

## *AUT3*, a Serine/Threonine Kinase Gene, Is Essential for Autophagocytosis in *Saccharomyces cerevisiae*

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**Autophagocytosis is a starvation-induced process, carrying proteins destined for degradation to the lysosome. In the yeast *Saccharomyces cerevisiae*, the autophagic process is visualized by the appearance of autophagic vesicles in the vacuoles of proteinase yscB-deficient strains during starvation. *aut3-1* mutant cells which exhibit a block in the autophagic process have been isolated previously. By using the drastically reduced sporulation frequency of homozygous *aut3-1* diploid cells, the *AUT3* gene was cloned by complementation. The Aut3 protein consists of 897 amino acids. The amino-terminal part of the protein shows significant homologies to serine/threonine kinases. *aut3* null mutant cells are fully viable on rich media but show a reduced survival rate upon starvation. They are unable to accumulate autophagic vesicles in the vacuole during starvation. Starvation-induced vacuolar protein breakdown is almost completely impaired in *aut3*-deficient cells. Vacuolar morphology and acidification are not influenced in *aut3*-deficient cells. Also, secretion of invertase, endocytic uptake of Lucifer Yellow, and vacuolar protein sorting appear wild type like in *aut3*-deficient cells, suggesting autophagocytosis as a novel route for the transport of proteins from the cytosol to the vacuole. By using a fusion of Aut3p with green-fluorescent protein, Aut3p was localized to the cytosol.**

For eukaryotic cells, sorting and transport of various proteins to different organelles are very important processes. Most of these protein transport processes, like secretion (40, 42), vacuolar protein sorting (6, 46, 47), endocytosis (36), or mitochondrial protein uptake (9, 29, 39), are well known, and a lot of work has been done to characterize these processes genetically and biochemically. The yeast *Saccharomyces cerevisiae* has turned out to be an ideal model organism for the study of these processes, but little attention has been given to the transport of cytoplasmic proteins destined for degradation into the vacuole, the counterpart of the mammalian lysosome. The molecular signals for regulation of autophagocytosis are unknown. For mammalian cells, Dice (10) and Cuervo and Dice (8) proposed a selective pathway using a KFERQ-related pentapeptide motif. Furthermore unselective, starvation-induced autophagocytosis is well documented in mammalian cells (for reviews see references 14 and 43), but the molecular mechanisms and the genes involved in this process are still unknown. Detailed electron microscopic studies have suggested that during autophagocytosis, parts of the cytoplasm are engulfed by restricted areas of the rough endoplasmic reticulum (12, 13, 16, 55), thus forming double- or multilayered, nascent autophagic vesicles or autophagosomes. These vesicles mature to degradative autophagic vesicles or autolysosomes (12, 13, 30, 33).

In the methylotrophic yeast *Pichia pastoris*, peroxisomes are degraded by a microautophagic process upon shifting of the cells from methanol to a glucose-containing medium or by a macroautophagy-like process after shifting of cells from methanol to ethanol (54). In *S. cerevisiae*, evidence of autophagocytosis has been detected by the visualization of autophagic vesicles inside the vacuole of cells deficient for proteinase B during starvation for nitrogen (48, 51). The accumulation of these vesicles can also be induced by application of the proteinase B inhibitor PMSF (phenylmethane sulfonyl fluoride) to

cells starving for nitrogen. Electron microscopic studies have suggested the existence of autophagic vesicles in the cytoplasm (3). Recently, attempts to genetically dissect the autophagic pathway in *S. cerevisiae* have been started by isolating mutants (*aut* or *apg* mutants) with a defect in this process (51, 53). The overlap of autophagy with other protein transport processes in the cell is not understood. Surprisingly, however, a genetic and phenotypic overlap of autophagocytosis with the selective and rapid (half time of about 45 min) vacuolar uptake of aminopeptidase I (API) directly from the cytoplasm (25) has been detected. Nearly all *aut* mutants exhibited a block in the vacuolar uptake of API. A variety of *cvt* mutants with a defect in the cytoplasm-to-vacuole targeting of API genetically overlap the *aut* mutant strains. *aut3* was found to be allelic with *cvt10* (19).

Here we describe the isolation and characterization of the *AUT3* gene. Aut3p shows significant homologies to serine/threonine kinases. A chromosomal *aut3* null mutant strain is viable on rich media but shows a complete block in the autophagic process, demonstrated by its inability to accumulate autophagic vesicles in the vacuole and a block of starvation-induced vacuolar protein breakdown.

### MATERIALS AND METHODS

**Chemicals.** Yeast media were purchased from Difco, Detroit, Mich. [<sup>35</sup>S]methionine and NCS-II tissue solubilizer were from Amersham, Braunschweig, Germany. The liquid scintillator Rotiszint eco plus was from Roth, Karlsruhe, Germany. Zymolyase-100T was from Seikagaku, Tokyo, Japan. DNA-modifying enzymes were from Boehringer Mannheim, Germany, and oligonucleotides were from MWG Biotech, Ebersberg, Germany. All other chemicals were of analytical grade and were purchased from Sigma, Deisenhofen, Germany, or Merck, Darmstadt, Germany.

**Strains and media.** The yeast strains used in this study are listed in Table 1. Yeast strains were grown either in complete medium (YPD [1% yeast extract, 2% peptone, 2% glucose]) or in complete minimal dropout medium (CM [2% glucose, 0.67% yeast nitrogen base without amino acids, supplemented with the appropriate auxotrophic nutrients]) (2). For radiolabelling, strains were grown in sulfur-free labelling medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% proline, 2% glucose, supplemented with the appropriate auxotrophic nutrients except methionine and cysteine). Starvation medium contained 1% potassium acetate only, and sporulation medium contained 1% potassium acetate and auxotrophic nutrients. For solid media, 2% agar was added.

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TABLE 1. Yeast strains used in this study

| Strain | Genotype                                                                                                                                     | Source or reference |
|--------|----------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| WCG4a  | <i>mat<math>\alpha</math> his3-11,15 leu2-3,112 ura3</i>                                                                                     | 21                  |
| YMTA   | <i>mat<math>\alpha</math> pra1<math>\Delta</math>:HIS3 leu2-3,112 ura3</i>                                                                   | 51                  |
| YMS30  | <i>mat<math>\alpha</math> aut3<math>\Delta</math>:KAN<sup>R</sup> his3-11,15 leu2-3,112 ura3</i>                                             | This work           |
| YMS31  | <i>mat<math>\alpha</math> aut3-1 his3-11,15 leu2-3,112 ura3</i>                                                                              | 51                  |
| YMS32  | <i>mat<math>\alpha</math> aut3-1 pra1<math>\Delta</math>:HIS3 his3-11,15 leu2-3,112 ura3</i>                                                 | This work           |
| YMS33  | <i>mat<math>\alpha</math> aut3-1 his3-11,15 leu2-3,112 ura3 ade2</i><br><i>mat<math>\alpha</math> aut3-1 his3-11,15 leu2-3,112 ura3 ADE2</i> | This work           |

For DNA manipulation and sequencing, yeast transformation, and tetrad dissection, standard protocols were used (2).

**Isolation of *AUT3*.** The *aut3-1* homozygous diploid strain YMS33 was transformed with a pCS19-based genomic library (44), spread on CM-ura plates, and grown for 3 days at 30°C. About 18,000 transformants were washed from the plates and pooled. Aliquots (1/20) were taken, grown for 4 h in 20 ml of YPD, washed twice with sporulation medium, and resuspended in 20 ml of sporulation medium. After 5 days of incubation at 23°C, random spores were isolated by lysis of unsporulated diploid cells by Zymolyase digestion of the cell wall in hypoosmotic solution (2), spread on CM-ura plates, and grown for 4 days at 30°C. Red colonies were picked, grown overnight in 750  $\mu$ l of CM-ura at 30°C, and shifted to 750  $\mu$ l of starvation medium containing 1 mM PMSF. After 3 to 4 h of incubation at 30°C, cells were examined by light microscopy by using a Zeiss AxioScope with Nomarski optics. Plasmids from cells showing wild-type-like accumulation of autophagic vesicles were rescued and characterized by restriction analysis. Only one type of plasmid (pCSA), with a 7.4-kb genomic insert, was observed. By using the same procedure, 25,000 transformants from a Ycp50-based genomic library (38) were screened and two types of plasmids (YcpA and YcpC, with inserts of 18 and 11.4 kb, respectively) were isolated.

*Hind*III fragments of 0.7, 2.0, 2.5, and 4.0 kb containing the complete genomic insert from pCSA were subcloned in centromeric vector pRS316 (45) and sequenced. Also, a 3.7-kb *Pvu*II-*Bsa*AI fragment containing the open reading frame (ORF) *YGL180w* was subcloned in the *Sma*I site of yeast centromeric shuttle vector pRS316 and integrative plasmid pRS306.

By using primers ccctgtaatacatctttctctgttcgtttctgtatctgtcagctgaagctctgacgc and cccttagcactgtaccaacacgttactcttagagaacagcattagccactagtgatctg and plasmid pUG6 (18), a DNA fragment for the chromosomal replacement of ORF *YGL180w* with a *LoxP*-KAN<sup>R</sup>-*LoxP* cassette was created by PCR.

**Survival rate during starvation.** Cells were grown to early stationary phase, harvested, and resuspended in starvation medium to an optical density at 600 nm of 0.05. At the indicated times, samples were taken, diluted, and plated on YPD. After 48 h at 30°C, the number of growing colonies was determined.

**Invertase secretion.** Secretion of invertase was assayed as previously described (34) without shifting the cells to 37°C.

**Protein turnover.** Cells were grown in 10 ml of labelling medium at 30°C to approximately  $5 \times 10^7$ /ml. For the last 14 h of growth, 3.7 MBq of L-[<sup>35</sup>S]methionine was added to the culture. Cells were then harvested, washed three times with 10 ml of starvation medium, resuspended in 10 ml of starvation medium containing 10 mM nonradioactive methionine, and further incubated at 30°C. At the times indicated in Results, 1-ml samples were taken, mixed with 100  $\mu$ l of 110% (wt/vol) trichloroacetic acid (TCA), and incubated on ice for at least 4 h. For determination of the released acid-soluble radioactivity, the samples were centrifuged for 5 min at  $14,000 \times g$ . A 900- $\mu$ l volume of the supernatant was mixed with 5 ml of liquid scintillator. For determination of the total incorporated radioactivity, the pellets of the 0-h samples were washed five times with starvation medium containing 10% TCA and twice with ethanol-ether (1:1). The pellets were air dried and dissolved in 1 ml of NCS-II (containing 10% water) at 40°C. A 900- $\mu$ l volume of the solution was mixed with 5 ml of liquid scintillator. Radioactivity was determined with a Wallac 1410 liquid scintillation counter (Pharmacia). Protein breakdown was calculated as the increase of TCA-soluble activity divided by the TCA-insoluble activity of the 0-h sample. Different quenching effects of TCA and NCS were corrected by using an internal standard.

**Endocytosis.** Lucifer Yellow accumulation was assayed as previously described (35), by using 4 mg of Lucifer Yellow per ml and 8 mM sodium orthovanadate without shifting the cultures to 37°C.

**Construction of GFP-Aut3 fusion protein.** A 2.8-kb *Xmn*I-*Hpa*II fragment from pCSA was ligated in frame into the *Sma*I site which is part of the multicloning site of *CEN6/ARSH4* vector pRN295 (pRN295 further contains *URA3* and *Amp* as selectable markers [20a]), which contains the green-fluorescent protein (GFP) gene under the control of the inducible *Met25* promoter. In the resulting fusion protein, the first 30 amino acids of Aut3p are replaced with GFP. The fusion construct was introduced into strains WCG4a and YMS31. For induction of the *Met25* promoter, the strains were incubated overnight in methionine-free CM. Localization of GFP was examined by fluorescence microscopy using a Zeiss AxioScope.

**Nucleotide sequence accession number.** The sequence characterized in this study has been assigned GenBank accession number Z72702x1.

## RESULTS

The *aut3-1* mutant strain YBK26 was isolated by ethyl methanesulfonate mutagenesis for its inability to degrade the cytoplasmic enzyme fatty acid synthase and its defect in accumulating autophagic vesicles inside the vacuole during starvation on nitrogen-free media in the presence of PMSF (Fig. 1) (51). The existence of a single, recessive gene mutation responsible for the *aut3-1*-associated phenotype was confirmed by analyzing the tetrads of three successive backcrosses with wild-type strain WCG4a.

**Isolation of the *AUT3* gene.** Like other *aut* mutants, a homozygous *aut3-1 aut3-1* diploid strain was found to be nearly unable to sporulate. In our attempt to identify the *AUT3* gene, we transformed this diploid strain with a plasmid-encoded yeast genomic library. After shifting of the transformed cells to sporulation medium, predominantly those cells with a plasmid-encoded *AUT3* gene should be able to form asci. A significant enrichment of ascospores was achieved by lysing most of the diploid cells in a hypoosmotic solution after Zymolyase digestion of the cellular wall (2). The use of a heterozygous *ade2 ADE2* diploid strain allowed the rapid recognition of ascospores surviving this procedure. Only *ade2* haploid ascospores are able to form the typical red pigment. The red colonies were subsequently checked for accumulation of autophagic vesicles inside the vacuole after a 3-hour starvation period in the presence of PMSF. By using a centromeric pCS19-derived genomic library (44), 18,000 transformants were collected and subjected to the random-spore procedure described in Materials and Methods. After streaking on plates, 150 red colonies were checked for restoration of the ability to accumulate autophagic vesicles inside the vacuole. The genomic plasmids of 12 positive colonies were rescued. All plasmids contained the same 7.4-kb genomic insert. Partial sequencing of a 4.0-kb *Hind*III subclone localized this pCSA fragment to a region of chromosome VII left of *MTP5* (Fig. 2A). By using a Ycp50-derived genomic library (38), 25,000 additional transformants were checked. They yielded 10 positive clones with two different types of genomic inserts: Ycp50A (18.6 kb) and YcpC (10.2 kb), respectively. A 5-kb fragment was common to all three genomic fragments (Fig. 2A).

Four *Hind*III fragments of the pCSA genomic insert were subcloned (Fig. 2A) in the centromeric yeast shuttle vector pRS316. All were unable to complement the defect in the accumulation of autophagic vesicles seen in an *aut3-1* mutant strain. Sequencing of these genomic fragments identified a single ORF of 2.8 kb, which was incomplete in all four subclones (Fig. 2B). Recently, the established sequence was confirmed by the systematic yeast sequencing project. The ORF was designated *YGL180w* (G1615). A 3.7-kb *Pvu*II-*Bsa*AI fragment carrying only ORF *YGL180w* and its promoter was subcloned and found to complement an *aut3-1* mutation.

**Chromosomal deletion of *AUT3*.** To generate *YGL180w* chromosomal null mutant strain YMS30, we used a PCR-based deletion method and plasmid pUG6 (18) to replace a 2.8-kb fragment containing the promoter and most of the ORF with a *LoxP*-KAN<sup>R</sup>-*LoxP* cassette (58) conferring kanamycin resistance on yeast (Fig. 2A). The correct gene replacement was confirmed by Southern blot analysis (data not shown). YMS30 showed the defect in the accumulation of autophagic vesicles already known from the *aut3-1* mutant strain. An integrative plasmid was constructed by inserting the 3.6-kb *Bsa*AI-*Pvu*II fragment into the *Sma*I site of pRS306. The plasmid was lin-

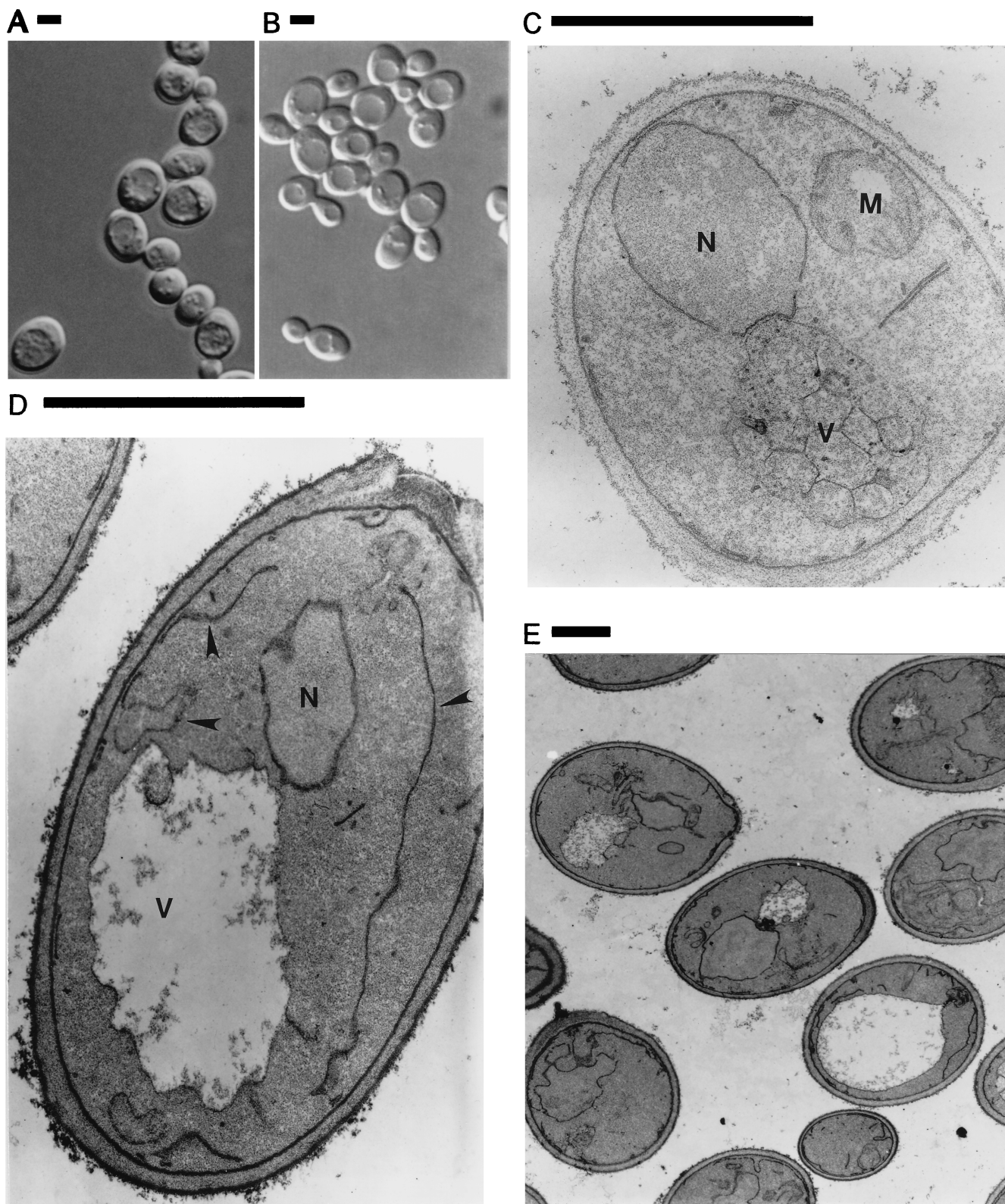


FIG. 1. Accumulation of autophagic vesicles in the vacuolar lumen is blocked in *aut3Δ* cells. After 4 h of starvation in the presence of 1 mM PMSF, the vacuole of wild-type strain WCG4a appears to be filled with vesicles (A). Under the same conditions, no vesicles are evident in vacuoles of *aut3* null mutant strain YMS30 (B). Compared to wild-type cells (C), *aut3-1* mutant cells (D and E) show an elongated endoplasmic reticulum. Yeast cells were prepared for electron microscopy by permanganate fixation as described elsewhere (57). N, nucleus; V, vacuole; M, mitochondrion; arrowhead, endoplasmic reticulum. A and B, Nomarski optics; B, C, and D, electron micrographs. Bars, 2  $\mu$ m.

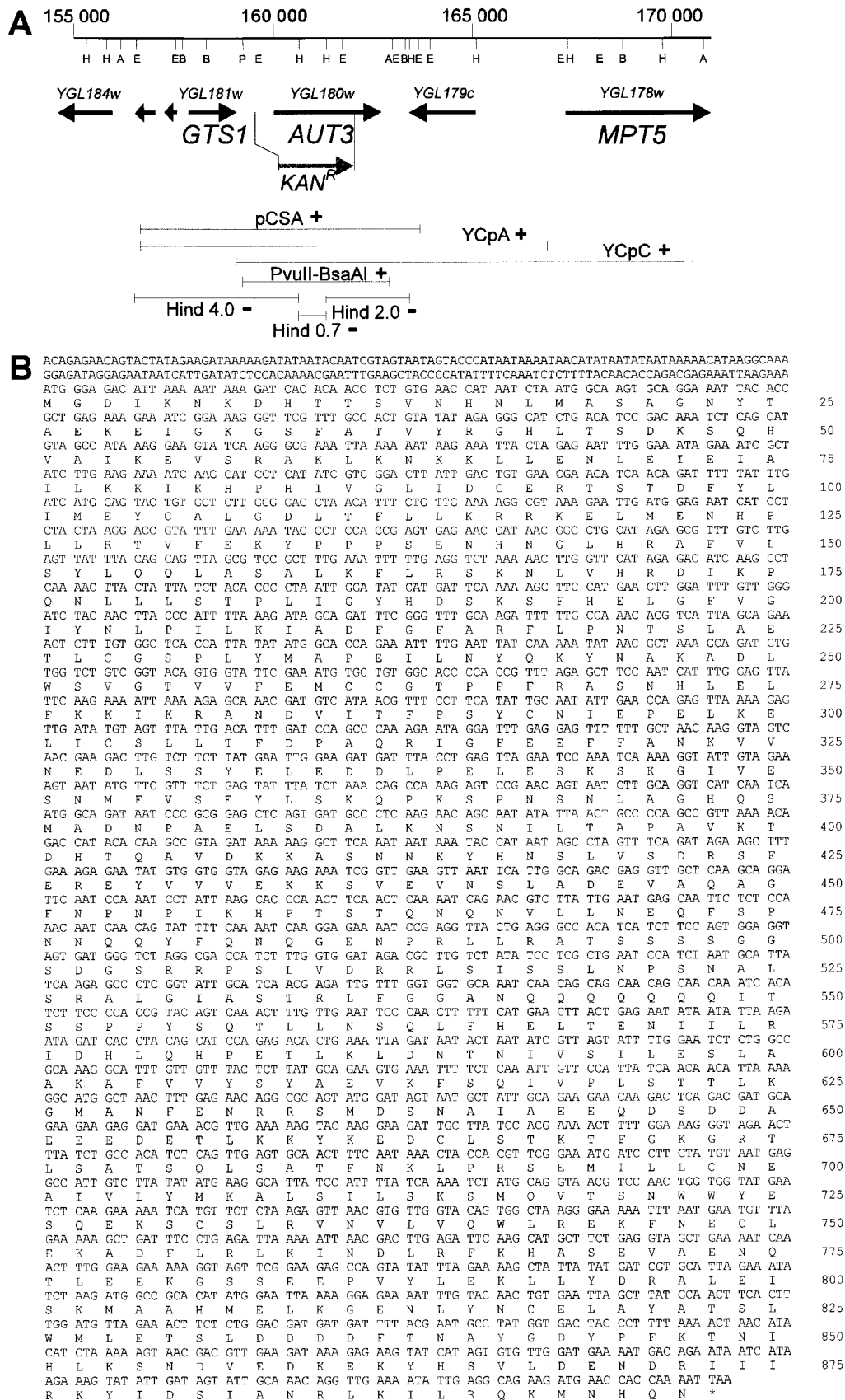


FIG. 2. (A) Restriction map of part of the genomic DNA fragment *SCVIIGENE\_10* surrounding *AUT3* (Restriction sites: A, *Bsa*AI; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pvu*II). The genomic fragments obtained from library plasmids pCSA, YCpA, and YCpC are shown. Plus and minus signs indicate the ability and inability, respectively, to complement the defect in vesicle accumulation. The fragments used for subcloning and sequencing of the *AUT3* gene and the chromosomal region replaced by the *KAN<sup>R</sup>* gene in *aut3* null mutant strain YMS30 are also shown. (B) Sequence of the *AUT3* gene. The nucleotide sequence of *AUT3* is identical to the published sequence of ORF *YGL180w*. Amino acids are shown in the single-letter code.

earized with *Bgl*II and chromosomally integrated into *aut3-1* mutant cells. The resulting strain was crossed with wild-type strain WCG4a. Analysis of 22 tetrads confirmed the identity of ORF *YGL180w* with the *aut3-1* locus.

**Homologs of Aut3p.** *AUT3* encodes a protein of 897 amino acids with a calculated molecular mass of 102 kDa. The amino-terminal region of Aut3p shows significant homologies with other known serine/threonine kinases (Fig. 3A). The highest homologies were found with *CEUNC51MR\_1*, an ORF of *Caenorhabditis elegans* with an unknown function (31), and *KMLC\_DICDI*, a myosin light-chain kinase homolog of *Dictyostelium discoideum* (49). The N-terminal 300 amino acids of Aut3p share 35% identity with the *C. elegans* protein and 28% identity with the *Dictyostelium* myosin light-chain kinase. A detailed analysis showed all of the characteristic features of serine/threonine kinases, like a glycine loop (amino acids 30 to 35), which forms part of the  $Mg^{2+}$ -ATP binding site, residues Lys54, Asp71, and Glu211, which recognize the  $\gamma$ -phosphate of  $Mg^{2+}$ -ATP and the catalytic domain (amino acids 168 to 180) (26, 27).

The carboxy-terminal part of Aut3p shows no significant homologies to other proteins in the databases. A serine-rich region (amino acids 495 to 526) may be a sign of phosphorylation, or probably autophosphorylation, of Aut3p. Other features of Aut3p are clusters of charged amino acids (Fig. 3B) and a cluster consisting of seven glutamines, from amino acids 542 to 548.

**Phenotypes of an *aut3* mutant strain.** Chromosomal deletion of the *AUT3* gene did not impair growth compared to a wild-type strain at 23, 30, or 37°C. The lack of autophagic vesicles accumulating inside the vacuole confirmed the autophagic defect of *aut3Δ* cells.

During the autophagic process, cytosolic proteins are transported to the vacuolar lumen and subjected to proteolysis. We therefore measured the ability of cells with *aut3* deleted to degrade proteins during nitrogen starvation. All cellular proteins were radiolabelled by growth for 16 h in the presence of [<sup>35</sup>S]methionine. After shifting of the cells to a nonradioactive starvation medium, nondegraded proteins were precipitated with TCA and the amount of acid-soluble small peptides generated by the action of proteinases was determined (Fig. 4). A wild-type strain showed an initial breakdown rate of 1.8% of all cellular proteins per h. Vacuolar proteolysis is almost completely blocked in strains deficient in the major vacuolar endoprotease yscA (*PEP4 PRA1*) (1, 24, 56, 59). A strain with *pra1* deleted exhibited an 82% reduction in the total protein degradation rate compared to a wild-type strain (Fig. 4) (50). The residual proteolysis rate is due to the action of the cytosolic proteasome (data not shown). An identical 82% reduction was found in an *aut3-1* mutant strain. A *pra1Δ aut3-1* double mutant strain showed no further decrease in the protein degradation rate (Fig. 4). This confirms the vacuolar localization of the protein degradation defect seen in *aut3-1* mutant cells and illustrates the unselective nature of autophagic protein breakdown. The block of the transport of cytosolic proteins to the vacuole under starvation conditions in *aut3-1* mutant cells has been shown by immunofluorescence localization of fatty acid synthase (51). Autophagocytosis and vacuolar protein breakdown are known to be induced by nutrient limi-

tation. Under these conditions, we found Aut3p to be essential for cellular function. Like a *pra1*-deficient strain, a haploid *aut3*-deficient strain has a significantly reduced ability to survive periods of nitrogen starvation (Fig. 5). Diploid wild-type cells normally form asci if they are subjected to starvation. Homozygous *aut3Δ* diploid cells, in contrast, are unable to sporulate.

Deletion of *AUT3* did not influence the wild-type-like morphology of the vacuole as checked by light microscopy with Nomarski optics and by applying the vacuolar membrane stain MDY-64 (Fig. 6A). The wild-type-like accumulation of Quinacrine (37) in the vacuole of *aut3Δ* cells is a good indication of normal acidification of this organelle (Fig. 6B). Electron microscopic studies demonstrated an elongated endoplasmic reticulum (Fig. 1).

We analyzed the influence of the block of autophagocytosis in *aut3Δ* cells on several other vesicle-mediated processes. We used invertase, a well-known marker protein (41), to check the integrity of the secretory pathway. As shown in Fig. 7A, *aut3Δ* cells secreted invertase with the same rate as a wild-type strain. Fluid phase endocytosis was measured by monitoring the uptake of the fluorescent dye Lucifer Yellow into the vacuole (11). No difference from a wild-type strain could be detected (Fig. 7B). Most recently, the correct sorting of resident vacuolar proteins like proteinase yscA and carboxypeptidase yscY was confirmed in *aut3-1* cells by using immunoblots and pulse-chase kinetic experiments (19). The selective import of API from the cytoplasm to the vacuole is blocked in *aut3Δ* cells, and recently, *AUT3* has been shown to be allelic with *CVT10* (19).

**Localization of Aut3p.** We constructed an in-frame fusion of GFP (5, 7) and Aut3p under the control of the inducible *Met25* promoter to localize the *AUT3* gene product in the cell. The GFP fusion protein, in which the first 32 amino acids of Aut3p are replaced with GFP, was unable to restore the mutant phenotypes in *aut3-1* mutant cells. Expression of the fusion protein in wild-type and *aut3Δ* cells showed a cytosolic localization in logarithmically growing cells (Fig. 8). In wild-type cells starved for nitrogen, some of the fusion protein was detected inside the vacuole; no fusion protein was detectable in vacuoles of starved *aut3Δ* cells (data not shown).

## DISCUSSION

For a better understanding of the importance of the autophagic process for cellular function and the adaptation of the cell to nutrient limitation and to get more detailed insights into the mechanistic principles of this process, we used a genetic approach. In a previous study, several autophagocytosis-defective *aut* mutants have been isolated (51). These *aut* mutants are unable to degrade the cytosolic fatty acid synthase and to accumulate autophagic vesicles inside the vacuole during periods of nitrogen starvation. By using the *aut3-1* mutant strain, we isolated the *AUT3* gene with the aid of a newly developed screening procedure based on the sporulation defect of homozygous *aut* diploid mutant strains.

We constructed a chromosomal *aut3* null mutant strain. The expected block in the autophagic pathway in this strain was demonstrated by the lack of autophagic vesicles that normally

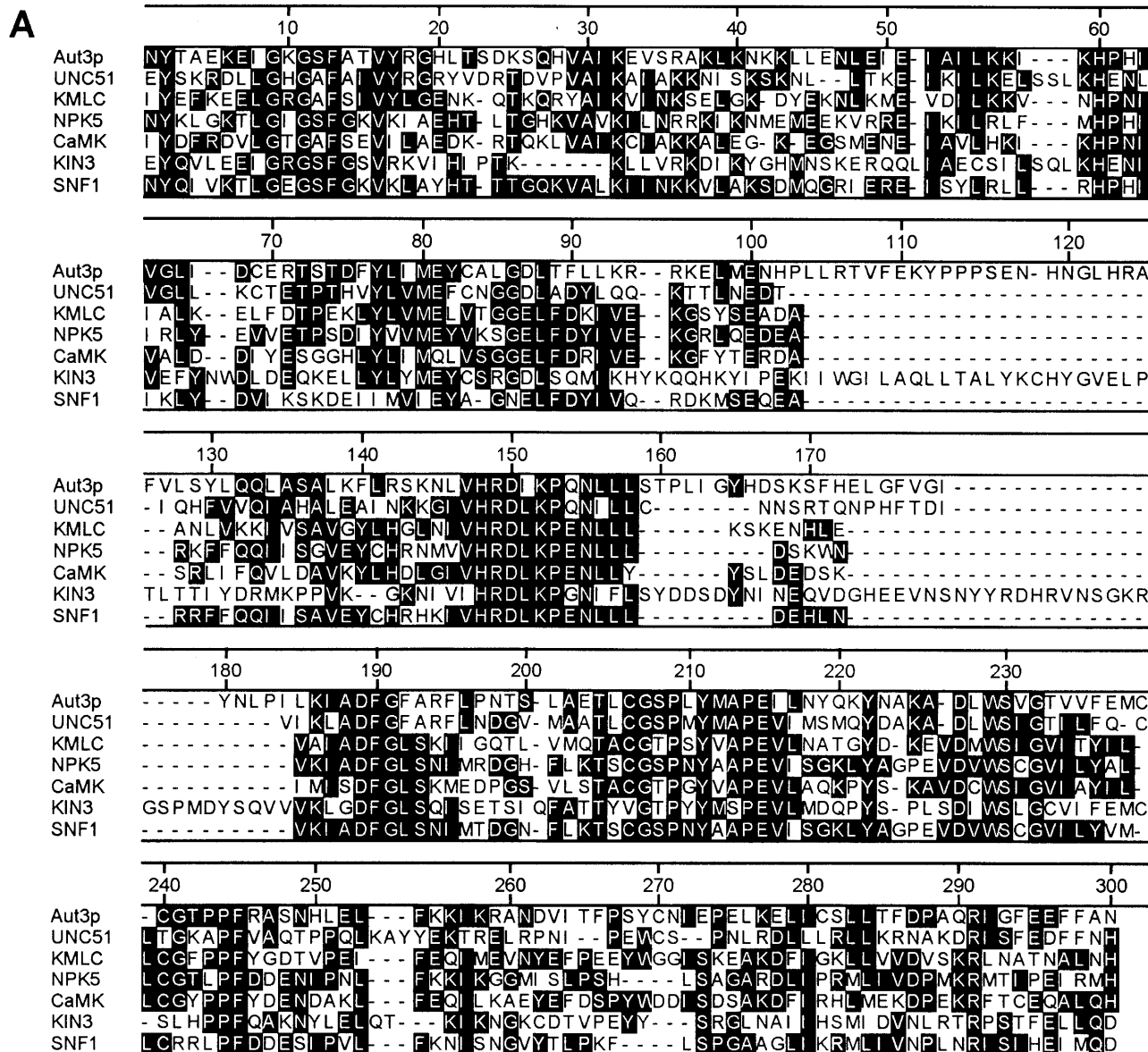


FIG. 3. (A) Homology of the N-terminal part of Aut3p to other kinases. UNC51, *CEUNC51MB\_1*; KMLC, *KMLC\_DICD1* (MLCK); NPK5, *JC1446*; CaMK, *A49682* ( $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase); KIN3, *YSCKIN3\_1*; SNF1, *SCSNF1*. Amino acids 168 to 180 are essential for the kinase activity. (B) Clusters of positively and negatively charged amino acid residues within Aut3p.

accumulate inside the vacuole during starvation for nitrogen in the presence of PMSF.

The high importance of autophagocytosis for the degradation of intracellular proteins was demonstrated by determining the total protein breakdown rate under starvation conditions in *aut3-1* mutant cells. The measured 82% reduction of proteolysis in *aut3-1* mutant cells illustrates the unspecific nature of autophagocytosis in *S. cerevisiae*. This is in good agreement

with previous findings obtained with mammalian (28) and yeast (15) cells. An isogenic strain with *pra1* deleted exhibited the same 82% reduction in the total protein breakdown rate; a *pra1* *aut3-1* double mutant strain showed no further cumulative reduction in the protein degradation rate. This confirms the vacuolar origin of the *aut3*-dependent autophagic protein degradation defect. By using indirect immunofluorescence microscopy, it has already been shown that *aut3-1* mutant cells

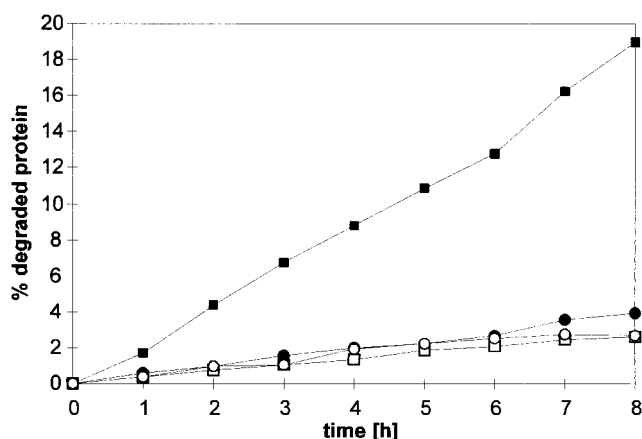


FIG. 4. Degradation of total cellular proteins during starvation. *pra1* deletion strain YMTA (□) and *aut3-1* mutant strain YMS31 (●) showed drastically reduced turnover rates compared to wild-type strain WCG4a (■). No further decrease in the turnover rate of *aut3-1 pra1* double mutant strain YMS32 (○) was observed.

are unable to transport cytoplasmic fatty acid synthase to the vacuolar lumen (51). Taken together, these results demonstrate that the *AUT3*-dependent autophagic process in yeast is responsible for the unspecific transport of cytoplasmic proteins to the vacuole. The residual 18% protein breakdown under these conditions is due to nonvacuolar proteolysis.

The block of autophagic protein uptake into the vacuole in *aut3Δ* cells had no significant influence on vacuolar morphology as checked by Nomarski optics in a light microscope and by electron microscopy. Also, vacuolar acidification, checked by accumulation of the fluorescent dye Quinacrine, appeared normal.

Besides these morphological criteria, we used an *aut3*-deficient strain to investigate the fidelity of other vesicle-mediated protein transport pathways. The vacuolar protein-sorting pathway is responsible for proper sorting of resident vacuolar proteinases like proteinase yscA and carboxypeptidase yscY (6, 46, 47). Kinetic analysis of CPY maturation and steady-state immunoblot analysis of PrA maturation in *aut3-1* mutant cells showed correct sorting, maturation, and glycosylation of these enzymes (19). We checked the efficiency of the secretory pathway (40, 41) by monitoring the appearance of

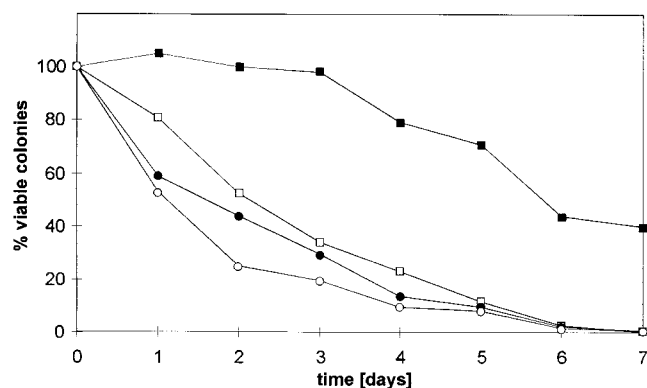


FIG. 5. Survival during starvation in 1% potassium acetate. Like the *pra1* mutant strain (□), *aut3* null mutant strain YMS30 (●) and *aut3-1 pra1* double mutant strain YMS32 (○) showed a drastically reduced survival rate compared to wild-type strain WCG4a (■).

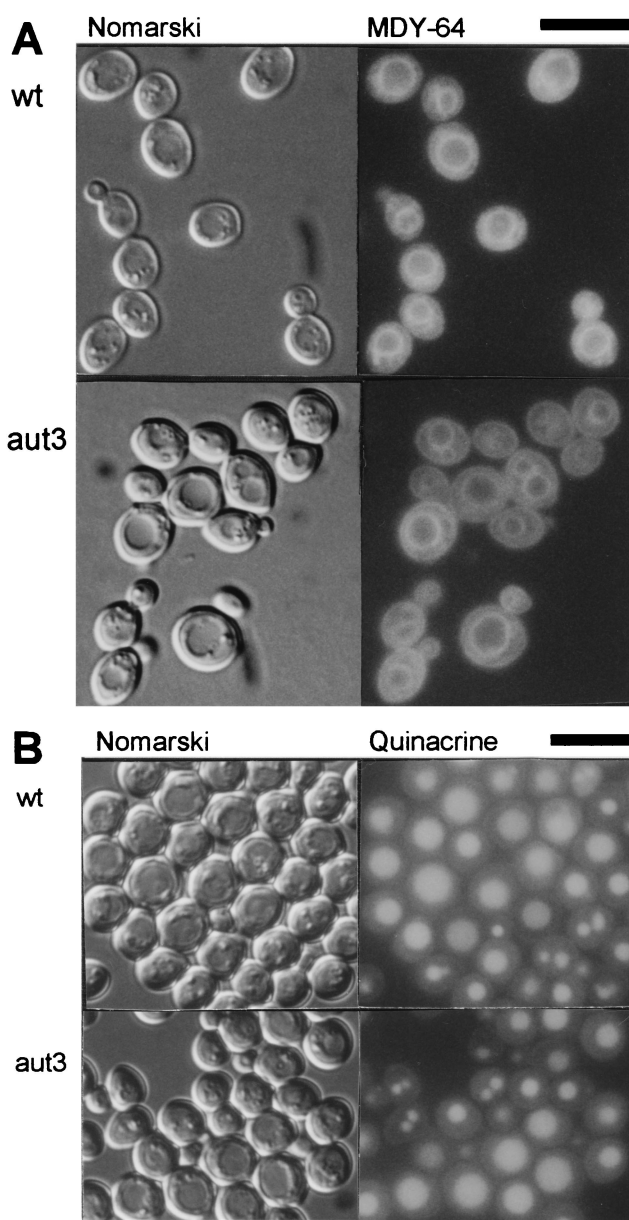


FIG. 6. (A) The vacuole of *aut3* null mutant strain YMS30 is morphologically similar to the vacuoles of wild-type (wt) strain WCG4a as checked by light microscopy with Nomarski optics and by applying the vacuolar membrane stain MDY-64. (B) pH-dependent accumulation of the fluorescent dye quinacrine in the vacuolar lumen. Vacuolar staining of *aut3Δ* cells is not distinguishable from that of wild-type cells, indicating that acidification of the vacuole occurs normally in *aut3* mutant cells. Bars, 5  $\mu$ m.

invertase activity in the extracellular medium. Fluid phase endocytosis (36) was checked by monitoring the uptake of the fluorescent dye Lucifer Yellow. All of these processes showed wild-type characteristics. These findings suggest that autophagocytosis is no essential prerequisite for vacuolar protein sorting, endocytosis, or secretion but constitutes a new route delivering cytosolic proteins to the vacuole. In mammalian cells, the appearance of endocytosed material in autophagic vacuoles has been demonstrated (17, 32, 52), but the normal pathway of autophagocytosis seems to be the fusion of autophagic vacuoles with lysosomes (12, 13, 30).

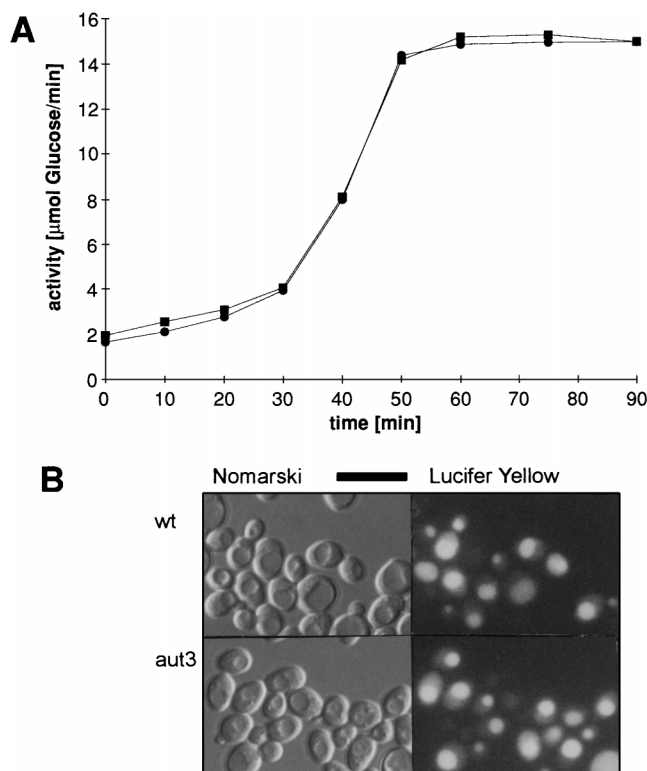


FIG. 7. (A) Secretion of invertase at wild-type level in *aut3* mutant cells. In the supernatants of wild type (■) strain WCG4a and *aut3* deletion strain YMS30 (●), invertase activity increased with similar kinetics after induction. (B) Vacuolar staining of *aut3* null mutant cells with the fluid phase endocytosis marker Lucifer Yellow is indistinguishable from that of wild-type (wt) cells. Bar, 5 μm.

Under nutrient limitation conditions, autophagocytosis constitutes an essential process for cellular function. Under these conditions, *aut3Δ* cells had a significantly lower survival rate than a wild-type strain and sporulation of homozygous *aut3Δ* diploid cells was abolished.

The resident vacuolar aminopeptidase I is synthesized as an inactive cytoplasmic precursor, which is taken up by the vacuole with a half time of about 45 min independently of the secretory and vacuolar protein-sorting pathway. Inside the vacuole, precursor API becomes mature API in a proteinase yscB-

dependent manner (25). Seventeen complementation groups of *cvt* mutant strains with a defect in the selective cytoplasm-to-vacuole targeting of precursor API have been isolated (19, 20). *Aut3-1* mutant cells are impaired in the uptake of the cytoplasmic API precursor into the vacuole, and an allelism of the *aut3-1* mutant strain with the *cvt10-1* mutant strain has been found (19). This demonstrates an essential function of Aut3p in the selective and rapid translocation of precursor API from the cytoplasm to the vacuole.

On the basis of the use of a GFP-Aut3 fusion protein, a cytoplasmic localization of Aut3p was proposed. The localization of some fusion protein in the vacuole of wild-type cells after starvation is most likely due to the action of the autophagic process itself.

Aut3p shows significant homologies to a large number of serine/threonine kinases. An indication of kinase involvement in the autophagic process in mammalian cells has been found (4, 22, 23). In those studies, an inhibitory effect due to the action of kinases was suggested. A chromosomal deletion of *AUT3*, in contrast, leads to a recessive block of autophagocytosis. This suggests that phosphorylation of a target protein through Aut3p activates the autophagic process. One probable function of Aut3p might be involvement in a signal transduction cascade, probably nutrient sensing. A serine-rich region in Aut3p (amino acids 495 to 526) might also imply regulation of this kinase by phosphorylation.

Kinases have also been found to be implicated in the vacuolar protein-sorting pathway (46). The elongation of the ER seen in *aut3*-deficient cells may indicate a function of Aut3p in the biogenesis of autophagic vesicles at the ER. Further studies to clarify the exact function of *AUT3* in autophagocytosis are underway.

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#### REFERENCES

- Ammerer, G., C. P. Hunter, J. H. Rothman, G. C. Saari, L. A. Valls, and T. H. Stevens. 1986. *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. *Mol. Cell. Biol.* 6:2490-2499.
- Ausubel, F. M., R. Brent, R. E. Kingston, and D. D. Moore. 1987. Current protocols in molecular biology. Greene Publishing Associates, New York, N.Y.
- 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.
- Blommaert, E. F., J. J. Luiken, P. J. Blommaert, G. M. van Woerkom, and A. J. Meijer. 1995. Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. *J. Biol. Chem.* 270:2320-2326.
- Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263:802-805.
- Conibear, E., and T. H. Stevens. 1995. Vacuolar biogenesis in yeast: sorting out the sorting proteins. *Cell* 83:513-516.
- Cubitt, A. B., R. Heim, S. R. Adams, A. E. Boyd, L. A. Gross, and R. Y. Tsien. 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* 20:448-455.
- Cuervo, A. M., and J. F. Dice. 1996. A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* 273:501-503.
- Cyr, D. M., C. Ungermann, and W. Neupert. 1995. Analysis of mitochondrial protein import pathway in *Saccharomyces cerevisiae* with translocation intermediates. *Methods Enzymol.* 260:241-252.
- Dice, J. F. 1990. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. *Trends Biochem. Sci.* 15:305-309.
- Dulic, V., M. Egerton, I. Elguindi, S. Rath, B. Singer, and H. Riezman. 1991. Yeast endocytosis assays. *Methods Enzymol.* 194:697-710.

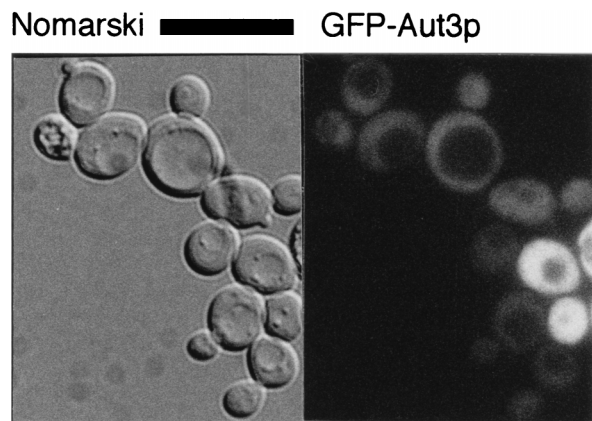


FIG. 8. Intracellular localization of the GFP-Aut3 fusion protein. Bar, 5 μm.

12. Dunn, W. J. 1990. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. *J. Cell Biol.* **110**:1923–1933.
13. Dunn, W. J. 1990. Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. *J. Cell Biol.* **110**:1935–1945.
14. Dunn, W. J. 1994. Autophagy and related mechanisms of lysosome-mediated protein degradation. *Trends Cell Biol.* **4**:139–143.
15. Egner, R., M. Thumm, M. Straub, A. Simeon, H. J. Schüller, and D. H. Wolf. 1993. Tracing intracellular proteolytic pathways. Proteolysis of fatty acid synthase and other cytoplasmic proteins in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268**:27269–27276.
16. Furuno, K., T. Ishikawa, K. Akasaki, S. Lee, Y. Nishimura, H. Tsuji, M. Himeno, and K. Kato. 1990. Immunocytochemical study of the surrounding envelope of autophagic vacuoles in cultured rat hepatocytes. *Exp. Cell Res.* **189**:261–268.
17. Gordon, P. B., H. Hoyvik, and P. O. Seglen. 1992. Prelysosomal and lysosomal connections between autophagy and endocytosis. *Biochem. J.* **283**:361–369.
18. Güldener, U., S. Heck, T. Fielder, J. Beinhauer, and J. H. Hegemann. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* **24**:2519–2524.
19. Harding, T. M., A. Hefner-Gravink, M. Thumm, and D. J. Klionsky. 1996. Genetic and phenotypic overlap between autophagy and the cytoplasm to vacuole targeting pathway. *J. Biol. Chem.* **271**:17621–17624.
20. 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.
- 20a. Hegemann, J. Personal communication.
21. Heinemeyer, W., A. Gruhler, V. Mohrle, Y. Mahe, and D. H. Wolf. 1993. PRE2, highly homologous to the human major histocompatibility complex-linked *RING10* gene, codes for a yeast proteasome subunit necessary for chymotryptic activity and degradation of ubiquitinated proteins. *J. Biol. Chem.* **268**:5115–5120.
22. Holen, I., P. B. Gordon, and P. O. Seglen. 1992. Protein kinase-dependent effects of okadaic acid on hepatocytic autophagy and cytoskeletal integrity. *Biochem. J.* **284**:633–636.
23. Holen, I., P. B. Gordon, and P. O. Seglen. 1993. Inhibition of hepatocytic autophagy by okadaic acid and other protein phosphatase inhibitors. *Eur. J. Biochem.* **215**:113–122.
24. Jones, E. W. 1991. Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266**:7963–7966.
25. Klionsky, D. J., R. Cueva, and D. S. Yaver. 1992. Amino peptidase I of *Saccharomyces cerevisiae* is localized to the vacuole independent of the secretory pathway. *J. Cell Biol.* **119**:287–299.
26. Knighton, D. R., J. H. Zheng, E. L. Ten, V. A. Ashford, N. H. Xuong, S. S. Taylor, and J. M. Sowadski. 1991. Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**:407–414.
27. Knighton, D. R., J. H. Zheng, E. L. Ten, N. H. Xuong, S. S. Taylor, and J. M. Sowadski. 1991. Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**:414–420.
28. Kopitz, J., G. O. Kisen, P. B. Gordon, P. Bohley, and P. O. Seglen. 1990. Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes. *J. Cell Biol.* **111**:941–953.
29. Kubrich, M., K. Dietmeier, and N. Pfanner. 1995. Genetic and biochemical dissection of the mitochondrial protein-import machinery. *Curr. Genet.* **27**:393–403.
30. Lawrence, B. P., and W. J. Brown. 1992. Autophagic vacuoles rapidly fuse with pre-existing lysosomes in cultured hepatocytes. *J. Cell Sci.* **102**:515–526.
31. Ogura, K., C. Wicky, L. Magnenat, H. Tobler, I. Mori, F. Muller, and Y. Ohshima. 1994. *Caenorhabditis elegans unc-51* gene required for axonal elongation encodes a novel serine/threonine kinase. *Genes Dev.* **8**:2389–2400.
32. Punnonen, E. L., S. Autio, H. Kaija, and H. Reunanen. 1993. Autophagic vacuoles fuse with the prelysosomal compartment in cultured rat fibroblasts. *Eur. J. Cell Biol.* **61**:54–66.
33. Rabouille, C., G. J. Strous, J. D. Crapo, H. J. Geuze, and J. W. Slot. 1993. The differential degradation of two cytosolic proteins as a tool to monitor autophagy in hepatocytes by immunocytochemistry. *J. Cell Biol.* **120**:897–908.
34. Rath, S., J. Rohrer, F. Crausaz, and H. Riezman. 1993. *end3* and *end4*: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*. *J. Cell Biol.* **120**:55–65.
35. Riezman, H. 1985. Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis. *Cell* **40**:1001–1009.
36. Riezman, H. 1993. Yeast endocytosis. *Trends Cell Biol.* **3**:273–277.
37. Roberts, C. J., C. K. Raymond, C. T. Yamahiro, and T. H. Stevens. 1991. Methods for studying the yeast vacuole. *Methods Enzymol.* **194**:644–661.
38. Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink. 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* **60**:237–243.
39. Schatz, G., and B. Dobberstein. 1996. Common principles of protein translocation across membranes. *Science* **271**:1519–1526.
40. Schekman, R. 1992. Genetic and biochemical analysis of vesicular traffic in yeast. *Curr. Opin. Cell Biol.* **4**:587–592.
41. Schekman, R., B. Esmon, S. Ferro-Novick, C. Field, and P. Novick. 1983. Yeast secretory mutants: isolation and characterization. *Methods Enzymol.* **96**:802–815.
42. Schekman, R., and L. Orci. 1996. Coat proteins and vesicle budding. *Science* **271**:1526–1533.
43. Seglen, P. O. P., and P. Bohley. 1992. Autophagy and other vacuolar protein degradation mechanisms. *Experientia* **48**:158–172.
44. Sengstag, C. 1993. The sequence of *Saccharomyces cerevisiae* cloning vector pCS19 allowing direct selection for DNA inserts. *Gene* **124**:141–142.
45. Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**:19–27.
46. Stack, J. H., W. D. De, K. Takegawa, and S. D. Emr. 1995. Vesicle-mediated protein transport: regulatory interactions between the Vps15 protein kinase and the Vps34 PtdIns 3-kinase essential for protein sorting to the vacuole in yeast. *J. Cell Biol.* **129**:321–334.
47. Stack, J. H., B. Horadzovsky, and S. D. Emr. 1995. Receptor-mediated protein sorting to the vacuole in yeast: roles for a protein kinase, a lipid kinase and GTP-binding protein. *Annu. Rev. Cell Dev. Biol.* **11**:1–33.
48. Takeshige, K., M. Baba, S. Tsuboi, T. Noda, and Y. Ohsumi. 1992. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J. Cell Biol.* **119**:301–311.
49. Tan, J. L., and J. A. Spudich. 1991. Characterization and bacterial expression of the *Dictyostelium* myosin light chain kinase cDNA. Identification of an autoinhibitory domain. *J. Biol. Chem.* **266**:16044–16049.
50. Teichert, U., B. Mechler, H. Müller, and D. H. Wolf. 1989. Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation, and cell survival. *J. Biol. Chem.* **264**:16037–16045.
51. Thumm, M., R. Egner, B. Koch, M. Schlumpberger, M. Straub, M. Veenhuis, and D. H. Wolf. 1994. Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae*. *FEBS Lett.* **349**:275–280.
52. Tooze, J., M. Hollinshead, T. Ludwig, K. Howell, B. Hofflack, and H. Kern. 1990. In exocrine pancreas, the basolateral endocytic pathway converges with the autophagic pathway immediately after the early endosome. *J. Cell Biol.* **111**:329–345.
53. Tsukada, M., and Y. Ohsumi. 1993. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett.* **333**:169–174.
54. Tuttle, D. L., and W. J. Dunn. 1995. Divergent modes of autophagy in the methylotrophic yeast *Pichia pastoris*. *J. Cell Sci.* **108**:25–35.
55. Ueno, T., D. Munro, and E. Kominami. 1991. Membrane markers of endoplasmic reticulum preserved in autophagic vacuolar membranes isolated from leupeptin-administered rat liver. *J. Biol. Chem.* **266**:18995–18999.
56. van den Hazel, H. B., M. C. Kielland-Brandt, and J. R. Winther. 1996. Review: biosynthesis and function of yeast vacuolar proteases. *Yeast* **12**:1–16.
57. Veenhuis, M., I. Keiser, and W. Harder. 1979. Characterization of peroxisomes in glucose-grown *Hansenula polymorpha* and their development after the transfer of cells into methanol-containing media. *Arch. Microbiol.* **120**:167–177.
58. Wach, A., A. Brachat, R. Pohlmann, and P. Philippsen. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**:1793–1808.
59. Woolford, C. A., L. B. Daniels, F. J. Park, E. W. Jones, A. J. Van, and M. A. Innis. 1986. The *PEP4* gene encodes an aspartyl protease implicated in the posttranslational regulation of *Saccharomyces cerevisiae* vacuolar hydrolases. *Mol. Cell. Biol.* **6**:2500–2510.