Structure-based analyses reveal distinct binding sites for Atg2 and phosphoinositides in Atg18*

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*Running Title: Crystal structure of Kluyveromyces marxianus Hsv2

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Keywords: autophagy; Hsv2; Atg18; Atg2; crystal structure

Background: Atg18 plays a critical role in autophagy as a complex with Atg2 and PtdIns(3)P. Results: The structure of the Atg18 paralog was determined and important residues in Atg18 were identified. Conclusions: Atg18 recognizes Atg2 and membranes using distinct regions. Significance: This study will be a basis for elucidating the function of Atg18 in autophagy.

Autophagy is an intracellular degradation system by which cytoplasmic materials are enclosed by an autophagosome and delivered to a lysosome/vacuole. Atg18 plays a critical role in autophagosome formation as a complex with Atg2 and phosphatidylinositol 3-phosphate (PtdIns(3)P). However, little is known about the structure of Atg18 and its recognition mode of Atg2 or PtdIns(3)P. Here, we report the crystal structure of Kluyveromyces marxianus (Km) Hsv2, an Atg18 paralog, at 2.6 Å resolution. The structure reveals a seven-bladed β-propeller without circular permutation. Mutational analyses of Atg18 based on the KmHsv2 structure suggested that Atg18 has two phosphoinositide binding sites at blades 5 and 6, whereas the Atg2 binding region is located at blade 2. Point mutations in the loops of blade 2 specifically abrogated autophagy without affecting another Atg18 function, the regulation of vacuolar morphology at the vacuolar membrane. This architecture enables Atg18 to form a complex with Atg2 and PtdIns(3)P in parallel, thereby functioning in the formation of autophagosomes at autophagic membranes.

Macroautophagy (hereafter referred to as autophagy) is an intracellular degradation system conserved among eukaryotes from yeast to mammals. During autophagy, a double membrane structure called an autophagosome sequesters a portion of the cytoplasm and fuses with a vacuole (or lysosome in the case of mammalian autophagy) to deliver its inner contents to the lumen of the organelle (1). Autophagy is important in a wide range of physiological processes such as adaptation to starvation, quality control of intracellular proteins and organelles, embryonic development, elimination of intracellular microbes, and prevention of neurodegeneration and tumor formation (2-4).

Currently, more than 30 genes involved in autophagy have been isolated in yeast and termed autophagy-related (ATG) genes. Among these genes, ATG1–10, 12–14, 16–18, 29, and 31 are essential for autophagosome formation during starvation-induced autophagy, and the 18
Atg proteins they encode are classified into six functional groups (1,5): (i) starvation-responsive Atg1 kinase complex; (ii) class III phosphatidylinositol (PtdIns) 3-kinase complex I; (iii) proteins involved in the ubiquitin-like conjugation of Atg12 with Atg5; (iv) proteins involved in the ubiquitin-like conjugation of Atg8 with phosphatidylethanolamine; (v) multimembrane-spanning protein Atg9; and (vi) Atg2-Atg18 complex. These Atg proteins localize, at least in part, to the pre-autophagosomal structure (PAS), which is proximal to the vacuole and plays a central role in autophagosome formation (6). The characterization of each of these proteins is ongoing, and the interrelationships among these functional groups have also been studied systematically. However, except for the proteins involved in ubiquitin-like conjugation (7), structural studies on the Atg proteins involved in autophagosome formation have been limited.

Atg18 plays a critical role in autophagy (8,9) by forming a protein complex with Atg2 (10). Besides Atg2, Atg18 also binds to PtdIns 3-phosphate (PtdIns(3)P) and PtdIns (3,5)-bisphosphate (PtdIns(3,5)P2), and is therefore considered to be one of the effectors of these molecules (11,12). Atg18 and Atg2 localize to the PAS in an interdependent manner, for which the ability of Atg18 to bind PtdIns(3)P is required (10,13). Furthermore, the production of PtdIns(3)P at the PAS by PtdIns 3-kinase complex I is required for autophagosome formation (14,15). These observations suggest that the interaction of Atg18 with Atg2 and PtdIns(3)P at the PAS is essential for the formation of autophagosomes. In addition to autophagy, Atg18 also has a role in regulating the vacuolar morphology of yeast, for which Atg18 localizes to the vacuolar membrane through its interaction with PtdIns(3,5)P2, but not with PtdIns(3)P (11,16). Previous studies predicted the structure of Atg18 and its homologs as a seven-bladed β-propeller fold, and identified a putative phosphoinositide binding motif, FRRG, within the predicted β-propeller structure (11,17,18). However, little is known about the molecular mechanisms underlying how Atg18 recognizes Atg2 and PtdIns(3)P via its β-propeller structure and how the Atg2-Atg18 complex functions in the formation of autophagosomes.

In yeast, two Atg18 paralogs have been identified: Atg21 and Hsv2 (11,12,19). Although Atg21 and Hsv2 are not essential for autophagy, they contain an FRRG motif and bind to PtdIns(3)P and PtdIns(3,5)P2 (12,17,20). Moreover, they were also predicted to have a seven-bladed β-propeller fold. In order to reveal the architecture of Atg18, we herein report the crystal structure of Hsv2 from a thermotolerant yeast, Kluyveromyces marxianus (Km), at a resolution of 2.6 Å. The structure reveals a seven-bladed β-propeller fold. Mutational analyses of Atg18 based on the structure of KmHsv2 showed that Atg18 possesses two binding sites for phosphoinositides at blades 5 and 6, while the loop regions of blade 2 are specifically required for recognizing Atg2 and thus for autophagy. These results suggest that Atg18 tethers Atg2 to the PAS and autophagic membranes through its simultaneous interaction with Atg2 and PtdIns(3)P, thus playing a critical role in the formation of autophagosomes.

EXPERIMENTAL PROCEDURES

Protein expression and purification—K. marxianus Hsv2 was amplified by polymerase chain reaction and cloned into the pGEX-6P-1 vector to produce glutathione S-transferase (GST) fusion proteins (GE Healthcare). The construct was sequenced to confirm its identity and expressed in Escherichia coli strain BL21 (DE3) cells that were cultured in 2× YT medium (yeast extract, 10 g/L; tryptone, 16 g/L; and sodium chloride, 5 g/L). After cell lysis by sonication, GST-fused proteins were purified by affinity chromatography using a glutathione-Sepharose 4B column (GE Healthcare). The GST tag was then cleaved with PreScission protease (GE Healthcare) and removed by affinity chromatography using a glutathione-Sepharose 4B column. This process left a Gly-Pro-Leu-Gly-Ser sequence at the N-terminus of Hsv2. Further purification was performed using a Superdex 200 gel filtration column (GE Healthcare) and elution with 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl.

X-ray crystallography—Crystallization of Hsv2 was performed using the sitting drop vapor diffusion method at 20°C. Drops of 10
mg/mL Hsv2 in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 2 mM dithiothreitol were mixed with equal amounts of reservoir solution (1.2 M (NH₄)₂SO₄ and 100 mM acetate buffer at pH 5.5), and equilibrated against 100 μL of the same reservoir solution by vapor diffusion. Crystals, typically with dimensions of 0.30 × 0.25 × 0.25 mm, were obtained within a week. Diffraction data of the native and selenomethionine-labeled crystals were collected on an ADSC Quantum 210 charge-coupled device detector using beamline AR-NW12A (KEK). The diffraction data were indexed, integrated, and scaled using the HKL2000 program suite (21). The initial phasing was performed by the multi-wavelength anomalous dispersion method using the peak, edge, and remote data from the selenomethionine-labeled crystals. After the 28 selenium sites were identified using the SHELXD program, the initial phase and density modification were calculated using the SHELXE program (22). Automated model building was performed using the Buccaneer program (23) in the CCP4 program suite (24). Further model building was performed manually using the COOT program (25), and crystallographic refinement was performed using the crystallography and nuclear magnetic resonance system software (26). Data collection, phasing, and refinement statistics are summarized in Table 1.

**Yeast strains and media**—We utilized standard methods for yeast manipulation (27). The *Saccharomyces cerevisiae* strains used in this study are listed in Table 2. Yeast cultures were incubated in rich YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% D-glucose) or SDCA medium (0.17% yeast nitrogen base w/o amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.5% casamino acid, and 2% D-glucose) containing appropriate amino acids. Gene disruption or epitope tagging was carried out as reported previously (28,29). To induce autophagy, the cells were grown to mid-log phase in YPD or SDCA medium and then incubated in SD (-N) medium (0.17% yeast nitrogen base w/o amino acids and ammonium sulfate, and 2% D-glucose) for 4 h or treated with rapamycin (final concentration, 0.2 μg/mL; Sigma) for 1–3 h.

**Plasmid construction for the yeast experiments**—The pRS316-based plasmid for Atg18-3×HA-EGFP was generated as reported previously (30). Point mutations were introduced by PCR-based site-directed mutagenesis using the pRS316-based plasmid for Atg18-3×HA-EGFP as a template. The successful introduction of the point mutations was confirmed by sequencing.

**Microscopic observations**—The intracellular localization of mRFP- or GFP-tagged proteins (including GFPplus, EGFP, and yeGFP) was visualized using an inverted fluorescence microscope (IX-71; Olympus) equipped with an EM-CCD digital camera (ImagEM; Hamamatsu Photonics K.K.). Images were acquired using Aquacosmos 2.6 software (Hamamatsu Photonics K.K.) and processed using Photoshop CS4 software (Adobe Systems). To observe the PAS, yeast cells were treated with rapamycin (final concentration, 0.2 μg/mL; Sigma) for 1 h to induce autophagy.

**FM4-64 staining**—Cells at the logarithmic phase were loaded with 2 μg/mL FM4-64 (Invitrogen) for 30 min, washed, and chased with FM4-64 free media for 30 min.

**Pho8Δ60 alkaline phosphatase assay**—To quantify bulk autophagic activity, we utilized the Pho8Δ60 alkaline phosphatase assay as described previously (31).

**Preparation of total lysates and immunoblotting**—Yeast protein extracts were prepared as reported previously (30). Immunoblotting was performed using anti-Ape1 (API-2), anti-HA (3F10), anti-myc (9E10), or anti-Pgk1 antisera (Invitrogen/Molecular Probes). Chemiluminescence detection was performed using Pierce Western Blotting Substrate (Thermo Scientific) and detected using an LAS-4000 mini image analyzer (GE Life Sciences).

**Co-immunoprecipitation**—Cells were treated with 200 μg/mL zymolyase-100T (07665-55, Nacalai Tesque) for 45 min at 30°C in spheroplasting buffer (50 mM HEPES-KOH, pH 7.2, 1 M sorbitol, 1% yeast extract, 2% bacto-peptone, 1% glucose, and 10 mM DTT). The spheroplasts were washed once with spheroplasting buffer, grown for 20 min at 30°C, and then treated with 0.5 μg/mL rapamycin for 1 h at 30°C. The spheroplasts were harvested and
treated with 0.5% Triton X-100 for 30 min on ice in lysis buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl₂, and protease inhibitor cocktail (P8340; Sigma)). The total lysate was centrifuged at 17,400 x g for 20 min at 4°C, and the resulting supernatant was incubated with GFP-Trap_M (gtm-20; ChromoTek) or rabbit IgG-conjugated magnetic beads (Dynabeads M-270 epoxy; Invitrogen) for 3 h at 4°C. The bound materials were washed three times with lysis buffer, and then eluted with SDS-PAGE sample buffer for 15 min at 65°C.

RESULTS

Overall structure of KmHsv2—We first tried to crystallize S. cerevisiae Atg18, but failed to obtain good diffracting crystals. So, we adopted a strategy to obtain crystals from various Atg18 homologs/paralogs that included: S. cerevisiae Atg21, Pichia pastoris Atg18, Arabidopsis thaliana Atg18b, and Homo sapiens Atg18 homologs (HsWIP1-4). However, these trials also failed. Recently, we succeeded in determining the solution structure of Atg10 by switching the target from ScAtg10 to K. marxianus Atg10 (32). K. marxianus is a thermotolerant yeast, so that the homologs in this yeast could be expected to have higher stability than those in other eukaryotes. So, we attempted to crystallize K. marxianus Atg18 paralogs and succeeded in obtaining good diffracting crystals of KmHsv2 (referred to simply as Hsv2 hereafter), and we determined the crystal structure of Hsv2 by the multi-wavelength anomalous dispersion method using a selenomethionine-substituted crystal (Supplementary Fig. S1). The structure was refined against 2.6 Å data to an R-factor of 0.224 and a free R-factor of 0.252 (Table 1).

The asymmetric unit of the crystal contains two Hsv2 molecules (Hsv2A and Hsv2B). The obtained Hsv2 model lacks 13 N-terminal residues and some loop regions (residues 164–181 in Hsv2A, and residues 163–181 and 268–284 in Hsv2B) due to undefined electron density. Hsv2 possesses a seven-bladed β-propeller fold, in which each blade consists of a 4 β-stranded, anti-parallel β-sheet, resembling each other (Fig. 1A). Circular permutation, which is frequently observed in β-propeller proteins, is not observed in this fold. Residues 263–289 of Hsv2A form a large extended loop connecting the C and D β-strands (CD loop) in blade 6, which protrudes from the β-propeller fold as far as ~25 Å. The ordered conformation of this loop is stabilized by crystal packing. In contrast, the electron density of the equivalent residues of Hsv2B was disordered, suggesting that these residues have a flexible conformation in solution.

Two binding sites for phosphoinositides—The phosphoinositide-binding FRRG motif (residues 229–232) of Hsv2 is located at the β-strand D of blade 5 and the loop connecting blades 5 and 6. Intriguingly, the side chains of the two arginine residues of the motif, Arg230 and Arg231, point in opposite directions (Fig. 1B). In the proximity of Arg230, there is a basic pocket composed of Ser209, Thr213, and Arg216 from blade 5 and His189, Thr190, and Asn191 from the loop connecting blades 4 and 5. A sulfate ion is bound to this basic pocket, suggesting it has a role in accommodating phosphoinositides; thus, this pocket was named site 1. The structure of site 1 is very similar between Hsv2A and Hsv2B (Fig. 1B). Arg231 is involved in the construction of another basic pocket composed of Ser254, Lys256, Thr258, and His260 from blade 6. The structure of this second pocket is somewhat distinct between Hsv2A and Hsv2B. In Hsv2A, the loop connecting blades 5 and 6 has a conformation closer to blade 6, and the side chain of Asp234 on the loop is bound deeply in the pocket, whereas in Hsv2B, the pocket is occupied by the side chain of Glu336 of the crystallographically adjacent Hsv2 molecule. In both cases, the basic pocket is occupied by a negatively-charged carboxyl group, suggesting it has a similar role to site 1 in accommodating phosphoinositides; thus, the second pocket was named site 2. The residues constituting sites 1 and 2 are highly conserved among the Atg18 homologs/paralogs, suggesting the possibility that Atg18 and its relatives possess two binding pockets for phosphoinositides. During the preparation of this manuscript, Baskaran et al. and Krick et al., reported the crystal structure of K. lactis Hsv2 (33,34). The overall structure of K. lactis Hsv2 is similar to that of KmHsv2, and it possesses two basic pockets similar to sites 1 and 2 in KmHsv2. They showed that both
pockets are important for recognizing phosphoinositides according to in vitro mutational analyses. We also observed that single mutation at either Arg230 (site 1) or Arg231 (site 2) resulted in a partial defect, and simultaneous mutation at both residues resulted in a more severe defect in autophagy (Supplementary Fig. S2), suggesting that both sites are important for recognizing PtdIns(3)P. Besides two binding pockets, Baskaran et al. also showed that a long loop in blade 6 of KIHsv2, which is equivalent to the long loop (residues 263–289) of KmHsv2, is important for its association with membranes.

Effect of mutating the conserved residues of Atg18 on vacuolar morphology—Structurally annotated multiple sequence alignment of KmHsv2 with Atg18 orthologs (S. cerevisiae, K. marxianus, P. pastoris, and H. sapiens WIPI-1) showed that the residues in blades 2, 3, 5, and 6 are highly conserved among the Atg18 orthologs (Fig. 2A). Fig. 2B shows the location of the conserved residues in the Atg18 orthologs on the structure of KmHsv2. Conserved, exposed residues are especially clustered at blades 2 and 3. In order to evaluate the functional significance of these conserved residues in the regulation of vacuolar morphology, we introduced point mutations at these sites, especially those conserved among Atg18 orthologs but not in Hsv2, and prepared the following six mutants: F54A/S55A, S57A/L58A (both at the AB loop of blade 2), I49K/L96K (at strand A in blades 2 and 3), P72A/R73A (at the BC loop of blade 2), M121A/R122A/L123A (at the CD loop of blade 3), and T126R/N132R (at strand D of blade 3). As mentioned above, many of the residues in sites 1 and 2 are conserved among Atg18 homologs/paralogs and are responsible for the recognition of phosphoinositides. We selected His244 from site 1 and His315 from site 2 and prepared an Atg18 mutant with an alanine substitution at both histidine residues (H244A/H315A). These seven mutants and wild-type Atg18 were expressed as fusion proteins with a 3xHA-EGFP tag (hereafter referred to as Atg18-HG) in atg18Δ cells using the pRS316 centromeric plasmid and visualized by fluorescence microscopy (Fig. 3, Atg18-HG lane). At the same time, the vacuoles were visualized using FM4-64 staining (Fig. 3, FM4-64 lane). While wild-type Atg18-HG localized to the vacuolar membrane and properly regulated the vacuolar morphology, Atg18R244A/H315A-HG was not recruited to the vacuolar membrane and cells expressing Atg18R244A/H315A-HG showed abnormally enlarged vacuoles. We confirmed that the H244A/H315A mutations abrogated the binding affinity of Atg18 for PtdIns(3,5)P2 by an in vitro pull-down assay using PIP beads (data not shown). These results are consistent with previous reports showing that the binding activity of Atg18 to PtdIns(3,5)P2 is required for the localization of Atg18 to the vacuolar membrane and for the maintenance of vacuolar morphology (11,16). Conversely, all of the six mutants at blades 2 and 3 showed normal localization to the vacuolar membrane, and cells expressing these mutants showed normal vacuolar morphology. These data indicate that the mutations at blades 2 and 3 did not affect the affinity of Atg18 for phosphoinositides nor abrogate its role in the regulation of vacuolar morphology.

Effect of mutating the conserved residues of Atg18 on autophagy—Next, we studied the significance of the conserved residues of Atg18 in autophagy using the same panel of mutants. Autophagic activity was estimated using the Pho8Δ60 assay (31). This method utilizes a genetically engineered cytosolic form of alkaline phosphatase, Pho8 (Pho8Δ60), which is delivered into the vacuole exclusively by autophagy and activated. Thus, autophagic activity correlates well with alkaline phosphatase activity. As shown in Fig. 4A, atg18Δ cells expressing Atg18P72A/R73A-HG (Atg18P72A/R73A-HG cells) showed almost no autophagic activity. Atg18F54A/S55A-HG, Atg18S57A/L58A-HG, and Atg18M121A/R122A/L123A-HG cells showed mildly, but significantly, reduced autophagic activity, among which that of Atg18S54A/S55A-HG cells was the lowest. Conversely, Atg18I49K/L96K-HG and Atg18T126R/N132R-HG cells showed autophagic activity comparable with that of wild-type Atg18-HG cells. Atg18R244A/H315A-HG cells showed approximately half of the autophagic activity of wild-type Atg18-HG cells, suggesting that this mutant retained weak PtdIns(3)P binding ability that is sufficient for
the partial progression of autophagy.

Next, we monitored aminopeptidase I (Ape1) maturation. The preform of Ape1 (prApe1) is transported to the vacuole via the cytoplasm-to-vacuole targeting pathway under nutrient-rich conditions and by autophagy in response to starvation or rapamycin treatment (35). In the vacuole, prApe1 is processed into a mature form (mApe1) that can be monitored by western blotting for Ape1. As shown in Fig. 4B, cells expressing wild-type and mutant Atg18-HGs, except for Atg18P72AR73A-HG, showed a strong mApe1 band and a weak prApe1 band. In contrast, prApe1 maturation is more sensitive to rapamycin treatment. The prApe1 band in response to rapamycin treatment was attenuated, whereas that of Atg18P72AR73A-HG cells was slightly attenuated. Similar results were obtained when Atg2 fused to a TAP tag was pulled down using IgG-conjugated magnetic beads (Fig. 6B). These results show that the AB and BC loops in blade 2 are important for the interaction of Atg18 with Atg2.

**DISCUSSION**

In this study, we determined the crystal structure of *K. marxianus* Hsv2 and revealed the seven-bladed β-propeller architecture conserved among the Atg18 family of proteins. Furthermore, we showed, using in vivo mutational analyses of Atg18, that the loop regions in blade 2 play a critical role in autophagy through their interaction with Atg2, whereas the two basic pockets in blades 5 and 6 are responsible for phosphoinositide binding and play an essential role in the regulation of vacuolar morphology. Immunoprecipitation experiments showed that the AB and BC loops in blade 2, which are located on opposite surfaces of the ring-like structure of Atg18, are important for its interaction with Atg2. These observations suggest that Atg2 recognizes both surfaces of the ring simultaneously, which might be achieved by gripping the ring from the side.
of blade 2 (Fig. 7). It appears to be easy for Atg2 to perform such interactions since its size is very large (~180 kDa). Very recently, Baskaran et al. and Krick et al. showed that Hsv2 could interact edge-on to the membrane such that the two binding pockets for phosphoinositides in blades 5 and 6 might contact the membrane surface, while the CD loop of blade 6 might penetrate the membrane (Fig. 7) (33,34). According to this model, the membrane will not interfere with the interaction between Atg18 and Atg2 since blade 2 of Atg18 will be located distally from the membrane, thus enabling the simultaneous interaction of Atg18 with both Atg2 and the membrane. This model of Atg18-Atg2 interaction appears to be conserved in their human counterparts since the residues responsible for the interaction with Atg2 are conserved between ScAtg18 and HsWIPI-1, an Atg18 ortholog. This hypothesis is supported by the report that HsAtg2A directly interacted with ScAtg18 (36).

In addition to the autophagic function of Atg18, it also has a role in the maintenance of vacuolar morphology. The interaction between Atg18 and Vac14, one of regulators of PtdIns(3,5)P₂, appears to be required for the proper regulation of PtdIns(3,5)P₂ and vacuolar morphology (37). In the present study, Atg18P72A/R73A-HG cells had normal sized vacuoles (Fig. 3), indicating that Atg18P72A/R73A-HG could be bound to Vac14. This observation suggests that the Vac14-binding site of Atg18 is distinct from its Atg2-binding site. Since the function of Atg18 in the regulation of vacuolar morphology is not conserved among the homologs in higher eukaryotes, such as mammals, the Vac14 binding site may be located in the non-conserved region of Atg18.

We recently showed that Atg18 is most likely to be important for the localization of Atg2 to the PAS (30). Conversely, the interaction of Atg18 with Atg2 was demonstrated to be essential for the PAS targeting of Atg18 (Fig. 5). This kind of interdependency between two Atg proteins for PAS targeting is also observed for Atg6-Atg14 and Atg5-Atg16 complexes (38,39). However, the mechanisms underlying these interdependent targeting of Atg proteins to the PAS are yet to be elucidated. After targeting to the PAS, the Atg2-Atg18 complex plays an absolutely critical role in autophagosome formation together with other Atg groups. However, the specific roles of this complex are also yet to be established. A structural study of the Atg2-Atg18 complex is required to uncover these critical issues and will create a path toward understanding the molecular mechanisms of autophagosome formation.

FOOTNOTES

We are grateful to Ms Hiromi Kirisako and Chika Kondo-Kakuta for technical supports. The synchrotron radiation experiments were performed at the beamline NW12A at KEK, Japan. This work was supported in part by JSPS KAKENHI Grant Number 23687012 (NNN), 10J01988 (YW), 23000015 (YO), MEXT KAKENHI 24113725 (NNN), MEXT Targeted Proteins Research Program (FI, YO) and Leave a Nest grant BioGarage award (TK).

The abbreviations used are: 3xHA-EGFP, HG; Atg, autophagy-related; GST, glutathione S-transferase; PAS, pre-autophagosomal structure; phosphoinositide, PtdIns; phosphoinositide 3-phosphate, PtdIns(3)P; phosphoinositide 3,5-bisphosphate, PtdIns(3,5)P₂; tandem affinity purification, TAP.

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**FIGURE LEGENDS**

**Fig. 1.** Crystal structure of Hsv2. A, ribbon diagram of the Hsv2 structure. The seven blades and β-strands are labeled. B, phosphoinositide binding sites of Hsv2A (left) and Hsv2B (right). The side chains of the site 1 and 2 residues and the bound sulfate are shown with a stick model. The crystallographically adjacent Hsv2 molecule bound to Site 2 is shown in yellow. Sites 1 and 2 are surrounded by broken-line circles.

**Fig. 2.** A, structurally annotated sequence alignment of Atg18 homologs. Gaps are introduced to
maximize the similarity. The conserved residues are shaded in black. The secondary structural elements of Hsv2 are shown above the sequence. B, mapping of the residues conserved among the Atg18 orthologs on the KmHsv2 structure. The conserved residues are shaded in black. The residues in parentheses are the Atg18 residues that are the structural equivalent of KmHsv2 residues. Blade 2 AB and BC loops as well as sites 1 and 2 are surrounded by broken-line circles.

Fig. 3. Localization of Atg18-HG mutants and vacuolar morphology. Exponentially growing atg18Δ cells (TKY1001) expressing the indicated Atg18-HG mutants were labeled with FM4-64 and subjected to fluorescence microscopy. The scale bar indicates 2 μm.

Fig. 4. Mutational effect of the conserved residues of Atg18 on autophagy. A, autophagic activity was estimated using an ALP assay (see Experimental Procedures). The white and black bars indicate ALP activity at 0 and 4 h after starvation, respectively. The values and the error bars are the means and the standard deviations of three independent experiments, respectively. B, Total lysates from atg18Δ cells carrying the indicated plasmids were subjected to western blotting using anti-Ape1, anti-HA, or anti-Pgk1 (loading control) antiserum. In order to induce autophagy, the cells were treated with rapamycin for 3 h.

Fig. 5. The BC loop of blade 2 is essential for the PAS targeting of Atg18. atg18Δ cells carrying integrated mRFP-Ape1 and Atg18-HG mutants were subjected to microscopic observations after rapamycin treatment for 3 h. The arrows indicate the PAS, and the scale bar indicates 2 μm.

Fig. 6. Analysis of the interaction between Atg18 and Atg2. Co-immunoprecipitation experiments were performed as described in Experimental Procedures. A, Atg18-HGs were pulled down using GFP-trap magnetic beads. The protein bands for Atg2 and Pgk1 were detected using rabbit IgG or an anti-Pgk1 antibody, respectively. The protein bands for Atg18 were detected using an anti-GFP or anti-HA antibody. B, Atg2 fused to a TAP tag was pulled down using IgG-conjugated magnetic beads. The protein bands for Atg2, Atg18, and Pgk1 were detected using an anti-Atg2, anti-HA, or anti-Pgk1 antibody, respectively.

Fig. 7. A schematic representation of the Atg2-Atg18 complex on the membrane. Atg2 (yellow) grips the ring-like structure of Atg18 (green) at blade 2, while Atg18 interacts with membrane (dark red) at blades 5 and 6.
Table 1. Data collection, phasing, and refinement statistics

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<td>0.106 (0.618)</td>
<td>0.110 (0.684)</td>
</tr>
<tr>
<td><strong>Phasing statistics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>50-3.1</td>
<td></td>
</tr>
<tr>
<td>No. of Se sites</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Figure of merit (initial)</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Figure of merit (after SHELXE)</td>
<td>0.70</td>
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<tr>
<td><strong>Refinement statistics</strong></td>
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<tr>
<td>Resolution range (Å)</td>
<td>50-2.6</td>
<td></td>
</tr>
<tr>
<td>No. of protein atoms</td>
<td>5,031</td>
<td></td>
</tr>
<tr>
<td>No. of sulfate ions</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>$R / R_{	ext{free}}$</td>
<td>0.224 / 0.252</td>
<td></td>
</tr>
<tr>
<td>rms deviation from ideality bond length (Å)</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>angle (°)</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses refer to the highest resolution shell.

Table 2. Cell strains used in this study

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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>SEY6210</td>
<td>MATa leu2-3,112 ura3-52 his3Δ200 trp1Δ901 lys2-801 suc2-Δ9</td>
<td>(40)</td>
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<tr>
<td>BJ2168</td>
<td>MATa pro1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52</td>
<td>Yeast Genetic Stock Center</td>
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<tr>
<td>KOY192</td>
<td>SEY6210 pho8Δ::PGPD pho8Δ60::kanMX</td>
<td>(39)</td>
</tr>
<tr>
<td>TKY1001</td>
<td>SEY6210 atg18Δ::kanMX</td>
<td>(30)</td>
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<tr>
<td>TKY1051</td>
<td>KOY192 atg18Δ::natNT2</td>
<td>(30)</td>
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<tr>
<td>TKY1498</td>
<td>TKY1001 mRFP–APE1::HIS</td>
<td>(30)</td>
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<tr>
<td>TKY1732</td>
<td>BJ2168 ATG2–TAP:TRP1 atg18Δ::natNT2</td>
<td>This study</td>
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<tr>
<td>ScHY-2569</td>
<td>BJ2168 atg18Δ::kanMX6</td>
<td>This study</td>
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</tbody>
</table>
Figure 3

Bright vector M121A/R122A/L123A T126R/N132R H244A/H315A wild-type I49K/L96K F54A/S55A S57A/L58A P72A/R73A Atg18-HG FM4-64

Table: Bright vector M121A/R122A/L123A T126R/N132R H244A/H315A wild-type I49K/L96K F54A/S55A S57A/L58A P72A/R73A Atg18-HG FM4-64
Figure 4

A

Bar graph showing ALP activity (% of wild-type cells) for different constructs under starvation conditions. The constructs are labeled as follows:

- Vector
- Wild-type
- I49K/L96K
- S57A/L58A
- F54A/S55A
- P72A/R73A
- M121A/R122A/L123A
- T126R/N132R
- H244A/H315A

B

Western blot analysis showing the effects of rapamycin on the expression of various proteins. The proteins analyzed are:

- prApe1
- mApe1
- Atg18–HG
- Pgk1

The blot shows the expression levels of these proteins under different conditions with and without rapamycin treatment.
Figure 5

Comparison of wild-type vector, P72A/R73A, H244A/H315A, F54A/S55A, and 1 hr rapamycin treated cells showing Atg18–HGBright mRFP–Ape1 Merged images.
Figure 6

A

IP: GFP

Input | Bound
--- | ---
Atg2-TAP | 220
Atg18-HG | 120
Atg18-HG | 120
Pgk1 | 50

[Atg18-HG] αGFP

Bound

IP; GFP

B

IP: TAP

Input | Bound
--- | ---
Atg2-TAP | 220
Atg2-TAP | 220
Atg18-HG | 120
Atg18-HG | 120
Pgk1 | 50

[Atg18-HG] αHA

Bound

IP; TAP

Wild-type | + + + +
Wild-type | + + + +
P72A/R73A | + + + +
P72A/R73A | + + + +
H244A/H315A | + + + +
H244A/H315A | + + + +
Figure 7
Membrane
Atg2
Atg18
Site 2
Site 1
3
4
5 6
7
1
2
PtdIns(3)P CD loop (Blade 6)
Structure-based analyses reveal distinct binding sites for Atg2 and phosphoinositides in Atg18

Yasunori Watanabe, Takafumi Kobayashi, Hayashi Yamamoto, Hisashi Hoshida, Rinji Akada, Fuyuhiko Inagaki, Yoshinori Ohsumi and Nobuo N. Noda

J. Biol. Chem. published online July 31, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.397570

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