Three-dimensional Structure of Guanylyl Cyclase Activating Protein-2, a Calcium-sensitive Modulator of Photoreceptor Guanylyl Cyclases*

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We report here NMR spectroscopic studies of the three-di-
mensional structure of the Ca\(^{2+}\)-bound form of GCAP-2 as a
step toward understanding the molecular mechanism of regu-
lation of photoreceptor guanylyl cyclases. Ideally, one would
like to solve the structures of the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound
form of myristoylated GCAP-2, the physiologic species, but this
is not yet feasible because of the low solubility of the myristoy-
lated protein. We chose instead to solve the structure of Ca\(^{2+}\)-
bound unmyristoylated GCAP-2, which is soluble and gives
clearly resolved NMR spectra. Moreover, the structure of the
unmyristoylated form of GCAP-2 is likely to be biologically
pertinent. Unmyristoylated GCAP-2 is nearly as effective as
myristoylated GCAP-2 in activating guanylyl cyclase at low
Ca\(^{2+}\) and inhibiting it at high Ca\(^{2+}\) (29). Hence, structural
studies of unmyristoylated GCAP-2 should reveal the Ca\(^{2+}\)-
induced conformational changes underlying its regulation of
cyclase.

EXPERIMENTAL PROCEDURES

Sample Preparation—Unmyristoylated recombinant GCAP-2 pro-
tein uniformly labeled with nitrogen-15 and carbon-13 was expressed in
Escherichia coli strain BL21(DE3)pLysS using pET11d vector (Nova-
gen) grown in M9 minimal medium (containing 15N-labeled NH\(_4\)Cl and
\([^{13}C_6]\)glucose) according to previously published procedures (29). Spe-
cific labeling of GCAP-2 with valine or leucine, whose methyl carbons
were stereospecifically labeled with \([^{13}C]\), was prepared as described
previously (30). Recombinant GCAP-2 protein expressed in E. coli forms
insoluble inclusion bodies that were conveniently isolated and solubi-
ized using 8M urea (29). The urea-solubilized protein was then dia-
lyzed extensively to remove urea. More than 80% of the refolded
GCAP-2 (after dialysis of urea) remained soluble. The soluble GCAP-2
was then further purified using gel filtration chromatography described
previously (29). In addition, anion-exchange chromatography (DEAE-
Sepharose, Amersham Pharmacia Biotech) was performed at pH 6.1
and at room temperature. GCAP-2 eluted from the DEAE-Sepharose
column (50-ml bed volume) using a salt gradient (0–0.5M KCl over 60
min at flow rate, 2 ml min\(^{-1}\)).

Samples for NMR experiments were prepared by dissolving 15N-
labeled or \([^{13}C/^{15}N]\)-labeled GCAP-2 (1 mM) in 0.5 ml of a 95% H\(_2\)O, 5%
\(2\)H\(_2\)O or 99% \(2\)H\(_2\)O solution containing 50 mM KCl, 10 mM CaCl\(_2\),
10 mM \([^{2}H_{10}]\)dithiothreitol, 25 mM \([^{2}H_{28}]\)octyl
b-glucoside at pH 6.8.

NMR Spectroscopy—All NMR experiments were performed at 45 \(^{\circ}\)C
on a Varian UNITY-plus 500 or UNITY-600 spectrometer equipped
with a four channel interface and a triple resonance probe with an
actively shielded z gradient together with a pulse field gradient
accessory.

The 15N-1H HSQC spectra (Fig. 2) (and heteronuclear multiple quan-
tum coherence-J) were recorded on the uniformly 15N-labeled GCAP-2
sample (95% H\(_2\)O, 5% \(2\)H\(_2\)O) at pH 6.1 and 6.8.

Structure of Guanylyl Cyclase Activating Protein-2

![Amino acid sequence alignment of bovine GCAP-2 (accession no. U32856) with bovine GCAP-1 (accession no. P46065),
human GCAP-3 (accession no. AF110002), frog GCIP (accession no. AF047884), and bovine recoverin (accession no. P21457). The
29-residue EF-hand motifs are highlighted in color: green, EF-1; red, EF-2; cyan, EF-3; yellow, EF-4. Regions of regular secondary structure
(a-helices and \(b\)-strands) are indicated schematically.](image-url)
All triple resonance experiments were performed as described previously (31) on the uniformly 13C/15N-labeled GCAP-2 sample in 95% H2O with the following number of complex points and acquisition times: HNCO (1H( F1) 32, 23.7 ms; 15C( F2) 64, 42.7 ms; 1H (F3) 512, 64 ms), HNCACB (13N( F1) 32, 23.7 ms; 13C( F2) 48, 6.3 ms; 1H (F3) 512, 64 ms), CBCACOAH (15C( F1) 32, 6.8 ms; 13CO (F2) 64, 42 ms; 1H (F3) 512, 64 ms), CBCACOAH (15C( F1) 32, 6.8 ms; 13CO (F2) 64, 42 ms; 1H (F3) 512, 64 ms), and HHBACONHN (15N( F1) 32, 23.7 ms; 1H (F3) 64 21 ms; 1H (F3) 512, 64 ms). The triple resonance spectra were analyzed as described previously (31) and provided a nearly complete sequence-specific assignment of the backbone resonances.

The side chain resonances were assigned as described (30) by analyzing three-dimensional HCCH-total correlation spectroscopy spectra (7 and 14 ms mixing time) recorded on 13C/15N-labeled GCAP-2 (99% H2O) with the following number of complex points and acquisition times: 'H (F1) 128, 36.5 ms; '13C (F2) 32, 10.6 ms; 'H (F3) 416, 52 ms.

Structure calculations from residues 2–190 were performed using the YASAP protocol (32) within X-PLOR (33) as described previously (34). A total of 1791 interproton distance restraints (552 intrasidue, 457 sequential, 250 short range, and 350 long range) was obtained as described (30) by the analysis of 13C- and 15N-edited nuclear Overhauser effect spectroscopy-HSQC spectra (100 ms mixing time) recorded on 13C/15N-labeled GCAP-2 (99% H2O) for 13C-edited experiments) of 13C- and 15N-labeled GCAP-2 (95% H2O for 13N-edited with the following number of complex points and acquisition times: 'H (F1) 128, 25.6 ms; 13C (F2) 64, 16.6 ms; 15N (F3) 32, 32.10.6 ms; 1H (F3) 416, 52 ms.

In addition to the nuclear Overhauser effect-derived distance restraints, 18 distance restraints involving Ca2+ bound to loop residues 1, 3, 5, 7, and 12 in EF-2, EF-3, and EF-4 (24, 35, 36); 136 distance restraints for 68 hydrogen bonds; and 216 dihedral angle restraints (114 ϕ and 102 ϕ) were included in the structure calculation. 50 independent structures were calculated, and 22 of those with the lowest total energy were selected. The average total and experimental distance and angle restraints were violated by more than 0.40 Å and 4.0°, respectively. The average root mean square (RMS) deviations from an idealized geometry for bonds, angles, and improper torsions are 0.0073 Å, 2.04°, and 0.91°, respectively.

Ca2+-binding Measurements—Tryptophan fluorescence titrations (Fig. 3) were performed with 1 μM GCAP-2 in 2 ml of 0.1 M KCl, 50 mM HEPES (pH 7.5), 1 mM dithiothreitol at 25 °C. The free calcium concentration (30 nm to 2 μM) was set using an EGTA buffer system. The protein samples initially contained an equal molar ratio of total Ca2+ and EGTA (2 mM); the free Ca2+ concentration was adjusted by adding aliquots of 0.1 mM EGTA. The free Ca2+ concentration was calculated based on the total amount of Ca2+ and EGTA present using the computer algorithm by Brooks and Storey (37). The calculated free Ca2+ concentrations agreed closely with measured Ca2+ concentrations using fluorescent indicator dyes fluo-3 and rhod-2 (Molecular Probes, Eugene, OR) with Kd of 0.4 and 1.0 μM, respectively (38).

Ca2+-binding curves (Fig. 3) were obtained by the equilibrium dialysis method using a DiaSpro-equilibrium Dialyzer (Sialomed, Columbia, MD). The apparatus consisted of two fluid-containing chambers (protein and buffer chambers) separated by a thin dialysis membrane (molecular mass cutoff, 10 kDa). The protein chamber contained 100 μl of 50 μM GCAP-2 in the same buffer used in the fluorescence titration above plus the addition of 1 μM 45Ca2+ (total radioactivity, 1.4 μCi). The buffer chamber contained 100 μl of buffer (excluding any GCAP-2) plus the addition of a known amount of cold Ca2+. The fluid in the two chambers was allowed to come to equilibrium after 12 h at 25 °C. Fifteen different dialysis experiments were performed at various cold Ca2+ concentrations (0, 1, 2, 10, 25, 35, 45, 65, 100, 125, 140, 150, 160, 170 μM). At equilibrium, the free Ca2+ concentration is defined by

\[
Ca^{2+}_{\text{free}} = Ca^{2+}_{\text{tot}} - Ca^{2+}_{\text{bound}} \quad (\text{Eq. 1})
\]

where \(Ca^{2+}_{\text{bound}}\) is the total Ca2+ concentration in the system, \(r_{p}\) is the radioactivity (counts/min) of 45Ca2+ measured from an aliquot of the buffer chamber, and \(r_{b}\) is the radioactivity measured from an equal aliquot of the protein chamber. The concentration of Ca2+ bound to protein is as follows.

\[
Ca^{2+}_{\text{bound}} = Ca^{2+}_{\text{tot}} - Ca^{2+}_{\text{free}} \quad (\text{Eq. 2})
\]

The fractional saturation is then defined as

\[
Y = \frac{P_{\text{bound}}}{P_{\text{tot}}} = \frac{(Ca^{2+}_{\text{b}})^n}{(Ca^{2+}_{\text{tot}} + K_d)} \quad (\text{Eq. 3})
\]

where \(P_{\text{tot}}\) is the total protein concentration in the system, \(P_{\text{bound}}\) is the concentration of protein species bound by Ca2+, \(n\) is the Hill coefficient, and \(K_d\) is the apparent dissociation constant.

RESULTS

The structure of recombinant GCAP-2 uniformly labeled with carbon-13 and nitrogen-15 was studied by heteronuclear NMR spectroscopy. Two-dimensional heteronuclear single quantum coherence (15N-H HSQC) NMR spectra, which serve as fingerprints of the conformation of main chain and side chain amide groups, were obtained. The HSQC spectra of unmyristoylated GCAP-2 are presented in Fig. 2. The Ca2+-bound unmyristoylated protein exhibits many sharp and well resolved peaks. In contrast, the Ca2+-free form exhibits broad and poorly resolved peaks, suggesting that Ca2+-free GCAP-2 may represent an unfolded, aggregated protein. However, circular dichroism studies (data not shown) indicate that Ca2+-free GCAP-2 is well folded with greater than 60% helical content. In addition, the Ca2+-free GCAP-2 sample used in the NMR study is biologically active and was shown to activate photoreceptor guanylate cyclase. Hence, Ca2+-free GCAP-2 in our study represents a well defined and folded protein. The observed broadening of the NMR peaks suggests that Ca2+-free GCAP-2 most likely forms a dimer or other multimeric species under the conditions of the NMR experiment. The HSQC spectrum of Ca2+-free myristoylated protein (data not shown) is similar to that of Ca2+-free unmyristoylated protein. The low solubility of the Ca2+-bound myristoylated protein prevented us from obtaining its HSQC spectrum.

The striking Ca2+-induced spectral differences point to a large Ca2+-induced structural change in the unmyristoylated protein. The characteristic NMR peaks of the Ca2+-bound form saturate on addition of three molar equivalents of Ca2+ to the protein. Ca2+-binding measurements using equilibrium dialysis and tryptophan fluorescence titrations also showed that three Ca2+ bind to unmyristoylated GCAP-2 (Fig. 3). The apparent affinity is 300 ± 40 nM, and the Hill coefficient is 2.1 ± 0.2. A stoichiometry of three Ca2+ bound to GCAP-2 is also supported by site-directed mutagenesis studies of the EF-hand motifs (16). Substituting glutamine for glutamate at position 12 of the EF-hand loops (EF-2, EF-3, EF-4) prevents the binding of Ca2+ and produces a constitutively active form of GCAP-2.

The strong and well resolved peaks observed in the HSQC spectrum of Ca2+-bound, unmyristoylated GCAP-2 (Fig. 2B) suggested that it would be feasible to determine its three-dimensional structure. To elucidate the structure, resonances in the NMR spectrum were assigned to specific amino acid residues. Triple resonance experiments correlating 15N, 13C, and 1H were performed to facilitate making assignments. Over 95% of the backbone resonances were assigned as indicated in Fig. 2B. These backbone assignments served as the basis for assigning about 80% of the side chain resonances. Nuclear Overhauser effect spectroscopy experiments were analyzed to establish nearly 2000 proton-proton distance relationships (~11 nuclear Overhauser effects/residue) throughout the protein. In addition, 216 dihedral angle restraints (ϕ and ψ) were deduced from J-coupling and chemical shift data. Finally, the three-dimensional structure was calculated by distance geometry and restrained molecular dynamics.

A superposition of 22 structures of Ca2+-bound unmyristoylated GCAP-2 consistent with the NMR data is shown in Fig. 4, and their average is depicted as a ribbon diagram (Fig. 5A) and a space-filling model (Fig. 5B). The entire polypeptide chain has been traced except for the disordered region at the carboxyl
The structure near the amino terminus (residues 2–18) and the region between EF-3 and EF-4 (residues 132–144) are rather poorly defined (the RMS deviation of the main chain atoms is greater than 2 Å) because of a relatively small number of nuclear Overhauser effect contacts observed in these regions. Also, chemical shift data indicate a structurally disordered, random coil secondary structure in most of these regions.

GCAP-2 is a compact protein (radius of gyration, 17 Å) made of two domains separated by a flexible linker (Fig. 5). Each domain contains a pair of EF-hands, the 29-residue helix-loop-helix motifs (highlighted in color in Fig. 1) found in calmodulin, troponin C, parvalbumin, recoverin, and other members of the superfamily (39). The EF-hands are defined from the amino terminus: EF-1 (Ala22-Val51), EF-2 (Thr58-Leu87), EF-3 (Leu96-Lys126), and EF-4 (Glu147-Arg176). EF-1 and EF-2 interact in-
FIG. 4. Superposition of the main chain atoms of 22 NMR-derived structures of unmyristoylated GCAP-2 with three Ca\(^{2+}\) bound. The four EF-hands (green, red, cyan, yellow) and three bound Ca\(^{2+}\) (orange) are highlighted. The RMS deviation of the NMR-derived structures relative to the mean structure is 0.88 ± 0.1 Å for main chain atoms and 1.44 ± 0.1 Å for all non-hydrogen atoms in the regions of regular secondary structure. This figure was generated by MIDAS (48).

Figure 4: Superposition of the main chain atoms of 22 NMR-derived structures of unmyristoylated GCAP-2 with three Ca\(^{2+}\) bound. The four EF-hands (green, red, cyan, yellow) and three bound Ca\(^{2+}\) (orange) are highlighted. The RMS deviation of the NMR-derived structures relative to the mean structure is 0.88 ± 0.1 Å for main chain atoms and 1.44 ± 0.1 Å for all non-hydrogen atoms in the regions of regular secondary structure. This figure was generated by MIDAS (48).

Three Ca\(^{2+}\) are bound to GCAP-2, as anticipated on the basis of its amino acid sequence and site-directed mutagenesis. The structure of EF-3 is strikingly similar to that of EF-3 in Ca\(^{2+}\)-bound recoverin and calmodulin. The RMS deviations of the 116 main chain atoms of EF-3 are 0.66 Å in comparing GCAP-2 with recoverin and 0.80 Å in comparing GCAP-2 with calmodulin. Likewise, the coordination of Ca\(^{2+}\) is virtually identical in all three. The interhelical angle or helix packing angle of EF-3 is 94° (GCAP-2), 95° (recoverin), and 96° (calmodulin).

The structures of EF-1, EF-2, and EF-4 from GCAP-2 are somewhat different from the corresponding EF-hands of recoverin. The RMS deviations of the main chain atoms of these EF-hands are 1.9 Å (EF-1), 1.4 Å (EF-2), and 1.9 Å (EF-4) in comparing GCAP-2 with recoverin. The interhelical angles are 108° (EF-1), 109° (EF-2), and 98° (EF-4) for GCAP-2 compared with 115° (EF-2), 118° (EF-3), and 92° (EF-4) for Ca\(^{2+}\)-bound recoverin. The four EF-hands of GCAP-2 assume the “open conformation” of Ca\(^{2+}\)-occupied EF-hands seen in recoverin, calmodulin, and troponin C.

The structures of the 12-residue Ca\(^{2+}\)-binding loop of the EF-hands are depicted in Fig. 7. The loop of EF-1 is quite similar to that of recoverin and again shows why this motif does not bind Ca\(^{2+}\). EF-1 is distorted from a favorable Ca\(^{2+}\)-binding geometry by Pro\(^{106}\) at the fourth position of the 12-residue loop. Also, the third residue in the loop (Cys\(^{85}\)) is not suitable for ligating Ca\(^{2+}\). The bulky sulfhydryl group sterically blocks the entry of Ca\(^{2+}\). The EF-2 loop adopts a favorable structure for binding Ca\(^{2+}\), despite the tight turn centered at Asn\(^{174}\) (position 6 of the loop). Normally, a glycine residue is conserved at position 6 in most other EF-hands (Fig. 1). The loop of EF-3 is very typical of Ca\(^{2+}\)-occupied EF-hands and closely resembles the EF-3 loop of recoverin and calmodulin. The EF-4 loop of GCAP-2 is quite different from that of recoverin. In recoverin, the second residue in the loop (Lys\(^{162}\)) forms a salt bridge with residue 12 (Glu\(^{171}\)) that disables Ca\(^{2+}\) binding. In GCAP-2, the second residue of the EF-4 loop (Glu\(^{159}\)) is negatively charged and cannot form a salt bridge that would impede Ca\(^{2+}\) binding. Furthermore, residues 1 and 3 of the EF-4 loop (Asp\(^{158}\) and Asn\(^{160}\)) contain oxygen atoms in their side chains that can ligate Ca\(^{2+}\), in contrast with the corresponding residues of recoverin (Gly\(^{160}\) and Lys\(^{162}\)). Thus, Ca\(^{2+}\) binds to EF-4 similarly to EF-2 and EF-3.

GCAP-2 has a solvent-exposed, hydrophobic surface formed by residues from EF-1 and EF-2 (Fig. 8A). The exposed patch of hydrophobic residues is formed by the clustering of several aromatic side chains (Trp\(^{27}\), Phe\(^{31}\), Phe\(^{45}\), Phe\(^{48}\), Phe\(^{59}\), and Tyr\(^{81}\)) and several aliphatic residues (Leu\(^{24}\), Leu\(^{40}\), Ile\(^{76}\), Val\(^{82}\), Leu\(^{85}\), and Leu\(^{89}\)) (Fig. 8B). These exposed hydrophobic residues are highly conserved in members of the family (22) and form a similar nonpolar patch in Ca\(^{2+}\)-bound recoverin (24, 25). In Ca\(^{2+}\)-free recoverin, these residues make close contacts with the highly sequestered myristoyl group (26). Ca\(^{2+}\)-induced extrusion of the myristoyl group causes these residues to become solvent exposed, suggesting that they may serve as a target-binding site.

DISCUSSION

In this study we present the three-dimensional structure of unmyristoylated GCAP-2 with three Ca\(^{2+}\) bound. This structure is an important step toward 1) understanding the regulatory mechanism of photoreceptor guanylyl cyclases and 2) elucidating the novel membrane-targeting mechanism of GCAPs. Although the precise structure of the amino-terminal myristoyl group of GCAP-2 could not be studied, our structure shows the amino-terminal region (residues 2–18) to be solvent exposed, suggesting that the covalently attached myristoyl group may be extruded as in Ca\(^{2+}\)-bound recoverin (25). Recent NMR
studies on the myristoyl group of GCAP-2 also suggest that the myristoyl group may be solvent exposed (41). An extruded myristoyl group of Ca\(^{2+}\)-bound GCAP-2 may not necessarily interact with bilayer membranes (as demonstrated for recoverin), because the Ca\(^{2+}\)-bound, myristoylated GCAP-2 appears to be cytosolic at low ionic strength (29). Instead, the myristoyl group of GCAP-2 might interact with the cyclase or perhaps with itself to form a soluble dimer. Structural studies of the myristoylated GCAPs are needed to more rigorously determine the structural role of the myristoyl group and to test whether the myristoyl group can be sequestered in Ca\(^{2+}\)-free GCAP-2 as was seen for Ca\(^{2+}\)-free recoverin (26).

The exposed hydrophobic patch of GCAP-2 (Fig. 8) may serve a role in regulating guanylyl cyclase. Recent site-directed mutagenesis studies reveal that many of these exposed residues are important in the cyclase interaction (42). In particular, replacement of residues 78–110 (that includes the exiting helix of EF-2) with corresponding residues of neurocalcin results in a chimeric protein that fails to inhibit guanylyl cyclase at low Ca\(^{2+}\) levels but activates it at high Ca\(^{2+}\). Also, the replacement of residues in EF-1 (residues 24–49) with the corresponding residues of neurocalcin renders the chimera completely inactive. It will be interesting to make point mutations of individual residues in the exposed patch to more precisely map their effect on the cyclase interaction.

The hydrophobic patch of GCAP-2 may also serve as a possible dimerization site. The crystal structures of Ca\(^{2+}\)-bound unmyristoylated recoverin (24) and neurocalcin (36) both show the presence of a stable dimer in the asymmetric unit. Dimerization of GCAP-2 might enable a Ca\(^{2+}\)-bound monomer to tie up a Ca\(^{2+}\)-free monomer to prevent activation of the cyclase. Alternatively, a dimer of Ca\(^{2+}\)-bound GCAP-2 might bind directly to the cyclase and inhibit it. However, GCAP-2 does not appear to dimerize in our NMR experiments perhaps because

**Fig. 5.** Schematic ribbon representation (A) and space-filling model (B) of the energy-minimized average structure of unmyristoylated GCAP-2 with three Ca\(^{2+}\) bound. The side chain atoms of residues at the domain interface (Ala\(^{63}\), Ala\(^{67}\), Ile\(^{103}\), and Ile\(^{120}\)) are shown in A and the color scheme is as in Fig. 4. The figure was generated using Molscript (49) and Raster3d (23).
detergent (20 mM octyl glucoside) was present in our samples to dramatically sharpen the peaks in the NMR spectrum. This detergent does not appear to denature or inactivate GCAP-2 as was demonstrated in the original purification of GCAP-2 from the retina. Additional studies are needed to test whether GCAP-2 forms a functional dimer under physiological conditions.

The structure of GCAP-2 near the amino terminus (residues 2–18) appears different from that of recoverin. There is virtually no sequence similarity between recoverin and the GCAPs in this region. Recoverin contains a long, amphipathic helix (residues 4–18) that packs against the sequestered myristoyl group (26). This amino-terminal helix is considerably shorter in bovine GCAP-2 (residues 7–13) because four residues have been deleted in this region (Fig. 1). The orientation of the

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A. M. Dizhoor, personal communication.
The amino-terminal helix is different in recoverin and GCAP-2. This helix in recoverin extends close to the interdomain linker, whereas it interacts primarily with the entering helix of EF-1 in GCAP-2. The helix orientation in GCAP-2 is also characterized by contacts between Ser⁶ and Leu⁷⁹. We note, however, that these apparent structural differences in the amino-terminal region between recoverin and GCAP-2 may result from the very low precision of our structure in this region (RMS deviation, 4 Å) because of dynamical disordering. Substitution of this amino-terminal region with the corresponding residues of neurocalcin has little effect on the function of GCAP-2 (42), consistent with our finding that this region is structurally

**Fig. 8.** Space-filling representation (A) and ball-and-stick model (B) of side chain atoms of the exposed hydrophobic patch of GCAP-2. Hydrophobic, negatively charged, and positively charged residues are highlighted in yellow, red, and blue, respectively. Solvent-exposed hydrophobic residues from EF-1 and EF-2 are indicated.
disordered.

The carboxyl-terminal helix (residues 180–186, highlighted in white in Fig. 5) interacts with the helices of EF-3 and EF-4, similar to that seen for recoverin (43). The association of the COOH-terminal helix with these EF-hands resembles the interaction of calmodulin with its helical target peptides (44). The carboxyl-terminal helix may enhance the specificity of GCAP-2 and recoverin by blocking their adventitious binding to targets of calmodulin.

The GCAP-2 structure is likely to be similar to that of GCAP-1 (40% sequence identity), GCAP-3 (35% identity), and GCIP (37% identity), because the overall main chain structure appears so similar to recoverin (RMS deviation, 2.2 Å; identity, 30%) and to neurocalcin (RMS deviation, 2.0 Å; identity, 40%). Most of the hydrophobic residues in the hydrophobic core and in the exposed patch (Fig. 8) are highly conserved. Also conserved are the residues that ligate Ca<sup>2+</sup> in the EF-hand loops (Fig. 7). Interestingly, important residues in the entering helix of EF-2 at the domain interface (Ala<sup>57</sup>, Ala<sup>63</sup>, and Ala<sup>67</sup>) are not conserved. Other structurally important and nonconserved residues include Asn<sup>74</sup>, Leu<sup>92</sup>, Thr<sup>93</sup>, His<sup>95</sup>, and Thr<sup>100</sup>. Considerable differences are also found in the amino-terminal (residues 2–18) and carboxyl-terminal (residues 191–204) regions. These differences suggest that the interaction and/or orientation between the NH<sub>2</sub>-terminal and COOH-terminal domains might be different in GCAP-1, GCAP-3, and GCIP. Indeed, a point mutation at the domain interface causes very different phenotypes in GCAP-1 and GCAP-2. The mutation (Y99C) causes GCAP-1 to be constitutively active (45, 46), resulting in autosomal dominant cone dystrophy in humans (47). In contrast, the corresponding mutation in GCAP-2 (Y104C) does not alter its Ca<sup>2+</sup> sensitivity and partially inactivates GCAP-2 (45).

In summary, we have determined the structure of unmyristoylated GCAP-2 with three bound Ca<sup>2+</sup> by NMR spectroscopy. The overall main chain structure of GCAP-2 is similar to that of Ca<sup>2+</sup>-bound recoverin except for structural differences near the amino terminus (residues 2–18) and the binding of Ca<sup>2+</sup> to EF-4. We see an exposed hydrophobic patch of residues belonging to EF-1 and EF-2 that may play a role in regulating guanylyl cyclase. Our next goal is to solve the structure of Ca<sup>2+</sup>-free GCAP-2, a formidable challenge because of its lower stability and solubility, to fully elucidate the Ca<sup>2+</sup>-induced structural changes that allow GCAP-2 to activate guanylyl cyclases in the absence of Ca<sup>2+</sup>.

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