Interactions within the Coiled-coil Domain of RetGC-1 Guanylyl Cyclase Are Optimized for Regulation Rather than for High Affinity*

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RetGC-1, a member of the membrane guanylyl cyclase family of proteins, is regulated in photoreceptor cells by a Ca2+-binding protein known as GCAP-1. Proper regulation of RetGC-1 is essential in photoreceptor cells for normal light adaptation and recovery to the dark state. In this study we show that cGMP synthesis by RetGC-1 requires dimerization, because critical functions in the catalytic site must be provided by each of the two polypeptide chains of the dimer. We also show that an intact α-helical coiled-coil structure is required to provide dimerization strength for the catalytic domain of RetGC-1. However, the dimerization strength of this domain must be precisely optimized for proper regulation by GCAP-1. We found that Arg838 within the dimerization domain establishes the Ca2+ sensitivity of RetGC-1 by determining the strength of the coiled-coil interaction. Arg838 substitutions dominantly enhance cGMP synthesis even at the highest Ca2+ concentrations that occur in normal dark-adapted photoreceptor cells. Molecular dynamics simulations suggest that Arg838 substitutions disrupt a small network of salt bridges to allow an abnormal extension of coiled-coil structure. Substitutions at Arg838 were first identified by linkage to the retinal degenerative disease, autosomal dominant cone rod dystrophy (adCORD). Consistent with the characteristics of this disease, the Arg838-substituted RetGC-1 mutants exhibit a dominant biochemical phenotype. We propose that accelerated cGMP synthesis in humans with adCORD is the primary cause of cone-rod degeneration.

Photoexcitation of vertebrate photoreceptor cells reduces the intracellular concentration of cGMP and closes cGMP-gated cation channels in the plasma membrane of the photoreceptor cell outer segment. This hyperpolarizes the photoreceptor cell and lowers the free Ca2+ concentration in the outer segment (1, 2). Guanylyl cyclase is stimulated by the lowered [Ca2+]i to synthesize cGMP and enhance photoreceptor cell recovery and light adaptation. There are two forms of photoreceptor cell membrane guanylyl cyclases in humans, RetGC-1 and RetGC-2. Immunofluorescence studies suggest that RetGC-1 is primarily expressed in cones and to a lesser extent in rods (3, 4). Mutations in the RetGC-1 gene have been linked to Leber congenital amaurosis, a severe retinopathy inherited in an autosomal recessive mode (5).

RetGC-1 belongs to the membrane GC1 family that includes natriuretic peptide receptor-GCs (NPR-A/GC-A and NPR-B/GC-B), heat stable enterotoxin (Star/GC-C), and sea urchin sperm GCs (6–8). Each of the membrane GC family is a type I transmembrane protein that has an extracellular domain linked by a single transmembrane domain to an intracellular catalytic domain. In contrast to most other membrane GCs that are stimulated by the binding of a ligand to extracellular domain, RetGC-1 is stimulated intracellularly by a Ca2+-binding protein, GCAP-1 (3, 9, 10). Both GCAP-1 and a related protein, GCAP-2, have four EF-hand-like domains, including three functional EF-hands that bind Ca2+. At low levels of Ca2+, the Ca2+-free form of GCAP-1 activates photoreceptor cell membrane guanylyl cyclase, but at high (~1 μM) Ca2+ concentrations, the Ca2+-bound form of GCAP-1 inhibits it. This mechanism of regulation was established by demonstrating that mutations that prevent Ca2+ binding to the EF-hands turn GCAPs into constitutive activators of photoreceptor cell membrane guanylyl cyclase (11, 12). Therefore, the effect of Ca2+ on photoreceptor cell membrane guanylyl cyclases is mediated solely through Ca2+ binding to the GCAP protein and not through a direct interaction of Ca2+ with the cyclase. This type of Ca2+-sensitive regulation of photoreceptor cell membrane guanylyl activity by GCAPs occurs both with photoreceptor cell homogenates and with recombinant RetGC-1 expressed in HEK-293 or COS-7 cell lines (9, 13, 14).

Several lines of evidence suggest that membrane guanylyl cyclase function as dimers. A recent report showed that the structure of the extracellular domain of NPR-A is a dimer (15), and modeling studies of guanylyl cyclases based on the known structure of adenyl cyclases suggest that the catalytic do

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1 The abbreviations used are: GC, guanylyl cyclase; GCAPs, guanylyl cyclase-activating proteins; adCORD, autosomal dominant cone-rod dystrophy; HA, hemagglutinin tag; ERF, triple mutant of RetGC-1 that causes adCORD; ER, double mutant of RetGC-1 that causes adCORD; CMV, cytomegalovirus; PCR, polymerase chain reaction; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; MD, molecular dynamics simulation; DD, dimerization domain.
mains function as dimers (16–20). A cross-linking study by Yu et al. (21) also suggested that dimerization of RetGC-1 may be an essential step in regulation by GCAPs.

A putative coiled-coil structure that is near the catalytic domains of all membrane GCs enhances dimerization of GC-A (22). The α-helical coiled-coil is the most widespread subunit oligomerization motif found in proteins (23). However, the role of the coiled-coil domain in regulation of RetGC-1 has not been previously established.

Recent genetic linkage studies have shown that mutations at the C-terminal end of the coiled-coil domain of RetGC-1 cause a form of autosomal dominant cone-rod dystrophy (adCORD), a human disease in which cones and then rods degenerate (24–26). Those studies identified four independent mutations in RetGC-1 linked to adCORD. Each of them involves a substitution at Arg838, highlighting the critical role of this residue. We showed previously that one of these mutations, R838C, alters the Ca²⁺ sensitivity of GCAP-1-stimulated RetGC-1 activity (27). Here we report additional biochemical and structural analysis of this and other Arg838 mutations. Surprisingly, our analyses reveal that Arg838 may be part of a critical network of salt bridges that normally destabilizes the active state of the cyclase. Arg838 substitutions disrupt this network. Consequently, the coiled-coil structure is extended in the mutant enzymes resulting in abnormally enhanced stability of the dimerized catalytic domain. In other words, the normal molecular interactions within this structure are in fact not optimized for maximum extension and stability of the coiled-coil, and instead Arg838 represents a stop signal for dimerization. Thus, the coiled-coil domain of RetGC-1 appears to be finely tuned for optimal regulation by physiological concentrations of Ca²⁺.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Bacteriophage, and Media—**AKH54, xim², and the Escherichia coli strain AG1688 were kindly provided by Dr. J. C. Hu (Texas A&M University). AG1688, which carries the lacI gene for maintaining low expression level of the fusion protein, was used to assay immunity to the tester phage AKH54. AKH54 carries a deletion of cl repressor. xim² contains a substitution of the immunity region of lambda such that the lambda repressor is not able to repress the phage growth. The E. coli strain XL-1 blue (Stratagene, La Jolla, CA), was used for cloning of the plasmids. E. coli strains used in this study were grown routinely in Luria broth (LB) (28) supplemented with appropriate antibiotics. Antibiotics were added to cultures at the following concentrations: ampicillin (100 μg/ml) and kanamycin (30 μg/ml). All strains were stored at −70°C in LB containing 20% (v/v) glycerol.

**Mutagenesis—**HA-tagged RetGC-1 was generated by single-stranded mutagenesis (29). Briefly, single-stranded DNA was made from a plasmid, pBluescript (Stratagene, La Jolla, CA), containing the 1.35-kb NcoI fragment (L824S,Y827S RetGC-1, and pRC-CMV L831S,L834S RetGC-1) were each amplified by PCR from pKH101, and pJH391-DDcoil2, and pJH-DDcoil3. The negative control, pKH101, expressed the wild-type RetGC-1 we used in our experiments. The expressed hGCAP-2 was slightly more effective than hGCAP-2 at stimulating human RetGC-1. Bovine GCAP-2 protein was prepared as described previously. Expression in Human Embryonic Kidney (HEK) 293 Cells—4—5 μg of the constructs was transiently transfected into 50–60% confluent HEK-293 cells using Fugene-6 reagent (Roche Molecular Biochemicals, Indianapolis, IN). The cells were grown in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (50/50) (Life Technologies Inc., Rockville, MD), 10% heat-treated fetal calf serum, 10 mM HEPES, pH 7.4, in 100-mm tissue culture dishes. Cells were harvested after 48 h, washed with phosphate-buffered saline, and removed from dishes by rocking for 10 min at room temperature in phosphate-buffered saline containing 0.2% EDTA. Cells were pelleted by centrifugation and were swollen in hypotonic lysis buffer (10 mM Tris, pH 7.5/5 mM MgCl₂/1 mM ATP/5 mM 2-mercaptoethanol) for 10 min on ice. Cells were lysed by passing four times through a 26-gauge needle. The lysates were pelleted at 2000 rpm for 4°C in a Beckman table centrifuge to remove large debris and unbroken cells. The supernatant from this spin was pelleted at 14,000 rpm for 10 min, resuspended in lysis buffer, frozen on dry ice, and stored as aliquots at −70°C. Protein content was measured in the presence of 0.1% SDS using Bio-Rad protein assay with bovine serum albumin as a standard.

**Western Blotting—**HEK-293 membranes containing equal amounts of total membrane protein (40 μg) were electrophoresed on a 7.5% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked in TTBS (Tris-buffered saline, 0.05% Tween 20) containing 5% dry milk for 1 h at room temperature. For detection of RetGC-1 protein, membranes were probed using a mix of rabbit polyclonal CAT-Ab (corresponding to Met747-Ser1052 of RetGC-1) and KHD-Ab (corresponding to Met643-Ser746 of RetGC-1) for an hour in blocking buffer (31). After extensive washing with TTBS, the membrane was probed for 1 h at room temperature with a donkey anti-rabbit antibody coupled to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). After several washes with TTBS, the membrane was developed using chemiluminescent reagents (Amersham Pharmacia Biotech). Hemagglutinin (HA)-tagged RetGC-1 was detected using a horseradish peroxidase-linked anti-HA (3F10) from Roche Molecular Biochemicals.

**GC Assays—**Membranes containing equal amounts of total protein were reuspended in GC buffer (100 mM KCl/50 mM MOPS, pH 7.4/7.7 mM 2-mercaptoethanol/10 mM MgCl₂/6 mM NaCl/0.5 mM ATP). All reactions also contained 10 μg/ml ‘EGTA’ buffers. Measurement of guanylyl cyclase activity was carried out at 30°C for 20 min as described previously (27). Stimulated reactions contained recombinant human GCAP-1 or bovine GCAP-2. Assays measuring Mn²⁺/Triton X-100 activity contained 1% Triton X-100 and 10 mM MnCl₂ instead of MgCl₂. All experiments shown were repeated at least twice from independent transfections with similar results.

**EGTA—**Ca²⁺-EGTA buffers were prepared from solutions of EGTA (Sigma) and EGTA saturated with CaCl₂ (Fluka, Milwaukee, WI) by pH titration as described previously (32). Free Ca²⁺ concentrations under the assay conditions were calculated using a multifactor program (33) and verified by Ca²⁺ electrode analysis and by titration with Rhod-2 fluorescent dye (Calbiochem, La Jolla, CA).

**Lamba Retrpressor (cl) Fusion Constructs—**All repressor chimeric proteins were expressed from a lacUV5 promoter on a pBR322-based plasmid called pJH391 (34). The wild-type, coi2 (L824S,Y827S) and coi3 (L831S,L834S) RetGC-1 gene were each amplified by PCR from primary cDNA using designed primers and the phage DNA as a template. The DNA isolated from the lysis buffer was then amplified by PCR. The plasmid construct was then obtained by a HindIII/xbaI restriction of the construct into an Inframe fusion to the N-terminal 117 amino acids of the Lambda phage cl protein, generating plasmids pJH391-DDwt, pH391-DDoII2, and pH-DDoII3. The negative control, pH101, expresses only the first 101 amino acids of the DNA binding domain of the lambda cl repressor in E. coli. The construct expresses a fusion between the repressor and the leucine zipper of GCN4 (34).

**λ Repressor-derived Dimerization Tests—**Bacterial cells expressing different lambda repressor N-terminal fusions were assessed for immunity to AKH54 by cross-streak analysis. AKH54 lysate (10⁻⁹ plaque-forming units), prepared as described by Miller (1982), was spread down a corner of a Petri dish, and bacterial colonies were streaked...
across the phage. Immunity was scored as the ability to grow after contact with the phage on the far side of the streak. A second control phage lysate, λmm\textsuperscript{24}, with different immunity was spread down the Petri dish adjacent to phage AKH54. All strains of bacteria, including those that express oligomeric repressor fusions would be sensitive to λmm\textsuperscript{24}, making two different host ranges was to ensure discrimination of cells that are immune because of λ repressor dimerization from cells that have lost the lambda receptor.

**E. coli Cellular Fractionation and Western Blotting of λ Repressor Fusions—**E. coli (AG1688) expressing different repressor fusions were grown in 3 ml of Luria broth overnight. Cells were harvested, and soluble proteins were extracted with 500 μl of B-Per Bacterial protein extraction reagent, according to the manufacturer’s protocol (Pierce, Rockford, IL). Equal amounts of total soluble protein (40 μg), as determined by a Bio-Rad assay, were electrophoresed on 15% SDS-PAGE gels and transferred to nitrocellulose membranes. Lambda repressor-dimerization domain fusions were detected by an affinity-purified DD antibody. The antibody was raised against Met\textsuperscript{55}-Ser\textsuperscript{59} catalytic domain of RetGC\textsubscript{1} and affinity-purified using a glutathione S-transferase dimerization domain (amino acids 817–857) RetGC-1 fusion (31). Western blots were performed as described earlier.

**Molecular Dynamics Simulations—**MD simulations were performed using the program ENCAD (35). The potential energy function and MD protocols have been described elsewhere (36, 37). An all-atom representation was used for both the protein and solvent. Side-chain protonation states for the protein were represented neutral (the manufacter’s protocol), and Asp and Glu residues were positively charged and Asp and Glu were negatively charged. Because a crystal structure is not available for the dimerization domain of RetGC\textsubscript{1}, the initial starting structures for the simulations were modeled as parallel coiled-coils spanning residues 823–857. The models were designed to keep the residues in register with respect to sliding along the helix axis while aligning nonpolar and complementary-charged groups at the interface. An antiparallel coiled-coil was also investigated, but complementarity of the residues along the interface was not as good as in the parallel arrangement, resulting in a ~37 kcal/mol higher (less favorable) potential energy after 1000 steps of in vacuo minimization. Amino acid mutations were made to the minimized parallel structure to yield mutant homodimers: R838C and R838S.

The proteins were then each solvated using a box extending at least 8 Å in all directions, resulting in the addition of ~3870 water molecules. The water density was set to the experimental value at 25 °C of 0.997 g ml\textsuperscript{-1}, and periodic boundary conditions were employed to reduce edge effects. The solvent was then subjected to 1000 cycles of minimization, 2 ps of MD, and another 1000 steps of minimization. This water preparation was followed by 1000 steps of minimization of the protein in the field of the solvent and then 1000 steps of MD minimization of the full protein-solvent system. After the preparatory steps described above, the system was heated to 25 °C. Each independent simulation was carried out for 5 ns using a 2-fs integration time step (5000 ps, or 2.5 × 10\textsuperscript{9} iterations). An 8-Å smooth, nonbonded cutoff was used, and the nonbonded list was updated every 2 cycles; this procedure has been shown to yield stable and reasonable simulations (35, 36). 25,000 structures were saved for analysis (5 per ps) from each trajectory.

**RESULTS**

The purpose of this study is to investigate the structural basis by which a coiled-coil dimerization domain plays an essential role in regulation of RetGC-1 by Ca\textsuperscript{2+}. In the first step of our analysis we establish that the catalytic domain of RetGC-1 must form a dimer to synthesize cGMP. We show this by demonstrating that the catalytic site is made up of essential functional groups contributed separately by each polypeptide chain of the dimer. We then demonstrate that the coiled-coil domain is essential to bring the subunits of the catalytic domain together. Finally, we analyze how mutations linked to adCORD alter the Ca\textsuperscript{2+} sensitivity of cGMP synthesis by affecting the dimerization structure of the coiled-coil domain.

**Guanylyl Cyclases Function as Dimers—**Molecular modeling studies based on the structure of adenylyl cyclase suggest that synthesis of cGMP by RetGC-1 requires an intramolecular nucleophilic attack by a deprotonated 3′-hydroxyl of GTP on the α-phosphate (P\textsubscript{2}′) of GTP (16, 38, 39). A metal ion-A (Mg\textsuperscript{2+}) appears to be involved in the activation of the 3′-OH group (Fig. 1a). This is followed by stabilization of the transition state by two metal ions (Mg\textsuperscript{2+} and Mg\textsuperscript{2+}) (39–41). A recent report of an adenylyl cyclase crystal structure in complex with metal ions conclusively shows that two metal ions bind in the active site (20). Two highly conserved aspartic acid residues from the catalytic C1a domain of adenylyl cyclase coordinate both metal ions (16, 20, 42).

A structure of the RetGC-1 guanylyl cyclase catalytic domain has not yet been determined, but a model for it has been proposed (16, 43). In this model the two metal ions essential for activation and conversion of GTP to cGMP in RetGC-1 are coordinated by aspartates (Asp\textsuperscript{885} and Asp\textsuperscript{929}) from one subunit of the RetGC homodimer (Fig. 1a). Residues that interact with the guanine ring appear to be on the opposite subunit (16). This proposed mechanism predicts that dimerization of the catalytic domains is necessary for cGMP formation.

To confirm this dimeric model experimentally, we used two types of RetGC-1 mutations that inactivate separate functions of the catalytic site: 1) We substituted Asp\textsuperscript{885} and Asp\textsuperscript{929} with alanine to prevent Mg\textsuperscript{2+} binding. We then expressed the mutant cyclases in human embryonic kidney cells (HEK-293) and assayed their abilities to produce cGMP from GTP. Both mutants, D885A and D929A failed to synthesize cGMP either in the presence or absence of GCAPs, consistent with the role of these amino acids in coordinating essential metal ions (Fig. 1, a and c). These mutants also were inactive in the presence of Mn\textsuperscript{2+} and Trichot, which constitutively activates normal membrane GCs and has been used to quantitate general catalytic activity (44). This finding confirmed that the mutations affect the catalytic site and do not specifically interfere with GCAP-1-mediated activation. Other studies using adenylyl cyclase reached similar conclusions about the essential role of these aspartic acids in cyclic nucleotide synthesis (16, 20, 40). 2) Guanylyl cyclases can be converted to adenylyl cyclases by two specific amino acid substitutions (19, 43). In RetGC-1 this can be achieved by combining E925K and C997D substitutions to produce a protein with virtually no guanylyl cyclase activity (Fig. 1, b and c) (43). However, we know that these mutants are folded properly, because they have appreciable adenylyl cyclase activity and retain responsiveness to GCAPs (43). Similar types of mutations have also been studied using a soluble guanylyl cyclase isoform (19).

The homodimer model of RetGC-1 predicts that there are two active sites in the catalytic domain (Fig. 1b, panel i). At each site, one polypeptide chain provides the aspartates required for metal binding and the other chain provides the residues required for guanine specificity. The model predicts that a heterodimer of an aspartate mutant polypeptide with an adenine-specific polypeptide would produce one functionally active site (Fig. 1b, panels iv and v). We confirmed this prediction by co-expressing either D885A or D929A RetGC-1 together with the adenine specific form (AC) of RetGC-1. Expression of either type of mutant alone produced no GC activity (Fig. 1b, panels ii, iii Fig. 1c). But co-expression created a heterodimer that synthesizes cGMP and retains GCAP sensitivity (Fig. 1b, panels iv, v and Fig. 1c). The co-expressed mutants (D885A/AC or D929A/AC) also had appreciable Mn\textsuperscript{2+}/Triton-stimulated activity confirming that the inactivating effects of the mutations do not represent specific effects only on sensitivity to GCAP-1-mediated activation. In summary, in the heterodimer, the D885A or D929A GC-1 chain provides the guanine ring binding site, and the partner chain (AC) provides aspartates that coordinate metal ions needed for catalysis. The result of this study confirms experimentally that dimerization of the catalytic domain is absolutely required for synthesis of cGMP by RetGC-1.

**An Intact Coiled-Coil Domain Is Essential for RetGC-1 Activation by GCAPs—**Because dimerization is required for...
Role of Coiled-coil in Regulation of Guanylyl Cyclase Activity and adCORD

RetGC-1 catalytic activity, we analyzed the region in RetGC-1 most likely to stabilize catalytic domain dimerization. A highly conserved hinge region between the kinase homology domain and the catalytic domain GC-A (NPR-A) is responsible for dimerization of the GC-A intracellular domain (Fig. 2a) (22). This region is also required for GC-A enzymatic activity (22). The role of the corresponding hinge region in RetGC-1 has not yet been established (45). Structure prediction analysis suggests that this region of RetGC-1 forms an amphipathic α-helical coiled-coil structure (Fig. 2b) (46). Coiled-coils share a characteristic heptad repeat, (abcdefg)ₙ, in which the first (a) and fourth (d) positions are usually hydrophobic residues. Analysis of the hinge region sequence from RetGC-1 by the COILS2 secondary structure prediction program (46), suggests that it has a very high probability to form four heptads from amino acid residues 817–844 (Fig. 2b). To test the role of these coils in RetGC-1 activity, we selectively disrupted either the second (L824S,Y827S) or third (L831S,L834S) heptad repeat by changing conserved hydrophobic “a” and “d” residues to serines. We then expressed the mutant and wild-type cyclases in HEK-293 cells. As shown in the Western blot, both the mutants and wild-type were expressed at similar levels (Fig. 2c). Both expressed mutants (coil2 or coil3 mutant) could not be stimulated by hGCAP-1 (Fig. 2c) or by bGCAP-2 (results not shown) to synthesize cGMP. To be certain that the mutations did not cause nonspecific aggregation of the expressed proteins, we performed centrifugation of the HEK-293 membranes through a 50% sucrose cushion. We found both coil mutant forms of RetGC-1 to be associated with membranes to the same extent (90% of total RetGC-1) as wild-type RetGC-1 (results not shown) (47). Therefore, the lack of guanylyl cyclase activity in coil2 or coil3 mutant is not due to its nonspecific aggregation into insoluble complex.

To confirm the ability of the hinge region of RetGC-1 to mediate protein dimerization, we used an E. coli system that was first developed to demonstrate that the leucine zipper motif of GCN4 is responsible for dimerization of that protein. Since then, this system has also been used to extensively study the sequence requirements for α-helical coils from various molecules, including the leucine zipper of yeast GCN4 and coiled-coil domains of the APC protein (product of the APC (adenomatous polyposis coli) gene) (34, 48). This method involves using the N terminus of phage λ repressor as a reporter for the dimerization capabilities of various amino acids that are fused downstream. The λ repressor consists of an N-terminal domain that binds DNA and a C-terminal domain that mediates dimerization (49). Heterologous domains that are able to dimerize can functionally substitute for the C-terminal domain. A functional dimeric repressor blocks the transcription of genes that are responsible for the phage lytic cycle and confers immunity to lambda phage.

We fused the putative dimerization domain (DD) from amino acid residue 817 to 844 of RetGC-1 (DD-wt), coils 2 (DD-coils2), and coils 3 (DD-coils3) mutants to the N-terminal DNA binding domain of λ repressor. We then assessed the ability of the hybrid protein to render E. coli immune to lambda phage.

AC mutant (4 µg) alone or in combination as indicated (each construct 2 µg). The AC mutant is E925K,C997D RetGC-1 (43). The immunoblot with antibody to RetGC-1 kinase homology domain and catalytic domain shows equivalent levels of expression (inset). The control lane labeled pBC-CMV contained membranes from HEK-293 cells transfected with the plasmid pBC-CMV that had no insert. Equal amounts of membrane protein were assayed for 20 min. for basal-, GCAP-1-, or GCAP-2-stimulated activities (43). For GCAP-stimulated samples, 3.4 µM GCAP-1 or GCAP-2 and 1 mM EGTA were added. Mn²⁺/Triton X-100 samples contained 1% Triton X-100 and 10 mM MnCl₂ instead of MgCl₂.
transmembrane (ECD) membrane guanylyl cyclases. They are the extra cellular domain (of RetGC-1. a type dimerization domain (DD-Coils2 DD-WT expression of d probed with an antibody against KHD and CAT regions of RetGC-1. Inset at the indicated concentrations of hGCAP-1. RetGC-1. HEK-293 cells were transiently transfected with 4 mutant (L824S,Y827S) and coils 3 (L831S,L834S) and wild-type served among these membrane GCs. c to toxin receptor (). The amino acid residues in atrial natriuretic peptide receptor (GC-D) RetGC-1, the human, rat (r) (RetGC-2, the olfactory guanylyl cyclase (GC-D) RetGC-1, the human, rat (r) include the human (h), rat (r), bovine (r) RetGC-2, the olfactory guanylyl cyclase (GC-D), the atrial natriuretic peptide receptor (GC-A), and the heat-stable enterotoxin receptor (GC-C). The amino acid residues in boldface are conserved among these membrane GCs. c, GCAP-1 titrations of coils 2 mutant (L824S,Y827S) and coils 3 (L831S,L834S) and wild-type RetGC-1. HEK-293 cells were transiently transfected with 4 µg of wild-type Ret GC-1, Coils 2, or Coils 3 mutant constructs. Equal amounts of membrane protein (40 µg) were assayed for cyclase activity at the indicated concentrations of hGCAP-1. Inset, an immunoblot probed with an antibody against KHD and CAT regions of RetGC-1. d, expression of λ repressor chimeric proteins containing RetGC-1 wild-type dimerization domain (DD-WT), Coils 2 mutant (DD-Coils2), and infection by formation of active dimeric λ repressor protein. The E. coli strains expressing the λ repressor fusions were cross-streaked against the λKH54, a phage carrying a deletion in the functional repressor that prevents lysogenization. Immunity to phage was determined visually (Fig. 2e). As a negative control, an E. coli strain expressing just the λ repressor DNA binding domain (pKH101) was used (50). A well-characterized λ repressor-leucine zipper fusion (GCN4) was used as a positive control (34). As revealed by cross-streak analysis, bacteria expressing λ repressor hybrids DD-wt and GCN4 (positive control) were clearly more immune to AKH54 infection than the negative control (Fig. 2e). These results indicate a successful reconstitution of λ DNA binding activity promoted by the normal dimerization domain (DD-wt) of RetGC-1. We also confirmed that the immunity to λ KH54 depends specifically on the functional repressor and is not a result of interference with other cellular pathways (e.g. lack of λ receptor). This was demonstrated by showing that the cells immune to λKH54 are still permissive to lysis by λimm21 (Fig. 2e). λimm21 is a related phage that lyses E. coli by the same mechanism as λKH54. However, it can specifically escape the control exerted by the λ repressor protein.

In contrast, both the DD-coils 2 and DD-coils 3 mutants behaved like the λ repressor DNA binding domain alone (pKH101) and were unable to confer immunity to cells. We also analyzed repressor activity quantitatively by measuring plating efficiency of phage AKH54 on cells expressing the different repressor fusions. The DD-coils2 and the DD-coils3 were 1000 times less efficient than the DD-wt of RetGC-1 at restoring λ repressor activity (Fig. 2e). Western blot analysis using an affinity-purified antibody against the dimerization domain (DD) of RetGC-1 showed that the soluble repressor fusion proteins were present at nearly identical levels (Fig. 2d). By comparing the RetGC-1 immunoreactivity of these antibodies with antibodies against the catalytic domain we confirmed that the DD antibodies recognized the wild-type, coils2, and coils3 RetGC-1 mutants equally well (data not shown). These immunological controls rule out the possibility that the inability of DD-coils2 and DD-coils3 to dimerize λ repressor is merely due to low level expression.

Taken together our guanylyl cyclase assays and E. coli λ repressor fusion analyses of the DD-coils2 and DD-coils3 mutants show that an intact coiled-coil domain is required to produce a functional dimeric state of the catalytic domain in RetGC-1. adCORD Mutants Are More Sensitive to Activation by GCAP-1—Several naturally occurring mutations at the end of the putative coiled-coil domain in RetGC-1 have been linked to adCORD in humans (Fig. 3r) (24–26). This dominantly inherited disease is characterized by early degeneration of cones, decreased visual acuity, central vision defects, loss of color vision, and photophobia. At later stages, rods also degenerate.
resulting in progressive night blindness and peripheral visual field loss.

A previous study reported that a conservative E837D substitution in RetGC-1 was linked to adCORD (26). However, reappraisal of the original human mutation showed that it is instead a double mutant (E837D,R838S) in all affected family members (25). To identify the relevant biochemical phenotype of this and other RetGC-1 mutations that cause adCORD, we first expressed the adCORD double mutant (E837D,R838S, "ERT-HA") in HEK-293 cells. We also expressed the wild-type GC-1 ("GC1-HA") and an adCORD triple mutant (E837D,R838S,T839M, "ERT-HA"). Each protein also contained an N-terminal hemagglutinin tag (HA). Western blots using both anti-HA and RetGC-1 antibodies showed that the mutants ERT-HA, ER-HA, and wild-type GC1-HA are expressed at similar levels (Fig. 3b, lanes 1–3). The activity of ERT-HA in the absence of GCAP and the Mn2+/Triton activity were low (2-fold reduction) compared with wild-type GC-1, whereas the intrinsic activity of ER-HA was unaltered (Fig. 3c).

Both mutants were analyzed for their ability to be activated by increasing amounts of human GCAP-1 at low [Ca2+]1. The concentrations of GCAP-1 required for half-maximal activation (K1/2) were reduced for both mutants (Fig. 3d, Table I). The increased apparent affinity toward RetGC-1 mutants was specific for GCAP-1. The sensitivity to stimulation by hGCAP-2 was unaltered (data not shown).

**Table I**

| Mutant          | K1/2 μM | IC50 μM | IC50 μM |
|-----------------|---------|---------|---------|
| GC-1 (HA)       | 9.1 ± 0.5 | 580     | 620     |
| ERT (HA)        | 5.3 ± 0.2 | 800     | 770     |
| ER (HA)         | 2.5 ± 0.2 | 950     | 910     |
| GC-1 (HA)/GC-1  | 9.6 ± 0.9 | 580     | 600     |
| ERT (HA)/GC-1   | 5.9 ± 0.6 | 1000    | 820     |
| ER (HA)/GC-1    | 2.8 ± 0.3 | 1200    | 930     |

*a* K1/2 represents the concentration of GCAP-1 required for half-maximal activation of guanylyl cyclase.

*b* The half-maximal [Ca2+]1 for inhibition of cyclase activity at 3.4 μM GCAP-1.

*c* The half-maximal [Ca2+]1 for inhibition of cyclase activity at 17 μM GCAP-1.
The mutations in the coiled-coil domain of RetGC-1 cause a dominant disease phenotype in humans. To determine if the increased apparent affinity for hGCAP-1 is also dominant, we co-expressed either the HA-tagged ER or ERT mutant with untagged GC-1 at a 1:1 ratio. As a control, we also co-expressed HA-tagged GC-1 with untagged GC-1. Comparison of immunoblots using antibodies against the HA-tag and against RetGC-1 revealed that HA-tagged and untagged cyclase proteins are present at similar levels when co-expressed (Fig. 3b).

When ERT-HA was expressed alone, its basal activity was low, but in the co-expression experiments both ERT and ER mutants had levels of basal and Mn2+/Triton stimulated activity that were the same as wild-type RetGC-1 (Fig. 3c). It appears that co-expression with the wild-type RetGC-1 enhances the folding or stability of the mutant proteins. The co-expressed mutants, like the ERT and ER mutants expressed alone, are more sensitive to GCAP-1 (Fig. 3e, Table I) confirming that this biochemical phenotype is dominant.

Ca2+ Sensitivity Is Altered in adCORD Mutants—About 3% of the cGMP-gated Na+/Ca2+ channels in photoreceptor cell plasma membranes are open in darkness (51). Because these channels allow Ca2+ to flow into the photoreceptor cell, intracellular [Ca2+]i is highest in the dark. Under those conditions, Ca2+ binds to GCAPs, which inactivate RetGC to slow cGMP synthesis. When light stimulates phototransduction, the channels close and there is a net efflux of Ca2+. At lower levels of Ca2+, Ca2+ dissociates from GCAPs, which then stimulate RetGC to increase production of cGMP, reopen the channels, and restore sensitivity to light (52, 53). The free Ca2+ concentrations in rods and cones of humans are not precisely known, but, based on studies with other species, they probably vary within the sub-micromolar range (54).

We showed previously that one of the adCORD mutations (R838C) shifts the Ca2+ sensitivity of RetGC-1 (27). Therefore, we also examined this property for the ERT and ER adCORD mutants. Although the Ca2+ concentration (IC50) required for half-maximal inhibition by 3.4 μM GCAP-1 was 580 nM for wild-type GC-1, both mutants, ERT and ER, required significantly higher concentrations of Ca2+ for inactivation (Fig. 4a, Table I). The Ca2+ sensitivities were the same when 3.4, 6.8, or 17 μM GCAP-1 was used (Fig. 4a, results not shown, Fig. 4b and Table I). The mutant cyclases also retained nearly 15–30% of their maximal activity at Ca2+ concentrations higher than 10 μM (Fig. 4, a and b). This biochemical effect, like the change in apparent affinity for GCAP-1, was dominant (Fig. 4, c and d, and Table I). The co-expressed cyclases also retained nearly 15–30% of their maximal cyclase activity at Ca2+ concentrations higher than 10 μM (Fig. 4, a and b). These effects of altered Ca2+ sensitivity were also specific for GCAP-1.

The combined effects of increased sensitivity to activation by GCAP-1 and decreased sensitivity to inhibition by Ca2+ are most clearly demonstrated when the cyclase activities of WT and mutants are compared at low (100 nM) and high (1.6 μM) Ca2+ concentrations (Fig. 4e). Both ERT and ER have higher activity than GC-1 at micromolar levels of Ca2+ (Fig. 4e). This difference in activity is most pronounced when the co-expressed ERT/GC-1 and ER/GC-1 are compared with GC-1 (Fig. 4e). Taken altogether, our results show that the Ca2+ feedback that controls cyclase activity through GCAP-1 is altered in adCORD patients with mutations in RetGC-1.

Arg838 Is Critical for Proper Regulation—The only amino acid that is altered in all four RetGC-1 adCORD mutations is Arg838. R838C alone has been linked to adCORD (26) and produces a similar biochemical phenotype (27). Both R838C and R838S GC-1 mutants have enhanced sensitivity to hGCAP-1 and reduced Ca2+ sensitivity (data not shown, 27). To determine if neighboring amino acid residues also contribute to this phenotype, we made the E837D and T839M single mutants. E837D behaved identically to wild-type GC-1 in our assays (data not shown). The E837D substitution was initially described to cause adCORD (26). But our biochemical studies led to a reinvestigation of the original mutation, which was subsequently redefined as a double mutation: E837D,R838S (25). T839M had a higher basal activity (2-fold) and increased sensitivity (2-fold) to both hGCAP-1 and GCAP-2. However, the Ca2+ sensitivity with hGCAP-1 in the co-expressed T839M mutant was only slightly altered (IC50 = 694 nM). Collectively, these results show that altered Ca2+ sensitivity is linked to adCORD and that only mutations that alter Arg838 cause shifts in Ca2+ sensitivity.

A Network of Salt Bridges in the Dimerization Domain May Be Required for Proper Regulation of RetGC-1 by GCAP-1—Mutations that disrupt the coiled-coil domains of structural proteins, such as keratin and spectrin, have also been linked to autosomal dominant human diseases (55, 56). Most substitutions in keratin that cause the skin disorder, epidermolytic hyperkeratosis, occur at the beginning or end of a coiled-coil rod domain (57). This finding is consistent with another study showing that proline mutations at the end of the rod domain in keratin are more disruptive than internal mutations (58). Similarly, it is interesting to note that the crucial arginine at residue 838 in RetGC-1 is predicted to lie exactly at the edge of the third heptad of the coiled-coil domain (Fig. 2a).

To understand the relationship between structural changes caused by arginine mutations in RetGC-1 and the observed altered regulation of cyclase activity, we modeled the sequence fully as a dimeric coiled-coil and then performed molecular dynamics simulations of wild-type and the R838C and R838S mutants in water (Fig. 5a). Molecular dynamics simulations of the WT peptide show that the residues after Arg838 splay apart, separating the C-terminal regions of the two α-helices (Fig. 5, a and b). In contrast, the α-helices stay together in the R838C and R838S mutants (Fig. 5, a and b). Separation of the helices in the WT peptide can be attributed to inter-molecular salt bridges (α-g- and α-d-type) between arginine and a highly conserved glutamic acid from the opposite chain (Fig. 5c). A similar arrangement of salt bridges was recently observed in the crystal structure of the coiled-coil “trigger site” of cortexillin I (59).

In WT RetGC-1, the Arg838 salt bridges pull the coiled-coil domains together and constrain the mobility of the helices in the C-terminal end. Consequently, acidic side chains at the dimerization interface are forced to interact so that electrostatic repulsion between them drives apart the polypeptide chains downstream from the Arg salt bridges (Fig. 5, a–c). Note, however, that the chains separate to relieve charge repulsion while remaining close enough to prevent a major influx of water to the interface, thereby retaining a relatively nonpolar environment for the leucines. This phenomenon is similar to that recently observed in the atomic structure of the rod domain of the intermediate filament protein vimentin, where the C-terminal consensus motif (YRKKLEGEE) bends away from the coiled-coil axis in the region of three conserved glutamic acids (60). This conserved motif has been shown to control the filament width, and any minor alteration in that domain causes severe epidermolytic diseases in humans (61).

When arginine is replaced by cysteine or serine in RetGC-1, the constraint produced by the salt bridge interaction is lost. The C-terminal glutamates are not pulled toward the interface and instead are free to orient themselves toward the water; compare the Glu residues in the wild-type and R838S structures in Fig. 5c. The mutant helices better optimize nonpolar
FIG. 4. Altered Ca$^{2+}$ sensitivity of ERT and ER mutants to GCAP-1. a, Ca$^{2+}$ titration of ERT, ER, and wild-type RetGC-1 stimulated with 3.4 μM GCAP-1. GC activity was measured in transiently transfected membranes expressing ERT, ER, or wild-type RetGC-1 as described in Fig. 3d. The half-maximal [Ca$^{2+}$] for inhibition (IC$_{50}$) was 580 nM for wild-type GC-1, 800 nM for ERT-HA, and 950 nM for ER-HA. b, Ca$^{2+}$ titration of ERT, ER, and wild-type RetGC-1 stimulated with 17 μM GCAP-1. GC activity was measured in transiently transfected membranes expressing ERT, ER, or wild-type RetGC-1, as described in Fig. 3d. The half-maximal [Ca$^{2+}$] for inhibition (IC$_{50}$) was 620 nM for wild-type GC-1, 770 nM for ERT-HA, and 910 nM for ER-HA. c, GC activity measured in co-expressed membrane, as described in Fig. 3e. All reactions contained 3.4 μM GCAP-1. The half-maximal [Ca$^{2+}$] for inhibition (IC$_{50}$) was 580 nM for wild-type GC-1(HA)/GC-1, 1000 nM for ERT-HA/GC-1, and 1190 nM for ER-HA/GC-1. d, GC activity measured in co-expressed membrane, as described in Fig. 3e. All reactions contained 17 μM GCAP-1. The half-maximal [Ca$^{2+}$] for inhibition (IC$_{50}$) was 620 nM for wild-type GC-1(HA)/GC-1, 820 nM for ERT-HA/GC-1, and 930 nM for ER-HA/GC-1. e, GC activity measured at low calcium (124 nM) representing the maximal activity and at high calcium (1680 nM). All reactions contained 3.4 μM GCAP-1. Assays were performed as described in Fig. 1. The results shown are representative of at least two independent experiments, each with duplicate data points.
packing interactions at the dimerization interface (Fig. 5b).
Ser838 satisfies its side-chain hydrogen bonding potential via interactions with the carbonyl oxygen of Leu834 on the previous turn of the helix (Fig. 5c).

To test the involvement of Arg838 in forming such an intermolecular salt bridge in Ret GC-1, we selectively mutated it to a similarly charged lysine, or to a negatively charged glutamate or aspartate. The R838E and R838D substitutions should destroy the salt bridges formed by arginine in wild-type Ret GC-1. This would allow the α-helices to stay together and stabilize the C-terminal end of the coiled-coil domain. As expected, high concentrations of Ca\(^{2+}\) are required to inhibit R838E and R838D mutants activated by 3.4 μM GCAP-1. Furthermore, the activity remains at 50% of its maximum even at...
Ca\textsuperscript{2+} concentrations as high as 27 \( \mu \)M (Fig. 5d). Similar results were obtained using 17 \( \mu \)M GCAP-1 (Fig. 5e). Conservative substitution of arginine to lysine did not alter the Ca\textsuperscript{2+} sensitivity (Fig. 5, d and e). Western blotting confirmed that all RetGC-1 mutants were expressed at similar levels (results not shown).

These findings support the idea that intermolecular salt bridges formed by Arg or Lys at position 838 pull Glu\textsuperscript{387} and Glu\textsuperscript{641} from the other helix into the dimerization interface. Although these Glu residues interact favorably with Arg\textsuperscript{838} they are then in close proximity to other Glu residues in the C-terminal region of this domain and charge repulsion leads to disruption of the dimer (Fig. 5c). Arg\textsuperscript{838} simultaneously acts to stabilize the coiled-coil interface and serves as a stop signal. Thus, the Arg\textsuperscript{838} salt bridges may either keep the catalytic domains far enough apart or shift their orientation sufficiently so that RetGC-1 can be activated or inhibited by GCAP-1 at the physiologically appropriate Ca\textsuperscript{2+} concentrations. More direct structural studies are necessary to verify the existence of the salt bridges and their role in controlling cyclase activity.

**DISCUSSION**

In this study we demonstrated that the native structure of a coiled-coil domain within RetGC-1 is critical for proper regulation by GCAP-1 and Ca\textsuperscript{2+}. We first established that the minimal functional stoichiometry of the RetGC-1 catalytic domain is a dimer. We then demonstrated that formation of a functional RetGC-1 catalytic dimer requires the presence of a structurally intact coiled-coil. Finally, we showed how the GCAP-mediated Ca\textsuperscript{2+} sensitivity of cGMP synthesis is altered by disease-linked mutations that enhance the dimerization strength of the coiled-coil domain.

**The RetGC-1 Catalytic Domain Functions Only as a Dimer**—Previous evidence has suggested that guanylyl cyclases function as dimers (15–20). Models of the guanylyl cyclase catalytic domain (16, 18) predict that aspartates from one polypeptide chain (Asp\textsuperscript{385} and Asp\textsuperscript{928} in RetGC-1) coordinate essential metal ions that interact with the phosphates in GTP and catalyze phosphodiester formation. Along with metal ions, Arg\textsuperscript{976}, Arg\textsuperscript{1008}, and Lys\textsuperscript{1048} help stabilize the pentavalent phosphate (Fig. 1a). The models also predict that specific residues (Glu\textsuperscript{466} and Cys\textsuperscript{397} in RetGC-1) on the other polypeptide chain in the dimer are required for specific interactions with the guanine ring.

In this report we confirmed these predictions experimentally by showing that RetGC-1 polypeptides with mutations at Asp\textsuperscript{385} or Asp\textsuperscript{928} cannot catalyze phosphodiester formation when expressed alone. We also showed that RetGC-1 polypeptides with mutations at Glu\textsuperscript{925} and Cys\textsuperscript{397} cannot synthesize cGMP. However, coexpression of these two types of mutant polypeptide chains reconstitutes a functional catalytic site (Fig. 1d). This can happen only if the two different mutant chains form a mixed dimer in which the catalytic function is provided by one polypeptide chain and the guanine ring binding function is provided by the other chain (Fig. 1c).

**Dimerization Induced by the RetGC-1 Coiled-Coil Is Required for Catalysis**—We also showed that an intact coiled-coil structure adjacent to the catalytic domain of RetGC-1 is essential for dimerization and catalytic activity. Substitution of critical hydrophobic amino acids within this structure reduced its ability to dimerize a repressor (Fig. 2c) and, in the context of RetGC-1, abolished catalytic activity (Fig. 2c).

**A Shift in Ca\textsuperscript{2+} Sensitivity of GCAP-1-mediated RetGC-1 Regulation Is Linked to adCORD but Changes in Intrinsic Activity Are Not**—Mutations in the human RetGC-1 gene have been linked to adCORD, an inherited retinal dystrophy. Substitutions at Arg\textsuperscript{838} near the end of the coiled-coil closest to the catalytic domain occur in all five forms of adCORD linked to RetGC-1 that have been reported. We analyzed effects of these mutations on overall activity, sensitivity to GCAP-1 and sensitivity to Ca\textsuperscript{2+}. In agreement with reports by Duda et al. (62, 63), we initially found that the “ERT” mutation linked to adCORD causes reduced catalytic activity when the mutant polypeptide is expressed alone. But our subsequent analyses of this and other adCORD mutants showed that the effect on intrinsic activity is not sufficient to explain the disease phenotype. Most importantly, co-expression of the ERT mutant with wild-type RetGC-1, as occurs in patients with adCORD, produces an enzyme with normal intrinsic activity.

The biochemical phenotype that is linked to adCORD is altered sensitivity to Ca\textsuperscript{2+} in the presence of GCAP-1. This phenotype is apparent both when the ERT mutant is expressed alone and when it is co-expressed with normal RetGC-1. Further studies with additional adCORD mutants also confirmed that it is the shifted Ca\textsuperscript{2+} sensitivity, not the altered intrinsic activity, that is linked to the adCORD mutations.

Why do Arg\textsuperscript{838} mutations alter the intrinsic activity of the mutant enzymes when they are expressed alone? We found by sucrose density gradient centrifugation (results not shown) that only a fraction of normal RetGC-1 in the cultured cell systems, widely used for expression of guanylyl and adenylyl cyclases, associates with the plasma membrane. The remainder appears to be not transported or folded properly. Therefore, any mutation introduced into the cyclase enzyme has the potential to favor or disfavor proper folding. Because of this observation, effects on the apparent intrinsic level of activity of mutants expressed alone should be discounted. In contrast, we found that the shifted Ca\textsuperscript{2+} sensitivity occurs independently of folding efficiency. It occurs at both low activity levels when some of the mutants are expressed alone and at high intrinsic levels when they are co-expressed with normal RetGC-1.

**Insights into the Mechanisms by Which GCAPs Regulate RetGCs**—The effect of Ca\textsuperscript{2+} on RetGCs is initiated entirely by Ca\textsuperscript{2+} binding to the EF-hand structures in GCAPs (11, 12). Evidence suggests that RetGCs and GCAPs bind to each other independently of [Ca\textsuperscript{2+}] (11, 12, 31). Therefore, it is not the GCAP-RetGC binding interaction that is affected by Ca\textsuperscript{2+}. Instead Ca\textsuperscript{2+} binding to the GCAP-RetGC complex induces structural changes that stabilize the inactive state relative to the active state of the RetGC catalytic dimer.

Our findings show that the dimerization strength of the coiled-coil domain must be fine-tuned for proper regulation by Ca\textsuperscript{2+}. The coiled-coil poises the catalytic domain in a state...
where its dimerization energy is regulated by physiological Ca\(^{2+}\) concentrations. Mutations linked to adCORD enhance dimerization, i.e. they stabilize the active state. Therefore, higher Ca\(^{2+}\) concentrations are required to overcome the increased stability of the active state. The shifted Ca\(^{2+}\) sensitivities in the mutants is accompanied by an apparent increase in sensitivity to GCAP-1 in the absence of Ca\(^{2+}\) (Fig. 3, d and e). It is unlikely that this represents increased affinity of RetGC-1 for GCAP-1. Instead when GCAP-1 binds to RetGC-1 in the absence of Ca\(^{2+}\) the active state is stabilized by the mutations so that abnormally high activity is detected. We have reported previously (27) that the apparent increase in GCAP-1 sensitivity is directly predicted from an analysis of coupled equilibria that describe regulation of RetGCs by GCAPs.

All of the adCORD mutations in RetGC-1 that have been reported include a substitution at Arg\(^{838}\). Why is this residue so critical? Our molecular dynamics-based modeling of the RetGC-1-coiled-coil strongly suggests that Arg\(^{838}\) forms critical salt bridges that force the helices into a configuration that produces electrostatic repulsion between the two polypeptide chains C-terminal to Arg\(^{838}\). This causes the chains to splay apart as they extend toward the catalytic domain. This normal situation should precisely determine the dimerization strength at the junction between the coiled-coil and the catalytic domain. Mutations at Arg\(^{838}\) that disrupt the salt bridges allow the coiled-coil structure in the model to extend closer to the catalytic domain. Therefore, more energy should be required to pull apart the catalytic dimer. Further structural studies will be required to confirm this model experimentally.

Based on these findings we propose in Fig. 6 a simplified model for regulation of RetGCs by GCAPs and Ca\(^{2+}\). In a photoreceptor cell in darkness, free [Ca\(^{2+}\)] is as high as 700 nM (54). In this state Ca\(^{2+}\) binds to GCAP-1, which pulls apart the polypeptide chains of the catalytic dimer, thereby reducing cGMP synthesis. When [Ca\(^{2+}\)] falls after light stimulation, Ca\(^{2+}\)-free GCAP-1 activates RetGC-1 by forcing the catalytic domains closer together (Fig. 6, top panel).

**Insights into the Molecular and Physiological Basis of adCORD—Mutations linked to adCORD enhance the stability of the active dimer. Dimerization is facilitated by increased stability of the C-terminal end of the coiled-coil (Fig. 6, lower panel).**

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Interactions within the Coiled-coil Domain of RetGC-1 Guanylyl Cyclase Are Optimized for Regulation Rather than for High Affinity

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