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*In vivo* and *in vitro* Reconstitution of Atg8 conjugation essential for autophagy

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Running title: Reconstitution of Atg8 lipidation mediated by ubiquitin-like system.
Summary

In an analogous manner to protein ubiquitination, carboxy terminus of Atg8p, yeast protein essential for autophagy, conjugates to a head group of phosphatidylethanolamine via an amide bond. Though physiological role of this reaction is assigned to membrane organization during autophagy, its molecular details are still unknown. Here, we showed that E. coli cells co-expressed Atg8p, Atg7p (E1), and Atg3p (E2) allowed to form conjugate of Atg8p with endogenous PE. Further, we established an in vitro Atg8p-PE reconstitution system using purified Atg8pG116, Atg7p, Atg3p, and PE-containing liposomes, demonstrating that the Atg7p and the Atg3p are minimal catalysts for Atg8p-PE conjugate reaction. Efficiency of this lipidation reaction depends on the state of the substrate, PE (phospholipid bilayer and its lipid composition). It is also suggested that the lipidation induces a conformational change in the N-terminal region of Atg8p. In vitro system developed here will provide a powerful system for further understanding the precise role of lipidation and interaction of two ubiquitin-like systems essential for autophagy.
Introduction

Autophagy, an intracellular bulk degradation system in the lysosomes/vacuoles of eukaryotic cells is necessary for the recycling of cytoplasmic components for survival during nutrient starvation conditions (1-3). Membrane dynamics in autophagy are distinct from classical membrane trafficking. In particular, the mechanism by which this new double membrane-bounded compartment, the autophagosome, is formed has remained poorly understood. Genetic screens of the budding yeast, *Saccharomyces cerevisiae*, have identified 16 genes potentially involved in this process, named *ATG* genes (formerly *APG/AUT*) (4). Dissection of autophagosome biogenesis through intensive characterization of these gene products Atgps (1) has revealed that the overall process of autophagy in yeast is similar to that in higher eukaryotes, exhibiting conservation of the molecular machinery involved (5).

Our recent studies have identified two ubiquitin-like conjugation systems essential for autophagosome formation; these systems utilize approximately half of the Atg protein members (6-8). Atg12p, a ubiquitin-like protein, is covalently linked to Atg5p through an isopeptide bond between the C-terminal glycine of Atg12p and Lys149 of Atg5p by sequential reactions catalyzed by Atg7p (E1) and Atg10p (E2) (6,9,10). Atg12p-Atg5p conjugates then associate with Atg16p to form a multimeric complex, Atg12p-Atg5p-Atg16p, mediated by Atg16p homo-oligomerization (11).

A second ubiquitin-like protein, Atg8p, that was originally reported as a microtubule-associated protein (Aut7p) (12), is conjugated to a membrane phospholipid,
phosphatidylethanolamine (PE) (7). The C-terminal arginine of newly synthesized Atg8p (Atg8pR117) is initially removed by the Atg4p protease to expose a C-terminal glycine residue (Atg8pG116) (13). Atg8pG116 is then activated by Atg7p (E1) and transferred to Atg3p (E2) (7). Finally, the Atg8pG116 conjugates to PE through an amide bond between its C-terminal glycine and the amino group of PE (7). Atg8p-PE is tightly associated with membranes, behaving as an integral membrane protein. Notably, Atg8 conjugation system, while similar to ubiquitination mechanically, utilizes a ubiquitous phospholipid, not a protein, as a target. Liberation of Atg8p moiety from Atg8p-PE by the action of Atg4p (deconjugation) is required for the normal progression of autophagy (13). The mammalian Atg8p homologues, MAP1-LC3, GATE-16, and GABARAP, have been implicated in membrane dynamics, including autophagy, intra-Golgi transport, and GABA receptor sorting to the postsynaptic membrane (14-16). These Atg8p homologues are also modified via a ubiquitin-like system analogous to Atg8p lipidation (14,17-19).

We have observed a small number of autophagosome-like structures of abnormal morphology in the null mutant of \( ATG8 \) (20). While Abeliovich et al. reported that significantly smaller vesicles than normal autophagosome were observed in a similar \( \Delta atg8 \) strain (21). These observations imply that the Atg8p-PE may function in normal development of autophagosomal membrane. In fact, previous immunoelectron microscopic analyses demonstrated the specific localization of Atg8p to the isolation membrane (intermediate structure of autophagosome) under the starvation conditions (20).
The precise roles of the two conjugates during autophagosome formation remain unclear. Atg8p-PE conjugation is severely reduced in the absence of the Atg12p-Atg5p conjugate. Thus, it appears likely that the functions of these two conjugates, Atg8p-PE and Atg12p-Atg5p, are closely related. To identify the molecular events governing autophagosome formation, we need to address not only the function of each of the two conjugates, but also the interrelationship between Atg8p-PE and Atg12p-Atg5p.

In this study, to address the molecular machinery involved in membrane dynamics, we focused on Atg8p lipidation, and developed in vitro reconstitution system of Atg8p-PE.
Experimental Procedures

Strains media and standard molecular genetical methods

The *Eschericia coli* strains XL1Blue and BL21 (DE3) were used for plasmid construction and protein expression, respectively. Total lipid extracts from the *E. coli* strain W3011 and a PE-deficient strain, GN10, were used to make liposomes. *E. coli* transformants were grown in Luria-Bertani (LB) medium at 37 °C, supplemented with the appropriate antibiotics (ampicillin, 50 µg/ml; chloramphenicol, 20 µg/ml). GN10 was grown in LB medium containing 50 mM MgCl₂ at 37 °C (22). Total cell lysates from the *Saccharomyces cerevisiae* strain SEY6210 (*MATα*leu2-3 112 ura3-52 trp1-Δ901 his3-Δ200 ade2-101 lys2-801 suc2-Δ9) and its derivatives (*Δatg7::HIS3* and *Δatg3::TRP1*) were used as Atg8p-PE mobility standards in western blot analyses. Total lipid extracts from SEY6210 was used for making liposomes. Yeast cells were grown in YEPD (1% yeast extract, 2% polypepton, 2% glucose) at 30 °C. Molecular biological procedures were performed in accordance with standard procedures (23).

Plasmid construction and protein expression in *E. coli*

To construct *E. coli* expression plasmids encoding Atg7-Myc (C-terminal 3x Myc-tagged Atg7), Atg3, Atg8G116 (C-terminal glycine-exposed form of Atg8), and Atg8R117 (C-terminal arginine-containing form of Atg8), the appropriate genes were amplified from the following plasmids, pRS424Atg7-Myc, pRS426Atg3, and pRS426Atg8, by PCR using the following primers: (ATG7-MYC) KpnI-SD (Fw), 5’-
GGGGTACCCCAGGAGGAATTCACCATGTCGTCAGA-3’, SmaI (Rv), 5’-
TCCCCCGGGGAATGCAAAATATTA-3’, (ATG3) SmaI-SD (Fw), 5’-
TCCCCGGGGAAGGAGGAATTACCATGTTAGATC-3’, BamHI (Rv), 5’-
CGGGATCCCGTTACCAAACCTTC-3’, (ATGS8G116) BglII-SD (Fw), 5’-
GAAGATCTTCAGGAGGAATTACCATGAATGTCTAC-3’, XbaI (Rv), 5’-
GCTCTAGAGCTAGCCAAATGTATTTTC-3’. (ATGS8G117) BglII-SD (Fw), 5’-
GAAGATCTTCAGGAGGAATTACCATGAATGTCTAC-3’, HindIII-SD (Rv), 5’-
CCCAAGTTGGGCTAGCCAAATGTATTTTC-3’. The resulting PCR products all contained a Shine-Dalgarno sequence (SD) upstream of the start codon to facilitate efficient translation in E. coli. The amplified genes were subcloned into pUC18, then inserted into the multiple cloning sites of the arabinose-inducible plasmids, pBAD18 and pBAD33 (24). pBAD18 includes gene expression via the PBAD promoter, expresses AraC, confers Amp’ (ampicillin resistance), and contains a pBR origin. pBAD33 includes gene expression via the PBAD promoter, expresses AraC, confers Cm’ (chloramphenicol resistance), and contains a pACYC origin (Diagram showed in Fig. 1A). Atg7<sup>C507S</sup>-Myc and Atg3<sup>C234S</sup> mutant forms were encoded by derivatives of the pBAD plasmids, containing single amino acid substitutions from cysteine to serine within the active centers of Atg7 and Atg3, respectively. These plasmids were constructed using a QuikChange site-directed mutagenesis kit (Stratagene) using the following mutagenesis oligonucleotides; (ATG7<sup>C507S</sup>) (Fw), 5’-
ACTTTGGATCAAATGTCGACAGTAACTAGACC-3’, (Rv), 5’-
GGGTCTAGTTACTGTCGACATTGGAGTCCAAAGT-3’, (ATG3<sup>C234S</sup>) (Fw), 5’-
GTTTCCATTCTCAGGAAGCAAGCATGCTAATGTA-3', (Rv), 5’-
TACATTAGCATGCT TGCTTGGATGAATGGAAAC-3'. BL21 (DE3) cells were
transformed with pBAD-Atg7-Myc and either pBAD-C-Atg8G116, pBAD-C-Atg3Atg8R117,
or pBAD-C-Atg3Atg8G116. Transformed cells were grown to an OD600 of 0.5 prior to
the addition of 0.2% arabinose to the cultures. After induction for 1 h, cells were
harvested and lysed in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1 mM
DTT and 1 mM PMSF by sonication (2.0 Odunit cells/100 μl). For western blotting,
cell lysates (equivalent to 0.04 Odunit cells) were subjected to SDS-PAGE containing 6
M urea (urea-SDS-PAGE).

Protein purification

To purify the recombinant Atg proteins, we constructed Glutathion-S-
transferase (GST) fusions of the desired Atgs (GST-Atg7-Myc, GST-Atg3, GST-
Atg3C234S and GST-Atg8G116) by insertion of PCR products into the cloning site (BamHI/
SalI) of pGEX4-T-1 (Amersham Pharmacia Biotech). Following introduction of the
obtained plasmids into BL21 (DE3) E.coli, expression of each protein was induced by
addition of 0.05 mM isopropyl β-D(-)-thiogalactopyranoside (IPTG) to the medium.
After induction, cells were disrupted by sonication in TBST (50 mM Tris-HCl, pH 7.5,
150 mM NaCl, 1% Triton X-100) containing 2 mM DTT, and 1 mM
phenylmethylsulfonylfluoride (PMSF). After removal of cell debris by centrifugation
at 10,000 rpm for 10 min, obtained cell lysates were incubated with 1 ml glutathion-
Sepharose 4B (50% slurry in TBST) (Amersham Pharmacia Biotech) for 30 min at 4 °C.
to collect recombinant proteins. The proteins bound to the Sepharose beads were washed with TBST, then suspended in 400 μl TBS containing 2 mM DTT. The GST fused Atg proteins were treated with thrombin (10 U) at 20°C for 1 h; the glutathion-Sepharose 4B/GST complexes were then removed by centrifugation. Protein concentrations were determined by Bradford’s methods (PIERCE) using bovine serum albumin as a standard. The purified Atg proteins were then diluted in TBS containing 50% glycerol for storage at -30°C.

**Extraction of total lipids and liposome preparation**

W3011 and GN10 *E. coli* strains were grown in LB medium containing 50 mM MgCl₂ to stationary phase. SEY6210 yeast strain was grown in YEPD to stationary phase. Both types of cells were harvested (cells wet-weight 10 g) and total lipids were extracted by Bligh and Dyer’s methods (25). The total lipids were dissolved in 10 ml chloroform, and the concentration of total phospholipids was quantified by a phosphorus assay using phospho-molybdate reaction (26). Individual phospholipids were separated by two-dimensional thin-layer chromatography, and the composition of phospholipids was determined by phosphorus assay (W3011; PE/PG/CL/PA = 75.4 : 16.6 : 4.3 : 3.57, GN10; PE/PG/CL/PA = < 0.01 : 52.6 : 33.6 : 11.4, SEY6210; PE/PC/PS/PI= 16.6 : 51.7 : 13.7 : 18.0).

Total lipids from *E. coli* and yeast were used to generate *E. coli* and yeast total lipid liposomes, respectively. To prepare liposomes with various phospholipid compositions, phospholipids were mixed in the appropriate ratios from stocks dissolved.
in chlorohorm as described above. Phosphatidylethanolamine (PE) from *E. coli*,
dioleoylphosphatidylethanolamine (DOPE), 1-palmitoyl-2-oleoylphoshatidyldcholine
(POPC), phosphatidylglycerol (DOPG), phosphatidylinositol (PI) from Bovine liver,
and dioleoylphosphate (DOPA), dioleoylphosphatidylerine (DOPS) were purchased
from Avanti Polar Lipids. After transfer to a glass tube, the chloroform solvent was
removed by rotary evaporation. Samples were dried further in a desiccator under
vacuum for 12 h. The resulting lipid film was suspended in a buffer (25 mM Tris-HCl,
pH 7.5, 137 mM NaCl, 2.7 mM KCl) at a final concentration of 1 mM phospholipids by
vortexing at room temperature. Samples were then subjected to sonication for 5 min to
obtain small unilamellar liposomes.

**Reconstitution of Atg8p-PE conjugation system in vitro**

*In vitro* reconstitution of Atg8 system was performed using purified Atg
proteins and liposomes. Liposomes were mixed with purified Atg7p-Myc, Atg3p (or
Atg3p^{C^{234}S}), and Atg8p^{G^{116}} in reconstitution buffer (50 mM Tris-HCl, pH 8.0, 100 mM
NaCl, 0.2 mM DTT) in the presence of an ATP regeneration system (1 mM ATP
(Sigma), 1 mM MgCl$_2$, 5 mM phosphocreatine (Sigma), and 2.5 μg creatine kinase
(Roche), pH 7.0). The reaction mixture (with a final pH of 7.6) was incubated at 30 °C
for 10-60 min.

**Antibodies and immunoblotting**

Polyclonal antibodies against full-length Atg3p and against the Atg8p N-
terminal peptide have been previously described (7). A polyclonal antibody against full-length Atg8p and a monoclonal antibody against Myc (9E10) were purchased from Rockland and BabCo, respectively.

SDS-PAGE was performed according to Laemmli’s method. SDS-PAGE separation in the presence of 6 M urea (urea-SDS-PAGE gel) was used to distinguish Atg8p and Atg8p-PE conjugates, as described (13). To visualize the protein bands within the gel, SDS-PAGE gels were stained using GelCode Blue (PIERCE). For immunoblot analyses, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobolon-PSQ, Millipore). Immunoblotting analysis was performed using rabbit or mouse antibodies against the specified proteins, then visualized using either peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG. The immunoreactive protein bands were detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences)

Immunoprecipitation

In vitro Atg8p-PE conjugation was performed as described in the above. The Atg8p-PE bound liposomes were collected by centrifugation at 15,000 rpm for 10 min. The harvested Atg8p-PE liposomes were mixed with the nearly equal amount of Atg8p, and treated with 2% CHAPS for 1 h on ice. The solubilized Atg8p-PE and Atg8p were suspended in IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2% CHAPS, and 0.01% BSA (w/w)), and subjected to immunoprecipitation analysis according to previously reported methods with the following modifications (7). Briefly, samples
were incubated at 4°C for 2 h in the presence or absence of the anti-N-terminal 14 peptide of Atg8p antibody. Protein G Sepharose 4 Fast Flow beads (Amersham Biosciences) were subsequently added to the samples and further incubated at 4°C for 1 h. Immune complexes were washed with TBS containing 2% CHAPS and suspended in SDS-PAGE sample buffer. The resulting immunoprecipitates were separated by urea-SDS-PAGE and visualized by SyproOrange staining (Amersham Pharmacia Biotech).

Trypsin digestion

*In vitro* Atg8-PE conjugation was performed as described in the above. Then, Atg8-PE conjugated liposomes were recovered by centrifugation at 15,000 rpm for 10 min, and solubilized in TBS containing 1 mM DTT and 1% CHAPS for 1 h on ice. The mixture of Atg8p-PE (2.0 μg) and Atg8p (1.5 μg), or each of them was treated with trypsin (0.01 μg) (Sigma T8802) in 20 μl TBS containing 1 mM DTT and 1% CHAPS at 30°C for indicated time (0, 1, 3, 7, and 15 min). The digestion was stopped by boiling in SDS-PAGE sample buffer. Samples were subjected to urea-SDS-PAGE and stained by CBB.
Results

Expression of the components of Atg8 system in *Escherichia coli*

Previous studies of the Atg8 system revealed that the C-terminal glycine of Atg8p is covalently conjugated to an amino group of PE through ubiquitination-like reactions requiring Atg7p and Atg3p, functioning as E1- and E2-like enzymes, respectively (7). We have not, however, excluded the possibility that other factor(s), such as an E3, is required for conjugation.

To address this issue, we applied an *E. coli* expression system. For this purpose, expression plasmids encoding the components of the Atg8 system, Atg7p bearing a Myc epitope tag, Atg3p, and either the processed or unprocessed form of Atg8p, under the control of inducible arabinose-promoter were constructed (Fig. 1A). *E. coli* BL21 (DE3) cells harboring these expression plasmids were cultured to logarithmic growth phase, then cultured for an additional 60 min in the presence of 0.2% arabinose. Total cell lysates from the *E. coli* were then subjected to SDS-PAGE containing 6 M urea (urea-SDS-PAGE). Subsequent immunoblot analyses with anti-Myc and anti-Atg3p antibodies detected Atg7p-Myc and Atg3p at the expected molecular masses of 78 and 36 kDa, respectively (Fig. 1B, lanes 1-3). Atg8p\(^\text{R117}\), the nascent, unprocessed form of Atg8p, was detected as a single band of approximately 13.5 kDa, the predicted molecular mass; Atg8p\(^\text{G116}\), the processed form of Atg8p, migrated similarly to Atg8p\(^\text{R117}\) (Fig. 1B, lanes 1 and 2).

Interestingly, only upon simultaneous expression of Atg7p-Myc, Atg3p, and
Atg8p$^{\text{G}_{116}}$, an additional Atg8p band appeared (Fig. 1B, lane 3). This band, migrating faster than the Atg8p$^{\text{G}_{116}}$ form, was not observed in *E. coli* co-expressing the unprocessed Atg8p$^{\text{R}_{117}}$ form of Atg8p (Fig. 1B, lane 2). The faster migrating band is not an Atg8p degradation product, but a modified form, since only one band for Atg8p was observed by conventional SDS-PAGE (data not shown) (13). Furthermore, the mobility of the modified Atg8p by urea-SDS-PAGE corresponded to that observed for Atg8p-PE derived from yeast cells (Fig. 1B, lanes 3 and 6).

During Atg8p lipidation, the C-terminal glycine of Atg8p first links to Cys$^{507}$ of Atg7p, then is transferred to Cys$^{234}$ of Atg3p through generation of a thioester bond (7). The mutant proteins Atg7p$^{\text{C}_{507S}}$-Myc and Atg3p$^{\text{C}_{234S}}$ possess serine residues at the active site cysteines. Expression of the Atg7p$^{\text{C}_{507S}}$-Myc or Atg3p$^{\text{C}_{234S}}$ mutants in *E. coli* in place of the wild-type Atg7p-Myc or Atg3p, respectively, abolished the appearance of the modified Atg8p form (Fig.1C, lanes 5 and 6). Instead, expression of Atg3p$^{\text{C}_{234S}}$ generated a novel 50-kDa band, detectable with both anti-Atg8p and anti-Atg3p antibodies (data not shown and Fig.1C, lane 6). Appearance of the band was resistant to high concentrations of dithiothreitol (100 mM DTT) and dependent upon the Atg8p C-terminal glycine and active Atg7p (Fig.1C, lanes 7 and 8). These results suggest that this species is an Atg8p-Atg3p ester conjugate formed between the C-terminal glycine (Gly$^{116}$) of Atg8p and the Atg3p$^{\text{C}_{234S}}$ serine residue (Fig.1C, lane 6). We concluded that sequential enzymatic reactions occur in *E. coli* cells in a manner similar to that observed in yeast cells. Thus, these co-expression experiments indicate that the E1 and E2 enzymes, Atg7p and Atg3p, are sufficient for the modification of the
processed form of Atg8p.

**PE is the target of Atg8p in *E. coli***

The above results prompted us to attempt the complete *in vitro* reconstitution of Atg8p conjugation using purified Atg proteins and liposomes. Plasmids encoding GST fusions of each of the Atg proteins were constructed using the pGEX4T-1 vector, to produce GST-Atg7-Myc, GST-Atg3, and GST-Atg8<sup>G116</sup>, then introduced into the BL21 (DE3) *E. coli* strain. The recombinant proteins were induced by IPTG and purified as described in the Experimental Procedures. Purified Atg proteins exhibited high purity, as demonstrated by Coomassie Brilliant Blue (CBB) staining in SDS-PAGE (Fig. 2A, lanes 3-5). Liposomes were prepared using *E. coli* total phospholipids, as described in the Experimental Procedures. After mixing the purified Atg7p-Myc, Atg3p, and Atg8p<sup>G116</sup> with *E. coli* phospholipid-containing liposomes, the mixture was incubated at 30°C in the presence of an ATP regeneration system. The reaction products were then subjected to urea-SDS-PAGE and visualized by CBB staining. The modified form of Atg8p was generated in this *in vitro* reaction in an ATP-dependent manner, as determined by the presence of the faster migrating, Atg8p-specific band (Fig. 2A, lanes 6 and 7).

*E. coli* membrane phospholipids consist primarily of PE, phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidic acid (PA). PE is the major glycerophospholipid (~75% of total lipids). *E. coli* PE is produced exclusively from phosphatidylserine (PS) via decarboxylation. An *E. coli* strain, GN10, possesses a null
mutant of the pssA gene that encodes PS synthase; thus, this strain completely lacks PE (22). When this in vitro reaction was performed using liposomes made from the total lipids of the GN10 strain, the modified Atg8p could not be detected (Fig. 2A, lane 8). Liposomes were generated from a mixture of 30% GN10 total lipids and 70% purified E. coli PE; when these liposomes were subjected to in vitro reaction with purified Atg proteins, we observed the successful modification of Atg8p at levels similar to those seen with wild-type E. coli liposomes (Fig. 2A, lanes 6 and 9). These results clearly demonstrate that PE is the target of Atg8p modification. We also investigated Atg8p-PE formation using liposomes made from pure phospholipids. Atg8p-PE conjugation could be reproduced with the liposomes composed of 70% dioleoylphosphatidylethanolamine (DOPE) and 30% 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) (Fig. 2B). Taking together, we concluded that the PE-containing liposome is sufficient for conjugation to Atg8p; biological membranes containing additional components do not appear to be necessary.

**Characterization of in vitro Atg8p lipidation**

While in vitro conjugation studies of ubiquitin-like proteins, such as SUMO have been performed, conjugation reactions in the absence of E3 have always demonstrated low efficiencies. Such reactions were improved by the addition of excess amounts of E1 and E2 to SUMO (27). To examine the efficiency of in vitro Atg8p-PE conjugation, we titrated the amounts of Atg8pG116 and PE against fixed amounts of Atg7p-Myc and Atg3p. The results clearly indicated that the amount of
Atg8p-PE was augmented with increasing Atg8p and PE ranging from a 5-fold to a 20-fold molar excess of these targets to Atg7p-Myc and Atg3p (Fig. 2B). These results suggest that Atg7p and Atg3p induce in vitro Atg8p-PE conjugation in a catalytic manner. Hence, we conclude that the minimal essential factors necessary for Atg8p-PE conjugation are Atg7p, Atg3p, Atg8pG116, PE-containing membranes, and ATP.

It is an important question what physical state of lipid molecules is required for lipidation reaction. To investigate this problem, we pretreated 70% DOPE/30% POPC liposomes with an amphoteric detergent, 20 mM CHAPS, on ice for 30 min. We then subjected these lipids to the in vitro reconstitution assay in the presence of 10 mM CHAPS, the critical micelle concentration (CMC). As assessed by subsequent urea-SDS-PAGE analysis, the detergent completely prevented Atg8p lipidation (Fig. 2C, lane 2). Since the Atg8p-Atg3pC234S ester conjugate could be formed in the presence of detergent (Fig. 2C, lanes 3-5), activation of Atg8p and the thioester transferring of Atg8p from E1 to E2 occur normally in the presence of detergent. The formation of the amide bond between Atg8p and PE, however, requires PE in the lipid bilayer phase.

**Lipid composition of target membrane**

We prepared liposomes with differential PE contents by combination of GN10 total lipids and purified *E. coli* PE. Interestingly, the yield of Atg8p-PE increased in a dose-dependent manner with increasing PE content up to 70% of the total lipids, despite an equal amount of total PE (70 nmoles) present in each reaction (Fig. 3A). Liposomes containing more than 90% PE, however, resulted in a remarkably diminished
quantity of Atg8p-PE conjugates (Fig. 3A). PE has a small hydrophilic headgroup responsible for its tendency to form non-bilayer structures (28). Thus, liposomes with excess PE may possess an unfavorable structure for Atg8p lipidation. We confirmed these results using two synthetic phospholipids, DOPE and POPC. When DOPE content was increased in DOPE/POPC liposomes, the yield of Atg8p-PE conjugate peaked at approximately 70% DOPE liposome content (Fig. 3B). By western blotting analysis with an anti-Atg8p antibody, we could detect the Atg8p-PE species, even when the reaction was performed with 20% or 100% PE liposomes (Fig. 3B). The fast migrating band is Atg8p-PE, as it could be detected with 100% DOPE liposomes in the absence of additional lipids (Fig. 3B). From these results, we concluded that the PE content of membranes is an important factor governing the efficiency of Atg8p-PE formation.

The lipid composition of E. coli membranes is quite different from that of yeast. E. coli membranes contain more than 70% PE, while yeast organelle membranes contain approximately 15-30% PE (29). We examined in vitro Atg8p-PE formation using yeast total lipid liposomes, containing a ratio of PE/PC/PS/PI = 16.6/51.7/13.7/18.0. Atg8p-PE conjugation in yeast liposomes was slightly more efficient than that seen in 20% DOPE/80% POPC liposomes, despite a lower PE content (Fig. 4). To determine the effect of other phospholipids on reaction efficiency, we mixed negatively charged phospholipids at final concentrations of 10 or 20% to the 20% DOPE/80% POPC liposomes. The level of Atg8p-PE formed in the 20% DOPE liposomes was enhanced by increases in either PI or PG (Fig. 4). A similar
enhancement was also obtained by the addition of either PS or PA (data not shown). The Atg8p-PE conjugation reaction thus appears to be sensitive to membrane lipid composition.

**High sensitivity of Atg8p-PE in western blotting**

We have two anti-Atg8p polyclonal antibodies, one generated by immunization with the N-terminal 14 amino acids (Met¹-Arg¹⁴) of Atg8p (hereafter anti-N-peptide antibody) (20) and the other a commercially available antibody against full-length Atg8p (Rockland). In both the *E. coli* and *in vitro* reconstitution experiments with Atg8p, the signal intensity of Atg8p-PE by immunoblot analysis was much higher than that of Atg8p<sup>G116</sup> alone when using the anti-N-peptide antibody (see Fig. 3C). We quantified the differences in the signal intensities of Atg8p<sup>G116</sup> and Atg8p-PE by immunoblot analysis with the anti-N-peptide antibody following the *in vitro* reaction at 30 °C for 1 h in the absence or presence of ATP. By CBB staining, Atg8p<sup>G116</sup> was significantly converted to Atg8p-PE in the presence (lane 1), but not the absence (lane 5), of ATP (Fig. 5A). By CBB staining, the two bands representing Atg8p-PE (lane 1) and Atg8p<sup>G116</sup> (lane 5) were of similar intensities (Fig. 5A). Immunoblot analysis, however, with the anti-N-peptide antibody detected the Atg8p-PE band with a greater intensity than that of Atg8p<sup>G116</sup> (Fig. 5A). Even when the mixture was prepared with Atg8p<sup>G116</sup> and Atg8p-PE at a ratio 19:1, the signal for Atg8p-PE exhibited higher in intensity than that for Atg8p by the anti-N-peptide antibody immunoblotting (Fig. 5B). In contrast, western analysis using the antibody against the whole Atg8 protein gave
signals corresponding faithfully to the amounts of Atg8p and Atg8p-PE detected by CBB staining (Fig. 5B). These results indicate that the Atg8p-PE conjugate exhibits a higher affinity for the anti-N-peptide antibody.

Next, we examined the affinity of the two Atg8p forms for these antibodies in the absence of SDS denaturation using a direct dot-blot assay. The in vitro reaction samples (examined in Fig. 5A) were added to a dilution buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2 mM DTT, 2% glycerol, 10 mM CHAPS, and 0.5 mg/ml BSA) and spotted onto PVDF membranes after serial dilution (27.5 -0.05 ng Atg8p). Subsequently, each spot was washed by spotting with TBS and subjected to the immunoblot analysis with both the anti-N-peptide and anti-whole Atg8p antibodies. The resulting immunoblot signals demonstrated the high affinity of Atg8p-PE for the anti-N-peptide antibody, at least 12-fold higher than that of Atg8pG116 for this antibody by quantification using NIH image (Fig. 5C). In contrast, the affinity of Atg8p and Atg8p-PE for the antibody against whole Atg8p was equivalent (Fig. 5C).

We confirmed the high affinity of Atg8p-PE for the anti-N-peptide antibody by immunoprecipitation experiments. A mixture of equal amounts of Atg8p and Atg8p-PE was subjected to immunoprecipitation using the anti-N-peptide antibody in the presence of 2% CHAPS (Fig. 5D, lane 1). Following harvest using protein G Sepharose, eluted molecules were separated by urea-SDS-PAGE. By sypro orange staining, no precipitates were detectable in control samples lacking antibody. The resulting immunoprecipitates using the anti-N-peptide antibody contained Atg8p-PE, but only minimal Atg8p (Fig. 5D, lane 3). Thus, the lipidated form of Atg8p...
preferentially binds the anti-N-peptide antibody (Fig. 5D, lanes 2 and 3). These results strongly suggest that conformation of lipidated Atg8p at the N-terminal is distinct from that of free Atg8p.

Further, to ascertain the conformation change of Atg8p-PE, Atg8p and Atg8p-PE were subjected to limited proteolysis by trypsin. First, the mixture of Atg8p and Atg8p-PE was treated with trypsin at 30°C for the indicated time (Fig. 5E lanes 1-5). The resulting products were separated by SDS-PAGE, and visualized by CBB staining. As shown in Fig. 5E, Atg8p-PE (2.0 μg; lane 1) was rapidly degraded (lane 4) and completely digested at 15 min (lane 5). On the other hand, Atg8p (1.5 μg; Fig. 5E lane 1) was relatively resistant to trypsin digestion (Fig. 5E lane 4) and still detectable at 15 min (Fig. 5E lane 5). The remaining Atg8p (Fig. 5E lanes 1-5) was not derived from cleavage of Atg8p-PE, since Atg8p and Atg8p-PE were digested in the same time course when treated individually (Fig. 5E lanes 6-11). These results suggested that Atg8p becomes highly sensitive to trypsin digestion after lipidation. Hence, we concluded that PE conjugation may induce a conformation change in the Atg8p N-terminus.
Discussion

*E. coli* cell provides a suitable system for co-expression of multiple proteins under strict control. Here, we applied this expression system to the ubiquitin-like modification of Atg8p, successfully accomplishing the sequential reactions *in vivo* necessary for Atg8p conjugation. Co-expression of multiple components involved in a concerted mechanism provides a fruitful abstraction facilitating the elucidation of the mechanisms of complicated reaction systems. We have also succeeded making Atg12p-Atg5p conjugate in *E. coli* cells (unpublished results). As the final target of Atg8p modification is phosphatidylethanolamine, we expected that this lipid acceptor endogenously resided in *E. coli* membranes. The *E. coli* inner membrane turned out to be an unexpectedly efficient target membrane, which contains approximately 70% PE.

Complete *in vitro* reconstitution of Atg8p lipidation with purified Atg8p$^{G116}$, Atg7p, Atg3p, and PE-containing liposome was established. Conjugation could proceed quite efficiently in multiple catalytic cycles in the presence of ATP, indicating that Atg8p, E1, E2 and liposomes are sufficient for chemical reactions of conjugate formation. PE-deficient liposome could not accept Atg8p, but exogenous supplementation with PE revealed an optimum PE content for the reaction at approximately 70% PE.

The most critical unknown remaining in this process is the site of Atg8p lipidation in yeast cells during autophagosome formation. We showed that lipid bilayer structure is indispensable for Atg8p lipidation. The most simple explanation from the above results is a membrane with high PE content is preferable for the Atg8p
lipidation. Generally, yeast organelle membranes contain approximately 15-30% PE (29). However, phospholipids in biomembranes are distributed asymmetrically throughout lipid bilayers; PE is primarily exposed on the cytoplasmic surface of organelle membranes (30). Therefore, small regions with high PE content may not be unrealistic within yeast cells. Negatively charged phospholipids also preferentially modulate the efficiency of the lipidation reaction, suggesting that the site of Atg8p-PE modification in vivo may be membrane microdomains containing substantial PE and additional negatively charged lipids.

While we do not know which proteins recognize phospholipids during Atg8p lipidation, previous studies have demonstrated that Atg7p and Atg3p form an E1/E2 complex in yeast (31). In the in vitro reaction mixture, Atg7p and Atg3p could be recovered from the liposome fraction (data not shown). Thus, the Atg7p/Atg3p complex may interact with the membrane surface, facilitating the transfer of Atg8p from Atg3p to PE. Further studies on lipid composition and its relevance for the membrane-binding of the Atg7p/Atg3p complex will aid in our understanding of the site of lipidation.

In vitro lipidation reaction is highly efficient; the final molar ratio of Atg8p-PE to free PE could reach 1:14, when the reaction mixture contains 5.0 μM Atg8pG116 and 70 μM PE (shown in Fig. 2). Assuming that PE is evenly distributed across the inner and outer leaflets of the liposomes and that all liposomes are unilamellar, one out of every seven PE molecules in the outer surface must be modified by Atg8p. This result suggests that Atg8p molecules heavily cover the liposome surface. In the in vitro
reaction, the reaction mixture became increasingly turbid through the formation of large liposome aggregates. The aggregate formation clearly correlated with the extent of Atg8p-PE. Treatment of the reaction mixture with the deconjugating enzyme Atg4p dissociated these aggregates, suggesting that Atg8p-PE causes liposome aggregation.

While we have not yet solved the tertiary structure of Atg8p, the structures of the mammalian Atg8p homologues, GABARAP, GATE-16, and LC3, have been determined. The structure of these Atg8p homologues contain a ubiquitin-fold and additional N-terminal two α-helices, which are not found in other members of the ubiquitin superfamily (32-34). We generated an antibody against N-terminal peptide of Atg8p, which covers the first α-helix of Atg8p (20). By western blotting, we showed that the affinity of the anti-N-peptide antibody for Atg8p-PE was much higher than that for Atg8p. While several factors can affect the efficiency of western blotting of small protein like ubiquitin-like molecules, the reactivity of an additional anti-Atg8p did not appear to be affected by PE-modification, ruling out potential technical problems, such as transfer efficiency, as the reason for this differential recognition. Instead, it is more likely that the differences of reactivity of anti-N-terminal antibody could be accounted for by differential accessibility of the N-terminal region following PE-conjugation. We confirmed the induction of a conformation change in Atg8p by dot-blot assay, immunoprecipitation experiments, and protease sensitivity without denaturing procedures. These results propose that the lipidation of Atg8p induces the conformation change of Atg8p under native conditions.

Previously, we reported the presence of Atg8p in autophagic bodies by
detection of HA-Atg8p with an anti-HA antibody, concluding that Atg8p-PE is cleaved off after autophagosome formation (20). Use of the anti-N-terminal antibody to detect Atg8p for immuno EM analysis, however, could detect Atg8p-specific signals on the autophagosome and its intermediate structure, but not within the autophagic bodies (Baba., unpublished result). This result may also reflect differences in Atg8p and the lipidated form.

Based on the crystal structure of Atg8p homologues, Atg8p is predicted to place the first $\alpha$-helix ($\alpha_1$) (Tyr$^4$-Glu$^8$) in the N-terminal helical domain in the face of the central $\beta$ sheet ($\beta_1$) (Arg$^{28}$-Lys$^{35}$) by hydrogen bonding between the backbone NH groups at the N terminus of helix $\alpha_1$ and Glu$^{34}$, conserved throughout the Atg8p family. The crystal structure of GABARAP is proposed to adopt two distinct conformations, with the N-terminal $\alpha_1$ region in either a closed or open state, corresponding to the monomer and oligomer, respectively (35). This transition from the closed to the open conformation has been observed in high salt conditions. For Atg8p, a similar conformational change may be induced by Atg8p lipidation. As in the open form of GABARAP, movement of the N-terminal region of Atg8p-PE would expose the hydrophobic surface of the central $\beta$ sheets of $\beta_1$ and $\beta_2$. This change to an open structure of Atg8p-PE may facilitate the homodimeric interaction of conjugates with each other, may causing the membrane aggregation discussed above. Alternatively, the conformational change may induce interaction of the open form of Atg8p-PE with other molecules. Legesse-Miller et al. has reported that Atg8p may be involved in fusion process mediated by SNARE (soluble N-ethylmaleimide sensitive factor attachment
protein receptor) molecule (36). Thus, it is possible that Atg8p-PE participates in membrane fusion event by interacting with certain protein.

Another question that remains to be answered is the relationship between the Atg12p and Atg8p conjugation systems. We have previously reported only minimal formation of the Atg8p-PE conjugate in the absence of Atg12p-Atg5p (37). Preliminary experiments demonstrated an enhancement of the \textit{in vitro} lipidation reaction by the presence of Atg12p-Atg5p conjugate. \textit{In vitro} analysis of this interaction will give us critical information on the relationship between Atg8p lipidation and Atg12p-Atg5p conjugation systems and their roles in autophagy.
References

1. Klionsky, D. J., and Ohsumi, Y. (1999) Annu Rev Cell Dev Biol 15, 1-32
2. Huang, W. P., and Klionsky, D. J. (2002) Cell Struct Funct 27, 409-420
3. Wang, C. W., and Klionsky, D. J. (2003) Mol Med 9, 65-76
4. Klionsky, D. J., Cregg, J. M., Dunn, W. A., Jr., Emr, S. D., Sakai, Y., Sandoval, I. V., Sibirny, A., Subramani, S., Thum, M., Veenhuis, M., and Ohsumi, Y. (2003) Dev Cell 5, 539-545
5. Mizushima, N., Ohsumi, Y., and Yoshimori, T. (2002) Cell Struct Funct 27, 421-429
6. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M., and Ohsumi, Y. (1998) Nature 395, 395-398
7. Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000) Nature 408, 488-492
8. Ohsumi, Y. (2001) Nat Rev Mol Cell Biol 2, 211-216
9. Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., and Kominami, E. (1999) Mol Biol Cell 10, 1367-1379
10. Shintani, T., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T., and Ohsumi, Y. (1999) Embo J 18, 5234-5241
11. Kuma, A., Mizushima, N., Ishihara, N., and Ohsumi, Y. (2002) J Biol Chem 277, 18619-18625
12. Lang, T., Schaeffeler, E., Bernreuther, D., Bredschneider, M., Wolf, D. H., and Thumm, M. (1998) Embo J 17, 3597-3607
13. Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000) J Cell Biol 151, 263-276
14. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000) Embo J 19, 5720-5728
15. Sagiv, Y., Legesse-Miller, A., Porat, A., and Elazar, Z. (2000) Embo J 19, 1494-1504
16. Wang, H., Bedford, F. K., Brandon, N. J., Moss, S. J., and Olsen, R. W. (1999) Nature 397, 69-72
17. Tanida, I., Komatsu, M., Ueno, T., and Kominami, E. (2003) *Biochem Biophys Res Commun* **300**, 637-644
18. He, H., Dang, Y., Dai, F., Guo, Z., Wu, J., She, X., Pei, Y., Chen, Y., Ling, W., Wu, C., Zhao, S., Liu, J. O., and Yu, L. (2003) *J Biol Chem* **278**, 29278-29287
19. Hemelaar, J., Lelyveld, V. S., Kessler, B. M., and Ploegh, H. L. (2003) *J Biol Chem* **278**, 51841-51850
20. Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. (1999) *J Cell Biol* **147**, 435-446
21. Abeliovich, H., Dunn, W. A., Jr., Kim, J., and Klionsky, D. J. (2000) *J Cell Biol* **151**, 1025-1034
22. Saha, S. K., Nishijima, S., Matsuzaki, H., Shibuya, I., and Matsumoto, K. (1996) *Biosci Biotechnol Biochem* **60**, 111-116
23. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY,
24. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) *J Bacteriol* **177**, 4121-4130
25. Bligh, E. G., and Dyer, W. J. (1959) *Can J Med Sci.* **37**, 911-917
26. Bartlett, G. R. (1958) *Ann N Y Acad Sci* **75**, 110-114
27. Takahashi, Y., Kahyo, T., Toh, E. A., Yasuda, H., and Kikuchi, Y. (2001) *J Biol Chem* **276**, 48973-48977
28. Cullis, P. R., and de Kruijff, B. (1979) *Biochim Biophys Acta* **559**, 399-420
29. Tuller, G., Nemec, T., Hrastnik, C., and Daum, G. (1999) *Yeast* **15**, 1555-1564
30. Verkleij, A. J., and Post, J. A. (2000) *J Membr Biol* **178**, 1-10
31. Komatsu, M., Tanida, I., Ueno, T., Ohsumi, M., Ohsumi, Y., and Kominami, E. (2001) *J Biol Chem* **276**, 9846-9854
32. Paz, Y., Elazar, Z., and Fass, D. (2000) *J Biol Chem* **275**, 25445-25450
33. Stangler, T., Mayr, L. M., and Willbold, D. (2002) *J Biol Chem* **277**, 13363-13366
34. Sugawara, K., Suzuki, N. N., Fujioka, Y., Mizushima, N., Ohsumi, Y., and Inagaki, F. (2003) *Acta Crystallogr D Biol Crystallogr* **59**, 1464-1465
35. Coyle, J. E., Qamar, S., Rajashankar, K. R., and Nikolov, D. B. (2002) *Neuron* **33**, 63-74
36. Legesse-Miller, A., Sagiv, Y., Glozman, R., and Elazar, Z. (2000) *J Biol Chem* **275**, 32966-32973

37. Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T., and Ohsumi, Y. (2001) *Embo J* **20**, 5971-5981
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Figure legends

Fig. 1. Expression of the Atg8 system components in *E. coli*. (A) Diagram of expression plasmids. To construct expression plasmids for Atg7p-Myc, Atg3p, Atg8p\textsuperscript{G116} (the processed form of Atg8p), and Atg8p\textsuperscript{R117} (nascent form of Atg8p) in *E. coli*, each gene was introduced into pBAD (pBAD18) or pBADC (pBAD33) plasmids. The inserted DNA fragments contain a Shine-Dalgarno (SD) sequence. (B) Modified Atg8p in *E. coli* expression system. Expressed proteins in *E. coli* cells were detected by immunoblotting with anti-Myc, anti-Atg3p, and anti-Atg8p antibodies. The mobility of the Atg8p bands in *E. coli* cells concurrently expressing Atg7p-Myc, Atg3p, and Atg8p\textsuperscript{G116} was compared with Atg8p and Atg8p-PE forms derived from Δatg7, Δatg3 and wild-type (wt) yeast by urea-SDS-PAGE. Yeast cell lysates were prepared by glass bead disruption, as previously reported (20). R indicates Atg8p\textsuperscript{R117}. (C) Atg8p intermediates in *E. coli* expression system. Atg7p-Myc, Atg3p, and Atg8p\textsuperscript{G116} were induced in *E. coli* cells as indicated; CS indicates Atg7p\textsuperscript{C507S} or Atg3p\textsuperscript{C234S}, while R designates Atg8p\textsuperscript{R117} expressed by addition of 0.2% arabinose to cultures for 1 h. Each product was analyzed by the western blotting as described above.

Fig. 2. *In vitro* reconstitution of Atg8 system. (A) *In vitro* assay for Atg8p-PE. *E. coli* lipid liposomes, an ATP regeneration system (1 mM ATP, 1 mM MgCl\textsubscript{2}, 5 mM phosphocreatine, and 2.5 μg creatine kinase (CK)), and purified Atg proteins were visualized on CBB stained urea-SDS-PAGE gels (lanes 1-5). The *in vitro* reaction mixtures (100 μl) containing *E. coli* lipid liposomes, 1.0 μM Atg7p-Myc, 1.0 μM Atg3p,
and 5.0 μM Atg8pG116 were incubated at 30 °C for 30 min in the presence (+) or absence (-) of the ATP regeneration system (ATP) (lanes 6 and 7). 20 μl of each sample was then subjected to urea-SDS-PAGE. Separated bands were visualized by CBB staining. Liposomes were prepared from the total lipids isolated from W3011 (wild-type; wt) (lanes 6 and 7), GN10 (ΔpssA; 0% PE) (lane 8) or the mixture of 30% GN10/70% E. coli PE (lane 9). Equal amount of PE (7 nmoles), however, was present in each reaction mixture containing PE liposomes.  

**B** Atg8p-PE is generated by the catalytic action of Atg7p and Atg3p. Increasing amounts of Atg8p, ranging from 5.0 to 20 μM, were mixed with a constant quantity of 70% DOPE/30% POPC liposomes (100 μM-400 μM phospholipids) in the presence of 1.0 μM Atg7p-Myc and 1.0 μM Atg3p. After incubation at 30 °C for 1 h, generated Atg8p-PE was detected by urea-SDS-PAGE with CBB staining.  

**C** Lipid bilayer is essential for Atg8p-PE conjugation. 70% DOPE/30% POPC liposomes were suspended in reconstitution buffer in the presence or absence of 20 mM CHAPS on ice for 30 min. Following the addition of 5.0 μM Atg8p, 1.0 μM Atg7p-Myc, and either 1.0 μM Atg3p or Atg3pC234S, the in vitro reaction was performed at 30 °C for 1 h in the presence or absence of the ATP regeneration system (ATP). The in vitro products were detected by CBB stained urea-SDS-PAGE gel. Atg8p-Atg3pC234S indicates the ester linkage product.

**Fig. 3. In vitro assay of Atg8p-PE conjugation with variable PE membrane content.**  

**(A)** In vitro Atg8p-PE with E. coli liposome. In vitro reconstitution of Atg8 system was performed at 30 °C for 20 min as described in Fig. 2 in the presence (+) or absence
(-) of the ATP regeneration system. The products were then analyzed by CBB-stained urea-SDS-PAGE gel. Liposomes were prepared from W3011 total lipids (wild-type; wt), GN10 total lipids (0% PE), a lipid mixture of GN10/E. coli PE with the indicated PE content (10-90%), and E. coli PE alone (100% PE). Each reaction mixture (100 μl) contained the equal amount of PE (7 nmoles), with the exception of PE-negative liposomes. (B) In vitro Atg8p-PE generation from pure phospholipid liposomes. In vitro reactions were performed as described above using DOPE/POPC liposomes with the indicated PE content. The in vitro samples (20 μl) were then subjected to urea-SDS-PAGE, and visualized by CBB staining. (C) In addition, these samples (2 μl) were also analyzed by western blotting with an antibody against N-terminal peptide of Atg8p.

**Fig. 4. Effect of negatively charged phospholipids.** Liposomes were prepared from either yeast total lipids or the pure phospholipids (DOPE, POPC, DOPI, DOPG) at the indicated compositions. Using the generated liposomes, Atg8p-PE was generated for 30 min as in Fig. 2. The in vitro products were visualized by CBB-stained urea-SDS-PAGE. Each reaction mixture (100 μl) contained equal amount of PE (7 nmoles), with the exception of PE-negative liposomes.

**Fig. 5. Conformation change of Atg8p by lipidation.** (A) High signal intensity of Atg8p-PE in western blot with anti-N-terminal peptide antibody. The in vitro reaction was performed as in Fig. 2 in the presence (lanes 1-4) or absence of ATP (lanes 5-8).
After incubation for 1 h, 1.5 µg Atg8 protein from the *in vitro* mixture was subjected to urea-SDS-PAGE and visualized by CBB staining (CBB) (lanes 1 and 5). Samples (Atg8 protein amounts; lanes 2 and 6 (140 ng), lanes 3 and 7 (70 ng), lanes 4 and 8 (35 ng)) were then detected by western blotting (WB) with anti-N-peptide antibody (lanes 2-4 and 6-8). **(B)** Atg8p-PE exhibits a high affinity for the N-terminal peptide antibody. Atg8p (95 ng) and Atg8p-PE (5 ng), derived from *in vitro* reaction samples, were mixed and subjected to urea-SDS-PAGE. Subsequently, western blot was performed on these samples using antibodies against either the N-terminal peptide (N) or the whole (W) Atg8 protein. **(C)** Dot-blot assay. The *in vitro* products (shown in a) were suspended in dilution buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2 mM DTT, 2% glycerol, 10 mM CHAPS, and 0.5 mg/ml BSA (Sigma 7030)) and spotted onto PVDF membranes (Immobilon-PSQ) as indicated (dilution series of x10-x5,120 includes 27.6-0.05 µg Atg8p (or Atg8p-PE)). The signals for Atg8p and Atg8p-PE in dot-blot assays were detected according to the immunoblot procedures detailed above with antibodies against either the N-terminal peptide (P) or the whole protein (W) of Atg8p. The resulting signal intensities were evaluated by densitometry using NIH Image. **(D)** Immunoprecipitation with anti-N-peptide antibody. Equal amounts of Atg8p-PE and Atg8p from *in vitro* reaction were mixed in IP buffer (lane 1) prior to the addition of the anti-N-peptide antibody to the sample (lane 3). The immunoprecipitation was performed as described in the Experimental Procedures, and each sample was analyzed by urea-SDS-PAGE with SyproOrange staining. As a control, a sample lacking added antibody is shown in lane 2. **(E)** Trypsin digestion of
Atg8p and Atg8p-PE. Mixture of Atg8p (1.5 μg) and Atg8p-PE (2.0 μg) was incubated at 30 °C for the indicated time in the presence of trypsin (0.01 μg) (lanes 1-5). Further, Atg8p (1.5 μg) (lanes 6, 8, and 10) and Atg8p-PE (2.0 μg) (lanes 7, 9, and 11) were treated individually with trypsin (0.005 μg) at 30 °C for the indicated time (Lanes 6-11). Samples were analyzed by urea-SDS-PAGE followed by CBB staining. Asterisk indicates proteolytic products.
Fig. 1. Ichimura et al.

A

| pBAD-Atg7-Myc | amp' | araC | ATG7-Myc |
|---------------|------|------|---------|
| pBADC-Atg8\(G^{116}\) | cm' | araC | ATG8\(G^{116}\) |
| pBADC-Atg3Atg8\(G^{116}\) | cm' | araC | ATG3 > ATG8\(G^{116}\) |
| pBADC-Atg3Atg8\(R^{117}\) | cm' | araC | ATG3 > ATG8\(R^{117}\) |

\[\square\] PBAD promoter, \[\blacksquare\] AGGAGGAATTACCATGXXX \(\text{(SD sequence)}\) \(\text{(start codon)}\)

B

| Atg7-Myc | + | + | + |
| Atg8 \(\neg\) | - | + | + |
| Atg8 \(G^{116}\) | + | R | + |

Atg7p-Myc

| Atg3p |
|-------|

| Atg8p |
|-------|

| Modified Atg8p |
|----------------|

\(E.\text{coli} | Yeast\)

| 1 | 2 | 3 | 4 | 5 | 6 |

C

| Atg7-Myc | + | + | - | + | CS | + | + | - |
| Atg3 | + | + | + | - | + | CS | CS | CS |
| Atg8 \(G^{116}\) | + | R | + | + | + | R | + |

Atg7p-Myc

| Atg8p-Apg3p |
|-------------|

| Atg3p |
|-------|

| Atg8p |
|-------|

| Modified Atg8p |
|----------------|

\(1 | 2 | 3 | 4 | 5 | 6 | 7 | 8\)
Fig. 2. Ichimura et al.
Fig. 3. Ichimura et al.

A

E. coli (wt) liposome PE (E. coli) content of liposome (%) ATP

Atg7p-Myc Atg3p Atg8p + - 0 10 20 30 40 50 60 70 80 90 100

Atg7p-Myc Atg3p

Atg8p

Atg8p-PE

B

PE (DOPE) content of liposome (%) 0 10 20 30 40 50 60 70 80 90 100

Atg8p

Atg8p-PE

C

Western blot
Fig. 4. Ichimura et al.

[Image of a gel with bands labeled Atg8p and Atg8p-PE, and a list of conditions: PE:PC:PG / 50:50, PE:PC:PG / 20:60:20, PE:PC:PG / 20:70:10, PE:PC:PG / 20:80, PE:PC / 80, PC:PG / 20:80, Yeast total lipids]
Fig. 5. Ichimura et al.
