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This division aims to understand the autophagy in respects to its molecular mechanism and its physiological role in yeast and higher eukaryotes. Cells execute degradation processes of their constituents together with biosynthetic processes. These two processes are well coordinated to regulate the biological activities. In other word, we must shed light on degradation process to fully understand the cell, because the study on the degradation has been retarded compared to the biosynthetic process. Autophagy is a major route for bulk degradation of cytoplasmic constituents and organelles in lysosome/vacuole, and is well conserved in eukaryotes.

I. Background

Upon nutrient starvation, autophagic process starts as building up a membrane structure, an autophagosome, in the cytoplasm. The autophagosome sequesters cytosol and organelles nonselectively. Then it is delivered to the vacuole/lysosome, and the cytoplasmic materials inside are degraded by vacuolar/lysosomal hydrolases. We had discovered autophagy in a simple model organism, *S. cerevisiae* and morphologically and genetically defined the whole process.

II. In vitro reconstitution of Atg8-PE conjugation system

In yeast, *S. cerevisiae*, Atg8 plays an important role during autophagosome formation. We have previously reported that the Atg8 is covalently attached to phosphatidylethanolamine (PE) via a ubiquitin-like conjugation system. The C-terminal Arg of newly synthesized Atg8 ($Atg8^{R117}$) is removed by Atg4 protease to expose a Gly residue at the C-terminus ($Atg8^{G116}$). The $Apg8^{G116}$ is then activated by Atg7 (E1 enzyme) and transferred to Atg3 (E2 enzyme). Following these reactions, the $Apg8^{G116}$ conjugates to PE through an amide bond between its C-terminal Gly and the amino group of PE. The subsequent deconjugation reaction by Atg4 is necessary for the normal progression of autophagy. We developed *in vitro* Atg8-PE reconstitution system. The Atg8-PE was successfully reconstituted simply with $Atg8^{G116}$, Atg7 and Atg3 by using *in vivo* in *E. coli* and *in vitro* system. These results suggested that Atg7 and Atg3 are necessary and sufficient for the Atg8-PE conjugation reaction. The *in vitro* Atg8-PE reconstitution system using recombinants and liposomes demonstrated that the efficiency of Atg8-PE conjugation was strongly affected by lipid composition. Further, the Atg8 was linked to the PE in liposomes, but not to the PE in the presence of detergent, suggesting that the lipid bilayer of membrane is essential for the Atg8-PE conjugation.

III. Molecular dissection of Atg12

Atg12 is activated by Atg7, transferred to Atg10 and attached to Atg5 in a manner similar to the ubiquitination. Although Atg12 has scarcely sequence similarity with ubiquitin, its secondary structure was predicted to have ubiquitin-like domain in the C-terminus region. We prepared N-terminally truncated Atg12 mutant of the yeast *S. cerevisiae* according to the predicted secondary structure. Truncated form of Atg12 having only a predicted ubiquitin-like domain conjugated with Atg5 was still active in autophagy. While a truncated Atg12 mutant lacking the first beta strand in the ubiquitin-like domain didn't conjugate with Atg5. These results showed that the ubiquitin-like domain of Atg12 is necessary and sufficient for conjugation and autophagy. Furthermore, we altered several hydrophobic amino acid residues in the ubiqutin-like domain of Atg8 and found a certain amino acid residue is critical for autophagy, even though still active for conjugation with Atg5.

IV. Preferential degradation by autophagy

In contrast to the ubiqutin/proteasome pathway, autophagy is thought to be non-selective protein degradation process. We surveyed the changes of protein profiles after nitrogen starvation of wild-type and Δ*atg7* cells using 2D-PAGE, and we found that cytosolic acetaldehyde dehydrogenase (Ald6p; Figure 1) was degraded more rapidly than other cytosolic proteins in an autophagy dependent manner. We observed that Ald6p was enclosed in the autophagosome and delivered to the vacuole preferentially. The Ald6p may be harmful to cells during nitrogen starvation, since disruption of *ALD6* improved the loss of viability of the Δ*atg7* mutant. This is the first report of the preferential degradation of a detrimental protein via autophagy.

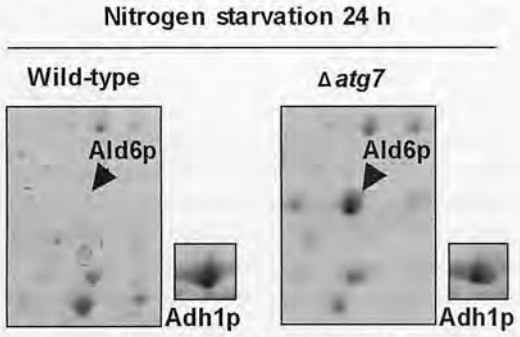


Figure 1. Ald6p was degraded by autophagy. Protein spots from 2D-PAGE using soluble lysates of nitrogen-starved cells were shown. Ald6p, cytosolic acetaldehyde dehydrogenase; Adh1p, alcohol dehydrogenase (control).

V. Involvement of early secretory pathway

In addition to Atg proteins, we have previously identified the involvement of secretory proteins in autophagy. Autophagosome formation is completely blocked when some early Sec proteins such as Sec24p, are defective, which are involved in the formation of the COPII coated vesicles from ER to Golgi. The autophagic defect in *sec24* deleted mutant cells was, however, suppressed upon the recovery of ER-Golgi secretory flow by the overexpression of its homologue, Sfb2p. We found that the autophagic defect is not observed in *sec13* and *sec31* mutants, a phenomenon that can be explained by the fact that starvation stress suppresses the secretory defect of these mutants. These observations indicate that the active flow in the early secretory pathway plays an important role in autophagy. Both autophagy and its closely related cytoplasm to vacuole-targeting (Cvt) pathway occur through a pre-autophagosomal structure (PAS), and since the PAS and the functional Cvt pathway exist in all *sec* mutants, the early secretory pathway must be involved specifically in autophagy, subsequent to PAS formation.

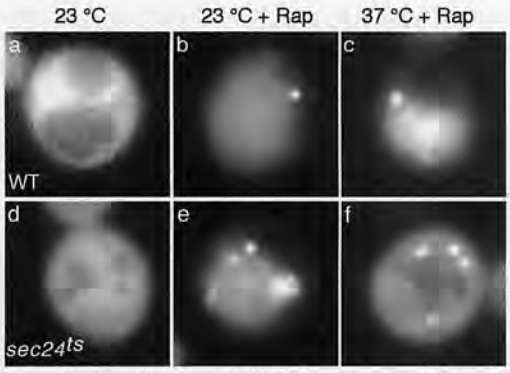
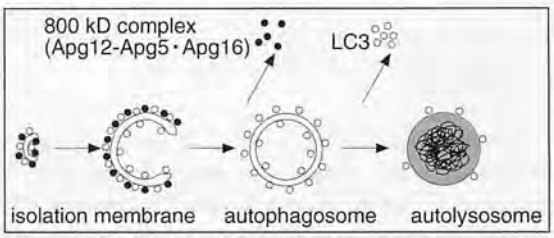


Figure 2. Localization of GFP-Atg8p in *sec24* mutant cells. Wild-type (KVY55) and *sec24* (MHY24) cells haboring pRS316 GFP-Atg8p were used. GFP-Atg8p was visualized under growing condition (a, d), starvation condition at permissive temperature (b, e) and starvation condition at non-permissive temperature (c, f).

VI. Mammalian Atg16-like protein: a novel WD-repeat protein

We have shown that, in yeast and mammalian cells, the Atg12-Atg5 protein conjugate, which is formed by a ubiquitin-like system, is essential for autophagosome formation. In yeast, the Atg12-Atg5 conjugate interacts with a small coiled-coil protein, Atg16, to form a ~350-kDa multimeric complex. We demonstrate that the mouse Atg12-Atg5 conjugate forms a ~800-kDa protein complex containing a novel WD repeat protein (Fig. 3). As the N-terminal region of this novel protein shows homology with yeast Atg16, we have designated it mouse Atg16-like protein (Atg16L). Atg16L, however, has a large C-terminal domain containing seven WD repeats, absent from yeast Atg16. Atg16L interacts with both Atg5 and additional Atg16L monomers; neither interaction, however, depends on the WD-repeat domain. In conjunction with Atg12-Atg5, Atg16L associates with the autophagic isolation membrane for the duration of autophagosome formation. As these features are similar to yeast Atg16, we concluded Atg16L is the functional counterpart of the yeast Atg16. We also found that membrane targeting of Atg16L requires Atg5, but not Atg12. As WD repeat proteins provide a platform for protein-protein interactions, the ~800-kDa complex is expected to function in autophagosome formation, further interacting with other proteins in mammalian cells.



Figgure 3. Model of autophagosome formation in mammalian cells.
The Atg12-Atg5 conjugate and Atg16L localize to the isolation membrane throughout its elongation process. LC3 is recruited to the membrane in the Atg5-dependent manner. Atg12-Atg5 and Atg16L dissociate from the membrane upon completion of autophagosome formation, while LC3 remains on the autophagosome membrane. Atg5 and its modification by Atg12 are required for elongation of the isolation membrane.

VII. Mice with a fluorescent marker for autophagy

In yeast, autophagy is required for cell survival during starvation and is necessary for spore formation. In contrast, the role of autophagy in mammals is still poorly understood. Although the possible involvement of autophagy in development, cell death and pathogenesis has been repeatedly pointed out, systematic analysis has not been performed, mainly due to a limitation of monitoring methods. Our recent studies have made available several marker proteins for autophagosomes. To understand where and when autophagy occurs in vivo, we have generated transgenic mice systemically expressing GFP fused to LC3, which is a mammalian homologue of yeast Aut7/Atg8. Cryosections of various organs were prepared and the occurrence of autophagy was examined by fluorescence microscopy (Fig. 4). Active autophagy was observed in various tissues, such as the skeletal muscle, liver, heart, exocrine glands, thymic epithelial cells, lens

epithelial cells and podocytes. Autophagy is differently induced by nutrient starvation in most tissues. In some tissues, autophagy even occurs spontaneously. Our results suggest that the regulation of autophagy is organ-dependent and the role of autophagy is not restricted to the starvation response. This transgenic mouse is a useful tool to study mammalian autophagy.

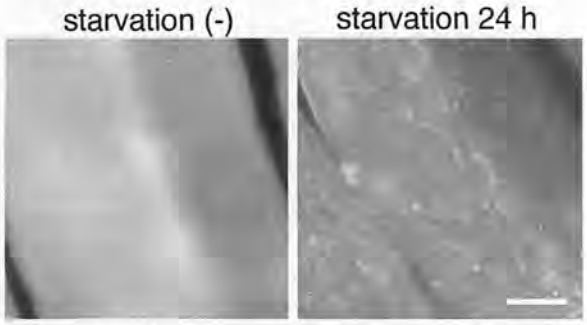


Figure 4. Muscle autophagy in response to food withdrawal. GFP images of gastrocnemius muscles at 0 h and 24 h starvation. Small dots indicate autophagosomes. Bar, 10 μm.

VIII. Monitoring of autophagic process in a whole plant

So far, autophagy in plant has been described by morphological studies. Recent genome-wide search revealed significant conservation in autophagy genes between yeast and plant. But little is known about the physiological roles and molecular mechanisms underlying autophagy in higher plants. To elucidate the plant autophagy, we focused ubiquitination-like Atg8 lipidation system. In yeast, Atg8 binds to autophagosomes and is delivered to the vacuole through autophagic process.

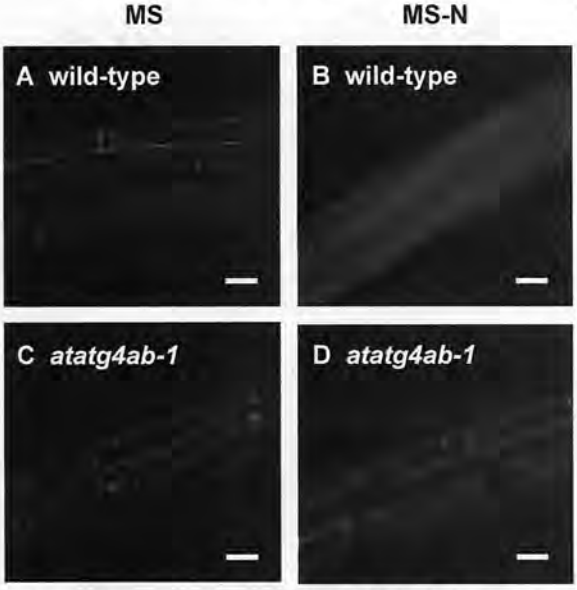


Figure 5. Localization of GFP-AtATG8a fusion protein in Arabidopsis roots. The roots of 1-week-old transgenic seedlings were grown on MS and MS-N medium. For starvation, seedlings grown on MS medium were transferred to MS-N medium for additional 7 days. (A, B) Wild-type Arabidopsis roots stably expressing GFP-AtATG8a. (C, D) *atatg4ab-1* disruption mutant roots stably expressing GFP-AtATG8a. Bar: 20 μm.

Therefore, Atg8 is a useful molecular marker for monitoring autophagic process. To establish a system monitoring autophagy in a whole plant, we generated transgenic Arabidopsis expressing GFP-AtATG8 fusion protein. In wild-type plants, GFP-AtATG8s were observed as ring structures in the cytoplasm (Figure 5 panel A), and delivered to the lumens of vacuole under nitrogen-starvation condition (Figure 5 panel B). On the basis of analogy of yeast, we regarded these ring structures as autophagosomes. In contrast, in double disruptant of *AtATG4*s which are required for C-terminal cleavage of Atg8, GFP-AtATG8s were not localized on autophagosomes (Figure 5 panel C) and were not delivered to the vacuole under nitrogen-starvation condition (Figure 5 panel D). These results indicate that AtATG8 is a suitable marker for monitoring autophagy in plant.

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