Endocytosis and Degradation of the Yeast Uracil Permease Under Adverse Conditions*

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Yeast uracil permease follows the secretory pathway to the plasma membrane and is phosphorylated on serine residues in a post-Golgi compartment. The protein was found to be rather stable in growing cells, but its turnover rate (half-life of about 7 h) was much faster than that of most yeast proteins. Several adverse conditions triggered the rapid degradation of uracil permease, and so a loss of uracil uptake. Turnover was rapid when yeast cells were starved of either nitrogen, phosphate, or carbon, and as they approached the stationary growth phase. Rapid permease degradation was also promoted by the inhibition of protein synthesis.

The degradation of uracil permease in response to several stresses was strikingly slower in the two mutants, end3 and end4, that are deficient in the internalization step of receptor-mediated endocytosis. Thus, internalization is the first step in the permease degradative pathway. Uracil permease is degraded in the vacuole, since pep4 mutant cells lacking vacuolar protease activities accumulated large amounts of uracil permease, which was located within the vacuole by immunofluorescence. We have yet to determine whether adverse conditions enhance permease endocytosis and subsequent degradation or divert internalized uracil permease from a recycling to a degradative pathway.

Endocytosis plays a key role in the physiology of eucaryotic cells. In mammalian, it is involved in nutrition, removal of unwanted molecules from the plasma membrane, and in the cell response to hormones. Many polypeptide hormones and their receptors are cleared from the cell surface by endocytosis (1). The intracellular fates of a number of receptors and their ligands have been described in detail. Some receptors undergo endocytosis regardless of their bound/free state, while other receptors only undergo endocytosis when bound to ligand (2). Internalized receptors pass through complex vesicular and tubular structures, defined morphologically and biochemically as early and late endosomes (1). From these compartments, the endocytic pathway branches, allowing either recycling to the cell surface or delivery to the lysosomes for proteolysis. Permanent internalization followed by recycling to the plasma membrane has also been described for the GLUT4 glucose transporter (3). The internalization signals for this protein and for several receptors have been identified (2, 3). In contrast with the knowledge about endocytic pathways or signals, information relative to the endocytic machinery is still rather limited. Biochemical studies have focused mainly on the role of clathrin and its associated proteins, adaptins, in the internalization step (4), and more recently on the role of rab proteins in the vesicular traffic (5).

The use of a genetic approach of endocytosis has been developed in recent years in Saccharomyces cerevisiae in order to identify new proteins involved in this complex process and to investigate their in vivo function. Two markers were initially developed. Lucifer yellow was used to follow fluid-phase endocytosis, and a-factor was used to follow receptor-mediated endocytosis (6, 7). α-Factor is a pheromone that binds to its specific receptor, the product of the STE2 gene, and triggers its endocytosis, as judged from the clearance of receptor activity from the cell surface (8). This ligand is internalized and passes through endocytic compartments before being delivered to the vacuole where it is degraded (9). Two genes, END3 and END4, necessary for the internalization of α-factor linked to its receptor, have recently been identified (10). A vesiculation step requiring the SEC18 gene product would be involved in the targeting of α-factor from early to late endosomes (11). The delivery of α-factor to the vacuole depends upon the YPT7 gene product, which is homologous with the mammalian rab7p (12). The intracellular fate of a plasma membrane protein when passing along the endocytic pathway was recently described for the first time in yeast, both at the biochemical and morphological level (13). This study showed that the a-factor receptor may undergo two modes of endocytosis depending on the presence or absence of its ligand. Both pathways deliver the receptor to the vacuole for degradation. The occurrence of a recycling pathway in yeast was proposed as a way to explain certain features of the mutant ren1 impaired in a-factor receptor endocytosis (13).

The number of endocytic markers in yeast presently available remains very limited. Hence, following the fate of other proteins would help both to define hypothetical endocytic pathways alternative to that followed by the pheromone receptors, and to clarify the functions of endocytosis in yeast. The permeases, which catalyze the entry of essential nutrients, are likely to be good candidates for use as tools to probe endocytosis and its role in the turnover of plasma membrane proteins. Nutrient uptake is indeed very sensitive to environmental changes. Yeast cells growing on non-fermentable carbon sources respond to the addition of glucose by inactivating the galactose, maltose, and high affinity glucose transport systems (14, 15). Similarly, several amino acid permeases are inactivated by ammonia (16). The observation that these catabolite inactivations are reversible, except after the inactivation of protein synthesis, has led to the suggestion that they result from proteolytic degradation of the permeases (14). But although more than 20 genes encoding plasma membrane transporters have been cloned and sequenced to date in S. cerevisiae, the biochemical information on these proteins is very limited (17, 18). Apart from data on the targeting of some permeases at the plasma membrane (19, 20), the intracellular fate of yeast permeases is poorly documented, and the molecular events of their turnover have not yet been analyzed.
One of the difficulties in studying permeases at the protein level is that yeast cells contain very little of these proteins. Overexpression of the FUR4-encoded uracil permease (21) in an active form without deleterious effect for yeast cells enabled us to undertake molecular analysis of the intracellular fate of this permease. Newly synthesized uracil permease is delivered to the cell surface via the secretory pathway and is phosphorylated in a post-Golgi compartment on its way to and/or within the plasma membrane (20). We noticed in that study that uracil permease has a short half-life at high temperatures (20). We therefore investigated the turnover of uracil permease to define the situations triggering proteolysis of the permease and the pathway involved in degradation. The data presented here indicate that uracil permease is rather stable in growing cells. However, under adverse circumstances, uracil permease is selected for degradation. The permease is internalized by endocytosis before it undergoes proteolysis, since proteolysis is severely delayed in the two mutants end3 and end4 that are deficient in receptor-mediated endocytosis. These experiments are the first to directly examine the turnover of a permease in S. cerevisiae at the molecular level and provide an example of endocytosis of a non-receptor plasma membrane protein in yeast.

**Materials and Methods**

**Strains, Plasmids, and Growth Conditions**—The *S. cerevisiae* strains were W303-1B/D (MATa, ade2-1, ura3-1, his3, leu2, trp1, can1-100) (22), NC122-6p6 (MATa, leu2, ura2, ura3, his4, trp1) (18) derived from the parental strain FL100 (23); RH144-3D (MATa, ura3-1, his4, leu2, bar1-1) (10) and its derivative strains RH144-3D end3 (MATa, end3:URA3, ura3, his4, leu2, bar1-1) and RH268-1C (MATa, ura3, his4, leu2, bar1-1) (10); and W6-AA (Mats, pep4-3, ura3-1, his3-11, leu2-3-112, trp1) which resulted from a cross between W303-1B/D and W06-12 (Mats, pep4-3, trp1) (24). These strains were transferred according to Ref. 25 with multiplicity plasmids carrying the FUR4 gene and either the LEU2 or the URA3 gene as selection marker. The plasmids pEPG and pEPG-2 (20) both contain the LEU2 gene and the FUR4 gene, which is under the control of its own promoter in pEP and of the GAL10 promoter in pEPF. The plasmid p195Gφ was constructed by inserting an EcoRV-BamHI fragment from the plasmid pEG, containing the FUR4 gene under the control of its own promoter of the GAL10 promoter, in the multiple cloning site of the plasmid YEp352 (25) containing the URA3 gene as a selection marker. The plasmid YEp352 (27) carrying the URA3 gene was also introduced in the strains RH144-3D and RH268-1C to make them prototrophic for uracil.

Cells were grown at 30 °C, or 25 °C for thermosensitive mutants, in minimal synthetic (YNB) medium containing 0.67% yeast nitrogen base, supplemented with the required nutrients. Carbon sources were 2% glucose for cells transformed with the plasmid pEPF or 4% galactose plus 0.02% glucose for cells transformed with the plasmids pEPF and p195Gφ.

**Measurement of Uracil Uptake**—One ml of yeast culture was incubated with $5 \mu$g [3H]uracil (Amersham) for 20 s at 30 °C and then quickly filtered through Whatman GPC filters. Filters were washed twice with ice-cold water and counted for radioactivity.

For all the data reported in the present paper, under conditions of exponential growth ($R < 1 \times 10^6$ cells/ml), the uracil uptake activity was raised up to $25-26 \mu$g/min/10$^6$ cells for multiplicity plasmid-encoded permease, compared with 0.2-1 $\mu$g/min/10$^6$ cells for chromosomal encoded permease.

**Yeast Cell Extracts and Immunoblotting**—2-4 $\times 10^6$ cells were harvested and cell extracts were prepared by lysis with 0.5 ml of 0.2 M NaOH, 0.2% mercaptoethanol for 10 min on ice. Trichloroacetic acid was added to a final concentration of 5%, and the samples were incubated for an additional 10 min on ice. Precipitates were collected by centrifugation at 12,000 $\times g$ for 5 min. The pellets were neutralized and dissolved in 35 ml of dissociation buffer (4% sodium dodecyl sulfate, 0.1 M Tris hydrochloride, pH 6.8, 4 mM EDTA, 20% glycerol, 2% 2-mercaptoethanol, 0.02% bromphenol blue) and 15 ml of 1 M Tris base, and heated at 37 °C for 15 min. 0.5 to 1 $\times 10^6$ cells were loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis in a Tricine system (28). Proteins were transferred to a nitrocellulose membrane and visualized, and cell extracts were prepared by lysis with 0.5 ml of 0.2 M NaOH, 0.2% mercaptoethanol for 10 min on ice. Trichloroacetic acid was added to a final concentration of 5%, and the samples were incubated for an additional 10 min on ice. Precipitates were collected by centrifugation at 12,000 $\times g$ for 5 min. The pellets were neutralized and dissolved in 35 ml of dissociation buffer (4% sodium dodecyl sulfate, 0.1 M Tris hydrochloride, pH 6.8, 4 mM EDTA, 20% glycerol, 2% 2-mercaptoethanol, 0.02% bromphenol blue) and 15 ml of 1 M Tris base, and heated at 37 °C for 15 min. 0.5 to 1 $\times 10^6$ cells were loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis in a Tricine system (28). Proteins were transferred to a nitrocellulose membrane and visualized.

**RESULTS**

**Localization of Overproduced Uracil Permease**—The fate of uracil permease, once it had arrived at the plasma membrane, was investigated in cells which produced this permease from high copy number plasmids bearing the FUR4 gene under the control of its own promoter, or of the inducible GAL10 promoter. The turnover of the permease was followed by checking, in addition to the loss of uracil uptake, the decrease in the amount of permease on Western immunoblots. Pulse-chase experiments showed previously that the protein is phosphorylated in a post-Golgi compartment. As the permease species detected on the immunoblots were mainly phosphorylated species (20), overexpressed permease is likely to be mostly at the plasma membrane. This point was checked by immunofluorescence. Cells disrupted for the FUR4 gene were devoid of any staining (Fig. 1A). While a low level of synthesis gave no immuno-labelling (not shown), the cell surface of transformed cells was clearly

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1 The abbreviation used is: Tricine, N-tris(hydroxymethyl)methylglycine.
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Fig. 1. Location of overproduced uracil permease. The strain NC122-sp6 disrupted for the FUR4 gene (A) and the strain W303-1B/D transformed with the plasmid pFp (B) or pgF (C) were grown in YNB medium with glucose (A and B) or galactose (C) as a carbon source. Cells harvested during logarithmic growth were fixed and processed for immunofluorescence using a purified specific antibody to uracil permease as described under "Materials and Methods." They were examined for phase contrast and indirect immunofluorescence using a confocal laser microscope.

decorated by the specific antibodies. Staining seemed to be mainly over the plasma membrane, and immunofluorescence was occasionally seen in vesicle-like bodies that seemed to lie under the inner surface of the plasma membrane (Fig. 1, B and C). There was no perinuclear-labeled material unlike the situation observed for most proteins that accumulate in the endoplasmic reticulum in yeast (29). The distribution of overproduced uracil permease appeared very similar to that of the few plasma membrane proteins detected so far by immunofluorescence in yeast (30), including non-overproduced H+-ATPase (19, 31). Thus, overproduction probably does not alter the expected location of uracil permease.

Turnover of Uracil Permease after Inhibition of Protein Synthesis—The turnover of uracil permease was estimated by following uracil uptake and the level of immunodetected permease after inhibiting protein synthesis with cycloheximide. The experiment was performed on cells grown on either glucose (Fig. 2, A and B) or galactose (Fig. 2, A and C). The loss of uracil uptake (Fig. 2A) followed the time course expected for a first-order reaction. The calculated half-life was 2.5 h for glucose-grown cells. The drop in immunodetected permease appeared to parallel the loss of uracil uptake whatever the carbon source used, indicating that degradation of the permease is responsible for the drop in uracil uptake, and that permease turnover was faster in galactose-grown cells. Adding glucose together with cycloheximide failed to slow the turnover (not shown), indicating that glucose is not involved in degradation per se.

Staining the blots with Ponceau Red showed that there was no significant decrease in the amount of total proteins after cycloheximide treatment (not shown). The behavior of another plasma membrane protein, H+-ATPase, was used as a control in galactose-grown cells, which have a rapid permease turnover. The overexpression of the permease had no effect on either the amount or the profile of the H+-ATPase (compare lanes 1 and 2, Fig. 2C). Incubation for 2 h with cycloheximide resulted in only a slight decrease in the ATPase, while the permease had almost completely disappeared (Fig. 2C, lanes 2-5). This behavior of H+-ATPase is in agreement with the half-life of 11 h described for this protein after inhibition of protein synthesis (32).

The turnover of the uracil permease was therefore far higher than that of most of cell proteins and of the control plasma membrane protein, H+-ATPase. This suggests that there is a specific degradation after inhibition of protein synthesis. The rate of permease degradation was found to be dependent upon the carbon source for growth. The degradation required energy metabolism, as the immunodetected permease was not lost when the medium contained sodium azide (Fig. 2C, lane 6).
Fig. 2. Loss of uracil uptake and degradation of uracil permease after inhibition of protein synthesis. NC122-sp6 cells transformed with the plasmids pF or pG were grown to logarithmic growth phase on glucose or galactose medium, respectively. Cycloheximide (100 μg/ml) was then added to the medium. A, uracil uptake was measured at different times. Results are percent of initial activities. B, protein extracts were prepared from glucose grown cells removed at the times indicated, and the uracil permease was visualized by Western blot analysis. C, protein extracts were prepared from galactose grown cells and probed with specific antisera for uracil permease and plasma membrane H+-ATPase. Lane 1, control extract from untransformed cells; Lanes 2-5, extracts from cells removed at the times indicated after the addition of cycloheximide. Lane 6, extract from cells to which 10 mM sodium azide was added at the same time as cycloheximide.

Turnover of Uracil Permease in Exponentially Growing Cells—Cells expressing the FUR4 gene under the control of the inducible GAL10 promoter were used to follow the fate of uracil permease in actively growing cells. Glucose was added to galactose growing cells to stop further transcription of the FUR4 gene. As FUR4 mRNA has a very rapid turnover (t½ = 2 min) (33), adding glucose indeed rapidly blocked further synthesis of the permease. It led to a sharp drop in the amount of labeled immunoprecipitable permease within minutes (control not shown). Uraicil uptake increased with time in control cells, under conditions of ongoing permease synthesis (Fig. 3A). After glucose repression of permease synthesis in growing cells, uracil uptake exhibited only a slight decrease (20%) within 4 h (Fig. 3A). The kinetics of this loss of activity followed a first-order reaction, with a half-time of approximately 7 h. Thereafter, there was a rapid inactivation before cells arrived at stationery phase. In contrast to this biphasic loss of uracil uptake during cell growth, cycloheximide immediately triggered a rapid loss of uracil uptake (Fig. 3A), as observed previously (Fig. 2A). The half-life was 30 min in the presence of this inhibitor in the present experiment. The time courses of the amount of immunodetectable permease in the presence or the absence of cycloheximide were qualitatively in agreement with the kinetic data. The loss of uracil uptake (Fig. 3B). However, degradation seemed to proceed before any significant decrease in uracil uptake, and to be greater, especially in the absence of cycloheximide. Several independent factors might explain such a discrepancy. The uracil permease activity is dependent upon the activity of the H+-ATPase, which is influenced by several environmental changes. For instance it is activated as the cells approach the stationary phase (34). This might explain why the permease degradation was transiently offset by the activation of the remaining protein during this period. A difference in the state of phosphorylation of uracil permease might also influence its specific activity.

This experiment revealed several striking properties of uracil permease turnover. The kinetics of the loss of uracil uptake and of permease degradation appeared very different in growing and non-growing cells. The sharp drop in uracil permease brought on by cycloheximide was probably due to the stress induced by the total inhibition of protein synthesis. The same
observation was made in cells expressing the FUR4 gene under the control of the repressible PHO5 promoter: repression of FUR4 gene expression with phosphate left the permease activity rather stable in growing cells, whereas it rapidly decreased when cycloheximide was added (data not shown).

Uracil permease appeared to be degraded only slowly in early exponential growing cells shifted from galactose to glucose. A rapid degradation was then triggered about one generation before cells arrived at the stationary growth phase (Fig. 3). Similarly, uracil uptake declined and permease was lost during late exponential growth of transformed cells having different genetic background whatever the carbon source for growth. Such a decrease of permease at the approach of the stationary phase cannot be due to the overproduction of the permease, since uracil uptake declined in a similar fashion during the growth of untransformed strains, which carried 20-50-fold less permease activity (data not shown).

**Turnover of Uracil Permease upon Nutrient Starvation.**—The decline in permease activity during the late exponential growth phase suggested that permease breakdown might also be triggered by the limitation of a required nutrient. To test this possibility cells were starved of nitrogen, phosphorous, or carbon source after shutting off new permease synthesis with glucose. The growth began to slow after 1 h in a nitrogen or phosphorous-free medium compared with that in standard medium, whereas the lack of carbon source led to an immediate arrest of growth. In parallel, uracil uptake was rather stable during the first hour of nitrogen or phosphorous deprivation and thereafter it declined with a half-time of 90 min, whereas it hardly decreased in standard medium (Fig. 4A). The inactivation triggered by phosphate or nitrogen starvation was accompanied by enhanced permease degradation (data not shown). Carbon starvation led to the immediate loss of all permease activity as did azide (35), since uracil uptake is energy-dependent. Immunoblots of extracts from glucose-starved cells showed extensive degradation after 15 min of glucose starvation, and the loss of most of the permease within 45 min (Fig. 4B). These data indicate that the turnover rate of the permease increased greatly upon nutrient deprivation.

As nutrient deprivation triggers an arrest in the G1 phase of the cell cycle (36), we checked to see whether the increase in the turnover of uracil permease could be linked to the cell cycle arrest. α-Factor and glucose were added to a cells, which expressed uracil permease under galactose control, and permease activity was followed over time. While α-factor indeed promoted the formation of shmoo, it had no influence on permease turnover (data not shown). The lifetime of uracil permease therefore seems to be sensitive to the stress induced by nutrient starvation, but not to arrest in the G1 phase of the cell cycle.

**Uracil Permease Degradation Is Subsequent to Internalization By Endocytosis.**—Proteolysis of a plasma membrane protein could be achieved by a direct breakdown of a selected protein at the plasma membrane, or by selective internalization and transport to the vacuole for non-specific proteolysis (37). *end3* and *end4* mutant cells were used to determine whether uracil permease was removed from the cell surface before its degradation. Those mutants are thermosensitive for growth (10). They fail to internalize α-factor, its receptor, or to accumulate a fluid-phase marker in the vacuole. The *end4* mutant has a thermosensitive endocytic defect: the *end4* mutant protein is partially functional at 24 °C, and irreversibly inactivated at 37 °C. The *end4* mutation affects an early step in the internalization pathway (10). The loss of uracil uptake and the degradation of uracil permease upon inhibition of protein synthesis by cycloheximide were compared in isogenic *end3, end4*, and wild type cells. In the wild type cells, raising the temperature from 25 to 37 °C accelerated (x 3) the loss of uracil uptake (Table I). This effect was found in all the wild type strains tested. Thus, heat stress further enhanced the rate of degradation triggered by cycloheximide. A mild heat shock also resulted in an increase in permease turnover, even in growing cells.

The disruption of the *END3* gene conferred partial protection against the inactivation of uracil permease (Table I and Fig. 5A). The time required to lose 50% of uptake was 2-fold greater than that of wild type cells at 25 °C and 4-fold greater at 37 °C (Table I). The coordination between the drop in uracil uptake and the loss of immunodetected permease for both *end3* and wild type strain (Fig. 5B) clearly showed that the absence of the *END3* gene product slowed permease degradation. The *end4* mutation provided a striking protection against both permease inactivation and degradation (Fig. 5, A and B). This effect was already seen at 25 °C. The half-time of uracil uptake was twice that of wild type strain (Table I). Increasing the temperature from 25 to 37 °C, which accelerated inactivation in all wild type strains, and even in the *end3* strain, almost completely prevented any drop in uracil uptake in the *end4* strain (Table I and Fig. 5A). It was less than 10% during the 2-h treatment with

\[ J. M. Galan, personal communication. \]
cytochrome c at 37 °C, compared with over 90% in the wild-type strain (Fig. 5A). In the meantime, the immunodetected permease, which disappeared in the wild-type strain, was still present at high level in the end4 mutant strain (Fig. 5B). However, some degradation still occurred.

These data clearly indicate that the END3 and END4 gene products are involved in the degradation of uracil permease triggered by cycloheximide. Internalization of uracil permease is therefore required prior to its degradation.

We checked that internalization also preceded the degradation of the permease induced by nutritional stress by depriving the same wild type and end cells of nitrogen source at a restrictive temperature. After 2 h at 37 °C, the level of immunodetected permease had not dropped in end4 cells, while it had dropped to zero in wild type cells (Fig. 6). The behavior of the permease in end3 cells was intermediate (not shown). Therefore, the degradation triggered by nitrogen starvation required the same endocytosis pathway as that induced by an arrest of protein synthesis. In addition, the holding of immunodetected permease at a high level in end4 cells submitted to various kinds of stress further confirmed that a high percentage of the protein was located upstream of the end4 block, i.e., at the plasma membrane, in transformed cells overexpressing uracil permease.

Uracil Permease Is Degraded in the Vacuole—We examined the fate of permease in pep4 cells lacking vacuolar protease activities (38, 39) to determine where the permease was degraded. Pep4 mutants cells overexpressing uracil permease under the control of its own promoter carried the same permease activity as wild type-transformed cells, but about 10 times more permease was detected on immunoblots (Fig. 7A). The amounts of permease labeled in a 10-min pulse with [35S]methionine in cells resulted from a decrease in the rate of permease proteolysis. Indirect immunofluorescence in pep4 cells allowed detection of uracil permease at both the plasma membrane and in the vacuole (Fig. 7C). These data would be consistent with vacuolar degradation of uracil permease.

**DISCUSSION**

Uracil permease has a rather "slow" turnover (half-life of ~7 h) in growing cells. The protein appears to reside for several hours at the cell surface before being sorted out for degradation. This relative stability is seen in galactose grown cells transferred to glucose medium. However, the turnover of the "stable" uracil permease in growing cells is much faster than that of the bulk of yeast proteins (t1/2 > 150 h for up to 90% of total proteins) (40), or even that of the H+-ATPase (t1/2 > 20 h in glucose-growing cells) (32).

Several adverse conditions can trigger a rapid degradation of uracil permease, and thus a loss of uracil uptake. Turnover is rapid when yeast cells are starved of either nitrogen, phosphate, or carbon source, and as they approach the stationary phase. Permease degradation is also induced by other stresses, such as heat stress, or the inhibition of protein synthesis, two conditions that were also reported to affect the turnover rate of the plasma membrane H+-ATPase (32, 41). Blocking protein synthesis promoted the most spectacular effect on permease degradation: it can induce up to a 15-fold increase in the permease turnover rate. Various stresses can obviously have additional effects: the rate of permease degradation triggered by cycloheximide increases with an upshift in temperature, and as the cells approach the stationary phase. It should be emphasized that the time course of permease inactivation and degradation appear to depend also upon numerous other factors, such as the genetic background of the strains used, or the carbon source for growth.

The acceleration of permease turnover rate by stressful situations is not due to bulk endocytosis, as the receptor-mediated internalization of α-factor is not altered by stress (42). Uracil permease degradation seems to be rather specifically regulated. The permease is entirely degraded after inhibition of protein synthesis for 2 h, whereas the bulk of yeast proteins and the plasma membrane H+-ATPase hardly decrease. Uracil permease is rather stable in growing cells supplemented with glucose. In addition, the catabolite inactivation of several sugar transporters (14, 43, 44). In contrast, uracil permease is rapidly lost upon carbon starvation, a stress known to stabilize sugar transporters in yeast (14, 43, 44). The physiological relevance of the regulation of uracil permease degradation is not clear. Uracil permease activity is required to provide uracil to pyrimidine-deficient yeast strains or to pump the uracil excreted in the medium (35, 45). Internal uracil is used, via UMP (45) as an alternative pathway to provide pyrimidine for RNA synthesis, i.e., mainly for ribosomal RNA synthesis. The rate of rRNA genes transcription, and more generally ribosomal synthesis, is believed to be a sensitive parameter of the cell’s prospects (46). It drops during heat stress (47), upon arrival at the stationary phase, or upon inhibition of protein synthesis (48). The release of uracil permease from the cell surface and its subsequent proteolysis under adverse conditions might therefore be correlated with the regulation of ribosomal synthesis.

It will be necessary to identify the signals involved in the regulation of uracil permease turnover rate. As observed for several receptors, variations in the phosphorylation state of the permease might regulate its stability. When compared with galactose grown cells, glucose grown cells appear to be enriched in highly phosphorylated permease species. In parallel, the rate of permease degradation triggered by cycloheximide is more rapid in galactose grown cells than in glucose grown cells. Identification of the phosphorylated residues of uracil permease and their in vitro mutagenesis should indicate whether permease phosphorylation is indeed involved in the control of its turnover rate.

On the other hand, uracil permease includes a nine amino acid sequence (RALGSLTLD) that is very similar to the "destruction box" consensus sequence (RXALGXXINX) present in mitotic cyclins (49). It has been demonstrated that this sequence is required for the ubiquitin-dependent proteolysis of a sea urchin B cyclin (50). Although to date, ubiquitin-dependent degradation has been shown to involve cytosolic proteolysis by the proteasome (51), there are rare examples in mammals of the conjugation of ubiquitin to plasma membrane proteins (52, 53). Furthermore, ubiquitinated membrane-bound proteins have been found in the yeast vacuole (54) and mammalian lysosome (55). A role of this nine amino acid sequence in the regulation of permease turnover rate would agree with the enhancement of permease degradation under stress conditions. Such conditions are known to induce transcription of the polyubiquitin gene and synthesis of several ubiquitin-conjugating enzymes (56). Current investigations are examining...
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Fig. 5. Turnover of uracil permease in wild type and end mutant cells after inhibition of protein synthesis at a non-permissive temperature. RH144-3D (WT), RH268-1C (end4), and RH144-3Dend3 (end3) cells which overexpressed the FUR4 gene under the control of its own promoter were grown at 25 °C with glucose as a carbon source to a same cell density (10^7 cells/ml). At t = 0 cultures were transferred to 37 °C, and cycloheximide was added to the medium. A, uracil uptake was measured at various times. Results are percent initial activity. B, protein extracts were prepared at the times indicated. Protein extracts from 0.2-ml cultures were analyzed for permease by Western immunoblotting.

Fig. 6. Effect of nitrogen deprivation upon permease degradation in wild type and end4 mutant cells. RH144-3D (WT) and RH268-1C (end4) cells which overexpressed the FUR4 gene under the control of its own promoter were grown at 25 °C up to 1 x 10^7 cells/ml. They were transferred to 37 °C in a medium lacking nitrogen source. Protein extracts were prepared before and after a 2-h deprivation. Proteins from 0.2 ml of culture were analyzed for immunodetectable permease.

whether the destruction box sequence indeed plays a role in uracil permease degradation.

The degradation of uracil permease in response to several stresses is slowed in end3 mutant and severely reduced in end4 mutant strains at 37 °C. Uracil permease is therefore degraded after it has been internalized by endocytosis, a process which requires the End3p and End4p proteins (10). Whatever the function of the End3p, it is partially bypassed, since the defect in permease internalization was only partial in an endd-disrupted strain. The defect in uracil permease internalization is immediate in the end4 thermosensitive mutant upon upshift to a non-permissive temperature, as observed for both α-factor internalization and for clearance of α-factor receptor (10). The permease activity is almost completely maintained in end4 mutant cells at non-permissive temperature although some degradation still occurs. This indicates that end4 deficiency completely blocks internalization and that a certain amount of the protein is present in an internal pool within the endocytic pathway prior to the onset of degradation. This pool, however, would represent only a small percentage of the immunodetected permease in these glucose grown cells.

Preliminary experiments let us to show that the degradation of uracil permease is partially inhibited in a sec18 mutant at non-permissive temperature. The Sec18p, the yeast homolog of the mammalian NSF (57), is required in the secretory and endocytic pathways when vesiculation steps proceed. It would be necessary for the transit of internalized α-factor from early to late endosomes (11). Thus, the partial defect in permease degradation observed in a sec18 mutant implies that at least part of internalized permease requires a vesiculation step to proceed in the degradative pathway.

The degradation of endocytosed uracil permease probably occurs in the vacuole. Pep4 mutant cells lacking vacuolar protease activities (38) accumulate large amounts of uracil permease within the vacuole. However, this accumulation occurs even in the exponential growth phase. This appears to contradict other data indicating that uracil permease has a rather long lifetime in exponentially growing cells, at least in some experimental conditions (Fig. 3). We have yet to determine whether uracil permease undergoes continuous endocytosis,
followed by vacuolar degradation in some strains or under some growth conditions, or whether part of newly synthesized permease undergoes endocytosis and subsequent proteolysis in some strains or under some stress situations. This event is dependent upon permease and its endocytic pathway for transporters. Uracil permease appears as an appropriate marker for studies of the endocytic pathway for transporters in yeast. Uracil permease can be either stable or rapidly degraded, a situation which might be representative of that of other permeases. It will be important to understand whether stable permeases remain in a stable location at the plasma membrane, or undergo constant recycling through an early-like endosome. Analysis of the mechanisms triggering the degradation of uracil permease which is highly sensitive to environmental changes will expand our understanding of endocytosis in yeast, especially under stress conditions.

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Addendum—After this paper was submitted for publication, it was reported that proteolytic degradation accounts for catabolite inactivation of the maltose transporter (Lucero, P., Herweijer, M., and Laguna, R. (1993) FEBS Lett. 333, 165-168).

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