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Expression, crystallization and preliminary crystallographic data analysis of filamin A repeats 14–16

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Human filamin A is a 280 kDa protein involved in actin-filament cross-linking. It is structurally divided into an actin-binding headpiece (ABD) and a rod domain containing 24 immunoglobulin-like (Ig) repeats. A fragment of human filamin A (Ig repeats 14–16) was cloned and expressed in *Escherichia coli* and the purified protein was crystallized in 1.6 *M* ammonium sulfate, 2% PEG 1000 and 100 m*M* HEPES pH 7.5. The crystals diffracted to 1.95 Å and belong to space group $P2_12_12_1$, with unit-cell parameters $a = 50.63$, $b = 52.10$, $c = 98.46$ Å, $\alpha = \beta = \gamma = 90^\circ$.

1. Introduction

Actin-filament architecture and dynamics contribute to the morphology and motility of cells in processes that are tightly regulated by a vast and diverse set of actin-binding proteins (ABPs). The distinct properties of ABPs have allowed classification by function as capping, severing, nucleating, sequestering and cross-linking proteins. The mechanisms of action of members of several of these classes have been clarified by the elucidation of their X-ray structures in complex with actin. However, ABPs that cross-link filamentous actin (F-actin), including filamins, α-actinin and spectrin, have not been amenable to this approach owing to the implicit heterogeneity of F-actin.

Filamins were first discovered over 30 years ago as a group of F-actin cross-linking proteins that exist in *Dictyostelium*, chicken and mammals (Shizuta *et al.*, 1976; Wang *et al.*, 1975). In humans, three different filamin genes, *flna*, *flnb* and *flnc*, have been identified, encoding the proteins filamins A, B and C, respectively. Filamins A and B are ubiquitously expressed (Gorlin *et al.*, 1990; Takafuta *et al.*, 1998), while filamin C is predominantly expressed in cardiac and skeletal muscles (Xie *et al.*, 1998). The filamins are 280 kDa proteins consisting of an N-terminal actin-binding domain (ABD) headpiece followed by a C-terminal rod region (Hock *et al.*, 1990). The ABD comprises tandem calponin-homology domains (CH1 and CH2) that are responsible for F-actin interaction. Each 110-residue CH domain consists of six or seven α-helices, four of which contain 11–18 residues. Filamin headpieces show sequence similarity to other α-actinin-like CH domains and are expected to adopt similar structures (Carugo *et al.*, 1997).

The rod region consists of repeating 100-residue immunoglobulin-like domains that are rich in β-structure. In human filamins there are 24 Ig-like domains; there are 20 in *Drosophila* and six in *Dictyostelium* (Fucini *et al.*, 1997; Popowicz *et al.*, 2006). Domain 24 in humans is able to homodimerize, which gives the filamin dimer a V-shape speculated to be crucial in F-actin cross-linking (Feng & Walsh, 2004; Pudas *et al.*, 2005; Weihing, 1988). The Ig-like domains are connected by short stretches of amino acids, with larger insertions between domains 15–16 and 23–24 that form hinges 1 and 2, respectively (Davies *et al.*, 1978; van der Flier & Sonnenberg, 2001). Filamins also interact with a number of transmembrane receptors that enable the actin cytoskeleton to respond to extracellular stimuli, thereby casting filamin as a scaffolding protein that bridges signalling to the cytoskeleton. Consequently, mutations in the filamin genes have been linked to various diseases, including periventricular heterotropia and the otopalatodigital (OPD) spectrum disorders (Fox *et al.*, 1998; Robertson *et al.*, 2003; Zenker *et al.*, 2004). Furthermore, aberrant

filamin cleavage is associated with several conditions, including Graves' disease, myasthenia gravis, glomerulonephritis and platelet activation (Leedman *et al.*, 1993; Peutz-Kootstra *et al.*, 2000; Yamamoto *et al.*, 1987).

Crystal structures of several Ig-like domains of human filamins have been reported, including domains 17, 21, 23 and 24. These structures have revealed the dimerization interface and the interactions with Von Willebrand factor (Nakamura *et al.*, 2006; Pudas *et al.*, 2005). However, these structures cover only a small proportion of the Ig-like domains and many more structures are required to provide a comprehensive description of the rod region and its interaction with binding partners. We have identified rod domains 14–16 of filamin A as a key region for structural characterization. This triplet of domains interacts with the extracellular calcium receptor (CaR), FILIP, TRAF 1 and 2 and FAP 52 and also contains the flexible hinge 1 (Awata *et al.*, 2001; Popowicz *et al.*, 2006; Taveau *et al.*, 2003). Here, we report the expression, purification, crystallization and preliminary crystallographic data analysis of filamin A repeats 14–16.

2. Materials and methods

2.1. Cloning, expression and purification

The DNA segment encoding human filamin A residues 1534–1875 was amplified from a human foetal kidney cDNA (Clontech). The 5′-primer AAAGTCGACCCCTTCAAGGTCAAGGTG and 3′-primer AAAGCGGCCGCTTAGAGGCCAGGCCCATA were used to amplify a 1 kbp PCR product that was inserted into the *Sal*I and *Not*I sites of the pGEX-4T-3 plasmid (GE Healthcare). The DNA fragment was verified by DNA sequencing and subsequently transformed into *Escherichia coli* BL21 (DE3) expression cells (Invitrogen). The expressed protein has an N-terminal GST tag followed by a thrombin protease cleavage site and an extra PNSRVD linker between the thrombin cleavage site and the protein.

6 l LB medium supplemented with 100 µg ml^{-1} ampicillin was inoculated with 300 ml of a fresh overnight culture and grown in a glass fermenter (Bellco Glass Inc., USA) at 310 K with stirring at 150 rev min^{-1} until the OD_{600} reached 0.8. Induction was achieved with 1 m*M* IPTG and expression was allowed to continue overnight at 303 K.

Cells were harvested by centrifugation in a Beckman J6-MI centrifuge at 4000 rev min^{-1} for 45 min at 277 K. Pellets were resuspended in GST lysis buffer (50 m*M* Tris pH 7.6, 150 m*M* NaCl and 1 m*M* DTT) and frozen at 193 K. Pellets frozen in lysis buffer were thawed and further lysed by sonicating on ice using a Sonicator 3000 (Misonix Incorporated).

Cell debris was pelleted by centrifugation at 18 500 rev min^{-1} in a Sorvall SA-300 rotor for 45 min at 277 K and the supernatant was further clarified by filtration through a 0.45 µm filter (Sartorius). The supernatant was then loaded onto an ÄKTA Xpress purification system (GE Healthcare) for passage through a GST-Trap FF 5 ml affinity column with thrombin (20 units per millilitre of beads) in GST-binding buffer (50 m*M* Tris pH 7.6, 150 m*M* NaCl and 1 m*M* DTT) for 10 h to remove the GST tag. The tag-free protein was then passed through a Hi-Prep 26/10 desalting column pre-equilibrated in 20 m*M* Tris pH 7.6 and a Resource Q anion exchanger (binding buffer, 20 m*M* Tris pH 7.6; elution buffer, 20 m*M* Tris pH 7.6, 1 *M* NaCl). A final Superdex 75 gel-filtration step was performed in 50 m*M* Tris pH 7.6, 150 m*M* NaCl (all columns were from GE Healthcare). Fractions containing filamin A repeats 14–16 were run on SDS–PAGE (Fig. 1) and those judged to be pure were subsequently pooled and concentrated to 10 mg ml^{-1} with buffer exchange into 10 m*M* Tris pH 7.2, 50 m*M* NaCl using a 10 kDa cutoff Vivaspin membrane (Vivascience). The size on the gel corresponded to the expected 36.3 kDa tag-free protein. Prior to crystallization, the sample was checked for homogeneity by dynamic light scattering using a DynaPro MSX/TC Instrument (Protein Solutions Ltd).

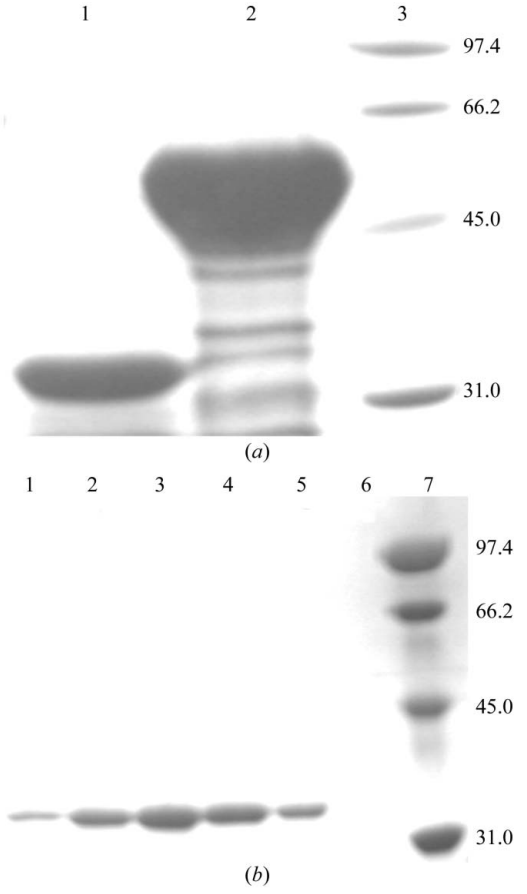

Figure 1
SDS–PAGE showing tag-cleavage and gel-filtration fractions of human filamin A repeats 14–16. (*a*) Cleaved protein (lane 1), uncleaved protein (lane 2) and molecular-weight standards (lane 3) with sizes labelled in kDa. (*b*) Fractions from gel filtration (lanes 1–6) showing the 36.3 kDa protein and molecular-weight standards (lane 7).

Figure 2
Crystal (dimensions 0.3 × 0.2 × 0.05 mm) of filamin A repeats 14–16 in 1.6 *M* ammonium sulfate, 2% PEG 1000 and 100 m*M* HEPES pH 7.5. Alhough lacking distinct edges, this lens-shaped crystal diffracted to 1.7 Å.

Table 1
Statistics of preliminary data analysis.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	24.62–1.95 (2.06–1.95)
Wavelength (Å)	1.5418
Unit-cell parameters (Å, °)	a = 50.63, b = 52.10, c = 98.46, $\alpha = \beta = \gamma = 9$
Space group	$P2_12_12_1$
Observed reflections	204864 (26685)
Unique reflections	18692 (2598)
R_{merge} (%)	0.071 (0.330)
Completeness (%)	95.4 (92.9)
Redundancy (%)	11.0 (10.3)

2.2. Crystallization and data collection

The search for crystallization conditions for concentrated filamin A repeats 14–16 was carried out *via* sitting-drop vapour-diffusion experiments with the aid of an Innovadyne 96+8 screenmaker (Innovadyne). 0.2 µl protein solution was mixed with 0.2 µl reservoir solution and equilibrated over 60 µl reservoir solution. Plate-like crystals were observed in several conditions from Wizard (Emerald Biosciences) and JB HTS II L (Jena Bioscience) screens at 288 K.

For cryoprotection, the crystal was soaked in a cryosolution containing mother liquor supplemented with 15% glycerol for 30 s. The crystal was then quickly mounted and cryocooled in a nitrogen cryostream at 100 K on a Rigaku/MSC FR-E Superbright X-ray source operating at 40 kV and 80 mA with a copper anode. 280 image frames were collected (120 s exposure time per frame, 0.5° oscillations) to a resolution of 1.7 Å on an R-AXIS IV^{++} detector at a wavelength of 1.542 Å.

The collected data were indexed using *MOSFLM* (Collaborative Computational Project, Number 4, 1994) and scaling was carried out to 1.95 Å using *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The DNA segment encoding human filamin A repeats 14–16 with an N-terminal GST tag was cloned and expressed in *E. coli* BL21 (DE3) cells. Crystals first appeared after four weeks of incubation at 288 K and grew to final dimensions of 0.3 × 0.2 × 0.05 mm over a further three weeks (Fig. 2). Data were collected from a crystal that grew in a well corresponding to 1.6 *M* ammonium sulfate, 2% PEG 1000 and 100 m*M* HEPES pH 7.5.

The space group was deduced to be $P2_12_12_1$, with unit-cell parameters a = 50.63, b = 52.10, c = 98.46 Å, $\alpha = \beta = \gamma = 90°$. The unit-cell volume is 259 747 Å^3 and one protein molecule is assumed per asymmetric unit (see Table 1).

The quality of the data indicates the possibility of solving the structure of filamin A repeats 14–16 by molecular replacement using the crystal structure of domain 24 of human filamin C (PDB code 1v05) as a start model in a multicopy search. We expect to achieve a solution from molecular replacement and refinement using the programs *AMoRe* and *REFMAC* v.5.0 (Collaborative Computational Project, Number 4, 1994), respectively. Viewing of the molecule and manual rebuilding will be achieved with the aid of the program *O* (Jones *et al.*, 1991).

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