Formation Process of Autophagosome Is Traced with Apg8/Aut7p in Yeast

Takayoshi Kirisako,* Misuzu Baba,† Naoiada Ishihara,* Kouichi Miyazawa,† Mariko Ohsumi,§ Tamotsu Yoshimori,* Takeshi Noda,* and Yoshinori Ohsumi*†

*D epartment of Cell Biology, National Institute for Basic Biology, O kazaki 444-8585, Japan; †D epartment of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan; and §D epartment of Biosciences, Teikyo University of Science and Technology, Yamanashi 409-0193, Japan

Abstract. We characterized A pg8/Aut7p essential for autophagy in yeast. A pg8p was transcriptionally upregulated in response to starvation and mostly existed as a protein bound to membrane under both growing and starvation conditions. Immunofluorescence microscopy revealed that the intracellular localization of A pg8p changed drastically after shift to starvation. A pg8p resided on unidentified tiny dot structures dispersed in the cytoplasm at growing phase. During starvation, it was localized on large punctate structures, some of which were confirmed to be autophagosomes and autophagic bodies by immuno-EM. Besides these structures, we found that A pg8p was enriched on isolation membranes and in electron less-dense regions, which should contain A pg8p-localized membrane- or lipid-containing structures. These structures would represent intermediate structures during autophagosome formation. Here, we also showed that microtubule does not play an essential role in the autophagy in yeast. The result does not match with the previously proposed role of A pg8/Aut7p, delivery of autophagosome to the vacuole along microtubule. Moreover, it is revealed that autophagosome formation is severely impaired in the apg8 null mutant. A pg8p would play an important role in the autophagosome formation.

Key words: autophagy • autophagosome • Apg8/Aut7p • Saccharomyces cerevisiae • vacuole

AU TOPH AGY is responsible for intracellular bulk protein degradation in the lytic organelle, lysosome/vacuole (Kopitz et al., 1990; Dunn, 1994). It is induced when cells cannot ingest nutrients from the extracellular environment. By autophagy, cytoplasmic components are sequestered to the lysosome/vacuole to be degraded. In yeast, the defect in autophagy results in loss of viability during starvation, indicating that this protein degradation provides the minimal nutrients required for starved cells to survive (Tsukada and Ohsumi, 1993). In addition, autophagy-deficient mutants are defective in sporulation in yeast, suggesting that it is necessary for the cell differentiation (Tsukada and Ohsumi, 1993).

A unique membrane dynamic is observed in the process of autophagy. The whole process seems to be well conserved through eukaryotes from yeast to mammal. In the case of yeast, autophagosome, a double-membrane structure surrounding a portion of the cytoplasm, is formed (Baba et al., 1994). Then, its outer membrane fuses to the vacuolar membrane depending upon the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (S N A R E) molecules (Baba et al., 1994; Darsow et al., 1997; Sato et al., 1998). Subsequently, autophagic body is released into the vacuolar lumen (Takeshige et al., 1992; Baba et al., 1994). The membrane of autophagic body is disrupted in the vacuole depending on proteinase A/proteinase B and the digestion of its contents, cytosolic materials, follows (Takeshige et al., 1992).

We isolated a group of autophagy-defective mutants (apg) in the yeast Saccharomyces cerevisiae (Tsukada and Ohsumi, 1993). Harding et al. (1995) isolated the cvt mutants defective in Cvt pathway, transport of precursor form of aminopeptidase I (proA PI) from the cytosol to the vacuole under nutrient-rich conditions (H ard ing et al., 1995). Its whole process is topologically the same as autophagy (Baba et al., 1997; Scott et al., 1997). Most cvt mutants are allelic to apg mutant (cvt5 is allelic to apg8/aut7), and every apg mutant is defective in Cvt pathway (Scott et al., 1996). Thus, A pg proteins participate in both autophagic and Cvt pathways.

We have characterized 13 A pg gene products for these years. APG1/AUT3 encodes a protein kinase, whose kinase activity is essential for autophagy (Matsuura et al., 1997; Straub et al., 1997). Its overexpression suppresses

A ddress correspondence to Y oh sumi O hsumi, D epartment of Cell Biology, National Institute for Basic Biology, O kazaki 444-8585, Japan. Tel.: 81-564-55-7513. Fax: 81-564-55-7516. E-mail: yohsumi@nibb.ac.jp

© The Rockefeller University Press, 0021-9525/99/10/435/12 $5.00
The Journal of Cell Biology, Volume 147, Number 2, October 18, 1999 435–446
http://www.jcb.org

1. A bbr eviations used in this paper: A LP, alkaline phosphatase; A PI, aminopeptidase I; H A, hemagglutinin; O R F, open reading frame; proA PI, precursor form of aminopeptidase I.
the defect in autophagy of the *apg13* mutant (Funakoshi et al., 1997). A pg6V/p530p and A pg14p form a protein complex on yet unidentified membrane structures (Kametaka et al., 1998). A pg12p is conjugated to A pg5p in a similar manner as ubiquitin (Mizushima et al., 1998). A pg7p and A pg10p are the enzymes catalyzing this conjugation reaction (Mizushima et al., 1998; Kim et al., 1999; Shintani et al., 1999; Tanida et al., 1999). A pg16p forms homooligomer and is bound to A pg5p–A pg12p conjugate, resulting in forming multimeric protein complex (Mizushima et al., 1999). It was reported that A UT1, A UT2, and A UT7 are essential for the autophagy (Schlumberger et al., 1997; Lang et al., 1998). A ut2p is physically associated with A ut7p (Lang et al., 1998).

In the process of autophagy, formation of autophagosome has been poorly characterized so far. In mammalian cells, several groups proposed the potential source of the membrane of autophagosome such as ER, post-Golgi membrane, or yet uncharacterized organelle, phagophore (Dunn, 1990; Seglen et al., 1990; Yamamoto et al., 1990a,b). The origin of autophagosome is still controversial. Moreover, it is unknown how the double membrane structure of autophagosome is constructed. This confusing situation may be due to lack of a specific marker for autophagosomal membrane. Even in yeast cells, a specific marker protein localized on autophagosome has not been identified, although many A pg proteins have been characterized so far. Since autophagosome is a transient structure in autophagic process, a marker molecule is prerequisite to trace the formation process.

Here, we report on the expression and intracellular localization of A pg8p, one of A pg proteins essential for autophagy, previously reported as A ut7p (Lang et al., 1998). A pg8p is localized on the autophagosomes and their precursor structures during starvation. Based upon the examination of the intermediate structures of autophagosome, we present a novel model for the formation process of autophagosome. We also report that *apg8* mutant is defective in autophagosome formation. A pg8p may play an important role during autophagosome formation.

### Materials and Methods

#### Yeast Strains and Media

Yeast strains used in this study are shown in Table I. To construct the *Δapg8* strains, TRP1, LEU2, and HIS3, which was obtained from pJJ 288, pJ 252, and pJ 215, respectively, was substituted for the A ccl1-H pal fragment containing 98% of the APG8 open reading frame (ORF). TK 402, TK 404, TK 405, TK 407, and KIV 5 were obtained by selection on appropriate amino acid drop-out medium. Media used in this study were described previously, and SD medium supplemented with 0.5% casamino acid was referred as SD + CA medium (Shirahama et al., 1997).

#### Cloning of APG8 and Plasmid Construction

APG8 was cloned according to the method previously reported (Kametaka et al., 1996). pAG8317 was generated by cloning 2.6 kbp of Xbal–Xbal fragment, including APG8, into pBleuropsiturum1. Using pAG8317 as a template DNA, the Spel–Ecor1 fragment, including the 0.9 kbp of HindIII–HindIII sequence, was generated by PCR using following primers: Apg8F1, 5′-GATATGATGCTGTCCGCTAGAGAAG-3′; Apg8R1, 5′-GGAATTCAGAATCTTGGCTCCGTTG-3′; pTK101 and pTK102 were generated by inserting this Spel–Ecor1 fragment into the yeast centromeric and multicopy vectors, pRS316 and pRS426 (Sikorski and Hieter, 1989), respectively. For construction of 3× HA (hemagglutinin)-tagged APG8 plasmids, a BamHI site was generated at the 5′ terminus of the APG8 ORF by PCR using Apg8F1, Apg8R1, and the following primers: 5′-GATATGATGCTGTCCGCTAGAGAAG-3′ and 5′-AGAATCTTGGCTCCGTTG-3′. The resulting fragment was cloned into pBleuropsiturum1. A pg8::HIS3, which was obtained from pJJ288, was amplified by PCR, and inserted in the BamHI site of the 3× HA and Apg8 plasmids, pTK110 and pTK108, were generated by cloning this Spel–Ecor1 fragment containing the 3× HA-tagged APG8 gene into yeast centromeric plasmids, pRS314 and pRS316 (Sikorski and Hieter, 1989), respectively.

#### Alkaline Phosphatase Assay

A alkaline phosphatase (ALP) assay was performed as previously described (Noda et al., 1995; Noda and Ohashi, 1998).

#### Nocodazole Treatment

Cells growing in YE PD medium were treated with nocodazole (Sigma Chemical Co.) for 3 h at a final concentration of 10 μg/ml. Cells were transferred to 0.17% yeast nitrogen base without amino acids and ammonium sulfate supplemented with 1 mM PM SF and 10 μg/ml nocodazole, and further incubated for 4.5 h. The accumulation of autophagic bodies was observed under a microscope. For ALP assay, the nocodazole-treated

| Table I. Strains Used in this Study |
|-----------------------------------|
| **Strain** | **Genotype** | **Source** |
| YW5-1B | MATa leu2 ura3 trp1 | Noda et al., 1995 |
| TK404 | MATa leu2 ura3 trp1 Δapg8::TRP1 | This study |
| TK201 | MATa leu2 ura3 trp1 Δapg8::TRP1 pTK201 | This study |
| TK405 | MATa leu2 ura3 trp1 Δapg8::LEU2 | This study |
| TK114 | MATa leu2 ura3 trp1 Δapg8::LEU2 pTK110 | This study |
| STY1 | MATa leu2 ura3 trp1 Δpep4::URA3 | Nakamura et al., 1997 |
| TK407 | MATa leu2 ura3 trp1 Δpep4::URA3 Δapg8::LEU2 | This study |
| TK116 | MATa leu2 ura3 trp1 Δpep4::URA3 Δapg8::LEU2 pTK110 | This study |
| TN124 | MATa leu2 ura3 trp1 Δpho8::PHO8Δ60Δpho13::LEU2 | Noda et al., 1995 |
| TK402 | MATa leu2 ura3 trp1 Δpho8::PHO8Δ60Δpho13::LEU2Δapg8::TRP1 | This study |
| TK301 | MATa leu2 ura3 trp1 Δpho8::PHO8Δ60Δpho13::LEU2Δapg8::TRP1 pTK101 | This study |
| T303 | MATa leu2 ura3 trp1 Δpho8::PHO8Δ60Δpho13::LEU2Δapg8::TRP1 pRS316 | This study |
| TK307 | MATa leu2 ura3 trp1 Δpho8::PHO8Δ60Δpho13::LEU2Δapg8::TRP1 pTK108 | This study |
| TK415 | MATa leu2 ura3 trp1 Δpho7::LEU2 | This study |
| TK411 | MATa leu2 ura3 trp1 Δpho7::LEU2 Δapg8::TRP1 | This study |
| KIV5 | MATa leu2 ura3 trp1 lys2 his3 suc2-Δ9 Δapg8::HIS3 | This study |
cells in YEPD were starved in SD (-N) medium containing 10 μg/ml nocardazole for 4.5 h.

**Production of Antiserum against Apg8p**

A peptide including the NH2-terminal sequence of Apg8p, M KSTF-K-SEY PF EK C, was synthesized by a Model 433A peptide synthesizer (PE Applied Biosystems). The peptide was conjugated to Keyhole Limpet Hemocyanin (Sigma Chemical Co.) with sulfo-N-succinimidyl 4-(p-maleimido-phenyl)butyrate (Pierce Chemical Co.) according to the method previously reported (Iwai et al., 1988). The resulting conjugates were immunized to a rabbit and anti-Apg8p antiserum was obtained.

**Western Blotting of Apg8p**

Cell lysates were prepared by breaking cells with the glass beads in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM PMF, and the protease inhibitor cocktail™ (Boehringer Mannheim Corp.). Total protein (20 μM) was subjected to SD-PAGE and transferred to PVDF (polyvinylidene fluoride) membrane (Millipore Corp.). The resulting membrane was incubated with a 1:10,000 dilution of the anti-Apg8p antibody for 1 h, followed by a 1:10,000 dilution of HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 30 min. Signals were detected by the ECL kit (New England Nuclear, Inc.), which was used throughout this study. For the analysis of rapamycin treatment, growing cells were treated with 0.2 μg/ml rapamycin (Sigma Chemical Co.) for 2 h at 30°C in YEPD medium. Lysate preparation, followed by Western blotting, were performed as described above.

**Northern Blotting**

Cells were suspended in TES solution (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS) and treated with acid phenol at 65°C for 1 h. A queuos phase was collected and RNA was recovered by ethanol precipitation. mRNA was recovered by Oligotex-dT30 super (> TaKaRa) according to the manufacturer’s protocol, separated on 1.2% agarose containing 2.2 M formaldehyde by electrophoresis, and transferred to NYTRAN membrane (Schleicher & Schuell, Inc.). 32P-labeled probes for Apg8 and Act1 mRNA were prepared from each ORF fragment using Megaprime DNA labeling system (Nycomed A mersham, Inc.) according to the appended protocol. The mRNA transferred membrane was incubated with each probe at 42°C overnight, and signals were detected by autoradiography.

**Subcellular Fractionation and Solubilization**

Subcellular fractionation was performed as previously described by Horazdovsky and Emr (1989). Y W5-1B cells were cultured in YEPD medium at 30°C to 1-2 × 107 cells/ml, shifted to SD (-N) medium for 3 h and converted to spheroplasts. Spheroplasting medium was composed of YEP, 1.2 M sorbitol, 20 mM Tris-HCl, pH 7.5, 1% glucose, and 25 μg/ml of Zymolyase 100T (Seikagaku Kogyo) for growing cells, or 0.17% Zymolyase 100T for yeast cells in SD(-N) medium for 4.5 h and converted to spheroplasts. Spheroplasts were lysed in 20 mM Pipes/KOH, pH 6.8, 0.2 M sorbitol, 0.5 mM PMF, and the protease inhibitor cocktail. Cleared lysates were generated by centrifugation at 500 g for 5 min, and treated with 100 μg/ml of proteinase K with or without 2% Triton X-100 on ice for 30 min. Equal volume of 20% TCA was added to the lysates to stop the reaction. Precipitant was collected by centrifugation, suspended in SDS sample buffer, and subjected to immunoblotting. To detect the signal of A PI, 1:5,000 diluted anti-A PI antibody (gift from D. Kionysky, Section of Microbiology, UC Davis, CA) was used.

**Immunofluorescence Microscopy**

Immunofluorescent staining was performed according to the method previously described by Nishikawa et al. (1994). In this experiment, Δapg8 and Δapg8Δapet4 cells harboring 3 × HA -tagged Apg8 plasmid were used. Spheroplasts prepared from the cells fixed with formaldehyde were coated on the multwell slide glass (Cel-line A associates, Inc.) coated by poly-lysine (mol wt > 300,000; Sigma Chemical Co.), and permeabilized by treatment with 0.5% Triton X-100 in PBS for 10 min. Permeabilized cells were incubated in PBS containing 1% BSA at room temperature for 10 min and treated with a 1:1,000 dilution of anti-HA mAb, 16B12 (B erkeley A nitobio Co., Inc.), in the same buffer at room temperature for 1 h. The cells were then incubated with 10 μg/ml of anti-mouse Ig-fluorescein F(ab’2) fragment (Boehringer Mannheim Corp.) at room temperature for 1 h and observed under a confocal microscope, LSM 510 (Zeiss).

**Electron Microscopy**

Cells were subjected to rapid freezing and freeze-substitution fixation, and observed as previously reported (Baba et al., 1997). For immunoelectron microscopy, ultrathin sections were collected onto formvar-coated nickel grids and blocked in PBS containing 2% BSA at room temperature for 15 min. Incubations were carried out by floating grids on a 20 μl drop of a 1:1,500 dilution of anti-HA mAb, 16B12, at room temperature for 1.5 h. After washing, the grids were incubated for 1 h with 5- or 10-nm gold-conjugated goat anti-mouse IgG (Bio Cell Lab.). The grids were washed several times in PBS followed by several drops of distilled water and fixed with 1% glutaraldehyde for 3 min. The sections were stained with 4% uranyl acetate for 7 min and examined.

**Proteinase K Treatment**

Cells were starved in SD (-N) medium for 4.5 h and converted to spheroplasts. Spheroplasts were lysed in 20 mM Pipes/KOH, pH 6.8, 0.2 M sorbitol, 0.5 mM PMF, and the protease inhibitor cocktail. Cleared lysates were generated by centrifugation at 500 g for 5 min, and treated with 100 μg/ml of proteinase K with or without 2% Triton X-100 on ice for 30 min. Equal volume of 20% TCA was added to the lysates to stop the reaction. Precipitant was collected by centrifugation, suspended in SDS sample buffer, and subjected to immunoblotting. To detect the signal of A PI, 1:5,000 diluted anti-A PI antibody (gift from D. Kionysky, Section of Microbiology, UC Davis, CA) was used.

**Results**

**APG8, an Essential Gene for Autophagy**

We reported an apg8-1 mutant defective in the autophagy in yeast (Tsukada and Ohsumi, 1993). To identify the APG8, cloning was done as previously described (Kame-taka et al., 1996). Subcloning and sequencing revealed that APG8 is identical to the ORF named YBL078c, and encodes a hydrophilic protein of 117 amino acids. During this study, Lang et al. (1998) reported A U T7 as a novel autophagy-responsible gene, which was revealed to be identical to APG8.

We constructed the apg8 null mutant and ascertained morphologically that Apg8p is essential for autophagy, judging from no accumulation of autophagic bodies in the vacuole (data not shown). Furthermore, using the A LP assay system (Noda et al., 1995), we confirmed biochemically that Apg8p is essential for autophagy. The cells used for this assay have a truncated form of Pho8p, Pho8Δ60p, in the cytosol as an inactive precursor form. During starvation, Pho8Δ60p is sequestered to the vacuole, depending upon autophagy, where it is processed by the vacuolar enzymes and acquires phosphatase activity. So, we can monitor the progress of the autophagy by the increase of the ALP activity. Wild-type (T N124), Δapg8 mutant, and the Δapg8 mutant harboring APG8 on a centromeric plasmid
were grown in SD + CA medium until 1–2 × 10^7 cells/ml, and then shifted to SD (–N) medium for three hours. The ALP activity in the cell lysates prepared from each culture was measured (Fig. 1). In wild-type cells, the ALP activity increased in response to starvation, whereas in the Δapg8 cells its elevation was considerably reduced. The autophagic defect in the Δapg8 mutant was recovered by introducing Apg8 on a centromeric plasmid. The result clearly shows that deletion of Apg8 causes severe defect in autophagy.

No Requirement of Microtubule for Autophagy

A homology search showed that Apg8p and its homologues make a large gene family through yeast to higher eukaryotes (Lang et al., 1998). One of the homologues has been reported as rat MAP1-LC3, which is directly bound to microtubule in vitro (Mann and Hammarback, 1994). Furthermore, Lang et al. (1998) proposed that Apg8p is bound to microtubule via another protein, Aut2p, and functions on the delivery of autophagosomes to the vacuole. We have already cloned 13 Apg genes, and realized that Apg8p physically interacts with Apg4p. However, Apg4p has an entirely different function from what they proposed (Kirisako, T., and Y. Ohsumi, manuscript in preparation).

So far, there is no evidence showing that microtubules play a role in the autophagy in yeast. Nocodazole is a microtubule depolymerizing drug, which affects both spindle and cytoplasmic microtubules (Solomon, 1991). We asked, morphologically and biochemically, whether autophagy proceeds normally in the presence of this drug. Wild-type cells were cultured until 10^7 cells/ml in YEPD medium at 30°C. Nocodazole was added to the culture and it was incubated at 30°C for three hours. After this treatment, >70% of the cells were arrested at G2 stage with a large bud. Then the cells were transferred to starvation medium containing 1 mM PM SF and 10 μg/ml nocodazole, and incubated further at 30°C for 4.5 h. A s shown in Fig. 2 A, most large budded cells accumulated the autophagic bodies in their vacuole. TN124 cells were treated the same way, and the ALP activity was measured. Nocodazole treatment did not affect the increase of ALP activity (Fig. 2 B). Furthermore, in a tub2 mutant, autophagic bodies were normally accumulated in the vacuole (data not shown). These results indicate that depolymerization of microtubule does not affect the progress of autophagy. Hence, we concluded that microtubule is not necessary for the autophagy in yeast.

Expression of Apg8

We raised an antiserum against Apg8p using a synthetic peptide of the NH2-terminal 13 amino acids of Apg8p. Using the anti-Apg8p antibody, we carried out Western blotting of the cell lysate prepared from logarithmically growing cells. In wild-type cells, the anti-Apg8p antibody brought out a single band at ~15 kD (Fig. 3 A, lane 1), which is close to the predicted molecular mass of Apg8p (13.6 kD). Intensity of the band was enhanced in the cells harboring Apg8 on a multicopy plasmid (Fig. 3 A, lane 2), whereas no signal was detected in the Δapgs cells (Fig. 3 A, lane 3). The result revealed that Apg8p is expressed in the wild-type cells at growing phase. Since Apg8 is required also for the transport of proAPI from the cytosol to the vacuole (Scott et al., 1996), Apg8p expressed during vegetative growth should play a role in this pathway. Next, we examined Apg8p levels before and after shift to nitrogen starvation. The wild-type cells growing in YEPD medium were shifted to SD (–N) medium for various periods of time, and then the lysates prepared from these cell cultures were subjected to immunoblotting with the anti-Apg8p antibody. As shown in Fig. 3 B, the amount of Apg8p started to increase 30 min after shift to starvation and remained at a high level after two hours. Finally, the amount of Apg8p increased about eightfold in response to starvation (Fig. 3 B).

Next, we performed Northern blotting analysis. Total mRNA was prepared from wild-type cells growing in YEPD medium, and the cells shifted to SD (–N) medium for various periods of time. As shown in Fig. 3 C, the amount of Apg8 mRNA increased drastically in response to starvation, and reached at a maximum level within 30 min after shift to starvation. After 30 min, the amount...
Kirisako et al. Formation Process of Autophagosome Traced with Apg8p/Aut7p

prepared from growing and starved wild-type cells. Cell lysates were

correlation at transcription level. Among the 12
cates that the increase of Apg8p during starvation is regu-

ggradually decreased, but at six hours, it was still several-
-fold higher than that of the growing cells. This result indi-

cates that the increase of A pg8p during starvation is regu-

Autophagy is known to be induced by inactivation of
Tor, a phosphatidylinositol kinase homologue (Noda and
Ohsumi, 1998). We investigated the effect of Tor function
on the expression of A pg8p. Wild-type cells were treated
with rapamycin in Y EPD medium for two hours, and the
lysates prepared from the cells were subjected to immuno-
blotting with the anti-A pg8p antibody. Apg8p was recov-
ered mostly in LSP and HSP fractions under both growing
and starvation conditions, although in starved cells it was
more detectable in HSS fraction than in growing cells (Fig.
4 A ). Next, the cell lysates were vigorously sonicated to
exclude luminal proteins out of organelles or other mem-
brane structures, and was spun at 100,000 g for one hour to
generate a pellet fraction. Under both growth conditions,
A pg8p mostly remained in the pellet fraction, in contrast
CPY, a vacuolar luminal protein, was completely recov-
ered in supernatant fraction (Fig. 4 B ). It suggests that
A pg8p is bound to membrane or associated with a pellet-
able large protein complex. The pellet fractions generated
from the sonicated lysates were treated with 2% Triton
X-100, incubated for 30 min on ice, and then separated
to supernatant and pellet fractions by centrifugation at

cel Fractionation of Apg8p

We carried out subcellular fractionation. Cell lysates were
prepared from growing and starved wild-type cells. Cell ly-
sates (T) were centrifuged at 13,000 g for 15 min and the
pellet (LSP) fractions were obtained. The resulting super-
natant fractions were spun at 100,000 g for one hour to
generate supernatant (HSS) and pellet (HSP) fractions,
and the distribution of A pg8p was examined by immuno-
blotting with the anti-A pg8p antibody. A pg8p was recov-
ered mostly in LSP and HSP fractions under both growing
and starvation conditions, although in starved cells it was
more detectable in HSS fraction than in growing cells (Fig.
4 A ). Next, the cell lysates were vigorously sonicated to
exclude luminal proteins out of organelles or other mem-
brane structures, and was spun at 100,000 g for one hour to
genenerate a pellet fraction. Under both growth conditions,
A pg8p mostly remained in the pellet fraction, in contrast
CPY, a vacuolar luminal protein, was completely recov-
ered in supernatant fraction (Fig. 4 B ). It suggests that
A pg8p is bound to membrane or associated with a pellet-
able large protein complex. The pellet fractions generated
from the sonicated lysates were treated with 2% Triton
X-100, incubated for 30 min on ice, and then separated
to supernatant and pellet fractions by centrifugation at

cel Fractionation of Apg8p

We carried out subcellular fractionation. Cell lysates were
prepared from growing and starved wild-type cells. Cell ly-

Figure 3. Expression of A PG 8. A, The lysates prepared from the
growing cells were subjected to immunoblotting with the anti-
A pg8p antibody. Lane 1, wild-type cells (YW5-1B); lane 2, Δapg8
cells harboring A PG 8 on a multicopy vector (TK 201); lane 3,
Δapg8 cells (TK 404). B, The wild-type (YW5-1B) cells were cul-
tured in Y EPD medium until 1-2 × 10^7 cells/ml (0 h) and shifted
to SD(-N) medium for 0.5, 1, 2, 3, 4, and 6 h at 30°C. The
amount of A pg8p was estimated by immunoblotting with anti-
A pg8p antibody. Each lane has 10 μg of total protein. C, Y WS-
1B cells were cultured in Y EPD medium until 1-2 × 10^7 cells/ml
(0 h) and shifted to SD(-N) medium for 0.5, 1, 2, and 6 h at 30°C,
and mRNA was prepared from each culture as described in Ma-
terials and Methods. A PG 8 mRNA and ACT1 mRNA were de-
tected by Northern blotting with each specific probe. Each lane
has 4 μg of total mRNA. D, The wild-type (YW5-1B) cells were cul-
tured in Y EPD medium until 1 × 10^7 cells/ml at 30°C. Rapamycin
was added to the culture at a final concentration of 0.2 μg/ml and the
cells were further incubated in Y EPD medium for two hours at
30°C. Western blotting was performed with the anti-A pg8p anti-
body. Each lane has 20 μg of total protein.

Figure 4. Subcellular fractionation and solubilization of A pg8p.
The wild-type cells (YW5-1B) growing logarithmically in Y EPD
medium or starved in SD(-N) medium for three hours were
lysed. A, The lysates (T) were centrifuged at 13,000 g for 15 min
to generate pellet (LSP) and supernatant (LSS). LSS fraction was
further centrifuged at 100,000 g for one hour and separated to
pellet (HSP) and supernatant (HSS). LSP and HSP were resus-
pended in an equal volume of the lysis buffer to the original ly-
sates. Equal volume of each sample was applied to each well, and
immunoblotting was performed with anti-A pg8p antibody. B, The
lysates (T) were sonicated and centrifuged at 100,000 g for one hour to separate pellet (P) and supernatant (S). The dis-
100,000 g for another one hour. As shown in Fig. 4 C, 2% Triton X-100 efficiently solubilized Apg8p, indicating that most pelletable Apg8p is bound to some membrane structures under both growing and starvation conditions.

**Drastic Change of Localization of Apg8p in Response to Starvation**

To examine intracellular localization of Apg8p, immunofluorescence microscopy was performed. First, we carried out this experiment using the anti-Apg8p antibody, but sufficient signal was not obtained. Then, we constructed a single copy 3×HA-tagged Apg8p plasmid and introduced it into the Δapg8 mutant. We ascertained that the expression level of the 3×HA-tagged Apg8p gene in the resulting transformant, TK114, was similar to that of authentic Apg8 (data not shown), and that the transformant recovered the autophagic activity up to 70% of the wild-type cells (Fig. 1). The cells were grown in SD+CA medium until 2×10^7 cells/ml, and diluted fourfold in YEPD medium. The cells were grown for two generations, transferred to SD(-N) medium, and incubated for 0, 0.5, and 3 h. The localization of 3×HA-Apg8p was examined with anti-HA mAb. In the cells growing in YEPD medium

**Figure 5. Immunofluorescent staining of Δapg8 cells expressing 3×HA-Apg8p.**

A, The Δapg8 cells harboring 3×HA-tagged Apg8 plasmid, TK114 cells, were grown until logarithmic phase. The cells were fixed by formaldehyde and were treated with Zymolyase 100T to generate spheroplasts. The spheroplasts were permeabilized by 0.5% Triton X-100, and incubated with the anti-HA antibody, 16B12, followed with the FITC-conjugated anti-mouse IgG. Left, Fluorescence image of 3×HA-Apg8p; right, Nomarski image of the cells. Arrows show punctate signals that are a little larger than tiny dot signals. Bars, 10 μm. B, Immunofluorescent staining of YW5-1B cells before (0 h) and after (3 h) shift to starvation (negative control). Left, Fluorescence image; right, Nomarski image. Bars, 10 μm. C, The cells were shifted to starvation for 0, 0.5, and 3 h. Upper panels, Fluorescence images of 3×HA-Apg8p; lower panels, Nomarski images of the cells. Bars, 5 μm.
(t = 0), the fluorescence image consisted of many tiny dots dispersed throughout the cytoplasm, which was distinct from well-known organelles such as ER, Golgi body, nucleus, vacuole, or plasma membrane (Fig. 5, A and C, 0 h). In addition, we occasionally observed punctate signals distinct from the tiny dots (Fig. 5 A, arrows). 30 min after the shift to the starvation condition, the staining pattern drastically changed; one or two bright, large, punctate signals appeared in the cytoplasm (Fig. 5 C). These large punctate signals always resided in the cytoplasm, just beside the vacuole (Fig. 5 C). The number of these punctate signals was constantly 1–3/cell during starvation.

We further examined the localization of Apg8p in the background of Δpep4 mutant, in which the autophagic bodies accumulate in the vacuole during starvation (Takeshige et al., 1992). TK 116 strain was generated by introducing the 3 × HA-tagged APG8 plasmid into the Δapg8Δpep4. As shown in Fig. 6 A, under growing condition, the fluorescence pattern was observed as tiny dots dispersed in the cytoplasm as TK 114 cells. At 30 min after shift to the starvation condition, 1–3 large punctate signals per cell appeared next to the vacuole, also like wild-type cells (Fig. 6 B). At one hour, more punctate signals were observed than wild-type cells, and >50% were detected in the vacuole (Fig. 6 C). The punctate signals in the vacuole gradually increased and completely filled the vacuole at six hours later (Fig. 6, C–E). The signals coincided with autophagic bodies (Fig. 6, C and H, arrows). As autophagic bodies are derived from autophagosomes, at least some population of Apg8p might be localized on or in the autophagosomes and delivered to the vacuole during starvation. Some of the large punctate signals in the cytoplasm (Fig. 5 C, 0.5 and 3 h, and Fig. 6) may represent autophagosomes.

Intermediate Structures Traced with Apg8p

To obtain further information about the intracellular localization of Apg8p during starvation, immunoelectron microscopic analysis was performed using the Δpep4 cells, TK 116. The logarithmically growing cells were shifted to SD(–N) medium for one, two, or three hours. The localization of 3 × HA-Apg8p in the starved cells was examined with the anti-HA antibody. In the autophagic bodies, density of the gold particles was significantly higher than that in the cytoplasm (Fig. 7, arrows). This fact was in good agreement with the result obtained from immunofluorescence analysis. Moreover, it was revealed that most of Apg8p was in the lumen, but not on the membrane of autophagic bodies.

Next, we examined localization of Apg8p in the cytoplasm of the starved cell. As expected from the result of immunofluorescence microscopy, gold particles were associated with the autophagosomes (Fig. 8). Most mature autophagosomes with clear unit membranes and uniform intramembrane space were certainly stained, but not heavily (Fig. 8, A and B), and, in some cases, the gold particles were mostly detected in their lumen (Fig. 8 B). As shown in Fig. 8, C and D, some autophagosomes were heavily stained with the gold particles. In these autophagosomes, the gold particles were mostly detected along the double membrane. However, their intramembrane space was not homogenous, but partly swollen (Fig. 8, C and D, arrows) not like the mature autophagosome. These structures may represent nascent autophagosomes or the latest structures in the autophagosome formation.

Besides autophagosomes, we found heavily stained

(t = 0), the fluorescence image consisted of many tiny dots dispersed throughout the cytoplasm, which was distinct from well known organelles such as ER, Golgi body, nucleus, vacuole, or plasma membrane (Fig. 5, A and C, 0 h). In addition, we occasionally observed punctate signals distinct from the tiny dots (Fig. 5 A, arrows). 30 min after the shift to the starvation condition, the staining pattern drastically changed; one or two bright, large, punctate signals appeared in the cytoplasm (Fig. 5 C). These large punctate signals always resided in the cytoplasm, just beside the vacuole (Fig. 5 C). The number of these punctate signals was constantly 1–3/cell during starvation.

We further examined the localization of Apg8p in the background of Δpep4 mutant, in which the autophagic bodies accumulate in the vacuole during starvation (Takeshige et al., 1992). TK 116 strain was generated by introducing the 3 × HA-tagged APG8 plasmid into the Δapg8Δpep4. As shown in Fig. 6 A, under growing condition, the fluorescence pattern was observed as tiny dots dispersed in the cytoplasm as TK 114 cells. At 30 min after shift to the starvation condition, 1–3 large punctate signals per cell appeared next to the vacuole, also like wild-type cells (Fig. 6 B). At one hour, more punctate signals were observed than wild-type cells, and >50% were detected in the vacuole (Fig. 6 C). The punctate signals in the vacuole gradually increased and completely filled the vacuole at six hours later (Fig. 6, C–E). The signals coincided with autophagic bodies (Fig. 6, C and H, arrows). As autophagic bodies are derived from autophagosomes, at least some population of Apg8p might be localized on or in the autophagosomes and delivered to the vacuole during starvation. Some of the large punctate signals in the cytoplasm (Fig. 5 C, 0.5 and 3 h, and Fig. 6) may represent autophagosomes.

Intermediate Structures Traced with Apg8p

To obtain further information about the intracellular localization of Apg8p during starvation, immunoelectron microscopic analysis was performed using the Δpep4 cells, TK 116. The logarithmically growing cells were shifted to SD(–N) medium for one, two, or three hours. The localization of 3 × HA-Apg8p in the starved cells was examined with the anti-HA antibody. In the autophagic bodies, density of the gold particles was significantly higher than that in the cytoplasm (Fig. 7, arrows). This fact was in good agreement with the result obtained from immunofluorescence analysis. Moreover, it was revealed that most of Apg8p was in the lumen, but not on the membrane of autophagic bodies.

Next, we examined localization of Apg8p in the cytoplasm of the starved cell. As expected from the result of immunofluorescence microscopy, gold particles were associated with the autophagosomes (Fig. 8). Most mature autophagosomes with clear unit membranes and uniform intramembrane space were certainly stained, but not heavily (Fig. 8, A and B), and, in some cases, the gold particles were mostly detected in their lumen (Fig. 8 B). As shown in Fig. 8, C and D, some autophagosomes were heavily stained with the gold particles. In these autophagosomes, the gold particles were mostly detected along the double membrane. However, their intramembrane space was not homogenous, but partly swollen (Fig. 8, C and D, arrows) not like the mature autophagosome. These structures may represent nascent autophagosomes or the latest structures in the autophagosome formation.

Besides autophagosomes, we found heavily stained
structures with immunogold in the cytoplasm. In Fig. 9 A, the gold particles are located along the surface on a curved membrane sac. Fig. 9 B shows that the gold particles were arranged in a rough circle, and some of them resided along membrane structures, which partly appear as clear double membrane. In Fig. 9 C, a semicircular isolation membrane (large arrow) and its open region (small arrow) were stained with gold particles. The gold particles were apparently more concentrated in the open region rather than on the isolation membrane. In addition, we found that gold particles were concentrated in a limited area close to the vacuole (Fig. 9 D). The electron density in this area was less than that of the cytosol. The image of this Apg8p-enriched area is similar to that of the open region of the semicircular isolation membrane shown in Fig. 9 C. We suppose that all these structures shown in Fig. 9 represent intermediate structures of autophagosome.

**Crucial Function of Apg8p in Autophagosome Formation**

To elucidate the step at which Apg8p functions in the autophagic pathway, we used Ypt7 mutant as a control. Ypt7, a Rab family protein, is responsible for the fusion events to the vacuole (Schimmoller and Riezman, 1993; H aas et al., 1995). It was reported that in Ypt7, Cvt vesicles become detectable (K im et al., 1999). We studied by electron microscope, using rapid freezing and freeze-substitution-fixation method, whether Ypt7 is also required for the fusion of autophagosome to the vacuole. A was shown in Fig. 10 A, Ypt7 cells under starvation had many fragmented vacuoles, and autophagosomes were much more detectable in the cytoplasm than in wild-type cells. Thus, the depletion of YPT7 causes the accumulation of autophagosomes in the cytoplasm.

The Δapg8 cells were examined also by EM. They were mostly normal with a few large vacuoles, but autophagosomes could be hardly detected in their cytoplasm (Fig. 10 B). However, at low frequency, membrane structures, having enclosed a portion of cytosol, were observed only under starvation conditions. Some were indistinguishable from the autophagosome (Fig. 10 C), but others showed more complicated structures, distinct from typical autophagosome, such as the structure having condensed contents or multivesicular structure (Fig. 10, D and E). Next, Δapg8 cells having the background of Δypt7 were examined. The Δypt7Δapg8 cells did not accumulate autophagosomes in the cytosol, and at low frequency autophagosome-like structures were detected in their cytoplasm, like Δapg8 cells (data not shown). We counted the number of autophagosomes and autophagosome-like structures in Δypt7 cells and Δypt7Δapg8 cells. In the sections of 500 cells, we found 269 autophagosomes in Δypt7 cells, whereas in Δypt7Δapg8 cells, only 16 autophagosome-like structures were detected (5.9% of Δypt7 cells). The result clearly shows that autophagosomes are not accumulated in Δapg8 cells.
We reported that autophagy is not only responsible for nonselective degradation of cytosolic components, but also highly selective proAPI sequestration to the vacuole during starvation (Baba et al., 1997). ProAPI could be a marker as a cargo of the autophagosomes under starvation, just like Cvt vesicles under growing condition (Kim et al., 1999). Cell lysate was prepared from starved \textit{D}ypt7 cells and analyzed by immunoblotting with anti-API anti-

Figure 9. Possible intermediate structures of autophagosome. A, A membrane sac under construction. Arrows show gold particles. B and C, Isolation membranes. B, Isolation membrane is detected along the arrows. C, The small arrow shows a semicircular isolation membrane and a large arrow marks its open area. D, Apg8p-residing structures gathered in the area close to the vacuole. TK 116 cells were incubated in SD (–N) medium for one hour (A and C), or two hours (B and D). The localization of 3 × HA–Apg8p was detected with anti-HA antibody, followed by the incubation with 5-nm gold- (A) or 10-nm gold- (B–D) conjugated goat anti-mouse IgG. V, vacuole.

Figure 10. Fine morphology of \textit{D}ypt7 and \textit{D}apg8 cells and proteinase-K accession to proAPI in these mutants. A, EM image of \textit{D}ypt7 cell starved in SD (–N) medium for four hours. Autophagosomes (AP, arrows) were accumulated. B, EM image of the starved \textit{D}apg8 cell. C–E, The representatives of the membrane structures detected in the starved \textit{D}apg8 cells. C, A autophagosome-like structure indistinguishable from autophagosome. D and E, Aberrant multivesicular structures. N, nucleus; V, vacuole. F, Proteinase K-sensitivity of proAPI. Cell lysates were prepared from \textit{D}ypt7 and \textit{D}apg8 cells starved in SD (–N) medium for 4.5 h. The lysates were treated with 100 µg/ml proteinase K in the presence or absence of Triton X–100. A asterisk shows mature Apg8p and digestion product of proAPI by proteinase K.
Here, we report characterization of Apg8p, one of the APG gene products essential for autophagy. Apg8p turned out to be identical to AUT7, recently reported (Lang et al., 1998). They proposed that Apg8p functions in the delivery of autophagosome to the vacuole along microtubule. However, we found that autophagy proceeds normally, even if the microtubule is depolymerized (Fig. 2). This result suggests that microtubule does not play an essential role in the autophagy in yeast. The proposal by Lang et al. (1998) was based upon the physical interactions of Apg4/Aut2p with tubulin and Apg8/Aut7p by in vitro and two-hybrid analyses. But, they did not demonstrate that Apg8p is bound to microtubule via Apg4/Aut2p. We have found that recombinant GST-Apg4p is bound to glutathione-Sepharose column nonspecifically (our unpublished result). It may be due to the acidic nature of Apg4p (predicted isoelectric point is 4.4). One possible interpretation of their result is that Apg4p is bound to tubulin nonspecifically. They also described qualitatively that the Δapg8 cells accumulate autophagosomes in the cytoplasm. Here, we demonstrated quantitatively that the Δapg8 null mutant does not accumulate autophagosomes in the cytoplasm. So, we concluded that Apg8p participates in the autophagosome formation. This conclusion is not affected whether Apg4/Aut2p is indeed bound to tubulin.

Intermediate structures of autophagosome formation have been poorly characterized in both yeast and mammalian cells because of their dynamic feature and the lack of specific marker of autophagosome. In yeast, only a cup-shaped structure was detected as a possible intermediate structure of autophagosome (Baba et al., 1994). In this study, we found that most Apg8p is bound to membrane (Fig. 4) and localized in autophagosomes and autophagic bodies (Figs. 6-8). Apg8p must be a key molecule to identify intermediate structures of autophagosome formation. Actually, we found that Apg8p is localized on premature autophagosomes (Fig. 8, C and D) and isolation membranes (Fig. 9, A - C). In addition, an Apg8p-enriched region was observed in the cytoplasm close to the vacuole (Fig. 9 D), and a similar image was obtained in the open region of the semicircular isolation membrane (Fig. 9 C, small arrow). These regions were always electron less-dense, indicating that they contain Apg8p-localized membrane- or lipid-containing structures, possible precursor structures of autophagosomal membrane. Based on our observations, we propose a model for the scheme of autophagosome formation as follows. The isolation membrane is formed by sequential assembly of the precursor structures. As the isolation membrane becomes spherical (Fig. 8, C and D), its intramembrane space gradually becomes thin and homogenous, and finally a mature autophagosome is formed (Fig. 8, A and B). Our assembly model is distinct from the one that autophagosome is formed by enclosing the cytosol with preexisting membrane cisterna, such as ER or Golgi body. A utophagosomal membrane shows a unique feature morphologically distinct from well-known organelles (Baba et al., 1994, 1995). It is observed as a thinner membrane and has a much lower density of intramembrane particles in freeze-fracture images than the membrane of other organelles. These features may be derived from the nature of the precursor structures. The dot structures observed under growing condition may be the same to them and to one of the sources of autophagosomal membrane.

Immunoelectron microscopy and biochemical analyses have shown that Apg8p is bound to the cytoplasmic faces of mature autophagosomes and intermediate structures (Lang et al., 1998). These data indicate that Apg8p bound to membrane plays its role in the formation of autophagosome. In mature autophagosomes, Apg8p was observed less than in those intermediate structures (Fig. 8, A and B). In some typical autophagosomes, it was detected in the cytoplasm close to the vacuole membrane in freeze-fracture images (Fig. 9 D). Furthermore, we found that Apg8p was detected in the vacuolar membrane to which the outer membrane of the autophagosome had fused (Fig. 7). These results suggest that Apg8p starts to dissociate from the membrane of autophagosome after finishing its role.

**Discussion**

Here, we report characterization of Apg8p, one of the APG gene products essential for autophagy. Apg8p turned out to be identical to AUT7, recently reported (Lang et al., 1998). They proposed that Apg8p functions in the delivery of autophagosome to the vacuole along microtubule. However, we found that autophagy proceeds normally, even if the microtubule is depolymerized (Fig. 2). This result suggests that microtubule does not play an essential role in autophagy in yeast. The proposal by Lang et al. (1998) was based upon the physical interactions of Apg4/Aut2p with tubulin and Apg8/Aut7p by in vitro and two-hybrid analyses. But, they did not demonstrate that Apg8p is bound to microtubule via Apg4/Aut2p. We have found that recombinant GST-Apg4p is bound to glutathione-Sepharose column nonspecifically (our unpublished result). It may be due to the acidic nature of Apg4p (predicted isoelectric point is 4.4). One possible interpretation of their result is that Apg4p is bound to tubulin nonspecifically. They also described qualitatively that the Δapg8 cells accumulate autophagosomes in the cytoplasm. Here, we demonstrated quantitatively that the Δapg8 null mutant does not accumulate autophagosomes in the cytoplasm. So, we concluded that Apg8p participates in the autophagosome formation. This conclusion is not affected whether Apg4/Aut2p is indeed bound to tubulin.
starvation (Fig. 4 A). A pg8p dissociated from the inner membrane would be entrapped in the lumen of autophagosome and transported to the vacuole, and A pg8p detached from the outer membrane may be recycled for the next autophagosome formation. Since A pg8p is not an integral membrane protein, it would be able to attach and detach from membrane. We found that some portion of A pg8p is tightly bound to membrane (data not shown). However, it is still unclear how A pg8p interacts with membrane structures, particularly autophagosomal membrane. The interaction of A pg8p with membrane is now under investigation.

The apg8 null mutation severely impairs autophagosome formation, leading to the defect in bulk nonselective protein transport and degradation (Figs. 1 and 10). However, a small amount of mature A PI was detected during starvation. ProA PI may be transported to the vacuole via structures rarely observed in Δapg8 cells (Fig. 10, C–E). Among them, there are the structures indistinguishable from autophagosome (Fig. 10 C), suggesting that a small number of autophagosomes are built up in the absence of A pg8p. Thus, A pg8p may modulate the efficiency of autophagosome formation. In addition, we found the starvation-induced structures showing abnormal morphology in the Δapg8 cells (Fig. 10, D and E). Alternatively, A pg8p might regulate the morphology of autophagosome. In any case, A pg8p would play an important role in the assembly of the precursor structures into autophagosomal membrane.

The Cvt pathway proceeds with topologically the same membrane dynamics to the autophagy (Baba et al., 1997; Scott et al., 1997). Proteinase K is accessible to proA PI without detergent in the growing cvt5/apg8 mutant cells (Harding et al., 1995), indicating that A pg8p also is required for the formation of Cvt vesicle. Since A pg8p is localized on autophagosomes, it would be reasonable to speculate that it is localized on the Cvt vesicles also. In the growing cells, small punctate signals of 3 × HA–A pg8p may represent the Cvt vesicles (Fig. 5 A; arrows). Between the two pathways, the most apparent difference is the size of vesicles. The autophagosomes are 300–900 nm in diameter, while the Cvt vesicles are 140–160 nm (Baba et al., 1994, 1997). Surface area of autophagosomal membrane is calculated at ~16-fold of Cvt vesicle, on average. Therefore, the autophagosome formation would require more A pg8p than the Cvt vesicle formation. Moreover, a significant amount of A pg8p is transported to the vacuole upon autophagy during starvation. These may be the reasons why A pg8p increases during starvation. However, it is still an open question whether the increase of A pg8p is actually necessary for the autophagy. At least, it is clear that the increase of A pg8p is not sufficient for the induction of autophagy, because overexpression of A pg8p did not induce autophagy under growing condition (data not shown). In addition, we showed that A pg8p is transcriptionally upregulated by inactivation of Tor signaling cascade. We found STRE-like sequences in the promoter region of APG8. STREs are found in the genes induced in response to various stresses, such as CTTL1, encoding cytosolic catalase T, and play roles as positive control element (Belazzi et al., 1991; Schuller et al., 1994; Moskvina et al., 1998). Marchler et al. (1993) reported the mutation in the STREs of CTTL1 (CTTL1-23), which causes loss of induction activity. The corresponding mutation was introduced in the STRE-like sequences of APG8. However, it led to the increase of A pg8p at growing phase instead of the loss of the induction during starvation (data not shown), suggesting that a negative regulator is bound to the cis-elements to repress the expression under nutrient rich condition.

A pg8p is the first identified molecule that is localized on the autophagy-related membrane structures. Now it becomes a useful marker for further analysis on the whole process of membrane dynamics in autophagy. Primary structures of A pg8p are highly conserved among homologues in other organisms (Lang et al., 1998). We anticipate that some A pg8p homologues may function in the process of formation of autophagosome in higher eukaryotes. Further study of A pg8p will provide a breakthrough for elucidating the molecular mechanism of autophagy.

We are grateful to Dr. Masako Osumi for the use of EM facilities, Dr. Klionsky for the gift of anti-A PI antibody, and Dr. Mizushima for the gift of the Δapg8 null mutant.

This work was supported in part by Grants-in-Aid for the Ministry of Education, Science, and Culture of Japan.

Submitted: 7 July 1999
Revised: 25 August 1999
Accepted: 7 September 1999

References

Baba, M., K. Takeshige, N. Baba, and Y. Ohsumi. 1994. Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization. J. Cell Biol. 124:903–913.
Baba, M., M. Osumi, and Y. Ohsumi. 1995. Analysis of the membrane structures involved in autophagy in yeast by freeze- replica method. Cell Struct. Funct. 20:465–471.
Baba, M., M. Osumi, S.V. Scott, D.J. Klionsky, and Y. Ohsumi. 1997. Two distinct pathways for targeting proteins from the cytoplasm to the vacuole/lysosome. J. Cell Biol. 139:1687–1695.
Belazzi, T., A. Wagner, R. Wieser, M. Schanz, G. Adam, A. Hartig, and H. Ruš. 1991. Negative regulation of transcription of the Saccharomyces cerevisiae catalase T (CTT1) gene by cAMP is mediated by a negative control element. EMBO J. (Eur. Mol. Biol. Organ.) 10:585–592.
Darrow, T., S.E. Rieder, and S.D. Emr. 1997. A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. J. Cell Biol. 138:517–526.
Dunn, W.A., Jr. 1990. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. J. Cell Biol. 110:1923–1933.
Dunn, W.A., Jr. 1994. A putative role of the Vam3p Rab GTPase in targeting autophagy. Trends Cell Biol. 4:139–143.
Funakoshi, T., A. Matsuura, T. Noda, and Y. Ohsumi. 1997. A putative PAPG13 gene involved in autophagy in yeast, Saccharomyces cerevisiae. Gene 192:207–212.
Haas, A., D. Scheidmann, T. Lazor, D. Gallwitz, and W. Wickner. 1995. The GTPase Ypt7p of Saccharomyces cerevisiae is required on both partner vacuoles for the homotypic fusion step of vacuole inheritance. EMBO J. (Eur. Mol. Biol. Organ.) 14:5258–5270.
Harding, T.M., K.A. Morano, S.V. Scott, and D.J. Klionsky. 1995. Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. J. Cell Biol. 131:591–602.
Hozakovsky, B.F., and S.D. Emr. 1993. The VPS16 gene product is associated with a sedimentable protein complex and is essential for vacuolar protein sorting in yeast. J. Biol. Chem. 268:4953–4962.
Iwai, K., S. Fukuoka, T. Fushiki, K. Kido, Y. Sengoku, and T. Semba. 1988. Preparation of a verifiable peptide-protein immunogen: direction-controlled conjugation of a synthetic fragment of the monitor peptide with myoglobin and application for sequence analysis. Anal. Biochem. 171:277–282.
Kametaka, S., A. Matsuura, Y. Wada, and Y. Ohsumi. 1996. Structural and functional analyses of APG5, a gene involved in autophagy in yeast. Gene 178:139–143.
Kametaka, S., T. Okano, M. Osumi, and Y. Ohsumi. 1998. A pg14p and A pg6/ Vps33p form a protein complex essential for autophagy in the yeast, Saccharomyces cerevisiae. J. Biol. Chem. 273:22894–22901.
Kim, J., V.M. Dalton, K.P. Eggerton, S.V. Scott, and D.J. Klionsky. 1999. A pg7p/Cvt2p is required for the cytoplasm-to-vacuole targeting, macroautophagy, and peroxisome degradation pathways. Mol. Biol. Cell. 10:1337–1351.

Kirisako et al. Formation Process of Autophagosome Traced with Apg8/Aut7p

445

Kim, J., V.M. Dalton, K.P. Eggerton, S.V. Scott, and D.J. Klionsky. 1999. A pg7p/Cvt2p is required for the cytoplasm-to-vacuole targeting, macroautophagy, and peroxisome degradation pathways. Mol. Biol. Cell. 10:1337–1351.
