Crystallization and preliminary X-ray crystallographic analysis of human myotubularin-related protein 1

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Myotubularin-related protein 1 is a phosphatase that dephosphorylates phospholipids such as phosphatidylinositol 3-phosphate or phosphatidylinositol 3,5-bisphosphate. In this study, human MTMR1 was overexpressed in *Escherichia coli*, purified and crystallized at 277 K using polyethylene glycol 20 000 as a precipitant. Diffraction data were collected to 2.0 Å resolution using synchrotron radiation. The crystals belonged to space group *P*1, with unit-cell parameters $a = 67.219$, $b = 96.587$, $c = 97.581$ Å, $\alpha = 87.597$, $\beta = 86.072$, $\gamma = 77.327^\circ$. Assuming the presence of four molecules in the asymmetric unit, the calculated Matthews coefficient value was $2.61 \text{ Å}^3 \text{ Da}^{-1}$ and the corresponding solvent content was 52.9%.

1. Introduction

Phosphatidylinositol (PtdIns) in eukaryotic membranes can be phosphorylated at the D3, D4 and D5 positions of the inositol ring, resulting in the formation of seven different phosphoinositides (PIs; Robinson & Dixon, 2006). PIs play important roles in signal transduction, membrane trafficking, cell growth and survival, cell division and cellular motility (Robinson & Dixon, 2006). The phosphate at the D3 position of phosphatidylinositol 3-phosphate [PtdIns(3)P] and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P₂] can be dephosphorylated by myotubularin and myotubularin-related proteins (MTM1 and MTMRs; Clague & Lorenzo, 2005; Robinson & Dixon, 2006).

The myotubularin family is a subgroup of the tyrosine/dual-specificity phosphatase (PTP/DSP) superfamily (Laporte *et al.*, 2003). While the PTP domains in MTM1, MTMR1, MTMR2, MTMR3, MTMR4, MTMR6, MTMR7 and MTMR8 possess catalytically active PTP with a CX₅R consensus motif, the other six members of the MTMRs (MTMR5 and MTMR9–MTMR13) have inactive PTP domains (Robinson & Dixon, 2006; Nandurkar & Huysmans, 2002; Laporte *et al.*, 2003). These inactive MTMRs interact with the catalytically active MTMRs *via* their coiled-coil domain, regulating the function of the MTMRs (Kim *et al.*, 2003; Hnia *et al.*, 2012).

Human MTMR1 contains 665 amino acids, a PH-GRAM domain (Doerks *et al.*, 2000), an active PTP domain, a coiled-coil domain and a PDZ-binding motif. Although MTMR1 was one of the first myotubularin-related proteins to be identified,

Table 1
Macromolecule-production information.

Source organism	<i>Homo sapiens</i>
DNA source	Open Biosystems (catalogue No. MHS1010-7295807)
Forward primer†	5'-GGAATTCATATGGAAGAGGCTCCACTTTT-AGAGGCTCCACTTTT-3'
Reverse primer‡	5'-CCGCCGCTCGAGCCGTGGATTCCATCGTAC-TGATTCCATCGTACATA-3'
Expression vector	Modified pET-32
Expression host	<i>E. coli</i> Rosetta2 (DE3)
Complete amino-acid sequence of the construct produced§	MEEAPLFPGESIKAIVKDVMIICPFMGAVSGT-LTVDTFKLYFKNVERDPHFILDVPLGVISR-VEKIGAQSHGDNSCGIEIVCKDMRNLRLAY-KQEEQSKLGIFENLNKHAFPLSNGQALFAF-SYKEKFPINGWKVYDPVSEYKRQGLPNESW-KISKINSNYEFCDTYPAIIIVPTSVDKDDL-SKVAAFRAKGRVPVLSWIHPESQATITRCS-QPLVGPNDKRCKEDEKYLQTIMDANAQSHK-LIIFDARQNSVADTNKTKGGGYESESAYPN-AELVFLEIHNHVMRESLRKLEIVYPSID-EARWLSNVDGTHWLEYIRMLLAGAVRIADK-IESGKTSVVVHSSDGDWRTAQLTSLAMLML-DSYYRTIKGFETLVEKEWISFGHRFALRVG-HGNDNHADADRSPIFLQFVDCVWQMTRQFP-SAFEFNELFLITILDHLYSCLFGTFLCNCE-QQRFKEDVYTKTISLWSYINQLDEFNPNF-FVNYENHVLYPVASLSHLELWVNYVRWNP- <u>RL</u> LEHHHHHHHH

† The NdeI site is underlined. ‡ The XhoI site is underlined. § The C438S mutation site and the ten-residue cloning artefact are underlined.

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	MRC 96-well plate
Temperature (K)	277
Protein concentration (mg ml ⁻¹)	13.5
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM Na ₂ HPO ₄ , 5 mM dithiothreitol
Composition of reservoir solution	100 mM Na HEPES pH 7.0, 15%(v/v) polyethylene glycol 20 000
Volume (µl) and ratio of drop	2 (1:1 ratio of protein and reservoir)
Volume of reservoir (µl)	70

it remains poorly studied. It displays high amino-acid sequence similarity to MTM1 and MTMR2. It has been reported that aberrant expression of MTMR1 leads to myotonic dystrophy (DM; Buj-Bello *et al.*, 2002; Hnia *et al.*, 2012).

Some structural studies on MTMRs have been performed. Crystal structures of MTMR2 have been elucidated, revealing the substrate specificity and the catalytic mechanism (Begley *et al.*, 2003, 2006). The crystal structure of MTMR6 has also been determined and deposited in the Protein Data Bank. However, structural information on MTMR1 is not yet available. Structural studies of various MTMR proteins might be helpful in order to understand the detailed function of individual MTMRs at the molecular level. In order to improve our understanding of MTMR1 by comparing it with other MTMR structures, we initiated this study aimed at the structural determination of MTMR1. As the first step towards structural determination, we overexpressed and crystallized recombinant MTMR1. Diffraction data were then collected to 2.0 Å resolution using synchrotron radiation.

2. Materials and methods

2.1. Macromolecule production

The constructed MTMR1 gene (encoding amino-acid residues 95–607) was amplified using the polymerase chain reaction and inserted into modified pET-32 vector (Novagen). The active-site cysteine was then replaced with serine (C438S) using the QuikChange method. The resulting recombinant MTMR1 protein contains ten extra residues at its C-terminus (LEHHHHHHHH) for purification (Table 1). The recombinant protein was overexpressed in *Escherichia coli* strain Rosetta2 (DE3) cells (Novagen). The transformed cells were grown in Luria–Bertani medium containing 50 µg ml⁻¹ kanamycin at 310 K. Expression of recombinant MTMR1 was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the cells had reached an optical density (OD_{600 nm}) of 0.5. The cell cultures were induced with IPTG

Figure 1
(a) SDS-PAGE analysis of purified MTMR1. The left lane contains molecular-weight marker (labelled in kDa). (b) SEC elution profile of MTMR1.

for 20 h at 291 K. The cells were then harvested by centrifugation at 8000g for 20 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer (20 mM Tris-HCl pH 8.0, 10 mM Na₂HPO₄, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and the cells were disrupted by sonication. The lysate was centrifuged at 13 000g for 1 h at 277 K. The supernatant was loaded by gravity onto a Glass Econo-Column (Bio-Rad)

packed with Ni²⁺-chelated Ni-NTA agarose resin (Qiagen) which had previously been equilibrated with buffer A (20 mM Tris-HCl pH 8.0, 10 mM Na₂HPO₄, 300 mM NaCl) and was subsequently washed with buffer A containing 50 mM imidazole. The protein was eluted with buffer A containing 400 mM imidazole. Final purification of the protein was achieved by size-exclusion chromatography. The sample was injected onto

Protein Name				Protein Score	Mass	Peptides matched
Myotubularin-related protein 1				138	74631	37
Peptide Information						
Observed	Mr(expt)	Mr(calc)	Delta	Start	End Peptide Sequence	
506.28	505.28	505.30	-0.03	481	484 FALR	
506.28	505.28	505.30	-0.03	481	484 FALR	
563.30	562.29	562.32	-0.03	279	283 VAAFR	
572.27	571.26	571.29	-0.03	604	607 WNPR	
830.45	829.45	829.48	-0.04	415	422 MLLAGAVR	
830.45	829.45	829.48	-0.04	415	422 MLLAGAVR	
847.46	846.46	846.50	-0.04	337	343 LIIFDAR	
847.46	846.46	846.50	-0.04	337	343 LIIFDAR	
861.42	860.41	860.45	-0.04	222	228 FPINGWK	
861.42	860.41	860.45	-0.04	222	228 FPINGWK	
1031.46	1030.46	1030.50	-0.04	473	480 EWISFGHR	
1047.53	1046.52	1046.58	-0.06	194	202 LGIFENLNK	
1255.58	1254.57	1254.62	-0.06	229	238 VYDPVSEYKR	
1255.58	1254.57	1254.62	-0.05	229	238 VYDPVSEYKR	
1291.60	1290.59	1290.65	-0.06	389	399 EIVYPSIDEAR	
1291.60	1290.59	1290.65	-0.06	389	399 EIVYPSIDEAR	
1313.60	1312.60	1312.66	-0.06	305	316 CSQPLVGPNDKR	
1313.60	1312.60	1312.66	-0.06	305	316 CSQPLVGPNDKR	
1532.76	1531.76	1531.82	-0.07	387	399 LKEIVYPSIDEAR	
1677.85	1676.84	1676.92	-0.08	142	156 DPHFILDVPLGVISR	
1677.85	1676.84	1676.92	-0.08	142	156 DPHFILDVPLGVISR	
1888.84	1887.83	1887.93	-0.10	400	414 WLSNVDGTHWLEYIR	
1888.84	1887.83	1887.85	-0.01	160	177 IGAQSHGDNSCGIEIVCK + Carboxymethyl (C)	
1897.87	1896.86	1896.95	-0.09	203	219 HAFPLSNGQALFAFSYK	
1933.96	1932.95	1933.04	-0.09	288	304 VPVLSWIHPESQATITR	
1933.96	1932.95	1933.04	-0.09	288	304 VPVLSWIHPESQATITR	
1976.89	1975.88	1975.97	-0.09	445	461 TAQLTSLAMLMLDSYYR	
1976.89	1975.88	1975.97	-0.09	445	461 TAQLTSLAMLMLDSYYR	
1992.89	1991.89	1991.97	-0.08	445	461 TAQLTSLAMLMLDSYYR + Oxidation (M)	
1992.89	1991.89	1991.97	-0.08	445	461 TAQLTSLAMLMLDSYYR + Oxidation (M)	
2045.87	2044.87	2045.06	-0.19	604	619 WNPRMRPQMPIHQNLIK	
2147.09	2146.08	2146.16	-0.09	286	304 GRVPVLSWIHPESQATITR	
2147.09	2146.08	2146.16	-0.09	286	304 GRVPVLSWIHPESQATITR	
2176.10	2175.09	2175.18	-0.09	138	156 NVERDPHFILDVPLGVISR	
2176.10	2175.09	2175.18	-0.09	138	156 NVERDPHFILDVPLGVISR	
3032.32	3031.31	3031.45	-0.14	355	381 GGGYESESAYPNAELVFLEIHNHVMR	
3032.32	3031.31	3031.45	-0.14	355	381 GGGYESESAYPNAELVFLEIHNHVMR	

Figure 2
Identification of the purified protein by MALDI-TOF/TOF analysis.

a HiLoad 16/600 Superdex 200 pg (GE Healthcare) column that had been pre-equilibrated with buffer *B* (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 mM Na₂HPO₄, 5 mM dithiothreitol). The purified protein was concentrated to 13.5 mg ml^{−1} using an Amicon Ultra-15 ultrafiltration device (Millipore). The homogeneity of the purified protein was determined by SDS–PAGE under denaturing conditions using 12% (v/v) polyacrylamide gels. The protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. Dynamic light-scattering (DLS) experiments were performed on a DynaPro Titan instrument (Wyatt Technology) at 298 K and 20% laser power. Purified protein was identified by MALDI-TOF/TOF mass spectrometry. Analysis was performed using a 4700

(a)

(b)

Figure 3
(a) Crystals of human MTMR1. (b) X-ray diffraction pattern of a human MTMR1 crystal.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline 5C, PLS
Wavelength (Å)	0.9796
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	3
Space group	<i>P</i> 1
Unit-cell parameters (Å, °)	<i>a</i> = 67.219, <i>b</i> = 96.587, <i>c</i> = 97.581, <i>α</i> = 87.597, <i>β</i> = 86.072, <i>γ</i> = 77.327
Mosaicity (°)	0.4
Resolution range (Å)	50.0–2.0 (2.03–2.00)
Total No. of reflections	343072
No. of unique reflections	136981
Completeness (%)	84.7 (63.2)
Multiplicity	2.5 (2.0)
<i>I</i> / <i>σ</i> (<i>I</i>)	18.9 (2.6)
<i>R</i> _{int} † (%)	8.3 (39.2)
Overall <i>B</i> factor from Wilson plot (Å ²)	44.6

† As this value was not available, it was estimated by multiplying the *R*_{merge} value by the factor $[N/(N-1)]^{1/2}$, where *N* is the data redundancy.

Proteomics Analyzer (AB Sciex). The acquired MS/MS spectra were analyzed against the Swiss-Prot database using *ProteinPilot* v.5.0 (AB Sciex).

2.2. Crystallization

The initial crystallization screening was performed using the ProPlex kit (Molecular Dimensions) by the sitting-drop vapour-diffusion method in 96-well plates at 277 K. The best crystals were grown in reservoir solution consisting of 100 mM Na HEPES pH 7.0, 15% (v/v) polyethylene glycol 20 000 (PEG 20K) and used for X-ray diffraction experiments (Table 2).

2.3. Data collection and processing

Several cryoprotectants were tested for data collection under cryogenic conditions, including glycerol, ethylene glycol, polyethylene glycol 400 and (±)-2-methyl-2,4-pentane-diol. These results confirmed glycerol to be the best cryoprotectant. The crystal was transferred into reservoir solution containing 25% (v/v) glycerol for data collection under cryogenic conditions before being flash-cooled in liquid nitrogen. X-ray diffraction data were collected using an ADSC Q315r detector on beamline 5C at Pohang Light Source (PLS), Republic of Korea. The crystal was rotated through a total angle of 360° with 1.0° oscillation per frame. The exposure time was 3 s per frame (Table 3). The raw data were processed and scaled using the *HKL-2000* software package (Otwinowski & Minor, 1997). *MOLREP* from the *CCP4* program package was used for molecular replacement (Vagin & Teplyakov, 2010; Winn *et al.*, 2011).

3. Results and discussion

In this study, MTMR1 was overexpressed in *E. coli* in a soluble form, purified and crystallized for structural studies. The

purification yield was about 14.6 mg purified protein per litre of *E. coli* culture. SDS-PAGE analysis of purified MTMR1 showed that the protein was highly purified and suitable for crystallization (Fig. 1). The elution profile of MTMR1 on a HiLoad 16/600 Superdex 200 prep-grade column (120 ml column volume) exhibited a large single peak at 87 ml elution volume (Fig. 1). The native molecular weight of the recombinant protein as estimated by dynamic light-scattering analysis was ~69 kDa, with a polydispersity of 13.7%, suggesting that purified MTMR1 exists as monomers in solution owing to truncation of the coiled-coil domain at the C-terminus. When the purified protein was analysed by MALDI-TOF/TOF, it was identified as MTMR1 (Fig. 2). Crystals were formed under various crystallization conditions, but most of them were not suitable for X-ray diffraction data collection owing to poor diffraction quality. The best crystals were obtained using a reservoir solution consisting of 100 mM Na HEPES pH 7.0, 15% (v/v) PEG 20K. The crystals used in the X-ray diffraction experiments grew to maximum dimensions of approximately $0.2 \times 0.2 \times 0.1$ mm within six weeks (Fig. 3). Although the best crystals still exhibited diffraction patterns with smeared spots in some frames, it was possible to collect diffraction data. X-ray diffraction data were collected to 2.0 Å resolution (Fig. 3). The native crystals belonged to space group *P*1, with unit-cell parameters $a = 67.219$, $b = 96.587$, $c = 97.581$ Å, $\alpha = 87.597$, $\beta = 86.072$, $\gamma = 77.327^\circ$. Assuming the presence of four molecules in the asymmetric unit, the calculated Matthews coefficient (V_M) was $2.61 \text{ Å}^3 \text{ Da}^{-1}$ and the corresponding solvent content was 52.9% (Matthews, 1968). Data-collection statistics are shown in Table 3. Molecular replacement using the MTMR2 structure (PDB entry 1lw3; Begley *et al.*, 2003) as a search model was successful and allowed the identification of four protein molecules in the asymmetric unit. Although the diffraction data exhibited low completeness, the resulting electron-

density map was interpretable. Further refinement of the model structure is in progress.

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