

# **Studies on the ubiquitin-like conjugation reaction of Atg8 required for autophagosome formation**

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## Abbreviations

ALP	alkaline phosphatase
CBB	coomassie brilliant blue
DTT	dithiothreitol
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-protein ligase
EDTA	ethylenediaminetetraacetic acid
GST	glutathione S-transferase
IPTG	isopropyl $\beta$ -D-thiogalactopyranoside
ME	$\beta$ -mercaptoethanol
NEM	N-ethylmaleimide
PA	phosphatidic acid
PAS	pre-autophagosomal structure
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PG	phosphatidylglycerol
PI	phosphatidylinositol
PIC	protease inhibitor cocktail
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
PVDF	polyvinylidene difluoride
TCA	trichloroacetic acid
Tor	target of rapamycin
Ubl	ubiquitin-like protein

## Summary

Autophagy is a major self-degradative process in eukaryotic cells that plays fundamental roles in cellular and organismal homeostasis, and is involved in many physiological and pathological situations. When autophagy is induced, cytoplasmic materials and organelles are sequestered into newly emerging double-membrane vesicles called autophagosomes, and delivered to the lysosome or the vacuole for degradation.

In the past decade, many *ATG* (autophagy-related) genes have been identified by genetic approaches using the yeast *Saccharomyces cerevisiae*. Atg8, a ubiquitin-like protein (Ubl), is one of the proteins essential for autophagosome formation. The cysteine protease Atg4 first removes the C-terminal arginine of Atg8 to expose the glycine as the new terminus. This glycine forms a thioester bond with Atg7, an activating enzyme (E1), and transfers to and also forms a thioester bond with Atg3, a conjugation enzyme (E2). Atg8 is eventually conjugated to the amino group in the hydrophilic head of phosphatidylethanolamine (PE). Atg8 is anchored to isolation membrane and autophagosomal membranes probably as this lipid-modified form, and thought to directly participate in the formation of these membranes. Atg4 also catalyzes the deconjugation of Atg8-PE after it has fulfilled its role in autophagosome formation, thus Atg8 is reused. Because the details of this sequential reaction of Atg8 lipidation are unclear, I focus on the mechanism of Atg8 lipidation, in this study.

The Atg8 conjugation system was reconstituted using purified proteins expressed in *Escherichia coli* and PE-containing liposomes in vitro. First, I successfully capture authentic thioester intermediates, Atg8-Atg7 and Atg8-Atg3, which can not have been detected because of their lability. This allows me to analyze the sequential reaction of Atg8 lipidation.

It was shown that Atg8 could be conjugated with phosphatidylserine (PS) as efficiently as PE in vitro. However, PE was identified as the sole lipid conjugated to the C-terminal glycine of Atg8 in vivo. It suggests that there exists a

mechanism that directs Atg8 conjugation preferentially to PE in the cell. In this study, I show that, in contrast to PE conjugation, the PS conjugation of Atg8 is markedly suppressed at physiological (neutral) pH. Then, I show that both of the Atg8-Atg7 and the Atg8-Atg3 intermediates are formed in the presence of PS liposomes as rapidly as in the presence of PE liposomes, and transfer of Atg8 from Atg3 to PS is specifically retarded at neutral pH. Furthermore, the addition of acidic phospholipids to liposomes is also suggested to result in the preferential formation of the Atg8-PE conjugate. I also show that the acidic phospholipids specifically promote the recruitment of the Atg8-Atg7 and the Atg8-Atg3 thioester intermediates to the membrane. Furthermore, it was reported that the Atg12-Atg5 conjugate, which is formed by ubiquitin-like conjugation reaction, is indispensable for Atg8-PE production in vivo, and that recombinant Atg12-Atg5 indeed stimulates Atg8-PE and Atg8-PS production in vitro.

The preferential formation of Atg8-PE can be achieved by combination of neutral pH, acidic phospholipids, and the Atg12-Atg5 conjugate. Furthermore, I show that PS is not essential for autophagosome formation even if Atg8 is conjugated to PS in vivo, because the autophagic activity of cells deficient for the PS synthesis enzyme-deficient (*pss1Δ*) cells was normal. However, in vitro, the less efficient but significant production of Atg8-PS was still observed, suggesting that the exclusive formation of Atg8-PE requires precise in vivo settings for these factors and/or other factor(s). Alternatively, this result may imply the production of Atg8-PS in vivo, although its amount should be much less than the PE conjugate. Previously, PE was detected as the sole lipid conjugated to Atg8 and its mammalian homolog LC3 (microtubule-associated protein light chain 3) in vivo. However, lipidated Atg8 and LC3 were forced to accumulate by mutation or treatment with lysosomal inhibitors under nutrient-replete conditions. Therefore, there also remains an alternative possibility that Atg8-PS is formed in starved cells undergoing autophagy, so, I try to purify lipidated Atg8 from the cells under starvation conditions for detailed analysis of lipids conjugated to Atg8 by LC-MS/MS.

Next, I perform gel filtration chromatography to know the interaction of the components in the Atg8 conjugation reaction in vitro. Atg3 interacts with Atg7 as it shown, and Atg3 also interacts with the Atg8–Atg7 thioester intermediate. Furthermore, I find the Atg8–Atg3 thioester intermediate releases from Atg7. It is reasonable because Atg7 is reused rapidly for the next cycle of the reaction. Furthermore, I show that Atg3 and Atg7 are interacted via the disulfide bond between active center cysteines in Atg3 and Atg7. I show the model of the sequential reaction of Atg8 lipidation.

## **Introduction**

The cellular homeostasis is based on the proper balance between the synthesis and the destruction of proteins. It is thought that the short-lived proteins are selectively degraded by the ubiquitin-proteasome pathway (Pickart *et al.* 2004), while the long-lived proteins are non-selectively delivered to and degraded in the vacuole/lysosome by the autophagy pathway (Xie and Klionsky. 2007). In yeast, the major function of autophagy is that cells adjust to various starvation conditions. In mammalian cells, the roles of autophagy have not been implicated in only cellular processes but also nutrient recycling (Kuma *et al.* 2004), development (Scott *et al.* 2004, Juhász *et al.* 2003, Tsukamoto *et al.* 2008), differentiation (Otto *et al.* 2003, 2004, Meléndez *et al.* 2003), intracellular clearance (Komatsu *et al.* 2007b), cell death (Schweichel *et al.* 1973, Clarke *et al.* 1990), pathogen clearance (Nakagawa *et al.* 2004), and antigen presentation (Dengjel *et al.* 2005), and has also been linked to pathological processes such as cancer progression (Karantza-Wadsworth *et al.* 2007) and neurodegenerative disease (stern *et al.* 2008).

### **The autophagic pathway**

Autophagy refers to any cellular degradative pathway that involves the delivery of cytoplasmic cargo to the vacuole/lysosome. At least three forms have been identified – chaperone-mediated autophagy, microautophagy, and macroautophagy – that differ with respect to their physiological functions and the mode of cargo delivery to the vacuole/lysosome. Chaperone-mediated autophagy occurs when substrate proteins with the KFERQ motif bind to a chaperone molecular complex containing Hsc70, Hsp90, and other co-chaperones. The substrate/chaperone complex then associate directly with the lysosomal surface receptor, Lamp2, and with the help of chaperones, substrate proteins are transported through the lysosomal membrane into the lumen, where they are degraded (Dice. 2007). Microautophagy involves sequestration of a portion of cytoplasm through invaginations of the

vacuole/lysosome. Microautophagy; however, this occurs at the surface of the vacuole/lysosome. Macroautophagy – hereafter simply referred to as autophagy – is the major regulated catabolic mechanism to degrade proteins and organelles non-selectively. In the yeast *Saccharomyces cerevisiae*, when cells are starved of various nutrients or upon inhibition of Target of Rapamycin (Tor) (Noda and Ohsumi. 1998, Díaz-Troya *et al.* 2008), a cup-shaped membranous structure called an isolation membrane appears in the cell. The isolation membrane expands to form the autophagosome, a double-membraned vesicle that sequesters cytoplasmic materials and organelles (Kirisako *et al.* 1999, Mizushima *et al.* 2001). The autophagosome is followed by fusion with the vacuole (Baba *et al.* 1994), where the captured materials, together with the inner membrane, are degraded (Fig. 1, Takeshige *et al.* 1992, Baba *et al.* 1994).

### **Molecular mechanism of autophagy in yeast**

The discovery of autophagy in yeast allowed us to introduce genetic approaches into this research field. Two sets of mutants with defects in autophagy were isolated by independent approaches. Takeshige *et al.* found that the accumulation of the inner membrane-bound structures called autophagic bodies in the vacuoles of the starved cells was induced in the presence of phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, which was assumed to inhibit the activity of the vacuolar processing enzyme, Prb1 (Takeshige *et al.* 1992). Therefore, in yeast, defects in autophagy can be monitored by observing autophagic bodies under a light microscope. Genome-wide screen were performed using a collection of 5000 yeast single-gene deletion mutants in the BY4741 background by a loss of viability under starvation conditions and the accumulation of autophagic bodies. On the basis of these criteria, 14 complementation *APG* (autophagy) mutants were obtained (Tsukada *et al.* 1993). Further genes, *APG16* and *APG17*, were obtained by two-hybrid screening using Atg12 and Atg1 as baits, respectively (Mizushima *et al.* 1999, Kamada *et al.* 2000). Whereas, 6 *AUT* mutants were isolated from cell mutagenized with ethyl methanesulfonate as those defective in

the degradation of fatty acid synthase, Fas1 and Fas2, under starvation conditions (Thumm *et al.* 1994). It was known that these enzymes accumulate inside the vacuole during starvation of strains defective in the vacuolar endoproteinases. These independent screens have led to a range of names, then the nomenclature of these gene names was unified and termed *ATG* genes (Klionsky *et al.* 2003). Further morphological and statistical methods identified Atg29 (Kawamata *et al.* 2008) and Atg31 (Kabeya *et al.* 2007) and over 20 genes are involved in autophagy presently.

The characterization of these gene products, the Atg proteins, revealed that they consist of five functional groups; (i) the Atg1 kinase and its regulators, (ii) the autophagy-specific phosphatidylinositol 3-kinase complex, (iii) the membrane protein complex including Atg9, Atg2 and Atg18, (iv) the Atg8 lipid conjugation system (v) the Atg12–Atg5 protein conjugation system. In response to nutrient starvation, when the Atg proteins are expressed as fusion with a fluorescent protein such as green fluorescent protein, and observed under a microscope, most of the Atg proteins assemble and form a dot in the vicinity of the vacuole. The dot is called pre-autophagosomal structure (PAS) (Suzuki *et al.* 2001, 2007).

#### (i) The Atg1 kinase and its regulators

The Tor, the conserved Ser/Thr kinase, signaling pathway regulates the induction of autophagy (Noda *et al.* 1998). Under nutrient-rich conditions, active Tor function leads to hyperphosphorylation of Atg13 (Kamada *et al.* 2000), although it is not known whether Tor directly phosphorylates Atg13 (Fig. 2A). Inhibition of Tor upon nutrient starvation or treatment with rapamycin results in rapid dephosphorylation of Atg13 (Kamada *et al.* 2000). The dephosphorylated Atg13 binds to Atg1, accompanied by elevation of the kinase activity of Atg1. Atg17 forms a complex with Atg29 and Atg31, which is specifically required for starvation-induced autophagy (Kabeya *et al.* 2007, Kawamata *et al.* 2008), further binds to Atg13, and plays an important role in activating the Atg1 kinase (Kabeya *et al.* 2005).

(ii) The autophagy-specific phosphatidylinositol 3-kinase complex

Vps (vacuolar protein sorting) 34 is a sole PI3-kinase in yeast cells. Vps34 forms part of two distinct protein complexes (Kihara *et al.* 2001). One complex including Vps30, Vps38, Vps34 and Vps15 is responsible for vacuolar protein sorting via endosome, and other is composed of Vps30, Atg14, Vps34 and Vps15 (Fig. 2B). The another complex is responsible for autophagy. Atg14 is required for localization of complex to PAS (Obara *et al.* 2006). It is suggested that the production of PI3-phosphate (P) at PAS is necessary for autophagosome formation via recruitment of proteins associating with PI3P.

(iii) The membrane protein complex including Atg9, Atg2 and Atg18

Atg9 is a sole transmembrane protein among known the Atg proteins (Noda *et al.* 2000). Atg9 fused with GFP localizes to the PAS and also exists as cytosolic locomoting punctate structures (Monastyrska *et al.*, 2008). Atg9 is physically associated with Atg2 (Wang *et al.* 2001), which forms a complex with Atg18 (Suzuki *et al.* 2007). It was proposed that the Atg2-Atg18 complex contributes to the release of Atg9 from the PAS because Atg9 is accumulated at PAS in the  $\Delta atg2$  or the  $\Delta atg18$  cells (Reggiori *et al.* 2004). Atg18 binds to PI3P produced by the autophagy-specific PI3-kinase complex, which is essential for full activity in autophagy (Strømhaug *et al.* 2004, Obara *et al.* 2008). Atg18 also binds to PI3,5-bisphosphate (PI3,5P<sub>2</sub>), which is necessary for the regulation of vacuolar morphology (Dove *et al.* 2004).

(iv) The Atg8 lipid conjugation system

In yeast *Saccharomyces cerevisiae*, the Atg proteins contain two ubiquitin-like proteins (Ubls), Atg12 and Atg8. Unlike ubiquitin and other ubiquitin-like modifiers, the Ubl protein Atg8 is attached to the lipid PE. The C-terminal Arg117 residue of Atg8 is removed by a cysteine protease, Atg4, to expose Gly116 (Kirisako *et al.* 2000). This exposed glycine is activated by the common E1 of Atg7 (Ichimura *et al.* 2000), then transferred to the specific E2

Atg3 (Ichimura *et al.* 2000), and finally conjugated to the amino group in the hydrophilic head of PE (Fig. 3B, Ichimura *et al.* 2000). This lipidation of Atg8 is reversible; Atg4 cleaves Atg8-PE, which results in the release of Atg8 (Kirisako *et al.* 2000). While Atg8 exists as the unconjugated form under nutrient-rich conditions, when autophagy is induced, its conjugation with PE is stimulated (Huang *et al.* 2000). This Atg8-PE system as well as the Atg12-Atg5 system is highly conserved in both mammals and plants (Tanida *et al.* 2001, Nemoto *et al.* 2003, Tanida *et al.* 2002, Tanida *et al.* 2004, Kabeya *et al.* 2000, Yoshimoto *et al.* 2004, Fujioka *et al.* 2008).

(v) The Atg12-Atg5 protein conjugation system

Similarly to protein ubiquitination, the C-terminal glycine of Atg12 is activated by the E1 enzyme Atg7 with consumption of ATP and forms a thioester bond with Cys507 of Atg7 (Tanida *et al.* 1999), transferred to Cys133 of the E2 enzyme Atg10 (Shintani *et al.* 1999), and eventually forms an isopeptide bond with Lys149 of Atg5 (Mizushima *et al.* 1998). Atg12-Atg5 interacts with Atg16 (Mizushima *et al.* 1999), and forms a complex of about 350 kDa through homo-oligomerization of Atg16 (Kuma *et al.* 2002).

Few Atg8-PE molecules can be detected by Western blot in mutants affecting the formation of the Atg12-Atg5 protein conjugation (Mizushima *et al.* 2001, Suzuki *et al.* 2001). It has been reported that the Atg12-Atg5 conjugate has an E3-like activity for Atg8 lipidation (Hanada *et al.* 2007, Fujita *et al.* 2008a). Canonical E3 enzymes bind to both the E2 enzyme and the substrate, and stimulate the transfer of ubiquitin from the E2-ubiquitin intermediate to the substrate. Similarly, Atg12-Atg5 directly interacts with both the E2 enzyme Atg3 and PE-containing liposomes *in vitro* (Hanada *et al.* 2007); Atg12-Atg5 accelerates the transfer of Atg8 from Atg3 to PE (Hanada *et al.* 2007). In this way, Atg12-Atg5 meets the criteria for the E3 enzyme, it, however, lacks a domain conserved in other known E3s such as HECT-type and RING-type ones (Suzuki NN *et al.* 2005). The stimulation of Atg8 lipidation by Atg12-Atg5 does

not involve Atg16 *in vitro* (Hanada *et al.* 2007), which, however, is required for the production of Atg8-PE *in vivo* because in yeast, Atg16 directs Atg12-Atg5 to PAS (Suzuki *et al.* 2007). These results suggest that essential factors for autophagosome formation are concentrated to appropriate site is significant *in vivo*, and that Atg16 is essential for specification of the site of Atg8 lipidation. In addition, in mammalian cells, ectopic expression of an Atg16 variant with a plasma membrane-targeting sequence directs Atg12-Atg5 to that membrane, where lipidation of LC3 occurs (Fujita *et al.* 2008a). Therefore, it is conceivable that Atg16 also decides the site of Atg8 lipidation in mammalian cells.

The formation of Atg12-Atg5-Atg16 and Atg8-PE is essential for autophagy. Electron-microscopy analyses showed that Atg12-Atg5 and Atg8 are required for the expanding isolation membrane but not for its initiation (Mizushima *et al.* 2001). In mammalian cells, Atg12-Atg5-Atg16 is localized on the outer surface of the expanding isolation membrane and dissociated from the membrane immediately after or before autophagosome completion (Mizushima *et al.* 2001, 2003). On the basis of these phenomena, Atg12-Atg5-Atg16 was proposed as a candidate for a coatmer involved in autophagosome formation (Kuma *et al.* 2002). However, a quantitative study shows that the number of Atg12-Atg5-Atg16 molecules at the PAS is not sufficient to coat the forming vesicle (Geng *et al.* 2008). Atg8 is distributed on the both sides of the isolation membrane probably as a lipidated form, and dissociated from the membrane by Atg4 after autophagosome completion (Kirisako *et al.* 1999, Huang *et al.* 2000). However, part of Atg8 remain inside the autophagosome, and delivered to and degraded in the vacuole (Kabeya *et al.* 2000).

In selective autophagy, Atg8 also functions in cargo recognition. For example, in yeast, the vacuolar aminopeptidase 1, Ape1, is selectively and constitutively transported into the vacuole via an autophagy-like process called the cytoplasm-to-vacuole targeting (Cvt) pathway (Scott *et al.* 2001). Immediately after translated in the cytoplasm, Ape1 self-assembles into a huge complex (Oda *et al.* 1996, Kim *et al.* 1997) and interacts with the receptor protein Atg19 (Shintaini *et al.* 2001, Suzuki *et al.* 2002). Atg19 further interacts with Atg8

(Chang *et al.* 2007, Noda *et al.* 2008). In mammalian cells, p62 assembles via its N-terminal PB1 domain (Ponting *et al.* 2002), and thereby can form large aggregates containing ubiquitinated proteins (Vadlamudi *et al.* 1996, Bjorkoy *et al.* 2005, Komatsu *et al.* 2007a). P62 further interacts with LC3 (Pankiv *et al.* 2007, Komatsu *et al.* 2007a). These interactions are thought to be important for efficient engulfment of Ape1 or p62 into Cvt vesicles or autophagosome membrane.

Ichimura *et al.* reconstituted the Atg8 conjugation system using purified proteins and PE-containing liposomes (Ichimura *et al.* 2004). In addition, Nakatogawa *et al.* found that Atg8 forms an oligomer in response to PE conjugation, leading to tethering and hemifusion of liposomes (Nakatogawa *et al.* 2007). The electron microscopic analyses indicate that these functions of Atg8 are involved in the expansion of autophagosome membranes *in vivo*. It was also indicated that there is a quantitative relationship between the amounts of Atg8 and the vesicle size. In yeast cells, when the expression of Atg8 lowered, the size of autophagosome membranes was reduced (Geng *et al.* 2008). Furthermore, it was shown that Atg8 function in the complete closure of autophagosome formation in mammalian cells (Fujita *et al.* 2008b, Sou *et al.* 2008). Electron microscopic analyses showed that the isolation membranes impaired elongation and closure were accumulated in the cytosol in Atg3-deficient mice (Sou *et al.* 2008). These results suggest a possible role of Atg8 as a component of the scaffold that supports the expanding and complete closure of isolation membranes.

## **The aim of this study**

The insights into lipid composition of autophagosomal membranes as well as their origins are poor because it is difficult to purify autophagosomal membranes. While the electron microscopic analyses showed that the membranes of the autophagosome are thinner than other intracellular membrane; the ER, the Golgi, the vacuoles and plasma membrane (Baba. 1996). It is thought that the abundance of proteins in autophagosomal membranes may be low, or the lipids constituting autophagosomal membranes may be special compared with other membranes. Because Atg8 localizes on the autophagosomal membranes probably as a lipidated form, the lipid is a constitutive of the membranes. Then, I have tried to analyze Atg8-PE.

Ichimura *et al.* identified PE as the sole lipid conjugated to the C-terminal glycine of Atg8 purified from yeast cells (Ichimura *et al.* 2000). In vitro, however, Atg8 also forms a conjugate with PS (Ichimura *et al.* 2004), which like PE has an amino group in the head moiety (Fig. 4). Likewise, LC3 also conjugated to PS as efficiently as PE in vitro system, even though the lipidated form of LC3 purified from cultured cells contained only LC3-PE (Sou *et al.* 2006). These results suggest that there exists a mechanism that directs Atg8 conjugation preferentially to PE in the cell. Hanada *et al.* showed that neither Atg12-Atg5 nor its complex with Atg16 is involved in this mechanism; they stimulated both Atg8-PE and Atg8-PS formation to a similar degree (Hanada *et al.* 2007). In this study, I showed that the preferential conjugation of Atg8 to PE can be reproduced in vitro either by physiological pH or the addition of acidic phospholipids to liposomes (Oh-oka *et al.* 2008). Further analyses suggest that physiological pH conditions evoke the intrinsic substrate specificity of Atg3 and that acidic phospholipids facilitate the binding of the Atg8-Atg7 and the Atg8-Atg3 thioester intermediates to the membrane (Oh-oka *et al.* 2008). However, the formation of Atg8-PE and Atg8-PS were accelerated by Atg12-Atg5, thus the less efficient but significant production of Atg8-PS was still observed (Oh-oka *et al.* 2008). Therefore, I have tried to know whether Atg8 conjugates not only to PE but also to PS in vivo. Furthermore, I wanted to know

whether Atg8-PS is essential for autophagy, but it is difficult to remove only Atg8-PS. Then, I investigated the autophagic activity using *pss1Δ* cells. Pss1 is known to as an only enzyme to generate PS (Letts *et al.* 1983), and *PSS1* is not a lethal gene. I showed that Atg8-PS is not essential for autophagosome formation if Atg8 conjugates to PS in vivo.

Atg8 is activated by the E1 enzyme Atg7 and the E2 enzyme Atg3 then conjugated to PE. Atg7 is essential for both Atg12 and Atg8 conjugation reactions. Atg7 forms a homodimer via the C-terminal region, which is essential for the activity as the E1 enzyme and the interaction with Atg3 (Komatsu *et al.* 2001). Furthermore, analyses based on the crystal structure of Atg3 revealed its binding sites for Atg8 and Atg7 (Yamada *et al.* 2007). However, the detail of protein interactions in the Atg8 conjugation reaction is still unknown. Therefore, I performed gel filtration chromatography to gain insights into this issue.

## Results

### **The Atg8-PS formation is severely suppressed under neutral pH conditions**

The conjugation of Atg8 to phosphatidylethanolamine (PE) can be reproduced in vitro by mixing purified proteins, Atg8, Atg7, Atg3, with liposomes containing PE and incubating this mixture in the presence of ATP (Ichimura *et al.* 2004). Ichimura *et al.* examined optimal conditions in establishing this system and accordingly set the pH of the reaction buffer at 8.0 (Ichimura *et al.* 2004). Under these conditions, the same system also efficiently mediates the conjugation of Atg8 to phosphatidylserine (PS) (Fig. 5A, left panels) (Ichimura *et al.* 2004). The intracellular pH of yeast cells, however, has been estimated at ~6.0-7.0 (Valli *et al.* 2005). I found that PS conjugation barely occurred at pH 7.0, even with liposomes containing high concentrations of PS; in contrast, PE conjugation occurred efficiently (Fig. 5A, right panels). I performed conjugation reactions at various pH, using liposomes composed of 50 mol% of phosphatidylcholine (PC) and either 50 mol% of PE or PS (Fig. 5B, C). The results clearly showed that PS conjugation is more sensitive than PE conjugation to lowering pH. Thus, the formation of Atg8-PS is markedly attenuated under physiological pH conditions. I also found that the Atg8-PE was not formed at pH 7.0 for 30 min, and the PE conjugation reaction then rapidly proceeded (see Discussion).

### **Atg8 transfer from Atg3 to PS is restricted at neutral pH**

I investigated which step(s) in the Atg8-PS formation was affected at neutral pH. To this end, I aimed to capture authentic thioester intermediates Atg8-Atg7 and Atg8-Atg3, whereas E1 and E2 mutants with the active center cysteines replaced with serines have been conventionally used to fix the intermediates in studies of ubiquitin and other Ubl systems. However, probably because of their alkali lability, these intermediates could not be detected by standard basic SDS-PAGE (Laemmli *et al.* 1970). Therefore, I tried the NuPAGE

system (Invitrogen) that allows separation of proteins in a neutral pH environment. Atg8, Atg7, and Atg3 were incubated with ATP in the absence of liposomes and subjected to the NuPAGE separation without reducing reagents. Two additional bands of ~85 and 55 kDa appeared upon incubation, accompanied by a concomitant decrease of Atg7 and Atg3 (Fig. 6A, lanes 1-5). These bands were sensitive to the reductant  $\beta$ -mercaptoethanol (ME) (Fig. 6A, lane 6), and their production depended on ATP (Fig. 6A, lane 7). Neither of these products appeared if Atg7 was excluded (Fig. 6A, lane 8), and only the 85-kDa band appeared in the absence of Atg3 (data not shown and Fig. 11A, lanes 16-21). Immunoblotting analyses showed that the 85- and 55-kDa products both contained Atg8 and exclusively contained Atg7 and Atg3, respectively (Fig. 6B, the 100- kDa band contained Atg3 and vanished upon incubation). Taken together, these results suggest that these products are thioester intermediates of the conjugation reaction, Atg8–Atg7 and Atg8–Atg3.

I also analyzed reactions containing PE liposomes using this system (Fig. 7). I found that both the 85- and 55-kDa products significantly decreased upon the completion of the Atg8–PE formation, accompanied by a concomitant increase in free Atg3 (Fig. 7, left panels, free Atg7 was somehow upshifted after long incubation), suggesting that these products are bona fide reaction intermediates. I showed that both of the intermediates were formed in the presence of PS liposomes as rapidly as with PE liposomes even at pH 7.0 and stably persisted during incubation; the conjugation of Atg8 with PS hardly occurred in this condition. These results suggest that transfer of Atg8 from Atg3 to PS is specifically impeded at neutral pH.

### **Acidic phospholipids specifically stimulate conjugation reactions with PE liposomes**

Ichimura *et al.* reported that the Atg8–PE formation was accelerated by the addition of acidic phospholipids to liposomes (Ichimura *et al.* 2004). This stimulative effect was clearly observed when the basal reaction efficiency was lowered by decreasing the PE concentration to 25 mol%. In addition, to examine

the effect of acidic phospholipids on the Atg8–PS formation, I used a reaction buffer of pH 8.0. I examined the PE and PS conjugation of Atg8 under these conditions, with liposomes containing various concentrations of phosphatidylinositol (PI), a major acidic phospholipid in eukaryotic cells (Fig. 8). Consistent with the previous report, Atg8 was efficiently conjugated with PE as the PI concentration increased, saturating at ~10 mol% (Fig. 8A, left panel). In contrast, I found that PS conjugation was not at all stimulated by the addition of PI (Fig. 8A, right panel). This was also the case for other acidic phospholipids, phosphatidic acid (PA) and phosphatidylglycerol (PG); these lipids promoted PE conjugation to similar extents but did not promote PS conjugation at all (Fig. 8B). Similar results were obtained in experiments at pH 7.0 (data not shown). I conclude that the PE conjugation of Atg8 occurs even more preferentially in the presence of negatively charged lipids.

In addition, it has been recently reported that PI3P, whose production by the PI3-kinase Vps34 is essential for autophagy, is enriched in autophagosomal membranes as well as vacuolar and endosomal membranes (Gillooly *et al.* 2000, Obara *et al.* 2008). I showed that PI3P also stimulates the Atg8–PE formation *in vitro*; its extent, however, was comparable with that of PI (Fig. 9).

### **Acidic phospholipids facilitate the membrane binding of thioester intermediates**

I next investigated how acidic phospholipids facilitated the production of Atg8–PE. The addition of PI to liposomes did not affect the formation rates of either the Atg8–Atg7 or the Atg8–Atg3 intermediates (Fig. 10). I then examined the binding of the proteins to liposomes, by cosedimentation experiments (Fig. 11). Atg8, Atg7, and Atg3 were incubated with ATP to form the thioester intermediates, chilled, and then mixed with liposomes, followed by ultracentrifugation to sediment the liposomes and bound proteins. A reaction buffer at pH 7.0 was used in these experiments. I confirmed that lipidation of Atg8 did not occur during these manipulations (data not shown) and that no significant protein precipitation occurred in the absence of liposomes (Fig. 11A,

lanes 1-3). Similarly, no significant cosedimentation was observed when the proteins were mixed with PE liposome that did not contain PI (Fig. 11A, lanes 4-6). However, the addition of PI to this liposome significantly increased the levels of Atg8-Atg7 and Atg8-Atg3 cosedimented with the liposomes (Fig. 11A, lanes 7-9, B). In addition, the experiments without ATP clearly showed that Atg8, Atg7, and Atg3 alone did not bind to the PE liposome, irrespective of the presence of PI (Fig. 11A, lanes 10-15). These results suggest that acidic phospholipids specifically promote the recruitment of the thioester intermediates to the membrane, thereby facilitating the conjugation reaction of Atg8. I also showed that the binding of Atg8-Atg7 to PE liposomes containing PI depends on Atg3 (Fig. 11A, lanes 16-21).

The binding of the Atg proteins to PS liposomes was also examined. The thioester intermediates did bind to PS liposomes as substantially as PE liposomes containing PI, and this binding was not enhanced by PI addition (Fig. 11A, lanes 22-27, B). This insensitivity to PI is consistent with what I observed in the conjugation reaction (Fig. 8). These results suggest that PS itself also behave as an acidic phospholipid. I used liposomes containing 25 mol% PS for examination of PS conjugation. These PS liposomes are probably saturated for the effect of acidic phospholipids, which might explain their insensitivity to other acidic phospholipids. Furthermore, I want to examine using the PE and PS containing liposome. However, it is difficult to distinguish between Atg8-PE and Atg8-PS produced in the same reaction mixture, because the reaction efficiency is changed dependently on the structure of fatty acid, although I tried to use fluorescence labeled PS. Then, I performed the conjugation reactions with liposomes containing 25 mol% PE and either 0, 5, 15 or 25 mol% PS at pH 7.0. Under these conditions, the conjugation of Atg8 to PS hardly occurs (Fig. 8). It appeared that the formation of Atg8-PE was promoted by PS (Fig. 12); the presence of PS on the same membrane with PE leads to the stimulation of the Atg8-PE formation.

### **In vitro reproduction of the preferential formation of Atg8-PE**

I examined the combination of the buffer pH and the lipid compositions of liposomes that evoked the preferential formation of Atg8-PE. These conditions are thought to be closer to the physiological situation. However, neither PE nor PS conjugation occurred (Fig. 13, upper panels). It has been reported that the Atg12-Atg5 conjugate is indispensable for Atg8-PE production in vivo (Suzuki *et al.* 2001) and that recombinant Atg12-Atg5 indeed stimulates it in vitro (Hanada *et al.* 2007). Therefore, Atg12-Atg5 was included in the reaction under these conditions. As reported previously, the formation of both Atg8-PE and Atg8-PS was accelerated by Atg12-Atg5; however, the acceleration was more prominent for PE conjugation under these conditions (Fig. 13, lower panels).

These results suggest that preferential formation of Atg8-PE can be achieved by combination of the neutral pH, acidic phospholipids, and the Atg12-Atg5 conjugate (Fig. 14).

In addition to controlling conjugate formation, it is also conceivable that rapid deconjugation of Atg8-PS results in the predominant accumulation of Atg8-PE in vivo. However, I showed in vitro that Atg4 deconjugates Atg8-PS similarly or rather inefficiently compared with Atg8-PE (Fig. 15).

### **Purification of the lipidated form of Atg8 from yeast cells**

As described above, the preferential formation of Atg8-PE was reproduced in vitro, however, the less efficient but significant production of Atg8-PS was still observed, suggesting that the exclusive formation of Atg8-PE requires precise in vivo settings for these factors and/or an additional factor(s). Alternatively, this result may imply the production of Atg8-PS in vivo, although its amount should be much less than the PE conjugate. In the previous study, Ichimura *et al.* purified the membrane anchored form of Atg8 using yeast cells lacking *ATG4* and *ATG8*, and expressing His<sub>6</sub>-tagged, Gly116-exposed Atg8, His<sub>6</sub>-Atg8<sup>G116</sup>, and identified PE but not PS as a lipid conjugated to Atg8 (Ichimura *et al.* 2000). However, this analysis included several issues: (i) In *atg4Δ* cells, Atg8-PE was accumulated independently of autophagy; it is possible that purified Atg8-PE might be different from that actually functioning in autophagy. (ii) The purification

of lipidated Atg8 was performed with cells grown to stationary phase ( $OD_{600}=7.0$ ) in nutrient-rich media, where autophagy was not induced. (iii) The membrane fraction was solubilized with 1% TritonX-100; however, I showed that only ~50% of lipidated Atg8 could not be solubilized in this condition (data not shown). There remained the possibility that some specific species of lipidated Atg8 were overlooked in the previous analyses. In this study, I tried to purify lipidated Atg8 paying attention to these points, and to analyze of lipids conjugated to Atg8. (Hereafter, I call lipidated Atg8 instead of Atg8-PE.) First, with regard to solubilization condition, I found that almost all lipidated Atg8 were solubilized with 1% SDS (Fig. 18A). Then, I constructed yeast strains genomically expressing tagged Atg8 such as  $HIS_8$  (H) -Atg8,  $HIS_8$ -TEV (HT) -Atg8,  $HIS_8$ -TEV- 3 × FLAG (HTF) -Atg8, and 3 × FLAG-GST (FG) -Atg8 (Fig. 16A), and examined their autophagic activity by the alkaline phosphatase (ALP) assay. It was shown that all the tagged Atg8 except for FG-Atg8 was almost fully functional (Fig. 16B). The activity of autophagy can also be assessed by examining the maturation of a vacuolar enzyme, Ape1. Ape1 is synthesized in a pro-form (pr Ape1) and delivered from the cytoplasm to the vacuole via the Cvt pathway (Scott *et al.* 2001). Under starvation conditions, Ape1 is more efficiently transported to the vacuole via the autophagic pathway. Upon delivery to the vacuole, Ape1 is cleaved to be matured (mApe1). Ape1 was matured in cells expressing the tagged Atg8 except for FG-Atg8 as efficiently as wild type cells (Fig. 16C). I also examined lipidation of the Atg8 variants by immunoblotting analyses (Fig. 16C). Lipidation of Atg8 was stimulated when autophagy is induced. Lipidated Atg8 was accumulated under starvation conditions in wild type cells and cells expressing H- and HTF-Atg8, although the expression level of HT-Atg8 was somehow reduced (Fig. 16C). I could not see lipidated Atg8 in FG-Atg8 cells (Fig. 16C). Because I expected that two successive purifications were required to purify lipidated Atg8, I decided to use the cells expressing HTF-Atg8. It is known that the affinity of Atg8-PE to antibodies against the N-terminal region of Atg8 is ~12-fold higher than that of unlipidated Atg8 (Ichimura *et al.* 2004), and I showed that the affinity of Atg8-PS produced by the

in vitro reaction was similar to that of Atg8-PE (data not shown). Therefore, the amount of lipidated HTF-Atg8 was estimated at about 20% of total Atg8.

Cells were starved in SD (-N) media for 4.5 h to induce autophagy. It is known that lipidated Atg8 is deconjugated by the Cysteine protease Atg4 (Kirisako *et al.* 2000). To suppress the possible activity of Atg4 in cell lysates, the cells were suspended with the buffer containing 20 mM N-ethylmaleimide (NEM), a cysteine modification reagent, and disrupted by multi-beads shocker (Yasui-kikai). The lysates were diluted with the buffer containing 4 M urea, and centrifuged at 500  $\times g$  at 4°C for 10 min to remove cell debris. The supernatant was centrifuged at 100,000  $\times g$  for 30 min to sediment membranes containing lipidated Atg8. It is notable that lipidated Atg8 was sedimented by centrifugation at 500  $\times g$ , when the lysate was diluted with the buffer containing 2 M urea or without urea. Oppositely, lipidated Atg8 could not be sedimented by centrifugation at 100,000  $\times g$ , when the lysate was diluted with the buffer containing 6 M urea (data not shown). The precipitate was solubilized with 1% SDS, which was then diluted to 10 fold with the buffer containing protease inhibitor cocktail (PIC) and 20 mM NEM, followed by centrifugation at 100,000  $\times g$  for 30 min. I showed that it is important to add PIC and NEM in this step to stabilize lipidated Atg8 (data not shown). The supernatant was subjected to purification with Ni-NTA agarose. The elution of bound proteins was executed with the buffer containing 250 mM imidazole. This eluate was further subjected to purification with anti-FLAG M2 affinity agarose. The bound proteins were eluted with the buffer containing 1 mg/ml 3 $\times$ FLAG peptide. These processes were carried out by the batch method, because the column method somehow did not allow elution of lipidated Atg8 from the column (data not shown). The efficiency of purification of lipidated Atg8 was examined by immunoblotting with anti-Atg8 antibodies, showing that almost all lipidated Atg8 could be purified (Fig. 18A). The final eluates obtained from 1500 O.D.unit cells expressing H-Atg8 and HTF-Atg8 were subjected to urea-SDS-PAGE and analyzed by silver staining (Fig. 18A, lane17). Two specific bands of 18 and 20 kDa appeared in the sample of HTF-Atg8 compared with that of H-Atg8 (Fig. 18B). These products should be

lipidated and unlipidated HTF-Atg8. Now, I am trying to analyze the head group and the structures of the fatty acid of this purified lipidated HTF-Atg8 by LC-MS/MS.

### **PS is not essential for autophagy**

As aforementioned, there remains the possibility that a small amount of Atg8-PS is produced in the cell. Although this will be directly addressed by analyses of lipidated Atg8, which I purified, I also examined the possible significance of Atg8-PS in autophagy using the *pss1Δ* strain. Pss1 is essential for biosynthesis of PS (Letts *et al.* 1983), and its deletion is not lethal. Several descriptions were found in *pss1Δ* cells. (i) The growth of *pss1Δ* cells were retarded. (ii) In the *pss1Δ* cells, the intensified fragmentation of vacuoles was observed, and the viability was reduced under nitrogen starvation conditions. Therefore, rapamycin was added to the YEPD medium to induce autophagy, although the fragmentation of vacuoles was observed (Fig. 19A). (iii) The expression of Atg8 and the vacuolar protease Pra1 were elevated prominently, and the maturation of Ape1 was promoted even under growing conditions in the *pss1Δ* strains (Fig. 19C). The *pss1Δ* cells may be subjected to stress of starvation even under growing conditions. (iv) The membrane transport of the Carboxypeptidase Y (CPY), which is translocated into endoplasmic reticulum membranes and transported to the vacuole via the golgi apparatus (Stevens *et al.* 1982), is normal (Fig. 19C).

In wild type cells, autophagic bodies accumulated in the vacuole in the presence of phenylmethylsulfonyl fluoride (PMSF), which prevents the disintegration of autophagic bodies. In *atg8Δ* cells, no autophagic body was observed, and in *pra1Δ* cells, autophagic bodies were accumulated even under growing conditions. In *pss1Δ* cells, autophagic bodies accumulated as well as wild type cells, in the presence of PMSF (Fig. 19A). I also examined the autophagic activity by the ALP assay, and showed that the activity of *pss1Δ* cells was similar to that of wild-type cells (Fig. 19B). In *pss1Δ* cells treated with rapamycin, the transport of prApe1 to the vacuole and the formation of Atg8-PE

occurred normally (Fig. 19C). Taken these results together, I concluded that PS is not essential for autophagy even if Atg8 conjugates to PS in vivo.

### **Membrane fusion does not occur between liposomes tethered by Atg8-PS**

It is known that Atg8 forms an oligomer in response to PE conjugation, leading to the tethering together and hemifusion of the liposomes (Nakatogawa *et al.* 2007). I examined whether Atg8-PS has similar activities in vitro. This reaction mixtures became turbid during the incubation, which under a light microscope, was found to be a result of forming aggregates (data not shown). I also examined whether membrane fusion occurred between the liposomes connected by Atg8-PS using lipid mixing assay. As shown in Fig. 20A, this method is based on energy transfer from NBD (Excitation: 460 nm, Emission: 534 nm) to Rho (Excitation: 550 nm, Emission: 590 nm), each of which is conjugated to PE. Because of the amino group of the ethanolamine moiety is modified with the dyes, these lipids cannot be conjugated with Atg8. If both of the conjugated dyes are present at appropriate concentrations in the same liposome, the fluorescence of NBD is effectively quenched by Rho. If this “NBD+Rho” liposome is fused with a “nonlabelled” liposome, which results in an increase of the average distance between the two dyes on the membrane, the NBD fluorescence will be dequenched. A mixture of the nonlabeled and “NBD+Rho” liposomes were subjected to the conjugation reaction (Fig. 20B). The resulting liposome clusters were dissociated by proteinase K treatment, followed by fluorescence measurements (Excitation: 460 nm, Emission: 530 nm). As reported previously, a significant ATP-dependent increase of the fluorescence was observed in PE containing liposomes (Fig. 20C, columns 2, 3), suggesting that the membrane fusion was caused by Atg8-PE (Nakatogawa *et al.* 2007). The increase of the fluorescence was also confirmed dependently on polyethylene glycol (PEG), which causes complete fusion (Akiyama *et al.* 2003), in PE containing liposomes (Nakatogawa *et al.* 2007, Figure 20C, columns 4, 5). The increase of the fluorescence was confirmed in PS containing liposomes (Fig. 20C, columns 9 and 10). Whereas, the fluorescence hardly increase

dependently on ATP in PS containing liposomes, which was incubated with Atg8, Atg7 and Atg3 proteins (Fig. 20C, columns 6, 7). These results suggest that membrane fusion does not occur between liposomes tethered by Atg8-PS, although the PS containing liposomes could fuse each other dependently on PEG. However, it should be noted that lipid contents of liposomes affect the efficiency of their fusion; the possibility, PE containing liposome tends to fuse each other, but PS containing liposome does not, is remained. Therefore, more detailed analyses need to assert that 8-PS does not have membrane fusion activity.

### **Gel filtration analyses of components involved in the Atg8 conjugation reaction**

It is known that Atg8 is activated by Atg7, and then transferred to Atg3, followed by conjugation with PE. However, the details of protein interaction in the sequential reaction of Atg8 lipidation are unclear. Therefore, I performed gel filtration chromatography to address this issue. The purified proteins were incubated with or without ATP at 30°C for 10 min. The solution was subjected to size exclusion chromatography on a Sephadex 200 column and eluted from the column 0.8-ml/fraction. Atg8 was detected in fractions 14 and 15, Atg7 was detected in fraction 8, and Atg3 was detected in fraction 11 (Fig. 21A, I~III). It is known that Atg7 forms a homodimer via the C-terminal region, which is essential for the activity as the E1 enzyme and for the interaction with Atg3 (Komatsu *et al.* 2001). Furthermore, analyses based on the crystal structure of Atg3 revealed its binding sites for Atg8 and Atg7 (Yamada *et al.* 2007). In these analyses, a small amount of Atg8 were detected in the same fraction with Atg7, but Atg8 were hardly detected in the same fraction with Atg3 (Fig. 21A, IV, V). These interactions of proteins were too weak to be examined by gel filtration. Atg7 and Atg3 were detected in the same fractions 7 and 8, then it was suggested that the complex was formed by Atg7 and Atg3 (Fig. 21A, VI). When Atg8 and Atg7 were incubated with ATP, the Atg8-Atg7 intermediate was formed and detected in fraction 8 (Fig. 21B, I), although it was unclear whether Atg8-Atg7 interacted

with Atg7. When Atg8, Atg7 and Atg3 were incubated with ATP, the Atg8-Atg7 and the Atg8-Atg3 intermediates were formed predictably. It was predicted that Atg8-Atg7 interacted with Atg3, because Atg8-Atg7 was detected in fraction 7 with Atg3. Interestingly, Atg8-Atg3 was released from Atg7 and was detected in fraction 11 (Fig. 21B, II). I thought that Atg8-Atg3 was released from Atg7 because Atg7-Atg3 complex was dissociated for the active center in Atg3 was used by thioester conjugation. Then, I prepared the Atg7 and Atg3 mutant proteins with the active center cysteines replaced with serines. Atg7-Atg3 complex was dissociated using these protein mutants, and Atg7 and Atg3 was reinstated in fractions 8 and 11 (Fig. 21C, II-V). Furthermore, this complex was not formed by NEM or dithiothreitol (DTT) treatment (Fig. 21C, VI, VII). The immunoblotting analyses using anti-Atg7 and anti-Atg3 antibodies showed that the band of Atg7-Atg3 complex was vanished by adding DTT to reaction buffer (Fig. 21D). These results suggest that this complex was formed via the disulfide bond between active center cysteines in Atg7 and Atg3. Several possibilities for the formation of this complex were considered (see discussion).

## Discussion

Neither of the Atg8 nor the Atg12 systems includes typical E3 enzymes. This is reasonable, considering that both systems target single substrates, which are directly recognized by the E2 enzymes Atg3 and Atg10. In the Atg8 system, Atg3 specifically mediates PE conjugation of Atg8 *in vivo*, however, *in vitro* studies showed that Atg3 can recognize PS in addition to PE (Ichimura *et al.* 2004). Although the existence of some proteinaceous factors has been assumed *in vivo*, here I have shown that intracellular environments such as pH and lipid compositions of membranes can direct the conjugation of Atg8 to PE.

I found that PS conjugation was severely suppressed at neutral pH at the step of Atg8 transfer from Atg3 to lipid, suggesting that substrate specificity intrinsic to Atg3 is displayed under those conditions. Because protonation states of the lipid head groups do not vary significantly among the pH conditions I examined (Cevc. 1993), some conformational change might occur in Atg3 such that PS is not acceptable. I showed that lowering the pH also delayed Atg8 transfer to PE, although to a lesser extent than PS. Therefore, the structural configuration of the substrate-binding pocket of Atg3 (Yamada *et al.* 2007) may be tightened at lower pH.

I also highlighted a unique aspect of Atg3; it is an enzyme whose activity is lowered under physiological conditions. In addition, observation of conjugate formation with the thioester intermediates clearly showed that the conjugase activity of Atg3 is rate-limiting (Fig. 7); Atg12–Atg5 drastically enhances this activity through a direct interaction with Atg3 (Hanada *et al.* 2007). It is known that most autophagy-related proteins, including Atg8 and Atg12–Atg5, localize to a specific perivacuolar structure, preautophagosomal structure (PAS). Therefore, the intrinsically low activity of Atg3 may be important to prevent mislocalized reaction and thus ensure the PAS-limited formation of Atg8–PE.

The liposome binding of the thioester intermediates was increased by acidic phospholipids, and binding of Atg8–Atg7 required Atg3. Because Atg3 conjugated to Atg8 then released from Atg7 (Fig. 21B), Atg8–Atg7 binds to the

liposomes containing acidic phospholipids would depend on free Atg3. I found that Atg8-Atg7 and Atg8-Atg3 bound to liposomes containing acidic phospholipids in the absence of PE and PS (data not shown). In addition, the Atg8-PE formation was stimulated by structurally different acidic phospholipids. These results suggest that Atg8-Atg3 and Atg3 in complex with Atg8-Atg7 could recognize the negatively charged surface of the membrane. Because neither Atg8 nor Atg3 binds to the liposome by itself, I speculate synergistic cooperation of these proteins, which may involve a conserved, positively charged region on Atg8 (Paz *et al.* 2000, Coyle *et al.* 2002, Sugawara *et al.* 2004) and/or some conformational changes induced upon thioester bond formation.

While the stimulative effect of PI was saturated at ~10 mol% in liposomes composed of the nearly electroneutral lipids PC and PE, I used liposomes containing 25 mol% PS for examination of PS conjugation. Because PS itself should also behave as an acidic phospholipid, these PS liposomes are probably saturated for the effect of acidic phospholipids, which might explain their insensitivity to other acidic phospholipids, and also suggests that the efficiency of PS conjugation we observed includes the self-stimulation effect.

The site of the Atg8-PE formation, and thus the lipid composition of the target membrane, remains to be elucidated. However, most intracellular membranes of yeast contain substantial amounts of PI (~10-20%) as well as PE (~20-25%) and PS (~5-10%) (Zinser and Daum. 2005). Therefore, it is probable that acidic phospholipids contribute to the selective conjugation of Atg8 with PE in the cell.

I also found that the reaction curve of Atg8-PE conjugation reaction was drawn like sigmoid (Fig. 5B). Whereas, the graph of the formation of Atg8-PE using liposomes containing PI was drawn linearly (Fig. 8A) as well as the graph of Atg8-PS conjugation reaction (Fig. 5B). These results suggest that the reaction efficiency of Atg8-PE was low initially, because thioester intermediates could not bound to liposomes. The liposome may become acidic gradually as the progression of Atg8-PE conjugation reaction then thioester intermediates

become to bind to the liposomes, then the Atg8-PE formation may be stimulated radically. This is, positive-cooperatively is seen in the Atg8-PE formation. As a result, it is predictable that in vivo, the Atg8-PE formation is accelerated than the Atg8-PS formation on the isolation membrane because it is known that Atg8 is localized on the isolation membrane.

The preferential formation of Atg8-PE was reproduced in vitro when the conjugation reaction is performed at physiological pH in the presence of acidic phospholipids and Atg12-Atg5. However, the less efficient but significant production of Atg8-PS was still observed under the same condition. Then, more careful in vivo analyses will be needed to address these possibilities. As shown in this study, I purified lipidated Atg8 from starved cells. Now, I am trying to identify the lipid molecule conjugated to Atg8 by LC-MS/MS.

The Atg7-Atg3 complex was formed via the disulfide bond between cysteines in their active centers. Several possibilities were considered for this complex formation. First, the disulfide bond was formed unmeaningly, because the cysteine residues in Atg7 and Atg3 of the complex are in close proximity. To verify this possibility, I have to examine whether the Atg7-Atg3 complex is dead-end or intermediate of reaction, although there is a possibility that the bond was meaningless if the bond is not dead end. Secondly, the Atg7-Atg3 complex may stimulate the transfer of Atg8 from Atg7 to Atg3 because Atg7 and Atg3 are held in close proximity. Furthermore, the disulfide bond formation may regulate the Atg8 conjugation reaction. Recently, it was shown that Uba2 (E1) forms a disulfide bond with Ubc9 (E2) for sumoylation when the environment is shifted toward a more oxidizing condition (Bossiss *et al.* 2006). Dr. Ichimura examined optimal conditions in establishing the Atg8 conjugation reaction system and accordingly set the buffer containing 0.2 mM DTT. The Atg8-PE formation is suppressed in the buffer containing 5 mM DTT or without DTT. It is conceivable that the Atg7-Atg3 complex is fixed in the DTT free buffer and the Atg8 conjugation reaction is restricted. Conversely, this complex may dissociate in the buffer containing 5 mM DTT, then Atg8 lipidation is retarded, although there is a possibility that Atg8-Atg7 and Atg8-Atg3 intermediates dissociate. Furthermore,

the stabilization of the Atg7-Atg3 complex may change dependently on the nutrient status. Onodera *et al.* showed that the reductant, acetaldehyde dehydrogenase 6, which is well known as glucose-6-phosphate dehydrogenase (glucose-6-phosphate + NADP<sup>+</sup> → 6-phosphogluconolactone + NADPH) (Grabowska and Chelstowska. 2003), was eliminated selectively by autophagy under starvation conditions. They suggested that intracellular environment is fully reduced under starvation conditions (Onodera *et al.* 2004). It is possibility that Atg7-Atg3 complex is stabilized under growing conditions, this complex may be unstabled just as it should be under starvation conditions. When the disulfide bond becomes unstable, Atg7 might interact with Atg3 weakly besides active center in Atg7 and Atg3 because the less Atg7-Atg3 complex but a small amount was still observed using inactive proteins, Atg7<sup>C507A</sup>, Atg7<sup>C507S</sup>, Atg3<sup>C234A</sup> and Atg3<sup>C234S</sup>. As a result, the conjugation reaction may progresses rapidly under starvation conditions.

When Atg8, Atg7 and Atg3 were incubated with ATP, Atg3 conjugated to Atg8 then released from Atg7 (Fig. 21D). Such a reaction is reasonable, because Atg7 can be reused rapidly for the next cycle of the reaction. Atg7 is also an E1 enzyme in the Atg12-Atg5 conjugation reaction. The rapid release of Atg8-Atg3 from Atg7 is significant for that the Atg8 conjugation reaction system doesn't disturb the Atg12-Atg5 conjugation reaction system. Similarly, E1 of Ubl NEDD8 binds to free E2 and releases NEDD8-E2 thioester intermediate by conformational change of E1 (Huang *et al.* 2007).

I hypothesize the model of the sequential reaction of Atg8 lipidation as shown in Fig 22. Atg7 forms a homodimer with the C-terminal region, and also forms a complex with Atg3 via a disulfide bond between catalytic cysteine residues. The disulfide bond is cleaved, and Atg8 sequentially forms thioester intermediates with Atg7 and Atg3. Atg3 binds to Atg8-Atg7 weakly via a non-covalent interaction (s), thus, the transfer of Atg8 from Atg7 to Atg3 occurs efficiently. Atg7 detached from Atg8-Atg3, then, Atg7 is reused rapidly for the next cycle of the reaction.

In this study, I revealed molecular details of the Atg8 conjugation reaction.

Next, I have to show the details in vivo based on the many findings led from in vitro analyses. Because Atg8 localizes on the isolation membrane and the autophagosome probably as a lipidated form. A lipid(s) conjugated to Atg8 is suggested to be one of lipids those are constituents of autophagosome membranes. Therefore, more detailed analyses of lipids contributed to Atg8, including determination of not only head group species but also structures of fatty acid moieties may provide us with insights into lipid compositions of autophagosomal membranes as well as their origins. Further studies will improve our understanding of the molecular mechanisms of autophagosome formation.

## Materials and Methods

### Protein purification

*Escherichia coli* BL21 cells that carried pGEX-4T encoding Atg8 (exposed for C-terminal glycine) or pGEX6P encoding Atg7, Atg3, Atg4, Atg7<sup>C507A</sup>, Atg7<sup>C507S</sup>, Atg3<sup>C234A</sup> and Atg3<sup>C234S</sup> were grown in 500 ml of LB media (10 mg/ml tryptone, 5 mg/ml yeast extract, 10 mg/ml NaCl, and 1 mM NaOH) containing 50 µg/ml ampicillin to OD<sub>600</sub> = ~0.5 at 37°C, at which point they were chilled on ice for 15 min. The cells were then shaken at 15°C for 20 h in the presence of 0.05 mM isopropylβ-D-thiogalactopyranoside (IPTG) and harvested by centrifugation. The pellets were suspended in 15 ml of buffer A (20 mM HEPES-KOH (pH 7.2) and 0.5 M NaCl) containing 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 g of 0.1 mm chilled glass beads (Yasui-kikai) were added to this suspension. After the cells were disrupted by multi beads shocker (2,500 rpm for 30 sec × 4 times with 30 sec interval at 0°C, Yasui-kikai), the lysates were centrifuged at 13,000 × *g* for 20 min. The supernatants were mixed with 1 ml (bed volume) of Glutathione Sepharose 4B resin (Amersham Bioscience) and rotated at 4°C for 45 min. The resins were subjected to three successive washes with 10 ml of buffer A, and two washes with 10 ml buffer B (20 mM HEPES-KOH (pH 7.2) and 150 mM NaCl) containing 1 mM DTT. The sample of Atg8 was suspended in 1 ml of buffer B containing 1 mM DTT and 20 units of thrombin and incubated at 4°C for 1.5 h. The samples of other proteins were suspended buffer B containing 1 mM DTT and 80 units of PreScission protease (Amersham Biosciences) at 4°C for 4 h. Thrombin and PreScission protease release the glutathione S-transferase (GST) moiety from these proteins. The eluates were appropriately concentrated with VIVASPIN (VIVAScience), and glycerol was added to a final concentration of 25% to Atg8<sup>G116</sup> and 50% to other proteins for storage at -80°C. The Atg12–Atg5 conjugate was kindly gifted from Dr. Hanada (Hanada *et al.* 2007).

### Liposome preparation

Liposomes were prepared using dioleylephosphatidylethanolamine (DOPE), dioleylphosphatidylserine (DOPS), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), phosphatidylinositol purified from yeast (yPI), dioleoylphosphatidic acid (DOPA), dioleylephosphatidylglycerol (DOPG) phosphatidylinositol purified from bovine liver (bPI), Dipalmitoyl PE-7-nitro-2,1,3-benzoxadiazol-4-yl (NBD-PE) and Dioleoyl PE-Lissamine Rhodamine B Sulfonyl (Rho-PE) dissolved in chloroform (Avanti), dipalmitoyl PI (DPPI) (Echelon), dipalmitoyl PI 3-phosphate (DPPI3P) (Sigma) were mixed in various combination in glass tubes. The chloroform was then evaporated, resulting in lipid films. The films were hydrated in distilled water at room temperature for 45 min, and homogeneously sized unilamellar vesicles were prepared by a rapid extrusion procedure (Mayer *et al.* 1986) using a Mini-extruder set in combination with polycarbonate filters with a pore size of 400 nm (Avanti).

### **In vitro conjugation reaction**

Liposomes (350  $\mu$ M lipids) were mixed with purified proteins (5  $\mu$ M Atg8, 0.5  $\mu$ M Atg7, 0.5  $\mu$ M Atg3) in reconstitution buffer (either 50 mM MES-NaOH (pH 6.0) or 50 mM Tris-HCl (pH 7.0, 7.5, 8.0, 8.5, or 9.0), 1 mM ATP, 1 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.2 mM DTT) and incubated at 30°C. The pH of the reaction mixtures was measured by pH/ion meter F-53 (Horiba). The reaction was stopped by mixing with 1/4 volume of SDS sample buffer composed of 250 mM Tris-HCl (pH 7.5), 10% SDS, 40% glycerol, and 0.5 M DTT or 25%  $\beta$ -mercaptoethanol (ME) ; the samples were then boiled for 3 min before urea-SDS-PAGE analysis. For detection of thioester intermediates, SDS sample buffer without reducing reagents was added to the reaction mixtures, which were incubated at 42°C for 5 min before NuPAGE separation.

### **SDS-PAGE and immunoblotting analysis**

SDS-PAGE was performed with 13.5% polyacrylamide gels containing 6 M urea to separate Atg8-PE from unlipidated Atg8, or 10% polyacrylamide gels to detect other proteins. GelCode blue stain reagent (Pierce) was used for

coomassie brilliant blue (CBB)-staining, and silver stain MS kit (Wako) was used for silver staining of the proteins. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) and detected with the polyclonal antibodies against to N-terminal region of Atg8 (Kirisako *et al.* 2000), Atg3 (Ichimura *et al.* 2000), Atg7 (Santa Cruz), Alkaline phosphatase 8 (Pho8) (laboratory stock), Aminopeptidase I (Ape1) (laboratory stock), carboxypeptidase Y (CPY) (Suntory), proteinase A1 (Pra1) (Suntory) by the ECL system (Amersham Pharmacia Biotech). For detection of thioester intermediates, electrophoresis was performed using NuPAGE 12% Bis-Tris gels (Invitrogen).

### **Yeast strains and media.**

The *Saccharomyces cerevisiae* strains used in this study are listed in Table I. SEY6210 and BY4741 were used as hosts for the various plasmids. Cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) medium at 30°C. Autophagy was induced at 30°C for 4 h, in SD(-N) (0.67% yeast nitrogen base without amino acids and 0.5% ammonium sulfate, 2% glucose) or in growth media containing 0.5 µg/ml rapamycin (Sigma).

### **Plasmid Construction**

The sequence of the open reading frame (ORF) of the *ATG8* gene excluding initiation codon (Met) and including sites of restriction enzyme EcoRV and BamHI was amplified by polymerase chain reaction (PCR) using the following primers: 5' – CGGGGTACCGATATCAAGTCTACATTTAAGTCTGA -3' and 5' – CGCGGATCCCTACCTGCCAAATGTATTTT - 3'. The resulting fragments were cloned into EcoRV-BamHI site in pBluescript KS<sup>+</sup>/TEF<sup>T</sup>-TEF<sup>P</sup>-kanMX4 (pBS/T-P-kanMX4, Fig. 11A), which was gifted from Dr. Yamamoto. The following plasmids were constructed based on this plasmid. To construct tagged Atg8, the sequences of His<sub>8</sub> (H) and His<sub>8</sub>-TEV (HT) including Met were cloned into restriction enzyme sites KpnI and EcoRV, the DNA fragments were generated by PCR with oligonucleotides 5' – CATGCATCACCA TCATCACCACCACCACGAT - 3' and 5' – ATCGTGGTGGTGGTGGTGGTGGT



cocktail (PIC) and 20 mM N-ethylmaleimide (NEM), and disrupted using multi-beads shocker (2,500 rpm for 30 sec × 8 times with 30 sec interval at 0°C). The lysates were diluted 10 fold with buffer A containing 4 M urea, and centrifuged at 500 × *g* for 10 min to remove cell debris. The supernatants were centrifuged at 100,000 × *g* for 30 min to sediment membranes containing lipidated Atg8. The precipitates were solubilized with 1% SDS at r.t. for 1 h, which was then diluted to 10 fold with buffer B (buffer A containing 1% Tritonx-100), PIC and 20 mM NEM followed by centrifugation at 100,000 × *g* for 30 min. The supernatant was subjected to purification with Ni-NTA agarose (Invitrogen) at 4°C for 1 h. The resins were washed with buffer B followed by buffer B containing 20 mM imidazole, and the elution of bound proteins was executed with the buffer containing 250 mM imidazole. This eluates were further subjected to purification with anti-FLAG M2 affinity agarose (Sigma) with buffer C (buffer B containing 1 mM ethylenediaminetetraacetic acid (EDTA)), PIC and rotated at 4°C for 1.5 h. The resins were washed with buffer C, and the bound proteins were eluted with buffer C containing 1 mg/ml 3×FLAG peptide. These processes were carried out by the batch method.

### **Preparation of cell lysates.**

Yeast cell lysates were prepared by alkali lysis and glass beads disruption. For the alkali lysis, 5.0 OD<sub>600</sub> units cells were harvested by centrifugation and directly resuspended in 100 µl of an alkaline solution (0.2 N NaOH and 0.5% ME) and incubated on ice for 15 min. The solution was added with 100 µl of cold trichloroacetic acid (TCA), then incubated on ice for 15 min, and collected by centrifugation at 15000 rpm for 5 min at 4°C. The precipitates were washed twice with cold acetone, and suspended in SDS sample buffer (5.0 OD<sub>600</sub> units/100 µl), and boiled for 5 min. The lysates were subjected to SDS-PAGE and immunoblotting. Yeast cells disrupted by Glass beads were used in alkaline phosphatase (ALP) assay as described below section.

### **Assay of autophagy**

Cells grown YEPD ( $OD_{600}=3.0$ ) were incubated further in the presence of rapamycin for 4 h with or without 1 mM PMSF. Under these conditions, accumulation of autophagic bodies in the vacuole was analyzed under light microscope (Takeshige *et al.* 1992).

For the biochemical analyses of autophagy, ALP activity was measured by overexpression of Pho8 $\Delta$ 60 with pTN3 in SEY6210, or using KOY137 (BY4741 *pho8::GPD<sup>P</sup>-PHO8 $\Delta$ 60-kanMX4*) back ground cells, this strain was a gift from Dr. Okamoto. The principle of ALP assay system; ALP is transported to the vacuole from endoplasmic reticulum via secretory pathway, and then processed by Pra1 in the vacuole lumen to become active. SEY6210 expressing Pho8 $\Delta$ 60 or KOY137 has an artificial defect of the ALP transport, subsequently remains proform of ALP (prALP) in the cytosol. The prALP is transported to the vacuole dependently on autophagy. Hence, ALP activity indicates the autophagic activity. Cells grown in YEPD to log phase ( $OD_{600}=1.5\sim 2.0$ ) were incubated further in the presence of rapamycin for 4 h. Harvested cells were washed with distilled water and suspended in 0.1 ml ALP assay buffer (250 mM Tris-HCl (pH 9.0), 10 mM MgSO<sub>4</sub>, 10  $\mu$ M ZnSO<sub>4</sub>) and disrupted by vortexing with glass beads. After centrifugation at 15000 rpm for 5 min, 10  $\mu$ l of the supernatants and 50  $\mu$ l of 55 mM  $\alpha$ -naphthylphosphate were added to 0.5 ml of assay buffer. After incubation for 10 min at 30°C, 0.5 ml of 2 M glycine-NaOH (pH 11.0) was added to stop the reaction. ALP activity was measured by fluorescence spectrometer (emission at 472 nm and excitation at 345 nm), and autophagic activity was expressed by unit ( $\Delta F_{472}/\text{min}/\text{mg protein}/10.7$ ). Protein concentration was determined by the Bicinchoninic Acid (BCA) method (Pierce).

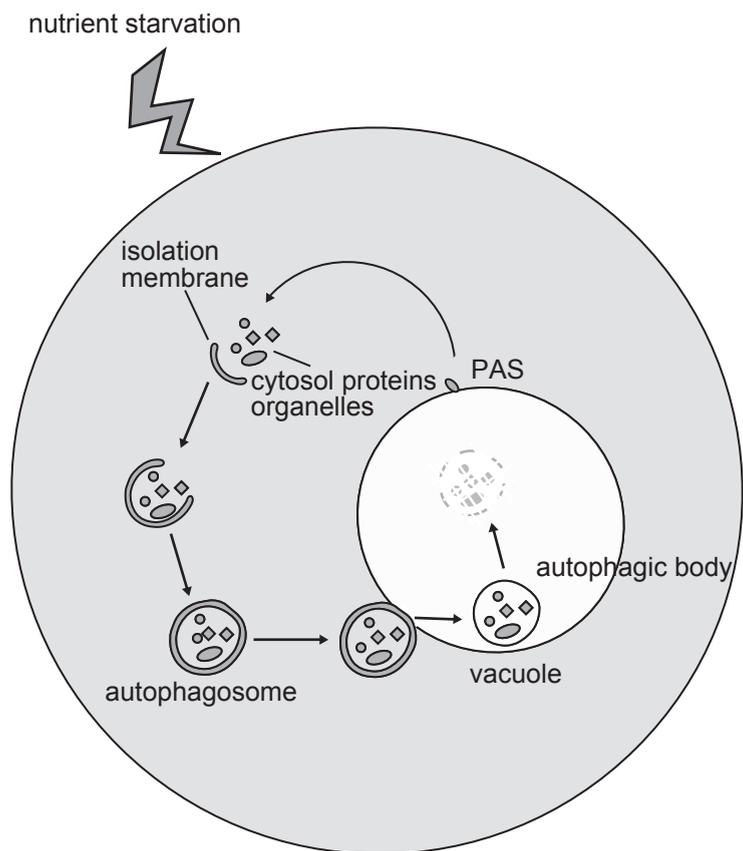
### **Gel filtration**

The purified proteins (2  $\mu$ M Atg8, 2  $\mu$ M Atg7 (Atg7<sup>C507A</sup>, Atg7<sup>C507S</sup>) and 2  $\mu$ M Atg3 (Atg3<sup>C234A</sup>, Atg3<sup>C234S</sup>), were incubated with Tris-HCl (pH 7.0) buffer with or without ATP, NEM, DTT at 30°C for 10 min. The solution was subjected to size exclusion chromatography on a Sephadex 200 column (Amersham Biosciences), equilibrated with 50 mM Tris-HCl (pH 7.0) and 150 mM NaCl. And about 0.05 mg

of protein in 200  $\mu$ l was applied on and eluted from the column at a flow rate of 0.5 ml/min, and 0.8-ml fractions were collected. The column was calibrated with both high and low molecular mass gel filtration standard proteins (Amersham Biosciences) containing Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa). These samples were concentrated with TCA and washed with acetone. These precipitates were suspended in SDS sample buffer with or without DTT and subjected to 10% SDS-PAGE or NuPAGE and analyzed by CBB staining or immunoblotting.

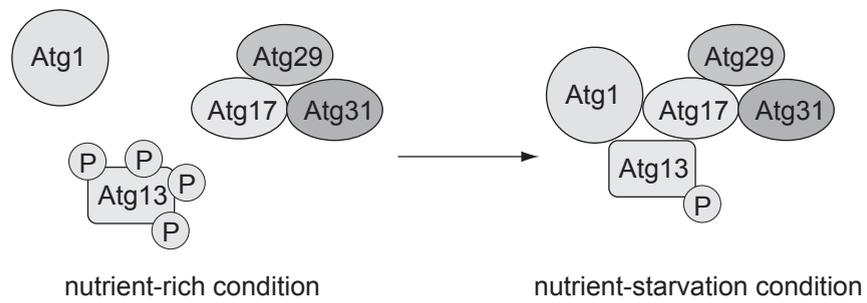
Strain	Genotype	Source
SEY6210	<i>MAT<math>\alpha</math> leu2-3,112 ura3-52 his3<math>\Delta</math>200 trp1<math>\Delta</math>901 lys2-801 suc2-<math>\Delta</math>9</i>	Robinson <i>et al.</i> 1988
KVY5	SEY6210 <i>atg8<math>\Delta</math>::HIS3</i>	Kirisako <i>et al.</i> 1999
KYO23	SEY6210 <i>ATG8::ATG8-kanMX4</i>	This study
KYO25	SEY6210 <i>ATG8::His<math>_{\delta}</math>-ATG8-kanMX4</i>	This study
KYO27	SEY6210 <i>ATG8::His<math>_{\delta}</math>-TEV-ATG8-kanMX4</i>	This study
KYO31	SEY6210 <i>ATG8::His<math>_{\delta}</math>-TEV-3 <math>\times</math> Flag-ATG8-kanMX4</i>	This study
KYO32	SEY6210 <i>ATG8::3 <math>\times</math> Flag-GST-ATG8-kanMX4</i>	This study
KYO13	BY4741 <i>atg8<math>\Delta</math>::kanMX4</i>	This study
YLR423C	BY4741 <i>atg17<math>\Delta</math>::kanMX4</i>	ResGen
YPL154C	BY4741 <i>pra1<math>\Delta</math>::kanMX4</i>	ResGen
YDR481C	BY4741 <i>pho8<math>\Delta</math>::kanMX4</i>	ResGen
KYO51	BY4741 <i>pss1<math>\Delta</math>::nutNT2</i>	This study
KOY137	BY4741 <i>pho8<math>\Delta</math>::GPD<sup>P</sup>-PHO8<math>\Delta</math>60-kanMX4</i>	Dr. Okamoto
KOY190	BY4741 <i>pho8<math>\Delta</math>::GPD<sup>P</sup>-PHO8<math>\Delta</math>60-kanMX4 atg7::NutNT2</i>	Dr. Okamoto
KYO49	BY4741 <i>pho8<math>\Delta</math>::GPD<sup>P</sup>-PHO8<math>\Delta</math>60-kanMX4 pss1::NutNT2</i>	This study

**Table I. Strains used in this study.**

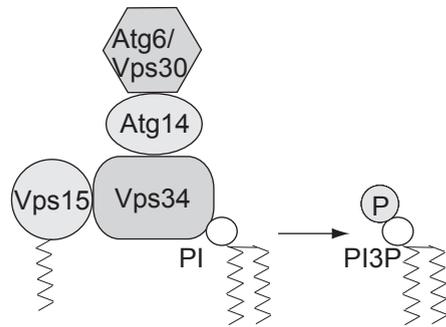


**Fig. 1 A schematic overview of autophagy in yeast.**

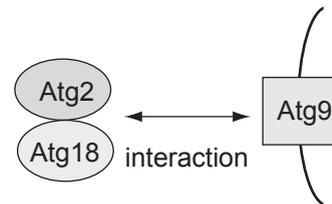
A



B



C

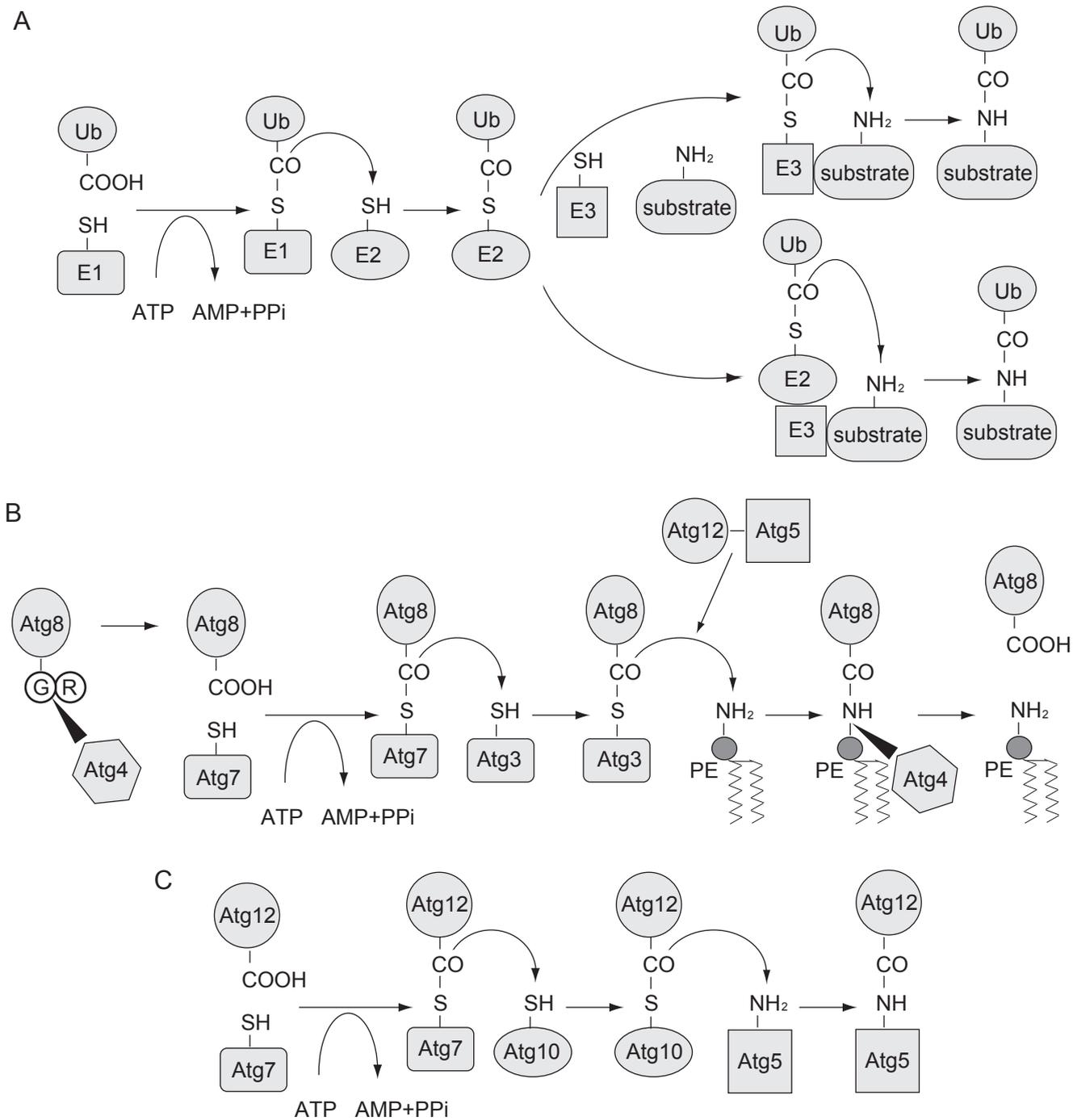


**Fig. 2 Functional subgroups of Atg proteins.**

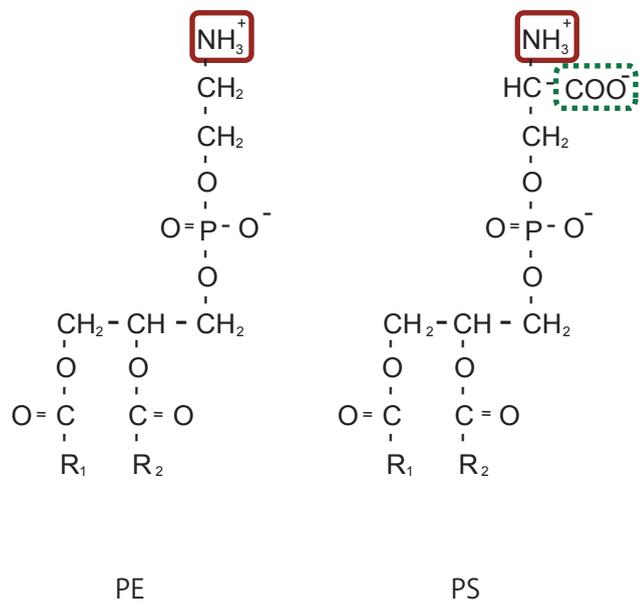
A. The Atg1 kinase and its regulator.

B. The autophagy-specific phosphatidylinositol 3-kinase complex.

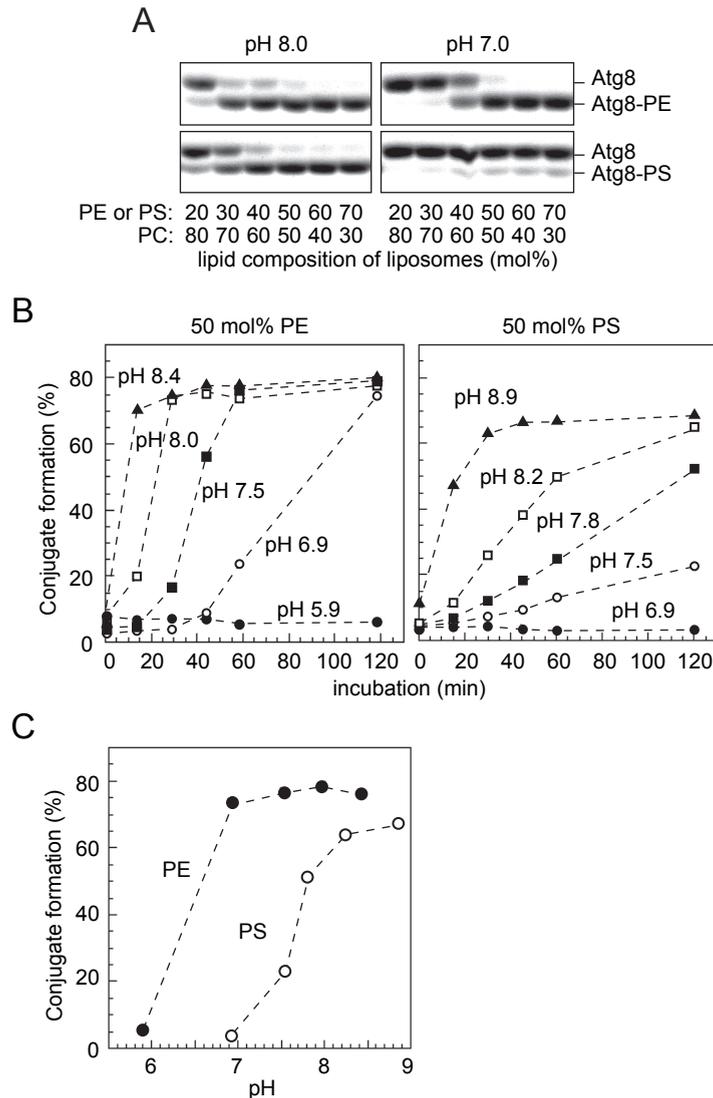
C. The membrane protein complex including Atg9, Atg2 and Atg18.



**Fig. 3 A schematic representation of an ubiquitin and an ubiquitin-like conjugation system.**



**Fig. 4 The molecular structure of PE and PS.**

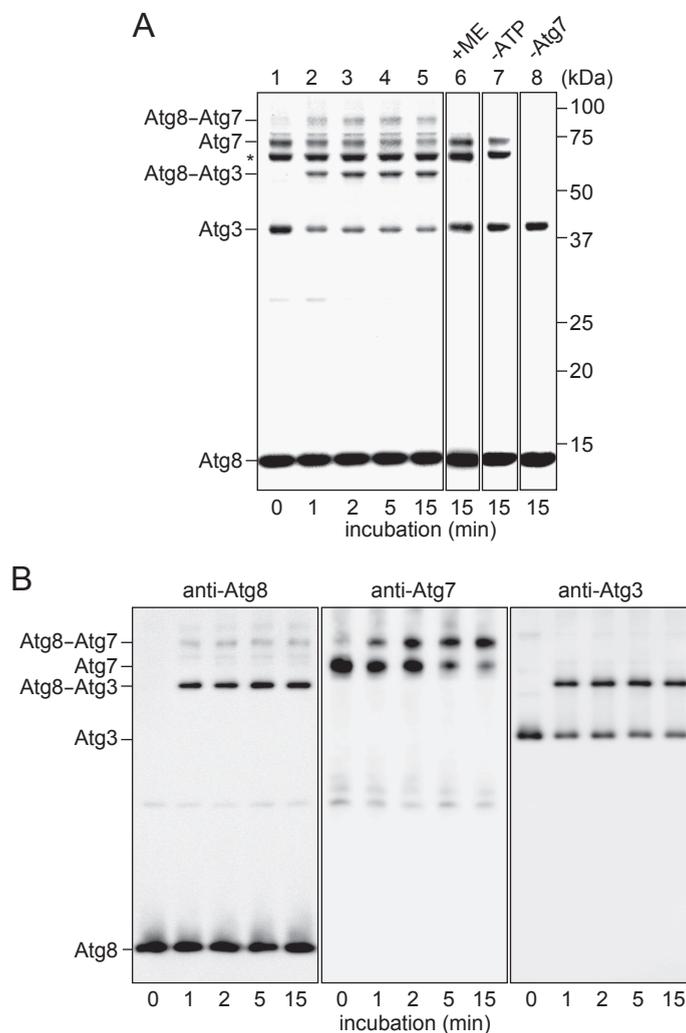


**Fig. 5 Atg8-PS formation is severely suppressed under neutral pH conditions.**

A. Atg8 (5  $\mu$ M), Atg7 (0.5  $\mu$ M), and Atg3 (0.5  $\mu$ M) were incubated with liposomes (350  $\mu$ M) consisting of the indicated concentrations of PC and either PE or PS and 1 mM ATP at 30°C for 120 min using Tris-HCl (pH 8.0 or 7.0) as the reaction buffer. These samples were subjected to urea-SDS-PAGE, followed by CBB staining.

B. The reactions were similarly performed with liposomes consisting of 50 mol% of PE or PS at various pH. The pH of each reaction mixture was determined by a pH meter. After incubation for the indicated time periods, the reaction was stopped by mixing with SDS sample buffer, followed by urea-SDS-PAGE separation. The intensities of the CBB-stained protein bands were measured using the Scion Image software and used to calculate conjugate formation efficiencies (%), in which the intensities of lipidated Atg8 were divided by intensities of total Atg8 protein.

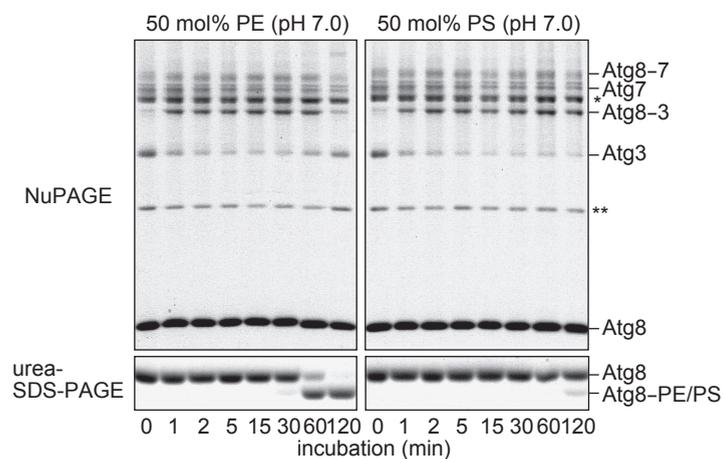
C. The conjugation formation efficiencies after 120 min of incubation shown B are also presented, with the *horizontal* axes showing the pH of the reaction mixtures.



**Fig. 6 Detecting thioester intermediates of the Atg8 conjugation reaction.**

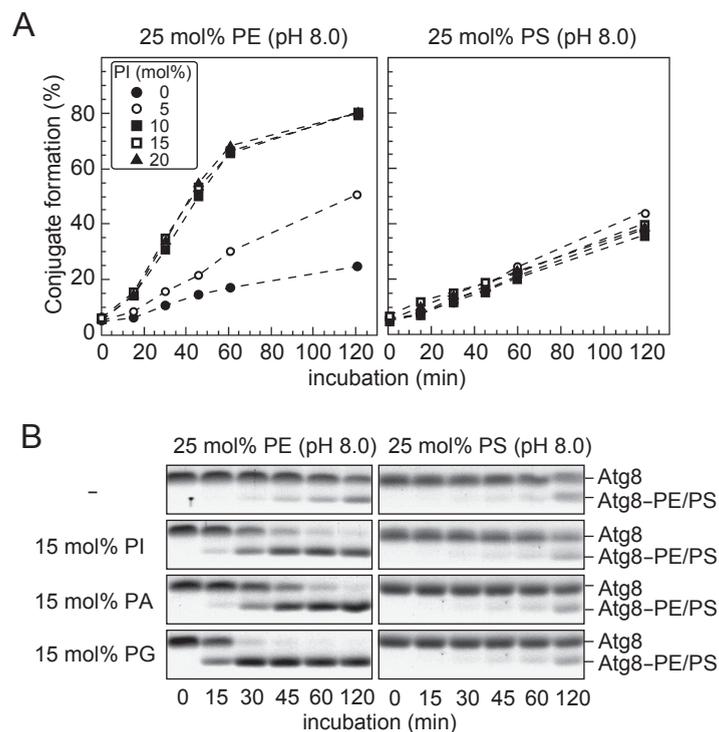
A. Atg8, Atg7, and Atg3 were incubated with ATP in the absence of liposomes at pH 7.0 at 30°C. The samples were mixed with sample buffer without DTT and subjected to NuPAGE separation, followed by CBB staining (lanes 1-5). The sample as in lane 5 was also treated with 0.2 M DTT before NuPAGE (lane 6). The reaction mixtures, incubated without ATP (lane 7) or Atg7 (lane 8), were similarly analyzed in the absence of DTT. The asterisk represents a contaminant protein present in the purified preparation of Atg7.

B. The samples corresponding to lanes 1-5 in A were analyzed by immunoblotting using antibodies against Atg8, Atg7, and Atg3.



**Fig. 7 Transfer of Atg8 from Atg3 to PS is retarded at neutral pH.**

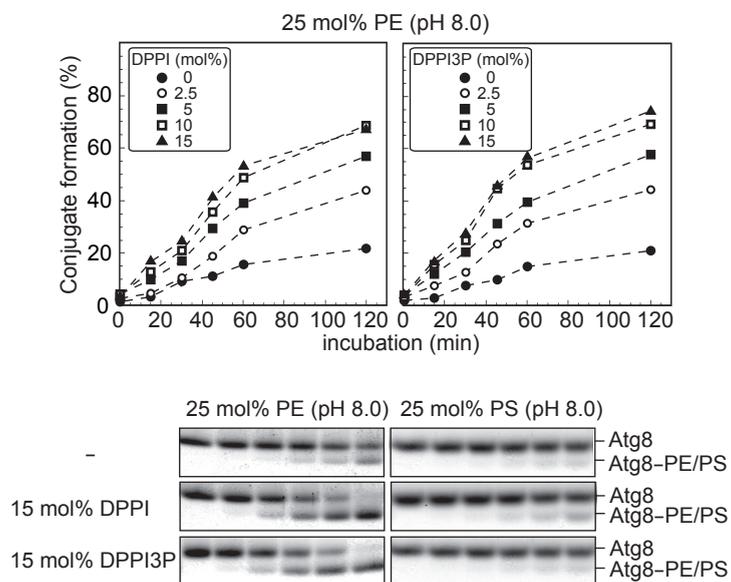
The conjugation reactions performed at pH 7.0 as described in Fig. 5B were analyzed using the NuPAGE gel system as described in Fig. 6A. The same samples were also analyzed using urea-SDS-PAGE to assess lipidation of Atg8. The double asterisks show the bands containing Atg8 that do not appear in the presence of DTT.



**Fig. 8 Acidic phospholipids contribute to the preferential formation of Atg8-PE.**

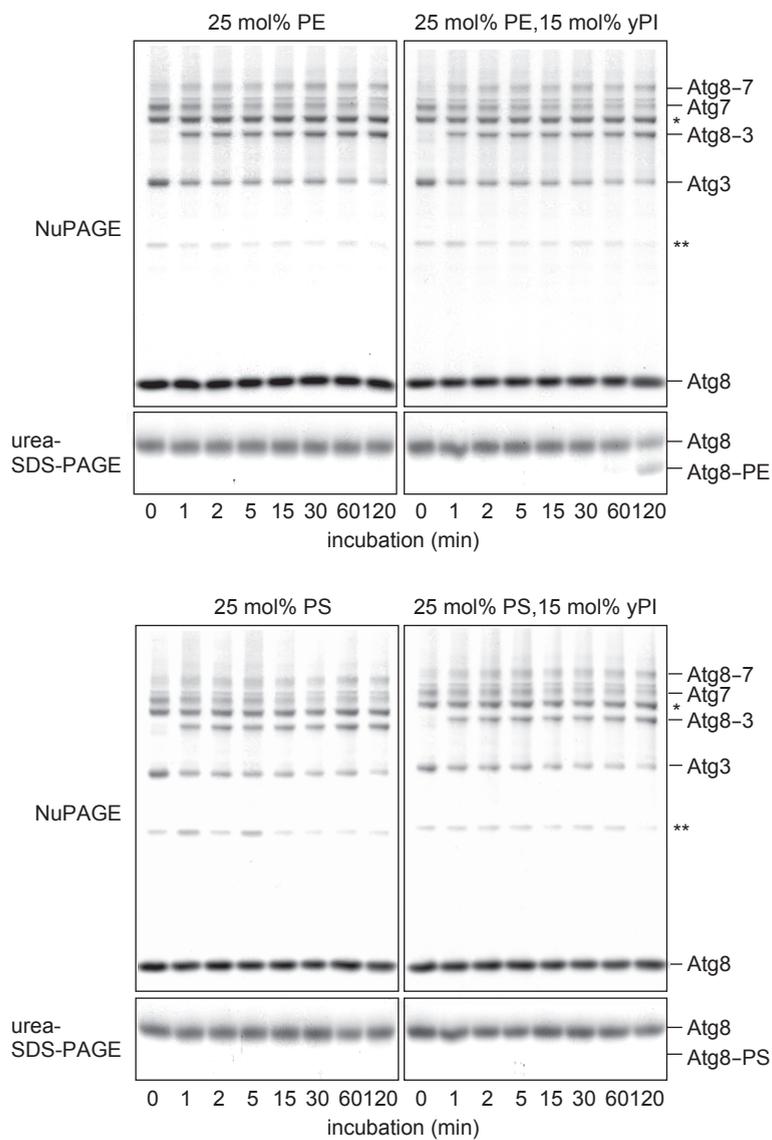
A. The conjugation reactions were performed at pH 8.0 with liposomes composed of either 25 mol% of PE or PS and the indicated and remaining concentrations of PI and PC, respectively, which were analyzed as described in Fig. 5B.

B. The PE and PS conjugation of Atg8 was similarly examined in the presence of various acidic phospholipids.



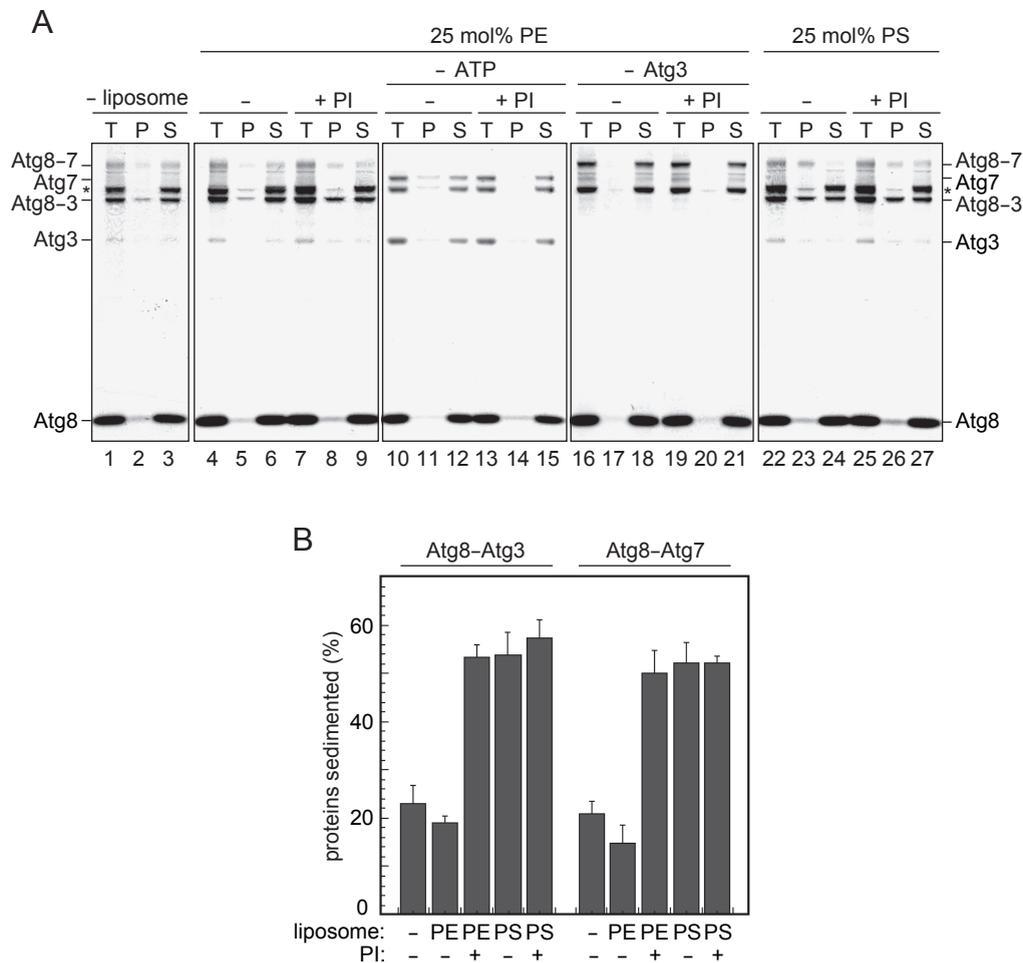
**Fig. 9 PI3P does not stimulate the formation of Atg8-PS.**

The conjugation reactions were performed at pH 8.0 with liposomes composed of 25 mol% PE and the indicated and remaining concentrations of either DPPI or DPPI3P and PC, respectively, which were analyzed as described in Fig. 5B.



**Fig. 10 Acidic phospholipids do not affect formation of thioester intermediates.**

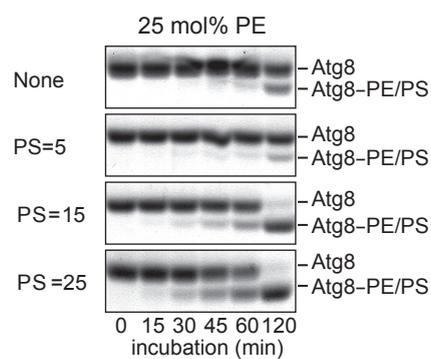
The formation of Atg8-Atg7 and Atg8-Atg3 intermediates at pH 7.0 was examined in the presence of liposomes composed of 75 mol% PC and either 25 mol% PE or PS, or liposomes composed of 60 mol% PC, 15 mol% PI, and either 25 mol% PE or PS, as described in Fig. 7.



**Fig. 11 PI facilitates membrane binding of thioester intermediates.**

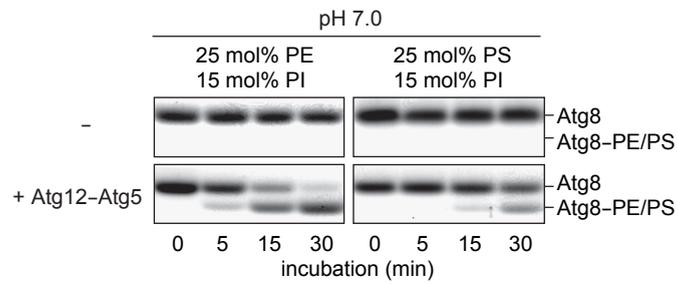
A. The thioester intermediates were formed as described in Fig. 6A (lanes 1-9 and 22-27). The reactions were also performed without ATP (lanes 10-15) or Atg3 (lanes 16-21). These mixtures were chilled on ice for 5 min and then further incubated on ice in the presence (lanes 4-27) or absence (lanes 1-3) of liposomes composed of 75 mol% of PC and either 25 mol% of PE (lanes 4-6, 10-12 and 16-18) or PS (lanes 22-24), or liposomes composed of 60 mol% of PC, 15 mol% of PI, and either 25 mol% of PE (lanes 7-9, 13-15 and 19-21) or PS (lanes 25-27) for 10 min (T, total). The samples were subjected to ultracentrifugation at 100,000  $\times g$  for 30 min at 4°C to generate pellets (P) and supernatants (S). These samples were analyzed by NuPAGE as described in Fig. 6A.

B. The experiments were repeated three times, and the intensities of the bands of Atg8-Atg3 and Atg8-Atg7 were quantified. The average values for the ration of the proteins in the pellet fractions to those in the total samples are shown with error bars for the standard deviations.



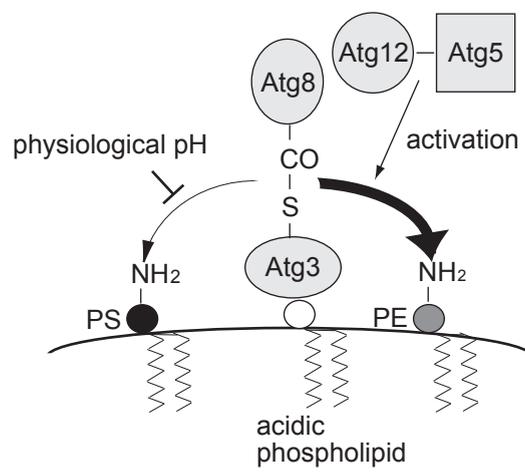
**Fig. 12 PS behaves also as an acidic phospholipid.**

The conjugation reactions were performed at pH 7.0 with liposomes composed of 25 mol% PE and the indicated and remaining concentrations of PS and PC, respectively.

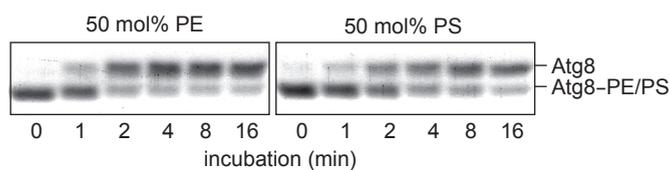


**Fig. 13 In vitro reproduction of preferential Atg8-PE formation.**

The formation of Atg8-PE and Atg8-PS was examined as described in the legend to Fig. 5A using liposomes composed of 60 mol% of PC, 15 mol% of PI, and either 25 mol% of PE or PS at pH 7.0 in the presence or absence of 0.02  $\mu$ M Atg12-Atg5.

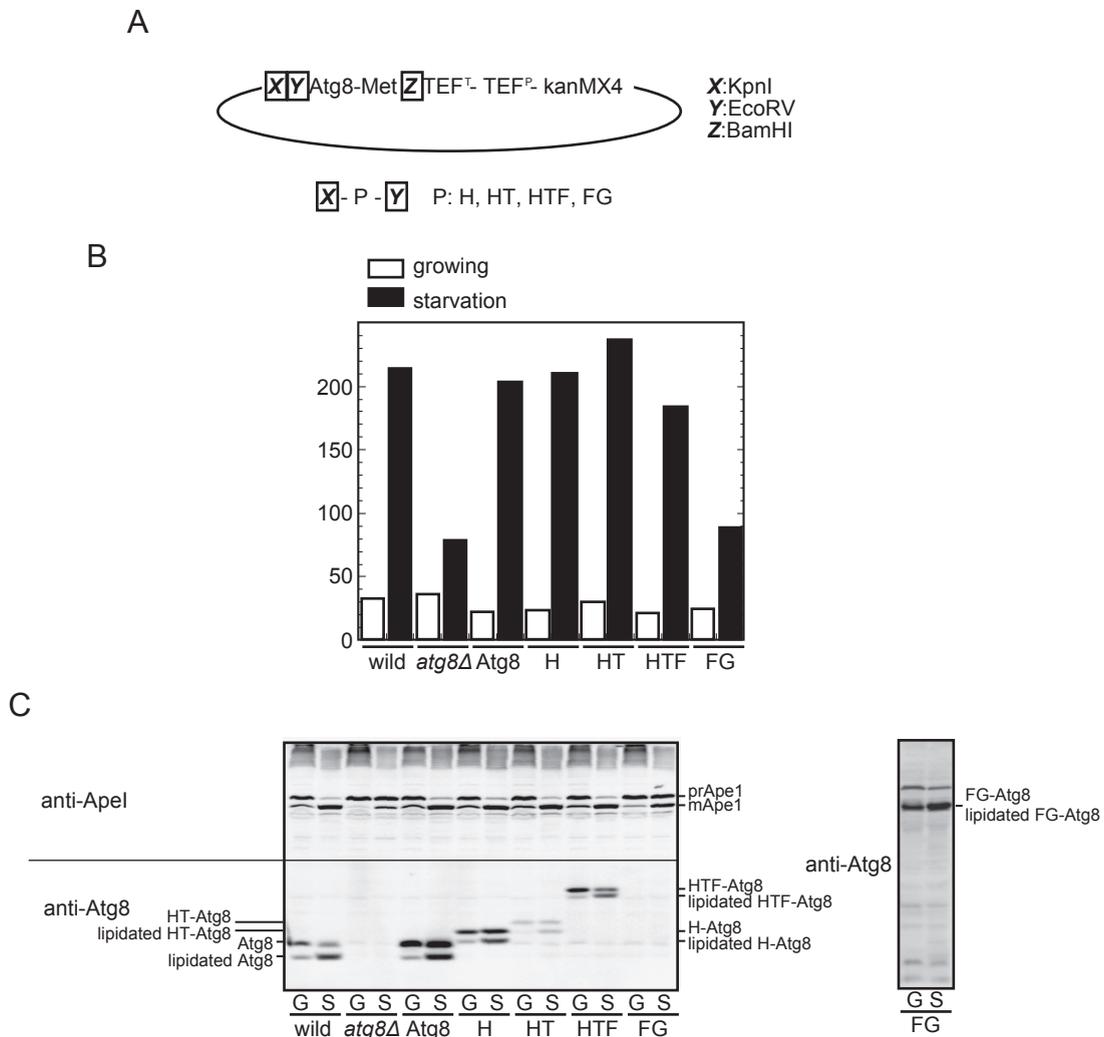


**Fig14. Regulatory mechanism of the Atg8 conjugation reaction**



**Fig. 15 Atg4 mediates deconjugation of Atg8-PS similarly to that of Atg8-PE.**

The PE and PS conjugation reactions of Atg8 were performed at pH 9.0 for 30 min using liposomes composed of 50 mol% PC and either 50 mol% PE or PS, as described in Fig. 5A. The remaining ATP was depleted by incubation with 1U/ml apyrase for 3 min, and the pH of the reaction mixture, was adjusted to 7.0 with 0.5 M MES-NaOH (pH 5.0). Atg4 (0.5  $\mu$ M) was then added, and these samples were incubated at 30°C for the indicated time periods.



**Fig.16 Autophagic activity of the cells expressing tagged Atg8.**

A. Plasmid construction of tagged Atg8. X, Y, and Z were restriction enzyme site, KpnI, EcoRV and BamHI. His<sub>8</sub> (H), His<sub>8</sub>-TEV (HT), His<sub>8</sub>-TEV-3×Flag (HTF), 3×Flag-GST (FG) were cloned into KpnI-EcoRV sites with initiation codon (Met). The sequence of ORF of Atg8 excluding Met was cloned into EcoRV-BamHI sites.

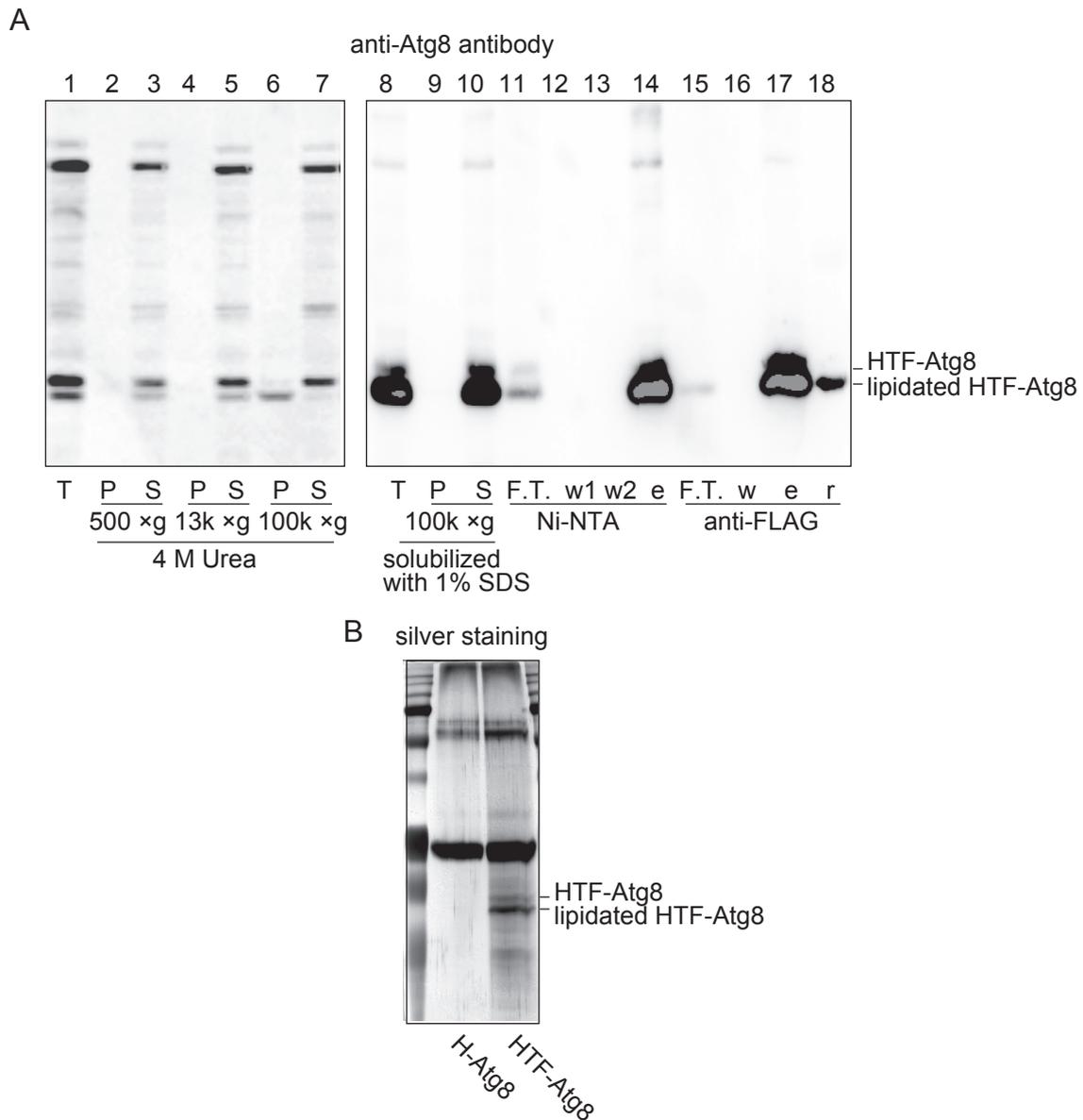
B. ALP activity of the cells overexpressing Pho8Δ60 with pTN3 was measured. Several cells were grown on SD (-ura) medium (O.D.<sub>600</sub>=1.5) (open bars), and starved in SD (-N) medium for 4.5 h (closed bars).

C. Immunoblotting by anti-Ape 1 and anti-Atg8. SEY6210 cells genomically expressing tagged Atg8 were grown on YEPD medium (O.D.<sub>600</sub>=0.8) (G), and starved in SD (-N) medium for 4.5 h (S).

yeast cells were grown in YPD to  $OD_{600} = \sim 1.5$   
 ↓  
 starved in SD(-N) media for 4.5 hr  
 ↓  
 cells were suspended with buffer A (50 mM Tris-HCl (pH 7.0), 150 mM NaCl), 1 mM PMSF, PIC and 20 mM NEM  
 ↓  
 cells were disrupted by beads shocker (2500 rpm, 30 s, 8 times)  
 ↓  
 dilution of lysate with buffer A containing 4 M urea  
 ↓  
 cfg. 500 ×g at 4°C for 10 min  
 ↓  
 cfg. 100,000 ×g at 4°C for 30 min  
 ↓  
 solubilization with 1% SDS at r.t. for 1 hr  
 ↓  
 dilution with buffer B (buffer A containing 1% TritonX-100), PIC and 20 mM NEM  
 ↓  
 cfg. 100.000 ×g at 4°C for 30 min  
 ↓  
 add Ni-NTA agarose and incubation at 4°C for 1 hr  
 ↓  
 remove sup.  
 ↓  
 washed with buffer B  
 ↓  
 washed with buffer B containing 20 mM imidazol  
 ↓  
 eluted with buffer B containing 250 mM imidazol  
 ↓  
 add EDTA (final 1 mM) and anti-FLAG M2 affinity gel and incubation at 4°C for 1.5 hr  
 ↓  
 washed with buffer C (buffer B containing 1 mM EDTA)  
 ↓  
 eluted with buffer C containing 1 mg/ml 3×Flag peptide

batch method

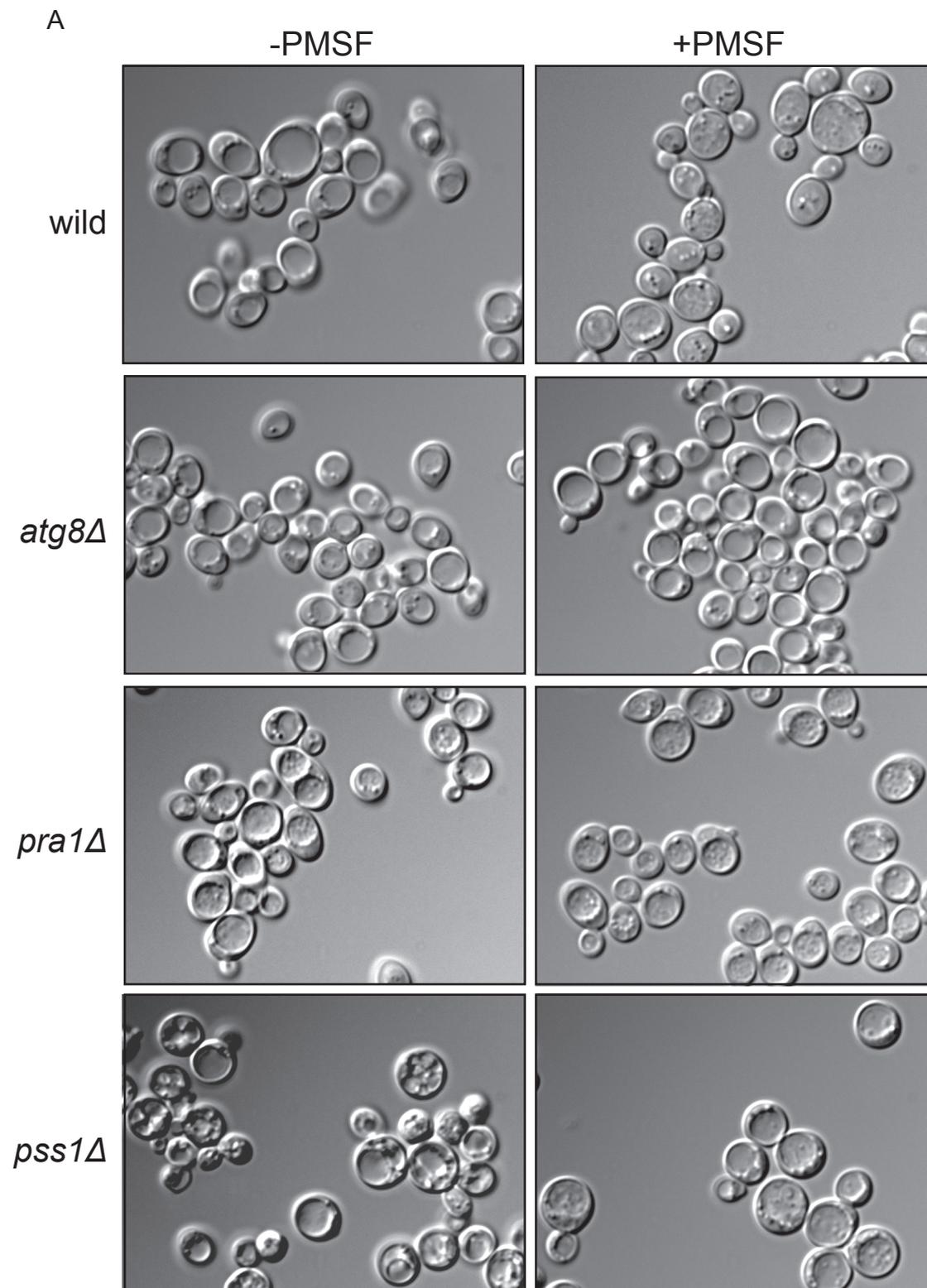
**Fig. 17 The outline of the isolation of lipidated Atg8.**

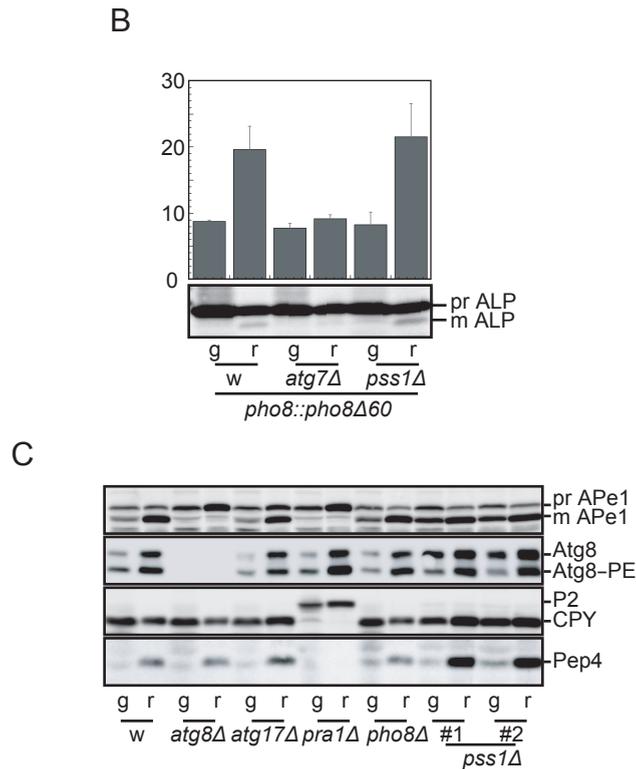


**Fig. 18 Purification of lipidated Atg8.**

A. Lipidated Atg8 were purified as shown in Fig. 17 from SEY6210 *ATG8::HTF-ATG8* cells. The purification efficiency was checked by immunoblotting with anti-Atg8 antibody. 0.2 O.D.cells / lane was injected to lanes 1-7, 1 O.D.cells / lane was injected to lanes 8-13, 5 O.D.cells / lane was injected to lanes 14-16, 20 O.D.cells / lane was injected to lanes 17, 18.

B. Lipidated Atg8 were purified from 1500 O.D.cells genomically expressing H-Atg8 or HTF-Atg8, and detected by silver staining.



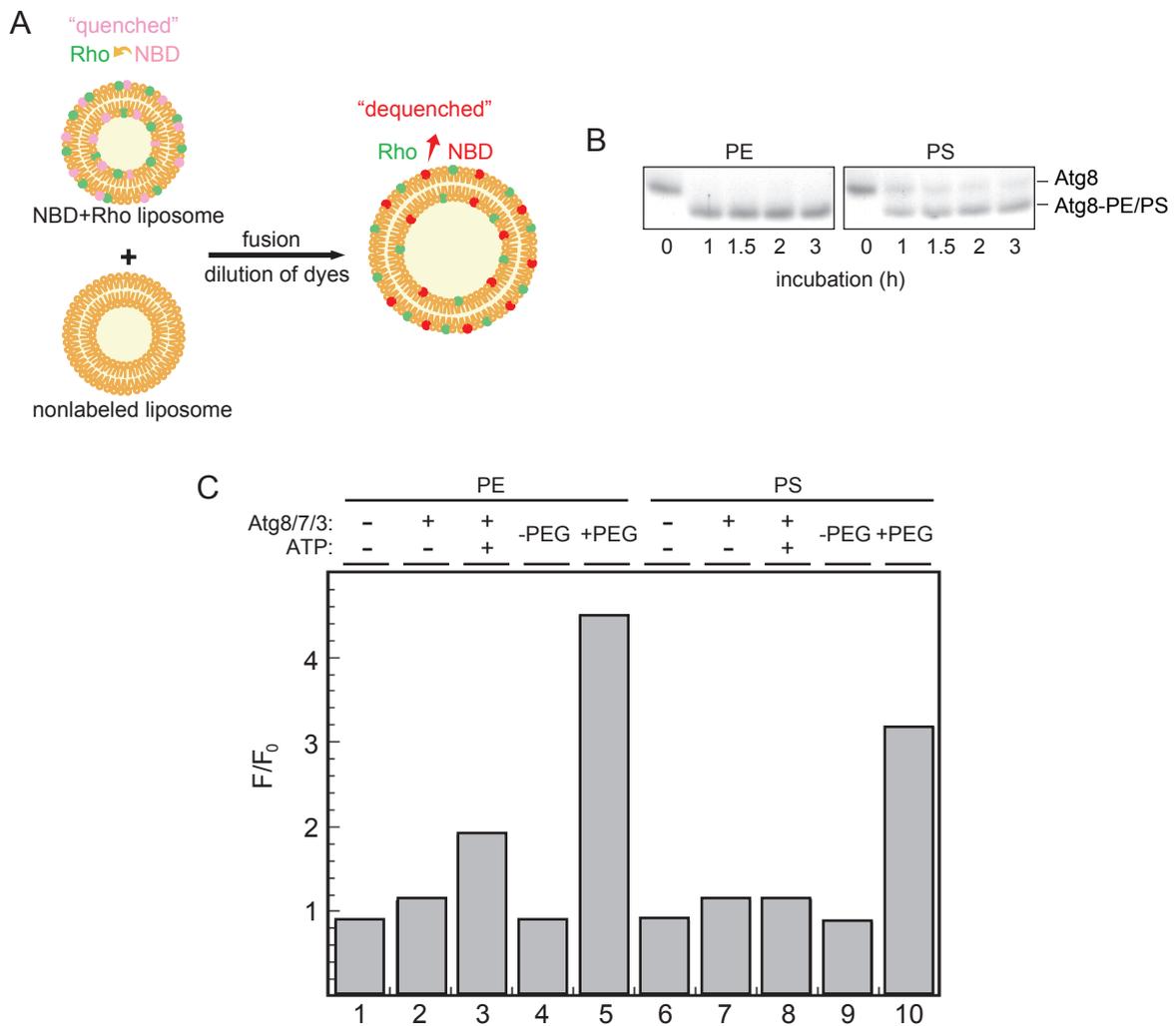


**Fig. 19 PS is not essential for autophagy.**

A. BY4741 wild type cells, *atg8Δ::kanMX4*, *pra1Δ::kanMX4*, *pss1Δ::natNT2* grown in YEPD ( $OD_{600}=3.0$ ) were treated with rapamycin ( $0.5 \mu\text{g/ml}$ ) for 4 h with or without 1 mM PMSF, and observed by microscopy.

B. Cells overexpressing cytosolic Pho8 (*pho8Δ::GPD-PHO8Δ60-KanMX4*) wild type, *atg7Δ::natNT2*, *pss1::natNT2* grown in YEPD ( $OD_{600}=2.0$ ) (g) were treated with rapamycin for 4 h (r). The autophagic activity of the cells was measured by ALP assay. The expression level of ALP was analyzed by immunoblotting using anti-ALP antibody.

C. Wild type cells, *atg8::kanMX4*, *pho8::kanMX4*, *pss1::natNT2* #1, 2 grown on YEPD ( $OD_{600}=1.5$ ) (g) were treated with rapamycin for 4 h (r). Total lysates were prepared from each strain by alkaline-TCA method. The expression level of various proteins were analyzed by immunoblotting using anti-Atg8, CPY, Pra1 antibody.

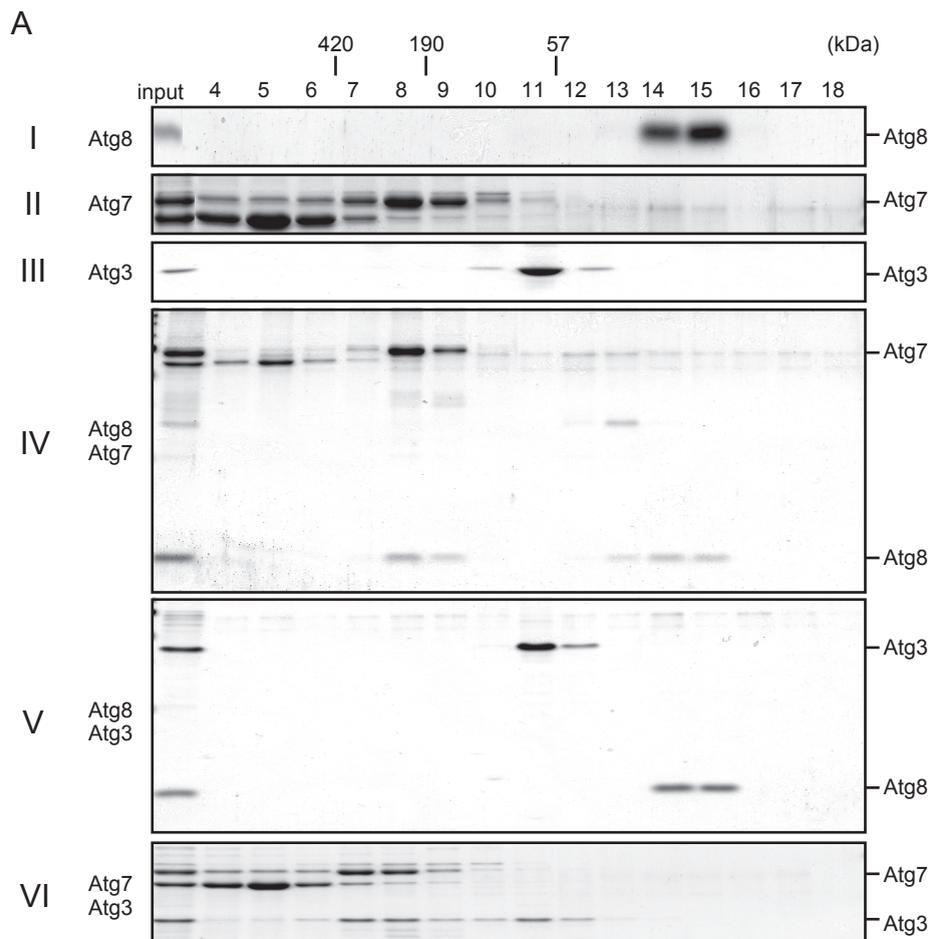


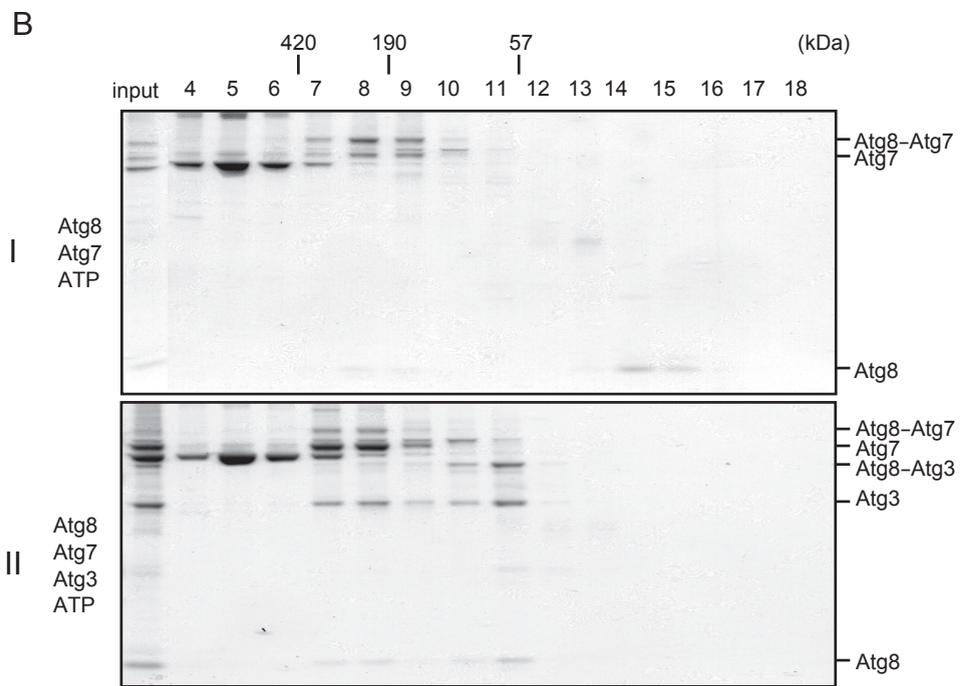
**Fig. 20 Membrane fusion does not occur between liposomes tethered by Atg8-PS.**

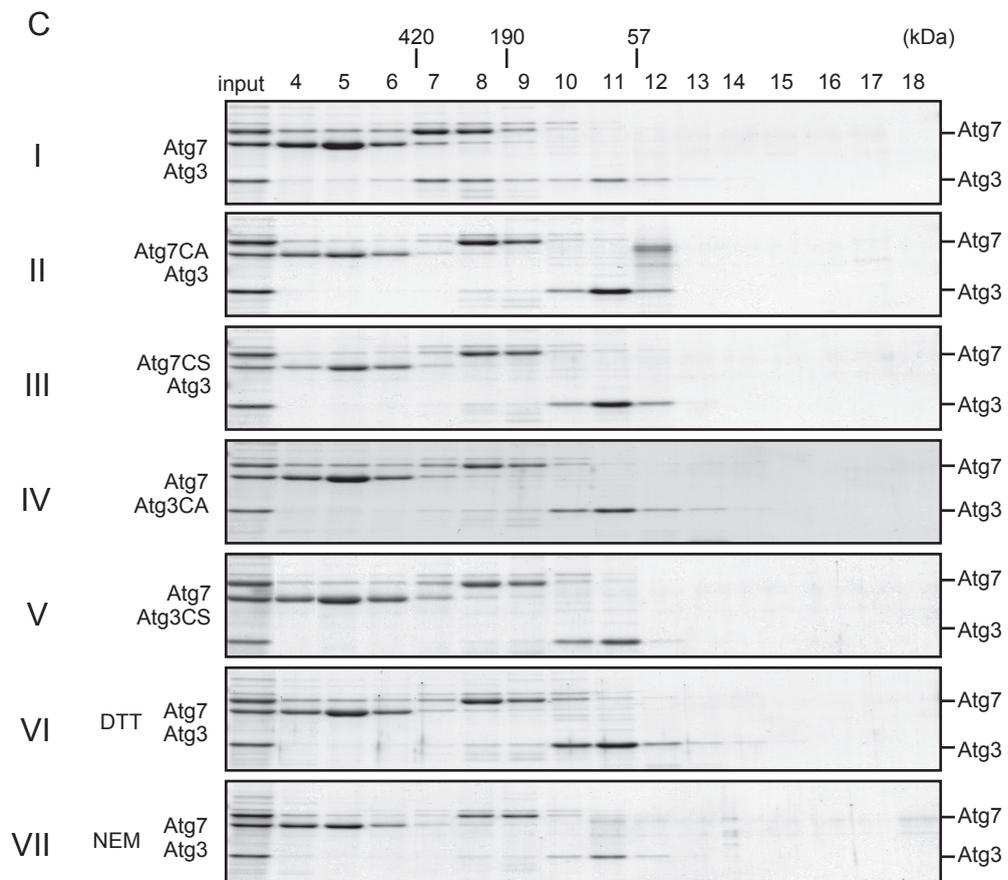
A. The principle of the lipid mixing assay.

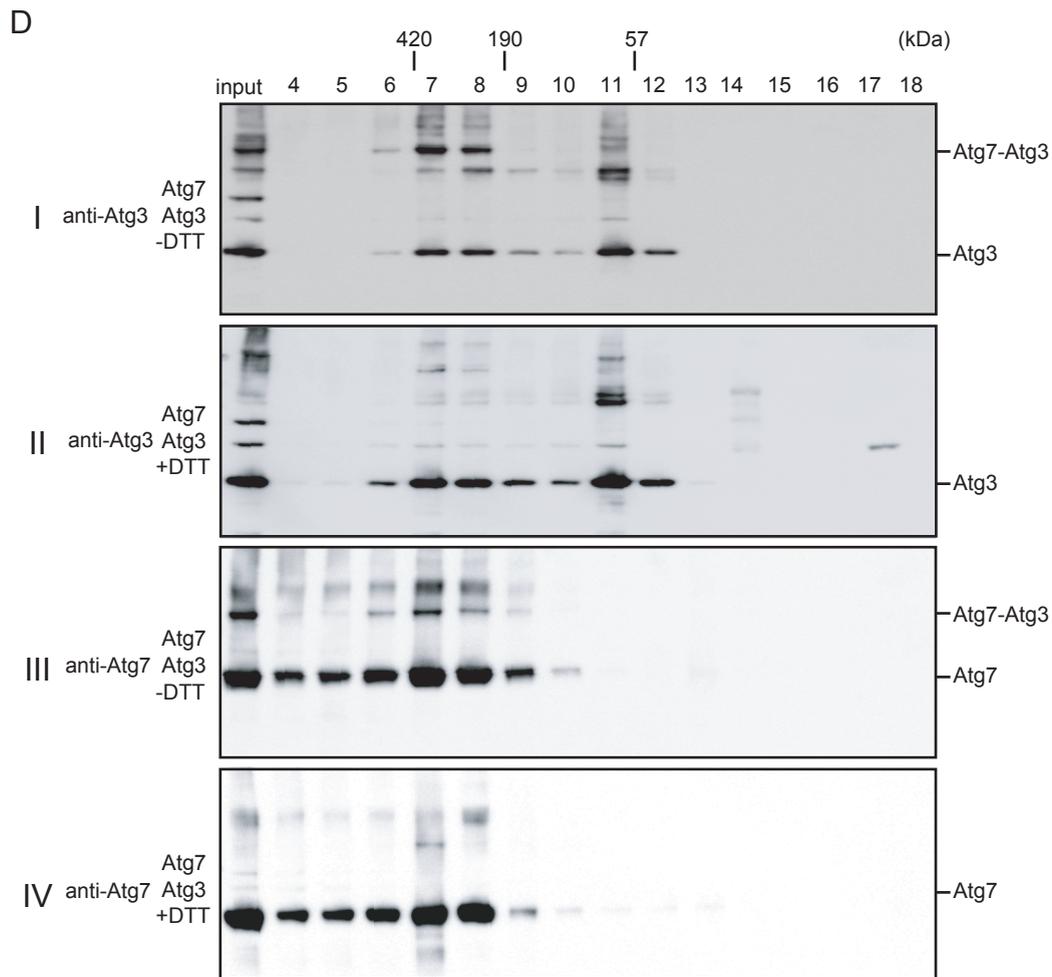
B. Atg8 (2  $\mu$ M), Atg7 (1  $\mu$ M), and Atg3 (1  $\mu$ M) were incubated with 4:1 mixture of nonlabeled (55 mol% PE, 30 mol% PC and 15 mol% bPI) and NBD+Rho-labeled (55 mol% PE, 27.5 mol% PC, 15 mol% bPI, 1 mol% NBD-PE and 1.5 mol% Rho-PE) liposomes or (55 mol% PS, 30 mol% PC and 15 mol% bPI) and NBD+Rho-labeled (55 mol% PS, 27.5 mol% PC, 15 mol% bPI, 1 mol% NBD-PE and 1.5 mol% Rho-PE) liposomes and 1 mM ATP at 30°C for the indicated time periods with Tris-HCl (pH 8.0) buffer. These samples were subjected to urea-SDS-PAGE, followed by CBB staining.

C. The lipid mixing assay was performed with proteins and PE or PS mixture liposomes in the presence or absence of ATP (columns 3, 2 and 8, 7, respectively) for 2 hr as described in B. For PEG-induced fusion reactions, the mixture liposomes were incubated at 37°C for 30 min in the presence or absence of 12.5% PEG3350 (columns 5, 4 and 10, 9, respectively).









**Fig. 21 Gel filtration analyses of the components involved in Atg8 lipidation.**

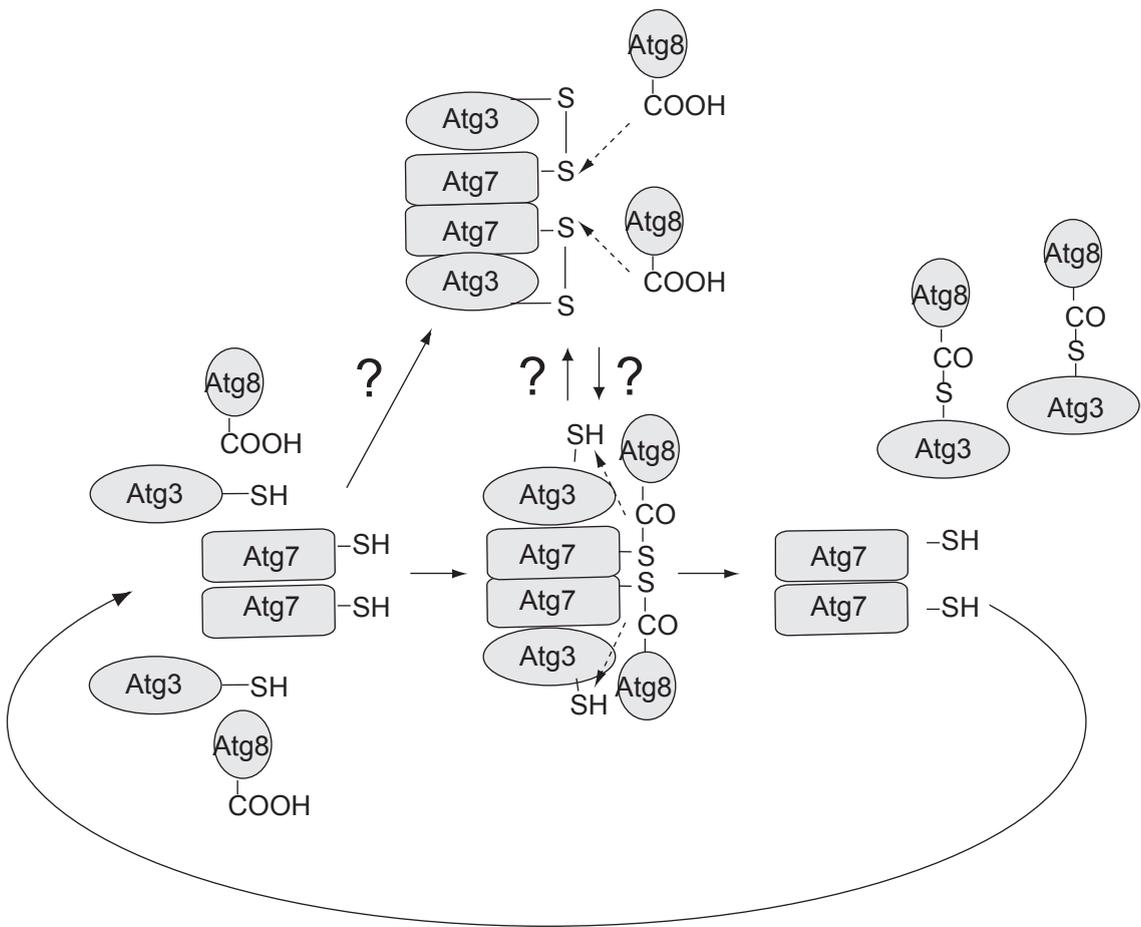
A. Atg8 (2  $\mu$ M) (I), Atg7 (2  $\mu$ M) (II), Atg3(2  $\mu$ M) (III), Atg8 and Atg7 (IV), Atg8 and Atg3 (V), and Atg7 and Atg3 (VI) were incubated at pH7.0 at 30°C for 10 min. These samples were subjected to gel filtration analyses with buffer A containing 50 mM Tris-HCl (pH7.0) and 150 mM NaCl.

B. Atg8 and Atg7 (I) Atg8, Atg7 and Atg3 (II) were incubated with ATP and subjected to gel filtration analyses with buffer A.

C. Atg7 and Atg3 (I), Atg7<sup>C507A</sup> and Atg3 (II), Atg7<sup>C507S</sup> and Atg3 (V), Atg7 and Atg3<sup>C234A</sup> (VI), Atg7 and Atg3<sup>C234S</sup> (V) and Atg7 and Atg3 with 10 mM DTT were incubated at 30°C for 10 min. (VI), Atg7 and Atg3 were incubated with 5 mM NEM (N-ethylmaleimide) individual at 30°C for 10 min, added 10 mM DTT and incubated at 30°C for 10 min (VII), and subjected to gel filtration analyses with buffer A (I~VI) or buffer B containing 50 mM Tris-HCl (pH7.0), 150 mM NaCl and 10 mM DTT.

D. Atg7 and Atg3 were incubated at 30°C for 10 min. and subjected to gel filtration analyses with buffer A.

These samples were concentrated with TCA, mixed with SDS sample buffer with DTT (A, C and D II, IV) or without DTT (B, D I, III) and subjected to 10% SDS-PAGE (A, C), or NuPAGE (B, D), and analyzed by CBB staining (A, B and C) or immunoblotting using antibodies against Atg3 and Atg7 (D).



**Fig22. The model of Atg8 conjugation reaction.**

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# Physiological pH and Acidic Phospholipids Contribute to Substrate Specificity in Lipidation of Atg8\*<sup>§</sup>

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Yeast Atg8 and its mammalian homolog LC3 are ubiquitin-like proteins involved in autophagy, a primary pathway for degradation of cytosolic constituents in vacuoles/lysosomes. Whereas the lipid phosphatidylethanolamine (PE) was identified as the sole *in vivo* target of their conjugation reactions, *in vitro* studies showed that the same system can mediate the conjugation of these proteins with phosphatidylserine as efficiently as with PE. Here, we show that, in contrast to PE conjugation, the *in vitro* phosphatidylserine conjugation of Atg8 is markedly suppressed at physiological pH. Furthermore, the addition of acidic phospholipids to liposomes also results in the preferential formation of the Atg8-PE conjugate. We have successfully captured authentic thioester intermediates, allowing us to elucidate which step in the conjugation reaction is affected by these changes in pH and membrane lipid composition. We propose that these factors contribute to the selective formation of Atg8-PE in the cell.

Various cellular activities involve post-translational modifications of proteins, among which ubiquitin and ubiquitin-like protein (Ubl)<sup>2</sup> systems are remarkably versatile both in regard to the processes they regulate and their mechanisms (1). Autophagy is an evolutionarily conserved pathway that mediates bulk degradation of cytosolic proteins and organelles in lysosomes/vacuoles (2). This process requires the formation of double membrane-bound structures, called autophagosomes, that sequester the materials to be degraded. Two UbLs, Atg12 and Atg8, are involved in autophagosome formation in yeast *Saccharomyces cerevisiae*. Atg12 is activated by the E1 enzyme Atg7, then transferred to the E2 enzyme Atg10, and finally conjugated to the Lys<sup>149</sup> of Atg5 (3). The Atg12-Atg5 conjugate further interacts with Atg16 and forms a large complex of ~350 kDa (4). For the conjugation reaction of Ubl Atg8, the cysteine protease Atg4 first removes the C-terminal arginine of Atg8 to expose the glycine essential for the reaction (5,

6). Atg8 then sequentially forms thioester intermediates with the common E1 Atg7 but then a specific E2, Atg3 (7). The conjugation target of Atg8 is the amino group in the hydrophilic head of dioleoylphosphatidylethanolamine (PE) (see Fig. 1A). Atg4 also catalyzes the deconjugation of Atg8-PE and thus recycles and/or regulates Atg8 (5). Both of these Ubl conjugates are localized on intermediate structures of autophagosomes and are thought to play essential roles in their formation. These Ubl systems are highly conserved in both mammals and plants (8–14).

Recently, we reconstituted the Atg8 conjugation system using purified proteins and PE-containing liposomes (15) and found that Atg8 forms an oligomer in response to PE conjugation, leading to the tethering together and hemifusion of the liposomes (16). This function of Atg8 was suggested to be involved in the expansion of autophagosomal membranes. Moreover, we used this *in vitro* system to show that the Atg12-Atg5 conjugate enhances the conjugase activity of Atg3, suggesting that Atg12-Atg5 has an E3-like function (17). It was also shown that Atg16 is dispensable for this function of Atg12-Atg5 *in vitro* (17).

We identified PE as the sole lipid conjugated to the C-terminal glycine of Atg8 purified from yeast cells (7). *In vitro*, however, Atg8 also forms a conjugate with phosphatidylserine (PS), which like PE has an amino group in the head moiety (17). Likewise, mammalian homologs of Atg8, including LC3, are also conjugated to PS as efficiently as PE in a similar *in vitro* system, even though the lipidated form of LC3 purified from cultured cells contained only PE (18). These results suggested that there exists a mechanism that directs Atg8 conjugation preferentially to PE in the cell. We showed that neither Atg12-Atg5 nor its complex with Atg16 is involved in this mechanism; they stimulates both Atg8-PE and Atg8-PS formation to a similar degree (17).

In this study, we show that the preferential conjugation of Atg8 to PE can be reproduced *in vitro* either by physiological pH or the addition of acidic phospholipids to liposomes. Further analyses suggest that physiological pH conditions evoke the intrinsic substrate specificity of Atg3 and that acidic phospholipids facilitate the binding of the Atg8-Atg3 thioester intermediate to the membrane.

## EXPERIMENTAL PROCEDURES

**Protein Purification and Liposome Preparation**—*S. cerevisiae* proteins Atg8 (truncated for the C-terminal arginine), Atg7, Atg3, and Atg4, were expressed in *Escherichia coli* and purified as described previously (16). The Atg12-Atg5 conjugate was formed in *E. coli* and purified as reported previously (17). Liposomes were also prepared as previously reported (16) using PE,

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S3.

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<sup>2</sup> The abbreviations used are: Ubl, ubiquitin-like protein; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; ME,  $\beta$ -mercaptoethanol; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; MES, 2-(N-morpholino)ethanesulfonic acid.

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dioleoylphosphatidylserine (PS), 1-palmitoyl-2-oleoylphosphatidylcholine (PC), phosphatidylinositol purified from yeast (PI), dioleoylphosphatidic acid, dioleoylphosphatidylglycerol, and polycarbonate filters with a pore size of 400 nm (Avanti).

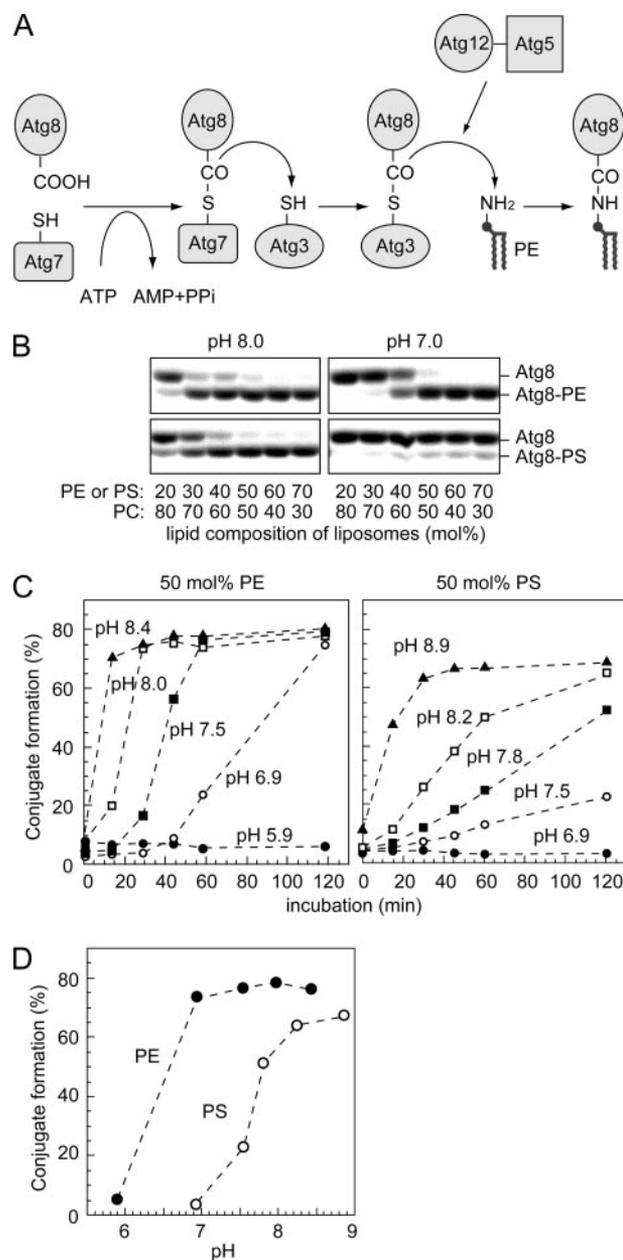
**In Vitro Conjugation Reaction**—The reaction mixtures including liposomes (350  $\mu\text{M}$  lipids), purified proteins (5  $\mu\text{M}$  Atg8, 0.5  $\mu\text{M}$  Atg7, 0.5  $\mu\text{M}$  Atg3, and 0.02  $\mu\text{M}$  Atg12-Atg5), 1 mM ATP, 1 mM  $\text{MgCl}_2$ , 100 mM NaCl, 0.2 mM dithiothreitol, and either 50 mM MES-NaOH (pH 6.0), 50 mM Tris-HCl (pH 7.0, 7.5, 8.0, 8.5, or 9.0) were incubated at 30  $^\circ\text{C}$ . The pH of the reaction mixtures was measured by pH/ion meter F-53 (Horiba). The reaction was stopped by mixing with  $\frac{1}{4}$  volume of SDS sample buffer composed of 250 mM Tris-HCl (pH 7.5), 10% SDS, 40% glycerol, and 25%  $\beta$ -mercaptoethanol (ME); the samples were then boiled for 3 min before urea-SDS-PAGE analysis. For detection of thioester intermediates, SDS sample buffer without ME was added to the reaction mixtures, which were incubated at 42  $^\circ\text{C}$  for 5 min before NuPAGE separation.

**SDS-PAGE and Immunoblotting Analysis**—The separation of the lipidated and unlipidated forms of Atg8 using urea-SDS-PAGE as well as the methods for Coomassie Brilliant Blue staining and immunoblotting analyses were described previously (16). Polyclonal antibodies against Atg8 (5) and Atg3 (7) and a monoclonal antibody to Atg7 yN-16 (Santa Cruz) were used. For detection of thioester intermediates, electrophoresis was performed using NuPAGE 12% Bis-Tris gels (Invitrogen).

## RESULTS

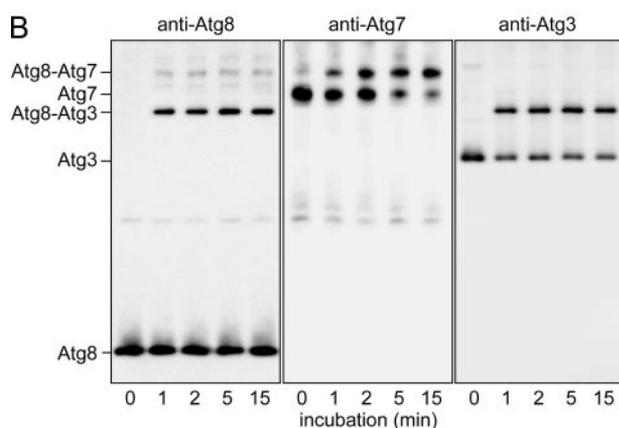
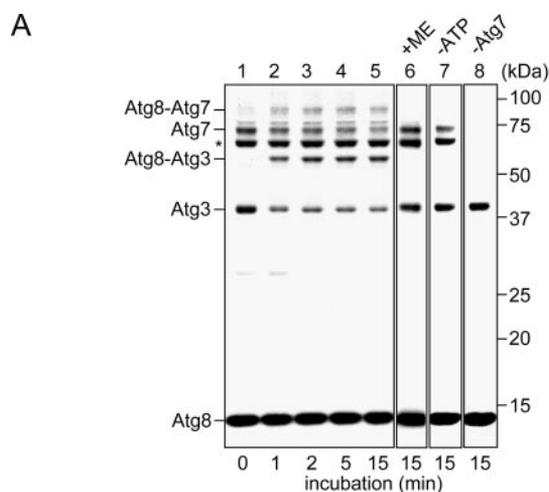
**Atg8-PS Formation Is Suppressed under Neutral pH Conditions**—The conjugation of Atg8 to PE can be reproduced *in vitro* by mixing purified proteins, C-terminal glycine-exposed Atg8 (hereafter called Atg8), Atg7, and Atg3, with liposomes containing PE and incubating this mixture in the presence of ATP (15). We examined optimal conditions in establishing this system and accordingly set the pH of the reaction buffer at 8.0 in the previous studies. Under these conditions, the same system also efficiently mediates the conjugation of Atg8 to PS (Fig. 1B, left panels) (17). The intracellular pH of yeast cells, however, has been estimated at  $\sim 6.0$ – $7.0$  (19). We found that PS conjugation barely occurred at pH 7.0, even with liposomes containing high concentrations of PS; in contrast, PE conjugation occurred efficiently (Fig. 1B, right panels). We performed conjugation reactions at various pH, using liposomes composed of 50 mol % of PC and either 50 mol % of PE or PS (Fig. 1, C and D). The results clearly showed that PS conjugation is more sensitive than PE conjugation to lowering pH. Thus, the formation of Atg8-PS is markedly attenuated under physiological pH conditions.

**Atg8 Transfer from Atg3 to PS Is Restricted at Neutral pH**—We investigated which step(s) in Atg8-PS formation was affected at neutral pH. To this end, we aimed to capture authentic thioester intermediates Atg8-Atg7 and Atg8-Atg3, whereas E1 and E2 mutants with the active site cysteines replaced with serines have been conventionally used to fix the intermediates in studies of ubiquitin and other Ubl systems. However, probably because of their alkali lability, these intermediates could not be detected by standard basic SDS-PAGE (20). Therefore, we tried the NuPAGE system (Invitrogen) that allows separation of proteins in a neutral pH environment. Atg8, Atg7, and



**FIGURE 1. Atg8-PS formation is severely suppressed under neutral pH conditions.** A, a schematic representation of a ubiquitin-like conjugation system that mediates lipidation of Atg8. B, Atg8 (5  $\mu\text{M}$ ), Atg7 (0.5  $\mu\text{M}$ ), and Atg3 (0.5  $\mu\text{M}$ ) were incubated with liposomes (350  $\mu\text{M}$  lipids) consisting of the indicated concentrations of PC and either PE or PS and 1 mM ATP at 30  $^\circ\text{C}$  for 120 min using Tris-HCl (pH 8.0 or 7.0) as the reaction buffer. These samples were subjected to urea-SDS-PAGE, followed by Coomassie Brilliant Blue staining. C, the reactions were similarly performed with liposomes consisting of 50 mol % of PC and either 50 mol % of PE or PS at various pH. The pH of each reaction mixture was determined by a pH meter. After incubation for the indicated time periods, the reaction was stopped by mixing with SDS sample buffer, followed by urea-SDS-PAGE separation. The intensities of the Coomassie Brilliant Blue-stained protein bands were measured using the Scion Image software and used to calculate conjugate formation efficiencies (%), in which the intensities of lipidated Atg8 were divided by intensities of total Atg8 protein. D, the conjugate formation efficiencies after 120 min of incubation shown in C are also presented, with the horizontal axis showing the pH of the reaction mixtures.

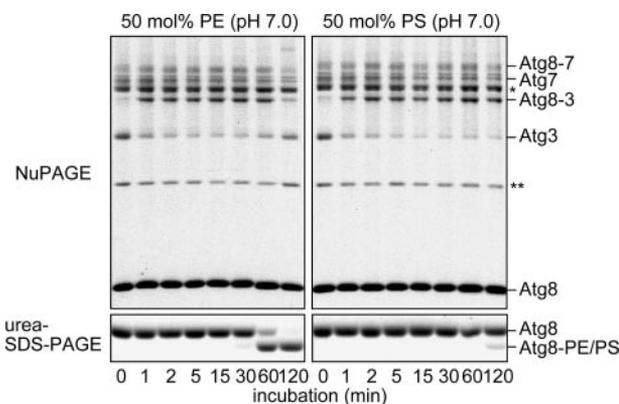
Atg3 were incubated with ATP in the absence of liposomes and subjected to the NuPAGE separation without reducing reagents. Two additional bands of  $\sim 85$  and 55 kDa appeared upon incubation, accompanied by a concomitant decrease of



**FIGURE 2. Detecting thioester intermediates of the Atg8 conjugation reaction.** A, Atg8, Atg7, and Atg3 were incubated with ATP in the absence of liposomes at pH 7.0 at 30 °C. The samples were mixed with sample buffer without ME and subjected to NuPAGE separation, followed by Coomassie Brilliant Blue staining (lanes 1–5). The same sample as in lane 5 was also treated with 5% ME before NuPAGE (lane 6). The reaction mixtures, incubated without ATP (lane 7) or Atg7 (lane 8), were similarly analyzed in the absence of ME. The asterisk represents a contaminant protein present in the purified preparation of Atg7. B, the samples corresponding to lanes 1–5 in A were analyzed by immunoblotting using antibodies against Atg8, Atg7, and Atg3.

Atg7 and Atg3 (Fig. 2A, lanes 1–5). These bands were sensitive to the reductant  $\beta$ -mercaptoethanol (Fig. 2A, lane 6), and their production depended on ATP (Fig. 2A, lane 7). Neither of these products appeared if Atg7 was excluded (Fig. 2A, lane 8), and only the 85-kDa band appeared in the absence of Atg3 (data not shown and Fig. 5A, lanes 16–21). Immunoblotting analyses showed that the 85- and 55-kDa products both contained Atg8 and exclusively contained Atg7 and Atg3, respectively (Fig. 2B). Taken together, these results suggest that these products are thioester intermediates of the conjugation reaction, Atg8-Atg7 and Atg8-Atg3.

We also analyzed reactions containing PE liposomes using this system (Fig. 3). We found that both the 85- and 55-kDa products significantly decreased upon the completion of Atg8-PE formation, accompanied by a concomitant increase in free Atg3 (Fig. 3, left panels; free Atg7 was somehow upshifted after long incubation), suggesting that these products are *bona fide* reaction intermediates. We showed that both of the intermediates were formed in the presence of PS liposomes as rapidly as with PE liposomes even at pH 7.0 and stably persisted during incubation; the conjugation of Atg8 with PS hardly



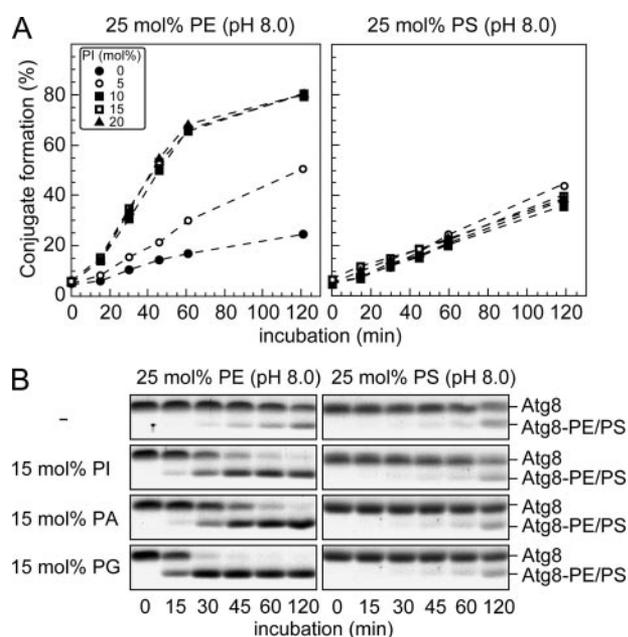
**FIGURE 3. Transfer of Atg8 from Atg3 to PS is retarded at neutral pH.** The conjugation reactions performed at pH 7.0 as described in Fig. 1C were analyzed using the NuPAGE gel system as described in Fig. 2A. The same samples were also analyzed using urea-SDS-PAGE to assess lipidation of Atg8. The double asterisks show the bands containing Atg8 that do not appear in the presence of ME.

occurred in this condition. These results suggest that transfer of Atg8 from Atg3 to PS is specifically impeded at neutral pH.

**Acidic Phospholipids Specifically Stimulate Conjugation Reactions with PE Liposomes**—We reported that Atg8-PE formation was accelerated by the addition of acidic phospholipids to liposomes (15). This stimulative effect was clearly observed when the basal reaction efficiency was lowered by decreasing the PE concentration to 25 mol %. In addition, to examine the effect of acidic phospholipids on Atg8-PS formation, we used a reaction buffer of pH 8.0. We examined the PE and PS conjugation of Atg8 under these conditions, with liposomes containing various concentrations of phosphatidylinositol (PI), a major acidic phospholipid in eukaryotic cells (Fig. 4). Consistent with the previous report, Atg8 was efficiently conjugated with PE as the PI concentration increased, saturating at ~10 mol % (Fig. 4A, left panels). In contrast, we found that PS conjugation was not at all stimulated by the addition of PI (Fig. 4A, right panels). This was also the case for other acidic phospholipids, phosphatidic acid and phosphatidylglycerol; these lipids promoted PE conjugation to similar extents but did not promote PS conjugation at all (Fig. 4B). Similar results were obtained in experiments at pH 7.0 (data not shown). We conclude that the PE conjugation of Atg8 occurs even more preferentially in the presence of negatively charged lipids.

**Acidic Phospholipids Facilitate the Membrane Binding of Thioester Intermediates**—We next investigated how acidic phospholipids facilitated the production of Atg8-PE. The addition of PI to liposomes did not affect the formation rates of either the Atg8-Atg7 or the Atg8-Atg3 intermediates (Supplemental Fig. S1). We then examined the binding of the proteins to liposomes, by cosedimentation experiments (Fig. 5). Atg8, Atg7, and Atg3 were incubated with ATP to form the thioester intermediates, chilled, and then mixed with liposomes, followed by ultracentrifugation to sediment the liposomes and bound proteins. A reaction buffer at pH 7.0 was used in these experiments. We confirmed that lipidation of Atg8 did not occur during these manipulations (data not shown) and that no significant protein precipitation occurred in the absence of liposomes (Fig. 5A, lanes 1–3). Similarly, no significant cosedi-

## Substrate Specificity in Atg8 Lipidation



**FIGURE 4. Acidic phospholipids contribute to the preferential formation of Atg8-PE.** *A*, the conjugation reactions were performed at pH 8.0 with liposomes composed of either 25 mol % of PE or PS and the indicated and remaining concentrations of PI and PC, respectively, which were analyzed as described in Fig. 1C. *B*, the PE and PS conjugation of Atg8 was similarly examined in the presence of various acidic phospholipids. *PA*, dioleoylphosphatidic acid; *PG*, dioleoylphosphatidylglycerol.

mentation was observed when the proteins were mixed with PE liposome that did not contain PI (Fig. 5A, lanes 4–6). However, the addition of PI to this liposome significantly increased the levels of Atg8-Atg7 and Atg8-Atg3 cosedimented with the liposome, especially for the latter (Fig. 5A, lanes 7–9). In addition, the experiments without ATP clearly showed that Atg8, Atg7 and Atg3 alone did not bind to the PE liposome, irrespective of the presence of PI (Fig. 5A, lanes 10–15). These results suggest that acidic phospholipids specifically promote the recruitment of the thioester intermediates to the membrane, thereby facilitating the conjugation reaction of Atg8. We also showed that the binding of Atg8-Atg7 to PE liposomes containing PI depends on Atg3 (Fig. 5A, lanes 16–21; see “Discussion”).

The binding of the Atg proteins to PS liposomes was also examined. The thioester intermediates did bind to PS liposomes as substantially as PE liposomes containing PI, and this binding was not enhanced by PI addition (Fig. 5A, lanes 22–27). This insensitivity to PI is consistent with what we observed in the conjugation reaction (Fig. 4). These results with the PS liposome could be explained by the acidic nature of PS itself (see “Discussion”).

**In Vitro Reproduction of the Preferential Formation of Atg8-PE**—We finally examined the combination of the buffer pH and the lipid compositions of liposomes that evoked the preferential formation of Atg8-PE. These conditions are thought to be closer to the physiological situation (also see “Discussion”). However, neither PE nor PS conjugation occurred (Fig. 6, upper panels). We have reported that the Atg12-Atg5 conjugate is indispensable for Atg8-PE production *in vivo* (21) and that recombinant Atg12-Atg5 indeed stimulates it *in vitro* (17). Therefore, Atg12-Atg5 was included in the

reaction under these conditions. As reported previously, the formation of both Atg8-PE and Atg8-PS was accelerated by Atg12-Atg5; however, the acceleration was more prominent for PE conjugation under these conditions (Fig. 6, lower panels). These results suggest that preferential formation of Atg8-PE can be achieved by combination of the neutral pH, acidic phospholipids, and the Atg12-Atg5 conjugate.

In addition to controlling conjugate formation, it is also conceivable that rapid deconjugation of Atg8-PS results in the predominant accumulation of Atg8-PE *in vivo*. However, we showed *in vitro* that Atg4 deconjugates Atg8-PS similarly or rather inefficiently compared with Atg8-PE (Supplemental Fig. S2).

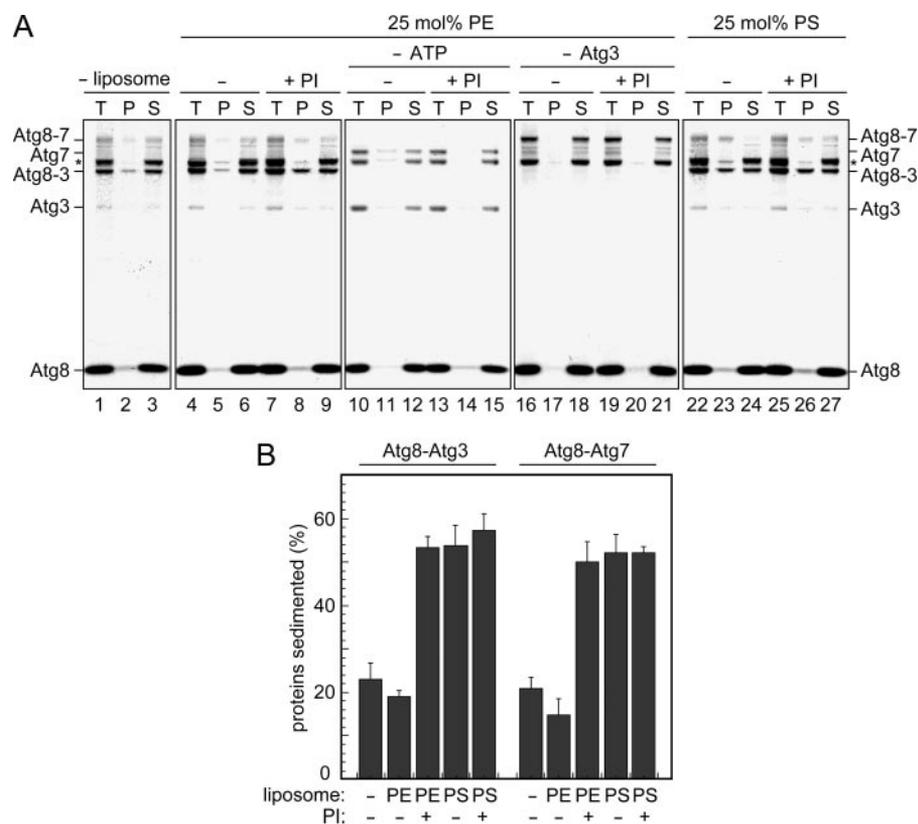
## DISCUSSION

Neither of the two Ubl systems involved in autophagy includes typical E3 enzymes. This is reasonable, considering that both systems target single substrates, which are directly recognized by the E2 enzymes. However, *in vitro* studies showed that Atg3 can recognize PS in addition to PE. Although the existence of some proteinaceous factors has been assumed *in vivo* (18), here we have shown that intracellular environments such as pH and lipid compositions of membranes can direct the conjugation of Atg8 to PE.

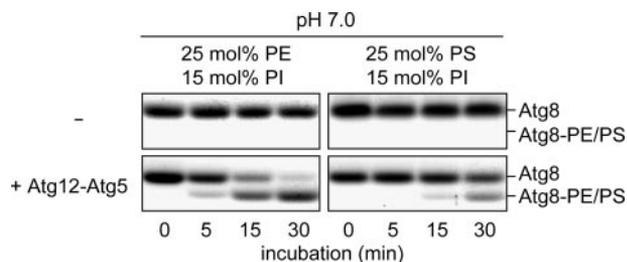
We found that PS conjugation was severely suppressed at neutral pH at the step of Atg8 transfer from Atg3 to the lipid, suggesting that substrate specificity intrinsic to Atg3 is displayed under those conditions. Because protonation states of the lipid head groups do not vary significantly among the pH conditions we examined (22), some conformational change might occur in Atg3 such that PS is not acceptable. We showed that lowering the pH also delayed Atg8 transfer to PE, although to a lesser extent than PS. Therefore, the structural configuration of the substrate-binding pocket of Atg3 (23) may be tightened at lower pH.

Our results also highlighted a unique aspect of Atg3; it is an enzyme whose activity is lowered under physiological conditions. In addition, observation of conjugate formation with the thioester intermediates clearly showed that the conjugase activity of Atg3 is rate-limiting (Fig. 3); Atg12-Atg5 drastically enhances this activity through a direct interaction with Atg3 (17). It is known that most autophagy-related proteins, including Atg8 and Atg12-Atg5, localize to a specific perivacuolar structure called the preautophagosomal structure, where autophagosome formation should take place (21). Therefore, the intrinsically low activity of Atg3 may be important to prevent mislocalized reaction and thus ensure the preautophagosomal structure-limited formation of Atg8-PE.

The liposome binding of the thioester intermediates was increased by acidic phospholipids, and binding of Atg8-Atg7 required Atg3. Because Atg3 alone did not bind to the liposomes, the liposome binding of Atg8-Atg7 could occur through an interaction with Atg8-Atg3, although it should be noted that an increased amount of free Atg3 also binds to the liposomes in the presence of Atg8-Atg7 and Atg8-Atg3 (Fig. 5A, lanes 7–9), suggesting that the membrane-bound complexes may exist in multiple states. We found that Atg8-Atg3 binds to liposomes containing acidic phospholipids in the absence of PE and PS (data not shown). In addition, Atg8-PE formation is stimulated by structurally different acidic phospholipids. Therefore, Atg8-



**FIGURE 5. PI facilitates membrane binding of thioester intermediates.** *A*, the thioester intermediates were formed as described in Fig. 2*A* (lanes 1–9 and 22–27). The reactions were also performed without ATP (lanes 10–15) or Atg3 (lanes 16–21). These mixtures were chilled on ice for 5 min and then further incubated on ice in the presence (lanes 4–7) or absence (lanes 1–3) of liposomes composed of 75 mol % of PC and either 25 mol % of PE (lanes 4–6, 10–12 and 16–18) or PS (lanes 22–24), or liposomes composed of 60 mol % of PC, 15 mol % of PI, and either 25 mol % of PE (lanes 7–9, 13–15 and 19–21) or PS (lanes 25–27) for 10 min (*T*, total). The samples were subjected to ultracentrifugation at  $100,000 \times g$  for 30 min at  $4^\circ\text{C}$  to generate pellets (*P*) and supernatants (*S*). These samples were analyzed by NuPAGE as described in Fig. 2*A*. *B*, the experiments were repeated three times, and the intensities of the bands of Atg8-Atg3 and Atg8-Atg7 were quantified. The average values for the ratio of the proteins in the pellet fractions to those in the total samples are shown with error bars for the standard deviations.



**FIGURE 6. *In vitro* reproduction of preferential Atg8-PE formation.** The formation of Atg8-PE and Atg8-PS was examined as described in the legend to Fig. 1*C* using liposomes composed of 60 mol % of PC, 15 mol % of PI, and either 25 mol % of PE or PS at pH 7.0 in the presence or absence of  $0.02 \mu\text{M}$  Atg12-Atg5.

Atg3 could be recognizing the negatively charged surface of the membrane. Because neither Atg8 nor Atg3 binds to the liposome by itself, we speculate synergistic cooperation of these proteins, which may involve a conserved, positively charged region on Atg8 (24–26) and/or some conformational changes induced upon thioester bond formation.

Although the stimulative effect of PI was saturated at  $\sim 10$  mol % in liposomes composed of the nearly electroneutral lipids PC and PE, we used liposomes containing 25 mol % of PS for examination of PS conjugation. Because PS itself should also

behave as an acidic phospholipid, these PS liposomes are probably saturated for the effect of acidic phospholipids, which might explain their insensitivity to other acidic phospholipids and also suggests that the efficiency of PS conjugation we observed includes the self-stimulation effect. Although it will be important to distinguish Atg8-PE from Atg8-PS produced in the same reaction mixture, we still assume that the presence of PS on the same membrane also leads to the stimulation of Atg8-PE formation.

The site of Atg8-PE formation, and thus the lipid composition of the target membrane, remains to be elucidated. However, most intracellular membranes of yeast contain substantial amounts of PI ( $\sim 10$ – $20\%$ ) as well as PE ( $\sim 20$ – $25\%$ ) and PS ( $\sim 5$ – $10\%$ ) (27). In addition, it has been recently reported that phosphatidylinositol 3-phosphate, whose production by the phosphatidylinositol 3-kinase Vps34 is essential for autophagy, is enriched in autophagosomal membranes as well as vacuolar and endosomal membranes (28, 29). Therefore, it is probable that these acidic phospholipids contribute to the selective conjugation of Atg8 with PE in the cell. We showed that phosphatidylinositol 3-phosphate also stimulates Atg8-PE formation *in vitro*; its extent, however, was comparable with that of PI (Supplemental Fig. S3). Although phosphatidylinositol 3-phosphate is possible to promote the production of Atg8-PE as well as other acidic phospholipids, it should primarily function as an effector for its specific binding proteins required for autophagosome formation (30–32).

The preferential formation of Atg8-PE was reproduced *in vitro* by combining the neutral pH and the addition of acidic phospholipids to liposomes in the presence of the Atg12-Atg5 conjugate. However, the less efficient but significant production of Atg8-PS was still observed, suggesting that the exclusive formation of Atg8-PE requires precise *in vivo* settings for these factors and/or an additional factor(s). Alternatively, this result may imply the production of Atg8-PS *in vivo*, although its amount should be much less than the PE conjugate. More careful *in vivo* analyses will be needed to address these possibilities.

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