DIVISION OF BIOENERGETICS

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This division aims to understand the autophagy in respects to its molecular mechanism and its physiological role in 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 bio-synthetic process. Autophagy is well conserved in eukaryotes and is a major route for bulk degradation of cytoplasmic constituents and organelles in a lytic compartment, lysosome/vacuole.

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 proteases. We had discovered autophagy in a simple eukaryotic model organism, *Saccharomyces cerevisiae* and morphologically defined the whole process. We have isolated a set of autophagy-deficient mutants (*apg*), and have cloned most of the *APG* genes essential for autophagy. We are now characterizing these gene products.

II. Apg1-Apg13 protein kinase complex mediates induction of autophagy in response to starvation signal

We previously reported that Tor protein represses induction of autophagy during growing condition, since we observed that rapamycin, a Tor-specific inhibitor, induced autophagy even under such a condition. Next question is how inhibition of Tor function leads autophagy induction. Among 15 Apg proteins Apg1p and Apg13p show close relations with Tor. Apg1p is a protein kinase, and Apg1 kinase activity is enhanced by nutrient starvation or rapamycin-treatment. This activation requires *APG13*. Apg13p is highly phosphorylated in a Tor-dependent manner. In starved or rapamycin-treated cell, Apg13p is immediately dephosphorylated. And defect of autophagy by deletion of *APG13* is rescued by overexpression of *APG1*. These suggest that Apg13p plays a key role in signal transduction from Tor to Apg1p. We found that only dephosphorylated form (but not hyper-phosphorylated form) of Apg13p associates to Apg1p under starved condition. And we demonstrated that this association confers Apg1 activation which is required for the induction of autophagy. We also identified a couple of Apg1-binding proteins Apg17p and Cvt9p suggesting that Apg1 makes a large protein complex.

III. Two Distinct Phosphatidylinositol 3-Kinase Complexes Function in Autophagy and Carboxypeptidase Y Sorting

Vps30p/Apg6p is required for both autophagy and sorting of carboxypeptidase Y (CPY). Although Vps30p is known to interact with Apg14p, its precise role has remained unclear. We found that two proteins copurify with Vps30p. By mass spectrometry they were identified to be Vps38p and Vps34p, a phosphatidylinositol (PtdIns) 3-kinase. Vps34p, Vps38p, Apg14p, and Vps15p, an activator of Vps34p, were coimmunoprecipitated with Vps30p. These results indicate that Vps30p functions as a subunit of a Vps34 PtdIns 3-kinase complex. Phenotypic analyses indicated that

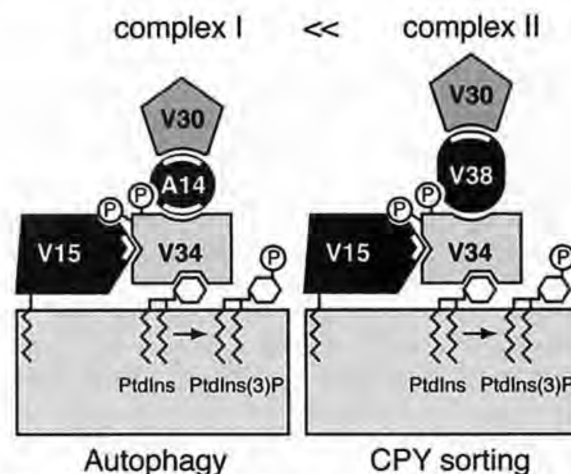

Fig.1 PI3 kinase complex is required for autophagy.
PI3 kinase(vps34) and its activator (vps15) form at least two complex. Complex I (Vps34, Vps15, Apg14, and Vps30) is essential for autophagy, and Complex II (Vps34, Vps15, Vps38, and Vps30) functions for vacuolar enzyme CPY sorting.

Apg14p and Vps38p are required for autophagy and CPY sorting, respectively, whereas Vps30p, Vps34p, and Vps15p are required for both processes. Coimmunoprecipitation using anti-Apg14p and anti-Vps38p antibodies and pull-down experiments showed that two distinct Vps34 PtdIns 3-kinase complexes exist: one, containing Vps15p, Vps30p, and Apg14p, functions in autophagy and the other containing Vps15p, Vps30p, and Vps38p functions in CPY sorting. The *vps34* and *vps15* mutants displayed additional phenotypes such as defects in transport of proteinase A and proteinase B, implying the existence of another PtdIns 3-kinase complex(es). We propose that multiple Vps34p-Vps15p complexes associated with specific regulatory proteins might fulfil their membrane trafficking events at different sites (Fig. 1).

VI. Discovery of novel lipidation reaction mediated by ubiquitin-like system

Apg8/Aut7, which plays an important role in the formation of autophagosome, tends to bind to membranes in spite of its hydrophilic nature. We showed that the mode of the association of Apg8 with membranes changes depending on a series of modifications of the protein itself. First, the carboxy-terminal Arg of newly synthesized Apg8 is removed by Apg4/Aut2, a novel cysteine protease, and a Gly residue becomes the carboxy-terminal residue of the protein that is now designated Apg8FG. Subsequently, Apg8FG forms a conjugate with an unidentified molecule "X" and thereby binds tightly to membranes. This modification requires the carboxy-terminal Gly residue of Apg8FG and Apg7, a ubiquitin E1-like enzyme. Finally, the adduct Apg8FG-X is reversed to soluble or loosely membrane-bound Apg8FG by cleavage by Apg4. The mode of action of Apg4, which cleaves both newly synthesized Apg8 and Apg8FG-X, resembles that of deubiquitinating enzymes.

We then succeeded to identify molecule X and discovred a novel mode of protein lipidation. Apg8 is covalently conjugated to phosphatidylethanolamine (PE) through an amide bond between the C-terminal glycine and the amino group of PE. This lipidation is mediated by a ubiquitination-like system (Fig. 2). Apg8 is a ubiquitin-like protein that is activated by an E1 protein, Apg7, and is transferred subsequently to an E2 enzyme Apg3/Aut1. Apg7 activates two different ubiquitin-like proteins, Apg12 and Apg8, and assigns them to specific E2 enzymes, Apg10 and Apg3, respectively. This reversible lipidation of Apg8 appears to be coupled to the membrane dynamics of autophagy and the Cvt pathway.

V. Mammalian Apg proteins; localization and function

Autophagy was first described in mammalian cells in the 1960s and its morphology and regulation have been extensively investigated. However, molecular mechanism underlying its process is poorly understood. Recent studies on yeast Apg proteins revealed that

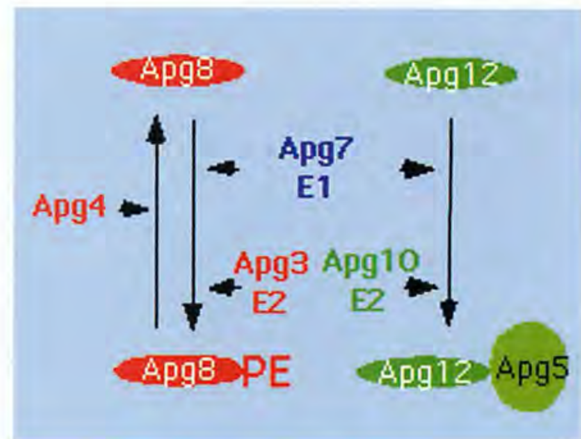

Fig.2 Two Ubiquitin-like systems essential for autophagy
Apg12 and Apg8 are activated by common El enzyme, Apg7. Then they are assigned to different conjugating enzymes, Apg10 and Apg3, respectively. Finally Apg12 forms a conjugate with a single target molecule, Apg5. Apg8 conjugate with a membrane phospholipid, phosphatidylethanolamine (PE). Apg8-PE is reversed to Apg8 by Apg4.

molecular machinery of autophagy seems highly conserved among eukaryotes. We are analyzing the mammalian orthologues of Apg proteins.

Apg8 has at least three mammalian homologues, one of which, LC3, was identified to be involved in autophagy. Newly synthesized rat LC3, a 142 amino acid protein, was immediately processed to remove the C-terminal 22 amino acids to generate the LC3-I form, which was further converted to the LC3-II form. While LC3-I was cytosolic, LC3-II was membrane bound and enriched in the autophagic vacuole fraction (Figure 3A and B). LC3-II may correspond to yeast Apg8-PE (the PE-conjugated form of Apg8). Immunoelectron microscopy revealed LC3 was present both inside and outside of autophagosomes in addition to cytoplasm. LC3 was less detected on autolysosome, suggesting that some LC3 dissociated from the membrane after autophagosome formation (Figure 3B). Consistent with such localization, the amount of LC3-II was correlated with the extent of the autophagosome formation. Although LC3 was originally identified as microtubule-associated protein 1 light chain 3, involvement of microtuble in LC3 function and autophagy has remained to be clarified. LC3-II is the first mammalian protein identified that specifically associates with the autophagosome membranes.

The Apg12-Apg5 protein conjugation system is another well conserved machinery. Most mammalian Apg5 was present in the Apg12-conjugated form in the cytoplasm. However, a small fraction of the Apg12-Apg5 conjugate localized to the isolation membranes when autophagy proceeds (Figure 3A and B). Using GFP-tagged Apg5, we revealed that the cup-shaped isolation membrane is developed from a small crescent-shaped compartment. These small structures have never been recognized unless labeled with Apg5, but they are

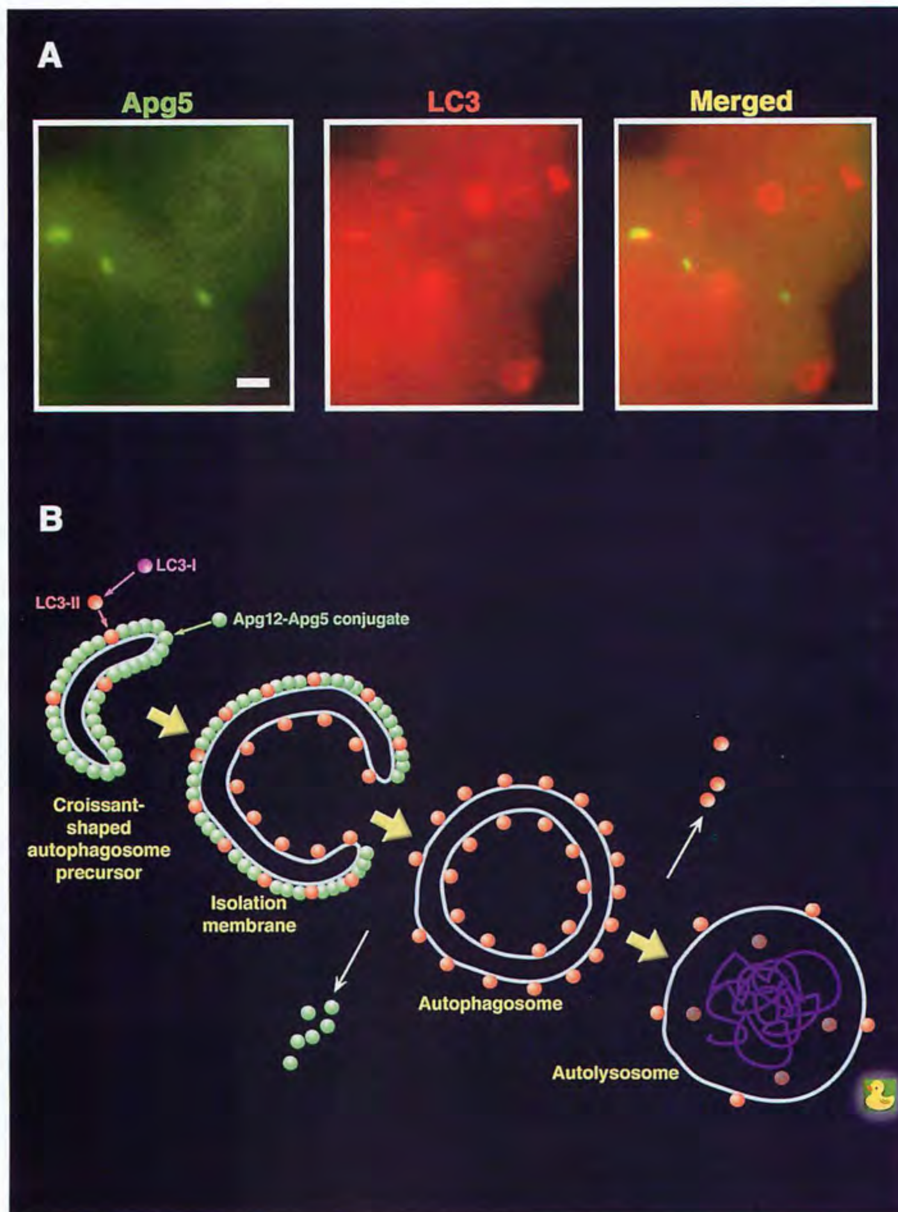

Fig.3.
A. Distribution of Apg5 and LC3 in mouse embryonic stem cells.
YFP-LC3 (middle panel) localizes to spherical autophagosomes and semi-spherical isolation membranes, whereas CFP-Apg5 (left panel) localizes to only isolation membranes. Bar, 2 µm.

B. Model of dynamic association of Apg12-Apg5 and LC3 with autophagic membranes.
Apg12-Apg5 conjugate localizes to the crescent-shaped autophagosome precursors. While these structures elongate and maturate into cup-shaped isolation membranes, cytosolic LC3-I is recruited to the membrane in the Apg12-Apg5-dependent manner and accumulated as the LC3-II form. Apg5 biases its localization to the outer side of the membrane. Apg5 plays an essential role in this membrane development. Immediately before or after the completion of autophagosome formation, Apg5 detaches from the membrane. Some LC3 also dissociate from the autophagosomal membrane thereafter.

likely to be direct precursors of the autophagosome. Apg5 localized on the isolation membrane throughout its elongation process. Apg5 was preferentially distributed in the outer side of the membrane and detaches from it immediately before or after autophagosome formation is completed (Figure 3B). To examine the role of Apg5, we generated Apg5-deficient ES cells. *APG5* $^{-/-}$ cells are viable but bulk protein degradation was significantly reduced. Morphological analysis revealed that autophagosome formation was impaired in *APG5* $^{-/-}$ cells. We also showed that the covalent modification of Apg5 with Apg12 was not required for its membrane targeting but is essential for involvement of Apg5 in elongation of the isolation membranes. Intriguingly, Apg12-Apg5 was required for targeting of LC3 to the isolation membranes and LC3-II generation.

Therefore, the Apg12-Apg5 conjugate plays essential roles in isolation membrane development in cooperation with LC3. In addition, our studies provided good molecular markers, LC3 and Apg12-Apg5, for autophagic membrane at all stages and isolation membranes, respectively, which so far have been defined only by morphology.

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