Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary crystallographic analysis of human Atg4B–LC3 complex

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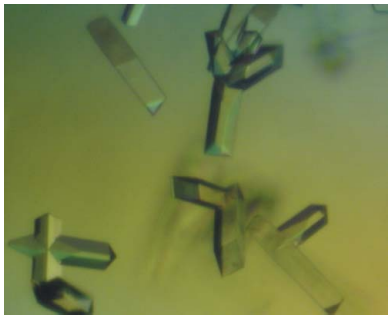
Received 14 November 2006
Accepted 28 December 2006



The reversible modification of Atg8 with phosphatidylethanolamine (PE) is crucial for autophagy, the bulk degradation process of cytoplasmic components by the vacuolar/lysosomal system. Atg4 is a cysteine protease that is responsible for the processing and deconjugation of Atg8. Human Atg4B (HsAtg4B; a mammalian orthologue of yeast Atg4) and LC3 (a mammalian orthologue of yeast Atg8) were expressed and purified and two complexes, one consisting of HsAtg4B(1–354) and LC3(1–120) (complex I; the product complex) and the other consisting of HsAtg4B(1–354) and LC3(1–124) (complex II; the substrate complex), were crystallized using polyethylene glycol 3350 as a precipitant. In both complexes His280 of HsAtg4B was mutated to alanine. The crystals belong to the same space group $P2_12_12_1$, with unit-cell parameters a = 47.5, b = 91.8, c = 102.6 Å for complex I and a = 46.9, b = 90.9, c = 102.5 Å for complex II. Diffraction data were collected to a resolution of 1.9 Å from both crystals.

1. Introduction

Autophagy is a starvation-induced response that mediates the bulk degradation of cytoplasmic components in lysosomes/vacuoles (Seglen & Bohley, 1992; Takeshige *et al.*, 1992) and plays a critical role in numerous biological processes such as neurodegeneration and pathogen infection as well as in survival response during neonatal starvation (Hara *et al.*, 2006; Komatsu *et al.*, 2006; Ogawa *et al.*, 2005; Nakagawa *et al.*, 2004; Kuma *et al.*, 2004). 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 lysosomal/vacuolar lumen. Genetic approaches in *Saccharomyces cerevisiae* have isolated autophagy-defective (*Atg*) genes that are essential for autophagosome formation (Tsukada & Ohsumi, 1993; Klionsky *et al.*, 2003). Among them, four Atg proteins, namely Atg3, Atg4, Atg7 and Atg8, have been shown to be involved in a novel ubiquitin-like conjugation system named the Atg8 system, which mediates protein lipidation (Ichimura *et al.*, 2000, 2004). Atg8 is cleaved at its C-terminal arginine residue by Atg4, a novel cysteine protease (Kirisako *et al.*, 2000), and the exposed C-terminal glycine is conjugated to phosphatidylethanolamine (PE) by Atg7, an E1-like enzyme, and Atg3, an E2-like enzyme (Ichimura *et al.*, 2000). Atg8–PE is further deconjugated, again by Atg4. This reversible modification of Atg8 is crucial for the normal progression of autophagy (Kirisako *et al.*, 2000).

In mammals, an Atg8-like conjugation system, called the LC3 system, has been shown to exist (Kabeya *et al.*, 2000; Tanida *et al.*, 2001, 2002). Like the Atg8 system in yeast, the C-terminal region of LC3, a mammalian orthologue of Atg8, is cleaved by mammalian Atg4 homologues (Kabeya *et al.*, 2000). The processed form, called LC3-I, has a glycine residue at the C-terminus (Kabeya *et al.*, 2000) and resides in the cytosol. After activation by mammalian Atg7 and mammalian Atg3 homologues (Tanida *et al.*, 2001, 2002), LC3-I is further modified to another form, called LC3-II, which is likely to be the PE-conjugated form (Kabeya *et al.*, 2004; Sou *et al.*, 2006) similar to the case in the Atg8 system. Since LC3-II is localized to the autophagosomal membrane, it is now widely used as a key molecule to monitor autophagosome formation and autophagy activity in

mammalian systems (Kabeya *et al.*, 2000). In mammals, at least four homologues of yeast Atg4 are found, HsAtg4A/autophagin-2, HsAtg4B/autophagin-1, autophagin-3 and autophagin-4 (Kirisako *et al.*, 2000; Marino *et al.*, 2003), of which HsAtg4B cleaves LC3 most efficiently (Kabeya *et al.*, 2004). Recently, the crystal structure of HsAtg4B was reported in its free form (Sugawara *et al.*, 2005; Kumanomidou *et al.*, 2006). Cys74, Asp278 and His280 of HsAtg4B form the catalytic triad and have been shown to be essential for the catalytic activity of HsAtg4B (Sugawara *et al.*, 2005). Intriguingly, Cys74 of HsAtg4B is auto-inhibited by the inhibitory loop, suggesting that LC3 may induce conformational changes of HsAtg4B upon complex formation with the enzyme. In order to elucidate the molecular basis of recognition and processing of LC3 by HsAtg4B, we started crystallographic studies of the HsAtg4B–LC3 complex. In this report, we describe the crystallization and preliminary crystallographic analysis of HsAtg4B in complex with both unprocessed and processed forms of LC3.

2. Expression and purification

The expression and purification of full-length HsAtg4B [HsAtg4B(1–393)] have been described previously (Sugawara *et al.*, 2005). In addition to full-length HsAtg4B, we prepared two HsAtg4B mutants: a C-terminally truncated HsAtg4B [HsAtg4B(1–354)] and HsAtg4B(1–354) with a mutation of His280 to alanine [HsAtg4B(1–354,H280A)]. Using *Nde*I–*Bam*HI restriction sites, residues 1–354 of HsAtg4B were inserted into a pGEX-6P vector (GE Healthcare). The H280A mutation was introduced by PCR-mediated site-directed mutagenesis. Both constructs were sequenced in order to confirm their identities. They were expressed in *Escherichia coli* BL21 (DE3) with glutathione *S*-transferase (GST) fused at the N-terminus and a hexahistidine tag attached at the C-terminus. After cell lysis, the GST-fusion proteins were first purified by affinity chromatography using a glutathione Sepharose 4B column (GE Healthcare Biosciences) and GST was cleaved with PreScission protease (GE Healthcare Biosciences). A Gly-Pro-His sequence remained at the N-terminus of HsAtg4B. The proteins were then applied onto an Ni–NTA column (Qiagen) equilibrated with 0.5 *M* NaCl and 10 m*M* imidazole in 20 m*M* Tris–HCl pH 8.0 and were eluted with 200 m*M* imidazole and 0.1 *M* NaCl in 20 m*M* Tris–HCl pH 8.0. The eluted proteins were further applied onto a Resource Q anion-exchange column (GE Healthcare Biosciences) equilibrated with 20 m*M* Tris–HCl pH 8.0 and the proteins were eluted with a 0–500 m*M* NaCl gradient in the same buffer. Further purification was carried out on a Superdex 75 gel-filtration column (GE Healthcare Biosciences) eluted with 20 m*M* Tris–HCl pH 8.0 and 150 m*M* NaCl. The purified protein, which retained a hexahistidine tag at the C-terminus, was used for crystallization.

LC3(1–120), a processed form that exposes Gly120 at the C-terminus, was prepared as described previously (Sugawara *et al.*, 2003). LC3(1–124), an unprocessed form that has four residues C-terminal of Gly120, was prepared as follows. Using *Nde*I–*Bam*HI restriction sites, residues 1–124 of rat LC3-1B were inserted into a pGEX-6P vector and expressed in *E. coli* BL21 (DE3) with GST fused at the N-terminus. After cell lysis, the GST-fusion protein was purified by affinity chromatography using a glutathione Sepharose 4B column and GST was cleaved with PreScission protease. A Gly-Pro-His sequence remained at the N-terminus of the protein. LC3(1–124) was then applied onto a HiTrap CM cation-exchange column (GE Healthcare Biosciences) equilibrated with 20 m*M* Tris–HCl pH 8.0 and the protein was eluted with a 0–500 m*M* NaCl gradient in the same buffer. Further purification was performed on a Superdex 75 gel-filtration column eluted with 20 m*M* Tris–HCl pH 8.0 and 150 m*M* NaCl.

3. Crystallization

All crystallization trials were performed by the sitting-drop vapour-diffusion method at 293 K. Initial screening was performed using Wizard I and Wizard II (Emerald Biostructures) and Crystal Screen and Crystal Screen II (Hampton Research). Typically, 0.3 µl drops of protein solution were mixed with equal amounts of reservoir solution and equilibrated against 100 µl of the same reservoir solution by vapour diffusion. We first tried to crystallize the HsAtg4B(1–393)–LC3(1–120) complex, but failed. Since the C-terminal region (residues 355–393) of HsAtg4B seems to be flexible (Sugawara *et al.*, 2005) and unfavourable for crystallization, we next tried to cocrystallize a C-terminally truncated form of HsAtg4B [HsAtg4B(1–354)] with LC3(1–120), but again no crystals were obtained. It should be noted here that LC3(1–120) is not a substrate but a reaction product of HsAtg4B. Compared with the substrate LC3, the negatively charged carboxyl group of Gly120 in LC3(1–120) seemed to be unfavourable for complex formation with wild-type HsAtg4B, the

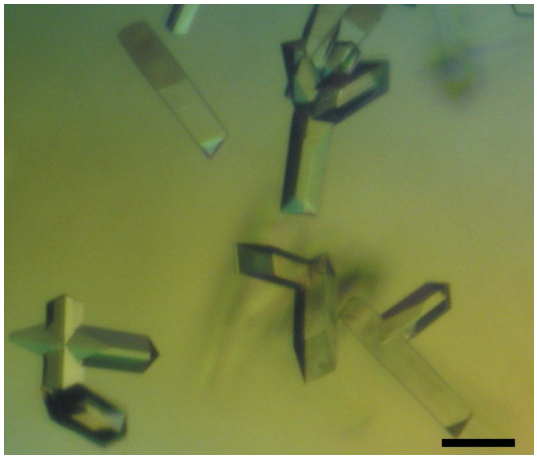


Figure 1
Crystals of the HsAtg4B(1–354,H280A)–LC3(1–120) complex. The scale bar is 100 µm in length.

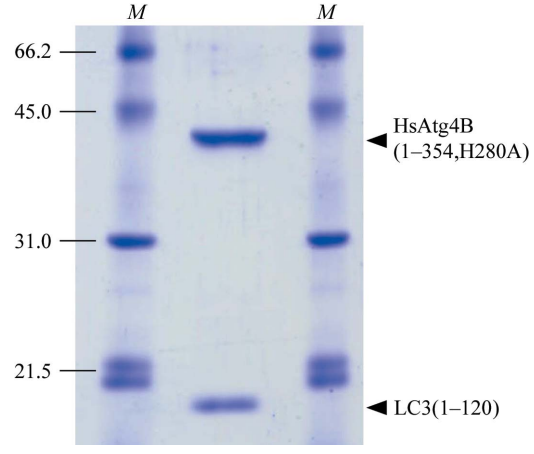


Figure 2
SDS–PAGE of complex I crystals on 15% gel. Lanes *M* contain molecular-weight markers (labelled in kDa). Proteins were stained with Coomassie Brilliant Blue.

Table 1
Diffraction data statistics.

Values in parentheses are for the highest resolution shell.

Data set	1	2
Protein complex	HsAtg4B(1–354,H280A)–LC3(1–120)	HsAtg4B(1–354,H280A)–LC3(1–124)
Beamline	NW12A	BL41XU
Wavelength (Å)	1.00	1.00
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters		
a (Å)	47.5	46.9
b (Å)	91.8	90.9
c (Å)	102.6	102.5
Resolution range (Å)	50–1.9	50–1.9
Observed reflections	183688	222721
Unique reflections	34586	34039
Completeness (%)	95.6 (92.5)	96.4 (69.5)
$R_{\rm merge}(I)$†	0.056 (0.330)	0.079 (0.343)
$I/\sigma(I)$	15.6 (3.9)	8.7 (1.9)

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

catalytic cysteine of which (Cys74) is highly nucleophilic. Therefore, we introduced a point mutation of His280 of HsAtg4B(1–354) to alanine, which destroys the catalytic triad and thus reduces the nucleophilicity of Cys74. We used this mutant for cocrystallization with LC3(1–120). Crystals of the HsAtg4B(1–354,H280A)–LC3(1–120) complex (complex I) were initially obtained by mixing 0.3 µl 10 mg ml^{-1} HsAtg4B(1–354,H280A) and 3.5 mg ml^{-1} LC3(1–120) (the two proteins are in an equimolar ratio) in 150 m*M* NaCl, 20 m*M* Tris–HCl pH 8.0 and 2 m*M* DTT with an equal amount of a reservoir solution consisting of 20% PEG 3000 in 0.1 *M* citrate pH 5.5 and equilibrating against 100 µl reservoir solution. After optimization of reservoir conditions, crystals suitable for data collection were obtained with a reservoir solution consisting of 20% PEG 3350 in 0.1 *M* citrate pH 5.8 (Fig. 1). These crystals were confirmed to contain both HsAtg4B and LC3 by SDS–PAGE analysis (Fig. 2). Using these crystals as microseeds, crystals of the HsAtg4B(1–354,H280A)–LC3(1–124) complex (complex II) were obtained by mixing 10 mg ml^{-1} HsAtg4B(1–354,H280A) and 3.5 mg ml^{-1} LC3(1–124) in 150 m*M* NaCl, 20 m*M* Tris–HCl pH 8.0 and 2 m*M* DTT with an equal amount of reservoir solution consisting of 20% PEG 3350 in 0.1 *M* citrate pH 5.8.

4. Preliminary X-ray analysis

Crystals were immersed into reservoir solution supplemented with 15% glycerol as a cryoprotectant for several seconds and were then flash-cooled and kept in a stream of nitrogen gas at 90 K during data collection. Diffraction data were collected from crystals of complex I using an ADSC Quantum 315 charge-coupled device detector on beamline BL-5A, KEK, Japan at a wavelength of 1.00 Å (data set 1). Diffraction data were collected from crystals of complex II using an ADSC Quantum 315 charge-coupled device detector on beamline BL41XU, SPring-8, Japan at a wavelength of 1.00 Å (data set 2). All diffraction data were processed using the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1. The crystals belong to the same space group $P2_12_12_1$, with unit-cell parameters a = 47.5, b = 91.8, c = 102.6 Å for complex I and a = 46.9, b = 90.9, c = 102.5 Å for complex II. The acceptable range of the volume-to-weight ratio ($V_{\rm M}$) values (Matthews, 1968) indicates that both crystals contain one HsAtg4B–LC3 complex per asymmetric unit (the $V_{\rm M}$ values are 2.02 and 1.96 Å^3 Da^{-1} for complexes I and II, respectively). Molecular replacement was performed using the program *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 software suite (Collaborative Computational Project, Number 4, 1994) for data set 1. The crystal structures of HsAtg4B (PDB code 2cy7; Sugawara *et al.*, 2005) and LC3 (PDB code 1ugm; Sugawara *et al.*, 2004) were used as search models. The R and $R_{\rm free}$ values after initial refinement using *CNS* (Brünger *et al.*, 1998) were 0.272 and 0.356, respectively. Crystallographic refinement is now in progress for both complexes.

We thank the staff at beamline NW12, KEK, Japan and at beamline BL41XU, SPring-8, Japan for data-collection support. This work was supported by a Grant-in-Aid for Young Scientists (B) 17790048 and for Scientific Research on Priority Areas and by the National Project on Protein Structural and Functional Analyses from the Ministry of Education, Culture, Sports, Science and Technology, Japan. This work was carried out under the NIBB Cooperative Research Program (4-148).

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