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Apg16p is required for the function of the Apg12p–Apg5p conjugate in the yeast autophagy pathway

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Autophagy is an intracellular bulk degradation system that is ubiquitous for eukaryotic cells. In this process, cytoplasmic components are enclosed in autophagosomes and delivered to lysosomes/vacuoles. We recently found that a protein conjugation system, in which Apg12p is covalently attached to Apg5p, is indispensable for autophagy in yeast. Here, we describe a novel coiled-coil protein, Apg16p, essential for autophagy. Apg16p interacts with Apg12p-conjugated Apg5p and less preferentially with unconjugated Apg5p. Moreover, the coiled-coil domain of Apg16p mediates self-multimerization that leads to cross-linking of Apg5p molecules and formation of a stable protein complex. Apg16p is not essential for the Apg12p–Apg5p conjugation reaction. These results suggest that the Apg12p–Apg5p conjugate requires Apg16p to accomplish its role in the autophagy pathway, and Apg16p is a key molecule as a linker to form the Apg12p–Apg5p–Apg16p multimer.

Keywords: autophagy/coiled-coil/protein complex/protein conjugation/*Saccharomyces cerevisiae*

Introduction

Autophagy is an intracellular degradation system in which cytoplasmic components are sequestered to the lysosome/vacuole by a membrane-mediated process (Seglen and Bohley, 1992; Dunn, 1994). There are two major classes of autophagy: microautophagy and macroautophagy. Microautophagy is a process of incorporation of cytoplasmic components by invagination of the lysosomal/vacuolar membrane. In macroautophagy, cytoplasmic components are first enclosed in double membrane structures termed autophagosomes, and then delivered to the lysosome/vacuole to be degraded. Autophagy is a relatively non-selective bulk process. Cytoplasmic proteins, carbohydrates, lipids, nucleic acids and even organelles such as mitochondria and the endoplasmic reticulum are sequestered. In mammalian hepatocytes, autophagy is regulated by extracellular nutrients and hormones, and is suggested to be essential for cellular homeostasis (Mortimore and Pösö, 1987). It has also been reported to be responsible for various physiological processes such as degradation of phenobarbital-induced endoplasmic reticulum in hepatocytes (Bolender and Weibel, 1973; Masaki *et al*., 1987) and post-partum regression of luteal cells (Paavola, 1978) and of uterine smooth muscle cells (Henell *et al*., 1983). Therefore, autophagy has been thought to be crucial also for cellular remodeling, differentiation and removal of obsolete cellular components.

The molecular basis of the autophagic pathway has been poorly characterized in higher eukaryotes. We have used the yeast *Saccharomyces cerevisiae* as a model system since we found that macroautophagy in this yeast proceeds in a manner quite similar to that in animal cells (Takeshige *et al*., 1992; Baba *et al*., 1994). Taking advantage of yeast genetics, we isolated 14 autophagy-defective (*apg*) mutants (Tsukada and Ohsumi, 1993). Cloning of *APG* genes revealed that they were novel genes (Kametaka *et al*., 1996; Funakoshi *et al*., 1997; Matsuura *et al*., 1997), except *APG6*, which is allelic to *VPS30* involved in vacuolar protein sorting (Kametaka *et al*., 1998). The *apg* mutants partially overlap with *aut* mutants that are also based on defects in autophagy (Thumm *et al*., 1994; Schlumpberger *et al*., 1997; Straub *et al*., 1997; Lang *et al*., 1998).

These *apg* mutants have a common phenotype: (i) loss of bulk protein degradation during starvation; (ii) loss of viability during starvation; (iii) a defect in sporulation of the homozygous diploid; and (iv) normal vacuolar function (Tsukada and Ohsumi, 1993). A vacuolar enzyme, aminopeptidase I (API), is delivered from the cytoplasm to vacuoles constitutively by a non-classical vesicular mechanism to yield a mature active enzyme (Klionsky *et al*., 1992; Scott *et al*., 1997). This 'Cvt pathway' is distinct from but closely related to the autophagic pathway (Baba *et al*., 1997), and all the *apg* mutants also show defects in this pathway (Scott *et al*., 1996).

During the systematic characterization of Apg proteins, we discovered a new protein conjugation system essential for autophagy (Mizushima *et al*., 1998a). Apg12p, a 186 amino acid protein, is covalently attached to Apg5p, a 294 amino acid protein, through an isopeptide bond between the C-terminal glycine of Apg12p and Lys149 of Apg5p. This conjugation reaction requires ATP and two other factors, Apg7p and Apg10p. Apg7p shows significant homology to the ubiquitin-activating enzyme (E1), and indeed it was shown to be an Apg12p-activating enzyme (Kim *et al*., 1999; Tanida *et al*., 1999; Yuan *et al*., 1999). Apg10p is another conjugating enzyme (T.Shintani, N.Mizushima, Y.Ogawa, A.Matsuura, T.Noda and Y.Ohsumi, in preparation). These four Apg proteins (Apg5p, 7p, 10p and 12p) function together in the protein conjugation system. Although Apg12p does not have apparent homology to ubiquitin, the Apg12 conjugation system is similar to that of ubiquitination (Hochstrasser, 1996; Varshavsky, 1997; Ciechanover, 1998; Hershko and Ciechanover, 1998), Recently, these types of covalent modifications were discovered for other ubiquitin-like

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MGNFIITERKKAKEERSNPQTDSMDDLLIRRLTDRNDKEA   40
HLNELFQDNSGAIGGNIVSHDDALLNTLAILQKELKSKEQ   80
EIRRLKEVIALKNKNTERLNDELISGTIENNVLQQKLSDL  120
KKEHSQLVARWLKKTEKETEAMNSEIDGTK            150
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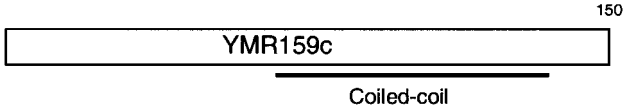

Fig. 1. Sequence and structure of YMR159c. (**A**) Amino acid sequence of YMR159c. (**B**) Structural analysis of YMR159c. The amino acid sequence was analyzed using the COILS program in the 28 residue window setting (obtained from http://www.isrec.isb-sib.ch/software/COILS_form.html) (Lupas *et al*., 1991). A putative coiled-coil region (probability of 1.0) was shown.

molecules such as SUMO-1 (Matunis *et al*., 1996; Kamitani *et al*., 1997; Mahajan *et al*., 1997), Smt3p (Johnson and Blobel, 1997; Johnson *et al*., 1997), NEDD8 (Osaka *et al*., 1998), Rub1p (Lammer *et al*., 1998; Liakopoulos *et al*., 1998), UCRP (Haas *et al*., 1987) and Fau (Olvera and Wool, 1993; Nakamura *et al*., 1995). Accordingly, the importance of protein conjugation systems has increasingly been recognized.

Since the Apg12 conjugation system is well conserved in human (Mizushima *et al*., 1998b), further analysis of the system will provide important insights into the molecular basis of autophagy in eukaryotes. In this report, we describe the cloning and characterization of a novel Apg protein, Apg16p, that binds to Apg5p and is required for Apg12p–Apg5p function.

Results

Two-hybrid screening with Apg12p identifies YMR159c

To identify proteins related to the Apg12p conjugation system, a yeast two-hybrid screening was carried out with Apg12p as bait. This screening was aimed at obtaining proteins that bind to Apg12p and/or the Apg12p–Apg5p conjugate. The two-hybrid system we used has three criteria for positive interaction: (i) growth on Ade$^-$ plates; (ii) growth on His$^-$ plates; and (iii) positive for β-galactosidase activity (James *et al*., 1996). Thirty four Ade$^+$ colonies were obtained from 2×10^7 transformants screened. Among them, 33 colonies were also His$^+$ and β-gal$^+$. DNA sequencing and restriction enzyme analysis revealed that 28 clones contained an entire open reading frame (ORF), YMR159c (Figure 1). Four contained only the N-terminal third of YMR159c. To test the specificity of the interaction, the prey plasmid containing the entire YMR159c and pGBD-APG12 or pGBD vector alone were co-transformed. Only the cells carrying both the prey plasmid and pGBD-APG12 grew on Ade$^-$ Trp$^-$ Leu$^-$ plates (see Figure 5) and His$^-$ Trp$^-$ Leu$^-$ plates [with 5 mM 3-amino-triazole (AT)], and were positive for β-gal activity (data not shown). These results suggest that the product of YMR159c specifically interacts with Apg12p.

APG16 is an essential gene for autophagy

YMR159c encodes a 17 kDa hydrophilic protein (150 amino acids) (Figure 1). It contains a coiled-coil motif

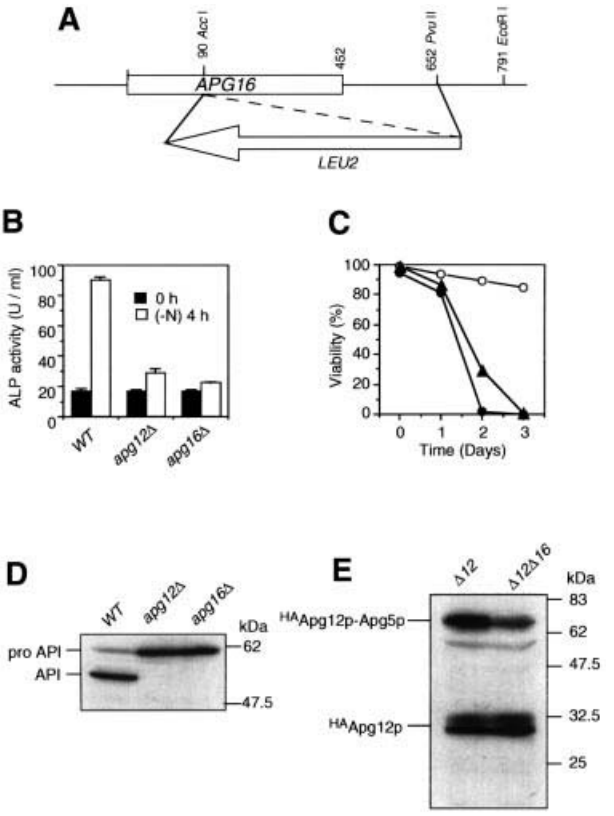

Fig. 2. Phenotype of the *apg16*Δ strain. (**A**) Disruption constructs of YMR159c (*APG16*). Disruption of YMR159c was achieved by insertion of the *LEU2* marker gene at the *Acc*I site or by replacing an *Acc*I–*Pvu*II fragment with the *LEU2* gene. (**B**) Autophagy-negative phenotype of the *apg16*Δ strain. The autophagic ability of wild-type (TN125), *apg12*Δ (YNM107) and *apg16*Δ cells (YNM114) was measured by the alkaline phosphatase assay before (black bars) and after (white bars) nitrogen starvation for 4 h. Error bars indicate the standard deviation of three independent experiments. (**C**) Loss of viability during starvation. Wild-type (KA311B, ○), *apg12*Δ (YNM101, ●) and *apg16*Δ cells (YNM124, ▲) were cultured in nitrogen starvation medium, and their viability was determined by phloxine B staining. (**D**) Defect in API maturation in *apg16*Δ cells. Transport of pro-API to the vacuole was examined by immunoblotting with anti-API antiserum. The positions of precursor and mature API are indicated. (**E**) The Apg12p–Apg5p conjugation in *apg16*Δ cells. Total lysates were prepared by the NaOH/2-mercaptoethanol extraction method from *apg12*Δ (YNM107) and *apg12*Δ*apg16*Δ cells (YNM115), both carrying pHA-APG12 (CEN). Western blot analysis was performed using anti-HA antibody.

at the C-terminal half. To determine the null phenotype of the YMR159c gene, we disrupted the ORF by insertion of the *LEU2* gene at the *Acc*I site in the KA31 diploid background (Figure 2A) The *YMR159/ymr159*Δ*::LEU2* diploid cells were allowed to sporulate and seven tetrads were dissected. All spores were viable and the segregation pattern of Leu$^+$ and Leu$^-$ was 2:2. We next examined their autophagic ability by detecting autophagic body accumulation (Takeshige *et al*., 1992). When cultured under nitrogen starvation conditions in the presence of phenylmethylsulfonyl fluoride (PMSF), autophagic bodies accumulated in the vacuoles of all the Leu$^-$ segregants as well as of wild-type cells. On the other hand, all the Leu$^+$ segregants showed no accumulation of autophagic bodies during starvation, indicating that they are autophagy negative. When we disrupted YMR159c by replacement of an *Acc*I–*Pvu*II fragment with the *LEU2* gene in KA31 haploid

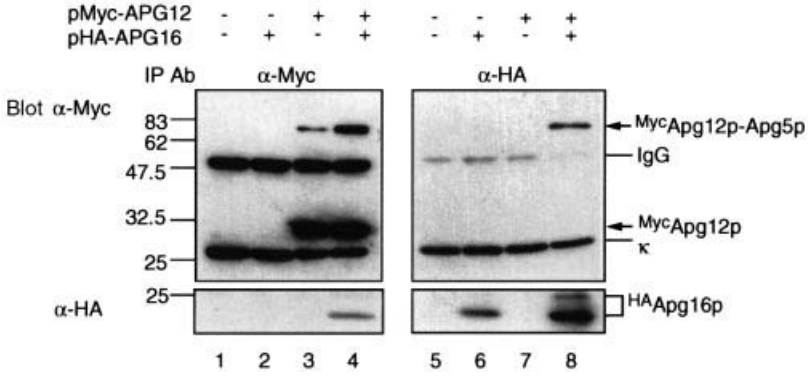

Fig. 3. Apg16p interacts with the Apg12p–Apg5p conjugate but not with the Apg12p monomer. The *apg12Δapg16Δ* cells (YNM115) were transformed with high copy (2μ) plasmids encoding ^{Myc}Apg12p and/or ^{HA}Apg16p as indicated. Total lysates were immunoprecipitated with anti-Myc (lanes 1–4) or anti-HA antibody (lanes 5–8) and detected by immunoblotting using anti-Myc or anti-HA antibody. The positions of the IgG heavy chain and κ light chain are indicated.

cells, similar results were obtained. We used the latter version of the disruptant (YNM124) for further analyses. The loss of autophagic activity was also confirmed by an assay system, in which a truncated form of pro-alkaline phosphatase (ALP) expressed in the cytoplasm was delivered to the vacuoles in an autophagy-dependent manner, and processed to an active enzyme (Noda *et al*., 1995). The *ymr159Δ pho8Δ60* strain (YNM114) showed no significant elevation of phosphatase activity after 4 h nitrogen starvation (Figure 2B). Thus, YMR159c turned out to be essential for autophagy. Since it is not allelic to the other *APG* genes, this gene was designated as *APG16*.

The *apg16Δ* cells grew normally in a nutrient-rich medium, YEPD (data not shown), but lost their viability during starvation (Figure 2C). The homozygous diploid cells of *apg16Δ* were defective in sporulation (data not shown). The vacuolar enzyme API is delivered from the cytoplasm to vacuoles to yield a mature active enzyme in a manner similar to macroautophagy (Klionsky *et al*., 1992; Baba *et al*., 1997; Scott *et al*., 1997). As with all the other *apg* mutants, the *apg16Δ* cells showed a complete defect in this pathway (Figure 2D). These results show that the *apg16Δ* strain shares the common characters with other *apg* mutants, and that *APG16* is a typical *APG* gene.

As we have shown previously, hemagglutinin (HA)-tagged Apg12p exists as two forms. One is a ^{HA}Apg12p monomer (31 kDa), and the other is covalently conjugated to Apg5p (the 70 kDa ^{HA}Apg12p–Apg5p conjugate). The conjugate is completely lost in the conjugation enzyme-deficient strains *apg7Δ* and *apg10Δ* (Mizushima *et al*., 1998a). In the *apg16Δ* cells, although it was reduced, the Apg12p–Apg5p conjugate was clearly detected (Figure 2E, and see also below), indicating that Apg16p is not essential for the conjugation reaction. Nonetheless, the *apg16Δ* cells showed complete defects in the Apg and Cvt pathways (Figure 2B and D). These results suggest that Apg16p functions after the conjugation reaction as an Apg12p- or Apg12p–Apg5p conjugate-interacting protein, rather than in the conjugation pathway as a conjugation-promoting factor.

Apg16p interacts with the Apg12p–Apg5p conjugate, but not with the Apg12p monomer

For biochemical analysis of Apg16p, we tagged Apg16p with three HA epitopes at the C-terminus. The C-terminal tagged Apg16p complemented the phenotype of the *apg16Δ* strain (data not shown). ^{HA}Apg16p was detected as a 21 kDa band (see Figure 7), a size compatible with the predicted molecular mass (17 kDa) plus the tagged HA peptide (40 amino acids). In addition, there was another faint band at 25 kDa. Both signals of 21 and 25 kDa are intensified in a strain overexpressing Apg16p (Figure 3, lane 8). It is possible that Apg16p is partially subjected to a post-translational modification, although we have not yet characterized this. The expression level and the band pattern were not significantly changed after 3 h of nitrogen starvation (data not shown).

To prove the interaction between Apg12p and Apg16p *in vivo*, co-immunoprecipitation analysis was performed. Total lysates of cells transformed with high copy (2μ) plasmids expressing ^{Myc}Apg12p and/or ^{HA}Apg16p from their own promotors were immunoprecipitated with anti-Myc or anti-HA antibody. In the precipitates with anti-Myc, ^{HA}Apg16p was co-precipitated specifically only when cells expressed both ^{Myc}Apg12p and ^{HA}Apg16p (Figure 3, lane 4). The 25 kDa Apg16p was also co-precipitated, but to a lesser extent. These results suggest that Apg12p interacts with Apg16p *in vivo*.

As mentioned above, the Apg12p–Apg5p conjugate was generated in the absence of Apg16p, but its amount was reduced. This reduction is observed more readily when Apg12p and Apg16p are overexpressed by 2μ plasmids (Figure 3, lane 3). In the immunoprecipitation analysis, only the Apg12p–Apg5p conjugate and not free Apg12p was co-precipitated by the anti-HA antibody (compare Figure 3, lane 4 with lane 8). These data suggest that Apg16p interacts with the Apg12p–Apg5p conjugate but not with the Apg12p monomer, and that Apg16p may stabilize the conjugate. The interaction between Apg16p and Apg12p–Apg5p was not affected by nitrogen starvation (data not shown).

Apg16p directly interacts with Apg5p

The immunoprecipitation analysis in Figure 3 indicates that Apg16p either interacts with Apg12p only when it is conjugated to Apg5p or interacts directly with Apg5p. Using the two-hybrid system, we examined the relationship between these Apg proteins. The two-hybrid interaction between Apg12p and Apg16p that is seen in the wild-type strain was not observed in an *apg5Δ* strain (Table I).

Table I. Two-hybrid interaction in *apg5*Δ and *apg*12Δ

BD	AD	Growth on Trp⁻ Leu⁻ Ade⁻		
		WT	*apg5*Δ	*apg12*Δ
APG12	APG16	+	–	+
APG12ΔG	APG16	–	n.d.	n.d.
APG5	APG12	+	+	+
APG5	APG16	+	+	+
APG16	APG16	+	+	+

n.d., not determined.

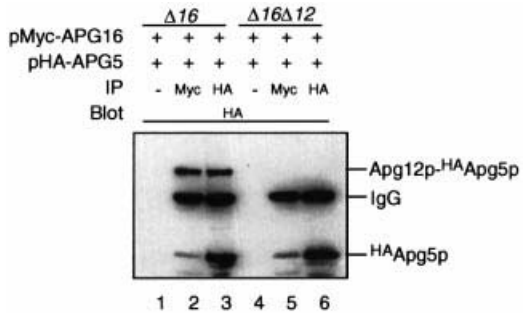

Fig. 4. Apg16p interacts preferentially with Apg12p-modified Apg5p. Total lysates were prepared from *apg16*Δ cells (YNM124) or *apg12*Δ*apg16*Δ cells (YNM115) harboring both pHA-APG5 and pMyc-APG16 CEN plasmids. The lysates were immunoprecipitated and analyzed as described in Figure 3.

The C-terminal glycine of Apg12p is essential for the Apg12p–Apg5p conjugation, since Apg12ΔGp in which the C-terminal glycine is deleted cannot be conjugated to Apg5p (Mizushima *et al*., 1998a). In the two-hybrid system, Apg16p did not interact with Apg12ΔGp. These results suggest that the interaction between Apg12p and Apg16p is indirect and depends on the conjugation of Apg12p to Apg5p. In agreement with these results, when we tested Apg16p and Apg5p, we detected a strong two-hybrid interaction (Table I). Furthermore, growth of the strains bearing the Apg16p and Apg5p two-hybrid plasmids was not affected by the absence of Apg12p. This indicates that Apg16p interacts directly with Apg5p. Strong interaction was detected between pGAD-APG16N and pGBD-APG12 or pGBD-APG5 (Figure 5A), suggesting that Apg5p associates with the N-terminal region of Apg16p and that their interaction does not require the coiled-coil domain.

Apg16p interacts preferentially with Apg12p-modified Apg5p

We next examined the *in vivo* interaction between Apg5p and Apg16p. ^{HA}Apg5p and ^{Myc}Apg16p were expressed by CEN plasmid (single copy) in *apg16*Δ cells. In these cells, about one-fifth of Apg5p was conjugated by Apg12p (Figure 4, lane 3). Immunoprecipitation with anti-Myc showed that both Apg5p monomer and Apg12-modified Apg5p were precipitated (Figure 4, lane 2), confirming that Apg16p interacts with Apg5p. However, it is noteworthy that only a very small portion of unconjugated Apg5p was brought down by anti-Myc antibody, and most of the Apg16p-interacting Apg5p was modified by Apg12p (Figure 4, compare the level of Apg5p in lanes 2 and 3). In *apg12*Δ*apg16*Δ cells in which Apg5p exists only as an unmodified form, a very small amount of Apg5p interacted with Apg16p (Figure 4, lanes 5 and 6). These data indicate that Apg16p associates preferentially with Apg12p-modified Apg5p and, without Apg12p modification, Apg16p interacts with Apg5p only weakly.

The coiled-coil domain of Apg16p mediates self-multimerization

As shown in Figure 5A, the interaction of Apg5p and Apg16p does not require the coiled-coil domain. Since coiled-coil structures are known to be involved in protein–protein interaction, we examined whether Apg16p interacts with protein(s) other than Apg5p through its coiled-coil region. Two-hybrid study revealed that Apg16p interacted with not only Apg5p, but also with Apg16p itself (Figure 5A and Table I). Full-length Apg16p interacted with the C-terminal half of Apg16p containing the coiled-coil region, but not with the N-terminal region (Figure 5A). The Apg16p–Apg16p interaction did not require Apg5p or Apg12p (Table I). These results suggest that the coiled-coil domain mediates self-multimerization of Apg16p.

To detect Apg16p multimerization *in vivo*, lysate from *agp16*Δ cells carrying both HA-APG16 and Myc-APG16 plasmids was subjected to immunoprecipitation analysis. As shown in Figure 5B, ^{HA}Apg16p was precipitated by anti-Myc antibody, indicating that Apg16p forms a multimer *in vivo*.

Apg16p cross-links the Apg12p–Apg5p conjugates

Because Apg16p forms a multivalent complex, we hypothesized that Apg16p may act as a linker, and Apg16p, Apg5p and Apg12p constitute a large protein complex. Cell lysate from *apg12*Δ cells expressing both ^{HA}Apg12p and ^{Myc}Apg12p was subjected to immunoprecipitation. In the immunoprecipitates with anti-Myc antibody, we detected ^{HA}Apg12p–Apg5p in addition to ^{Myc}Apg12p–Apg5p and ^{Myc}Apg12p (Figure 6A, lane 3). ^{HA}Apg12p monomer was not detected. Similarly, in the immunoprecipitates with anti-HA, ^{Myc}Apg12p–Apg5p but not ^{Myc}Apg12p was detected (data not shown). These results suggest that at least two Apg12p–Apg5p conjugates exist in the same complex. In contrast, ^{HA}Apg12p–Apg5p was not precipitated with anti-Myc antibody in the absence of Apg16p (Figure 6A, lane 4), indicating that the interaction between the HA- and Myc-tagged Apg12p–Apg5p conjugates is mediated by Apg16p (as illustrated in Figure 6A, lower panel).

Although Apg16p interacted preferentially with the Apg12p–Apg5p conjugate, it also interacted with unconjugated Apg5p (Figure 4). We thus examined whether unconjugated Apg5p is also included in the Apg16p-mediated complex. Cell lysate was prepared from cells expressing both ^{HA}Apg5p and ^{Myc}Apg12p, and immunoprecipitated. A small but significant amount of unconjugated Apg5p was precipitated with anti-Myc antibody (Figure 6B, lane 2). As expected, this was not observed in the absence of Apg16p (Figure 6B, lane 5). These results indicated that a portion of unconjugated Apg5p was assembled with conjugated Apg5p through Apg16p (Figure 6B, lower panel). Taken together, these results suggest that Apg16p functions as a linker to form the (Apg12p–)Apg5p–Apg16p multimeric complex.

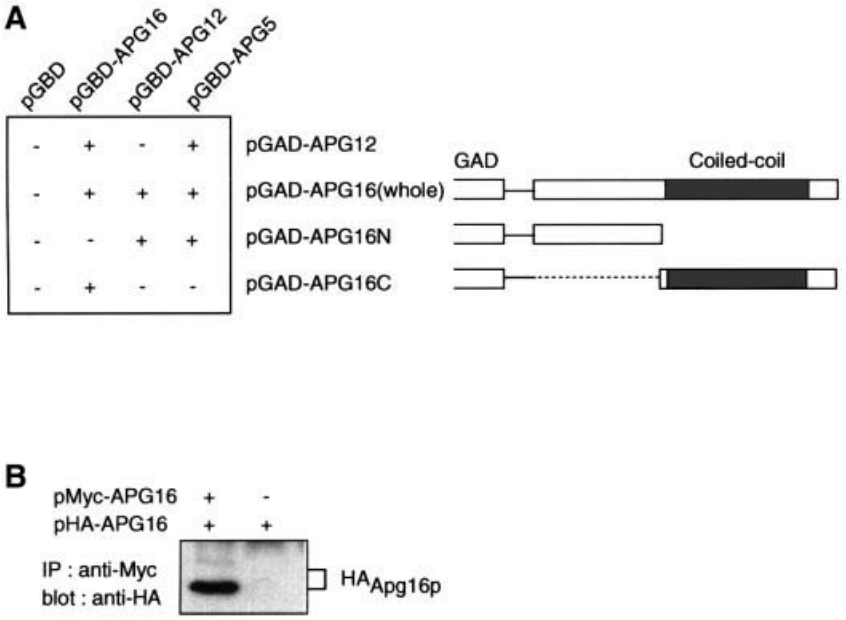

Fig. 5. The N-terminal region of Apg16p is required for interaction with Apg5p, and the C-terminal region is required for self-multimerization. (**A**) PJ69-4A cells were co-transformed with each pGBD and pGAD plasmid as indicated. Transformants were selected on Trp$^-$ Leu$^-$ plates, and then two-hybrid interaction (+ or –) was assessed for growth on Ade$^-$ Trp$^-$ Leu$^-$ plates. (**B**) Self-multimerization of Apg16p *in vivo*. Lysates of *apg16*Δ cells (YNM124) transformed with pMyc-APG16 (2μ) and/or pHA-APG16 (2μ) were immunoprecipitated with anti-Myc antibody and detected with anti-HA antibody.

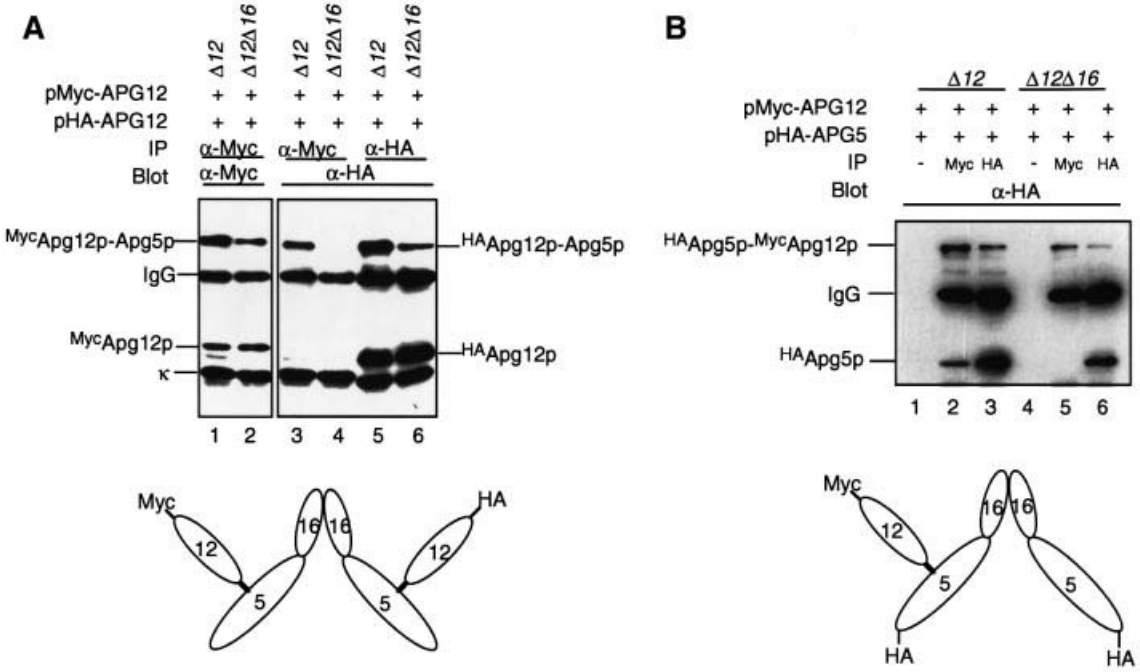

Fig. 6. Apg16p mediates Apg5p assembly. Immunoprecipitation was carried out as described in Figure 3, using *apg12*Δ (YNM107) and *apg12*Δ*apg16*Δ cells (YNM115) carrying pMyc-APG12 (CEN) and pHA-APG12 (CEN) (**A**), or pMyc-APG12 (CEN) and pHA-APG5 (CEN) (**B**). Following SDS–PAGE, immunoprecipitates were subjected to immunoblot analysis using anti-HA or anti-Myc antibody as indicated. A model of the Apg12p–Apg5p–Apg16p complex is illustrated in each panel. Apg16p forms a homo-oligomer through its C-terminal coiled-coil region. It is not clear whether Apg16p forms a dimer or a larger multimer. The N-terminal region of each Apg16p interacts with Apg5p; most of these complexes are conjugated to Apg12p (A) but a small population are unmodified (B). In the absence of Apg16p, separate Apg5ps do not assemble.

The Apg12p–Apg5p conjugate and Apg16p depend on each other for their expression level

As shown above (Figure 2E, Figure 3, lane 3, Figure 6A and B), the amount of the Apg12p–Apg5p conjugate was reduced in the absence of Apg16p, suggesting that the Apg12p–Apg5p conjugate is stabilized by interacting with Apg16p. In the immunoprecipitation analysis, the amount of Apg16p is also decreased in the absence of Apg12p (Figure 3, lane 6). To exclude the possibility that Apg16p was degraded during the immunoprecipitation procedures, we prepared the total lysates directly by the NaOH/2-mercaptoethanol extraction method from cells harboring

CEN plasmids, and examined the proteins by Western blot analysis. The amount of Apg16p was still reduced in Δ*apg5* cells (Figure 7A). Furthermore, it was also reduced in Δ*apg12* cells (Figure 7 B), suggesting that the stability of Apg16p depends on the Apg12p–Apg5p conjugate rather than on Apg5p itself. Together with the data in Figure 2E, these results show that the apparent steady-state levels of the Apg12p–Apg5p conjugate and Apg16p are co-dependent, supporting the idea that Apg16p interacts preferentially with Apg12p-modified Apg5p, resulting in formation of a stable complex.

Membrane association of Apg16 depends on Apg5p

Apg5p is a hydrophilic protein that is found in pelletable complexes (Mizushima *et al*., 1998a). Apg16p is also a hydrophilic protein but, because of its interaction with Apg5p, it may interact with membranes. We examined the subcellular localization of Apg5p and Apg16p by fractionation analysis. Total cell lysates were prepared in 0.2 M sorbitol, 20 mM triethanolamine (pH 7.2) and 1 mM EDTA, and were centrifuged at 100 000 *g* for 60 min to generate pellet and supernatant fractions. Unconjugated Apg5p existed mainly in the pellet fraction, with a low level found in the supernatant fraction (Figure 8A, Δ*5*). The Apg12p–Agp5p conjugate was detected exclusively in the pellet fraction. Most of the Apg16p was also in the pellet and a smaller amount was in the supernatant fraction (Figure 8B). It has been demonstrated that Apg5p is a peripheral membrane protein (M.D.George and D.J.Klionsky, in preparation). Thus, these findings suggested that Apg16p also exists mostly on the same membrane structures. These fractionation patterns were not affected significantly by nitrogen starvation (data not shown).

As shown in Figure 8A, both the Apg12p–Apg5p conjugate and Apg5p monomer were still pelletable in Δ*apg16* cells (Figure 8A, Δ*5*Δ*16*). In contrast, most of the Apg16p shifted to the soluble fraction in Δ*apg5* cells (data not shown). Since the amount of Apg16p was reduced in Δ*apg5* cells (as in Figure 7B), we fractionated the lysates of cells expressing these proteins from 2μ plasmids. Similarly to the results with centromeric expression, when Apg5p was overexpressed, overexpressed Apg16p still localized to the pellet fraction (Figure 8C). However, when Apg5p was absent, most of the Apg16p was recovered in the supernatant fraction, suggesting that membrane binding of Apg16p is Apg5p dependent.

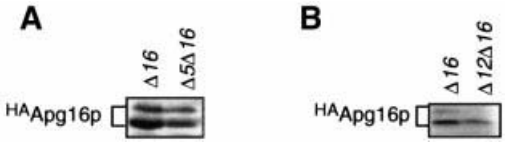

Fig. 7. Expression levels of Apg16p are dependent on the Apg12p–Apg5p conjugate. Total lysates were prepared by the NaOH/2-mercaptoethanol extraction method from *apg16*Δ (YNM124), *apg5*Δ*apg16*Δ (YNM126) and *apg12*Δ*apg16*Δ cells (YNM115) carrying pHA-APG16 (CEN). Western blot analysis was performed using anti-HA antibody.

Discussion

By our previous morphological screening for macroautophagy-defective mutants, 14 *apg* mutants (*apg*7 and *apg*11 are allelic) were isolated. Here we identify a novel *APG* gene, *APG16*. The studies on the *apg16*Δ strain showed that it demonstrates the typical Apg$^-$ phenotype: negative for autophagic body accumulation, loss of viability during starvation, and defects in sporulation and API maturation. Apg16p is the first Apg protein identified after the mutant screening. This apparently indicates that our former screening was not saturated. We now have 15 *APG* genes, but there might be additional genes involved in autophagy.

The amino acid sequence of Apg16p revealed that it has a coiled-coil motif encompassing most of its C-terminal half, but no other known characteristic sequences. BLAST searches have failed to identify any proteins with significant homology in other species. Previously, Apg16p was proposed as a yeast Sap18 counterpart, only because it has a

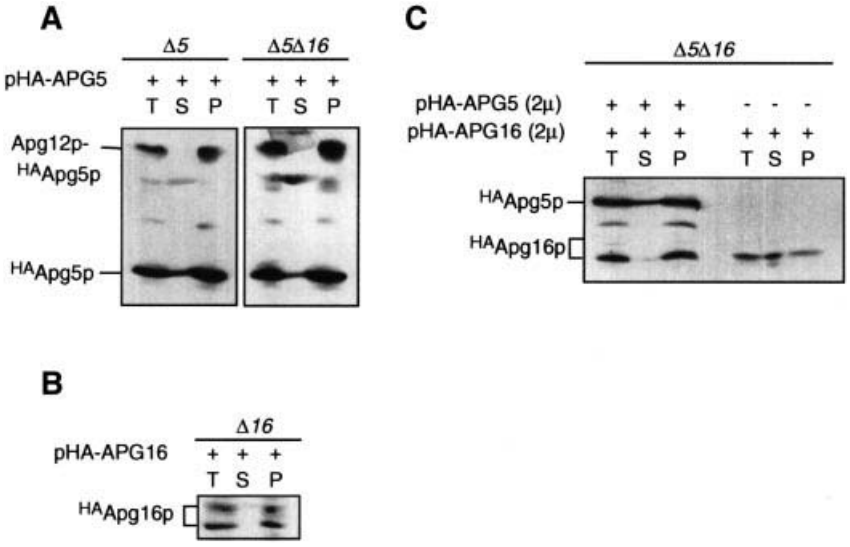

Fig. 8. Apg16p exists in a pellet fraction in an Apg5p-dependent manner. Spheroplasts were generated from cells harboring pHA-APG5 (**A**), pHA-APG16 (**B**) or pHA-APG16 (2μ) with or without pHA-APG5 (2μ) (**C**). They were lysed by Dounce homogenization. After unbroken spheroplasts were removed by low-speed centrifugation, the supernatant (T) was fractionated by 100 000 *g* centrifugation for 60 min to generate a supernatant (S) and pellet (P) fraction. Each fraction was subjected to immunoblot analysis using anti-HA antibody.

weak homology to human Sap18 (16.1% identity) (Zhang *et al*., 1997). In human cells, Sap18 is a component of a Sin3-containing complex which contains histone deacetylases, HDAC1 and HDAC2. It has been thought that histone deacetylation leads to transcription repression. Sap18 interacts with Sin3 and enhances Sin3-mediated repression of transcription (Zhang *et al*., 1997). However, our data argue against this proposal. First, Sap18 is suggested to be responsible for general transcription events, whereas our *apg16*Δ strain showed a specific defect in autophagy. Second, if Apg16p is a functional Sap18 homolog, its function would be Sin3p-mediated. However, autophagy proceeded normally in a *sin3* disruptant (data not shown). Third, Apg16p is a coiled-coil protein but the human Sap18 is not. Therefore, we believe that this protein is not a functional counterpart of human Sap18 in yeast, and the nomenclature 'Apg16p' is appropriate.

Although Apg16p was isolated originally as an Apg12p-interacting protein by a two-hybrid screening, the interaction between Apg12p and Apg16p turned out to be indirect and mediated by Apg5p (Table I). Further two-hybrid and immunoprecipitation analyses strongly suggest that Apg16p interacts directly with Apg5p. However, we cannot rule out the possibility that other proteins may mediate the interaction between Apg5p and Apg16p.

Is Apg16p an enzyme that acts at the last step of the conjugation reaction like ubiquitin ligases? This is unlikely because a significant amount of the Apg12p–Apg5p conjugate is generated in *apg16*Δ cells, although the level is reduced (Figure 2E). We have observed that cells with a smaller amount of the conjugate can proceed with autophagy (Mizushima *et al*., 1998a). Thus, the decrease of the conjugate seen in the *apg16*Δ strain cannot account for the complete block of both Apg and Cvt pathways. Instead, the data suggest that without Apg16p, Apg5p cannot function even if it is conjugated to Apg12p. Taken together, we conclude that Apg16p functions as an essential partner of the Apg12p–Apg5p conjugate, but not in the conjugation reaction.

Further studies revealed that most of the Apg5p interacting with Apg16p is conjugated to Apg12p. Thus, there is a preferential interaction between the Apg12p–Apg5p conjugate and Apg16p at steady state. A possible explanation is that Apg5p first interacts with Apg16p, and this accelerates the Apg12p conjugation. This may account for the decrease in the amount of the Apg12p–Apg5p conjugate in the *apg16*Δ cells (Figure 8A). However, we consider this is less likely because the amount of unconjugated Apg5p that interacts with Apg16p remains very small even in the absence of Apg12p.

Alternatively, we prefer the model where Apg12p modification of Apg5p facilitates Apg16p recruitment. Generally, post-translational modification by protein conjugation alters metabolic stability, complex formation or subcellular localization of target proteins. Ubiquitination of cellular proteins targets them to a large multisubunit protease, the 26S proteasome, for degradation (Hochstrasser, 1996; Varshavsky, 1997; Ciechanover, 1998; Hershko and Ciechanover, 1998). Substrate recognition by the proteasome is thought to be mediated by ubiquitin receptors (S5a in human, Rpn10p in yeast). However, the interaction between Apg12p–Apg5p and Apg16p is different from that of ubiquitinated protein and ubiquitin receptors. S5a binds to polyubiquitin chains but does not recognize the proteins (Deveraux *et al*., 1994), whereas Apg16p directly binds to Apg5p although the binding is inefficient without Apg12p modification. Another well characterized system is that of SUMO-1 conjugation. The major SUMO-1 substrate in mammalian cells is Ran GTPase-activating protein, Ran-GAP1. SUMO-1-modification of Ran-GAP1 targets the otherwise cytosolic protein to the nuclear pore complex, where it forms a stable complex with RanBP2 (Matunis *et al*., 1996; Kamitani *et al*., 1997; Mahajan *et al*., 1997). Binding to RanBP2 is specific for SUMO-1-modified Ran-GAP1 (Matunis *et al*., 1998). It is suggested that SUMO-1 modification exposes or creates a RanBP2-binding site in Ran-GAP1. Since Apg16p interaction is preferential for Apg12p-modified Apg5p, a similar mechanism may be involved.

An interesting feature of Apg16p is its ability to form a homo-oligomer through its coiled-coil region. Proteins with coiled-coil segments usually form homo- or heterodimers, but recent structural analysis disclosed that in these cases there are three, four or five helices (Lupas, 1996). We have not determined whether Apg16p forms a dimer or a larger multimer. While each Apg16p associates with (Apg12p–)Apg5p, its multimerization confers the capacity to cross-link (Apg12p–)Apg5p. As shown in Figure 7, two or more (Apg12p–)Apg5ps are involved in a protein complex, and this depends on Apg16p. In the *apg16*Δ cells, individual Apg5ps are not assembled. Apg16p thus is a key molecule in constitution of the Apg12p–Apg5p–Apg16p complex. This is in agreement with our observation that the levels of the Apg12p–Apg5p conjugate and Apg16p depend on each other.

Where does the Apg12p–Apg5p–Apg16p complex function? All *apg* mutants do not accumulate autophagosomes in the cytoplasm under starvation conditions (M.Baba and Y.Ohsumi, unpublished data). This means that Apg proteins act at or before the step of autophagosome formation, such as starvation sensing and signal transduction. We have suggested that Apg5p is a membrane-associating protein since it is pelletable (Mizushima *et al*., 1998a). Apg16p also exists in a P100 fraction in an Apg5p-dependent manner. These data suggest that multivalent Apg16 proteins bind to and cross-link (Apg12p–)Apg5p on some membrane structures. Recently, direct evidence was obtained showing that Apg5p is a peripheral membrane protein and is required for the step of vesicle formation/completion in the Cvt pathway (M.D.George and D.J.Klionsky, in preparation). We thus speculate that the Apg12p–Apg5p–Apg16p complex functions in the step of autophagosome formation and/or completion. Morphological analysis to examine the precise localization of this complex, whether it is on the autophagosome, its precursor or other structures, is in progress.

Materials and methods

Yeast strains and media

Saccharomyces cerevisiae strains used in this study are listed in Table II. Cells were grown either in YPD medium (1% yeast extract, 2% peptone and 2% glucose) or in SD medium containing nutritional supplements. For nitrogen starvation, SD(–N) medium (0.17% yeast nitrogen base without amino acid and ammonium sulfate and 2% glucose) was used.

Plasmid construction

For two-hybrid screening, the bait plasmid (pGBD-APG12) encoding Apg12p fused in-frame to the C-terminal end of the Gal4p DNA-binding

Table II. Strains used in this study

Strain	Genotype	Source
KA311B	*MAT*α *ura3 leu2 his3 trp1*	Irie *et al.* (1993)
YNM105	*MAT*α *ura3 leu2 his3 trp1 apg16Δ::LEU2*	this study
YNM124	*MAT*α *ura3 leu2 his3 trp1 apg16Δ::LEU2*	this study
YNM101	*MAT*α *ura3 leu2 his3 trp1 apg12Δ::HIS3*	Mizushima *et al*. (1998a)
YNM115	*MAT*α *ura3 leu2 his3 trp1 apg16Δ::LEU2 apg12Δ::HIS3*	this study
YNM126	*MAT*α *ura3 leu2 his3 trp1 apg5Δ::HIS3 apg16Δ::LEU2*	this study
TN125	*MAT*a *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 pho8::pho8Δ60*	Noda *et al.* (1998)
YNM114	*MAT*a *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 pho8::pho8Δ60 apg16Δ::LEU2*	this study
YNM107	*MAT*a *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 pho8::pho8Δ60 apg12Δ::HIS3*	Mizushima *et al*. (1998a)
PJ69-4A	*MAT*a *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James *et al*. (1996)
YNM120	*MAT*a *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ apg5Δ::HIS3*	this study
YNM116	*MAT*a *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ apg12Δ::HIS3*	this study

domain was constructed as follows: the entire *APG12* ORF was PCR-amplified and inserted into the *Pst*I site of pGBD-C1 (James *et al*., 1996). pGAD-APG16 is the original plasmid in the pGAD-C1 vector containing the entire YMR159c gene isolated in the two-hybrid screening. Parts of *APG16* encoding the N-terminal half (amino acids 1–87) and the C-terminal half (88–150) were amplified by PCR and inserted into the *Eco*RI site of pGAD-C1 to generate pGAD-APG16N and pGAD-APG16C, respectively. pGBD-APG5 was created by subcloning of the entire *APG5* coding sequence into the *Bam*HI and *Pst*I sites of pGBD-C1. pGBD-APG16 and pGAD-APG12 contain the same insert sequences as pGAD-APG16 and pGBD-APG12, respectively. A genomic DNA fragment containing *APG16* was amplified by PCR and was cloned into the *Bam*HI and *Xho*I sites of pRS314, whose *Not*I site was destroyed. A *Not*I site was created immediately before the stop codon, where 3× HA or 3× Myc epitopes were introduced to generate pHA-APG16(316) and pMyc-APG16(316). Their 1.5 kb *Spe*I–*Xho*I fragments were also cloned into the same sites in pRS316, and in 2μ vectors pRS426 and pRS424. The plasmids encoding 3×Myc-Apg12p (pMyc-APG12) and 1×HA-Apg5p (pHA-APG5) were described previously (Mizushima *et al*., 1998a).

Two-hybrid analysis

The two-hybrid screening was performed using the system described by James *et al*. (1996). The strain PJ69-4A was transformed sequentially with pGBD-APG12 and a mixture of yeast genomic two-hybrid libraries fused to the GAL activation domain (Y2HLA-C1, -C2 and -C3) (James *et al*., 1996). Transformants were selected for growth on Ade^- Trp^- Leu^- plates. Transformation efficiency was estimated by counting the number of Trp^+ Leu^+ colonies. Ade^+ colonies were tested for growth on His^- Trp^- Leu^- plates containing 5 mM 3-AT, and for β-galactosidase activity by colony lifting assay. Yeast DNA was recovered from Ade^+ His^+ gal^+ colonies and transformed into *Escherichia coli*. Prey plasmids containing genomic DNA clones were identified by restriction mapping and characterized further by DNA sequencing. Positive interaction was verified by co-transformation of pGBD-APG12 and each of the recovered prey plasmids. Subsequent two-hybrid interaction analyses were carried out by co-transformation of each pGBD and pGAD plasmid into PJ69-4A or its mutant (*apg12*Δ or *apg5*Δ). Transformants were selected on Trp^- Leu^- plates, and then tested for growth on Ade^- Trp^- Leu^- plates.

Immunoprecipitation

Yeast cells were grown in appropriate medium to early log phase, and 200 OD_{600} cells were harvested. They were resuspended in 1 ml of a lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 2 μg/ml pepstatin and 1× protease inhibitor cocktail (Complete™, Boehringer Mannheim)]. Cells were disrupted with 0.5 volumes of glass beads by vortexing for 10 min at 4°C. After removal of cell debris by centrifugation at 3000 *g* for 5 min, total cell lysate was obtained. To each sample, 0.1 volume of 10% NP-40 was added. The samples were pre-cleared with 5 μl of protein G–Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) for 1 h at 4°C. Then, aliquots (~5 mg of protein) were incubated with or without 1 μl of monoclonal anti-Myc antibody (9E10) or anti-HA antibody (16B12, BAbCo) for 2 h. To each sample, 5 μl of protein G–Sepharose 4 Fast Flow was added and then the samples were incubated for an additional 2 h. The Sepharose beads were washed three times with the lysis buffer and the bound proteins were eluted with 50 μl of SDS–PAGE sample buffer. Samples (10 μl) were analyzed by SDS–PAGE and immunoblotting.

Immunoblot analysis

For Western analysis, the NaOH/2-mercaptoethanol extraction protocol was used with slight modification (Horvath and Reizman, 1994). Ten OD_{600} cells were resuspended directly in 0.2 M NaOH and 0.5% 2-mercaptoethanol and incubated for 15 min on ice. A 1 ml aliquot of cold acetone was added to each sample and then these were incubated at –20°C for 1 h. After centrifugation at 15 000 *g* for 5 min, the pellets were resuspended in 200 μl of SDS sample buffer and boiled for 5 min. Cell lysates (10 μl) were separated by SDS–PAGE and were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). Immunoblot analysis was performed with mouse monoclonal anti-HA antibody (16B12), anti-Myc antibody (9E10) or anti-API (gift from Dr Klionsky), and developed by an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

Differential centrifugation

Cells were grown to an OD_{600} of 1.0. Fifty OD_{600} units of cells were collected and spheroplasts were generated in a nutrient-rich medium, YEPD-SPM, as described previously (Kametaka *et al*., 1998). Cells were lysed by douncing in 1 ml of cold buffer containing 0.2 M sorbitol, 20 mM triethanolamine (pH 7.2), 1 mM EDTA, 1 mM PMSF, 2 μg/ml pepstatin and 1× Complete™. After centrifugation at 500 *g* for 5 min, the supernatant (total lysate) was centrifuged at 100 000 *g* for 60 min to generate a pellet and supernatant. Each fraction was subjected to SDS–PAGE and immunoblotting using anti-HA antibody (16B12).

Other methods

To monitor the autophagic activity, the ALP assay was performed as described previously (Noda and Ohsumi, 1998). Cell viability was determined by phloxine B staining (Tsukada and Ohsumi, 1993).

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