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Structural study on Atg8-receptor complexes that mediate ER-phagy

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1 Introduction

Autophagy is an intracellular degradation system and plays a critical role in the degradation of various organelles [1]. Selective degradation of endoplasmic reticulum (ER) by autophagy is named ER-phagy, which is mediated by an ER-phagy receptor Atg40 in budding yeast and several ER-phagy receptors that include Sec62 in mammals [2, 3]. These ER-phagy receptors possess a transmembrane region and is embedded in the ER membrane. Upon ER-phagy induction, ER-phagy receptors interact with Atg8 on the newly generated isolation membrane (IM), a precursor of autophagosomes, using Atg8-family interacting motif (AIM) [4], thereby tethering a portion of the ER to the IM and promoting ER-phagy. In this study, we studied the interaction of Atg8 with two distinct ER-phagy receptors, Atg40 and Sec62, by X-ray crystallography.

2 Experiment

Plasmids for expression of fusion proteins for crystallization were constructed by inserting the genes encoding Atg40 (237-252) ($Atg40^{AIM}$) and human SEC62 (361-376) with the T367D mutation ($Sec62^{AIM}$) into upstream of the sequence encoding $Atg8^{K26P}$ of pGEX6P-$Atg8^{K26P}$ (the K26P mutation was introduced for stabilizing Atg8 [5]) or upstream of the sequence encoding GABARAP of pGEX6P-GABARAP (with the F3S V4T mutations for enhancing crystallization). Proteins were expressed in E. coli BL21 (DE3). Bacteria were cultured at 37°C until OD600 became 0.8-1.0 and further cultured at 16°C with 100 μM IPTG overnight. After centrifugation, the pellets were resuspended with 20 ml PBS with 0.5 mM EDTA and lysed by sonication for 10 min. After centrifugation, the supernatants were incubated with GST-accept resin (Nacalai tesque). After 3 times wash by PBS, the proteins were eluted with glutathione buffer (10 mM glutathione and 50 mM Tris pH 8.0). The buffer of the eluates was exchanged with PBS by a Bio-Scale Mini BioGel P-6 desalting column (Bio-Rad). After incubation with GST-fused HRV 3C protease at 4°C overnight, the samples were subjected to a GST-accept resin column to remove excised GST and the protease.

All crystallization trials were performed by the sitting-drop vapor-diffusion method at 20°C. For crystallization of $Atg40^{AIM}$-Atg8 fusion, 41 mg/ml protein was incubated with 10% PEG 8000, 0.1 M HEPES pH 7.5, 8% ethylene glycol as a reservoir and C6 well of silver bullets bio (0.04% Cortisone, 0.04% (±)-Epinephrine, 0.04% Protoporphyrin disodium salt, 0.04% Pyridoxine, 0.04% Thymidine 5'-monophosphate disodium salt hydrate, and 0.02 M HEPES buffer pH 6.8) (Hampton Research) as an additive. The volume ratio of protein, reservoir, and additive was 2:1:1. For crystallization of $SEC62^{AIM}$-GABARAP fusion, 40 mg/ml protein was mixed with a reservoir solution consisting of 1.5 M ammonium sulfate and 0.1 M Tris pH 8.5. The mixing volume ratio of protein and reservoir solution was 1:1 and mixed solution was equilibrated against the reservoir solution. All crystals were obtained within 4 days.

Crystals were soaked in cryoprotectant and frozen in liquid nitrogen. The cryoprotectant for $Atg40^{AIM}$-Atg8 fusion was prepared by adding 25% ethylene glycol to the reservoir solution. The cryoprotectants for $SEC62^{AIM}$-GABARAP fusions were prepared by adding 33% glycerol to each reservoir solution. The flash-cooled crystals were kept in a stream of nitrogen gas at -178° C during data collection. Diffraction data collections of the crystals for Atg40 and Sec62 were performed at the beamlines BL-1A and NE3A at KEK, Japan, with the wavelength of 1.1000 and 1.0000 Å, respectively. The diffraction data were indexed, integrated, and scaled using XDS [6]. The structures of $Atg40^{AIM}$-Atg8 and $SEC62^{AIM}$-GABARAP were determined by the molecular replacement method using Phenix [7]. Atg8 (PDBID: 2ZPN) and GABARAP (PDBID: 1GNU) structures were used as a search model. Crystallographic refinement was done by using Phenix and COOT programs [7, 8]. All structural images in the manuscript were prepared by PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.). Parameters of diffraction data collection and crystallographic refinement are summarized in Table 1.

3 Results and Discussion

The core of the AIM is composed of four amino-acid residues [W/F/Y]-X-X-[L/I/V]. The first aromatic residue and the fourth aliphatic residue bind to two hydrophobic pockets in Atg8 (the W- and L-sites, respectively), and the main chain of the motif forms an intermolecular parallel β-sheet with β2 of Atg8 [4]. The crystal structure of $Atg40^{AIM}$-Atg8 revealed that as with canonical AIMs, the first and fourth residues Y242 and M245 of $Atg40^{AIM}$ bound to the W- and L-sites of Atg8, respectively. In addition, the second residue D243 formed salt bridges with the R67 residue of Atg8. Moreover, the Atg40 region C-terminal to M245 assumed a helical conformation, and D247 in this helix formed salt bridges with R67 of Atg8. Residue F238 on the N-terminal side of the core motif also interacted with I21 and the aliphatic portion of R20 of Atg8.

We also determined the crystal structure of GABARAP (a mammalian Atg8 homologue) bound to $SEC62^{AIM}$. Remarkably, the AIM containing region of Sec62 is structurally similar to that of Atg40, consisting of the core AIM and its C-terminal short helix. The core AIM residues

of SEC62 (F363 and I366) interacted with GABARAP in a similar manner. In addition, as with Atg40, the second AIM residues (E364 of SEC62) and acidic residues in the short helices (E370 of SEC62) interacted with R67 of GABARAP. Thus, the C-terminal helix-assisted AIM is a unique structural basis commonly seen in the interactions of ER-phagy receptors with Atg8-family proteins [9].

Table 1: Data collection and refinement statistics

	Atg40^{AIM}-Atg8 (PDB 7BRN)	Sec62^{AIM}-GABARAP (PDB 7BRT)
Data collection		
Space group	$P3_221$	$P2_12_12_1$
Cell dimensions		
a, *b*, *c* (Å)	74.7, 74.7, 57.1	42.9, 42.9, 144.0
α, β, γ (°)	90, 90, 120	90, 90, 90
Resolution (Å)[a]	37.4-2.23 (2.31-2.23)[a]	41.1-2.00 (2.07-2.00)
R_{merge}	0.141 (0.595)	0.164 (0.920)
$I/\sigma(I)$	13.78 (2.44)	9.27 (2.02)
Completeness (%)	99.58 (97.91)	99.73 (98.34)
Redundancy	9.6 (7.3)	6.5 (5.8)
Refinement		
Resolution (Å)	37.4-2.23	41.1-2.00
No. reflections	9227	18676
R_{work} / R_{free}	0.2031/0.2187	0.1656 / 0.1836
No. atoms		
Protein	1076	2172
Water	44	291
B factors		
Protein	45.99	30.61
Water	45.06	34.55
R.m.s deviations		
Bond lengths (Å)	0.008	0.007
Bond angles (°)	1.29	0.91

[a]Values in parentheses are for highest-resolution shell.

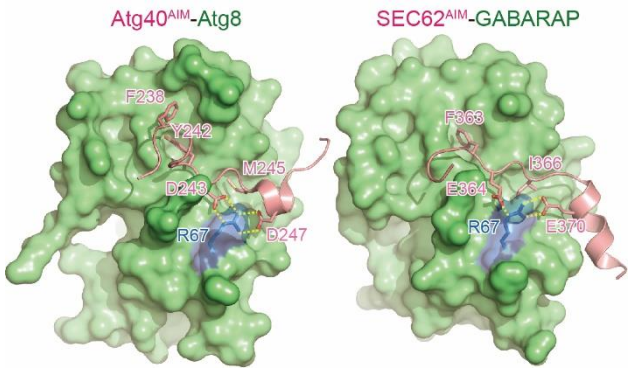

Fig. 1: Structure of Atg40^{AIM}-Atg8 complex (left) and Sec62^{AIM}-GABARAP complex (right).

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