PHYSIOLOGICAL PH AND ACIDIC PHOSPHOLIPIDS CONTRIBUTE TO SUBSTRATE SPECIFICITY IN LIPIDATION OF ATG8

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Running head: Substrate specificity in Atg8 lipidation

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 these proteins’ conjugation with phosphatidylserine (PS) as efficiently as with PE. Here, we show that, in contrast to PE conjugation, the in vitro PS 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 (Ub)⁵ and ubiquitin-like protein (Ubl) 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 \textit{Saccharomyces cerevisiae}. Atg12 is activated by the E1 enzyme Atg7, then transferred to the E2 enzyme Atg10, and finally conjugated to the Lys149 of Atg5 (3). The Atg12-Atg5 conjugate further interacts with Atg16 and forms a large complex of about 350kDa (4). For the conjugation reaction of the other ubiquitin-like protein 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 phosphatidylethanolamine (PE) (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 \textit{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 \textit{in vitro} (17).

We identified PE as the sole lipid conjugated to the C-terminal glycine of Atg8 purified from yeast cells (7). \textit{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 \textit{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 stimulate 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 E. 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 dioleoylphosphatidylethanolamine (PE), dioleoylphosphatidylserine (PS), 1-palmitoyl-2-oleoylphosphatidylcholine (PC), phosphatidylinositol purified from yeast (PI), dioleoylphosphatidic acid (PA), dioleoylphosphatidylglycerol (PG), and polycarbonate filters with a pore size of 400 nm (Avanti).

In vitro conjugation reaction—The reaction mixtures including liposomes (350 μM lipids), purified proteins (5 μM Atg8, 0.5 μM Atg7, 0.5 μM Atg3, and 0.02 μM Atg12-Atg5), 1 mM ATP, 1 mM MgCl2, 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°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 25% β-mercaptoethanol (ME); 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°C for 5 min before NuPAGE separation.

SDS-PAGE and immunoblotting analysis—The separation of the lipitated and unlipitated forms of Atg8 using urea-SDS-PAGE as well as the methods for CBB-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) (17). The intracellular pH of yeast cells, however, has been estimated at ~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). We performed conjugation reactions at various pH, using liposomes composed of 50 mol% phosphatidylcholine (PC) and either 50 mol% PE or PS (Fig. 1C, 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 Ub and other Ubl systems. However, probably due to 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 Atg3 were incubated with ATP in the absence of liposomes, and subjected to the NuPAGE separation without reducing reagents. Two additional bands of about 85 and 55 kDa appeared upon incubation, accompanied by a concomitant decrease of Atg7 and Atg3 (Fig. 2A, lanes 1-5). These bands were sensitive to the reductant β-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. 5, 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 of 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; 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 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, in order 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 about 10 mol% (Fig. 4A, left). In contrast, we found that PS conjugation was not at all stimulated by the addition of PI (Fig. 4A, right). 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. 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 cosedimentation 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. While 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 directly 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 (PAS), where autophagosome formation should take place.
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 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-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.

While the stimulative effect of PI was saturated at ~10 mol% in liposomes composed of the nearly electroneutral lipids PC and PE, we 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. 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 (~10-20%) as well as PE (~20-25%) and PS (~5-10%) (27). In addition, it has been recently reported that PI3-phosphate (PI3P), whose production by the PI3-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 PI3P also stimulates Atg8-PE formation \textit{in vitro}, its extent, however, was comparable to that of PI (Supplemental Fig. S3). Although PI3P is possible to promote the production of Atg8-PE as well as other acidic phospholipids, its 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 \textit{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 \textit{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 \textit{in vivo} analyses will be needed to address these possibilities.

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\footnote{The abbreviations used are: Ub, ubiquitin; Ubl, ubiquitin-like protein; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol; ME, \(\beta\)-mercaptoethanol; PI3P, phosphatidylinositol 3-phosphate; PAS, preautophagosomal structure.}

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**LEGENDS TO FIGURES**

**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 μM), Atg7 (0.5 μM), and Atg3 (0.5 μM) were incubated with liposomes (350 μM lipids) consisting of the indicated concentrations of PC and either PE or PS, and 1 mM ATP at 30°C for 2 hrs 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. 

*C*, The reactions were similarly performed with liposomes consisting of 50 mol% PC and either 50 mol% 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.

_**D**_ The conjugate formation efficiencies after 120-min incubation shown in _C_ are also presented, with the horizontal axis showing the pH of the reaction mixtures.

**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 β-mercaptoethanol (ME) and subjected to NuPAGE separation, followed by CBB-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.

**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 asterisk shows bands containing Atg8 that do not appear in the presence of ME.

**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% PE or PS and the indicated and remaining concentrations of PI and PC, respectively, which were analyzed as described in Fig 1C. _B and C_, The PE and PS conjugation of Atg8 was similarly examined in the presence of various acidic phospholipids.
FIGURE 5. PI facilitates membrane binding of thioester intermediates. A, The thioester intermediates were formed as described in Fig 2A (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, then further incubated on ice in the presence (lanes 4-27) or absence (lanes 1-3) of liposomes composed of 75 mol% PC and either 25 mol% PE (lanes 4-6, 10-12 and 16-18) or PS (lanes 22-24), or liposomes composed of 60 mol% PC, 15 mol% PI, and either 25 mol% PE (lanes 7-9, 13-15 and 19-21) or PS (lanes 25-27) for 10 min (T, total). Samples were subjected to ultracentrifugation at 100,000 ×g for 30 min at 4°C to generate pellets (P) and supernatants (S). These samples were analyzed by NuPAGE as described in Fig 2A. 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 Fig 1C using liposomes composed of 60 mol% PC, 15 mol% PI, and either 25 mol% PE or PS at pH 7.0 in the presence or absence of 0.02 μM Atg12-Atg5.
Oh-oka et al. Figure 2
Oh-oka et al. Figure 3
Oh-oka et al. Figure 4
Oh-oka et al. Figure 5
Oh-oka et al. Figure 6
Physiological pH and acidic phospholipids contribute to substrate specificity in lipidation of Atg8
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