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Expression, purification, crystallization and preliminary crystallographic analysis of human myotubularin-related protein 3

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Myotubularin-related proteins are a large family of phosphatases that have the catalytic activity of dephosphorylating the phospholipid molecules phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5-bisphosphate. Each of the 14 family members contains a phosphatase catalytic domain, which is inactive in six family members owing to amino-acid changes in a key motif for the activity. All of the members also bear PH-GRAM domains, which have low homologies between them and have roles that are not yet clear. Here, the cloning, expression, purification and crystallization of human myotubularin-related protein 3 encompassing the PH-GRAM and the phosphatase catalytic domain are reported. Preliminary X-ray crystallographic analysis shows that the crystals diffracted to 3.30 Å resolution at a synchrotron X-ray source. The crystals belonged to space group $C2$, with unit-cell parameters a = 323.3, b = 263.3, c = 149.4 Å, β = 109.7°.

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1. Introduction

Phosphatidylinositol (PtdIns) is a major inositol lipid in eukaryotic membranes and its conversion to derivatives plays important roles in a variety of intracellular signalling mechanisms. The D3, D4 and D5 positions of the inositol ring of PtdIns can be phosphorylated to give derivatives known as phosphoinositides. In response to environmental changes, the phosphoinositide profile is regulated by several phospholipases, PtdIns kinases and phosphoinositide phosphatases. Among the phosphoinositides, phosphatidylinositol 3-phosphate [PtdIns(3)P] and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] are known to regulate endosomal trafficking events and the degradation step of autophagy (Ferguson *et al.*, 2009; Huang *et al.*, 2011; Nicot & Laporte, 2008; Robinson & Dixon, 2006).

The myotubularin-related proteins (MTMRs) are a large family of conserved proteins with the specific activity of dephosphorylating PtdIns(3)P and PtdIns(3,5)P_2 to give PtdIns and PtdIns(5)P, respectively (Blondeau *et al.*, 2000). MTMR families share a common structural core containing a PH-GRAM domain, a phosphatase catalytic domain and a coiled-coil domain (Begley & Dixon, 2005). The coiled-coil domain is critical for homodimerization or heterodimerization of MTMRs, by which MTMR functions are regulated (Lorenzo *et al.*, 2006; Kim *et al.*, 2003). The PH-GRAM domain has been predicted to function in intracellular protein–protein or lipid–protein interactions (Doerks *et al.*, 2000). Several studies have demonstrated that this domain mediates the localization of MTMRs to different subcellular compartments by binding to phosphoinositides and determines the functional specificity of MTMRs by specific protein–protein interactions (Berger *et al.*, 2003; Choudhury *et al.*, 2006; Tsujita *et al.*, 2004). The sequence homologies of the PH-GRAM domains in MTMRs are very low, with less than 20% identity, implying a role as a determinant of the specific functions of each MTMR. Among the 14 myotubularin-related proteins, eight members (MTM1, MTMR1–4 and MTMR6–8) have the catalytic activity of dephosphorylating PtdIns(3)P and PtdIns(3,5)P2 at the D3 position with the consensus $C(X)_5R$ motif in the active sites of their

Table 1
Macromolecule-production information.

Source organism	*Homo sapiens*
DNA source	Open Biosystems (catalogue No. MHS1010-99823267)
Forward primer†	5′-GGG CCC CAT ATG AAG CAG CTG ATC CG-3′
Reverse primer‡	5′-GGG CCC GCG GCC GCT CAG GAT GGG CAG GG-3′
Expression vector	pET-28a
Expression host	*E. coli* BL21(DE3)
Complete amino-acid sequence of the construct produced§	GSHMKQLIREDENLQVPFLELHGESTEFVGRAEDAIIALSNY-RLHIKFKESLVNVPLQLIESVECRDIFQLHLTCKDCKVIR-CQFSTFEQCQEWLKRLNNAIRPPAKIEDLFSFAYHAWCME-VYASEKEQHGDLCRPGEHVTSRFKNEVERMGFDMNNAWRI-SNINEKYKLCGSYPQELIVPAWITDKELESVSSFRSWKRI-PAVIYRHQSNGAVIARCGQPEVSWWGWRNADDEHLVQSVA-KACASDSRSSGSKLSTRNTSRDFPNGGDLSDVEFDSSLSN-ASGAESLAIQPQKLLILDARSYAAAVANRAKGGGCECPEY-YPSCEVVFMGMANIHSIRRSFQSLRLLCTQMPDPGNWLSA-LESTKWLHHLSVLLKSALLVVHAVDQDQRPVLVHCSDGWD-RTPQIVALAKLLLDPYYRTIEGFQVLVEMEWLDFGHKFAD-RCGHGENSDDLNERCPVFLQWLDCVHQLQRQFPCSFEFNE-AFLVKLVQHTYSCLFGTFLCNNAKERGEKHTQERTCSVWS-LLRAGNKAFKNLLYSSQSEAVLYPVCHVRNLMLWSAVYLP-CPS

† *Nde*I site underlined. ‡ *Xho*I site underlined. § Cloning artefact underlined.

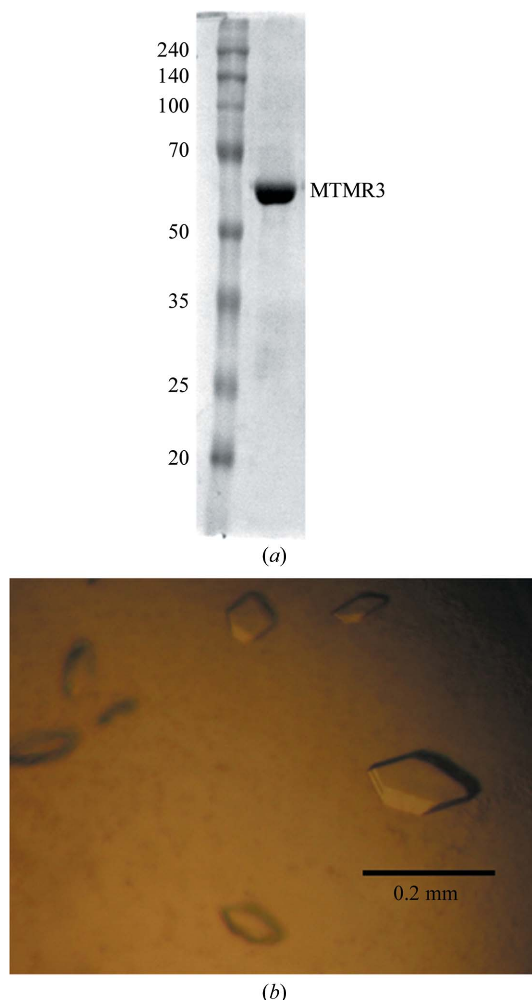

Figure 1
SDS–PAGE and crystals of MTMR3. (*a*) SDS–PAGE analysis of the purified MTMR3 containing the PH-GRAM and phosphatase domains with a molecular weight of 64 kDa. The left lane contains molecular-weight marker (labelled in kDa). (*b*) Crystals of human MTMR3 grown in 0.1 *M* sodium acetate pH 5.5, 36%(*v*/*v*) 2-methyl-2,4-pentanediol, 0.01 *M* taurine at 20°C.

phosphatase catalytic domains, which is the characteristic motif of protein tyrosine phosphatases (PTPs; Denu & Dixon, 1998; Blondeau *et al.*, 2000). The remaining six members (MTMR5 and MTMR9–13) are catalytically inactive as they bear inactivating mutations within the active-site C(*X*)$_5$R motif (Laporte *et al.*, 2003). Human MTMR3 is an active phospholipid phosphatase with 1198 amino acids and a molecular weight of 134 kDa. In addition to an N-terminal PH-GRAM domain, a phosphatase domain and a coiled-coil domain, MTMR3 contains a C-terminal FYVE domain. Recent studies have shown that MTMR3 plays a critical role in regulating autophagy and cancer cell migration, suggesting MTMR3 may be a novel therapeutic target in metastatic cancer (Oppelt *et al.*, 2013, 2014; Taguchi-Atarashi *et al.*, 2010). Structural studies of MTMR2 revealed that the phosphatase domain is structurally unique among PTPs, providing the substrate specificity, and the PH-GRAM domain is a larger motif with a PH domain fold, which is known as a phosphoinositide-binding module (Begley *et al.*, 2003, 2006).

Here, we report the cloning, expression, purification and crystallization of human MTMR3 as well as preliminary crystallographic analysis of X-ray diffraction data collected to 3.30 Å resolution. Comprehensive structural studies of the MTMR family should provide insight into their specific functions and regulation mechanisms.

2. Materials and methods

2.1. Macromolecule production

The gene encoding the PH-GRAM domain and the catalytic phosphatase domain (amino acids 21–581) of human MTMR3 was PCR-amplified from the MTMR3 cDNA purchased from Open Biosystems (USA). The forward primer contained an *Nde*I restriction site (bold) and had the sequence 5′-GGG CCC **CAT ATG** AAG CAG CTG ATC CG-3′, while the reverse primer contained an *Not*I restriction site (bold) and had the sequence 5′-GGG CCC **GCG GCC GC**T CAG GAT GGG CAG GG-3′. The PCR product was cloned into pET-28a vector (Novagen) using *Nde*I and *Not*I restriction sites, and the construction of the plasmid contained a 6×His tag fused to the N-terminus of the expressed protein for purification purposes. The correct sequence of the insert gene was verified by DNA sequencing using T7 promoter primer. The protein was overexpressed using plasmid-transformed *Escherichia coli* BL21 (DE3) (Novagen) cells. The cells were first grown at 37°C in LB medium supplemented with 25 µg ml^{-1} kanamycin. Protein expression was induced by adding 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) when the cells reached an optical density at 600 nm of about 0.6 and cell growth continued for 16 h at 20°C prior to harvesting by centrifugation at 3000*g* (30 min, 4°C). The cell pellet was resuspended in lysis buffer (20 m*M* Tris, 10 m*M* Na_2HPO_4, 300 m*M* NaCl pH 8.0) and disrupted by sonication on ice. The crude lysate was centrifuged at 25 000*g* for 1 h at 4°C.

The supernatant containing the soluble protein was applied onto a nickel–nitrilotriacetic acid (Ni–NTA) column (Qiagen) and was washed with five column volumes of wash buffer (20 m*M* Tris, 10 m*M* Na_2HPO_4, 300 m*M* NaCl, 50 m*M* imidazole pH 8.0). The protein was eluted with elution buffer (20 m*M* Tris, 10 m*M* Na_2HPO_4, 300 m*M* NaCl, 400 m*M* imidazole pH 8.0). The eluted protein was buffer-exchanged into 20 m*M* Tris, 10 m*M* Na_2HPO_4, 300 m*M* NaCl pH 8.0 by dialysis and was treated with bovine thrombin (Invitrogen) to remove the 6×His tag (16 h, 4°C). To remove the 6×His-uncleaved form, the protein was further applied onto an Ni–NTA column and the nonbinding fractions were concentrated for gel-filtration

Table 2
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline 5C, PLS
Wavelength (Å)	1.0000
Temperature (°C)	−173
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	1.0
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	*C*2
Unit-cell parameters (Å, °)	a = 323.3, b = 263.3, c = 149.4, β = 109.7
Resolution range (Å)	50.0–3.30 (3.38–3.30)
Total No. of reflections	173782
No. of unique reflections	48275
Completeness (%)	99.7 (99.8)
Multiplicity	3.6 (3.4)
$\langle I/\sigma(I)\rangle$	22.0 (2.4)
$R_{\rm r.i.m.}$†	0.094 (0.48)
Overall B factor from Wilson plot (Å^2)	53.2
Mosaicity (°)	0.71

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

chromatography using a Superdex 200 HR26/60 column (GE Healthcare, USA). The column had previously been equilibrated with gel-filtration buffer (20 m*M* Tris, 10 m*M* Na_2HPO_4, 300 m*M* NaCl, 5 m*M* DTT pH 8.0). The eluted fractions were concentrated to 12 mg ml^{-1} and the purity of the protein was examined by 12% SDS–PAGE and determined to be >95%. The recombinant protein contains additional amino-acid residues at the N-terminus (GSHM) originating from the plasmid, giving a total of 565 residues, as analyzed by SDS–PAGE. Macromolecule-production information is summarized in Table 1.

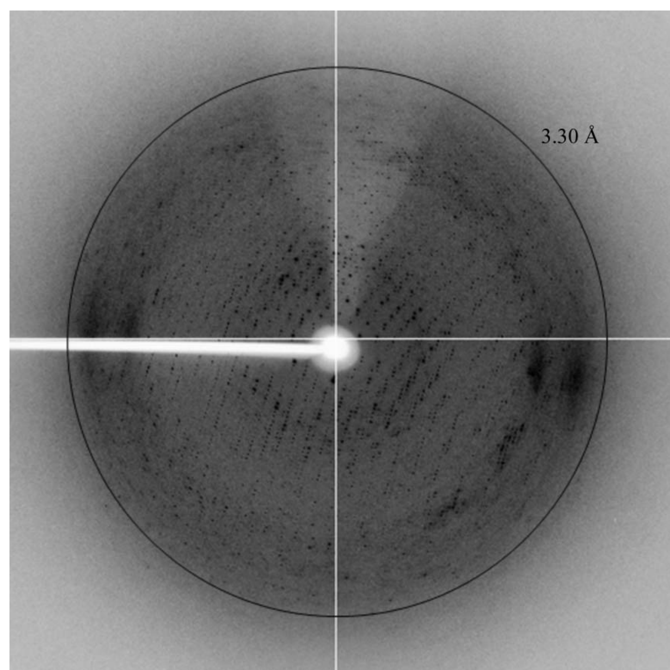

Figure 2
Typical diffraction image of a crystal of human MTMR3. The resolution limit (3.30 Å) is indicated by a circle.

2.2. Crystallization

Conditions for obtaining the protein crystals were screened using commercial screening kits by the hanging-drop vapour-diffusion method in 24-well VDX plates (Hampton Research, USA) at 20°C. Crystallization drops were prepared by mixing 0.8 µl protein solution and 0.8 µl reservoir solution. Each hanging drop was equilibrated over 400 ml reservoir solution. Tiny microcrystals appeared after 5 d in the condition 0.1 *M* sodium acetate pH 5.0, 40%(*v*/*v*) 2-methyl-2,4-pentanediol. For further optimization of crystal quality, additive screens (Hampton Research, USA) were used. Crystals of maximum size were obtained from the condition 0.1 *M* sodium acetate pH 5.5, 36%(*v*/*v*) 2-methyl-2,4-pentanediol, 0.01 *M* taurine within 7 d (Fig. 1). As this crystallization condition itself is cryoprotective, no additional cryoprotectant was required for data collection.

2.3. Data collection and processing

X-ray diffraction data were collected at −173°C on beamline 5C of the Pohang Light Source (PLS), Republic of Korea. A total rotation range of 360° was covered with 1.0° oscillation and 1 s exposure per frame. The wavelength of the synchrotron X-ray beam was 1.0000 Å and the crystal-to-detector distance was set to 300 mm. X-ray diffraction data were collected to 3.30 Å resolution (Fig. 2). Data were indexed, integrated, scaled and merged using *DENZO* and *SCALEPACK* from the *HKL*-2000 software package (Otwinowski & Minor, 1997). The crystals belonged to space group *C*2, with unit-cell parameters a = 323.3, b = 263.3, c = 149.4 Å, β = 109.7°.

3. Results and discussion

Human MTMR3 encompassing the PH-GRAM and the phosphatase domain was cloned, expressed, purified and crystallized for structural studies. Crystals of optimal size for X-ray diffraction experiments were obtained using a reservoir solution consisting of 0.1 *M* sodium acetate pH 5.5, 36%(*v*/*v*) 2-methyl-2,4-pentanediol, 0.01 *M* taurine and their approximate dimensions were 200 × 100 × 20 µm. X-ray diffraction data were collected to 3.30 Å resolution. X-ray diffraction data from the crystal indicated that it belonged to space group *C*2, with unit-cell parameters a = 323.3, b = 263.3, c = 149.4 Å, β = 109.7°. Data-collection statistics are provided in Table 2. It was ambiguous how many protein molecules were contained in the asymmetric unit. According to Matthews coefficient calculations with the molecular weight of 64 kDa, the crystallographic structure might contain ten to 22 protein molecules in the asymmetric unit with a $V_{\rm M}$ of 2.13–4.68 Å^3 Da^{-1} and a solvent content of 42.2–73.7% (Matthews, 1968). Molecular replacement (MR) was performed using the crystal structure of human MTMR2 (PDB entry 1lw3; 37% sequence identity; Begley *et al.*, 2003) or MTMR6 (PDB entry 2yf0; 34% sequence identity; Structural Genomics Consortium, unpublished work) as a search model. As the relative positions of the PH-GRAM domain and the phosphatase domain are different in the two MTMR structures, each domain was also used as a search model for MR. However, all of the MR trials failed to solve the structure, probably owing to the high copy number in the asymmetric unit. Experiments with selenomethionine-substituted protein and heavy-atom derivative crystals are now in progress to obtain experimental phases.

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