Starvation Induces Vacuolar Targeting and Degradation of the Tryptophan Permease in Yeast

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Abstract. In Saccharomyces cerevisiae, amino acid permeases are divided into two classes. One class, represented by the general amino acid permease GA P1, 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 GA P1.

Key words: GA P1 • 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 (Sophianopoulos and Diallinas, 1995). Permeases of one class, including the general amino acid permease, GA P1, and the proline permease, PUT4, are regulated in response to the available nitrogen source (Jauliaux et al., 1987; Jauliaux and Grenson, 1990; Vandenberg 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 GA P1 is complex, occurring at the levels of GA P1 transcription and GA P1 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 GA P1, it has recently been shown that transport from the Golgi to the plasma membrane is not a default process. Depending on the nitrogen source, GA P1 is delivered from the Golgi to either the vacuole or the plasma membrane. In the presence of a good nitrogen source, GA P1 is delivered to the vacuole and degraded, whereas, in the presence of a poor nitrogen source, GA P1 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; Springael and Andere, 1998). The NP1/RSP5 ubiquitin-protein ligase is required for GA P1 ubiquitination. NP1/RSP5 was originally identified as the nitrogenase repressor for GA P1 (Hiein et al., 1995; Juniaux et al., 1987). A further internalization, GA P1 is transported to the vacuole for degradation.

The immunosuppressant rapamycin induces a starvation state in yeast cells, stationary phase or G0, by binding and inhibiting target of rapamycin (TOR) 1 and TOR 2 (Hietman et al., 1991; Stan et al., 1994; Zheng et al., 1995; Barket et al., 1996). The TOR proteins, two highly homologous phosphatidylinositol kinase-related kinases (Kunz et al., 1993; Helliwell et al., 1994), normally control cell growth by signaling activation of translation initiation and early G 1 progression in response to nutrients (Barbet et al., 1996; Di Como and Andreadis, 1996; Berset et al., 1998). TOR 2 also activates a Rho-type GTPase switch and thereby controls the cell cycle-dependent organization of the actin cytoskeleton (Schmidt et al., 1996, 1997; Bickle et al., 1998). However, this TOR 2 unique function is not rapamycin sensitive (Zheng et al., 1995; Barbet et al., 1996).

Here we show that the constitutive permease TAT 2 is indeed regulated. Inversely to GA P1, TAT 2 is targeted to the vacuole and degraded upon nutrient deprivation. Surprisingly, an internal pool of TAT 2 appears to be routed directly to the vacuole independently of the plasma membrane. Furthermore, the regulated sorting of TAT 2 is controlled by the TOR proteins.

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 described (Sherman, 1991). Yeast transformation was performed by the lithium acetate procedure (Ito et al., 1983).

DNA Manipulations

Restriction enzyme digestes and ligations were done by standard methods. Enzymes and buffers were obtained commercially (Boehringer Mannheim). DNA was sequenced by the dyeoxy-chain termination method with the T7 sequencing system (Pharmacia).

Construction of HA-tagged and Mutant TAT 2

HA-TAT 2 (in pA S55, pA S64, or pTB 287) encodes an NH 2-terminally HA-tagged, fully functional TAT 2 protein under control of its own pro-moter. HA-TAT 2 was constructed by ligating a 0.65-kb PstI-XbaI PCR product, containing the TAT 2 promoter, 5′ untranslated region, initiation codon, and double HA tag, to a 2.3-kb XbaI-EcoRI PCR product containing the TAT 2 open reading frame and 3′ noncoding region. The PstI and EcoRI sites were natural sites flanking the TAT 2 gene. The PCR primers used to generate the 0.65-kb fragment were 5′-CGCTACATGATGCAATTCGCGGACGTCATAGGGATAGCCCGCATAGTCAGGAACAAATGAAGCTG-3′ (XbaI site in italics) and the 3′-CCATGGAGCGAAAGATCAGCTG-3′ (XbaI site in italics) and the –20 primer (New England Biolabs). To create deletion variants, the following primers were used in combination with the –20 primer (New England Biolabs) to generate alternative 2.3-kb fragments: 5′-GGTCTCGAAGATGCTGCCGCTGAAATCAGGACGGCTCG-3′ (TAT 2Δ210), 5′-GGTCTAGATGACGGCTCGGATAAGCAGGATACGGTGCCATG-3′ (TAT 2Δ171), 5′-GGTCTAGAAGATGCTGCCGCTGAAATCAGGACGGCTCG-3′ (TAT 2Δ240), 5′-GGTCTAGAAGATGCTGCCGCTGAAATCAGGACGGCTCG-3′ (TAT 2Δ299). A t 2 deletion (TAT 2Δ171–31) was constructed in a two-step PCR with the complementary primers 5′-CTAGTCATGATGCAATTCGCGGACGTCATAGGGATAGCCCGCATAGTCAGGAACAAATGAAGCTG-3′ (antisense) and 5′-GGTCTAGAAGATGCTGCCGCTGAAATCAGGACGGCTCG-3′ (sense), each in combination with a flanking primer and pTB 287 as the template. A silent nucleotide substitution that created a SacI site (italics) is indicated in bold, and point mutations that changed lysine codons to arginine codons are underlined. V ent DNA Polymerase (New England Biolabs) or Taq polymerase (Boehringer Mannheim) was used in PCR. Mutations, deletions, and introduction of the HA tag-encoding sequence in TAT 2 were verified by sequencing.

Amino Acid Import Studies

The import rates of radiolabeled amino acids (L-[5,5-3H]tryptophan (33 Ci/mmol), L-[4,5,5-3H]leucine (58 Ci/mmol), and L-[2,5,5-3H]-histidine (42 Ci/mmol; Amersham) were measured as described (Hietman et al., 1993), with the following modifications. Wild-type (K9-3a) cells were grown to early logarithmic phase in YPD medium at 30°C, and the culture was split into six equal aliquots. Individual aliquots were incubated at 30°C 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 each aliquot. For the graph in Fig. 1 A the 10-min timepoint of each curve was plotted over the rapamycin preincubation time of the given aliquot.

Table I. Strains

| Strain | Genotype |
|--------|----------|
| J K9-3D | MATα his4 leu2 ura3 trp1 mel1HMLa |
| MH684  | JK9-3da pep4::URA3 |
| 2375c  | MATα ade2 |
| 27083a | MATα npi1 ura3 |
| 27002d | MATα dsh4 his2 ura3 |
| RH1552 | MATα sec4-1 his4 lys2 ura3 |
| RSY255 | MATα leu2 ura3 |
| RSY271 | MATα sec18-1 his4 ura3 |
| RSY281 | MATα sec23-1 his4 ura3 |
| MT16-9B| MATα apg1-1 leu2 ura3 |
| RH1602 | MATα his4 leu2 ura3 bar1 |
| RH1597 | MATα end4-1 his4 leu2 ura3 bar1 |
| RH2378 | MATα VPS27 his4 leu2 ura3 |
| RH2379 | MATα vps27 his4 leu2 ura3 |
| SEY6210| MATα PEP12 his3 leu2 ura3 trp1 lys2 suc2 |
| CBY9/5C| MATα pep12 ade2 his2 leu2 ura3 trp1 lys2 suc2 |
**Western Analysis**

To prepare whole cell extracts for SDS-PAGE 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 PMSE, and 1% NP-40) and lysed with glass beads in a Mini Beadbeater (Bio-Spec 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-PAGE (10% acrylamide) and Western analysis (A usubel et al., 1998). For detection of tagged proteins, a rat anti-HA antibody (clone 3F10; Boehminger Mannheim) and mouse anti-myc antibody (9E10; kindly provided by H.-P. Hauri, 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 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 NaHPO4, 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 12CA5; Boehringer Mannheim) and mouse anti-myc antibody (9E10; kindly provided by H.-P. Hauri, Biozentrum, University of Basel, Basel, Switzerland) were used. For signal detection, the A mersham 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 auxotrophic 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), followed by a 500-fold decrease in cold methionine import (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), followed by a 500-fold decrease in cold methionine import.

**Cell Labeling and Immunoprecipitation**

Logarithmically growing wild-type cells (J K 9-3d) expressing HA-TAT2 were diluted in 400 ml SC medium (starting OD660 = 0.1) containing 2 mM of L-[35S]methionine (E as tagg prot e in labeling system; NE). A fier 4 h (OD660 = 0.3), a 1,000-fold excess of cold methionine was added. The culture was split, and rapamycin or empty drug vehicle was added. Temperature-sensitive sec4 cells (R H 1952) grown at 24°C in early logarithmic phase and expressing HA-TAT2 or HA-TAT25·K were chilled on ice, harvested by centrifugation, and immediately resuspended at an OD660 = 5 in 12 ml of SC medium containing 2 mCi of L-[35S]methionine instead of cold methionine. Cells were incubated for 3 min at 24°C, then shifted to 37°C for an additional 5 min. The cultures were chilled on ice, harvested, and resuspended at an OD660 = 0.5 in prewarmed (37°C) SC medium containing cold methionine and rapamycin (200 ng/ml). Culture aliquots equal to 15 OD660 of cells were removed after 0, 30, 60, and 90 min, and kept on ice for 10 min in the presence of 10 mM NaN3 and 10 mM NaF. Cells were harvested and cell extracts prepared as described above. Extracts were precleared for 30 min with Sepharose CL-4B, and incubated for 2 h at 4°C with protein G immobilized on Sepharose CL-4B (Sigma) and monoclonal anti-HA antibodies (12CA5 or 16B12; Babco), in a volume of 1 ml. The beads were collected and washed extensively with extraction buffer. Bound protein was solubilized by incubating in SDS sample buffer (A usubel et al., 1998) for 10 min at 37°C. Gels were scanned and signals quantified using a PhosphorImager (Molecular Dynamics) and ImageQuant software. Ubiquitinated TAT2 was visualized in Western blots by use of monoclonal anti-myc antibody (clone 9E10). For this purpose, myc-tagged ubiquitin was expressed as described (Ellison and Hochstrasser, 1991), in wild-type cells (J K 9-3d) also expressing HA-TAT2, HA-TAT25·K, or untagged TAT2. The permease was immunoprecipitated from extracts of these cells (from a total of 1 mg of protein) as described above.

**Table II. Plasmids**

| Plasmid | Description |
|---------|-------------|
| pTAT2   | pAS8; 3.7-kb EcoRI fragment containing TAT2 in pSEY18 (2μ URA3) |
| pHA-TAT2| pAS55; TAT2 promoter-HA-TAT2 in YEpplac185 (2μ URA3) |
|         | pAS64; TAT2 promoter-HA-TAT2 in YEpplac181 (2μ LEU2) |
| pHA-TAT2Δ29 | pTR287; TAT2 promoter-HA-TAT2 in YEpplac33 (CEN URA3) |
| pHA-TAT2Δ17·31 | pTR289; pAS55 containing a deletion of codons 17 through 31 of TAT2 |
| pHA-TAT2Δ10 | pTR306; pAS55 containing a deletion of codons 2 through 10 of TAT2 |
| pHA-TAT2Δ17 | pTR307; pAS55 containing a deletion of codons 2 through 17 of TAT2 |
| pHA-TAT2Δ20 | pTR313; pAS55 containing a deletion of codons 2 through 20 of TAT2 |
| pHA-TAT2Δ29 | pTR348; pAS55 containing a deletion of codons 2 through 29 of TAT2 |
| pHA-TAT2Δ29·KISR | pTR373; pTB348 with a nucleotide substitution changing lysine codon 31 of TAT2 to arginine |
| pHA-TAT2Δ31 | pTR359; pAS55 containing a deletion of codons 2 through 31 of TAT2 |
| pHA-TAT2Δ3·K-R | pTR355; pAS55 with nucleotide substitutions changing lysine codons 10, 17, 20, 29, and 31 of TAT2 to arginine codons |
| pPL230 | pRS316::SHRT·HA (Ljungdahl et al., 1992) |
| pPL257 | pRS316::GAPI·HA (Ljungdahl et al., 1992) |
| pPL321 | pRS316::HIP1·myc (Kuehn et al., 1996) |
| YEp105 | encodes myc-tagged ubiquitin under control of the copper-inducible CUP1 promoter (Ellison and Hochstrasser, 1991) |
tion. Wild-type cells grown to early logarithmic phase in medium containing glucose, ammonium, and required amino acids (SC medium) were harvested by filtration and resuspended in normal SC medium and in nutrient-modified SC medium. TAT2 protein levels were then examined after 60 min of incubation in the new media. First, the medium was modified by increasing, decreasing, or removing tryptophan. Whereas increasing (20×; 800 μg/ml) and decreasing (0.1×; 4 μg/ml) the tryptophan concentration had little or no effect, tryptophan starvation resulted in an almost complete loss of TAT2 protein (Fig. 1 B, upper panel). Second, cells were shifted to medium modified in the nitrogen source, i.e., medium containing a reduced amount of ammonium (0.1×; 1 mM), no ammonium, or proline instead of ammonium. A similar effect was observed in Fig. 1 B (middle panel), nitrogen limitation correlated with a decrease in the level of TAT2 protein, up to a complete loss of the permease in ammonium-free and proline media. In contrast to TAT2, GAP1 is activated upon shift from ammonium to the poor nitrogen source proline, and inactivated (degraded) upon shift from proline to ammonium (Magasanik, 1992; Springael and André, 1998), suggesting that TAT2 and GAP1 are invertedly regulated in response to the nitrogen source. Third, cells were shifted to media with reduced (0.1×; 0.2%) or no glucose. The levels of TAT2 protein again correlated with nutrient availability, dropping to undetectable levels in the absence of glucose (Fig. 1 B, lower panel). Thus, the downregulation of TAT2 protein is a novel starvation response, caused by either rapamycin treatment or amino acid, nitrogen or carbon deprivation.

To determine whether the nutrient regulation observed for TAT2 also occurs for other high affinity constitutive permeases, we measured histidine import in rapamycin-treated cells. The effect of rapamycin on histidine uptake was similar to that observed for tryptophan uptake (not shown). We also examined the abundance of myc-tagged histidine permease HIP1 (Tanaka and Fink, 1985; Kuhn et al., 1996) in rapamycin-treated cells. Like TAT2, HIP1 almost completely disappeared within 60 min of rapamycin treatment (Fig. 1 C). Thus, starvation-induced downregulation is not unique to TAT2, but also applies to another, and possibly to all, so-called constitutive amino acid permeases. The starvation program in yeast may involve a global downregulation of high specificity permeases.

In contrast to TAT2 and HIP1, rapamycin treatment caused a significant increase in GAP1 protein, even in the presence of nutrients (Fig. 1 C). The abundance of SHR3, an ER-resident amino acid permease chaperone (Ljungdahl et al., 1992), was not significantly affected by rapamycin treatment (Fig. 1 C). Thus, the rapamycin-sensitive TOR proteins appear to regulate inversely the high specificity permeases, such as TAT2 and HIP1, and the broad specificity permease GAP1, in response to nutrient availability.

**Starvation Induces Turnover 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 radiolabeled 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. A similar effect was observed in Fig. 2, the half-life of TAT2 in the rapamycin-treated cells was ~30 min, whereas in the rapamycin-untreated cells the half-life was >90 min. Thus, the TAT2 protein is significantly more stable in exponentially grow-

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**Figure 1. Starvation induces downregulation of TAT2.**

(A) Wild-type cells (JK 9-3d) treated with rapamycin for different times were assayed for tryptophan uptake (open circles), as described in Materials and Methods. Wild-type cells expressing HA-TAT2 (JK 9-3d/pAS55) and treated with rapamycin for different times were assayed for the level of HA-TAT2 protein (TAT2, closed circles), by Western analysis as described in Materials and Methods. (B) Wild-type cells expressing HA-TAT2 (JK 9-3d/pAS55) 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.
ing cells than in starved cells, indicating that starvation induces degradation of TAT2.

**Turnover of TAT2 Requires Ubiquitination, Endocytosis, and Vacuolar 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 Andre, 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. 3A). 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 Andre, 1998), the amount of TAT2 protein was significantly increased in logistically 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 (Jouniaux et al., 1987; Papa and Hochstrasser, 1993), has a ubiquitination deficiency (Galan and Haguenaert-Tsapis, 1997; Lucero and Lagunas, 1997), possibly due to a defect in replenishing the pool of free ubiquitin (Galan and Haguenaert-Tsapis, 1997; Singer et al., 1996). Likewise 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-treated wild-type cells 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 

and 

pep4 

cells, the 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-

The Journal of Cell Biology, Volume 146, 1999 1232
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 NH$_2$ Terminal of TAT2 Is Required for Ubiquitination and Degradation**

Ligand-induced ubiquitination and internalization of the pheromone receptor STE2 involves the sequence SINDA KSS 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. A according to a structure prediction by the TOP PRED algorithm, both the NH$_2$-terminal and COOH-terminal sequences of TAT2 face the cytoplasm. In the extreme NH$_2$ terminus of TAT2, two ExKS motifs, similar to the DxKS core of the STE2 SINDA KSS motif, are present (Fig. 5 A, amino acids 18–21 and 27–30).

To determine whether the NH$_2$ terminus of TAT2 is required for the degradation of the permease, we expressed several NH$_2$-terminal deletion variants of the permease, and assayed their stability in rapamycin-treated cells (Fig. 5 A). Deletion of up to amino acid 29 (TAT2Δ29) had no effect, deletion of the NH$_2$-terminal 31 amino acids (TAT2Δ31) 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 NH$_2$-terminal 31 amino acids was constructed (TAT2Δ17-31, 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 NH$_2$-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 TAT2Δ29, and data not shown). Stabilization of TAT2 was observed only when all five lysine residues were exchanged for arginines. Furthermore, the TAT2Δ29K31R 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 TAT2Δ29K31R 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 TAT2Δ29K31R in the plasma membrane, with no detectable signal elsewhere in the cell (Fig. 5 B, and data not shown). A similar pattern for rapamycin-treated and -untreated cells may reflect an abnormally high accumulation of the stabilized permease in the plasma membrane even in growing cells. The TAT2Δ29K31R and TAT2Δ31 proteins behaved identically to TAT2Δ29K31R, with regard to both abundance and localization (data not shown). Finally, probing immunoprecipitated TAT2Δ29K31R for ubiquitin failed to reveal any ubiquitinated forms of TAT2Δ29K31R (Fig. 3 B). Taken together, these results suggest that at least one lysine residue within the NH$_2$-terminal 31 amino acids is required for TAT2 degradation.
Acids of TAT2 is necessary for ubiquitination and degradation of at least the plasma membrane pool of the permease.

**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 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 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 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

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### Figure 6.

Upon starvation, TAT2 is sorted from internal compartments to the vacuole independently of the plasma membrane. A parental wild-type strain (RSY255), and sec4 (RH1552), sec18 (RSY271), sec23 (RSY281), apg1 (MT16-9B), pep12 (CBY9/5C), and vps27 (RH2379) mutants expressing HA-TAT2 (PA555) were grown at 24°C to early logarithmic phase and shifted to 37°C for 3 min (10 min for the pep12 mutant). Rapamycin was added to half of each culture. A further 60 min of further incubation, cells were harvested and processed for Western analysis.

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### Figure 7.

NH2-terminal lysine residues of TAT2 are required for rapamycin-induced degradation of newly made TAT2. Temperature-sensitive sec4 mutant cells expressing HA-TAT2 (PA555) or HA-TAT25K (pTB355) were grown in SC medium at 24°C to early logarithmic phase, labeled with 35S-methionine for 3 min and shifted to 37°C for 5 min. Labeled cells were harvested and chased at 37°C in SC medium containing 200 ng/ml of rapamycin. A liquid was taken at the indicated times of chase, and cell extracts were prepared. HA-TAT2 or HA-TAT25K was immunoprecipitated, eluted from the washed protein G-Sepharose beads at 37°C, subjected to SDS-PAGE, and visualized by fluorography (for details, see Materials and Methods).
Beck et al. TOR Controls Amino Acid Permeases

TOR Controls Amino Acid Permeases

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 mechanism by which ubiquitination may divert the internalization of a mutant STE2 pheromone receptor from an intracellular site to the vacuole. The 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 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 targeted to the vacuole and degraded in nonstarved cells (Roberg et al., 1997a; Springael and Andre, 1998). A rapamycin kinase protects GAP1 (Springael and Andre, 1998). NPI1, a ubiquitin ligase, presumably ubiquitinates GAP1 and thereby triggers the internalization and degradation of the permease (Springael and Andre, 1998). NPI1 is also responsible for inactivation of GAP1 upon starvation. We consider an inverse regulation 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, NPR1 and NPI1. NPR1, a ubiquitin ligase, presumably ubiquitinates GAP1 and thereby triggers the internalization and degradation of the permease (Springael and Andre, 1998). NPR1, a Ser/Thr protein kinase, is a positive regulator of GAP1 (Vandenbol et al., 1987). The mechanism by which NPR1 activates GAP1 is unknown, but the finding that GAP1 is phosphorylated when active suggests that NPR1 directly phosphorylates GAP1 and thereby protects it from NPI1-dependent degradation (Stanbrough and Magasanik, 1995). NPR1 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 NPR1 is controlled by TOR and the TOR downstream effector TA42 (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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References

A usubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1995. Current Protocols in Molecular Biology. John Wiley & Sons, Inc. New York.

Barbet, N.C., U. Schneider, S.B. Hellwille, K. Stansfield, M.F. Tuite, and M.N. Hall. 1996. TOR1 controls translation initiation and early G1 progression in yeast. Mol. Biol. Cell. 7:725–42.

Beccher, K.A., S.E. Rieder, S.D. Emr, and E.W. Jones. 1996. Novel syntaxin homologue, Pep2p, required for the sorting of luminal hydrolases to the lysosome-like vacuole in yeast. Mol. Biol. Cell. 7:579–594.

Berset, C., H. Trachsel, and M. Altmann. 1998. The TOR (target of rapamycin) signal transduction pathway regulates the stability of translation initiation factor eIF4G in the yeast Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA. 95:4264–4269.

Bickle, M., P.A. Delley, A. Schmidt, and M.N. Hall. 1998. Cell wall integrity modulates RHO1 activity via the exchange factor ROM2. EMBO J. 17:2225–2235.

Coults, C.R., S.D. Emr, and F.B. Horoszky. 1994. Mutations in the SEC1 homologue, required for the sorting of lumenal hydrolases to the lysosome-like vacuole in yeast. Mol. Biol. Cell. 7:725–42.

Di Como, C.J., and K.T. Arndt. 1996. Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. J. Biol. Chem. 271:643–650.

Elakie, K.A., M. Bernstein, and S.D. Emr. 1988. Characterization of a component of the yeast secretion machinery: identification of the SEC18 gene product. Mol. Cell. Biol. 8:4098–4109.

Ellison, M.J., and M. Hochstrasser. 1991. Epitope-tagged ubiquitin. A new probe for analyzing ubiquitin function. J. Biol. Chem. 266:21150–21157.

Galán, J.M., V. Moreau, B. Andre, C. Volland, and R. Haguenauer-Tsapis. 1997. Ubiquitin is involved in ubiquitination of a yeast plasma membrane protein. EMBO (Euro. Mol. Biol. Organ.) J. 16:5847-5854.

Galán, J.M., V. Moreau, B. Andre, C. Volland, and R. Haguenauer-Tsapis. 1997. Ubiquitination mediated by the Npi1p/Syp ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. J. Biol. Chem. 271:10946–10951.

Goud, B.A., A. Salminen, N.C. Walworth, and P.J. Novick. 1988. A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. J. Cell Biol. 103:753–786.

Gerson, M., C. Hou, and M. Crabbe. 1970. M. ulceris of the amino acid permeases in Saccharomyces cerevisiae. IV. Evidence for a general amino acid permease. J. Biol. Chem. 245:10227–10235.

Hein, C.J., S.Y. Springael, C. Volland, R. Haguenauer-Tsapis, and B. Andre. 1995. NPL1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Mol. Microbiol. 17:297–305.

Heitel, J., N.R. Moova, and M.N. Hall. 1991. Targets for cell cycle arrest by TOR2, a nutrient-sensitive vacuolar mutants of Saccharomyces cerevisiae. Mol. Microbiol. 5:2417–2426.

Ljungdahl, P.O., C.J. Gimeno, C.A. Styles, and G.R. Fink. 1992. SHR3: a novel homologue of the human tre-2 oncogene. J. Cell Biol. 117:1215–1226.

Lucero, P., R. Llagunes. 1995. Catabolite inactivation of the yeast maltose transporter requires ubiquitin-ligase Npi1p and ubiquitin-hydrolase Npi2p. FEMS Microbiol. Lett. 147:273–277.

Maganaskin, B. 1992. Regulation of nitrogen utilization. In The Molecular and Cellular Biology of the Yeast Saccharomyces cerevisiae. Vol. 2. Gene Expression. E.W. Jones, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 263–317.

Matsaura, A., M. Sutakuda, Y. Wada, and Y. Osuhm. 1997. A gol1p, a novel protein kinase required for the autophagic process in Saccharomyces cerevisiae. Gene. 192:245–250.

Nelissen, B., R. De Wachter, and A. Goffeau. 1997. Classification of all putative permeases and other membrane plasmid transporters of the yeast Saccharomyces cerevisiae. J. Bacteriol. 179:1541–1549.

Piper, R.C., W. Whitters, and T.H. Stevens. 1996. Yeast vac1p is a Sec1-like protein required for the consumption of vacuole-targeted, post-Golgi transport vesicles. Eur. J. Cell Biol. 65:305–318.

Piper, V.G., R.A. Cooper, H. Yang, and T.H. Stevens. 1995. Vps57 controls vacuolar and endocytic traffic through a prevacuolar compartment in Saccharomyces cerevisiae. J. Cell Biol. 131:603–617.

Raths, S., J. Rohrer, F. Crauss, and H. Riezman. 1993. end1 and end2: two mutants defective in receptor-mediated and fluid-phase endocytosis in Saccharomyces cerevisiae. J. Cell Biol. 120:55–65.

Roberg, K.J., N. Rowley, and C.A. Kaiser. 1997a. Control of amino acid permease sorting in the late secretory pathway of Saccharomyces cerevisiae. Mol. Gen. Genet. 253:587–768.

Schmidt, A., T. Beck, A. Koller, J. Kunz, and M.N. Hall. 1997. The yeast phosphatidylinositol kinase homolog required for G1 progression. EMBO J. 16:4597–4606.

Schmidt, A., M.N. Hall, and A. Koller. 1994. Two FK 506 resistance-confering genes in Saccharomyces cerevisiae, TAT1 and TAT2, encode amino acid permeases mediating tyrosine and tryptophan uptake. Mol. Cell. Biol. 14:6597–6606.

Schmidt, A., J.K. Kunz, and M.N. Hall. 1996. TOR1 is required for organization of the actin cytoskeleton in yeast. Proc. Natl. Acad. Sci. USA. 93:13780–13785.

Schmidt, A., M. Bickle, T. Beck, and M.N. Hall. 1997. The yeast phosphotyrosine-specific phosphatase gene doa4 activates R Ho1 and R Ho2 via the exchange factor ROM2. EMBO J. 16:531–542.

Schmidt, A., T. Beck, A. Koller, J. Kunz, and M.N. Hall. 1998. The TOR nutrient-signal pathway mobilizes the phosphatidylinositol kinase Yps1p to the plasma membrane. J. Biol. Chem. 273:2694–2698.
Springael, J.Y., and B. Andre. 1998. Nitrogen-regulated ubiquitination of the Gap1 permease of Saccharomyces cerevisiae. Mol. Biol. Cell. 9:1253–1263.

Stan, R., M.M. McLoughlin, R. Cafferkey, R.K. Johnson, M. Rosenberg, and G.P. Livi. 1994. Interaction between FKBP12–rapamycin and TOR involves a conserved serine residue. J. Biol. Chem. 269:32027–32030.

Stanbrough, M., and B. Magasanik. 1995. Transcriptional and posttranslational regulation of the general amino acid permease of Saccharomyces cerevisiae. J. Bacteriol. 177:94–102.

Tanaka, J., and G.R. Fink. 1985. The histidine permease gene (HIP1) of Saccharomyces cerevisiae. Gene 38:205–214.

Vandenbol, M., J.C. Jauniaux, S. Vissers, and M. Grenson. 1987. Isolation of the NPR1 gene responsible for the reactivation of ammonia-sensitive amino acid permeases in Saccharomyces cerevisiae. RNA analysis and gene dosage effects. Eur. J. Biochem. 164:607–612.

Vandenbol, M., J.C. Jauniaux, and M. Grenson. 1990. The Saccharomyces cerevisiae NPR1 gene required for the activity of ammonia-sensitive amino acid permeases encodes a protein kinase homologue. Mol. Gen. Genet. 222:393–399.

Zheng, X.F., D. Florentino, J. Chen, G.R. Crabtree, and S.L. Schreiber. 1995. TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin. Cell. 82:121–130.