Abstract. In Saccharomyces cerevisiae, amino acid permeases are divided into two classes. One class, represented by the general amino acid permease GAP1, contains permeases regulated in response to the nitrogen source. The other class, including the high affinity tryptophan permease, TAT2, consists of the so-called constitutive permeases. We show that TAT2 is regulated at the level of protein stability. In exponentially growing cells, TAT2 is in the plasma membrane and also accumulates in internal compartments of the secretory pathway. Upon nutrient deprivation or rapamycin treatment, TAT2 is transported to and degraded in the vacuole. The ubiquitination machinery and lysine residues within the NH$_2$-terminal 31 amino acids of TAT2 mediate ubiquitination and degradation of the permease. Starvation-induced degradation of internal TAT2 is blocked in sec18, sec23, pep12, and vps27 mutants, but not in sec4, end4, and apg1 mutants, suggesting that, upon nutrient limitation, internal TAT2 is diverted from the late secretory pathway to the vacuolar pathway. Furthermore, our results suggest that TAT2 stability and sorting are controlled by the TOR signaling pathway, and regulated inversely to that of GAP1.

Key words: GAP1 • TOR • rapamycin • ubiquitin • endocytosis

Amino acid permeases and homologues in yeast form a family of 23 members with demonstrated or suspected transport activity, and an additional member with a presumed regulatory function (Nelissen et al., 1997; Didion et al., 1998). Based on their function and regulation, the amino acid permeases are divided into two classes (Sophianopoulou and Diallinas, 1995). Permeases of one class, including the general amino acid permease, GAP1, and the proline permease, PUT4, are regulated in response to the available nitrogen source (Jauniaux et al., 1987; Jauniaux and Grenson, 1990; Vandenbol et al., 1990). In the presence of a good nitrogen source, such as ammonium or glutamine, the uptake activity by these permeases is low, whereas in media containing a poor nitrogen source, such as proline or urea, transport activity is strongly induced. Both permeases transport amino acids that can be used as a nitrogen source. The nitrogen-dependent regulation of GAP1 is complex, occurring at the levels of GAP1 transcription and GAP1 sorting and degradation (Stanbrough and Magasanik, 1995; Roberg et al., 1997b; Springael and Andre, 1998). The second class of amino acid permeases consists of mainly high affinity transporters that are specific for single amino acids or a small set of structurally related amino acids. These permeases, including the histidine permease, HIP1 (Tanaka and Fink, 1985), and the tryptophan permease, TAT2 (Schmidt et al., 1994), import amino acids primarily for use in protein synthesis. They are thought not to be regulated and are thus often referred to as the constitutive permeases.

Newly made amino acid permeases, despite lacking an NH$_2$-terminal signal sequence, are inserted into the membrane of the endoplasmic reticulum (Ljungdahl et al., 1992). Assisted by the ER-resident protein SHR3, the permeases are then packaged into coatomer protein (COP)-II vesicles (Kuehn et al., 1996), and routed through the secretory pathway to the plasma membrane, where they actively import their amino acid substrates. For GAP1, it has recently been shown that transport from the Golgi to the plasma membrane is not a default process. Depending on the nitrogen source, GAP1 is delivered from the Golgi to either the vacuole or the plasma membrane. In the presence of a good nitrogen source, GAP1 is targeted to the plasma membrane (Roberg et al., 1997a,b).
The GA P1 permease, like many other integral plasma membrane proteins, is removed from the plasma membrane via ubiquitin-triggered internalization (Hicke and Riezman, 1996; Hicke, 1997; Sprinzak and A ndre, 1998). The NPl/RSp5 ubiquitin-protein ligase is required for GA P1 ubiquitination. NPl/RSp5 was originally identified as the nitrogen permissive inactivator for GA P1 (H e in et al., 1995; J uniaus et al., 1987). A fter internalization, GA P1 is transported to the vacuole for degradation.

The immunosuppressant rapamycin induces a starvation state in yeast cells, stationary phase or G 0, by binding and inhibiting target of rapamycin (TOR) 1 and TOR 2 (H eitman et al., 1991; Stan et al., 1994; Z heng et al., 1995; B arbet et al., 1996). The TOR proteins, two highly homologous phosphatidylinositol kinase-related kinases (K uz et al., 1993; H ellowell et al., 1994), normally control cell growth by signaling activation of translation initiation and early G 1 progression in response to nutrients (B arbet et al., 1996; D i Como and A rndt, 1996; B erset et al., 1998). TOR 2 also activates a Rho-type GTPase switch and thereby controls the cell cycle-dependent organization of the actin cytoskeleton (Como and Arndt, 1996; B erset et al., 1998). TOR2 also activates a Rho-type GTPase switch and thereby controls the cell cycle-dependent organization of the actin cytoskeleton (Como and Arndt, 1996; B erset et al., 1998). TOR2 also activates a Rho-type GTPase switch and thereby controls the cell cycle-dependent organization of the actin cytoskeleton (Como and Arndt, 1996; B erset et al., 1998). TOR2 also activates a Rho-type GTPase switch and thereby controls the cell cycle-dependent organization of the actin cytoskeleton (Como and Arndt, 1996; B erset et al., 1998).

Here we show that the constitutive permease T A T2 is indeed regulated. Inversely to G A P1, T A T2 is targeted to the vacuole and degraded upon nutrient deprivation. Surprisingly, an internal pool of T A T2 appears to be routed inversely to GAP1, T A T2 is targeted to this TOR2 unique function is not rapamycin sensitive (S chmidt et al., 1996, 1997; Bickle et al., 1998). However, cell cycle–dependent organization of the actin cytoskeleton (Como and Arndt, 1996; B erset et al., 1998). TOR2 also activates a Rho-type GTPase switch and thereby controls the cell cycle–dependent organization of the actin cytoskeleton (Como and Arndt, 1996; B erset et al., 1998). TOR2 also activates a Rho-type GTPase switch and thereby controls the cell cycle–dependent organization of the actin cytoskeleton (Como and Arndt, 1996; B erset et al., 1998). TOR2 also activates a Rho-type GTPase switch and thereby controls the cell cycle–dependent organization of the actin cytoskeleton (Como and Arndt, 1996; B erset et al., 1998).

Materials and Methods

Strains, Plasmids, and Media

S. cerevisiae strains used in this work are listed in Table I. Plasmids used in this work are listed in Table II. Rich medium (YPD) was prepared as described (Sherman, 1991). Synthetic complete medium (SC) lacking the appropriate nutrients for plasmid maintenance was as described (Sherman, 1991) with the following modifications. Tryptophan was added to 40 mg/ml, leucine to 100 mg/ml, and glutamic acid, aspartic acid, and serine were added 10 mM ammonium sulfate or 1 mg/ml proline, or no ammonium or proline. Rapamycin (gift of Sandoz Pharmaceutical) was dissolved at 1 mg/ml tryptophan, 20 mg/ml, and added to liquid media to a final concentration of 200 ng/ml.

Genetic Techniques

Yeast transformation was performed by the lithium acetate procedure (Ito et al., 1983). E. coli strain DH 5α was used for propagation and isolation of plasmids as described (A usubel et al., 1998).

DNA Manipulations

Restriction enzyme digestes and ligations were done by standard methods. Enzymes and buffers were obtained commercially (B oehringer M annheim). DNA was sequenced by the dyeoxy-chain termination method with the T 7 sequencing system (Pharmacia).

Construction of HA-tagged and Mutant T A T2

HA-T A T2 (in pA S55, pA S64, or pT B287) encodes an NH2-terminally HA-tagged, fully functional T A T2 protein under control of its own pro-

Table I. Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tr>
<td>JK9-3D</td>
<td>MATα his4 leu2 ura3 trp1 mel1HML1a</td>
</tr>
<tr>
<td>MH684</td>
<td>JK9-3da pep1::URA3</td>
</tr>
<tr>
<td>23344c</td>
<td>MATα his4 leu2 ura3</td>
</tr>
<tr>
<td>27038a</td>
<td>MATα apg1 leu2 ura3</td>
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<tr>
<td>27000d</td>
<td>MATα dne4hip2 ura3</td>
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<td>RH1552</td>
<td>MATα sec4-1 his4 lys2 ura3</td>
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<td>RSY255</td>
<td>MATα leu2 ura3</td>
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<td>RSY271</td>
<td>MATα sec18-1 his4 ura3</td>
</tr>
<tr>
<td>RSY281</td>
<td>MATα sec23-1 his4 ura3</td>
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<tr>
<td>MT16-9B</td>
<td>MATα apg1-1 leu2 ura3</td>
</tr>
<tr>
<td>RH1602</td>
<td>MATα his4 leu2 ura3 bar1</td>
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<td>RH1597</td>
<td>MATα end4-1 his4 leu2 ura3 bar1</td>
</tr>
<tr>
<td>RH2378</td>
<td>MATα VPS27 his4 leu2 ura3</td>
</tr>
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<td>RH2379</td>
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<td>SEY6210</td>
<td>MATα PEPI2 his3 leu2 ura3 trp1 lys2 suc2</td>
</tr>
<tr>
<td>CBY9/5C</td>
<td>MATα pep12 ade2 his2 leu2 ura3 trp1 lys2 suc2</td>
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</table>

Amino Acid Import Studies

The import rates of radiolabeled amino acids (L-[3,5-3H]tryptophan (33 Ci/mmol), L-[4,5-3H]leucine (58 Ci/mmol), and L-[2,5-3H]histidine (42 Ci/mmol; A merham) were measured as described (H etman et al., 1993), with the following modifications. Wild-type (JK9-3a) cells were grown to early logarithmic phase in 1X PD medium at 30 ºC, and the culture was split in six equal aliquots. Individual aliquots were incubated at 30 ºC in the presence of rapamycin for either 0, 15, 30, 45, 60, or 90 min. Uptake of labeled amino acids (% import/OD over time) was determined for cells of the presence of rapamycin for either 0, 15, 30, 45, 60, or 90 min. Uptake of labeled amino acids (% import/OD over time) was determined for cells of the presence of rapamycin for either 0, 15, 30, 45, 60, or 90 min. Uptake of labeled amino acids (% import/OD over time) was determined for cells of the presence of rapamycin for either 0, 15, 30, 45, 60, or 90 min. Uptake of labeled amino acids (% import/OD over time) was determined for cells of the presence of rapamycin for either 0, 15, 30, 45, 60, or 90 min.
Table II. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
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<tbody>
<tr>
<td>pTAT2</td>
<td>pAS8; 3.7-kb EcoRI fragment containing TAT2 in pSEY18 (2µ URA3)</td>
</tr>
<tr>
<td>pH-A-TAT2</td>
<td>pAS55; TAT2 promoter-HA-TAT2 in YEp195 (2µ URA3)</td>
</tr>
<tr>
<td>pH-A-TAT2</td>
<td>pAS64; TAT2 promoter-HA-TAT2 in YEp181 (2µ LEU2)</td>
</tr>
<tr>
<td>pH-A-TAT2Δ29</td>
<td>pTR287; TAT2 promoter-HA-TAT2 in YEp133 (CEN URA3)</td>
</tr>
<tr>
<td>pH-A-TAT2Δ17-31</td>
<td>pTR288; PS55 containing a deletion of codons 2 through 29 of TAT2</td>
</tr>
<tr>
<td>pH-A-TAT2Δ10</td>
<td>pTR294; PS55 containing a deletion of codons 17 through 31 of TAT2</td>
</tr>
<tr>
<td>pH-A-TAT2Δ17</td>
<td>pTR306; PS55 containing a deletion of codons 2 through 10 of TAT2</td>
</tr>
<tr>
<td>pH-A-TAT2Δ20</td>
<td>pTR307; PS55 containing a deletion of codons 2 through 17 of TAT2</td>
</tr>
<tr>
<td>pH-A-TAT2Δ20</td>
<td>pTR313; PS55 containing a deletion of codons 2 through 20 of TAT2</td>
</tr>
<tr>
<td>pH-A-TAT2Δ29</td>
<td>pTR348; PS55 containing a deletion of codons 2 through 29 of TAT2</td>
</tr>
<tr>
<td>pH-A-TAT2Δ29K31R</td>
<td>pTR373; PTB348 with a nucleotide substitution changing lysine codon 31 of TAT2 to arginine</td>
</tr>
<tr>
<td>pH-A-TAT2Δ31</td>
<td>pTR359; PS55 containing a deletion of codons 2 through 31 of TAT2</td>
</tr>
<tr>
<td>pPL230</td>
<td>pRS166::SH3-HA (Ljungdahl et al., 1992)</td>
</tr>
<tr>
<td>pPL257</td>
<td>pRS166::GAP1-HA (Ljungdahl et al., 1992)</td>
</tr>
<tr>
<td>pPL321</td>
<td>pRS166::HIP1-myc (Kuehn et al., 1996)</td>
</tr>
<tr>
<td>Yepl105</td>
<td>encodes myc-tagged ubiquitin under control of the copper-inducible CUP1 promoter (Ellison and Hochstrasser, 1991)</td>
</tr>
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</table>

**Western Analysis**

To prepare whole cell extracts for SDS-PA GE and Western analysis, cells were grown in SC medium to early logarithmic phase, resuspended in ice-cold extraction buffer (120 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM PMSF, and 1% NP-40) and lysed with glass beads in a Mini-Beadbeater (BioSpec Products). Unbroken cells and debris were removed by a 500-g spin, and protein concentrations were determined using the BioRad microassay. Samples were denatured at 37°C for 10 min. A total of 50 µg protein was loaded per lane for standard SDS-PA GE (10% acrylamide) and Western analysis (A usubel et al., 1998). For detection of tagged proteins, a rat anti-HA antibody (clone 3F10; Boehringer Mannheim) and mouse anti-myc antibody (9E10; kindly provided by H.-P. Hausr, Biozentrum, University of Basel, Basel, Switzerland) were used. For signal detection, the Amersham ECL kit was used; stripping of blots and reprobing were performed as recommended by the manufacturer.

**Indirect Immunofluorescence**

Untreated logarithmically growing cells or cells treated with rapamycin for 60 min were fixed for 2 h in the growth medium supplemented with formaldehyde (3.7% final) and potassium phosphate buffer (100 mM final, pH 6.5). Cells were washed and resuspended in sorbitol buffer (1.2 M sorbitol and 100 mM potassium phosphate, pH 6.5). Cell walls were digested for 30 min at 37°C in sorbitol buffer supplemented with β-mercaptoethanol (20 mM final) and recombinant lyticase (5 mg/ml) or zymolase 20T (12.5 mg/ml; Seigagaku Corporation), yielding identical results. Spheroplasts were fixed on poly-L-lysine-coated glass slides and permeabilized with PBT (53 mM NaH₂PO₄, 13 mM NaH₂PO₄, 75 mM NaCl, 1% BSA, and 0.1% Triton X-100). Immunofluorescence directed against the HA-epitope was performed by application of a high affinity monoclonal anti-HA antibody (clone 16B12; Boehringer Mannheim) and mouse anti-myc antibody (9E10; kindly provided by H.-P. Hausr, Biozentrum, University of Basel, Basel, Switzerland) were used. For signal detection, the Amersham ECL kit was used; stripping of blots and reprobing were performed as recommended by the manufacturer.

**Results**

*Starvation Induces Downregulation of TAT2*

We observed that amino acid prototrophic yeast strains are less sensitive to rapamycin than auxotropic strains (Beck, T., A. Schmidt, and M.N. Hall, unpublished observation). Because auxotrophic strains rely on the uptake of externally added amino acids for growth, this suggested that rapamycin inhibits amino acid import. We measured the import of radiolabeled tryptophan in cells treated with rapamycin for various times, and found a significant and early decrease in tryptophan uptake (Fig. 1A). The time-dependent decrease in tryptophan import was followed, with a short time lag, by a downregulation in the level of TAT2 protein (Fig. 1A). Tryptophan import and TAT2 protein dropped to almost undetectable levels after 60 min of rapamycin treatment.

As rapamycin causes starvation in yeast cells, the rapamycin-induced downregulation of TAT2 appeared to be a novel starvation response. To confirm that the downregulation of TAT2 is indeed a starvation response, we examined if nutrient deprivation causes a similar downregulation.
3d/pA S55) were grown in SC medium and shifted to SC medium modified in the concentration of tryptophan, ammonium (NH₄⁺), proline or glucose, as described in the text. After 60 min of incubation in the modified media, protein extracts were prepared and analyzed for HA-TAT2 by Western analysis. (C) Wild-type cells expressing HA-TAT2 (JK9-3d/pAS55), HIP1-myc (JK9-3d/pPL321), GAP1-HA (JK9-3d/pPL257), or SHR3-HA (JK9-3d/pPL250) were grown in SC medium to early logarithmic phase. Rapamycin (+) or empty drug vehicle (−) was added. After 60 min, cells were harvested and processed for Western analysis.

Starvation induces downregulation of TAT2

Rapamycin also causes a downregulation in translation initiation (Barbet et al., 1996). Because the downregulation of translation is earlier than the observed downregulation of TAT2, the decrease in TAT2 levels could reflect a loss of de novo protein synthesis in combination with a normally short half-life of the permease. We performed a pulse-chase experiment to determine whether the loss of TAT2 resulted from increased turnover or solely from reduced synthesis of the protein. Cells expressing HA-tagged TAT2 were grown to early logarithmic phase in the presence of radiolaabeled methionine, and chased with an excess of cold methionine in the presence or absence of rapamycin. Culture aliquots were taken at 15-min intervals and processed for immunoprecipitation of the permease. As shown in Fig. 2, the half-life of TAT2 in the rapamycin-treated cells was ~30 min, whereas in the rapamycin-ununtreated cells the half-life was >90 min. Thus, the TAT2 protein is significantly more stable in exponentially grow-
ing cells than in starved cells, indicating that starvation induces degradation of TAT2.

Turnover of TAT2 Requires Ubiquitination, Endocytosis, and Vacular Proteases

Degradation of TAT2 is likely to occur in a manner similar to that of other permeases such as FUR4, GAP1, MAL61, and GA L2. Upon NPI1/RSP5-dependent ubiquitination, these proteins are internalized, transported to the vacuole, and degraded by vacuolar hydrolases (Galan et al., 1996; Hicke, 1997; Horak and Wolf, 1997; Lucero and Lagunas, 1997; Springael and A ndre, 1998). To investigate the requirements of TAT2 degradation, we analyzed the steady state levels of HA–TAT2 in mutant and wild-type cells treated with rapamycin or drug vehicle alone (Fig. 3 A). In wild-type cells, TAT2 was almost completely degraded within 60 min after addition of rapamycin, as described above. In an npi1/rsp5 mutant, defective in the ubiquitin-protein ligase NPI1/RSP5 (Springael and A ndre, 1998), the amount of TAT2 protein was significantly increased in logarithmically growing cells, as compared with a wild-type strain (NPI1), and the permease was still present in rapamycin-treated cells. To confirm the involvement of ubiquitin in the rapamycin-induced degradation of TAT2, we examined another ubiquitination-deficient mutant. The doa4/npi2 mutant, defective in a ubiquitin hydrolase (J auniaux et al., 1987; Papa and Hochstrasser, 1993), has a ubiquitination deficiency (Galan and A guenauer-T sapis, 1997; Lucero and Lagunas, 1997), possibly due to a defect in replenishing the pool of free ubiquitin (Galan and A guenauer-T sapis, 1997; Singer et al., 1996). Like in npi1/rsp5 cells, TAT2 was resistant to rapamycin-induced degradation in the doa4/npi2 mutant. In an end4 mutant (Raths et al., 1993), defective for endocytosis of several plasma membrane proteins, TAT2 was weakly (∼10%) resistant to rapamycin-induced degradation. In a pep4 mutant lacking vacuolar proteases, TAT2 was completely resistant to rapamycin-induced degradation.

To further examine the requirements of TAT2 degradation, we analyzed the cellular distribution of TAT2 in the above mutants and wild-type cells treated or untreated with rapamycin (Fig. 4). Visualization of HA–TAT2 in rapamycin-unlabeled wild-type cells by immunofluorescence revealed a strong ER signal, a punctate signal reminiscent of Golgi-localized proteins, and only weak plasma membrane staining. This localization of TAT2 differs from that of the uracil permease FUR4, which is found mainly in the
plasma membrane (Volland et al., 1992), but is similar to that of GAP1 which can be found mainly in the ER and Golgi (Roberg et al., 1997b). Furthermore, the distribution of TAT2 was the same, although the signal differed in intensity, when HA-TAT2 was expressed from a single copy plasmid (pTB287) or a multicopy plasmid (pA S55 or pA S64) (data not shown). In rapamycin-treated wild-type cells, the TAT2 signal became very faint, as expected, due to degradation of the TAT2 protein, but did not change significantly in pattern (data not shown). In rapamycin-treated npi1/rsp5 cells, TAT2 accumulated in the plasma membrane and the vacuolar membrane. The TAT2 protein behaved similarly in the doa4/npi2 mutant as in the npi1/rsp5 mutant (data not shown). The accumulation of TAT2 in the vacuolar membrane of the doa4/npi2 and npi1/rsp5 mutants is possibly due to a constitutive vacuolar protease defect in these nonconditional mutants (Latterich and Watson, 1991). In rapamycin-treated end4 cells (shifted to nonpermissive temperature before drug treatment), the TAT2 signal was faint and corresponded mainly to the plasma membrane, indicating that an endocytosis defect protects plasma membrane TAT2 but not internal TAT2 from rapamycin-induced degradation (note that the exposure time for the TAT2 signal in the end4 cells in Fig. 4 was sixfold longer as compared with the exposure time for the npi1 cells). The stabilization of only the plasma membrane pool of TAT2, a small portion of the total pool, by an endocytosis defect accounts for why TAT2 was only weakly resistant to degradation in the end4 mutant (see above). In rapamycin-treated pep4 cells, TAT2 accumulated mainly in the vacuolar membrane.

The cellular distribution of TAT2 in rapamycin-untreated doa4/npi2, npi1/rsp5, and pep4 cells was the same as described above for these cells when rapamycin treated. This may reflect a basal level of TAT2 turnover in exponentially growing (rapamycin-untreated) cells. Because the doa4/npi2, npi1/rsp5, and pep4 mutations are constitutive, a defect in basal internalization and degradation of TAT2 may result in an aberrant accumulation of the permease even in growing cells.

To investigate further the involvement of ubiquitination in TAT2 degradation, we examined if TAT2 itself is ubiquitinated. The permease was immunoprecipitated from rapamycin-treated cells expressing HA-TAT2 alone or coex-

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**Figure 4.** Cellular distribution of HA-TAT2 by indirect immunofluorescence. Wild-type (wt, JK9-3d) and npi1 (27038a) and pep4 (MH 684) mutant strains transformed with pH A-TAT2 (pA S55 or, in the case of pep4, pA S64) were grown in SC medium at 30°C to early logarithmic phase. The shown wild-type cells were not treated with rapamycin. All other cells shown were treated with rapamycin for 60 min. The conditional end4 mutant (RH 1597/pA S55) was grown at 24°C and shifted to 37°C for 5 min before the addition of rapamycin. Cells were fixed and processed for detection of HA-TAT2 by immunofluorescence (see Materials and Methods). The cellular distribution of HA-TAT2 is shown in the left column (TAT2). The same field of cells is shown in the middle (Normarski) and right columns (DNA). The right column shows the cells stained with the DNA specific stain DAPI. The endoplasmic reticulum corresponds to the area encircling the DAPI-staining nucleus (see arrowheads in wt cells). The vacuole appears as a large crater in cells visualized by Nomarski optics (see arrowheads in npi1 and pep4 cells). The exposure time to visualize HA-TAT2 in end4 cells was approximately sixfold longer than for other strains shown.
pressing HA-TAT2 and myc-tagged ubiquitin (myc-Ub) (Ellison and Hochstrasser, 1991). Probing with an anti-HA antibody revealed the signal for TAT2 (∼50 kD) in both immunoprecipitates, whereas probing with an anti-myc antibody revealed only >50 kD proteins solely in the immunoprecipitate derived from cells coexpressing myc-Ub (Fig. 3 B). Neither signal was detected in immunoprecipitates from cells expressing an untagged version of TAT2. This suggests that TAT2 is ubiquitinated. Taken together, the above results indicate that TAT2 degradation involves ubiquitination, vacuolar proteases, and, in part, endocytosis.

The NH2 Terminus of TAT2 Is Required for Ubiquitination and Degradation

Ligand-induced ubiquitination and internalization of the pheromone receptor STE2 involves the sequence SINDAKSS within the cytosolic tail of the receptor (Hicke et al., 1998). Replacement of the aspartic acid to alanine or the lysine residue to an arginine in the DAKS core of this motif impairs the internalization of a truncated form of the receptor, whereas mutation of the aspartic acid to glutamic acid, or substitution of the first serine by an alanine, did not interfere with endocytosis. According to a structure prediction by the TOP PRED algorithm, both the NH2-terminal and COOH-terminal sequences of TAT2 face the cytoplasm. In the extreme NH2 terminus of TAT2, two ExKS motifs, similar to the DAKS core of the STE2 SINDAKSS motif, are present (Fig. 5 A, amino acids 18–21 and 27–30).

To determine whether the NH2 terminus of TAT2 is required for the degradation of the permease, we expressed several NH2-terminal deletion variants of the permease, and assayed their stability in rapamycin-treated cells (Fig. 5 B). Whereas deletion of up to amino acid 29 (TAT2 D29) had no effect, deletion of the NH2-terminal 31 amino acids (TAT2 D31) completely stabilized the protein (Fig. 5 A). To further characterize the sequence required for degradation, a smaller deletion that completely removed the two ExKS motifs within the NH2-terminal 31 amino acids was constructed (TAT2 D17–31, Fig. 5 A). However, this deletion did not result in a degradation-resistant variant of TAT2, suggesting that the ExKS motifs are not essential for TAT2 degradation.

Ubiquitin is attached to lysine residues in target proteins. There are five lysine residues in the NH2-terminal 31 amino acids of TAT2, and the above analysis suggests that all five lysines must be deleted to prevent TAT2 degradation. To determine if one or more of the lysines is required for degradation, possibly as an acceptor for ubiquitin, all five lysines were changed to arginines. Single lysine mutants and several combinations, including a combination of all five substitutions, were examined for stability upon rapamycin treatment (Fig. 5 A for TAT2 D29K31R, and data not shown). Stabilization of TAT2 was observed only when all five lysine residues were exchanged for arginines. Furthermore, the TAT2 D29K31R protein was approximately threefold more abundant (in rapamycin-treated and untreated cells) than wild-type TAT2 in rapamycin-untreated cells (data not shown). This increase in the level of TAT2 D29K31R was similar to the increase of wild-type TAT2 caused by a defect in the ubiquitination machinery, as described above, and may again reflect a loss of basal turnover of the permease. Immunofluorescence on rapamycin-treated and untreated cells revealed a pronounced accumulation of TAT2 D29K31R in the plasma membrane, with no detectable signal elsewhere in the cell (Fig. 5 B, and data not shown). A defective wild-type TAT2 in ubiquitination mutants, the similar pattern for rapamycin-treated and untreated cells may reflect an aberrantly high accumulation of the stabilized permease in the plasma membrane even in growing cells. The TAT2 D29K31R and TAT2 D31L proteins behaved identically to TAT2 D29K31R with regard to both abundance and localization (data not shown). Finally, probing immunoprecipitated TAT2 D29K31R for ubiquitin failed to reveal any ubiquitinated forms of TAT2 D29K31R (Fig. 3 B). Taken together, these results suggest that at least one lysine residue within the NH2-terminal 31 amino acids of TAT2 is required for degradation, possibly as an acceptor for ubiquitin.
Upon Starvation, TAT2 Is Sorted from Internal Compartments to the Vacuole Independently of the Plasma Membrane

If internal TAT2 is transported to the vacuole via the plasma membrane, TAT2 should be completely stabilized in an end4 mutant, due to a block in plasma membrane internalization. However, results presented above indicate that the large intracellular pool of TAT2 is degraded in an end4 mutant, suggesting that internal TAT2 is sorted to the vacuole independently of the plasma membrane. To investigate if TAT2 is targeted from the ER and the Golgi to the vacuole without passing through the plasma membrane, we examined the stability of TAT2 in sec4, sec18, and sec23 mutants. SEC4 (Goud et al., 1988) is a small GTPase required for fusion of Golgi-derived vesicles with the plasma membrane. SEC23 (Hicke and Schekman, 1989) is a subunit of the COPII complex required for ER to Golgi transport. SEC18 (Eakle et al., 1988) is the yeast NSF homologue and is generally required for fusion of vesicles with acceptor membranes (reviewed in Kaiser et al., 1997). The amounts of TAT2 in extracts of rapamycin-treated wild-type cells and the sec mutants were compared by Western analysis (Fig. 6). In the sec4 mutant, TAT2 was degraded like in wild-type cells. In contrast, TAT2 was completely stable in the sec18 mutant and mostly stable in the sec23 mutant. Taken together, the above results suggest that TAT2 is targeted from the ER and the Golgi to the vacuole independently of the plasma membrane. Thus, upon starvation, TAT2 appears to be diverted from the secretory pathway to the vacuolar pathway.

Recently, TOR has been implicated in the control of autophagy, a starvation-induced process in which bulk cytoplasm, including organelles, is membrane enclosed and transported to the vacuole (Noda and Ohsumi, 1998). Since the effect of rapamycin on TAT2 is a starvation response, and rapamycin exerts its effects by inhibiting the TOR proteins, we asked whether the degradation of TAT2 is an autophagic event. TAT2 stability was examined in an apg1 mutant defective for autophagy (Matsuura et al., 1997). In the presence of rapamycin, HA-TAT2 was completely turned over in the apg1 strain, indicating that autophagy is not required for the degradation of TAT2 in response to starvation (Fig. 6).

Because starvation-induced degradation of intracellular TAT2 does not rely on passage through the cell surface or on autophagy, a likely transport route to the vacuole is via the PEP12-, VPS45-, and VPS27-dependent pathway (Cowles et al., 1994; Piper et al., 1994, 1995; Becherer et al., 1996). In this VPS pathway, proteins are delivered from the Golgi to the vacuole via a prevacuolar/endosomal compartment. If TAT2 is transported to the vacuole via the VPS pathway, the permease should be stable in a rapamycin-treated mutant defective in this pathway. Indeed, TAT2 was still present in extracts of rapamycin-treated pep12, vps27, and vps45 cells (Fig. 6, data not shown). Taken together, the above results suggest that upon nutrient depletion, TAT2 is transported from the ER and Golgi to the vacuole via the VPS pathway, without passing through the cell surface.

Mutation of the five NH2-terminal lysine residues in TAT2 results in an apparently complete stabilization of the permease (see Fig. 5 A), suggesting that the intracellular pool of TAT2, in addition to the plasma membrane pool (see above), requires ubiquitination for rapamycin-induced targeting to the vacuole and degradation. To investigate whether the NH2-terminal lysine residues are required for degradation of the internal pool of TAT2, temperature-sensitive sec4 cells expressing either HA-TAT2 or HA-TAT25K-R were pulse labeled for 3 min at permissive temperature, shifted to nonpermissive temperature, and chased in the presence of rapamycin (see Materials and Methods). This experiment was designed to investigate the fate of newly made intracellular TAT2 and TAT25K-R proteins that are unable to reach the plasma membrane due to the sec4 mutation. Whereas newly made HA-TAT2 was rapidly degraded in rapamycin-treated cells, HA-TAT25K-R was stable over a 90-min chase period (Fig. 7), suggesting that the NH2-terminal lysine residues of TAT2 and ubiquitination are also required for rapamycin-induced degradation of the internal pool of TAT2.

Discussion

We have shown that the tryptophan permease TAT2, a constitutive amino acid permease, is indeed regulated, at
Figure 8. Model for inverse regulation of specific (TAT2) and broad-range (GAP1) amino acid permeases by TOR in response to nutrients. (A) In the presence of nutrients, active TOR mediates the sorting of TAT2 to the plasma membrane and the routing of GAP1 to the vacuole for degradation. (B) Upon nutrient deprivation, TOR is inactive and TAT2 is targeted to the vacuole for degradation whereas GAP1 is routed to the plasma membrane. TOR is associated with the plasma membrane.

The level of protein sorting and stability. In starved cells, rapamycin-treated or nutrient-deprived, TAT2 is ubiquitinated, targeted from the plasma membrane, the ER, and the Golgi to the vacuole, and then degraded. The internal TAT2 is routed to the vacuole independently of the plasma membrane. The regulation of the specific amino acid permease TAT2 is inverse to that of the broad-range permease GAP1. GAP1 is routed to the vacuole and degraded in nonstarved cells (Roberg et al., 1997a; Springael and A ndré, 1998). A rapamycin inhibits the TOR proteins, and induces both the downregulation of TAT2 and the upregulation of GAP1. Thus, TOR and presumably the TOR nutrient-signaling pathway mediate the inversely regulated sorting and stability of the two types of permeases. A model summarizing our findings is shown in Fig. 8.

In growing cells, TAT2 follows the secretory pathway to the plasma membrane. In starved cells, TAT2 is targeted to the vacuole. How is this regulated sorting of TAT2 controlled? Ubiquitination is important in targeting TAT2 from the plasma membrane to the vacuole, as TAT2 accumulates in the plasma membrane in ubiquitination mutants. Furthermore, mutant TAT2 (TAT2K>R) altered in five NH2-terminal lysine residues required for ubiquitination accumulates in the plasma membrane in wild-type cells. Ubiquitination may also be required for diverting intracellular TAT2 to the vacuole, as newly made intracellular TAT2K>R is not degraded upon rapamycin treatment (Fig. 7). Jenness et al. (1997) have suggested that ubiquitination is required for delivery of a mutant STE2 pheromone receptor from an intracellular site to the vacuole. The mechanism by which ubiquitination may divert the internal pool of TAT2 from the secretory pathway to the vacuolar pathway upon nutrient deprivation remains to be determined. The alternative routing of GAP1 to the cell surface or the vacuole appears to be controlled at the level of GAP1 packaging into specialized vesicles leaving the Golgi (Roberg et al., 1997b).

TAT2, and at least the histidine permease HIP1, are turned over upon starvation. This is a novel aspect of the starvation response in yeast cells. Why are specific permeases turned over upon starvation? The specific permease TAT2 and the broad-range permease GAP1 are regulated inversely, at least in response to the quality and quantity of the nitrogen source. There are several specific permeases but only two known broad-range permeases, GAP1 and A GP1, in yeast (G renson et al., 1970; Jauniaux and Grenson, 1990; N elissen et al., 1997; S chreve et al., 1998). We suggest that specific amino acid permeases are expressed and functionally maintained under nutrient-rich conditions, and are probably fine-regulated by the availability of their substrates. Once nutrients become limiting, cells may express and maintain only a couple broad-range permeases, instead of the several specific permeases, as a means to reduce energy consumption. The inverse regulation of specific and broad-range permeases provides a mechanism to optimize import with regard to the quality and quantity of nutrients.

Upon rapamycin treatment or upon shift from a good nitrogen source (ammonium) to a poor nitrogen source (proline), GAP1 is stabilized and TAT2 is degraded. How is this inverse regulation of TAT2 and GAP1 achieved? To date, two posttranslational regulators of GAP1 are known, N P11 and N P R1. N P11, a ubiquitin ligase, presumably ubiquitinates GAP1 and thereby triggers the internalization and degradation of the permease (Springael and A ndré, 1998). N P R1 is a Ser/T hr protein kinase, a positive regulator of GAP1 (V andenbol et al., 1987). The mechanism by which N P R1 activates GAP1 is unknown, but the finding that GAP1 is phosphorylated when active suggests that NPR1 directly phosphorylates GAP1 and thereby protects it from N P11-dependent degradation (Stanbrough and Magasanik, 1995). N P11 is also responsible for inactivation of TAT2. Thus, we consider an inverse regulation via this ubiquitin ligase unlikely. However, the inverse regulation may be achieved through NPR1. Indeed, we have found that NPR1 mediates the destruction of TAT2 under conditions when the kinase protects GAP1 (Schmidt et al., 1998). Furthermore, we have shown that N P R1 is controlled by TOR and the TOR downstream effector T A P42 (Schmidt et al., 1998). Because TAT2 is regulated in response to nitrogen, carbon and amino acid availability, it is of interest to determine whether GAP1 is also regulated in response to nutrients other than nitrogen. Furthermore, it remains to be determined how the TOR nutrient–signaling pathway senses the availability of nutrients.

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