The Crystal Structure of Atg3, an Autophagy-related Ubiquitin Carrier Protein (E2) Enzyme that Mediates Atg8 Lipidation*

Yuya Yamada, Nobuo N. Suzuki, Takao Hanada, Yoshinobu Ichimura, Hiroyuki Kumeta, Yuko Fujioka, Yoshinori Ohsumi, and Fuyuhiko Inagaki

From the Department of Structural Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, N-12, W-6, Kita-ku, Sapporo 060-0812, Japan and Division of Molecular Cell Biology, National Institute for Basic Biology, 38 Nishigonaka, Myodaiji, Okazaki 444-8585, Japan

Atg3 is an E2-like enzyme that catalyzes the conjugation of Atg8 and phosphatidylethanolamine (PE). The Atg8-PE conjugate is essential for autophagy, which is the bulk degradation process of cytoplasmic components by the vacuolar/lysosomal system. We report here the crystal structure of *Saccharomyces cerevisiae* Atg3 at 2.5-Å resolution. Atg3 has an α/β-fold, and its core region is topologically similar to canonical E2 enzymes. Atg3 has two regions inserted in the core region, one of which consists of ~80 residues and has a random coil structure in solution and another with a long α-helical structure that protrudes from the core region as far as 30 Å. In vivo and in vitro analyses suggested that the former region is responsible for binding Atg7, an E1-like enzyme, and that the latter is responsible for binding Atg8. A sulfate ion was bound near the catalytic cysteine of Atg3, suggesting a possible binding site for the phosphate moiety of PE. The structure of Atg3 provides a molecular basis for understanding the unique lipidation reaction that Atg3 carries out.

Ubiquitin is conjugated to its target proteins by a series of reactions. Initially, the C terminus of ubiquitin is processed by a specific protease, exposing a glycine residue at the C terminus. Next, the exposed glycine is activated by an E1 enzyme and transferred to an E2 enzyme. Finally, ubiquitin is conjugated to its target proteins by an E3 enzyme (1). Many ubiquitin-like modifiers have been reported, and they all seem to be conjugated to their targets via a mechanism similar to ubiquitination (2).

Atg8, a 14-kDa protein, is one such ubiquitin-like modifier, although its target is not a protein but rather a phospholipid, phosphatidylethanolamine (PE) (3). Despite having little sequence homology with ubiquitin, Atg8 has a similar structure (4) and is thought to be conjugated to PE by a mechanism similar to ubiquitination. Specifically, the carboxyl-terminal arginine of Atg8 is first processed by Atg4, a cysteine protease structurally related to deubiquitinating enzymes (5, 6). The exposed glycine residue of Atg8 is then activated by Atg7, an E1-like enzyme (7), and then transferred to Atg3, an E2-like enzyme (3). Finally, Atg8 is conjugated to PE (3).

The formation of the Atg8-PE conjugate is essential for autophagy, the bulk degradation process of cytosolic components by the vacuolar/lysosomal system (8). Although autophagy was originally identified as a response to starvation, recent studies show that it plays a critical role in many biological processes such as neurodegeneration, infection, and cellular survival during neonatal starvation (9–13). In *in vitro* reconstitution studies revealed that Atg3, Atg7, Atg8, ATP, and PE-containing membranes are necessary and sufficient for the conjugation of Atg8 with PE (14). Although most E2 enzymes require E3 enzymes, Atg3 by itself is catalytically competent for Atg8-PE formation.

We report here the crystal structure of Atg3 at 2.5-Å resolution. The core of Atg3 is topologically similar to canonical E2 enzymes. In addition to the core region, Atg3 has two characteristic insertions, both of which are essential for the function of Atg3 *in vitro* and *in vivo*. These results provide important information for understanding the unique lipidation reaction mediated by Atg3.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Wild-type Atg3 was expressed and purified for crystallization as described previously (15). For *in vitro* assays, wild-type Atg3 and three Atg3 mutants (Atg3ΔFR, Atg3FR, and Atg3ΔHR) were prepared as follows. The regions encoding amino acids 1–310 (wild-type), 1–82 and 163–310 (Atg3ΔFR), 1–245 and 281–310 (Atg3ΔHR), and 84–161 (Atg3FR) of Atg3 were inserted into pGEX-6P-1 (GE Healthcare) and were expressed in *Escherichia coli* strain BL21 (DE3) cells. The cells were lysed, and glutathia-
one S-transferase (GST)-fused Atg3s were purified by affinity chromatography using a glutathione-Sepharose 4B column (GE Healthcare). For in vitro pulldown assays, GST-fused Atg3s were further purified by a Resource Q anion-exchange column (GE Healthcare) followed by a Superdex 200 gel filtration column (GE Healthcare) without excision of GST. For in vitro conjugation assays, GST was excised from GST-fused Atg3 with PreScission protease (GE Healthcare), and the excised GST and PreScission protease were removed from Atg3 by repurification on a glutathione-Sepharose 4B column. Atg7 and a processed form of Atg8 used for in vitro conjugation and pulldown assays were prepared as follows. The region coding residues 1–116 of Atg8 was inserted into pGEX-6P-1 and was expressed in E. coli BL21 cells. After cell lysis, GST-fused Atg8 was purified by affinity chromatography using a glutathione-Sepharose 4B column, and GST was excised from Atg8 with PreScission protease. Atg7 was further purified by a Resource Q column followed by a Superdex 200 column. The region encoding amino acids 1–116 of Atg8 was inserted into pGEX-6P-1 and was expressed in E. coli BL21 cells. After cell lysis, GST-fused Atg8 was purified by affinity chromatography using a glutathione-Sepharose 4B column, and GST was excised from Atg8 with PreScission protease. Atg8 was further purified by a Resource S cation-exchange column (GE Healthcare) followed by a Superdex 75 gel filtration column (GE Healthcare). All constructs were sequenced to confirm their identities.

Preparation of Derivative Crystals and Data Collection—Crystallization of Atg3 was carried out as described previously (15). Platinum and two mercury derivatives were prepared by soaking the crystals of Atg3 for 30 min in a solution of 1.8 M lithium sulfate and 0.1 M citrate buffer (pH 6.6) containing 10 mM K₂PtCl₄, 1 mM HgCl₂, or 0.1 mM thimerosal. The selenomethionine derivative was expressed in E. coli B834 (DE3) using an amino acid medium containing selenomethionine instead of methionine, and it was crystallized under the same conditions used for native crystals. Derivative crystals were immersed in a reservoir solution supplemented with 25% glycerol for several seconds, flash-cooled, and kept in a stream of nitrogen gas at 100 K during data collection. Diffraction data for the native crystal was collected on the ADSC Quantum 315 charge-coupled device detector at beamline BL41XU, Spring-8 (Japan) at a wavelength of 1.00 Å. Diffraction data for derivatives were collected on Rigaku R-AXIS IV or VII imaging plate detectors using copper Kα radiation from an in-house Rigaku rotating anode x-ray generator. Diffraction data were processed with the HKL2000 program (16). The statistics for the diffraction data are summarized in Table 1.

Structure Determination and Refinement—The structure of Atg3 was determined by the multiple isomorphous replacement method. Heavy atom sites were found and refined, and the initial phases were calculated using the program SOLVE and RESOLVE (17). Approximately 40% of the residues were automatically modeled as a polyalanine chain by RESOLVE. Further model construction was performed manually using the molecular modeling program Turbo-Frodo (18), and the refinement was performed using the CNS program (19). The refinement statistics are summarized in Table 1. The coordinates and structure factors of Atg3 have been deposited in the Protein Data Bank (PDB code 2DYT).

NMR Spectroscopy—Proteins uniformly labeled with ¹⁵N were prepared by growing bacteria in M9 medium containing 1 g/liter ¹⁵NH₄Cl (Shoko). The purified Atg3FR and full-length Atg3 were concentrated to 0.5 mM in 92% H₂O/8% D₂O con-
Crystall Structure of Atg3

taining 20 mM phosphate (pH 6.5), 150 mM NaCl, and 5 mM dithiothreitol. 1H-15N heteronuclear single quantum correlation (HSQC) spectra were obtained with Varian UNITY INOVA 500 spectrometer equipped with a cryogenic probe. Spectra were processed by NMRPipe (20), and data analysis was carried out using the SPARKY program (21).

In Vitro Pulldown Assay—The purified GST-fused Atg3 mutants and GST (control) (0.5 nmol) were mixed with purified Atg7 or Atg8 (0.5 nmol) in phosphate-buffered saline (PBS) and incubated at 277 K for 1 h. Next, glutathione-Sepharose 4B slurry was added, and the mixture was further incubated at 277 K for 1 h. After washing the beads three times with PBS, proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl buffer (pH 8.0). The eluates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were detected by staining with Coomassie Brilliant Blue (CBB).

In Vitro Conjugation Assay—In vitro conjugation was performed according to the previous report (14). Liposomes were prepared as follows. All phospholipids were purchased from Avanti Polar Lipids. Dioleoylphosphatidylethanolamine, 1-palmitoyl-2-oleoylphosphatidylcholine, and dioleoylphosphatic acid were mixed in a glass tube at the ratio of 7:1:2 in chloroform. The chloroform solvent was removed by rotary evaporation. A

RESULTS

Overall Structure of Atg3—The crystal structure of Atg3 was refined against 2.5 Å data to an R-factor of 0.207 and a free R-factor of 0.244. The region corresponding to amino acids 21–306 was modeled along with two sulfate and 46 water molecules. The 20 amino-terminal and 4 carboxyl-terminal residues as well as three regions corresponding to residues 84–128, 143–162, and 271–278 lacked defined electron density and were omitted from the model (Fig. 1A). The overall structure of Atg3 has a unique hammer-like shape consisting of a head and a handle. The head moiety is composed of a central six-stranded β-sheet (strands 1–6) with five α-helices (A, B, D, E, G) surrounding it, and it has a globular-fold with approximate dimensions of 45 × 40 × 35 Å. The handle moiety is composed of a long α-helix (F) and a partially disordered loop region, and it protrudes from the head moiety as far as 30 Å. At the interface between the head and handle moieties, Atg3 has a “floating” helix C in which the amino and carboxyl termini are disordered and not connected to the other regions of Atg3 in the model.

Structural Comparison with Canonical E2s—Although Atg3 is thought to be an E2-like enzyme based on its function, it has little sequence homology with canonical E2 enzymes, so it is not clear whether Atg3 has structural similarity with them. For comparison, the structure of Ubc9, an E2 enzyme for small ubiquitin-like modifier (SUMO), is shown in the same orientation as Atg3 (Fig. 1B) (22). Although the structure of Atg3 seems to be significantly different from that of Ubc9, structural comparison using the Dali search engine revealed that the topology of the head moiety of Atg3 is very similar to canonical E2 enzymes including Ubc9. All E2 enzymes reported so far have a conserved E2 core composed of a central four-stranded β-sheet and four α-helices surrounding it (Fig. 1B, right) (22, 23). The head moiety of Atg3 has the corresponding four β-strands (1–4) and two α-helices (B and G) but lacks two carboxyl-terminal α-helices (Fig. 1B, left). Intriguingly, ubiquitin E2 variant domains, which have a canonical E2 core-fold but do not have E2 activities, also lack these two carboxyl-terminal α-helices (24). Although Ubc9 has only four strands, it has a short β-hairpin-like loop that topologically corresponds to strands 5 and 6 of Atg3 (Fig. 1B).

The Catalytic Site Structure of Atg3—Although more secondary structures are inserted at the amino- and carboxyl-terminal sides of Atg3 Cys-234 compared with Ubc9 Cys-93, both catalytic cysteines are located at the carboxyl terminus of the β-sheet (strand 5 and 6) or the similarly located β-hairpin-like structure (Fig. 1B). Fig. 1C shows the detailed structure around the catalytic cysteine of Atg3 (left) and Ubc9 (right). The catalytic cysteine of Ubc9, Cys-93, is located at the carboxyl terminus of the hairpin-like loop and forms a hydrogen bond with Asn-85 at the amino terminus of the same hairpin. Thus, the side chains of Cys-93 and Asn-85 are in close proximity. Asn-85 of Ubc9 is conserved among all canonical E2s and is essential for the conjugation reaction because it functions as an oxyanion hole. Cys-234 of Atg3 is located at the carboxyl terminus of strand 6 in a similar fashion as Cys-93 of Ubc9, but there is no residue corresponding to Asn-85 of Ubc9 at the amino terminus of strand 5. Furthermore, Pro-233 forms a hydrogen bond with Val-239 on helix F, so that the side chain of Cys-234 faces helix F but not strand 5. As a result, the side chain of Cys-234 is surrounded by the side chains of Gln-27 at the loop between helices A and B and Asn-238 at the amino terminus of the helix F, neither of which is conserved among Atg3 homologs (Fig.
Further studies are required to identify the residue of Atg3 that functions like Asn-85 of Ubc9 as an oxyanion hole.

Two Unique Inserted Regions in Atg3—As mentioned above, the handle moiety, which consists of a long α-helix F and a loop, is a structural feature unique to Atg3. We named this the handle region (HR). Fig. 2 shows a sequence alignment of three Atg3 homologs. The sequence of human Ubc9 is also aligned based on the crystal structures. The amino-terminal half of helix F is conserved among Atg3 homologs, but its carboxyl-terminal half and downstream loop region are not conserved. The long helical structure of the HR seems to be unique to yeast Atg3. In addition to the HR, Atg3 has a second unique inserted region, a long inserted region between strands 2 and 3 that is present in all three Atg3 homologs. Although the sequence identity of this region is low among the Atg3 homologs, its length (80–100 residues) and highly acidic character (calculated pI = 3.6–3.7) are conserved. In the yeast Atg3 crystal, this region takes a flexible conformation, and most residues are not modeled because of their poor electron density. The conformation of residues 129–142, which are modeled as helix C, also seems to be flexible because the average B-factor of these residues is rather high (89.9 and 54.0 Å² for helix C and all Atg3 residues, respectively). Helix C forms weak interactions with three helices (B, F, G), which may partially stabilize its conformation in the crystal. We named the inserted region between strands 2 and 3 the flexible region (FR).

FR Has a Random Coil Structure in Solution—Because most residues of FR are disordered in the crystal, we could not obtain further structural information on FR from the crystal structure. To obtain structural information on FR in solution, we expressed the FR moiety of Atg3 in E. coli, purified it, and analyzed it by NMR spectroscopy. Fig. 3A shows the 1H-15N HSQC spectrum of the FR moiety of Atg3. The pattern of the spectrum indicates a random coil structure and does not contain any signs of secondary structures. We also measured the HSQC spectrum of full-length Atg3 (Fig. 3B). Intriguingly, the spectrum of full-length Atg3 was strikingly similar to that of FR, suggesting that most observed signals for full-length Atg3 were derived from the FR moiety. This observation indicates that the mobility of FR is much higher than that of other regions of Atg3 because the residues located at the flexible regions give strong NMR signals because of long T2 relaxation times compared with those located at the rigid regions. These results suggest that the FR moiety of Atg3 has a random coil structure and...
is highly mobile in solution, not only in the isolated state but also in intact Atg3. Although residues 129–142 of FR have a helical structure in the crystal (Fig. 1, A and B), they may have a more flexible conformation in solution.

**Conjugation Activity of Atg3 Mutants**—To identify the roles of FR and HR in Atg3 in the Atg8-PE conjugation system, we generated mutants lacking FR (ΔFR) and HR (ΔHR). These proteins were expressed in *E. coli*, purified, and used for *in vitro* conjugation assays. Because the amino-terminal conserved residues (239–245) of HR form hydrophobic interactions with the core region of Atg3, they are included in the ΔHR construct to avoid destroying the overall fold of Atg3. Wild-type, ΔFR, and ΔHR mutants of Atg3 were incubated for 1 h at 30°C with Atg7, ATP, and PE-containing liposomes, separated by urea-SDS-PAGE, and stained with CBB (Fig. 4A). Compared with wild-type Atg3, the conjugation activity of the Atg3ΔHR mutant was moderately reduced, and that of Atg3ΔFR was greatly reduced.

We next analyzed the conjugation activity of these mutants *in vivo*. Similar levels of wild-type, ΔHR, and ΔFR Atg3s were expressed in Δatg3 yeast strains (Fig. 4B). Cell extracts were separated by urea-SDS-PAGE, and Atg8 was visualized by immunoblotting with an anti-Atg8 antibody (Fig. 4C). Atg3ΔHR had a moderate ability to mediate Atg8-PE conjugation, whereas Atg3ΔFR completely lacked the ability to mediate Atg8-PE formation. These results agree well with the *in vitro* results (Fig. 4A).

Finally, we examined the autophagy activity of these Atg3 mutants by monitoring the maturation of the proform of Ape1, which was transported into vacuoles via the cytoplasm-to-vacuole targeting pathway under nutrient-rich conditions or by
autophagy under condition of nutrient starvation. The protein is then processed into a mature form within the vacuoles. Cells expressing Atg3ΔHR showed a moderate level of Ape1 maturation, whereas Ape1 was not processed to the mature form in cells expressing Atg3ΔFR (Fig. 4D). These results suggest that HR is partly necessary and that FR is crucial for the function of Atg3 in autophagy.

Identification of Binding Partners for HR and FR—Our in vitro and in vivo analyses showed that HR and FR of Atg3 play some roles in the formation of Atg8-PE. We next performed an in vitro pulldown assay to determine whether HR and FR of Atg3 are responsible for the recognition of Atg7 and Atg8. Wild-type Atg3, Atg3ΔHR, Atg3ΔFR, and Atg3FR fused to GST were mixed with either Atg7 or Atg8 and then applied to glutathione-Sepharose 4B. As expected, wild-type Atg3 was bound to both Atg7 and Atg8 (Fig. 5, A and B). Atg3ΔHR also bound to Atg7, indicating that HR is not necessary for Atg7 binding. In contrast, Atg3ΔFR could not bind Atg7. Interestingly, Atg3FR bound to Atg7 as strongly as wild-type Atg3. These results indicate that FR of Atg3 is necessary and sufficient for Atg7 binding. On the other hand, Atg3ΔFR bound to Atg8 as strongly as wild-type Atg3, whereas Atg3ΔHR bound only weakly to Atg8. This indicates that HR of Atg3 mediates the binding of Atg8.

DISCUSSION

In the current study, we showed that, despite having little sequence homology, Atg3 and canonical E2 enzymes have similar structures. There are four classes of E2 enzymes (I–IV). Class I consists of the conserved E2 core and lacks large extensions (25), whereas classes II and III have carboxyl- and amino-terminal extensions, respectively (26, 27), and class IV has both.
Recently, the structure of Ubc9 bound to the SUMO-RanGAP1 conjugate was reported (Fig. 6C) (30). In this structure, SUMO is bound to the second helix of Ubc9, which corresponds to helix G of Atg3. The structure of Ubc1 bound to ubiquitin was also reported, in which ubiquitin is bound similarly to the second helix of Ubc1 (31). These two structures suggest that the second helix of canonical E2s is the conserved binding site for modifier proteins. HR of Atg3 is inserted at the amino terminus of helix G and occupies a similar location as the SUMO/ubiquitin site in Ubc9/Ubc1 (Fig. 6, A and C). Notably, an in vitro pulldown assay showed that Atg8 binds to HR. A sulfate ion derived from the crystallization reservoir solution binds in the groove near Cys-234 (Figs. 1C and 6A). This sulfate ion and HR are located on the opposite sides of Cys-234, just as RanGAP1 and SUMO are located on the opposite sides of Ubc9 Cys-93 (Fig. 6, A and C). Anions often mimic phosphate groups, and in the case of PE-binding protein, acetate and cacodylate ions were observed at the binding site of the phosphate moiety of PE (32, 33). Accordingly, the sulfate ion observed in the Atg3 crystal may also mimic the phosphate group of PE and bind to the PE-binding site. Three basic residues (Lys-183, His-232, Lys-235), which surround the sulfate ion, might recognize the phosphate moiety of PE. However, the recognition of PE by Atg3 seems to be more complex because PE is incorporated into membranes in vivo.

Although Atg8-PE conjugation does not require E3 enzymes in vitro, the Atg12-Atg5 conjugate, the other conjugate required for autophagy, is crucial for Atg8-PE conjugation in vivo (34). However, the molecular details of this reaction remain unknown. Atg8-PE conjugation is the only lipidation reaction known to be mediated by a ubiquitin-like modification system. Because PE does not exist alone in solution but is instead incorporated into membranes, recognition of PE as a target for conjugation is expected to require specific machinery not utilized by canonical protein-protein conjugation systems. Therefore, the role of the Atg12-Atg5 conjugate in the Atg8-PE conjugation reaction must be elucidated.

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