Activation and Inhibition of Photoreceptor Guanylyl Cyclase by Guanylyl Cyclase Activating Protein 1 (GCAP-1)

THE FUNCTIONAL ROLE OF Mg$^{2+}$/Ca$^{2+}$ EXCHANGE IN EF-HAND DOMAINS*

Received for publication, March 20, 2007, and in revised form, May 18, 2007 Published, JBC Papers in Press, June 1, 2007, DOI 10.1074/jbc.M702368200

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Guanylyl cyclase activating protein 1 (GCAP-1), a Ca$^{2+}$/Mg$^{2+}$ sensor protein that accelerates retinal guanylyl cyclase (RetGC) in the light and decelerates it in the dark, is inactive in cation-free form. Binding of Mg$^{2+}$ in EF-hands 2 and 3 was essential for RetGC activation in the conditions mimicking light adaptation. Mg$^{2+}$ binding in EF-hand 2 affected the conformation of a neighboring non-metal binding domain, EF-hand-1, and increased GCAP-1 affinity for RetGC nearly 40-fold compared with the metal-free EF-hand 2. Mg$^{2+}$ binding in EF-hand 3 increased GCAP-1 affinity for RetGC 5-fold and its maximal RetGC stimulation 2-fold. Mg$^{2+}$ binding in EF-hand 4 affected neither GCAP-1 affinity for RetGC, nor RetGC activation. Inactivation of Ca$^{2+}$ binding in EF-hand 4 was sufficient to render GCAP-1 a constitutive activator of RetGC, whereas the EF-hand 3 role in Ca$^{2+}$-dependent deceleration of RetGC was likely to be through the neighboring EF-hand 4. Inactivation of Ca$^{2+}$ binding in EF-hand 2 affected cooperativity of RetGC inhibition by Ca$^{2+}$, but did not prevent the inhibition. We conclude that 1) Mg$^{2+}$ binding in EF-hands 2 and 3, but not EF-hand 4, is essential for the ability of GCAP-1 to activate RetGC in the light; 2) Mg$^{2+}$ or Ca$^{2+}$ binding in EF-hand 3 and especially in EF-hand 2 is required for high-affinity interaction with the cyclase and affects the conformation of the neighboring EF-hand 1, a domain required for targeting RetGC; and 3) RetGC inhibition is likely to be primarily caused by Ca$^{2+}$ binding in EF-hand 4.

Calcium is the major regulator of the physiological responses in photoreceptor cells. Calcium enters outer segments of vertebrate photoreceptors through cGMP-gated Na$^{+}$/Ca$^{2+}$ channels in the outer segment plasma membrane and is continuously removed from the outer segment by a light-independent Na$^{+}$/K$^{+}$, Ca$^{2+}$ exchanger (for review, see Refs. 1–4). In the dark, cGMP keeps a small percentage of the Na$^{+}$/Ca$^{2+}$ channels open, and the hydrolysis of cGMP by a light-activated phosphodiesterase, PDE6, generates photoreponses in rods and cones. When light triggers cGMP hydrolysis, it also, through the closure of the channels, lowers the intracellular concentration of Ca$^{2+}$ from near 250 nM in the dark to near 25 nM in the light (5–7). Guanylyl cyclase activating proteins (GCAPs) are Ca$^{2+}$/Mg$^{2+}$-sensor proteins that impart Ca$^{2+}$ sensitivity to retinal guanylyl cyclase (RetGC), the enzyme that supplies the photoreceptor cell with cGMP (8–11). GCAPs become RetGC activators at low Ca$^{2+}$ concentrations and inhibit it at high Ca$^{2+}$, such that when the Ca$^{2+}$ concentration drops upon the illumination, GCAPs activate RetGC to quickly restore the level of cGMP in photoreceptors and thus accelerate their recovery from excitation. Conversely, when RetGC produces enough cGMP to reopen the Na$^{+}$/Ca$^{2+}$ channels, Ca$^{2+}$ re-enters the outer segments and the Ca$^{2+}$-bound GCAPs decelerate cGMP synthesis.

GCAPs are recoverin-like neuronal calcium-binding proteins, also referred to as the neuronal calcium sensors (NCS) family, a part of a larger superfamily of EF-hand Ca$^{2+}$-binding proteins (12–16). Like all other members of that superfamily GCAPs have Ca$^{2+}$-binding EF-hand domains of the helix-loop-helix structure. The metal-binding loop in the NCS proteins is traditionally defined as 12 sequential amino acid residues, of which 6 residues provide the oxygen atoms required for the metal coordination, including the invariant first and the last coordinating residues, Asp and Glu, respectively. The N-terminal EF-hand domains in GCAP-1 and GCAP-2 lack some of the oxygen-containing groups required for coordinating the metal ion (17), but are instead required for their interaction with RetGC (18–20). We previously found that in the conditions that mimic light-adapted or dark-adapted photoreceptors, three other EF-hands in GCAP-1 are predominantly filled by either Mg$^{2+}$ or Ca$^{2+}$, respectively (21, 22).

There are multiple reports that substitutions of the first and last coordinating amino acids inactivate EF-hands in NCS proteins (17, 22–25). Among these are the observations that inactivation of all three metal-binding EF-hands in GCAP-2 by substitution of the last Glu in EF-hands 2 and 3 with Gln and the first Asp in the EF-hand 4 with Asn make GCAP-2 a constitutive, insensitive to Ca$^{2+}$ activator of RetGC (24). A similar effect was observed for GCAP-1, whose EF-hands were disabled by substitution of the last Glu in the Ca$^{2+}$-binding loop with Asp (25). Those observations lead to the perception that metal-free GCAPs are the activators of RetGC and that GCAPs undergo transition from Ca$^{2+}$-bound to the metal-free form between dark and light. However, our recent study of metal-binding

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* This work was supported in part by National Institutes of Health Grant EY11522 and the Pennsylvania Lions Sight Conservation and Eye Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: GCAP, guanylyl cyclase activating protein; RetGC, photoreceptor membrane guanylyl cyclase; MOPS, 4-morpholinopropanesulfonic acid; NCS, neuronal calcium sensors.
Functional States of GCAP-1 Controlled by Ca\(^{2+}\) and Mg\(^{2+}\) Binding

Mutations that disable both Ca\(^{2+}\) and Mg\(^{2+}\) binding to EF-hand

|    | N | 100 | 144 |
|----|---|-----|-----|
|    | 64 | 102 | 148 |
|    | EF2 | EF3 | EF4 |
|    | Q | 75 | 111 | 155|

FIGURE 1. EF-hands of bovine GCAP-1 and mutations used in this study. Mutations introduced in EF-hands of GCAP-1: D64N, D100N/D102G, and D144N/D148G to disable both Ca\(^{2+}\) and Mg\(^{2+}\) binding to EF-hands 2, 3, and 4, respectively; E79Q, E111Q, and E155Q to disable only Ca\(^{2+}\) binding to EF-hands 2, 3, and 4, respectively.

EXPERIMENTAL PROCEDURES

Recombinant GCAP-1 and Its Mutants—All mutations were incorporated into bovine GCAP-1 cDNA by PCR using a “splicing by overlap extension” technique (27). Wild type GCAP-1 and its mutants used in this study also carried a D6S substitution that creates a recognition site for the yeast N-myristoyl transferase (28), and does not interfere with the RetGC regulation by the recombinant GCAP-1 (29, 30). GCAP-1 cDNA was expressed under control of the isopropyl β-D-thiogalactopyranoside-regulated T7 promoter in a BL21(DE3) pLysS Escherichia coli strain (Novagen/Calbiochem) harboring a pBB131 plasmid for a yeast N-myristoyl transferase expression as described (29, 30). Cells were grown in standard LB medium containing 40 μg/ml kanamycin and 100 μg/ml ampicillin. Free myristic acid was added from a concentrated ethanol solution to the suspension of bacterial cells to a final concentration of 100 μg/ml 20 min prior to the induction with 0.5 mM isopropyl β-D-thiogalactopyranoside. Three hours after the induction, the bacterial pellet was harvested and the recombinant GCAPs were purified as described previously in detail (22). The concentration of GCAP-1 and its mutants was determined in 20 mM Na-phosphate buffer (pH 6.5) containing 6 mM guanidine hydrochloride using extinction coefficients at 280 nm computed for the individual mutants from their amino acid composition (31) utilizing the ExPASY proteomic WWW server software from the Swiss Institute of Bioinformatics (32).

Tryptophan Fluorescence Measurements—Fluorescence emission at 332 nm (excitation at 290 nm) was recorded at 23 °C using 4 μM GCAP-1 in 0.6 ml of 100 mM MOPS/KOH (pH 7.2), 40 mM KCl, and 1 mM EGTA. Small aliquots of concentrated MgCl\(_2\) solution were added to obtain the desired free Mg\(^{2+}\) concentrations. The free Ca\(^{2+}\) and Mg\(^{2+}\) concentrations in solution were calculated according to the method of Brooks and Stoney (33), utilizing the algorithm of Marks and Maxfield (34). The data shown are representative from three to four independent experiments producing virtually identical results.

Guanylyl Cyclase (GC) Assay—Wild type RetGC-1 was expressed in HEK 293 human embryonic kidney cells as previously described (35). The assay mixture (25 μl) contained 30 mM MOPS/KOH (pH 7.2), 60 mM KCl, 4 mM NaCl, 1 mM dithiothreitol, 5 mM free Mg\(^{2+}\), 2 mM Ca/EGTA buffer, 0.3 mM ATP, 4 mM cGMP, 1 mM GTP, 1 μCi of [α-\(^{32}\)P]GTP, 0.1 μCi of [8-\(^{3}\)H]cGMP, GCAP-1, and HEK 293 cell membranes. The reaction mixture was incubated for 40 min at 30 °C, stopped by heating for 2.5 min at 95 °C, and the aliquots were analyzed by TLC using fluorescent plastic-backed polyethyleneimine cellulose plates (Merck) as described previously (9, 35). The data shown are representative from three to seven independent experiments producing virtually identical results.

RESULTS

Mutations That Inhibit Cation Binding in EF-hands of GCAP-1 and May or May Not Result in Constitutive Activation of RetGC—In our previous study we found that disabling all three metal-binding EF-hands in GCAP-1 by mutations that nonselectively hampered both Ca\(^{2+}\) and Mg\(^{2+}\) binding failed to produce a constitutive, Ca\(^{2+}\)-insensitive activation of RetGC-1 (22). That was contrary to what one would expect if the apo form of the GCAP were the activator form and argued that the substitution of Ca\(^{2+}\) by Mg\(^{2+}\), rather than merely loss of Ca\(^{2+}\), was required to make GCAP-1 undergo the inhibitor-to-activator transition in the light. If this hypothesis were true, then to make GCAP-1 a constitutive activator of RetGC it should be made unable to bind Ca\(^{2+}\), yet still retain the ability to bind Mg\(^{2+}\) in its EF-hand(s). We found that the replacement of the last Glu in Ca\(^{2+}\)-binding loops with Gln (Fig. 1) prevented binding of Ca\(^{2+}\), but had little effect on Mg\(^{2+}\) binding (22). Predictably, EF(2,3,4) mutant generated by such a substitution in all three metal-binding EF-hands, GCAP-1(E75Q/E111Q/E155Q) activated RetGC-1 at low Ca\(^{2+}\) and continued to activate it even at high concentrations of Ca\(^{2+}\), which turn wild type GCAP-1 into a RetGC-1 inhibitor (Fig. 2). Contrary to that, a different EF(2,3,4) mutant, GCAP-1(D64N/D100N/D102G/D144N/D148G), which binds neither Ca\(^{2+}\) nor Mg\(^{2+}\) (22), failed to activate RetGC within the same free Ca\(^{2+}\) range (Fig. 2). Evidently, for GCAP-1 to become a RetGC-1 activator under the conditions typical for light-adapted photoreceptors one or more of its EF-hands must be filled with Mg\(^{2+}\).

EF-hands 2 and 3 Require Mg\(^{2+}\) Binding for Activation of RetGC under Light-adapted Conditions—To identify the EF-hands that need to bind Mg\(^{2+}\) to maintain the activator state of GCAP-1, we disabled Mg\(^{2+}\) binding in the individual EF-hands by point mutations and tested their ability to activate RetGC-1 in the absence of Ca\(^{2+}\) in comparison with the wild type.
Functional States of GCAP-1 Controlled by Ca\(^{2+}\) and Mg\(^{2+}\) Binding

![Graph: The Ca\(^{2+}\) sensitivity of RetGC-1 regulation by wild type GCAP-1 and its EF(2,3,4)\(^{-}\) mutants.](image)

FIGURE 2. The Ca\(^{2+}\) sensitivity of RetGC-1 regulation by wild type GCAP-1 and its EF(2,3,4)\(^{-}\) mutants. Recombinant RetGC-1 was assayed at various free Ca\(^{2+}\) concentrations in the presence of 5 \(\mu\)M wild type GCAP-1 (C), E75Q/E111Q/E155Q GCAP-1 (A), or D64N/D100N/D102G/D144N/D148G GCAP-1 (D), at saturating 5 \(\mu\)M free Mg\(^{2+}\). The data for wild type GCAP-1 were fitted by the equation, \(A = (A_{max} - A_{min})/(1 + ([Ca]/K_{1/2})^n) + A_{min}\), where \(A\) is the activity of RetGC-1, \(A_{max}\) and \(A_{min}\) are the maximal and minimal activity of RetGC in the assay, respectively, \([Ca]_{1/2}\) is the free Ca\(^{2+}\) concentration required for half-maximal inhibition of RetGC-1 by GCAP, \(n\) is the cooperativity coefficient. For other conditions of the assay, see "Experimental Procedures."

GCAP-1 or EF-hand mutants that retained Mg\(^{2+}\) binding (Fig. 1 and Ref. 22).

A substitution in EF-hand 2, D64N, that prevents both Ca\(^{2+}\) and Mg\(^{2+}\) binding (22) was compared with the wild-type and E75Q substitution, which selectively preserves Mg\(^{2+}\) binding (22). Whereas the GCAP-1(E75Q) showed dose dependence \((K_{1/2})\) and the maximal activity of RetGC-1 stimulated by GCAP-1 \((A_{max})\) similar to the wild type, disabling of Mg\(^{2+}\) binding dramatically reduced the apparent affinity of GCAP-1(D64N) for RetGC-1, but had little effect on the \(A_{max}\) (Fig. 3A, Table 1).

Disabling Mg\(^{2+}\) binding in EF-hand 3 by a double mutation, D100N/D102G (22), noticeably reduced both the apparent affinity of GCAP-1 for RetGC-1 and its maximal activation. Opposite to that, a substitution that preserves Mg\(^{2+}\) binding, E111Q, affected neither \(A_{max}\) nor \(K_{1/2}\) (Fig. 3B, Table 1).

Unlike EF-hands 2 or 3, disabling Mg\(^{2+}\) binding in EF-hand 4 by a double substitution, D144N/D148G (22), had no effect on \(A_{max}\) or \(K_{1/2}\) (Fig. 3C, Table 1). These data strongly indicate that among the three metal-binding EF-hands of GCAP-1, binding of Mg\(^{2+}\) in EF-hands 2 and 3 is important for RetGC-1 activation, whereas Mg\(^{2+}\) binding in EF-hand 4 does not significantly contribute to creating the activator state of GCAP-1.

Indeed, simultaneous disabling of Mg\(^{2+}\) binding in both EF-hands 2 and 3 in GCAP-1(D64N/D100N/D102G) diminished its \(A_{max}\) and \(K_{1/2}\) even stronger than in the individually disabled EF-hands (Fig. 3D, Table 1). Unlike that, a different EF(2,3)\(^{-}\) mutant, E75Q/E111Q, that retained Mg\(^{2+}\) binding in EF-hands 2 and 3 (22) demonstrated \(A_{max}\) and \(K_{1/2}\) similar to the wild type (Fig. 3D, Table 1). However, because the value for \(K_{1/2}\) was difficult to determine more accurately due to the low activity of the mutant, we additionally verified that the GCAP-1(D64N/D100N/D102G) lost its ability to interact with RetGC-1 in a competition experiment, where RetGC-1 was activated by 3 \(\mu\)M wild type GCAP-1 at low free [Ca\(^{2+}\)] in the presence of increasing concentrations of the GCAP-1(D64N/D100N/D102G) (Fig. 3E). Even at 20-fold excess of the GCAP-1(D64N/D100N/D102G) over the wild type we find no evidence for its interference with the activation of RetGC-1. We therefore conclude that once GCAP-1 is lacking Mg\(^{2+}\) binding in both EF-hands 2 and 3, it becomes unable to properly interact with RetGC-1.

Mg\(^{2+}\) Binding in EF-hand 2 Affects Conformation of EF-hand-like Domain 1—Although both EF-hands 2 and 3 are involved in activation of RetGC-1 at low free Ca\(^{2+}\), it appeared that Mg\(^{2+}\) binding in EF-hand 2 was the most critical for GCAP-1 affinity for the cyclase. There is a significant conformational difference between the Mg\(^{2+}\)-bound and metal-free GCAP-1 (21, 22). In particular, binding of Mg\(^{2+}\) by EF-hand 2 and 3 causes structural changes in EF1, an EF-hand-like domain that cannot bind metal ion but is required for interaction of GCAPs with RetGC (18–20). These changes can be monitored by a change in fluorescence of Trp\(^{21}\) located in the middle of the GCAP-1 EF1 domain (22). Because in addition to the Trp\(^{21}\) GCAP-1 contains two more Trp residues, Trp\(^{51}\) and Trp\(^{94}\), we replaced them with Phe and used the resultant GCAP-1(W51F/W94F) mutant (22) as a template for creating two other mutants, GCAP-1(D64N/W51F/W94F) and GCAP-1(E75Q/W51F/W94F), both with additional mutations affecting EF-hand 2. Each contained a single Trp\(^{21}\) residue, but only the latter mutant was able to bind Mg\(^{2+}\) in EF-hand 2. The GCAP-1(W51F/W94F) exhibited a prominent Mg\(^{2+}\)-dependent decrease in Trp\(^{21}\) fluorescence caused by conformational changes in the EF1 domain (Fig. 4 and Ref. 22). Similar results were observed with the GCAP-1(E75Q/W51F/W94F), whose EF-hand 2 was modified by the E75Q mutation that preserved Mg\(^{2+}\) binding (Fig. 4). Contrary to that, disabling Mg\(^{2+}\) binding in EF-hand 2 by the D64N substitution in GCAP-1(D64N/W51F/W94F) prevented decrease in the Trp\(^{21}\) fluorescence. These results suggest that Mg\(^{2+}\) binding in EF-hand 2 creates proper conformation of the neighboring EF-hand-like domain, known to be required for GCAPs interaction with RetGC-1 (18, 19). The exact reason why Trp\(^{21}\) fluorescence does not stay completely flat but slightly increases in the D64N mutant is unknown, but one possible explanation can be that when EF-hand 2 is in its apo (non-physiological) form, cation binding to the neighbor EF-hand 4, through conformational change in the rest of the molecule, affects the environment for Trp\(^{21}\) and thus produces the small increase in fluorescence. It is therefore only possible to see this effect when the EF-2 is inactivated and the rest of the Trp residues in the molecule are removed. In the presence of the functional EF-hand 2 the influence from the rest of the molecule on the environment of Trp\(^{21}\) is strongly opposed by cation binding in the neighboring EF-hand 2 that results in a decrease of fluorescence.

Ca\(^{2+}\) Binding and Inhibition of RetGC—In photoreceptor cells, GCAP-1 undergoes transition between the Mg\(^{2+}\)- and Ca\(^{2+}\)-bound forms as the concentration of intracellular Ca\(^{2+}\) changes between light and dark (21, 22), a process that turns GCAP-1 from RetGC activator into RetGC inhibitor. The replacement of Mg\(^{2+}\) by Ca\(^{2+}\) occurs in all three EF-hands (22), however, it is still unclear how individual EF-hands of GCAP-1 contribute to its transition to the RetGC inhibitor form. Another group
Functional States of GCAP-1 Controlled by Ca\(^{2+}\) and Mg\(^{2+}\) Binding

**FIGURE 3.** The effect of various substitutions in GCAP-1 EF-hands on RetGC-1 activation. Mutations that either prevented or preserved Mg\(^{2+}\) binding were introduced in the individual EF-hands of GCAP-1 and the activity of RetGC-1 was measured in the presence of 2 mM EGTA, 5 mM free Mg\(^{2+}\), and increasing concentrations of GCAP-1 as described under "Experimental Procedures." 

| Mutants | \(A_{\text{max}}\) \((\text{nmol cGMP/min})\) | \(K_{1/2}\) \((\text{M})\) |
|---------|----------------------------------|---------|
| Wild type | 25.6 ± 2.4 (\(n=3\)) | 1.56 ± 0.5 (\(n=3\)) |
| EF(2)* | D64N | 26.8 ± 0.7 (\(n=3\)) | 1.05 ± 0.04 (\(n=3\)) |
| EA111Q | 12.6 ± 2.4 (\(n=4\)) | 5.0 ± 1.5 (\(n=4\)) |
| EF(3)* | D100N/D102G | 25.6 ± 1.1 (\(n=3\)) | 1.3 ± 0.7 (\(n=3\)) |
| E111Q | 25 ± 0.3 (\(n=3\)) | 0.6 ± 0.1 (\(n=3\)) |
| EF(2,3)* | D64N/D100N/D102G | 7.5 ± 0.6 (\(n=3\)) | 65 ± 22 (\(n=3\)) |
| E75Q/E111Q | 26.2 ± 1.9 (\(n=3\)) | 1.5 ± 0.25 (\(n=3\)) |

\(A_{\text{max}}\) represents the maximal level of RetGC-1 activation by GCAP at low free Ca\(^{2+}\) (mean ± S.D.). 

\(K_{1/2}\) represents the concentration of GCAP required for half-maximal activation of RetGC-1 (mean ± S.D., \(n\) is the number of independent measurements).

Previously reported that disabling of Ca\(^{2+}\) binding in EF-hands 3 and 4 by substitution of the last Glu with Asp in the 12-amino acid Ca\(^{2+}\)-binding loop converted GCAP-1 into a constitutive activator of RetGC (25). That mutant showed a similar to wild type GCAP-1 dose dependence of RetGC stimulation at low free Ca\(^{2+}\) but did not inhibit cyclase at high free Ca\(^{2+}\) (25). However, the mutations used in Ref. 25 do not disable binding of Mg\(^{2+}\) (22, 36–38). Therefore, to revisit this question, we used an EF(3,4)* mutant with EF-hands 3 and 4 disabled by different mutations, D100N/D102G and D144N/D148G, respectively. These mutations prevented both Mg\(^{2+}\) and Ca\(^{2+}\) binding to EF-hands 3 and 4, but preserved high-affinity Ca\(^{2+}\) and Mg\(^{2+}\) binding in EF-hand 2 (22). At low free Ca\(^{2+}\) the D100N/D102G/D144N/D148G EF(3,4)* mutant was able to activate RetGC-1, but much less efficiently than wild type GCAP-1 (Fig. 5 and Table 2). Surprisingly, Ca\(^{2+}\) sensitivity of RetGC-1 regulation by this mutant was reversed: increase in Ca\(^{2+}\) concentrations further increased RetGC-1 stimulation (Fig. 5A), instead of inhibiting it. Such an increase could only result from Ca\(^{2+}\) binding in EF-hand 2, because EF-hands 3 and 4 in this mutant do not bind Ca\(^{2+}\), even at the highest concentration used in the assay (22). Its apparent affinity for RetGC-1 was also reduced at low Ca\(^{2+}\) concentrations but increased with the rise of free Ca\(^{2+}\) in the assay (Table 2).

The EF(2,3,4)* mutant, D64N/D100N/D102G/E75Q/E111Q, activated RetGC-1 in the absence of Ca\(^{2+}\) similarly to
the GCAP-1 EF(3,4)− mutant, D100N/D102G/D144N/D148G, because the E75Q mutation preserves Mg2+ binding in EF-hand 2 (22). However, it did not regulate RetGC-1 in a Ca2+-sensitive manner (Fig. 5A), because none of its EF-hands could now bind Ca2+ (22). The concentration of free Mg2+ in the assay was 5 mM, which saturates binding to EF-hand 2 in wild type GCAP-1 and in the EF(2,3,4)− mutant, E75Q/D100N/D102G/D144N/D148G (22). The transition of EF-hand 2 from the Mg2+-bound to the Ca2+-bound form substantially increased the apparent affinity of the GCAP-1(D100N/D102G/D144N/D148G) for RetGC-1 (Table 2). The GCAP-1(E75Q/D100N/D102G/D144N/D148G) that could not exchange Mg2+ in EF-hand 2 for Ca2+ exhibited almost no change in its apparent affinity for RetGC-1 between the low and high free Ca2+ (Fig. 5 and Table 2). Thus, in this artificial situation, when cation binding in EF-3 and EF-4 is completely disabled, the transition of EF-hand 2 from the Mg2+-bound to the Ca2+-bound form does not make GCAP-1 a RetGC-1 inhibitor and can even mimic RetGC-1 activator conformation.

Consistent with the earlier observations (25), this finding argues that EF-hand 2 is not essential for the Ca2+-dependent inhibition of RetGC-1 by GCAP-1. Indeed, inactivation of EF-hand 2 by a mutation, E75Q, did not make GCAP-1 a constitutive activator of RetGC-1 (Fig. 6 and Ref. 25). However, unlike the total lack of effect in the Ref. 25, we also found that disabling of Ca2+ binding in EF-hand 2 by the E75Q mutation in the conditions of saturation by Mg2+ to some extent affected both the cooperativity (n decreased from 2.34 ± 0.1 (n = 5) in wild type to 1.7 ± 0.06 (n = 3) in E75Q) and Ca2+ sensitivity of RetGC-1 regulation by GCAP-1 (Fig. 6). One possible explanation for the difference between the two observations is that the E75Q mutation in EF-hand 2 blocks Ca2+ binding more efficiently than the E75D, as we showed previously (22).

Contrary to the EF-hand 2, inactivation of both EF-hands 3 and 4 did make GCAP-1 a constitutive activator of RetGC-1, and so did the inactivation of individual EF-hands 3 or 4 (Fig. 6 and Ref. 25). Yet, at this point we cannot determine the direct contribution of EF-hand 3 in RetGC-1 inhibition by GCAP-1. Although our present findings do not exclude that this indeed may be the case, we found that disabling of EF-hand 3 by various mutations always dramatically decreased the affinity of wild type EF-hand 4 for Ca2+ (22). In other words, we were unable to find a mutation that would disable Ca2+ binding in EF-hand 3 without also hampering Ca2+ binding in EF-hand 4 at the same time. In addition to that, the results shown in Fig. 6 can argue that Ca2+ binding in EF-hand 3 itself has a relatively small additional effect when compared with EF-hand 4. Hence, even in its Ca2+-bound form EF-hand 3 mostly preserves the activator form of the GCAP-1 rather than provides a conformational switch for RetGC inhibition. Therefore, it is more likely that the main role of EF-hand 3 in

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**TABLE 2**

Effect of Ca2+ binding in EF hand 2 on activation of RetGC-1 by EF(3,4)− mutant

| Mutants | Amax | K1/2 | μM |
|---------|------|------|----|
| GCAP-1 EF(3,4)− in EGTA | 16 ± 2.6 (n = 4) | 4.4 ± 0.8 (n = 4) |
| GCAP-1 EF(3,4)− at 10 μM [Ca2+] | 22 ± 3.5 (n = 3) | 1.2 ± 0.3 (n = 3) |
| GCAP-1 E75Q/EF(3,4)− in EGTA | 18 ± 0.0 (n = 3) | 6.3 ± 0.3 (n = 3) |
| GCAP-1 E75Q/EF(3,4)− at 10 μM [Ca2+] | 15 ± 1.0 (n = 3) | 4.2 ± 0.3 (n = 3) |

* Amax, the maximal level of RetGC-1 activation by GCAP at indicated free Ca2+ in the presence of 5 mM free Mg2+ (mean ± S.D. n is the number of independent measurements).

* K1/2, the concentration of Ca2+ required for half-maximal activation of RetGC-1 (mean ± S.D. n is the number of independent measurements).

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**FIGURE 4.** Effect of disabling of Mg2+-binding in EF-hand 2 on the single Trp21 fluorescence in EF-hand 1. Mutations, E75Q (A) or D64N (C), were introduced to inactivate EF-hand 2 in a GCAP-1 mutant, W51F/W94F (D), that has a single remaining Trp21 residue (22). The fluorescence of the Trp21 was recorded as a function of Mg2+ (mean ± S.D., n is the number of independent measurements).

**FIGURE 5.** Effect of EF-hand 2 transition between its Mg2+- and Ca2+-bound state on RetGC-1 activation. A, recombinant RetGC-1 was assayed for GC activity at 5 mM free Mg2+ and variable free Ca2+ concentrations in the presence of 2 μM GCAP-1; C, wild type; B, D100N/D102G/D144N/D148G; D, E75Q/D100N/D102G/D144N/D148G. B and C, dose dependence of RetGC-1 activation by GCAP-1(D100N/D102G/D144N/D148G) (8) or GCAP-1(E75Q/D100N/D102G/D144N/D148G) (C) in the presence of 1 mM EGTA (open symbols) or 10 μM free Ca2+ (filled symbols). The concentration of free Mg2+ in the GC assay was 5 mM. The data were fitted as described in the legend to Fig. 3. The values of Amax and K1/2 are summarized in Table 2.
Ca$^{2+}$-dependent inhibition of RetGC can be indirect, through regulation of Ca$^{2+}$ binding to the neighboring EF-hand 4.

**DISCUSSION**

The Role of EF-hands in RetGC Regulation by GCAP-1—GCAP-1 contains four EF-hand structures of which three can bind either Ca$^{2+}$ or Mg$^{2+}$ under physiologically relevant conditions (Fig. 7A). We find in this study that both EF-hands 2 and 3 in GCAP-1 must be occupied by Mg$^{2+}$ to maintain GCAP-1 in its RetGC activator state under the conditions that exist in photoreceptors in the light (Fig. 3, Table 1). The apo EF-hands 2 and 3 do not support the GCAP-1 conformation required for the interaction with the cyclase (Fig. 3). We consider one of the most important findings of this study that the EF-hand 2, whose role in RetGC regulation was previously deemed unclear (25), is in fact a crucial element in RetGC regulation by GCAP-1, and the cation binding in EF-hand 2 is required for high-affinity interaction with RetGC (Figs. 3 and 5 and Tables 1 and 2). Another EF-hand that cannot effectively maintain the activator conformation of GCAP-1 in its apo form is EF-hand 3. It also note that both EF-hands 2 and 3 retain the activator conformation required for RetGC stimulation. The apo forms of these EF-hands do not create the proper conformation for GCAP-1. In the dark, all three EF-hands of GCAP-1 are predominantly occupied by Ca$^{2+}$, however, the main requirement for converting GCAP-1 into the "RetGC inhibitor" is binding of Ca$^{2+}$ in EF-hand 4. Ca$^{2+}$ binding in EF-hands 2 and 3 is primarily required for GCAP-1 to preserve binding to RetGC and to facilitate the high-affinity binding of Ca$^{2+}$ to EF-hand 4. B. Ca$^{2+}$/Mg$^{2+}$ exchange in EF-hands of GCAP-1 between light and dark provides functional switch between its "RetGC activator" and RetGC inhibitor states. The apo form of GCAP-1 has no function. Other explanations are under "Discussion."

FIGURE 6. Effect of individual EF-hand inactivation in GCAP-1 on Ca$^{2+}$ sensitivity of RetGC-1 regulation. RetGC activity was measured as a function of free Ca$^{2+}$ concentrations at 5 mM free Mg$^{2+}$ in the presence of 2 μM wild type GCAP-1 (c), E75Q (©), E11Q (▪), E155Q (●), D144N/D148G (▲), or E111Q/D144N/D148G (Δ). The data for wild type GCAP-1 and E75Q mutant were fitted by the equation, $A = (A_{\text{max}} - A_{\text{min}})/(1 + ([\text{Ca}] / [\text{Ca}_{1/2}])) + A_{\text{min}}$, where $A$ is the activity of RetGC-1, $A_{\text{max}}$ and $A_{\text{min}}$ are the maximal and minimal activity of RetGC in the assay, respectively, and $[\text{Ca}]_{1/2}$ is the free Ca$^{2+}$ concentration required for half-maximal inhibition of RetGC-1 by GCAP, $n$ is the Hill coefficient. For other conditions of the assay see "Experimental Procedures."
it was always suppressed when Ca\(^{2+}\) binding in the EF-hand 3 was affected (but not vice versa). Therefore, it is likely that EF-hand 3 contributes to switching off the cyclase primarily through its indirect effect on the affinity of the EF-hand 4 for Ca\(^{2+}\), rather than in directly providing a conformational switch for RetGC inhibition. This would also suggest that Ca\(^{2+}\) binding in the EF-3/EF-4 globular domain occurs sequentially, first in EF-hand 3 and only after that in EF-hand 4.

The EF-hand domain 1, which cannot itself coordinate metal, has been shown to be crucial for GCAP-1 and GCAP-2 interaction with RetGC (18–20, 30, 42) and in some other NCS proteins for interaction with their targets (43–45). We found a striking effect of EF-hand 2 occupation by Mg\(^{2+}\) on the conformation of the EF-hand 1 domain, revealed by its fluorescence spectra (Fig. 4). It is therefore tempting to speculate that EF-hand domain 1 is not only directly involved in high-affinity binding to RetGC, but also that high-affinity binding is directly controlled via the conformation of its neighboring metal-binding EF-hand 2.

To summarize, based on this and the number of previous studies (19, 20, 30, 42), we can propose the following functions to the EF-hands in GCAP-1 (Fig. 7A). (i) EF-hand-like domain 1: no metal binding, and contributes to the high-affinity interaction with RetGC. (ii) EF-hand 2: binding of Mg\(^{2+}\) in the light maintains the activator conformation of GCAP-1, controls the high-affinity binding of GCAP-1 to RetGC-1, presumably through involving EF-hand-like domain 1; it is not essential for the maximal level of stimulating activity of GCAP-1; and replacement of Mg\(^{2+}\) by Ca\(^{2+}\) in this EF-hand has only a small effect on inhibition of RetGC. (iii) EF-hand 3: binding of Mg\(^{2+}\) in the light maintains the optimal conformation of GCAP-1 for RetGC activation and, although to a lesser extent, contributes to the high-affinity GCAP/RetGC interaction; binding of Ca\(^{2+}\) fails to create the inhibitory conformation of GCAP-1, but most likely contributes to inactivation of the cyclase indirectly, by facilitating Ca\(^{2+}\)-binding in the neighboring EF-hand 4. (iv) EF-hand 4: binding of Mg\(^{2+}\) in this EF-hand contributes to neither binding nor activation of RetGC in the light; binding of Ca\(^{2+}\) in this EF-hand provides a potent functional switch to turn the cyclase off.

**Mg\(^{2+}\)/Ca\(^{2+}\) Cycle in GCAP-1 Controls RetGC-1 Regulation**—Free Ca\(^{2+}\) concentrations in rods and cones change nearly 10-fold in response to illumination, in mammals between 250 nM in the dark and 25 nM in the light (5–7). This provides a potent feedback for RetGC that accelerates the recovery of rods and cones (reviewed in Refs. 1, 4, and 46). Contrary to the previous view on GCAPs role in RetGC regulation via release and binding of Ca\(^{2+}\) to the apo form of GCAPs, we now argue that the actual process of switching the GCAP-1 between the activator and the “inhibitor” state is instead based on Mg\(^{2+}\)/Ca\(^{2+}\) exchange, summarized in the schematics presented in Fig. 7.B. The apo form of GCAP-1 is neither activator nor inhibitor of RetGC-1 and has no physiological role. Much like GDP/GTP exchange is required to regulate the conformation of a G-protein, the cation exchange in GCAP-1 is required to regulate RetGC-1. The fundamental differences from the GDP/GTP exchange in G-proteins is nevertheless, apparent: no external receptor is required for GCAP-1 to release or bind the divalent cations, and this can be accomplished solely as a result of the free Ca\(^{2+}\) concentration change relative to the free Mg\(^{2+}\) concentrations in the dark versus light.

The critical difference between the activator and the inhibitor conformation of GCAP-1 must be very subtle compared, for example, with that of recoverin (49–51). This is not a difference between the apo protein and the metal bound form, but between the two metal-bound forms, Mg\(^{2+}\) versus Ca\(^{2+}\). Moreover, EF-hands 1, 2, and 3 are even less likely to undergo a major change between Mg\(^{2+}\) and Ca\(^{2+}\) forms, because EF-2 and EF-3 are both capable of maintaining the high-affinity interaction with the cyclase in either Mg\(^{2+}\) or Ca\(^{2+}\) form. To date, only a partial Ca\(^{2+}\)-bound structure of two other GCAPs was established (47, 48), but to properly understand the mechanism and the structural basis for the RetGC regulation, one would need to compare, potentially rather subtle, differences between Mg\(^{2+}\)- and Ca\(^{2+}\)-bound GCAP-1.

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