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Minireview

Molecular machinery of autophagosome formation in yeast, *Saccharomyces cerevisiae*

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Abstract Autophagy is a degradation process accompanied by dynamic membrane organization. In the yeast, *Saccharomyces cerevisiae*, about 30 *ATG* (autophagy-related) genes have been identified as important genes for autophagy. Among them, 17 are indispensable for formation of the autophagosome, an organelle enclosed by a double lipid bilayer during starvation-induced autophagy. Recently, a central structure for autophagosome generation, termed the pre-autophagosomal structure, was identified. Despite intensive study, many questions regarding the mechanisms underlying autophagosome formation remain unanswered. In this review, we will give an overview of recent studies on the mechanisms of autophagosome formation and discuss these unresolved questions.

Keywords: Autophagy; Autophagosome; *ATG* genes; Pre-autophagosomal structure; Nutrient starvation

1. Introduction

Cellular homeostasis is balanced between the synthesis and degradation of proteins, nucleic acids, ribosomes, and organelles. The ubiquitin-proteasome pathway is a selective protein degradation pathway. In contrast, macroautophagy, hereafter referred to as autophagy, is a major pathway of bulk and non-selective degradation. In the yeast, *Saccharomyces cerevisiae*, autophagy has been studied as a cellular response for survival during nutrient-limited conditions [1]. Recent studies have shown that during nutrient-rich growth, autophagy contributes to the transport of vacuolar hydrolases to the vacuolar lumen in yeast [2], and the clearance of protein aggregates and defense against invasion of viruses or bacteria in mammalian cells [3]. Moreover, autophagy has a role in anti-aging in plants [4]. Breakdown of the autophagic system has maladaptive and often fatal pathological effects in higher eukaryotes; protein folding diseases, neurodegenerative diseases, and neonatal death immediately after birth have been associated with defective autophagy [3]. This process is required for development and differentiation in yeasts, slime molds, nematodes, and flies; that is, autophagy is required for remodeling of cells accompanied with bulk degradation of cellular components [5]. Thus, recent studies have revealed several aspects of cellular processes involving autophagy during vegetative growth conditions.

2. Process of autophagy

Because autophagy is dramatically activated in such severe environments to adapt and survive, the process of autophagy long has been studied under nutrient-limited conditions. Hereafter, we mainly focus on autophagy that has been described in the yeast *S. cerevisiae* exposed to nutrient-starvation conditions. Recent studies have revealed that autophagy proceeds through the following steps (Fig. 1): (i) detection of starvation signals; (ii) transmission of these signals to the autophagosome-generating apparatus known as the pre-autophagosomal structure (PAS); (iii) generation of an isolation membrane (IM) from the PAS; (iv) expansion of the IM; (v) fusion of the leading edges of the IM to complete autophagosome formation; (vi) fusion of the outer membrane of the autophagosome with the vacuolar membrane and subsequent release of autophagic bodies, whose membranes are derived from the inner membrane of the autophagosome; (vii) disintegration of the autophagic body and degradation of the contents by vacuolar hydrolases; and (viii) transport of the resulting amino acids and lipids to the cytoplasm for recycling.

Starvation signals are sensed and transmitted to the Tor signaling pathway, which is one of the master regulators of cell growth [6]. During vegetative growth, Tor kinase is active, and it phosphorylates several downstream proteins. When Tor kinase is inactivated by nutrient limitation or treatment with rapamycin, several cellular responses, including autophagy, are induced. This response results in autophagosome formation.

3. Autophagosome

The autophagosome in the yeast *S. cerevisiae* is encircled by double lipid bilayers and is about 400–900 nm in diameter [7].

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Abbreviations: AS-PI3K, autophagy-specific phosphatidylinositol 3-kinase; *ATG* genes, autophagy-related genes; COP, coatmer; Cvt, cytoplasm-to-vacuole targeting; ER, endoplasmic reticulum; GFP, green fluorescent protein; IM, isolation membrane; NSF, *N*-ethylmaleimide-sensitive fusion protein; PAS, pre-autophagosomal structure; PE, phosphatidylethanolamine; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(3,5)P_2, phosphatidylinositol 3,5-bisphosphate; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor

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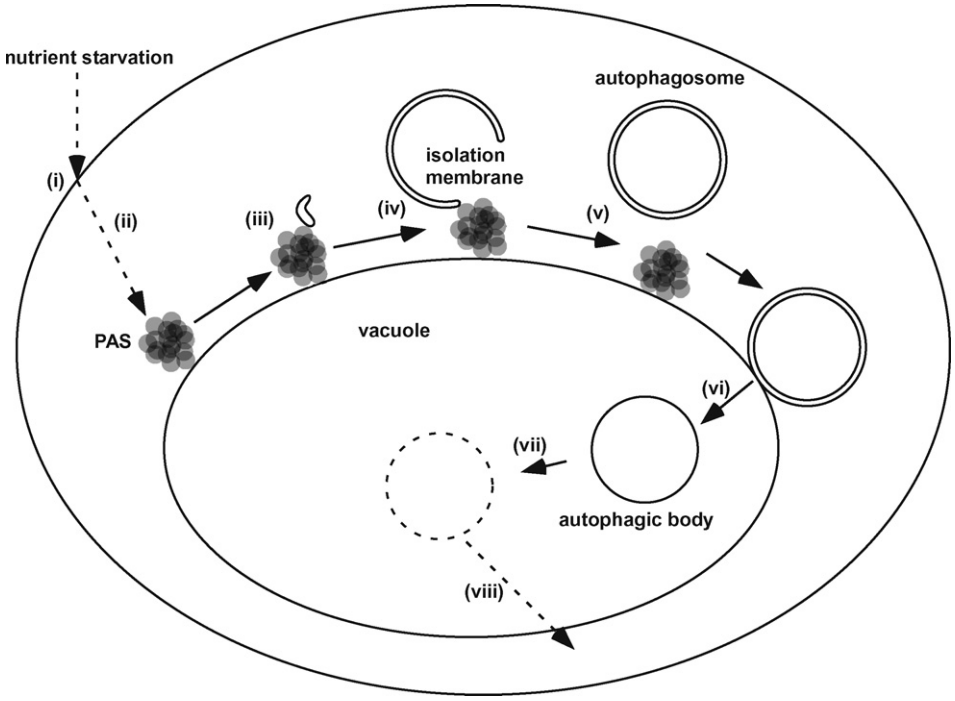

Fig. 1. Autophagy in the yeast *Saccharomyces cerevisiae*. Autophagy proceeds through the following steps: (i) sensation of starvation signals; (ii) transmission of these signals to the autophagosome-generating machinery known as the PAS (pre-autophagosomal structure); (iii) generation of an isolation membrane (IM) from the PAS; (iv) expansion of the IM; (v) fusion of the leading edges of the IM to complete autophagosome formation; (vi) fusion of the outer membrane of the autophagosome with the vacuolar membrane and subsequent release of autophagic bodies, whose membrane is derived from the inner membrane of the autophagosome; (vii) disintegration of the autophagic body and degradation of its contents by vacuolar hydrolases; and (viii) transport of the resulting amino acids and lipids to the cytoplasm for recycling.

The autophagosomal membrane is rich in lipids and poor in proteins [8], and is much larger than typical secretory vesicles, suggesting that it is composed of the minimal components necessary to load the maximum material destined to be degraded. The outer membrane of the autophagosome fuses with the vacuolar membrane by using general fusion machinery [9]. The autophagic body, the structure surrounded by the inner lipid bilayer, is released into the lumen of the vacuole [7]. Consequently, the autophagic body is degraded by vacuolar hydrolases.

Interestingly, the machinery employed in autophagosome formation is common to that in a biosynthetic pathway under vegetative growth conditions. This pathway, called the cytoplasm-to-vacuole targeting (Cvt) pathway, delivers a few vacuolar hydrolases to the vacuole in about 150-nm vesicles, named Cvt vesicles, which are also composed of double lipid bilayers. Aminopeptidase I (Ape1) [10] and α-mannosidase [11] are among the proteins transported by the Cvt pathway. Ape1 forms dodecamers [12], which are organized into large complexes that are about 150 nm in diameter; ultimately, this complex is enwrapped by a Cvt vesicle and delivered to the vacuole [2].

4. *ATG* genes required for autophagy

Using yeast genetic techniques, mutants deficient in the autophagic and the Cvt pathways were obtained in *S. cerevisiae*. *APG* (autophagy) [1] and *AUT* (autophagy) [13] genes were identified as essential genes for autophagy, and *CVT* genes were identified as those required for the Cvt pathway [10]. Interestingly, these groups of genes showed considerable overlap [14]. Moreover, other types of autophagy have been described by several groups, and the genes involved in each of these types had been named independently: *GSA* (glucose-induced selective autophagy) [15], *PAZ* (pexophagy zeocin-resistant) [16] and PDD (peroxisome degradation-deficient) [17]. To avoid confusion, the nomenclature was consolidated; all genes were referred to as *ATG* (autophagy-related) genes [18].

Currently, 27 *ATG* have been identified in *S. cerevisiae*. Most are grouped into three large groups by their function: non-selective autophagy (17 genes), selective autophagy (7 genes), and degradation of autophagic bodies (2 genes) [19]. One can regard selective autophagy as a type of macroautophagy in which cargo proteins and organelles are selectivity enwrapped. Thus, autophagosome formation mediated by genes involved in non-selective autophagy is essential for selective autophagy. Among all of the genes involved in this pathway, the 17 *ATG* genes compose the machinery of autophagosome formation. The proteins they encode make up the autophagosome at a perivacuolar and restricted site called the PAS [20,21]. Hereafter, we will focus on these 17 genes and their role in the molecular basis of autophagosome formation.

5. *ATG* genes involved in autophagosome formation

The contents of the autophagosome are sequestered by double lipid bilayers. Despite their role in biosynthesis rather than degradation, we consider Cvt vesicles to be variants of autophagosomes, owing to similarities in their topology and

formation. An autophagosome is formed through nucleation and expansion steps (Fig. 2A) [22,23]. The nucleation step is sufficient for generating a minimum-size autophagosome, such as a Cvt vesicle. Subsequent membrane expansion is needed to form a normal-sized autophagosome. Because genes involved in the expansion step are not necessary for Cvt vesicle formation, they can bypass the *cvt* phenotype upon nitrogen starvation or rapamycin treatment, whereas these in the nucleation step cannot, resulting in the emergence of mature Ape1 [22].

The 17 Atg proteins are categorized into six functional units [21]: (i) the Atg1 protein kinase and its regulators [24]; (ii) the Atg2·Atg18 complex [21]; (iii) the Atg8 system [25]; (iv) the Atg12 system [26]; (v) the autophagy-specific phosphatidylinositol 3-kinase (AS-PI3K) complex [27]; and (vi) Atg9 [28]. Atg8 and Atg12 are ubiquitin-like proteins [29]. Atg8 is conjugated to phosphatidylethanolamine (PE) by serial reactions of Atg4 (an Atg8 processing enzyme), Atg7 (an E1-like protein), and Atg3 (an E2-like protein). Atg12 is activated by Atg7 and conjugated to Atg5 by Atg10 (an E2-like protein) to become the Atg12–Atg5 conjugate [30]. This conjugate is organized into a complex by associating with Atg16 to form the Atg16 · Atg5–Atg12 complex [31]. Formation of Atg8-PE and the Atg16 · Atg5–Atg12 complex is essential for autophagosome formation. As a consequence, autophagosome formation is accomplished by six functional complexes or modified proteins: the Atg1 protein kinase and its regulators (Atg1, Atg13, and Atg17), the Atg2 · Atg18 complex, Atg8-PE, the Atg16 · Atg5–Atg12 complex, the AS-PI3K complex (Vps30/Atg6 and Atg14), and Atg9.

Among these, the Atg2 · Atg18 complex, the Atg16 · Atg5–Atg12 complex, the AS-PI3K complex, and Atg9 are required for the nucleation step, whereas Atg8-PE is involved in the expansion step (Fig. 2B). The Atg1 kinase and its regulators showed a complicated phenotype: Atg1 is essential for nucleation, but its regulators (Atg13 and Atg17) are required for the expansion step. Recently, Atg29 has been found to be required for the expansion step [32]. The role of Atg29 needs to be investigated further.

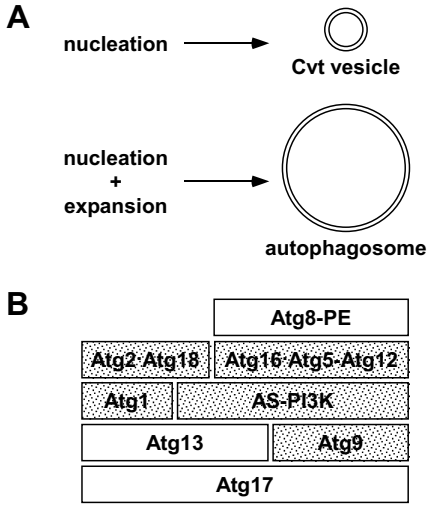

Fig. 2. *ATG* genes required for autophagosome formation in the yeast *Saccharomyces cerevisiae*. (A) The 17 *ATG* genes are distinguished by the step they are involved in. The nucleation step is required for formation of the Cvt vesicle, a minimum closed vesicle. In addition to the nucleation step, an expansion step is needed to form the autophagosome, a larger membrane-bound structure. (B) A hierarchy diagram of the 17 Atg proteins. The Atg proteins/complexes are recruited to the PAS on the basis of this hierarchy [21]. Atg17 behaves as a scaffold protein in PAS organization. Dotted proteins/complexes function in the nucleation step, and the others are involved in the expansion step.

6. Atg proteins involved in expansion

Atg13 and Atg17, the regulators of the Atg1 serine/threonine protein kinase, are required for the expansion step [33–35]. As noted, inactivation of Tor kinase induces autophagy. Which Atg protein receives the autophagy-inducing signal from Tor? Atg13 is hyper-phosphorylated under growing conditions, and our group found that dephosphorylation of Atg13p is closely related to Tor inactivation. Upon Tor kinase inactivation by rapamycin treatment or nitrogen starvation, Atg13p is rapidly dephosphorylated within several minutes [24]. The identities of the phosphatases involved in this reaction are unknown, but inactivation of Tor kinase directly or indirectly leads to dephosphorylation of Atg13p. Dephosphorylated Atg13p binds to Atg1p [36]. This binding activates the Atg1p kinase activity from a basal level. Atg17 is also involved in this activation step, but its involvement is possibly indirect (see below). One study demonstrated that a conformational change of Atg1p initiate autophagosome formation [33]. To elucidate the mechanisms of autophagy induction by Atg1 kinase and its regulators, the substrates of Atg1 kinase must be determined.

Atg8-PE is another molecule involved in the expansion step [22]. Atg8 is highly expressed during autophagy [37]. This expression is essential for the expansion of the IM; inhibition of protein synthesis by cycloheximide immediately affects the size of autophagosomes [22]. In the process of autophagosome formation, Atg8-PE localizes to the IMs [37]. This also suggests a role for Atg8-PE in IM expansion. Cleavage of Atg8-PE to Atg8 by Atg4 is also required for generation of normal autophagosomes [38]. In the absence of Atg8-PE cleavage, the curvature of the IM and the direction of IM expansion are abnormal (our unpublished observation). The responses of Atg13 and Atg8 to rapamycin are independent of each other [22]. At least two signaling pathways are activated upon initiation of autophagosome formation.

7. Atg proteins required for nucleation

The AS-PI3K complex (Vps30/Atg6, Atg14, Vps15, and Vps34) and the Atg2 · Atg18 complex are essential for nucleation. The functions of both complexes are closely related to phosphoinositide processing. In *S. cerevisiae*, Vps34 is the sole PI3K [39] and is essential for autophagosome formation [27]. The role of the AS-PI3K complex is apparently to produce phosphatidylinositol 3-phosphate (PtdIns(3)P) at the PAS. The localization of this complex at the PAS is controlled by Atg14 [40]. Our systematic localization analysis of Atg proteins suggests that one role of the AS-PI3K complex is to recruit other effector proteins to the PAS [21].

Atg18 has been reported to be an effector protein bound to phosphatidylinositol 3, 5-bisphosphate (PtdIns(3, 5)P_2) [41]. In yeast, PtdIns(3, 5)P_2 is synthesized by Fab1, the sole

PtdIns(3)P 5-kinase, from PtdIns(3)P produced by Vps34 [42]. Thus, in the absence of PI3K activity, yeast cells are able to produce neither PtdIns(3)P nor PtdIns(3, 5)P_2. Fab1 is dispensable for autophagosome formation [41]; thus, PtdIns(3, 5)P_2 is not required for autophagic activity. PtdIns(3)P and PtdIns(4)P have also been suggested to be binding partners of Atg18 [41,43]. Our recent study showed that Atg18 is recruited to the PAS in a manner dependent upon the AS-PI3K complex, suggesting that PtdIns(3)P is a key player in autophagosome formation by recruiting Atg18.

Atg2 forms a complex with Atg18, but the localization patterns of these proteins are different [21]; whereas Atg2 localizes only to the PAS, Atg18 additionally localizes to the vacuolar membrane. In the absence of Atg2, Atg18 disappears from the PAS, but it is still seen at the vacuolar membrane [21]. This pattern may exist because of PtdIns(3)P produced by the PI3K complex required for the vacuolar protein sorting pathway or PtdIns(3, 5)P_2 generated by Fab1. Atg2 and Atg18 reciprocally require each other to localize to the PAS [21]. Presumably, formation of the Atg2 · Atg18 complex acquires the ability to bind to phosphoinositides required for PAS localization. However, the functions of Atg2 and Atg18 in the nucleation step remain to be elucidated.

The Atg16 · Atg5–Atg12 complex is another complex required for the nucleation step. In mammalian cells, Atg5 is localized to the outer face of expanding IMs and detaches just after completion of autophagosome formation; this observation suggests that this complex plays a coatmer-like role in autophagosome formation [44]. Like the Atg2 · Atg18 complex, localization of the Atg16 · Atg5–Atg12 complex to the PAS depends on the AS-PI3K complex [21], suggesting that this complex interacts with PtdIns(3)P.

Among Atg proteins, Atg9 is known as the only integral membrane protein [28]. Fluorescence microscopy reveals that Atg9-GFP exists as two populations: one in the PAS and another in the periphery, composed of a few dozen small punctate structures moving rapidly around the cytoplasm. Lack of Atg1 activity leads to Atg9 accumulation to the PAS; Atg9 is abundant in the PAS at non-permissive temperatures in the *atg1*^{ts} strain, but this accumulation is not seen at permissive temperatures. This study proposed the hypothesis that Atg9 is recycled between the PAS and the cytoplasmic pool [45,46]. The most important issue to be addressed at the present time is the relationship between the PAS and peripheral pools. Does Atg9 really cycle between these two pools? If this cycle exists, is it essential for autophagosome formation? Regardless of these details, however, it is clear that Atg9 is deeply involved in the nucleation step. In the absence of Atg9, PAS organization is severely damaged, with a number of Atg proteins missing from the PAS [21]. This phenotype is compatible with Atg9 mainly playing a role in recruiting the AS-PI3K complex to the PAS.

8. Machinery involved in expansion of the isolation membrane

PtdIns(3)P is a lipid involved in autophagosome formation, but it does not seem to be sufficiently abundant to account for the large amount of lipids required for the expanding IM. It is one possibility that lipids are carried by a structure containing the transmembrane protein Atg9. In an Atg9-recycling model, the Atg9-structure provides lipids to the PAS or expanding IMs and recycles back to the cytoplasmic pool to recruit lipids to be delivered [45]. It has been proposed that mitochondria may serve as a lipid source [47]. Direct evidence of involvement of Atg9 recycling in expansion of the IM, however, has not yet been obtained.

Atg8-PE and the Atg16 · Atg5–Atg12 complex are known to localize to the IM [20,44]. Atg8-PE is tightly associated with membranes [38]; if it plays a role in delivering lipids to the expanding IM, then Atg8-PE containing structures should be targeted to the PAS or IM. Because PE is a cone-shaped phospholipid, it cannot be the only constituent of a vesicle; incorporation of other lipids is required. The route of Atg8-PE trafficking has not been described fully; however, it is likely that Atg8-PE is ultimately delivered to the PAS or the expanding edge of the IMs. Atg8-PE is localized on both of the IM membranes, but the Atg16 · Atg5–Atg12 complex resides only on the outer face. The association of Atg8-PE with the Atg16 · Atg5–Atg12 complex possibly underlies the curvature of the IM [48]. Once autophagosome formation is complete, the Atg16 · Atg5–Atg12 complex is uncoated immediately. This reaction may be mediated by cleavage of Atg8-PE by Atg4, resulting in a fusion-competent autophagosome.

9. Source of the isolation membrane

The nascent autophagosome is a curved membrane sac called the IM (Fig. 1). The autophagosome becomes mature upon fusion of the leading edges of the expanding IMs. During expansion of the IMs, the outer and the inner faces of unit membranes appear differentiated (see above) [44,49]. The properties underlying the difference between the outer and inner faces remain to be elucidated. Localization analysis of the other Atg proteins on the IMs will help reveal the mechanisms by which these differences are established.

The mechanisms of IM expansion are not fully understood. Although it is likely that multiple organelles are involved in supplying membranes to the IM, the possible main source of the IM is the endoplasmic reticulum (ER) membrane. Involvement of conventional vesicular transport was suspected by a negative observation with electron microscopy: typical secretory vesicles were not found at the leading edge of the expanding IMs [37]. However, the ER membrane plays an important role in the expansion of IMs. Formation of the COPII vesicle is essential for ER-to-Golgi transport. A mutant defective in the subcomplex required for COPII vesicle formation (*sec23*/24) participates in autophagosome formation, but other mutants (*sec12* and *sec13*/31) do not [9]. Interestingly, the autophagy defect of the *sec24* mutant is rescued by overexpression of the Sec24 homologue Sfb2; deletion of the gene encoding this protein does not affect autophagic activity [50]. Conventional COPII vesicles may not be involved in expansion of the IMs, but some COPII proteins may mediate flow in the early secretory pathway required for autophagosome formation.

To examine the possible involvement of the ER in autophagosome formation, the ER was labeled with an HDEL (His-Asp-Glu-Leu) signal peptide-tagged GFP (HDEL-GFP), which has a retention sequence to the ER lumen [51]. A fraction of HDEL-GFP was transported to the vacuole upon starvation and stained the vacuolar lumen. This transport was blocked in the autophagy-defective mutants, suggesting that the HDEL-GFP transport depends on autophagy. On

the other hand, a protein on the Golgi membrane was not delivered to the vacuole. Immunoelectron microscopy revealed that fragmented ER membranes were enclosed within autophagosomes; yet, involvement of ER membranes the in expansion of IMs has not been shown to date. This approach has been difficult, because very few marker proteins of the IM have been identified. More such markers must be identified in order to further investigate the membrane sources of the IM.

It is unlikely that canonical vesicular transport is involved in expansion of the IM (see above). Similarly, conventional fusion machinery used in the endomembrane system, NSF (*N*-ethylmaleimide-sensitive fusion protein), SNAP (soluble NSF attachment protein), and SNARE (SNAP receptor) [52–54], is not involved in expansion of the IM and completion of autophagosome formation. In the mutant of *SEC18*, a gene encoding the sole yeast NSF, most vesicular trafficking events, including autophagosome fusion to the vacuole, are blocked, but autophagosomes are normally formed [9]. Also, *sec17* (SNAP) and *vti1* (SNARE) mutants are defective in autophagosome fusion to the vacuole, but not in autophagosome formation [9]. However, we cannot exclude the possibility that residual activity of these proteins at non-permissive temperatures is sufficient for the fusion event during expansion of the IM, or that these mutants are not defective in the fusion event required for expansion of the IM. Because the essential proteins for conventional vesicular trafficking are not required for expansion of the IM, there likely exists an unknown trafficking system from the ER to the IM.

10. Role of the pre-autophagosomal structure in expansion of the isolation membrane

The six Atg complexes/proteins concentrate and work at the PAS [20]. Although specific targeting mechanisms have not yet been identified, it is likely that the main role of the PAS is to generate IMs by concentrating Atg proteins to a restricted site. Two models are possible regarding the relationship between the PAS and the IM (Fig. 3). In one model, the PAS generates IMs (Fig. 3A); in the second, the PAS is directly converted into IMs (Fig. 3B). In yeast, Atg8 and most other Atg proteins still localize to the PAS even after production of autophagosomes [20]; this seems to support the former model. In the case of yeast, the PAS seems to associate with the IM until it becomes a mature autophagosome; this suggests that the PAS is responsible for sealing the IM. On the other hand, in mammals, a crescent-shaped membrane labeled with Atg5 is transformed to a mature autophagosome, after which Atg5 leaves the membrane of the autophagosome [44]. This observation suggests the PAS matures and transforms into the autophagosome. These hypotheses raise the significant question of whether the crescent-shaped membrane is a counterpart of the PAS in yeast. It is still possible to hypothesize the existence of the multiple PAS structures in mammals. Recently, our group found that Atg17 determines the site of PAS organization (Fig. 2B) [21], but unfortunately the homologue of Atg17 has not yet been identified in mammals. In order to characterize the mammalian PAS equivalent, a functional homologue of Atg17p must be identified.

11. Concluding remarks

Juhasz and Neufeld have discussed the origins of autophagic membranes in their review [55]. Two models of IM expansion were presented: one is a maturation model, in which pre-existing organelle membranes are delivered to or mature into the IM. If this model is true, the PAS might play a converter-like role from the ER membrane to the IM. The PAS pumps IMs out of the ER by coating the membranes with Atg8-PE and the Atg16 · Atg5–Atg12 complex, generating the correct curvature. There is the possibility that the PAS is a uniquely differentiated region of the ER. A second proposed model is the assembly model; non-vesicular transport or de novo synthesis of membrane is responsible for IM expansion. However, as enzymes required for lipid synthesis do not occur among the Atg proteins, general lipid synthetic pathways would have to be involved in this synthesis. Determining the lipid composition

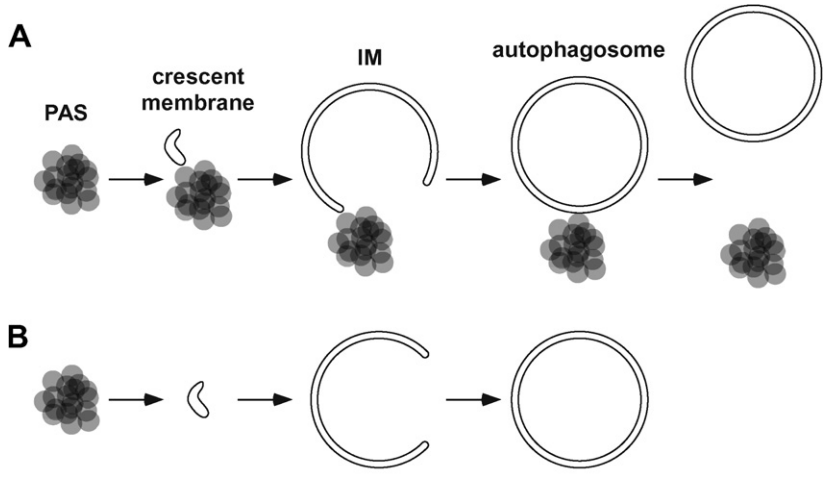

Fig. 3. Two models for the relationship between the PAS and the IM. (A) The PAS plays a role in producing the IMs and persists after the autophagosome matures. (B) The PAS is converted directly into the IM. Proteins required during autophagosome formation disassemble and disperse to the cytoplasm immediately after the autophagosome matures.

of IMs and autophagosomes should provide insight into this issue.

Recent studies have elucidated the process of autophagy at the molecular level; parts of the machine (Atg proteins) and the factory (PAS) have already been found. The most important outstanding mystery concerns the precise materials that make up these structures. To identify them may require the isolation of IMs or autophagosomes and their subsequent material analysis. Quite recently, *ATG31* has been characterized as a gene required for IM expansion [56].

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