Bacterial expression and preliminary crystallographic studies of a 149-residue fragment of human Caprin-1

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Caprin-1 is an RNA-binding protein which plays critical roles in several important biological processes, including cellular proliferation, the interferon-mediated antiviral innate immune response, the maintenance of synaptic plasticity and the formation of RNA stress granules. Caprin-1 has been implicated in the pathogenesis of several human diseases, including osteosarcoma, breast cancer, viral infections, hearing loss and neurodegenerative disorders. Despite the emerging biological and physiopathological significance of Caprin-1, no structural information is available for this protein. Moreover, Caprin-1 does not have sequence similarity to any other protein with a known structure. It is therefore expected that structural studies will play a particularly crucial role in revealing the functional mechanisms of Caprin-1. Here, a protein fragment of human Caprin-1 consisting of residues 112–260 was expressed, purified and crystallized. Native and Se-SAD data sets were collected to resolutions to 2.05 and 2.65 Å, respectively, in different space groups.

1. Introduction

Caprin-1 (cytoplasmic activation/proliferation-associated protein-1) and Caprin-2 are members of the Caprin protein family. These two proteins contain two homologous regions (HR1 and HR2; Grill *et al.*, 2004; Shiina *et al.*, 2005; Fig. 1). Human Caprin-1 and Caprin-2 are 51% identical and 73% similar in their HR1 regions and 36% identical and 54% similar in their HR2 regions. These two regions are highly conserved in vertebrates. The sequences of HR1 and HR2 do not conform to any consensus sequence for known protein domains suggestive of functions. Caprin-2 has an identifiable structural domain at its C-terminus (CRD; C1q-related domain) which is homologous to the globular head domain of the complement protein C1q (Shapiro & Scherer, 1998; Gaboriaud *et al.*, 2003; Garlatti *et al.*, 2010; Venkatraman Girija *et al.*, 2013; Miao *et al.*, 2014). Caprin-1 and Caprin-2 also contain RGG boxes and RG-rich sequences characteristic of RNA-binding proteins near the C-terminal region of HR2 (Fig. 1).

Caprin-1 and Caprin-2 are both multifunctional proteins. Caprin-1 has been studied much more intensively than Caprin-2. The available biochemical data indicate that the functions of Caprin-1 and Caprin-2 are independent and nonredundant.

Caprin-1 has many established functions. Firstly, Caprin-1 plays an essential role in cellular proliferation. High levels of Caprin-1 have been observed in proliferating T or B lymphocytes and hemopoietic progenitors (Grill *et al.*, 2004). At the tissue level, Caprin-1 is highly expressed in the thymus

and the spleen and its level is lowest in tissues with a low proportion of dividing cells (such as kidney or muscle). The adult brain is an exception, in which Caprin-1 is highly expressed and assumes a different function (see below for details). The absence of endogenous Caprin-1 in *CAPRIN1*-null DT40 cells led to a marked reduction in the proliferation rate, with a prolongation of the G1 phase of the cell cycle (Wang *et al.*, 2005). Conditional expression of human Caprin-1 restored the proliferation rate to the wild-type level. These experiments show that Caprin-1 is a positive regulator of cell proliferation and is required for the G1/S transition in the cell cycle. The molecular mechanism is largely unknown. It has been shown that Caprin-1 forms a stable complex with Ras-GAP SH3 domain-binding protein 1 (G3BP1; Solomon *et al.*, 2007). The Caprin-1–G3BP1 complex is localized in cytoplasmic RNA granules associated with microtubules. This complex selectively binds mRNAs of c-Myc and cyclin D2, two proteins with essential roles in the G1/S transition (Chiles, 2004; Kaczmarek *et al.*, 1985; Pardee, 1989). It is possible that the Caprin-1–G3BP1 complex regulates the transport and translation of mRNAs encoding proteins involved in cellular proliferation. In contrast to Caprin-1, the Caprin-2 level is increased at the stage of differentiation (Aerbajinai *et al.*, 2004), suggesting its involvement in the developmental transition from rapid proliferation towards terminal differentiation.

Caprin-1 is also involved in the regulation of synaptic plasticity. As a highly expressed protein in the brain, Caprin-1 is localized in the neuronal RNA granules in dendrites of hippocampal and neocortical pyramidal neurons (Shiina *et al.*, 2005). These RNA granules contain mRNAs of key proteins for synaptic plasticity, such as Ca^{2+} /calmodulin-dependent kinase II α (CaMKII α ; Burgin *et al.*, 1990), brain-derived neurotrophic factor (BDNF; Tongiorgi *et al.*, 1997), tyrosine receptor kinase B (TrkB; Tongiorgi *et al.*, 1997) and cAMP response element-binding protein (CREB; Crino *et al.*, 1998). Local translation of these mRNAs plays important roles in long-term synaptic plasticity (Kiebler & Bassell, 2006). Caprin-1 can bind directly to mRNAs and repress their translation *in vitro* and *in vivo* (Shiina *et al.*, 2005). Synaptic stimulation by BDNF induced dissociation of Caprin-1 from the neuronal RNA granules. The mRNAs were released and shifted to polysomes for local translation. Caprin-1 knockout mice died soon after birth owing to respiratory failure, suggesting defects in the brain stem (Shiina *et al.*, 2010). Caprin-2 is also highly expressed in brain and localized to neuronal RNA granules (Shiina & Tokunaga, 2010). However, the Caprin-1-containing and Caprin-2-containing neuronal

granules are different (for example, G3BP1 and ribosome components are found in the Caprin-1-containing granules but not in the Caprin-2-containing granules). Suppression of either Caprin-1 or Caprin-2 by siRNA knockdown reduces dendrite length and spine density in cultured neurons (Shiina & Tokunaga, 2010). Rescue experiments showed that Caprin-1 knockdown was rescued by Caprin-1 expression but not by Caprin-2 expression and that Caprin-2 knockdown was rescued by Caprin-2 expression but not by Caprin-1 expression. These results indicate that Caprin-1 and Caprin-2 both participate in neuron development but play distinct roles (Shiina & Tokunaga, 2010).

Caprin-1 also participates in the cellular stress response mediated by cytoplasmic stress granules (SGs). It has been shown that Caprin-1 (together with G3BP1) localized into SGs in response to oxidative stress induced by arsenite treatment (Solomon *et al.*, 2007). Like many RNA-binding proteins (RBPs) found in SGs, Caprin-1 promotes SGs when it is overexpressed. In cochlear hair cells, *CAPRIN1* transcription is down-regulated by the transcription factor Pou4f3 (Towers *et al.*, 2011), which is essential for the survival of auditory sensory hair cells (Xiang *et al.*, 1997). Several mutations in the human *Pou4f3* gene cause hearing loss (Collin *et al.*, 2008; Pauw *et al.*, 2008). In hair cells damaged by aminoglycosides, Pou4f3 mRNA levels decreased and Caprin-1 mRNA and protein levels increased, leading to the formation of Caprin-1-containing SGs. Temporal formation of the Caprin-1-containing SGs may constitute a protective response for the damaged hair cells to recover. In patients with mutations in the *Pou4f3* gene, mutated Pou4f3 may be defective in its ability to down-regulate *CAPRIN1* transcription. Persistent overexpression of Caprin-1 may lead to prolonged SG formation, which causes apoptosis of the hair cells. Caprin-1-containing SGs are also involved in the host antiviral response against *Japanese encephalitis virus* (JEV; Katoh *et al.*, 2013). To counter the host antiviral SG response, JEV utilizes its core protein to bind Caprin-1 and recruit several effector molecules that promote SG assembly (including G3BP1) to the perinuclear region, leading to the suppression of SG formation.

Caprin-1 has also been shown to participate in the interferon (IFN)-mediated antiviral innate immune response. IFN stimulation activates the transcription of hundreds of antiviral IFN-stimulated genes (ISGs; Wilson & Brooks, 2013). ISG proteins establish an antiviral state by targeting various steps of the viral life cycle. It was found that Caprin-1 and G3BP1 and G3BP2 mediated the IFN response against Dengue virus 2 (DENV-2; Bidet *et al.*, 2014). The three proteins were required for the translation of ISG mRNAs. To counter the IFN

Figure 1

Schematic diagram showing the putative domain structures of human Caprin-1 and Caprin-2. HR1 and HR2, homologous regions 1 and 2; CRD, C1q-related domain. The thin red lines indicate the locations of the RGG boxes.

response, DENV-2 utilizes its abundant noncoding sub-genomic flaviviral RNA (sRNA) as a decoy to bind Caprin-1 and G3BP1 and G3BP2, leading to a profound inhibition of ISG mRNA translation. How Caprin-1, G3BP1 and G3BP2 regulate the translation of ISG mRNAs and especially how specificity for ISG mRNAs is achieved remain to be elucidated.

While Caprin-1 and G3BP1 participate in antiviral responses against JEV and DENV2, the Caprin-1–G3BP1 complex is actually required for the intermediate-stage transcription of *Vaccinia virus* (VACV) DNA (Katsafanas & Moss, 2007). VACV is a poxvirus which contains double-stranded DNA genomes and replicates in the cytoplasm of host cells. The VACV transcriptional program is divided into three sequentially regulated stages: early, intermediate and late. The intermediate-stage transcription requires host factors, which were identified as Caprin-1 and G3BP1 (Katsafanas & Moss, 2007). The mechanism of action remains elusive.

Besides viral infection and hearing loss as discussed above, Caprin-1 has also been implicated in the pathogenesis of some other human diseases. Caprin-1 ectopic overexpression has been correlated with tumorigenesis and metastasis in osteosarcoma (OS), the most common primary bone malignancy in children and adolescents (Sabile *et al.*, 2013). More than 30% of OS patients develop lung metastasis, which is the leading cause of mortality. In cultured OS cells, Caprin-1 interacts with Cyr61, an OS malignancy-promoting protein. Overexpression of Caprin-1 led to the formation of Caprin-1/Cyr61-containing SGs and decreased the sensitivity of the OS cells to cisplatin-induced apoptosis. In SCID mice, overexpression of Caprin-1 dramatically enhanced primary tumor growth and remarkably increased lung metastasis and mortality. In a group of 59 OS patients, increased Caprin-1 expression was correlated with shortened survival. Moreover, patients with tumors co-expressing Caprin-1 and Cyr61 showed poorer survival than patients expressing one or neither of the proteins.

Ectopic overexpression of Caprin-1 has also been linked to breast cancer. Caprin-1 mRNA is a target of the microRNAs miR-223 and miR-16 (Kaddar *et al.*, 2009; Gong *et al.*, 2013). In a study on the expression of Caprin-1 and miR-223 in several breast cancer cells and normal breast cells, miR-223 expression levels were significantly lower in cancer cells than in normal cells, while Caprin-1 expression was higher in cancer cells than in normal cells (Gong *et al.*, 2013). Overexpression of Caprin-1 promoted the proliferation and invasion of breast cancer cells, which was inhibited by miR-223.

Caprin-1 may also play a role in the pathogenesis of Huntington's disease (HD). HD is a neurodegenerative disorder caused by an expansion of a polyglutamine (polyQ) repeat within huntingtin, the HD gene product (MacDonald *et al.*, 1993; Ross *et al.*, 1997). PolyQ expansion causes a conformational change in huntingtin, leading to protein misfolding/aggregation, aberrant protein interactions and neuronal cell death (Ross & Poirier, 2005; Li *et al.*, 1995). It was found that expanded huntingtin interacted with Caprin-1 and was redistributed to Caprin-1/G3BP1-containing SGs (Ratovitski *et al.*, 2012).

In spite of the emerging biological and physiopathological significance of Caprin-1, no structural study on this protein has been reported. This lack of structural knowledge has hampered research efforts in related fields, especially in view of the fact that Caprin-1 does not have sequence similarities to any other protein with a known structure. We have initiated a structural study on Caprin-1. Here, we report our progress on the bacterial expression, purification and preliminary crystallographic study of a 149-residue fragment of Caprin-1.

2. Materials and methods

2.1. Cloning

A plasmid containing the cDNA for full-length human Caprin-1 (GenBank BC001731, IMAGE clone 3355481) was purchased from the DNASU Plasmid Repository. The DNA encoding the protein construct for crystallization (residues 112–260) was amplified from the plasmid by PCR using Pfu DNA polymerase with the forward primer 5'-CAAGGACC-GAGCAGCCCCCTCATTACAGAGGAGTTTCATGGCA-3' and the reverse primer 5'-ACCACGGGGGAACCAACCCT-TATTCTTCCTCACACAGCCCCAT-3'. Both primers contain a specific 21-nucleotide sequence at the 5'-end for ligation-independent cloning (LIC). The PCR product was purified by agarose gel electrophoresis and processed by T4 DNA polymerase in the presence of 2.5 mM dATP (25°C for 40 min followed by 75°C for 20 min) to generate 5'-overhangs at both ends. The cloning vector is an in-house-modified LIC vector that contains DNA sequences encoding the Halo tag (Los *et al.*, 2008), a His tag, an eight-residue recognition sequence for *Human rhinovirus* (HRV) 3C protease and a specific LIC sequence 5'-TCAAGGACCGAGCAGCCCCGGGTGGTT-CCCCGTGGTA-3'. This LIC sequence contains an SmaI site in the middle. After digestion by SmaI, the cut vector was processed by T4 DNA polymerase in the presence of 2.5 mM dTTP (25°C for 40 min followed by 75°C for 20 min) to generate 5'-overhangs at both ends. The processed PCR insert and vector were mixed together at room temperature and incubated for 20 min. After annealing, the mixture was transformed into *Escherichia coli* DH5 α competent cells. A single colony was picked to grow overnight cultures to amplify the plasmid. Protein expression was carried out using *E. coli* NiCo21 (DE3) cells (New England Biolabs).

2.2. Protein expression and purification

The human Caprin-1 construct, residues 112–260, was expressed as a fusion protein with N-terminal Halo and His tags followed by a HRV 3C protease cleavage site (LEVLFQGP). After protease digestion, the target protein contains an artificial sequence GPSSP (from the protease cleavage site and LIC cloning sequence) at the N-terminus. Bacterial cultures were grown in LB medium until the cell density reached an absorbance of 0.6–0.8 measured at a wavelength of 600 nm. Protein expression was induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) to the culture to a final concentration of 0.4 mM. To obtain soluble

expression of the Caprin-1 protein construct, it was necessary to lower the temperature of the cell cultures to 10°C after induction by IPTG. The cell cultures were allowed to grow for 16 h before harvesting. The overexpressed protein was purified using cobalt agarose affinity resin from Gold Biotechnology. After the purified fusion protein has been eluted from the resin with an elution buffer consisting of 25 mM Tris pH 7.0, 200 mM NaCl, 200 mM imidazole, His-tagged HRV 3C protease was added to the eluted fusion protein. The protein solution (~20 ml) was dialyzed in 5 l of a buffer consisting of 25 mM Tris pH 7.0, 200 mM NaCl at 4°C overnight. Complete cleavage at the HRV 3C recognition site was achieved after dialysis for 12 h (confirmed by SDS-PAGE). The cleaved Halo and His tags were separated from the target protein by a reverse immobilized metal-affinity chromatography (IMAC) process with cobalt agarose affinity resin. The flowthrough from the reverse IMAC column contains the purified target protein. The purified protein was concentrated to a final concentration of ~10 mg ml⁻¹ in a buffer consisting of 25 mM Tris pH 7.0, 200 mM NaCl.

To prepare SeMet-labeled protein samples, the bacterial cells were grown in M9 minimal culture until the cell density reached an absorbance of 0.6–0.8 measured at a wavelength of 600 nm. The six amino acids leucine, isoleucine, lysine, phenylalanine, threonine and valine were added to the culture to a final concentration of 50–100 mg l⁻¹ to inhibit the methionine-biosynthesis pathway. The cell cultures were grown for a further 3 min at 37°C. The temperature was then lowered to 10°C. L-Selenomethionine was added to the cell cultures to a final concentration of 50 mg l⁻¹. Protein expression was induced by IPTG at a final concentration of 0.4 mM. All other steps were identical to the preparation of unlabeled protein samples.

2.3. Crystallization, data collection and processing

Crystallization trials were carried out using 96-well plates. Seven sets of in-house crystallization screening solutions (each containing 96 different conditions) were used in the initial screen. The crystallization solutions used various polyethylene glycols (PEGs) as the precipitants. Other variables included different buffers, pHs, salts, small organic molecules and cryoprotectants. Diffraction-quality protein crystals were obtained in several different crystallization conditions within a few hours. Crystallization optimization was carried out using 96-well plates. The protein crystals used for data collection were obtained at 22°C by sitting-drop vapor diffusion. For native crystals in space group C121, the well solution (50 µl) consisted of 22% PEG 750 MME, 0.1 M Tris pH 8.0, 0.1 M potassium fluoride, 10% glycerol. For SeMet-labeled crystals in space group P3₁21, the well solution consisted of 12% PEG 600, 0.1 M Tris pH 8.0, 0.1 M calcium acetate, 10% glycerol. The crystallization drops consisted of 1 µl protein sample mixed with 1 µl well solution. Using 10% glycerol as the cryoprotectant in the crystallization conditions, the crystals could be directly flash-cooled in liquid nitrogen.

Data collection was carried out on beamline 21ID-G of LS-CAT at the Advanced Photon Source, Argonne National Laboratory. The data were processed, integrated and scaled with the programs *MOSFLM* and *SCALA* in *CCP4* (Battye *et al.*, 2011; Winn *et al.*, 2011).

3. Results and discussion

The initial identification of the HR1 and HR2 regions was solely based on sequence conservation between Caprin-1 and Caprin-2 and among different species (Grill *et al.*, 2004). These regions may not really define globular protein domains. In fact, a different putative domain structure of Caprin-1 has been proposed which contained coiled-coil, NLS, NES, E-rich, Q-rich, RGG-box and RG-rich motifs or regions (Shiina & Tokunaga, 2010; Shiina *et al.*, 2005). The sequence of Caprin-1 has no similarity to any other protein of known structure. Therefore, possible structural domains of Caprin-1 and their boundaries cannot reliably be inferred from analysis of sequence and structural similarities.

We have employed a divide-and-conquer approach to study the structure of Caprin-1. This approach should be appropriate because many published studies have used truncation and/or deletion Caprin-1 constructs to study protein–protein interactions between Caprin-1 and its binding partners (Solomon *et al.*, 2007; Katoh *et al.*, 2013; El Fatimy *et al.*, 2012; Grill *et al.*, 2004; Shiina *et al.*, 2005). The N-terminus of Caprin-1 (about 50 residues) has a strong bias for Gly/Pro/Ala/Ser residues (accounting for 78%); apart from a valine and a methionine, there are no other hydrophobic residues. Therefore, this region has a high propensity to be disordered. The C-terminal RGG/RG-rich sequence (residues 608–709) is involved in RNA binding (Grill *et al.*, 2004). Low-complexity and RGG/RG repeat sequences are present in a large number of proteins (Thandapani *et al.*, 2013). To date, only a solution structure of the complex between a human fragile X mental retardation protein (FMRP) RGG peptide and a G-quadruplex RNA has been determined (Phan *et al.*, 2011). The study shows that the RGG peptide changes from a random coil to a well ordered conformation upon RNA binding. We therefore have focused our structural study on the Caprin-1 region spanning residues 51–608. We have been working on several different protein constructs containing various fragments within this region. To date, we have made good progress with a 149-residue Caprin-1 fragment consisting of residues 112–260.

We have been able to express the Caprin-1 fragment consisting of residues 112–260 in a soluble form as a fusion protein with N-terminal Halo and His tags to facilitate the expression and purification of the protein. An eight-residue HRV 3C protease cleavage site is present between the tags and the Caprin-1 fragment. After purification of the fusion protein using cobalt-affinity resin, the Halo and His tags were severed from the target protein using His-tagged HRV 3C protease. The tags and the added His-tagged HRV 3C protease were subsequently removed by reverse IMAC. The purified target protein was concentrated to ~10 mg ml⁻¹.

(Fig. 2). A native protein sample and an SeMet-labeled protein sample were prepared. Both protein samples yielded diffraction-quality crystals (Fig. 3). Data sets (at the peak wavelength of selenium, 0.9787 Å) were collected from crystals containing native or SeMet-labeled protein, which diffracted to 2.05 and 2.65 Å resolution, respectively. Data-collection and processing statistics are shown in Table 1. The protein used for crystallization has a molecular weight of ~16.5 kDa. We also carried out an SDS–PAGE analysis of the protein in the crystals. The result indicated that the protein in the crystal became slightly smaller (Fig. 2). It is possible that the Caprin-1 fragment consisting of residues 112–260 contains a stable domain of ~15–16 kDa, with the flanking N-terminal and/or C-terminal sequences being flexible. These flexible sequences might be digested by residual protease activity of the protein preparation. For the crystals in space group *C*121, the asymmetric unit is most likely to contain six protein molecules, with a solvent content of 47.3%. For the crystals in space group *P*3₁21, the asymmetric unit is most likely to contain two protein molecules, with a solvent content of 43.5%.

Figure 2
SDS–PAGE of the purified crystallization construct of Caprin-1. The protein bands are stained with Coomassie Blue. Lane *M* contains molecular-weight marker (labeled in kDa). Lane 1, protein sample used for crystallization. Lane 2, protein sample from washed and redissolved crystals.

Figure 3
Crystals of the HR1 domain of the human Caprin-1 protein. The crystals contain SeMet-labeled protein. The crystal in the center has dimensions of about 120 × 50 × 50 µm.

Table 1
Data-collection and processing statistics.

	Native	SeMet
Space group	<i>C</i> 121	<i>P</i> 3 ₁ 21
Unit-cell parameters		
<i>a</i> (Å)	106.2	66.0
<i>b</i> (Å)	77.3	66.0
<i>c</i> (Å)	58.0	117.7
α (°)	90.0	90.0
β (°)	90.0	90.0
γ (°)	90.0	120.0
Resolution (Å)	42.6–2.05 (2.16–2.05)	41.0–2.65 (2.79–2.65)
No. of unique reflections	29397 (4258)	9085 (1292)
Completeness (%)	99.0 (98.8)	100 (100)
<i>R</i> _{merge} (%)	9.6 (54.4)	7.6 (60.6)
$\langle I/\sigma(I) \rangle$	5.4 (1.6)	11.5 (2.8)
Multiplicity	3.1 (3.0)	5.4 (5.4)
Anomalous completeness (%)	N/A	100 (100)
Anomalous multiplicity	N/A	2.9 (2.8)

Because no homologous structure is available, we will use the SAD phasing method to determine the structure of this Caprin-1 fragment. In the SAD data set that we collected, useful anomalous signal extended to ~4.2 Å resolution, which might be sufficient to obtain an initial set of experimental phases to solve the structure using the SAD data set. The native data set has a higher resolution. A structure can be solved from this data set using the molecular-replacement method with the SAD structure as a search model. In view of the fact that the native and SeMet-labeled proteins crystallized in different space groups (Table 1), the structures may also provide insights into possible alternative protein–protein interactions involving this Caprin-1 fragment. To date, no structural information about Caprin-1 is available. Therefore, it is fully expected that structural studies will play a particularly crucial role in deciphering the functional mechanisms of this physiopathologically important protein.

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