Instead of Binding Calcium, One of the EF-hand Structures in Guanylyl Cyclase Activating Protein-2 Is Required for Targeting Photoreceptor Guanylyl Cyclase*

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Guanylyl cyclase activator proteins (GCAPs) are calcium-binding proteins closely related to recoverin, neurocalcin, and many other neuronal Ca2+-sensor proteins of the EF-hand superfamily. GCAP-1 and GCAP-2 interact with the intracellular portion of photoreceptor membrane guanylyl cyclase and stimulate its activity by promoting tight dimerization of the cyclase subunits. At low free Ca2+ concentrations, the activator form of GCAP-2 associates into a dimer, which dissociates when GCAP-2 binds Ca2+ and becomes inhibitor of the cyclase. GCAP-2 is known to have three active EF-hands and one additional EF-hand-like structure, EF-1, that deviates from the EF-hand consensus sequence. We have found that various point mutations within the EF-1 domain can specifically affect the ability of GCAP-2 to interact with the target cyclase but do not hamper the ability of GCAP-2 to undergo reversible Ca2+-sensitive dimerization. Point mutations within the EF-1 region can interfere with both the activation of the cyclase by the Ca2+-free form of GCAP-2 and the inhibition of retGC basal activity by the Ca2+-loaded GCAP-2. Our results strongly indicate that evolutionary conserved and GCAP-specific amino acid residues within the EF-1 can create a contact surface for binding GCAP-2 to the cyclase. Apparently, in the course of evolution GCAP-2 exchanged the ability of its first EF-hand motif to bind Ca2+ for the ability to interact with the target enzyme.

Light-induced hyperpolarization of the vertebrate photoreceptor plasma membrane inhibits the release of the neurohormone, glutamate, from the synaptic termini of rods and cones and thus generates the signal for the secondary neurons of the retina. As the first step in visual signal transduction, photosomized rhodopsin triggers hydrolysis of cGMP by activating a G-protein, transducin, that subsequently stimulates a cGMP phosphodiesterase, PDE6, and this causes cGMP-gated cation channels to close (see Refs. 1–3 for review). Both rods and cones can quickly recover to their resting potential after the excitation induced by a non-saturating flash of light. Exposure of photoreceptors to a constant illumination at first saturates their response, but through a complex process of light adaptation, the cells can reopen cGMP-gated cation channels and partially restore their light sensitivity. Ca2+-sensitive synthesis of cGMP by membrane guanylyl cyclase (retGC) plays one of the major roles among multiple reactions that result in opening of the cGMP-gated channels during recovery and light adaptation (4, 5). A Na+/Ca2+-exchanger continuously extrudes Ca2+ ions from the photoreceptor outer segment; therefore, when cGMP is hydrolyzed and the Na+/Ca2+ influx is stopped, the intracellular Ca2+ concentrations in rods and cones can decrease from near 500–600 nM in the dark down to 50 nM in the light (6, 7). In response to the decrease in free Ca2+ concentrations, Ca2+-binding proteins, GCAPs (reviewed in Refs. 8–10), accelerate synthesis of cGMP by retGC (reviewed in Refs. 11 and 12).

Two homologous GCAPs (GCAP-1 and -2) have been directly isolated from the retina, and the existence of a gene for the third homologue, GCAP-3, has been revealed by cDNA cloning in some vertebrate species (13–15). GCAP-2 is highly expressed in rods, whereas GCAP-1 is expressed at high levels in cones and at lower levels in rods (16–19). Disruption of both GCAP-1 and GCAP-2 genes results in abnormally slow recovery in mouse rods, especially in response to strong flash of light, consistently with the expected slower accumulation of cGMP in the absence of GCAPs. Although the relative contribution of GCAP-1 and -2 to the kinetics of dim flash responses in rods and cones remains unclear, GCAP-2 in vitro stimulates the activity of both known isoforms of retGC (retGC-1 and -2) present in photoreceptor membranes (20), and expression of GCAP-2 alone in GCAP-1/GCAP-2 knockout mice can restore the rate at which rods recover after a bright flash of light (21).

GCAPs are closely related to other recoverin-like proteins (22, 23) within the EF-hand superfamily. Similar to other members of this family, GCAPs are 24-kDa N-fatty-acylated proteins that contain four helix-turn-helix EF-hand structures (EF-1 through EF-4, Fig. 1), of which three (EF-2, -3, and -4) determine Ca2+ sensitivity of GCAPs. Similarly to other proteins of this group, in the first EF-hand domain, EF-1, the amino acid sequence corresponding to the Ca2+-binding loop is disrupted and does not have all of the proper side chain residues required for binding Ca2+ ion (22, 23). In their Ca2+-free form GCAPs stimulate the activity of guanylyl cyclase, but upon binding Ca2+ they undergo an activator-to-inhibitor transition (24, 25).

GCAPs activate retGC by enhancing dimerization of the cyclase subunits (26) required for the cyclase catalytic activity (27). Ca2+-free GCAP-2 can itself form a stable homodimer that

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‡ The abbreviations used are: retGC, photoreceptor membrane guanylyl cyclase; GCAP, guanylyl cyclase activating protein; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid.
can be detected by high resolution gel chromatography (28). Its dimerization is highly Ca\(^{2+}\)-sensitive so that Ca\(^{2+}\)-loaded GCAP-2 quickly dissociates into monomers (28). Previous results indicate that dimerization of the Ca\(^{2+}\)-free GCAP-2 is likely to be a part of a mechanism (“dimer-adapter” hypothesis) by which it modulates the interaction between the cyclase subunits (9, 28).

The three-dimensional structure of Ca\(^{2+}\)-loaded GCAP-2 is very similar to that of recoverin and neurocalcin (29), although the structure of the Ca\(^{2+}\)-free (activator) form of GCAP-2 remains undetermined. Potential binding sites for GCAP-2 in retGC have been studied using synthetic peptides, chemical cross-linking, and deletion analyses. According to these studies, there are several fragments in retGC kinase homology and catalytic domains that can make contact with GCAPs (30–32). However, the location of the binding sites for retGC within the GCAP-2 molecule remains rather obscure. Previous efforts to map functionally significant regions in GCAP-2 using GCAP-2/neurocalcin and GCAP-2/recoverin chimera or deletion mutants revealed that in addition to the three Ca\(^{2+}\)-binding EF-hand loops, there were three segments of the molecule that could not be exchanged for the corresponding regions from other recoverin-like proteins without loss of GCAP function as a Ca\(^{2+}\)-sensitive cyclase regulator (33). Surprisingly, one of those three regions, Lys\(^{295}\)–Phe\(^{468}\), included the first EF-hand-like motif that lost its ability to bind Ca\(^{2+}\). In the present paper, we demonstrate that various point mutations in this region can specifically inhibit interactions of GCAP-2 with the target enzyme. All tested EF-1 mutants of GCAP-2 that were unable to stimulate or inhibit the cyclase were still able to form dimers in the absence of Ca\(^{2+}\), similar to the wild type GCAP-2. We conclude that the first EF-hand-like motif in GCAP-2 participates in targeting retGC. These results are consistent with the model according to which GCAP-2 must have two independent functional contact surfaces, one for binding to the effector enzyme and the other for dimerization of the Ca\(^{2+}\)-free GCAP-2. We speculate that the ability to bind Ca\(^{2+}\) within the first EF-hand of GCAP-2 was lost in exchange for the ability to interact with the target cyclase.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis—** Mutant DNAs were constructed by using polymerase chain reaction (PCR). DNA fragments were first amplified by PCR using \(P_{fu}\) polymerase (Stratagene), bovine GCAP-2 cDNA as a template, and PCR primers containing mutations of the choice. The fragments were purified from agarose gel using a Promega Wizard kit template, and PCR primers containing mutations of the choice. The resulting Mutant DNAs were constructed by using PCR using specific for each particular member of the family (23). Also, assayed as described previously (16, 35). Experiments were conducted under infrared illumination using two 15-watt safety lights equipped with Kodak No. 11 infrared filters at a distance of \(50 \text{ cm}\) and an Excilab-2 dual high performance GEN II+ tube (PVS-3C) galleys. A typical receptor mixture contained \(0.2 \mu\text{M}\) GCAP-2 or its mutants in 25 mM 50 mM MOPS-KOH (pH 7.5), 60 mM KCl, 8 mM NaCl, 10 mM MgCl\(_2\), 2 mM Ca-EGTA buffer, 10 \(\mu\text{M}\) each of dipyridamole and zaprinast, 1 mM ATP, 1 mM GTP, 4 mM cGMP, 1 \(\mu\text{M}\) of \(\alpha\text{-[gamma-32P]}\text{GTP}, 0.1 \mu\text{M}\) of \(\text{H}[\text{cGMP}],\) and washed bovine outer segment membranes (approximately 3.5 \(\mu\text{g}\) of rhodopsin). The free Ca\(^{2+}\) concentrations were buffered at \(6 \text{ nM}\) or added to saturation (\(10^{-6} \text{ M}\)). The assay mixtures were incubated for 60 min at 30 °C, heated for 2 min at 95 °C, chilled on ice, centrifuged for 5 min at 10,000 \(g\), and analyzed by TLC on polyethylene imine cellulose plastic-backed plates with fluorescent background (Merck). The TLC plates were developed in 0.2 mM LiCl, cGMP spots were visualized under UV illumination, cut from the plate, eluted with 1 ml of 2 M LiCl, mixed with 10 ml of an Ecolume scintillation mixture (ICN), and both 2H and 3P radioactivity was counted. [3H]cGMP was used as the internal standard to ensure the absence of cGMP hydrolysis by phosphodiesterase. In all experiments the time course of the reaction was linear within the time of assay, and less than 10% of the GTP substrate was converted into cGMP. Basal activity of retGC in different preparations of washed photoreceptor outer segment membranes typically varied between 2 and 4 nmol of cGMP/min/mg of rhodopsin. Data shown in figures pertain to each individual experiment with the same preparation of washed photoreceptor outer segment membranes representative of at least two or three independent experiments giving similar results. In the absence of protein activators, the difference between retGC basal activity measured at 6 \(\text{nM}\) versus \(\geq 1 \text{ \muM}\) did not exceed 20%.

**High Resolution Gel Chromatography—** Recombinant proteins were injected in a volume of 200 \(\mu\text{l}\) into a Superdex 200 HR10/30 column (Amersham Pharmacia Biotech) using an automated FPLC system and eluted at 0.5 \(\text{ml/min}\) in buffer A (20 mM Tris-HCl, 50 mM KCl, 10 mM NaCl, 10 mM MgCl\(_2\), 1 mM dithiothreitol) containing either 400 \(\mu\text{M}\) EGTA or 300 \(\mu\text{M}\) CaCl\(_2\). The column was pre-equilibrated with two volumes of corresponding buffer between the runs. Free Ca\(^{2+}\) concentrations in the samples were adjusted prior to injection by adding EGTA or CaCl\(_2\), respectively. The standards used for calibration of the gel filtration column were blue dextran (2000 \(\text{kDa}\)), a-amylase (200 \(\text{kDa}\)), alcohol dehydrogenase (150 \(\text{kDa}\), Stokes radius = 45.5 \(\text{Å}\)), bovine serum albumin (66 \(\text{kDa}\), Stokes radius = 36.1 \(\text{Å}\)), carbonic anhydrase (29 \(\text{kDa}\), Stokes radius = 20.1 \(\text{Å}\)), and cytochrome c (12.4 \(\text{kDa}\)) (all from Sigma). Retention time corresponded to 29 \(\text{kDa}\) for the peak of GCAP-2 monomer and approximately 58–63 \(\text{kDa}\) for the dimer (29).

**RESULTS AND DISCUSSION**

Based on its NMR structure determined by Ames et al. (29), GCAP-2 is similar to recoverin and neurocalcin and consists of two globular pairs of EF-hand structures connected by a “hinge” region between EF-2 and EF-3 (Fig. 1). Three EF-hands (EF-2, -3, and -4) contribute to the functional switch that causes GCAP-2 to undergo an “activator-to-inhibitor” transition when it binds Ca\(^{2+}\) (\(E_{\text{Ca}}\)) \(200–300 \text{ ns}\), Hill coefficient \(1.7–2.1\), Ref. 8). Three Ca\(^{2+}\) ions bind to GCAP-2 with an apparent \(K_{\text{Ca}}\) of \(\sim 300 \text{ ns}\) and the cooperativity factor of 2 (29). Unlike these three EF-hands, the first EF-hand-related motif is short of several key side chain residues necessary for high affinity binding of Ca\(^{2+}\). To efficiently coordinate Ca\(^{2+}\) ion in the EF-hand loop by five side chain residues and one carboxyl oxygen of the main chain (36–38), it is required that the first Ca\(^{2+}\)-coordinating position (X) of the 12-amino acid consensus motif be occupied by Asp, and both the positions 3 (Y) and 9 (X) include oxygen-containing side chain residues (36). The absence of the first invariant Asp (replaced by Glu\(^{35}\)) of the consensus motif and replacement of the obligatory oxygen-containing side chain residues by Cys\(^{35}\) (Y) and Phe\(^{45}\) (X) prohibit Ca\(^{2+}\) binding within the loop structure of the EF-1 domain (16, 29).

Although the EF-1 domain is unable to bind Ca\(^{2+}\), its amino acid sequence remains highly homologous between recoverin-like proteins, except for several variable residues that are specific for each particular member of the family (23). Also,
regardless of its deviation from the EF-hand Ca\(^{2+}/\)H11001 binding consensus motif, EF-1 retains an overall shape of a helix-loop-helix domain similar to other EF-hands (29).

A previous observation (33) indicated that the EF-1 was an essential part of the GCAP-2 molecule. Substitution of that domain in GCAP-2 with the corresponding segment from neurocalcin resulted in a loss of GCAP-2 activity. Yet the functional role of the EF-1 remained unclear. The uncertainty has been 2-fold. First, the retGC activator form of GCAP-2 can associate as a homodimer and dissociate in the presence of Ca\(^{2+}/\)H11001. The inability of several GCAP-2 chimera mutants to form Ca\(^{2+}/\)H11001-free dimers correlated with the lack of their ability to promote retGC activation in the absence of Ca\(^{2+}\) (28). Therefore, the EF-1 could be involved in either target binding or dimerization of GCAP-2 (or both). Second, it could not be completely excluded that substitutions of a relatively large segment in the molecule, even with the corresponding fragment from a homologous protein, causes general misfolding of the protein. To further elucidate the possible functional role of the EF-1 domain in GCAP-2, we probed this region by single mutations that substituted some highly conserved (Cys35, Glu44) or variable (Lys30, Glu33, Phe41, His43, Phe48) amino acids (Fig. 1).

We have found that activation of retGC at low Ca\(^{2+}\) concentrations is highly sensitive to even single substitutions in most of these amino acid residues (Fig. 2, A–F). One of the invariant residues within the EF-1 motif of recoverin-like proteins is Cys35. Substitution of Cys35 with Ser (Fig. 2B) or Thr (data not shown) noticeably affects retGC activation, and replacing it with positively or negatively charged amino acids strongly suppresses the ability of GCAP-2 to activate the cyclase. The most dramatic effect has been observed in the case of negatively charged Asp that renders the mutant protein virtually inactive. Another fairly highly conserved amino acid residue, Glu44, is also essential for retGC activation because its substitution even with another acidic residue, Asp, causes a prominent decrease in activity of GCAP-2, whereas its substitution with a non-charged oxygen-containing side chain, Ser, completely inactivates the retGC stimulating activity of the Ca\(^{2+}\)-free GCAP-2 (Fig. 2E).

Similar to that, substitutions of several GCAP-2-specific amino acids had a profound negative effect on retGC stimulation by the Ca\(^{2+}\)-free GCAP-2 (Fig. 2, A, C, D, and F). Some...
of the substitutions markedly or even completely suppressed cyclase stimulation (for example, K30G, F41I, H43E, H43Q, F48V, F48S).

Apparently, some amino acid side chain residues within the EF-1 domain are not essential for the interaction with the cyclase (Fig. 2, G and H). For example, we did not find evidence that replacement of Glu33 with Gln, or even positively charged Lys, can seriously affect retGC activation. The side chain of Tyr81 (which is a part of the exiting helix in the second EF-hand) is located in close proximity to the amino acid residues of the EF-1 structure (Ref. 29). It can be replaced with the amino acids not found in this position in GCAPs, Ile or Phe, without a prominent effect on cyclase activation (Fig. 2I).

Why would different mutations within the EF-1 domain affect retGC activation? If the amino acid residues in the EF-1 were required for GCAP-2 binding to the cyclase, then one would expect to find mutations within this domain that reduce the apparent affinity of GCAP-2 in our retGC activation assay. Indeed, several substitutions that reduced GCAP-2 activity apparently decreased its affinity for the cyclase. For example, the C35T and the C35S GCAP-2 mutants had their EC50 values increased 2.5- and 6-fold, respectively, compared with the wild type GCAP-2, whereas at saturation (above 2.5 μM, data not shown) both GCAP-2 mutants fully activated retGC (to 103 and 100% of the wild type control level, data not shown). Similarly to that, the H43Q mutant had at least 10-fold higher EC50, but at saturation (above 10 μM GCAP-2) the cyclase stimulation reached 88% of the wild type control level.

In several cases (i.e. C35D, C35K, F41I, E44S, F48S) retGC activation by the GCAP-2 mutants remained very low and did not reach saturation in the conditions of the assay. For that reason, we were unable to reliably evaluate the exact values of their EC50, but it appeared increased at least 5–10-fold. In addition to that, we found that some mutations affected the maximal level of retGC activation rather than EC50. The EC50 increase in the case of the F48V mutant was only within 2-fold, but the cyclase activation at saturation did not exceed 30% of the wild type control.

The right shift of a dose dependence curve in case of the EF-1 mutants could be in a most simplified manner interpreted as a decrease in retGC binding affinity. It would be more difficult to explain the lower level of maximal cyclase activation found in some cases. At this point, we cannot offer any conclusive explanation for this phenomenon. Activation of the cyclase may require multiple steps, and various conformational changes in GCAP-2 may have to occur before retGC is activated. Perhaps the EF-1 domain may not only be involved in binding interactions with the cyclase but also through the intramolecular interactions influence other functional domains of GCAP-2 (for instance, EF-2) and thus affect the overall conformational switch in GCAP-2. However, our present results also strongly indicate that despite its inability to bind Ca2+, the N-terminal EF-hand domain in GCAP-2 is an essential part of the molecule that is important for targeting retGC.

GCAP-2 can form a complex with guanylyl cyclase both in its Ca2+-free and Ca2+-loaded form (8, 39). Instead of activating the cyclase, Ca2+-loaded GCAP-2 inhibits basal activity of retGC in washed photoreceptor membranes (8), arguably by interfering with reversible dimerization of retGC (26, 28). Therefore, we tested the ability of some of the GCAP-2 mutants in the EF-1 domain to inhibit cyclase basal activity at saturating Ca2+ concentrations (Fig. 3). We have found that several mutants that have lower ability to stimulate retGC in their Ca2+-free form are also less efficient as inhibitors of the cyclase basal activity. This appears to be consistent with the EF-1 domain of GCAP-2 being involved in the interaction with the cyclase both in the absence and in the presence of Ca2+.

It seems highly unlikely that different single amino acid substitutions within the EF-1 would all be a result of a general nonspecific misfolding of the protein. It is much more likely that these mutations directly affect the cyclase-binding site. However, both wild type and EF-1 mutants of GCAP-2 used in this study were isolated as Ca2+-loaded monomers (data not shown) using a previously described technique (28). At the same time, according to our previous observations, Ca2+-free GCAP-2 undergoes dimerization, which is likely to contribute to the cyclase regulation by promoting the interaction between two retGC subunits (28). This Ca2+-sensitive dimerization of GCAP-2 apparently involves multiple regions in the GCAP-2 molecule (28). Hence, the GCAP-2 mutants tested in the present study could be inactive because of a nonspecific misfolding of the protein. On the other hand, if the EF-1 were specifically required for the Ca2+-sensitive dimerization of GCAP-2, rather than retGC binding, that could also account for the absence of their activity. However, in both cases we would expect that the inactive GCAP-2 mutants failed to form dimers in the absence of Ca2+. Contrary to that, all tested EF-1 mutants, even those that completely lost their ability to regulate the cyclase, were able to dimerize at low Ca2+ concentration (Fig. 4, results with the rest of the GCAP-2 mutants are not shown, but all of them formed dimers in a Ca2+-free solution). These results strongly argue that 1) individual point mutations within the EF-1 domain do not cause general nonspecific misfolding of the protein and 2) these mutations specifically affect GCAP-2 interactions with retGC rather than the GCAP/GCAP interactions (as illustrated in Fig. 5).

Because various single mutations within the EF-1 domain can interfere with the cyclase regulation, it would be quite reasonable to expect that some of the variable amino acid residues within this region can determine the specificity of GCAP-2 as a cyclase regulator compared with other recoverin-
Phe41, which is exposed in GCAP-2 (29), with another hydro-
retGC stimulation (Fig. 2). Interestingly, substitutions of
(for example, Ser or Ile, respectively) only partially affect
substitutions that are found in other recoverin-like proteins
other members of the recoverin family. On the other hand, the
ations are normally not found in the corresponding positions in
F48S almost completely inactivate GCAP-2, these substitu-
GCAP-2 is less obvious. Although substitutions such as F41I or
lesser extent Phe41 and Phe48 (but not Glu33) indeed contribute
(Fig. 5). Inactivation of GCAP-2 can be achieved by various
like proteins or at least contribute to such specificity. The
results shown in Fig. 2 demonstrate that there are at least several such amino acids: Lys30, Phe41, His43, Phe48.
In many recoverin-like proteins (22, 23), Gly substitutes for
Lys30. A corresponding mutation in GCAP-2, K30G, strongly
decreases the efficiency of GCAP-2 as a retGC activator. Sub-
stitution of His43 with the amino acid residues commonly found
in many recoverin-like proteins, Glu or Gin, also strongly inhibits GCAP-2 activity. The F48V, a substitution derived from
Caenorhabditis elegans NCS-2 and calmodulin (22, 23), also markedly decreases retGC activation. Compared with that,
 contribution of Phe41 and Phe48 to the overall specificity of
GCAP-2 is less obvious. Although substitutions such as F41I or
F48S almost completely inactivate GCAP-2, these substitu-
tions are normally not found in the corresponding positions in
other members of the recoverin family. On the other hand, the
substitutions that are found in other recoverin-like proteins
(for example, Ser or Ile, respectively) only partially affect
retGC stimulation (Fig. 2). Interestingly, substitutions of
Phe41, which is exposed in GCAP-2 (29), with another hydro-
phobic amino acid, Ile, causes a much stronger effect than
substitution with a hydrophilic residue, Ser, found in S-modu-
lin and neurocalcin (40–42). Hence, Lys30, His43, and to a
lesser extent Phe41 and Phe48 (but not Glu33) indeed contribute
to the GCAP-2 specificity as a cyclase activator (33). The over-
all activity of GCAP-2 as a cyclase regulator apparently re-
quires both evolutionary conserved and specific variable resi-
dues within the EF-1 domain to optimize its interaction with
the target enzyme.

Our data strongly indicate that the N-terminal EF-hand-like
domain of GCAP-2, which is unable to bind Ca2+, creates a
contact surface for retGC (or at least a part of such surface)
(Fig. 5). Inactivation of GCAP-2 can be achieved by various
single mutations in this region and primarily affects the inter-
action between GCAP-2 and the cyclase. At the same time,
these mutations do not affect Ca2+-sensitive dimerization of
GCAP-2. Hence, different parts in GCAP-2 molecule are re-
 sponsible for the binding to the cyclase versus GCAP/GCAP
interaction. This seems to be consistent with the simplified
model according to which activation of retGC is a result of its
binding to the cyclase and subsequent stimulation of the cy-
lase dimerization via conformational switch in GCAP-2 and
enhanced GCAP/GCAP interactions (a dimer-adapter model,
Ref. 28). At present, it remains unclear how large the whole
retGC-binding surface in GCAP-2 could be or how many other
sites may be present. Further detailed studies of other regulat-
ory regions in GCAP-2 (33) will be required to answer this
question.

As an alternative hypothesis, one could propose that the
EF-1 domain does not necessarily directly interact with the
cyclase but rather influences those contacts with the target
enzyme that are made by a different part of the GCAP-2 mol-
eculc. Such a possibility can hardly be excluded, especially
because the EF-1 interacts very closely with the exiting helix of
the EF-2 domain, another region in GCAPs known to be essen-
tial for retGC regulation (33, 43). If there are other contact
regions for the cyclase in GCAP-2, the EF-1 may potentially
affect these other sites through the intramolecular interac-
tions. Moreover, the properties of some mutants (i.e. F48V in
Fig. 2) do indicate that the EF-1 may have an additional func-
tion other than binding to the target enzyme. However, a

Fig. 4. Ca2+-free GCAP-2 mutants within the EF-1 region re-
main capable of forming dimers. Purified recombinant proteins
were analyzed using high resolution gel chromatography as described
under "Experimental Procedures." a and b, wild type GCAP-2; c, F41I;
d, E44S; e, F48S; f, C35D. Sample and elution buffer contained either
300 μM CaCl2 (a) or 500 μM EGTA (b-f).

Fig. 5. Putative role of EF-1 domain in GCAP-2 in retGC reg-
ulation. A, GCAP-2 binds to the cyclase both at high and low free Ca2+
concentrations. The release of Ca2+ provides a conformational switch
that transforms GCAP-2 into its activator form and also stimulates
GCAP/GCAP interactions. The activator GCAP-2 dimer enhances asso-
ciation between retGC subunits required for its catalytic activity
dimer-adapter hypothesis, Ref. 28). B, mutations within the EF-1
domain of GCAP-1 inactivate retGC regulation but do not abolish
GCAP/GCAP dimer formation; therefore, it is most likely that the
specific role of EF-1 is to make a contact surface for binding for the
cyclase (or at least a part of such surface) in GCAP-2. Once the GCAP/
retGC interactions are affected by the mutations within the EF-1, it is
no longer able to regulate the cyclase even though Ca2+-sensitive
GCAP/GCAP interactions are not suppressed by these mutations. C, the
side chain residues within the EF-1 in GCAP-2 that are likely to
interact with retGC are marked in red, those that do not appear essen-
tial for targeting retGC are marked in yellow, Ca2+ ion bound within
EF-1 is shown in cyan (software used: “Swiss PdbViewer,” Ref. 45).
strong dependence of the GCAP-2 affinity to retGC on the side chains residues that do not directly contact the EF-2 (such as Cys35 or His43) would be more consistent with the EF-1 domain itself interacting with the cyclase.

It seems that in the course of the evolution the ability of the first EF-hand in GCAP-2 to bind Ca2+ ions was “traded” for its ability to interact with the target enzyme, retGC. Even when the Ca2+-binding consensus sequence in the EF-1 is only partially restored by placing oxygen-containing side chains at the positions corresponding to the Y or –X coordinates (Fig. 1), each substitution alone can inhibit GCAP-2 interaction with retGC (Fig. 2).

However, it is important to emphasize that inactivation of GCAP-2 by point mutations within the EF-1 domain described in this paper cannot be attributed to the restoration of Ca2+ binding within the EF-1 loop and does not require such restoration. First, Lys30 and Phe48 are both outside the Ca2+-binding loop, and His43 does not substitute for an oxygen-containing residue of the EF-hand consensus sequence (36). Second, although the replacement of Cys35 by oxygen-containing side chains (Ser or Asp) inhibits the interaction with retGC, such substitution cannot possibly restore Ca2+ binding within the EF-1 loop because the coordinating position (–X) still lacks the required oxygen-containing group (36). On the other hand, a mutation, F41I, at the (–X) coordinating position inhibits retGC activation stronger than the F41S, the substitution that partially restored by placing oxygen-containing side chains at the Ca2+ coordinating residues (Fig. 1).

It seems that—perhaps, in other recoverin-like proteins. If true, that could explain why the N-terminal EF-hand, despite its inability to function as a Ca2+-binding domain, remains well preserved among different members of the diverse family of recoverin-like proteins.

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