Ald6p Is a Preferred Target for Autophagy in Yeast, *Saccharomyces cerevisiae*

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Macropathology is the process of intracellular bulk protein degradation induced by nutrient starvation and is generally considered to be a nonselective degradation of cytosolic enzymes and organelles. However, it remains a possibility that some proteins may be preferentially degraded by autophagy. In this study, we have performed a systematic analysis on the substrate selectivity of autophagy in *Saccharomyces cerevisiae*, using two-dimensional PAGE. We performed a differential screen on wild-type and Δatg7/Δapg7 autophagy-deficient cells and found that cytosolic acetaldehyde dehydrogenase (Ald6p) decreased under nitrogen starvation. As assessed by immunoblot, Ald6p was reduced by greater than 92% after 24 h of nitrogen starvation. This reduction was dependent on Atg/Apg proteins and vacuolar proteases but was not dependent on the proteasome or the cytoplasm to vacuole targeting (Cvt) pathway. Using pulse-chase and subcellular fractionation, we have also demonstrated that Ald6p was preferentially transported to vacuoles via autophagosomes. Δatg7 Δald6 double mutant cells were able to maintain higher rates of viability than Δatg7 cells under nitrogen starvation, and Ald6p-overexpressing cells were not able to maintain high rates of viability. Furthermore, the Ald6pC306S mutant, which lacks enzymatic activity, had viability rates similar to Δald6 cells. Ald6p enzymatic activity may be disadvantageous for survival under nitrogen starvation; therefore, yeast cells may preferentially eliminate Ald6p via autophagy.

Cellular activities require the maintenance of a balance between the synthesis and degradation of proteins. Macropathology (hereafter referred to as autophagy) is an intracellular bulk degradation system that is well conserved in eukaryotes; autophagy transports cytoplasmic components to the lysosome/vacuole for degradation (1). This degradation is a cellular response to starvation and also plays a role in the recycling of cytoplasmic components, which may be important for cellular remodeling, development, and differentiation (2–4). A total of 16 genes that are essential for autophagy and that are named *APG* and *AUT* (current nomenclature is *ATG*) (5) have been identified by genetic screens in yeast, *Saccharomyces cerevisiae*. Much progress has been made in the functional analysis of these genes (6–8). In eukaryotic cells, there is another major degradation system, the ubiquitin proteasome pathway, which mediates the selective ubiquitination and subsequent degradation of proteins by the proteasome. This pathway serves mainly to degrade short-lived proteins, such as transcription factors, cell cycle regulators, and defective proteins. However, more than 99% of cellular proteins are long-lived, and autophagic degradation contributes to the turnover of these proteins. In contrast to selective degradation by the ubiquitin proteasome pathway, autophagy is generally thought to be nonspecific. Autophagy is initiated by the sequestration of cytoplasmic components in a double-membrane structure termed the autophagosome. Immunoelectron microscopy has shown that ribosomes and typical cytosolic marker enzymes, such as alcohol dehydrogenase (ADH) and phosphoglycerate kinase (PGK), are present in the autophagosome and autophagic bodies at the same densities as in the cytosol (9). The measurement of the enzymatic activities of these proteins also supports this conclusion (10). If degradation of long-lived proteins is exclusively mediated by autophagy, all proteins might be expected to have similar lifetimes. However, long-lived proteins have a variety of lifetimes; therefore, the autophagic pathway might have some selectivity. It is known that fructose-1,6-bisphosphatase (via the vacuolar import and degradation, the Vid pathway) (6, 11) and the peroxisome (via pexophagy) (12) are selectively transported from the cytoplasm to the vacuole and degraded.

To investigate the possibility of selective autophagic degradation, we attempted to compare the amounts of each intracellular protein under growth and starvation conditions in yeast, *S. cerevisiae*. We performed a systematic analysis using two-dimensional PAGE and MALDI-TOF mass spectrometry to detect the autophagy-dependent degradation of intracellular proteins. During these analyses, we observed an interesting behavior of the Mg²⁺- and NADPH-dependent cytosolic acetaldehyde dehydrogenase (Ald6p), which catalyzes the conversion of acetaldehyde to acetate in the cytosol (acetate + NADPH → NADP⁺ + acetate) (13). The *S. cerevisiae* genome encodes five or more different members of the aldehyde dehydrogenase family. Ald4p is the major K⁺- and NADPH-dependent cytosolic acetaldehyde dehydrogenase (Ald6p), which catalyzes the conversion of acetaldehyde to acetate in the cytosol (acetate + NADPH → NADP⁺ + acetate) (13). Ald5p is a minor K⁺-dependent mitochondrial acetaldehyde dehydrogenase (14), and Ald5p is a minor K⁺-dependent mitochondrial acetaldehyde dehydrogenase, which is induced when cells are grown in ethanol-containing medium (15). Ald2p and Ald3p are closely related cytosolic enzymes that are required for in vivo pantothentic acid biosynthesis via conversion of 3-aminopropanol to β-alanine (16). Ald4p and Ald6p function in...
the conversion of acetaldehyde to acetate, which is a key intermediate during fermentation of sugars and growth on ethanol and are consequently important for acetyl-CoA production. In contrast, Ald2p, Ald3p, and Ald5p may not contribute to the oxidation of acetaldehyde in vivo. Therefore, Ald6p is the only cytosolic acetaldehyde dehydrogenase in the yeast cell. We demonstrate here that Ald6p is degraded preferentially by autophagy and that reduction of Ald6p may improve viability rates under nitrogen starvation.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—The *S. cerevisiae* strains used in this study are listed in Table I. Standard techniques were used for yeast manipulation (17). Yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or SD + CA medium (0.17% casamino acid, 0.5% ammonium sulfate, and 2% glucose) supplemented with 0.002% adenine sulfate, 0.002% uracil, and 0.002% tryptophan if necessary. For nitrogen starvation, SD–N medium (0.17% yeast nitrogen base without ammonium sulfate and amino acids with and without 2% glucose) was used.

**Plasmid Construction**—To create the glutathione S-transferase-Ald6p fusion construct (pJO1), the open reading frame of ALD6 (YPLO61w) lacking the initiation codon (1.5 kb) was amplified by genomic PCR using the following primers: 5′-GGCGGATCCCTTACTTGTATAGTTCATCCATG-3′ and 5′-AGAACGCTGGTCAAATTTCTTCCTCTGGTT-3′. The site of BamHI sites at the open reading frame of modified GFP struct (pJO203), XhoI and BamHI sites were added to a sequence containing the ald6d′ fragment lacking the stop codon (1.5 kb) by genomic PCR amplification using the following primers: 5′-CCGCTCGAGCACCGACCATGTGGG-3′ and 5′-CCGCTCGAGCAACTTAATTCTGACAGCTTTTAC-3′. The resulting DNA fragments were cloned into the pRS426 multicopy plasmid (18) to yield pJO2. To create the Ald6p overexpression construct (pJO1), the open reading frame of modified Ald6p was amplified by PCR using the following primers: 5′-AACCAGAGGAGAATTTGACCAGCGTTCT-3′ and 5′-AACCAGAGGAGAATTTGACCAGCGTTCT-3′. The site of mutagenesis in pJO213 was confirmed by automated DNA sequencing.

**Two-dimensional PAGE and Peptide Mass Fingerprinting**—The strains were prepared by breaking yeast cells with glass beads in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science). The lysates were centrifuged at 100,000 × g for 1 h, and the supernatant was desalted with NAP-10™ (Amersham Biosciences). The protein concentrations were determined using a BCA assay kit (Pierce), and 300 μg of each lysate was applied to the gel. Isoelectric focusing was performed with IEFphor™ (Amersham Biosciences) and a 13 cm Immobiline™ DryStrip pH 4–7 (Amersham Biosciences) as described (19). The gel strip was subjected to SDS-PAGE (12.5% acrylamide), and the gel was stained with Coomassie Brilliant Blue R-250. The protein spots were picked, washed with 100 mM ammonium bicarbonate, dehydrated with acetonitrile, and dried in an evaporator. The spots were digested in the gel with 0.5 mg/ml of trypsin (Promega) in 100 mM ammonium bicarbonate for 12 h at 30°C. The digested peptides were extracted from the gel with 10% formic acid and 50% acetonitrile and desalted with the ZipTip™ C-18 (Millipore). The samples were mixed with α-cyano-4-hydroxy-cinnamic acid (Fluka) in a 2:1 ratio and were analyzed by MALDI-TOF mass spectrometry, REFLEX III (Bruker). The proteins were identified by searching the ProFound data base (www.129.85.19.192).

**Antibodies**—Ald6p-specific antibodies were prepared as follows. The pJO1 plasmid was transformed into *Escherichia coli* (DH5α), and transformants were grown in LB medium containing 50 μg/ml ampicillin to an A600 of 0.6. Recombinant protein expression was induced with 1 mM isopropyl-β-D-thiogalactosidase for an additional 6 h at 37°C. The recombinant protein was separated by SDS-PAGE and simultaneously stained with gel code (Pierce). The protein band was excised from the gel and eluted with an electric current. The eluted protein-dye complex was used to immunize rabbits. Anti-Ald4p antibodies were purchased from Rockland. Anti-ADH antibodies have been described previously (9). Anti-PGK antibody was purchased from Molecular Probes. Anti-aminopeptidase I (API) antibodies were our laboratory stock.

**Immunoblotting of Total Cell Lysates**—Whole cell lysates were prepared by disrupting cells with glass beads in lysis buffer. SDS-PAGE and immunoblotting were performed as described (20).

**Pulse-Chase Experiments**—The cells were cultured in YPD medium to an A600 of 1.0 at 30°C and were then washed twice and suspended in SD–N medium. The cells were pulse-labeled for 30 min by adding 1 MBq of [35S]methionine/1 A600 unit and chased by adding 0.004% methionine and 0.003% cysteine at 30°C. Immunoprecipitation was performed as described (21).

**Light Microscopy**—Fluorescence microscopy was performed using a Delta Vision microscope (Applied Precision) as described (22).

**Subcellular Fractionation and Proteinase K Protection Assays**—Yeast cells were converted to spheroplasts by treatment with 25 unit/ml of Zymolase-100T (Seikagaku Corporation). The spheroplast lysates were separated into cell fractions as described previously (21). To examine proteinase K sensitivity, each fraction without protease inhibitors was treated with 2 mg/ml proteinase K on ice for 30 min with or without 1% Triton X-100. The samples were precipitated with 10% trichloroacetic acid, washed once with cold acetone, resuspended in SDS sample buffer, and analyzed by SDS-PAGE and immunoblotting.

**Table I**

| Strain     | Genotype Source | Source |
|------------|-----------------|--------|
| SEY6210    | MATα his3Δ200 leu2-3,112 lys2–801 trp1Δ50 ura3–52 sue2Δ9 | Ref. 40 |
| KCV118     | SEY6210 Δarg4::HIS3 | Ref. 41 |
| JOY617     | SEY6210 Δarg4::HIS3 ald6d::kanMX4 | This study |
| KY617      | SEY6210 Δarg4::HIS3 ald6d::kanMX4 | This study |
| KY616      | SEY6210 Δarg4::HIS3 | This study |
| TTV1       | SEY6210 Δpap4::LEU2 | Ref. 42 |
| JOY6005    | SEY6210 Δpap4::LEU2 ald6d::ALD6-GFP | This study |
| JOY6006    | SEY6210 Δpap4::LEU2 ald6d::ALD6-GFP | This study |
| JOY66      | SEY6210 ald6d::kanMX4 | This study |
| JOY64      | SEY6210 Δoldd::URA3 | This study |
| JOY89      | SEY6210 Δoldd::URA3 | This study |
| JOY99      | SEY6210 ald6d::kanMX4 | This study |
| KY64       | SEY6210 Δpap4::LEU2 | Ref. 43 |
| YAK1       | SEY6210 Δpap4::HIS3Δarg4::LEU2 | This study |
| WCG4a      | MATα leu2–3,112 lys2–801 his3–11,15 | Ref. 28 |
| WCG4–1a    | WCG4a; pre1–1 | Ref. 28 |
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Results

Ald6p degradation was measured by pulse-chase experiments. Wild-type (WT, SEY6210) and ∆atg7 (KVY118) cells were grown to $A_{600} = 1.0$, preincubated in SD(−N) medium for 1 h, pulse-labeled with [35S]methionine for 30 min, and chased in SD(−N) medium for 0, 3, 6, and 9 h. The lysates were prepared and subjected to immunoprecipitation with anti-Ald6p or anti-ADH serum. The proteins were eluted and analyzed by SDS-PAGE followed by autoradiography.

A nearly linear manner and was ultimately reduced to 18% of the original level after 24 h of starvation (Fig. 1B). In contrast, Ald6p levels decreased only slightly in ∆atg7 mutant cells (KVY118). We next investigated whether the amount of Ald6p was reduced in various yeast strains that are defective in various steps of autophagy. ∆atg7 (KVY118), ∆atg17/apg17 (JOY617), and all ∆atg/apg mutant cells tested showed a similar defect in the loss of Ald6p (parts shown in Fig. 1C, lanes 1–6). The decrease of Ald6p also required Ypt7p, a protein that is essential for the fusion of autophagosomes to vacuoles (20), and Pep4p, vacuolar proteinase A (Fig. 1C, lanes 1, 2, and 7–10).

The selective transport of vacuolar enzymes (via the Cvt pathway), such as API and α-mannosidase, is known to utilize all of the Apg/Atg proteins except Apg17p (6, 23). Apg11p/Cvt9p and Apg19p/Cvt19p function only in the Cvt vesicle formation and do not play a role in autophagosome formation (24, 25). In ∆atg11 (JOY69) and ∆atg19 mutant cells, Ald6p was reduced in a manner similar to that of wild-type cells under nitrogen starvation (Fig. 1C, lanes 1, 2, 11, and 12; data for ∆atg19 not shown). As expected, another system of vacuolar transport, the Vid pathway (6, 11, 26), was not involved in this phenomenon (Fig. 1C, lanes 1, 2, 13, and 14). One mutant allele of the proteasome subunit PRE1 is pre-1-1, which is frequently used for the following reasons: the pre-1-1 mutation causes a defect in the degradation of short-lived proteins, ubiquitinated proteins (27, 28) and N-end rule substrates (29, 30) at 30 °C. In pre-1 mutant cells (WCG4–11a), Ald6p was decreased similarly to wild-type cells (WCG4a) under nitrogen starvation, indicating that Ald6p is not a substrate for proteasome-mediated degradation (Fig. 1D). Taken together, these mutant studies indicate that the reduction of Ald6p requires all of the Atg/Apg proteins and the processes of vacuolar proteolysis. However, Apg/Cvt proteins, Vid proteins, and proteasomal degradation are not involved in this phenomenon.

Reduced Ald6p Levels Implied a Rapid Degradation under Nitrogen Starvation—We hypothesized that the decrease in Ald6p levels was the result of rapid degradation during nitrogen starvation. To examine this possibility, the kinetics of Ald6p degradation was measured by pulse-chase experiments. Wild-type (SEY6210) and ∆atg7 (KVY118) cells were pulse-labeled for 30 min with [35S]methionine and chased with cold methionine and cysteine for 0, 3, 6, and 9 h. In wild-type cells, the Ald6p was rapidly degraded and was barely detectable after 6 h of chase (Fig. 2). In contrast, the degradation rate of Ald6p was clearly slower in ∆atg7 mutant cells. In addition, ADH, a known nonelective marker of autophagy (9), did not show rapid degradation like Ald6p (Fig. 2). The reduction of Ald6p levels implied a rapid degradation dependent on Atg7p during nitrogen starvation. These results suggest that Ald6p is transported to the vacuole and degraded much more rapidly than typical cytosolic proteins.
Visualization of Ald6p during Nitrogen Starvation—The process of Ald6p vacuolar transport was visualized by expressing physiological levels of an Ald6p-GFP fusion protein from the authentic ALD6 promoter. Upon starvation, the vacuoles gradually became fluorescent. In addition, in \( \Delta \text{pep4} \) cells (JOY6005), many bright dots, which were presumably autophagic bodies, were observed moving around in the vacuole (Fig. 3). In \( \Delta \text{pep4} \Delta \text{atg7} \) double mutant cells (JOY6006), no fluorescence was observed in the vacuoles, but rather, the cytosol was evenly stained (Fig. 3). Because Ald6p-GFP was transported to the vacuole in autophagic bodies during nitrogen starvation, we hypothesized that transport of Ald6p from the cytosol to the vacuole occurred via the autophagosome.

\[ \text{Ald6p Was Preferentially Transported to the Vacuole through the Autophagosome} \]

We previously reported that \( \text{ypt7} \) cells accumulate autophagosomes in the cytosol under nitrogen starvation (20). Using proform of API as a selective cargo marker of autophagosomes, Ishihara et al. (21) showed the low speed pellet (P13) fraction enriches the autophagosomes. So next we studied the behavior of Ald6p in \( \text{ypt7} \) cells (KV44). Under growing conditions proform of API was exclusively resided in the high speed supernatant (S100), but under nitrogen starvation conditions a significant portion was recovered in the P13 fraction as reported (Ref. 21 and Fig. 4A). Similarly Ald6p was recovered in the P13 fraction only under nitrogen starvation condition (Fig. 4A). This fraction completely diminished in \( \text{ypt7} \Delta \text{atg1/} \text{apg1} \) mutant (YAK1; Fig. 4B, lanes 5–8), indicating that a certain amount of Ald6p is in the autophagosomes. As shown in Fig. 4D, Ald6p and proform of API in P13 fraction were resistant to proteinase K treatment but digested in the presence of 1% Triton X-100. This also supported the possibility that Ald6p is sequestered into autophagosomes.

\[ \text{Ald6p Enzymatic Activity May Be Disadvantageous during Nitrogen Starvation} \]

Next, we examined the physiological relevance of the preferential degradation of Ald6p during nitrogen starvation. Autophagy-defective mutants cannot maintain viability under long span nitrogen starvation (2). \( \Delta \text{atg7} \Delta \text{ald6} \) mutant (JOY676) cells also started to die after 2 days of nitrogen starvation, but its viability decreased more slowly than
We surveyed the changes of soluble proteins before and after nitrogen starvation using two-dimensional PAGE. One protein, Ald6p, showed a clear reduction, which was dependent on Atg/Atg proteins, under nitrogen starvation for 24 h (Fig. 1). Previous morphological studies have indicated that autophagy degrades about 2% of the cytosol/h in yeast (9, 10). Scott et al. (33) showed by [35S]methionine pulse-chase experiments that the rate of vacuolar delivery of cytosolic Pho5Ald6p by autophagy was 4% h during the initial 6 h of nitrogen starvation. Autophagy proceeds linearly during the first 6 h of starvation and then gradually slows (33). We know that both diploid and haploid cells induce autophagy in sporulation medium, 2% potassium acetate (10). In a previous report, Betz and Weisner (34) showed that protein degradation in haploid cells occurred at a slower rate than in diploid cells in sporulation medium. Diploid cells degraded 2.5% of the cellular protein/h in sporulation medium (34). Taken together, these results indicate that most proteins should not decrease below 70% of their original levels because of autophagy, even after 24 h of starvation. In wild-type cells, the amount of Ald6p was reduced to 15% of the initial level after 24 h of nitrogen starvation (Fig. 1B). This large decrease in Ald6p levels reflects preferential autophagic degradation.

The result shown in Fig. 4C indicates that the specificity of Ald6p degradation may be achieved by a step of sequestration to the autophagosome. Suzuki et al. (22, 31) indicated that the vacuolar targeting of the proform of API (via the Cvtpathway) required localization with the preautophagosomal structure, which plays a central role in autophagosomal formation. In both Δatg11/cvt9 and Δatg19/cvt19 mutant cells, the proform of API localized to the cytosol away from the preautophagosomal structure and was not targeted to the vacuole (31, 35). It was expected that Atg11p and Atg19p would be membrane receptors for the proform of API (24, 25). However, Ald6p degradation was not dependent on Atg11p (Fig. 1C, lanes 1, 2, 11, and 12) and Atg19p (data not shown). During nitrogen starvation, the half-life (t1/2) of Ald6p was 100 min (Fig. 2), and the half-life of API was 30 min (6). These results indicate that Ald6p is not likely to be a cargo of the general Cvtpathway. One factor contributing to protein targeting is the existence of a membrane receptor; it is possible that the selective sequestration of Ald6p is mediated by a yet unknown molecule(s) on the autophagosome. Further studies of the molecular mechanisms underlying targeted autophagy are now in progress to investigate these possibilities.

To address the physiological significance of this selective degradation, we analyzed the viability of Δald6 or ALD6 over-expressing cells. We have demonstrated that Ald6p enzymatic activity might be disadvantageous for the survival of yeast cells during nitrogen starvation (Fig. 5). Brening and Jespersen (36) have previously reported that Ald6p levels increased during lag phase, the first hours after inoculation of the culture. Meaden et al. (13) reported that the growth of Δald6 mutant cells is slower than that of wild-type cells in both YPD and synthetic medium. It is known that acetaldehyde dehydrogenase is closely related to lipid biosynthesis through the intermediary of acetyl-CoA synthase and fatty acid synthase in the cytosol. Because lipid biosynthesis is a critical process, the expression of Ald6p would be necessary during growth under nutrient conditions. Why is cytosolic Ald6p acetaldehyde dehydrogenase activity harmful under nitrogen starvation conditions? One possible explanations might be that Ald6p may disturb NADPH flux during nitrogen starvation. It is well known that glucose-6-phosphate dehydrogenase (Zw1p; glucose-6-phosphate + NADP⁺ → 6-phosphogluconolactone + NADPH) is the greatest contributor to the reduction of NADP⁺

**DISCUSSION**

that of Δatg7 mutant cells (KVY118; Fig. 5A). The viability of AtgΔald6 cells (JOY66) also improved slightly from that of wild-type cells (AtgΔALD6; SEY6210) under nitrogen starvation. However, disruption of mitochondrial acetaldehyde dehydrogenase (Ald4p), AtgΔald4 (JOY64), and Δatg7 Δald4 (JOY674) cells had no effect on the viabilities of wild-type (SEY6210) and Δatg7 (KVY118; open circle), Δald6 (JOY66; closed square), and Δatg7 Δald6 (JOY676; open square) cells were used. B, wild-type (SEY6210; closed circle), Δatg7 (KVY118; open circle), Δald6 (JOY64; closed triangle), and Δatg7 Δald4 (JOY674; open triangle) cells were used. C, wild-type cells (SEY6210) harboring pRS426 plasmid (closed circle), Δald6 cells (JOY66) harboring pRS426 plasmid (closed square), and Δald6 cells (JOY66) harboring pRS426-ALD6 (closed triangle) were used. D, Δald6 cells (JOY66) harboring pRS426 plasmid (closed square), Δald6 cells (JOY66) harboring pRS426-ald6ΔCys306 plasmid (closed diamond), Δatg7 Δald6 cells (JOY676) harboring pRS426 plasmid (open square), and Δatg7 Δald6 cells (JOY676) cells harboring pRS426-ald6ΔCys306 plasmid (open diamond) were used.

**Fig. 5.** Loss of viability during nitrogen starvation. The cells were grown in YPD medium to an A660 = 1.0, washed twice in SD (−N) medium, and suspended in SD (−N) medium. The viability was determined as described under “Experimental Procedures.” The data are the averages of three independent experiments. 2μ, overexpression using multicopy plasmid. A, wild-type (SEY6210; closed circle), Δatg7 (KVY118; open circle), Δald6 (JOY66; closed square), and Δatg7 Δald6 (JOY676; open square) cells were used. B, wild-type (SEY6210; closed circle), Δatg7 (KVY118; open circle), Δald6 (JOY64; closed triangle), and Δatg7 Δald4 (JOY674; open triangle) cells were used. C, wild-type cells (SEY6210) harboring pRS426 plasmid (closed circle), Δald6 cells (JOY66) harboring pRS426 plasmid (closed square), and Δald6 cells (JOY66) harboring pRS426-ALD6 (closed triangle) were used. D, Δald6 cells (JOY66) harboring pRS426 plasmid (closed square), Δald6 cells (JOY66) harboring pRS426-ald6ΔCys306 plasmid (closed diamond), Δatg7 Δald6 cells (JOY676) harboring pRS426 plasmid (open square), and Δatg7 Δald6 cells (JOY676) cells harboring pRS426-ald6ΔCys306 plasmid (open diamond) were used.

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in the yeast cell. Grabowska and Chelstowska (37) have recently demonstrated that Δald6 Δzwf1 double mutant cells are not viable under normal growth conditions or under anaerobic growth conditions even in the presence of glutathione. It is suggested that Ald6p plays an important role in maintaining a high rate of NADPH/NADP+ cycling in the yeast cell. However, upon nitrogen starvation, both fatty acid and deoxyribonucleoside biosynthesis, which consume large amounts of NADPH, shut down immediately (38, 39). We speculate that the reduction of NADP+ by Ald6p might be excessive in nitrogen-starved cells. An excessive amount of NADPH might inhibit the enzymatic activity of Zwf1p, which catalyzes the initial reaction of the synthesis of ribose-5-phosphate, which is an essential material for the generation of some amino acids and ribonucleotides (39). Ald4p, the mitochondrial acetaldehyde dehydrogenase, utilizes mainly NAD+ as a co-enzyme (14) and is induced during nitrogen starvation (38). In our experiments, Δatg7 Δald4 mutant cells were not able to maintain high rates of viability like Δatg7 ald6 cells (Fig. 5B). It is likely that the down-regulation of Ald6p by preferential autophagic degradation may optimize NADPH/NADP+ levels in the cytosol. Thus, Ald6p may have a bilateral character: it is beneficial in growth under nutrient conditions but disadvantageous to survival under nitrogen starvation.

Here, we show that Ald6p is one example of a preferential substrate for autophagic degradation. Ald6p was the only major protein on the two-dimensional PAGE gel to decrease during starvation; however, it is still possible that other minor proteins behave like Ald6p. If we are able to find such proteins, it would help clarify the molecular mechanisms of selective autophagy and the physiological significance of the preferential degradation.

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REFERENCES

1. Ashford, T. P., and Porter, K. R. (1962) J. Cell Biol. 12, 198–202
2. Tsukada, M., and Ohsumi, Y. (1993) FEBS Lett. 333, 169–174
3. Otto, G. P., Wu, M. Y., Kazgan, N., Anderson, O. R., and Kessin, R. H. (2003) J. Biol. Chem. 278, 17636–17645
4. Melendez, A., Tallocoy, Z., Seaman, M., Eskelinen, E. L., Hall, D. H., and Levine, B. (2003) Science 301, 1387–1391
5. Kloniosky, D. J., Crepp, J. M., Dunn, W. A., Emr, S. D., Sakai, Y., Sanedoval, I. V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., and Ohsumi, Y. (2003) Dev. Cell 3, 539–545
6. Kloniosky, D. J., and Ohsumi, Y. (1999) Ann. Rev. Cell Dev. Biol. 15, 1–32
7. Ohsumi, Y. (2001) Nat. Rev. Mol. Cell. Biol. 2, 211–216
8. Barth, H., Meiling-Wesse, K., Epple, U. D., and Thumm, M. (2001) FEBS Lett. 508, 23–28
9. Baba, M., Takeshige, K., Baba, N., and Ohsumi, Y. (1994) J. Cell Biol. 124, 993–913
10. Takeshige, K., Baba, M., Tsuchi, S., Noda, T., and Ohsumi, Y. (1992) J. Cell Biol. 119, 301–311
11. Hoffman, M., and Chiang, H. L. (1996) Genetics 143, 1555–1566
12. Mukaiyama, H., Oku, M., Baba, M., Samizo, T., Hammond, A. T. G., Glick, B. S., Kita, N., and Sakai, Y. (2002) Genes Cells 7, 75–90
13. Meaden, P. G., Dickinson, F. M., Misfud, A., Tessier, W., Westerland, J., Bussey, H., and Midgley, M. (1997) Yeast 13, 1319–1327
14. Tessier, W. D., Meaden, P. G., Dickinson, F. M., and Midgley, M. (1998) FEBS Microbiol. Lett. 164, 29–34
15. Kurita, O., and Nishida, Y. (1999) FEBS Microbiol. Lett. 181, 281–287
16. White, W. H., Skatrud, P. L., Xue, Z., and Toyn, J. H. (2003) Genetics 163, 69–77
17. Bude, D., Dawson, D., and Stearn, T. (2000) Method in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
19. Gerg, A., Obermaierl, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., and Weiss, W. (2000) Electrophoresis 21, 1037–1053
20. Kiriaka, T., Baba, M., Ishihara, M., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. (1999) J. Cell Biol. 147, 435–446
21. Ishihara, M., Hayama, S., Yokota, S., Suzuki, K., Kamada, Y., Ishihara, N., Yoshimori, T., Noda, T., and Ohsumi, Y. (2001) Mol. Cell. 7, 3690–3702
22. Suzuki, K., Kiriaka, T., Kamada, Y., Mizushima, N., Noda, T., and Ohsumi, Y. (2001) EMBO J. 20, 5971–5981
23. Khafan, W. A., and Klonisky, D. J. (2002) Curr. Opin. Cell Biol. 14, 468–475
24. Kim, J., Kamada, Y., Stromhaug, P. E., Guan, J., Hefner-Gravink, A., Baba, M., Scott, S. V., Ohsumi, Y., Dunn, W. A., Jr., and Klonsky, D. J. (2001) J. Cell Biol. 153, 381–396
25. Scott, S. V., Guan, J., Hutchins, M. U., Kim, J., and Klonsky, D. J. (2001) Mol. Cell 7, 1131–1141
26. Botin, C. R., McCann, J. A., Hung, G. G., Eleo, C. P., and Chiang, H. L. (2002) J. Cell Sci. 115, 655–666
27. Heinemeyer, W., Kleinschmidt, J., Saidowsky, J., Escher, C., and Wolf, D. H. (1991) EMBO J. 10, 555–562
28. Heinemeyer, W., Gruber, A., Mohrle, V., Mahe, Y., and Wolf, D. H. (1993) J. Biol. Chem. 268, 5115–5120
29. Richter-Ruoff, B., Heinemeyer, W., and Wolf, D. H. (1992) FEBS Lett. 302, 192–196
30. Seufert, W., and Jentsch, S. (1992) EMBO J. 11, 3077–3080
31. Suzuki, K., Kamada, Y., and Ohsumi, Y. (2002) Dev. Cell 3, 815–824
32. Farres, J., Wang, T. T., Cunningham, S. J., and Weiner, H. (1995) Biochemistry 34, 2592–2598
33. Scott, S. V., Hefner-Gravink, A., Morano, K. A., Noda, T., Ohsumi, Y., and Klonsky, D. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12304–12308
34. Botin, C. R., and Weiner, H. (1996) Eur. J. Biochem. 243, 385–395
35. Shintani, T., Huang, W. P., Stromhaug, P. E., and Klonsky, D. J. (2002) Dev. Cell 3, 825–837
36. Breijning, J., and Jespersen, L. (2002) Int. J. Food Microbl. 75, 27–38
37. Grabowska, D., and Chelstowska, A. (2003) J. Biol. Chem. 278, 13984–13988
38. Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) Mol. Cell 11, 4241–4257
39. Murray, R. K., Graner, D. K., Mayes, P. A., and Rodwell, V. M. (2003) Harper's Illustrated Biochemistry, 26th Ed. McGraw-Hill Co., Columbus, OH
40. Robinson, J. S., Klonsky, D. J., Banta, L. M., and Egd, S. D. (1988) Mol. Cell. Biol. 8, 4936–4948
41. Kiriaka, T., Ichimura, Y., Okada, H., Kabea, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000) J. Cell Biol. 151, 263–276
42. Gerhardt, B., Kordas, T. J., Thompson, C. M., Patel, P., and Vida, T. (1998) J. Biol. Chem. 273, 15818–15829
43. Kiriaka, T., Baba, M., Ishihara, N., and Ohsumi, Y. (2001) J. Cell Biol. 152, 519–530
