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Ca^{2+}-dependent Regulation of Phototransduction†

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Abstract

Photon absorption by rhodopsin triggers the phototransduction signaling pathway that culminates in degradation of cGMP, closure of cGMP-gated ion channels and hyperpolarization of the photoreceptor membrane. This process is accompanied by a decrease in free Ca^{2+} concentration in the photoreceptor cytosol sensed by Ca^{2+}-binding proteins that modulate phototransduction and activate the recovery phase to reestablish the photoreceptor dark potential. Guanylate cyclase-activating proteins (GCAPs) belong to the neuronal calcium sensor (NCS) family and are responsible for activating retinal guanylate cyclases (retGCs) at low Ca^{2+} concentrations triggering synthesis of cGMP and recovery of the dark potential. Here we review recent structural insight into the role of the N-terminal myristoylation in GCAPs and compare it to other NCS family members. We discuss previous studies identifying regions of GCAPs important for retGC1 regulation in the context of the new structural data available for myristoylated GCAP1. In addition, we present a hypothetical model for the Ca^{2+}-triggered conformational change in GCAPs and retGC1 regulation. Finally, we briefly discuss the involvement of mutant GCAP1 proteins in the etiology of retinal degeneration as well as the importance of other Ca^{2+} sensors in the modulation of phototransduction.

The phototransduction pathway links absorption of light to a decrease in cytosolic cGMP. Depletion of the cGMP pool induces closure of cGMP-gated cation channels and hyperpolarization of photoreceptor cells with a consequent decrease in glutamate neurotransmitter release detected by secondary neurons (1). Briefly, phototransduction is initiated with absorption of a photon by the chromophore 11-*cis*-retinal that is covalently linked to G-protein-coupled receptors known as opsins. Isomerization of 11-*cis*-retinal to all-*trans*-retinal and dissociation of the chromophore produces a conformational change in the opsin and consequent activation of the coupled heterotrimeric G-protein transducin (2). Activated transducin then activates a retina-specific phosphodiesterase (PDE6) that cleaves cGMP, depleting cytoplasmic cGMP and closing cGMP-gated cation channels. The level of cGMP is then restored to dark levels by activation of retina-specific guanylate cyclases (retGCs: retGC1 or GC-E; and retGC2 or GC-F) (3,4). retGC1 appears to be critical for cone

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function (5). Both disruption of normal phototransduction and recovery of the dark state by photoreceptors exposed to light are associated with a variety of cone-rod retinopathies (6).

The plasma membrane potential in photoreceptors is determined, in part, by open cGMP-gated cation channels. The fraction of open channels depends on the level of cGMP in the cytoplasm, which is controlled by the opposing effects of PDE6 and retGCs. RetGCs are transmembrane proteins located in the disk membranes of photoreceptor cells. An extracellular domain lies within the lumen of the disk membranes, but unlike homologous GCs in other tissues that are activated by ligand binding to the extracellular domain, no ligand has been identified for retGC1. In fact, they can be activated even when the extracellular domain has been truncated (7–9). Instead, retGCs are regulated by Ca^{2+}, which plays a crucial role in the regulation of phototransduction in photoreceptors. The concentration of free Ca^{2+} in the cytoplasm drops from ~550 to ~50 nM during activation due to the closing of cGMP-gated cation channels that block the influx of Ca^{2+}, as well as to the action of Na^{+}/Ca^{2+}-K^{+} exchangers which export Ca^{2+} from the cell (10). Retina-specific neuronal calcium sensor (NCS) proteins bind Ca^{2+} at the higher concentrations characteristic of the dark state but not at the lower concentrations achieved after prolonged activation and link phototransduction to Ca^{2+} signaling (3,4).

NCS proteins belong to the EF-hand superfamily of Ca^{2+}-binding proteins. They are expressed in neurons and have four EF-hands but only two or three of them are able to bind Ca^{2+} (11). Most NCS proteins are myristoylated at the N-terminus. Two classes of myristoylated NCS proteins are expressed in photoreceptors and are active in phototransduction—(1) guanylate cyclase-activating proteins (GCAPs) that regulate retGCs in response to Ca^{2+} and mediate the restoration dark levels of cGMP; and (2) recoverin, which plays a role in prolonging the photoresponse.

GUANYLATE CYCLASE-ACTIVATING PROTEINS

GCAPs modulate cGMP production by retGCs, inhibiting the cyclase at high Ca^{2+} concentrations typical of the dark state, and activating the cyclase when the Ca^{2+} concentration drops after phototransduction. Two GCAPs, GCAP1 and GCAP2, are conserved among most vertebrates, while a third isoform, GCAP3, has been identified in humans and fish (12). Additional GCAPs (GCAP4–8) are present in teleosts and likely arise from gene duplication (13). All GCAPs are approximately 23 kDa proteins with four EF-hands. However, only the last three EF-hands are capable of binding Ca^{2+}. Each GCAP can activate either of the two retGCs identified in rod and cone cells. Therefore, the specific roles of individual GCAP isoforms are unclear (14,15). GCAP isoforms have ~40% identity to each other with the greatest variation noted at the N- and C-termini; these polypeptide segments might play an important role in GCAP function as suggested by a related protein, guanylate cyclase-inhibitory protein (GCIP), which diverges from GCAPs in these regions. Identified in frogs, GCIP possesses the inhibitory function of GCAPs at high Ca^{2+} concentrations (~500 nM) but does not activate retGC1 (16).

Structures of unmyristoylated GCAP2 and GCAP3 have been determined by NMR and X-ray crystallography respectively (17,18). Very recently, the first structure of a myristoylated

GCAP (myrGCAP1) was solved crystallographically (Fig. 1) (19). The structures show that GCAPs are compact α-helical proteins with two domains, each with two EF-hands, similar to other NCS proteins. The members of the NCS family share substantial sequence identity (30–50%) and a similar overall fold. Several structures of NCS proteins with Ca^{2+} bound have been determined and a superposition of representative NCS proteins onto GCAP1 reveals that the overall structure is well conserved in this family (Fig. 2). The root-mean-square deviations for the structurally conserved α-carbons between myrGCAP1 and the NCS proteins with known structures oscillate between 3.3 and 3.9 Å (summarized in Table 1). In spite of the sequence and overall structural similarity among NCS family members, their functions are generally not interchangeable. The main differences arise from the length and conformation of the N- and C-terminal helices and, to a lesser extent, the relative orientation of the N-terminal domain with respect to the C-terminal domain. These structural differences and amino acids conserved only within each class of the NCS family determine the target specificity of these proteins. For GCAPs, conserved residues that do not participate in Ca^{2+}-binding are highlighted in the surface representation of GCAP1 in Fig. 3 as possible sites of interaction with retGC1.

GCAPS AND RETINAL DISEASE

GCAP1 is of particular interest due to its association with autosomal-dominant cone-rod dystrophies. Point mutations in five separate codons give rise to amino acid changes observed in families with cone degeneration including P50L, Y99C, I143NT, L150F and E155G. The mutations Y99C, I143NT, L150F and E155G each alter the Ca^{2+} sensitivity of GCAP1 (20–22). The E155G mutation eliminates the glutamate at position 12 of the Ca^{2+}-binding loop of EF-hand 4 (23). The glutamate side chain at this position of the EF-hand-binding loop helps coordinate Ca^{2+} and is conserved in all EF-hands. Elimination of the corresponding glutamate in EF-hand 3 produces a similar phenotype to E155G, *i.e.* a constitutively active GCAP1 (24). Y99 stabilizes a kink in the C-terminal helix of GCAP1 that enables the C-terminal helix to contact the N-terminal domain and the myristoyl group (19). The side chain of Y99 hydrogen bonds with a main chain carbonyl within the C-terminal helix forcing a kink that is further stabilized by other hydrogen bonds and a salt bridge. Also, Y99 has hydrophobic interactions with surrounding residues and is the last residue of the entering helix to EF-hand 3. Of particular note, I107 within the EF-hand 3-binding loop interacts with Y99. Thus the Y99C mutation likely has two effects—(1) it destabilizes the C-terminal helix kink by removing a hydrogen bond and (2) it introduces a void volume in the hydrophobic core as a result of the smaller Cys side chain (19).

The effect of mutations in residues I143 and L150 is not entirely clear. I143 is located just before the Ca^{2+}-binding loop of EF-hand 4 and substitution of a residue capable of hydrogen bonding might alter the geometry of the EF-hand, preventing Ca^{2+} binding. Likewise, substitution of L150 by the larger phenylalanine residue might disrupt the conformation of EF-hand 4 enough to prevent proper Ca^{2+} binding (18). All these GCAP1 mutations share a similar phenotype wherein retGC1 is inadequately inhibited. Finally, the P50L mutation appears to affect GCAP function by a different mechanism. This mutation has been shown to increase the susceptibility of GCAP1 to trypsin proteolysis, suggesting that a shorter half-

life of GCAP1 might reduce GCAP1 levels in cones to the point that retGC activity is insufficiently inhibited in the dark state (25).

All mutations in GCAP1 are linked to increased levels of cGMP in photoreceptors leading to degeneration of the retina. One speculative possibility for the mechanism of degeneration is that the increased levels of cGMP in cones open a higher percentage of cGMP-gated cation channels leading to a prolonged increase in intracellular Ca^{2+} sufficient to trigger apoptosis (26).

N-TERMINAL ACYLATION OF NCS PROTEINS

The structures of myristoylated recoverin in both Ca^{2+}-bound and Ca^{2+}-free forms have been determined by NMR (27,28). Like other NCS proteins, recoverin is a compact α-helical protein with each pair of EF-hands connected by a central loop. The Ca^{2+}-bound structure has the myristoyl group exposed whereas the Ca^{2+}-free structure has the myristoyl group bound in a hydrophobic cleft on the surface of the protein. The exposure of the myristoyl group in Ca^{2+} is essential for anchoring the protein to the membrane. Upon Ca^{2+} release, the myristoyl group is sequestered in a deep hydrophobic cleft of the protein allowing free movement of recoverin through the cytoplasm. This behavior has been termed the "Ca^{2+}-myristoyl switch" (27,29,30). Other NCS proteins including hippocalcin and the visinin-like proteins (ViLiPs) show myristoyl switch behavior (31–33). However, some NCS proteins including GCAPs do not appear to have a canonical myristoyl switch (34,35). Therefore, the role of the N-terminal acylation in these proteins has remained obscure (11).

The recently determined structure of Ca^{2+}-bound myrGCAP1 shows that, in sharp contrast to Ca^{2+}-bound recoverin, the myristoyl group is completely buried within the N-terminal domain of GCAP1 (Fig. 1) (19). Moreover, fluorescence quenching experiments using a 16-NBD-palmitoyl group attached to GCAP1 indicate that the myristoyl group remains buried in both the Ca^{2+}-bound and Ca^{2+}-free forms of the protein (19). Therefore, the myristoyl group is "nonswitching" in GCAP1 and instead has a stabilizing, structural role in this protein. The published structures of GCAP2 and GCAP3 are from unmyristoylated proteins and it is likely that these structures are affected by the absence of the natural myristoyl group. In particular, the N- and C-terminal regions of both GCAP2 and GCAP3 are poorly defined or extremely flexible in the unmyristoylated structures. In myrGCAP1 however, the N- and C-terminal helices are clustered together with the buried myristoyl group, which stabilizes their conformation. Importantly, removal of the C-terminal helix residues contacting the myristoyl group prevents GCAP1 activation of retGC1 (36). The contacts between the C-terminal helix with the N-terminal helix and myristoyl group stabilize the orientation of the N- and C-terminal domains relative to each other. Whereas myristoylation has been shown to be necessary for proper Ca^{2+} sensitivity of GCAP1, it appears to be less important for Ca^{2+} sensitivity of GCAP2 (35).

GCAP REQUIREMENTS FOR RETGC REGULATION

The interface(s) of interaction between retGCs and GCAPs have not been determined with certainty. GCAP1 and GCAP2 are bound to the intracellular part of retGC1 in both the Ca^{2+}-free (activating) and Ca^{2+}-bound (inhibiting) forms (8). Several experiments have been

performed to identify the regions of GCAP important for the interaction with retGC1 (Fig. 4). Chimeras of bovine GCAP1 and GCIP have shown that the N-terminal region of GCAP1 is crucial for retGC regulation. A chimera composed of the N-terminal 20 residues from GCIP and the remaining residues from GCAP1 did not activate retGC1 while a chimera of the N-terminal 43 residues of GCAP1 along with the remaining residues from GCIP activated retGC1 in a Ca^{2+}-dependent manner (37). Additionally, chimeric proteins containing sequences from the NCS family protein neurocalcin (~35% identity to GCAPs) were substituted into bovine GCAP1; these experiments identified the polypeptides M157-R182 and W21-T27 in bovine GCAP1 as necessary for activation of retGC1 (36). Also, N-terminal deletions of the myristoyl group and the first 25 residues of GCAP1 showed that this region is essential for proper Ca^{2+}-dependent retGC1 activation (38).

Point mutations in the EF-hand 1 region of GCAP2 prevent activation of retGC1, confirming the importance of this region for GCAP activity (39). Moreover, experiments with bovine GCAP2 identified residues K29 through F48 as required for both activation and inhibition of retGC1 whereas V171-N189 was only required for activation. Curiously, the region between EF-hand 2 and EF-hand 3 (F78-D113) in GCAP2 determined the direction of Ca^{2+} regulation of retGC1. Substituting this region for the corresponding sequence of neurocalcin created a chimera that activated retGC1 at high Ca^{2+} and inhibited retGC1 at low Ca^{2+} (40).

Alternative approaches to pinpoint GCAP residues important for retGC1 regulation have provided additional information. Peptide competition experiments suggest that the region between F73 and K87 in GCAP1 is important for interaction with retGC1 (41). Moreover, experiments in which GCAP and retGC1 were crosslinked, trypsin digested and the fragments analyzed by mass spectrometry, revealed that the regions around C17 and C105 on GCAP1 were in close proximity to the kinase homology domain on retGC1 (42).

MODELS OF THE CONFORMATIONAL CHANGE IN GCAPS AND RETGC REGULATION

The structure of the Ca^{2+} free, activating form of a GCAP is not yet available. However, it is thought that the conformational change induced by Ca^{2+} release involves relatively little change in the domain structure but a significant rotation of the N-terminal domain with respect to the C-terminal domain. Such a conformational change has been experimentally observed in recoverin (Fig. 5a,b) (27–29). Furthermore, tryptophan fluorescence experiments with GCAP1 support a conformational change involving reorientation of the N- and C-terminal domains because W91, located on the central helix, is exposed only under low Ca^{2+} conditions (43).

The chimera experiments described above suggest that the N- and C-terminal helices of GCAP1 are the most important for retGC1. The structure of myristoylated chicken GCAP1 shows these regions in close proximity to each other and clustered with the myristoyl group in the Ca^{2+} is bound, inhibitory conformation of the protein (Figs. 1 and 4 and schematically in Fig. 5c). Any rotation of the N- and C-terminal domains relative to each other would necessarily pull the terminal helices apart (schematically in Fig. 5d). We propose that this

separation of the N- and C-terminal helices in GCAP1 is crucial to induce the activated conformation of retGC1.

A separate model of GCAP activation of retGC1 involving dimerization of the GCAP has been proposed based on the observation that GCAP2 migrates as a dimer in the absence of Ca^{2+} but entirely as a monomer in the presence of Ca^{2+} during gel chromatography (40,44,45). However, this behavior was not observed for GCAP1 (40). Structures of retGC and its complex with GCAPs will be necessary to unravel the mechanistic details of GCAP regulation of retGCs. These structures would also allow the mechanistic integration of other modulatory signals like retGC phosphorylation (46) as well as understanding the roles of the noncatalytic retGC domains such as the extracellular, kinase homology and dimerization domains (47).

OTHER CA^{2+} SENSORS IN PHOTOTRANSDUCTION

Recoverin is another member of the NCS family active in phototransduction. In mouse rods, recoverin prolongs the photoresponse, thereby increasing rod sensitivity in dim light. (48). One model of recoverin mechanism suggests that it functions as a Ca^{2+}-sensitive regulator of rhodopsin kinase (RK) in the outer segment (49,50), a function that does not require recoverin myristoylation (51,52). However, inhibition of RK is not specific to recoverin as other NCS proteins are capable of Ca^{2+}-sensitive regulation of RK as well (53). Also, recoverin is present in the rod inner segment and synaptic terminals and it has an effect on the photo-response that must be downstream of its outer segment action in addition to any outer segment effects it may have. Recoverin has also been proposed to act either as a Ca^{2+} buffer, a regulator of the conductance in the inner segment of photoreceptors, or by an interaction with the neurotransmitter release machinery at the synapse (48).

The ubiquitous Ca^{2+}-binding protein calmodulin (CaM) might also have a role in recovery of the dark state after photoexcitation. It has been reported that Ca^{2+}-bound CaM decreases the sensitivity of cGMP-gated cation channels to cGMP in rods (54). Moreover, the β subunit of rod CNG channels has a CaM-binding site (55). While CaM dramatically alters sensitivity of many types of cyclic-nucleotide channels, the magnitude of the effect in rods is small, arguing against this being a significant mechanism (56). Additionally, a study of catfish cone channels concluded that, unlike in rods, Ca^{2+}/CaM did not alter cone CNG channel activity at all (57).

While progress has been made on the mode of Ca^{2+}-dependent regulation of phototransduction, much remains unknown. The mechanism of activation of retGCs by GCAPs is still poorly understood. Work on the structure of Ca^{2+}-free GCAPs and GCAPs in complex with retGCs is needed to assess the role of different GCAPs in photoreceptors and the mechanism of retGC regulation. Finally, the contributions of additional Ca^{2+} sensors, such as recoverin and CaM, to the regulation of phototransduction *in vivo* remain to be fully addressed.

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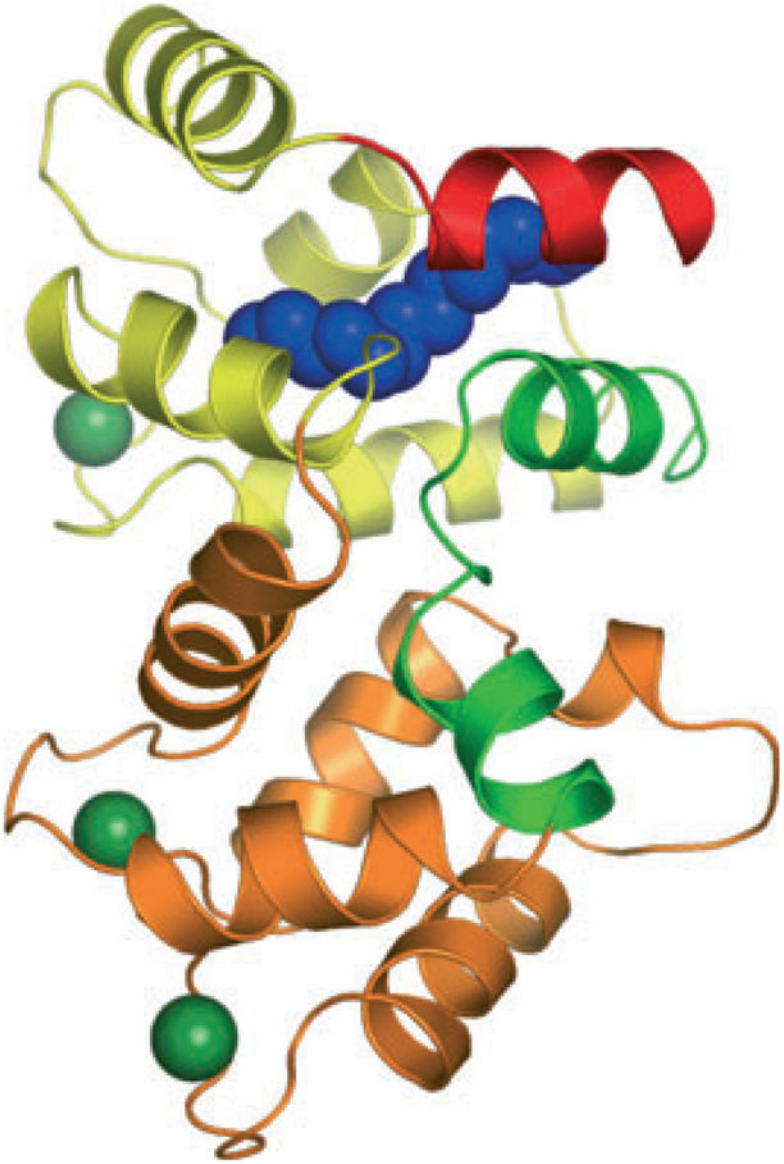

Figure 1.

Structure of myristoylated chicken GCAP1 with Ca^{2+} bound. The cartoon representation is colored yellow for EF-hands 1 and 2 and orange for EF-hands 3 and 4. The N-terminal helix is colored red, the kinked C-terminal helix is green and the myristoyl group is shown as a space-filling model in blue. Bound Ca^{2+} ions are shown in dark green.

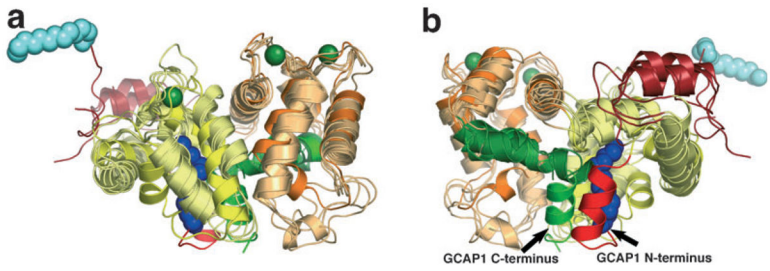

Figure 2.

Superposition of representative NCS protein structures in the Ca^{2+}-bound form. Front (a) and back (b) cartoon representations of myrGCAP1 superimposed onto the NCS family members myr-recoverin, neurocalcin, NCS-1 and KChIP1. The coloring of myrGCAP1 is as in Fig. 1 with the N- and C-terminal helices in bright red and green, respectively. The other NCS proteins are colored light yellow for EF-hands 1 and 2 and light orange for EF-hands 3 and 4, dark red for the N-terminal helix and dark green for the C-terminal segment. The recoverin myristoyl group is shown as a space-filling model in light blue. Ca^{2+} ions bound to GCAP1 are shown in dark green for reference but are omitted for the other NCS proteins for clarity.

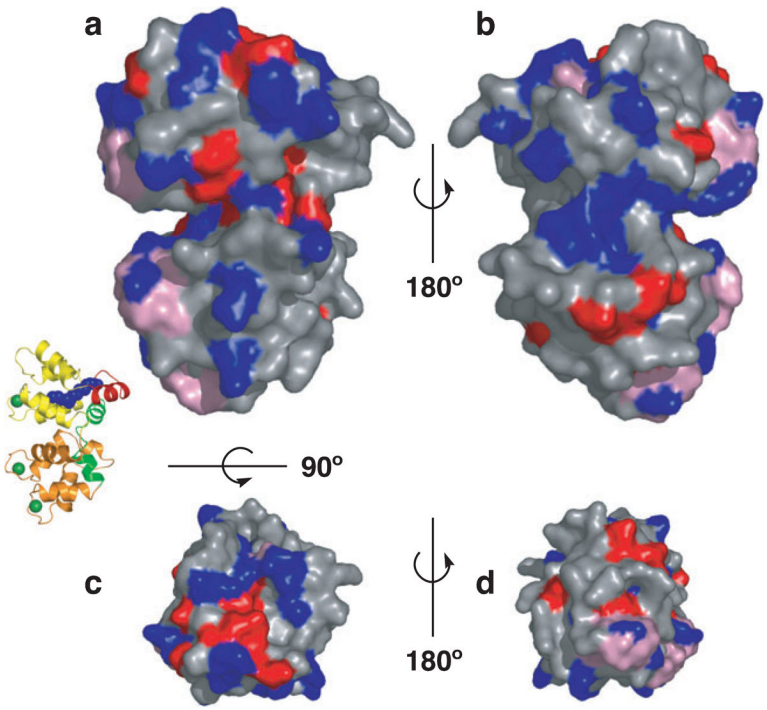

Figure 3.

Surface residue conservation in GCAPs. Surface representations of myrGCAP1 in front (a), back (b), top (c) and bottom (d) orientations (a cartoon model oriented as in [a] is shown for reference). Residues conserved in GCAP1 across species, but not GCAP2, are colored blue. Residues conserved in GCAP1 and GCAP2, but not across the NCS family, are in red. Residues required for myristoylation and Ca^{2+} coordination across NCS family are shown in pink.

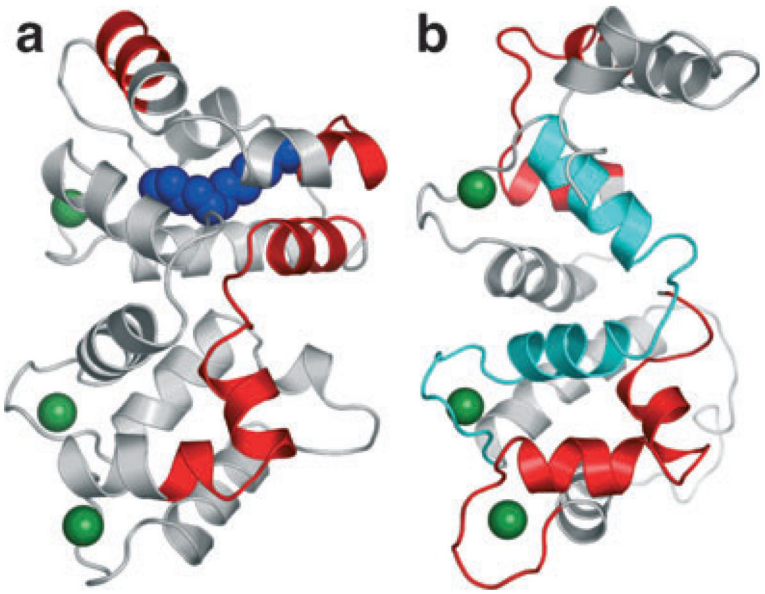

Figure 4.

Regions of GCAP1 (a) and GCAP2 (b) identified in chimera studies as important for retGC regulation. Regions affecting retGC regulation are shown in red. The region of GCAP2 that reverses Ca^{2+} dependency is shown in cyan. The myristoyl group in GCAP1 is shown as a space-filling model in blue.

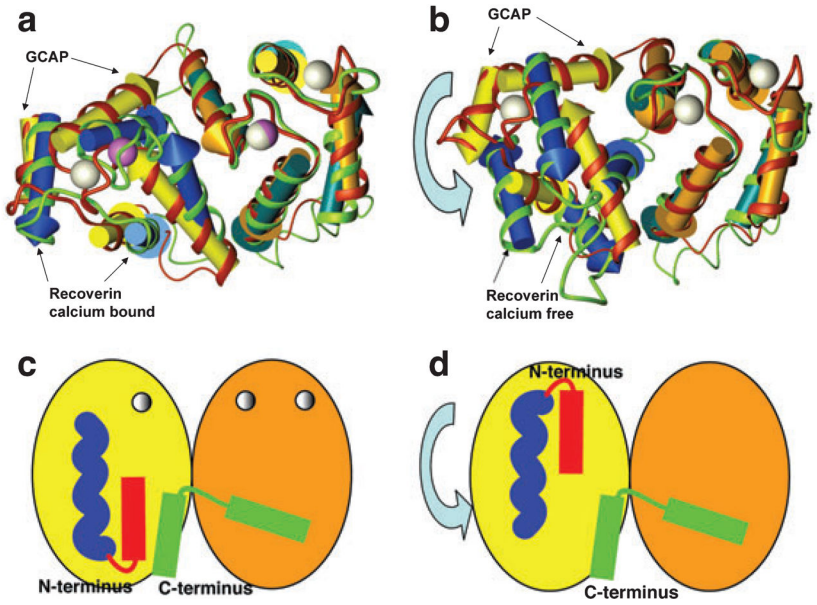

Figure 5.

Model of the Ca^{2+}-induced conformational change in GCAP1. (a) EF-hands of Ca^{2+}-bound GCAP represented by yellow and orange arrowed cylinders, red ribbon and white calcium ions, superimposed on Ca^{2+}-bound recoverin represented by blue and green-blue arrowed cylinders, green ribbon and violet Ca^{2+} ions. N- and C-terminal parts of the structure were removed for clarity. Superimposition was done on C-terminal EF-hand pairs (orange and green-blue cylinders). (b) EF-hands of Ca^{2+}-bound GCAP superimposed on Ca^{2+}-free recoverin. Superimposition was done on C-terminal EF-hand pairs. Coloring as in (a). (c, d) Schematic representation of a possible Ca^{2+}-induced conformational change in GCAP1 where the N-terminal (red) and C-terminal (green) helices are clustered together in the Ca^{2+}-bound state (c), but separated by domain rotation in the Ca^{2+}-free form (d). The myristoyl group is represented in blue.

Table 1

Structures of Ca^{2+}-bound NCS family members.

Protein (species)	Resolution (Å)	RSMD–GCAP1 (Å)	Myr	PDB ID (Ref.)
GCAP1 (chicken)	2.00	–	Yes	2r2i (19)
Recoverin (bovine)	NMR	3.7*	Yes	1jsa (27)
Recoverin (bovine)	1.9	3.4	No	1rec (58)
Recoverin (human)	2.2	3.6	No	2d8n (S. Kamo, S. Kishishita, K. Murayama, M. Shirouzu and S. Yokohama, unpublished)
GCAP2 (bovine)	NMR	3.6*	No	1jba (17)
GCAP3 (human)	3.00	3.9	No	2ggz (18)
Neurocalcin (bovine)	2.40	3.8 (chain A) 3.5 (chain B)	No	1bjf (59)
NCS-1 (human)	1.90	3.9 (chain A) 3.7 (chain B)	No	1g8i (60)
Frequenin (yeast)	NMR	3.7*	No	1fpw (61)
KChIP (human)	2.3	3.3	No	1s1e (62)

* For NMR structures the root-mean-square deviations with GCAP1 was calculated using the “central” structure of the NMR family.