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 multicellular organisms. 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 well conserved in eukaryotes and is a major route for bulk degradation of cytoplasmic constituents and organelles.

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 already isolated a set of autophagy-deficient mutants (*apg*), and have cloned most of *APG* genes essential for autophagy. We are now characterizing these gene products.

II. Apg10 is a unique protein conjugating enzyme

Last year, we reported on novel protein conjugation system required for autophagy. Apg12, one of Apg proteins, is terminated with glycine at its C-terminus. The C-terminal glycine is bound to a lysine residue of Apg5, another Apg protein, via an isopeptide bond. This conjugation reaction is distinct from but quite similar to ubiquitination and the other related reaction such as SUMO-1 and Rub1 system. All of these ubiquitination-like (Ubl) reactions are catalyzed by a series of enzyme system.

First, C-terminal glycine of Ubl protein is activated by binding to active center of E1-like enzyme via a thioester bond. E1-like enzymes are ATPase which share sequence similarity each other, and the ATPase activity is prerequisite for the reaction. Collaborating with Dr. Kominami's laboratory(Juntendo Med. Univ.), we have reported that Apg7 is an E1-like enzyme involved in Apg12 conjugation reaction.

Next, C-terminal glycine of Ubl protein is conjugated to the active center of E2-like enzyme via thioester bond. We characterized Apg10, which is required for conjugation reaction of Apg12. Apg10 binds to Apg12 depending on Apg7 function. We identified active center, Cys residue, of Apg10. C-terminal Gly of Apg12 bind to this Cys via a thioester bond. The active Apg10 is required for Apg12-Apg5 conjugation, and necessary for autophagy. Most notably, all previously known E2-like enzymes show sequence similarity each other, however Apg10 does not show the similarity to any known E2 enzymes. Apg10 is a quite unique E2-like enzyme. These findings prompted us the idea that Ubl conjugation system is more prevailing than ever expected from sequence similarity.

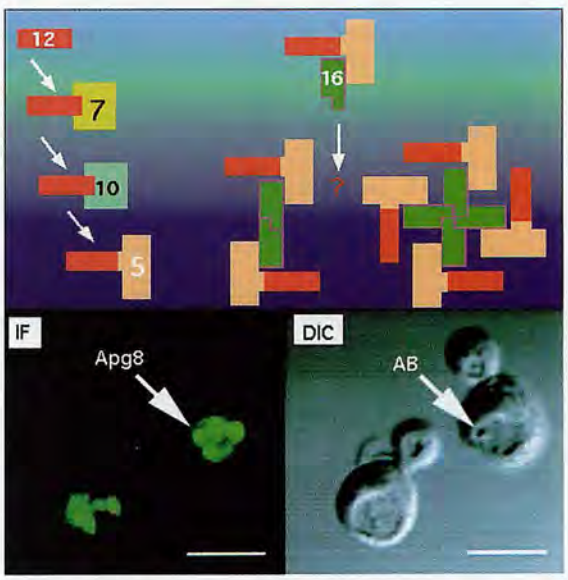

Figure 1 Molecular apparatuses for autophagy in yeast. **Upper Panel**: The diagram of Apg12 system. The numerals in the boxes denotes the corresponding Apg proteins. The number of each subunits in the final complex is still not determined. Arrow related to Apg16 does not necessarily represent the order of the events, rather do show the concept of the organization. **Lower Panel:** Immunofluorescence (Left) and Nomarsky Images (Right) of Apg8 localization. The vacuolar protease deficient mutants were starved and the tagged Apg8 is observed as the FITC signal (the arrow in left panel). Autophagic body is indicated by the arrow in the right panel

III. Apg16 crosslinks multiple Apg12-Apg5 conjugates

Despite these findings of novel Ubl system, the mechanism how the system is involved in autophagic mechanism is still to be determined. However, an accessory part related to this system, which will aid us understanding the point, was discovered.

In a two-hybrid screen with Apg12 as a bait, we identified a novel protein, Apg16, which is required for autophagy. Thereafter, we noticed that the Apg16 is not bound to Apg12 but Apg12-Apg5 conjugate. Moreover, Apg16 is bound to Apg5 alone, but more preferentially to Apg12-Apg5 conjugate. The binding occurs at the N-terminal region of Apg16. On the other hand, Apg16 have a coiled coil domain at its C-terminus, which generally functions for protein interaction. Actually Apg16 forms an homooligomer via the coiled coil domain. In accordance with this scheme, two or more independent Apg12-Apg5 conjugates were shown to be cross-linked via Apg16. Hence, this hetero-oligomeric large protein complex may play a role in autophagic process.

IV. Apg8 as a tracer of membrane flow in autophagosome formation

Autophagosome is a double membrane structure. The outer membrane fuses with the vacuolar membrane, and the inner membrane structure is released into the vacuolar lumen. The released membrane structure, autophagic body, is disintegrated depending on the vacuolar protease activity, and the cytosolic materials inside are degraded. Therefore, when vacuolar protease activity is blocked, autophagic bodies are accumulated inside the vacuole.

Apg8, one of Apg proteins, is transcriptionally upregulated upon starvation via Tor-kinase dependent signaling pathway, which controls induction of autophagy. Indirect immunofluorescence study showed that during a nutrient-rich growing phase, Apg8 is mostly dispersed throughout the cytoplasm as tiny dot structures. Upon starvation, Apg8-positive structure appears adjacent to the vacuole, which represents autophagosome or its intermediate. When vacuolar protease activity is blocked, Apg8 was observed to be accumulated inside autophagic bodies. According to immuno EM analysis in collaboration with Dr. M. Baba (Japan Women's Univ.), Apg8 is mostly enriched on the membrane of forming intermediate of autophagosome. Upon completion of the formation, Apg8 is detached from the membrane and some are released to the cytosol and the others are entrapped within the autophagosome. Thus, Apg8 could be a useful tracer for membrane flow in autophagosome formation. More detailed EM observation clarified that Apg8-positive electron lucent small structures are gathering around forming autophagosome. We postulate that these structures are one of the precursors of autophagosomal membrane. If this is the case, formation process of autophagosome is accompanied with multiple fusion of small precursor structures to the

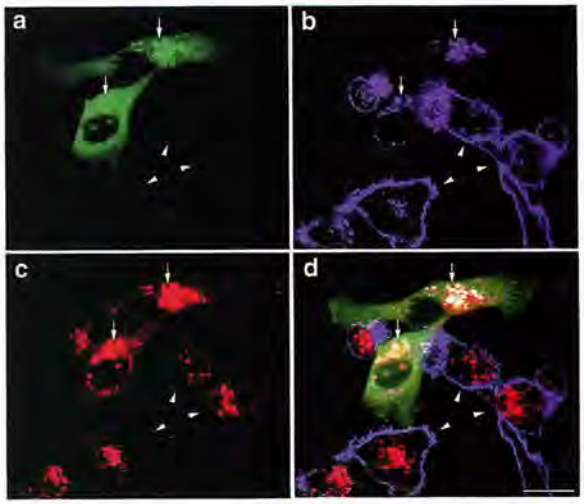

Figure 2. TfR and the endocytosed dextran are accumulated in the compartments where the mutant SKD1 is localized. HeLa cells transfected with the mutant SKD1 fused with GFP were incubated in the presence of 1 mg/ml TRITC-dextran at 37 °C for 8 hours. The cells were then fixed, permeabilized, and subjected to immunofluorescence confocal microscopy for TfR. GFP-mutant SKD1 labeling (a), TfR staining (b), TRITC-dextran labeling (c), and a merged image (d) of a same field are shown. Arrows heads and arrows indicate the untransfected cells and the mutant SKD1-positive compartments in the transfected cells, respectively. Bar, 20 μm.

intermediate structure. This contradicts to the generally accepted scheme that autophagosome is formed by engulfing of the preexisting large membrane structure, such as ER. This hypothesis will be tested in our ongoing project.

V. SKD1 regulates morphology of endosomes and membrane traffic through them in mammalian cells

We also engage in the study of mammalian to pursue cell biological and physiological issues of autophagy. In the course of the study, we encountered an interesting molecule, which regulates membrane dynamics in endosomal system.

The mouse SKD1 is an AAA-type ATPase homologous to the yeast Vps4p/ Csc1p implicated in both autophagy and transport from endosomes to the vacuole. To elucidate a possible role of SKD1 in mammalian endocytosis, we generated a mutant SKD1, harbouring a mutation (E235Q) that is equivalent to the dominant negative mutation (E233Q) in Vps4p/Csc1p. Overexpression of the mutant SKD1 in cultured mammalian cells caused defect in uptake of transferrin and low-density lipoprotein. This was due to loss of their receptors from the cell surface. The decrease of the surface transferrin receptor (TfR) was correlated with expression levels of the mutant protein. The mutant protein displayed a perinuclear punctate distribution in contrast to a diffuse pattern of the wild type SKD1. TfR, the lysosomal protein lamp-1, endocytosed dextran and epidermal growth factor (EGF), but not markers for the secretory pathway were accumulated in the mutant

SKD1-localized compartments. Degradation of EGF was inhibited. Electron microscopy revealed that the compartments were exaggerated multivesicular vacuoles with numerous tubulo-vesicular extensions containing TfR and an endocytosed horseradish peroxidase. The early endosome antigen EEA1 was also redistributed to these aberrant membranes. Taken together, our findings suggest that SKD1 regulates morphology of endosomes and membrane traffic through them.

Publication List:

Original Articles

Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y. and Kominami, E. (1999). Apg7p/Cvt2p: A novel protein-activating enzyme essential for autophagy. *Mol Biol Cell.* **10**, 1367-1379.

Shintani, T., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T. and Ohsumi, Y. (1999). Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. *EMBO J.* **18**, 5234-5241.

Mizushima, N., Noda, T. and Ohsumi, Y. (1999). Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. *EMBO J.* **18**, 3888-3896.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T. and Ohsumi, Y. (1999). Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J Cell Biol.* **147**, 435-446.

Tagawa, Y., Yamamoto, A., Yoshimori, T., Masaki, R., Omori, K., Himeno, M., Inoue, K. and Tashiro, Y. (1999). A 60 kDa plasma membrane protein changes its localization to autophagosome and autolysosome membranes during induction of autophagy in rat hepatoma cell line, H-4-II-E cells. *Cell Struct Funct.* **24**, 59-70.

Yabe, I., Horiuchi, K., Nakahara, K., Hiyama, T., Yamanaka, T., Wang, P. C., Toda, K., Hirata, A., Ohsumi, Y., Hirata, R., Anraku, Y. and Kusaka, I. (1999). Patch clamp studies on V-type ATPase of vacuolar membrane of haploid *Saccharomyces cerevisiae*. Preparation and utilization of a giant cell containing a giant vacuole. *J Biol Chem.* **274**, 34903-34910.

Noda T., Kim J., Huang WP., Baba M., Tokunaga C., Ohsumi Y., and Klionsky D.J., (2000) Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the cvt and autophagy pathways. *J Cell Biol.l* **148**, 465-480

Yoshimori T., Yamagata F., Yamamoto A., Mizushima N., Kabeya Y., Nara A., Miwako I., Ohashi M., Ohsumi M., Ohsumi Y., (2000) The mouse SKD1, a homologue of yeast Vps4p, is required for normal endosomal trafficking and morphology in mammalian cells. *Mol Biol Cell.* **11**, 747-763

George, M.D., Baba M., Scott, S.V., Mizushima N., Garrison B., Ohsumi Y., Klionsky D.J., (2000) Apg5p functions in the sequestration step in the cytoplasm to vacuole targeting and macroautophagy pathways. *Mol Biol Cell.* **in press**

Furukawa, K. Mizushima, N., Noda, T., Ohsumi, Y., (2000) A protein conjugation system in yeast with homology to biosynthtic enzyme reaction of prokaryotes. *J Biol Chem.* **275**, 7462-7465

Review Articles

Klionsky, D. J. and Ohsumi, Y. (1999). Vacuolar import of proteins and organelles from the cytoplasm. *Annu Rev Cell Dev Biol.* **15**, 1-32.

Ohsumi, Y. (1999). Molecular mechanism of autophagy in yeast, *Saccharomyces cerevisiae. Philos Trans R. Soc. Lond, B* **354**, 1577-1580; discussion 1580-1581.

Ohsumi, Y. (1999). Pictures in cell biology. Autophagosomes in yeast. *Trends Cell Biol.* **9**, 162.