The autophagy-related protein (Atg) 8 conjugation system is essential for the formation of double-membrane vesicles called autophagosomes during autophagy, a bulk degradation process conserved among most eukaryotes. It is also important in yeast for recognizing target vacuolar enzymes through the receptor protein Atg19 during the cytoplasm-to-vacuole targeting (Cvt) pathway, a selective type of autophagy. Atg3 is an E2-like enzyme that conjugates Atg8 with phosphatidylethanolamine (PE). Here, we show that Atg3 directly interacts with Atg8 through the WEDL sequence, which is distinct from canonical interaction between E2 and ubiquitin-like modifiers. Moreover, NMR experiments suggest that the mode of interaction between Atg8 and Atg3 is quite similar to that between Atg8/LC3 and the Atg8-family interacting motif (AIM) conserved in autophagic receptors, such as Atg19 and p62. Thus, the WEDL sequence in Atg3 is a canonical AIM. In vitro analyses showed that Atg3 AIM is crucial for the transfer of Atg8 from the Atg8–Atg3 thioester intermediate to PE, but not for the formation of the intermediate. Intriguingly, in vivo experiments showed that it is necessary for the Cvt pathway, but not for starvation-induced autophagy. Atg3 AIM attenuated the inhibitory effect of Atg19 on Atg8 lipidation in vitro, suggesting that Atg3 AIM may be important for the lipidation of Atg19-bound Atg8 during the Cvt pathway.
Atg8 is processed by Atg4 (a cysteine protease) to expose Gly at its C terminus (6). The exposed Gly of Atg8 is activated by Atg7 (an E1-like enzyme) to form an Atg8–Atg7 thioester intermediate (7), and is then transferred to Atg3 (an E2-like enzyme) to form an Atg8–Atg3 thioester intermediate (8). Atg8 is finally conjugated to the amino group of a phosphatidylethanolamine (PE) (8). Although these factors are sufficient for the formation of Atg8-PE conjugates in vitro (9), the Atg12-Atg5 conjugate, which is formed by the Atg12 system, is required in vivo (10). Recently, in vitro studies have shown that the Atg12-Atg5 conjugate promotes the conjugation reaction between Atg8 and PE by directly interacting with Atg3 (11,12). The Atg8-PE conjugate mediates membrane tethering and hemifusion, and is responsible for the expansion of autophagosomal membranes (13).

In the Atg8 system, Atg3 receives Atg8 from Atg7 and transfers it to PE. We previously reported the crystal structure of Atg3 and its interaction mode with Atg8 and Atg7 (14,15). Atg3 is composed of three characteristic regions: the E2 core region, the flexible region, and the handle region (HR). The latter two regions are necessary for the interaction with Atg7 and Atg8, but the detailed interaction mode is unknown (14). Recently, we have shown that Atg8 and its mammalian ortholog LC3 recognize the WXXL sequence conserved in Atg19 and p62, autophagic receptors for Ape1 and ubiquitinated protein aggregates, respectively, in a similar manner (15,16). Therefore, the WXXL sequence is considered to be an Atg8-family interacting motif (AIM) (17). Atg8-Atg19AIM and LC3-p62AIM interactions are crucial for the selective transport of Ape1 and protein aggregates to the vacuole/lysosome through the Cvt pathway and autophagy, respectively (16,18). Since Atg3 HR, which is responsible for the interaction with Atg8, also has a WXXL sequence (Trp270-Glu271-Asp272-Leu273), we postulated that Atg3 also interacts with Atg8 through the sequence.

Here, we show by NMR spectroscopy that Atg3 directly interacts with Atg8 through the WEDL sequence in HR, and that the interaction is quite similar to Atg8-Atg19AIM and LC3-p62AIM interactions. Thus, the WEDL sequence of Atg3 is a canonical AIM. In vitro analyses showed that Atg3AIM contributes to the efficient formation of Atg8-PE conjugates particularly under the existence of Atg19, and in vivo analyses showed that Atg3AIM is necessary for the Cvt pathway. These results suggest that AIM identified in autophagic receptors is also utilized in a non-receptor protein, Atg3, and plays a crucial role in autophagic processes.

**EXPERIMENTAL PROCEDURES**

**Protein expression and purification**—Plasmid construction, expression and purification of Atg7, Atg3, Atg8, and Atg12-Atg5 conjugate bound to the N-terminal region (residues 1-46) of Atg16 (Atg16N) were performed as described previously (14,19). The Atg19 genes were amplified by polymerase chain reaction and cloned into pGEX6P-1 (GE Healthcare). Mutations leading to the specific amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis. All constructs were sequenced to confirm their identities and were expressed in *Escherichia coli* BL21 (DE3). The cells were lysed and glutathione S-transferase (GST)-fused Atg proteins were purified by affinity chromatography using a glutathione-Sepharose 4B column (GE Healthcare). GST-fused Atg3 and GST-fused Atg19 proteins for pull-down assay were further purified using a HiTrap Desalting column (GE Healthcare). For NMR spectroscopy, GST was excised from Atg8 K26P (a fully active mutant of Atg8) (20) and Atg3HR with a PreScission protease (GE Healthcare). Atg8 K26P was further purified using a CM cation-exchange column (GE Healthcare), followed by a Superdex75 gel filtration column (GE Healthcare). Atg3 HR was further purified using a Superdex30 gel filtration column (GE Healthcare). For in vitro conjugation assays, GST was excised from Atg3 and Atg19 with a PreScission protease (GE Healthcare). Atg3 was further purified using a DEAE anion-exchange column (GE Healthcare), followed by a Superdex75 gel filtration column. Atg19 was further purified with a glutathione-Sepharose 4B column to remove GST followed by a Superdex200 gel filtration column. $^{15}$N labeled and $^{13}$C-
15N-double labeled proteins were prepared by growing E. coli in M9 media using 15NH4Cl and 13C-glucose as the sole nitrogen and carbon sources, respectively. 2H- 15N-double labeled proteins for the transferred cross-saturation experiment were prepared by growing E. coli in 99.8% D2O M9 media using 15NH4Cl and 97% 2H6-glucose as the sole nitrogen and carbon sources.

NMR spectroscopy– NMR experiments were carried out at 298 K on a Varian UNITY INOVA 600 spectrometer. 0.3 mM 13C, 15N-labeled Atg3 HR was prepared and 1H, 13C, 15N resonance assignments of Atg3 HR were performed using following sets of spectra: [1H- 15N] HSQC, HNCO, HN(CO)CA, HNCA, H(N)COHA, HBBH(15N)H, [1H- 13C] HSQC, C(CO)NH, CCH-TOCSY, HCCH-TOCSY, HbCbCgCdHd and HbCbCgCdCeHe. Spectra were processed by NMRpipe (21) and data analyses were conducted using the Sparky program (22). The sample solution of the 15N-labeled Atg3 HR complexed with a 1.5 molar equivalent of non-labeled Atg8 K26P dissolved in 20 mM phosphate buffer (pH 6.8), 100 mM NaCl, and 5 mM dithiothreitol was prepared for chemical shift perturbation and steady state NOE measurements. Likewise, the sample solution of 15N-labeled Atg8 K26P complexed with a 1.5 molar equivalent of non-labeled Atg3 HR dissolved in 20 mM phosphate buffer (pH 6.8) and 100 mM NaCl was prepared for chemical shift perturbation studies. Chemical shift perturbations (Δppm) were calculated using a following equation: Δppm = [(ΔδHN)2 + (ΔδN/5)2]1/2, where ΔδHN and ΔδN are the differences in chemical shift between the free and complex states along the 1H and 15N axes, respectively. The transferred cross-saturation experiment was carried out using the pulse scheme (23) at 298 K on a Varian UNITY INOVA 800 spectrometer. A 0.87 mM 2H, 15N-labeled Atg8 K26P in complex with 2 mol % of the nonlabeled Atg3 was dissolved in 50 mM phosphate buffer (pH 6.8), 100 mM NaCl, and 5 mM dithiothreitol containing 10% H2O/ 90% D2O. Saturation of the aliphatic protons of Atg3 was made using the WURST-2 decoupling scheme (24). The measurement time was set at 0 ppm, and the maximum radiofrequency amplitude was 0.26 kHz for WURST-2. The measurement time was 14 h, with a relaxation delay of 1.0 s and a saturation time of 2.0 s. To evaluate the effect of the residual aliphatic protons within Atg8 K26P, a transferred cross-saturation experiment without Atg3 was also performed under the same conditions. Spectra were processed by the NMRpipe program (21), and data analyses were performed using the Sparky program (22). Intensity ratio was calculated as sat (+)/ sat (-).

In vitro pull-down assay– For the interaction study between Atg3 and Atg8, the purified GST-Atg3s (wild-type and mutants) were incubated with glutathione-Sepharose 4B beads for 15 min at 277 K, then Atg8s (wild-type and mutants) were loaded to the beads and they were further incubated for 15 min at 277 K. After washing the beads with phosphate-buffered saline (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 Coomassie Brilliant Blue (CBB) staining. For the competition study, the purified GST-Atg19 was incubated with glutathione-Sepharose 4B beads for 15 min at 277 K, then Atg8s (wild-type and mutants) were loaded to the beads and they were further incubated for 15 min at 277 K. After washing the beads with PBS, 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 CBB staining. Quantification of the results were performed using ImageJ software (25).

In vitro conjugation assay– In vitro conjugation assays were performed according to a previous report (9). For detecting Atg8–Atg3 thioester intermediates, Atg7 (1 μM), Atg3s (5 μM) and Atg8 (10 μM) were incubated in reaction buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1mM ATP, 1 mM MgCl2, and 1 mM dithiothreitol) at 303 K for 15 min. The reaction was stopped by mixing with SDS-PAGE sample buffer and boiling. The samples were subjected to Nu-PAGE 4-12% Bis-Tris gels (Invitrogen) (26) and proteins were detected by CBB staining (Fig. 3A) or immunoblotting using anti-Atg8 antibody (Fig. 6B). Liposomes were prepared as follows. All lipids were purchased from Avanti...
Polar Lipids. Dioleoylphosphatidylethanolamine and 1-palmitoyl-2-oleoylphosphatidylcholine were mixed in a glass tube at molar ratio of 2:8 in chloroform. The chloroform solvent was removed by rotary evaporation. The mixture was further dried in a desiccator under vacuum for 12 h. The resulting lipid film was hydrated in 20 mM Tris-HCl buffer (pH 8.0) at a final concentration of 1 mM phospholipid for 30 min, and suspended by sonication for 5 min to obtain small unilamellar liposomes. For detecting Atg8-PE, Atg7 (1 μM), Atg3 variants (1 μM), Atg8 (5 μM), and liposomes (400 μM lipids) were incubated in reaction buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM ATP, 1 mM MgCl₂, and 1 mM dithiothreitol) at 303 K for 1 h. The reaction was stopped by boiling the sample with SDS-PAGE sample buffer. The samples were subjected to Urea-SDS-PAGE (8), and proteins were detected by CBB staining. Quantification of the lipidation levels of Atg8 was carried out using ImageJ software.

In vivo analyses of Atg3 mutants – The ATG3 gene was disrupted with a TRP1 marker in SEY6210 (MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9) (27). This strain was transformed with centromeric plasmids, pRS315-ATG3, pRS315-ATG3 W270A, and the empty vector pRS315. These cells were grown in synthetic dextrose media supplemented with appropriate nutrients to mid-log phase and incubated in SD-N media (2% glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulfate) for 4 h to induce autophagy. The preparation of samples for SDS-PAGE and immunoblotting analyses were performed as described previously (13). ALP assay was performed with the indicator strain YTH115 (SEY6210, pho8Δ::TDH3p-pho8Δ60 atg3Δ::TRP1) transformed with the above plasmids, as described previously (28). For autophagic body observation, the vacuolar proteinase-deficient strain YTH118 (BJ2168 (Yeast Genetic Stock Center, University of California, Berkeley, CA), atg3Δ::TRP1) was used.

Vacuole preparations - Yeast vacuoles were isolated on a Ficoll step gradient as described previously (29) with minor modifications. Cells were grown to early log phase, and then 550~800 A₆₀₀ units were harvested and spheroplasted. Spheroplasts then suspended in 2.5 ml of 15% Ficoll solution were disrupted with a polycarbonate filter (3.0 μm pore, 47 mm), and the lysed cell solution was loaded in the bottom of an ultraclear SW41 tube (Beckman Coulter, Fullerton CA) and overlaid with 8%, 4% and 0% Ficoll solutions. Vacuoles were collected from the 0%/4% float interface with a Pasteur pipette after spinning for 1.5 h at 110,000 x g at 4 ºC. Vacuoles were checked under a microscope.

Activity assay of α-mannosidase (Ams1)- Cell lysates and harvested vacuoles were assayed for Ams1. Ams1 activity was determined based on the established protocol (30) with minor modifications. Samples were treated with Triton X-100 (2.5% final concentration), and the volume was then brought up to 400 μl with distilled H₂O. 100 μl of 5 x substrate mix (200 mM sodium acetate, pH 6.5, 2 mM 4-methylumbelliferyl-α-D-mannopyranoside) was added to start the reaction and the samples were then incubated for 1 h at 37 ºC. The reaction was stopped with 200 μl of 10% trichloroacetic acid, and any particulates were spun down in a microcentrifuge for 5 min. An equal volume of 1 M glycine, pH 10.4, was added to neutralize the reaction prior to fluorescence measurement at 450 nm. Results from assays for each strain were tabulated from three independent vacuole preparations.

RESULTS

Atg3 directly interacts with Atg8 through the WEDL sequence in HR – Atg8 directly interacts with Atg3 through HR (14), and Atg3 HR has a WXXL (Trp 270-Glu 271-Asp 272-Leu 273) sequence (Fig. 1A), which was shown to be a common motif, AIM, recognized by Atg8/LC3 (16,17). Thus, we postulated that the WEDL sequence in HR might be responsible for the direct interaction with Atg8. To confirm the hypothesis, we first performed an in vitro pull-down assay using wild-type and mutant Atg3s fused to GST and wild-type Atg8. GST-Atg3 can bind to Atg8 without a thioester bond (14) (Fig. 1B, lane 1). However, Atg3 W270A (Trp 270 was substituted with alanine), D272A, and L273A mutants, particularly W270A, lost their ability to bind to Atg8 (Fig. 1B, lanes 2-5; Supplementary Fig.
S4A). These data suggest that the WEDL sequence in HR is critical for Atg3-Atg8 interaction. To confirm this interaction, we performed an NMR titration experiment using 15N-labeled Atg3 HR and a non-labeled K26P mutant of Atg8 (a stable mutant with full autophagic activity) (20). A number of HN cross-peaks originated from the main-chains of Asp272 and Leu273, and the side-chain of Trp270 shifted significantly (Fig. 1C; Supplementary Fig. S1). Moreover, we measured steady-state NOE of the Atg3 HR complexed with Atg8 K26P, which suggested that residues 266-275, including the WEDL sequence, were mainly involved in the interaction with Atg8 K26P (Fig. 1D). Next, NMR titration experiments were performed using 15N-labeled Atg8 K26P and non-labeled Atg3 HR to show the interaction mode between Atg3 HR and Atg8. In the case of the interaction between Atg8 and an Atg19 peptide (Trp412-Glu413-Glu414-Leu415), the side chains of Trp412 and Leu415 interact with the two hydrophobic pockets of Atg8; one is composed of Glu17, Ile21, Pro30, Ile32, Lys48, and Leu50, and the other is composed of Tyr49, Val51, Pro52, Leu55, Phe60, and Val63 (16). In addition to these hydrophobic interactions, the side chains of Glu413 and Glu414 form ionic interactions with those of Arg28 and Arg26 of Atg8, respectively (16). When non-labeled Atg3 HR was titrated into the solution containing 15N-labeled Atg8 K26P, a number of HN cross-peaks in the HSQC spectrum of Atg8 K26P, including Val31, Lys48, Lys48, Tyr49, Leu50, Val51, Val53, and Ile64 shifted significantly (Fig. 2A; Supplementary Fig. S2). Residues with large chemical shift perturbations are plotted on the crystal structure of the Atg8-Atg19AIM complex (Fig. 2A) (16). The residues that showed large chemical shift perturbations were similar to those involved in the interaction with Atg19AIM, and all cross-peaks were perturbed in a dose-dependent manner. These NMR data suggest that the Atg8-Atg3 HR interaction is quite similar to the Atg8-Atg19AIM interaction (16). Moreover, to show the interaction mode between intact Atg3 and Atg8, a transferred cross-saturation experiment (31) was performed using D2H, 15N-labeled Atg8 K26P, and non-labeled Atg3. Signal intensity ratio was calculated based on the spectra with and without irradiation. The result showed that the intensities of the residues in β1, β2, and α3, including Val41, Lys48, Tyr49, Val51, Phe60, and Val53 were significantly decreased (Fig. 2B; Supplementary Fig. S3). No residues were affected on the back of the molecule. These attenuated residues coincided with the residues that showed large chemical shift perturbations in the titration experiments using 15N-labeled Atg8 K26P and non-labeled Atg3 HR (Fig. 2, A,B), clearly showing that the direct interaction between Atg3 HR and Atg8 is also maintained in the full-length Atg3-Atg8 interaction. We further performed in vitro pull-down assays to determine which residue of Atg8 is important for the interaction. As expected, Atg8 R28A, L50A, P52A, and R67A mutants, which were defective in the interaction with Atg19AIM (16) (our unpublished results), were severely impaired in binding to Atg3 (Fig. 2C; Supplementary Fig. S4B). These results clearly indicate that the WEDL sequence of Atg3 HR is a functional AIM, and that Atg3AIM directly interacts with Atg8 in a manner similar to Atg8-Atg19AIM and LC3-p62AIM interactions. Atg3AIM is responsible for the lipidation of Atg8 but not for the formation of the Atg8-Atg3 thioester intermediate—After Atg3 receives Atg8 from the Atg7~Atg8 thioester to form Atg3~Atg8 thioester, it transfers Atg8 to the amino group of PE. Although Atg3AIM was shown to be essential for the direct interaction between Atg3 and Atg8, its significance in Atg3 functions is elusive. To elucidate the significance of Atg3AIM in the Atg8 system, in vitro conjugation assays were performed using wild-type and W270A mutant Atg3s. Purified Atg7, Atg8, and wild-type or mutant Atg3 were incubated with ATP at 303 K for 15 min. Atg3 W270A formed Atg8~Atg3 thioester intermediates, which reached a plateau within 15 min as in the case of wild-type Atg3 (Fig. 3A; Supplementary Fig. S4C). These results show that the interaction of Atg8 with Atg3AIM is by guest on November 7, 2017 http://www.jbc.org/ Downloaded from
crucial for the efficient transfer of Atg8 from Atg3 to PE. *Atg3*<sup>AIM</sup> is dispensable for Atg8 lipidation in the presence of Atg12-Atg5 conjugates. To investigate the *in vivo* significance of *Atg3*<sup>AIM</sup> in Atg8 lipidation, we next examined the accumulation of Atg8-PE conjugates in *atg3*<sup>Δ</sup> cells expressing Atg3 W270A. Unexpectedly, the accumulation of Atg8-PE conjugates in *atg3*<sup>Δ</sup> cells expressing Atg3 W270A was comparable with that expressing wild-type Atg3 under both nutrient-rich and starvation conditions (Fig. 4A, Supplementary Fig. S4D). This suggests that Atg3<sup>AIM</sup> is dispensable for Atg8 lipidation *in vivo*. Since *in vivo* conditions contain Atg12-Atg5 conjugates that promote Atg8 lipidation, we next performed *in vitro* conjugation assays in the presence of Atg12-Atg5 conjugates, which showed that the lipidation level of Atg8 using Atg3 W270A was comparable with that using wild-type Atg3 (Fig. 4B, Supplementary Fig. S4E). These data suggest that Atg3<sup>AIM</sup> is dispensable for Atg8 lipidation in the presence of Atg12-Atg5 conjugates.

*Atg3*<sup>AIM</sup> is important for the Cvt pathway but not for starvation-induced autophagy. We next examined whether Atg3<sup>AIM</sup> has a role in autophagic processes. The activity of starvation-induced autophagy was estimated by an alkaline phosphatase (ALP) assay (28). The ALP assay monitors the transport of ALP from the cytoplasm into the vacuole via autophagy by measuring the activity of alkaline phosphatase, which is artificially expressed in the cytosol as a proform and transported via autophagy into the vacuole, where it is processed into the mature form. *atg3*<sup>Δ</sup> cells expressing Atg3 W270A showed autophagic activity comparable with that expressing wild-type Atg3 under starvation (Fig. 5A), suggesting that Atg3<sup>AIM</sup> is dispensable for starvation-induced autophagy. Similar results were obtained when autophagy was induced by rapamycin treatment (Supplementary Fig. S5A). We then observed the autophagic bodies accumulated in vacuoles under starvation using an optical microscope, which also suggested that Atg3<sup>AIM</sup> is dispensable for starvation-induced autophagy (Supplementary Fig. S5B). Next, we monitored the maturation of the proform of Ape1, which is selectively transported into the vacuole via the Cvt pathway under nutrition-rich conditions. The protein is then processed into a mature form within the vacuole. Ape1 maturation in *atg3*<sup>Δ</sup> cells expressing Atg3 W270A was significantly lower than in those expressing wild-type Atg3 (Fig. 5B). Moreover, we measured the activity of Ams1 recovered in the vacuole, which is another selective cargo protein transported to the vacuole via the Cvt pathway. The activity of Ams1 recovered in the vacuole of *atg3*<sup>Δ</sup> cells expressing Atg3 W270A was much lower than that expressing wild-type Atg3 (Fig. 5C). These results suggest that Atg3<sup>AIM</sup> is important for the Cvt pathway.

*Atg3*<sup>AIM</sup> is important for Atg8 lipidation in the presence of Atg19. *In vivo* experiments showed that Atg3<sup>AIM</sup> is dispensable for Atg8 lipidation and starvation-induced autophagy. Nevertheless, Atg3<sup>AIM</sup> was shown to be important for the Cvt pathway. In the Cvt pathway, Atg19, the receptor protein for Ape1, directly interacts with Atg8 through the C-terminal AIM (Trp<sup>412</sup>-Glu<sup>413</sup>-Glu<sup>414</sup>-Leu<sup>415</sup>) and this interaction is crucial for the Cvt pathway. Since Atg19<sup>AIM</sup> may compete with Atg3<sup>AIM</sup> for Atg8 binding, we then examined the effects of Atg19 on Atg3-Atg8 interaction and on Atg8 lipidation *in vitro*. *In vitro* pull-down assays showed that Atg8 binding to Atg19 was attenuated by Atg3 in a dose-dependent manner (Fig. 6A, left; Supplementary Fig. S4F). This attenuation was less observed when Atg3 W270A instead of wild-type Atg3 was used (Fig. 6A, right; Supplementary Fig. S4F). These results indicate that Atg19<sup>AIM</sup> competes with Atg3<sup>AIM</sup> for Atg8 binding. Next, *in vitro* conjugation reactions were performed using wild-type or the W270A mutant of Atg3 in the presence of Atg19. Formation of the Atg8~Atg3 thioester intermediate was not affected by Atg19 using both wild-type and W270A Atg3 (Fig. 6B). In contrast, lipidation of Atg8 using wild-type Atg3 was slightly inhibited by Atg19, and that using Atg3 W270A was significantly inhibited by Atg19 in a dose-dependent manner (Fig. 6C,D). These results suggest that Atg3<sup>AIM</sup> is important for Atg8 lipidation in the presence of Atg19.

**DISCUSSION**

In this study, we examined the direct interaction...
between Atg8 and Atg3 by NMR analyses. NMR data, together with in vitro pull-down assays, showed that the direct Atg8-Atg3 interaction is mediated by AIM in Atg3 HR, and that the interaction between Atg8 and Atg3\textsuperscript{AIM} is quite similar to the Atg8-Atg19\textsuperscript{AIM} and LC3-p62\textsuperscript{AIM} interactions. Furthermore, in vitro conjugation assays showed that this interaction is important for the formation of Atg8-PE conjugates but not for the formation of Atg8-Atg3 thioester intermediates. Because the Atg8-Atg3 thioester intermediate is formed on Atg7, and Atg7 should directly interact with both Atg8 and Atg3, the Atg8-Atg3\textsuperscript{AIM} interaction may be not required for Atg8-Atg3 thioester formation. In contrast, the Atg8-Atg3\textsuperscript{AIM} interaction contributed to the efficient formation of Atg8-PE conjugates, suggesting that the interaction promotes the transfer of Atg8 from the Atg8-Atg3 thioester intermediate to PE. In the case of SUMO, a ubiquitin-like modifier, Nup358/RanBP2 acts as an E3 by binding both SUMO and Ubc9, the E2 enzyme for SUMO, to position the SUMO-Ubc9 thioester at optimal orientation to enhance the conjugation of SUMO to its target protein (32). The SUMO interacting motif in the N-terminal region of Nup358/RanBP2 forms an antiparallel \(\beta\)-sheet with SUMO \(\beta\)2, resulting in reduced conformational flexibility of the SUMO-Ubc9 complex, thus preventing non-productive SUMO-Ubc9 conformation and aligning the complex and thioester for SUMO transfer. Similarly, Atg3\textsuperscript{AIM} forms a parallel \(\beta\)-sheet with Atg8 \(\beta\)2, indicating the possibility that Atg8-Atg3\textsuperscript{AIM} interaction may reduce the conformational flexibility of the Atg8-Atg3 thioester intermediate and regulate the orientation of each protein for the efficient lipidation of Atg8.

In contrast to the reduced conjugating activity of Atg3 W270A mutant in vitro, the same mutant retained activity comparable with that of wild-type Atg3 in vivo. This discrepancy can be attributed to the existence of Atg12-Atg5 conjugates in vivo. Atg12-Atg5 conjugates are constitutively expressed and facilitate the lipidation of Atg8 in vivo. An in vitro conjugation assay in the presence of Atg12-Atg5 conjugates showed that promotion of Atg8 lipidation by Atg3\textsuperscript{AIM} was superseded by Atg12-Atg5 conjugates (Fig. 4B). This observation suggests that the E3-like functions of Atg12-Atg5 conjugates may include the E3-like activity of Atg3\textsuperscript{AIM}, reducing the conformational flexibility of Atg8-Atg3 thioester and regulating the orientation of each protein for the efficient lipidation of Atg8.

Although the importance of Atg3\textsuperscript{AIM} in Atg8 lipidation was not validated in vivo, it was unexpected to find that Atg3\textsuperscript{AIM} is important for the Cvt pathway. This observation is consistent with the fact that Atg3\textsuperscript{AIM} is conserved among yeasts (Fig. 1A) but not among higher eukaryotes, and that the Cvt pathway has been identified only in yeasts. In vitro conjugation assays in the presence of Atg19 suggest that Atg3\textsuperscript{AIM} attenuates the inhibitory effect of Atg19 on Atg8 lipidation (Fig. 6C, D). This is attributed to the competition between Atg3\textsuperscript{AIM} and Atg19\textsuperscript{AIM} for Atg8 binding (Fig. 6A; Supplementary Fig. S4F). Then, what is the role of Atg3\textsuperscript{AIM} in the Cvt pathway? During the Cvt pathway, the cargo proteins (mainly Ape1) facilitate receptor recruitment and vesicle formation rather than the forming vesicle concentrates the cargo proteins (33). This is in contrast to the case of starvation-induced autophagy, in which the formation of autophagosomes is independent of cargo proteins. Therefore, it is likely that Cvt vesicle formation proceeds along the surface of the Ape1-Atg19 complex. During this process, Atg8 plays two pivotal roles: one is recognizing Atg19, and the other is expanding the membranes of the Cvt vesicle. It is not clear how Atg8 exerts these two functions simultaneously when the active sites overlap (13,16,34). Although current knowledge is restricted, the following model can be proposed to clarify these observations and the crucial role of Atg3\textsuperscript{AIM} in the Cvt pathway (Fig. 7). First, a PE-free form of Atg8 binds to the Atg19-Ape1 complex. Next, Atg3 and unknown membranes are recruited to the complex and Atg3 conjugates Atg8 to PE in these membranes, during which Atg3\textsuperscript{AIM} plays a crucial role by releasing Atg8 from Atg19\textsuperscript{AIM}. Finally, Atg8-PE functions for membrane expansion. A low concentration of Atg19 around forming autophagosomes may be the reason why Atg3\textsuperscript{AIM} is dispensable for starvation-induced
autophagy. Whatever the case, further studies are required to elucidate the molecular events during the Cvt pathway and autophagy. The most critical issues are when and where Atg8-PE conjugates form.

AIMs were initially identified in the autophagic receptors Atg19 and p62 (16,18,35), and later they were also identified in mitophagic receptors Atg32 and Nix, as well as NBR1, another receptor for ubiquitinated protein aggregates (36-38). Therefore, AIMs appear to be widely utilized in autophagic receptors for interaction with Atg8/LC3. We have recently shown that LC3 specifically recognizes the YEDL sequence in the N-terminal tail of Atg4B in a similar manner to Atg8-Atg19AIM and LC3-p62AIM interactions, showing that the YEDL sequence in Atg4B is an AIM (39). Here, we showed that Atg3 also has an AIM, and utilizes the motif for interaction with Atg8. These observations suggest that AIMs are utilized not only in autophagic receptors, but also in enzymes modifying Atg8/LC3, and exert crucial roles in autophagosome formation.

REFERENCES


FOOTNOTES

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Abbreviations: AIM, Atg8-family interacting motif; E1, ubiquitin-activating enzyme; E2, ubiquitin-carrier protein; E3, ubiquitin-protein isopeptide ligase; PE, phosphatidylethanolamine; GST, glutathione-S-transferase; HSQC, heteronuclear single quantum correlation; PBS, phosphate-buffered saline; CBB, Coomassie Brilliant Blue; HR, the handle region; SUMO, small ubiquitin-like modifier.

FIGURE LEGENDS

Fig. 1. The WEDL sequence of Atg3 HR is responsible for the Atg8-Atg3 interaction. *A*. Multiple sequence alignment of Atg3 HR among yeast Atg3 homologues. Asterisk, colon, and period indicate perfectly, highly, and moderately conserved residues, respectively. The WEDL sequence is enclosed by a square. Sc, *Saccharomyces cerevisiae*; Kl, *Kluyveromyces lactis*; Ag, *Ashbya gossypii*; Pp, *Pichia pastoris*. *B*. *In vitro* pull-down assay between GST-fused Atg3s and Atg8. *C*. 15N-labeled Atg3 HR was titrated with non-labeled Atg8 K26P up to 1.5 molar equivalent, and chemical shift perturbations (Δppm) were calculated using following equation: Δppm = [(ΔδHN)² + (ΔδN/5)²]1/2. The main-chain of Trp270, Glu271, Asp272 and Leu273 is colored red and that of the other HR residues is colored gray. The side-chain of Trp270 is colored cyan. *D*. 1H-15N steady state NOE of Atg3 HR. The coloring is the same as in *C*.

Fig. 2. The Atg8-Atg3AIM interaction is similar to the Atg8-Atg19AIM interaction. *A*. Chemical shift perturbation experiments between Atg8 and Atg3 HR. 15N-Atg8 K26P was titrated with non-labeled Atg3 HR up to 1.5 molar equivalent, and chemical shift perturbations (Δppm) were calculated using the equation in Fig. 1C. The residues with high Δppm values are colored red (>0.4) and yellow (>0.2), respectively, and mapped on the structure of the Atg8-Atg19AIM complex (PDB ID 2ZPN), in which the Atg19 peptide is shown with a stick model. The overlaid seven HSQC spectra are shown in Fig. S2. *B*. Plot of the intensity ratios of the crosspeaks in the transferred cross-saturation experiments...
between Atg8 and full-length Atg3. The intensity ratio was calculated as sat (+) / sat (-). The ratios for Thr4, Phe5, Lys6, Arg47, Asp102, and Phe104 were not available. The residues with low intensity ratio are mapped on the structure of the Atg8-Atg19\textsuperscript{AIM} complex, in which intensity ratios below 0.5, 0.6, and 0.7 are colored red, orange, and yellow, respectively. 

\textit{C}. In vitro pull-down assay between GST-fused Atg3 and Atg8 mutants.

\textbf{Fig. 3.} Atg8-Atg3\textsuperscript{AIM} interaction is responsible for the formation of Atg8-PE conjugates in vitro. \textit{A}. Purified Atg3s, Atg7, and Atg8 were incubated with ATP for 15 min at 303K. Then, samples were separated by NuPAGE without reduction, and Atg8--Atg3 thioester intermediates were detected by CBB staining. \textit{B}. Purified Atg3s, Atg7, Atg8, and PE-containing liposomes were incubated with ATP for 1 h at 303K. Then, samples were separated by Urea-SDS-PAGE, and Atg8-PE was detected by CBB staining. \textit{C}. The Atg8 and Atg8-PE bands at 60 min in \textit{B} were quantified and the conjugation formation was calculated by dividing the Atg8-PE amount by the total Atg8 amount. The values and the error bars are the means and the standard deviations of three independent experiments, respectively.

\textbf{Fig. 4.} Atg8-Atg3\textsuperscript{AIM} interaction is dispensable for Atg8 lipidation in the presence of Atg12-Atg5 conjugates. \textit{A}. The expression level of the Atg3 mutant protein, and lipidation of Atg8 in vivo. \textit{atg3}\textsuperscript{Δ} yeast cells carrying centromeric plasmids that expressed wild type Atg3 (WT) or its mutant form (W270A) or the empty vector were grown to mid-log phase (nutrient rich), and then incubated in nitrogen-deprived media for 4.5 h (starvation). Proteins were extracted by alkaline treatment and separated by SDS-PAGE, followed by immunoblotting with antibodies against Atg3 and Atg8. \textit{B}. Purified Atg3s, Atg7, Atg8, and PE-containing liposomes were incubated with ATP for 1 h at 303 K, with or without 0.2 µM Atg12-Atg5-Atg16N. Samples were separated by Urea-SDS-PAGE, and detected by CBB staining.

\textbf{Fig. 5.} Atg8-Atg3\textsuperscript{AIM} interaction is crucial for the Cvt pathway but not for starvation-induced autophagy. \textit{A}. The ALP assay of the Atg3 mutant. Wild type Atg3 and the W270A mutant were expressed from centromeric plasmids in an indicator strain for the ALP assay, and their autophagic activities were measured under nutrient rich (starvation 0 h) and starvation (4.5 h) conditions. The experiments were repeated three times, and the averages of the results are shown with error bars for the standard deviations. \textit{B}. The Cvt pathway in the Atg3 mutant. The maturation of Ape1 in yeast cells expressing wild type Atg3 or the W270A mutant under nutrient rich conditions was examined by immunoblotting with anti-Ape1 as described in Fig. 4\textit{A}. The bands of the precursor and mature forms of Ape1 were quantified to calculate the maturation efficiency, and the average values obtained from three independent experiments are shown with error bars for the standard deviations. \textit{C}. Activity assay of Ams1. Vacuoles were isolated on Ficoll step gradients and the Ams1 activity was measured as described in “Experimental Procedures”. The percentage of recovery was calculated by dividing the total enzyme activity recovered in the vacuole fraction by the total activity loaded on the gradient to obtain the vacuole fraction. The experiments were repeated three times, and the averages of the results are shown with error bars for the standard deviations.

\textbf{Fig. 6.} Atg3\textsuperscript{AIM} is required for efficient Atg8 lipidation in the presence of Atg19. \textit{A}. In vitro pull-down assay between GST-fused Atg19 and Atg8 in the presence of various amounts of wild-type Atg3 (left) or Atg3 W270A (right). Values in eq above the panels mean the molar ratio of Atg3 to GST-Atg19. \textit{B}. Purified Atg3s, Atg7 and Atg8 were incubated for 15 min at 303 K in the presence or absence of 50 µM Atg19. Samples were subjected to NuPAGE followed by immunoblotting using anti-Atg8 antibody. \textit{C}. Purified Atg3s, Atg7, Atg8, and PE-containing liposomes were incubated with ATP for 1 h at 303 K in the presence of 0–10 µM Atg19. Then samples were subjected to Urea-SDS-PAGE, and the protein bands were detected by CBB staining. \textit{D}. The Atg8 and Atg8-PE bands at 1 hr in \textit{C} were quantified and the conjugation formation was calculated by dividing the Atg8-PE amount by the total.
Atg8 amount. The values and the error bars are the means and the standard deviations of three independent experiments, respectively.

**Fig. 7.** Proposed model of Atg3\textsuperscript{AIM} function in the Cvt pathway. After translation in the cytosol, precursor Ape1 (prApe1) self-assembles into a huge complex (Ape1 complex; gray), and Atg19 (cyan) and Atg8 (green) coat the complex through direct prApe1-Atg19 and Atg19\textsuperscript{AIM}-Atg8 interactions. After activation of Atg8 by Atg7 (red), Atg8-Atg19\textsuperscript{AIM} interaction is replaced by Atg8-Atg3\textsuperscript{AIM} interaction, and Atg8 is conjugated to PE in the membrane of unknown origin, which might play a crucial role in expanding the isolation membrane in the proximity of the Ape1 complex.
FIGURE 1.

A

α-helix F

ScAtg3 241 ILLDVRVVRQRRKE----LQEMLQDGVSDDLQDIDDSLR 282
KlAtg3 238 VLMEKVRSRSR--------ARKVTDQTDKDEDQDVDDGLR 274
AgAtg3 217 VLMEKAAKQKP--------VEEEQPDQREDQIDVEVDSILR 254
PpAtg3 241 VLMARATQRSIQUALASSTGVKHLQIAHNDNLQWEDDNTSDETTQGIR 287

B

Elute

Atg3 mutants

GST-Atg3

Atg8

Input

C

D

$\Delta$ppm

0

0.2

0.4

0.6

0.8

1.0

1.2

W

ε

K

E

E

E

L

Q

Q

D

D

D

G

V

W

I

S

270 280 260

Elute by guest on November 7, 2017 http://www.jbc.org/ Downloaded from

Wc
FIGURE 2.

A

B

C

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</tr>
<tr>
<td>R28A</td>
</tr>
<tr>
<td>K46A</td>
</tr>
<tr>
<td>L50A</td>
</tr>
<tr>
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</tr>
<tr>
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FIGURE 4.

A

nutrient rich

starvation

WT

vector

W270A

WT

vector

W270A

B

Atg12-Atg5-Atg16N

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</table>

Atg8

Atg8-PE

Atg8

Atg8-PE
FIGURE 5.

A

B

C

WT vector W270A

ALP activity (U/mg-protein)

WT  vector  W270A

nitrogen starvation

0 hr  4.5 hr

Ape1 maturation (%)

pApe1  mApe1

Recovery in vacuole fraction (%)

WT  vector  W270A
FIGURE 6.

A

Atg3 WT
GST-Atg19
Elute(Atg8)

Atg3 W270A
GST-Atg19
Elute(Atg8)

B

WT
W270A

Atg3
Atg19

incubation time [min]

IB: α-Atg8

C

WT
W270A

Atg3
Atg19 [µM]

Conjugation formation [%]

D

- Atg19
+ 1µM Atg19
+ 2µM Atg19
+ 5µM Atg19
+ 10µM Atg19

Conjugation formation [%]
FIGURE 7.

Apel complex

PE conjugation

Membrane expansion
Autophagy-related protein (Atg) 8-family interacting motif in Atg3 mediates the Atg3-Atg8 interaction and is crucial for the cytoplasm-to-vacuole targeting pathway
Masaya Yamaguchi, Nobuo N. Noda, Hitoshi Nakatogawa, Hiroyuki Kumeta, Yoshinori Ohsumi and Fuyuhiko Inagaki

J. Biol. Chem. published online July 8, 2010

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