In yeast, loss of Hog1 leads to osmosensitivity of autophagy

Tanja PRICK^{*1}, Michael THUMM[†], Karl KÖHRER[‡], Dieter HÄUSSINGER^{*} and Stephan vom DAHL^{*}

^{*}Division of Gastroenterology, Hepatology and Infectious Diseases, Heinrich-Heine-University, Moorenstrasse 5, D-40225 Düsseldorf, Germany, [†]Center of Biochemistry and Molecular Cell Biology, Georg-August-University, Heinrich-Dueker-Weg 12, D-37073 Göttingen, Germany, and [‡]Biomedical Research Center, Heinrich-Heine-University, Universitätsstrasse 1, D-40225 Düsseldorf, Germany

In mammalian liver, proteolysis is regulated by the cellular hydration state in a microtubule- and p38^{MAPK} (p38 mitogen-activated protein kinase)-dependent fashion. Osmosensing in liver cells towards proteolysis is achieved by activation of integrin receptors. The yeast orthologue of p38^{MAPK} is Hog1 (high-osmolarity glycerol 1), which is involved in the hyperosmotic-response pathway. Since it is not known whether starvation-induced autophagy in yeast is osmosensitive and whether Hog1 is involved in this process, we performed fluorescence microscopy experiments. The *hog1*Δ cells exhibited a visible decrease of autophagy in hypo-osmotic and hyperosmotic nitrogen-starvation medium as compared with normo-osmolarity, as determined by GFP (green fluorescent protein)–Atg8 (autophagy-related 8) fluorescence. Western blot analysis of GFP–Atg8 degradation showed that WT (wild-type) cells maintained a stable autophagic activity over a broad osmolarity range, whereas *hog1*Δ cells showed an impaired autophagic activity during hypo- and hyper-osmotic stress. In [³H]leucine-pre-labelled yeast cells, the

proteolysis rate was osmodependent only in *hog1*Δ cells. Neither maturation of pro-aminopeptidase I nor vitality was affected by osmotic stress in either yeast strain. In contrast, rapamycin-dependent autophagy, as measured by degradation of GFP–Atg8, did not significantly respond to hypo-osmotic or hyperosmotic stress in *hog1*Δ or WT cells. We conclude that Hog1 plays a role in the stabilization machinery of nitrogen-deprivation-induced autophagy in yeast cells during ambient osmolarity changes. This could be an analogy to the p38^{MAPK} pathway in mammalian liver, where osmosensing towards p38^{MAPK} is required for autophagy regulation by hypo-osmotic or amino-acid-induced cell swelling. A phenotypic difference is observed in rapamycin-induced autophagy, which does not seem to respond to extracellular osmolarity changes in *hog1*Δ cells.

Key words: autophagy, high-osmolarity glycerol 1 gene (*HOG1*), liver, p38 mitogen-activated protein kinase (p38^{MAPK}), osmosensitivity, rapamycin.

INTRODUCTION

In mammalian liver, bulk protein degradation occurs by two routes: ubiquitin-dependent proteolysis and lysosomal proteolysis. Protein degradation via lysosomal proteolysis is induced by nitrogen starvation, involves the formation of autophagosomes and represents the major breakdown process for protein degradation, yielding amino acids for *de novo* protein synthesis or energy expenditure (reviewed in [1–6]). In liver, autophagosomes have a half life of 6–8 min and can digest up to 5% of cytosolic protein per hour [7]. Autophagic proteolysis is stimulated by glucagon, oxidative stress, hyperosmotic exposure [8] or deprivation of amino acids [9]. On the other hand, autophagic proteolysis in liver is inhibited by insulin, IGF-1 (insulin-like growth factor 1), amino acids, bile acids [10] and cell swelling [11]. Besides hormones and amino acids, cell hydration has been identified as a major regulatory principle of autophagic proteolysis, involving integrin receptors, Src kinase, p38^{MAPK} (p38 mitogen-activated protein kinase) activation and, as yet unidentified, elements of the microtubular cytoskeleton [12–15]. Cell swelling activates p38^{MAPK} in liver and subsequently leads to down-regulation of sequestration, i.e. the initial step in the formation of autophagosomes [12]. Cell swelling partially mediates the insulin-induced inhibition of proteolysis in liver [13].

In yeast, the formation of autophagosomes has been well characterized at the molecular level, involving a cascade of

events leading to the formation of autophagosomes from the pre-autophagosomal structure. Autophagosomes are finally delivered to the vacuole, the lysosomal compartment of yeast cells (reviewed in [2,16,17]). Yeast protein Hog1 (high-osmolarity glycerol 1) is the orthologue of p38^{MAPK} that controls the hyperosmotic response. The hyperosmotic response of yeast cells involves activation of Hog1 with consecutive induction of glycerol-synthesizing genes, resulting in an increased production of glycerol [18–20].

It is unknown whether, like in liver, yeast autophagy responds to osmotic stress and whether this is controlled by *HOG1*. The present study shows that nitrogen-starvation-induced autophagy of yeast responds to osmotic stress in *hog1*Δ cells. Hog1 stabilizes this type of autophagy during osmotic stress in WT (wild-type) cells. A loss of Hog1 causes osmosensitivity of the autophagy.

MATERIALS AND METHODS

Yeast strains and media

The *Saccharomyces cerevisiae* strains used in the present study are listed in Table 1. Yeast cells were grown either in complete liquid medium YPD (1% yeast extract, 2% peptone and 2% glucose) or CM (complete minimal) medium [0.67% YNB (yeast nitrogen base) (without amino acids) and 2% glucose

Abbreviations used: Ape1, aminopeptidase I; Atg, autophagy-related; *AUT*, autophagy gene; CM medium, complete minimal medium; Cvt, cytoplasm-to-vacuole targeting; GFP, green fluorescent protein; Hog1, high-osmolarity glycerol 1; MAPK, mitogen-activated protein kinase; PE, phosphatidylethanolamine; PGK, 3-phosphoglycerate kinase; SD(–N), synthetic starvation medium without nitrogen; TCA, trichloroacetic acid; WT, wild-type; YPD, yeast/peptone/dextrose; YNB, yeast nitrogen base.

¹ To whom correspondence should be addressed (email tapr@arcor.de).

Table 1 Yeast strains used in the present study

EUROSCARF, European *Saccharomyces cerevisiae* Archive for Functional Analysis (Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany).

Strain	Genotype	Reference
WCG4a	<i>Matα ura3 his3-11,15 leu2-3,112</i>	[45]
YTP1	<i>Matα ura3 his3-11,15 leu2-3,112 hog1Δ::KAN^R</i>	The present study
YSR2	<i>Matα ura3 his3-11,15 leu2-3,112 aut9Δ::KAN^R</i>	[46]
<i>atg8Δ</i>	<i>WCG4a atg8Δ::KAN^R</i>	[47]
<i>atg13Δ</i>	<i>Matα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 atg13Δ::KAN^R</i>	EUROSCARF

and supplemented with 40 mg/l adenine sulphate, 200 mg/l L-leucine, 80 mg/l L-tryptophan, 45 mg/l L-histidine and 150 mg/l L-lysine]. For solid medium, 2–3 % agar was added. Autophagy was induced by starving cells of nitrogen in SD(–N) (synthetic starvation medium without nitrogen) (0.17 % YNB without amino acids or ammonium sulphate, and 2 % glucose) or by adding 0.2 μ g/ml rapamycin to the growth medium. Hypo-osmotic starvation medium was achieved by dilution of SD(–N) with sterile water, whereas hyperosmolarity was produced by adding the respective amounts of NaCl, raffinose, sorbitol or ammonium sulphate. The osmolality of these solutions was measured by freezing-point depression with an Osmomat 030 from Gonotec.

Antibodies and chemicals

Monoclonal anti-GFP (green fluorescent protein) antibodies were from Clontech, anti-PGK (3-phosphoglycerate kinase) antibodies were from Molecular Probes, antibodies against Atg8 (autophagy-related 8) are described in [21,22], and anti-Atg13 antibodies were raised by immunization of rabbits using the synthetic peptides QKYNQLGVEEDDDNDRLN and HNEDDDQDDDLV coupled to KLH (keyhole-limpet haemocyanin). The horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody was from Medac, and horseradish-peroxidase-conjugated goat anti-mouse antibody was from Dianova. The antibody against proApe1 (pro-aminopeptidase I) is described in [23].

Oligonucleotides were from MWG-Biotec, rapamycin was from Alexis, PMSF was from Sigma, liquid-scintillation cocktail (Ultima Gold) was from PerkinElmer, CompleteTM protease inhibitor cocktail (EDTA-free) was from Roche, and glass beads (G-8722) from Sigma. Other analytical chemicals were of analytical grade and were purchased from Sigma or Merck.

Chromosomal deletion of *HOG1*

For gene disruption, two oligonucleotides (Δ *hog1*-1, 5'-ATG-ACCACTAACGAGGAATTCATTAGGACACAGATATTCCA-GCTGAAGCTTCGTACGC-3', Δ *hog1*-2, 5'-TTACTGTTGGA-ACTCATTAGCGTACTGTATGGCCTGGTTGCATAGGCCAC-TAGTGGATCTG-3') were used that carry at their 3'-end a segment homologous with sequences left and right of the loxP-kanMX-loxP module on plasmid pUG6 and at their 5'-end a segment homologous with the open reading frame to be disrupted [24]. This PCR fragment conferring Geneticin[®] resistance was used to chromosomally replace the gene in the WT strain WCG4a, generating YTP1 (*hog1 Δ*). For selection for G-418 resistance after yeast transformation, the YPD agar plates were supplemented with 200 mg/l Geneticin[®]. G-418 sulphate was from

Gibco BRL. Gene replacement was confirmed by Southern blot analysis (results not shown).

GFP-Atg8 fusion protein

WT and *hog1 Δ* cells were transformed with the plasmid pRS316:GFP-ATG8 [25] to visualize the localization of Atg8 and to measure the GFP-Atg8 degradation. The centromeric plasmid pGFP-ATG8 carries a GFP-Atg8 fusion protein under control of the native ATG8 promoter.

Direct fluorescence microscopy

The pGFP-Atg8 cells were grown to stationary phase in CM medium without uracil, transferred to either normo-osmotic (150 mosmol/l), hypo-osmotic (30 mosmol/l) or hyperosmotic (500 mosmol/l, 175 mM NaCl added) SD(–N) medium in the presence of the proteinase B inhibitor PMSF (1 mM) for 4 h and visualized with a Zeiss Axioskop 2 plus and an Axiocam image system.

GFP-Atg8 degradation

WT and *hog1 Δ* cells expressing pGFP-Atg8 were grown in CM medium without uracil to the exponential growth phase, harvested, washed twice with SD(–N) medium and then shaken at 30 °C in either normo-osmotic (150 mosmol/l) or hypo-osmotic (30–60 mosmol/l) or hyperosmotic (200–500 mosmol/l, e.g. 25–175 mM NaCl added) SD(–N) medium. Each hour, one D_{600} unit of cells was collected, lysed and prepared for Western blotting. The samples were resuspended in Laemmli buffer and an equal amount of each sample was separated by standard SDS/10 % PAGE, and electroblotted on to PVDF membranes (Pall Corporation). Subsequently, GFP fusion proteins and free GFP were detected with antibodies against GFP and analysed by densitometric analysis. The level of free GFP corresponds to the autophagic rate, since GFP is rather proteolysis-resistant and thus accumulates inside the vacuole. Finally, the membranes were probed with antibodies against PGK as a loading control [26].

Proteolysis rate analysis

The proteolysis rate of WT and *hog1 Δ* cells was determined by measurement of ³H-label release from yeast cells by a method related to that of Takeshige et al. [27]. The cells were incubated with 1 μ Ci/ml of L-[4,5-³H]leucine for 16 h before nitrogen starvation in SD(–N) medium. After washing the cells with SD(–N) medium, they were exposed to normo-osmotic (150 mosmol/l), hypo-osmotic (30 mosmol/l) or hyperosmotic (500 mosmol/l, 175 mM NaCl added) SD(–N) medium for 4 h. At hourly intervals, one D_{600} unit of cells was harvested. After separating cells from medium by centrifugation at 16100 g for 2 min at room temperature (20–25 °C), the pellets and the supernatants were collected separately and stored at –80 °C. After thawing the samples, the pellets (cell fractions) were resuspended in 1 ml of water. After ultrasonic treatment, in each case, 200 μ l of 50 % TCA (trichloroacetic acid) was added. After a 10 min incubation on ice, the samples were collected by centrifugation (9300 g for 5 min at 4 °C). The supernatant from each sample was removed and collected separately. The TCA pellets were resuspended in 1 ml of water. Both the supernatants and the pellets underwent liquid-scintillation counting, before the proteolysis rate was calculated as the fractional TCA-soluble radioactivity of the supernatant. Counts were related to total radioactivity and expressed as the percentage proteolysis over the indicated time period. The sum of intra- and extra-cellular radioactivity at the respective time point was set to 100 % [27].

Generation of aniso-osmotic incubation media

Hypo-osmotic starvation medium was generated by dilution of SD(–N) medium with sterile water to achieve 30 mosmol/l. This medium was complemented with either 60 mM NaCl or 120 mM raffinose to reach normo-osmolarity (150 mosmol/l). Mid-exponential phase pGFP-Atg8 cells grown in CM medium without uracil were starved in normo-osmotic (150 mosmol/l), hypo-osmotic (30 mosmol/l) or in either NaCl- or raffinose-complemented (150 mosmol/l) medium for 4 h. Cells were withdrawn hourly similar to the measuring of the GFP-Atg8 degradation rate.

If indicated, hyperosmolar incubation media were obtained by adding the respective amounts of NaCl, raffinose, sorbitol or ammonium sulphate. The normo-osmotic medium was complemented with 175 mM NaCl, 350 mM raffinose, 350 mM sorbitol or 110 mM ammonium sulphate to reach hyperosmolarity (500 mosmol/l). Mid-exponential phase pGFP-Atg8 cells grown in CM medium without uracil were starved in either normo-osmotic (150 mosmol/l) or hyperosmotic (500 mosmol/l) medium complemented with raffinose, sorbitol or ammonium sulphate for 4 h. Cells were withdrawn hourly similar to the procedure described for measuring of the GFP-Atg8 degradation rate.

Phenotypic characterization of osmosensitivity of *hog1Δ* cells

WT and *hog1Δ* cells were grown to stationary growth phase in YPD medium. In serial dilution (1:100–1:10⁷), equal amounts of cells were plated on standard YPD agar plates or on agar plates with either 0.1 M or 0.5 M NaCl added. After 3 days of incubation at 30°C, viable cells were visible as colonies.

Survival analysis

An equal amount of cells, either starved in normo-osmotic (150 mosmol/l), hypo-osmotic (30–60 mosmol/l) or hyperosmotic (200–500 mosmol/l) SD(–N) medium was plated hourly on YPD agar plates for 4 h. After 3 days of incubation at 30°C, the number of growing colonies was determined. The relative survival rate was calculated by comparison with the number of colonies appearing before starvation.

Analysis of the Cvt (cytoplasm-to-vacuole targeting) pathway

To determine whether the Cvt pathway proceeds in *hog1Δ* cells, the cells were cultured either in YPD or hypo- and normo-osmotic SD(–N) medium and then shaken at 30°C. At hourly intervals, for 4 h, one *D*₆₀₀ unit of cells was taken, prepared for Western blotting and processed for immunoblots with antibodies against Ape1 [28].

Atg8 lipidation immunoblot analysis

For analysis of Atg8 lipidation, WT, *hog1Δ* and *atg8Δ* cells were grown to a *D*₆₀₀ of 0.5–0.8 in YPD medium, harvested, washed twice with SD(–N) medium and then starved for 4 h in either normo-osmotic (150 mosmol/l), hypo-osmotic (30–60 mosmol/l) or hyperosmotic (200–500 mosmol/l) SD(–N) medium. Samples were collected at the indicated time points and subjected to immunoblot analysis as described previously using antibodies against Atg8 [21]. Separation of Atg8 from Atg8-PE (phosphatidylethanolamine) was obtained by adding 6 M urea to standard SDS/15% polyacrylamide gels [29].

Atg13 phosphorylation immunoblot analysis

To determine the Atg13 phosphorylation state during osmotic stress, WT and *hog1Δ* cells containing overexpressed Atg13 or *atg13Δ* cells were grown in selective medium. Then normo-

Figure 1 Direct fluorescence microscopy of WT and *hog1Δ* cells during osmotic stress

A fusion protein of Atg8 with GFP was expressed from a centromeric plasmid under control of the native *ATG8* promoter was expressed in WT (A, C, E) or *hog1Δ* (B, D, F) cells. After starving the cells for nitrogen in normo-osmotic (150 mosmol/l), hypo-osmotic (30 mosmol/l) or hyperosmotic (500 mosmol/l) SD(–N) medium for 4 h in the presence of PMSF, the cells were analysed by Nomarski optics and epifluorescence. Hypo-osmolarity was achieved by diluting SD(–N) medium with sterile water, whereas hyperosmolarity was produced by the addition of the corresponding amount of NaCl. Strains used were isogenic to the WT strain WCG4ac. Scale bar, 10 μ m.

osmotic (150 mosmol/l), hypo-osmotic (30 mosmol/l) or hyperosmotic (500 mosmol/l) nitrogen-starvation conditions [SD(–N)] were installed at zero time. At hourly intervals, for 4 h, five *D*₆₀₀ units of cells were taken. The samples were collected by centrifugation (1550 *g* for 5 min at 4°C), and the supernatant was removed. The cell pellet was resuspended in lysis buffer (PBS, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM Na₃VO₄, 50 mM KF, 15 mM sodium pyrophosphate, 15 mM *p*-nitrophenylphosphate, 20 μ g/ml leupeptin, 20 μ g/ml benzamidin, 10 μ g/ml pepstatin A, 40 μ g/ml aprotinin, 1 mM PMSF, 0.5% Tween 20 and CompleteTM protease inhibitor cocktail) [30] and 100 μ l of glass beads were added. After 30 min of vigorous shaking at 4°C, the cell lysates were centrifuged at 1550 *g* for 10 min at 4°C. Finally, the supernatants were removed, collected on ice and prepared for Western blotting. The resulting immunocomplex was subjected to immunoblotting using antibodies against Atg13 (1:5000). The horseradish-peroxidase-conjugated anti-rabbit goat antibody (1:10000) was used as a secondary antibody. Immunodetection was carried out with the ECL[®] (enhanced chemiluminescence) system (Amersham Biosciences).

Statistical analysis

Results from independent experiments are expressed as the means $\pm$ S.E.M. Results were compared using Student's *t* test, with *P* < 0.05 considered to be statistically significant.

RESULTS

Cytoplasmic GFP-Atg8 does not reach the vacuole in *hog1Δ* cells

Using the centromeric plasmid, pRS316-GFP-Atg8, encoding an in-frame fusion protein consisting of GFP and Atg8, the occurrence of autophagic vesicles in transformed yeast cells upon starvation was followed by Nomarski optics and fluorescence microscopy. Under normo-osmotic nitrogen-starvation conditions in the presence of the proteinase B inhibitor PMSF, numerous vesicles accumulated inside the vacuole of these cells, either in WT or *hog1Δ* cells (Figures 1A and 1B). Under hypo-osmotic (30 mosmol/l; Figures 1C and 1D) or hyperosmotic (500 mosmol/l; Figures 1E and 1F) incubation conditions for 4 h,

HOG1 deletion led to osmosensitivity of autophagy in yeast cells, as shown by a significantly decreased intravacuolar GFP level.

Osmosensitivity of Atg8 degradation in *hog1Δ* cells

Mid-exponential phase cells expressing GFP–Atg8 from pGFP–Atg8 were starved in SD(–N) media with various osmolarities, and the autophagy rate was determined by immunoblotting. In WT and *hog1Δ* cells, nitrogen deprivation for 4 h led to a strong induction of GFP–Atg8 degradation, as shown by an increased formation of free GFP over time (Figure 2A). The level of free GFP corresponds to the autophagic rate, since GFP is rather proteolysis-resistant and thus accumulates inside the vacuole. Under normo-osmotic culture conditions (150 mosmol/l), there was no significant difference in GFP–Atg8 degradation between WT or *hog1Δ* cells. Under conditions of moderate osmotic stress, *hog1Δ* cells showed a decreased ability to degrade GFP–Atg8 via autophagy either during hypo-osmotic (30 mosmol/l) or hyperosmotic (500 mosmol/l) incubation conditions (Figure 2A). In *hog1Δ* cells, the GFP–Atg8 degradation rates under hypo-osmotic (30 mosmol/l) and hyperosmotic (500 mosmol/l) incubation conditions were $56 \pm 9\%$ ($n = 7$) and $56 \pm 14\%$ ($n = 4$) after a 4 h aniso-osmotic incubation period (Figure 2B), and there was a close correlation between the degree of osmotic stress and the impairment of GFP–Atg8 degradation in *hog1Δ* cells under aniso-osmotic conditions.

In *hog1Δ* cells, proteolysis is modulated by ambient osmolarity

Proteolysis rates from WT cells, *hog1Δ* cells and the autophagy-deficient strain *aut9Δ* (*atg9Δ*) were determined under hypo- and hyper-osmotic incubation conditions. In comparison with an autophagy-deficient yeast strain (*aut9Δ*) as negative control, WT and *hog1Δ* cells showed an induction of autophagic proteolysis during nitrogen starvation for 4 h (Figures 3A and 3B), reflected by a stable release of ^3H -associated TCA-soluble radioactivity from the cells after an equilibration period of 1 h. During either hyper- or hypo-osmotic stress, the proteolysis rate of *hog1Δ* cells fell from $1.3 \pm 0.2\%$ per 3 h ($n = 8$) under normo-osmotic conditions (150 mosmol/l) to $0.5 \pm 0.2\%$ per 3 h ($n = 5$) under hypo-osmotic incubation conditions (30 mosmol/l) and $0.2 \pm 0.2\%$ per 3 h ($n = 3$) under hyperosmotic conditions (500 mosmol/l). The corresponding proteolysis rates per 3 h in WT cells were $1.3 \pm 0.2\%$ ($n = 8$), $1.0 \pm 0.1\%$ ($n = 5$) and $0.8 \pm 0.3\%$ ($n = 3$) under normo-osmotic (150 mosmol/l), hypo-osmotic (30 mosmol/l) and hyperosmotic (500 mosmol/l) incubation conditions (Figure 3C) respectively.

Viability of *hog1Δ* cells is not impaired by moderate osmotic stress

In order to study whether the osmolarity changes that modulated autophagy did affect the viability of *hog1Δ* cells, these cells were exposed to different osmolarities for 4 h and growth on rich medium was examined. Whereas increments of osmolarity from 250 to 450 mosmol/l did not affect growth, an extracellular osmolarity of 1400 mosmol/l was lethal not to the WT cells, but to the *hog1Δ* cells, as described by others [20,31]. Moderate aniso-osmotic conditions (30–500 mosmol/l), as used in the experiments described in Figures 1–3, did not affect the viability of WT cells or that of *hog1Δ* cells (results not shown).

Intact Cvt of Ape1 in *hog1Δ* cells

In order to study whether intracellular transport mechanisms were generally disturbed in osmosensitive cells (*hog1Δ*), the *hog1Δ*

Figure 2 Osmosensitivity of GFP–Atg8 degradation in *hog1Δ* cells

WT and *hog1Δ* cells expressing GFP–Atg8 were grown to the exponential growth phase in selective medium and were starved of nitrogen in normo-osmotic (150 mosmol/l), hypo-osmotic (30–60 mosmol/l) or hyperosmotic (200–500 mosmol/l) SD(–N) medium for the indicated time period. Degradation of the GFP–Atg8 fusion protein leads to the formation of free GFP. Representative immunoblots from 31 experiments are shown in (A). To normalize for the amount of loaded protein, Western blots, as shown in (A), were analysed for PGK levels. PGK levels in samples from WT or *hog1Δ* cells were quantified using densitometric analysis, compared with control protein levels after 4 h of normo-osmotic nitrogen-starvation in WT cells (relative protein level = 1) and results are expressed as relative protein levels. The GFP–Atg8 degradation rates were calculated by comparison with control experiments from WT cells [4 h of normo-osmotic SD(–N) medium] as shown in (B), and the GFP values were normalized to that of PGK. The amount of free GFP in WT cells, 4 h after incubation with normo-osmotic SD(–N) medium was set to 100%. ●, WT cells; ○, *hog1Δ* cells. Results are means $\pm$ S.E.M. for six to 31 separate determinations; * $P < 0.05$, *** $P < 0.001$, compared with respective WT control.

strain was examined for a defect in maturation of proApe1 by immunoblot analysis. Under nutrient-rich conditions, the Cvt pathway constitutively transports Ape1 to the vacuole, whereas, under starvation conditions, transport of Ape1 to the vacuole is achieved by the autophagic pathway [32,33]. *Hog1Δ* cells from the stationary growth phase, starved for 4 h in SD(–N) medium, showed normal maturation of Ape1 from proApe1 (Figure 4,

Figure 3 Proteolysis rate analysis

The proteolysis rate of WT and *hog1*Δ cells was determined by the measurement of ^3H label release from yeast cells, which had been labelled with $1 \mu\text{Ci/ml}$ of [^3H]leucine 16 h before the shift to aniso-osmotic incubation conditions. After washing with SD(–N) medium, cells were exposed to various osmolarities. Each hour, for a total of 4 h, $1 D_{600}$ unit of cells was harvested. The proteolysis rate (C) was determined by liquid-scintillation counting and was calculated as fractional TCA-soluble radioactivity in the supernatant. Counts were related to total radioactivity (A and B). (A, B) ●, WT cells; ○, *hog1*Δ cells; ▼, *atg9*Δ cells. (C) Black bars, WT cells; white bars, *hog1*Δ cells; grey bars, *atg9*Δ cells. Results are means $\pm$ S.E.M. for three separate determinations * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with respective WT control.

lane 8). These results show that Hog1 is not essential for the maturation of Ape1. Maturation of Ape1 was also unaffected if *hog1*Δ cells were exposed to hypo-osmotic incubation conditions for 4 h (Figure 4, lane 9).

Figure 4 Maturation of Ape1 in *hog1*Δ cells

Maturation of proApe1 (proAPI, 60 kDa); mature Ape1 (mAPI, 51 kDa) was determined in WT cells (lanes 4–6), *hog1*Δ cells (lanes 7–9) and *pep4*Δ cells (defective in Ape1 maturation, lanes 1–3) under normo-osmotic (lanes 2, 5 and 8) and hypo-osmotic (lanes 3, 6 and 9) nitrogen-starvation conditions.

Figure 5 Osmosensitivity of autophagy in *hog1*Δ cells is dependent on extracellular osmolarity, but not on availability of nutrients

Hypo-osmotic starvation medium [30 mosmol/l, obtained by dilution in water of SD(–N) medium] was complemented with either 60 mM NaCl or 120 mM raffinose to reach an osmolarity of 150 mosmol/l. Mid-exponential phase cells with plasmid pGFP-Atg8 were grown in selective medium and then starved in normo-osmotic control SD(–N) medium (150 mosmol/l), hypo-osmotic (30 mosmol/l) or complemented medium (150 mosmol/l) for 4 h. Normo-osmotic starvation medium (150 mosmol/l) was supplemented with 150 mM NaCl, 340 mM raffinose, 340 mM sorbitol or 110 mM ammonium sulphate to reach an osmolarity of 500 mosmol/l. Cells were withdrawn hourly, and the GFP-Atg8 degradation rate was determined as described in the text. Black bars, WT cells; white bars, *hog1*Δ cells. Results are means $\pm$ S.E.M. for three to five separate determinations; * $P < 0.05$, *** $P < 0.001$, compared with respective WT control.

Osmosensitivity of autophagy in *hog1*Δ cells is reversible and is independent of nutrient availability

In order to demonstrate reversibility and that the decreased rate of autophagy in *hog1*Δ cells was related to aniso-osmolarity rather than to changes of the concentrations of NaCl or nutrients, hypo-osmotic SD(–N) medium was complemented with 60 mM NaCl or 120 mM raffinose to normo-osmolarity (150 mosmol/l). Both the complementation of hypo-osmotic starvation medium with NaCl or raffinose led to a restoration of autophagy to control levels (150 mosmol/l) in *hog1*Δ cells (Figure 5). On the other hand, supplementation of the medium to hyperosmolarity with raffinose, sorbitol or ammonium sulphate led to a decreased autophagy rate in *hog1*Δ cells (Figure 5). It can be concluded that the decreased ability of *hog1*Δ yeast cells to degrade GFP-Atg8 via autophagy is dependent on the extracellular osmolarity and not an effect

Figure 6 Lipidation of Atg8 is not influenced by osmotic stress

To analyse the lipidation state of Atg8 (14 kDa) in WT and *hog1Δ* cells during osmotic stress, cells were grown to exponential phase and were shifted to normo-osmotic (A), hypo-osmotic (B) or hyperosmotic (C) SD(–N) medium for the indicated times. The osmolarity was varied by dilution of SD(–N) with water or by the addition of NaCl. Samples were taken after 0, 1, 2, 3 and 4 h, lysed and prepared for Western blotting, and extracts were separated by SDS/PAGE containing 6 M urea. The gels were then immunoblotted and probed with antibodies against Atg8. WT cells (●) were used as a positive control, *hog1Δ* cells (○) and *atg8Δ* cells (▼) were used as negative controls. Representative immunoblots of three experiments are shown. The blots shown in (A) were quantified by densitometry (B) (Table 2).

that is dependent on the lack of NaCl/nutrients or an increase of extracellular NaCl.

Osmotic stress in *hog1Δ* cells does not involve differential Atg8 lipidation or Atg13 phosphorylation status

In order to identify the molecular function of Hog1 for the autophagic machinery, the lipidation status of Atg8 was investigated. Atg8 is a soluble ubiquitin-like protein that undergoes a lipid conjugation step with PE. The covalent attachment to PE results in a form of Atg8 that is tightly membrane-associated. The membrane bound form of Atg8 plays a role in autophagosome formation [33]. Yeast cells were starved for 4 h in SD(–N) media with different osmolarities, and samples were subjected to immunoblot analysis. The two bands representing Atg8 and Atg8–PE were of similar intensity in WT and *hog1Δ* cells in normo-, hypo- and hyper-osmotic starvation media (Figure 6). Dephosphorylation of Atg13 upon installation of starvation conditions or by addition of rapamycin is, besides the conjugation step of Atg5, necessary for the formation of the autophagosome [34,35]. The phosphorylation status of Atg13 was unchanged in WT or *hog1Δ*

cells during conditions of hypo-, normo- and hyper-osmolarity, either in starved yeast cells or in rapamycin-stimulated cells (results not shown). The results show that neither differences in the Atg8 lipidation nor the Atg13 phosphorylation status may explain the different response of starvation-induced autophagy of *hog1Δ* cells towards osmotic stress as compared with that of WT cells.

Rapamycin-induced Atg8 degradation does not respond to osmotic stress

Mid-exponential phase cells expressing GFP–Atg8 were exposed to rapamycin (0.2 µg/ml) in CM medium without uracil, and the autophagy rate was determined by immunoblotting (Figure 7A). In WT and *hog1Δ* cells, addition of rapamycin for 4 h led to a strong induction of GFP–Atg8 degradation, as shown by an increased formation of free GFP over time (Figures 7A and 7B). After 4 h, under normo-osmotic incubation conditions and under conditions of moderate osmotic stress, *hog1Δ* cells showed an unaltered ability to degrade GFP–Atg8 via autophagy (Figures 7A and 7C). The results differ from those seen in starvation-induced

Figure 7 Rapamycin-induced autophagy is not osmosensitive in *hog1*Δ cells

WT and *hog1*Δ cells expressing GFP-Atg8 were grown to the exponential growth phase in selective medium, and 0.2 μg/ml rapamycin was added to either normo-osmotic (150 mosmol/l), hypo-osmotic (30 mosmol/l) or hyperosmotic (500 mosmol/l) selective medium for the indicated time. Representative immunoblots of six to 16 experiments are shown (A). The blots shown in (A) were quantified as described in Figure 2 (B and C). To normalize for the amount of loaded protein, Western blots, as shown in (A), were analysed for PGK levels. PGK levels in samples from WT (●) or *hog1*Δ (○) cells were quantified via densitometric analysis, compared with control protein levels after 4 h normo-osmotic nitrogen-starvation in WT cells (relative protein level = 1), and results are expressed as relative protein levels. The free GFP values were normalized to PGK. ●, WT cells; ○, *hog1*Δ cells. Results are means ± S.E.M. for six to 16 separate determinations.

autophagy (Figure 2) and point to different signalling mechanisms controlling nitrogen-starvation- or rapamycin-induced autophagy respectively.

DISCUSSION

The *S. cerevisiae* Hog1 is a orthologue of the mammalian p38^{MAPK}. Since it is known that autophagic proteolysis in mammalian liver is regulated by the cellular hydration state in a microtubule- and p38^{MAPK}-dependent fashion [36], this may suggest a specific function of Hog1 for yeast autophagy. Osmosensing and signalling towards p38^{MAPK} is required for proteolysis regulation by hypo-osmotic or amino-acid-induced hepatocyte swelling in mammalian liver [15,37]. In yeast, Hog1 is known to be a protein with MAPK activity which is involved in the hyperosmotic response pathway [20]. The cellular response to stress is obviously aimed at protecting cells from the detrimental effect of stress and at repairing possible damage. *HOG1* has not yet been described as a gene required for starvation-induced autophagy or maturation of proApe1.

The present study shows that the starvation-induced autophagy is osmosensitive in yeast cells lacking Hog1 and is stable in WT cells (Figures 1–3). Furthermore, Hog1 is not essential for proApe1 maturation in the Cvt pathway (Figure 4). Therefore GFP-Atg8, which is specifically associated with autophagosomes, was employed as a tool to directly monitor autophagic protein transport [25,26]. Autophagy is responsible for intracellular bulk protein degradation in the vacuole. It is induced when cells lack nutrients from the extracellular environment. Via autophagy, cytoplasmic components are sequestered and are transported to the vacuole for degradation. In yeast, a defect in autophagy results in a loss of viability during starvation, indicating

that protein degradation provides the minimal nutrients that are required for survival of starved cells [38]. The deletion of *HOG1* in yeast cells leads to osmosensitivity [39]. In comparison with the WT, *hog1*Δ cells have a reduced ability to survive during osmotic stress of more than 0.5 M NaCl. The range of aniso-osmotic incubation conditions employed in the experiments shown in the present study (30–500 mosmol/l) did not affect the viability of *hog1*Δ cells (results not shown), excluding a non-specific impact of loss of Hog1 on general cellular function in the presence of moderate aniso-osmolar conditions.

Using direct fluorescence microscopy, *hog1*Δ cells expressing GFP-Atg8 show a visible decrease of autophagy in hypo-osmotic and hyperosmotic nitrogen-starvation medium in comparison with normo-osmolarity (Figure 1). The GFP-Atg8 degradation rate in WT and *hog1*Δ cells under normo-osmotic starvation conditions is similar (Figure 2), indicating that there is no autophagy defect in *hog1*Δ cells under normo-osmotic conditions. The data show that Hog1 is not essential for the starvation-induced autophagy under normo-osmotic conditions. In cells lacking Hog1, autophagy is not blocked, but is reduced in its rate under aniso-osmotic incubation conditions (Figures 1, 2 and 3). Therefore Hog1 stabilizes the autophagy during phases of osmotic stress in WT cells. A loss of Hog1 causes osmosensitivity of autophagy.

The complementation of hypo-osmotic starvation medium with NaCl or raffinose led to a restoration of autophagy to control level of normo-osmotic conditions in *hog1*Δ cells (Figure 5). Therefore the osmosensitivity of autophagy does not depend on availability of nutrients or Na⁺ ions in *hog1*Δ cells. Furthermore, supplementation of the incubation medium to hyperosmolarity with raffinose, sorbitol or ammonium sulphate instead of NaCl led to a reduced GFP-Atg8 degradation rate in *hog1*Δ cells (Figure 5). This suggests that the decreased ability of *hog1*Δ cells to degrade GFP-Atg8 via autophagy is dependent on extracellular osmolarity

Table 2 Lipidation state of Atg8 during aniso-osmotic nitrogen starvation

WT, *hog1Δ* and *atg8Δ* cells were grown to exponential phase and were transferred to normo-osmotic (150 mosmol/l), hypo-osmotic (30 mosmol/l) or hyperosmotic (500 mosmol/l) SD(–N) medium for 4 h. Samples were taken after 0, 1, 2, 3 and 4 h, lysed and prepared for Western blotting as described in the text. Blots (Figure 6A) were quantified (Figure 6B), and the relative lipidation state of Atg8 (control: WT cells, 4 h normo-osmotic, 100 %) after 2 and 4 h exposure to starvation medium of different osmolarities is shown. n.d., not detectable.

Strain	Incubation time (h)	Relative lipidation state of Atg8 (%)		
		Normo-osmotic	Hypo-osmotic	Hyperosmotic
WT	2	102.5	103.8	108.2
<i>hog1Δ</i>	2	106.7	91.9	101.9
<i>atg8Δ</i>	2	n.d.	n.d.	n.d.
WT	4	100.0	90.2	66.6
<i>hog1Δ</i>	4	83.8	82.4	94.8
<i>atg8Δ</i>	4	n.d.	n.d.	n.d.

and is not an effect dependent on a lack of nutrients, NaCl or other ions.

Currently, the molecular mechanism by which Hog1 influences autophagy is incompletely understood. Neither Atg8 lipidation (Figure 6 and Table 2) nor Atg13 phosphorylation were affected by osmotic stress. Atg8 is a ubiquitin-like protein which is conjugated to PE to become Atg8–PE through a series of post-translational modifications owing to Atg4 [29], Atg7 (E1) [40] and Atg3 (E2) [41]. WT cells do not accumulate Atg8–PE because the protein is degraded after vacuolar delivery [42]. There is no lipidation defect of Atg8 in *hog1Δ* cells (Figure 6). *Hog1Δ* cells did not show significantly reduced Atg8–PE levels compared with the WT cells during incubation under aniso-osmotic conditions. In a similar way, the phosphorylation state of Atg13 was not changed during osmotic stress in *hog1Δ* cells. Normally, the level of Atg13 phosphorylation is regulated by the availability of nitrogen sources. In rich medium conditions that support transport of proApe1 by the Cvt pathway, Atg13 is hyperphosphorylated. Under nitrogen-starvation conditions, the autophagy pathway is activated, and the phosphorylation of Atg13 is decreased [30].

The results show that rapamycin-induced autophagy is not osmosensitive (Figure 7). It can therefore be concluded that, although phenotypically similar, autophagy induction by starvation or rapamycin differs biochemically. In liver, autophagy is dependent on mTOR (mammalian target of rapamycin) [3], but TOR (target of rapamycin)-independent mechanisms have been identified as well [37,43]. In yeast, it could also be possible that the two pathways are similar and that rapamycin is acting further downstream of Hog1. Currently, it is not clear, how far autophagy mechanisms induced by either starvation or rapamycin differ biochemically in yeast.

Based on our findings, it is speculated that Hog1 has a different function in the regulation of yeast autophagy than the mammalian p38^{MAPK} for proteolysis. In liver, activation of p38^{MAPK} by hypo-osmolarity leads to an inhibition of autophagy [37], whereas inhibition of p38^{MAPK} leads to a relative stimulation of autophagy during hypo-osmotic stress. In yeast, loss of *HOG1* leads to inhibition of autophagy upon hypo- and hyper-osmotic stress (the present study). Obviously, the two species share in common the presence of osmosignalling mechanisms linked to Hog1/p38^{MAPK}, but differ in the biochemical outcome. One explanation could be the presence of integrin receptors in liver cells, which are not present in yeast cells and have developed later in evolution [22]. In liver cells, osmosensing occurs via integrin receptors and Src kinase [15,44]. In yeast, the exact mechanisms of sensing

changes of osmotic environment are not yet fully understood. It could be speculated that, in yeast, Hog1 serves to help cells survive and maintain autophagy during osmotic stress, whereas mammalian cells use p38^{MAPK} as a key regulatory protein to modulate autophagy via the hydration state [30]. Cell hydration in liver cells is constantly modulated and is challenged by a variety of environmental conditions, such as hormones, amino acids and cytokines.

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