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Crystallization and preliminary X-ray analysis of the C-terminal domain of CCM2, part of a novel adaptor protein involved in cerebral cavernous malformations

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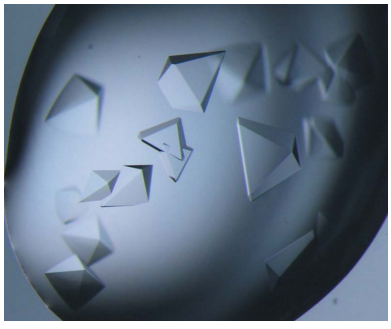
Cerebral cavernous malformation 2 (CCM2) is a novel two-domain adaptor protein which participates in multiple cellular signalling pathways. Loss-of-function mutations in the gene encoding CCM2 are the cause of common human vascular lesions called cerebral cavernous malformations. Here, the purification, crystallization and preliminary X-ray crystallographic studies of the C-terminal domain of CCM2 (CCM2-Ct) are reported. Diffraction data were collected from native and selenomethionine-substituted crystals of CCM2-Ct to resolutions of 2.9 and 2.7 Å, respectively. Both crystals belonged to space group $I4_122$ with similar unit-cell parameters. The native crystals had unit-cell parameters $a = b = 113.29$, $c = 101.62$ Å.

1. Introduction

Cerebral cavernous malformations (CCMs) are sporadic or inherited vascular lesions in the brain that affect 0.1–0.5% of individuals in the general population, often leading to headaches, seizures and stroke owing to focal haemorrhages (Kleaveland *et al.*, 2009). Familial CCMs have been linked to autosomal dominant mutations in three genes, *ccm1–3* (Akers *et al.*, 2009). The encoded CCM proteins have been characterized to interact with each other, forming a large CCM signalling complex (Revencu & Vikkula, 2006). This observation, in association with their involvement in pathology, raised speculation that all CCM proteins participate in common or related pathways that are required for vascular integrity (Pagenstecher *et al.*, 2009).

CCM2 has been predicted to be an adaptor protein which lacks any known catalytic domains but contains a defined phosphotyrosine-binding (PTB) domain at the N-terminus that is responsible for binding partner proteins. As an adaptor protein, CCM2 was first characterized to function as a scaffold involved in small GTPase Rac-dependent p38 mitogen-activated protein kinase (MAPK) activation when the cell is under hyperosmotic stress (Uhlik *et al.*, 2003). In the signalling cascades that regulate vascular integrity, CCM2 associates with CCM1 and participates in HEG1 (the transmembrane receptor heart of glass 1) mediated endothelial cell junctions (Faurobert & Albiges-Rizo, 2010). Both CCM proteins also inhibit the activation of small GTPase RhoA and its downstream effector Rho kinase (ROCK) to limit vascular permeability (Whitehead *et al.*, 2009). Recently, the function of CCM2 has been extended to cell death. By specifically interacting with receptor tyrosine kinase TrkA *via* its N-terminal PTB domain, CCM2 mediates TrkA-dependent cell death in pediatric neuroblastic tumours. The C-terminal domain of CCM2, defined as an independent domain named a Karet domain, links to the death pathway by an unknown mechanism (Harel *et al.*, 2009).

The increasing understanding of the physiological role of CCM2 in multiple signalling pathways has led to great interest in exploring the structural basis of the function of this novel two-domain adaptor protein. However, the structure of CCM2, including those of its individual domains, has not yet been reported. Here, we report the recombinant expression, purification and crystallization of the



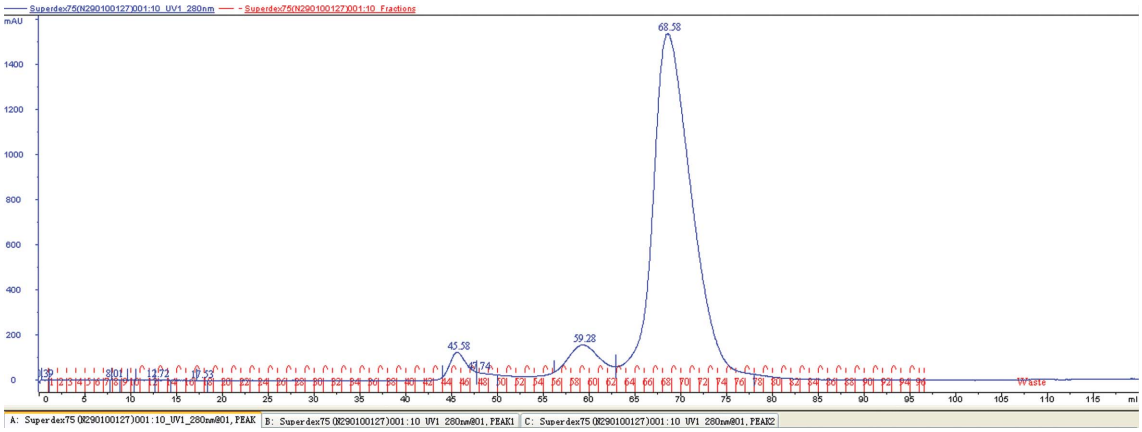


Figure 1
Chromatogram (Superdex 75) of native CCM2-Ct.

C-terminal domain of CCM2, which provide a solid foundation for uncovering its structural characteristics and understanding its functional roles based on the crystal structure.

2. Materials and methods

2.1. Cloning, expression and purification

The gene encoding human full-length CCM2 was purchased from Proteintech (Wuhan, People's Republic of China; NCBI reference sequence NP_113631.1/gi:13899275). A construct for the C-terminal domain comprising residues 290–444 of human CCM2 (designated CCM2-Ct) was amplified by PCR and subcloned into pET22b(+) vector (Novagen) using *Nde*I and *Xho*I restriction sites, resulting in a six-histidine tag fused to the C-terminus of recombinant CCM2-Ct. The plasmids containing the CCM2-Ct construct were amplified in DH5α-competent *Escherichia coli* cells and confirmed by DNA sequencing.

The recombinant plasmid was transformed into *E. coli* host strain BL21 (DE3) competent cells, which were then grown on an LB plate containing 100 µg ml^{-1} ampicillin overnight at 310 K. 5 ml LB medium was inoculated with a single colony and cultured overnight. 1.5 ml bacterial culture was transferred into 50 ml fresh LB medium and cultured for 3 h; 8 ml of the 50 ml bacterial culture was then transferred into 800 ml fresh LB medium. All LB media were supplemented with 100 µg ml^{-1} ampicillin. When the OD_{600} of the culture reached 0.8, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 m*M* to induce the expression of recombinant CCM2-Ct protein. The bacteria were grown at 310 K for an additional 3 h and were harvested by centrifugation (Hitachi Himac CRT, R5S2 rotor) at 4000 rev min^{-1} for 30 min. Purification was completed using an Ni–NTA affinity column (Novagen) and an ÄKTA purification system (Amersham Pharmacia). The cell pellet was resuspended in 25 ml lysis buffer (50 m*M* phosphate buffer pH 8.0, 300 m*M* NaCl, 10 m*M* imidazole, 10 m*M* β-mercaptoethanol) with 1 m*M* phenylmethanesulfonyl fluoride (PMSF). The cells were lysed by sonication and centrifuged at 12 000 rev min^{-1} for 30 min (Sigma 3K30, 12150 rotor). The supernatant was loaded directly onto an Ni–NTA column (Novagen) which was pre-equilibrated with lysis buffer. The column was washed with wash buffer (50 m*M* phosphate buffer pH 8.0, 300 m*M* NaCl, 20 m*M* imidazole, 10 m*M* β-mercaptoethanol). The protein was eluted with elution buffer (50 m*M* phosphate buffer pH 8.0, 300 m*M* NaCl, 250 m*M* imidazole, 10 m*M* β-mercaptoethanol) and further concentrated by ultrafiltration (Millipore). After concentration, the protein was loaded onto a Superdex 75 HiLoad 16/60 column (Amersham Pharmacia) pre-equilibrated with buffer consisting of 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 0.2 m*M* EDTA, 5 m*M* DTT at 277 K. The purified protein was concentrated prior to crystallization. Selenomethionine-substituted (SeMet) CCM2-Ct was produced by expression in *E. coli* methionine-auxotrophic strain B834 (DE3). The purification procedure used for the SeMet derivative was the same as that used for the native protein.

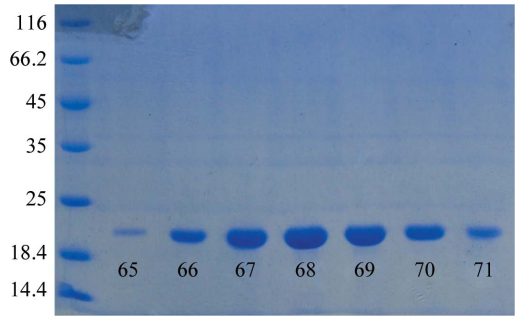


Figure 2
SDS–PAGE of purified native CCM2-Ct. The lane numbers correspond to the fraction numbers in the Superdex 75 chromatogram.

Figure 3
Crystals of native CCM2-Ct (0.2 × 0.2 × 0.2 mm).

2.2. Crystallization

Both native and SeMet CCM2-Ct were concentrated to approximately 30 mg ml^{-1}. Crystallization experiments were set up using the hanging-drop vapour-diffusion method by mixing equal volumes (1 µl) of protein solution and reservoir solution and suspending the drop over 0.5 ml reservoir solution at 293 K (McPherson, 1999). Initial crystallization screening was performed using Crystal Screen and Index kits from Hampton Research (Jancarik & Kim, 1991). The crystallization condition was optimized by varying the type and the concentration of the precipitant and the pH. The optimization of the crystallization conditions for the SeMet derivative was based on that for the native protein with minor modifications.

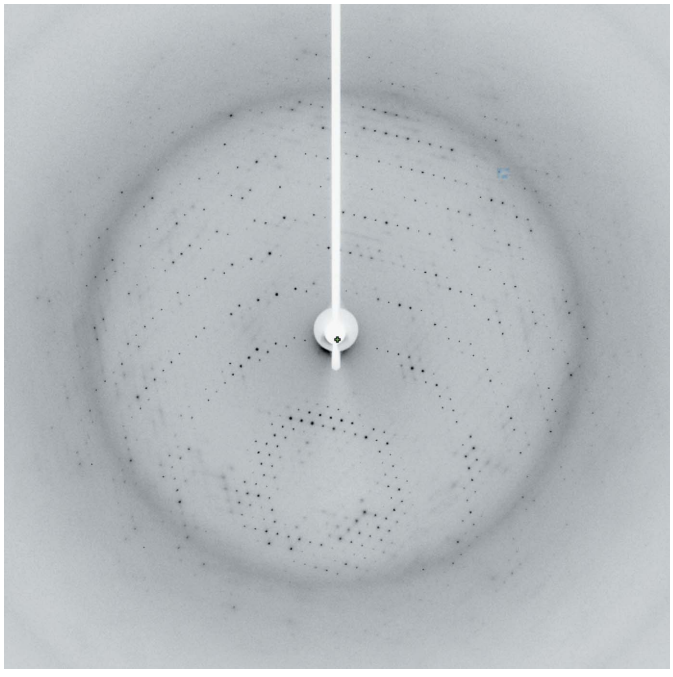


Figure 4
Diffraction pattern of the native CCM2-Ct crystal.

2.3. Data collection and processing

A data set for native CCM2-Ct was collected on beamline NW12A at the Photon Factory (KEK, Tsukuba, Japan). A total of 180 frames were collected at a wavelength of 0.96397 Å with a crystal-to-detector distance of 291 mm, 1° oscillation and 20 s exposure per frame. A single-wavelength anomalous diffraction (SAD) data set was collected for the SeMet derivative on beamline BL17U of the Shanghai Synchrotron Radiation Facility (Shanghai, People's Republic of China). A total of 180 frames were collected at the peak wavelength of 0.97916 Å with a crystal-to-detector distance of 250 mm, 1° oscillation and 1.2 s exposure per frame. *MOSFLM* (v.6.2.2; Leslie, 1992) and *SCALA* from the *CCP*4 program suite (v.4.2.2; Winn *et al.*, 2011) were used for the processing, reduction and scaling of the diffraction data sets.

3. Results

CCM2-Ct was expressed in *E. coli* host strain BL21 (DE3) and purified using an Ni–NTA affinity column (Novagen) followed by a Superdex 75 HiLoad 16/60 column (Amersham Pharmacia; Fig. 1). Purified protein was obtained (Fig. 2) and concentrated to approximately 30 mg ml^{-1}.

In the initial screening, tiny single native crystals were observed from several conditions of the Index kit after 3–4 d. As the result of optimization, well shaped native crystals (0.2 × 0.2 × 0.2 mm) of CCM2-Ct were obtained using 24–28% Tacsimate pH 7.0 (Hampton Research; Fig. 3). SeMet-derivative crystals (0.2 × 0.2 × 0.2 mm)

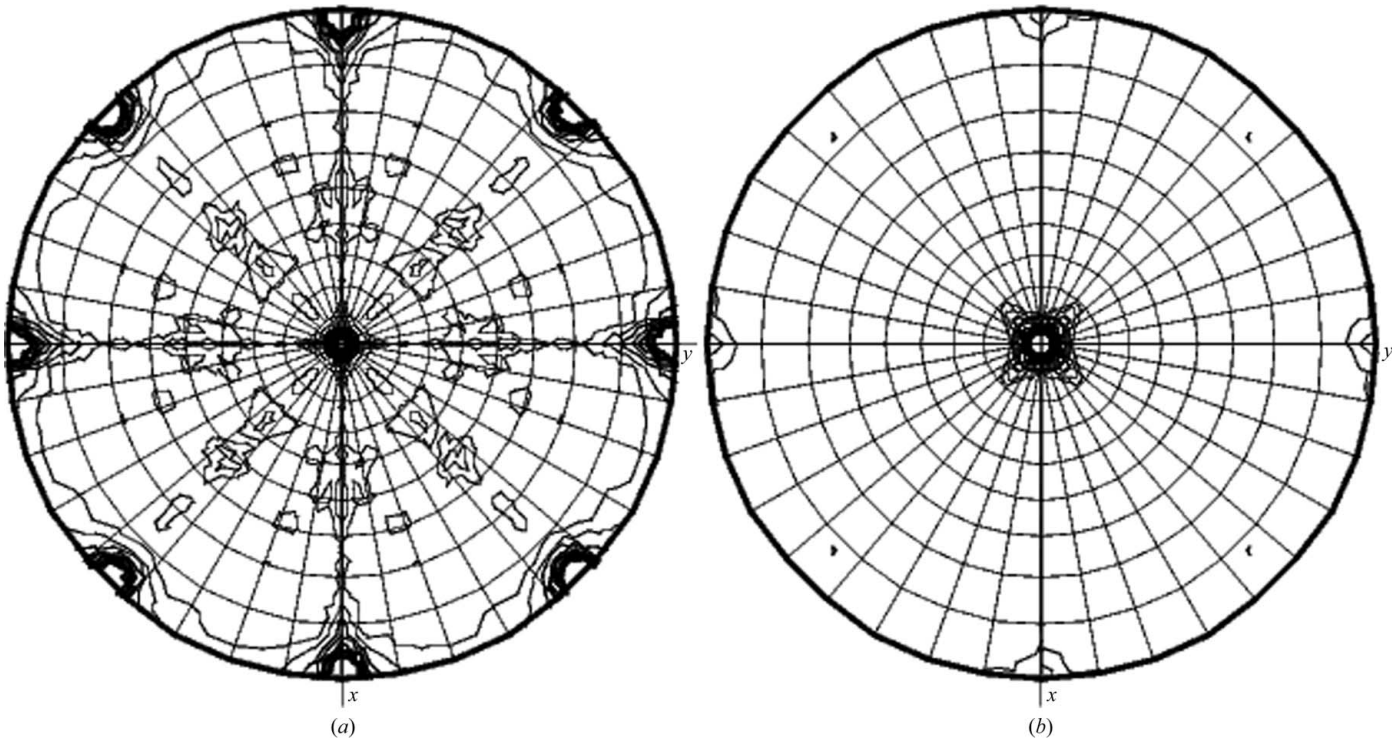


Figure 5
Stereographic plots of the (*a*) $\chi = 180°$ and (*b*) $\chi = 90°$ sections of the self-rotation function. The radius of integration was 43.19 Å and the upper resolution limit was 2.97 Å.

Table 1
Statistics of the X-ray data sets for native and SeMet CCM2-Ct crystals.

Values in parentheses are for the highest resolution shell.

Data set	Native	SeMet (peak)
Space group	$I4_122$	$I4_122$
Unit-cell parameters (Å, °)	$a = b = 113.29$, $c = 101.62$, $\alpha = \beta = \gamma = 90$	$a = b = 113.30$, $c = 102.55$, $\alpha = \beta = \gamma = 90$
Resolution (Å)	50.0–2.90 (3.06–2.90)	50.0–2.70 (2.85–2.70)
Wavelength (Å)	0.96397	0.96916
No. of reflections	106809	135047
Completeness (%)	99.9 (100)	99.7 (100)
Multiplicity	14.0 (14.5)	14.3 (14.7)
Average $I/\sigma(I)$	23.5 (8.1)	23.1 (6.9)
$R_{\rm merge}$† (%)	8.1 (31.9)	7.7 (38.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean of the observations $I_i(hkl)$ of reflection hkl.

were obtained using 45% Tacsimate pH 7.0. Prior to data collection, both the native and the SeMet-derivative crystals were transferred to reservoir solution supplemented with 10%(*v*/*v*) glycerol as a cryoprotectant.

The data-collection statistics for crystals of both native and SeMet CCM2-Ct are shown in Table 1. The native crystals diffracted to 2.9 Å resolution (Fig. 4). The crystals belonged to space group $I4_122$, with unit-cell parameters $a = b = 113.29$, $c = 101.62$ Å. Calculations showed that there were probably one or two CCM2-Ct monomers in the asymmetric unit, with Matthews coefficients of 4.57 or 2.29 Å^3 Da^{-1} and solvent contents of 73.11 or 46.21%, respectively. Self-rotation functions calculated by *MOLREP* in the *CCP*4 program suite at various resolutions all had strong twofold or fourfold rotation axes (Fig. 5). Noncrystallographic twofold symmetric axes could not be observed in stereographic plots of the self-rotation function. Therefore there is one molecule of CCM2-Ct in the asymmetric unit, although the solvent content is rather high. The SeMet-derivative crystals diffracted to a resolution of 2.7 Å and belonged to the same space group, with similar unit-cell parameters to those of the native crystals. Based on the quality of the X-ray data sets, structural determination is being carried out.

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