverin. The length of this chimera exceeds the lengths of ΔVQ, ΔSL, and ΔRI.

Since the EF4 chimera does not activate RetGC (Fig. 3*A*) a specific sequence in the COOH-terminal region is required for activation, not simply any sequence of a suitable length. More precisely the presence of the sequence RIVRR flanked by Arg^{177} and Arg^{182} appears to be crucial for activation but not for inhibition. The residues COOH-terminal of Arg^{182} are not essential for stimulating RetGC. The actual structural requirements provided by the RIVRR structure are not yet clear. Results of a preliminary alanine scanning mutagenesis study

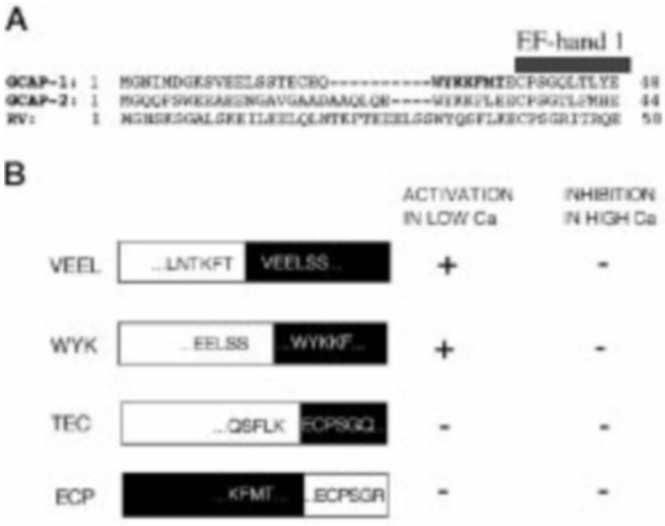

FIG. 4. **NH_2-terminal constructs.** *A*, an alignment of the NH_2-terminal sequences of GCAP-1, GCAP-2, and recoverin. *B*, *black* denotes recoverin sequences, *white* denotes GCAP-1. A minimum 2-fold difference from the negative control with BSA was considered as a positive effect (+). Less than 2-fold difference was considered as no effect (−). The sequence WYKKFMT appears to be critical for the ability to activate GC.

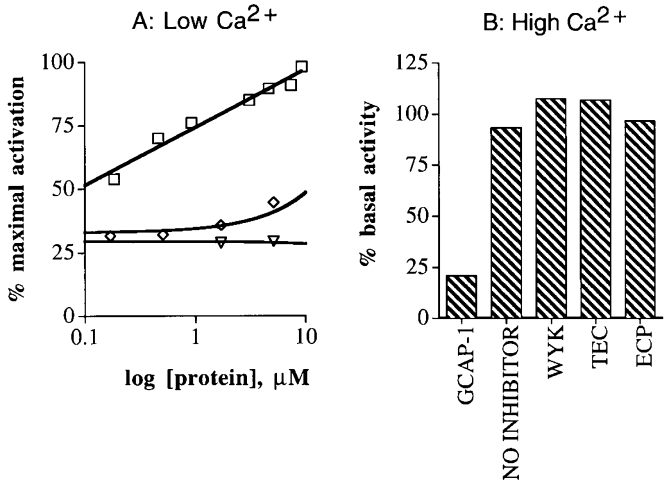

FIG. 5. *A*, effects of NH_2-terminal chimeras on RetGC activity in low Ca^{2+}. □ denotes GCAP-1, ◇ WYK, ▽ TEC. The constructs were tested in a GC assay using washed ROS membranes. WYK and TEC were purified by affinity chromatography and GCAP-1 was purified on a gel filtration column. All assays were performed in a 25-μl volume in the presence of 1 mM EGTA. The data shown are the average of duplicate data points in one experiment. They are representative of two or more independent experiments. *B*, inhibition of RetGC in high Ca^{2+} by NH_2-terminal chimeras. The constructs were tested in a GC assay using ROS washed membranes. All assays were performed in a 25-μl volume in the presence of >10 μM free Ca^{2+}. The data shown are the average of duplicate data points in one experiment. They are representative of two or more independent experiments. The following concentrations were used: GCAP-1 at 4 μM, WYK at 15 μM, TEC at 6 μM, ECP at 11 μM.

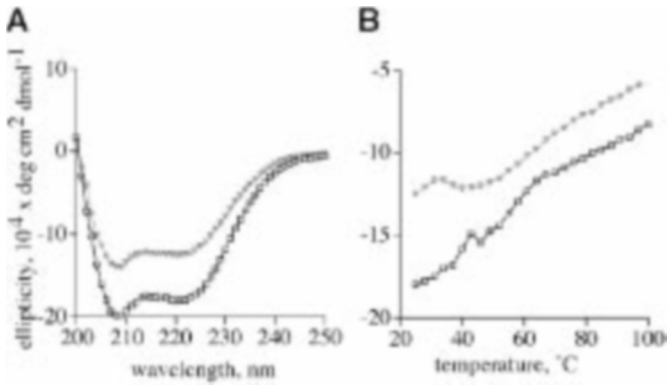

FIG. 6. **Circular dichroism of TEC and GCAP-1.** *A*, ellipticity was monitored at 25 °C. □ denotes GCAP-1, ▽ TEC. *B*, ellipticity at 222 nm was monitored as a function of temperature. The data were normalized according to the formula described under "Experimental Procedures."

suggest that none of the specific residues within the RIVRR sequence are essential for RetGC regulation (data not shown).

A chimera, EF3–4$^-$, has the region between EF-hands 3 and 4 substituted with the corresponding recoverin sequence (see Fig. 7 and discussion on core sequences below). It can stimulate RetGC as shown in Fig. 8*A*. Based on the EF3–4$^-$ chimera and the ΔLQ truncation mutant we conclude that all elements essential for RetGC activation that lie in the COOH terminus are localized within residues Glu^{155} and Arg^{182}.

The Role of the NH_2 Terminus—We consider the NH_2 terminus of GCAP-1 as residues from Gly^2 to Thr^{27}. It is 31% identical to the corresponding region of recoverin. Since recoverin does not regulate RetGC, we constructed and analyzed chimeras that have increasing portions of the GCAP-1 NH_2 terminus replaced by recoverin (Fig. 4). The "VEEL" chimera with sequence from the NH_2 terminus to Val^{10} replaced by recoverin stimulates RetGC in low Ca^{2+} (data not shown). The chimera referred to as "WYK" has recoverin sequence from the NH_2 terminus to Trp^{21}. This chimera also stimulated RetGC as shown in Fig. 5*A*. However, replacing only 6 more residues of the native GCAP-1 sequence produced a chimera, "TEC," that was completely inactive (Fig. 5*A*). This can be because TEC lacks sequence elements necessary to activate RetGC. Alternatively, misfolding could cause TEC to be inactive.

In order to evaluate the folding state of the TEC chimera we used circular dichroism (Fig. 6). GCAP-1 displayed a spectrum with an ellipticity 1.5 times higher than that of TEC. However, the shape of the two spectra are virtually indistinguishable and both are characteristic of a folded protein (Fig. 6*A*). The ellipticity at 222 nm decreased as a function of temperature in a similar fashion for TEC and wt GCAP-1 (Fig. 6*B*). Ellipticity at this wavelength is indicative of the helical content of a protein. It is routinely used to monitor temperature denaturation of proteins.

These data suggest the α-helical content of TEC is similar to that of GCAP-1 at room temperature. The smaller ellipticity of TEC may be explained by the tendency of the recoverin NH_2 terminus to stay unfolded (22, 23). However, the CD spectra indicate that most, if not all, of TEC is indeed folded. Since WYK activates RetGC and TEC does not, we conclude that the GCAP-1 sequence from Trp^{21} to Thr^{27}, WYKKFMT, is required for activation. Even though this sequence is essential, other residues in the core also contribute to activation. This is apparent from the properties of core substitution mutants we describe in the following section. A summary of the NH_2-terminal chimeras and their properties is presented in Fig. 4*B*.

An essential inhibitory structure also resides within the GCAP-1 NH_2 terminus. None of the recoverin/GCAP-1 chimeras VEEL, TEC, and WYK inhibit RetGC in high Ca^{2+} (Fig. 5*B*). Despite its ability to stimulate, WYK did not block activation of Ret GC by a Ca^{2+}-insensitive GCAP-1 mutant in high Ca^{2+} at up to 30-fold molar access (data not shown). The NH_2 terminus is not in itself sufficient for inhibition, however. A chimera, "ECP," consisting of the complete NH_2 terminus from GCAP-1 up to EF-hand 1 and the rest of the sequence from recoverin fails to inhibit RetGC (Fig. 5*B*).

The Role of the Core Sequences—We consider the sequence between Glu^{28} and Met^{157} as the core of GCAP-1; it includes EF-hands 1 through 4 (Fig. 7A). We constructed chimeras that replaced native GCAP-1 core sequences with the corresponding

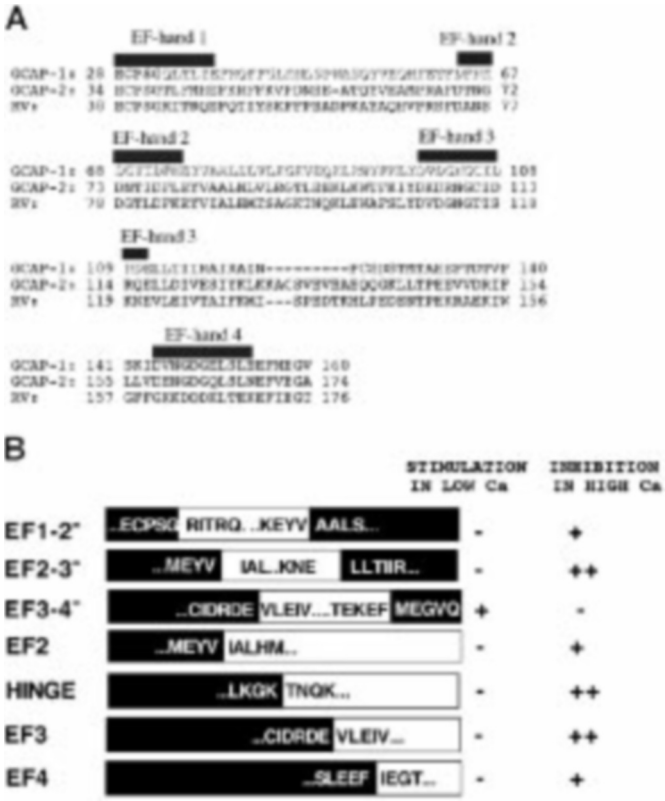

FIG. 7. **The core chimeras.** *A* shows an alignment of the core sequences of GCAP-1, GCAP-2 and recoverin. Highlighting represents the region necessary for activation. *B*, EF2, EF3, and EF4 chimeras consist of an NH_2-terminal GCAP-1 stretch (shown in *black*) and a COOH-terminal recoverin stretch (shown in *white*). For stimulation and inhibition at least a 2-fold effect on the basal activity of GC was considered as positive (+). Less than 2-fold difference was considered to be no effect (−).

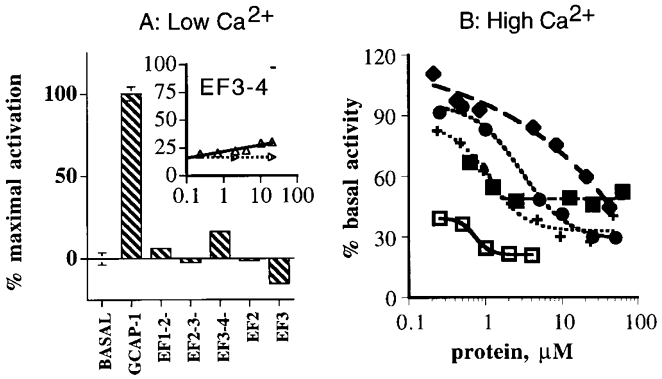

FIG. 8. **Effects of core chimeras on RetGC activity in low Ca^{2+}.** *A*, the chimeras were tested in a GC assay using washed ROS membranes in the presence of 1 mM EGTA. Basal GC activity in the presence of a nonspecific protein (BSA) was taken to be 0 and % maximal activation was calculated using the formula: %(X) = ((activity X − basal activity)/(maximal stimulated activity − basal activity))*100%. The following concentrations were used: GCAP-1 at 1 μM, EF1–2^- at 100 μM, EF2–3^- at 50 μM, EF3–4^- at 58 μM, EF2 21 μM, EF3 at 26 μM. Chimeras EF3, EF2–3^-, and EF2 suppressed RetGC below the basal level. EF3–4^- displayed the ability to stimulate RetGC 2-fold above the basal level. The *inset* shows a titration of ROS with EF3–4^- protein in low Ca^{2+}. △ denotes EF3–4^-, ▷ denotes basal activity. The data shown are the average of duplicate data points in a one experiment. They are representative of two or more independent experiments. *B*, inhibition in high Ca^{2+} by core chimeras. △ denotes EF2, ● EF3, ■ EF4, + denotes HINGE, □ GCAP-1. The chimeras were tested in a GC assay using washed ROS membranes. All assays were performed in the presence of >10 μM free Ca^{2+}. The data shown are the average of duplicate data points in one experiment. They are representative of two or more independent experiments. Basal GC activity in the presence of BSA was taken to be 100%.

sequences of recoverin. The "EF4" chimera was spliced at Phe^{156} at the end of EF-hand 4 giving it the least recoverin and most GCAP-1 sequence. We also produced chimeras spliced after Glu^{111} at the end of EF-hand 3 ("EF3") and at Val^{77} at the end of EF-hand 2 ("EF2").

The chimera EF1–2^- has the region between EF-hands 1 and 2 replaced by recoverin. Similarly, EF2–3^- and EF3–4^- have recoverin sequences between the corresponding EF-hands.

EF4, EF3, and EF2 inhibit RetGC in high Ca^{2+} although the concentrations required for inhibition are higher than for wt-GCAP-1 (Fig. 8*B*). The EF2 chimera, which has the least GCAP-1 sequence, also blocked activation of RetGC in high Ca^{2+} by a Ca^{2+}-insensitive GCAP-1 mutant (data not shown). Out of EF1–2^-, EF2–3^-, and EF3–4^-, only EF3–4^- failed to inhibit RetGC (data not shown). This may suggest that the region between EF-hands 3 and 4 is involved in inhibiting RetGC. Alternatively, the recoverin sequence introduced into this chimera may interfere with the correct conformation required for inhibition.

None of the chimeras with the C terminus replaced by recoverin (EF4, EF3, and EF2) activate RetGC (Fig. 8*A*). This agrees with our finding described above that the COOH-terminal RIVRR structure is needed for activation. This sequence is not present in recoverin.

The chimera EF3–4^- activates RetGC in low Ca^{2+} by 2-fold above the basal level. This constitutes 28% of the wt GCAP-1 level of activation in this experiment (Fig. 8*A*, *inset*). In this chimera the GCAP-1 sequence between Glu^{111} and Phe^{156} is replaced by recoverin. Since the conservation between GCAP-1 and recoverin is quite low here, we suggest that this region is not essential for RetGC activation.

The regions between EF-hands 1 and 2 and between 2 and 3 could not be replaced without complete loss of the ability to activate RetGC. It appears unlikely that this whole 71-amino acid stretch interacts with the cyclase. Rather, it may provide for the proper configuration of the activating elements that we identified in the NH_2 and COOH termini. Since both EF1–2^- and EF2–3^- can inhibit RetGC in high Ca^{2+} it appears that they can bind to the cyclase but fail to activate it.

Effects of Key Mutants on Recombinant RetGC1 and RetGC2—To study the effects our mutants may have on the known retinal guanylyl cyclases we tested several mutant proteins on bovine recombinant RetGC1 and GC2 referred to as OS GC1 and OS GC2. Fig. 9 shows the effects of key mutants in low Ca^{2+}. The key COOH-terminal truncations, ΔLQ and ΔRI, exhibited regulatory properties toward recombinant RetGC1 and GC2 that are similar to those of the total ROS guanylyl cyclase activity. The longer mutant ΔLQ stimulated RetGC1 by 10-fold, while the shorter mutant ΔRI which lacks the critical structure represented by "RIVRR" sequence had a less than 2-fold effect on GC1. For the less active recombinant RetGC2 the effects were: 2.6-fold for ΔLQ and under 2-fold for ΔRI. Similarly the key NH_2-terminal chimeras WYK and TEC affected the recombinant cyclases much like the RetGC activity in ROS preparations. Both for expressed RetGC1 and GC2 WYK stimulated the activity and TEC did not have a significant effect (Fig. 9). We conclude that the essential GCAP-1 stimulatory sequences we identified in the native system are also essential for activation of recombinant RetGC1 and GC2. We were not able to assess the inhibitory effects of our mutants on recombinant RetGC1 and GC2 due to the very low basal activity of the bovine recombinant cyclases.

DISCUSSION

In this study we evaluated the ability of deletion mutants and GCAP-1/recoverin chimeras to regulate RetGC. The ability

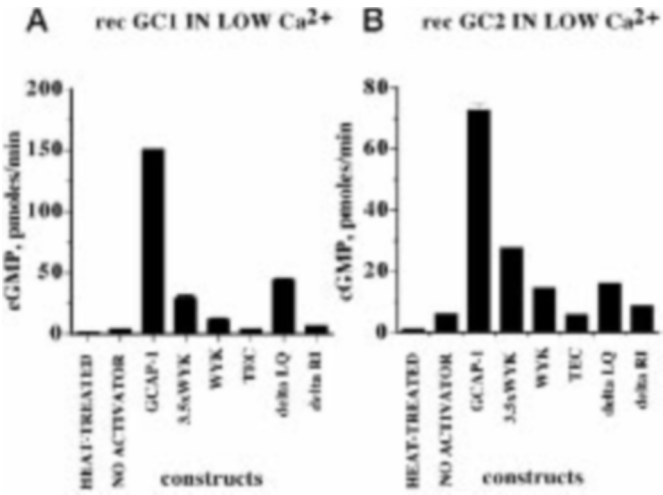

FIG. 9. **Effect of mutants on recombinant GC1 and GC2.** GCAP-1 and critical mutants were added to the membrane fraction of HEK293 cells expressing (*A*) RetGC1 and (*B*) RetGC2 and cyclase activity was assayed. All assays were performed in the presence of 1 mM EGTA. The following protein concentrations were used: GCAP-1 at 18 μM, WYK at 57 and 16 μM, TEC at 16 μM, ΔLQ at 78 μM, ΔRI at 76 μM. The data shown are representative of three independent experiments. Each value is the average of two measurements. The *error bars* are negligible where not visible.

TABLE I
IC_{50} and fold maximal inhibition for COOH-terminal core constructs

The values were calculated from fitted curves within the range of collected data points. IC_{50} concentrations were calculated for each construct individually, *i.e.* based on its maximal fold inhibition.

Name of construct	IC_{50}	Fold maximal inhibition
	μM	
wt GCAP-1	0.014	5.0
ΔSL	2.064	4.0
ΔVQ	12.655	2.2
EF4	0.003	2.2
EF3	2.500	3.5
HINGE	0.943	3.6
EF2	6.423	2.3

FIG. 10. **Summary of GCAP-1 sequences necessary for activation and inhibition of RetGC.** Regions necessary for inhibition are labeled with $-$; regions needed for activation are underscored with $+$. $\times$ represents regions whose role in inhibition or activation is tentative. *Bold script* highlights essential activation or inhibition sequences. The functional EF-hands are *boxed* with a *solid line*, the non-functional EF-hand is *boxed* with a *dotted line*.

of each protein to stimulate RetGC was studied at low free Ca^{2+} levels buffered by EGTA. The ability to inhibit was assayed at >10 μM free Ca^{2+}. Here we correlate the effects of the mutants on RetGC activity with the presence of specific GCAP-1 sequences. We use this correlation to map GCAP-1 sequences critical for RetGC regulation. We do not distinguish here between sequences that directly interact with RetGC and those required for any other reason, *e.g.* for proper scaffolding of non-contiguous interacting side chains.

Experimental Strategy—In order to simplify the analysis of the mutants, we broke down the sequence into three major stretches: the NH_2 terminus (Gly^2 to Thr^{27}), the core (Glu^{28} to Phe^{156}), and COOH terminus (Met^{157} to Gly^{205}). In this discussion we take a qualitative approach to describing the ability of each protein to activate and inhibit RetGC. When a mutant was able to regulate RetGC we frequently found that its apparent affinity for RetGC was altered (Table I). This suggests that the chimeras may not reproduce all features of the wild type GCAP-1 conformation correctly. Nonetheless, the stimulation and inhibition of RetGC by these mutant proteins were reproducible. We only considered 2-fold or greater effects as significant. This cut-off clearly differentiated between a specific effect and background variation that we routinely observe with nonspecific proteins (*e.g.* BSA, recoverin) in our assay. The effect of these proteins on GC activity typically does not exceed 10% of the basal level.

The presence of two distinct guanylyl cyclases, RetGC1 and RetGC2, is established in ROS of humans and other species (1–5). These cyclases are referred to as ROS GC1 and ROS GC2 in the literature. At present it is not clear if they account for all cyclase activity in ROS or if other cyclases are also present. In this study we have focused on the regions of GCAP-1 which are essential for the interaction with cyclases. We therefore used ROS preparations to make all cyclases that are regulated by GCAP-1 *in vivo* available to the GCAP-1 mutants in our assays. However, we have also confirmed that the key COOH-terminal deletion mutants and NH_2-terminal chimeras affect recombinant ROS GC1 and ROS GC2 the same way they affect GC activity in ROS preparations (Fig. 9).

The Role of the COOH Terminus—In the COOH terminus of GCAP-1 a structure represented by the sequence RIVRR appears crucial for activation. A mutant truncated immediately after this sequence activates RetGC whereas a truncation that stops immediately before it does not. Paradoxically, none of the residues in the RIVRR sequence seems essential for RetGC activation based on the results of a preliminary point mutagenesis study. Our results localize all elements essential for activation in the COOH terminus to residues from Glu^{155} to Arg^{182}.

None of the structures in the COOH terminus of GCAP-1 are required to inhibit RetGC. All the truncation mutants as well as chimeras with the COOH terminus of recoverin inhibit the cyclase.

The Role of the NH_2 Terminus and the Core—As evident from the TEC chimera the NH_2 terminus is critical for activating RetGC. TEC displays CD spectra resembling those of wt GCAP-1 (Fig. 6, *A* and *B*) arguing that it is a folded protein. It does not, however, stimulate RetGC in low Ca^{2+} (Fig. 5*A*) nor does it block stimulation by wt GCAP-1 (data not shown). Another chimera, WYK, that included only 7 more residues of GCAP-1 than TEC activates RetGC by over 2-fold. We conclude that these 7 residues, WYKKFMT, are essential for activation.

Replacing the NH_2 terminus of GCAP-1 with recoverin sequence to Ser^9 (as in the VEEL chimera, Fig. 5*B*) abolishes inhibition but not activation. This shows that a structure within GCAP-1 between Gly^2 and Ser^9 is specifically required for inhibition. It has been shown in a different study that an NH_2-terminal peptide derived from GCAP-1 blocks activation of RetGC by GCAP-1 (IC_{50} of 10 μM) (4, 8). The role of the NH_2 terminus is summarized in Fig. 4*B*.

We identified no GCAP-1-specific sequences within the core of the protein (Glu^{28} to Phe^{156}) that are required for inhibition. The chimera EF2 with all GCAP-1 sequence from EF-hand 2 to the COOH terminus replaced by recoverin sequence inhibits RetGC (Fig. 8*B*). EF1–2^- with GCAP-1 sequence between EF-hand 1 and EF-hand 2 replaced by the corresponding recoverin sequence also inhibits RetGC (data not shown). Based on the ability of the EF1–2^- chimera to inhibit we conclude that the region of GCAP-1 from Gln^{33} to Val^{77} is not specifically required for inhibition. To summarize, the first 9 amino acids of GCAP-1 are specifically required for inhibition of RetGC in high Ca^{2+}. Other residues in the NH_2 terminus and the core, however, are likely to contribute to inhibition in a nonspecific way, *e.g.* by providing scaffolding for inhibitory structures. For example, chimera ECP that contains the whole NH_2 terminus of GCAP-1 down to Thr^{27}, with the rest of it derived from recoverin, fails to inhibit RetGC in high Ca^{2+}.

Chimeras EF1–2^- and EF2–3^- do not stimulate RetGC. That shows that the GCAP-1 region between EF-hands 1 and 3 is necessary for RetGC activation. Since this is a long stretch, it appears unlikely that all of it is involved in a direct contact with RetGC. This region of GCAP-1 may provide for the correct scaffolding of activating sequences, while the corresponding region of recoverin does not fulfill this role. The role of the core sequences is summarized in Fig. 7*B*.

Activation Versus Inhibition—A summary of our findings is shown in Fig. 10. The inhibitory and stimulatory effects of GCAP-1 on RetGC appear to require different GCAP-1 structures. Stimulation requires both the COOH-terminal RIVRR and the NH_2-terminal WYKKFMT sequences, whereas inhibition appears to require the first 9 amino acids which are distinct from either of the stimulatory determinants. Moreover, structures between EF-hands 1 and 3 are required for activation but not for inhibition.

Comparison with GCAP-2—Both GCAP-1 and GCAP-2 inhibit and stimulate RetGC, yet there are substantial differences in their sequences. In particular the NH_2 and the COOH termini of the two proteins have few common primary sequence features. A parallel study using chimeras of GCAP-2 with neurocalcin (see accompanying article, Ref. 25) showed that a sequence near the COOH terminus of GCAP-2 is specifically required for activation. This correlates with our finding that a specific sequence in the GCAP-1 COOH terminus is essential for activation but not inhibition of RetGC.

The GCAP-2 study also identified a sequence flanking EF1 of GCAP-2 as important for activation and inhibition. According to our results a sequence, WYKKFMT, which flanks EF1 in GCAP-1 is necessary for activation of RetGC. In fact part of this stretch, WYKKF, is conserved between the two proteins.

There are also significant differences between the findings in the GCAP-1 and GCAP-2 studies. GCAP-1 but not GCAP-2 appears to require the 9 NH_2-terminal residues for inhibition and the region between EF-hands 1 and 3 for activation of RetGC. We have produced a GCAP-1 chimera whose region between Ser^{53} and Ile^{122} is replaced by the corresponding recoverin sequence. A similar GCAP-2/neurocalcin chimera displayed reversed Ca^{2+} sensitivity in the GCAP-2 study. GCAP-1/recoverin chimera we have constructed, however, does not exhibit a reversed Ca^{2+} sensitivity. It inhibits RetGC in high Ca^{2+} and does not stimulate it in low Ca^{2+} (data not shown). These differences between GCAP-1 and GCAP-2 may reflect the divergence of the primary sequences of the two proteins. For instance, a mutation in GCAP-1 and GCAP-2 has been shown to affect their activity differently (13–15). Alternatively, the differences may arise from the experimental systems used to make chimeras in the two studies since recoverin has less homology to GCAP-1 than neurocalcin to GCAP-2. Further structural analyses will be required to clarify the precise functions of the regions identified in these studies for binding, activation, and inhibition of RetGCs.

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REFERENCES

1. Yarfitz, S., and Hurley, J. B. (1994) *J. Biol. Chem.* **269,** 14329–14332
2. Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, Q., Spencer, M., Laura, R., Lu, L., and Hurley, J. B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5535–5539
3. Goraczniak, R. M., Duda, T., Sitaramayya, A., and Sharma, R. K. (1994) *Biochem. J.* **302,** 455–461
4. Yang, R-B., Foster, D. C., Garbers, D. L., and Fulle, H-J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 602–606
5. Kawamura, S., Cox, J. A., and Nef, P. (1994) *Biochem. Biophys. Res. Commun.* **203,** 121–127
6. Koch, K. W., and Stryer, L. (1988) *Nature* **334,** 64–71
7. Dizhoor, A. M., Lowe, D. G., Olshevskaya, E. V., Laura, R. P., and Hurley, J. B. (1994) *Neuron* **12,** 1345–1352
8. Gorczyca, W. A., Polans, A. S., Surgucheva, I. G., Subbaraya, I., Baehr, W., and Palczewski, K. (1995) *J. Biol. Chem.* **270,** 22029–22036
9. Palczewski, K., Subbaraya, I., Gorczyca, W. A., Helekar, B. S., Ruiz, C. C., Ohguro, H., Huang, J., Zhao, X., Crabb, J. W., and Johnson, R. S. (1994) *Neuron* **13,** 395–404
10. Dizhoor, A. M., Olshevskaya, E. V., Henzel, W. J., Wong, S. C., Stults, J. T., Ankoudinova, I., and Hurley, J. B. (1995) *J. Biol. Chem.* **270,** 25200–25206
11. Dizhoor, A. M., and Hurley, J. B. (1996) *J. Biol. Chem.* **271,** 19346–19350
12. Rudnicka-Nawrot, M., Surgucheva, I., Hulmes, J. D., Haeseleer, F., Sokal, I., Crabb, J. W., Baehr, W., and Palczewski, K. (1998) *Biochemistry* **37,** 248–257
13. Payne, A. M., Downes, S. M., Bessant, D. A., Taylor, R., Holder, G. E., Warren, M. J., Bird, A. C., and Bhattacharya, S. S. (1998) *Hum. Mol. Genet.* **7,** 273–277
14. Dizhoor, A. M., Boikov, S. M., and Olshevskaya, E. V. (1998) *J. Biol. Chem.* **273,** 17311–17314
15. Sokal, I., Li, N., Surgucheva, I., Warren, M. J., Payne, A. M., Bhattacharya, S. S., Baehr, W., and Palczewski, K. (1998) *Mol. Cell* **2,** 129–133
16. Gorczyca, W. A., Gray Keller, M. P., Detwiler, P. B., and Palczewski, K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4014–4018
17. Otto-Bruc, A., Buczylko, J., Surgucheva, I., Subbaraya, I., Rudnicka-Nawrot, M., Crabb, J. W., Arendt, A., Hargrave, P. A., Baehr, W., and Palczewski, K. (1997) *Biochemistry* **36,** 4295–4302
18. Ray, S., Zozulya, S., Niemi, G. A., Flaherty, K. M., Brolley, D., Dizhoor, A. M., McKay, D. B., Hurley, J. B., and Stryer, L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5705–5709
19. Horton, R. M., and Pease, L. R. (1991) *Directed Mutagenesis: A Practical Approach*, IRL Press, Oxford
20. Duronio, R. J., Rudnick, D. A., Adams, S. P., Towler, D. A., and Gordon, J. I. (1991) *J. Biol. Chem.* **266,** 10498–10504
21. Hurley, J. B., Dizhoor, A. M., Ray, S., and Stryer, L. (1993) *Science* **260,** 740
22. Ames, J. B., Tanaka, T., Stryer, L., and Ikura, M. (1994) *Biochemistry* **33,** 10743–10753
23. Flaherty, K. M., Zozulya, S., Stryer, L., and McKay, D. B. (1993) *Cell* **75,** 709–716
24. Pozdnyakov, N., Yoshida, A., Cooper, N. G., Margulis, A., Duda, T. Sharma, R. K., Sitaramayya, A. (1995) *Biochemistry* **34,** 14279–14283
25. Olshevskaya, E. V., Boikov, S., Ermilov, A., Krylov, D., Hurley, J. B., and Dizhoor, A. M. (1999) *J. Biol. Chem.* **274,** 10823–10832