Identification of Target Binding Site in Photoreceptor Guanylyl Cyclase Activating Protein 1 (GCAP1)*

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*Running title: Target binding interface on GCAP1

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Background: GCAP1 regulates cGMP synthesis in photoreceptors in response to light.

Results: Mutagenesis of the entire GCAP1 surface reveals its guanylyl cyclase interface.

Conclusion: The interface forms a compact patch that enables both primary binding to and allosteric activation of the target enzyme.

Significance: Guanylyl cyclase activation by GCAP1 is indispensable for vision and survival of photoreceptors.

ABSTRACT

Retinal guanylyl cyclase (RetGC) activating proteins (GCAPs) regulate visual phototransduction and trigger congenital retinal diseases in humans, but GCAP interaction with its target enzyme remains obscure. We mapped GCAP1 residues comprising the RetGC1 binding site by mutagenizing the entire surface of GCAP1 and testing the ability of each mutant to bind RetGC1 in a cell-based assay and to activate it in vitro. Mutations that most strongly affect activation of RetGC1 localize to a distinct patch formed by the surface of non-metal binding EF-hand 1, the loop and the exiting helix of EF-hand 2, and the entering helix of EF-hand 3. Mutations in the binding patch can completely block activation of the cyclase without affecting Ca²⁺ binding stoichiometry of GCAP1 or its tertiary fold. Exposed residues in the C-terminal portion of GCAP1 including EF-hand 4 and the helix connecting it with the N-terminal lobe of GCAP1 are not critical for activation of the cyclase. GCAP1 mutants that failed to activate RetGC1 in vitro were GFP-tagged and co-expressed in HEK293 cells with mOrange-tagged RetGC1 to test their direct binding in cyto. Most of the GCAP1 mutations introduced into the “binding patch” prevent co-localization with RetGC1, except for Met26, Lys85, and Trp94. With these residues mutated, GCAP1 completely fails to stimulate cyclase activity but still binds RetGC1 and competes with the wild type GCAP1. Thus, RetGC1 activation by GCAP1 involves establishing a tight complex through the “binding patch” with additional activation step involving Met26, Lys85 and Trp94.

Retinal membrane guanylyl cyclase (RetGC) and RetGC activating proteins
(GCAPs) play a critical role in physiology of vertebrate photoreceptors by producing the second messenger of phototransduction, cGMP, and regulating its synthesis in a light-sensitive manner (1, 2). Photoactivated pigment (e.g., rhodopsin), via G<sub>i</sub> protein-dependent stimulation of PDE6 phosphodiesterase, triggers the decay of cGMP and shuts off cGMP gated channels in the outer segment, thus hyperpolarizing the photoreceptors in response to light (reviewed in reference 3). Timely recovery of rods and cones from excitation requires activation of RetGC by GCAPs through a Ca<sup>2+</sup> feedback pathway (4-6). Once the cGMP channels close in response to light, the influx of Ca<sup>2+</sup> through the channels stops and the free Ca<sup>2+</sup> concentration in the outer segment rapidly declines (7-8). In the absence of Ca<sup>2+</sup>, GCAPs convert into a Mg<sup>2+</sup>-bound state (9-11) that stimulates RetGC to restore the cGMP levels in the outer segment and re-open the channels (reviewed in reference 12). Ca<sup>2+</sup>/GCAP-regulated RetGC isozymes RetGC1 and RetGC2 (13-15) are the only source for the cGMP synthesis in rod and cone outer segments - in their absence, neither Ca<sup>2+</sup>-sensitive cGMP synthesis nor visual light responses can be detected in the retina (16-17). GCAPs are present in various isoforms in different Vertebrata species (18-19), but only GCAP1 (20) and GCAP2 (21) are ubiquitously present throughout the subfilum and are the only two isoforms encoded by the genome in some mammalian species, such as mice and rats. Structures of Ca<sup>2+</sup>-liganded GCAP1 (Fig. 1A), GCAP2 and GCAP3 have been mostly solved by NMR spectroscopy and X-ray crystallography (22-24) and the principal role of GCAPs in physiology and retinal diseases is well understood (2, 25-26). GCAPs have different Ca<sup>2+</sup> sensitivities and target specificities. GCAP1 is present in mammalian rods and cones and primarily regulates RetGC1 (27), isozyme that comprises at least two-thirds of the total RetGC activity in rod outer segments (17), while GCAP2 regulates both RetGC1 and RetGC2 in rods (17, 20), but is virtually absent from cones (21, 28).

GCAPs (29-33) and RetGC1 (34-36) have been linked to multiple forms of human congenital blindness caused by mutations that either disable the synthetic activity of RetGC1 (37-39) or affect Ca<sup>2+</sup> sensitivity of its regulation by GCAPs (30, 40-46). Despite the importance of RetGC regulation for the normal retinal physiology and disease and the ample physiological and biochemical data about regulation of cGMP synthesis in the photoreceptor outer segment, the molecular mechanism of RetGC activation by GCAP remains obscure. To date, there were several attempts to identify the possible sites of target recognition in GCAPs using chimeras with other neuronal calcium sensor proteins (NCS) (47-49), implicating several regions in GCAP primary structure as likely parts of the cyclase-binding interface. However, the precise identity of the binding interface(s) with the cyclase could not be directly derived from the earlier low-resolution studies. In the present study, we describe a refined mapping of the residues in GCAP1 using global mutagenesis of the surface-exposed residues combined with functional tests that allow distinguishing between the primary binding to the cyclase versus its activation. We find that the residues required for GCAP1 binding to RetGC1 form a distinct “binding patch” on one side of the molecule that also contains at least two residues, Met26 and Trp94, that are not essential for the primary binding but affect secondary interactions required for RetGC1 activation.

**EXPERIMENTAL PROCEDURES**

**GCAP1 mutagenesis and purification.—**

Bovine D6S GCAP1 cDNA was mutated by conventional “splicing by overlap extension” approach utilizing mutations embedded in chemically synthesized PCR primers (Integrated DNA Technologies); the cDNA was amplified
and inserted into the NcoI/BamHI sites of pET11d vector (Novagen/Calbiochem) as previously described (10), except for using high-fidelity Phusion Flash polymerase (Fermentas/Thermo Fisher Scientific) instead of Pfu polymerase. The constructs were sequenced and transformed into the BLR(DE3) E. coli strain harboring pBB131 plasmid encoding yeast N-myristoyl transferase. The myristoylated GCAP1 expressed in cells cultured in the presence of myristic acid and induced with IPTG was isolated and refolded from inclusion bodies by urea extraction and then purified using hydrophobic and size-exclusion chromatography as described previously in full detail (10, 50-51), except 5 mM MgCl₂ was present during the urea extraction and the subsequent dialysis steps.

*eGFP-tagged at the C-terminus GCAP 1 for RetGC1 co-transfection experiments* was expressed in HEK293 cells from pQBIIN3 vector (Clontech) using calcium phosphate precipitation technique for transfection as described previously (38, 52).

**RetGC1 expression and activity assay.**—

The human RetGC1 cDNA was expressed in HEK293 cells from a modified pRCCMV vector (Invitrogen) using calcium phosphate precipitation for the transfection, and the membrane fraction containing expressed RetGC1 was isolated as previously described in detail (11, 50). The activity of the cyclase was assayed using [α-32P]GTP as a substrate and the [32P]cGMP product was quantified using TLC as described previously (11, 50). Briefly, the assay mixture (25 µL) incubated at 30°C contained 30 mM MOPS – KOH (pH 7.2), 60 mM KCl, 4 mM NaCl, 1 mM DTT, 2 mM Ca²⁺/EGTA buffer, 1 mM free Mg²⁺, 0.3 mM ATP, 4 mM cGMP, 1 mM GTP, and 1 µCi of [α-32P]GTP. The resultant [32P]cGMP product was analyzed by TLC using fluorescently-backed polyethyleneimine cellulose plates (Merck) developed in 0.2 M LiCl and eluted with 2 M LiCl, as described (11). Up to 20 µM GCAP in assays did not compromise TLC performance.

**Co-transfection experiments.**—

The mOrange-tagged RetGC1 (51) was used in this study because of brighter fluorescence and lower cytotoxicity compared to the dsRed-tagged RetGC1 (38, 52) in the conditions of our experiments. The mOrange-tagged RetGC1 for co-transfection experiments was constructed in two steps. The cDNA portion coding for the human RetGC1 (14) extracellular domain (which does not participate in GCAP binding – see references 38, 52-53) between Cys14 and Asp24 was substituted with a 31-b.p. fragment containing engineered *NheI* and *AgeI* restriction nuclease sites. The mOrange cDNA encoded by the pmOrange plasmid (Clontech) was then amplified using Pfu polymerase (Stratagene/Agilent Technologies) with the *NheI* and *AgeI* sites at the ends and inserted in the modified extracellular domain, in-frame with the leader sequence of RetGC1. The resultant construct was sequenced, expressed in HEK293 cells to verify preservation of RetGC1 activity and GCAP-dependent regulation, and then co-expressed with the SuperGlo (Clontech) enhanced green fluorescent protein (GFP)-tagged GCAP1 in HEK293 cells at the RetGC1:GCAP1 plasmid ratio ~125:1 to test their co-localization using confocal microscopy as described in detail previously (51, 52). Confocal images were taken using an Olympus FV1000 Spectral instrument and processed using Olympus FluoView FV10-ASW software.

**Ca²⁺ binding assay** was performed using Fluo-4FF Ca²⁺ indicator dye (Molecular probes/Life Technologies) as described previously in detail (10, 50-51). The readings of the fluorescence intensity were corrected for dilution caused by addition of CaCl₂. Free Ca²⁺ in the reaction mixture was calculated using the formula: 

$$[\text{Ca}^{2+}]_T = K_d \times (F - F_{\text{min}})/(100 - F),$$

where F is the fluorescence intensity of the Ca²⁺ indicator in the assay mixture expressed as a percentage of
the fluorescence of the Ca$^{2+}$-saturated indicator (recorded at the end of each experiment in 1 mM [Ca]), $F_{\text{min}}$ is the fluorescence intensity of the Ca$^{2+}$ indicator in the absence of Ca$^{2+}$ and also expressed as a percentage of the fluorescence of the Ca$^{2+}$-saturated indicator, and $K_d$ is a corrected constant of the indicator dye for Ca$^{2+}$ (10).

**Tryptophan fluorescence of GCAP1 mutants** was measured in the presence of 10 mM Mg$^{2+}$ and Ca$^{2+}$/EGTA mixtures as previously described in detail (10, 50-51). The free metal concentrations in assays containing Ca$^{2+}$/EGTA mixtures were calculated using Bound and Determined and MaxChelator software correcting for pH, salt and nucleotide concentrations, and temperature. The fluorescence data were fitted using a simplified saturating hyperbolic function: 

$$([\text{Ca}]_{\text{bound}}/\text{GCAP}) = B_{\text{max}} \times ([\text{Ca}] / ([\text{Ca}] + K_d)),$$

where $[\text{Ca}]_{\text{bound}}$ is the concentration of Ca$^{2+}$ bound to GCAP1 calculated as $[\text{Ca}]_{\text{bound}} = [\text{Ca}]_{\text{total}} - [\text{Ca}]_{\text{free}}$, $B_{\text{max}}$ is the mol of Ca$^{2+}$ ions bound per mol of GCAP at saturation, $K_d$ is the apparent dissociation constant for Ca$^{2+}$. The trace amounts of EDTA introduced into assay from the stock solutions of the proteins were negligible compared to the protein concentration in the assay.

**NMR spectroscopy.**—

$^{15}$N-labeled GCAP1 was expressed in BL21(DE3) E. coli strain, purified and analyzed as described by (54-56). Two-dimensional $^{15}$N-$^1$H HSQC spectra with 2048 ($^1$H) x 256 ($^{15}$N) data points were recorded for $^{15}$N-labeled samples of myristoylated GCAP1 mutants (M26R, S31Y and F73E) using 800 MHz Bruker Avance III NMR spectrometer equipped with a triple resonance cryogenic probe. Each NMR sample consisted of myristoylated GCAP1 protein dissolved in buffer containing 95% H$_2$O/5% D$_2$O, 10 mM Tris-d$_{11}$, 2 mM CaCl$_2$, 1 mM dithiothreitol-d$_8$ at pH 7.4 as described by (54). All NMR experiments were performed at 37 °C. Spectra were processed using NMRpipe software package (57) and analyzed using SPARKY.

**RESULTS**

**Mutagenesis and the primary screening for the residues affecting RetGC1 activation.**—

In total, 107 residues, surface-exposed based on the Ca$^{2+}$GCAP1 crystal structure (24), were altered, mostly using a single point mutation, although in some cases residues were substituted in pairs or in larger blocks as indicated in Fig. 1B and Supplemental Table S1. The residues substituted by the mutagenesis have been marked with colors and the asterisks in the primary structure of bovine GCAP1 presented in Fig. 1C. For the purpose of a more efficient disruption of GCAP1 interaction with the target enzyme by single point mutations at the surface, the side chains were substituted with those that had distinctly different properties: i.e., negatively charged — by positively charged and vice versa, short side chain — by a long chain and vice versa, and hydrophobic— by a hydrophilic. In the last case, hydrophobic residues we mutated were already exposed to the solution in the GCAP1 crystal structure and its replacement with a hydrophilic residue would not be expected to cause major re-arrangement of the protein fold. The opposite would not be necessarily true, and we generally avoided replacement of hydrophilic side chains with Ala and more hydrophobic residues, Trp or Phe, in order to minimize the possibility of a non-specific backbone misfolding. In some cases, when substitutions with Tyr were selected, the correct fold of the protein main chain was evaluated using NMR spectroscopy and other methods as described further in this section. For the same reason, we did not mutate the internal residues forming the hydrophobic core of the molecule. Additional reasons not to mutate residues embedded deeply in the hydrophobic core of the Ca$^{2+}$GCAP1 X-ray crystal structure (24) was the results of recent NMR
spectroscopy studies combined with the molecular modeling (56) arguing that conformational changes in GCAP1 upon its transition from the Ca\(^{2+}\) bound (inhibitor) state to the activator state do not involve major rearrangement of the overall backbone fold of the molecule. Lastly, we excluded from the mutagenesis of those side chains that participate in metal coordination in EF-hands 2 and 3 - in order to prevent the loss of Mg\(^{2+}\) binding, needed to maintain the activator state of GCAP1 (11, 52) as well as the first five amino residues at the N-terminus – to prevent the potential loss of recognition by N-myristoyl transferase.

Myristoylated GCAP1 mutants expressed in E. coli were purified and screened for their ability to activate RetGC1 in vitro in comparison with the wild type (WT) GCAP1 (Fig. 1B). Based on the WT GCAP1 \(K_{d} \approx 10^{-6}\) M for RetGC1 activation (38, 56), we used for the initial screening a subsaturating concentration of 5 µM GCAP1 and those mutants whose activator capacity was substantially compromised were selected using 50% decrease in the level of RetGC1 activation as the threshold for the primary screening. It also needs to be noted that, in order to optimize activation of the cyclase and to minimize any potential effects of the mutations on Mg\(^{2+}\) binding affinity of GCAP1, Ca\(^{2+}\) was chelated by EGTA and the assay was also saturated with Mg\(^{2+}\) added in a large excess (10 mM) over the GCAP1 \(K_{d, Mg} \approx 0.2\) mM (9-10).

Most of the mutations introduced at various positions on the surface did not critically affect RetGC1 activation based on the selected threshold (Table 1). Moreover, vast majority of the surface-exposed residues tolerated a major change in their properties with surprisingly little effect on the cyclase stimulation (Fig. 1B, C). A chimera protein containing the first two EF-hands from GCAP1 and the C-terminal half from GCAP2 (47) when tested in the conditions of the screening, activated RetGC1 just like the wild type (Table 1), despite the substantial diversity of the sequence between the two homologs in the C-terminal portion surrounding EF-hand 4. Moreover, a 19-residue truncation, Asn185–Gly205, of the GCAP1 molecule was did not suppress its activity (Fig. 1B). This was consistent with earlier observations that the region downstream from Arg182 tolerates replacement with the C-terminus from recoverin (48). However, we also found that contrary to earlier expectations based on substitution of larger fragments in GCAP primary structure (47-48), none of the individually tested surface residues in the exiting helix of EF-hand 4 or in alpha-helices 10 and 11 was essential for the cyclase activation.

Unlike most of the surface residues, there was a group of 24 mutants whose ability to activate RetGC1 fell to or below the 50% threshold. In 19 of them it was suppressed by \(\sim 80\)% or was even completely lacking (Fig. 1B): Y22D (<2% normal RetGC1 activation in primary screening); K23D (7%); K24D (2%); M26R (<1%); E28R (19%); P30Y (2%); S31Y (6%); Y37R (1%); E38R (<1%); G32N (7%); F73E (1%); M74K (18%); V77E (1%); A78E (<1%); K85E (4%); K87D (22%); K93E (6%); W94A (5%); K97S (18%) (Table 1). This suggested that cyclase-binding interface could be compromised in these mutants. These mutations, except for a few that did not show any measurable activity were further subjected to a comparative analysis for concentration dependence of the cyclase activation (Fig. 2), together with several mutations that did not show inactivating effect in the primary screen.

All mutants in EF-hand 1 that retained at least some residual activity, displayed markedly reduced apparent affinity for RetGC1 (Fig. 2). The K23D, E28R, P30Y, S31Y, G32D) all displayed a strong reduction of their apparent affinities for the cyclase, although in some cases (Y22D, M26R, P30Y, S31Y, G32D, Y37R, E38R) the activity was virtually lacking and therefore neither \(a_{max}\) nor \(K_{d}\) could be precisely determined from the analysis (Fig. 1B and Fig. 2). In contrast to that, T27K and T27E substitutions (56) had very little effect.
Some mutations in the part of GCAP1 surface formed by the exposed residues of EF-hand 2 and EF-hand 3 (Fig. 2B, C) also strongly reduced the apparent affinity for the cyclase (M74K, K87D, K97S) or virtually eliminated GCAP1 activity (F73E, V77E, A78E, K85E, W94A). The complete lack of activity in the latter cases made it impossible to determine the change in kinetic parameters from the dose response of RetGC1 activation.

Compared to the EF-hands 1-3, there was very little effect of mutations in the region that contained EF-hand 4 and the portion proximal to the C-terminus. None of the tested mutations in that region eliminated GCAP1 ability to activate the cyclase. Only one among the tested mutants, S152E, in EF-4 loop showed a relatively modest, to 40% wild type level, decrease in the primary screen (Fig. 1B). The decrease resulted from a 4-fold reduction in the apparent affinity for the cyclase (K_{1/2} 7 μM vs. 1.5 μM in WT) (Fig. 2D). Other substitutions had rather minor effect and even deletion of the entire C-terminal fragment, Asn185-G205, affected neither the A_{max} nor K_{1/2} (Fig. 2D).

Cys29 in EF hand 1, but not other Cys residues, contributes to GCAP1 interface for RetGC1. —

One of the residues in the non-metalligand EF-hand 1 sensitive to mutagenesis was Cys29. In contrast, other Cys residues located at the positions 18, 106 and 125 contribute little to the recognition of the target enzyme. We replaced all four Cys with a similar size residue, Thr (Fig. 3). The mutant lacking all Cys (Cys-) reduced the affinity for the cyclase GCAP1 almost 10-fold (K_{1/2} = 12 μM vs. 1.5 μM in WT). However, a single reverse replacement, T29C, thus restoring the Cys29 in EF-hand 1 (“Cys(-)T29C”) simultaneously restored the activity of GCAP1 by increasing its apparent affinity for RetGC1 back to normal (1.2 μM, Table 2). Conversely, alkylation of that single Cys29 in the C18,106,125T GCAP1 triple mutant Cys(-)T29C by N-ethyl maleimide (NEM) blocked RetGC1 activation.

**Effect of the inactivating mutations on colocalization of GCAP1 with RetGC1 in HEK293 cells. —**

Since RetGC1 complex with GCAPs is unstable in detergents, in order to directly verify whether or not the mutants that fail to activate RetGC1 in vitro retained their ability to bind with the target enzyme, we co-expressed GCAP1-GFP and mOrange-RetGC1 in HEK293 cells using previously developed cell-based assay (52) (Fig. 4, 5). It needs to be noted that the presence of the fluorescent tags at the C-terminus of GCAP1 or at the N-terminus of RetGC1 does not block the cyclase activation by GCAP1 (38, 52). When expressed alone in HEK293 cells, wild type GCAP1-GFP (Fig. 4A) spreads uniformly throughout the cytoplasm and the nucleus, excluding nucleoli and vacuoles (52). In contrast, when co-expressed with RetGC1, GCAP1 and its active mutants (Fig. 4, 5) become anchored to the membranes through the cytoplasmic segment of the cyclase. As a result, GCAP1-GFP fluorescence acquires a well-defined membrane (predominantly ER membranes – see reference 52) localization pattern and clears from the nucleus (38, 52). Fig. 4B demonstrate that the distribution of both fluorescent tags across the cell coincides with each other. Pearson’s correlation coefficient (PCC) 0.93 from the whole-cell image analysis in wild type indicates nearly perfect co-localization of GCAP1 with the cyclase (Fig. 6, Table 3).

In contrast to the wild type, mutations in different parts of EF-hand 1 (Y22D, K24D, S31Y, G32N, E38R) disrupted GCAP1/RetGC1 co-localization such that GCAP1 fluorescence was again mostly spread uniformly over the cytoplasm and the karyoplasm, unlike the fluorescence of the mOrange RetGC1 localized only to the membranes (Fig. 4B-C, F-H). Consequently, in all five EF-hand 1 mutants, the correlation coefficient for colocalization of the two fluorescent markers (Table 3) fell to and below the threshold (0.5, see reference 68) of
co-localization. Similar pattern was observed when the entire cytoplasmic portion of RetGC1 was truncated (52) and the red fluorescence in membranes was merely bleeding through the diffuse green fluorescence of GCAP1 in the cytoplasm (Fig. 6U). Hence, the lack of RetGC activation by these mutants can be explained by their failure to bind with the target enzyme. Conversely, the T27K GCAP1 that retained its ability to activate RetGC1 \textit{in vitro} (Fig. 1, 2) also displayed normal GCAP1/RetGC colocalization pattern typical for the wild type (Fig. 4F). One exception among the mutations in EF-hand 1 that eliminated GCAP1 activity by blocking its binding to the target was the M26R GCAP1, which clearly retained its ability to co-localize with RetGC1 (Fig. 4E, Fig. 6D, Table 3).

Similar heterogeneity was found for the mutations in the region containing EF-hands 2 and 3 (Fig. 5, 6 and Table 3). F73E, V77E, A78E, K93E and K97S substitutions that inactivated RetGC1 activation all disrupted co-localization pattern for GCAP1GFP and mOrange RetGC1 (Fig. 5A-C, E, G; Fig. 6I-K, M, O). However, the W94A GCAP1 (Fig. 5F, 6N) retained well-defined co-localization pattern, albeit less sharply defined than in WT GCAP1 (Table 3).

More complex pattern was observed with the K85E substitution located in the “hinge” – the region between the two semi-globules of GCAP1 connecting EF-hands 2 and 3 (Fig. 5D). In that case, some transfected cells showed no evidence for co-localization between the two proteins, while half of them (marked with \textit{asterisks}) still showed co-localization of the two proteins (Fig. 6L), although visibly less prominent than the wild type. Reflecting that variability, the PCC of 0.70 ± 0.14 (Table 3) averaged from multiple cells was above the colocalization criterion threshold (PCC = 0.5, ref. 68), but substantially lower than the 0.93 ± 0.02 in the wild type. Evidently, the K85E GCAP1 ability to associate with RetGC1 was compromised but not completely lost. Therefore, the complete loss of the cyclase activation by either W94A or K85E (Fig. 1B, Fig. 2C) could hardly be explained by disruption of RetGC1 binding.

In control experiments, mutations in EF-hand 3 (G103R, Fig. 5H), EF-hand 4 (S141Y, E158R, and D175R, Fig. 6Q-S) and the C-terminal fragment (deletion Asn185-Gly205, (Fig. 5I, Fig. 6T) that did not make GCAP1 lose its ability to activate RetGC did not affect its normal binding pattern, which remained indistinguishable from that of the wild type (Table 3).

\textbf{GCAP1 mutants that fail to bind RetGC1 remain structurally intact.} — We tested whether or not the GCAP1 mutants that completely stopped activating RetGC1 and were unable to bind with the target enzyme in a cell-based assay failed to do so because of a non-specific misfolding. We found that even completely inactive mutants retain the normal Ca\textsuperscript{2+} binding stoichiometry of three per molecule (Fig. 7A), thus arguing that the GCAP1 lack of binding to the target was not due to deterioration of the overall main chain fold. To further test the structural integrity, we tested in selected mutants the change in the intrinsic Trp fluorescence in response to the displacement of Mg\textsuperscript{2+} by Ca\textsuperscript{2+} in their EF-hands (9, 10, 50). In all cases, the increase of Trp94 fluorescence reflecting metal-dependent conformational change typical for the wild type remained intact even for the most severely inactivated mutants such as S31Y or V77E (Fig. 7B-D). Furthermore, we also recorded NMR spectra of two mutants that completely failed to activate RetGC and bind it in a cell-based assay, F73E and S31Y (Fig. 7E). Unlike CD spectroscopy, which only detects changes in protein secondary structure, NMR spectroscopy detects the overall tertiary fold and therefore provides a more sensitive probe of unfolding. NMR can determine the existence of a molten globule state that retains regular secondary structure but lacks important tertiary contacts in
the hydrophobic core, like what is observed for apo-GCAP1 (54). The NMR spectra of
myristoylated forms of M26R, S31Y and F73E in the Ca\(^{2+}\)-bound state are all similar to that of
wild type GCAP1 (54, 56), indicating that these
mutants are properly folded and structurally intact. The cluster of three downfield NMR
peaks near 10.5 ppm (assigned to Gly69, Gly105 and Gly149) indicate that Ca\(^{2+}\) is bound
at EF2, EF3 and EF4 in each of these mutants with a similar structural environment like that of
wild type (54, 56). We therefore conclude that
these point mutations that inactivate RetGC
stimulation must specifically affect GCAP1 at
the interface that binds to the cyclase and the inactivity of these mutants is not the result of
protein misfolding.

M26R, W94A and K85A compete with the wild
type GCAP1.—

Unlike other mutations in GCAP1 that
eliminated RetGC stimulation, M26R and
W94A and to a lesser extent K85E not only
displayed co-localization with RetGC1 in
HEK293 cells (Fig. 4-6), but were also able to
compete with WT GCAP1 in vitro (Fig. 8).
When RetGC1 in the assay was activated half-
way by 1 \(\mu\)M WT GCAP1, addition of the
M26R GCAP1 completely negated RetGC1
activation within the low micromolar range
(EC\(_{50}<1\mu\)M), thus arguing that the M26R
GCAP1 binds to the target almost as efficiently
as the wild type, even though such binding does
not result in activation of the cyclase. The
W94A and to a lesser extent K85E were also
able to displace the activator effect of the WT
GCAP1, even though it required much higher
concentrations of both mutants to achieve the
EC\(_{50}\) dose (5 \(\mu\)M and 10 \(\mu\)M, respectively).

Dissimilar to the M26R, W94A and K85E,
mutants that failed to activate RetGC1 in vitro
and co-localize with the target enzyme in
HEK293 cells (F73E, R93E, P30Y, E37R) were
unable to compete with the WT GCAP1 even at
concentrations as high as 20 \(\mu\)M (Fig. 7). Again,
neither M26R, nor W94A, nor K85E underwent
non-specific misfolding as a result of the point
mutations, because the stoichiometry of Ca\(^{2+}\)
binding remained normal (Fig. 7A). All three
mutants retained their ability to bind to the
cyclase and compete with the wild type,
consistent with proper folding. In addition, we
tested the M26R GCAP1 by NMR spectroscopy
(Fig. 7E) and found no evidence that its overall
backbone fold was compromised compared to
that of the myristoylated wild type GCAP1.
These results argue that Met26 contributes
rather little to the primary binding, while Trp94
and Lys85 contribute to the primary binding of
GCAP1 to RetGC1 less than many other
residues creating the cyclase-binding interface,
but all three residues are instead very important
for maintaining the proper secondary
interactions required for the cyclase activation.

DISCUSSION

The map of RetGC1 binding interface in
GCAP1.—

Functional analysis of several mutations in
different regions of the GCAP1 molecule
combined with the NMR spectroscopy (56)
previously demonstrated that the amplitude of
chemical shift changes (between the activator
and the inhibitor states) does not necessarily
correlate with the importance of a particular
residue for RetGC activation. Indeed, in spite of
relatively large chemical shift differences
observed for Thr27, Lys142 or Leu153, these
residues were not sensitive to mutations that
radically changed the properties of those side
chains (56). In contrast, Lys23, which shows
rather small chemical shift change, did not
tolerate substitution without a severe loss of the
activator capacity (56). Hence, functional
identification of the residues forming interface
with the cyclase requires direct mutagenesis of
the entire surface of the GCAP1 molecule.

In contrast to the Ca\(^{2+}\)-myristoyl switch in
recoverin (58, 59), GCAPs retain their fatty
moiety inside the protein globule (24, 60).
Moreover, GCAP1 does not undergo a major rearrangement of its main chain upon metal binding and displays rather modest Ca$^{2+}$-induced changes in chemical shifts detected by NMR spectroscopy for its inhibitor versus activator states (54, 56). Therefore, crystal structure of Ca$^{2+}$-bound GCAP1 (24) can be used to differentiate between the surface-exposed residues and those embedded into the hydrophobic core. It also needs to be noted that although several regions in GCAP1 have been previously shown to not tolerate substitution with corresponding residues from homologous NCS proteins (48), we mutated the surface residues in our present study in a non-biased fashion, not contingent on the previous mapping. The results of such mapping are summarized in Fig. 9, which depicts the color-marked residues in the cyclase-binding interface superimposed on a crystal model of GCAP1 structure (24). The surface residues that do not tolerate substitution without at least 50% loss in cyclase activation capacity (see Fig. 1B) or strong disruption of GCAP1 co-localization with RetGC1 in HEK293 cells (Fig. 4-6) are marked in red, while those residues that have little effect on the cyclase activity are marked in blue. The interface for the cyclase is located on one side of the GCAP1 molecule and is fairly compact. The most sensitive residues create a well-defined region on the surface of the semi-globule I formed by the EF-hands 1 (alpha helices $\alpha2$, $\alpha3$ and the non-metal binding loop) as well as the loop and the exiting helix $\alpha5$ of the EF-hand 2, extending through the “hinge” region into the entering helix $\alpha6$ of the EF-hand 3 in the semi-globule II (Fig. 1A, Fig. 9). Indications that the non-metal binding EF-hand 1 is likely involved in target recognition emerged in earlier mapping attempts using substitutions of larger fragments in GCAP1 and GCAP2 molecules with those from homologous NCS proteins (47-49) well as limited number of point mutations (61). The present analysis reveals that most of the target binding interface is created by nearly continuous patch on the surface of the adjacent portions of the EF-hand 1 (Tyr22, Lys23, Lys24, Met26, Glu28, Pro30, Ser31, Gly32, Tyr37, Glu38) and the metal-binding EF-hand 2 (Phe73, Met74, Val77, Ala78). Cys29 located in EF-hand 1 loop is the only Cys residue in GCAP1 contributing to the GCAP1 interface for the target enzyme (Fig. 3). This is consistent with the previous observations that Cys in the EF-hand 1 loop is important for the cyclase activation (49, 61). In a striking contrast to that, position 27 (blue) in the middle of EF-1 loop of the “binding patch” shows little involvement in cyclase binding and activation (56, and Fig. 1B, C; 3A, 4E). This indicates that when the complex between GCAP1 and RetGC1 is formed, Thr27 is likely facing an opening in the RetGC1 structure, rather than forms a tight contact with the target.

Our analysis further reveals that the loop of EF-hand 2 also contributes to the cyclase-binding interface. Two side chains in that loop, Phe73 and Met74, which face outward and do not participate in coordination of the metal ion (24), both affect the cyclase binding, such that the F73E GCAP1 completely loses its ability to bind and activate the target (Fig. 1B, 2B, 5A, Table 3). The “binding patch” in GCAP1 extends from the EF-2 loop to the exiting helix $\alpha5$ of EF-hand 2 and the entering helix $\alpha6$ of EF-3. The “hinge” region between the two semi-globules in GCAPs has been implicated in the cyclase regulation (47, 48). We find that three non-charged residues exposed on the surface of the exiting helix $\alpha5$ of EF-2, Met74, Val77 and Ala78, participate in the cyclase binding. Val77 and Ala78 are particularly important, because their replacement with the negatively charged Glu blocks RetGC1 binding entirely (Fig. 1B, 2B, and 5B, C). Lys85 in the “hinge” region, as well as Arg93, Trp94 and Lys97 in the entering helix $\alpha6$ of the EF-hand 3 all strongly contribute to the RetGC1 activation, but apparently through different mechanisms, as we discuss further in this section. It is also important to point that none of the tested mutations that caused complete loss of GCAP1 activation showed any evidence of a non-
specific unfolding of the GCAP1 molecule (Fig. 7). For example, even after reversing the charge of the side chain (such as in K85E and other mutants we tested) Ca$^{2+}$ binding stoichiometry and NMR spectra remain perfectly intact, arguing that inactivation of such mutants could rather result from breaking electrostatic interactions with the target enzyme. That could either shift the equilibrium between the activated versus inactivated states of the cyclase or may, given the severe inactivating effect of the K85E mutation, completely abolish the necessary activation step that utilizes a specific charge in that position.

Earlier studies using chemical cross-linking of Cys residues (62) indicated that the entering helix $\alpha2$ of EF-hand 1 and the entering helix $\alpha6$ of EF-hand 3 both come in proximity with the RetGC1 surface. This is consistent with our present findings, because Cys18, although not essential for the binding itself, is located fairly close to the residues of major importance for the interface, Tyr 22, Val77 and Ala78. The Cys106 in the loop of EF-3 itself is not essential for the binding to the target, but locates not far from the Ser152, which moderately contributes to the binding, and on the same side as Arg93 and Lys97 in the entering helix of EF-3. So it is possible that Cys106 becomes proximal to the RetGC1 surface once the complex is established through the binding patch in GCAP1. Interestingly, Cys29 was not reported among the products of chemical cross-linking between RetGC1 and GCAP1 (62), in contrast to our functional data indicating that Cys29 is an essential part of the cyclase-binding interface (Fig. 3). We reason that GCAP1 binding to the cyclase is so intolerant to alkylation of the Cys29 (Fig. 3) that modification of this residue by a cross-linking agent could itself prevent binding of the Cys29-modified GCAP1 to the cyclase (Fig. 3).

In contrast to other three EF-hands, the EF-4 and the surface of the helices $\alpha10$ and 11 (Fig. 1) show little involvement in target binding. Only one residue in this region, Ser152, responded to substitution by decreasing cyclase activation down to 40% of the WT level, just below the 50% criterion threshold (Fig. 1B). The S152E indeed displayed an apparent, although relatively modest, increase in the $K_{i}$ for RetGC1 activation (Fig. 2). Naturally, it is also important to remember that EF-4 still remains a critical Ca$^{2+}$-sensor part of the molecule, responsible for converting GCAP1 into its inhibitor state and affecting the neighboring EF-hand 3 (9, 10). Moreover, mutated EF-4 can even turn GCAP1 into a Ca$^{2+}$-insensitive constitutive activator of the cyclase causing photoreceptor degeneration (30, 43). Yet, in spite of being an essential EF-hand for switching the cyclase on and off by the conformational changes induced by Ca$^{2+}$ binding, the surface of the EF-hand 4 contributes rather little to the binding of RetGC1. Interestingly, a chimera protein comprised of the semi-globule I from GCAP1 and semi-globule II from GCAP2 (47) activates RetGC1 just like WT GCAP1, despite the substantial difference in the C-terminal sequence between the two GCAPs. This is fully consistent with the lack of strong inactivation of GCAP1 by various single-point mutations on the surface of the semi-globule II in GCAP1 and argues that those side chains in the semi-globule II that are drastically different between the two GCAPs are not essential for the binding interaction with the cyclase.

GCAP1 binding to the cyclase does not equal target activation: GCAP1 activates RetGC by a two-step mechanism. —

RetGC1 is active as a homodimer, because the two active sites in the catalytic domain of the enzyme are formed by two catalytic subunits, each providing one Mg$^{2+}$ binding and one GTP binding site for the two complementing each other active sites (45, 63). The binding of GCAP1 enhances RetGC dimerization in the complex to promote cyclase activation (64). Two possible thermodynamic mechanisms could explain the activation: (i) the
primary binding itself provides the necessary free energy to stimulate RetGC1 or (ii) primary binding itself is not sufficient for activation and some important secondary interactions or allosteric effects are provided in a second step once the complex with the cyclase is formed. Our mutational analysis of Met25, Lys85 and Trp94 provide evidence in favor of the latter scenario. The most intriguing observation of this study is that a few residues (Met26, Lys85 and Trp94, marked in magenta in Fig. 9) located near the “binding patch” are crucial for cyclase activation but are either not essential (Met26) or less critical for binding to RetGC1 than many other residues nearby. In other words, mutation of these residues (M26R, K85E, W94A) abolishes cyclase activation, but does not eliminate cyclase binding. Hence, they could indirectly affect cyclase activation by some secondary interaction. For example, GCAP1 activation of RetGC might occur in two steps, where the first step involves direct binding of GCAP1 to RetGC (see residues in red in Fig. 9) followed by a second step that promotes an induced-fit or secondary contact by Met26, Lys85 and Trp94 required for activation. In this two-step mechanism, mutation of Met26, Lys85 or Trp94 would have little effect on the first step of binding, but could strongly affect secondary contacts that form during the second step.

The involvement of secondary interactions would be consistent with the observation that GCAPs bind similarly to RetGC both in Ca\(^{2+}\) and Mg\(^{2+}\) - saturated forms, yet only Ca\(^{2+}\)-free/Mg\(^{2+}\)-bound GCAP activates the cyclase (11, 65, 66). The first binding step would be similar for both Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free GCAP1, but the secondary contacts induced in the second step could be different for activation (Ca\(^{2+}\)-free GCAP1) vs. inhibition (Ca\(^{2+}\)-bound GCAP1). To some extent, Trp94 and Lys85 might be involved in both primary binding and the secondary contacts, because the W94A GCAP1 and K85E GCAP1 compete with the wild type GCAP1 much less efficiently than M26R (Fig. 8). Thus, Met26 does not appear to be essential for primary binding at all and its replacement only affects the RetGC activation (Figs. 2A, 4D, 7). The precise mechanism of activation by Met26 during the second step remains unclear, because the structure of the RetGC1/GCAP1 complex is unknown. However, there is a possible structural link to metal binding in GCAP1. Met26 is in close proximity to the Ca\(^{2+}\)/Mg\(^{2+}\) binding site in EF-2 and the disposition of the M26 side chain could be affected by Ca\(^{2+}\) binding or replacement with Mg\(^{2+}\) in the process of cyclase regulation. Also, Trp94 fluorescence is strongly affected by the metal binding in the neighboring EF-hand 4 (10), together with the EPR spectroscopy (67), suggesting movement in this part of the molecule in response to metal binding. Moreover, molecular modeling predicts that Trp94 side chain would likely alter its orientation between the activator and the inhibitor states of GCAP1 (56). Conceivably, changing the orientation of the Trp side chain could provide a possible “push-button” action within the RetGC1/GCAP1 complex.

Previous mapping of the regions in GCAP primary structures by larger fragment substitutions also implicated a portion adjacent to the exiting helix in EF-hand 4 as a possible part of the interface for the cyclase (47-48). To our surprise, we find little evidence that either EF-4 or the adjacent C-terminal portions of the molecule are strongly involved in forming the interface. Why would then the replacement of EF-4 and adjacent C-terminal region with corresponding residues from other NCS proteins affect the ability of the Ca\(^{2+}\)-free GCAP1 to activate the cyclase? A possible explanation is that EF-4 is connected to the semi-globule I (Fig. 1A) via two alpha-helical stretches protruding to the opposite side of the molecule and contacting the myristoyl moiety inside the N-terminal semi-globule I (24). We recently demonstrated that this connection likely creates a “Calcium-myristoyl tug” action (50-51) improving the affinity of GCAP1 for RetGC1. Therefore, altering the “tug” portion in GCAP1...
could affect the interface with the cyclase formed by the semi-globule I, even though the “tug” portion of the molecule itself does not form a direct contact with the cyclase.

To conclude, our results identify the functional interface with the target enzyme in GCAP1 as a “binding patch” on one side of the molecule, and this region is solely responsible for the binding with RetGC1 that occurs via amino acid residues on the surfaces of EF-hand 1, the loop and the exiting helix of EF-hand 2 and the entering helix of EF-hand 3. Another important conclusion from this study is that a few residues near the binding patch (Met26, Lys85 and Trp94) are critically important for activation of the cyclase but mutations of these residues do not prevent binding to RetGC1. We propose a two-step mechanism, in which the first step involves primary binding to RetGC via the binding patch (highlighted red in Fig. 9) followed by a second step that induces secondary contacts important for cyclase activation involving Met26, Lys85 and Trp94.

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FOOTNOTES

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Abbreviations: EGTA – ethylene glycol-bis(2-aminoethyl)ether)-N,N,N’,N’-tetraacetic acid; GCAP – guanylyl cyclase activating protein; GFP – green fluorescent protein; NEM – N-ethyl maleimide; NCS – neuronal calcium-sensor protein; PCC – Pearson’s correleataion coefficient; RetGC – retinal membrane guanylyl cyclase.

FIGURE LEGENDS

**Figure 1. Effect of amino acid substitutions in GCAP1 on RetGC1 activation.** A, Three-dimensional model of Ca^{2+}-liganded GCAP1 (24) annotated as follows. Myr – N-myristoyl moiety; EF-1 through EF-4 – EF-hand domains; α1 through α11 -alpha helices, numbered beginning from the amino-terminus; “hinge” – the loop connecting two semi-globules (I and II) between α5 and α6 helices; Ca^{2+} ions bound in three metal binding EF-hand loops (EF-2 through EF4) are shown as spheres. B, RetGC1 activation by 5 μM GCAP1 mutants normalized to the wild type activation in control samples (mean ± SD, N = 3). The following mutations were tested: K8E, S9R; E11,12K; S15R,T16A; E17K,C18D; C18,106,125T; H19R; Q20R; Y22D; K23D; K24D; M26R; T27K; T27E; E28R; C29T; P30Y; S31Y; G32N; Q33R; T35R; L36E; Y37R; E38R; Q41R; ; K46D; N47R; P50G; W51N,S53R; E57R; Q58R; E61R; F65N; K67D; Y70A; F73E; M74K; V77E; A78E; S81A; L82S; K85E; K87D; V88R; E89R; Q90R; R93E; W94A; K97S; V101Y; G103R; C106D; R109D; D110R; R117D; R120D; D127R; A132R; E133R; E134R; D137R; F140A; S141Y; K142D; V145R; G147R; E150Y; S152E; L153R; E154C; E155G; M157R; E158R; K162E; Q164R; L166R; L167R; R172E; D175K; R178D; R181E; Q184R; deletion: ΔGln184—Gly205; additional substitutions, I122E, N1123A, P124E, C125Q, S126Q, D127G,S128K, T129L, M130L, T138R, S141L, V145E, and the Val160-Gly205 region replacement with the corresponding region from GCAP2 were tested as a single chimera construct (47). The assays contained 10 mM MgCl2 and 2 mM EGTA. The threshold level of 50% activation (dashed line) was selected for segregating the mutants for suspected damage of RetGC1-binding interface; the mutations that caused this decrease are shown in open circles and the substitutions are labeled next to the data points. C, Positions of the mutations causing major decrease in RetGC1 activating capacity in GCAP1 primary structure. All mutated side chains are marked in bold; those, whose replacement rendered RetGC1 activation ≤50% wild type level are marked in red and underlined, those whose mutations reduced activation below 80% are marked with red asterisks; the 12- residues loops of the EF-1 through EF-4 are shaded.

**Fig. 2. Dose-dependence of RetGC1 activation by GCAP1 mutants.** A, Mutations in EF-hand I: WT (●); E17K (◆); H19R (◊); K23D (▽); M26R (○); T27K (♦); T27E (▲); E28R (▼);
P30Y (●); S31Y (■); G32N (□). B. Mutations in EF-hand 2: WT (●); F73E (◇); M74K (▲); V77E (○); A78E (□). C. Mutations in the “hinge” region and EF-hand 3: WT (●); K85E (○); K87D (▲); W94A (□); K97S (△). D. Mutations in EF-hand 4 and C-terminal segment: WT (●); S141Y (○); S152E (○); L153R (△); L166R (▲); deletion, Asn185–Gly205 (●). Activation of recombinant RetGC1 by increasing concentrations of purified GCAP1 was assayed in the presence of 2 mM EGTA and 10 mM Mg²⁺; the data points (mean ± SD, N=5 for WT and P30Y, N=3 for V77E and A78E, and N=2 for other mutants) were fitted using Synergy KaleidaGraph 4 utilizing standard Levenberg-Marquardt algorithm of nonlinear least-squares routines assuming Michaelis function, $a = a_{max} \left[\text{GCAP} \right] / (K_{1/2GCAP} + [\text{GCAP}])$, where $a$ is the activity of RetGC in the assay, $a_{max}$ is the maximal activity of RetGC, [GCAP] is the concentration of GCAP, $K_{1/2GCAP}$ is the GCAP concentration required for half-maximal activation.

**Fig. 3.** Cys 29 contributes to the cyclase-binding interface of GCAP1. Dose dependence of RetGC1 activation by GCAP1 mutants: WT (○); WT, pre-treated with NEM (◇); C18,29,106,125T (“Cys(−)” , ▲); the Cys(−) mutant with Cys29 restored (“Cys(−)T29C”, ●); the Cys(−)T29C mutant, pre-treated with NEM (●). There, where NEM treatment is indicated, GCAP1 was fully reduced by 5 mM DTT, loaded on butyl-Sepharose chromatography column in 20 mM TrisHCl (pH 7.5) containing 1 M NaCl and eluted by a step of 5 mM Na-phosphate buffer, and divided into two equal portions, one of which was incubated with 2 mM NEM for 1 hour at room temperature, and the other contained 1 mM DTT instead. At the end of the reaction, 4 mM dithiothreitol was added to quench NEM, and both the alkylated and non-alkylated samples were desalted by several cycles of concentration/dilution using Amicon Ultra-4 membrane concentrator. Other conditions of the assay were the same as in Fig. 2. The data points (mean ± SEM, N=3) were fitted assuming the Michaelis hyperbolic function.

**Fig. 4.** Effect of mutations in EF-hand 1 on direct binding of GCAP1 to RetGC1 in HEK293 cells. A, GCAP1GFP expressed in HEK293 cells without RetGC1 diffuses throughout the cytoplasm and the nucleus (52): left panel – GCAP1GFP fluorescence, middle panel – fluorescence superimposed on differential interference contrast (DIC) image; right panel – distribution of GCAP1 fluorescence (arbitrary scale) across the cell scanned along the dashed line shown in the middle panel; B-I, Expression vectors coding for GCAP1-GFP (green) and mOrangeRetGC1 (red) were co-transfected in HEK293 cells at ~1:125 molar ratio using previously described method (52). Each row of panels presents (left to right) respective fluorescence image of GCAP1, fluorescence image of RetGC1, merged image of the two, and the distribution of the corresponding fluorochromes brightness (arbitrary scale) scanned across the cell along the dashed line shown in the merged image: B, Wild type; C, Y22D; D, K24D; E, M26R; F, T27K; G, S31Y; H, G32N; I, E38R. Minor gamma adjustment for better clarity of perception in some panels was applied to the whole view field; the fluorescence intensity distribution in all cases was recorded from the original image within proportional range of the photomultiplier, without any adjustments to the image itself; objective: ×60; the green fluorescence was excited by 488 nm and the red fluorescence – by 543 nm laser, respectively.

**Fig. 5.** Effect of mutations in EF-hands 2, 3, 4 and C-terminus on direct binding of GCAP1 to RetGC1 in HEK293 cells. The analysis was performed and the results are presented as described in Fig. 4. A, F73E; B, V77E; C, A78E; D, K85E (asterisks mark those cells that display
co-localization pattern); E, K93E; F, W94A; G, K97S; H, G103R; H, Asn185-Gly205 deletion mutant.

**Fig. 6.** mOrange RetGC1 (left) and GCAP1-GFP (middle) fluorescence intensities distribution in HEK293 cells and their colocalization test (right panels). Fluorescence images acquired as presented in Fig. 4 and Fig. 5 were processed without editing using the Olympus Fluoview FV10-ASW software to reconstruct the fluorescence intensities over the entire confocal cell image in each case. The examples of a cross-correlation test for the red (Y-axis) versus green (X-axis) pixels in each image are shown in the right panel and include Pearson’s correlation coefficient (PCC) (68). The PCC values for each mutant averaged from multiple cells were then summarized in Table 3. A-T, mOrange- RetGC1 coexpressed with the following variants of GCAP1-GFP: A – WT; B – Y22D; C – K24D; D – M26R; E – T27K; F – S31Y; G – G32N; H – E38R; I – F73E; J – V77E; K – A78E; L – K85E; M – K93E; N – W94A; O – K97S; P – G103R; Q – S141Y; R – E158R; S – D175R; T – deletion, Asn185-Gly205. U, wild type GCAP1-GFP was coexpressed with the extracellular portion of RetGC1 containing red fluorescent tag (52), but lacking the entire cytoplasmic segment required for GCAP binding (52, 53).

**Fig. 7.** GCAP1 mutants that cannot activate RetGC1 can still bind Ca\(^{2+}\). A, The normal Ca\(^{2+}\) binding stoichiometry of three per GCAP1 remains in the mutants that completely fail to activate the cyclase: WT (●); M26R (△); S31Y (□); G32N (∨); F73E (■); V77E (○); K85E (↑); W94A (◇). Ca\(^{2+}\) binding isotherms were obtained using Ca\(^{2+}\) fluorescent indicator dye Fluo-4FF (10, 48). The fluorescence data were fitted using a simplified saturating hyperbolic function: ([Ca]\(_{\text{bound}}/[\text{GCAP}]) = B_{\text{max}} \times [\text{Ca}]_{\text{free}}/([\text{Ca}]_{\text{free}} + K_d), where B_{\text{max}} is the mol of Ca\(^{2+}\) ions bound per mol of GCAP at saturation, K_d is the apparent dissociation constant, [Ca]\(_{\text{bound}}\) is the concentration of Ca\(^{2+}\) bound to GCAP1 calculated as [Ca]\(_{\text{bound}}\) = [Ca]\(_{\text{total}}\) – [Ca]\(_{\text{free}}\). The data shown are representative from 2-4 independent experiments producing in each case virtually identical Ca\(^{2+}\) binding stoichiometry. B – D, Ca\(^{2+}\)-dependent increase in Trp94 fluorescence (9, 10) was tested in WT, S31Y, and V77E GCAP1, respectively. The analysis was performed as described in Experimental Procedures in the presence of 10 mM MgCl\(_2\). E, NMR spectra of GCAP1 mutants. Uniform \(^{15}\)N-labeled samples of Ca\(^{2+}\)-bound and myristoylated forms of M26R (top), S32Y (middle) and F73E (bottom) were prepared as described in Experimental Procedures. All NMR spectra were recorded at 37 °C using a Bruker Avance III NMR spectrometer equipped with cryogenic TCI probe. Three downfield peaks at \(~\)10.5 ppm are assigned to Gly69, Gly105, and Gly149 (55), and indicate that Ca\(^{2+}\) is bound at EF2, EF3 and EF4. Minor spectral differences are observed for exposed residues in unstructured regions, most likely due to small differences in solvent conditions. However, the overall chemical shift patterns in these spectra are similar to that of wild type GCAP (54, 55), thus confirming that each of the mutants is properly folded and structurally intact.

**Fig. 8.** The M26R, W94A, and K85E GCAP1 compete with wild type in RetGC1 assay. RetGC1 activated half-way by 1 μM WT GCAP1 in the presence of 10 mM Mg\(^{2+}\) and 2 mM EGTA was assayed in the presence of increasing concentrations of the following GCAP1 mutants: M26R (○); F73E (●); P30Y (◇); Y37R (■); K85E (●); R93E (□); W94A (△), empirical curve fit.
Fig. 9. Functional map of the RetGC1-binding interface in GCAP1 three-dimensional structure. A. The RetGC-binding interface is located in a well-defined patch of amino acid residues on one side of the GCAP1 molecule. In the template based on the crystal structure of myristoylated GCAP1 (24), those mutated residues for which the RetGC1 activation fell to ≤ 50% threshold in Fig.1B are marked in red (near the threshold – dark red, below 25% normal - bright red and magenta) and those above the threshold are marked in blue. The non-mutated residues are shown in gray, Ca\(^{2+}\) ions - in cyan, and myristoyl moiety (myr) – in black. The model was rotated in three 90°-steps. EF1 through EF4 mark the respective EF-hand domains. B, close-up views of the cyclase-binding interface from panel “A” at two different angles. The residues comprising the “binding patch” are marked in red as above and labeled. The Met26, Trp94, and Lys85, marked in magenta show the position of the residues inside the binding patch that were critical for the cyclase activation but not (or at least not only) through the disruption of the primary binding - rather through the secondary interactions. The Thr 27 (blue) in the middle of the EF-hand 1 portion of the “binding patch” is very tolerant to the changes in charge and length of the side chain, unlike the surrounding residues. Other explanations are given in the Discussion section.
Table 1. Primary screening for RetGC1 activity in the presence of 5 μM GCAP1.

| Mutant          | GC activity | Efficacy, % | Mutant          | GC activity | Efficacy, % |
|-----------------|-------------|-------------|-----------------|-------------|-------------|
| D6S (WT)        | 19.2        | 100         | K85E            | 0.7         | 4           |
| K8E/S9R         | 19.1        | 99          | K87D            | 4.2         | 22          |
| E11,12K         | 22.7        | 118         | V88R            | 16.1        | 84          |
| S15R/T16A       | 16.1        | 84          | E89R            | 22.7        | 118         |
| E17K            | 6.5         | 34          | Q90R            | 16.8        | 87          |
| C18,106,125T    | 19.3        | 100         | R178D           | 13.6        | 71          |
| H19R            | 10.1        | 52          | A78E            | 0.0         | 0           |
| Q20R            | 20.0        | 104         | R181E/Q184R     | 15.7        | 82          |
| Y22D            | 0.3         | 2           | S81A            | 13.5        | 70          |
| K23D            | 1.3         | 7           | #N185-G205      | 19.2        | 100         |
| K24D            | 0.4         | 2           | L82S            | 11.2        | 58          |
| M26R            | 0.1         | 0           | Chimera XXIIc   | 23.8        | 124         |
| T27K            | 16.2        | 84          | R93E            | 1.2         | 6           |
| T27E            | 17.8        | 92          | W94A            | 1.0         | 5           |
| E28R            | 3.6         | 19          | K97S            | 3.5         | 18          |
| C29T            | 7.5         | 39          | V101Y           | 20.9        | 109         |
| P30Y            | 0.4         | 2           | C106D           | 19.9        | 103         |
| S31Y            | 1.1         | 6           | R109D           | 19.2        | 100         |
| G32N            | 1.3         | 7           | D110R           | 16.8        | 87          |
| Q33R            | 13.7        | 71          | R117D           | 18.1        | 94          |
| T35R            | 18.5        | 96          | E120D           | 19.1        | 99          |
| L36E            | 17.8        | 93          | D127R           | 17.8        | 92          |
| Y37R            | 0.3         | 1           | D137R           | 19.4        | 101         |
| E38R            | 0.0         | 0           | F140A           | 18.9        | 98          |
| Q41R            | 13.8        | 72          | S141Y           | 28.6        | 148         |
| K46D            | 10.0        | 52          | E142D           | 19.1        | 99          |
| N47R            | 13.9        | 72          | V145R           | 16.8        | 87          |
| P50G            | 17.9        | 93          | G147R           | 17.8        | 93          |
| W51N/S53R       | 20.1        | 104         | E150Y           | 18.0        | 93          |
| E57R            | 16.7        | 87          | E154C           | 17.3        | 90          |
| Q58R            | 17.4        | 90          | M157R           | 19.3        | 100         |
| E61R            | 12.8        | 67          | E158R           | 19.4        | 101         |
| F65N            | 21.5        | 112         | K152E           | 17.8        | 93          |
| K67D            | 18.6        | 97          | Q164R           | 17.3        | 90          |
| Y70A            | 19.0        | 99          | L166R           | 15.0        | 78          |
| F73E            | 0.2         | 1           | L167R           | 18.8        | 98          |
| M74K            | 3.4         | 18          | R172E           | 14.1        | 73          |
| V77E            | 0.2         | 1           | D175K           | 20.7        | 108         |
| A78E            | 0.0         | 0           | R181E/Q184R     | 15.7        | 82          |
| S81A            | 13.5        | 70          | ΔN185-G205      | 19.2        | 100         |
| L82S            | 11.2        | 58          | Chimera XXIIc   | 23.8        | 124         |

a Nanomol cGMP/min/mg; recombinant RetGC1 was reconstituted with GCAP1 mutants in the presence
of 10 mM MgCl$_2$ and 2 mM EGTA and assayed as described in Fig. 1B and Experimental Procedures.  

b Normalized per RetGC1 activity in the presence of wild type GCAP1 as a 100%-efficacy standard. The standard error from three independent measurements did not exceed 5% of the mean value for all tested mutants.

c The chimera containing the semi-globule I from GCAP1 and the semi-globule II from GCAP2 produced as described in reference (47).
Table 2. Activation of RetGC1 by selected GCAP1 mutants.

| GCAP1                | $K_{1/2\text{GCAP}}$ | $a_{max}$       |
|----------------------|-----------------------|-----------------|
| WT                   | 1.6 ± 0.1             | 27 ± 1.6 (N=5)  |
| E17K                 | 8.3 ± 0.1             | 18 ± 0.3 (N=2)  |
| C18, 29,106,125T     | 12 ± 2                | 29 ± 1 (N=3)    |
| C18, 106, 125T       | 1.2 ± 0.2             | 25 ± 0.2 (N=3)  |
| H19R                 | 4.5 ± 0.4             | 18 ± 1 (N=2)    |
| K23D                 | 25 ± 4                | 9 ± 0.9 (N=2)   |
| M26R                 | ND b                  | ND (N=2)        |
| T27K                 | 3.3 ± 0.5             | 28 ± 2 (N=2)    |
| T27E                 | 2.9 ± 0.1             | 28 ± 0.4 (N=2)  |
| E28R                 | 29 ± 4                | 28 ± 3 (N=2)    |
| P30Y                 | ND                    | ND (N=5)        |
| S31Y                 | 60 ± 19 c             | 10 ± 3 (N=2)    |
| G32N                 | 13 ± 3                | 39 ± 12 (N=2)   |
| K46D                 | 9.7 ± 0.9             | 31 ± 1.4 (N=2)  |
| F73E                 | ND                    | ND (N=2)        |
| M74K                 | 7.1 ± 0.5             | 17 ± 1 (N=2)    |
| V77E                 | ND                    | ND (N=3)        |
| A78E                 | ND                    | ND (N=3)        |
| K85E                 | ND                    | ND (N=2)        |
| K87D                 | 15 ± 1                | 19 ± 0.4 (N=2)  |
| W94A                 | ND                    | ND (N=2)        |
| K97S                 | 13 ± 1                | 12 ± 1 (N=2)    |
| S141Y                | 1.6 ± 0.1             | 27 ± 0.4 (N=2)  |
| S152E                | 7.2 ± 0.6             | 20 ± 0.6 (N=2)  |
| L153R                | 3.2 ± 0.1             | 27 ± 0.5 (N=2)  |
| L166R                | 3.9 ± 0.2             | 28 ± 0.7 (N=2)  |

\(^a\)Compiled from titrations exemplified in Fig. 2, 3 and (56). The $K_{1/2\text{GCAP}}$ and $a_{max}$ (mean ± standard error) are from the fit of the data sum from N independent trials, assuming Michaelis function: $a = a_{max} [\text{GCAP}] / (K_{1/2\text{GCAP}} + [\text{GCAP}])$, where $a$ is the activity of RetGC in the assay, $a_{max}$ is the maximal activity of RetGC, [GCAP] is the concentration of GCAP1, $K_{1/2\text{GCAP}}$ is the GCAP concentration required for half-maximal activation. The data were fitted using a Synergy Kleidagaph 4.0 software utilizing conventional Levenberg-Marquardt algorithm of nonlinear least-squares routines.

\(^b\)ND – not determined: the activity was too low to reliably extract the parameters.
Due to dramatic loss of the affinity for the target and limitations of the fit method, the error is larger for the those mutants whose $K_{1/2 \text{GCAP}}$ was similar to or above the maximal GCAP concentration in assay and the measured $a$ was much lower than $a_{\text{max}}$. 

*Target binding interface on GCAP1*
Table 3. RetGC1 and GCAP1 colocalization in HEK293 cells. Pearson’s correlation coefficient (PCC) for the mOrange-RetGC1 and GCAP1-GFP co-expressed in HEK293 cells was determined from the analysis of the fluorescence distribution in the respective red and green channels of the confocal images using Olympus FluoView FV10-ASW software.

| GCAP1-GFP | PCC, mean ± SD<sup>a</sup> | p<sup>b</sup> |
|-----------|---------------------------|-------------|
| WT        | 0.93 ± 0.02 (n = 32)      |             |
| Y22D      | 0.45 ± 0.11 (n = 24)      | <0.0001     |
| K24D      | 0.40 ± 0.14 (n = 23)      | <0.0001     |
| M26R      | 0.91 ± 0.03 (n = 19)      |             |
| T27K      | 0.92 ± 0.05 (n = 19)      |             |
| S31Y      | 0.48 ± 0.14 (n = 15)      | <0.0001     |
| G32N      | 0.54 ± 0.12 (n = 21)      | <0.0001     |
| E38R      | 0.32 ± 0.12 (n = 21)      | <0.0001     |
| F73E      | 0.26 ± 0.10 (n = 21)      | <0.0001     |
| V77E      | 0.41 ± 0.10 (n = 18)      | <0.0001     |
| A78E      | 0.45 ± 0.11 (n = 21)      | <0.0001     |
| K85E      | 0.70 ± 0.14 (n = 32)      | <0.0001     |
| K93E      | 0.49 ± 0.14 (n = 20)      | <0.0001     |
| W94A      | 0.88 ± 0.08 (n = 35)      |             |
| K97S      | 0.51 ± 0.18 (n = 20)      | <0.0001     |
| G103R     | 0.91 ± 0.05 (n = 20)      |             |
| S141Y     | 0.92 ± 0.03 (n = 17)      |             |
| E158R     | 0.92 ± 0.04 (n = 21)      |             |
| D175R     | 0.93 ± 0.04 (n = 13)      |             |
| D185-205  | 0.92 ± 0.04 (n = 22)      |             |

<sup>a</sup> The mOrange-RetGC1 and GCAP1-GFP were co-expressed in HEK293 cells and the confocal microscopy was performed as described in Experimental Procedures. Note that PCC values ≤ 0.5 generally indicate absence of colocalization and PC = 1.0 indicates perfect colocalization of all red and green pixels in the image (68).

<sup>b</sup> In comparison with WT, from one-way ANOVA/Bonferroni all-pairs comparison test (CL = 99%) processed using Synergy KaleidaGraph 4 software.
Target binding interface on GCAP1

FIGURES

Figure 1
Target binding interface on GCAP1

Figure 2

(A) RetGC activity, nmol G min⁻¹ mg⁻¹ versus [GCAP], µM for WT, T27K, and T27E.

(B) RetGC activity, nmol G min⁻¹ mg⁻¹ versus [GCAP], µM for WT, M74K, A78E, F73E, and V77E.

(C) RetGC activity, nmol G min⁻¹ mg⁻¹ versus [GCAP], µM for WT, K87D, K97S, K85E, and W94A.

(D) RetGC activity, nmol G min⁻¹ mg⁻¹ versus [GCAP], µM for WT, ΔN185-G205, S141Y, L153R, L155R, and S152E.
Figure 3
Figure 4

Target binding interface on GCAP1

A

GCAP1GFP

mOrangeGC1

merge

scan

B

WT

C

Y22D

D

K24D

E

M26R

F

T27K

G

S31Y

H

G32N

I

E38R
Target binding interface on GCAP1

Figure 5

[Image of fluorescence microscopy images showing different mutants and their effects on binding]

A: GCAP1GFP (F73E)
B: mOrangeGC1 (V77E)
C: merge of A and B
D: scan of A and B

E: GCAP1GFP (A78E)
F: mOrangeGC1 (K85E)
G: merge of E and F
H: scan of E and F

I: GCAP1GFP (K93E)
J: mOrangeGC1 (W94A)
K: merge of I and J
L: scan of I and J

M: GCAP1GFP (K97S)
N: mOrangeGC1 (G103R)
O: merge of M and N
P: scan of M and N

Q: GCAP1GFP (Δ185-205)
R: mOrangeGC1 (Δ185-205)
S: merge of Q and R
T: scan of Q and R
Target binding interface on GCAP1

Figure 6

[Diagram showing fluorescence distribution and colocalization test for various mutants of GCAP1 and RetGC1]
Figure 7

Target binding interface on GCAP1

A

B

C

D

E

Figure 7
Figure 8

![Graph showing RetGC1 activity vs. GCAP1 concentration for various mutants.](image-url)
Target binding interface on GCAP1

Figure 9
