Structure of the N-terminal Calcium Sensor Domain of Centrin Reveals the Biochemical Basis for Domain-specific Function

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Centrin is an essential component of microtubule-organizing centers in organisms ranging from algae and yeast to humans. It is an EF-hand calcium-binding protein with homology to calmodulin but distinct calcium binding properties. In a previously proposed model, the C-terminal domain of centrin serves as a constitutive anchor to target proteins, and the N-terminal domain serves as the sensor of calcium signals. The three-dimensional structure of the N-terminal domain of Chlamydomonas reinhardtii centrin has been determined in the presence of calcium by solution NMR spectroscopy. The domain is found to occupy an open conformation typical of EF-hand calcium sensors. Comparison of the N- and C-terminal domains of centrin reveals a structural and biochemical basis for the domain specificity of interactions with its cellular targets and the distinct nature of centrin relative to other EF-hand proteins. An NMR titration of the centrin N-terminal domain with a fragment of the known centrin target Sf11 reveals binding of the peptide to a discrete site on the protein, which supports the proposal that the N-terminal domain serves as a calcium sensor in centrin.

Many dynamic processes in cells are critically dependent on the microtubule-based cytoskeleton. The number, direction, and polarity of the microtubules are regulated by an organelle known as the microtubule-organizing center (MTOC). In higher eukaryotes, such as humans, the centrioles function as the MTOC. Lower organisms possess equivalent MTOCs, notably the basal body in algae and the spindle pole body in yeast. The composition of MTOCs is highly proteinaceous, and there exists significant structural heterogeneity between them in different eukaryotes. Despite this heterogeneity, MTOCs in all eukaryotes contain a number of conserved protein components. One of these proteins is centrin (also known as caltractin), an EF-hand calcium-binding protein that has been identified in organisms ranging from protozoa and yeast to plants and humans (1, 2). Genetic studies show that centrin is essential to proper cellular division because it regulates the cell cycle-dependent duplication and segregation of the MTOCs (3, 4). Other centrin functions include: (i) initiation of flagellar excision in Chlamydomonas reinhardtii through a fiber-based microtubule severing mechanism (5), (ii) forming part of the human heterotrim cemic DNA damage recognition complex required for global genome nucleotide excision repair (6), (iii) modulation of homologous recombination and nucleotide excision repair in Arabidopsis (7), and (iv) involvement with nuclear mRNA export machinery in yeast (8).

Centrin is closely related to the ubiquitous archetypal EF-hand calcium sensor protein calmodulin (CaM). Both proteins are composed of two structurally independent globular domains connected by a flexible linker. Each structural domain contains two helix-loop-helix "EF-hand" calcium-binding motifs (9). Unlike CaM, centrin has a long (~20 residue) positively charged N-terminal extension that is highly disordered in solution. This region has been shown to mediate calcium-dependent polymerization of human centrin2 (hCen2) in vitro (10).

Calcium-induced conformational change is central to the target activation mechanism of CaM and other EF-hand calcium sensor proteins (11). The mechanism for activation of CaM-like calcium sensors involves calcium-induced structural rearrangements within each domain, which lead to the formation of large hydrophobic cavities on the molecular surface that mediate the interaction with target proteins. The interactions of CaM with kinase targets were the first to be characterized structurally and these studies revealed binding through a wraparound mode where both domains envelop a single helical moiety from the target (12). As additional CaM targets were characterized, other binding mechanisms were observed including extended and dimerization modes (reviewed in Ref. 13). In the case of centrin, there is mounting evidence that the extended binding mode may be more prominent (14–17), and this subject remains an area of intense investigation.

Centrin and CaM have strikingly different calcium binding properties when measured in vitro. CaM has four high affinity calcium-binding sites (designated sites I–IV) with dissociation constants ($K_d$) in the range of 1–10 μM under physiological salt concentrations (9). The relatively high affinity of CaM for calcium enables it to respond effectively to physiologically relevant increases in the intracellular concentration of free calcium, which typically rises into the range of 1–10 μM. Most centrins, in contrast, have different calcium binding properties, including one or more non-functional calcium binding sites. Two examples emphasize these distinct centrin calcium binding characteristics. 1) The yeast homologue of centrin (cdc31p) has two defective calcium binding sites (sites II and III) based on both mutational studies and primary

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The atomic coordinates and structure factors (code 2AM) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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4 The abbreviations used are: MTOC, microtubule-organizing center; CRC-C, C77-residue C-terminal fragment of Chlamydomonas reinhardtii centrin; CRC-N, 94-residue N-terminal fragment of Chlamydomonas reinhardtii centrin; hCen2, human centrin isoform-2; CaM, calmodulin; TnC, troponin C; cdc31p, the yeast homologue of centrin; K$_{19}$, 19-residue cdc31p-binding domain of Kar1; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser effect.
sequence analysis of the residues comprising the EF-hand loops (15, 18). 2) The C-terminal domain of C. reinhardtii centrin (CRC-C) has substantially lower affinity for calcium in vitro than that observed for typical EF-hand calcium sensors because of the substitution of a highly conserved glutamate with an aspartate at position 12 in site III (16, 19). In canonical EF-hand loops, the glutamate at position 12 provides important bidentate oxygen coordination of calcium. Nevertheless, despite reduced calcium binding affinity in site IV, CRC-C is able to mediate the interaction with Kar1 and the other target proteins reported for centrin (20–24) using the fully functional site IV.

A number of centrin-binding partners have been identified, and the majority of those studied appear to bind primarily via the C-terminal domain. Recently, a peptide corresponding to the cdc31p-binding repeat of a centrin binding motif containing the consensus sequence AX2LX3FL/LEX6WK/R. It has been shown that the interaction of Sfi1 with centrin is essential for mitosis (20). A model has been proposed for calcium/centrin-dependent contraction of the Sfi1 filament (43), in which it is conceivable that both centrin domains are involved.

We report here the three-dimensional structure of CRC-N in the presence of calcium, determined by solution NMR spectroscopy. The domain occupies an open conformation similar to EF-hand calcium sensor domains. A comparative analysis of the structure reveals why CRC interacts with Kar1 only via its C-terminal domain and identifies structural features that distinguish CRC from CaM and other typical EF-hand calcium sensor proteins. To test the proposal that it serves as a calcium sensor, titrations of CRC-N with the seventh centrin-binding repeat of Sfi1 were performed, using intrinsic tryptophan fluorescence and NMR spectroscopy to characterize the interaction.

EXPERIMENTAL PROCEDURES

Recombinant C. reinhardtii centrin N-terminal domain was expressed and purified as described elsewhere (19). The 96-residue construct used in this study consists of residues Met1 through Gly9262. It has been shown that the interaction of Sfi1 (underlined) was synthesized by Sigma Genosys and further purified by high performance liquid chromatography.

Fluorescence Spectroscopy—All fluorescence experiments were performed on a Spex Fluorolog 1681 fluorimeter (Spex Industries Inc., Edison, NJ) at 20 °C. The excitation wavelength was 285 nm, with slit width set to 2.0 mm. Small aliquots of appropriate dilutions of a 1 mM CRC-N stock solution containing 150 mM KCl and 25 mM Tris at pH 7.1 were added to a 5 μM (initial concentration) Sfi1 peptide solution under identical conditions, then incubated with 1 mM EDTA or 5 mM Ca2+. Corrections for background fluorescence were made by subtracting the spectra from identical solutions without peptide.

NMR Spectroscopy—NMR data were acquired on five different samples of CRC-N with the following isotopic compositions: unlabeled; U-13C, U-15N; U-13C, U-15N,13C; and 10%13C. Buffers contained either 10% or 50% 2H2O as appropriate. Each sample typically had a protein concentration of 1–2 mM in a buffer of 25 mM Tris-d11 and 5–10 mM CaCl2 at pH 7.0.

All NMR data were recorded at 25 °C on Bruker DRX600 and DRX800 spectrometers equipped with triple-resonance probe heads and triple axis pulsed field gradient accessories. Backbone sequential assignments of CRC-N were achieved by the combined use of HNCA, CBCANH, CBCA(CO)NH, and HNCO experiments (NMR experiments reviewed in Ref. 25). Aliphatic side chain resonance assignments were obtained from (H)CC(CO)NH, (H)CCCO(NH), and HBHA(CO)NH experiments. 1H chemical shift assignments of aromatic side chains were primarily based on a two-dimensional homonuclear two-quantum experiment. Stereoscopic assignments of the valine and leucine methyl groups were achieved using the 10% 13C-enriched sample (26). To obtain NOE-based distance restraints, a two-dimensional homonuclear NOESY experiment was recorded on the unlabeled sample, a three-dimensional 15N NOESY-HSQC on the 15N-enriched sample, and three-dimensional 13C NOESY-HSQC and four-dimensional 13C HMQCN-NOESY-HMQC on the uniformly 13C-labeled sample. The mixing time used in all NOESY experiments was 100 ms. Standard 2D 15N-1H HSQC spectra were acquired under identical conditions for calcium-loaded U-15N CRC-N the absence and presence of a 3-fold excess of Sfi1 peptide. Data were processed in FELIX (Version 2000; Accelrys, San Diego, CA).

Structure Calculations—NOE assignments were made using FELIX and aided by the program SANE (27). Initial analysis of the NOE data indicated that the first 23 residues (Gly1–23) through Gly9251 of the 96-residue CRC-N construct were highly disordered. Consequently, the first 20 residues (Gly1–20) through Gly9250 were excluded from the structure calculations. For residues Leu9262 through Met9264, a total of 1108 NOE-based distance restraints (462 intraresidue, 220 sequential, 208 medium range, and 218 long range) were derived from the suite of NOEY experiments noted above. These restraints were initially assigned conservative upper bounds based on calibration of the NOESY cross-peak intensities against NOE correlations corresponding to known proton-proton distances or distance ranges. They were subsequently fine-tuned through a series of test structure calculations. The final restraint list had upper bounds of 3.5, 4.5, and 6.0 Å for the three-dimensional 15N NOESY-HSQC, three-dimensional 13C NOESY-HSQC, and four-dimensional 13C HMQCN-NOESY-HMQC data sets, and 4.5 and 6.0 Å for the two-dimensional NOEY experiment. In addition, 24 distance restraints were generated for 12 hydrogen bonds based on the observation of reduced amide exchange rates and characteristic NOEs (28), and 116 backbone torsion angle restraints, each with an assigned minimum range of ±30°, were obtained from 1Hα, 13Cα, 13Cβ, 15N, and 1H chemical shifts using the program TALOS (29).

Initial structures of CRC-N were generated using DYANA (30) and subsequently refined with the program AMBER (31) (Version 7.0) using a 20-ps simulated annealing protocol described previously (16). Out of the 50 structures generated, 45 converged properly to a single fold. The program FINDFAM (32) was then used to establish that at least 20 structures were needed to adequately represent the ensemble. The final representative ensemble of 20 structures was selected on the basis of minimal constraint violation energies. All of these structures also had favorable (low) molecular energies in the AMBER force field.

Structure Analysis—Graphical analysis of the structures was carried out using the program MOLMOL (33). The stereochemistry of the final family of structures was assessed using the software PROCHECK NMR (34). Inter-helical angles were calculated using the program INTERHLX (Kyoko Yap, University of Toronto). ClustalW (35) was used for multiple-sequence alignments. Homology models were constructed using
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To further test the ability of CRC-N to serve as a calcium sensor, experiments were performed to characterize CRC-N binding of a peptide fragment of Sfi1 corresponding to the seventh centrin binding repeat. This repeat was chosen because it is closest to the consensus AXi-LXXi/F/LX3WK/R sequence. Addition of calcium-free CRC-N to a solution of the Sfi1 peptide had no effect on the intrinsic tryptophan fluorescence. However, repeating this experiment using calcium-loaded CRC-N resulted in a substantial quenching of fluorescence and a shift in λmax from 355 to 335 nm (Fig. 2A). Fitting of the titration data to a standard binding curve indicates a dissociation constant in the micromolar range. Note that it is difficult to judge the significance of this value because centrin is anticipated to be pre-associated with Sfi1 via the C-terminal domain at the basal level of Ca2+.

To obtain further insight into the nature of the binding of the Sfi1 peptide to CRC-N, an NMR titration of the peptide into a solution of calcium-loaded, 15N-enriched CRC-N was performed. Consistent with the fluorescence experiments, perturbations in the NMR signals of CRC-N were observed only in the presence of calcium. Fig. 2B shows an overlay of the 15N-1H HSQC spectrum of calcium-loaded CRC-N in the absence and presence of the Sfi1 peptide. The observation of perturbations of only a subset of CRC-N signals indicates that the peptide binds to a discrete binding site on the protein.

Table 3 lists all residues whose chemical shifts can be positively identified as perturbed by the addition of the Sfi1 peptide. Remarkably, residues are perturbed in each of the structural elements of the protein, even the unstructured N-terminal region (Met1–Gly31). To analyze these data, it is important to recognize that the origin of perturbations in NMR chemical shifts is a combination of changes in the environment due to the presence of a ligand and any alterations in the conformation of the protein. In many cases, NMR chemical shift perturbations provide direct insights into the location of the ligand binding site because the structure of the protein is not significantly affected. However, Sfi1 binding to CRC-N, the widespread distribution of chemical shift perturbations suggests that the conformation of protein changes in concert with binding of the peptide and limits the ability to define the binding site in the absence of additional experimental data such as intermolecular NOEs.

### DISCUSSION

Previous studies of centrin have shown that its N- and C-terminal domains have a number of distinct features (10, 17, 19, 38 – 40, 44). For example, a number of centrin targets have been shown to interact.

#### RESULTS

The high resolution three-dimensional structure of calcium-loaded CRC-N was determined by multidimensional heteronuclear NMR spectroscopy. The favorable line widths and excellent dispersion exhibited in the NMR spectra greatly facilitated the chemical shift assignments and NOE analysis. Nearly complete 1H, 13C, and 15N resonance assignments were obtained for CRC-N. A total of 1248 (molar range. Note that it is difficult to judge the significance of this value due to the presence of a ligand

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like centrin, Sfi1 is an essential component of the yeast MTOC (20). Yeast Sfi1 contains 17 copies of a consensus repeat sequence that binds centrin. It has been shown that the interaction of Sfi1 with centrin is essential for mitosis, and a model has been proposed involving calcium-dependent contraction of the Sfi1 filament (43). We have previously obtained evidence suggesting that CRC-C can bind targets at the basal level of calcium in the cell (17). In this study we show that the consensus repeat 7 of Sfi1 binds to a specific site on CRC-N only in the presence of calcium. Thus, our results support the proposal by Salisbury (43) and suggest a specific model in which the centrin C-terminal domain serves as an anchor and the N-terminal domain serves as the calcium sensor.

Insights into the biochemical basis for differences between the two centrin domains are evident from sequence analysis combined with comparative analysis of their 3D structures. An alignment of CRC-N and CRC-C sequences is shown in Fig. 3A. A notable property of CRC-C is that it possesses only two of the four well conserved methionine residues found in CRC-N and other CaM-like calcium sensor domains, which are purported to play a key role in target binding (41, 42). Position 127, which is an alanine in CRC-C but a Met in CRC-N, is an important residue in CRC-C. This small residue is associated with the unique secondary hydrophobic pocket in the CRC-C binding site, into which the Leu13 side chain of K19 is deeply inserted. Fig. 3B shows that in CRC-N this pocket is filled in (by Met54). This difference in the CRC-N and CRC-C structures is fully consistent with the lack of high affinity binding to Kar1 and the concept of domain-specific binding targets for centrin.

The differences between CRC-N and CRC-C extend beyond the unique secondary hydrophobic binding pocket. To fully understand the properties that distinguish CRC-N, additional comparative structural analyses were carried out with other calcium sensor domains. Returning to inter-helical angles, although CRC-N clearly occupies an open conformation, careful inspection reveals significant differences relative to CRC-C and the domains of CaM and TnC (Table 2). Of note in these comparisons are the consistent differences in the critical I/II and III/IV inter-helical interfaces, which are larger by 28° and 17° in the case of CRC-N versus CRC-C and by 27° and 24° in the case of CRC-N versus CaM-N.

Sequence alignment of the domains of CRC and CaM provides further insight into the significant difference in the interface between helices I and II. One readily apparent difference is a well conserved valine in CRC-C and the CaM domains, which is substituted by a smaller alanine in CRC-N (Ala63) (Fig. 3A). Fig. 4 shows a comparison of the side chain packing in the inter-helical interface between helices I and II in CRC-N and CaM-N. The smaller Ala63 side chain in CRC-N allows the two helices to pack more closely against each other, which correlates with the observed larger inter-helical angle (Table 2). This contributes sig-

### TABLE 2
Comparison of inter-helical angles in CRC-N, CaM, TnC, and S100B

| Domain | I/II° | I/III° | I/IV° | II/III° | II/IV° | III/IV° |
|--------|-------|--------|-------|---------|--------|---------|
| CaM-N  | 113.0 | 111.9  | 114.5 | 112.0   | 115.3  | 94.8    |
| TnC-N  | 121.1 | 118.6  | 147.8 | 113.9   | 112.0  | 92.7    |
| S100B  | 132.7 | 80.6   | 124.1 | 100.0   | 100.0  | 102.7   |

* The standard deviations in the inter-helical angles for the three NMR structure ensembles (2AMI, 1OQP, 1CFC) are all less than 8°. Helices are defined as follows: helix I, 6–19 (CRC-N), 9–10 (CRC-C), 5–13 (CaM-N), 82–92 (CaM-C), 13–26 (TnC-N), 45–55 (TnC-C), 2–18 (S100B); helix II, 29–39 (CRC-N), 130–140 (CRC-C), 29–37 (CaM-N), 112–121 (CaM-C), 36–46 (TnC-N), 112–121 (TnC-C), 29–39 (S100B); helix III, 45–55 (CRC-N), 136–146 (CRC-C), 45–55 (CaM-N), 118–128 (CaM-C), 52–62 (TnC-N), 128–138 (TnC-C), 50–60 (S100B); helix IV, 65–74 (CRC-N), 156–165 (CRC-C), 65–75 (CaM-N), 138–146 (CaM-C), 72–81 (TnC-N), 148–158 (TnC-C), 70–86 (S100B).
significantly to CRC-N appearing to be “less open” than CRC-C, CaM-N, and other calcium sensor domains.

Molecular surface representations in Fig. 5 show that CRC-N has a unique, relatively flat binding surface, unlike the concave binding surfaces observed in other calmodulin family calcium sensor domains. In the case of CRC-N, the binding pocket is filled in by several long side chains, such as Met54, Met69, Ile70, Ile73, and Lys93. An analysis of multiple sequence alignments of 30 different centrins (structbio.vanderbilt.edu/chazin/cabp_database/seq/align/centrin.aln.html) reveals a high degree of conservation of these side chains. Indeed, a homology model of hCen2-N shows surface and charge characteristics very similar to CRC-N in Fig. 5 because many critical residues in the binding site are conserved. These observations suggest that the unique features observed for CRC-N are likely conserved among all centrins.

The electrostatic potential surface of CRC-N is compared with those of CRC-C, CaM-N, and CaM-C in Fig. 5. CRC-N is the least negatively charged among the four domains. This fact is most evident on the face of the putative CRC-N target-binding site, which contains a large basic patch. The concentration of basic residues in the CRC-N target binding surface contrasts starkly with the largely acidic nature of the binding site in CRC-C and the two CaM domains. The opposite charge properties of the two CRC domains are consistent with our proposal that the CRC-N has the potential to recognize targets that are distinct from CRC-C and the CaM domains.

The electrostatic potential surface of CRC-N is compared with those of CRC-C, CaM-N, and CaM-C in Fig. 5. CRC-N has an alanine at position 53 in place of a well conserved valine in CaM-N, CaM-C, and CRC-C. This sequence difference allows closer packing of helix II against Phe37 in helix I in CRC-N. The arrow indicates the shift in packing of this conserved Phe ring.

The electrostatic potential surfaces are displayed for CRC-N (2AMI), CRC-C (1OQP), CaM-N (1CLL), CaM-C (1CLL), hCen2-N (modeled on our CRC-N structure), and hCen2-C (2A4J). The gradient in the electrostatic charge potential is depicted as color gradients, red for negative charge and blue for positive charge.
Why then does the Sfi1 peptide interact with CRC-N but the Kar1 peptide does not? The critical factors are evidently due to differences elsewhere in the sequence. For example, residues immediately N-terminal to the Trp anchor are quite different between Kar1 and Sfi1, with the three-residue gap of particular importance (Fig. 6). The evidence from our NMR titration of the Sfi1 peptide with CRC-N reveals there are many residues perturbed that lie outside the archetypal target binding surface, including a number of residues in the unstructured N-terminal residues (1–21). The latter is intriguing because the sole functional property associated with this region to date is calcium-dependent oligomerization of human centrin 2 observed only in vitro in the absence of any other proteins (10). Since NMR chemical shift perturbations can arise from either direct contacts with ligand or allosteric structural perturbations, the structural implications cannot yet be specified from available data. Regardless of the structural details, the data do strongly imply that Sfi1 interacts with CRC-N in a manner different from the complex of K19 with CRC-C.

CONCLUDING REMARKS

The structure reported here along with the demonstration of calcium-dependent binding of the Sfi1 peptide and several other lines of evidence provide strong support for the proposal that the N-terminal domain of centrin serves as a calcium sensor. This hypothesis is consistent with the domain-specific functional model of centrin. The centrin C-terminal domain serves as an anchor that is constitutively bound even at the basal level of calcium in the cell (17). Our new data provide evidence supporting the role of the N-terminal domain as the calcium-sensitive regulatory element. Experiments to test and refine this hypothesis are urgently required and will enable the in-depth knowledge of centrin structure and biochemistry to be used to better understand its cellular functions.

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