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Crystallization and preliminary X-ray crystallographic analysis of the PH-GRAM domain of human MTMR4

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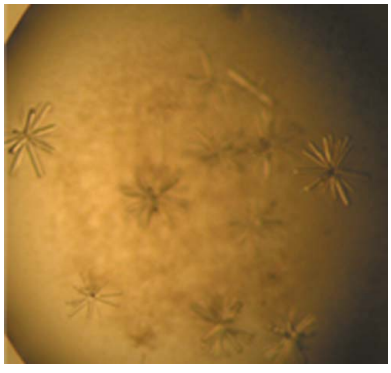

Phosphoinositide lipid molecules play critical roles in intracellular signalling pathways and are regulated by phospholipases, lipid kinases and phosphatases. In particular, phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5-bisphosphate are related to endosomal trafficking events through the recruitment of effector proteins and are involved in the degradation step of autophagy. Myotubularin-related proteins (MTMRs) are a large family of phosphatases that catalyze the dephosphorylation of phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5-bisphosphate at the D3 position, thereby regulating cellular phosphoinositide levels. In this study, the PH-GRAM domain of human MTMR4 was cloned, overexpressed in *Escherichia coli*, purified and crystallized by the vapour-diffusion method. The crystals diffracted to 3.20 Å resolution at a synchrotron beamline and belonged to either space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 109.10$, $c = 238.97$ Å.

1. Introduction

Myotubularins are a large family of conserved proteins with phosphoinositide phosphatase activity. The myotubularin gene (*MTM1*) has been identified as a gene that is mutated in X-linked myotubular myopathy (Laporte *et al.*, 1996). *MTM1* encodes a protein with an amino-acid sequence similar to the protein tyrosine phosphatases (PTPs), which are characterized by a $C(X)_5R$ motif in the active site (Denu & Dixon, 1998). In addition to *MTM1*, 13 related genes, named *MTM1*-related (*MTMR*) 1–13, have been identified in the human genome (Laporte *et al.*, 2003). It was discovered that mutations in the *MTMR2* and *MTMR13* genes cause two forms of Charcot–Marie–Tooth neuropathy (CMT4B1 and CMT4B2; Bolino *et al.*, 2000; Azzedine *et al.*, 2003). Recent studies have shown that several MTMRs are associated with human diseases, including cancers, obesity and metabolic syndrome, epilepsy and Creutzfeldt–Jakob disease (Hendriks & Pulido, 2013). Among the 14 members of this family, six proteins are catalytically inactive as they contain mutations in the key $C(X)_5R$ active-site motif (Wishart *et al.*, 2001). The active myotubularins have the catalytic activity of dephosphorylating phosphatidylinositol 3-monophosphate [PtdIns(3)P] and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] to give phosphatidylinositol (PtdIns) and phosphatidylinositol 3-monophosphate [PtdIns(5)P], respectively (Blondeau *et al.*, 2000). PtdIns(3)P and PtdIns(3,5)P_2 are known to regulate endocytosis and endosomal trafficking events by recruiting effector proteins, thus implying that MTMRs play critical roles in these processes (Nicot & Laporte, 2008; Robinson & Dixon, 2006). In particular, MTMR4 localizes to early endosomes and plays an important role in the attenuation of TGF-β and BMP signalling by dephosphorylating activated R-Smads that have been trafficked to early endosomes (Yu *et al.*, 2010, 2013). Moreover, MTMR4 has been identified as a regulator of the sorting of endosomal cargo, such as transferrin, into recycling endosomes in a Rab11-dependent manner (Naughtin *et al.*, 2010).

The myotubularin proteins share a common structural core consisting of a PH-GRAM domain, a PTP-like catalytic domain and a coiled-coil motif (Begley & Dixon, 2005). The coiled-coil domain is critical for the homodimerization or heterodimerization of MTMRs, *via* which MTMR functions are regulated (Lorenzo *et al.*, 2006; Kim *et*

al., 2003). The PH-GRAM domain was originally defined based on homology from bioinformatics analysis of three groups of proteins (glucosyltransferases, Rab-like GTPase activators and myotubularins) and has been predicted to function in intracellular protein–protein or lipid–protein interactions (Doerks *et al.*, 2000). The crystal structure of MTMR2 revealed that this domain is part of a larger motif that encompasses a PH domain, which is known to be a phosphoinositide-binding module that targets its host protein to specific cell membranes (Begley *et al.*, 2003). 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 that this domain may determine the specific functions of each MTMRs.

Here, we report the cloning, expression, purification and crystallization of the PH-GRAM domain of human MTMR4 as well as preliminary crystallographic analysis of diffraction data collected to 3.20 Å resolution. Although the functions of the PH-GRAM domain in MTMRs remain unclear, comprehensive structural studies of this domain of MTMRs may help to identify its role in MTMR functions.

2. Materials and methods

2.1. Macromolecule production

The gene encoding the PH-GRAM domain (amino acids 21–126) of human MTMR4 was PCR-amplified from MTMR4 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 GAA CTA GTG AAG GAG GAA-3′, while the reverse primer contained a *Xho*I restriction site (bold) and had the sequence 5′-GGG CCC **CTC GAG** TCA GTC TTC AGG CTT GGC AG-3′. The PCR product was cloned into pET-28a vector (Novagen) using the *Nde*I and *Xho*I restriction sites. The correct sequence of the insert gene was verified by DNA sequencing using the T7 promoter primer. The protein was overexpressed with an amino-terminal His_6 tag using plasmid-transformed *Escherichia coli* BL21 (DE3) cells (Novagen, USA). 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-thio-galactopyranoside (IPTG) when the cells reached an optical density at 600 nm of about 0.6 and the cells were grown 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 pH 8.0, 300 m*M* NaCl) 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 poured into an Ni–nitrilotriacetic acid (Ni–NTA) column (Qiagen, USA) and washed with five column volumes of wash buffer (20 m*M* Tris pH 8.0, 300 m*M* NaCl, 50 m*M* imidazole). The protein was then eluted with elution buffer (20 m*M* Tris pH 8.0, 300 m*M* NaCl, 400 m*M* imidazole). The eluted protein was buffer-exchanged into 20 m*M* Tris pH 8.0, 300 m*M* NaCl by dialysis and treated with bovine thrombin (Invitrogen, USA) to remove the His_6 tag (16 h, 4°C). To remove the His_6-uncleaved form, the protein was further applied onto an Ni–NTA column and the unbound fractions were concentrated for gel-filtration chromatography using a Superdex 200

Table 1
Macromolecule-production information.

Source organism	*Homo sapiens*
DNA source	Open Biosystems (catalogue No. MHS6278-202801286)
Forward primer†	5′-GGG CCC CAT ATG AAG GAA CTA GTG AAG GAG GAA-3′
Reverse primer‡	5′-GGG CCC CTC GAG TCA GTC TTC AGG CTT GGC AG-3′
Expression vector	pET-28a
Expression host	*E. coli* BL21(DE3)
Complete amino-acid sequence of the construct produced§	GSHMKELVKEEENLQVPFTVLQGEGVEFLGRAADALIAISNY-RLHIKFKDSVINVPLRMIDSVESRDMFQLHISCKDSKVVR-CHFSTFKQCQEWLSRLSRATARPAKPED

† The *Nde*I site is shown underlined. ‡ The *Xho*I site is shown underlined. § A cloning artifact is shown underlined.

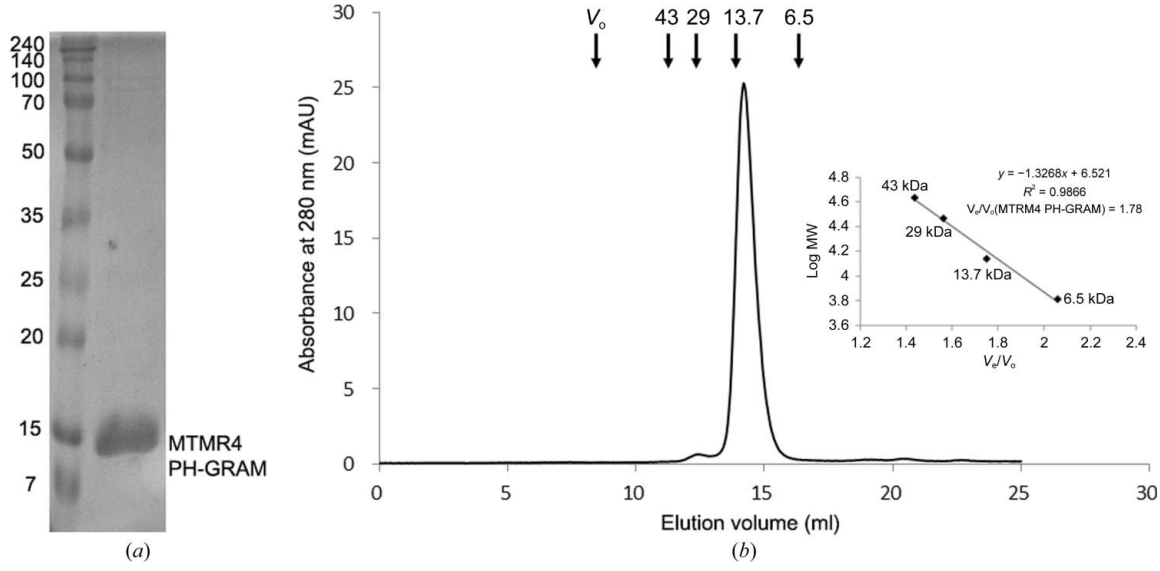

Figure 1
Purification of the PH-GRAM domain of human MTMR4. (*a*) SDS–PAGE of the purified protein with molecular-weight marker (labelled in kDa). (*b*) Size-exclusion chromatographic analysis of the purified protein using a Superdex 75 10/30 GL column. The arrows at the top indicate the elution volumes of proteins of known mass applied to the same column (indicated in kDa; V_o, void volume). The elution volume of the protein (14.2 ml) suggests that the molecule is present as a monomer in solution. The molecular-weight standards for size-exclusion chromatography are ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa).

HR 26/60 column (GE Healthcare, USA). The column had previously been equilibrated with gel-filtration buffer (20 m*M* Tris pH 8.0, 300 m*M* NaCl, 5 m*M* DTT). The elution profile of the protein showed a single major peak; the fractions comprising this peak were concentrated to 10 mg ml^{-1} and the purity of the protein was examined by 12% SDS–PAGE and determined to be >95% (Fig. 1*a*). The recombinant protein contained additional amino-acid residues at the N-terminus (GSHM) originating from the plasmid, giving a total of 110 residues, and the final yield of the purified protein was approximately 3 mg per litre of cell culture. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

Conditions for obtaining the protein crystals were screened using commercial screening kits with 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 µl reservoir solution. Tiny microcrystals appeared after 5 d in SaltRx condition 1-31 (Hampton Research, USA) consisting of 0.1 *M* bis-tris propane pH 7.0, 3.5 *M* sodium formate, and the crystallization condition was further optimized to obtain better crystals. Crystals of maximum size were obtained from 0.1 *M* bis-tris propane pH 6.8, 2.8 *M* sodium formate. The crystals grown in this condition were spoke-shaped and yielded single crystals when touched with a needle. The crystals were cryocooled in liquid nitrogen after soaking in cryoprotectant (20% glycerol added to the crystallization buffer).

2.3. Data collection and processing

X-ray diffraction data were collected at 100 K on beamline 7A of the Pohang Light Source (PLS), Republic of Korea. A total rotation range of 100° was collected with 1.0° oscillation and an exposure of 1 s per frame. The wavelength of the synchrotron X-ray beam was 0.9792 Å and the crystal-to-detector distance was set to 300 mm. X-ray diffraction data were collected to 3.20 Å resolution. Data were indexed, integrated, scaled and merged using *DENZO* and *SCALEPACK* from the *HKL*-2000 software package (Otwinowski & Minor, 1997). Given the systematic absence of the diffraction data, the crystals belonged to either space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 109.10$, $c = 238.97$ Å.

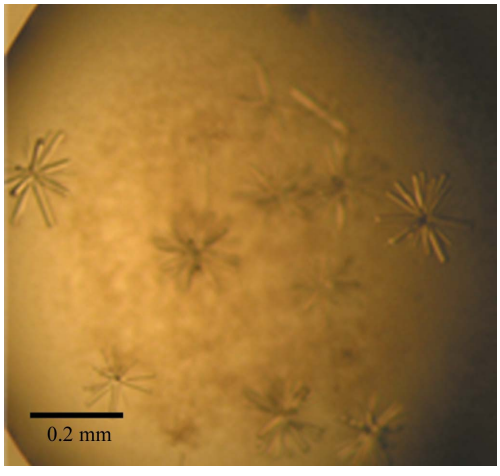

Figure 2
Crystals of the PH-GRAM domain of human MTMR4 grown in 0.1 *M* bis-tris propane pH 6.8, 2.8 *M* sodium formate at 20°C.

Table 2
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	7A, PLS
Wavelength (Å)	0.9792
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	1.0
Total rotation range (°)	100
Exposure time per image (s)	1
Space group	$P6_1$ or $P6_5$
Unit-cell parameters (Å)	$a = b = 109.10$, $c = 238.97$
Resolution range (Å)	50.0–3.20 (3.27–3.20)
Total No. of reflections	175273
No. of unique reflections	28224
Completeness (%)	99.4 (99.2)
Multiplicity	6.2 (6.0)
$\langle I/\sigma(I)\rangle$	22.0 (2.4)
$R_{\rm r.i.m.}$†	0.078 (0.44)
Overall *B* factor from Wilson plot (Å^2)	49.8

† 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.

3. Results and discussion

The PH-GRAM domain of human MTMR4 was cloned, expressed, purified and crystallized for structural studies. The purified protein eluted at approximately 14.2 ml from a Superdex 75 10/30 GL column (GE Healthcare, USA) as a monodisperse protein sample corresponding to a molecular weight of about 14.4 kDa (Fig. 1*b*). This suggests that the protein (theoretical molecular weight 12.6 kDa) exists as a monomer in solution. The best diffracting crystal was obtained using a reservoir solution consisting of 0.1 *M* bis-tris propane pH 6.8, 2.8 *M* sodium formate. Spoke-shaped crystals grew to an optimal size for X-ray diffraction experiments within 7 d and the approximate dimensions of the broken single crystals were 20 × 20 × 150 µm (Fig. 2). X-ray diffraction data were collected to 3.20 Å resolution (Fig. 3). X-ray diffraction data from the crystal indicated

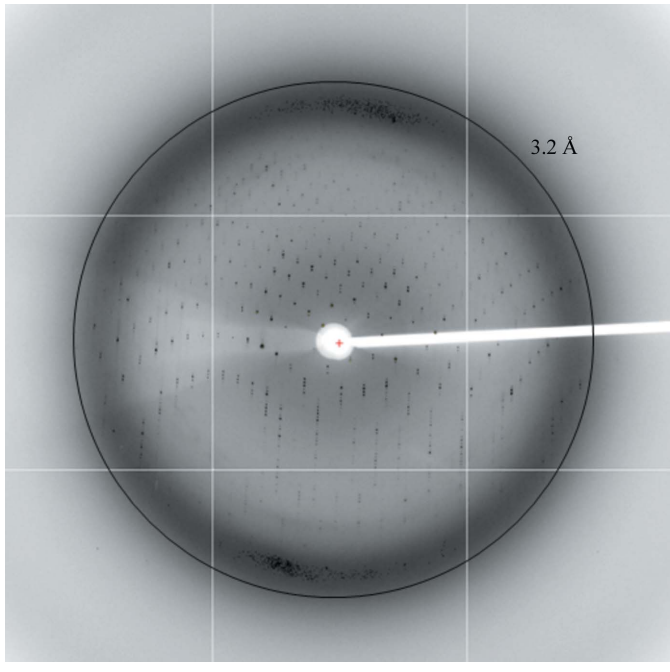

Figure 3
Typical diffraction image of a crystal of the PH-GRAM domain of MTMR4. The resolution limit (3.2 Å) is indicated by a circle.

that it belonged to either space group $P6_1$ or $P6_5$ on the basis of systematic absences, with unit-cell parameters $a = b = 109.10$, $c = 238.97$ Å. Data-collection statistics are provided in Table 2. The number of protein molecules contained in the asymmetric unit was ambiguous. According to Matthews coefficient calculations with the molecular weight of 12.6 kDa, the crystallographic structure might contain 8–16 protein molecules in the asymmetric unit with a V_M of 4.07–2.04 Å^3 Da^{-1} and a solvent content of 69.9–39.6% (Matthews, 1968). In spite of the presence of many molecules in the asymmetric unit, the self-rotation function and native Patterson did not reveal any noncrystallographic symmetry. Molecular replacement was performed using the PH-GRAM domain of human MTMR2 (PDB entry 1lw3; 19% sequence identity; Begley *et al*., 2003) or MTMR6 (PDB entry 2yf0; 13% sequence identity; Structural Genomics Consortium, unpublished work) as a search model. However, this failed to solve the structure, probably owing to the low homology of the search models and the high copy number of proteins per asymmetric unit. Experiments with SeMet-substituted protein are in progress to obtain experimental phases.

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