

DIVISION OF BIOENERGETICS

Professor:	Yoshinori Ohsumi
Associate Professor:	Tamotsu Yoshimori
Research Associate:	Takeshi Noda Yoshiaki Kamada
Post Doctoral Fellow:	Noboru Mizushima ¹ Takahiro Shintani ² (from April) Naotada Ishihara ¹ (from July)
Graduate Students:	Takayoshi Kirisako (Graduate Univ. of Advanced Studies) Yoshinobu Ichimura (Graduate Univ. of Advanced Studies)
Technical Staff:	Yukiko Kabeya
Visiting Fellow:	Fumi Yamagata (Teikyo Univ. of Sci. and Tech.)
Visiting Scientist:	Kazuaki Furukawa (from November) Satsuki Okamoto

This division aims to elucidate the physiological role and mechanism of intracellular protein degradation in a lytic compartment. Up to recently, molecular biological studies on the topic of cellular regulation have focused on protein synthesis, and (the control of) gene expression. However, it is clear that cellular activity is maintained by a delicate balance between synthesis and breakdown of individual proteins. For the last few years, protein degradation has become a hot field in cell biology, because it plays critical roles in many aspects of cellular functions. Most of the selective protein degradation is believed to occur in the cytosol through the ubiquitin/proteasome system. In contrast, major bulk protein degradation takes place in the lysosome/vacuole. Non-specific protein turnover has essential roles under nutrient stress or for cell differentiation of eukaryotic cells. Until now, the molecular details of protein degradation in the lytic compartment remained to be uncovered due to the lack of a sensitive monitoring system, and the dynamic features of the lysosomal/vacuolar membrane system.

Autophagy is defined as the process of bulk degradation of cytoplasmic proteins or organelles in the lytic compartment and seems to be a ubiquitous and basic cellular activity in all eukaryotic cell types. Autophagy has been described mostly in higher eukaryotic cells by using electron microscopy. The lysosome/vacuole is a single membrane bound compartment containing various hydrolytic enzymes. Segregation of lytic enzymes from the cytosol requires an obvious cell biological process how to deliver proteins or organelles destined for degradation into the lumen of this compartment. Macroautophagy is the major route to the lysosome/vacuole under nutrient starvation conditions. This process involves the formation of a double membrane structure called an autophagosome (AP). The AP fuses with a primary lysosome and matures to be an autophagolysosome, then its contents are digested and recycled to be reused.

Autophagy has been studied extensively using various kinds of mammalian tissues and cultured cells. It is highly regulated by various factors such as amino

acid or metabolites, hormones and growth factors, and second messengers. Because the modes of autophagy are so diverse and the physiological role and regulation of autophagy depend upon the cell type and physiological demands, it is difficult to present a general model for autophagy in higher eukaryotes. For this reason, it has been helpful to develop a simple system to elucidate the fundamental machinery of the autophagic process.

Yeast Induces Autophagy as Mammalian Cells

Recently we discovered yeast, *S. cerevisiae*, induces bulk protein turnover in the vacuoles under starvation conditions. This whole process corresponds to that of macroautophagy in higher eukaryotic cells. By electron microscopic analyses we succeeded in detecting double membrane structures in the cytoplasm enclosing a portion of cytosol. This autophagosome immediately fuses with vacuoles, delivering single inner membrane structures, autophagic body (AB) in the vacuole. When vacuolar proteinase activities are blocked genetically or by specific inhibitor ABs are accumulated in the vacuoles. They move around vigorously in the vacuoles by Brownian motion, and are easily detectable by light microscope. Thus we can follow the progression of autophagy as the accumulation of ABs in real time. Biochemical and immunoelectron microscopic analyses showed that starvation-induced sequestration is non-selective, that is, cytosolic enzymes and organelles are sequestered randomly to the vacuoles to be degraded.

Autophagy in yeast is a kind of general stress response to adverse environmental condition and is induced by various starvation conditions such as nitrogen, carbon, sulfate, and even amino acid depletion. Signal transduction of starvation for induction of autophagy is a key question to be solved. Recently we found rapamycin induces autophagy in the cell growing in a rich medium and that Tor, phosphatidylinositol kinase homologue plays essential role for the regulation of autophagy.

Another crucial and controversial question is the mechanism of formation of autophagosome. Still nothing is clear about the origin of the membrane. Freeze-fracture electron microscopy showed that autophagosomal membrane has quite characteristic feature and the density of intramembrane particles is extremely low as compared with other intracellular membrane.

Genetical dissection of autophagic process

Yeast has made great contribution to solve many fundamental problems in cell biology because of tractability of genetic and molecular biological techniques. In order to dissect the complex process of autophagy to its elementary steps we started to isolate mutants defective in the process of autophagy. Total 14 autophagy defective *agp* (Autophagy) mutants

were isolated. They cannot induce protein degradation upon shift from growth medium to the starvation medium. They grow normally in a rich medium, but start to die after 2 days in the starvation medium. This suggests that the autophagy is necessary for the maintenance of cell viability under starvation. Homozygous diploid of each *apg* mutant cannot undergo sporulation, indicating that this cell differentiation requires bulk protein degradation via autophagy.

APG genes are essential for Cvt pathway

One of vacuolar enzymes, aminopeptidase I, API is synthesized as a proform and sequestered to the vacuole directly from cytosol to the vacuole. All *apg* genes are necessary for this process. Electron microscopic analyses revealed that API first forms a complex in the cytosol and delivered to the vacuole by the distinct but topologically similar mechanism to the macroautophagy. Though this Cvt pathway is biosynthetic and constitutive, and kinetically quite different from the autophagy, but it must share most machinery with the autophagy.

Analyses of APG genes

Now we are focusing on characterization of *APG* genes, essential for autophagy. So far we have finished cloning and identification of twelve *APG* genes.

APG1 codes a novel Ser/Thr protein kinase essential for the induction of autophagy. This provides the first direct evidence for involvement of protein phosphorylation in the process of autophagy. Apg13p interacts with Apg1p and might function as a regulator of its kinase activity. So far most *APG* genes are novel except *APG6* and non-essential for vegetative growth. The *APG6* gene is allelic to *VPS30*, which is required for retrieval of the carboxypeptidase, CPY receptor to the late Golgi from the endosome. *APG14* encodes a novel hydrophilic protein of 40.5kDa. Subcellular fractionation experiments indicate that both Apg14p and Apg6p are peripheral membrane proteins and form a stable complex. The *apg14* mutant is normal for CPY sorting. While overexpression of Apg14p suppresses the autophagy defect of *apg6-1*, *APG14* does not function as a multicopy suppressor of the CPY sorting defect of this mutant. These results suggest that Apg6p has dual functions in autophagy and vacuolar protein sorting, and that Apg14p is specifically required for the autophagic process.

Using the assay system of autophagy we developed, we are studying genetic interaction among *APG* genes. Some *APG* gene products are starvation-inducible or change phosphorylation state by nutrient starvation. Analyses of intracellular localization of these Apgps may provide us specific marker to elucidate the membrane dynamics during autophagy.

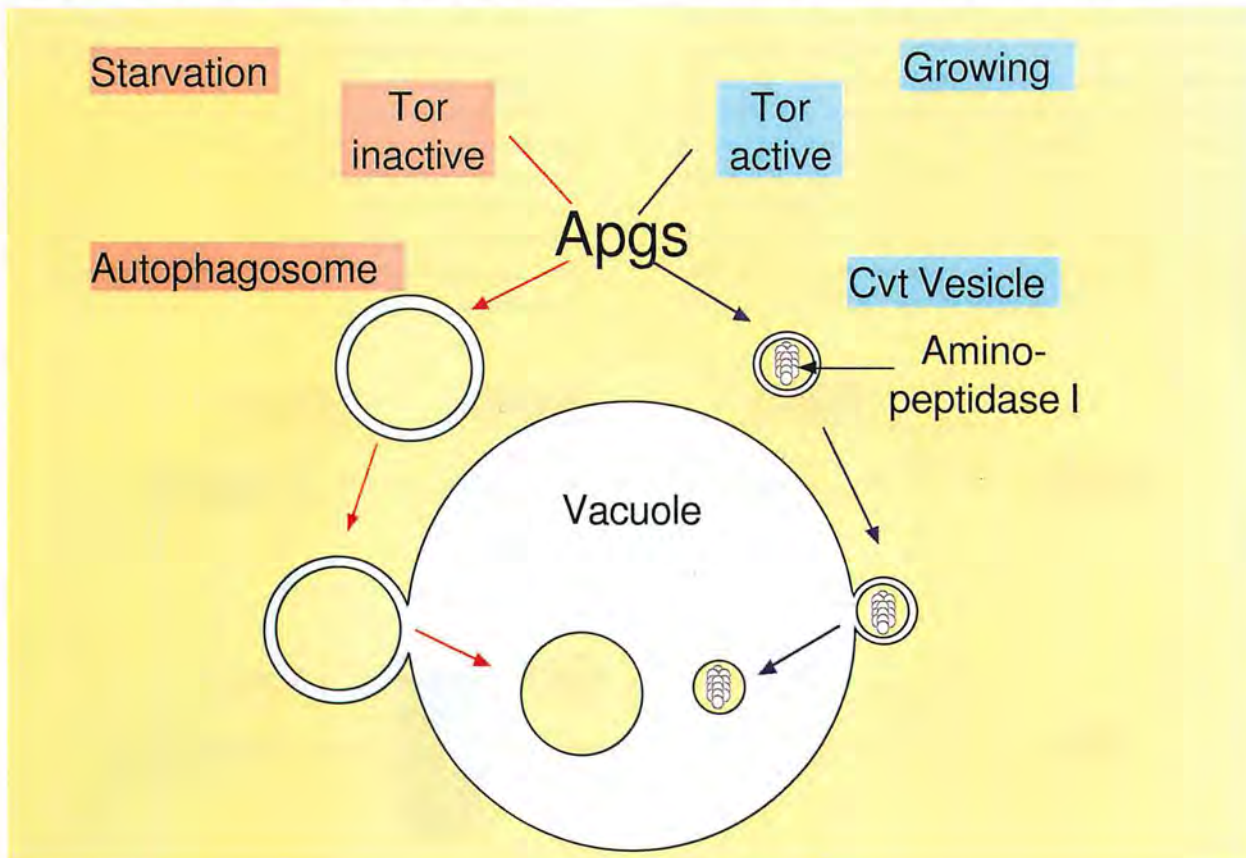


Fig. 1 Schema of autophagy in yeast. When yeast cells face to starvation, isolation membrane starts to enclose a portion of cytosol and form an autophagosome. Autophagosome fuses to the vacuolar membrane, delivering autophagic body in the vacuoles (Autophagic pathway). Under growing condition one of vacuolar enzymes, API is selectively sequestered to the vacuole (Cvt pathway). Two distinct pathways share most molecular machinery.

Novel protein conjugation system essential for autophagy

Recently we found that four *APG* genes are involved in a novel protein conjugation system essential for autophagy and the Cvt pathway. Apg12p is a 21kDa hydrophilic protein. Western blot analysis of HA-tagged Apg12p shows an additional band at 70kDa in addition to a band with the expected molecular mass. Sequence analysis of *APG5* shows that it encodes a hydrophilic protein of 34kDa. A western blot of Apg5p also shows the 70kDa band, suggesting covalent binding between Apg5p and Apg12p. Elimination of a single Gly at the C-terminus of Apg12p results in a complete loss of the higher molecular mass band. Similarly, substitution of Lys 149 of Apg5p, among 19 Lys residues, with Arg also abolishes this band. These results strongly suggest that the C-terminal Gly of Apg12p is conjugated to Lys149 of Apg5p via an isopeptide bond. Cells expressing these mutant forms of Apg12p or Apg5p are defective in autophagy and also Cvt pathway, indicating that the conjugate formation of Apg5p-Apg12p is essential for both pathways.

Furthermore, among the *apg* mutants, *apg7* and *apg10*, could not form the Apg5p-Apg12p conjugate. These two genes may encode enzymes required for the conjugate formation. From sequence data, Apg7p is predicted to be an E1 like activating enzyme for Apg12p. Recently, many ubiquitin-related molecules have been identified, and shown to function in various regulatory steps. However, Apg12p has no homology with ubiquitin or ubiquitin-related molecules. Apg12p is much larger than ubiquitin and seems to have a single target, Apg5p. The Apg5p-Apg12p conjugation reaction was reconstituted in vitro and shown to be ATP-dependent. Similarly, mutations in the ATP-binding site of Apg7p block conjugate formation. The function of this conjugate in the Apg and Cvt path-

ways is not known yet but that the conjugation may be necessary for autophagosome or Cvt vesicle formation.

Approach to mammalian autophagy

Then, there is no reason for us hesitating to challenge mammalian autophagy again, by taking advantage of identified genes involved in yeast autophagy. By searching databases, we have found several mammalian cDNA related to the yeast genes. Homologues of Apg12p and Apg5p are widely distributed from yeast to higher organisms. We cloned recently the human homologues of Apg12p and Apg5p and demonstrated a similar conjugation reaction in mammalian cultured cells. This result strongly suggests that the novel conjugation system is conserved in all eukaryotes. To elucidate roles of autophagy in development, differentiation, physiology, pathology in a multi-cellular system, we are making Apg12-deficient mice.

We also found that one of the Apgp homologues is specifically associated to the autophagosome membrane in human cultured cells. Post-translational processing of the molecule regulates the association. This is the first identified protein that is localized in mammalian autophagosomes. Yeast Csc1p is an AAA-type ATPase implicated in regulation of autophagy. We showed evidences that mouse SKD1 protein homologous to Csc1p plays a pivotal role on membrane transport via early endosomes in human cultured cells. This suggests involvement of endosomal system in the autophagic process.

Knowledge in yeast must give us key to uncover the mechanism of autophagy in higher eukaryotic organisms such as mammals. We are now developing an assay for monitoring autophagy in mammalian cultured cells to perform systematic evaluation of identified candidates for components in machinery of mammalian autophagy.

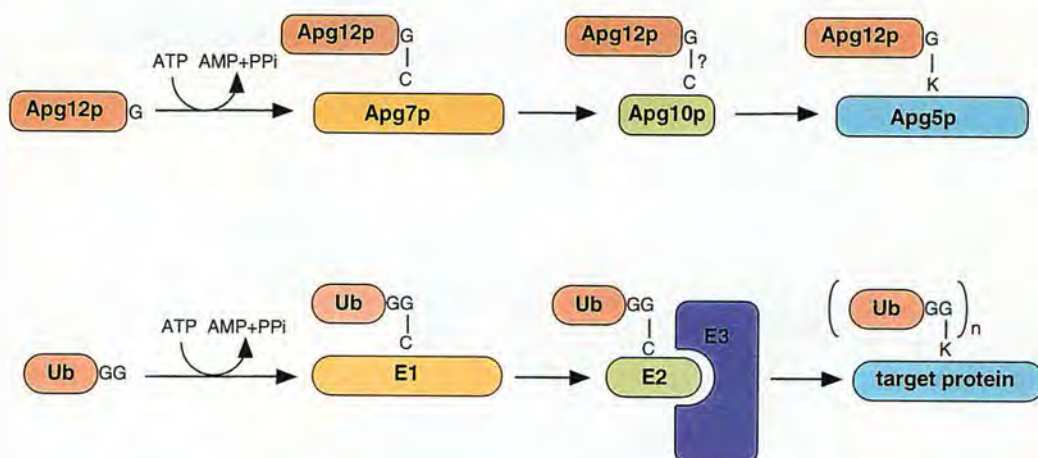


Fig. 2 The Apg12p conjugation system and ubiquitin system

Apg12p is activated by Apg7p in an ATP-dependent manner. After formation of a thioester with Apg7p, and then possibly with Apg10p, Apg12p is finally conjugated to Apg5p via an isopeptide bond (upper). Although Apg12p has no homology to ubiquitin, the Apg12p conjugation system is quite similar to that of ubiquitin (lower) which is involved in selective proteolysis.

Publication List:

- Kametaka, S., Okano, T., Ohsumi, M., and Ohsumi, Y. Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast, *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 22284-22291 (1998).
- Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., Geoge, M. D., Klionsky, D. J. Ohsumi, M., Ohsumi, Y. A protein conjugation system essential for autophagy, *Nature* 395, 395-398 (1998).
- Mizushima, N., Sugita, H., Yoshimori, T., and Ohsumi, Y. A New Protein Conjugation System in Human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. *J. Biol. Chem.* in press.
- Noda, T. and Ohsumi, Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem.* **273**, 3963-3966 (1998).
- Tagawa Y., Yamamoto A., Yoshimori T., Masaki R., Omori K., Himeno M., Inoue K., Tashiro Y. 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.* in press.
- Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., and Kominami, E. Apg7p/Cvt2p: a novel protein activating enzyme essential for autophagy. *Mol. Biol. Cell*, in press.
- Yamamoto A., Tagawa Y., Yoshimori T., Moriyama Y., Masaki R., Tashiro Y. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct. Funct.* **23**; 33-42 (1998).