**AUT1, a Gene Essential for Autophagocytosis in the Yeast *Saccharomyces cerevisiae***

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Autophagocytosis is a starvation-induced process responsible for transport of cytoplasmic proteins to the vacuole. In *Saccharomyces cerevisiae*, autophagy is characterized by the phenotypic appearance of autophagic vesicles inside the vacuole of strains deficient in proteasome yscB. The **AUT1** gene, essential for autophagy, was isolated by complementation of the sporulation deficiency of a diploid *aut1*-1 mutant strain by a yeast genomic library and characterized. **AUT1** is located on the right arm of chromosome XIV, 10 kb from the centromere, and encodes a protein of 310 amino acids, with an estimated molecular weight of 36 kDa. Cells carrying a chromosomal deletion of **AUT1** are defective in the starvation-induced bulk flow transport of cytoplasmic proteins to the vacuole. *aut1* null mutant strains are completely viable but show decreased survival rates during starvation. Homozygous *Δaut1* diploid cells fail to sporulate. The selective cytoplasm-to-vacuole transport of aminopeptidase I is blocked in logarithmically growing and in starved *Δaut1* cells. Deletion of the **AUT1** gene had no obvious influence on secretion, fluid phase endocytosis, or vacuolar protein sorting. This supports the idea of autophagocytosis as being a novel route transporting proteins from the cytoplasm to the vacuole.

Cells have developed sophisticated mechanisms to adapt to changes in the nutritional environment. One major process enabling cells to survive periods of nitrogen deprivation is degradation of large amounts of intracellular proteins. Eukaryotic cells contain two major systems for protein degradation, the proteasome and the lysosome (18, 21). While the proteasome seems to be responsible for the breakdown of regulated and short-lived proteins (17), the starvation-induced proteolytic breakdown is dependent mainly on the lysosome. An interesting question is how cytosolic proteins enter the lysosomal (vacuolar) lumen.

Morphological studies preferentially done with mammalian cells have demonstrated that this protein uptake process is due mainly to unselective bulk flow autophagocytosis (for reviews, see references 8 and 34). Detailed electron microscopic studies supported the idea that cytoplasm containing double or multilayered early autophagosomes are most likely formed from parts of the endoplasmic reticulum (9, 13, 45). These vesicles are further maturing to late autophagosomes and autolysosomes (10, 24, 26).

Another, more selective mechanism for vacuolar protein uptake during nutrient deprivation, based on a KFERQ-related pentapeptide motif, was proposed by Dice (6).

As a simple eukaryotic model organism which is easily amenable to genetic manipulations, we used the yeast *Saccharomyces cerevisiae* to study gene products involved in the uptake process of proteins from the cytoplasm into the vacuole. Under nitrogen starvation conditions in *Saccharomyces cerevisiae*, nearly half of the total cellular protein content is degraded during a 24-h period. More than 80% of this degradation takes place inside the vacuole, the counterpart of the mammalian lysosome (40). Following the concomitant uptake of several cytosolic enzymes into the vacuole, the unselective nature of protein entry into the vacuole in this organism was demonstrated (11). A detailed microscopic analysis showed the appearance of autophagic vesicles inside the vacuole, when cells with defects in the vacuolar endoproteinasises yscA or yscB are subject to starvation (37, 39). The accumulation of these vesicles can also be induced by incubating the cells in nitrogen-deficient media in the presence of the proteasome yscB inhibitor phenylmethylsulfonyl fluoride (PMSF) (39, 41). By indirect immunofluorescence microscopy, it has been shown that fatty acid synthase, a cytoplasmic marker protein, is localized inside these autophagic vesicles (41).

For genetic dissection of autophagocytosis, we isolated autophagocytosis mutants, i.e., mutants defective in the autophagic process (41). These *aut* mutants are defective in the breakdown of a cytoplasmic marker protein, fatty acid synthase, whose degradation during starvation was shown to be dependent mainly on the action of the vacuolar proteasises. Furthermore, *aut* mutants are unable to accumulate autophagic vesicles in the vacuolar lumen during periods of nitrogen starvation.

Another set of mutants (apg) with a defect in autophagocytosis was isolated due to their reduced ability to survive during starvation (44).

Here we report the isolation and sequencing of the **AUT1** gene, shown to be essential for the autophagocytotic process. Chromosomal deletion of **AUT1** does not influence growth on rich media but leads to a reduced survival rate of the mutant cells during periods of nitrogen starvation. Autophagocytosis seems to be an essential prerequisite for sporulation. Homozygously deleted *aut1* diploids are defective in the formation of ascii. The block of autophagocytosis in chromosomal *aut1*-deleted cells has no significant influence on endocytosis (29), secretion (31, 33), or vacuolar biogenesis (5, 38). Most recently, a phenotypic and genetic overlap of autophagocytosis and the selective import of aminopeptidase I from the cytoplasm into the vacuole (16, 20) has been found (15). We found *Δaut1* mutant strains to be impaired in the maturation of the precursor of aminopeptidase I. Our findings support the idea that autophagocytosis constitutes a new route of protein transport from the cytoplasm to the vacuole.
MATERIALS AND METHODS

Chemicals. PMSF was purchased from Serva, Heidelberg, Germany; zymolyase 100T was from Seikagaku Kyogo, Tokyo, Japan; Lucifer yellow and quina...er I were from Sigma or Roth (Karlsruhe, Germany), and all were of analytical grade.

Synthetic oligonucleotides were from Eurogentec, Ougrée, Belgium, and MW Biotech, Ebersberg, Germany.

Asparaginase. Antibodies directed against proteins yscA, yscB, and yscY as well as fatty acid synthase are described elsewhere (11, 12, 25); antibodies against aminopeptidase I were generously provided by D. J. Klionsky (20).

Media. Yeast strains were grown either in complete liquid medium YPD (1% yeast extract, 2% peptone, 2% glucose) or complete minimal dropout media (0.1% yeast nitrogen base [without amino acids and 2% glucose and supplemented with adenine, uracil, and amino acids) as described previously (1). For starvation or sporulation, cells were incubated in 1% potassium acetate.

Strains. For strains used, see Table 1. Strain WCG4a was obtained by chromosomal deletion of the ADE2 gene in WCG4a with a 2.3-kb BamHI fragment from pPL131. Strain YMS5a was made by transforming a 3.3-kb XhoI fragment from pAUT1A1:URA3 into the genome of WCG4a; strain YMS6a was made by transforming a 4.6-kb BamHI-SacI fragment from pAUT1A1:ADE2 into WCG4a; and YMS7a was made by transforming a 3.5-kb XbaI fragment from pAUT1A1:URA3 into WCG4a. YMS8 was obtained from a cross of YMS5 and YMTA. The end2 mutant strain RH932 was kindly provided by H. Riezman.

Plasmids. Standard DNA cloning and manipulation was done as described before (1). For DNA sequencing, we used the T7 DNA sequencing kit obtained from Pharmacia, Freiburg, Germany. Plasmid pPL131 used for chromosomal deletion was a gift from P. Ljungdahl, Stockholm, Sweden.

Plasmid pRS8 contains the S. cerevisiae genomic DNA including the AUT1 locus was selected from a YCplac111-based library (5a). For subcloning of a genomic fragment containing the functional AUT1 gene, a 3.3-kb XhoI fragment was isolated from pRS8 and cloned into the XbaI site of pRS315/AUT1 and replaced by a 1.1-kb URA3 fragment, yielding plasmid pAUT1A1::URA3. In a second approach, a 2.2-kb fragment containing the functional AUT2 marker was used to get pAUT1A1::ADE2.

Screening procedure. The ade2 deletion allele from pPL131 was chromosomally integrated into an end2-1 mutant strain, and the resulting strain was crossed with an end2-1 ADE2 mutant strain of the opposite mating type. The resulting diploid strain was transformed with yeast genomic libraries based on the CEN LEU2 shuttle vector YCplac111 (5a) or the 2µm URA3 shuttle vector YEp24 (3), and complementation of the nonsporulating phenotype of the diploid was searched for. The resulting colonies were washed from the plates, diluted into 1% potassium acetate at an optical density at 600 nm (OD600) of approximately 1.0, and incubated for 4 to 6 days at room temperature for sporulation. From 10 µl of sporulated cells, random spores were prepared by digestion with 0.1 mg of zymolyase 100T in 5 ml of sterile water, followed by the addition of 5 ml of 1.5% Nonidet P-40 solution and sonication. Thereafter, cells were washed and plated on selective media. The ratio between red (ade2) and white colonies (ADE2 or ade2::ADE2) was always near 1:1, indicating that very few diploid cells survived this procedure. Red colonies were picked and tested for the ability to accumulate autophagic vesicles upon starvation in the presence of PMSF as described previously (41).

Microscopy. Microscopic observations were done with a Zeiss Axioskop microscope. Visualization of autophagic vesicles inside the vacuole was done as described before (41). Vascular acidification was examined by observing accumulation of the fluorescent dye quinacrine inside the vacuole by standard procedures (30). Endoptylosis was examined as described previously (7) by accumulation of lucifer yellow. Staining with MDY-64 was done as described by Molecular Probes Europe BV, Leiden, The Netherlands.

Cell fractionation. Spheroplast formation, lysis, and preparation of vacuole-enriched and cytosolic fractions were performed as described by Harding et al. (15), except that cells were starved for 24 h at room temperature before spheroplast formation and preincubation in the presence of diithiothreitol was done for 30 min. Whole spheroplasts were harvested and washed at 1,000 × g.

Electrophoresis and immunoblotting. Cell extracts were prepared as described before (11), samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% or 12% acrylamide [23]) and blotted onto nitrocellulose membranes (43). To prevent unspecific binding of antibodies to nitrocellulose, membranes were incubated in PBS-T buffer (0.1% Tween 20, 100 µl NaCl, 20 mM potassium phosphate [pH 7.5]) containing 10% nonfat milk for at least 2 h.

For immunodetection, primary antisera against proteins yscA and yscB were used at a dilution of 1:5,000, and antisera against carboxypeptidase yscY, aminopeptidase I, and fatty acid synthase were used at a dilution of 1:10,000 in PBS-T and incubated with peroxidase-conjugated goat anti-rabbit secondary antibody at a dilution of 1:5,000 for 1 h, membranes were washed and the bands were visualized with the enhanced chemiluminescence detection kit provided by Amersham-Buchler.

Protein turnover. Cells were grown in 10 ml labeling medium (consisting of 0.17% yeast nitrogen base [without amino acids and ammonium sulfate], 2% proline, and 2% glucose and supplemented with the appropriate auxotrophic nutrients) at 30°C to approximately 5 × 10⁶ cells/ml. In the last 14 h of growth, 3.7 MBq of [1-¹⁴C]methionine was added to the culture. After labeling, the cells were collected by centrifugation, washed three times with starvation medium, resuspended in starvation medium containing 10 mM nonradioactive methionine, and further incubated at 30°C. At the indicated time, 1-ml samples were taken, mixed with 100 µl of 1% trichloroacetic acid, 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 × g. Nine hundred microliters of the supernatant was mixed with 5 ml of liquid scintillation mixture. For determination of the total incorporated radioactivity, the pellets of the 0-h samples were washed five times with starvation medium containing 10% trichloroacetic acid and two times with ethanol-ether (1:1). The pellets were air dried and dissolved in 1 ml of NCS-II-H₂O (9:1) at 40°C. Nine hundred microliters of the solution was mixed with 5 ml of liquid scintillator. Radioactivity was determined with a Wallace 1410 liquid scintillation counter (Pharmacia).

Survival during starvation. Cells of different strains were grown to equal ODs in YPD medium, harvested, and resuspended in 1% potassium acetate to an OD₆₀₀ of 0.02 to 0.05. Every 24 h, samples were taken, diluted, and plated, and the number of colonies growing was determined. The relative survival rate compared with the number of colonies recovering after the first 24 h of starvation was calculated.

Secretion of invertase. Secretion of invertase was assayed as described previously (27), without shifting the cells to 37°C.

RESULTS

The ade2-1 mutant strain FIM35 obtained by ethyl methane-sulfonate mutagenesis (41) was backcrossed four times with the wild-type strain WCG4a. Seventy-nine tetrads were analyzed for the phenotypic appearance of autophagic vesicles in the vacuole in the presence of PMSF to obtain the second segregation of vesicle accumulation. This demonstrates the existence of a recessive, single point mutation responsible for the ade2-1 phenotype. From the segregation pattern of the ade2-1 mutation and the centromere-linked auxotrophic marker trp1 (data not shown), the genetic distance (35) of
FIG. 1. (A) Part of the genomic DNA fragment SCN201952 surrounding the AUT1 gene. The chromosome XIV centromere sequence is located near position −7000. Also shown are the genomic fragments obtained from the library plasmids pRSp8 and pRSp34, the two subclones made from pRSp8, and the fragment used for subcloning of the AUT1 gene. (B) Sequence of the AUT1 gene. The nucleotide sequence of AUT1 is identical to the published sequence of ORF N2040. Amino acids are shown in a single-letter code. Recognition sites of several commonly used DNA restriction enzymes are indicated. (C) Clusters of positively and negatively charged amino acid residues within Aut1p.
AUT1 to the centromere was calculated to be 7.6 cM. A localization in direct neighborhood to TRP1 did not seem likely from these data.

**Isolation of the AUT1 gene.** In our attempt to isolate the AUT1 gene, we took advantage of the drastically reduced sporulation frequency of a homozygous aut1-1 mutant diploid strain. After transformation of this diploid strain with a plasmid-encoded yeast chromosomal library, predominantly those cells bearing a plasmid-borne AUT1 wild-type gene should be able to form asc. Most of the nonsporulated diploid cells were killed by following the established random spore protocol (1). The use of a heterozygous ade2/DE2 diploid strain allowed the rapid recognition of ascospores after this procedure. Only ade2 haploid ascospores exhibited the typical red pigment. A plasmid-encoded AUT1 gene should rescue not only the sporulation defect but also the defect in the autophagic pathway seen in an aut1-1 mutant strain. Therefore, the red colonies were further tested for restoration of their ability to accumulate autophagic vesicles inside the vacuole during a 4-h starvation period on nitrogen-free medium in the presence of PMSF.

In a typical experiment, batches of 3,000 clones transformed with the genomic library were pooled, sporulated, and subjected to the random spore procedure. After spreading on plates, 20 to 40 red colonies were checked from each batch for their ability to accumulate autophagic vesicles. By use of the centromeric YCplac111 genomic library, 45,000 colonies were analyzed in total. In one batch, five positive colonies were detected, and the respective plasmids were rescued. All plasmids were found to be identical and contained a 5.5-kb genomic insert (pRS8) (Fig. 1A).

In a similar approach, by use of an overexpressing high-copy Yep24-derived genomic library, 15,000 transformands were screened in five batches. Four positive colonies containing identical plasmids (pRS34) with a 7.5-kb genomic insert were found. Partial sequencing of the genomic inserts of pRS8 and pRS34 localized both of them to chromosome XIV, genomic fragment SCN201952 (Fig. 1A) (47). The genomic fragment SCN201952 is located on the right arm of chromosome XIV, directly flanking the centromere, which is in good agreement with the calculated genetic distance of the aut1-1 locus (7.6 cM) from the centromere. The only complete open reading frame (ORF) present in both of the plasmids pRS8 and pRS34 was the ORF N2040. A 3.3-kb XbaI fragment from pRS8 containing only ORF N2040 was subcloned into the vector pRS315 and found to be capable to restore the vesicle accumulation defect of the aut1-1 mutant strain. As expected, two subclones starting at the unique PstI site inside N2040 were unable to complement the aut1-1 mutation (Fig. 1A). The described 3.3-kb XbaI fragment was chromosomally integrated in an aut1-1 mutant, and the resulting strain was crossed with a wild-type strain. All ascospores exhibited a wild-type phenotype. This confirmed that the isolated ORF N2040 was indeed identical to AUT1. Resequencing of AUT1 did not uncover any discrepancies with the known sequence (47) in the databases (Fig. 1B). AUT1 encodes a protein with 310 amino acids and a calculated molecular size of 36 kDa. The AUT1 gene product Aut1p seems to be quite hydrophilic with clusters of charged amino acids (Fig. 1C) and a predicted isoelectric point of 4.4. A stretch of 23 amino acids (residues 130 to 152) seems to fulfill the definition given by Reali et al. (28) for KEKE motifs, although there is only one K present. KEKE motifs have been proposed to be involved in the assembly of proteins into larger complexes. The Aut1p contains no obvious transmembrane domains. The Aut1p shows no significant homologies to other proteins of known function in the databases.

**Chromosomal deletion of AUT1.** Chromosomal aut1 null mutant strains were constructed by deleting the AUT1 coding region between the BgII and NcoI restriction sites (Fig. 1B) and inserting the URA3 or ADE2 gene, respectively, as a selectable marker. The correct gene replacement was confirmed by Southern hybridization and PCR (data not shown).

The resulting strains YMS5 (aut1Δ::URA3) and YMS6 (aut1Δ::ADE2) were viable and did not show any growth phenotypes at 10, 30, or 37°C (data not shown). Vacular morphology was checked with Nomarski optics (Fig. 2A) and the vacuolar membrane dye MDY-64 (Fig. 2B). Delta1 cells exhibited a clearly visible vacuole, which appeared smaller than wild-type vacuoles. The membranes of autophagic vesicles accumulating inside the vacuoles of starved pral1-deficient cells could also be stained with MDY-64 (Fig. 2B). Quinacrine, a dye routinely used to detect the acidification of the vacuole (30), accumulated normally inside the vacuoles of Delta1 cells (Fig. 2C).

As expected, aut1 null mutant strains showed a block in the autophagic pathway, demonstrated by their inability to accumulate autophagic vesicles inside the vacuole during starvation for nitrogen in the presence of the protease inhibitor PMSF (Fig. 2A). For further confirmation of a defect in the uptake of cytoplasmic proteins into the vacuolar lumen of these strains, we checked the subcellular localization of a cytoplasmic protein, fatty acid synthase, after a 24-h starvation period for nitrogen. For cell fractionation, spheroplasted cells were hypotonically lysed without affecting the integrity of the vacuole. Thereafter, cytoplasmic and vacuolar enriched fractions were isolated in a centrifugation step (15). To prevent the degradation of cytoplasmic proteins inside the vacuole, strains defective in proteinase yscA (pral1/pep4) were used for this experiment. As a control for proper vacuolar enrichment, the localization of the resident vacuolar protease yscB was also checked (Fig. 3). As shown in Fig. 3A, lane P, in an AUT1 wild-type, pral1-deficient strain, significant amounts of both subunits of the cytoplasmic fatty acid synthase can be detected in the vacuolar enriched fraction by immunoblotting, whereas in the aut1/pral1 null mutant strain, no fatty acid synthase is localized to the vacuole (Fig. 3B, lane P).

Demonstration that not only the uptake of a single cytoplasmic protein into the vacuole but also the unspecific bulk flow of proteins to the vacuole is affected in aut1-deleted cells was brought about by determining the overall protein turnover rate. All cellular proteins were radiolabeled in growing cells with [35S]methionine. Thereafter, cells were shifted to a nitrogen-free, nonradioactive starvation medium. After precipitating nondegraded proteins with trichloroacetic acid, the amount of acid-soluble small peptides generated by the action of the intracellular proteasomes was determined (Fig. 4). Under these conditions, a wild-type strain exhibited an initial protein breakdown rate of 1.8% per h. The block of vacuolar proteolysis in a pral1-deficient strain reduces this rate to 18% of the level of a wild-type strain; the residual proteolysis rate is mainly due to the action of nonvacuolar proteasomes, most likely, the cytosolic and nuclear proteasomes (40). A nearly identical reduction of protein degradation to 18% of that of the wild type was detected in an aut1 null mutant strain (Fig. 4). This gives a strong indication for a block of unspecific protein uptake into the vacuole due to the defect in Aut1p.

Vacuolar proteolysis is most prominent in cells starving for nitrogen, and protease yscA-deficient cells cannot survive extended times of starvation (40, 46). We therefore measured the ability of haploid Delta1 cells to survive a starvation period (Fig. 5). Similar to a pral1-deficient strain, Delta1 cells exhibited a significantly reduced survival rate. After prolonged periods of
starvation, the survival rate of the Δaut1 strain was even lower than that of a pra1-deficient strain (Fig. 5).

Diploid cells respond to nutritional limitation by the differentiation process of sporulation and the formation of asc. Sporulation frequency was determined either by counting the asc under the microscope or by using ade2/ade2 heterozygous diploids and counting the number of red colonies formed after a random spore procedure. For calculation of sporulation frequencies, the viability of cells after sporulation was tested and only viable cells were taken into consideration to exclude any influence from mutant cells dying of starvation. Wild-type and heterozygous AUT1/Δaut1 diploids showed normal sporulation rates under various conditions (data not shown), whereas homozygous Δaut1/Δaut1 cells completely failed to sporulate. From several million viable diploid cells, we were unable to recover any haploid colonies or observe any asc microscopically (data not shown).

Overlap with other vesicle transport processes. We elucidated a potential overlap of autophagocytosis and other vesicle-mediated processes by checking the influence of a chromosomal aut1 deletion on several other protein transport processes.

Biogenesis of the vacuole. The correct sorting of soluble vacuolar proteinases to the vacuoles is a good indication of undisturbed vacuolar biogenesis. An analysis of the steady-state levels of the proteinases yscA and yscB and carboxypeptidase yscY by immunoblotting showed only the mature forms of these enzymes in Δaut1 cells after starvation (Fig. 6A) as well as in growing cells (data not shown). Recently, a more rigid kinetic pulse chase analysis of carboxypeptidase yscY maturation showed a wild-type-like sorting of this enzyme to the vacuole in an aut1-1 mutant strain (15).

Secretion. The efficiency of secretion was measured (Fig. 6B) by use of invertase as a well-known marker enzyme for secretion (32). The appearance of enzymatically active, extracellular invertase after induction of the enzyme by glucose deprivation was examined. There was no significant alteration in the time course of invertase secretion visible in an aut1-deficient strain as compared with that of a wild-type strain (Fig. 6B).

Endocytosis. In contrast to endocytosis-defective mutants, the ability of aut1 null mutant cells to take up lucifer yellow into the vacuole by fluid-phase endocytosis (7) was not affected (Fig. 6C).

Cytoplasm-to-vacuole targeting of aminopeptidase I. Aminopeptidase I was shown to be synthesized as a precursor in the cytoplasm, from where it is targeted directly to the vacuole without the detour through the secretory pathway. In the vacuole, the enzyme undergoes maturation by proteinase yscB (20). Cells deleted in the chromosomal aut1 gene are defective in maturation of preaminopeptidase I (Fig. 7). In crude extracts of logarithmically growing cells or cells starved for 4 h, no mature aminopeptidase I could be recognized (Fig. 7, lanes 4). A centromeric plasmid carrying the AUT1 gene almost completely cured this defect in logarithmically growing as well as in starved cells (Fig. 7, lanes 5). A small amount of preaminopeptidase I is still visible. Overexpression of AUT1 from a 2μ plasmid did not further reduce the amount of aminopeptidase I precursor (lanes 6).

**DISCUSSION**

The autophagic process of cytoplasmic protein uptake and delivery to the lysosome (vacuole) is not yet understood at the molecular level. To gain some understanding of this process, we followed a genetic approach with the model eukaryote *S. cerevisiae*. Analysis of an aut1-1 mutant strain, isolated by its inability to degrade a cytosolic marker protein and its inability to accumulate autophagic vesicles in the vacuole, sheds some first light on this process.

![FIG. 3. Cell fractionation. Cellular extracts of total spheroplasts (T), a vacuolar enriched pellet (P), and a cytosolic enriched supernatant (S) fraction were prepared as described after 24 h of starvation, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted, and probed with antibodies directed against the α- and β-subunits of fatty acid synthase and proteinase yscB. (A) The pra1 deletion strain shows accumulation of fatty acid synthase together with the vacuolar marker yscB in the vacuole-enriched fraction (P) after starvation. (B) In a pra1/aut1 double mutant strain under identical conditions, no fatty acid synthase is found in the fraction containing proteinase yscB, thereby demonstrating the block in autophagic transport due to deletion of the AUT1 gene.](http://jb.asm.org/)

![FIG. 4. Degradation of total cellular proteins during starvation. The pra1 deletion strain YMTA (C) showed drastically reduced turnover rates compared with that of the wild-type strain WCG4a (L). The same reduction in degradation could be observed in the aut1 null mutant YMS6 (C).](http://jb.asm.org/)
By use of the drastically reduced sporulation frequency of a homozygous aut1-1/aut1-1 diploid strain, we developed a new screening procedure which allows the rapid isolation of genes related to the autophagic process. This led us to the isolation of the AUT1 gene, which was sequenced and found to be identical to ORF N2040 on the right arm of chromosome XIV. The AUT1 gene encodes a protein of 310 amino acids that contains no obvious transmembrane domain. A search for homologs of Aut1p in the protein databases identified no significant similarities to other proteins of known function. One striking feature of Aut1p is the high content of charged amino acids.

We constructed chromosomal aut1 null mutant strains and confirmed the complete block of autophagocytosis in these strains by demonstrating the absence of any autophagic vesicles in the vacuolar lumen during a starvation period for nitrogen in the presence of PMSF. In mammalian tissues, autophagocytosis is well known as a starvation-induced transport process of cytoplasmic proteins into the lysosome. We therefore routinely used nitrogen starvation conditions with only 1% potassium acetate as the culture medium to fully induce the autophagic pathway. We performed cell fractionation experiments with pra1-deficient cells to prevent the degradation of proteins in the vacuolar lumen. In cells wild type for autophagocytosis, the cytosolic fatty acid synthase can be found in the vacuolar fraction after starvation as a result of the autophagic process. The lack of fatty acid synthase in the vacuolar enriched fraction of Δaut1 cells confirmed the inability of these cells to import cytosolic proteins into the vacuole.

Under starvation conditions in a strain wild type for protease and autophagocytosis, 40% of all cellular proteins were shown to be subject to vacuolar proteolysis during a 24-h period (40). We measured the overall protein breakdown rates in Δaut1 cells. Similar to a protease yscA-deficient strain, which is impaired in almost the complete protein breakdown inside the vacuole (19, 40, 46), an aut1 null mutant strain had a reduced proteolysis rate of 0.32% of all proteins per h compared with the rate of 1.8% per h found in a wild-type strain. This reduction very much supports the idea of autophagocytosis as being an unspecific bulk flow protein transport pathway.
vesicles with preexisting lysosomes (24). In (14,42), but the major route seems to be fusion of autophagic vesicles with preexisting lysosomes (24). In S. cerevisiae, an ultrastructural analysis showed double membrane-layered autophagosome-like structures in the cytosol. Sometimes, the outer membrane of these structures has been found in continuity with the vacuolar membrane (2). This proposes a membrane fusion event that could explain the appearance of single membrane-surrounded vesicles in the vacuolar lumen (37, 39).

Interestingly, we found that the membranes of autophagic vesicles, which accumulate inside vacuoles, could be stained with MDY-64, a dye routinely used to stain the vacuolar membrane. This might be a hint that the membranes of the autophagic vesicles in the vacuolar lumen to some extent resemble the vacuolar membrane.

We checked the effect of a block of autophagocytosis caused by an aut1 deletion on vacuolar morphology by using light microscopy with Nomarski optics and detected a wild-type-like vacuole with a slightly reduced size. Staining with the vacuolar membrane marker MDY-64 and vacuolar acidification probed with quinacrine (30) showed no difference between the aut1 deletion mutant and a wild-type strain. A steady-state analysis of the maturation of the proteinases yscA, yscB, and yscY, as well as a kinetic analysis of carboxypeptidase yscY sorting to the vacuole, showed no defect in the vacuolar protein sorting pathway. The time course of invertase secretion and the uptake of lucifer yellow by fluid phase endocytosis were wild type like. Taken together, these results suggest that Aut1p plays no essential function for other vesicle-mediated processes. This supports the idea that autophagocytosis constitutes a novel route to the vacuole, with no generally essential function for endocytosis and vacuolar biogenesis.

The action of Aut1p may function as a soluble cytosolic factor in the autophagic process by interacting with other specific proteins. This idea is supported by the existence of clusters of charged amino acids in the Aut1p. Such clusters have been proposed to be involved in the association of proteins (28). In an attempt to localize the Aut1p, we created epitope-tagged versions of the protein by inserting the HA epitope (hemagglutinin from Hae- mophilus influenzae) into the protein. Unfortunately, all constructs created to date were unable to complement the defects seen in an aut1-deleted strain and were therefore not useful to localize the Aut1p in the cells. Further studies to localize the Aut1p in the cells and to shed light on its precise function in the autophagic process are under way.

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