Two ubiquitin-like conjugation systems that mediate membrane formation during autophagy

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Abstract

In autophagy, the autophagosome, a transient organelle specialized for the sequestration and lysosomal or vacuolar transport of cellular constituents, is formed via unique membrane dynamics. This process requires concerted actions of a distinctive set of proteins named Atg (autophagy-related). Atg proteins include two ubiquitin-like proteins, Atg12 and Atg8 [LC3 (light-chain 3) and GABARAP (γ-aminobutyric acid receptor-associated protein) in mammals]. Sequential reactions by the E1 enzyme Atg7 and the E2 enzyme Atg10 conjugate Atg12 to the lysine residue in Atg5, and the resulting Atg12–Atg5 conjugate forms a complex with Atg16. On the other hand, Atg8 is first processed at the C-terminus by Atg4, which is related to ubiquitin-processing/deconjugating enzymes. Atg8 is then activated by Atg7 (shared with Atg12) and, via the E2 enzyme Atg3, finally conjugated to the amino group of the lipid PE (phosphatidylethanolamine). The Atg12–Atg5–Atg16 complex acts as an E3 enzyme for the conjugation reaction of Atg8; it enhances the E2 activity of Atg3 and specifies the site of Atg8–PE production to be autophagy-related membranes. Atg8–PE is suggested to be involved in autophagosome formation at multiple steps, including membrane expansion and closure. Moreover, Atg4 cleaves Atg8–PE to liberate Atg8 from membranes for reuse, and this reaction can also regulate autophagosome formation. Thus these two ubiquitin-like systems are intimately involved in driving the biogenesis of the autophagosomal membrane.

Keywords:
Atg protein, autophagosome formation, conjugation, deconjugation, ubiquitin-like protein.

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Introduction

A hallmark of the macroautophagy pathway (hereafter referred to as autophagy) is the biogenesis of the double-membrane vesicle autophagosome that sequesters material to be transported to the lysosome in mammalian cells or the vacuole in yeast and plant cells for degradation [1,2]. Among 36 Atg (autophagy-related) proteins identified in yeast, 15 ‘core’ Atg proteins play pivotal roles in the formation of the autophagosomal membrane and thus are required for any type of autophagy (i.e. both non-selective and selective autophagy). The core Atg proteins constitute six functional units: (i) the Atg1 protein kinase complex, (ii) the Atg9-containing membrane vesicle, (iii) the Atg14-containing PI3K (phosphoinositide 3-kinase) complex, (iv) the Atg2–Atg18 [WIPIs (WD-repeat protein interacting with phosphoinositides) in mammals] complex, (v) the Atg12 conjugation system, and (vi) the Atg8 [LC3 (light-chain 3) and GABARAP (γ-aminobutyric acid receptor-associated protein) in mammals] conjugation system. These units localize to a site for autophagosome formation in an ordered manner to organize the PAS (pre-autophagosomal structure), in which they are likely to produce a precursory membrane, followed by its expansion into the isolation membrane (or phagophore) and the completion of the autophagosome (Figure 1). Therefore analysis of the functions and regulation of the core Atg proteins is essential for understanding the molecular mechanism of autophagosome formation.

It is the intriguing fact that autophagy, a major proteolytic pathway comparable with the ubiquitin–proteasome pathway, employs two ubiquitin-like systems, the Atg12 and Atg8 systems, to which approximately half of the core Atg proteins are devoted. These systems have been extensively studied from different viewpoints, including those of cell biology, biochemistry and structural biology. Various unique and elaborate mechanisms underlying these systems have been unveiled, which attract researchers in not only autophagy, but also other fields. In the present chapter, I summarize our current knowledge on the functions and regulation of these ubiquitin-like systems in autophagosome formation and their mechanisms. Most of the important findings were first achieved by yeast studies and then confirmed in mammals; essentially, the same mechanisms are working. Therefore I describe the following mainly on the basis of the results of the yeast system, except for the case where there is a remarkable difference from the mammalian system.

![Figure 1. Process of autophagosome formation and localization of Atg12–Atg5–Atg16 and Atg8–PE](image)

Both the Atg12–Atg5–Atg16 complex and the Atg8–PE conjugate localize to the PAS. Although Atg8–PE is comparatively evenly distributed on the isolation membrane and retained on the complete autophagosome, Atg12–Atg5–Atg16 preferentially associates with the convex surface of the isolation membrane and dissociates from the membrane upon autophagosome completion.
Conjugation reactions of Atg12 and Atg8

Atg12 and Atg8 are conjugated to each specific target via a series of enzymatic reactions similar to protein ubiquitination (Figure 2). The C-terminal carboxyl group of Atg12 is activated by the E1 enzyme Atg7 with consumption of ATP to form a thioester bond with its catalytic cysteine residue, then transferred to the catalytic cysteine residue of the E2 enzyme Atg10, and eventually attached to the amino group of the lysine residue in Atg5 via an isopeptide bond [3]. Atg8 is synthesized with an additional sequence at its C-terminus, which is immediately removed by the processing/deconjugating enzyme Atg4 to expose the glycine residue essential for subsequent reactions [4]. The conjugation reaction of Atg8 is catalysed by Atg7 (Atg12 and Atg8 share the same E1 enzyme) and the specific E2 Atg3 (Figure 2) [5]. Remarkably, the target of Atg8 is not a protein, but the lipid PE (phosphatidylethanolamine); the C-terminal carboxyl group of Atg8 forms an amide bond with the amino group in the hydrophilic head moiety of PE, thereby Atg8 is anchored to membranes.

Structural analyses of Atg7 revealed that it has an adenylation domain similar to other E1 enzymes and forms a homodimer via this domain [6,7]. In addition, Atg7 has a unique domain

Figure 2. Atg12 and Atg8 conjugation systems

Similar to protein ubiquitination, the C-terminal carboxyl group of the ubiquitin-like protein Atg12 is activated by the E1 enzyme Atg7 using ATP, forms a thioester intermediate with its catalytic cysteine residue, is transferred to the catalytic cysteine residue of the E2 enzyme Atg10, and finally forms an isopeptide bond with the lysine residue in Atg5. Likewise, the ubiquitin-like protein Atg8 is activated by Atg7, transferred to the E2 enzyme Atg3, and finally conjugated to PE via an amide bond. Atg4 mediates both the C-terminal processing and deconjugation of Atg8. The Atg12–Atg5 conjugate enhances the E2 activity of Atg3.
in its N-terminal region, to which Atg10 and Atg3 bind in a mutually exclusive manner [6–9]. In combination with biochemical analyses, it was proposed that the Atg12 or Atg8 that has formed a thioester intermediate with a protomer in the Atg7 dimer is transferred to Atg10 or Atg3 bound to the other protomer in the same dimer respectively.

In typical E2 enzymes for ubiquitin, an Asn* (invariable asparagine residue) plays a pivotal role in the transfer of ubiquitin forming a thioester bond with the catalytic cysteine residue to a substrate lysine residue. However, in Atg3, a threonine residue, which is highly conserved among Atg3 homologues, is located at a position corresponding to Asn* [10]. Biochemical analysis suggested that this threonine residue is involved in Atg8 transfer to PE in a manner similar to Asn* in other E2 enzymes [11]. Atg3 may require the threonine residue to target not lysine residues in proteins, but the lipid PE.

Whereas E3 enzymes in the ubiquitin system determine substrate specificity and stimulate ubiquitin transfer to substrates, there is no E3 enzyme for the Atg12 system. Instead, the E2 enzyme Atg10 directly recognizes the substrate Atg5 [12]. This is reasonable considering that Atg5 is the sole substrate for the Atg12 conjugation reaction. Similarly, the E2 enzyme Atg3 itself recognizes the substrate PE in the Atg8 system, although in vitro reactions under non-physiological conditions allow Atg3 to conjugate Atg8 to phosphatidylserine, which has an amino group in its hydrophilic head as well as PE [13,14]. Although the Atg12–Atg5 conjugate enhances the E2 activity of Atg3 (see below), it does not affect the substrate preference of Atg3 [15].

**Functions of Atg12–Atg5 and Atg8–PE conjugates**

Cells defective in the formation of the Atg12–Atg5 conjugate (i.e. those lacking Atg12, Atg5 or Atg10) also exhibit a defect in the formation of Atg8–PE (called LC3/GABARAP-II in mammals) [16,17]. This indicated a linkage between the two conjugation systems. The conjugation reaction of Atg8 can be reconstituted in vitro using purified proteins, Atg8, Atg7 and Atg3, PE-containing liposomes (artificial membrane vesicles) and ATP [18]. On the other hand, the simultaneous expression of Atg12, Atg5, Atg7 and Atg10 allows the formation of Atg12–Atg5 in *Escherichia coli* cells, from which the conjugate can be purified. By adding it to the in vitro Atg8 system, it was clearly shown that Atg12–Atg5 itself has an ability to accelerate Atg8–PE formation [15]. It was further shown that Atg12–Atg5 interacts with the E2 enzyme Atg3 and stimulates the transfer of Atg8 forming a thioester bond with the catalytic cysteine residue of Atg3 to PE (Figure 2). Thus in the Atg8 conjugation reaction, Atg12–Atg5 exerts an activity like E3 enzymes in the ubiquitin system. In vivo, Atg12–Atg5 functions as a complex with Atg16 (Atg16L in mammals), which forms a dimer in its C-terminal region [19–21]. Since Atg16 interacts with Atg5 in the N-terminal region, the Atg12–Atg5–Atg16 complex is a 2:2:2 heterohexamer. Atg16 is dispensable for the E3-like activity of Atg12–Atg5, but is essential for the localization of the complex to autophagy-related membranes (see below).

Fluorescence and immunoelectron microscopy showed that Atg8–PE localizes to all the autophagy-related structures: the PAS, the isolation membrane, the complete autophagosome, the autophagic body (the inner membrane vesicle released into the vacuole on fusion between the autophagosomal outer membrane and the vacuole in yeast and plant cells) and the
autolysosome (the lysosome fused with the autophagosome in mammalian cells) \cite{22,23} (Figure 1). \textit{In vitro} studies also provided a clue to elucidate the molecular function of the Atg8–PE conjugate during autophagosome formation \cite{24}. In the \textit{in vitro} reaction, Atg8 is conjugated to PE in the outer leaflet of the liposomal lipid bilayer (Figure 3A). It was found that this leads to aggregation of the liposomes, associated with hemifusion of the membranes (fusion between the outer leaflets of two opposed membranes with the inner leaflets left intact) (Figure 3A). It was also shown that Atg8 forms oligomers when conjugated to PE. These results suggest that Atg8–PE conjugates on one membrane can interact with those on another membrane to tether the membranes together and cause their hemifusion. Most Atg8 mutants defective in autophagosome formation exhibited a significant decrease in membrane tethering and hemifusion, suggesting that these functions of Atg8 observed \textit{in vitro} are related to its \textit{in vivo} role during autophagosome formation \cite{24}.

How are the membrane tethering and hemifusion functions of Atg8–PE involved in autophagosome formation? Autophagosomes smaller than those in wild-type cells are formed in cells expressing Atg8 mutants partially defective in membrane tethering and hemifusion, indicating that the Atg8–PE functions are involved in the step that determines the size of the autophagosome, i.e. the step of membrane expansion (Figure 3B) \cite{24}. Consistently, decreased expression levels of Atg8 result in the formation of small autophagosomes \cite{25}. It is also possible that Atg8–PE plays an important role in an earlier event, the formation of an as yet unidentified precursory membrane at the PAS. In addition, it was reported that unclosed isolation membranes with abnormal morphology accumulate in mammalian cells deficient for PE conjugation of Atg8 homologues, suggesting that Atg8–PE is also involved in the closure of the isolation membrane (this step requires membrane fission) in addition to its normal

\textbf{Figure 3. Roles of Atg8–PE in autophagosome formation}

\textbf{(A)} Atg8–PE oligomerizes and causes tethering and hemifusion of liposomal membranes \textit{in vitro}. \textbf{(B)} Possible roles of Atg8–PE during autophagosome formation (see text for details).
development [26,27]. Further analyses are still required to understand precisely how Atg8–PE is involved in autophagosome formation.

It should be noted that Atg8–PE-mediated hemifusion, compared with liposome aggregation, is sensitive to the lipid composition of liposomes (H. Nakatogawa and Y. Ohsumi, unpublished results). It was also reported that hemifusion does not occur in liposomes containing PE at concentrations in typical organelle membranes [28]. Although this may indicate that Atg8–PE does not cause hemifusion in vivo, to clarify this point, it is essential to determine the lipid composition of membranes where Atg8–PE functions. At any rate, only hemifusion does not lead to the expansion of membranes; another protein(s) and/or a specific lipid composition may help complete fusion to occur in cells. It was recently reported that SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) proteins, which mediate membrane fusion in various endomembrane systems, are required for autophagosome formation, but it is unclear whether they co-operate with Atg8 [28,29].

Recently, on the basis of theoretical analysis, an intriguing model for curving of the isolation membrane was proposed [30]. The isolation membrane can spontaneously curve when it expands to a critical size, which is determined by three properties: the lateral dimension of the membrane, the molecular composition of the highly curved rims and an asymmetry between the two flat faces. Proteins that bind to membranes such as Atg8–PE can regulate the latter two properties of the sheet. Whereas a higher protein concentration at the rims suppresses curving of the isolation membrane and thus results in formation of a larger autophagosome, an asymmetric protein distribution between the two flat faces facilitates it, leading to small autophagosome formation. Therefore in these, Atg8–PE density and localization on the isolation membrane can contribute to determining the size of the autophagosome. These possible roles of Atg8–PE are not mutually exclusive with those discussed above, and it is tempting to speculate that Atg8–PE is involved in autophagosome formation in multiple ways.

**Mechanism of Atg3 activation by Atg12–Atg5**

Although the primary sequences of Atg8 and Atg12 show little similarity to ubiquitin, structural studies revealed that these proteins actually adopt a ubiquitin-like fold [10]. In addition, it was also revealed that Atg5 contains two ubiquitin-like folds [10,31,32]. These facts highlighted the peculiarity of the Atg conjugation systems; a ubiquitin-like protein conjugate with three ubiquitin-like folds serves as an E3 enzyme in the conjugation reaction of another ubiquitin-like protein. We recently succeeded in unveiling how Atg12–Atg5 enhances the E2 activity of Atg3 [11]. Atg3 has an E2 core domain similar to other E2 enzymes [10]. However, Atg3 adopts an inactive conformation in the absence of Atg12–Atg5; the side chain of the catalytic cysteine residue of Atg3 faces in the opposite direction from the aforementioned threonine residue, whereas that of typical E2 enzymes is directed towards Asn* (Figure 4A). Atg12–Atg5 causes the reorientation of the cysteine residue toward the threonine residue, resulting in the enhancement of Atg3 activity [11] (Figure 4B). How are three ubiquitin-like folds in Atg12–Atg5 involved in the rearrangement of the Atg3 catalytic centre? This is the intriguing question to be addressed next.
Spatial regulation of Atg8–PE formation by the Atg12–Atg5–Atg16 complex

In response to autophagy-inducing signals, the expression of Atg8 is transcriptionally up-regulated [22]. The Ume6–Sin3–Rpd3 complex binds to the promoter region of the ATG8 gene to repress its transcription under nutrient-rich conditions [33]. Rim15 phosphorylates Ume6 to disassemble the complex under starvation conditions, resulting in up-regulation of ATG8 transcription. Autophagy-inducing signals also stimulate the formation of Atg8–PE [24]. This is tightly linked to the localization of the Atg12–Atg5–Atg16 complex, which changes its localization from the cytoplasm to the PAS and the isolation membrane following autophagy-inducing signals [16,17] (Figure 1). It was also reported that Atg12–Atg5–Atg16 predominantly localizes to the convex surface of curved isolation membranes in mammalian cells [17]. Targeting Atg12–Atg5–Atg16 to those structures would lead to the production of Atg8–PE on the membranes via its E3 enzyme-like function that activates Atg3. Since PE is a major component in most intracellular membranes, the localized activation of Atg3 by Atg12–Atg5–Atg16 is likely to be important to produce Atg8–PE on autophagy-related membranes.
Atg5 and Atg16 co-operatively act to target the complex to the PAS [34]. In vitro analysis showed that Atg5 alone and the Atg12–Atg5–Atg16 complex, but neither Atg16 alone nor Atg12–Atg5 can bind to liposomes, suggesting that Atg16 increases the membrane-binding ability of Atg5, which is suppressed by Atg12 in the conjugate [35]. It is also known that the production of PI3P (phosphatidylinositol 3-phosphate) by the Atg14-containing PI3K complex is a prerequisite for the localization of Atg12–Atg5–Atg16 [34,36]. However, how Atg12–Atg5–Atg16 is localized to the PAS and how it is associated with the isolation membrane remain to be elucidated. Unlike Atg8, Atg12–Atg5–Atg16 is released from the membrane immediately before or on completion of the autophagosome [17]. This mechanism is also still elusive, but may involve PI3Ps [37].

**Significance of Atg8 deconjugation by Atg4**

As described above, Atg4 cleaves Atg8 at the peptide bond C-terminal to the glycine residue essential for the conjugation reaction. In addition, Atg4 also serves as a deconjugating enzyme that cleaves the amide bond between Atg8 and PE to release the protein from PE in membranes [4] (Figure 2). This reaction is thought to be important for reusing the Atg8 that has exerted its function for autophagosome formation and would occur on the complete autophagosome and the autolysosome or vacuole (Atg8–PE on the autophagosomal outer membrane can be transferred on to the lysosomal or vacuolar membrane following their fusion). Moreover, Atg8 deconjugation by Atg4 may also occur on the isolation membrane, which could positively or negatively affect membrane formation [38–40]. On the other hand, a mechanism by which the Atg8–PE that has not fulfilled its role is protected from deconjugation by Atg4 may also exist. Thus controlling Atg4-mediated deconjugation of Atg8 can regulate autophagosome formation, but further analyses are required to assess these possibilities.

As discussed above, the localized production of Atg8–PE on autophagy-related membranes should be achieved by the localization of the Atg12–Atg5–Atg16 complex to those membranes. However, recent studies suggested that this mechanism is not that strict: conjugation enzymes in the cytoplasm erroneously produce Atg8–PE on various intracellular membranes to a considerable degree [39–41]. Thus another new role of Atg4 was proposed; Atg4 deconjugates those non-productive Atg8–PE to maintain a cytoplasmic reservoir of unconjugated Atg8, which is required for Atg8–PE formation at correct sites (Figure 5).

**Conclusions**

In the present chapter, I have described the autophagy-related ubiquitin-like systems in the context of autophagosomal membrane biogenesis, but recent studies have also revealed their other aspects. The most prominent is a role of Atg8 homologues in selective types of autophagy, in which degradation targets, such as ubiquitin-positive protein aggregates, damaged mitochondria, superfluous peroxisomes and invasive bacterial cells, are exclusively enwrapped by the autophagosomal membrane [42,43]. Atg8 homologues bind to a consensus sequence named the AIM (Atg8 family-interacting motif) or LIR (LC3-interacting region) in receptor proteins, which specifically recognize each target, with highly conserved binding
pockets [44]. The interaction of Atg8–PE on the growing autophagosomal membrane with receptor proteins is likely to link the membrane to the targets. As another aspect, although there is a single Atg8 in yeast cells, many other organisms, including mammals and plants, have multiple Atg8 homologues. Although it had been suggested that they have different functions, actual cases have been reported recently [45,46]. It has also been reported that some viruses and bacteria target the Atg conjugation systems to interfere with autophagy for their proliferation in host cells. The roles of the Atg8 and Atg12 systems in diverse biological events, such as membrane traffic, the regulation of vacuolar morphology, phagocytosis and apoptosis, have also been implicated, which were suggested to be independent of autophagy. Studies on the Atg12 and Atg8 systems will continue to provide important insights into mechanisms, physiology and pathology not only within, but also beyond, autophagy.

Summary

- Autophagosome formation requires the Atg12 and Atg8 ubiquitin-like systems.
- The Atg12 and Atg8 conjugation reactions share Atg7 as an E1 enzyme and employ Atg10 and Atg3 as specific E2 enzymes.
- Atg4 serves as a processing/deconjugating enzyme for Atg8.
- Atg12 and Atg8 form conjugates with Atg5 and PE respectively.
- Atg12–Atg5 induces the rearrangement of the Atg3 catalytic centre to enhance its E2 activity.
- Atg16 forms a complex with Atg12–Atg5 and targets the complex to autophagy-related membranes, where Atg8–PE is produced.
- Atg8–PE is involved in autophagosome formation at multiple steps.
- Atg4-mediated deconjugation recycles the Atg8 that has fulfilled its role in membrane formation or has erroneously formed a conjugate with PE in autophagy-unrelated membranes.
- Thus the Atg12 and Atg8 systems are intimately involved in driving membrane formation during autophagy.
I thank Dr Yoshinori Ohsumi for critically reading this chapter. I apologize that many important references have been omitted due to the limitation on the number of citations.

References


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