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 Ca2+-sensitive manner. It contains four Ca2+-binding motifs, EF-hands, three of which are capable of binding Ca2+. GCAP-1 activates RetGC in low Ca2+ and inhibits it in high Ca2+. 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 Met157 to Arg182 is required for activation but not for inhibition of RetGC. We localized one essential stretch to 5 residues from Arg178 to Arg182. Another sequence essential for activation is within the N-terminal residues Trp21 to Thr27. 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 Gin33 to the COOH-terminal Gly205 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 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 GMP synthesis in high Ca2+ 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 Gly2 and Glu20, the second one is contiguous with the first; it runs from Glu28 to Glu57 (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 Ca2+-binding protein that does not regulate RetGC. Chimeras were used in cases where deletions were not desirable. For instance, deletions from the NH2 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 Ca2+, (ii) ability to inhibit it in high Ca2+, (iii) ability to block activation of RetGC by wt GCAP-1 in low Ca2+, or (iv) ability to block activation by a Ca2+-insensitive GCAP-1 mutant in high Ca2+.

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-
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: E7SQ, 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 NcoI or NdeI sites at the 5' and BamHI 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-D-galactopyranoside bacterial media were supplemented with free myristic acid. After expression (2–5 h) cells were harvested 48 h after transfection and lysed by passing three times through a 26-gauge needle in hypotonic buffer. A 500 \(\mu\)l of total membranes titrated with recombinant myristoylated recoverin (Fig. 1A). Recoverin did not stimulate RetGC even at concentrations of two or more independent experiments.

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 \(\mu\)M in 10 mM phosphate buffer (pH 7.0) and 50 \(\mu\)M EDTA. Denaturation curves were obtained by monitoring ellipticity at 222 nm. Ellipticities were normalized according to the formula: \(\theta_{	ext{norm}} = \theta_{\text{M}} M/LC\), where \(\theta_{\text{M}}\) is observed ellipticity in degrees, \(M\) 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 \(\mu\)Ci of \(\alpha\)-\(\beta\)-\(\gamma\)-\(\delta\)-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 GMP and 20,000 dpm of \(\alpha\)-\(\beta\)\(\gamma\)-\(\delta\)-GMP. For recombinant cyclases each assay point contained membranes with 10 \(\mu\)g 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. 1A). 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. 1B). Recoverin did not stimulate RetGC even at concentrations

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 and 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. \(\Delta\) denotes GCAP-1, \(\bigcirc\) 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 \(>10 \mu\)M Ca\(^{2+}\). The data shown are the average of duplicate data points in one experiment. They are representative of two or more independent experiments.
up to 30 μM whereas GCAP-1 stimulated it 4-fold at 10 μM concentration. Similarly, recoverin did not inhibit RetGC in >10 μM Ca2+ while GCAP-1 did (Fig. 1C).

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 Ca2+. 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 Phe156 at the end of EF-hand 4 to the very COOH-terminal Gly205. 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 Gly159 (ΔVQ) had only 7% of the stimulatory activity of wt GCAP-1 (low Ca2+ conditions) when its concentration in the assay was 25 μM (Fig. 3A). The stimulatory effect of GCAP-1 saturated below 10 μM (Fig. 3B).

The longer truncation mutants ΔSL (ends at Arg172) and ΔRI (ends at Thr176) also do not stimulate RetGC. In fact they suppress RetGC in low Ca2+ below the basal level (Fig. 3A).

The truncation mutant ΔLQ ends after Arg182. 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 Ca2+ (>10 μM) as shown in Fig. 3B. They also block activation by wt GCAP-1 in low Ca2+ 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 chimeran inhibits the cyclase in high Ca2+. The EF4 chimera contains GCAP-1 sequence from the NH2 terminus down to Phe156 following EF-hand 4 (Fig. 2). The rest of the chimera consists of lLe172 to Leu202 of recoverin. The length of this chimera exceeds the lengths of ΔVQ, ΔSL, and ΔRI.

Since the EF4 chimera does not activate RetGC (Fig. 3A) 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 Arg177 and Arg182 appears to be crucial for activation but not for inhibition. The residues COOH-terminal of Arg182 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. 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.
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. 8A. 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 Glu155 and Arg182.

The Role of the NH2 Terminus—We consider the NH2 terminus of GCAP-1 as residues from Gly2 to Thr27. 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 NH2 terminus replaced by recoverin (Fig. 4). The “VEEL” chimera with sequence from the NH2 terminus to Val10 replaced by recoverin stimulates RetGC in low Ca2+ (data not shown). The chimera referred to as “WYK” has recoverin sequence from the NH2 terminus to Trp21. This chimera also stimulated RetGC as shown in Fig. 5A. However, replacing only 6 more residues of the native GCAP-1 sequence produced a chimera, “TEC,” that was completely inactive (Fig. 5A). 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. 6A). The ellipticity at 222 nm decreased as a function of temperature in a similar fashion for TEC and wt GCAP-1 (Fig. 6B). 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 NH2 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 Trp21 to Thr27, 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 NH2-terminal chimeras and their properties is presented in Fig. 4B.

An essential inhibitory structure also resides within the GCAP-1 NH2 terminus. None of the recoverin/GCAP-1 chimeras VEEL, TEC, and WYK inhibit RetGC in high Ca2+ (Fig. 5B). Despite its ability to stimulate, WYK did not block activation of Ret GC by a Ca2+-insensitive GCAP-1 mutant in high Ca2+ at up to 30-fold molar access (data not shown). The NH2 terminus is not in itself sufficient for inhibition, however. A chimera, “ECP,” consisting of the complete NH2 terminus from GCAP-1 up to EF-hand 1 and the rest of the sequence from recoverin fails to inhibit RetGC (Fig. 5B).
sequences of recoverin. The “EF4” chimera was spliced at Phe\textsuperscript{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\textsuperscript{111} at the end of EF-hand 3 (“EF3”) and at Val\textsuperscript{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\textsuperscript{2+} although the concentrations required for inhibition are higher than for wt-GCAP-1 (Fig. 8B). The EF2 chimera, which has the least GCAP-1 sequence, also blocked activation of RetGC in high Ca\textsuperscript{2+} by a Ca\textsuperscript{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. 8A). 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\textsuperscript{2+} by 2-fold above the basal level. This constitutes 28% of the wt GCAP-1 level of activation in this experiment (Fig. 8A, inset). In this chimera the GCAP-1 sequence between Glu\textsuperscript{111} and Phe\textsuperscript{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\textsubscript{2} and COOH termini. Since both EF1–2 and EF2–3 can inhibit RetGC in high Ca\textsuperscript{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\textsuperscript{2+}. The key COOH-terminal truncations, ∆LO and ∆RI, exhibited regulatory properties toward recombinant RetGC1 and GC2 that are similar to those of the total ROS guanylyl cyclase activity. The longer mutant ∆LO stimulated RetGC1 by 10-fold, while the shorter mutant ∆RI 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 ∆LO and under 2-fold for ∆RI. Similarly the key NH\textsubscript{2}-terminal chimeras WIYK and TEC affected the recombinant cyclases much like the RetGC activity in ROS preparations. Both for expressed RetGC1 and GC2 the 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
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 \(\mu\)M 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 cyclase. The role of the NH\(_2\) terminus and the COOH-terminal core constructs is summarized in Fig. 4.

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. 5A) 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. 5B) 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 \(\mu\)M) (4, 8).
We identified no GCAP-1-specific sequences within the core of the protein (Glu28–Phe356) 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. 8B). 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 Gln33 to Val77 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 Ca2+. Other residues in the NH2 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 NH2 terminus of GCAP-1 down to Thr27, with the rest of it derived from recoverin, fails to inhibit RetGC in high Ca2+.

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. 7B.

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 NH2-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 NH2 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 NH2-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 Ser251 and Ile122 is replaced by the corresponding recoverin sequence. A similar GCAP-2/neurocalcin chimera displayed reversed Ca2+ sensitivity in the GCAP-2 study.

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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 Fulbe, H.-J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 602–606
5. Kawamura, S., Cox, J. A., and Nel, 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., Olsheskiyaya, 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. (1998) J. Biol. Chem. 273, 22029–22036
9. Palczewski, K., Subbaraya, I., Gorczyca, W. A., Helek, B. S., Ruiz, C. C., Ohguro, H., Huang, J., Zhao, X., Crabb, J. W., and Johnson, R. S. (1994) Neuron 13, 395–404
10. Palczewski, A. M., Olsheskiyaya, 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. Rudniska-Nawrot, M., Surgucheva, I., Herkols, J. D., Hasef, F., Sokol, 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., Bobok, S. M., and Olsheskiyaya, E. V. (1998) J. Biol. Chem. 273, 17311–17314
15. Sokol, I., Li, N., Surgucheva, I., Warren, M. J., Payne, A. M., Bhattacharya, S. S., Baehr, W., and Palczewski, K. (1998) Mol. Cell. Biol. 18, 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., Buczyklo, J., Surgucheva, I., Subbaraya, I., Rudniska-Nawrot, M., Crabb, J. W., Arendt, A., Hargrave, P. A., Baehr, W., and Palczewski, K. (1997) Biochemistry 36, 4295–4302
18. Ray, S., Zoloty, S., Niemi, G. A., Flaherty, K. M., Brolly, 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. L. (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., Zoloty, S., Stryer, L., and McKay, D. B. (1993) Cell 75, 709–716
24. Poznyakov, N., Yoshida, A., Cooper, N. G., Margulis, A., Duda, T. Sharma, R. K., Sitaramayya, A. (1995) Biochemistry 34, 14279–14283
25. Olsheskiyaya, E. V., Bobok, S., Ermilov, A., Krylov, D., Hurley, J. B., and Dizhoor, A. M. (1999) J. Biol. Chem. 274, 10823–10832