Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*

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Autophagy in the yeast is similar to that in mammalian cells. A mutant designated as *apg1* (autophagy) defective in accumulation of autophagic bodies in the vacuoles was isolated by selection using a light microscope from a mutagenized proteinase-deficient strain. In the *apg1* strain, which has normal vacuolar proteinases, nitrogen starvation did not induce protein degradation. The *apg1* mutant lost its viability faster than wild-type cells during nitrogen starvation. By using the loss of viability as a first screening test, 75 other *apg* mutants were selected. These *apg* mutants including *apg1* fell into 15 complementation groups. Genetic analyses of representative *apg* mutants revealed that they all had single recessive chromosomal mutations. Strains with each *apg* mutation were defective in protein degradation in the vacuoles induced by nitrogen starvation and homozygous diploids for each *apg* mutation did not sporulate. These results on the *apg* mutants suggest that autophagy via autophagic bodies is indispensable for protein degradation in the vacuoles under starvation conditions, and that at least 15 *APG* genes are involved in autophagy in yeast.

Yeast; Autophagy; Autophagic body; Vacuole; Protein degradation

1. INTRODUCTION

Recently, protein degradation has been found to be involved in various cellular functions. The lysosomal/vacuolar pathway is believed to be a non-selective pathway for bulk turnover of cytoplasmic components, primarily mediated by autophagy [1]. Autophagy has mainly been studied in mammalian cells by electron microscopy, and has been shown to be a general response of the cells to nutrient deprivation. During nutrient starvation, intracellular organelles and cytosol are sequestered in autophagosomes which subsequently fuse with lysosomes and are degraded within auto- phagolysosomes [2]. In mammalian cells, a lysosomal endomembrane system is highly developed, but it is too complicated to characterize autophagy biochemically. Various inhibitors have provided information on individual steps of the autophagic process, but many aspects of the mechanism and regulation of autophagy are still unknown.

Recently, we reported that a similar autophagic process to that in mammalian cell takes place in the yeast *Saccharomyces cerevisiae* [3]. Yeast cells lacking vacuolar proteinases accumulate spherical membrane structures named autophagic bodies (AB's) in vacuoles when transferred from a nutrient medium to various nutrient- deficient media. During incubation in nutrient-deficient media, AB's gradually increase in number and finally fill the vacuoles. Electron microscopic examination and biochemical analyses showed that AB's sequestered cytosolic components non-selectively in the vacuoles.

This yeast system has several advantages for studying autophagy at a molecular level. First, the autophagic process in yeast takes place synchronously in a homogeneous culture. Second, using a proteinase-deficient strain or the wild-type in the presence of PMSF, the autophagolysosomal stage can easily be determined on the basis of the extent of accumulation of AB's in the vacuoles. Third, with this system it is easy to apply classical and molecular genetic procedures. Isolation of mutants is an effective method for identifying individual steps in a complex pathway and has been used in studies on the cell division cycle [4] and the secretory pathway [5]. Thus with this system, the signal transduction and intracellular membrane flow involved in the autophagic process could be clarified in detail.

Here we report the first genetical studies on autophagy in yeast. Fifteen mutants showing defective accumulation of AB's in vacuoles in starvation conditions were isolated.

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Abbreviations: AB, autophagic body; EMS, ethyl methanesulfonate; LY, Lucifer yellow CH; PMSF, phenylmethylsulfonyl fluoride; PrA, proteinase A; PrB, proteinase B; TCA, trichloroacetic acid.

2. MATERIALS AND METHODS

2.1. Strains and media

Strains X2180-1A (*MATa SUC2 mal mel gal2 CUP1*), X2180-1B (*MATα SUC2 mal mel gal2 CUP1*), BJ3505 (*MATa pep4::HIS3 prb-Δ1.6R*) and BJ3501 (*MATα pep4::HIS3 prb-Δ1.6R*), constructed by

E.W. Jones [6] were from the Yeast Genetics Stock Center, Berkeley. The compositions of YEPD and the synthetic media [SD, SD(-N), SG, and SG(-N)] used were described in the previous paper [3]. For some YEP media, glucose was replaced by 2% potassium acetate, 2% ethanol and 3% glycerol. S (-C) was 0.67% dehydrated yeast nitrogen base. Solid media were prepared by adding 2% agar.

2.2. Isolation of mutants defective in autophagy

Two procedures for isolation of mutants were used. In the first, mutants that did not accumulate AB's in the vacuoles under carbon starvation conditions were isolated based on their morphological appearance. BJ3505 cells were grown in YEPD to stationary phase, and then suspended in 340 μl of 0.1 M sodium phosphate buffer (pH 7.0) and treated with 10 μl EMS for 60 min at 30°C; the survival rate was 40–50%. The mutagenized culture (20 μl) was diluted with 780 μl of 5% sodium thiosulfate and spread on YEPD plates. After 3 days culture at 30°C, the colonies were transferred to YEPD and SG plates with a sterile tooth-pick. After 3–4 h incubation on SG plates, cells in each colony were examined under a light microscope and those that had not accumulated AB's in the vacuoles were selected. These cells were grown to exponential phase in YEPD and transferred to liquid carbon starvation medium. At intervals of several hours, cells were observed by light microscopy and cells defective in accumulation of AB's were finally selected.

The second procedure for isolation of mutants was as follows. X2180-1A cells in stationary phase were mutagenized as described above. The mutagenized cells were grown in YEPD overnight at 30°C, diluted and spread on YEPD plates. After 3 days incubation at 30°C, colonies were replica-plated onto SD (-N) plates containing 20 μg/ml of phloxine B and incubated for 3–4 days at 30°C. Colonies that stained red were selected, grown again on YEPD plates and then transferred to liquid SD (-N) medium containing 1 mM PMSF. After 3–4 h incubation, cells were observed by light microscopy to select those that did not accumulate AB's in the vacuoles.

2.3. Measurement of protein degradation

Cells grown to logarithmic phase (2–4 × 10⁷ cells/ml) were collected by centrifugation and suspended in SD medium containing [¹⁴C]leucine at a final concentration of 0.011 MBq/ml. The cultures were labeled for 60–80 min and were chased for one further generation by adding 2 vols. of YEPD. The cells were then washed twice with starvation medium and incubated in starvation medium with or without 1 mM PMSF (in ethanol). At appropriate times, aliquots of the culture (100 μl) were withdrawn, mixed with 1 ml of 11% TCA and kept at 4°C overnight. The suspensions were then centrifuged at 12,500 × g for 10 min and the radioactivity of the supernatant was determined with Sintisol 500 (Dojin, Japan) in a Beckman LS 5800 liquid scintillation counter. Results were expressed as percentages of the initial total cellular radioactivity.

2.4. Determination of cell viability

Cultures (5 μl) were mounted on slide glasses, and then 0.5 μl of phloxine B (200 μg/ml) was added. The mixtures were examined by fluorescence microscopy with a blue filter. Brightly fluorescent cells were counted as dead cells. Similar results were obtained by determination of colony-forming ability on YEPD plates.

2.5. Vital staining

For quinacrine labeling, exponentially growing cells were transferred to an Eppendorf tube and collected by centrifugation. Quinacrine was added at a final concentration of 100 μM in 50 mM sodium phosphate buffer, pH 7.5 containing 2% glucose. After 5 min incubation at 30°C, the cells were collected by centrifugation, mounted on a microscope slide glass without washing and examined by fluorescence microscopy with a blue filter.

Cells were stained with Lucifer yellow CH (LY) as described by Riezman [7]. Cells of logarithmic phase (1–2 × 10⁷ cells/ml) in YEPD were incubated with 4 mg/ml LY at 30°C for 0.5–2 h and then examined by fluorescence microscopy with a blue filter.

2.6. Reagents

PMSF, phloxine B, and quinacrine were purchased from Sigma Chemical Co. (St. Louis, MO). Lucifer yellow CH was from Aldrich Chem. Co. (Milwaukee, WI). Aniline blue was from Chroma (Köngen, Germany). [¹⁴C]Leucine (9287 MBq/mmol) was from ICN Radiochemicals (Irvine, CA).

3. RESULTS

3.1. Isolation of mutants defective in accumulation of AB's by light microscopy

Various nutrient depletion induces autophagy in yeast cells. Cells lacking vacuolar proteinases accumulate autophagic bodies (AB's) in the vacuoles when transferred from a nutrient medium to various nutrient deficient media. The wild-type strain shows scarcely any
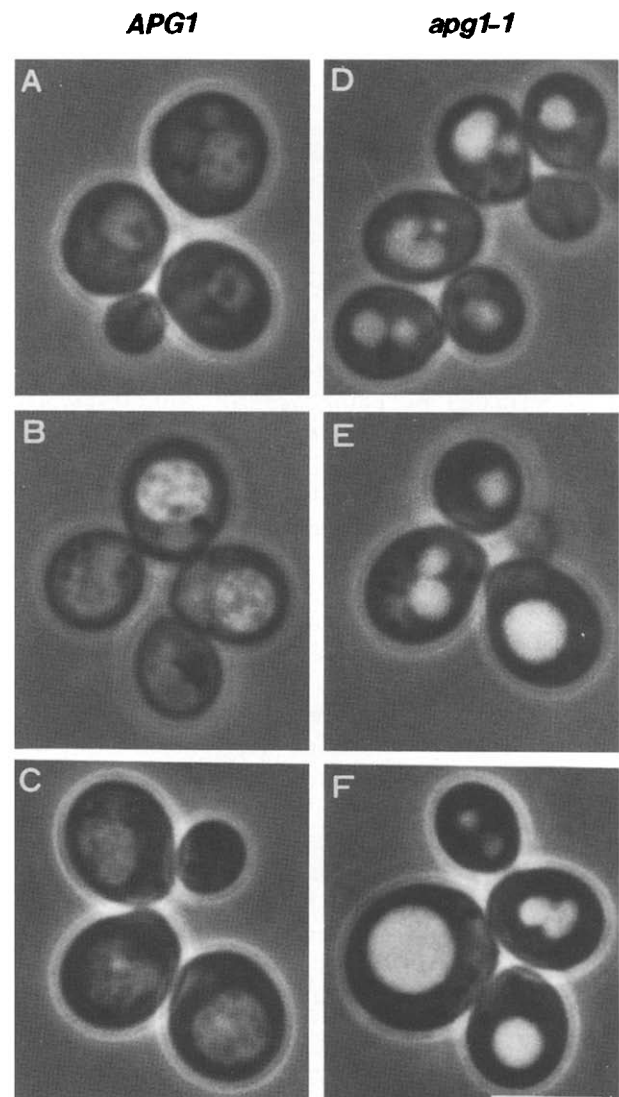

APG1   **apg1-1**

Fig. 1. Morphological changes of vacuoles during nitrogen starvation. Phase contrast microscopic images of cells of X2180-1A (*APG1*, left), and MT14-1B (*apg1-1*, right) incubated in SD (-N) medium containing 1 mM PMSF for 2 h (A,D), 4 h (B,E), and 8 h (C,F). Bar = 5 μm.

accumulation of AB's, probably because AB's are rapidly degraded by vacuolar hydrolases. However, in the presence of PMSF, wild-type cells accumulate AB's like vacuolar proteinase-deficient mutants.

The autophagolysosomal stage of yeast can be recognized under a light microscope by the accumulation of AB's in the vacuoles. Therefore, we attempted to isolate mutants defective in the autophagic process on the basis of the morphology of their vacuoles. A multiple proteinase-deficient strain, BJ3505, was mutagenized and cells that did not accumulate AB's in the vacuoles under starvation conditions were selected as described in section 2. We used SG medium, which is carbon deficient for BJ3505 [3] as starvation medium, because the vacuoles maintain a spherical shape and so detection of AB's in the vacuoles is easier in this medium than in nitrogen starvation medium.

By morphological screening of about 5000 mutagenized colonies, ten candidates were obtained and were crossed with BJ3501. The resulting diploids accumulated AB's in the vacuoles in SG medium, suggesting that all 10 mutations were recessive. Next, they were crossed with X2180-1B, the resulting diploids were sporulated and the resulting tetrads were dissected. Segregants were tested for accumulation of AB's in the presence of PMSF. In two mutants, the phenotype lacking accumulation of AB's segregated 2:2. Complementation analysis revealed that these two mutations were allelic. These two mutations were designated as apg1-1 and apg1-2 (for autophagy). The original apg1-1 mutant was crossed with X2180-1A or 1B four times to obtain a strain that was mostly isogenic to X2180-1A. The final
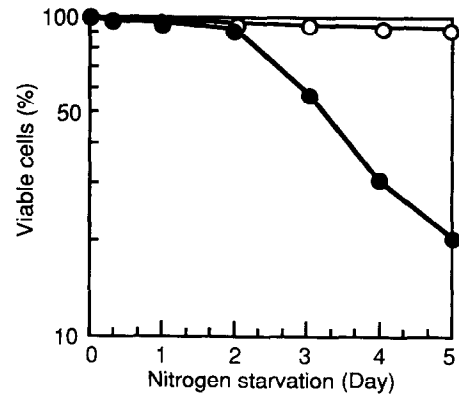
Fig. 3. Loss of viability of the apg1 mutant in nitrogen starvation medium. Exponentially growing cells of X2180-1A (APG1, o), and MT14-1B (apg1-1, ●) were transferred to SD (-N) medium. At the indicated times, viability was determined as described in section 2. 400–600 cells were counted at each time point. Data are averages of 3 determinations.

strain obtained, MT14-1B (MATa apg1-1) was characterized further.

3.2. Characterization of the apg1 mutant

X2180-1A and MT14-1B grown in YEPD were transferred to nitrogen starvation medium containing 1 mM PMSF, and after incubation for 2, 4 and 8 h, cells were observed by light microscopy. Accumulation of AB's in the vacuoles was observed in almost all wild-type cells and these AB's increased in number during 4–5 h incubation (Fig. 1A,B,C). The apg1-1 mutant scarcely accumulated any AB's during incubation for 8 h (Fig. 1D,E,F). Even after prolonged incubation for 24 h in SD (-N) medium containing PMSF, AB's were not detected in the apg1 cells. These results indicate that formation of AB's during nitrogen starvation was also blocked by the apg1-1 mutation.

Next, protein degradation induced by nitrogen starvation was examined. Total stable cellular proteins were labeled in SD medium containing [^{14}C]leucine. Protein degradation in vivo was determined as described in section 2. When the labeled wild-type cells were transferred to the starvation medium, radioactivity in the TCA-soluble fraction increased significantly. The rate of protein turnover was calculated to be about 1.3% of the total protein/h (data not shown). This protein degradation was induced by the starvation, because, when cells were transferred to SD medium, the radioactivity in the TCA-soluble fraction was negligible. Addition of 1 mM PMSF to the starvation medium suppressed about 60% of the protein degradation. The amount of degradation inhibited by addition of PMSF represents the amount of PMSF-sensitive protein degradation.

Using tetrads obtained from a heterozygous diploid (APG1/apg1-1), we examined the relationship between apg1 mutation and the rate of protein degradation. In every tetrad, two segregants showed high levels of pro-
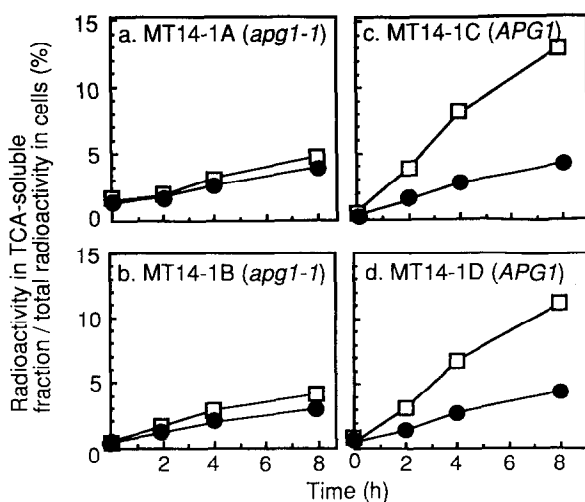

Fig. 2. Protein degradation in cells in nitrogen starvation medium. Radioactivities in the TCA-soluble fraction and in total cells were measured as described in section 2. With (●) and without 1 mM PMSF (□). (a) MT14-1A (apg1-1), (b) MT14-1B (apg1-1), (c) MT14-1C (APG1), and (d) MT14-1D (APG1) are segregants from X2180-1A/MT13-8C (APG1/apg1-1).

tein degradation comparable to that of X2180-1A cells, but two segregants showed low levels of protein degradation. Protein degradation in the former cells was depressed in the presence of PMSF, but the latter cells showed negligible PMSF-sensitive degradation and failed to accumulate AB's in the vacuoles in the presence of PMSF (Fig. 2). These results strongly suggest that PMSF-sensitive degradation takes place in the vacuoles and that the defect of protein degradation in the *apg1* mutant is caused by lack of formation of AB's.

Another characteristic feature of the *apg1* mutant was that it tended to lose viability under nitrogen starvation conditions. Cells of X2180-1A and MT14-1B grown to exponential phase in YEPD were transferred to SD (-N) medium, and then their viabilities were followed by stainability with phloxine B. Wild-type cells survived for more than 5 days without any significant loss of viability, but *apg1* cells began to lose viability about 2 days after the transfer (Fig. 3). Tetrad analysis revealed that loss of viability in SD (-N) medium co-segregated with the phenotype of lack of accumulation of AB's. Strains lacking proteinase A (PrA) or PrA and proteinase B (PrB) showed a similar time course of loss of viability to the *apg1* mutant when incubated in SD (-N) medium. This suggests that loss of viability in SD (-N) medium is due to a defect in protein degradation in the vacuoles.

3.3. Isolation of further apg mutants

Autophagy, a process for degrading self-components of the cytoplasm under adverse conditions, should be tightly regulated. In our laboratory, the existence of a double membrane structure, the autophagosome, in the

Table I

Distributions of *apg* mutations in 15 complementation groups

apg1	15
apg2	13
apg3	10
apg4	9
apg5	5
apg6	4
apg7	4
apg8	4
apg9	4
apg10	3
apg11	2
apg12	1
apg13	1
apg14	1
apg15	1
Total	77

cytosol and its fusion with the vacuolar membrane were demonstrated by electron microscopy (Baba, M. et al., manuscript in preparation). Membrane events involved in autophagy, such as sequestration of the cytosol into autophagosomes and fusion of autophagosomes with vacuolar membranes, must require many gene functions. Thus, isolation of further *apg* mutants was attempted.

For this purpose, the phenotype of loss of viability during nitrogen starvation shown by the *apg1* mutant was applied as a first screening test. As proteinase-deficient strains also lose viability faster than wild-type cells, X2180-1A cells were mutagenized with EMS and were grown on YEPD plates for 3 days at 30°C. Colonies were replica-plated onto SD (-N) plates containing 20 μg/ml of phloxine B. After incubation for 3–4 days at 30°C, red colonies stained with phloxine B were selected. As a second screening step, cells that did not accumulate AB's in vacuoles in SD (-N) medium containing PMSF were selected by light microscopy. In this selection, cells with abnormal vacuolar morphology were excluded.

From about 38,000 mutagenized cells, about 2700 red colonies were selected in the first screening. From these 2700 colonies, 99 *apg* mutants defective in accumulation of AB's were obtained by repeating the morphological selection at least 3 times. This means that about 4% of the candidates obtained by the first screening showed the Apg⁻ phenotype. These 99 mutants were crossed with the wild-type strain X2180-1B. Twelve of 99 strains were sterile and were not characterized further. All diploid strains obtained by crossing the other 87 strains with X2180-1B showed the Apg⁺ phenotype. In 12 of 87 strains, the mutant phenotype was not segregated 2:2, and these 12 mutants were not characterized further. Finally, 75 recessive *apg* mutations and *apg1-1* were crossed with each other and assigned to 15 complementation groups (Table I). Representatives from each of

Strain

X2180-1A (APG1)
MT14-1B (apg1-1)
MT23-1A (apg2-1)
MT33-1A (apg3-1)
MT43-1A (apg4-1)
MT53-1A (apg5-1)
MT63-1A (apg6-1)
MT73-1A (apg7-1)
MT83-1A (apg8-1)
MT93-1A (apg9-1)
MT103-1A (apg10-1)
MT113-1A (apg11-1)
MT123-1A (apg12-1)
MT133-1A (apg13-1)
MT143-1A (apg14-1)
MT153-1A (apg15-1)
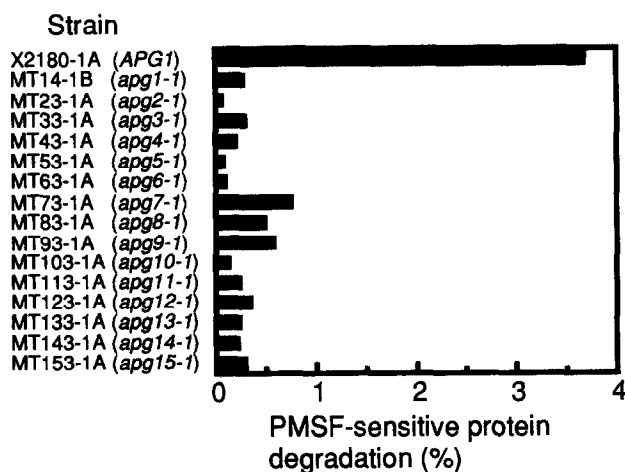
PMSF-sensitive protein degradation (%)

Fig. 4. PMSF-sensitive protein degradation of *apg* mutants in nitrogen starvation medium. PMSF-sensitive protein degradations in cells incubated in SD (-N) medium for 8 h are shown. PMSF-sensitive protein degradation was determined as described for Fig. 2. Total protein degradation in each *apg* mutants ranged to 28–36% of that in wild-type cells (see also Fig. 2). The strains used were representatives of each *apg* mutation.

the 14 newly isolated complementation groups were crossed 3 times with X2180-1A or -1B and named *apg2-1* to *apg15-1*.

3.4. Phenotypes of apg mutants

All *apg* mutants grew well in YEPD after successive crossing with wild-type cells. They could also grow on YEP plates containing a poor carbon source such as acetate, ethanol or glycerol. None of the *apg* mutants showed a *ts* or *cs* growth phenotype. Therefore, *apg* mutations do not seem to cause any apparent defect in mitotic cell cycle progression. However, they could not maintain their viability in nitrogen starvation conditions as a consequence of the first selection. The *apg* mutants showed rather characteristic time courses of loss of viability. Most cells were viable for 2 days after transfer to SD (-N) medium, and then gradually lost viability. After 5 days in SD (-N) medium, less than 20% of the cells were viable. Incubation of cells that had reached stationary phase in YEPD for 7 days did not cause cell death. Therefore, loss of viability was caused by nitrogen starvation.

All homozygous diploids for each *apg* mutant were sporulation-negative. None of the *apg* mutants formed AB's in their vacuoles in the presence of PMSF under carbon or single amino acid starvation as under nitrogen starvation . This indicates that each *apg* mutant has a defect in a certain common step in the process of
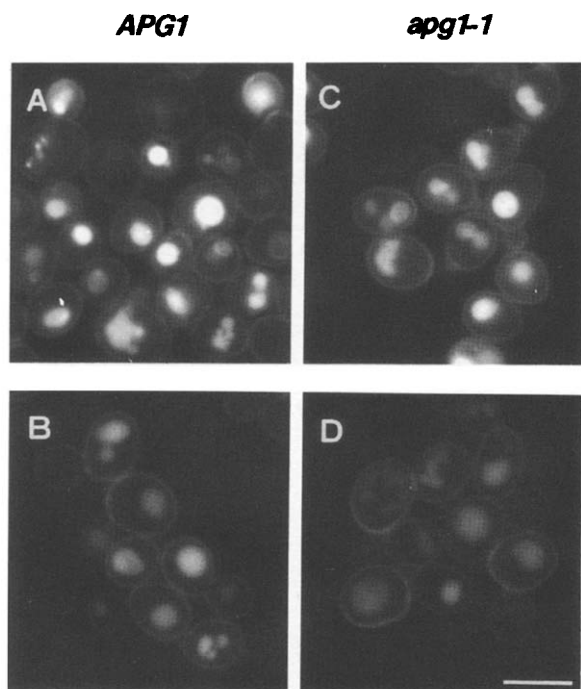

APG1   **apg1-1**

Fig. 5. Accumulations of quinacrine and Lucifer yellow CH in vacuoles. Cells of X2180-1A (*APG1*, left) and MT14-1B (*apg1-1*, right) grown to exponential phase in YEPD were stained with quinacrine (A,C) or Lucifer yellow CH (B,D). Cell walls were stained with 0.02% of Aniline blue to show the outline of the cells. Cells were observed and photographed under a fluorescence microscope. Bar = 5 μm.

autophagy induced by various nutrient starvation signals. Total protein degradation in these *apg* mutants was significantly reduced (less than 36% of that of the wild-type, data not shown). In accordance with the morphological characteristic of defective formation of AB's in the vacuoles, all *apg* mutants showed quite low levels of PMSF-sensitive protein degradation on nitrogen starvation (Fig. 4). These results ruled out the possibility that these *apg* mutants degrade AB's because of PMSF-resistance. The results also indicate that protein degradation in the vacuoles under starvation conditions takes place mostly via autophagy.

The inside of vacuoles is maintained at an acidic pH by vacuolar H^+-ATPase [8,9] and can be labeled with quinacrine which accumulates in a pH-dependent manner [10]. When exponentially growing *apg* mutants in YEPD were labeled with quinacrine, the dye accumulated in the vacuoles of most cells as in wild-type cells (Fig. 5A,C). These observations showed that acidification of the vacuoles was not impaired in any *apg* mutant. Yeast cells internalize LY by endocytosis [7]. This dye is subsequently transported to the vacuoles via presumptive endosomes [11]. As shown in Fig. 5B and D, both wild-type and *apg1* cells accumulated LY in the vacuoles. The vacuoles in all other *apg* mutants were also stained with LY, indicating that the endocytic process is functional in the *apg* mutants.

Furthermore, transport of PrA to the vacuoles occurred in all *apg* mutants (data not shown), suggesting that targeting of PrA to the vacuoles was normal. All these results suggest that vacuolar functions under growing conditions are normal in the mutants and that the Apg⁻ phenotype is not caused by a defect in the final destination compartment, the vacuole.

4. DISCUSSION

First, we isolated one mutant that has a defect in accumulation of AB's in the vacuoles by selection under a light microscope. This mutant was named *apg1* (autophagy). We found that the *apg1* mutant lost viability faster than wild-type cells in nitrogen-deficient medium. Using loss of viability during nitrogen starvation as a first screening test, we isolated 14 other recessive *apg* mutants.

All these mutants exhibited similar phenotypes. (1) They did not accumulate AB's under nitrogen-, carbon- or single amino acid-starvation conditions. (2) They had a defect in bulk protein degradation in the vacuoles induced by starvation. (3) Homozygous diploids with any *apg* mutation did not sporulate. (4) They lost viability rapidly during nitrogen starvation.

Strains lacking PrA and PrB lose viability rapidly under nitrogen starvation conditions, as reported previously [12]. This fact supports the idea that protein degradation in the vacuoles is essential for cell survival during nitrogen starvation. At present, the role of auto-

phagic protein degradation in maintenance of viability under starvation conditions is unknown. Several possible explanations are as follows. One is that autophagy may be responsible for supplying the materials necessary for entering and remaining in the resting state. That is, even in the resting state, minimum protein synthesis is necessary to keep cells viable. Another possibility is that not supply of materials, but reduced levels of cytosolic components such as numerous ribosomes and enzymes may be significant for survival under nutrient starvation conditions. A third possibility is that to enter the resting state, autophagy functions to lower the level of some specific factor(s) that is essential for progression of cell growth.

The present study suggests that at least 15 APG gene functions are required for the autophagic process. The facts that strains with apg mutations show defective accumulation of AB's in vacuoles and defective vacuolar protein degradation suggest that APG genes are involved in the step before the appearance of AB's in the vacuoles. APG gene products may be involved in the signal transduction system of nutrient starvation. They might cause defects in biogenesis of autophagosomal membranes, sequestration of cytosol in autophagosomes, transport of autophagosomes to the vacuoles, subsequent recognition of vacuolar membranes, and fusion of autophagosomes with vacuolar membranes.

Four of 15 apg genes contain only one allele (Table I), suggesting that our isolation procedure is not exhaustive and there may be more apg mutants. In our procedure for isolating mutants, mutagenized cells were recovered and grown in YEPD overnight. Therefore, cells with slow growth may have been eliminated. Although a mutant lacking PrB shows an apparent defect in the autophagic process, it does not lose viability under nitrogen starvation conditions. There may be another type of autophagy-defective mutant, such as a PrB-deficient mutant. In our selection procedure, mutants with morphologically abnormal vacuoles, such as fragmented vacuoles or undetectable vacuoles were eliminated during microscopic selection. If the molecular machinery involved in membrane phenomena in the autophagic process is coupled with a part of the biogenesis of vacuoles, these mutants may have a morpholog-

ical defect in vacuoles. None of the apg mutants showed a defect in the mitotic cell cycle, suggesting that vacuolar protein degradation is not a prerequisite for vegetative cell growth. But some of the machinery for autophagy may be common with that for other processes of membrane flow such as secretion or endocytosis. Mutants of these processes might be obtained only as conditional-lethal mutants.

The 15 apg mutants isolated here showed a similar phenotype, as described above. Electron microscopic analysis of the morphology of all these apg mutants will assist in characterization of each of them and in determination of the order of events. Determination of the order of events in autophagy and molecular biological analysis of individual APG genes will provide information on the mechanism and regulation of autophagy at a molecular level.

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