DIVISION OF MOLECULAR CELL BIOLOGY

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Most cellular activity is maintained by the balance between the synthesis and degradation of related proteins. Therefore, degradation processes as well as the regulation of gene expression play important roles in many physiological aspeets. Autophagy is a bulk degradation system for cytosolic proteins and organelles in lysosomes/vacuoles that is highly conserved in eukaryotic cells. Our division aims to understand the physiological roles and molecular mechanisms of autophagy in yeast and higher eukaryotes.

Ⅰ. Background

In response to nutrient starvation, the autophagic process starts as a building up of membrane structures called autophagosomes in the cytoplasm. The autophagosome sequesters a part of the cytosol and organelles non-selectively. It is then delivered to the vacuole/lysosome, and the cytoplasmic materials inside are degraded by vacuolar/lysosomal hydrolases. We discovered autophagy in a simple model organism, the budding yeast *Saccharomyces cerevisiae*, and have morphologically and genetically defined the whole process.

Ⅱ. Lap3 is a selective target of autophagy in yeast

Although autophagy is, in principle, a non-selective degradation process, several proteins and organelles were found to be selectively incorporated into the autophagosome. For instance, Ald6, a soluble cytoplasmic enzyme in yeast, is preferentially eliminated from the cytoplasm via autophagy; Little is known, however, about the mechanisms of Ald6 targeting to autophagosomes. Autophagy is also involved in the selective transport of Ape1, a vacuolar hydrolase, as a biosynthetic route. Recently, we discovered that leucine aminopeptidase III (Lap3), a soluble cytosolic cysteine protease, was spatially associated with Ape1 and selectively transported to the vacuole during nitrogen starvation (Figure 1). The rate of Lap3 transport was much higher than that of Ald6 and was similar to that of Ape1. Moreover, Atg11 and Atg19, essential factors for Ape1 transport, were important for Lap3 transport. Most of the Lap3 was degraded within a couple of hours in the vacuole, in contrast to Ape1; therefore, we concluded that the machinery required for Ape1 biosynthesis is used for the selective degradation of Lap3.

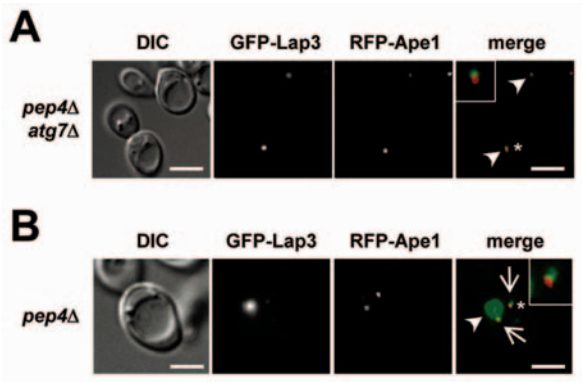

Figure 1. Microscopic analyses of Lap3. (A) Localization of GFP-Lap3 in *pep4Δ atg7Δ* (A) and *pep4Δ* (B) cells. Cells were grown in YPD media and then incubated in nitrogen starvation media (SD-N) for 3 h. The GFP and mRFP signals were observed simultaneously. Arrowheads indicate the co-localization of Lap3 and Ape1. Arrows point to intravacuolar structures. Insets are high-magnification images of dots marked with asterisks. GFP-Lap3 and mRFP-Ape1 dots do not merge, but are localized very closely to each other. Bars, 5 μm.

Ⅲ. Structural basis of cargo recognition by Atg8 homologs in selective autophagy

Atg8 homologs are ubiquitin-like proteins that localize on autophagosomal membranes and play crucial roles in the formation of the membranes. These proteins are also involved in the selective incorporation of specific cargo molecules into autophagosomes, in which Atg8 and its mammalian homolog, LC3, interact with Atg19 and p62, receptor proteins for a number of vacuolar enzymes including Ape1 and disease-related, ubiquitin-positive protein inclusions, respectively. In collaboration with Dr. Inagaki's group at Hokkaido University, we determined the structures of Atg8 and LC3 in complex with Atg19 and p62 peptides, respectively, which contain amino acid residues necessary and sufficient for complex formation. Remarkably, Atg8 and LC3 proved to interact with Atg19 and p62 in a quite similar manner, even though the entire sequences of Atg19 and p62 are unrelated to each other. Hydrophobic pockets conserved among Atg8 homologs recognized the side-chains of Trp and Leu in the WXXL motif in Atg19 and p62 (Figure 2). Mutational analyses of Atg8 and Atg19 showed that this interaction is indeed

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2008. The former title is indicated by an asterisk (*)

required for the vacuolar transport of Ape1. Thus, we revealed the fundamental interaction between receptors and Atg8 homologs by which specific cargo molecules are efficiently captured by autophagosomes.

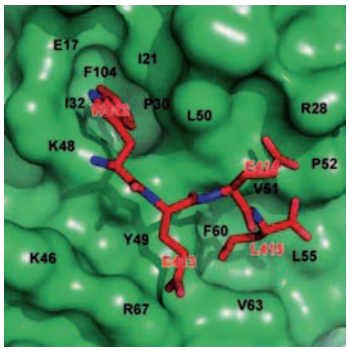

Figure 2. The structure of the interaction site of Atg8 with Atg19. The evolutionally conserved, hydrophobic binding pocket of Atg8 (surface model) accommodating a peptide corresponding to the Atg8-binding region of Atg19 (stick model) is shown.

Ⅳ. The determinants of substrate specificity in lipidation of Atg8

A ubiquitin-like system consisting of the E1 enzyme Atg7 and the E2 enzyme Atg3 mediates the conjugation of Atg8 with the lipid phosphatidylethanolamine (PE). The C-terminal carboxyl group of Atg8 is activated by Atg7, sequentially forms thioester intermediates with active site cysteine residues of Atg7 and Atg3, and eventually forms an amide bond with the amino group of PE. Probably as this lipid-modified form (Atg8–PE), Atg8 localizes to autophagosomal membranes and participates in their formation. The conjugation reaction of Atg8 can be reconstituted in vitro using purified proteins and liposomes containing PE. Whereas PE was identified as the sole in vivo target of Atg8, in vitro studies showed that the same system can mediate the conjugation of Atg8 with phosphatidylserine (PS) as efficiently as with PE. We found that, in contrast to PE conjugation, the PS conjugation of Atg8 is markedly suppressed at physiological pH (Figure 3). Furthermore, while the addition of acidic phospholipids to liposomes significantly promotes the production of Atg8–PE, this was not observed for Atg8–PS at all. Therefore, it was suggested that intracellular milieus, the pH of the cytosol and acidic phospholipids in membranes, contribute to the selective formation of Atg8–PE in the cell. We also showed that acidic phospholipids facilitate the binding of Atg8-Atg7 and Atg8-Atg3 thioester intermediates to the membrane (Figure 3).

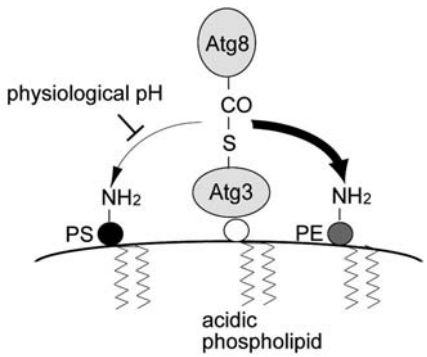

Figure 3. The neutral pH and the presence of acidic phospholipids result in the preferential formation of Atg8–PE.

Ⅴ. Amino-terminal region of Atg3 is essential for the association with PE in Atg8 lipidation

We previously showed using the in vitro system that the conjugate of the ubiquitin-like protein Atg12 with Atg5 (Atg12–Atg5) directly interacts with Atg3 and drastically stimulates its conjugating activity (transfer of Atg8 to PE). When PE-containing liposomes were absent from the reaction in the presence of Atg12–Atg5, Atg8 was mis-transferred to the serine residues in the N-terminal region of Atg3 from its active site cysteine, suggesting the involvement of the region in Atg8 lipidation. To examine the role of the N-terminal region of Atg3 in the formation of Atg8–PE, we prepared Atg3 mutants having deletions or mutations in this region. We found that the conjugating activities of the Atg3 mutants lacking N-terminal 7 amino acid residues or having the Leu-to-Asp mutation at position 6 were severely impaired both in vivo and in vitro. Furthermore, we revealed that the N-terminal region of Atg3 is responsible for the proper interaction with PE-containing membranes (Figure 4).

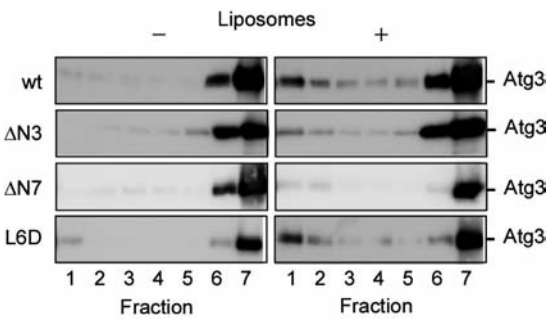

Figure 4. Floatation assay of Atg3 mutants. Purified Atg3 mutant proteins were incubated with liposomes containing PE, and then subjected to floatation assay. Fractions (fraction 1 represents the top fraction) were analyzed by western blotting with anti-Atg3 antibodies.

Ⅵ. The yeast Tor signaling pathway is involved in G2/M transition via Polo-kinase

The target of rapamycin (Tor) protein plays a central role in cell growth. Rapamycin inhibits cell growth and promotes cell cycle arrest at G1 (G0). Little is known, however, about whether or not Tor is involved in other stages of the cell division cycle. Unexpectedly, we found that the rapamycin-sensitive Tor complex 1 (TORC1) is involved in G2/M transition in *S. cerevisiae*. Strains carrying a temperature-sensitive allele of *KOG1* (*kog1-105*) encoding an essential component of TORC1, as well as yeast cells treated with rapamycin, show mitotic delay with prolonged G2 (Figure 5A). Overexpression of Cdc5, the yeast polo-like kinase,

rescues the growth defect of *kog1-105*, and in turn, Cdc5 activity is attenuated in *kog1-105* cells. The TORC1-Type2A phosphatase pathway mediates nucleocytoplasmic transport of Cdc5, which is a prerequisite for its proper localization and function (Figure 5B). The C-terminal polo-box domain of Cdc5 has an inhibitory role in nuclear translocation. Taken together, our results indicate a novel function of Tor in the regulation of cell cycle and proliferation.

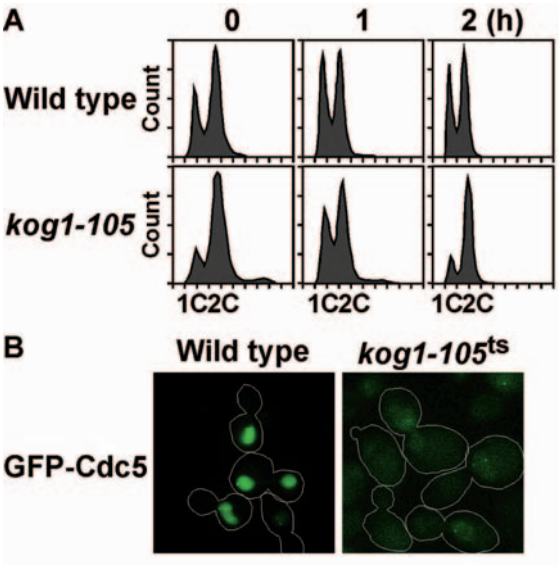

Figure 5. Tor regulates mitotic entry. (A) FACS analysis of wild type and *kog1-105*^{ts} strains. After temperature shift to 37°C, *kog1-105*^{ts} cells stop cell cycle progression with 2C DNA content, suggesting mitotic delay. (B) Localization of GFP-tagged Cdc5, the yeast polo-kinase. In wild type cells, Cdc5 localizes at the spindle pole body and the nucleus. In contrast, localization of Cdc5 is disrupted in the *kog1-105*^{ts} mutant.

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- Obara, K., Sekito, T., Niimi, K., and Ohsumi, Y. (2008). The Atg18-Atg2 complex is recruited to autophagic membranes via PtdIns(3)P and exerts an essential function. J. Biol. Chem. *283*, 23972-23980.
- Oh-oka, K., Nakatogawa, H., and Ohsumi, Y. (2008). Physiological pH and acidic phospholipids contribute to substrate specificity in lipidation of Atg8. J. Biol. Chem. *283*, 21847-21852.

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- Nakatogawa, H., Oh-oka, K., and Ohsumi, Y. (2008). Lipidation of Atg8: How is substrate specificity determined without a canonical E3 enzyme? Autophagy *4*, 911-913.
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