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

Ⅰ. Background

Upon nutrient starvation, the autophagic process starts as a building up of membrane structures called autophagosomes in the cytoplasm. The autophagosome sequesters cytosol and organelles nonselectively. 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 morphologically and genetically defined the whole process.

Ⅱ. Identification and characterization of autophagy-specific proteins, Atg17, Atg29, and Atg31

In S. *cerevisiae*, autophagy is induced under nutrient starvation. In growing conditions another autophagy-related transport, the Cvt pathway, is observed. While most of the Atg proteins are involved in both pathways, only a few Atg proteins, such as Atg17 and Atg29, are uniquely required for starvation-induced autophagy. Identification of novel autophagy-specific Atg protein(s) and their characterization are urgent issues for the elucidation of the induction mechanism of autophagy.

We identified Cis1/Atg31 as a third autophagy-unique protein. *ATG31* null mutant cells were defective in autophagy and lost viability under nitrogen-depleted conditions.

Atg29 and Atg31 localize to the pre-autophagosomal structure (PAS; a putative generation site of the autophagosome) in an *ATG17*-dependent manner (Figure 1), and Atg17-Atg29 and Atg17-Atg31 physical interactions are detected, suggesting that these three proteins make a ternary complex. We further observed that this autophagy-unique protein complex binds to Atg1, and this association is regulated by nutrient conditions. When the Cvt pathway is disrupted by deletion of *ATG11*, encoding a Cvt-specific protein, recruitment of the Atg17 complex to the PAS is stimulated by starvation. In addition, in *atg11Δ* cells, the trimeric complex is required for the PAS recruitment of other Atg proteins under starvation conditions. These findings indicate that the Atg17 complex plays an important role in the assembly of other Atg proteins to the autophagy-specific PAS to generate the autophagosome.

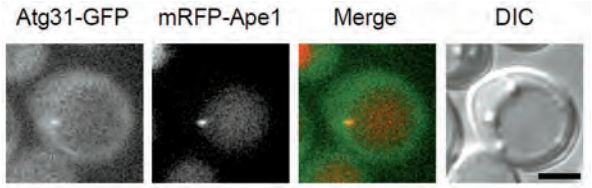

Figure 1. Atg31 localizes to the PAS. Yeast cells expressing Atg31-GFP and mRFP-Ape1 were incubated with rapamycin for 3 h. Fluorescence and DIC images are shown. Ape1 is used as a marker for the PAS. Bar: 2 μm.

Ⅲ. Functions of two ubiquitin-like conjugates in autophagosome formation

Autophagosome formation involves two ubiquitin-like proteins, Atg8 and Atg12. We showed that Atg12 forms a conjugate with another Atg protein, Atg5, whereas Atg8 is conjugated to a lipid, phosphatidylethanolamine (PE). We have reported on the functions of these conjugates using in vitro reconstitution systems consisting of purified protein components and PE-containing liposomes.

First, we showed that Atg8 mediates the tethering and hemifusion of membranes (liposomes), which are evoked by lipidation of the protein and reversibly modulated by Atg4 that catalyzes the deconjugation of Atg8-PE (Figure 2A). In addition, mutational analyses suggested that these functions of Atg8 observed *in vitro* are required for autophagosome formation *in vivo*; membrane tethering and hemifusion represent bona fide functions of Atg8. These results provide key insights into the unique membrane dynamics of autophagy, which cannot be explained by the mechanisms that have been elucidated in conventional vesicular trafficking systems.

Previous *in vivo* observations have suggested that two ubiquitin-like conjugation systems have an interrelationship:

the Atg12-Atg5 conjugate is required for effective formation of Atg8-PE. We purified the Atg12-Atg5 conjugate formed in *E. coli* and found that it dramatically accelerates the Atg8-PE conjugation reaction *in vitro*, in which transfer of Atg8 from the E2 enzyme Atg3 to PE is thought to be activated through the direct interaction between Atg12-Atg5 and Atg3 (Figure 2B). These results indicate that Atg12-Atg5 serves as a novel E3 enzyme in the ubiquitin-like conjugation reaction of Atg8. Further studies will shed light on understanding how these ubiquitin-like systems cooperate in autophagosome formation in the cell.

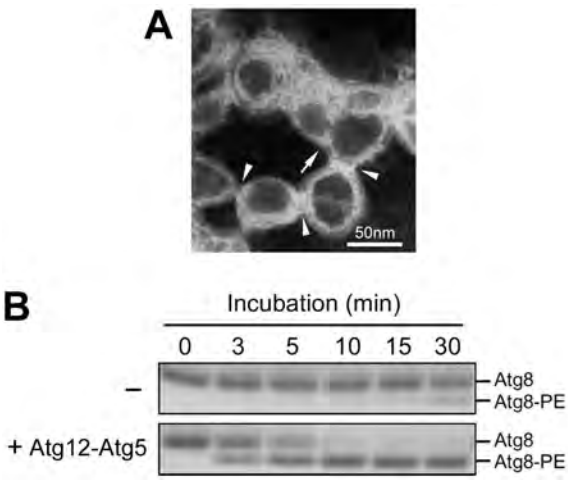

Figure 2. Functions of two ubiquitin-like protein conjugates. (A) Liposomes clustered upon in vitro production of Atg8-PE were stained with phosphotungsticacid and analyzed by electron microscopy. Arrowheads and an arrow, respectively, show junctions between liposomes and a structure suggested to represent hemifusion of liposomes. (B) In vitro conjugation reaction of Atg8-PE was performed in the presence or absence of the Atg12-Atg5 conjugate.

Ⅳ. Identification of another type of autophagy (microautophagy) under a stress condition

Our efforts to elucidate the molecular mechanism of autophagy have led to the accumulation of enormous amounts of knowledge regarding macroautophagy (macroautophagy is here simply described as autophagy). In contrast, little is known at present about the physiological functions and molecular machinery of another type of autophagic pathway, termed microautophagy (MIA). In MIA cargo components are sequestered directly by the vacuolar (lysosomal) membrane and transported into the vacuolar lumen after an inward pinch-off of the sequestering membrane (Figure 3A). In the yeast S. *cerevisiae*, MIA has been observed under some nutrient-starved conditions, but its biological significance remains unclear.

After phenotypic analyses of 18 *ATG*-gene disruptants, we found that disruptions of genes encoding a subset of phosphatidylinositol (PI) 3'-kinase complex led to severe viability defects when the cells were cultured on glycerol as a solo carbon source at 37ºC and eventually exposed to extensive oxidative stress. The frequency of the cells possessing vacuole invaginations increased under the culture condition, which was dependent on the PI 3'-kinase components necessary for the cell viability. Moreover, the product of the PI 3'-kinase, phosphatidylinositol 3'-monophosphate (PI3P), was found to be enriched at the site of vacuole invagination (Figure 3B). Subsequent morphological studies indicated that the vacuole invaginations are followed by a transport of lipid bodies into vacuole, thereby showing that MIA toward lipid bodies is induced under the stress condition.

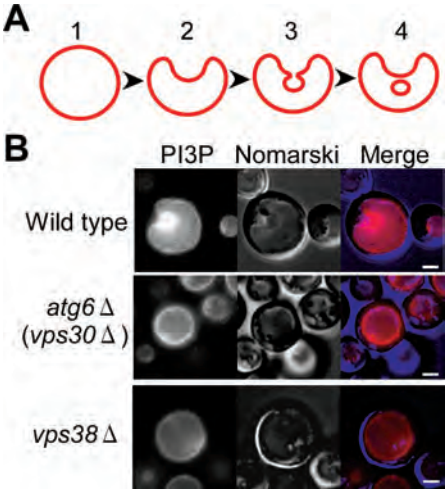

Figure 3. Membrane dynamics of MIA. (A) Schematic drawing of vacuolar membrane morphology undergoing MIA during glycerol culture at 37ºC. A part of a round vacuole (1) forms an invagination (2) dependent on some components of the PI 3'-kinase complex. Within the invagination a smaller vesicle structure emerges (3) and buds off into vacuolar lumen (4). (B) Localization of PI3P at the site of vacuole invagination. A tandem repeat of FYVE domain known to specifically bind to PI3P is expressed in fusion to monomeric RFP (mRFP). Fluorescence microscopy of the mRFP-(2x)FYVE was done for the denoted strains. Along with the mRFP signal (shown as PI3P), Nomarski images (Nomarski) and their superimposed images (Merge) are also shown. Bar, 2 μm.

Ⅴ. Lap3p is a novel cargo of selective autophagy during glycerol culture

Under growing conditions, a constitutive autophagy-like pathway, termed the Cvt (cytoplasm-to-vacuole-targeting) pathway, utilizes similar molecular machinery and vesicle structures to those of autophagy for a biosynthetic transport of aminopeptidase I (Ape1) into the vacuole. Recently, we discovered that in the cells grown on glycerol as a sole carbon source, the Cvt pathway was enhanced. Under this condition, we found that Leucyl aminopeptidase Ⅲ (Lap3), a neutral cysteine protease, was the selective cargo protein of the Cvt pathway (Figure 4A). Lap3 co-localized with Ape1 (Figure 4B), and Atg19 was involved in delivering Lap3 to the vacuole as a receptor protein. Further studies showed that a portion of Lap3 was degraded inside vacuoles, and thus suggested that the Cvt pathway might function as a degradative pathway and that Lap3 might be a cargo of the pathway.

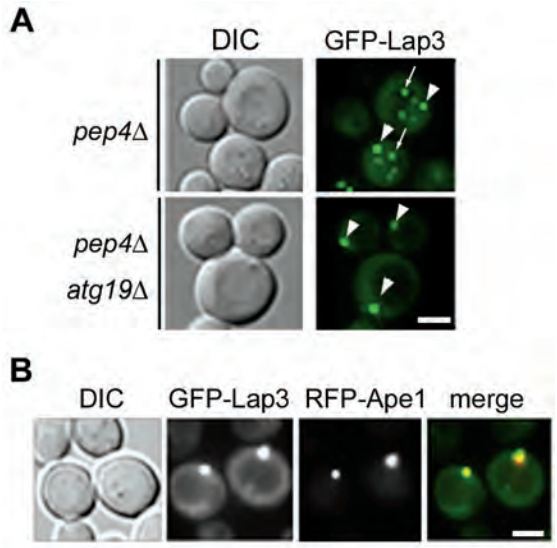

Figure 4. (A) A proteinase A deficient (*pep4*Δ) and *pep4*Δ *atg19*Δ cells expressing GFP-Lap3 were grown to logarithmic phase in the glycerol medium and examined by differential interference contrast (DIC) and fluorescence microscopy. Lap3 was selectively delivered to vacuole by the Cvt pathway. Arrows indicate the intravacuolar structures including GFP-Lap3 positive structures. Arrowheads indicate the punctate structures near vacuole. (B) Cells co-expressing GFP-Lap3 and RFP-Ape1 were examined by microscopy as described in (A). Lap3 co-localized with Ape1 near the vacuole. Bar, 4 μm.

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- Yamaguti, M., Suzuki, N.N., Fujioka, Y., Ohsumi, Y., and Inagaki, F. (2007). Crystallization and preliminary X-ray analysis of Atg10. Acta Crystallograph. Sect. F Struct. Biol. Cryst. Commun. *63*, 443-445.

〔Review article〕

- Suzuki, K., and Ohsumi, Y. (2007). Molecular machinery of autophagosome formation in yeast, *Saccharomyces cerevisiae*. FEBS Lett. *581*, 2156-2161.