Starvation-induced degradation of yeast hexose transporter Hxt7p is dependent on endocytosis, autophagy and the terminal sequences of the permease

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Abstract The yeast high-affinity glucose transporters Hxt6p and Hxt7p are rapidly degraded during nitrogen starvation in the presence of high concentrations of fermentable carbon sources. Our results suggest that degradation is mainly due to the stimulation of general protein turnover and not caused by a mechanism specifically triggered by glucose. Analysis of Hxt6p/7p stability and cellular distribution in *end4*, *aut2* and *apg1* mutants indicates that Hxt7p is internalized by endocytosis, and autophagy is involved in the final delivery of Hxt7p to the vacuole for proteolytic degradation. Internalization and degradation of Hxt7p were blocked after truncation of its N-terminal hydrophilic domain. Nevertheless, this fully functional and stabilized hexose transporter could not maintain fermentation capacity of the yeast cells under starvation conditions, indicating a regulatory constraint on glucose uptake.

Key words: Glucose uptake; Catabolite inactivation; Starvation; Endocytosis; Autophagy; *Saccharomyces cerevisiae*

1. Introduction

In the presence of high concentrations of glucose the high-affinity glucose transporters Hxt6p and Hxt7p of *Saccharomyces cerevisiae* were shown to be inactivated by proteolytic degradation in the vacuole after internalization by endocytosis, a process that is called 'catabolite inactivation' [1–3]. The available data suggest that degradation is specifically triggered by high concentrations of glucose, but not by e.g. raffinose, ensuring that the high-affinity transporters are rapidly removed from the plasma membrane under conditions where low-affinity transporters like Hxt1p are expressed. However, degradation of sugar transporters by glucose has almost exclusively been investigated using nitrogen-starved resting yeast cells to prevent the synthesis of new proteins [4–6]. In the case of maltose permease it has recently been proposed that the glucose-induced degradation might be mainly due to the stimulation of general protein turnover caused by the nitrogen starvation conditions and not by a specific mechanism controlled by glucose [6]. Moreover, as nitrogen starvation conditions also induce autophagy, a degradative process by which cells sequester bulk cytosol into double membrane vesicles and deliver them to the vacuole for degradation [7], we asked whether this pathway might also be involved in the degradation of hexose transporters.

Degradation of the sugar transport system is thought to play a crucial role in the decline of the fermentation rate of yeast cells during stages of starvation [1,8], and affects production efficiency and product quality in large scale production with yeasts of wine, beer, distilled liquor and baker's yeast. Therefore, we wanted to construct a stabilized and plasma membrane-localized Hxtp protein and investigate its influence on the fermentation capacity during nitrogen starvation conditions. Internalization of other plasma membrane proteins like the pheromone receptor Ste2p, the pheromone transporter Ste6p, and the amino acid permease Gap1p involves the sequence E/DXK within cytosolic domains of these proteins [9–11]. Remarkably, similar sequences can also be found in the Hxt7p protein. Moreover, S_{259} of Hxt7p within the $PES_{(257–259)}$ motif is highly conserved within the sugar transporter family, and the corresponding residue of the yeast maltose transporter Mal61p has been shown to be involved in its degradation [12]. Therefore, we have changed these and other residues of Hxt7p, and have analyzed the effects on stability, activity and fermentation capacity.

2. Materials and methods

2.1. Yeast strains and growth conditions

Yeast strains were: CEN.PK2-1C (*MATa leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2*), EBY.VW4000 (*MATa Δhxt1-17 Δgal2 Δstl1 Δagt1 Δmph2 Δmph3 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2*) [13], RKY#534 (*MATa bar1-1 leu2 his4 ura3*) and RKY#536 (*MATa bar1-1 leu2 his4 ura3 end4*) [3] (kindly provided by R. Kölling, Düsseldorf, Germany), and RE301 (*Δhxt3::LEU2::Δhxt6 Δhxt7::HIS3*) [14]. Rich media were based on 1% yeast extract and 2% peptone, minimal media (YNB) consisted of 0.67% Difco yeast nitrogen base without amino acids and supplemented for auxotrophic requirements, and nitrogen starvation medium (YNB−N) consisted of 0.17% Difco yeast nitrogen base without amino acids and ammonium. The media were supplemented with various carbon sources. Yeast cells were grown aerobically at 25°C (temperature-sensitive mutants) or 30°C on a rotary shaker. To induce catabolite inactivation, cells were harvested during exponential growth, washed with YNB−N medium and resuspended in YNB−N medium at 30°C or 37°C (temperature-sensitive mutants).

2.2. Glucose uptake and Western blot analysis

Glucose uptake was assayed as described previously [15] with modifications according to Walsh et al. [16]. Western blot analysis was done as described in [3]. Equal amounts of protein were loaded into each lane of a standard 10% acrylamide gel. Preparation of the Hxt6p/

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7p and Hxt1p antibodies is described in [3]. Pfk1p antibodies directed against both subunits of the yeast phosphofructokinase 1 enzyme were kindly provided by J. Heinisch, Hohenheim, Germany. The subunits appeared as one band in Western analysis.

2.3. Construction of yeast strains

CEN.PK2-1C-derived strains lacking the genes *AUT2* and *APG1* were constructed with the 'short flanking homology PCR' technology using a *loxP::kanMX::loxP* cassette [17], resulting in strains SKY41 and SKY51, respectively. The *AUT2* gene was also deleted in strain RKY#536, resulting in strain SKY43k (*end4^{ts} Δaut2*). The correct replacements were confirmed by PCR.

2.4. Construction of plasmids

An *HXT7-GFP* fusion gene including the *HXT7* promoter was constructed by fusion PCR essentially as described in [18]. The N- and C-terminal deletion constructs of *HXT7* were also constructed by fusion PCR, replacing the first 168 bp (without start codon) and the last 162 bp of the *HXT7* open reading frame, respectively, by the hemagglutinin (HA) sequence (TAC CCA TAC GAC GTT CCA GAC TAC GCT). The PCR products were cloned into plasmid YCplac33 [19] by homologous recombination in yeast cells, as described in [13]. *HXT7* mutant alleles were constructed according to a PCR mutagenesis protocol [20], and cloned into YCplac33.

3. Results and discussion

3.1. Degradation of hexose transporters is triggered by nitrogen starvation

To investigate whether carbon sources other than glucose might also influence the stability of the high-affinity glucose transporters Hxt6p and 7p, we examined their stabilities in media with either maltose or ethanol as the sole carbon sources. Because of the high similarity between Hxt6p and 7p it is not possible to distinguish between them in Western analysis. Both proteins are equally affected by degradation ([3]; unpublished results). However, because of its higher expression level Hxt7p is the more important transporter for yeast glucose uptake [14]. To induce *HXT6* and *HXT7* expression, yeast cells of strain CEN.PK2-1C were grown in rich medium with 3% raffinose [21]. They were harvested and incubated for up to 6 h in nitrogen starvation medium (YNB−N), supplemented with 5% glucose, 2% maltose or 3% ethanol. The relative amounts of hexose transporter proteins were determined by Western analysis with specific antibodies. Whereas Hxt6p/7p rapidly degraded in the presence of glucose (Fig. 1A; half-life about 45 min) and maltose (Fig. 1B; half-life about 2 h), ethanol did not induce degradation of Hxt6p/7p at all (Fig. 1C). In contrast, when raffinose-grown yeast cells were shifted to a YNB medium with 5% glucose in the presence of 0.5% ammonium sulfate as a nitrogen source the half-life value of Hxt6p/7p increased up to 5 h (Fig. 1D). Synthesis of the transporters in this medium is not expected because transcription is completely repressed by glucose [21,22].

Hxt1p is a low-affinity glucose transporter and its synthesis is specifically induced by high concentrations of glucose [23]. Surprisingly, even the Hxt1p proteins were rapidly degraded in the presence of 5% glucose when the cells were shifted to nitrogen starvation conditions (Fig. 1E). However, in contrast to Hxt6p/7p, degradation of Hxt1p was not complete. The results suggest that degradation of hexose transporters is triggered by nitrogen starvation, but only in the presence of high concentrations of fermentable carbon sources.

3.2. Turnover of Hxt6p/7p requires endocytosis and autophagy

Hxt6p/7p stability was examined in *aut2* (= *apg4*) and *apg1* (= *aut3*) mutants defective for autophagy [24,25]. Aut2p is a cysteine protease and is essential for delivery of autophagic vesicles to the vacuole, whereas Apg1p is a protein kinase involved in induction of autophagy after nutrient limitation. In YNB−N medium with 5% glucose, Hxt6p and 7p were clearly stabilized in the *aut2* (Fig. 1F) and *apg1* strains (Fig.

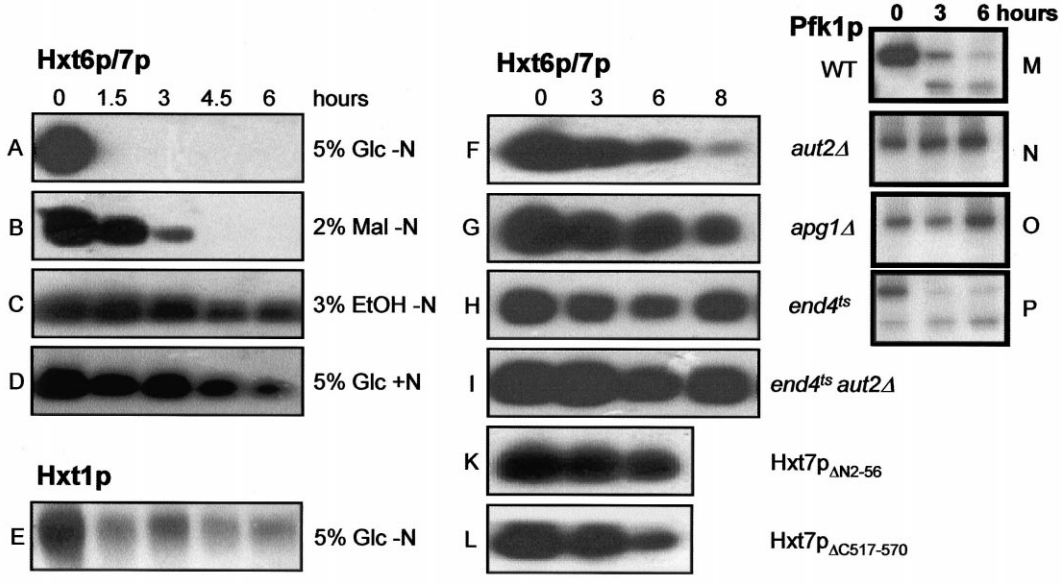

Fig. 1. Stability of *S. cerevisiae* hexose transporters during starvation conditions. The strains CEN.PK2-1C (wild type, A–E, M), SKY41 (*Δaut2*, F, N), SKY51 (*Δapg1*, G, O), RKY#536 (*end4^{ts}*, H, P), SKY43k (*end4^{ts} Δaut2*, I), RE301 (+*HXT7$_{ΔN2–56}$*) (K) and RE301 (+*HXT7$_{ΔC517–570}$*) (L) were grown to the early exponential phase in a medium with 3% raffinose (A–D, F–P) or 2% glucose (E). Cells were shifted to YNB−N (A–C, E–P) or YNB+N (D) with 5% glucose (A, D–P), 2% maltose (B) or 3% ethanol (C), and incubated at 30°C (A–G, K–O) or 37°C (H–I, P). At the indicated times, cells were harvested, membrane fractions were prepared and the proteins were subjected to Western analysis with Hxt6p/7p- (A–D, F–L), Hxt1p-specific (E) and Pfk1p-specific (M–P) antibodies. The experiments were repeated at least twice with similar results.

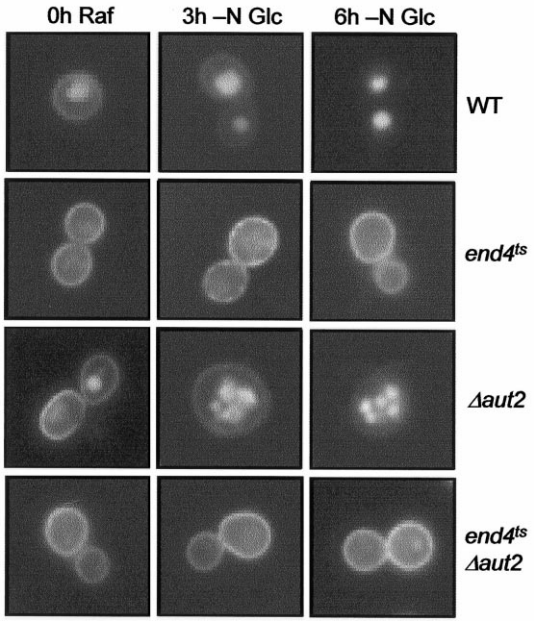

Fig. 2. Cellular distribution of Hxt7p-Gfp. Plasmid YCpHXT7-GFP was transformed into strains RKY#534 (wild type), RKY#536 (*end4^{ts}*), SKY44k (*Δaut2*) and SKY43k (*end4^{ts} Δaut2*), and Hxt7p-Gfp was visualized by fluorescence microscopy of raffinose-grown cells (25°C), and 3 h or 6 h after transferring the cells to YNB−N medium with 5% glucose at 37°C.

1G), as was the cytosolic enzyme phosphofructokinase 1 (Pfk1p), indicating that in response to starvation autophagy is required for both degradation of cytosolic proteins and the membrane proteins Hxt6p/7p. As Hxt6p/7p were also greatly stabilized in an *end4^{ts}* strain defective for endocytosis at the non-permissive temperature (Fig. 1H) (in contrast to Pfk1p) and in an *end4^{ts} aut2Δ* double mutant strain (Fig. 1I) our results indicate that both degradative pathways contribute to the delivery of Hxt6p/7p to the vacuole for proteolysis.

To further examine the contribution of endocytosis and autophagy for Hxt7p degradation, we analyzed the cellular distribution of a Hxt7p-Gfp fusion protein in the various mutants and wild type cells during growth or under nitrogen starvation conditions with 5% glucose (Fig. 2). Hxt7p-Gfp could restore the growth defect on glucose media of the glucose uptake-deficient hxt$^-$ strain EBY.VW4000 [13], indicating that the Hxt7p-Gfp fusion protein is able to mediate uptake of glucose across the plasma membrane. Western blot analysis with Hxt6p/7p-specific antibodies revealed that the fusion protein was rapidly degraded under nitrogen starvation conditions similar to the native Hxt7p protein (data not shown). Visualization of Hxt7p-Gfp by fluorescence microscopy in raffinose-growing wild type cells revealed a strong vacuolar signal and only weak plasma membrane staining (Fig. 2). This may reflect a high basal level of Hxt7p turnover in exponentially growing cells. Under nitrogen starvation conditions, the plasma membrane Hxt7p-Gfp signal became very faint and nearly all of the Hxt7p-Gfp protein was found in the vacuole. In growing as well as resting *end4^{ts}* cells (shifted to non-permissive temperature), the Hxt7p-Gfp signal corresponded mainly to the plasma membrane, indicating that an endocytosis defect protects Hxt7p from starvation-induced internalization.

In the *aut2* mutants during growth the Hxt7p-Gfp protein was found in the plasma membrane (Fig. 2), indicating that secretion of Hxt7p to the plasma membrane is not affected in autophagy mutants. However, after transferring the cells to nitrogen starvation conditions, the plasma membrane signal was lost, and Hxt7p-Gfp accumulated in relatively large intracellular vesicles but did not reach the vacuole. In an *end4^{ts} aut2* double mutant, the Hxt7p-Gfp protein was resistant to internalization as in the *end4^{ts}* single mutant. Thus it seems that Hxt7p is internalized by endocytosis, and autophagy is involved in the final delivery of Hxt7p to the vacuole.

3.3. The amino- and carboxy-terminal domains are required for degradation of Hxt7p

To determine whether specific amino acid motifs are required for the degradation of Hxt7p, we expressed several mutated variants of the permease on centromeric plasmids in strain RE301 (*Δhxt3-6-7*) [14], and assayed their stability under nitrogen starvation conditions. In the cytosolic N- and C-termini of Hxt7p and the cytosolic loop between transmembrane helices 6 and 7, four putative E/DXK motifs [9–11] are present (amino acids 38–40, 271–273, 515–517 and 558–560). Moreover, the $PES_{(257–259)}$ motif might be involved in sugar transporter degradation [12]. However, replacement of E_{271} by K, D_{558} by K or S_{259} by A had no effect on the degradation of Hxt7p (not shown). Nevertheless, replacement of the N-terminal amino acids 2–56 or the C-terminal amino acids 517–570 by an HA sequence significantly stabilized the proteins (Fig. 1K,L). Remarkably, the N-terminal domain of Hxt7p contains a proline/glutamic acid/serine/threonine-rich stretch of amino acids reminiscent of PEST sequences involved in the degradation of other plasma membrane proteins [26,27]. All these mutated alleles of *HXT7* except $HXT7_{\Delta C517–570}$ encode functional glucose transporters which are correctly targeted to the plasma membrane as they could complement the growth defect on glucose of the hxt$^-$ strain EBY.VW4000.

The kinetics of the wild type and the $Hxt7p_{\Delta N2–56}$ mutant

Table 1
Glucose uptake activities and ethanol production rates of yeast hxt$^-$ mutants (EBY.VW4000) expressing a wild type or an N-terminally truncated Hxt7p transporter

Conditions[a]		Glucose uptake activity		Ethanol production rate	
		V_{max} (nmol/min/mg dw)[b]	(%)	(mmol/g protein/h)	(%)
Hxt7p	+N	117	(100)	96	(100)
Hxt7p	−N	20	(17)	39	(41)
$Hxt7p_{\Delta N2–56}$	+N	76	(65)	28	(29)
$Hxt7p_{\Delta N2–56}$	−N	40	(34)	22	(23)

[a]Yeast cells were harvested during growth in YNB medium with ammonium sulfate and 3% raffinose (+N), or 6 h after incubation in YNB−N with 5% glucose (nitrogen starvation medium) (−N).
[b]dw = dry weight.

protein were compared after transformation of the corresponding plasmids into the glucose uptake-deficient hxt$^-$ strain EBY.VW4000. The affinity of the mutant permease did not differ from the wild type protein ($K_m \approx 1$ mM). However, during growth on raffinose the maximal transport activity was slightly reduced (Table 1). After incubation of the cells for 6 h in nitrogen starvation medium with 5% glucose, the glucose uptake activity of the strain expressing wild type Hxt7p greatly decreased (Table 1). In contrast, glucose uptake activity of the strain expressing the mutant protein decreased to only about half of the value before starvation. These results suggest that the N-terminally truncated Hxt7p protein resides in the plasma membrane in an active form during starvation conditions. We cannot explain the residual glucose uptake activity in the hxt$^-$ strain expressing wild type Hxt7p under starvation conditions, although no Hxt7p protein was detectable in Western analysis. Maybe, this is due to induction of the still unknown glucose transporting protein in *S. cerevisiae* [13].

3.4. The stabilized Hxt7p protein does not increase fermentation capacity under starvation conditions

The ethanol production rates of the hxt$^-$ strain expressing either the wild type or the mutant Hxt7p protein were compared (Table 1). Yeast cells were grown in YNB+N medium with 3% raffinose and harvested. One half of the cells was directly transferred into YNB+N medium with 1% glucose and the ethanol concentration was determined every 10 min for up to 1.5 h as described [28]. The other half of the yeast cells were first incubated for 6 h in YNB−N medium with 5% glucose before transferring them into the YNB+N medium with 1% glucose for ethanol determination. The ethanol production rates for the wild type Hxt7p decreased to about 40% after starvation (Table 1). Surprisingly, for the N-terminally truncated Hxt7p protein the ethanol production rates were very low before and after starvation. Therefore, as glucose uptake activity (as determined under *zero-trans* conditions) was higher for the mutant protein than for the wild type protein after starvation (Table 1), the lower ethanol production rates indicate a regulatory constraint on glucose transport during glucose metabolism which is lost in the N-terminally truncated mutant protein.

3.5. Conclusions

Our results indicate that during nitrogen starvation conditions, Hxt7p is internalized by endocytosis, and autophagy is involved in the final delivery of Hxt7p to the vacuole. This seems to be similar to mammalian cells where it has been shown that endosomes can fuse with autophagosomes prior to their entry into the lysosomal/vacuolar compartments [29–31]. In addition to nitrogen starvation conditions, a rich fermentable carbon source is necessary to trigger degradation. It is not yet clear whether this requirement indicates a specific signaling function of the carbon source or is connected to the energy requirements of the degradation process. Although the degradation of yeast sugar transporters seems to be due to the stimulation of general protein turnover, it is a specific process and not simply due to sequestration of bulky membrane fractions, as it is dependent on specific amino acid sequences of the proteins. Moreover, the extent of degradation varies between different hexose transporters.

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