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 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,[1] 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 routed to the vacuole and degraded, whereas, in the presence of a poor nitrogen source, GAP1 is targeted to the plasma membrane (Roberg et al., 1997a,b).

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1. *Abbreviations used in this paper*: COP, coatomer protein; GAP, general amino acid permease; HIP, histidine permease; PUT, proline permease; TAT, tryptophan permease; TOR, target of rapamycin.

The GAP1 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 Andre, 1998). The NPI1/RSP5 ubiquitin-protein ligase is required for GAP1 ubiquitination. NPI1/RSP5 was originally identified as the nitrogen permease inactivator for GAP1 (Hein et al., 1995; Jauniaux et al., 1987). After internalization, GAP1 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 TOR2 (Heitman et al., 1991; Stan et al., 1994; Zheng et al., 1995; Barbet 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 G1 progression in response to nutrients (Barbet et al., 1996; Di Como and Arndt, 1996; Berset et al., 1998). TOR2 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 TOR2 unique function is not rapamycin sensitive (Zheng et al., 1995; Schmidt et al., 1996).

Here we show that the constitutive permease TAT2 is indeed regulated. Inversely to GAP1, TAT2 is targeted to the vacuole and degraded upon nutrient deprivation. Surprisingly, an internal pool of TAT2 appears to be routed directly to the vacuole independently of the plasma membrane. Furthermore, the regulated sorting of TAT2 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 as described (Sherman, 1991) with the following modifications. Tryptophan was added to 40 μg/ml, leucine to 100 μg/ml, and glutamic acid, aspartic acid, and serine were omitted. Ammonium and proline media, or media without ammonium or proline as a nitrogen source, were prepared using yeast nitrogen base without ammonium sulfate and amino acids supplemented with 2% glucose, 40 μg/ml tryptophan, 20 μg/ml histidine, 100 μg/ml leucine, and either 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 in drug vehicle (90% ethanol and 10% Tween-20) and added to liquid media to a final concentration of 200 ng/ml.

Table I. Strains

Strain	Genotype
JK9-3D	*MAT***a** *his4 leu2 ura3 trp1 rme1HMLa*
MH684	JK9-3d**a** *pep4::URA3*
23344c	*MAT*α ura3
27038a	*MAT***a** *npi1 ura3*
27002d	*MAT***a** *doa4/npi2 ura3*
RH1552	*MAT*α *sec4-1 his4 lys2 ura3*
RSY255	*MAT*α *leu2 ura3*
RSY271	*MAT*α *sec18-1 his4 ura3*
RSY281	*MAT*α *sec23-1 his4 ura3*
MT16-9B	*MAT***a** *apg1-1 leu2 ura3*
RH1602	*MAT***a** *his4 leu2 ura3 bar1*
RH1597	*MAT***a** *end4-1 his4 leu2 ura3 bar1*
RH2378	*MAT*α *VPS27 his4 leu2 ura3*
RH2379	*MAT*α *vps27^{ts} his4 leu2 ura3*
SEY6210	*MAT*α *PEP12 his3 leu2 ura3 trp1 lys2 suc2*
CBY9/5C	*MAT***a** *pep12^{ts} ade2 his3 leu2 ura3 trp1 lys2 suc2*

Genetic Techniques

Yeast transformation was performed by the lithium acetate procedure (Ito et al., 1983). *Escherichia coli* strain DH5α was used for propagation and isolation of plasmids as described (Ausubel et al., 1998).

DNA Manipulations

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

Construction of HA-tagged and Mutant TAT2

HA-TAT2 (in pAS55, pAS64, or pTB287) encodes an NH_2-terminally HA-tagged, fully functional TAT2 protein under control of its own promoter. *HA-TAT2* was constructed by ligating a 0.65-kb PstI-XbaI PCR product, containing the *TAT2* promoter, 5′ untranslated region, initiation codon, and double HA tag, to a 2.3-kb XbaI-EcoRI PCR product containing the *TAT2* open reading frame and 3′ noncoding region. The PstI and EcoRI sites were natural sites flanking the *TAT2* gene. The PCR primers used to generate the 0.65-kb fragment were 5′-CG*TCTAGA*TGCATA-GTCCGGGACGTCATAGGGATAGCCCGCATAGTCAGGAACA-TCGTATGGGTA**CAT**ATGAGAGTGTGTTGCGTAATTTG-3′ (XbaI site in italics, antisense 2×HA open reading frame underlined and antisense initiation codon in bold) and the 1233 primer (New England Biolabs). The primers used to generate the 2.3-kb fragment were 5′-CG*TCTAGA*ACCGAAGACTTTATTTCTTCTGTC-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′-GG*TCTAGA*ATGCGTTCAAATGAGGAGCTG-3′ (*TAT2Δ10*), 5′-GG*TCTAGA*ATGGAGCGAAAATCTAACTTTGG-3′ (*TAT2Δ17*), 5′-CG*TCTAGA*ATGTCTAACTTTGGATTTGTAG-3′ (*TAT2Δ20*), 5′-CG*TCTAGA*TCCAAGCAATTAACATCATC-3′ (*TAT2Δ29*), 5′-CG*TCTAGA*TCCAGGCAATTAACATCATC-3′ (*TAT2Δ29^{K31R}*) and 5′-GG*TCTAGA*CAATTAATATCATCCTCATC-3′ (*TAT2Δ31*). An internal deletion (*TAT2Δ17-31*) was constructed in a two-step PCR with the complementary primers 5′-TCAAATGAGGAGCTGCAATTAACAT-CATCC-3′ and 5′-GGATGATGTTAATTGCAGCTCCTCATTTGA-3′, each in combination with a flanking primer and pTB287 as the template. *TAT2$^{5K>R}$* was reconstituted from two PCR fragments generated with primers 5′-GC*GAGCTC*CTCATTTGAACGCCTGACAGAAGA-3′ (antisense) and 5′-GAG*GAGCTC*AGGGAGCGAAGATCTAA-CTTTGGATTTGTAGAATACAGATCCAGGCAATTAACATCAT-CCTCATC-3′ (sense), each in combination with a flanking primer and pTB287 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. Vent 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 *TAT2* were verified by sequencing.

Amino Acid Import Studies

The import rates of radiolabeled amino acids (L-[5-^{3}H]-tryptophan (33 Ci/mmol), L-[4,5-^{3}H]-leucine (58 Ci/mmol), and L-[2,5-^{3}H]-histidine (42 Ci/mmol; Amersham) were measured as described (Heitman et al., 1993), with the following modifications. Wild-type (JK9-3d) 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 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 YEplac195 (2μ *URA3*)
	pAS64; *TAT2* promoter-*HA-TAT2* in YEplac181 (2μ *LEU2*)
	pTB287; *TAT2* promoter-HA-TAT2 in YCplac33 (*CEN URA3*)
pHA-TAT2Δ29	pTB288; pAS55 containing a deletion of codons 2 through 29 of *TAT2*
pHA-TAT2Δ17-31	pTB294; pAS55 containing a deletion of codons 17 through 31 of *TAT2*
pHA-TAT2Δ10	pTB306; pAS55 containing a deletion of codons 2 through 10 of *TAT2*
pHA-TAT2Δ17	pTB307; pAS55 containing a deletion of codons 2 through 17 of *TAT2*
pHA-TAT2Δ20	pTB313; pAS55 containing a deletion of codons 2 through 20 of *TAT2*
pHA-TAT2Δ29	pTB348; pAS55 containing a deletion of codons 2 through 29 of *TAT2*
pHA-TAT2Δ29^{K31R}	pTB373; pTB348 with a nucleotide substitution changing lysine codon 31 of *TAT2* to arginine
pHA-TAT2Δ31	pTB359; pAS55 containing a deletion of codons 2 through 31 of *TAT2*
pHA-TAT2Δ$^{5K>R}$	pTB355; pAS55 with nucleotide substitutions changing lysine codons 10, 17, 20, 29, and 31 of *TAT2* to arginine codons
pPL230	pRS316::*SHR3-HA* (Ljungdahl et al., 1992)
pPL257	pRS316::*GAP1-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)

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 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-PAGE (10% acrylamide) and Western analysis (Ausubel 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. 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 (1.2 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 zymolyase 20T (12.5 mg/ml; Seigagaku Corporation), yielding identical results. Spheroblasts were fixed on poly-L-lysine–coated glass slides and permeabilized with PBT (53 mM Na_2HPO_4, 13 mM NaH_2PO_4, 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; Babco) at a dilution of 1:1,000 in PBT for 2 h, and subsequently of a Cy3-conjugated rabbit anti–mouse IgG (Molecular Probes), diluted 1:1,000 in PBT, for 60 min. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) at a concentration of 1 μg/ml. Cells were visualized with a Zeiss Axiophot microscope (100× objective) and a video imaging system (MWG Biotech).

Cell Labeling and Immunoprecipitation

Logarithmically growing wild-type cells (JK9-3d) expressing HA-TAT2 were diluted in 400 ml SC medium (starting OD_{600} = 0.1) containing 2 mCi of L-[^{35}S]methionine (Easytag protein labeling system; NEN). After 4 h (OD_{600} = 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 (RH1552) grown at 24°C to early logarithmic phase and expressing HA-TAT2 or HA-TAT25$^{K>R}$ were chilled on ice, harvested by centrifugation, and immediately resuspended at an OD_{600} = 5 in 12 ml of SC medium containing 2 mCi of L-[^{35}S]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 OD_{600} = 0.5 in prewarmed (37°C) SC medium containing cold methionine and rapamycin (200 ng/ml). Culture aliquots equal to 15 OD_{600} of cells were removed after 0, 30, 60, and 90 min, and kept on ice for 10 min in the presence of 10 mM NaN_3 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 (Ausubel 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 (JK9-3d) also expressing HA-TAT2, HA-TAT25$^{K>R}$, or untagged TAT2. The permease was immunoprecipitated from extracts of these cells (from a total of 1 mg of protein) as described above.

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. 1 A). The time-dependent decrease in tryptophan import was followed, with a short time lag, by a downregulation in the level of TAT2 protein (Fig. 1 A). 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 downregula-

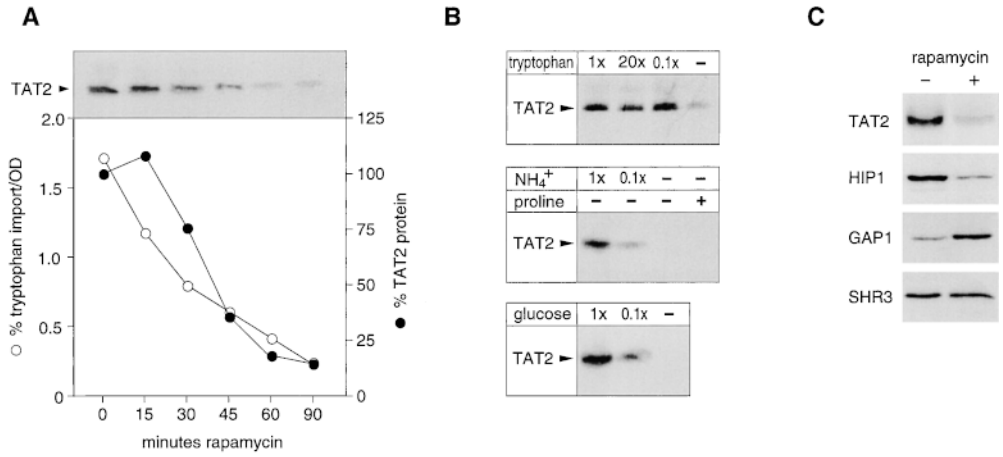

Figure 1. Starvation induces downregulation of TAT2. (A) Wild-type cells (JK9-3d) treated with rapamycin for different times were assayed for tryptophan uptake (open circles), as described in Materials and Methods. Wild-type cells expressing HA-TAT2 (JK9-3d/pAS55) and treated with rapamycin for different 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 (JK9-3d/pAS55) were grown in SC medium and shifted to SC medium modified in the concentration of tryptophan, ammonium (NH_4^+), 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/pPL230) 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.

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. As shown 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 Andre, 1998), suggesting that TAT2 and GAP1 are inversely 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; Kuehn 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. As shown 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-

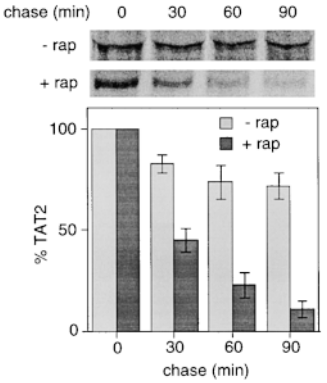

Figure 2. Starvation induces turnover of TAT2. Logarithmically growing wild-type cells expressing HA-TAT2 (JK9-3d/pAS55) were labeled with ^{35}S-methionine. A chase was performed by adding an excess of cold methionine, either alone or in combination with rapamycin (200 ng/ml). Aliquots of the untreated and rapamycin-treated cultures were removed at the indicated times of chase, and cell extracts were prepared. HA-TAT2 was immunoprecipitated from these extracts, eluted from the washed protein G–Sepharose beads at 37°C, subjected to SDS-PAGE, and visualized by fluorography (see Materials and Methods). Bars represent the average value from two independent experiments.

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 GAL2. 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. 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 Andre, 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 (Jauniaux et al., 1987; Papa and Hochstrasser, 1993), has a ubiqitination deficiency (Galan and Haguenauer-Tsapis, 1997; Lucero and Lagunas, 1997), possibly due to a defect in replenishing the pool of free ubiquitin (Galan and Haguenauer-Tsapis, 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

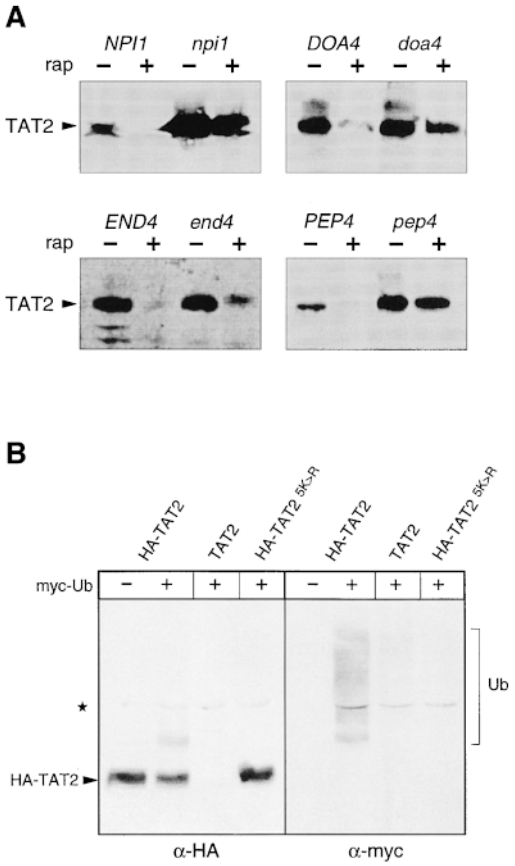

Figure 3. Turnover of TAT2 requires ubiquitination, endocytosis, and vacuolar proteases. (A) Parental wild-type strains (*NPI1*, 23344c; *DOA4*, 23344c; *PEP4*, JK9-3d) and *npi1* (27038a), *doa4* (27002d), and *pep4* (MH684) mutants expressing HA-TAT2 (from pAS55 or, in the case of *PEP4* and *pep4*, from pAS64), were grown in SC medium at 30°C to early logarithmic phase. Rapamycin (rap) or empty drug vehicle was added. The parental wild-type *END4* strain (RH1602) and the conditional *end4* mutant (RH1597) were grown at 24°C and shifted to 37°C for 5 min before the addition of rapamycin or empty drug vehicle. After 60 min in the presence of rapamycin, cells were harvested and processed for Western analysis of HA-TAT2 (TAT2). (B) Wild-type cells (JK9-3d) expressing either HA-TAT2 (pAS55), untagged TAT2 (pAS8), or HA-TAT2$^{5K>R}$ (pTB355), and containing a plasmid for copper-inducible expression of myc-tagged ubiquitin (YEp105) were grown in SC medium. Expression of myc-ub was not induced (−) or induced (+) with copper sulfate (100 μM) for 3 h. Before harvesting, cells were treated for 15 min with rapamycin (200 ng/ml). Cells were collected and processed for immunoprecipitation with anti-HA (from 1 mg of total protein, see Materials and Methods). Immunoprecipitated protein was subjected to Western analysis. The blot was probed with anti-HA antibody (α-HA), stripped and reprobed with anti-myc antibody (α-myc). The asterisk denotes a nonspecific band.

above mutants and wild-type cells treated or untreated with rapamycin (Fig. 4). Visualization of HA-TAT2 in rapamycin-untreated 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

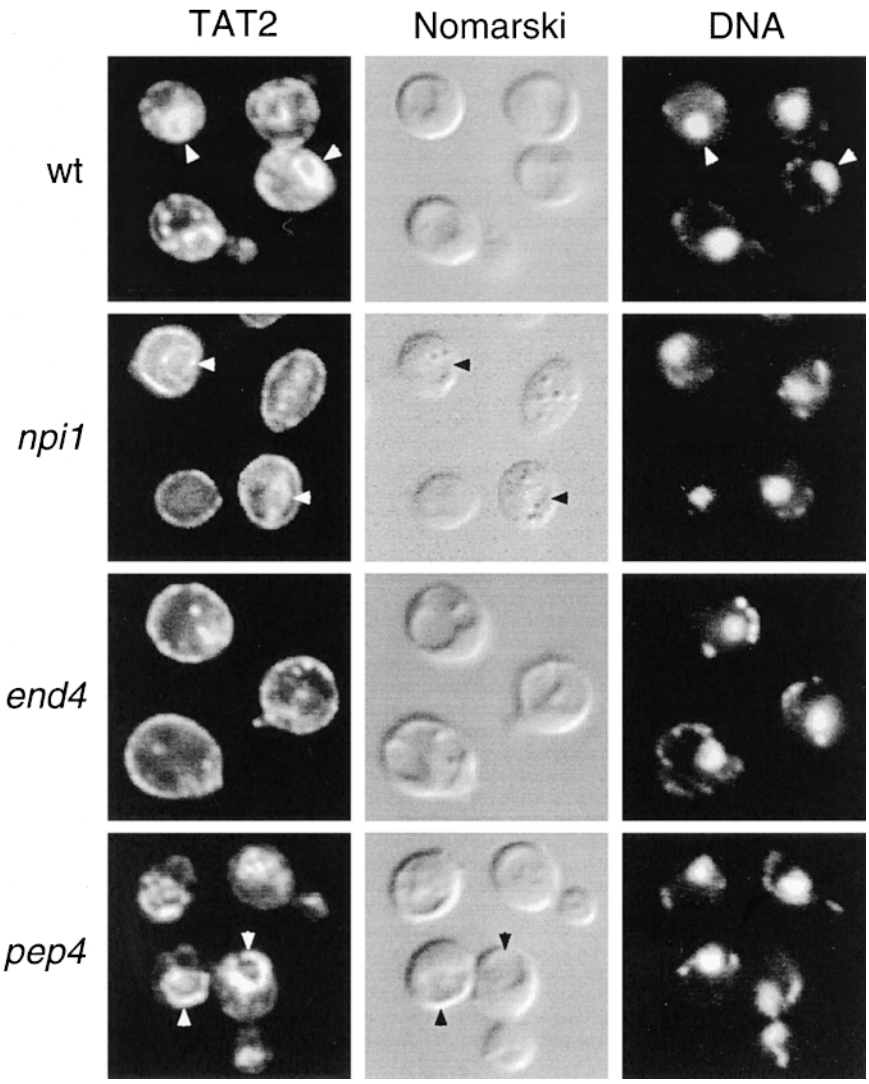

Figure 4. Cellular distribution of HA-TAT2 by indirect immunofluorescence. Wild-type (wt, JK9-3d) and *npi1* (27038a) and *pep4* (MH684) mutant strains transformed with pHA-TAT2 (pAS55 or, in the case of *pep4*, pAS64) were grown in SC medium at 30°C to early logarithmic phase. The shown wild-type cells were not treated with rapamycin. All other cells shown were treated with rapamcyin for 60 min. The conditional *end4* mutant (RH1597/pAS55) 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.

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 (pAS55 or pAS64) (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-

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 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 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 SINDAKSS 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). Whereas 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 $TAT2^{5K>R}$, and data not shown). Stabilization of TAT2 was observed only when all five lysine residues were exchanged for arginines. Furthermore, the $TAT2^{5K>R}$ 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 $TAT2^{5K>R}$ 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 $TAT2^{5K>R}$ in the plasma membrane, with no detectable signal elsewhere in the cell (Fig. 5 B, and data not shown). As observed for 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 $TAT2\Delta29^{K31R}$ and TAT2Δ31 proteins behaved identically to $TAT2^{5K>R}$ with regard to both abundance and localization (data not shown). Finally, probing immunoprecipitated $TAT2^{5K>R}$ for ubiquitin failed to reveal any ubiquitinated forms of $TAT2^{5K>R}$ (Fig. 3 B). Taken together, these results suggest that at least one lysine residue within the NH_2-terminal 31 amino

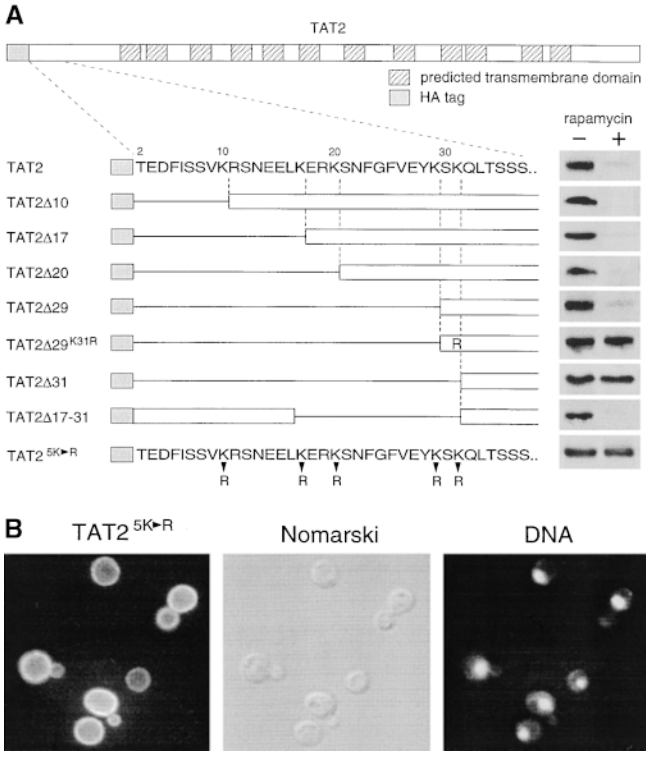

Figure 5. The NH_2 terminus of TAT2 is required for degradation. (A) Cells expressing HA-TAT2 containing NH_2-terminal mutations were grown to early logarithmic phase in SC medium (JK9-3d containing either pHA-TAT2Δ10, pHA-TAT2Δ17, pHA-TAT2Δ20, pHA-TAT2Δ29, pHA-$TAT2\Delta29^{K31R}$, pHA-TAT2Δ31, pHA-TAT2Δ17-31, or pHA-$TAT2^{5K>R}$). Rapamycin was added to one half of each culture. After 60 min of further incubation, extracts were prepared and assayed for HA-TAT2 by Western analysis. The levels of the mutant forms of HA-TAT2 are not normalized to the levels of wild-type HA-TAT2 (due to different exposure times), and thus an increased abundance for the stabilized proteins, as described in the text, is not reflected in these data. The numbering of amino acids refers to untagged TAT2. (B) $TAT2^{5K>R}$ accumulates in the plasma membrane. Wild-type cells (JK9-3d) expressing HA-$TAT2^{5K>R}$ were grown in SC medium to early logarithmic phase, treated with rapamycin for 60 min, and processed for visualization of HA-$TAT2^{5K>R}$ by immunofluorescence (see Fig. 4 legend).

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

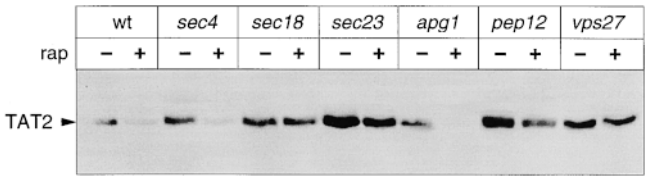

Figure 6. Upon starvation, TAT2 is sorted from internal compartments to the vacuole independently of the plasma membrane. A parental wild-type strain (RSY255), and *sec4* (RH1552), *sec18* (RSY271), *sec23* (RSY281), *apg1* (MT16-9B), *pep12* (CBY9/5C), and *vps27* (RH2379) mutants expressing HA-TAT2 (pAS55) were grown at 24°C to early logarithmic phase and shifted to 37°C for 5 min (10 min for the pep12 mutant). Rapamycin was added to half of each culture. After 60 min of further incubation, cells were harvested and processed for Western analysis.

to the vacuole via the VPS pathway, without passing through the cell surface.

Mutation of the five NH_2-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 NH_2-terminal lysine residues are required for degradation of the internal pool of TAT2, temperature-sensitive *sec4* cells expressing either HA-TAT2 or HA-TAT2$^{5K>R}$ 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 TAT2$^{5K>R}$ 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-TAT2$^{5K>R}$ was stable over a 90-min chase period (Fig. 7), suggesting that the NH_2-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

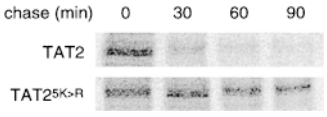

Figure 7. NH_2-terminal lysine residues of TAT2 are required for rapamycin-induced degradation of newly made TAT2. Temperature-sensitive *sec4* mutant cells expressing HA-TAT2 (pAS55) or HA-TAT2$^{5K>R}$ (pTB355) were grown in SC medium at 24°C to early logarithmic phase, labeled with ^{35}S-methionine 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. Aliquots were taken at the indicated times of chase, and cell extracts were prepared. HA-TAT2 or HA-TAT2$^{5K>R}$ 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).

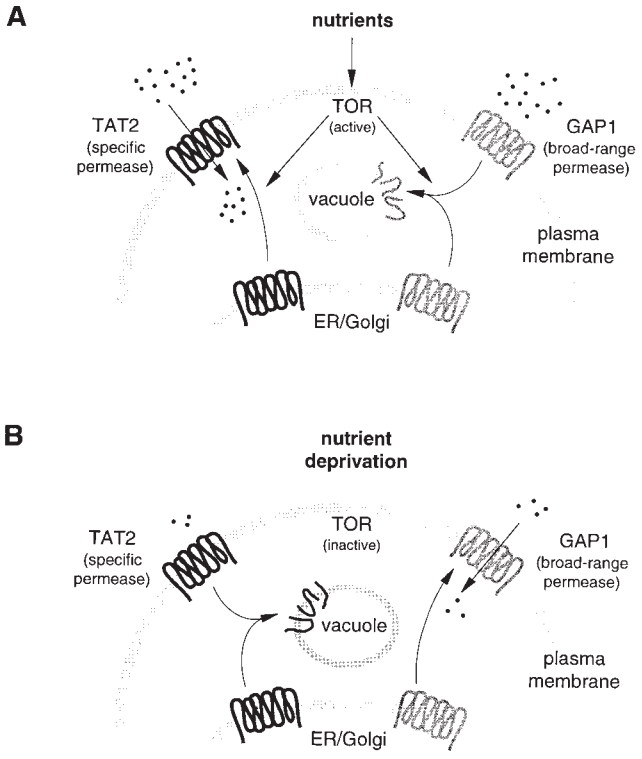

Figure 8. Model for inverse regulation of specific (TAT2) and broad-range (GAP1) amino acid permeases by TOR in response to nutrients. (A) In the presence of nutrients, active TOR mediates the sorting of TAT2 to the plasma membrane and the routing of GAP1 to the vacuole for degradation. (B) Upon nutrient 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 Andre, 1998). 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 ($TAT2^{5K>R}$) altered in five NH_2-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 $TAT2^{5K>R}$ 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 AGP1, in yeast (Grenson et al., 1970; Jauniaux and Grenson, 1990; Nelissen et al., 1997; Schreve 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, NPI1 and NPR1. NPI1, 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). NPI1 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 TAP42 (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

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1998. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York.

Barbet, N.C., U. Schneider, S.B. Helliwell, I. Stansfield, M.F. Tuite, and M.N. Hall. 1996. TOR controls translation initiation and early G1 progression in yeast. *Mol. Biol. Cell.* 7:25–42.

Becherer, K.A., S.E. Rieder, S.D. Emr, and E.W. Jones. 1996. Novel syntaxin homologue, Pep12p, required for the sorting of lumenal 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: 2235–2245.

Cowles, C.R., S.D. Emr, and B.F. Horazdovsky. 1994. Mutations in the *VPS45* gene, a *SEC1* homologue, result in vacuolar protein sorting defects and accumulation of membrane vesicles. *J. Cell Sci.* 107:3449–3459.

Di Como, C.J., and K.T. Arndt. 1996. Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. *Genes Dev.* 10:1904–1916.

Didion, T., B. Regenberg, M.U. Jorgensen, M.C. Kielland-Brandt, and H.A. Andersen. 1998. The permease homologue Ssy1p controls the expression of amino acid and peptide transporter genes in *Saccharomyces cerevisiae*. *Mol. Microbiol.* 27:643–650.

Eakle, 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.

Galan, J., and R. Haguenauer-Tsapis. 1997. Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:5847–5854.

Galan, J.M., V. Moreau, B. Andre, C. Volland, and R. Haguenauer-Tsapis. 1996. Ubiquitination mediated by the Npi1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. *J. Biol. Chem.* 271: 10946–10952.

Goud, B., 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. *Cell.* 53:753–768.

Grenson, M., C. Hou, and M. Crabeel. 1970. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. IV. Evidence for a general amino acid permease. *J. Bacteriol.* 103:770–777.

Hein, C., J.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.* 18:77–87.

Heitman, J., N.R. Movva, and M.N. Hall. 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science.* 253:905–909.

Heitman, J., A. Koller, J. Kunz, R. Henriquez, A. Schmidt, N.R. Movva, and M.N. Hall. 1993. The immunosuppressant FK506 inhibits amino acid import in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 13:5010–5019.

Helliwell, S.B., P. Wagner, J. Kunz, M. Deuter-Reinhard, R. Henriquez, and M.N. Hall. 1994. TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol. Biol. Cell.* 5:105–118.

Hicke, L. 1997. Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. *FASEB J.* 11:1215–1226.

Hicke, L., and R. Schekman. 1989. Yeast Sec23p acts in the cytoplasm to promote protein transport from the endoplasmic reticulum to the Golgi complex in vivo and in vitro. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1677–1684.

Hicke, L., and H. Riezman. 1996. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell.* 84:277–287.

Hicke, L., B. Zanolari, and H. Riezman. 1998. Cytoplasmic tail phosphorylation of the alpha-factor receptor is required for its ubiquitination and internalization. *J. Cell Biol.* 141:349–358.

Horak, J., and D.H. Wolf. 1997. Catabolite inactivation of the galactose transporter in the yeast *Saccharomyces cerevisiae*: ubiquitination, endocytosis, and degradation in the vacuole. *J. Bacteriol.* 179:1541–1549.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163–168.

Jauniaux, J.C., and M. Grenson. 1990. *GAP1*, the general amino acid permease gene of *Saccharomyces cerevisiae*. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. *Eur. J. Biochem.* 190:39–44.

Jauniaux, J.C., M. Vandenbol, S. Vissers, K. Broman, and M. Grenson. 1987. Nitrogen catabolite regulation of proline permease in *Saccharomyces cerevisiae*. Cloning of the PUT4 gene and study of PUT4 RNA levels in wild-type and mutant strains. *Eur. J. Biochem.* 164:601–606.

Jenness, D.D., Y. Li, C. Tipper, and P. Spatrick. 1997. Elimination of defective α-factor pheromone receptor. *Mol. Cell. Biol.* 17:6236–6245.

Kaiser, C.A., R.E. Gimeno, and D.A. Shaywitz. 1997. Protein Secretion, membrane biogenesis, and endocytosis. *In* The Molecular and Cellular Biology of the Yeast *Saccharomyces cerevisiae*. Vol. 3. J.R. Pringle, J.R. Broach, and E.W. Jones, editors. Cold Spring Harbor Laboratory Press, NY. 91–227.

Kuehn, M.J., R. Schekman, and P.O. Ljungdahl. 1996. Amino acid permeases require COPII components and the ER resident membrane protein Shr3p for packaging into transport vesicles in vitro. *J. Cell Biol.* 135:585–595.

Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N.R. Movva, and M.N. Hall. 1993. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell.* 73:585–596.

Latterich, M., and M.D. Watson. 1991. Isolation and characterization of osmosensitive 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 component of the secretory pathway specifically required for localization of amino acid permeases in yeast. *Cell.* 71:463–478.

Lucero, P., and R. Lagunas. 1997. Catabolite inactivation of the yeast maltose transporter requires ubiquitin-ligase npi1/rsp5 and ubiquitin-hydrolase npi2/doa4. *FEMS Microbiol. Lett.* 147:273–277.

Magasanik, B. 1992. Regulation of nitrogen utilization. *In* The Molecular and Cellular Biology of the Yeast *Saccharomyces cerevisiae*. Vol. 2. Gene Expression. E.W. Jones, J.R. Pringle, and J.R. Broach, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 283–317.

Matsuura, A., M. Tsukada, Y. Wada, and Y. Ohsumi. 1997. Apg1p, 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 plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 21:113–134.

Noda, T., and Y. Ohsumi. 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem.* 273:3963–3966.

Papa, F.R., and M. Hochstrasser. 1993. The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene. *Nature.* 366:313–319.

Piper, R.C., E.A. Whitters, and T.H. Stevens. 1994. Yeast Vps45p is a Sec1p-like protein required for the consumption of vacuole-targeted, post-Golgi transport vesicles. *Eur. J. Cell Biol.* 65:305–318.

Piper, R.C., A.A. Cooper, H. Yang, and T.H. Stevens. 1995. VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in *Saccharomyces cerevisiae*. *J. Cell Biol.* 131:603–617.

Raths, S., J. Rohrer, F. Crausaz, and H. Riezman. 1993. *end3* and *end4*: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*. *J. Cell Biol.* 120:55–65.

Roberg, K.J., S. Bickel, N. Rowley, and C.A. Kaiser. 1997a. Control of amino acid permease sorting in the late secretory pathway of *Saccharomyces cerevisiae* by SEC13, LST4, LST7, and LST8. *Genetics.* 147:1569–1584.

Roberg, K.J., N. Rowley, and C.A. Kaiser. 1997b. Physiological regulation of membrane protein sorting late in the secretory pathway of *Saccharomyces cerevisiae*. *J. Cell Biol.* 137:1469–1482.

Schmidt, A., M.N. Hall, and A. Koller. 1994. Two FK506 resistance-conferring 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. Kunz, and M.N. Hall. 1996. TOR2 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 phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell.* 88:531–542.

Schmidt, A., T. Beck, A. Koller, J. Kunz, and M.N. Hall. 1998. The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:6924–6931.

Schreve, J.L., J.K. Sin, and J.M. Garrett. 1998. The *Saccharomyces cerevisiae YCC5* (*YCL025c*) gene encodes an amino acid permease, Agp1, which transports asparagine and glutamine. *J. Bacteriol.* 180:2556–2559.

Sherman, F. 1991. Getting started with yeast. *In* Guide to Yeast Genetics and Molecular Biology. Vol. 194. C. Guthrie and G.R. Fink, editors. Academic Press, San Diego. 3–21.

Singer, J.D., B.M. Manning, and T. Formosa. 1996. Coordinating DNA replication to produce one copy of the genome requires genes that act in ubiquitin metabolism. *Mol. Cell. Biol.* 16:1356–1366.

Sophianopoulou, V., and G. Diallinas. 1995. Amino acid transporters of lower eukaryotes: regulation, structure and topogenesis. *FEMS Microbiol. Rev.* 16: 53–75.

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. McLaughlin, 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.