The RING domain of mitochondrial E3 ubiquitin ligase 1 and its complex with Ube2D2: crystallization and X-ray diffraction

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Mitochondrial E3 ubiquitin ligase 1 (MUL1) is located in the mitochondrial outer membrane and regulates various biological processes, including apoptosis, cell growth, mitophagy and mitochondrial dynamics. The C-terminal region of MUL1 faces the cytoplasm and contains the RING domain (MUL1-RING) where the Ub~E2 thioester binds. Unlike most RING-type E3 enzymes, MUL1-RING alone does not have an additional region that recruits a substrate protein, yet is still able to ubiquitylate the substrate, the p53 protein. Nevertheless, the exact mechanism of the ubiquitylation of p53 by MUL1-RING has not yet been elucidated. In order to understand this novel ubiquitylation mechanism, it is necessary to determine the three-dimensional structures of MUL1-RING and of its complex with the cognate E2 enzyme. Here, Ube2D2 was validated as a functional E2 enzyme for the ubiquitylation of the p53 transactivation domain (p53-TAD) by MUL1-RING, and purification and crystallization processes for MUL1-RING and the MUL1-RING–Ube2D2 complex are reported.

1. Introduction

Mitochondrial E3 ubiquitin ligase 1 (MUL1) is a mitochondrial E3 ligase that consists of a small cytoplasmic region (residues 1–8), a transmembrane-1 domain (TM-1; residues 9–29) domain, a mitochondrial intermembrane region (residues 30–238), a transmembrane-2 domain (TM-2; residues 239–259) and a C-terminal cytoplasmic region (residues 260–352) that contains the MUL1-RING domain. The ubiquitylation activity of MUL1 is important for the regulation of mitochondrial dynamics (fusion and fission), including mitophagy (Braschi *et al.*, 2009), and is also involved in various biological processes such as apoptosis and cell growth (Neuspiel *et al.*, 2008; Zhang *et al.*, 2008). MUL1 regulates the activation of NF- κ B signaling, which causes stress-induced hyperfusion of mitochondria (Zemirli *et al.*, 2014). The presence of MUL1-RING is critical for both the ubiquitylation (Li *et al.*, 2008) and SUMOylation (Braschi *et al.*, 2009) activities of MUL1. MUL1 promotes the degradation of ULK1 (unc-51-like autophagy activating kinase 1) and MFN-2 (mitofusin 2) through ubiquitylation (Yun *et al.*, 2014). On the other hand, MUL1 induces the SUMOylation of DNM1L/Drp1 to increase mitochondrial fission (Nunnari & Suomalainen, 2012) and delays mitochondrial fusion by inhibiting ubiquitylation (Deng *et al.*, 2008).

Ubiquitylation is mediated by an enzymatic cascade reaction involving a ubiquitin-activating enzyme (E1), a

Table 1
Production information for MUL1-RING and Ube2D2.

	MUL1-RING	Ube2D2
Source organism	<i>H. sapiens</i>	<i>H. sapiens</i>
DNA source	<i>H. sapiens</i> genomic DNA	<i>H. sapiens</i> genomic DNA
Forward primer† (5′–3′)	GAGGATCCAGTCTGAAGAGCGCCTG	GAGGATCCATGGCTCTGAAGAGAATC
Reverse primer‡ (5′–3′)	GACTCGAGTTAGCTGTTGTACAGGGGTATC	GACTCGAGTTACATCGCATACTTCTGAGTC
Cloning and expression vector	pGEX4T-3	pGEX4T-3
Cloning host	<i>E. coli</i> DH5α	<i>E. coli</i> DH5α
Expression host	<i>E. coli</i> Rosetta 2 (DE3)	<i>E. coli</i> Rosetta 2 (DE3)
Amino-acid sequence§	GSLKSACVVCLSSFKSCVFLECGHVCSCTECYRALPEPKKCPI CRQAITRVIPLYS	GSMALKRIHKEINLDLARDPPAQCSAGPVGDDMFHWQATIMGPN DSPYQGGVFFLTIFHPTDYPFKPPKVAFTTRIYHPNINSNG SICLDILRSQWSPALTISKVLLSICSLLCDPNPDDPLVPEI ARIYKTDREKYNRIAREWTQKYAM

† The BamHI site is underlined. ‡ The XhoI site is underlined. § After thrombin digestion.

ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase enzyme (E3) (Pickart & Eddins, 2004). The repetitive process of ubiquitin transfer leads to the polyubiquitylation of substrate proteins and their degradation by the 26S proteasome; the attached ubiquitin is then recycled, contributing to free ubiquitin (Komander & Rape, 2012). E3 ligases can be categorized into two main classes according to their mechanism of ubiquitin transfer. HECT-E3 ligases receive activated ubiquitin that is attached to E2 via a thioester (E2~UB) to form an intermediate, HECT-E3~UB, and then transfer ubiquitin to the target protein. In contrast, RING-E3 ligases directly deliver ubiquitin from E2~UB to the target protein (Pruneda *et al.*, 2012; Metzger *et al.*, 2014; Deshaies & Joazeiro, 2009), in which a lysine ϵ -amino group of the target protein attacks the carbonyl group of the E2~UB thioester and results in the formation of an isopeptide bond between the C-terminal Gly76 of ubiquitin and the lysine side chain of the target. The key feature of most RING-type E3 ligases is the presence of both a RING domain and another domain that recruit E2~UB and target protein, respectively (Plechanovová *et al.*, 2012).

MUL1-RING has been shown to play an important role in apoptosis, cell growth and the regulation of mitochondrial function. Interestingly, MUL1-RING alone is able to bind to and ubiquitylate the substrate protein p53 (Jung *et al.*, 2011; Peng *et al.*, 2016). We recently reported the solution structure of MUL1-RING and analyzed the interaction between MUL1-RING and the p53 transactivation domain (p53-TAD) using NMR spectroscopy (Lee *et al.*, 2019). However, the binding affinity between these two proteins is very weak, and the exact mechanism of MUL1-RING-mediated ubiquitylation of p53-TAD is still not clear. Therefore, it is important to determine the 3D structures of both free MUL1-RING and its complex with the cognate E2 enzyme in order to elucidate the unique ubiquitylation mechanism of MUL1-RING. Previous studies of the ubiquitylation activity of MUL1 with various E2 enzymes have shown that Ube2D1 and Ube2D3, but not Ube2D2, play a role in the ubiquitylation of p53 (Jung *et al.*, 2011). Since these human E2 enzymes belong to the yeast Ubc4 family and their amino-acid sequences are almost identical (Dou *et al.*, 2012; Ozkan *et al.*, 2005), it is necessary to validate the enzymatic activity of these E2 enzymes during the ubiquitylation of p53-TAD by MUL1-RING alone. Here, we

selected Ube2D2 for complex formation with MUL1-RING based on an *in vitro* ubiquitylation assay and report the purification and crystallization processes of MUL1-RING and the MUL1-RING–Ube2D2 complex in order to study the novel ubiquitylation mechanism of MUL1-RING alone.

2. Materials and methods

2.1. Expression and purification

To express the target proteins, the genes for human MUL1-RING (residues 298–352) and Ube2D2 (residue 1–147) were cloned into the pGEX4T-3 vector using the BamHI and XhoI restriction-enzyme sites (Table 1). The recombinant plasmid was transformed into *Escherichia coli* Rosetta 2 (DE3) cells. Expression of the glutathione *S*-transferase (GST)-tagged proteins was induced by incubation with 0.5 mM isopropyl β -D-1-thiogalactopyranoside for 6 h at 30°C. The GST-fusion proteins were purified by GST-affinity column chromatography with phosphate-buffered saline (PBS) buffer and the GST tag was removed by thrombin digestion. The MUL1-RING protein was further purified by size-exclusion chromatography (SEC) on a HiLoad 16/600 Superdex 75 column (GE Healthcare) with SEC buffer (50 mM MES, 50 mM NaCl, 5 μ M ZnSO₄, 10 mM DTT pH 6.5). The E2 protein was purified by SEC with a buffer consisting of 10 mM Tris–HCl, 100 mM NaCl, 1 mM DTT pH 7.5.

The formation of a complex between MUL1-RING and Ube2D2 was first confirmed by SEC high-performance liquid chromatography (HPLC) using a Biosep-SEC-S3000 column (Phenomenex) with SEC buffer. To obtain the MUL1-RING–Ube2D2 complex, a mixture of MUL1-RING and Ube2D2 in a 1:1 molar ratio was prepared using their UV extinction coefficients (Gill & von Hippel, 1989) and the mixture was further purified using the same SEC procedure as described for MUL1-RING alone. All purified proteins were concentrated using Amicon Ultra Centrifugal filters (Millipore) for crystallization setup.

2.2. In vitro ubiquitylation assay

The *in vitro* ubiquitylation of p53-TAD by MUL1-RING alone was assessed with four different E2 enzymes, Ube2D1, Ube2D2, Ube2D3 and Ube2L3 (UbcH7), following a

Table 2
Crystallization conditions.

	MUL1-RING	MUL1-RING–Ube2D2
Method	Sitting-drop vapor diffusion	Hanging-drop vapor diffusion
Plate type	96-well 2-drop MRC Crystallization Plates (Molecular Dimensions)	24-well VDX plate (Hampton Research)
Temperature (K)	293	293
Protein concentration (mg ml ⁻¹)	15	10
Buffer composition of protein solution	50 mM MES, 50 mM NaCl, 5 μ M ZnSO ₄ , 10 mM DTT pH 6.5	50 mM MES, 50 mM NaCl, 5 μ M ZnSO ₄ , 10 mM DTT pH 6.5
Composition of reservoir solution	0.4 M lithium sulfate monohydrate, 0.1 M bis-Tris pH 6.5, 25%(w/v) PEG 3350	4%(v/v) Tacsimate pH 6.0, 12%(w/v) PEG 3350
Volume and ratio of drop	2 μ l (1:1 ratio)	2 μ l (1:1 ratio)
Volume of reservoir (μ l)	100	1000

previously reported method (Choi *et al.*, 2015). Briefly, the *in vitro* ubiquitylation was performed with 20 μ M GST-fused MUL1-RING, 200 μ M ubiquitin, 1 μ M E1, 20 μ M E2 and 20 μ M p53-TAD in a buffer consisting of 50 mM Tris–HCl, 10 μ M ZnSO₄, 1 mM MgCl₂, 2 mM ATP pH 8.0 for 2.5 h at 25°C. The reaction samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the protein bands were visualized by Coomassie Blue staining. Ube2L3 was used as a negative control, since it does not have E3-independent reactivity with the lysine residue and has a preference for HECT-E3 ligases (Wenzel *et al.*, 2011).

2.3. Crystallization of MUL1-RING and the MUL1-RING–Ube2D2 complex

The MUL1-RING protein (15 mg ml⁻¹) was prepared in SEC buffer for crystallization setup. Initial sparse-matrix screening of the crystallization conditions was performed using commercial crystallization solution kits (Index, Crystal Screen, PEG/Ion, SaltRx and MembFac from Hampton Research and Wizard Classic from Molecular Dimensions) at 20°C. The crystals of MUL1-RING alone grew in five days using the sitting-drop vapor-diffusion method with 100 μ l reservoir buffer consisting of 0.1 M bis-Tris pH 6.5, 0.2–0.4 M lithium sulfate monohydrate, 25%(w/v) polyethylene glycol (PEG) 3350, in which 1 μ l protein solution and 1 μ l reservoir solution were mixed together (Table 2). The cryoprotectant solution for the crystal of MUL1-RING alone was prepared by adding 25% glycerol to the reservoir solution.

The MUL1-RING–Ube2D2 complex (10 mg ml⁻¹) was prepared in SEC buffer and sparse-matrix screening was then performed using a Mosquito Crystal nanolitre protein crystallization robot (TTP Labtech) with similar crystallization solution kits (Index, Crystal Screen and PEG/Ion from Hampton Research and ProPlex from Molecular Dimensions) at 20°C. The MUL1-RING (10 mg ml⁻¹) and Ube2D2 (10 mg ml⁻¹) proteins were also used separately to distinguish the complex crystals from crystals consisting of a single protein component during sparse-matrix screening. After various optimizations of the crystallization conditions, crystals of the MUL1-RING–Ube2D2 complex grew in three days using the hanging-drop vapor-diffusion method with 1 ml reservoir buffer consisting of 4%(v/v) Tacsimate pH 6.0, 12%(w/v) PEG 3350 (Table 2). The cryoprotectant solution

Table 3
X-ray diffraction and data-collection statistics for MUL1-RING and the MUL1-RING–Ube2D2 complex.

Values in parentheses are for the highest resolution shell.

	MUL1-RING	MUL1-RING–Ube2D2
Diffraction source	Beamline 7A, PAL	Beamline 7A, PAL
Wavelength (Å)	1.2837	0.9793
Temperature (K)	100	100
Detector	ADSC Q270r	ADSC Q270r
Crystal-to-detector distance (mm)	150	350
Rotation range per image (°)	1.0	1.0
Total rotation range (°)	360	360
Exposure time per image (s)	0.2	1.0
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	59.226, 66.593, 68.271	47.207, 141.241, 68.047
α , β , γ (°)	90.00, 90.00, 90.00	90.00, 104.81, 90.00
Mosaicity range (°)	0.707–1.103	0.850
Resolution range (Å)	50.00–1.80	50.00–2.70
Total No. of reflections	1034568	453307
No. of unique reflections	25532	21200
Completeness (%)	99.0 (96.8)	90.1 (74.8)
Multiplicity	8.4 (4.4)	5.1 (3.0)
<i>R</i> _{meas}	0.084 (0.407)	0.093 (0.337)
$\langle I/\sigma(I) \rangle$	24.5 (2.3)	20.2 (3.3)
Wilson <i>B</i> factor (Å ²)	16.9	51.6

for the crystal of the MUL1-RING–Ube2D2 complex was prepared by increasing the concentration of PEG 3350 in the reservoir solution to 40%(w/v).

2.4. Data collection and processing for MUL1-RING and the MUL1-RING–Ube2D2 complex

All X-ray diffraction data were recorded on beamline 7A at Pohang Accelerator Laboratory (PAL), Republic of Korea. Single crystals of MUL1-RING and the MUL1-RING–Ube2D2 complex were transferred into the cryoprotectant solution using a cryoloop and were then flash-cooled with cold nitrogen gas (100 K). The X-ray diffraction data were indexed, merged and scaled using the *HKL*-2000 software (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 3.

3. Results and discussion

We first examined whether Ube2D1, Ube2D2 and Ube2D3, the sequences of which are almost identical (Fig. 1*a*), were

able to support the ubiquitylation of p53-TAD by MUL1-RING alone, since (i) a previous report showed that Ube2D2 was not effective in the ubiquitylation of p53 by MUL1 (Jung *et al.*, 2011) and (ii) the backbone chemical shifts of Ube2D2 were already available, which may facilitate further NMR studies based on the crystal structure of the MUL1-RING–Ube2D2 complex. The *in vitro* ubiquitylation of p53-TAD by MUL1-RING alone was assessed for Ube2D1, Ube2D2, Ube2D3 and Ube2L3, in which Ube2L3 was used as a negative control. As predicted, all three E2 enzymes, but not Ube2L3, were able to support polyubiquitylation by MUL1-RING alone (Fig. 1*b*). Next, we also confirmed that MUL1-RING formed a stable complex with Ube2D2: SEC–HPLC and SDS–PAGE analyses showed that MUL1-RING co-eluted with Ube2D2 (Fig. 2).

MUL1-RING crystals were obtained using the sitting-drop method (Fig. 3*a*). X-ray diffraction data from a MUL1-RING crystal were collected to 1.80 Å resolution (Fig. 4*a*). The

crystal belonged to the orthorhombic space group $P2_12_12_1$ (unit-cell parameters $a = 59.22$, $b = 66.59$, $c = 68.27$ Å). The data set, with a resolution range of 50–1.80 Å, had 99% completeness and an R_{merge} of 8.4% (Table 3). Assuming the presence of four MUL1-RING molecules in the asymmetric unit, the corresponding Matthews coefficient (Matthews, 1968) and solvent content are $2.67 \text{ Å}^3 \text{ Da}^{-1}$ and 53.98%, respectively. To solve the crystal structure of MUL1-RING, we performed molecular replacement (MR) using *MOLREP* (Vagin & Teplyakov, 2010) and *Phaser* (McCoy *et al.*, 2007). In addition to the recent NMR ensemble structures of MUL1-RING (PDB entry 6k2k; Lee *et al.*, 2019), a homology model of MUL1-RING was calculated using the *Phyre2* web portal (Kelley *et al.*, 2015), in which the structure of baculoviral IAP repeat-containing protein 2 (PDB entry 3t6p; 41% identity; Dueber *et al.*, 2011) was used as a reference model. Unfortunately, neither model was able to derive a correct MR solution. The failure of MR with NMR ensemble structures of

Figure 1 An *in vitro* ubiquitylation assay by MUL1-RING alone. (a) Multiple sequence alignment of Ube2D1, Ube2D2, Ube2D3 and Ube2L3. Sequence alignment of Ube2D1, Ube2D2, Ube2D3 and Ube2L3 was carried out using *Clustal Omega* and *ESPrpt* 3.0 (Sievers & Higgins, 2018; Robert & Gouet, 2014). (b) The *in vitro* ubiquitylation of p53-TAD mediated by MUL1-RING was assessed for Ube2D1, Ube2D2 and Ube2D3. Ube2L3 (UbcH7) was used as a negative control. Molecular-weight markers are labeled in kDa.

MUL1-RING is interesting, since the structures were calculated with NH residual dipolar coupling data in addition to NOE distance and Talos angle restraints. The crystal structure of MUL1-RING might differ from the solution structure, or possible structural flexibility of MUL1-RING in solution may cause the failure of MR. To study the unique ubiquitylation mechanism of MUL1-RING, the structure of the complex of MUL1-RING with its cognate E2 enzyme is critical. Therefore, the crystal structure of MUL1-RING alone can simply be solved after determination of the complex structure.

The crystallization conditions for the MUL1-RING–Ube2D2 protein complex were screened using the sitting-drop method, and the final crystal form was obtained using the hanging-drop method after various crystal optimizations (Fig. 3*b*). In addition, we confirmed that the crystal actually contained MUL1-RING and Ube2D2 by SDS–PAGE of a dissolved crystal of the complex (Fig. 3*c*). X-ray diffraction data were collected from a crystal of the complex to 2.7 Å resolution (Fig. 4*b*). The crystal symmetry was determined to

be monoclinic (space group $P2_1$; unit-cell parameters $a = 47.21$, $b = 141.24$, $c = 68.05$ Å, $\alpha = \gamma = 90.00$, $\beta = 104.81^\circ$) and the statistics of the diffraction data are summarized in Table 3. The calculated Matthews coefficient (V_M) was 2.38 Å³ Da^{−1} with a solvent content of 48.26%, and thus four complex molecules appeared to be present in the asymmetric unit. We attempted to find an MR solution for the X-ray diffraction data from the MUL1-RING–Ube2D2 complex using the same NMR structures and the homologous model of MUL1-RING, but these trials were not successful. However, a *Phaser* MR analysis using a previous crystal structure of Ube2D2 (PDB entry 2esk; Ozkan *et al.*, 2005) clearly found four solutions with TFZ values of 13.0, 24.9, 26.0 and 30.0.

It would be interesting to observe whether there are any structural differences in MUL1-RING among the NMR ensemble structures of MUL1-RING, the crystal structure of MUL1-RING alone and the crystal structure of the MUL1-RING–Ube2D2 complex that may explain the reason why MR using the NMR ensemble structure of MUL1-RING failed and

Figure 2

SEC elution profile of the MUL1-RING–Ube2D2 mixture. MUL1-RING, Ube2D2 and their mixture were analyzed by (a) SEC–HPLC and (b) SDS–PAGE. In (b), lane *M* contains molecular-weight markers (labeled in kDa), lane 1 contains Ube2D2, lane 2 contains MUL1-RING and lane 3 contains MUL1-RING–Ube2D2.

Figure 3

(a, b) Crystals of human MUL1-RING (a) and of MUL1-RING complexed with Ube2D2 (b). (c) SDS–PAGE of a dissolved crystal of the MUL1-RING–Ube2D2 complex. Molecular-weight markers are labeled in kDa.

Figure 4
X-ray diffraction patterns of crystals of MUL1-RING (a) and of MUL1-RING complexed with Ube2D2 (b). The diffraction images were collected on beamline 7A at PAL.

may reveal unique structural features of the MUL1-RING–Ube2D2 complex compared with other RING–E2 proteins. The 3D structures of MUL1-RING and the MUL1-RING–Ube2D2 complex are likely to provide a structural basis for understanding the unique ubiquitylation mechanism of MUL1-RING alone that distinguishes it from other RING–E3 proteins.

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