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Cloning, expression, purification, crystallization and preliminary crystallographic analysis of γ-filamin repeat 23

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Human γ-filamin is a protein of 2705 amino-acid residues that localizes mainly in the myofibrillar Z-disc and to smaller extent in the subsarcolemmal region of striated muscle cells. γ-Filamin consists of an N-terminal actin-binding domain followed by a long rod-shaped region. The rod-shaped region consists of 24 immunoglobulin-like domains that form a platform for interaction with different transmembrane, cell-signalling and cytoskeletal proteins. γ-Filamin repeat 23 was indicated as being necessary for binding to the muscle-specific subsarcolemmal proteins γ- and δ-sarcoglycan and the myofibrillar protein FATZ1. The recombinant γ-filamin repeat 23 was crystallized using the hanging-drop vapour-diffusion method, which yielded needle-shaped diffraction-quality crystals. Diffraction data were collected to 2.05 Å resolution using 1.2 Å wavelength synchrotron radiation. Preliminary structural analysis shows one molecule, with predominantly β secondary-structure elements, per asymmetric unit.

1. Introduction

Filamins are a family of high-molecular-weight cytoskeletal proteins that organize filamentous actin in networks and stress fibres. Three human filamin gene paralogues have been identified: *flna*, *flnb* and *flnc*, which encode α-filamin, β-filamin and γ-filamin, respectively. Filamins bind filamentous actin through their amino-terminal actin-binding domain (ABD), which is composed of two calponin-homology domains in tandem (CH1, CH2). ABD is followed by a rod region that contains 24 filamin repeats of about 100 amino acids. The filamin repeats were predicted to consist of 7–8 β-strands of 6–9 amino acids connected by 3–4 amino-acid-long loops (Gorlin *et al.*, 1990), adopting an immunoglobulin-like fold. The three-dimensional structures of repeat 4 and of tandem repeats 5 and 6 of gelation factor (ABP120) from *Dictyostelium discoideum* (Fucini *et al.*, 1997; McCoy *et al.*, 1999) confirm the above prediction. Filamins form parallel homodimers through interactions between the C-terminal rod regions of two monomers (repeats 16–24), conferring a Y shape to the dimer (Gorlin *et al.*, 1990). This molecular architecture allows cross-linking of actin filaments *via* two ABDs located at the extremes of the two Y arms.

Human γ-filamin is a 280 kDa muscle-specific filamin that localizes mainly in the myofibrillar Z-disc and to smaller extent in the subsarcolemmal region of striated muscle cells. γ-Filamin repeats form a platform for interaction with androgen receptor (repeats 16–19), the transmembrane proteins γ- and δ-sarcoglycan (repeats 21–24) and the cytoskeletal proteins myotilin (repeats 19–21) and FATZ (repeats 20–24) (for details, see the review by van der Flier & Sonnenberg, 2001).

γ-Filamin repeat 23 (amino-acid residues 2475–2578) was identified as being necessary for binding to the muscle-specific subsarcolemmal proteins γ- and δ-sarcoglycan (Thompson *et al.*, 2000) and the myofibrillar protein FATZ1 (Faulkner *et al.*, 2000). Further studies should show whether repeat 23 is also sufficient for binding to these proteins. Here, we report the crystallization and preliminary crystallographic data analysis of human γ-filamin repeat 23.

2. Materials and methods

2.1. Cloning, expression and purification

The prokaryotic expression vector pAM3 was obtained by modifying vector pET28 (Novagen). It confers kanamycin resistance to the host bacteria and permits expression of proteins with an N- or C-terminal histidine tag (P. Steensgaard & A. Musacchio, personal communication). The DNA segment coding for repeat 23 (amino-acid residues 2475–2578) of human γ-filamin (*flnc* gene; GenBank accession No. AJ012737) was amplified by PCR from a human cDNA muscle library (Stratagene) using the 5′ primer AAA AAA GGA TCC CGC GTT GGG GAG CAG AGC CA and the 3′ primer AAA AAA GTC GAC TCA GGA CAG CCT CGG ACC AGT GA. The PCR product was cloned into the unique *Bam*HI and *Sal*I sites of the pAM3 plasmid,

producing GF23pAM3. The recombinant protein carries a 37-amino-acid residue N-terminal extension (MGSSHHHH-HHSSGLVPR^GSHMASMTGGQQMGR-GS) containing a histidine-tag and a thrombin-cleavage site. The linker is efficiently cleaved by thrombin at a site located between Arg and Gly (indicated above by ^).

Escherichia coli BL21(DE3) cells were transformed with GF23pAM3 and grown in 400 ml LB media supplemented with 50 µg ml^{-1} kanamycin in a 2 l Erlenmeyer flask at 310 K until the optical density (OD_{600}) reached 0.6; the cells were then induced with 0.2 m*M* IPTG (6 h, 291 K). The cells were harvested by centrifugation in a Beckman Coulter J6-MC centrifuge using a JS 4.2 rotor (4200*g*, 15 min, 277 K). The cell pellet was resuspended in 20 ml cold resuspension buffer (20 m*M* Tris, 50 m*M* NaCl, 20 m*M* imidazole pH 7.4) and lysed by sonication. Supernatant was separated from the cell debris by centrifugation in a Beckman Coulter Allegra 64R centrifuge using a F0650 rotor (30 000*g*, 20 min, 277 K). The supernatant was loaded onto a HiTrap Chelating HP column (1 ml; Amersham Biosciences) equilibrated with the resuspension buffer. The column was washed with 10 ml of this buffer and the sample was eluted with buffer composed of 20 m*M* Tris, 50 m*M* NaCl, 300 m*M* imidazole pH 7.4. Fractions containing γ-filamin repeat 23 were pooled and the histidine tag was cleaved off by the addition of bovine thrombin (10 units of thrombin per milligram of fusion protein, Amersham Biosciences), while dialysing against 1 l cold PBS (48 h, 277 K), in a 3500 Da molecular-weight cutoff dialysis tube (Spectra/Por 3.1; Spectrum Laboratories). The histidine tag was removed efficiently as shown from the inspection of SDS–PAGE gel of the sample after digestion (Fig. 1). To eliminate thrombin and possible traces of non-cleaved tagged protein (not observable by SDS–PAGE), dialyzed retentate was loaded onto a HiTrap Benzamidine FF column (1 ml; Amersham Biosciences) following the manufacturer's instructions. The flow-through of the benzamidine column was loaded onto a HiTrap Chelating HP column (1 ml) using the same protocol as for the uncleaved histidine-tagged γ-filamin repeat 23. The flowthrough fractions containing γ-filamin repeat 23 were concentrated to 200 µl using a Centricon YM-3 centrifugal device (Millipore) with a molecular-weight cutoff of 3000 Da and applied onto a Superdex 75 HR 10/30 size-exclusion chromatography column (Amersham Biosciences) equilibrated with 20 m*M* Tris, 150 m*M* NaCl, 5 m*M* DTT pH 7.4. γ-Filamin repeat 23 eluted in a single peak with an apparent molecular weight of about 16.6 kDa. Fractions containing the highly purified γ-filamin repeat 23 construct were dialyzed against 1 l 20 m*M* Tris, 50 m*M* NaCl pH 7.4 (48 h, 277 K) in a 3500 Da molecular-weight cutoff dialysis tube. The retentate was concentrated to 20 mg ml^{-1} using a Microcon YM-3 centrifugal filter device (Millipore). The concentrated protein sample was checked for homogeneity and purity by dynamic light scattering (Protein Solutions *DynaPro* v.6) and electrospray ionization mass spectroscopy.

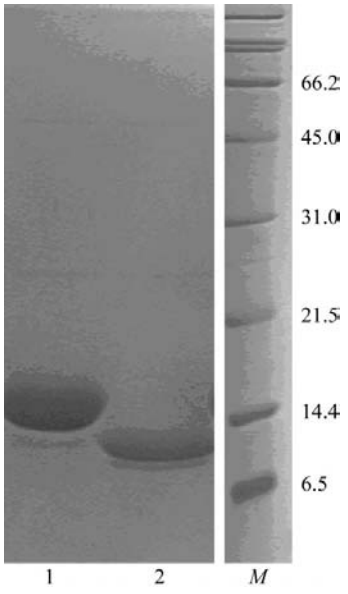

Figure 1
Thrombin cleavage of histidine-tagged γ-filamin repeat 23. Lane 1, before cleavage; lane 2, after thrombin cleavage. *M*, molecular-weight standards (kDa).

2.2. Crystallization

The hanging-drop vapour-diffusion method at 293 K was used for crystallization experiments. Drops were prepared by mixing 1 µl protein solution prepared as described above with 1 µl well solution suspended over 0.8 ml reservoir solution. The initial screening for crystallization conditions was performed using the Stura Footprint Screen No. 1 (Molecular Dimensions Ltd) and JBScreen 1 (Jena BioScience GmbH). Needle-shaped diffraction-quality crystals (Fig. 2) were observed after 16 h from the Stura Footprint Screen No. 1 under conditions containing 7.5, 17.5 and 22.5%(*w*/*v*) PEG 10K, 0.2 *M* imidazole malate pH 8.5.

Table 1
Data-collection and phasing statistics for the γ-filamin repeat 23 crystals.

Values in parentheses are for the highest resolution shell.

Crystal data	
Space group	$P3_12$
Unit-cell parameters	
a (Å)	52.1
b (Å)	52.1
c (Å)	69.2
Mosaicity (°)	0.5
Data collection and phasing	
Beamline	XRD1 (ELETTRA)
Wavelength (Å)	1.2
Resolution range (Å)	45.1–2.05 (2.16–2.05)
Observed reflections	238758
Unique reflections	7116
$R_{\rm merge}$†	0.061 (0.298)
Anomalous completeness (%)	97.1 (91.7)
$\langle I\rangle/\langle\sigma(I)\rangle$	7.1 (2.1)
Anomalous phasing power‡	1.6
Figure of merit (centric/acentric)	0.16/0.42
$R_{\rm Cullis}$(ano)§	0.70

† $R_{\rm merge} = \sum_h \sum_i |I_{hi} - I_h| / \sum_h \sum_i I_{hi}$, where I_h is the mean intensity of the scaled observation I_{hi}. ‡ Anomalous phasing power = $[\sum |F_H(\text{imag})|^2 / \sum ||\Delta F^{\pm}_{PH}(\text{obs})| - |\Delta F^{\pm}_{PH}(\text{calc})||^2]^{1/2}$, where $\Delta F^{\pm}_{PH}$ is the structure-factor difference between Bijvoet pairs and F_H(imag) is the imaginary component of the calculated structure-factor contribution by the anomalously scattering atoms. § $R_{\rm Cullis}(\text{ano}) = \sum ||\Delta F^{\pm}_{PH}(\text{obs})| - |\Delta F^{\pm}_{PH}(\text{calc})|| / \Delta F^{\pm}_{PH}(\text{obs})$.

2.3. Collection and processing of X-ray diffraction data

A crystal from the condition with intermediate PEG 10K concentration was picked up with a cryoloop (Hampton Research), passed briefly through a cryoprotectant solution consisting of 17.5% PEG 10K, 20% glycerol, 0.2 *M* imidazole malate pH 8.5 and rapidly mounted on the goniometer head and flash-cooled to 100 K in a nitrogen stream using a 700 Series Oxford Cryosystems cooling device (Oxford Cryosystems Ltd, 2000). A total of 228° of data with a 0.5° oscillation angle were collected to a maximum resolution of 2.05 Å on the tuneable XRD1 beamline at ELETTRA with

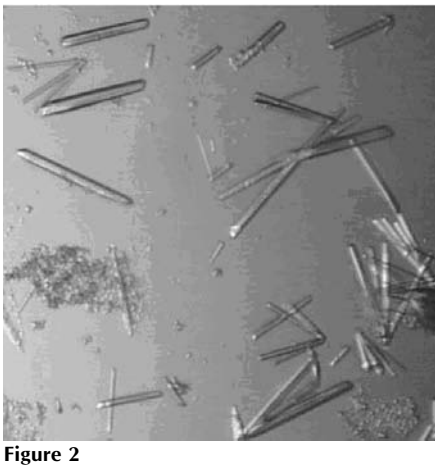

Figure 2
Crystals of γ-filamin repeat 23; approximate dimensions 250 × 20 × 20 µm.

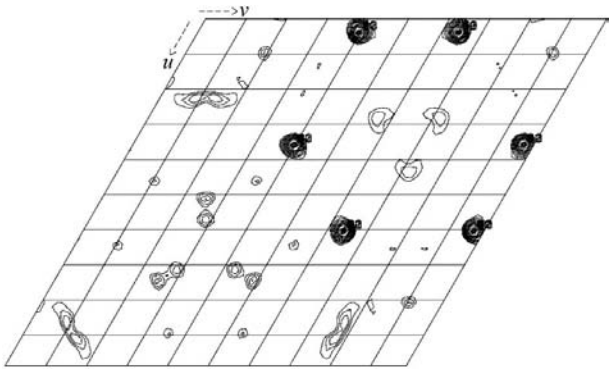

Figure 3
The Harker section at $w = 1/3$ of an anomalous Patterson map calculated with diffraction data from 30 to 3 Å resolution, contoured starting at 1.5σ in steps of 0.5σ.

1.2 Å wavelength using a MAR CCD detector. The data set was processed with *DENZO* (Otwinowski & Minor, 1997) and scaled with *SCALA* (Collaborative Computational Project, Number 4, 1994) (Table 1). An X-ray fluorescence spectrum around the nickel absorption edge was measured with a PIN diode mounted on a flexible arm and a multichannel analyser (VARRO Selenia); the scan was performed with steps of 2 eV.

2.4. Phasing

Inspection of the anomalous difference Patterson maps calculated with the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) showed the presence of an anomalous scatterer (Fig. 3). Its position was determined manually and confirmed by *SHELXD* (Schneider & Sheldrick, 2002) and then used as input to *SHARP* (de La Fortelle & Bricogne, 1997) for further heavy-atom refinement and phase calculation.

3. Results and discussion

The purity of the protein solution used for the crystallization experiments was checked by SDS–PAGE analysis and showed a single band of apparent molecular weight 14 kDa. Mass spectroscopy of the sample showed the presence of only one protein species with a molecular weight of 12 249 Da, closely matching the predicted calculated molecular weight of 12 247.7 Da. Dynamic light scattering was used to assess the monodispersity of the protein solution: a monomodal distribution with a polydispersity of 12.3% was observed and the gyration radius was estimated to be 19 Å, suggesting that the protein solution was homogenous and monomeric.

Needle-shaped crystals of γ-filamin repeat 23 nucleated and reached final average dimensions of $250 \times 20 \times 20$ µm within 16 h. The crystal lattice is primitive hexagonal, with unit-cell parameters $a = b = 52.1$, $c = 69.2$ Å. From the scaling statistics the space group was deduced to be either $P3_112$ or $P3_212$. The volume of the unit cell is 163 000 Å^3, which permits one protein molecule per asymmetric unit (V_M = 2.2 Å^3 Da^{-1}).

Trials to solve the phase problem with molecular replacement using the known structures of repeats 4, 5 and 6 from gelation factor (ABP120) as search models failed. Inspection of the anomalous difference Patterson map calculated with diffraction data to 3 Å resolution unexpectedly showed the presence of an anomalous scatterer (Fig. 3). Considering the purification protocol, crystallization conditions and wavelength of data collection (1.2 Å), this scatterer was presumed to be nickel. This assumption was corroborated by fluorescence scans performed on γ-filamin repeat 23 crystals, which clearly showed a signal at the nickel *K* absorption edge (Fig. 4). Nickel coordinates were determined from an anomalous difference Patterson map and verified with *SHELXD* (correlation coefficient 29.94/19.99 for all/weak reflections) using data truncated to 2.7 Å as suggested by the *XPREP* program (Bruker Nonius, Madison, Wisconsin, USA). The output of *SHELXD* was used as input to *SHARP* for heavy-atom refinement and phase calculation. The resolution of the diffraction data was gradually extended by small steps until the maximum resolution of 2.05 Å was reached. This procedure was repeated for both space groups and both 'hands' of the heavy-atom position, which resulted in an interpretable map for the $P3_112$ space group with an overall figure of merit (FOM) of 0.42 and 0.16 for acentric and centric reflections, respectively. The experimental electron-density map was not readily interpretable; therefore, it was solvent-flattened using an option in the WWW interface of *SHARP* 2.01 (*SUSHI* 3.0.15) to search for an optimal solvent envelope.

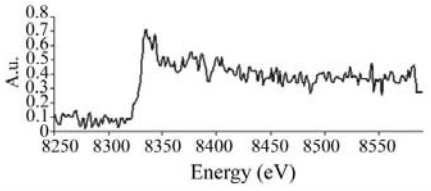

Figure 4
X-ray fluorescence spectrum of γ-filamin repeat 23 crystals.

The quality of the solvent-flattened electron-density maps suggests that it will be possible to ultimately determine the structure of γ-filamin repeat 23 from these data.

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