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Crystallization and preliminary X-ray analysis of LC3-I

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Aut7/Apg8 is located in the intermediate structures of the autophagosome and plays an essential role in autophagosome formation. The processed form, cleaved at a C-terminus of Gly120 and called LC3-I, was expressed, purified and crystallized in two crystal forms. One form belongs to space group $I4_1$, with unit-cell parameters a = 84.39, c = 36.89 Å. The other form belongs to space group $P4_1$ or $P4_3$, with unit-cell parameters a = 60.48, c = 35.28 Å. From the latter form, a complete diffraction data set was collected to 2.1 Å resolution.

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1. Introduction

Autophagy is a dynamic membrane-generation process for bulk protein degradation in the lysosome/vacuole (Baba *et al.*, 1994; Klionsky & Ohsumi, 1999). Cytoplasmic components of the cell are enclosed by double-membrane structures known as autophagosomes for delivery to lysosomes/vacuoles. This process is crucial for survival during starvation and cell differentiation. Aut7/Apg8 was first identified as a protein located in the intermediate structures of the autophagosome and is necessary for autophagosome formation in yeast (Kirisako *et al.*, 1999; Huang *et al.*, 2000). Aut7 exists in two forms, either in a free/peripherally membrane-bound form or in a tightly membrane-associated form (Kirisako *et al.*, 2000). In the latter form, the C-terminal region of Aut7 is cleaved and the remaining protein is covalently conjugated to phosphatidylethanolamine (PE) through ubiquitylation-like reactions (Ichimura *et al.*, 2000).

The protein LC3, which was the first identified homologue of Aut7, is a light chain of the microtubule-associated protein 1 in rat (Mann & Hammarback, 1994). Recently, LC3 has been shown to be proteolytically processed at the C-terminus of the conserved Gly120 to produce an active form, called LC3-I (Kabeya *et al.*, 2000). It is then further modified to another form, called LC3-II, which is localized to the autophagosomal membrane (Kabeya *et al.*, 2000). Although the target molecule has not been identified, LC3-II is possibly conjugated to PE in a manner similar to Aut7.

Recently, X-ray and NMR structures of two other mammalian Aut7 homologues, GATE-16, a 16 kDa Golgi-associated ATPase enhancer, and GABARAP, the $GABA_A$-receptor-associated protein, have been reported (Paz *et al.*, 2000; Knight *et al.*, 2002; Bavro *et al.*, 2002; Stangler *et al.*, 2002). While these structures revealed the adoption of ubiquitin-like folds, the structural basis for their functional differences remains to be elucidated. In this report, we expressed, purified and crystallized rat LC3-I. By determining the three-dimensional structure of LC3-I, the functional differences between the three mammalian Aut7 homologues and the molecular role of LC3 in autophagy can be studied.

2. Experimental

2.1. Expression and purification

As the C-terminal residues (121–142) of LC3 were susceptible to proteolysis in *Escherichia coli*, these residues were excluded from the construct. The region encoding residues 1–120, which corresponds to LC3-I, was inserted into a pGEX-6P vector (Amersham-Pharmacia) using *Bam*HI–*Eco*RI restriction sites. LC3-I was expressed in *E. coli* BL21(DE3) with glutathione *S*-transferase (GST) fused at the N-terminus. After cell lysis, the GST-fusion protein was purified by affinity chromatography using a glutathione-Sepharose 4B column. After cleavage of the GST protein from LC3-I with PreScission protease (Amersham-Pharmacia), gel-filtration chromatography was performed using HiLoad Superdex75 on an ÄKTA system (Amersham-Pharmacia). The purified protein was concentrated to 20–30 mg ml^{-1} in 0.15 M NaCl with 20 mM Tris–HCl pH 7.4.

2.2. Crystallization

Crystallization trials were performed using the sitting-drop vapour-diffusion method at 293 K. Initial screening was performed using sparse-matrix kits from Hampton Research and Emerald Biostructures. Typically, 0.5 µl drops of protein solution were mixed with equal amounts of reservoir solution. LC3-I was crystallized into two forms. Form I was

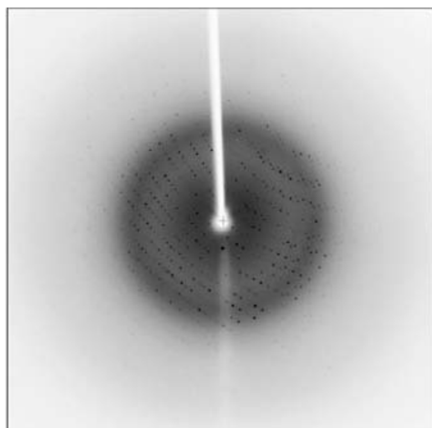

Figure 1
A diffraction image of LC3-I. The diffraction image was taken with a Rigaku R-AXIS IV imaging-plate detector using Cu $K\alpha$ radiation from an in-house Rigaku rotating-anode X-ray generator operating at 50 kV and 100 mA.

obtained with a reservoir solution consisting of 27% PEG 6000, 0.2 *M* cobalt chloride hexahydrate and 0.1 *M* acetic acid/sodium acetate pH 5.6. Form II was obtained with a reservoir solution consisting of 20% PEG 3350 and 0.05 *M* sodium citrate pH 5.5. Form I crystals reached dimensions of 30 × 30 × 350 µm after a few days and form II crystals reached dimensions of 50 × 50 × 250 µm after three weeks.

2.3. Preliminary X-ray analysis

All diffraction data were collected at 100–110 K on a Rigaku R-AXIS IV imaging-plate detector using Cu $K\alpha$ radiation from an in-house Rigaku rotating-anode X-ray generator operating at 50 kV and 100 mA. Crystals were immersed into reservoir solution which had been supplemented with 10–15% glycerol as a cryoprotectant for several seconds and were then flash-cooled and kept in a stream of nitrogen gas at 100–110 K during data collection. A complete diffraction data set was collected to 2.1 Å resolution (Fig. 1). Diffraction data were indexed, integrated and scaled with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). X-ray diffraction data statistics are summarized in Table 1. Attempts to obtain the phases for the structure of LC3-I are under way using the GATE-16 structure, which has 39% sequence identity (PDB code 1eo6), as a model for molecular replacement.

Table 1
Diffraction data statistics of LC3-I crystals.

Values in parentheses refer to the outer shell.

Crystal form	I	II
Space group	$I4_1$	$P4_1$ or $P4_3$
Unit-cell parameters (Å)		
a, b	84.39	60.48
c	36.89	35.28
Molecules per AU	1	1
Molecular weight (Da)	14555	14555
V_M† (Å^3 Da^{-1})	2.32	2.28
Resolution range (Å)	100–3.5 (3.63–3.5)	100–2.1 (2.18–2.1)
Observed reflections	3365	24966
Unique reflections	1590	7575
Completeness (%)	93.9 (93.9)	99.5 (100)
$R_{merge}(I)$‡	0.090 (0.37)	0.070 (0.313)
$I/\sigma(I)$	11.2 (2.4)	20.6 (4.0)

† Matthews coefficient (Matthews, 1968). ‡ $R_{merge}(I) = (\sum\sum|I_i - \langle I \rangle|)/\sum\sum I_i$, where I_i is the intensity of the ith observation and $\langle I \rangle$ is the mean intensity.

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