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Structure of the Atg12–Atg5 conjugate reveals a platform for stimulating Atg8–PE conjugation

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Atg12 is conjugated to Atg5 through enzymatic reactions similar to ubiquitination. The Atg12–Atg5 conjugate functions as an E3-like enzyme to promote lipidation of Atg8, whereas lipidated Atg8 has essential roles in both autophagosome formation and selective cargo recognition during autophagy. However, the molecular role of Atg12 modification in these processes has remained elusive. Here, we report the crystal structure of the Atg12–Atg5 conjugate. In addition to the isopeptide linkage, Atg12 forms hydrophobic and hydrophilic interactions with Atg5, thereby fixing its position on Atg5. Structural comparison with unmodified Atg5 and mutational analyses showed that Atg12 modification neither induces a conformational change in Atg5 nor creates a functionally important architecture. Rather, Atg12 functions as a binding module for Atg3, the E2 enzyme for Atg8, thus endowing Atg5 with the ability to interact with Atg3 to facilitate Atg8 lipidation.

Keywords: autophagy; Atg12; Atg5; Atg3; crystal structure

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INTRODUCTION

Ubiquitin (Ub) and ubiquitin-like proteins (Ubls) regulate various biological processes by covalently modifying substrate molecules [1]. Atg12 is one such Ubl and its C-terminal Gly 186 is covalently linked to the side chain of Atg5 Lys 149 through reactions catalysed by Atg7 (E1) and Atg10 (E2) [2]. The Atg12–Atg5 conjugate (– indicates a covalent bond) forms a stable complex with Atg16 [3] and has critical roles in autophagy, an intracellular degradation process conserved among most eukaryotes [4,5]. Autophagy requires another Ubl, Atg8, which is covalently linked

to the amino group of phosphatidylethanolamine (PE) through reactions catalysed by Atg7 and Atg3 (E2) [6].

Intriguingly, efficient Atg8–PE conjugate formation requires the Atg12–Atg5 conjugate *in vitro* [7,8], and Atg16 is required additionally *in vivo* [9]. These observations indicated that the Atg12–Atg5–Atg16 complex functions as an E3-like enzyme to facilitate Atg8–PE formation, which might be accomplished by the combination of at least two mechanisms: one is the activation of the conjugating activity of Atg3 by the Atg12–Atg5 conjugate and the other is the targeting of Atg3 to the proper membrane surface containing PE by Atg16.

Atg12 alone or the Atg5–Atg16 complex does not have E3-like activity for Atg8 lipidation, and conjugate formation between Atg12 and Atg5 is an essential step in this process [7,8]; therefore, it can be considered that Atg12 modification endows Atg5 with E3-like activity for Atg8 lipidation. This is partially reminiscent of the stimulation of the ubiquitination activity of cullin-RING ubiquitin ligases by the modification of the Ubl protein NEDD8. In this case, striking conformational rearrangements in cullin-RING ubiquitin ligase are induced by NEDD8 modification and are considered to be the main mechanism for the stimulation of ligase activity [10]. In the case of Atg12 modification, however, the molecular mechanism(s) underlying how the E3-like activity of Atg5 is regulated by Atg12 modification is totally unknown.

Here, we report the crystal structure of the Atg12–Atg5 conjugate bound to the N-terminal domain of Atg16. Structural information, together with *in vitro* and *in vivo* data, indicates that Atg12 modification does not induce a conformational change in Atg5, but simply endows Atg5 with the ability to bind to Atg3. These data will form the basis for establishing the molecular functions of the Atg12–Atg5–Atg16 complex in autophagy.

RESULTS AND DISCUSSION

Crystal structure of the Atg12–Atg5–Atg16N complex

Saccharomyces cerevisiae Atg12 consists of 186 amino acids, and contains a Ubl fold at its C-terminal region (residues 100–186). The N-terminal ~100 residues are not conserved among Atg12 homologues, and were reported to be dispensable for the function of Atg12 in autophagy [11]. Further, the C-terminal 10 residues of *S. cerevisiae* Atg5 were shown to have a flexible

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Table 1 | Refinement statistics

Resolution range (Å)	50.00–2.60
Reflections used	16,822
No. of protein atoms	2,868
No. of water molecules	27
R/R_{free}	0.231/0.258
Rmsd from ideality length (Å)	0.008
Angles (°)	1.3
Rmsd, root-mean-square deviation.	

conformation [12]. *S. cerevisiae* Atg16 consists of the N-terminal domain (Atg16N; residues 1–46) and the C-terminal coiled-coil domain that mediates homodimerization [13], and Atg16N was shown to be sufficient for Atg5 binding [12]. Therefore, we used Atg12 (100–186), Atg5 (1–284) and Atg16 (1–46) for the preparation and crystallization of the Atg12–Atg5 conjugate bound to Atg16N [14], and determined the structure at 2.6 Å resolution (Table 1).

The asymmetric unit contained one Atg12–Atg5–Atg16 complex (Fig 1A). Residues 100, 113–119 and 182–184 of Atg12, residues 1, 96–110, 135–136 and 242–246 of Atg5 and residues 1–21 of Atg16N were omitted from the model as they lacked a defined electron density. Atg5 consists of three domains: two Ubl domains (UblA and UblB) and a helix-rich domain between them. Atg16N consists of an α -helix followed by a tail region, which is bound to the groove formed between these Atg5 domains. These structures are completely similar to our previously determined structure of the Atg5–Atg16N complex, except for some loop regions (Fig 1D) [12]. These loop regions are displaced from the Atg12 modification site and are involved in distinct crystal-packing interactions. Thus, it is strongly suggested that Atg12 modification does not induce a large conformational change on the Atg5–Atg16N complex. The electron density of the side chain of Atg5 Lys 149 is abnormally large (Fig 1C), indicating that this residue is actually modified with the C-terminal Gly 186 of Atg12. Atg12 has a Ubl fold similar to our previously reported structure of plant Atg12b [15]. The electron density of the C-terminal tail region connecting the Ubl fold and Phe185 is disordered, suggesting that it has a flexible conformation.

Non-covalent interactions between Atg12 and Atg5

Besides the covalent linkage between Atg12 Gly 186 and Atg5 Lys 149, Atg12 forms many hydrophobic and hydrophilic interactions with Atg5 (Fig 1B). The side chains of Phe 154 and Phe 169 of Atg12 form hydrophobic interactions with those of Gln 150, Phe 153 and Ile 154 of Atg5. The side chain of Atg12 Gln 160 forms hydrogen bonds with those of Gln 150 and Gln 217 of Atg5. Further hydrophilic interactions are observed between the side chain of Atg12 Gln 159 and the main chain of Atg5 Thr 129, between the side chain of Atg12 Glu 164 and the main chain of Atg5 Ser 216, and between the main chain of Atg12 Ala 155 and the side chain of Atg5 His 146. These non-covalent interactions bury $\sim 650 \text{ Å}^2$ of the surface area of each protein.

The covalent linkage between Atg12 Gly 186 and Atg5 Lys 149 was shown to be essential for autophagy. To study the functional significance of the non-covalent interactions between Atg12 and Atg5 observed in the crystal, we introduced point mutations in

Atg12 and Atg5 to destroy the non-covalent interactions and studied the effects of these mutations on the formation of the Atg8–PE conjugate and autophagic activity *in vivo*. An alanine or arginine substitution was introduced at Gln 150 of Atg5 to diminish the hydrophilic and hydrophobic interactions (Atg5 Q150A) or to introduce a steric clash between Atg12 and Atg5 (Atg5 Q150R), respectively. An arginine substitution was introduced at Phe 154 or Gln 160 of Atg12 (Atg12 F154R, Q160R) to diminish the hydrophobic interactions (F154R) or to introduce a steric clash between Atg12 and Atg5 (F154R, Q160R). These Atg12 and Atg5 mutants were expressed using a centromeric plasmid in KYY142 cells, in which the *ATG12* gene was knocked out (*atg12Δ* cells) or KYY115 cells in which the *ATG5* gene was knocked out (*atg5Δ* cells), respectively, and the levels of Atg12–Atg5 and Atg8–PE conjugates were analysed by western blotting (Fig 1E, low-copy plasmid, top and middle panels). In these cells, the Atg12–Atg5 conjugate was formed similarly with wild-type cells, except for the cells expressing Atg12 F154R. Atg8–PE conjugate formation was also normal in these mutant cells, except for those expressing Atg12 F154R. Next, autophagic activity was examined by monitoring aminopeptidase I (Ape1) maturation. The preform of Ape1 (prApe1) is transported to the vacuole through the cytoplasm-to-vacuole targeting pathway under nutrient-rich conditions and by autophagy in response to starvation conditions or rapamycin treatment. In the vacuole, prApe1 is processed into a mature form (mApe1), which can be monitored by western blotting for Ape1. As shown in Fig 1E (low-copy plasmid, bottom panels), autophagic activity was normal in these mutant cells, except for those expressing Atg12 F154R. These data, except for F154R mutant, indicate that the non-covalent interaction between Atg12 and Atg5 is not necessary for the normal formation of the Atg8–PE conjugate or autophagic activity. As for the Phe 154 mutant, the expression level of the Atg12–Atg5 conjugate was too low to be detected by western blotting. Therefore, Atg12 mutants were also expressed using a 2μ-based, multicopy plasmid in *atg12Δ* cells (Fig 1E, multicopy plasmid). In this case, we could detect the weak band of Atg12(F154R)–Atg5 conjugate by western blotting even under starvation conditions, the quantity of which appears to be sufficient for the function of Atg12–Atg5 conjugate, as a very small amount of Atg12–Atg5 conjugate is sufficient for normal progression of autophagy [16]. Nevertheless, both Atg8–PE formation and autophagic activity were abrogated in these cells, suggesting that F154R mutation abrogated the function of Atg12. Phe 154 was previously reported to be a residue that was essential for the function of Atg12 in yeast [11], and its equivalent residue (Phe108) in human Atg12 was shown to be important for the interaction of Atg12 with Atg3 [9]. Therefore, it was speculated that the severe defects in autophagic activity and Atg8–PE conjugate formation observed in cells expressing this mutant could be attributed to the loss of the functional surface of Atg12, for example, that responsible for Atg3 binding, rather than the loss of the non-covalent interaction between Atg12 and Atg5.

Atg12 functions as a binding module for Atg3

Atg12 modification did not induce a conformational change in Atg5, and the determined structure of the Atg12–Atg5 conjugate itself appeared to be dispensable for its autophagic function. Then, we speculated that the Atg12 modification simply endows Atg5

Fig 1 | Crystal structure of the Atg12–Atg5 conjugate bound to Atg16N. (A) Ribbon representation of the Atg12–Atg5 conjugate bound to Atg16N. Atg12, Atg5 and Atg6 are coloured salmon pink, green and orange, respectively. Atg12 Phe 185 and Gly 186 and Atg5 Lys 149 are shown with a stick model. The disordered C-terminal region of Atg12 (residues 182–184) is indicated with a broken line. The left figure was obtained by a 90° rotation of the right figure along the vertical axis. The amino- and carboxy-termini are denoted as N and C, respectively. All of the figures representing the molecular structures were generated with PyMOL [26]. (B) Stereo view of the detailed interactions between Atg12 and Atg5. The side chains of the residues involved in the Atg12–Atg5 interaction are shown with a stick model, in which nitrogen and oxygen atoms are coloured red and blue, respectively. (C) Electron density map of the isopeptide linkage between Atg12 Gly 186 and Atg5 Lys 149. The simulated annealing $F_o - F_c$ difference Fourier map was calculated by omitting Atg12 Phe 185 and Gly 186 and Atg5 Lys 149, and is shown with black meshes at 5.0σ . (D) Superimposition of the Atg5–Atg16N complex structure (PDB ID 2DYM) on that of the Atg12–Atg5–Atg16N complex. The Atg12–Atg5–Atg16N complex is coloured red, while the Atg5–Atg16N complex is coloured grey. Atg12 Phe 185 and Gly 186 and the side chain of Atg5 Lys 149 are shown with a stick model. (E) *In vivo* analyses of the Atg12 and Atg5 mutants for studying the functional significance of the non-covalent Atg12–Atg5 interactions. Yeast cells with or without starvation were lysed and subjected to urea SDS–polyacrylamide gel electrophoresis (PAGE) followed by western blotting. Ape1, aminopeptidase I; mApe1, mature form of Ape1; PE, phosphatidylethanolamine; prApe1, preform of Ape1; WT, wild-type.

Fig 2 | Atg12 functions as a binding module for Atg3. (A) *In vitro* pull-down assay using recombinant plant Atg homologues. (B) Mapping of the mutated residues on the Atg12–Atg5–Atg16N complex structure. Atg5 and Atg16N are shown with a ribbon model, whereas Atg12 is shown with both surface and ribbon models. The side chains of Tyr 147, Tyr 149 and Phe 154 of Atg12 are shown with a stick model and coloured grey (Tyr 147) or blue (Tyr 149 and Phe 154). Corresponding AtAtg12b residues are indicated in parentheses. Atg12 Phe 185 and Gly 186 and the side chain of Atg5 Lys 149 are shown with a stick model. (C) Proposed model of Atg8–PE conjugate formation mediated by the Atg12–Atg5–Atg16 complex. CBB, Coomassie Brilliant Blue staining; GST, glutathione S-transferase; HA, haemagglutinin; PE, phosphatidylethanolamine; WT, wild-type.

with the ability to interact with other factor(s). Thus far, physical interactions between Atg12 and Atg3 have been reported in yeast and mammals; however, these interactions have never been studied in detail using purified proteins, one reason of which is the instability of Atg12. Although we could purify yeast Atg12–Atg5 conjugate for crystallization from *Escherichia coli*, we never succeeded in purifying yeast Atg12 alone, which suggests that Atg12 is intrinsically unstable and conjugate formation with Atg5 markedly stabilizes Atg12. However, we could purify a plant Atg12 homologue (AtAtg12b) as a free form and used it for structural and functional studies [8,15,17]. Furthermore, we previously showed, using an *in vitro* system, that the AtAtg12b–AtAtg5 conjugate has E3-like activity that enhances the conjugation of AtAtg8a with PE similarly with the yeast system [8]. Thus, we studied the direct interaction between Atg12 and Atg3 using plant homologues. As shown in Fig 2A, AtAtg12b bound directly to glutathione S-transferase (GST)–AtAtg3, but not to GST.

In contrast, AtAtg5 bound to GST nonspecifically and did not show specific binding to GST–AtAtg3. These data indicate that AtAtg3 specifically binds to AtAtg12b, but not AtAtg5. We also studied the interactions between AtAtg12b mutants and GST–AtAtg3. The substitution of Tyr 57 (corresponds to Tyr 149 of yeast Atg12) or Phe 62 (corresponds to Phe 154 of yeast Atg12) of AtAtg12b with alanine diminished the interaction with GST–AtAtg3, whereas substitution of Phe 55 (corresponds to Tyr 147 of yeast Atg12) with aspartate did not affect this interaction. Mapping of Atg12 Tyr 147, Tyr 149 and Phe 154 on the structure of the Atg12–Atg5–Atg16 complex is shown in Fig 2B. Atg12 Tyr 149 is partially, and Phe 154 is completely buried by Atg5, suggesting that the Atg3-binding surface of Atg12 is masked in the complex.

On the basis of our observations, we propose a model of Atg8 lipidation that is catalysed by Atg3 and the Atg12–Atg5–Atg16 complex (Fig 2C). Atg5 and Atg16 are targeted interdependently to the autophagic membrane [12,18,19],

for which Atg12 modification is dispensable [19]. Therefore, the Atg12–Atg5–Atg16 complex interacts with the membrane or some factor(s) on the membrane through the Atg16-bound side and exposes Atg12. The crystal structure suggests that the Atg3-binding surface of Atg12 (coloured cyan in Fig 2C) is masked by Atg5 in a conjugated state. Therefore, the Atg12–Atg5–Atg16 complex exists as a closed form when it is not working. The formation of the closed form might increase the stability of the complex, as the Atg3-binding surface of Atg12 is rather hydrophobic. Actually, recombinant Atg12–Atg5 conjugate is much more stable than free Atg12 [14,15]. When Atg3 thioester linked to Atg8 appears, the Atg3-binding surface of the Atg12 moiety is released from Atg5 and interacts with Atg3, which targets Atg3 to the membrane surface and facilitates the transfer of Atg8 from Atg3 to the PE in the membrane. We previously reported that the Atg12–Atg5 conjugate causes the artificial transfer of Atg8 from Atg3 to a serine residue in Atg3 itself *in vitro* [20], which indicates that the Atg12–Atg5 conjugate can enhance the reactivity of the Atg8 thioester linked to Atg3 directly. This enhancement will be mediated by Atg5 together with Atg12, although the elucidation of its molecular mechanism needs further structural and biochemical studies.

METHODS

X-ray crystallography. The expression, purification and crystallization of the Atg12–Atg5–Atg16N complex were reported previously [14]. Briefly, *S. cerevisiae* Atg12 (residues 100–186) with a hexa-histidine tag, Atg5 (residues 1–284), full-length Atg7 and Atg10 were coexpressed in *E. coli* BL21 (DE3), which resulted in the formation of the Atg12–Atg5 conjugate in *E. coli*. After purifying the Atg12–Atg5 conjugate through several chromatography steps, we mixed it with purified Atg16N and got the purified Atg12–Atg5–Atg16N complex by cation-exchange chromatography. Crystals of the Atg12–Atg5–Atg16N complex were obtained by the sitting-drop vapour diffusion method using 12% PEG10000, 0.5 M potassium thiocyanate and 0.1 M ADA, pH 6.5, as crystallization reagents. X-ray diffraction data up to 2.6 Å resolution were collected at the beamline (NW12A; KEK, Japan), as reported previously [14]. Its structure was determined by the molecular replacement method using the crystal structures of the Atg5–Atg16N complex (PDB ID 2DYM) and AtAtg12b (PDB ID 1WZ3) as search models. Molecular replacement and crystallographic refinement were performed using the crystallography and NMR system software [21]. Model building and modification were performed manually using the COOT programme [22]. The refinement statistics are summarized in Table 1.

In vitro pull-down assay. The preparation of recombinant AtAtg5–HA (haemagglutinin), AtAtg12b and GST-fused AtAtg3 was performed as reported previously [8]. Mutations leading to specific amino-acid substitutions were introduced by PCR-mediated site-directed mutagenesis and sequenced to confirm their identities. Purified GST–AtAtg3s (4 µg) and GST (4 µg) were mixed with AtAtg12b (6 µg) or AtAtg5–HA (20 µg) and incubated with glutathione-Sepharose 4B beads (GE Healthcare) at 4 °C. After washing the beads with phosphate-buffered saline, the proteins were eluted with 10 mM glutathione in 50 mM Tris–HCl buffer (pH 8.0). The eluates were subjected to SDS–PAGE, and the proteins were detected by Coomassie Brilliant Blue staining. They were also subjected to western blotting, and the AtAtg5–HA and

AtAtg12b bands were detected with anti-HA (16B12, COVANCE) and anti-AtAtg12b [15] antibodies, respectively.

In vivo assay. Point mutations were introduced by PCR-mediated site-directed mutagenesis using pRS316-based plasmids containing the *ATG5* or *ATG12* gene and pRS424-based plasmid containing the *ATG12* gene [2] as templates. Successful introduction of the point mutations was confirmed by sequencing. These plasmids were introduced into KUY142 (*MATα leu2 ura3 his3 trp1 lys2 suc2 atg5Δ::LEU2*) or KUY115 (*MATα leu2 ura3 his3 trp1 lys2 suc2 atg12Δ::HIS*) strains [18]. Cell growth, lysate preparation and immunoblot analyses were performed as described previously [11]. The Atg12–Atg5 conjugate, Atg8/Atg8–PE conjugate and prApe1/mApe1 were detected using anti-Atg12 [23], anti-Atg8 [24] and anti-Ape1 antibodies [25], respectively.

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Author contributions: N.N.N., Y.O. and F.I. designed the experiments. N.N.N. and Y.F. prepared recombinant proteins. N.N.N. performed crystallographic study and *in vitro* assays. T.H. performed *in vivo* assays. N.N.N. wrote the manuscript. All authors discussed the results and commented on the manuscript. N.N.N. and F.I. supervised the work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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