Characterization of self-assembly of *Euplotes octocarinatus* centrin

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Abstract

Centrin, an EF-hand calcium-binding protein with high homology to calmodulin (CaM), is an essential component for microtubule-organizing center (MTOC) in organisms ranging from algae and yeast to human. It plays an important structural role by contributing to the formation of Ca^{2+}-sensitive contractile filaments and some super-molecular assemblies. Previous work suggests that the N-terminal domain of centrin especially its first 20-residue fragment, is required for the self-assembly of protein. Native polyacrylamide gel electrophoresis (native-PAGE), pull-down assay, fluorescence resonance light scattering (RLS) and yeast two-hybrid assay indicate that the C-terminal domain of *Euplotes octocarinatus* centrin (EoCen) also contributes to the centrin self-assembly besides its N-terminal domain in vivo and in vitro. On the basis of our results, a self-assembly mode of centrin, which is C-to-C as well as N-to-N (between C- and C-terminal domains as well as between N- and N-terminal domains), is put forward providing maybe some insights into the molecular mechanism of centrin functions in the cell.

1. Introduction

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) [1]. As one of ubiquitous MTOC-associated proteins, centrins have been identified in diverse organisms including yeast, ciliates, green algae, higher plants, invertebrates, and vertebrates [2,3]. Accumulating evidence demonstrates that centrins are essential for signal transduction [2,4], proper cellular division [1], initiation of flagellar excision [5], forming complex for the recognition of damaged DNA [6], modulation of homologous recombination and nucleotide excision repair [7], involvement with nuclear mRNA export [8] and fibers contraction [2,4]. Schiebel and Bornens suggest that the fiber contraction is related with the polymerization, one of the characteristic features of this protein family, of centrin [2]. Studies on this subject have been well done. Almost all centrins have long (~20 residues) positively charged N-terminal extension that mediates the polymerization of this protein [9]. This fragment is the most distinctive and variable region of centrins and it has been suggested to be responsible for some functional diversity among centrin species [3,10,11]. Centrins from yeast, green algae and humans have been shown to form multimers under physiological conditions [9,11]. Studies on the polymerization properties of centrins indicate that the Ca^{2+}-induced polymerization of centrins, e.g., the formation of contractile centrin-fibers in green algae, is mainly dependent on the amino-terminal domain [9,11–13]. However, few investigations have focus on the role of C-terminal domain of centrin during the process of protein self-assembly.

Ciliate *Euplotes octocarinatus* centrin (EoCen), cloned firstly by our groups (GenBank accession number: Y18899), belongs to the EF-hand calcium-binding protein [21]. Like calmodulin (CaM), it is composed of two structurally independent globular domains connected by a flexible linker. Each structural domain contains two helix-loop-helix (known as EF-hand) calcium-binding motifs [14,15]. The conformational changes of EoCen induced by metal ions (Ca^{2+}; Tb^{3+}) have been characterized by spectral methods [16,17]. Cation binding to EoCen controls an important structural transition of protein from a closed state to an open state resulting in exposure of hydrophobic surface. In addition, binding of melittin with EoCen suggested that hydrophobic patches of EoCen mainly lie in the C-terminal domain of protein [18,19]. Up to now, no papers about the cell functions of EoCen have been reported.

Abbreviations: MTOC, microtubule-organizing center; CRC, Chlamydomonas reinhardtii caltractin; EoCen, *Euplotes octocarinatus* centrin; N-EoCen, N-terminal domain of *Euplotes octocarinatus* centrin; C-EoCen, C-terminal domain of *Euplotes octocarinatus* centrin; HsCen1, human centrin 1; HsCen2, human centrin 2; HsCen3, human centrin 3; PAGE, polyacrylamide gel electrophoresis; SPB, spindle pole body; RLS, resonance light scattering; CaM, calmodulin; EDTA, disodium ethylenediaminetetraacetatic acid; Hepes, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ANS, 8-anilino-naphthalene-1-sulfonate acid; GST, Glutathione S-transferase; TEMED, N,N,N’,N’-tetramethylethylenediamine; Trit, Tris-[hydroxyl-methyl]-aminomethane; IPTG, isopropyl-β-thiogalactopyranoside.

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quence analysis reveals that EoCen exhibits high sequence identity with human centrins (60%, 62% and 66%, respectively, with human centrin 1 HsCen1, human centrin 2 HsCen2 and human centrin 3 HsCen3). EoCen also contains the so-called N-terminal extension. Therefore, it can be inferred that EoCen maybe holds the characteristic of polymerization. Indeed, our previous work has proved the importance of the N-terminal domain in the process of EoCen self-association [20]. However, EoCen, truncated the first 23 residues, still has the propensity to self-assembly. It induces us to investigate whether the C-terminal domain of EoCen contributes to the protein self-association and whether this kind of association was participated by C-terminal domain of protein led to especial function of centrin. In order to confirm it, full length EoCen (1–168 aa) and two isolated domain C-EoCen (90–168 aa) as well as N-EoCen (1–101 aa) were constructed, expressed and purified by biological technology. By means of native-PAGE (polyacrylamide gel electrophoresis), pull-down assay, fluorescence resonance light scattering (RLS) and yeast two-hybrid assay, the self-assembly characterization of EoCen was studied under physiological conditions. Our results revealed the contribution of the C-terminal domain in EoCen self-assembly. On the basis of the present results, we put forward a new binding mode of centrin.

2. Materials and methods

2.1. Protein preparation

Three constructs of EoCen were used in this paper, namely, the full length protein (EoCen, 1–168 aa), isolated N-terminal domain of EoCen (N-EoCen, 1–101 aa) and isolated C-terminal domain of EoCen (C-EoCen, 90–168 aa). For the constructs of pGEX-6p-1-EoCen, pGEX-6p-1-N-EoCen and pGEX-6p-1-C-EoCen plasmids, the PCR products of EoCen, N-EoCen and C-EoCen were generated by using EoCen as template and then subcloned into expression vector pGEX-6p-1. After verification by DNA sequence analysis, the recombinant plasmid was transferred into E. coli. (DE3), which was incubated at 37 °C. At an optical density of 0.6–0.8 (at 600 nm), protein synthesis was induced using isopropyl-β-D-thiogalactopyranoside (IPTG 0.8 mM) for 3.5 h. Fusion proteins (EoCen, N-EoCen, and C-EoCen) were purified as a GST fusion protein using glutathione sepharose 4FF in PBS (KH2PO4 1.8, Na2HPO4 10, KCl 2.7 and NaCl 200 mM). The GST fusion proteins were then cleaved by PreScission Protease (PPase) and the cleavage protein fragments were further purified by HPLC [30]. The purity of the intermediate and final samples was assessed by SDS–PAGE and reverse-phase HPLC. After purification, the proteins were concentrated and kept at −80 °C.

The primers used for construction of recombinant plasmids were listed in Table 1.

2.2. Native- and SDS–PAGE assays

The electrophoretic mobility of protein was evaluated by non-denaturing polyacrylamide gel electrophoresis as a modified technique described by Beckingham [30]. Polyacrylamide gels contained 373 mM Tris (Tris-(hydroxyl-methyl)-aminomethane) (pH 8.5), 200 μM CaCl2, 1% ammonium persulfate and 30% acrylamide/bis (29:1). To induce polymerization, 0.1% TEMED (N,N,N',N'-tetramethylethylenediamine) was added. Tris-glycine electrophoresis running buffer contained PB buffer (in mM): KH2PO4 1.8, Na2HPO4 10, KCl 2.7 and 200 μM CaCl2 (pH 8.3). Samples in 0.1 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes) and 200 μM CaCl2 were diluted with non-denaturing buffer (150 mM Tris, 20% glycerol, 0.01% bromophenol blue) and loaded onto native-PAGE gels. Gels were run at a constant current of 11–12 mA for 2 h at room temperature. Then, it was stained with Coomassie blue R-250 for 2 h and washed in distilled water for 2 h.

For SDS–PAGE, keeping same experimental conditions as above described except that 1% SDS was added and running buffer contained 25 mM Tris (pH 8.3), 250 mM glycine, 1% SDS was used.

2.3. Glutathione S-transferase (GST) pull-down assay

In 10 mM Hepes buffer at pH 7.4, purified fusion proteins (5 mg) were mixed with 50 μL of glutathione Sepharose 4FF beads for 2 h at 4 °C (Amersham Pharmacia Biotech). Then 100 μL of supernatants containing recombinant EoCen or N-EoCen or C-EoCen (about 10−3 M) were added to the beads that were saturated by GST-proteins. The mixtures were incubated for 2 h at 4 °C with and end-over-end shaking. Then, beads were washed with 10 mM Hepes buffer to remove non-specific proteins and eluted by reductive glutathione. Bound proteins were analyzed by SDS–PAGE. Protein of the purified GST was used as negative control. Under same experimental conditions, purified proteins of GST (5 mg) were firstly added to the 50 μL of glutathione Sepharose 4FF beads. Then EoCen or N-EoCen or C-EoCen (1 mg) was added to the beads and the bound proteins were eluted by reductive glutathione. The results were analyzed by SDS–PAGE.

2.4. Resonance light scattering

Resonance light scattering (RLS) of samples was monitored by fluorescence in quartz cells of 1-cm optical path at room temperature (25 °C). The RLS was performed in 0.1 M Hepes at pH 7.4 with a fluorescence spectrometer (F-2500, Hitachi, Japan), using the same excitation and emission wavelengths. Samples (3 μM ca.) were prepared by gradually adding CaCl2 into solution of proteins. An equilibrium time of 5 min was used between each titration.

2.5. Yeast two-hybrid analysis

Yeast two-hybrid analysis was conducted described as previous [22-24,31,32]. Yeast cultures were grown using standard conditions [33] in YPD liquid medium [2% (w/v) bactopeptone, 1% (w/v) yeast extract, and 2% (w/v) glucose]. Yeast transformants were grown in synthetic minimal (SD) media [2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base without amino acids (Difco), supplemented with the required amino acids and cofactors] or in synthetic complete (SC) media [33].

<table>
<thead>
<tr>
<th>Genes</th>
<th>Vector</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>EoCen</td>
<td>pGBKTT7</td>
<td>GCGGAATTCATGATGAAGAAGCCAG</td>
<td>GCCCTGCAAGATTACCAAGTGGCC</td>
</tr>
<tr>
<td>EoCen</td>
<td>pGADTT7</td>
<td>GCGGAATTCATGATGAAGAAGCCAG</td>
<td>GCCCTGCAAGATTACCAAGTGGCC</td>
</tr>
<tr>
<td>N-EoCen</td>
<td>pGBKTT7</td>
<td>GCGGAATTCATGATGAAGAAGCCAG</td>
<td>GCCCTGCAAGATTACCAAGTGGCC</td>
</tr>
<tr>
<td>N-EoCen</td>
<td>pGADTT7</td>
<td>GCGGAATTCATGATGAAGAAGCCAG</td>
<td>GCCCTGCAAGATTACCAAGTGGCC</td>
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<tr>
<td>C-EoCen</td>
<td>pGBKTT7</td>
<td>GCGGAATTCATGACAGACATCTG</td>
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<tr>
<td>C-EoCen</td>
<td>pGADTT7</td>
<td>GCGGAATTCATGACAGACATCTG</td>
<td>GCCCTGCAAGATTACCAAGTGGCC</td>
</tr>
</tbody>
</table>

* The underlined sequences are EcoRI and Xhol, EcoRI and Pst restriction enzyme sites for vectors of pGADT7 and pGBKTT7, respectively.
The Matchmaker GAL-4-based two-hybrid system (Clontech) was employed in *E. octocarinatus*. *S. cerevisiae* strain. In the Two-hybrid system, there are two vectors. The first test proteins (bait) were fused into the GAL4 DNA-BD in the pGBK7T7 vector and the second test proteins (prey) were fused into the GAL4 DNA-AD in the pGADT7 vector. In the present works, the EoCen genes were, respectively, subcloned into pGBK7T7 and pGADT7 vectors with primers (Table 1). The sequences of the DNA inserts were verified by sequence analysis. AH109 was cotransformed with plasmids of pGBK7-EoCen (bait) and pGADT7-EoCen (prey). The transformants were selected on both tryptophan- and leucine-deficient media. *LacZ* reporter gene will be expressed, if there are interactions between two proteins of EoCen. Thus, the activated β-galactosidase (with X-gal as substrate) will be an indication of protein–protein interaction. Similarly, N-EoCen or C-EoCen was also subcloned into two vectors above mentioned, cotransformed into AH109 and investigated by *LacZ* reporter gene. The pairs of empty vector pGBK7T7 and gene encoded EoCen, pairs of empty vector pGADT7 and gene encoded EoCen were used as the negative controls.

3. Results

3.1. Native PAGE assay

Native-PAGE has been widely used to examine protein–protein interactions in various fields [25]. As shown in Fig. 1A, two bands of EoCen in 0.1 M Hepes and 200 µM CaCl2 (pH 8.0) with different mobility can be observed, which implied two different conformations of the protein. With the increasing of EoCen concentration (lane 2–5), the intensity of the two bands increased. However, no cooperativity during self-association, the system being described by mass balance equations for polymer and monomer of EoCen, the concentrations of species for polymer and monomer can be calculated by the following Formulas (1)–(5)

$$n \cdot \text{protein} = \text{polymer}$$  \hspace{1cm} (1)

$$[P]_{\text{mono}} = \frac{l_I}{I}$$  \hspace{1cm} (2)

$$[P]_{\text{poly}} = \frac{l_I}{I^n}$$  \hspace{1cm} (3)

$$K = \frac{[P]_{\text{poly}}}{[P]_{\text{mono}}} = \frac{i \cdot l_I}{n \cdot l_I^2}$$  \hspace{1cm} (4)

$$\frac{l_I}{l_I^2} = K \cdot \frac{n}{I}$$  \hspace{1cm} (5)

where $[P]_{\text{poly}}$ and $[P]_{\text{mono}}$ represent the concentration of polymer and monomer of EoCen, respectively. $l_I$ and $l_I^2$ are intensity of band I and II, respectively. And n represents the number of subunits in the multimeric complexes and i is the intensity of one unit of EoCen.

It can be seen from above formulas that the ratio of $l_I$ to $l_I^2$, relating with equilibrium constant is invariable nearly. In addition, EoCen, which was denatured in boiling water for 10 min at 95°C and dissolved in buffer containing 1% SDS, shows one electrophoresis band on the gel (Fig. 1A lane 1). The mobility of denatured EoCen is same to that of low band in lane 2–5 in Fig. 1A. This suggests that EoCen exists in the forms of monomer and multimer in the presence of Ca²⁺: Fig. 1B and Fig. 1C shows the monomer and multimer bands of purified N-EoCen and C-EoCen, respectively. Like full length protein EoCen, the ratios of the two forms of N-EoCen or C-EoCen distributes along two lines (curve b and c, respectively, in Fig. 1D).

3.2. In vitro self-assembly of EoCen

Fusion protein of GST-EoCen in the presence of 1.0 mM Ca²⁺ was firstly immobilized onto glutathione sepharose 4FF beads and then incubated with EoCen for 2 h at 4°C. After washing the beads mixtures with 10 mM Hepes buffer to remove unbound proteins, the sample was eluted by glutathione and analyzed by SDS–PAGE. It can be seen from Fig. 2A that the sample exhibited two bands of GST-EoCen and EoCen in the presence of Ca²⁺ (lane 2), suggesting

![Fig. 1. Native polyacrylamide gel electrophoresis of EoCen (A), N-EoCen (B), C-EoCen (C). A: lane 1: denatured EoCen (3.8 µg); concentration of EoCen is 5 (lane 2), 10 (lane 3), 20 (lane 4), 40 µg (lane 5), respectively. B: lane 1: denatured N-EoCen (2.3 µg); the concentration of N-EoCen is 2.3 (lane 2), 9.2 (lane 3), 37 (lane 4) µg, respectively. C: lane 1: denatured C-EoCen (5.3 µg); the concentration of C-EoCen is 18 (lane 2), 46 (lane 3), 64 (lane 4) µg, respectively. D: Plot of the ratio $l_I/l_I^2$ (intensity of lower band/intensity square of upper band) of EoCen (a), N-EoCen (b) and C-EoCen (c) against the concentrations of proteins.](image-url)
that the complex of GST-EoCen with EoCen has been formed under the conditions. While band of EoCen in the absence of Ca\(^{2+}\) could not be observed in lane 3 indicating the necessity of Ca\(^{2+}\) during EoCen self-assembly. At the same time, negative control experiment between purified GST and EoCen was carried out under same conditions. Results show that no band of EoCen has been observed (lane 4), demonstrating no non-specific interaction exist. This indicates that molecular interactions occur between two proteins of EoCen whereas GST alone failed to interact with EoCen. At pH 7.4, similar experimental protocols proved that GST-N-EoCen immobilized onto glutathione sepharose 4FF beads could bind with N-EoCen in the presence of 1.0 mM Ca\(^{2+}\) (Fig. 2B). But it could not bind with C-EoCen in spite that the concentration of Ca\(^{2+}\) reached to 2.0 mM and reaction time extended to 24 h at 4 °C. In addition, GST pull-down assay also indicated that one molecular of C-EoCen could bind to another molecular of EoCen in the presence of Ca\(^{2+}\) (1.0 mM) and Triton X-100 (0.1 mM) at pH 7.4. Whereas Ca\(^{2+}\) alone (Fig. 2C lane 1) or Triton X-100 alone (Fig. 2C lane 3) failed to induce the self-assembly of C-EoCen. It implies that both Ca\(^{2+}\) and Triton X-100 are necessary during the process of C-EoCen self-assembly. The interactions of GST-N-EoCen with C-EoCen, GST-C-EoCen with N-EoCen has not observed (data not shown). Negative controls revealed that GST could not bind with N-EoCen or C-EoCen (data not shown).

### 3.3. Resonance light scattering measurement

A series of fluorescence RLS was conducted at different wavelength between 250 nm and 600 nm to monitor the aggregation of EoCen in 0.1 M Hepes at pH 7.4. The RLS peak of EoCen located at near 366 nm. To correct dilution, the RLS intensity at 366 nm was converted to molar RLS intensity by dividing the RLS intensity (molar RLS) via the analytical concentration of EoCen. Titration curve of molar RLS versus [Ca\(^{2+}\)] was plotted. As shown in Fig. 3A, with the addition of Ca\(^{2+}\), molar scattering light intensity at 366 nm increased continuously and finally reached to a larger

![Fig. 2. In vitro binding of EoCen with GST-EoCen (A), binding of N-EoCen with GST-N-EoCen and GST-C-EoCen (B), and binding of C-EoCen with GST-C-EoCen (C).](image)

![Fig. 3. Resonance light scattering of Ca\(^{2+}\) titration EoCen or N-EoCen or C-EoCen. (A) Plot of the molar resonance light scattering intensity (molar RLS) at 366 nm of Ca\(^{2+}\) titrating EoCen ( ), N-EoCen ( ) and C-EoCen ( ) as a functions of the concentration of Ca\(^{2+}\) (millimolar) in 0.1 M Hepes at pH 7.4 and room temperature. (B) a: RLS titration curves of adding Ca\(^{2+}\) to the mixed solution of N-EoCen and C-EoCen (1:1) 0.1 M Hepes at pH 7.4. b: The sum of titration curves of Ca\(^{2+}\) titrating N-EoCen and C-EoCen individual 0.1 M Hepes at pH 7.4.)](image)
amplitude at [Ca$^{2+}$] = 0.7 mM, which indicated the presence of large scattering particles, with sizes comparable to the observation wavelength. Under same experimental conditions, we have also observed similar phenomena of scattering light intensity enhancement while Ca$^{2+}$ was added into the solution of N-EoCen (Fig. 3A). In 0.1 M Hepes at pH 7.4, Ca$^{2+}$ induced N-EoCen self-assembly forming larger scattering objects. While disodium ethylenediaminetetraacetic (EDTA) was added into the solution of Ca$^{2+}$-saturated N-EoCen, molar RLS at 366 nm was deduced significantly indicating the disappearance of scattering particles (data not shown). Hence, the self-assembly process is reversible. As far as C-EoCen, addition of Ca$^{2+}$ also led to the enhancement of scattering light in 0.1 M Hepes at pH 7.4 (Fig. 3A). Different from N-EoCen, final amplitude of C-EoCen induced by Ca$^{2+}$ are relatively low which maybe resulted from small molar extinction coefficient of C-EoCen [26]. In addition, RLS spectra of EoCen are different from that of N-EoCen alone, C-EoCen alone and the calculated sum of the individual N-EoCen and C-EoCen in the presence of 1.0 mM Ca$^{2+}$ (data not shown), which demonstrates the exist of cooperativity between two domains of EoCen. To identify whether there is interaction between the N-terminal and C-terminal domains of EoCen, the interaction was carried out by Ca$^{2+}$ titrating the mixed solution of C-EoCen and N-EoCen (1:1) in 0.1 M Hepes by RLS (Fig. 3B). It can be seen from titration curve that Ca$^{2+}$ titrating the mixed solution of N-EoCen and C-EoCen (1:1) also results in the enhancement of RLS at 366 nm. In addition, the titration curve of Ca$^{2+}$ vs. the mixture solution of N-EoCen and C-EoCen (1:1) is very close to the sum of the individual curves of Ca$^{2+}$ titrating N-EoCen and C$^{2+}$ titrating C-EoCen in the buffer of 0.1 M Hepes at pH 7.4 (Fig. 3B). This suggests that no interaction between N-EoCen and C-EoCen, which is in accordance with our previous results. All the spectral changes are reversible when either Ca$^{2+}$ or EDTA is eliminated.

3.4. In vivo assay for protein–protein interactions

Yeast two-hybrid assay is a very sensitive and unique technique for evaluating in vivo protein-protein interactions [22-24]. In the present yeast two-hybrid system, LacZ was used as a reporter gene. While one protein interacts with another protein, LacZ will be transcribed and then strains turn blue in medium containing a chromogenic substrate of 5-Bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-gal) [27]. Firstly, EoCen DNA was fused in-frame with GAL4BD (binding domain) to generate GAL4BD-EoCen while another EoCen DNA was fused in-frame with GAL4AD (activation domain) to generate GAL4AD-EoCen, respectively. Transformants expressing fusion proteins of GAL4BD-EoCen and GAL4AD-EoCen were tested for the expression of the reporter LacZ gene. Mating with cells containing an empty vector (GAL4BD or GAL4AD) were used as a negative control to determine whether there was an intrinsic transcriptional activation capacity, auto-activation activity or non-specific binding of EoCen to any unrelated proteins in yeast. As shown in Fig. 4a, expressions of cells co-transformed with plasmids of GAL4BD-EoCen and GAL4AD-EoCen turned blue in the presence of X-gal, indicating that there was interaction between two proteins EoCen in E. octocarinatus. On the other hand, there was no blue color for cells transformed with negative control vectors, indicating there were no auto-activation and non-specific binding of LacZ to yeast proteins (no cross-reaction of EoCen to the corresponding empty vector) (Fig. 4f and 4g). To identify the binding domain of two EoCen in vivo, AH109 yeast cells were co-transformed with plasmids expressing GAL4BD fused with N-EoCen and GAL4AD fused with N-EoCen or C-EoCen. At the same time, reactive pair of GAL4BD-C-EoCen with GAL4AD-C-EoCen has also been investigated by the yeast two-hybrid assay. Transformants were selected and measured for the expression of the reporter gene LacZ (Fig. 4b).

The results showed that expressions of cells co-transformed with plasmids of GAL4BD-C-EoCen and GAL4AD-C-EoCen, GAL4BD-N-EoCen and GAL4AD-N-EoCen turned blue in the presence of X-gal (Fig. 4b and 4c). In other word, N-EoCen and C-EoCen could bind with EoCen as well as the N-terminal domain of one molecular EoCen interacts with that of another EoCen as well as the N-terminal domain of one molecular EoCen interacts with that of another EoCen.

4. Discussion

Our results of biochemical, molecular biology and physicochemical experiments indicate that under the physiological conditions Euplotes centrin EoCen has the tendency to self-assembly whether in vivo or in vitro. Results indicate that it is Ca$^{2+}$-dependent and reversible. The C-terminal of EoCen also contributes to EoCen self-assembly besides its N-terminal domain. Addition of high concentration of NaCl led to the significant decrease of the RLS intensity of Ca$^{2+}$-saturated N-EoCen indicating that the molecular interactions of N-EoCen are mediated probably by electrostatic forces (Fig. 15). In addition, scattering light of Ca$^{2+}$-saturated C-EoCen was quenched to 70% by ANS (8-anilino-naphthalene-1-sulfonate acid) (Fig. 2S), which is a hydrophobic fluorescence probe and generally used to detect the exposed polar surfaces in protein structure [28]. The ANS inhibition effect demonstrates that ANS binding is in competition with the protein–protein interactions. The ensembles of experimental observations suggest that molecular interactions are maybe mediated by electrostatic and hydrophobic forces. Of course, other factors including hydrogen bond, the size, shape and conformation of EoCen and so on may have effects on the protein self-assembly [26,29]. Comparative sequence analysis (Fig. 5) reveals that both HsCen2 and EoCen contain a positively charged N-terminal extension, which is well in accordance with HsCen2. In spite of high sequence identity between EoCen and HsCen2, they...
still displays different assembly fashion. It can be analyzed from Fig. 5 that the N- and C-terminal domains of HsCen2 have pl values of 7.91 and 4.46, respectively. Its positively N-terminal domain recognizes, binds to negatively charged areas surrounding the C-terminal domain and forms polymerization [30]. Unlike human centrin, both domains of EoCen have low pl values (pl = 4.5 ca). Therefore, the N-terminal domain can not bind with its C-terminal domain. It has been reported that Ca2+-loaded centrins from different organisms including Euplotes, human and Chlamydomonas reinhardtii occupied open conformation [12–14]. However, this confirmation of centrin from different organisms may be different. And their subtle differences would possibly result in the various self-assembly properties between centrin clone from different organisms. In addition, there may exist unique hydrogen bond forming from particular amino acid alignment of EoCen, which contributes its characteristic of self-assembly. During EoCen polymerization, C-EoCen plays equal role to N-EoCen. The C-EoCen binds two Ca2+ via EF-hands III and IV and adopts an open conformation which mediates the binding with peptide [16]. In accordance with HsCen 2 [12] and Chlamydomonas centrin [31], the C-terminal fragment has the tendency to self-associate. EoCen aggregates in a mode of N-to-N (between two N-terminal domains) and C-to-C (between two C-terminal domains). The self-assembly of C-EoCen need the existence of both Ca2+ and Triton X-100 (0.1 mM). Spectroscopic experiments indicated a stronger Ca2+-affinity (105 M-1) 

![Fig. 5. Sequence comparison of EoCen (Euplotes octocarinatus centrin), CRC (Chlamydomonas reinhardtii caltractin), HsCen1 (human centrin 1), HsCen2 (human centrin 2), HsCen3 (human centrin 3), and CaM (calmodulin).](image)

tered mitosis [35]. And centrin aggregation fashions are unclear in the other phases of cell cycles. Different assembly mode of centrin for example N-to-N or N-to-C may lead to diverse cell morphologies or contractile behaviors of fibers. Otherwise, the identification of this new protein–protein interaction fashion will make for setting up structure–function relationships.

In summary, EoCen self-assembles in vivo and in vitro under the experimental conditions tested. Besides its N-terminal domain, the C-terminal domain of EoCen also attributes to the self-associate of protein. It is a Ca2+-dependent and reversible manner. EoCen self-associates in a mode of C-to-C as well as N-to-N, in which the C-terminal domain of one molecular EoCen interacts with that of another EoCen as well as the N-terminal domain of one molecular EoCen interacts with that of another EoCen. During this process, molecular interactions are mediated by both ionic and hydrophobic forces. The characterization of EoCen self-assembly will make for further studying biological functions.

Acknowledgements

We are very grateful to Dr. Zhepeng Wang for suggestions and revisions concerning the manuscript. We acknowledge to the financial support of this work by the National Natural Science Foundation of PR China (No. 200771068) and the National Science Foundation of Shanxi Province (No. 2007011024).

Appendix A. Supplementary material


References


