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Crystallization of the coiled-coil domain of Atg16 essential for autophagy

Atg16 is a scaffold protein that interacts with Atg12–Atg5 protein conjugates *via* its N-terminal domain and self-assembles *via* its coiled-coil domain, thus forming a multimeric Atg12–Atg5–Atg16 complex that is essential for autophagy. The coiled-coil domain of Atg16 was expressed, purified and crystallized. The crystal belonged to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = 127.7$, $c = 77.8$ Å. Self-rotation functions and volume-to-weight ratio values suggested that the crystal may contain six molecules per asymmetric unit. Since the domain does not contain a methionine residue, selenomethionine-labelled crystals were prepared with a leucine-to-methionine substitution in the coiled-coil domain and these crystals were used for the collection of single-wavelength anomalous dispersion data to 2.5 Å resolution.

1. Introduction

Autophagy is a starvation-induced response that mediates the bulk degradation of cytoplasmic components in the lysosome/vacuole (Seglen & Bohley, 1992; Takeshige *et al.*, 1992) and plays a critical role in fundamental biological processes such as intracellular clearance, differentiation, development, programmed cell death and antigen representation (Mizushima, 2007). In autophagy, a double-membrane structure called an autophagosome sequesters a portion of cytoplasm and fuses with the lysosome/vacuole to deliver its contents into the organelle lumen. The autophagosome-formation step requires at least 18 Atg proteins (Suzuki & Ohsumi, 2007; Kawamata *et al.*, 2008), of which eight constitute two ubiquitin-like conjugation systems, the Atg12 and Atg8 systems (Ohsumi, 2001).

In the Atg12 system, Atg12 is covalently linked to Atg5 by sequential reactions catalyzed by Atg7 (an E1-like enzyme) and Atg10 (an E2-like enzyme) (Mizushima *et al.*, 1998; Shintani *et al.*, 1999). The Atg12–Atg5 conjugate further interacts noncovalently with the N-terminal domain of Atg16 (Mizushima *et al.*, 1999). Since Atg16 self-assembles *via* the predicted coiled-coil motif (residues 67–123; Mizushima *et al.*, 1999), the Atg12–Atg5–Atg16 ternary complex behaves like a huge protein complex that has been shown by gel-filtration chromatography to be as large as 350 kDa (Kuma *et al.*, 2002). The Atg12–Atg5–Atg16 complex is essential for the formation of autophagosomes, for which the multimerization of the complex *via* the coiled-coil region of Atg16 is required (Kuma *et al.*, 2002). Little is known of the specific functions of the Atg12–Atg5–Atg16 complex in autophagosome formation; however, recent studies have shown that the complex functions as an E3-like enzyme in the Atg8 system (Hanada *et al.*, 2007; Fujioka *et al.*, 2008; Fujita *et al.*, 2008). In the Atg8 system, Atg8 is conjugated to phosphatidylethanolamine (PE) by sequential reactions catalyzed by Atg7 and Atg3 (another E2-like enzyme; Ichimura *et al.*, 2000), in which the Atg12–Atg5 conjugate has been shown to promote the transfer of Atg8 from Atg3 to PE by direct interaction with both Atg3 and PE (Hanada *et al.*, 2007). Furthermore, the mammalian Atg12–Atg5–Atg16L complex has been shown to specify the lipidation site of LC3, a mammalian Atg8 orthologue, through interaction with Atg3 and some unidentified factor(s) on the membrane (Fujita *et al.*, 2008).

We have previously reported the structures of plant Atg12 (Suzuki *et al.*, 2005) and Atg5 complexed with the N-terminal domain of

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Atg16 (Matsushita *et al.*, 2007) and have shown that both Atg12 and Atg5 are comprised of ubiquitin fold(s). However, the structure-determined region of Atg16 (residues 1–57) did not contain the predicted coiled-coil motif (residues 67–123). Furthermore, the predicted coiled-coil motif only has weak sequence homology to other coiled-coil proteins of reported structure. Therefore, in order to obtain structural information on the multimeric state of the Atg12–Atg5–Atg16 complex, structural study on the coiled-coil motif of Atg16 is required. In this report, we describe the expression, purification and crystallization of the coiled-coil domain (residues 50–123) of Atg16.

2. Experimental methods

2.1. Expression and purification

A fragment coding residues 50–123 of *Saccharomyces cerevisiae* Atg16 (NCBI Gene ID 855194) was PCR-amplified from the plasmid pGEX6P-1-ATG16 (Matsushita *et al.*, 2007) using 5'-GGGGATCCG-GCGCCATTGGTGGCAACATTG-3' as the forward primer and 5'-GGGGATCCTTATTCTTTTTCAGATCCGAGAG-3' as the reverse primer. The PCR product was cloned into the *Bam*HI site of a pGEX4T-1 vector (GE Healthcare). We hereafter refer to this region as the coiled-coil domain (CCD). The constructs were transformed into *Escherichia coli* BL21 (DE3) and expressed as a GST-fusion protein. After cell lysis, the GST-fused Atg16 CCD was purified by affinity chromatography using a glutathione-Sepharose 4B column (GE Healthcare). After cleaving a GST tag from the Atg16 CCD with thrombin protease (GE Healthcare; an artificial glycine remained at the N-terminus of the Atg16 CCD), the Atg16 CCD was again applied onto a glutathione-Sepharose 4B column in order to remove the excised GST. The Atg16 CCD was then applied onto a HiTrap DEAE FF column (GE Healthcare) equilibrated with 20 mM Tris–HCl pH 8.5 and eluted with a 0–300 mM NaCl gradient in the same buffer. Final purification was performed using a Superdex200 gel-filtration column (GE Healthcare) equilibrated with 500 mM NaCl, 20 mM Tris–HCl pH 8.5. The purified Atg16 CCD was concentrated and used for crystallization. For single-wavelength anomalous dispersion (SAD) phasing, a selenomethionine-labelled protein was prepared as follows. Since the Atg16 CCD lacked a methionine residue for selenomethionine labelling, one was introduced by PCR-mediated site-directed mutagenesis. Sequence alignment of the Atg16

Table 1

Diffraction data statistics for Atg16 CCD crystals.

Values in parentheses are for the outer shell.

	Native	Selenomethionine
Beamline	BL-5A (KEK)	BL41XU (SPring-8)
Wavelength (Å)	1.1000	0.9791
Resolution range (Å)	50–2.70 (2.80–2.70)	50–2.50 (2.59–2.50)
Observed reflections	240090	640275
Unique reflections	17961	42101
Completeness (%)	98.8 (94.8)	99.8 (99.9)
$R_{\text{merge}}(I)^{\dagger}$	0.060 (0.375)	0.054 (0.406)
Mean $I/\sigma(I)$	43.9 (4.3)	83.4 (10.6)
Matthews coefficient (Å ³ Da ^{−1})	3.17	3.14
Wilson B factor (Å ²)	64.6	59.5

$^{\dagger} R_{\text{merge}}(I) = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th observed amplitude of reflection hkl and $\langle I(hkl) \rangle$ is the mean amplitude for all observed reflections.

CCD with other structure-determined coiled-coil proteins with weak sequence homology suggested that Leu103 of Atg16 would have an ordered conformation but that its substitution with a methionine would not destroy the Atg16 CCD structure. Therefore, Leu103 of the Atg16 CCD was chosen to be replaced with a methionine and the mutant protein (L103M) was expressed in methionine-auxotroph *E. coli* B834 (DE3) using an amino-acid medium containing selenomethionine instead of methionine. The selenomethionine-labelled mutant protein was purified using the same procedure as that described for the native protein.

2.2. Crystallization

Crystallization trials were performed using the sitting-drop vapour-diffusion method at 293 K. Initial screening was performed using Crystal Screen and Crystal Screen 2 (Hampton Research), Wizard Screens I and II (Emerald BioStructures) and Structure Screens 1 and 2 (Molecular Dimensions Ltd) as reservoir solutions. Drops (0.3 µl) of 8 mg ml^{−1} Atg16 CCD in 20 mM Tris buffer pH 8.5 and 500 mM NaCl were mixed with equal amounts of each reservoir solution and equilibrated against 100 µl of the same reservoir solution by vapour diffusion. The Atg16 CCD crystallized using a reservoir solution consisting of 0.4–1.2 M ammonium chloride and 0.1 M acetate buffer pH 5.0 after incubation for a few months. Streak-seeding was used to accelerate the nucleation of the crystals and large

Figure 1
Atg16 CCD crystals. (a) Native crystal. (b) Selenomethionine crystal. The scale bar represents 100 µm.

Figure 2

Stereographic projections of the self-rotation functions from the data set for a native crystal. The self-rotation functions were calculated using a 30 Å radius of integration and data in the resolution range 15–4 Å.

crystals of $0.50 \times 0.25 \times 0.25$ mm in size were obtained after incubation for several months (Fig. 1a). Since the selenomethionine-labelled L103M mutant did not crystallize using the conditions under which native crystals were obtained, initial screening was again performed using the above-mentioned screening kits. The selenomethionine-labelled L103M mutant in 20 mM Tris buffer pH 8.5 and 500 mM NaCl was crystallized using a reservoir solution consisting of 1.5 M sodium chloride and 10% ethanol. A crystal with typical dimensions of $0.30 \times 0.15 \times 0.10$ mm (Fig. 1b) was obtained within a week.

2.3. Diffraction data collection and processing

Crystals were immersed in reservoir solution supplemented with 30% glycerol as a cryoprotectant for several seconds; they were then flash-cooled and kept in a stream of nitrogen gas at 90–100 K during data collection. Diffraction data from a native crystal were collected using an ADSC Quantum 315 charge-coupled device detector on the KEK beamline BL-5A at a wavelength of 1.1000 Å. The crystal belonged to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = 127.7$, $c = 77.8$ Å. Diffraction data from a selenomethionine-labelled crystal were collected using a Rayonix MX-225 charge-coupled device detector on SPring-8 beamline BL41XU at a wavelength of 0.9791 Å. The crystal was almost isomorphous to the native crystal, with unit-cell parameters $a = 127.3$, $c = 77.6$ Å. All diffraction data were processed using the *HKL-2000* program suite (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1.

3. Discussion

The acceptable range of the volume-to-weight ratio (V_M) value (Matthews, 1968) indicates that the crystal contains 5–10 molecules of Atg16 CCD per asymmetric unit ($V_M = 1.90$ – 3.80 Å³ Da^{−1}). Self-rotation functions were calculated using diffraction data from a native crystal. The sections at $\kappa = 90^\circ$, 120° and 180° (Fig. 2) showed that the crystal contains noncrystallographic twofold, threefold and fourfold axes. These data suggest that the crystal presumably contains six molecules per asymmetric unit ($V_M = 3.17$ Å³ Da^{−1}, calculated solvent content 61.2%). Using the SAD data, six Se positions were determined and good initial phases were obtained using *SOLVE/RESOLVE* (Terwilliger & Berendzen, 1999; Terwilliger, 2000). The number of Se positions and the electron-density map indicate that the

asymmetric unit actually contains six Atg16 CCD molecules. Model building and crystallographic refinement are now in progress.

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