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Purification, crystallization and preliminary crystallographic analysis of the globular domain of the human type V myosin Myo5a

Type V myosins constitute the main cargo-transporting class of myosin motors in higher eukaryotes. They are mainly defined by their C-terminal globular domain, which is required for cargo binding as well as for motor auto-inhibition in the absence of cargo. To date, high-resolution structures only exist for globular domains from yeast. Since the majority of cellular cargoes in yeast are very different from the cargoes in higher eukaryotes, structural insights into the domain organization of globular domains from human type V myosins are important. The globular domain of human Myo5a was cloned, expressed and crystallized and data sets were collected. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 75.04$, $b = 86.70$, $c = 131.41$ Å, $\alpha = \beta = \gamma = 90^\circ$, and diffracted with data-collection quality to 2.5 Å resolution.

1. Introduction

In eukaryotes, type V myosin motors move a range of cargoes along actin filaments to distinct sites in the cell (Hammer & Sellers, 2012). The number of type V myosin paralogues varies amongst different species. Whereas invertebrates only have a single paralogue and budding yeast has two paralogues (Jansen & Niessing, 2012), three paralogues are found in mammals (Hammer & Sellers, 2012). Although the mammalian type V myosins Myo5a, Myo5b and Myo5c share the same domain organization, their cargo specificities are different. Mutations in the *myo5a* and *myo5b* genes have been linked to the heritable Griscelli syndrome and microvillus inclusion disease, respectively (Müller *et al.*, 2008; Seabra *et al.*, 2002).

At their N-terminus, type V myosins have an actin-binding motor domain that hydrolyzes ATP and enables directional movement along actin filaments. The motor is followed by a lever arm, a coiled-coil dimerization domain and a C-terminal globular domain. The latter is mainly responsible for binding to cellular cargoes and for mediating autoinhibition in the cargo-free state (Trybus, 2008).

Two high-resolution structures of globular domains from yeast have previously been solved (Heuck *et al.*, 2010; Pashkova *et al.*, 2006). Since the corresponding proteins share only moderate sequence identities to vertebrate type V myosins, it remains unclear how similar the respective domains are in Myo5a–Myo5c. Thus, high-resolution structural information is required to understand the function of type V myosins in higher eukaryotes and their structural relationship to the previously published globular domains from yeast.

2. Materials and methods

2.1. Macromolecule production

Human Myo5a fragments were PCR-amplified from cDNA and ligated into the vector pGEX-6P-1 using the *Bam*HI and *Xho*I restriction sites (Table 1). The plasmids were sequenced, transformed into *Escherichia coli* BL21 (DE3) Star and cultured in LB medium. On reaching an OD₆₀₀ of 0.3–0.4, the cultures were cooled to 291 K, expression was induced with 0.25 mM IPTG and the cultures were further incubated in a shaker for 16 h. Cell pellets were resuspended and lysed by sonication. Cell debris was removed by centrifugation

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and the supernatant was loaded onto a GStrap FF column (GE Healthcare). After washing, the GST tag was cleaved off and the Myo5a protein was eluted and further purified by cation-exchange chromatography on an SP column (GE Healthcare). The protein was concentrated using centrifugal filter devices (Amicon Ultra, Millipore) and loaded onto a Superose 12 size-exclusion chromatography column (GE Healthcare) equilibrated with buffer consisting of 50 mM Tris pH 7.0, 140 mM NaCl, 2 mM DTT (Müller *et al.*, 2009). The peak fractions were analyzed by SDS-PAGE and concentrated for crystallization trials.

2.2. Crystallization

The protein solution was concentrated to 1–2.5 mg ml⁻¹ using centrifugal filter devices. Initial crystallization screens were set up as sitting-drop vapour-diffusion experiments using a Hydra II crystal-

lization robot and crystallization kits from Qiagen and Hampton Research. Screens were pipetted in 96-well plate format with a drop size of 0.5 µl and a 1:1 mixture of protein and crystallization solution. Crystallization drops were manually inspected at regular time intervals. Several crystals appeared in a range of conditions after 2–3 d (Fig. 1*a*). A subset of the initial crystallization conditions was manually refined in 24-well format using the hanging-drop vapour-diffusion technique, a final drop size of 2 µl and a 1:1 mixture of protein and crystallization solution.

2.3. Data collection and processing

Crystals were cryoprotected by adding different agents (glycerol, ethylene glycol or PEG 400) to the reservoir solution at different

Table 1

Macromolecule-production information.

Source organism	Human
DNA source	cDNA
Forward primer	AAA GGA TCC ATG CCC CGG AAA GAA AAG GAT TTC CAA (bold sequence: <i>Bam</i> HI)
Reverse primer	AAA CTC GAG TCA GAC CCG TGA AAT GAA GCC CAG (bold sequence: <i>Xho</i> I)
Cloning vector	pGEX-6P-1
Expression vector	pGEX-6P-1
Expression host	<i>E. coli</i> BL21 (DE3) Star
Complete amino-acid sequence of the construct produced	GPLGSPRKEKDFQGMLEYKKEDQKLVKNLIL- ELKPRGVAVNLIPGLPAYILFMCVRHADYLN- DDQKVRSLTSTINSIKKVLKRGDDFETVSF- WLSNTRCFLHCLKQYSGEFGFMKHNSTRQN- EHCLTNFDLAEYRQVLSDLAIQIYQQLVRVL- ENILQPMIVSGMLEHETIQGVSGVKPTGLRK- RTSSIADEGTYTLDLSILRQLNSFHSVMCQHG- MDPELIKQVVKQMFYIIGAITLNNLLLRKDM- CSWSKGMQIRYNVSQLLEEWLRDKNLMNSG- AKETLEPLIQAAQLLQVKKKTDDDAEAICS- MCNALTQAIVKVLNLYTPVNEFEERVSVSFI- RTIQMRLRDRKDSPLLMDAKHIFPVTFFPN- PSSLALETIQIPASLGLGFISRV

(a)

(b)

Figure 1
Initial crystallization attempts with the globular domain of human Myo5a. (a) Initial crystals grown in 0.1 M MES pH 6.2, 1.4 M MgSO₄ showed good morphology but only diffracted to approximately 8–10 Å resolution (b).

Figure 2
Diffraction image from a crystal of the Myo5a globular domain. Data sets collected from these crystals showed high-quality diffraction to a resolution of 2.5 Å. However, these diffraction patterns also showed some anisotropy.

Table 2

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline ID29, ESRF
Wavelength (Å)	0.98137
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	269.28
Rotation range per image (°)	0.5
Total rotation range (°)	198
Exposure time per image (s)	1
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	$a = 75.04$, $b = 86.70$, $c = 131.41$, $\alpha = \beta = \gamma = 90$
Mosaicity (°)	0.32
Resolution range (Å)	18–2.5
Total No. of reflections	175951 (19502)
No. of unique reflections	28947 (3195)
Completeness (%)	95.4 (97.2)
Multiplicity	6.1 (6.1)
$\langle I/\sigma(I) \rangle$	13.0 (4.8)
$R_{\text{r.i.m.}}$ (%)	10.5 (38.6)
Overall B factor from Wilson plot (Å ²)	39.2; no abnormalities such as ice rings were observed

concentrations and for a variety of incubation times. Diffraction-quality crystals were obtained using 20% glycerol as a cryoprotectant. Diffraction data were collected from crystals on beamline ID29 at the European Synchrotron Radiation Facility, Grenoble, France. Crystals showed diffraction to approximately 2.3 Å resolution (Fig. 2). Data sets were processed and scaled with the *XDS* package (Kabsch, 2010). The program *POINTLESS* and analysis of the self-rotation function were used to confirm the space group as $P2_12_12_1$ (total probability 80.5%, systematic absence probability 82.2%, Laue-group confidence 97.5% according to *POINTLESS*; Winn *et al.*, 2011; Evans, 2006). The data-collection statistics are summarized in Table 2.

3. Results and discussion

Different fragments of the globular domain of human Myo5a were expressed in fusion with GST. During purification, the tag was removed by cleavage with human rhinovirus 3C protease. Any truncation from the C-terminus resulted in rapidly degrading protein, whereas modulation of the N-terminal boundary eventually yielded nondegrading and well behaved protein. A fragment spanning from residue 1467 to the C-terminus (residue 1855) was successfully used for crystallization.

Figure 3

Two kinds of crystals of the globular domain of Myo5a were found in crystallization drops. The schematic drawing shows a crystal growing directly on the cover slide at the bottom of the drop. Such crystals only yielded poor diffraction, as shown in Fig. 1(b), or were highly anisotropic. In contrast, crystals growing in bundles from the surface of the drop diffracted with a quality suitable for data collection.

Table 3

Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	Qiagen EasyXtal 24-well plates
Temperature (K)	294 ± 2
Protein concentration (mg ml ⁻¹)	1.0–2.5
Buffer composition of protein solution	50 mM Tris pH 7.0, 140 mM NaCl, 2 mM DTT
Composition of reservoir solution	0.2 M ammonium sulfate, 0.1 M bis-tris pH 6.5, 25% (w/v) PEG 3350
Volume and ratio of drop	1 µl + 1 µl
Volume of reservoir (µl)	500

Although several crystallization conditions yielded crystals with good morphology (Fig. 1a), most of the corresponding diffraction patterns showed high anisotropy, high mosaicity and low resolution (Fig. 1b). Eventually, diffraction-quality crystals were grown at 294 K by hanging-drop vapour diffusion using a 1:1 mixture of protein solution (1–2.5 mg ml⁻¹) and the reservoir solution 0.2 M ammonium sulfate, 0.1 M bis-tris pH 6.5, 25% (w/v) PEG 3350 (Table 3). Surprisingly, we observed that only a minority of the crystals in a crystallization drop showed reasonable diffraction. After considerable effort, it was found that only those crystals that had grown from the drop surface into the drop solution diffracted well (Fig. 3). Crystals grown at the bottom of the drop yielded inferior diffraction patterns.

Obedying this rule, we were able to harvest crystals that diffracted to 2.3 Å resolution. Processing of the best data set yielded high-quality diffraction to 2.5 Å resolution. The corresponding space group was $P2_12_12_1$ and the unit-cell parameters were $a = 75.04$, $b = 86.70$, $c = 131.41$ Å, $\alpha = \beta = \gamma = 90^\circ$. The overall completeness of the data set was 95.4%, with a multiplicity of 6.1 and a mosaicity of 0.32°. The Matthews coefficient (Matthews, 1968; Kantardjieff & Rupp, 2003) was 2.47 Å³ Da⁻¹ for two molecules, with a solvent content of 50.1%, per asymmetric unit (the overall probability of this solution was 98%). Attempts to solve the structure by molecular replacement (Winn *et al.*, 2011) using the globular domains of yeast Myo2p (PDB entry 2f6h; Pashkova *et al.*, 2006) or Myo4p (PDB entry 3mmi; Heuck *et al.*, 2010) as templates failed. Molecular replacement with other orthorhombic space groups or with space groups of lower symmetry (monoclinic) was also not successful. Thus, experimental phasing will be required to solve this structure.

In summary, we found a C-terminal fragment comprising the globular cargo-binding domain of human Myo5a that crystallized. However, we also observed that most crystallization conditions were not suitable for obtaining diffraction-quality crystals. Furthermore, considerable variability in the diffraction quality of different crystals within the same crystallization drop was observed. We first had to identify which crystals in a drop yielded good diffraction before we could find sufficient well diffracting crystals to obtain high-resolution data sets. With such data sets and corresponding experimental phases, structure determination should be feasible.

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