THE ROYAL SOCIETY

Molecular mechanism of autophagy in yeast, *Saccharomyces cerevisiae*

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Bulk degradation of cytosol and organelles is important for cellular homeostasis under nutrient limitation, cell differentiation and development. This process occurs in a lytic compartment, and autophagy is the major route to the lysosome and/or vacuole. We found that yeast, *Saccharomyces cerevisiae*, induces autophagy under various starvation conditions. The whole process is essentially the same as macroautophagy in higher eukaryotic cells. However, little is known about the mechanism of autophagy at a molecular level. To elucidate the molecules involved, a genetic approach was carried out and a total of 16 autophagy-defective mutants (*apg*) were isolated. So far, 14 *APG* genes have been cloned. Among them we recently found a unique protein conjugation system essential for autophagy. The C-terminal glycine residue of a novel modifier protein Apg12p, a 186-amino-acid protein, is conjugated to a lysine residue of Apg5p, a 294-amino-acid protein, via an isopeptide bond. We also found that *apg7* and *apg10* mutants were unable to form an Apg12p–Apg5p conjugate. The conjugation reaction is mediated via Apg7p, E1-like activating enzyme and Apg10p, indicating that it is a ubiquitination-like system. These *APG* genes have mammalian homologues, suggesting that the Apg12 system is conserved from yeast to human. Further molecular and cell biological analyses of *APG* gene products will give us crucial clues to uncover the mechanism and regulation of autophagy.

Keywords: autophagy; Apg12p; protein conjugation; protein degradation

1. INTRODUCTION

Cellular activity is maintained by the continuous balance of synthesis and degradation of many proteins. Every protein has a definite lifetime, from several minutes to more than ten days. We do not know what the determinants of the lifetime of each protein are. It is now well accepted that short-lived proteins are degraded in the cytosol by the ubiquitin–proteasome system. Selective proteolysis in the cytosol should require a highly regulated recognition of the molecules to be degraded. This might be one of the reasons for the sophisticated ubiquitin conjugation system, while it has been widely believed that the acidic compartment, the lysosome–vacuole, is the main intracellular site of protein degradation. Since long-lived proteins account for more than 90% of total cellular protein, amino acids derived from digestion of these proteins play a more important role for maintenance of the amino-acid pool than the product from quick turnover of short-lived proteins.

It is already 50 years since C. de Duve discovered the lysosome lytic compartment in eukaryotic cells by biochemical analysis. Segregation of the degradation process from the cytosol, where biosynthesis takes place, has been thought to be important. But this raises another critical problem: how to sequester the cytoplasmic components to the lytic compartment. Autophagy was discovered more than 30 years ago by electron microscopy and is a major route of sequestration of cytoplasmic components to the lytic compartment (Dunn 1994). However, the molecular mechanism of autophagy is still poorly understood.

About ten years ago we started to study the mechanism of protein degradation in the vacuole–lytic compartment of yeast, *Saccharomyces cerevisiae*, as a model system. The vacuole is a relatively large compartment in yeast cells, which occupies about 25% of the total cell volume and contains various kinds of hydrolytic enzymes. At that time nothing was known about the mechanism of sequestration of substrates to be degraded to the vacuole. Bulk protein degradation occurs under starvation conditions. For example, sporulation in yeast is triggered by nitrogen starvation, i.e. this cell differentiation occurs without a supply of nitrogen compounds from outside. Therefore, bulk degradation of pre-existing proteins and nucleic acids must be essential for this cell differentiation.

2. AUTOPHAGY IN YEAST

I observed major vacuolar proteinase-deficient mutant cells under nitrogen starvation conditions by light microscopy, expecting that sequestered materials might be accumulated in the vacuoles without breaking down. An obvious morphological change of the vacuole was revealed (Takeshige *et al.* 1992). After about 30 min of starvation, spherical bodies appeared in the vacuole and gradually increased in number until they finally filled the vacuole. These structures moved around vigorously within the vacuole by Brownian motion. In wild-type cells we saw hardly any of this type of morphological change, but in the presence of phenylmethylsulphonyl fluoride, exactly the same morphological change was observed.

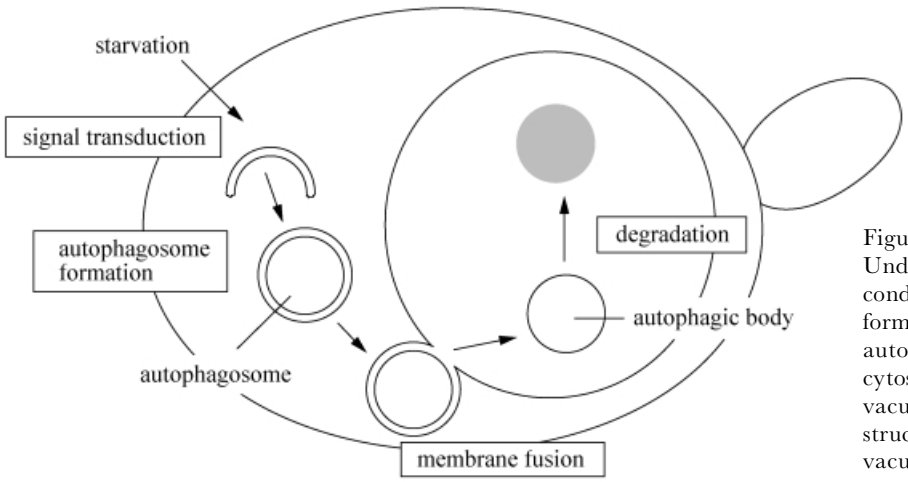

Figure 1. Scheme of autophagy in yeast. Under various nutrient-deprived conditions, a yeast cell induces the formation of a double-membrane structure, autophagosome, enclosing a portion of the cytosol. The autophagosome fuses with the vacuole, delivering a single-membrane structure, an autophagic body, in the vacuole.

Next, M. Baba and I began to analyse this starvation-induced morphological change by electron microscopy using the rapid-freezing and freeze-substitution fixation method (Takeshige *et al.* 1992). The vacuolar proteinase-deficient cell growing vegetatively had normal vacuoles, which contained no membranous structure inside, while in cells starved for 3 h in a nitrogen-free medium, every vacuole contained spherical structures regardless of their cell cycle or size of vacuoles. These spherical structures were single-membrane-bound organelles and their contents were exactly the same as cytosol itself, containing the same density of ribosomes as cytosol. Biochemical analysis of the isolated vacuoles containing these bodies revealed that active cytosolic enzymes are non-selectively incorporated into the vacuole. Membrane organelles such as mitochondria were also sequestered to the vacuole by chance (Baba *et al.* 1994). Not only nitrogen starvation, but carbon, sulphate, phosphate and even single auxotrophic amino-acid starvation induced exactly the same membrane phenomena. Therefore, we concluded that yeast cells sequester their own cytoplasmic components to the lytic compartment when they face adverse nutritional conditions.

The obvious question is how these single membrane structures appear in the vacuole. There are two possibilities. One is the invagination of vacuolar membrane and pinching off a portion of cytoplasm. Second is that a double-membrane structure appears in the cytosol first, then fuses with the vacuole. Further electron microscopy showed that a double-membrane structure encircled a portion of cytoplasm in the cytosol (Baba *et al.* 1994). The earliest membrane structure we could detect was a membrane sac enclosing a portion of cytosol. By sealing the membrane sac, a double-membrane structure—an autophagosome—may be formed. The autophagosome immediately targets the vacuole, makes contact with the vacuole and fuses with the vacuolar membrane, delivering an inner-membrane-bound structure to the vacuole. We named this single-membrane structure an autophagic body (Takeshige *et al.* 1992).

Autophagosomal membrane appears thinner than any other kind of membrane and it is very difficult to see the typical triple layer of the unit membrane structure in thin sections. To our surprise, the membrane of the autophagic body almost completely lacked intramembrane particles when observed by the freeze-replica method (Baba & Ohsumi 1995). The outer membrane of the autophagosomes contained a small number of particles, but the inner membrane, which corresponds to the autophagic body membrane, contained no particles at all. Autophagosomes must be specialized for delivery of a quantumized amount of cytoplasm to the vacuole.

From these morphological analyses, we proposed a scheme of autophagy in yeast, as shown in figure 1. Under various nutrient starvation conditions, yeast cells induce sequestration of their own cytoplasmic components to the vacuole. This whole process is topologically the same as macroautophagy known in mammalian cells.

3. GENETIC APPROACH TO THE AUTOPHAGY IN YEAST

There are many questions to be answered about the mechanism of autophagy. How does a cell recognize various kinds of nutrient conditions outside and transduce it to induce the dynamic membrane rearrangement? What is the origin of the autophagosomal membrane and how is the isolation membrane constructed and sealed to form a double-membrane structure? How does an autophagosome target the vacuole and fuse with the vacuolar membrane? To elucidate the molecular basis of autophagy we applied a genetic approach. Progress of autophagy in yeast can be easily monitored in real time as the accumulation of autophagic bodies in the vacuole under light microscopy. Using this method, M. Tsukada isolated about 100 autophagy-defective mutants (Tsukada & Ohsumi 1993). By genetic analysis, 14 complementation groups of *apg* mutants were obtained. These *apg* mutants showed quite similar phenotypes. Not all *apg* mutants can induce protein degradation under starvation. The homozygous diploid of each *apg* mutant is sporulation defective. All *apg* mutant cells start to die after one or two days of starvation, indicating that autophagy is essential for maintenance of cell viability under starvation. But they are normal in vegetative growth, vacuolar functions, secretion and endocytosis.

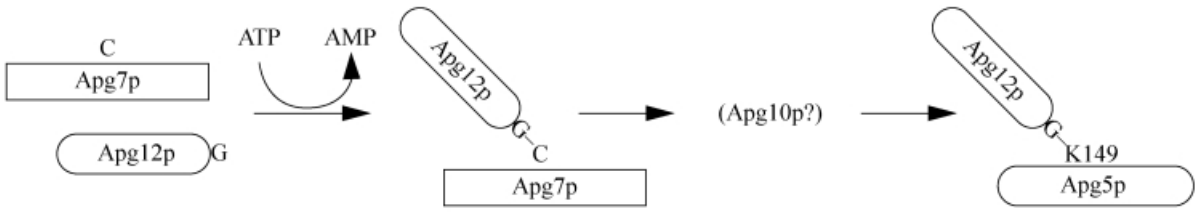

Figure 2. The mode of the Apg12 conjugation system. Apg12 is activated by Apg7p with ATP hydrolysis; C-terminal glycine is conjugated to Cys residue of Apg7p. Subsequent transfer of Apg12p to Apg10 results in formation of the Apg12p–Apg10p thioester. Finally, the carboxy terminus of Apg12p is covalently bonded to Lys of Apg5p via an isopeptide bond.

Klionsky and colleagues have been working on the biogenesis of one of the vacuolar enzymes aminopeptidase 1 (API) (Klionsky 1997). Most vacuolar enzymes target the vacuole via a secretory pathway, but API is synthesized as an inactive proform in the cytosol and targets the vacuole directly from the cytoplasm. Mutants defective in this pathway (*cvt*) were isolated by Klionsky's group (Hardy *et al.* 1995). API maturation is a constitutive process, but not starvation inducible, and its kinetics are completely different from that of autophagy. Autophagy defective mutants *apg* and *aut* isolated by Thumm's group (Thumm *et al.* 1994) were found to overlap with some *cvt* mutants (Harding *et al.* 1996; Scott *et al.* 1996). Furthermore, all *apg* mutants turned out to be defective in API maturation. In collaboration with Klionsky, we examined API transport by electron microscopy (Scott *et al.* 1997; Baba *et al.* 1997). ProAPI was not dispersed in the cytosol but instead localized almost exclusively in a small area of cytoplasm, while unidentified spherical particles associated with the API aggregate (Cvt complex). This API complex also existed as a double-membrane-bound form next to the vacuole. These Cvt vesicles were much smaller than autophagosomes, 140–160 nm in diameter, and selectively enclosed the Cvt complex but excluded cytosolic components such as ribosomes. They fuse with the vacuole and deliver Cvt bodies into the vacuole. These observations clearly indicated that API transport is mediated by the same membrane phenomena as macroautophagy. Moreover, under starvation conditions, proAPI was found to be enclosed by an autophagosome with cytosolic components such as ribosomes. Kinetic analyses indicate that ProAPI is selectively transported to the vacuole by autophagy under starvation. From these results we concluded that *APG* genes play a role in these two distinct pathways. It is likely that autophagic machinery was used for the biogenesis of a particular vacuolar enzyme. Another important suggestion from this study is that autophagy may become selective with a slight modification.

Recently we concentrated on the cloning and characterization of *APG* genes. So far we have obtained 12 *APG* genes, and by two-hybrid screening of these genes we obtained another two new *APG* genes (*APG16* and *APG17*). Therefore, autophagy in yeast requires at least 16 genes. To our surprise, almost all *APG* genes are novel genes, except *APG6*, which turned out to be allelic to *VPS30* required for vacuolar enzyme sorting (Kametaka *et al.* 1996). Gene disruption experiments indicated that none of the *APG* genes are essential for vegetative growth but all are essential for autophagy. By electron microscopy we could not detect any autophagosomes in the cytoplasm of *apg* mutants, suggesting that these genes function at or before autophagosome formation.

Apg1p is a protein kinase essential for autophagy (Matsuura *et al.* 1997). This kinase interacts with at least three other proteins, including Apg13p (Funakoshi *et al.* 1997) and Apg17p. We speculate that this protein complex may function as a molecular switch between the Apg and Cvt pathways. We now know of several genetic and physical interactions among *APG* genes: Apg6p binds to Apg14p (Kametaka *et al.* 1998), Apg4p interacts with Apg8p and so on.

The predicted amino-acid sequence of *APG* gene products seems to be soluble proteins, except Apg9p, which may encode a multispanning membrane protein. However, several Apgps behave as membrane-bound proteins. Cell fractionation experiments indicated that they are not residents of well-known organelles, such as the endoplasmic reticulum, Golgi, vacuole or nucleus.

4. NOVEL PROTEIN-CONJUGATION SYSTEM, ESSENTIAL FOR THE AUTOPHAGY

Quite recently we realized that four *APG* genes have a related function (Mizushima *et al.* 1998*a*). The *APG12* gene encodes a small hydrophilic protein of 186 amino acids with no homology. *APG12* disruptant showed a typical *apg* phenotype. N. Mizushima constructed an HA-tagged version of Apg12p and studied its expression by Western blot analysis. Almost equal amounts of two bands were found. The lower band corresponded to the authentic Apg12p but the other was much larger at 70 kDa, suggesting some unique protein modification reaction. We speculated that this higher band may be important for autophagy and tried to see Apg12p in all *apg* mutants. We found that in three *apg* mutants, *apg5*, *apg7* and *apg10*, the higher band is completely missing. Western blot with HA-tagged Apg5p also showed two bands at the predicted molecular mass of Apg5p and again at 70 kDa. Immunoprecipitation analysis confirmed that the 70 kDa band contains a one-to-one complex of Apg5p and Apg12p. Apg12p has a glycine residue at the C-terminus like ubiquitin. Deletion series of Apg12p clearly showed that elimination of a single glycine completely loses the formation of the high molecular band, suggesting that C-terminal glycine is a prerequisite for the conjugation, probably via an isopeptide bond with the ε-amino group of lysine of the target protein like

ubiquitin. Apg5p contains a total of 19 lysine residues, we systematically replaced each lysine residue of Apg5p with arginine. Among them only one lysine, at position 149, was shown to be essential for the conjugation. These Apgl2 ΔG186 and Apg5K149R mutants were autophagy- and Cvt-defective, strongly indicating that the conjugate formation is essential for autophagy and APl maturation (Mizushima *et al*. 1998*a*).

Apg7p and Apgl0p may consist of enzyme systems for this conjugation system. Significant sequence homology of Apg7p with Uba1p suggests that it may function as an activating enzyme for Apgl2p. Apgl0p may be an E2-like conjugation enzyme or a part of it (figure 2). We do not know at present whether a specific E3 molecule is involved in this conjugation system or not. Recently, several ubiquitin-related molecules were discovered and their important physiological roles were described. But Apgl2p has several unique features. Apgl2p has no homology to ubiquitin at all; so far Apg5p is the only target of this modifier, and is synthesized as an active molecule without any processing at the C-terminus. This is the first ubiquitin-unrelated protein modifier (Mizushima *et al*. 1998*a*).

Recently, we cloned a human homologue of Apgl2p, which has high homology in the C-terminal region with its yeast counterpart (Mizushima *et al*. 1998*b*). Previously, Apg5 human homologue was reported as an apoptosis-specific protein. We also cloned human Apg5. We transfected these two genes in COS cells and proved that human Apgl2 also conjugates to its human Apg5 counterpart. These findings indicate that the Apgl2-conjugation system is well conserved throughout all eukaryotes. We are now analysing the physiological role of this conjugate in autophagy and its related function in higher eukaryotes.

We could now have several molecular markers to investigate the molecular mechanism of autophagy. Further analyses of *APG* gene products will provide many clues to elucidate the physiological significance and new aspects of membrane dynamics in eukaryotic cells.

The above work was done by many people in my laboratory (M. Baba, T. Noda, Y. Kamada, N. Mizushima, T. Kirisako, S. Kametaka and T. Funakoshi), in M. Ohsumi's group at Teikyo University of Science and Technology, and in D. J. Klionsky's group at the University of California at Davis.

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Discussion

R. T. Hunt (*ICRF Clare Hall Laboratories, Hertfordshire, UK*). Does the system you describe constitute the signalling mechanism or is it part of the effector mechanism?

Y. Ohsumi. In all of the *apg* mutants we could not detect formation of autophagosomes, so we think the conjugation system may be involved in assembly of those kind of membrane structures.

R. T. Hunt (*ICRF Clare Hall Laboratories, Hertfordshire, UK*). Yes, it is hard to imagine how a membrane just forms spontaneously. Is it formed from 'nothing' in the middle of the cytoplasm?

Y. Ohsumi. That is a very good point. We are working at the moment on understanding how the autophagosome is assembled. It does not involve the secretory pathway.

A. Hershko (*Technion—Israel Institute for Technology, Haifa, Israel*). Where are these Apg proteins in the cell? Do you see any change in localization after conjugation, for example?

Y. Ohsumi. At this moment we see no change of the localization of Apg5p after conjugation. After starvation the ratio of the free Apgl2p to conjugated is similar. We haven't yet made GFP conjugates to carefully examine localization.

K. A. Nasmyth (*Research Institute of Molecular Pathology, Vienna, Austria*). What is known about autophagy in animal cells? Does it involve a similar cytological process?

Y. Ohsumi. Mammalian cells have lysosomes not vacuoles, but the process appears similar; autophagosomes fuse with primary lysosomes and inside the structure is digested, so exactly the same thing happens.