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Evaluating the Role of Retinal Membrane Guanylyl Cyclase 1 (RetGC1) Domains in Binding Guanylyl Cyclase-activating Proteins (GCAPs)*

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Background: GCAP1 and GCAP2 regulate cGMP synthesis by RetGC1 in photoreceptors.
Results: GCAPs compete for binding to RetGC1 in biochemical assays and in HEK293 cells co-expressing fluorescently labeled GCAPs with different forms of RetGC1.
Conclusion: The GCAP1 and GCAP2 binding site(s) overlaps within the kinase homology and/or dimerization domains of RetGC1.
Significance: RetGC1 and GCAPs contribute to normal vision and congenital blindness in humans.

Retinal membrane guanylyl cyclase 1 (RetGC1) regulated by guanylyl cyclase-activating proteins (GCAPs) controls photoreceptor recovery and when mutated causes blinding disorders. We evaluated the principal models of how GCAP1 and GCAP2 bind RetGC1: through a shared docking interface *versus* independent binding sites formed by distant portions of the cyclase intracellular domain. At near-saturating concentrations, GCAP1 and GCAP2 activated RetGC1 from HEK293 cells and RetGC2$^{-/-}$GCAPs1,2$^{-/-}$ mouse retinas in a non-additive fashion. The M26R GCAP1, which binds but does not activate RetGC1, suppressed activation of recombinant and native RetGC1 by competing with both GCAP1 and GCAP2. Untagged GCAP1 displaced both GCAP1-GFP and GCAP2-GFP from the complex with RetGC1 in HEK293 cells. The intracellular segment of a natriuretic peptide receptor A guanylyl cyclase failed to bind GCAPs, but replacing its kinase homology and dimerization domains with those from RetGC1 restored GCAP1 and GCAP2 binding by the hybrid cyclase and its GCAP-dependent regulation. Deletion of the Tyr^{1016}–Ser^{1103} fragment in RetGC1 did not block GCAP2 binding to the cyclase. In contrast, substitutions in the kinase homology domain, W708R and I734T, linked to Leber congenital amaurosis prevented binding of both GCAP1-GFP and GCAP2-GFP. Our results demonstrate that GCAPs cannot regulate RetGC1 using independent primary binding sites. Instead, GCAP1 and GCAP2 bind with the cyclase molecule in a mutually exclusive manner using a common or overlapping binding site(s) in the Arg^{488}–Arg^{851} portion of RetGC1, and mutations in that region causing Leber congenital amaurosis blindness disrupt activation of the cyclase by both GCAP1 and GCAP2.

Retinal membrane guanylyl cyclase (RetGC),[2] one of the key enzymes in photoreceptor physiology, produces a second messenger of phototransduction, cGMP, in mammalian rods and cones. During photoreceptor excitation and recovery, two RetGC isozymes, RetGC1 and RetGC2 (1–3) (also known as GC-E and GC-F or ROSGC1 and ROSGC2, respectively), are tightly regulated by calcium feedback (4) mediated by guanylyl cyclase-activating proteins (GCAPs) (5–9). Photoexcited visual pigments in rods and cones trigger rapid hydrolysis of cGMP by a G_t-coupled phosphodiesterase, PDE6, which shuts off cGMP-gated channels and causes hyperpolarization of the photoreceptor membrane. The interruption of Ca^{2+} influx through the cGMP-gated channels causes GCAPs to convert from their Ca^{2+}-bound state in the dark to a Mg^{2+}-bound state in the light and thus accelerates cGMP synthesis by RetGC, which speeds up the recovery of photoreceptors from excitation (7, 10). RetGC1 isozyme accounts for most of the cGMP synthetic activity in mammalian rods (11) and nearly all of it in cones (12, 13). In addition to that, the lack of RetGC1 activity or its abnormal regulation by GCAP1 causes retinal dysfunction in animals and blinding diseases in humans such as Leber congenital amaurosis (LCA) (14–16), congenital cone-rod degeneration, and dominant cone degeneration (17–22). Although it has been established that GCAPs activate RetGC1 by binding to its cytosolic or "intracellular" portion (23), major disparity exists in understanding where on the RetGC1 molecule this binding occurs (Fig. 1). The question remains whether or not GCAP1 and GCAP2 can activate the cyclase independently, *i.e.* by using different binding sites and different mechanisms. According to some of the previous studies, the regulatory properties of RetGC isoforms imparted by GCAP1 and GCAP2 depend on the cyclase kinase homology domain (KHD) (24), whereas other studies argued that the primary binding site for GCAPs could be located in the catalytic domain (25) or even that the two

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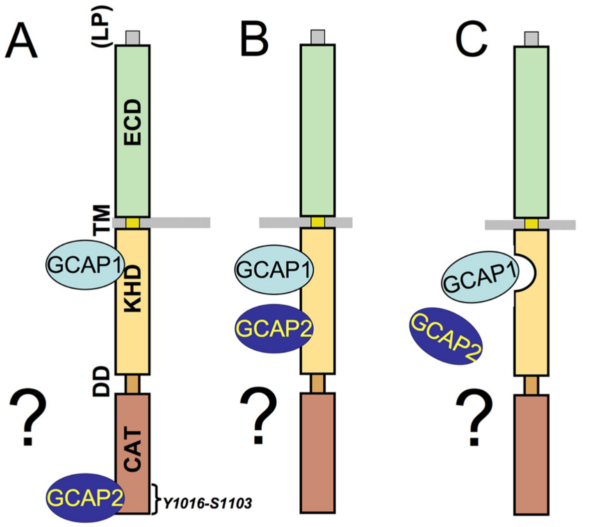
[2] The abbreviations used are: RetGC, retinal membrane guanylyl cyclase; BiFC, bimolecular fluorescence complementation; GCAP, guanylyl cyclase-activating protein; LCA, Leber congenital amaurosis; PCC, Pearson's correlation coefficient; NPRA, natriuretic peptide receptor A; KHD, kinase homology domain; DD, dimerization domain.



Independent binding sites
Independent activation

Overlapping binding site(s)
Competition

FIGURE 1. **Hypothetical scenarios for GCAP1 and GCAP2 binding with RetGC1.** *A*, GCAP1 and GCAP2 use remotely located binding sites on the same subunit of RetGC1 to activate it independently. GCAP1 binds to the KHD of the cyclase cytoplasmic segment in proximity to the transmembrane region, and GCAP2 binds to the Tyr^{1016}–Ser^{1103} fragment at the C terminus (here all residues are numbered from Met^{1} of the RetGC1 leader peptide (*LP*)) (27). *B*, GCAP1 and GCAP2 bind to non-overlapping sites in the same section of the cyclase primary structure involving the KHD and can independently activate RetGC. *C*, GCAP1 and GCAP2 compete for a common or overlapping binding site(s) such that only one GCAP can be bound to the same RetGC1 molecule at any given time. *ECD*, extracellular domain; *TM*, transmembrane region; *CAT*, catalytic domain. The schematics represent domains of primary structure, not the relative positions of the domains in a folded protein.

GCAPs use two binding sites very distant from each other in different cyclase domains (26–28). According to the latter model (27), regulation of the cyclase occurs by GCAP1 and GCAP2 independently bound to the same molecule of RetGC1 by GCAP1 bound at the cyclase KHD and GCAP2 bound at the C-terminal portion of the molecule on the opposite end of the intracellular segment (Fig. 1*A*). This model advocates the hypothesis that GCAP2 and another Ca^{2+}-binding protein, S100B, both bind the C-terminal region Tyr^{965}–Lys^{1054} in a bovine homolog of RetGC1 (equals Tyr^{1021}–Lys^{1110} if numbered starting from Met^{1} of the leader peptide; Refs. 26–28). In a human RetGC1, this region corresponds to the C-terminal fragment Tyr^{1016}–Ser^{1103} (residues numbered from the starting Met^{1} of the leader peptide coded by *GUCY2D* gene).

GCAPs shape the photoresponse by activating the cyclase in a sequential mode (29, 30). Therefore, how GCAPs bind to the cyclase (independently or by competing for the same subunit of RetGC1) would be critical for this relay mechanism. GCAP/RetGC interactions also contribute to photoreceptor survival itself. Multiple disease-related mutations in RetGC1 or GCAP1 shift Ca^{2+} sensitivity of cGMP synthesis, ultimately causing photoreceptor death (18–22, 31–36), but how these mutations affect the assembly and function of the active RetGC1-GCAP complex remains unclear. The major obstacles in studying RetGC remain a rather low content of RetGC in photoreceptor membranes (11, 37) and the notorious instability of the RetGC-GCAP complexes in detergents (38), making it impossible to directly isolate and/or quantify these complexes using conventional biochemical approaches such as a pulldown assay or immunoprecipitation. In the present study, we used RetGC1 activation analyses combined with a cell-based assay visualizing association of GCAPs with RetGC1 in HEK293 cells (39, 40) to demonstrate the following. (i) GCAP1 and GCAP2 regulate RetGC1 in a mutually exclusive fashion. (ii) Neither the catalytic domain nor the C-terminal portion, Tyr^{1016}–Lys^{1103}, of RetGC1 are essential for the primary binding of GCAP2. (iii) Instead, GCAP1 and GCAP2 compete over the same RetGC1 molecule using a common or overlapping primary binding site(s) located in a region that contains RetGC1 kinase homology and dimerization domains.

EXPERIMENTAL PROCEDURES

GCAP1 and GCAP2 Expression and Purification

Myristoylated bovine D6S GCAP1 (31) and GCAP2 (32) cDNAs were expressed from pET11d vector in the BLR(DE3) *Escherichia coli* strain (Novagen) harboring pBB131 plasmid encoding yeast *N*-myristoyltransferase. The cell cultures were incubated in the presence of myristic acid added prior to induction with isopropyl 1-thio-β-D-galactopyranoside, and both proteins were then isolated from inclusion bodies by urea extraction and purified using hydrophobic and size exclusion chromatography as described previously (42–44); 2 mM $MgCl_2$ was present during the urea extraction and subsequent dialysis steps. For co-transfection experiments in HEK293 cells, GCAP1 and GCAP2 were tagged at the C terminus with SuperGlo (Clontech) enhanced green fluorescent protein (GFP) by inserting each GCAP cDNA into pQBIfN3 vector (Clontech) as described previously (39). To produce untagged GCAP1 in HEK293 cells, the GFP-coding sequence was deleted by digesting the plasmid with EcoRI and ClaI, blunting with T4 polymerase, and self-ligation.

RetGC1 Expression and Activity Assay

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 described previously in detail (43, 45). The activity of the cyclase was assayed using [α-^{32}P]GTP (PerkinElmer Life Sciences) as a substrate, and the [^{32}P]cGMP product was quantified using TLC as described previously (43, 45). 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 Ca^{2+}/EGTA buffer, 1 or 6 mM free Mg^{2+} as indicated in the text, 0.3 mM ATP, 4 mM cGMP, 1 mM GTP, and 1 μCi of [α-^{32}P]GTP. The resultant [^{32}P]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.

RetGC1 Activity in Mouse Retinas

Experiments involving mice were conducted in accordance with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee. Triple gene knock-out RetGC2$^{-/-}$GCAPs1,2$^{-/-}$ mice were bred using the RetGC2$^{-/-}$ (46) and GCAPs1,2$^{-/-}$ double knock-out

(47) parental lines as described previously (11). All experiments utilizing mouse retinas were conducted in the dark under infrared illumination as described (11). Retinas were collected from mice dark-adapted overnight (four retinas per standard 1.5-ml Eppendorf tube), frozen in foil-wrapped tubes by immersion in liquid nitrogen, and kept at −70 °C before the experiment. RetGC1 activity was assayed in retinal homogenates containing the equivalent of 0.2 retina/25-μl assay.

Ca^{2+}/EGTA Buffers

Ca^{2+}/EGTA buffers maintaining defined free Ca^{2+} and Mg^{2+} concentrations in the RetGC assay were calculated, prepared, and verified by fluorescent Ca^{2+} indicator dyes as described previously in full detail (48).

Guanylyl Cyclases Constructs

All PCR-derived DNA fragments were amplified using PhusionFlash polymerase (Thermo Fisher); all intermediate and resulting DNA constructs were verified by sequencing on both strands. The constructs were expressed under control of the cytomegalovirus (CMV) promoter and utilized a bovine growth hormone polyadenylation signal.

mOrangeNPRA—A full-length cDNA clone for human natriuretic peptide receptor A (NPRA) membrane guanylyl cyclase coded by *GUCY2A* gene was obtained from Open Biosystems (catalogue number MHS1010-9204134ID, Thermo Scientific) and inserted between the CMV promoter and the bovine growth hormone polyadenylation sequence using the NotI/MluI sites of a modified pQBIfN3 plasmid (Clontech), thus eliminating the coding sequence for the fluorescent tag in the plasmid. The mOrange sequence (Clontech) was then amplified using 5′-AAAAAGGCGCGCCCCATGGTGAGCAAGGGCGAGGAGA and 5′-AAAAAACCGGTTTTGTACAGCTCGTCCATGCCGC primers, digested with AscI and AgeI endonucleases, and inserted into the AscI/SgrAI sites, thus substituting the Ala^{68}–Gly^{140} fragment in the extracellular domain of NPRA.

mOrangeRetGC1 and Hybrid Constructs—The cDNA portion coding for the human RetGC1 (2) extracellular domain (which does not participate in GCAP binding; see Ref. 23) between Cys^{14} and Asp^{24} was substituted with a 31-bp fragment containing engineered NheI and AgeI sites (44). The cDNA sequence coding for the His^{435}–Val^{971} RetGC1 fragment was modified to introduce a series of new restriction endonuclease sites (AgeI, BssHII, AvrII, AflII, KpnI, ClaI, NsiI, RsrII, and BsrGI) without changing the encoded protein sequence. The NheI and BstBI sites were added at the 5′-end of the DNA fragment produced by chemical synthesis (Integrated DNA Technologies). The chemically synthesized cDNA fragment was then inserted into the NheI/Bsu36I sites of the modified RetGC1 construct in a pRCCMV vector (Invitrogen) lacking the neomycin resistance-coding XhoI-XhoI fragment utilized for making mOrangeRetGC1 cDNA as described (40, 44) (this also eliminated the originally introduced AgeI site in the extracellular domain-coding fragment described in Refs. 44, 49, and 50). The resultant plasmid (pRetGC1NewRest2) was subsequently used for constructing mOrangeRetGC1 containing the new restriction sites, mOrangeRetGC1VenusN (with the deleted portion of RetGC1 C terminus), and a hybrid between RetGC1 and NPRA described below.

mOrangeRetGC1 plasmid encoding wild type intracellular segment of RetGC1 for expression in HEK293 cells was produced by inserting into the NheI/BstBI sites of the pRetGC1NewRest2 construct a fragment coding for the mOrange tag (Clontech) cDNA-amplified using forward 5′-AAAAAGCTAGCATGGTGAGCAAGGGCGA and reverse 5′-TCCTCCTTCTTCGAACTTGTACAGCTCGTCCATGC primers. The LCA-linked mutations, W708R and I734T, were introduced by replacing the KpnI/DraIII fragment of mOrangeRetGC1 plasmid with the respective PCR-generated fragments encoding these mutations.

The mOrangeGC1NPRAcat1 construct in which the C-terminal portion of NPRA substituted the catalytic domain of RetGC1 and the C-terminal peptide altogether was constructed as follows. The cDNA fragment coding for the Leu^{848}–Gly^{1061} fragment of NPRA was amplified using forward 5′-GCTGGAAAAGCAGAAGACGGACCGGCTGCTCTACCAGATCCTGCCTCA and reverse 5′-GAATGGCGGCCGGTCAGCCTCGGGTGCTACTCC primers and inserted into the RsrII/EagI sites of the pRetGC1NewRest2 plasmid, substituting the corresponding part of the intracellular segment of RetGC1 below the KHD (pGC1NPRAcat1). The mOrange cDNA was then amplified using two primers, 5′-AAAAAGCTAGCATGGTGAGCAAGGGCGA and 5′-TCCGGGCGAGTTCGAAACTTGTACAGCTCGTCCAT, and inserted into the NheI/BstBI restriction sites of the pGC1NPRAcat1 plasmid. The BstBI site for additional cloning purposes was removed while keeping the RetGC1 cDNA sequence in-frame with the mOrange by digesting with BstBI, blunting with T4 polymerase, and self-ligating.

mOrangeRetGC1VenusN and -C—DNA fragments coding for the Met^{1}–Gln^{158} (VenusN) and Lys^{159}–Lys^{239} (VenusC) (49) of the Venus yellow fluorescence protein (YFP; Ref. 50) both preceded by the 5′-CCGCGGTACTGCCTGTTTGGAGACACGGTCAACACCGCCTCGCGCATGGAGTCCACCGGGCTGGGAGGAGGAGGAGGAGGA sequence and followed by the 5′-TGACGGCCG sequence were chemically synthesized and inserted into the pUC-KAN vector (Genscript). They were then subcloned into the SacII/EagI sites of the pRetGC1NewRest2 plasmid. The W708R and I734T mOrangeRetGC1VenusN and -VenusC were produced by replacing the KpnI/SacII fragment in mOrangeRetGC1VenusN and -C vector plasmids with the respective fragments containing the LCA mutation.

Co-transfection Experiments

The mOrange-tagged guanylyl cyclase constructs were co-expressed with the SuperGlo GFP-tagged GCAP1 or GCAP2 in HEK293 cells at a cyclase:GCAP1 plasmid ratio of ~100:1 as described in detail previously (39, 40) except that instead of calcium phosphate precipitation a Promega FuGENE HD transfection reagent was used at ~3 μl/μg of DNA. Confocal images were taken utilizing an Olympus FV1000 Spectral instrument using 543- and 488-nm excitation for the red and the green fluorochromes, respectively, as described previously (39, 40) and processed using Olympus FluoView FV10-ASW software. In the case of YFP *versus* mOrange fluorescence recording, the emission intervals for recordings were adjusted to exclude bleeding of the fluorescence between the channels.

No changes to the original images were made except for occasional minor γ correction applied to the whole image. Quantitative analysis was performed using only original images without γ corrections.

Pearson's Correlation Coefficient (PCC)

The PCC for testing co-localization of GCAP-GFP with mOrange-tagged RetGC1 variants was calculated using Olympus FluoView FV10-ASW software as described previously (40), and the statistical difference between the PCC values was tested using the analysis of variance function in Synergy KaleidaGraph 4 software applying Bonferroni post hoc processing.

RESULTS

Testing Whether GCAP1 and GCAP2 Act Synergistically When They Activate RetGC1—The dose dependence of RetGC1 activation by either GCAP1 or GCAP2 presented in Fig. 2*A* shows that in line with the previous observations (11) 10 μM GCAP provided near-maximal activation of the cyclase with the maximal level of activity stimulated by GCAP1 being above that of GCAP2. A model assuming independent regulation of the cyclase by different GCAPs through independent binding sites on the same RetGC1 molecule (27) implies that the two GCAPs could act synergistically; *i.e.* the combined effect of both GCAPs together could exceed the levels of activation by saturating concentration of GCAP1 alone. However, adding GCAP2 to the assay nearly saturated with GCAP1 did not further stimulate the cyclase activity (Fig. 2*B*).

The same was true not only for a recombinant RetGC1 expressed in HEK293 cells but also for the native RetGC1 present in a mouse retina (Fig. 2*C*). Triple RetGC2$^{-/-}$ GCAPs1,2$^{-/-}$ (11) gene knock-out mice lack RetGC2 isozyme and both GCAPs altogether, but RetGC1 in these retinas is expressed at a normal ratio to rhodopsin and preserves its activation and normal Ca^{2+}-sensitive regulation in the presence of exogenously added GCAP1 and GCAP2 (11). Note that this cyclase assay in whole retina specifically measures activity of RetGC from photoreceptors (11, 33, 34). We found that the native RetGC1 in the triple knock-out retinas saturated by GCAP1 did not increase its activity when GCAP2 was also added in the same assay. These results argue that there is no synergy between the two GCAPs in activating RetGC1 and instead open a possibility that GCAPs may compete in binding to the same target enzyme.

GCAP1 and GCAP2 Compete for Binding to the Same RetGC1 Molecule—To test whether or not GCAPs directly compete for the same molecule of RetGC1, we used the M26R GCAP1 mutant (40), which does not activate RetGC1 even though it fully retains the ability to bind the cyclase (Ref. 40 and Fig. 3*A*, *inset*). The M26R GCAP1 not only blocked activation of the cyclase by the wild type GCAP1 but also blocked its activation by GCAP2 in a very similar fashion (Fig. 3*A*), thus arguing that GCAP1 and GCAP2 may compete for the same (or at least overlapping) binding site(s). To additionally verify that the suppression of RetGC1 activation by M26R mutant involves a direct displacement of GCAP from the RetGC1-GCAP complex, we used a cell-based assay (39, 40) utilizing fluorescently labeled GCAP1-GFP (Fig. 3, *B–D*). GCAP1-GFP when expressed alone is uniformly diffused throughout the cytoplasm and the nucleus of HEK293 cells but becomes anchored to membranes and no longer spreads to the nucleus when co-expressed with RetGC1 (39). As a result, GCAP1-GFP strongly co-localizes (Table 1) with mOrangeRetGC1 in endoplasmic reticulum and plasma membranes of HEK293 cells in a typical "tennis racket" pattern (Fig. 3*B* and Refs. 39 and 40). However, when the same co-transfection mixture included an excess of the DNA vector coding for the untagged GCAP1, the GCAP1-GFP displaced from its complex with mOrangeRetGC1 by the unlabeled GCAP1 acquired a uniform diffuse pattern of

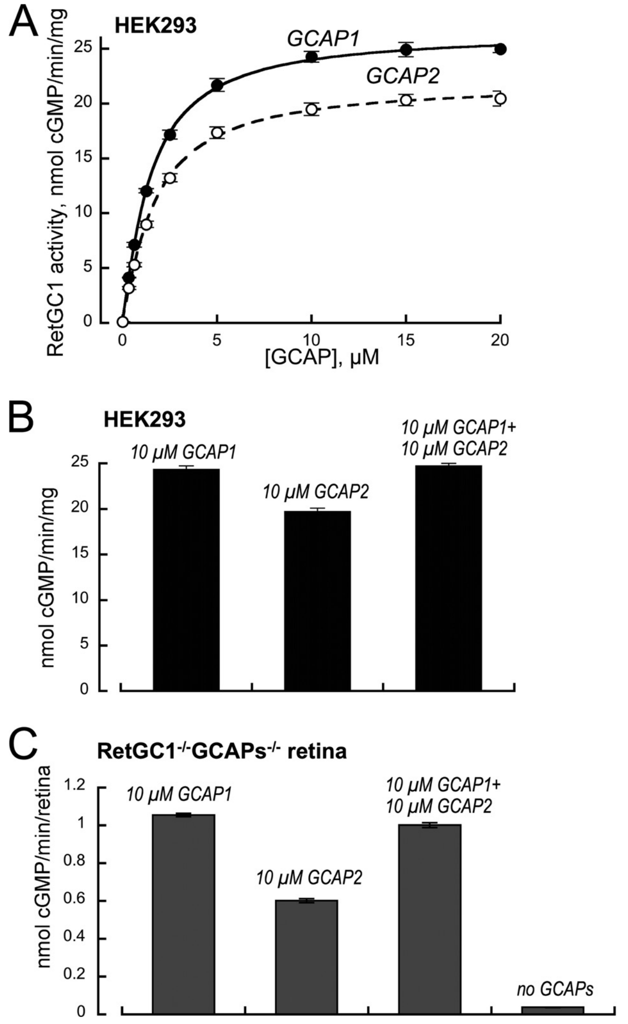


FIGURE 2. **Activation of RetGC1 by GCAP1 and GCAP2 lacks synergy.** *A*, dose dependence of RetGC1 expressed in HEK293 cells by GCAP1 and GCAP2. The data were fitted using Synergy KaleidaGraph 4 utilizing the standard Levenberg-Marquardt algorithm of nonlinear least square routines assuming a Hill function: $a = (a_{max} - a_{min})(1 + ([GCAP]/(K_{1/2GCAP})^h) + a_{min}$ where a is the activity of RetGC in the assay, a_{min} and a_{max} are the minimal and maximal activities (a_{max} = 26 and 22 nmol of cGMP/min/mg for GCAP1- and GCAP2-dependent stimulation, respectively), [GCAP] is the concentration of GCAP, $K_{1/2GCAP}$ is the GCAP concentration required for half-maximal activation (1.5 and 1.7 μM, respectively), and h is the Hill coefficient (1.18 and 1.16, respectively). A near-saturating concentration of 10 μM in each case was used in subsequent experiments described in *B* and *C*. *B*, lack of additive effect of GCAP2 on RetGC1 activity stimulated by GCAP1. RetGC1 expressed in HEK293 membranes was reconstituted with either 10 μM GCAP1 or 10 μM GCAP2 independently or with both GCAPs added together. C, same as *B* except that RetGC1 activity was measured in RetGC2$^{-/-}$GCAPs1,2$^{-/-}$ mouse retinas. The *rightmost column* shows the endogenous RetGC1 activity in the absence of GCAPs. *Error bars represent S.E.*

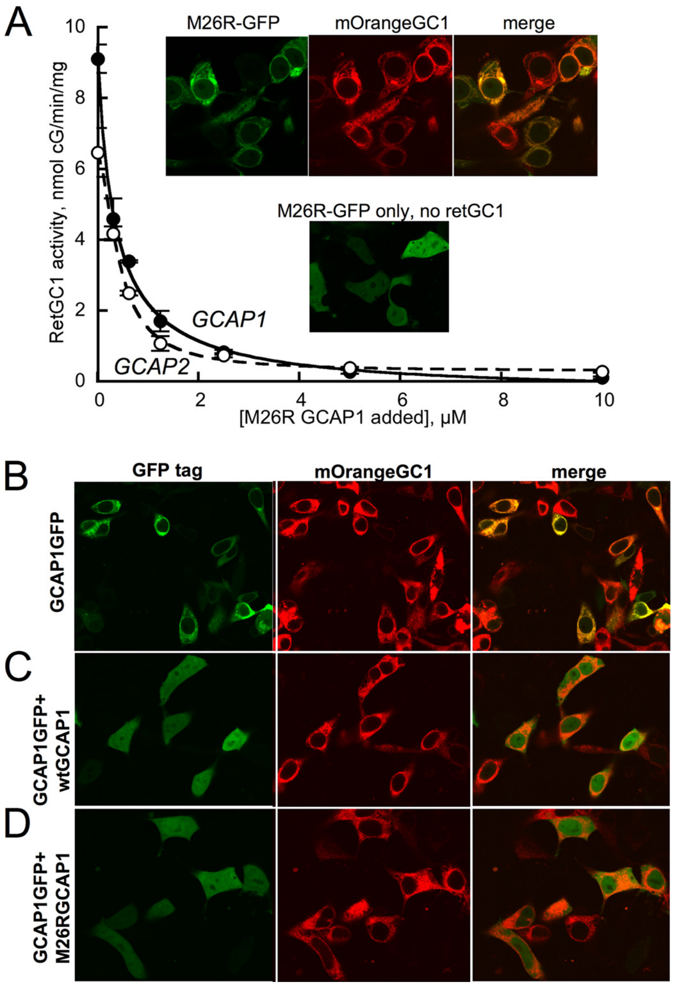


FIGURE 3. **M26R GCAP1 suppresses RetGC1 activation by GCAPs through direct competition for binding with the target enzyme.** *A*, inhibition of RetGC1 preactivated by 1 μM GCAP1 (●) or GCAP2 (○) to 9.4 and 6.5 nmol of cGMP/min/mg, respectively, by increasing concentrations of the M26R GCAP1. By empirical fitting, the $EC_{50M26RGCAP1}$ for the inhibition was 0.34 and 0.43 μM, respectively. *Inset*, M26R GCAP1-GFP expressed in HEK293 cells uniformly distributes through the cytoplasm and the nucleus (*bottom panel*) but co-localizes with the membranes when co-expressed with mOrangeRetGC1 (*upper panels*). *B* and *C*, M26R GCAP1 directly competes with GCAP1 binding to RetGC1. *B*, GCAP1-GFP co-expression with mOrangeRetGC1 in HEK293 cells at an expression vector ratio of 1:100 (0.01 and 1 μg, respectively). *C*, same as *B* but an excess (0.5 μg of DNA) of untagged GCAP1 was added in the co-transfection mixture. *D*, same as *B* but an excess (0.5 μg of DNA) of untagged M26R GCAP1 was added in the co-transfection mixture. *Error bars represent S.E.*

GCAP1-GFP expressed in the absence of the target (Fig. 3). Consequently, the PCC value falls from that indicating a strong co-localization (0.9) to below the threshold, indicating the lack of co-localization (51) (<0.5; Table 1). The untagged M26R GCAP1 displaced GCAP1-GFP in the same manner (Fig. 3*D*), causing PCC to fall to 0.32 ± 0.16 ($n = 36$), which is below the co-localization threshold (51). Hence, the M26R GCAP1 eliminated RetGC1 activation in Fig. 3*A* by displacing WT GCAP1 from the complex with the cyclase.

However, not only GCAP1- but also GCAP2-dependent activation of RetGC1 also becomes suppressed by the M26R GCAP1 in Fig. 3*A*, thus arguing that the mutant GCAP1 is likely to compete not only with WT GCAP1 but also with GCAP2 in a similar fashion. We confirmed that that was indeed the case using the experiments described below. We found that M26R GCAP1 mutant could directly compete with GCAP2 over RetGC1 in a functional RetGC1 activity assay (Fig. 4). At low micromolar concentrations of the M26R GCAP1, the dose dependence of the cyclase activation by either GCAP homolog was drastically shifted toward their higher concentrations (Fig. 4, *A* and *B*). Interestingly, relatively low cooperativity effectively makes the shape of the fit for either GCAP similar to a Michaelis function, but the cooperativity becomes more evident in the presence of the competing M26R GCAP1 possibly due to an allosteric effect(s) of the M26R as an inhibitor in the RetGC1 homodimer (see "Discussion"). Moreover, in the cell-based assay such as exemplified in Fig. 3 but utilizing GCAP2-GFP, the normal pattern of the GCAP2-GFP co-localization with the mOrangeRetGC1 (Fig. 4*C*) was disrupted in the presence of the untagged GCAP1 (Fig. 4*D* and Table 1). Last but not least, the M26R GCAP1 mutant blocked activation by both GCAP1 and GCAP2 not only of the recombinant RetGC1 but also of the native RetGC1 cyclase in the RetGC2$^{-/-}$GCAPs1,2$^{-/-}$ mouse retinas (Fig. 5).

Taken together, the experiments presented in Figs. 2–5 strongly argue that GCAP1 and GCAP2 cannot be bound to the same RetGC1 molecule at the same time using two independent non-overlapping primary binding sites. Instead, the two GCAPs by competing for the binding to the same RetGC1 molecule operate in a mutually exclusive manner so that only one GCAP isoform can bind to the same RetGC1 subunit at a time.

GCAP1 and GCAP2 Binding Sites on RetGC1 Overlap—A model proposed previously (27) advocates the idea of two sites for different GCAP isoforms being formed by portions of the cyclase primary structure on the opposite sides of the intracellular segment: for GCAP1-in KHD just near the transmembrane region and for GCAP2 at the C terminus in the Tyr^{1016}–Ser^{1103} fragment (here residues are numbered starting from the Met^{1} encoded by RetGC1 cDNA; Ref. 2). We therefore tested that hypothesis in experiments described in Figs. 6–9.

We replaced the C-terminal portion RetGC1 catalytic domain downstream from the Leu^{1014} (Fig. 6, *top*), and thus eliminated the entire putative Tyr^{1016}–Ser^{1103} GCAP2 binding site (26, 27), with a non-homologous sequence: the N-terminal portion of the YFP, VenusN (49). The chimera protein lacking the putative GCAP2 binding site was then tested for co-localization with GCAP2 in a cell-based binding assay (Fig. 6). It is important to emphasize that the mOrange fluorescence of the chimera remained properly associated with the endoplasmic reticulum and plasma membranes and that any detectable fluorescence produced by the residual VenusN part of the chimera (Fig. 6*A*) that could possibly interfere with the subsequent analysis was completely lacking. Because a portion of the cyclase catalytic domain was removed by this modification, we could not rely on the cyclase activity but were able to verify that the C-terminal portion of the chimera was not generally misfolded by applying a bimolecular fluorescence complementation (BiFC) (49) through co-expression with the VenusC-tagged RetGC1 (Fig. 6*B*). When both constructs containing the VenusN and VenusC portions of the YFP were co-transfected, prominent YFP fluorescence was registered, arguing against global unfolding of each construct. The mOrangeRetGC1VenusN chi-

TABLE 1

Co-localization of different variants of RetGC1 with GCAP1 in HEK293 cells

PCC for mOrangeRetGC1 and GCAP-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.

Co-transfection	PCC[a] (mean ± S.D.; *n*)	*p*[b]
GCAP1-GFP + mOrangeRetGC1	**0.90 ± 0.04**; 55	
GCAP1-GFP + mOrangeRetGC1 + untagged GCAP1	0.38 ± 0.16; 29	<0.0001[c]
GCAP1-GFP + mOrangeNPRA	0.24 ± 0.18; 21	<0.0001[c]
GCAP1-GFP + mOrangeRetGC1NPRAcat1	**0.90 ± 0.06**; 63	1[c]
GCAP1-GFP + mOrangeW708R	0.24 ± 0.16; 36	<0.0001[c]
GCAP1-GFP + mOrangeI734T	0.25 ± 0.09; 22	<0.0001[c]
GCAP2-GFP + mOrangeRetGC1	**0.88 ± 0.06**; 34	
GCAP2-GFP + mOrangeRetGC1 + GCAP untagged	0.49 ± 0.14; 34	<0.0001[d]
GCAP2-GFP + mOrangeGC1VenusN	**0.89 ± 0.05**; 47	1[d]
GCAP2-GFP + mOrangeGC1VenusN + untagged GCAP1	0.34 ± 0.17; 11	<0.0001[d]
GCAP2-GFP + mOrangeNPRA	0.48 ± 0.15; 21	<0.0001[d]
GCAP2-GFP + mOrangeRetGC1NPRAcat1	**0.90 ± 0.04**; 75	1[d]
GCAP2-GFP + mOrangeW708R	0.42 ± 0.15; 21	<0.0001[d]
GCAP2-GFP + mOrangeI734T	0.46 ± 0.12; 26	<0.0001[d]

[a] The mOrangeRetGC1 and GCAP1-GFP were co-expressed in HEK293 cells, and confocal microscopy was performed as described under "Experimental Procedures." PCC values indicating strong co-localization are highlighted in bold (note that PCC ≤ 0.5 generally means no co-localization, whereas PCC = 1.0 would indicate co-localization of all red and green pixels in the image (51)).

[b] From one-way analysis of variance/Bonferroni (α = 0.01) all-pairs comparison test (confidence level = 99%) processed using Synergy KaleidaGraph 4 software.

[c] Compared with GCAP1-GFP + mOrangeRetGC1.

[d] Compared with GCAP2-GFP + mOrangeRetGC1.

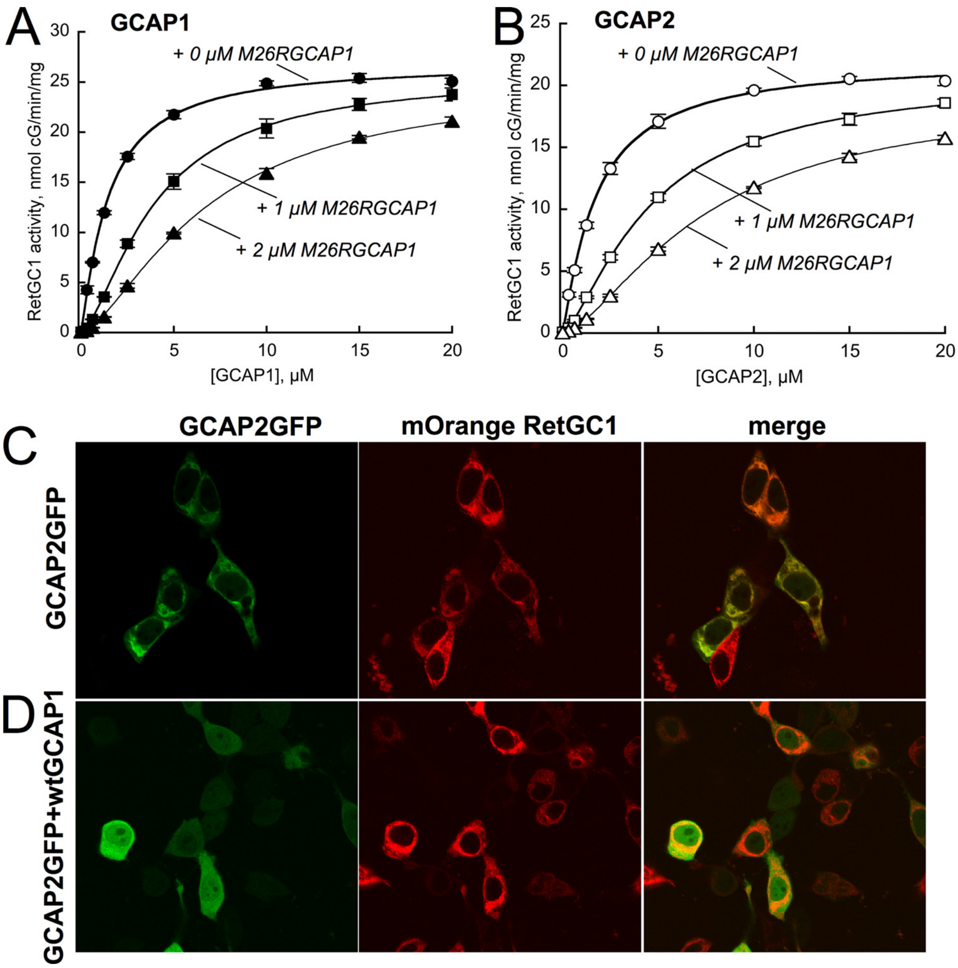


FIGURE 4. **GCAP1 directly competes with GCAP2 for binding with RetGC1.** *A* and *B*, the M26R GCAP1 shifts the dose dependence of RetGC1 activation by GCAP1 and GCAP2 toward higher concentrations. *A*, dose dependence of RetGC1 activation by GCAP1 in the absence (●) or presence of 1 (■) or 2 μM (▲) M26R GCAP1. *B*, dose dependence of RetGC1 activation by GCAP2 in the absence (○) or presence of 1 (□) or 2 μM (△) M26R GCAP1. The data points were fitted assuming the same Hill function as in Fig. 2; the a_{max} values for stimulation by GCAPs in the absence or presence of 1 and 2 μM M26R GCAP1 only slightly change for stimulation by GCAP1 (27, 26, and 25 nmol of cGMP/min/mg, respectively) or GCAP2 (22, 21, and 19 nmol of cGMP/min/mg, respectively), but the $K_{1/2GCAP}$ increased in the presence of M26R GCAP1 for wild type GCAP1 (1.5, 4, and 6.7 μM, respectively) and GCAP2 (1.7, 4.7, and 7.4 μM, respectively). The Hill coefficient increased for GCAP1 (from 1.17 in the absence to 1.53 in the presence of 2 μM M26R GCAP1) and for GCAP2 (from 1.14 in the absence to 1.54 in the presence of 2 μM M26R GCAP1). *C* and *D*, GCAP1 prevents binding of GCAP2 to RetGC1. *C*, GCAP2-GFP co-expressed with mOrangeRetGC1 acquires a typical membrane-bound pattern, co-localizing with the mOrangeRetGC1. *D*, same as *C* but 0.2 μg of untagged GCAP1-coding vector was also added in the co-transfection mixture. Note the diffuse uniform distribution of GCAP2-GFP displaced from the membrane complex with RetGC1. *Error bars represent S.E.*

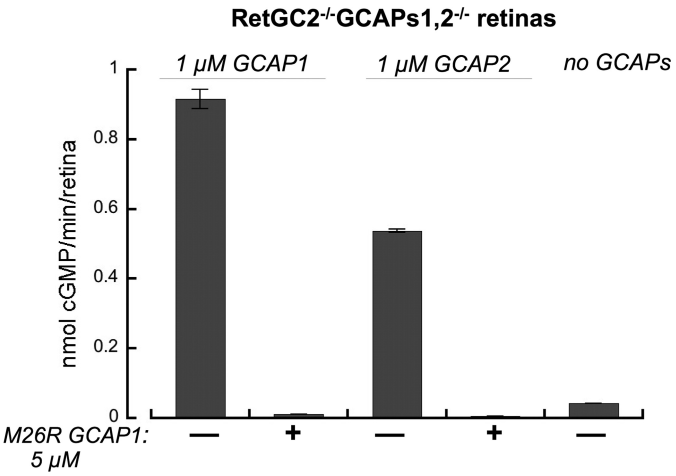


FIGURE 5. **M26R GCAP1 blocks activation of the native RetGC1 in RetGC2$^{-/-}$GCAPs1,2$^{-/-}$ mouse retinas by both GCAP1 and GCAP2.** Retinal homogenate from the RetGC2$^{-/-}$GCAPs1,2$^{-/-}$ retinas was reconstituted with purified 1 μM GCAP1 or GCAP2 in the absence or presence of 5 μM M26R GCAP1. The *rightmost column* indicates the endogenous activity of RetGC1 without added GCAPs. *Error bars represent S.E.*

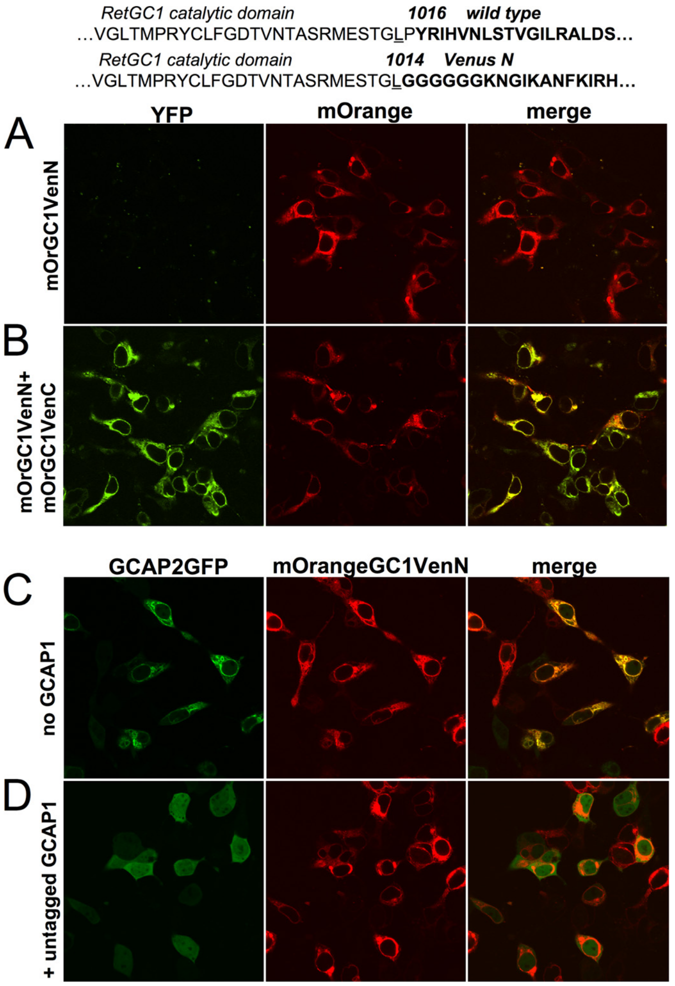


FIGURE 6. **Replacement of the Tyr^{1016}–Ser^{1103} region in RetGC1 with a non-homologous sequence does not eliminate binding of GCAP2.** *Top*, the mOrangeRetGC1 with the Pro^{1015}–Ser^{1103} C-terminal portion was substituted with six Gly residues followed by the N-terminal half of Venus YFP (*mOrangeGC1VenN*). *A*, mOrangeRetGC1VenusN (*mOrGC1VenN*) displays a normal membrane fluorescence pattern when expressed in HEK293 cells and does not emit YFP fluorescence when excited at 488 nm. *B*, BiFC experiment to probe for global unfolding of the Venus-containing constructs. The mOrangeRetGC1VenusN construct produces YFP fluorescence when co-expressed with mOrangeRetGC1VenusC (*mOrGC1VenC*) containing a shorter C-terminal portion of the YFP (49). No GCAP was present. *C*, GCAP2-GFP (0.01 μg of vector DNA) co-expressed with mOrangeRetGC1VenusN (1 μg of vector DNA) acquires a membrane binding pattern typical for GCAP2/RetGC1 co-localization (39) (compare with *Fig. 4C*). *D*, GCAP1 competes with GCAP2 for binding to mOrangeRetGC1VenusN. The untagged wild type GCAP1-coding vector (0.2 μg of DNA) was added in the same co-transfection mixture as in *C*. Note the pattern of GCAP2-GFP, which changes to diffusely spread through the cytoplasm and nuclei, dissimilar from that of the cyclase.

mera co-expressed with GCAP2-GFP co-localized with GCAP2 in a manner similar to the complete mOrangeRetGC1 (Fig. 6*C* and Table 1). Moreover, the untagged GCAP1 disrupted co-localization of GCAP2-GFP with the chimera (Fig. 6*C* and Table 1) just like it did with the mOrangeRetGC1 (Fig. 4*C*).

As an independent approach, we also constructed a chimera based on a distant homolog of RetGC1, a peptide hormone receptor guanylyl cyclase NPRA (*GUCY2A*) (52). RetGC1 and NPRA have a similar domain organization and homologous catalytic domains but share little identity between the other regions of their primary structures (2). When the two mOrange-tagged cyclase constructs are expressed in HEK293 cells, RetGC1 can be activated with either GCAP1 or GCAP2, but no activation of NPRA was ever detected with either GCAP isoform (Fig. 7). Consistent with that, neither GCAP1 nor GCAP2 could bind the NPRA in a cell-based assay (Fig. 8, *A* and *B*). Consequently, the intracellular segment of NPRA contains no functional binding sites for either GCAP. In striking contrast to that, the intracellular segment of the mOrangeGC1NPRAcat1 chimera containing the catalytic domain of NPRA and the KHD/DD portion derived from RetGC1 very effectively bound GCAP1-GFP and GCAP2-GFP in co-transfected cells (Fig. 8, *C* and *D*, and Table 1). Again, GCAP2-GFP was displaced from the complex with the mOrangeGC1NPRAcat1 by an excess of untagged GCAP1 (Fig. 8*E*).

Not only do NPRA and RetGC1 share little homology between their C-terminal portions, but the Tyr^{1016}–Ser^{1103} region in RetGC1 hypothesized to contain the GCAP2 binding site (26, 27) is one of the least homologous regions between the two cyclases (2), and most of it is merely absent from NPRA (Fig. 9, *top*). Nonetheless, GCAP2, which failed to activate the cytoplasmic portion of NPRA (Fig. 7), efficiently stimulated the chimera protein mOrangeGC1NPRAcat1 (Fig. 9) and regulated it within a submicromolar range of Ca^{2+} (Fig. 9, *inset*). In a manner typical for normal RetGC1 (11, 35), GCAP2 activated the chimera at free Ca^{2+} concentrations below 100 nM, but its activity became suppressed when Ca^{2+} concentrations increased above that level. Hence, the KHD/DD portion of RetGC1 but not its C-terminal portion defines regulation by GCAP2.

Disease-linked Mutations in RetGC1 KHD Block Binding of Both GCAP1 and GCAP2—Multiple mutations in human *GUCY2D* gene coding for RetGC1 cause LCA, a severe early onset loss of vision (14–16). Some LCA-linked mutations inactivate RetGC1 and/or suppress its activation by GCAP1 and GCAP2 (16). Here we tested two LCA-linked point mutations in the KHD, W708R and I734T, found in LCA patients and assigned the highest estimated pathogenic probability (15). Neither of the two mutants was active *in vitro* (not shown).

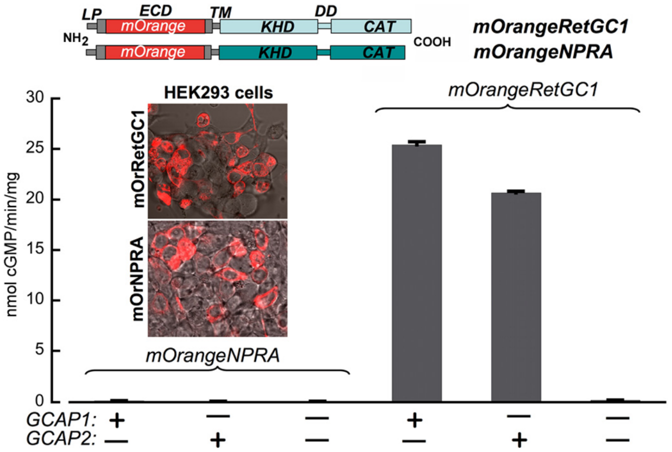


FIGURE 7. **GCAP1 and GCAP2 both stimulate mOrangeRetGC1 but not mOrangeNPRA cyclase.** The mOrangeRetGC1 (*mOrRetGC1*) (25 μg of DNA/100-mm dish) and mOrangeNPRA (*mOrNPRA*) (25 μg of DNA/100-mm dish) were expressed in HEK293 cells (*inset*). Membrane fractions containing fluorescently labeled cyclases were isolated as described under "Experimental Procedures" and reconstituted with 15 μM GCAP1 or GCAP2 in the presence of 10 mM $MgCl_2$. Although mOrangeRetGC1 becomes activated by GCAP1 and GCAP2, no activity of mOrangeNPRA can be detected in the presence of either GCAP. *Error bars represent S.E. LP*, leader peptide; *ECD*, extracellular domain; *TM*, transmembrane region; *CAT*, catalytic domain.

When co-transfected with the W708R or I734T mOrangeRetGC1 (Fig. 10, *A* and *B*) (positions of the residues are as encoded by *GUCY2D* in a non-modified RetGC1 cDNA), both GCAP1-GFP and GCAP2-GFP displayed a diffuse pattern, indicating lack of co-localization with the cyclase (PCC < 0.5; Table 1) (see Figs. 3*B* and 4*C* for comparison). When introduced in mOrangeRetGC1VenusN and -VenusC constructs, neither W708R (Fig. 10*C*) nor I734T (identical result; not shown) precluded the subunits harboring the same or two different LCA mutations (Fig 10*C*, *rightmost panel*) from forming a YFP fluorescent complex in the BiFC test. Evidently, these two mutations were less likely to cause unspecific global unfolding of the chimera protein and more likely to directly affect the portion of RetGC1 containing the GCAP1 and GCAP2 binding interface.

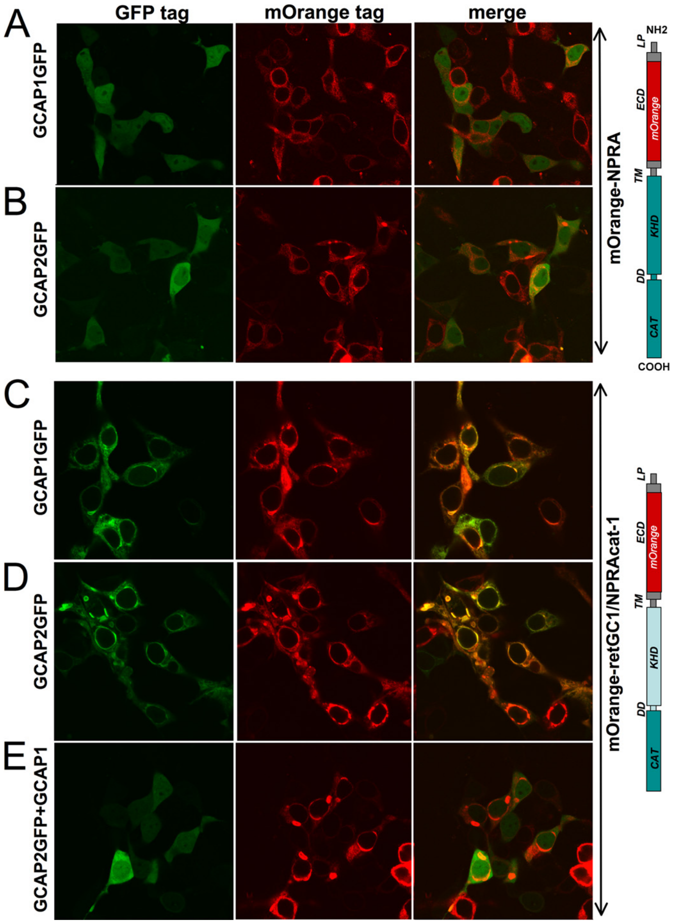


FIGURE 8. **GCAP1 and GCAP2 both bind to the site(s) formed by the KHD/DD region of the cytoplasmic portion of RetGC1.** *A* and *B*, neither GCAP1 nor GCAP2 binds to the cytoplasmic portion of NPRA. GCAP1-GFP (*A*) and GCAP2-GFP (*B*) fail to co-localize with the mOrangeNPRA in HEK293 cells. Note the diffuse uniform distribution of both GCAPs in contrast to the membrane localization of both cyclases. *C* and *D*, GCAP1-GFP (*C*) and GCAP2-GFP (*D*) co-localize with a chimera containing the catalytic domain and the C terminus from NPRA and the KHD/DD portion from RetGC1 (mOrangeRetGC1NPRAcat1). The respective co-transfection mixtures contained 0.01 μg of GCAP1-GFP DNA, 0.01 μg of GCAP2-GFP DNA, 1 μg of mOrangeNPRA DNA, or 1 μg of mOrangeRetGC1NPRAcat1 DNA. *E*, GCAP1 displaces GCAP2-GFP from its complex with RetGC1. Untagged GCAP1-coding vector (0.2 μg) was added to the GCAP2-GFP/mOrangeRetGC1NPRAcat1 transfection mixture. Note the change of membrane-bound GCAP2-GFP pattern in *D* to a diffuse uniform pattern in *E*. *LP*, leader peptide; *ECD*, extracellular domain; *TM*, transmembrane region; *CAT*, catalytic domain.

DISCUSSION

GCAP-RetGC Complexes and Regulation of Rod Photoresponse—The mechanisms of interaction between RetGC1 and its regulators, GCAPs, remain controversial and relatively poorly understood despite their critical role in retinal physiology and congenital diseases of photoreceptors. Activation of cGMP synthesis by GCAPs in photoexcited rods and cones is essential for their proper light sensitivities and response kinetics (10, 47, 53). In rods, GCAP1 and GCAP2 are recruited to activate RetGCs in a sequential or "relay" (29, 54) fashion based on the different Ca^{2+} sensitivities of GCAP1 and GCAP2 (11). GCAP1, which has lower sensitivity to inhibition by Ca^{2+}, starts stimulating RetGC activity early in response as soon as the free Ca^{2+} levels in the outer segment start to decline and continues to stimulate cGMP production until the end of the response. GCAP2 provides an additional boost of cGMP production to accelerate the recovery mainly in the midphase of the response when Ca^{2+} becomes depleted below 100 nM (30, 47, 55). *In vivo*, GCAP1 preferentially activates RetGC1, whereas GCAP2 can activate both RetGC1 and RetGC2 (55, 56). Therefore, knowing how GCAPs bind to the RetGC1 molecule can help better explain the mechanisms of Ca^{2+} feedback. Can GCAPs bind and regulate the same molecule of the cyclase independently? In Fig. 11, which illustrates the summary for our evaluation of different possible modes of GCAP/RetGC1 interaction, we present this as an unlikely scenario.

First, we found clear evidence that GCAP1 and GCAP2 fail to provide cumulative stimulation of the cyclase (Fig. 2). It needs to be pointed out that the lack of additional activation by two GCAPs together could hardly be explained by the cyclase reaching its maximal catalytic efficiency when stimulated by just the GCAP1 isoform because RetGC1, even at full saturation with GCAP1, remains a relatively low efficiency enzyme ($k_{cat}/K_m \sim 1$–1.6×10^5 $\text{M}^{-1}\,\text{s}^{-1}$; Ref. 11) at least 1000-fold less potent than a "perfect" diffusion-limited enzyme.

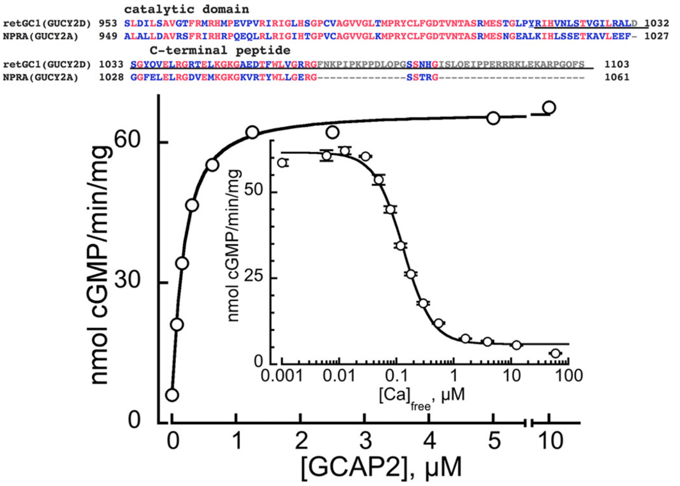


FIGURE 9. **The KHD/DD portion of RetGC1, Arg^{488}–Arg^{851}, enables activation and Ca^{2+}-sensitive regulation of the mOrangeRetGC1NPRAcat1 chimera by GCAP2.** The membrane fraction from the mOrangeRetGC1NPRAcat1-expressing HEK293 cells was reconstituted with purified GCAP2 at 10 mM Mg^{2+} in the presence of EGTA. The data were fitted assuming a Hill equation (see the legend to Fig. 2), yielding a_{max} = 66 nmol of cGMP/min/mg, $K_{1/2GCAP}$ = 0.2 μM, and Hill coefficient = 1.2. *Inset*, mOrangeRetGC1NPRAcat1 activation by 10 μM GCAP2 in the presence of 2 mM Ca^{2+}/EGTA buffer maintaining the indicated free concentrations of Ca^{2+} at 1 mM free Mg^{2+}. The data were fitted by KaleidaGraph 4 assuming the following function: $A = (A_{max} - A_{min})/(1 + ([Ca]/[Ca]_{1/2})^h) + A_{min}$ where A_{max} and A_{min} are the maximal and minimal activity of guanylyl cyclase, respectively, $[Ca]_{1/2}$ is the concentration of Ca^{2+} producing 50% inhibition (0.13 μM), and *h* is the Hill coefficient (1.7). RetGC activity was assayed as described under "Experimental Procedures." GCAP2 effectively activated the chimera in a Ca^{2+}-sensitive manner despite a very weak homology with the C-terminal portion of RetGC1 (*top*). The putative GCAP2 binding site in RetGC1 is *underlined*; identical amino acid residues in the alignment are shown in *red*, non-identical residues are shown in *blue*, and absent residues are shown in *gray*. The two sequences were aligned using NCBI Cobalt software. *Error bars represent S.E.*

Second and more importantly, our results argue that the two GCAPs cannot even occupy the same RetGC1 subunit at the same time (Figs. 4 and 5). Hence, our results directly contradict the model assuming independent binding of different GCAPs to the same cyclase molecule (27). Instead, we found clear evidence that GCAPs bind their target in a mutually exclusive manner so that only one isoform can occupy any given RetGC1 molecule at any given time. GCAP1 displays more than 5-fold higher affinity for the native RetGC1 isozyme in crude mouse retinal membranes than GCAP2 (11). Even assuming that each GCAP isoform is present in the outer segment at saturating concentration sufficient to activate all of the RetGC (11), one could expect a much larger population of RetGC1 molecules to be bound with GCAP1 and only a small fraction (less than 20%) to be bound with GCAP2. At the same time, nearly all RetGC2, which accounts for one-fourth to one-third of all RetGC activity (11), would remain almost exclusively in a complex with GCAP2 (56). Evidently, competition with GCAP1 would unlikely allow GCAP2 to bind RetGC1 *in vivo* at higher scale, although in the absence of GCAP1 such as in $GCAP1^{-/-}$ mouse rods, it can take over the entire pool of RetGC, and the total activity of the cyclase in the $GCAP1^{-/-}$ retina does not fall (30). RetGC1 in rods has to be involved in the relay regulation of cGMP synthesis by negative calcium feedback at both steps of the recovery as controlled by the two Ca^{2+} sensors but to a very different extent. Most of it, as a part of the RetGC1-GCAP1 complex, is activated by the early fall in Ca^{2+} levels, whereas the lesser portion bound with GCAP2 would provide, together with the RetGC2-GCAP2 complex, an additional boost of cGMP production in the midphase of recovery (30).

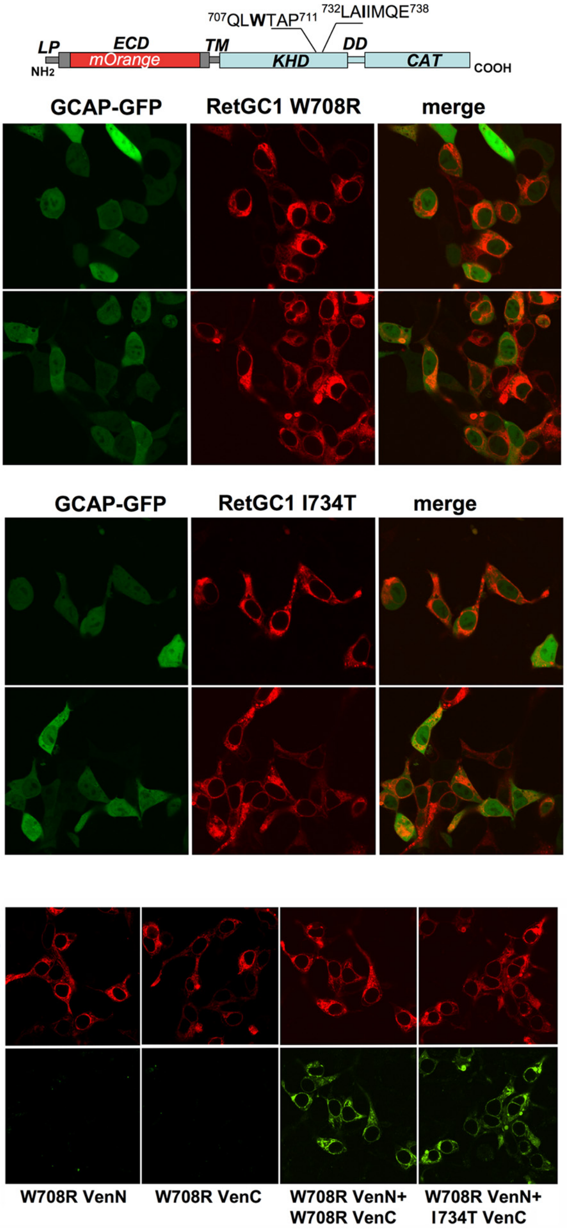


FIGURE 10. **Point mutations W708R and I734T in RetGC1 causing Leber congenital amaurosis inactivate binding of both GCAP1 and GCAP2.** *A* and *B*, the LCA-linked (45) substitutions W708R (*A*) and I734T (*B*) were introduced in the KHD of the mOrangeRetGC1, and each mutated cyclase was co-expressed with GCAP1-GFP or GCAP2-GFP as described under "Experimental Procedures" using 1 μg of each mutated mOrangeRetGC1 vector and 0.01 μg of GCAP1-GFP or GCAP2-GFP vector. Note the distinct lack of membrane co-localization of GCAP1 and GCAP2 with the W708R and I734T mutants. *C*, the W708R and I734T mOrangeRetGC1VenusN and -VenusC tested for global unfolding by BiFC. The YFP (*upper panels*) and mOrange (*lower panels*) fluorescence in transfected HEK293 cells was recorded as described in Fig. 6. *Left* to *right*, W708R mOrangeRetGC1VenusN construct (1 μg of DNA) expressed alone, W708R mOrangeRetGC1VenusC (1 μg) expressed alone, co-transfected W708R mOrangeRetGC1VenusN (0.5 μg) plus W708R mOrangeRetGC1VenusC (0.5 μg), and co-transfected W708R mOrange-RetGC1VenusN (0.5 μg) plus I734T mOrangeRetGC1VenusC (0.5 μg). Note that individually expressed VenusN (*VenN*)- and VenusC (*VenC*)-containing constructs do not produce YFP fluorescence, but co-expression of the two, either with the same or two different mutations, generates well defined BiFC. All images were taken using the same set of excitation and image acquisition settings.

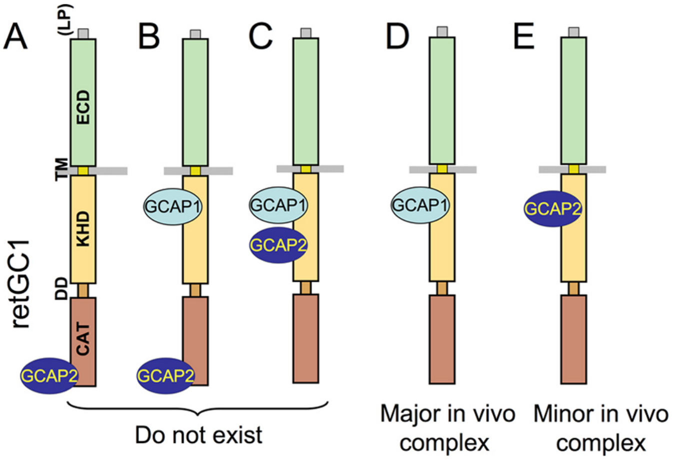


FIGURE 11. **Prohibited *versus* allowed complexes between individual RetGC1 subunit and different GCAP isoforms.** *A–C*, complexes that cannot exist. *A*, neither GCAP1 nor GCAP2 utilize the Tyr^{1016}–Ser^{1103} part of the human RetGC1 as its primary binding site. *B* and *C*, GCAP1 and GCAP2 cannot simultaneously and independently bind to the same subunit of RetGC1 either via two remotely positioned (*B*) or closely located (*C*) sites. *D* and *E*, complexes allowed *in vivo*. GCAPs compete for binding to the same RetGC1 molecule using either an overlapped or identical primary binding site(s) formed by the KHD/DD portion of the cyclase. Only one GCAP isoform, either GCAP1 (*D*) or GCAP2 (*E*), can occupy the same RetGC1 subunit at any given time. Note that the position of the GCAP binding site relative to the primary structure schematics of the cyclase domains is merely arbitrary because both KHD and DD could both participate in formation of the binding site(s) in a three-dimensional structure. Considering the greater affinity of the native RetGC1 isozyme for GCAP1 in crude mouse retina preparations (11), the main fraction of RetGC1 subunits must be in a complex with GCAP1, and a much lesser fraction (<20%) must be in a complex with GCAP2. Because the cyclase is catalytically active only as a homodimer (57–59), the main RetGC1-RetGC1 homodimer fraction should consequently contain GCAP1 as the Ca^{2+} sensor, and only a minor fraction should contain GCAP2 either as a homodimer or in a mixed RetGC1-GCAP1/RetGC1-GCAP2 dimer. The existence of the latter complex can be neither excluded nor confirmed at present. *LP*, leader peptide; *ECD*, extracellular domain; *TM*, transmembrane region; *CAT*, catalytic domain.

We should, however, consider certain limitations to that model not addressed in Fig. 11. First, the cyclase functions *in vivo* as a homodimer (57–59). The functional RetGC1 is likely to contain the cyclase homodimer with two activator molecules in it (60). The cooperativity for allosteric activation of the dimer by either GCAP becomes more evident when they have to compete with M26R GCAP1 (Fig. 4, *A* and *B*). Much like the competition between Ca^{2+}- and Mg^{2+}-liganded GCAPs makes Ca^{2+} dependence of cyclase regulation more cooperative than binding of Ca^{2+} to isolated GCAP1 (35, 48), creating the competing inhibitor/activator pair for Mg^{2+}-bound GCAP in the absence of Ca^{2+} accentuates the non-linear dose dependence. However, at this point, we do not know whether two different GCAPs can be bound to the two molecules of RetGC1 comprising the cyclase dimer or what Ca^{2+} sensitivity such a mixed complex would have. However, based on the higher affinity of the native RetGC1 for GCAP1 *versus* GCAP2 in crude retinal membranes (11), such a combination would unlikely represent a major fraction of RetGC1 in comparison with the $(GCAP1)_2$-$(RetGC1)_2$ tetrameric complex. Second, it needs to be noted that the use of the RetGC linear domain structure as a model is an oversimplification because in a three-dimensional structure different distal parts of the protein primary structure can come into proximity to each other to form the GCAP binding interface(s).

In regard to the relationship between the domain structure of RetGC1 and its function, our findings are at variance with the recently advocated hypotheses (26, 27) because we found that GCAP2 does not bind at the C terminus of RetGC1 (Figs. 6–8). Therefore, the model based on the two GCAPs having their binding sites independently formed by remote parts of the RetGC1 primary structure (27) is inconsistent with the experimental data from our study. Evidently, the Tyr^{1016}–Gly^{1103} region in RetGC1 defines neither GCAP2 binding (Figs. 6–8) nor Ca^{2+}-sensitive regulation of the cyclase (Fig. 9). Contrary to the expectations from the model (27), the $^{1016}RIHVNL^{1021}$ sequence at the end of the catalytic domain is not obligatory in making the cyclase interface with GCAP2 because the RetGC1NPRA chimera in which this sequence is replaced by mostly non-identical sequence ($^{1013}KIHLSS^{1018}$) of NPRA remained activated by GCAP2 and regulated by Ca^{2+} (Fig. 9), whereas the NPRA itself did not (Fig. 7). Even after the replacement, GCAP2 activated the hybrid cyclase with a high apparent affinity (Fig. 9). Moreover, we present clear evidence that the region of RetGC1 that imparts its ability to bind GCAP1 and GCAP2 (Figs. 6 and 8) in a mutually exclusive manner (Figs. 8 and 11, *D* and *E*) is the KHD/DD part of the molecule, not in the catalytic domain. Our results are also consistent with the early observations by Laura and Hurley (24) that the cyclase KHD rather than catalytic domain defines relative affinities of RetGC1 and RetGC2 isozymes for GCAP1 and GCAP2. A common or at least critically overlapping binding site(s) for GCAP1 and GCAP2 would also be more consistent with the structural similarity between the two GCAPs in both of which the cyclase binding interface includes closely homologous regions comprising EF-hands 1 and 2 (40, 61).

Mutations Causing a Blinding Disorder Destroy GCAP-RetGC1 Complex—Mutations in RetGC1 cause congenital LCA blindness through loss of photoreceptor function rather than photoreceptor degeneration (16). Considering that most of the cyclase activity in rods and nearly all of the activity in cones belongs to RetGC1 (11–13), blocking the cyclase activation by GCAPs could severely affect vision. The indication that binding sites for GCAP1 and GCAP2 are likely identical or overlapped also comes from the comparison of point mutations related to LCA in humans. Multiple mutations in *GUCY2D* gene found in LCA patients (15, 16) can affect RetGC1 activity and regulation by GCAPs in profoundly different ways (16), but the effect of the tested mutations in each case was virtually identical for both GCAP1- and GCAP2-dependent regulation (16). This would be consistent with the two GCAPs having common or overlapping rather than distantly separated binding sites formed by remotely separated portions of the cyclase primary structure. In the present study, we found that two previously uncharacterized LCA-linked point mutations in the KHD of RetGC1, W708R and I734T (15), completely abolish binding of GCAP1 and GCAP2 altogether (Fig. 10). These results are also surprising considering the hypothesis that the region $^{708}WTAPELL^{714}$ containing Trp^{708} would contribute to the regulation of the cyclase by GCAPs but not to their primary docking sites (41). Together with the data presented in Figs. 6–8, these results favor the possibility that GCAP1 and GCAP2 use a common structural interface(s) in RetGC1 formed by the KHD/DD portion of the cyclase primary structure. It also needs to be noted that in contrast to the lack of the cyclase (12) the lack of GCAPs does not suppress the amplitude of rods and cones response to light in a mouse model but instead hampers their adaptation to light (10, 47, 53). Hence, just disrupting GCAP binding to RetGC1 may or may not be sufficient to cause LCA on its own. We cannot exclude that some other interactions of RetGC1 in photoreceptors such as for example those related to its trafficking to the outer segment could be affected by LCA mutations. Subsequent *in vivo* studies may help reveal other potential effects for RetGC1 interactions and stability in photoreceptors. Our data also indicate that to pinpoint the side chains defining the actual docking site(s) for GCAP1 and GCAP2 will require a much more detailed mapping of the cyclase KHD and DD than it was previously believed.

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