DIVISION OF MOLECULAR CELL BIOLOGY

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This division aims to understand the physiological roles and molecular mechanism of autophagy in yeast and higher eukaryotes. All cellular activity is maintained by the balance between the synthesis and degradation of related proteins. It is now well known that the degradation process plays important roles in many physiological aspects. Autophagy is a bulk degradation system of cytosolic proteins and organelles in lysosome/vacuoles. Membrane dynamics during autophagy remain to be discovered.

I. Background

Upon nutrient starvation, the autophagic process starts as a building up of membrane structures (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, *Saccharomyces cerevisiae*, and morphologically and genetically defined the whole process.

II. Structural studies of Atg proteins

To understand the molecular basis of the functions of Atg proteins, structural information is crucial. For four years we have collaborated closely with Prof. F. Inagaki's lab at Hokkaido University. Our final goal is to elucidate the 3-D structures of every Atg protein, two conjugates and protein complexes involved in autophagy. We have succeeded in obtaining the crystal structures of LC3 (mammalian homolog of Atg8), human Atg4B, *Arabidopsis* Atg12 and the human Atg4B-LC3 complex, and yeast Ape1, Atg3, complex of Atg5-N-terminal Atg16 fragment, These results revealed the unique structure of Atg4 enzyme, a closed active site without binding of LC3. It was found that Atg5, the target of Atg12 conjugation, contains two ubiquitin-like domains and a helical region connecting them and Atg3, an E2-like enzyme for Atg8, has a distinct domain from other typical E2 enzymes. This structural data gives us many critical ideas to elucidate the mechanism of Atg protein functions. Atg proteins other than the two conjugation systems are not easy to crystallize, so we are now trying to determine the essential domains for their functions.

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). Although the functions of these conjugates have remained long-standing questions, we have recently made good progress on these issues using *in vitro* reconstitution systems consisting of purified protein components and PE-containing liposomes.

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

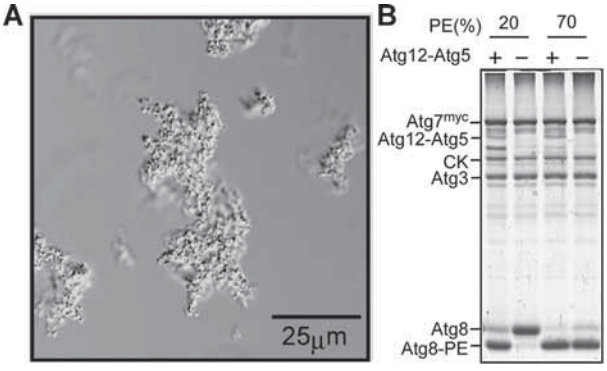

Figure 1. Functions of two ubiquitin-like protein conjugates. (A) The production of Atg8-PE *in vitro* leads to the clustering of liposomes, suggesting that Atg8-PE conjugates have an ability to tether together membranes to which they are anchored. The reaction mixture after incubation was observed under a light microscope. (B) The stimulation of the Atg8-PE conjugation reaction by the Atg12-Atg5 conjugate. If purified Atg12-Atg5 was added, Atg8 was effectively conjugated to PE even when liposomes containing a low concentration of PE (20%) were used.

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 also examined this possibility *in vitro* and found that the Atg12-Atg5 conjugate directly facilitates Atg8-PE formation, in which Atg3, an E2-like enzyme, is catalytically activated for Atg8 transfer to PE by the Atg12-Atg5 conjugate. Further studies will shed light on understanding how these ubiquitin-like systems cooperate in autophagosome formation in the cell.

IV. Assortment of phosphatidylinositol 3-kinase complexes ñ Atg14 directs association of complex I to the pre-autophagosomal structure in *S. cerevisiae* ñ

Phosphatidylinositol 3-kinase (PI3-K) is a lipid kinase which phospholyrates phosphatidylinositol to produce phosphatidylinositol 3-phosphate (PI3-P). The produced PI3-P often serves as a ìm arkî to concentrate downstream molecules to specific sites of the cell. This process is essential for autophagy and the transport of vacuolar hydrolases into the vacuole. We previously identified two distinct PI3-K complexes, complexes I and II, in yeast (Kihara et al., 2001). Complex I functions in autophagy, while complex II functions in the transport of vacuolar hydrolases. How the two complexes can function in distinct biological processes despite producing the same primary product, PI3-P, is unknown. Each complex contains a unique subunit, Atg14 for complex I and Vps38 for complex II, in addition to three common subunits shared by both complexes. We focused on the specific subunits and analyzed them in detail. Atg14 localized to the vacuolar membrane and the PAS, a possible site of autophagosome formation, whereas Vps38 localized to the vacuolar membrane and endosomes. We then monitored the localization of the other subunits and found that complex I localized to the vacuolar membrane and the PAS. The PAS-localization of complex I was entirely dependent on Atg14. In contrast, complex II localized to the vacuolar membrane and endosomes. Localization of complex II to the endosome was dependent on Vps38. From these results and other biochemical data, we proposed a model that the distinct functions of PI3-K complexes are acquired by the specific association of each complex to a distinct compartment that is mediated by the specific components (Figure 1). Next, we further dissected the key molecule, Atg14, by truncation analysis. Atg14 has three putative coiled-coil domains within the N-terminal half. Surprisingly, the region covering the first two coiled-coil domains was shown to be sufficient for the function of Atg14 in autophagy, if not fully active, which indicates that the primary role of Atg14 is protein-protein interaction. Through the protein-protein interaction at the coiled-coil domains, Atg14 was able to form functional PI3-K complex I and localize to the PAS.

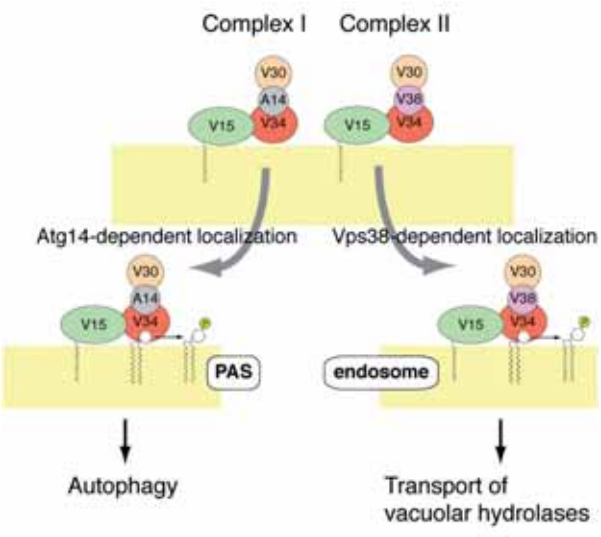

Figure 2. Function of PI3-K complexes is specified by distinct localization. PI3-K complex I localizes to the PAS in Atg14-dependent manner and functions in autophagy. In contrast, Vps38 mediates the localization of complex II to endosomes to function in sorting of vacuolar hydrolases. V30, Vps30; A14, Atg14; V34, Vps34; V15, Vps15; V38, Vps38.

V. Hierarchy of Atg proteins in pre-autophagosomal structure organization

Autophagy is a bulk degradation process that is conserved in eukaryotic cells and functions in the turnover of cytoplasmic materials and organelles. When eukaryotic cells face nutrient starvation, the autophagosome, a double-membraned organelle, is generated from the pre-autophagosomal structure (PAS). In the yeast *Saccharomyces cerevisiae*, at least 17 *ATG* (autophagy-related) genes are essential for autophagosome formation. Most of the Atg proteins are

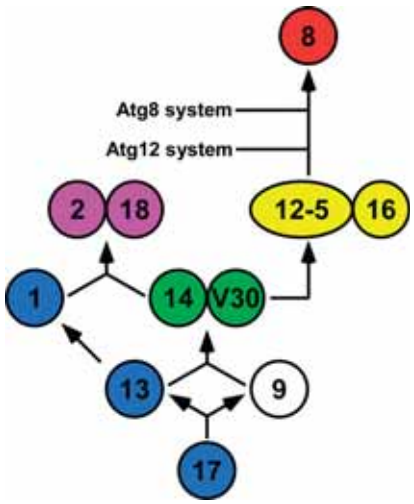

Figure 3. Hierarchy diagram of Atg proteins required for PAS organization. Tails of arrows are placed at Atg proteins that affect the PAS localization of other Atg proteins located at the heads of the arrows. Numbers: Atg proteins; V30: Vps30p/Atg6p.

localized to the PAS, leading to autophagosome production. However, the mechanism of PAS organization remains to be elucidated. Here, we performed a systematic and quantitative analysis by fluorescence microscopy to develop a hierarchy map of Atg proteins involved in PAS organization. This analysis suggests that Atg17p is the most basic protein in PAS organization: when it is specifically targeted to the plasma membrane, other Atg proteins are recruited to that location, suggesting that Atg17p acts as a scaffold protein to organize Atg proteins to the PAS.

VI. Arabidopsis Atg6/Vps30 is essential for pollen germination

Many *ATG* genes are conserved in plants and reverse genetic studies have demonstrated that Arabidopsis *atg* mutants were hypersensitive to nutrient starvation and exhibited accelerated senescence even under favorable growth conditions. In addition, we proposed that autophagy is involved in hypersensitive response cell death, an immune response of plants. In contrast to the previously characterized *AtATG* genes, all of which are fertile, we found that deletion of *AtATG6/VPS30* resulted in male sterility. Detailed microscopic observations have revealed that *AtATG6/VPS30* is essential for pollen germination (Figure 4). *AtATG6/VPS30* was able to restore vacuolar protein sorting as well as autophagy in yeast *atg6/vps30* mutant. Results have suggested that AtAtg6/Vps30 not only functions in autophagy but also has an autophagy-independent role, possibly in vesicle trafficking, which is responsible for pollen germination.

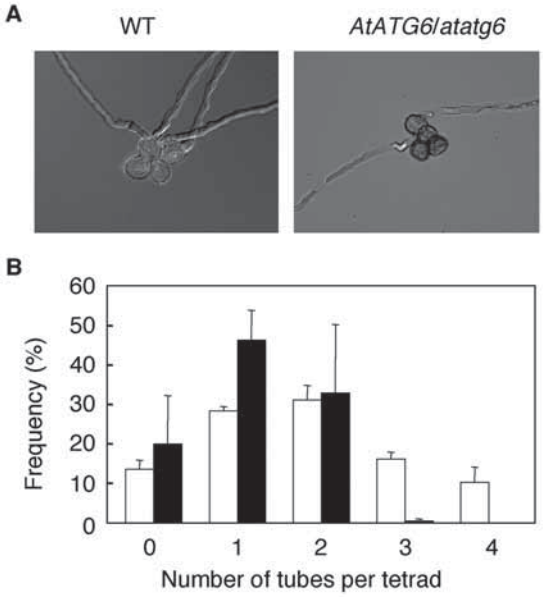

Figure 4. *AtATG6* is essential for pollen germination. (A), *In vitro* pollen germination of *AtATG6/atatg6* heterozygotes in the *qrt* background (right) and wild type (*qrt*, left). (B), Frequency (%) of wild type (*AtATG6/AtATG6/qrt/qrt*, open bars) and heterozygous mutant (*AtATG6/atatg6/qrt/qrt*, closed bars, $n > 500$) tetrads with zero to four pollen tubes. The values shown are the means of three different experiments (±SD).

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Original papers

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