Direct Sorting of the Yeast Uracil Permease to the Endosomal System Is Controlled by Uracil Binding and Rsp5p-dependent Ubiquitylation

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The yeast uracil permease, Fur4p, is downregulated by uracil, which is toxic to cells with high permease activity. Uracil promotes cell surface Rsp5p-dependent ubiquitylation of the permease, signaling its endocytosis and further vacuolar degradation. We show here that uracil also triggers the direct routing of its cognate permease from the Golgi apparatus to the endosomal system for degradation, without passage via the plasma membrane. This early sorting was not observed for a variant permease with a much lower affinity for uracil, suggesting that uracil binding is the signal for the diverted pathway. The FUI1-encoded uridine permease is similarly sorted for early vacuolar degradation in cells exposed to a toxic level of uridine uptake. Membrane proteins destined for vacuolar degradation require sorting at the endosome level to the intraluminal vesicles of the multivesicular bodies. In cells with low levels of Rsp5p, Fur4p can be still diverted from the Golgi apparatus but does not reach the vacuolar lumen, being instead missorted to the vacuolar membrane. Correct luminal delivery is restored by the biosynthetic addition of a single ubiquitin, suggesting that the ubiquitylation of Fur4p serves as a specific signal for sorting to the luminal vesicles of the multivesicular bodies. A fused ubiquitin is also able to sort some Fur4p from the Golgi to the degradative pathway in the absence of added uracil but the low efficiency of this sorting indicates that ubiquitin does not itself act as a dominant signal for Golgi-to-endosome trafficking. Our results are consistent with a model in which the binding of intracellular uracil to the permease signals its sorting from the Golgi apparatus and subsequent ubiquitylation ensures its delivery to the vacuolar lumen.

INTRODUCTION

Studies in the budding yeast, Saccharomyces cerevisiae, have made a major contribution to demonstrating the involvement of ubiquitylation in the trafficking of integral membrane proteins (Hicke, 2001). The endocytosis of numerous plasma membrane proteins has been shown to be mediated by ubiquitylation. The ubiquitin ligase Rsp5p seems to be the only ligase involved in the ubiquitylation of yeast plasma membrane proteins. This enzyme is the only member of the Nedd4 protein family of ubiquitin ligases known in yeast (Rotin et al., 2000). After removal from the plasma membrane, receptor and transporter proteins tagged with ubiquitin transit via endosomes and are ultimately delivered to the lumen of the vacuole, where they are degraded by vacuolar proteases. Delivery to the interior of the vacuole requires sorting in the late endosome compartment to internal vesicles resulting from invagination of the endosomal membrane, giving rise to multivesicular bodies (MVB; Piper and Luzio, 2001). Ubiquitin was recently shown to play a crucial role in this sorting step, in which proteins entering vesicles are separated from those that remain on the endosomal membrane. Those remaining on the membrane are found on the vacuolar membrane after fusion of the MVB with the vacuole (Katzmann et al., 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001). These studies essentially concerned biosynthetic proteins such as the carboxypeptidase S Cps1p and the polyphosphatase Phm5p, which are transported from the Golgi apparatus to the vacuolar lumen. The Golgi-located ubiquitin ligase Tul1p is required for the ubiquitylation of these cargoes, to ensure their correct delivery to the interior of the vacuole (Reggiori and Pelham, 2002).

Plasma membrane transporters are delivered to the cell surface by the secretory pathway. It was recently shown that the efficiency of delivery of some transporters may be regulated by nutritional conditions. Newly synthesized permeases may be routed, entirely or in part, to the degradative vacuolar pathway without passing via the cell surface (Arvan et al., 2002). One well-known example is that of the general amino acid permease, Gap1p, which can be diverted from the late secretory pathway to the vacuolar pathway, depending on the nitrogen source (Roberg et al., 1997; Soetens et al., 2001). The ubiquitylation of Gap1p is required for its delivery to the vacuole from the late secretory pathway (Arvan et al., 2002; Soetens et al., 2001). Ubiquitylation is also required for the endocytosis of this transporter under similar nutrient conditions (Springael and Andre, 1998), and the ubiquitin ligase Rsp5p plays an essential role in both processes. The tryptophan permease Tat2p is also sorted from the Golgi apparatus to the vacuole rather than the cell surface under conditions of nutrient deprivation.
Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>27061b</td>
<td>MATa ura3 trp1</td>
<td>Galan et al. (1996)</td>
</tr>
<tr>
<td>27064b</td>
<td>MATa ura3 trp1trp1p1</td>
<td>Galan et al. (1996)</td>
</tr>
<tr>
<td>27081a</td>
<td>MATa ura3 trp1trp1p1p2</td>
<td>Springael and Andre (1998)</td>
</tr>
<tr>
<td>33125a</td>
<td>MATa ura3 trp1pep1:KanMX4</td>
<td>De Craene et al. (2001)</td>
</tr>
<tr>
<td>OS27-1</td>
<td>MATa ura3 bul1::KanMX4 bul2::KanMX4</td>
<td>Sectens et al. (2001)</td>
</tr>
<tr>
<td>MOB51</td>
<td>MATa ura3 trp1 pep4::KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>MOB52</td>
<td>MATa ura3 trp1 end3::KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>MOB53</td>
<td>MATa ura3 trp1 doa4::KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>JM01</td>
<td>MATa ura3 trp1 np1::KanMX4</td>
<td>This study</td>
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<td>BY4742</td>
<td>MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</td>
<td>Euroscarf</td>
</tr>
<tr>
<td>BY4742tul1Δ</td>
<td>MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tul1::KanMX4</td>
<td>Euroscarf</td>
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All strains, with the exception of BY4742 and its derivative tul1Δ, are isogenic to the wild-type strain Y1278b except for the mutations stated.

(Beck et al., 1999) or in the presence of high tryptophan levels (Umebayashi and Nakano, 2003). A member of the Nramp family of metal transporters, Smf1p, is targeted to the vacuole in the presence of metal ions and accumulates at the cell surface under conditions of metal starvation (Liu and Cuclotta, 1999b). Similarly, trafficking of the ferrichrome transporter, Arn1p, depends on the exposure of the cells to ferrichrome (Kim et al., 2002).

Levels of the FUR4-encoded uracil permease are also subject to posttranslational control, with Fur4p levels decreasing in response to uracil (Séron et al., 1999). Downregulation has also been observed under adverse conditions, such as the inhibition of protein synthesis (Volland et al., 1994), suggesting that uracil of exogenous or catabolic origin downregulates the cognate permease to prevent the accumulation of excess intracellular uracil-derived nucleotides. We have shown that uracil accelerates degradation by increasing the efficiency of ubiquitylation of the permease, signaling its internalization (Galan et al., 1996; Séron et al., 1999). Permease ubiquitylation, which requires prior phosphorylation, is mediated by the Rsp5p ubiquitin ligase and occurs on two target lysines (Hein et al., 1995; Marchal et al., 1998; Marchal et al., 2000). We show here that uracil negatively controls the exocytic trafficking of its cognate permease by diverting it from the Golgi apparatus to the vacuole. Our results suggest that the Rsp5p-dependent ubiquitylation of Fur4p is required for the sorting of this protein to the internal vesicles of MVB, allowing its delivery to the vacuolar lumen.

MATERIALS AND METHODS

Media and Growth Conditions

The S. cerevisiae strains used in this study are listed in Table 1. Yeast strains were transformed as described by Gietz et al. (1992). Cells were grown at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in YNB minimal medium containing 0.5% ammonium sulfate, 0.17% yeast nitrogen base (Difco, Detroit, MI) without ammonium and amino acids and supplemented with 0.1% casamino-acids (Difco). The carbon source was 2% glucose, 2% galactose plus 0.02% glucose, or 2% lactose plus 0.05% glucose. Exogenous uracil and uridine were added at concentrations of 40 μg/ml and 80 μg/ml, respectively, corresponding to essentially identical millimolar concentrations.

Strain and Plasmid Construction

Yeast strains derived from the 27061b strain with deletions of the END3, PEP4, or DOA4 gene were obtained by replacement of the entire coding region with the genetically resistant gene KanMX4 (Wach, 1996). An ORF replacement cassette with long flanking homology regions was amplified by PCR and used to transform yeast cells. The integration of the KanMX marker into the correct locus in geneticin-resistant cells was confirmed by PCR analysis of chromosomal DNA.

The plasmid Yep46FUR4 (2 μm, TRP1, FUR4), carrying the FUR4 gene under the control of its promoter, was constructed from a 2.6-kb BamHI-KpnI fragment containing the FUR4 gene amplified by PCR, using genomic DNA as template, and inserted into the Yep46 vector. The centroplasmic plasmid pFL38GalFUR4-GFP, carrying the URA3 gene and a FUR4 fusion gene, encoding Fur4p with a C-terminal GFP tag, under the control of the GAL10 promoter, was constructed in a previous study and designated pFL38GFP-GFP (Marchal et al., 2002). To obtain Fur4-GFP variants, we used a PCR-based in vivo gap repair method (Papa et al., 1999). A DNA fragment including the mutations of interest in the FUR4 coding sequence and encompassing the GAL10 promoter, URA3 sequence downstream from the single 8su36 restriction site was amplified by PCR, using p195g derivative plasmids (Marchal et al., 1998) as templates. Yeast cells were then cotransformed with pFL38GalFUR4-GFP, cleaved at the 8su36 restriction site, and the fragments amplified by PCR. We obtained pFL38GalFUR4SA-GFP and pFL38GalFUR4SA-GFP, encoding Fur4-GFP variants in which K38, K41, and K60 were replaced by R and S 42, 43, 45, 35, and 56 were replaced by A. PFL38GalFUR4272-GFP was obtained in a similar manner, using the appropriate plasmid (Urban-Grimal et al., 1995) as a PCR template. It encodes a Fur4-GFP variant in which K272 is replaced by E. The K272E mutation was also introduced in the SA variant, giving rise to pFL38GalFUR4SA272-GFP. In all cases, the changes were confirmed by sequencing. To tag the FlI1-encoded uridine permease with GFP, we replaced the 3' flanking sequence of FUR4 at the chromosomal locus, including the stop codon, with a PCR-amplified fragment containing the sequence encoding the GFP(65T) variant and the KanMX module as a selection marker (Longtine et al., 1990). The PCR fragment was generated by amplification with appropriate oligonucleotides, using pFa5a-GFP(S65T)-kanMX as the template. The fragment was introduced into wild-type cells and clones resistant to geneticin were checked for correct integration by PCR analysis of chromosomal DNA. The FlI1-GFP fusion was then cloned, by gap repair, in pFL38, to put it under the control of the GAL10 promoter, thereby generating pFL38GalFlI1-GFP.

An in-frame ubiquitin was introduced at the N-terminus of Fur4-GFP in pFL38GalFUR4-GFP by the gap repair technique. The ubiquitin used for the fusion already contained mutations K6, K11, 27, 29, 48, and 63 R (Armason and Ellison, 1994) and the mutation G76V, to determine the rate of internalization of plasma membrane Fur4p. We obtained pFL38GalFUR4KR-GFP and pFL38GalFUR4SA272-GFP. In all cases, the changes were confirmed by sequencing. To tag the FUI4-encoded uridine permease with GFP, we replaced the 3' flanking sequence of FUR4 at the chromosomal locus, including the stop codon, with a PCR-amplified fragment containing the sequence encoding the GFP(65T) variant and the KanMX module as a selection marker (Longtine et al., 1990). The PCR fragment was generated by amplification with appropriate oligonucleotides, using pFa5a-GFP(S65T)-kanMX as the template. The fragment was introduced into wild-type cells and clones resistant to geneticin were checked for correct integration by PCR analysis of chromosomal DNA. The FUI4-GFP fusion was then cloned, by gap repair, in pFL38, to put it under the control of the GAL10 promoter, thereby generating pFL38GalFUI1-GFP.

Uracil Uptake

Uracil uptake was measured as previously described (Volland et al., 1994), to determine the rate of uptake of uracil by yeast cells. Cells were washed twice with ice-cold water and counted for radioactivity. Fur4p activity was measured various times after the addition of cycloheximide (100 μg/ml) previously described (Volland et al., 1994), to determine the rate of internalization of plasma membrane-located Fur4p.
Fluorescence Microscopy and Vacuole Staining
We added 2% glucose and 10 \(\mu\)M cell tracker blue CMAC (10 mM stock solution in DMSO, Molecular Probes, Eugene, OR) to cells cultured in YNB medium and growing. Cells were incubated for an additional 15 min, then centrifuged for 20 s to increase cell density by a factor of 10, and washed twice with YNB medium in the same conditions. Cells were viewed immediately, without fixing, under a fluorescence microscope and images were acquired with a digital camera.

Protein Extracts and Western Blotting
Total protein extracts were prepared by the NaOH-TCA lysis technique (Volland et al., 1994). Plasma membrane-enriched fractions were prepared as previously described (Dupre and Haguenauer-Tsapis, 2001) except that cells were broken in a “One Shot” Cell Disrupter (Constant Systems LTD, Divan-try, Northants, United Kingdom) at maximum pressure. Proteins in sample buffer were heated at 37°C, resolved by SDS-PAGE in 10% acrylamide gels using Tricine buffer, and transferred to nitrocellulose membranes. The mem- branes were probed with monoclonal anti-GFP antisera (Roche, Applied Sciences, Indianapolis, IN), polyclonal antibodies against the plasma mem- brane H+ ATPase Pma1p (a gift from C. Slayman) and monoclonal antibodies against Vat2p, a subunit of V-ATPase (Molecular Probes). Horseradish per- oxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G was used as the secondary antibody (Sigma, St. Louis, MO) and was detected by enhanced chemiluminescence (ECL).

Equilibrium Density Gradient
Membrane proteins were separated by equilibrium density centrifugation on a continuous 20–60% sucrose gradient, essentially as previously described (Kölling and Hollenberg, 1994). Exponentially growing cultures were arrested by adding 10 mM sodium azide. Cells (50 A600 U) were harvested by centrifugation, washed once in the presence of 10 mM azide, and disrupted in a “One Shot” Cell Disrupter with 0.5 ml of 10 mM Tris-HCl buffer, pH 7.6, containing 10% wt/wt sucrose, 10 mM EDTA, 25 mM freshly prepared N-ethylmaleimide and protease inhibitors (Complete Cocktail; Roche). Cell homogenates were broken in a Cell Disrupter with 0.5 ml of 10 mM Tris-HCl buffer, pH 7.6, containing 10% wt/wt sucrose, 10 mM EDTA, and centrifuged for 20 s to increase cell density by a factor of 10, and washed twice with ice-cold medium with galactose. Cells were incubated for an additional 15 min, then centrifuged again on ice for 60 min. Proteins were then pelleted by centrifuging at 100,000 × g in an SW41 rotor (Beckman, Berkeley, CA). We collected 0.8 ml fractions from the top of the gradient and made the volume up to 1.6 ml with distilled water, and proteins were precipitated by adding 160 \(\mu\)l of 100% TCA and incubating on ice for 60 min. Proteins were then pelleted by centrifugation and resuspended in 10 ml of 0.1 M Tris base + 40 \(\mu\)l of 2× sample buffer and heated for 10 min at 37°C. The proteins in each fraction were analyzed by Western blotting, as described above.

RESULTS
Newly Synthesized Uracll Permease Can Be Diverted from the Secretory Pathway to the Vacuole
The steady state level of plasma membrane transporters is highly regulated. It was recently shown that these proteins not only display regulated turnover at the plasma membrane, but also regulated delivery to the cell surface under specific physiological conditions. We investigated whether exogenous uracil, which downregulates Fur4p at the plasma membrane, also affected the fate of newly synthesized permease. Because GFP fusion proteins have proved very useful for the analysis of membrane protein distribution in living cells, we used a GFP-tagged version of the uracil permease placed under the control of a promoter that could be regulated, the GAL10 promoter, to follow the intracellular trafficking of this protein.

We first investigated whether end3Δ cells, impaired in the internalization step of endocytosis (Benedetti et al., 1994) and producing Fur4-GFP after galactose induction, were indeed protected against the endocytosis promoted by uracil. We determined the distribution of Fur4-GFP in wild-type and end3Δ cells cultured on galactose and then in the presence of glucose and uracil for a additional 2 h (Figure 1A). Exponentially growing end3Δ cells displayed fluorescence only at the cell surface, whereas wild-type cells displayed fluorescence both at the plasma membrane and within the vacuole. The presence of fluorescence in the vacuole probably re-

sulted from the basal endocytosis of Fur4p, which has been shown to occur (Séron et al., 1999). After glucose repression and further growth in the presence of uracil, the plasma membrane of wild-type cells no longer displayed fluores- cence, indicating that Fur4-GFP had been internalized and delivered to the vacuolar lumen, although not yet entirely degraded. In contrast, permease at the cell surface of end3Δ cells was not internalized further in the presence of uracil, showing that Fur4p was largely resistant to endocytosis in these conditions. We therefore used end3Δ cells to investi-
gate the effect of uracil on intracellular trafficking of the permease. End3Δ cells were cultured with lactate as the carbon source. Galactose was then added to the medium to induce permease synthesis in the presence or absence of uracil. Uracil was taken up by these cells at the very start of Fur4-GFP synthesis, albeit at a modest rate, thanks to the presence of the chromosomally encoded Fur4p in these cells. After 2 h of synthesis, Fur4-GFP was typically found almost exclusively on the plasma membrane in the absence of ura-

Ril, whereas substantial fluorescence within cells was also observed after exposure to uracil (Figure 1B). In cells exposed to uracil, intracellular permease was distributed simi-
larly to CMAC stain, a useful marker of the vacuolar lumen. Because the endocytic pathway is efficiently blocked in end3Δ cells, we concluded that uracil triggered the sorting of Fur4p from an internal compartment to the vacuole, without passage via the plasma membrane. Thus, uracil targets a portion of its specific transporter to the vacuole, ensuring regulated degradation without trafficking via the cell sur-
face. It should be noted that very similar patterns of vacuolar staining were obtained in PEP4 and pep12Δ cells, which are defective in vacuolar protease activities (our unpublished results). The vacuolar fluorescence observed is readily ac-
counted for by the relative resistance of GFP to vacuolar proteases, as shown by Western blot analysis (Figure 1C). We therefore used PEP4 cells for all subsequent experiments.

We investigated whether Fur4p was delivered to the vacuole via the vacuolar protein sorting (VPS) pathway. Dele-
tion of the endosomal SNARE protein Pep12p is known to block the VPS pathway by preventing the fusion of Golgi-derived vesicles with the late endosome (Gerrard et al., 2000). Pep12Δ cells were transformed with pFL38GalFUR4- GFP. All permease newly synthesized in pep12Δ cells in the absence of uracil was delivered to the plasma membrane, as in wild-type cells (Figure 1B). In pep12Δ cells exposed to uracil, no intracellular fluorescence was observed in the vacuole and instead, a punctate pattern of fluorescence was observed, probably corresponding to Golgi-derived vesicles. We checked that this fluorescent material indeed corre-
sponded to permease trafficking along the biosynthetic path-
way and not from the cell surface, by repeating the experi-
ment with a variant of Fur4p resistant to endocytosis. Fur4p-G2p, which lacks the lysines essential for ubiquitination at the cell surface, is resistant to endocytosis in wild-type cells (Marchal et al., 2000), as is the corresponding GFP fusion protein (see the control shown Figure 3A). Very simi-
lar fluorescence patterns were obtained with pep12Δ cells producing wild-type permease and endocytosis-resistant permease (Figure 1B). In cells exposed to uracil, Fur4p is therefore diverted from the Golgi apparatus to the vacuole via the VPS pathway.

Cell fractionation experiments were carried out to analyze in more detail the distribution of permease between intra-
cellular compartments and the cell surface. Extracts from end3Δ cells allowed to produce Fur4-GFP for 2 h in the presence or absence of exogenous uracil were subjected to

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centrifugation on sucrose density gradients and fractions were analyzed by immunoblotting with anti-GFP antibodies (Figure 1C). We detected a strong signal, corresponding to Fur4-GFP, in the highest density fraction, in the presence and absence of uracil. The H+ ATPase Pma1p, used as a plasma membrane protein marker, was also mainly detected in this fraction. The presence of Fur4-GFP in intermediate fractions can be accounted for in part by minor contamination with fragments of the plasma membrane, because Pma1p also gave a faint signal in these fractions. However, the presence of Fur4-GFP in these fractions is also consistent with the possible presence of this protein in endosomes or other intermediate organelles along the secretory or Golgi-to-vacuole pathways. The low-molecular-weight species present in the low-density fractions corresponds to GFP resistant to vacuolar proteases, as previously reported (Dupre and Hagnauer-Tsapis, 2003). This protein was present in much larger amounts in the presence of uracil than in its absence, because of the diversion of Fur4-GFP to the vacuole. We roughly estimated the proportions of material present in the vacuole and plasma membrane by western blotting, using several dilutions of the lowest and highest density fractions from this and another sucrose gradient experiment. We found that, in the presence of uracil, the intensity of the vacuolar GFP signal was similar to that of the plasma membrane Fur4-GFP signal, whereas it was only 10–20% as strong in the absence of uracil. Thus, ~30–40% of the permease appears to have been diverted by uracil to an early degradative pathway. This estimate was confirmed by an independent approach. We measured uracil uptake in end3Δ cells after 2 h of Fur4-GFP synthesis in the presence and absence of uracil. Although activity measurements are not entirely reliable for cells cultured in the presence of exogenous uracil, even if these cells are carefully washed, the addition of uracil appeared to decrease activity by 40–50%. Uracil may therefore decrease the delivery of its specific permease to the plasma membrane by a factor of up to two.

The FUII-encoded Uridine Permease Is Similarly Sorted for Early Vacuolar Degradation in Cells Exposed to Uridine

Uridine is another precursor of pyrimidine nucleotides that yeast cells are able to take up from the external environment (Wagner et al., 1998). We found that adding uridine to cells overproducing the specific FUII-encoded uridine permease resulted in a decrease in growth rate (unpublished re-
Uridine promoted the direct routing of newly synthesized uridine permease to the vacuole. (A) WT and end3Δ cells producing Fui1-GFP under control of the Gal10 promoter, were cultured on galactose to midexponential growth phase. Glucose (2%) and uridine were then added and cells cultured for an additional 2 h. Cells withdrawn from cultures before (0) and after (2 h) the addition of glucose and uridine were examined for fluorescence and with Nomarski optics. (B) Fui1-GFP was produced for 2 h in end3Δ cells in the absence or presence of uridine (urd). Permease distribution (GFP) and vacuolar staining (CMAC) were assessed.

Figure 2. Uridine promoted the direct routing of newly synthesized uridine permease to the vacuole.

We therefore thought that uridine might control Fui1p trafficking, preventing harmful uridine uptake. Galactose-induced Fui1-GFP accumulated at the cell surface in wild-type cells, in which only faint vacuolar fluorescence was observed, suggesting that the constitutive turnover of Fui1p is probably lower than that of Fur4p-GFP (Figure 2A and Figure 1A, respectively). After the addition of glucose to block permease synthesis and further growth for 2 h in the presence of uridine, plasma membrane fluorescence had disappeared and the vacuoles had become highly fluorescent, indicating that Fui1-GFP had been internalized and delivered to the vacuolar lumen (Figure 2A). End3Δ cells producing Fui1-GFP were subjected to the same glucose shut-off procedure. As expected, uridine permease, which was located entirely at the cell surface in these cells cultured on galactose, was not internalized further in the presence of uridine (Figure 2A). Thus, the plasma membrane-located Fui1p was turned over similarly to other permeases, by means of endocytosis and vacuolar degradation. We then investigated the possible effects of uridine on newly synthesized Fui1p by inducing the production of Fui1-GFP in the presence or absence of uridine in end3Δ cells. Fui1-GFP synthesized in the absence of uridine was present almost exclusively at the cell surface, whereas the presence of uridine resulted in additional fluorescence in the vacuole (Figure 2B). Uridine therefore routed a portion of Fui1p directly to the degradative pathway, without the necessity of passing via the plasma membrane. This effect is similar to that of uracil on Fur4p and appeared to be substrate specific as no direct targeting of Fui1p to the vacuole was observed in cells taking up large amounts of uracil (our unpublished results).

A Lack of Ubiquitylation Prevents Delivery of the Diverted Fur4p to the Vacular Lumen

Ubiquitylation was recently shown to be an important signal in post-Golgi sorting for both biosynthetic vacuolar proteins, such as Cps1p (Katzmann et al., 2001; Reggiori and Pelham, 2001), and plasma membrane transporters, such as Gap1p (Helliwell et al., 2001; Soetens et al., 2001), and Tat2p (Beck et al., 1999; Umebayashi and Nakano, 2003). The same cis- and trans-elements are involved in the ubiquitylation of Gap1p at the cell surface and in intracellular compartments (Soetens et al., 2001; Springael and Andre, 1998). We have previously shown that the cell surface ubiquitylation of Fur4p requires the ubiquitin ligase Rsp5p and that lysines 38 and 41 are the main ubiquitin acceptors (Galan et al., 1996; Marchal et al., 2000). Permease phosphorylation, occurring within an N-terminal PEST region of Fur4p, is also required for cell surface ubiquitylation. The SA variant of uracil permease, in which all five serine residues included in the PEST region are replaced by alanines, is very poorly phosphorylated, and is thus strongly stabilized at the plasma membrane (Marchal et al., 1998). We therefore investigated the possible involvement of Rsp5p and of the relevant serine and lysines of Fur4p in direct routing of the permease to the vacuole. We first checked that the GFP-tagged versions of the SA and KR variants were resistant to endocytosis in wild-type cells. Galactose-induced Fur4SA-GFP and Fur4KR-GFP accumulated only at the cell surface (Figure 3A). After the addition of glucose to block permease synthesis and further growth for 2 h in the presence of uracil, the plasma membrane remained fluorescent and no intracellular fluorescence was detected, showing that these variant permeases were indeed resistant to endocytosis. We then checked whether the SA variant was normally sorted to the endosomal pathway when synthesized in the presence of uracil. The SA variant was partly delivered to the vacuolar lumen (Figure 3B), and behaved very similarly to the wild-type uracil permease in end3Δ cells (Figure 1B). Because the SA variant is itself resistant to endocytosis, the fraction present within the vacuole of wild-type cells must have arrived at this location without passing via the plasma membrane. Thus, the phosphorylation of Fur4p, at least on the serine residues within the PEST sequence, is not important for the diverted pathway. This result is consistent with our previous observations that phosphorylation of the uracil permease occurs at a late stage in the secretory pathway (Volland et al., 1992), and depends on the casein kinase I isoforms, Yck1p and Yck2p (Marchal et al., 2000), two peripheral plasma membrane proteins (Vancura et al., 1994). We also found that uracil permease was generally less phosphorylated in cells cultured in the presence of uracil than in those cultured in its absence (Séron et al., 1999). This may be due to some of the permease not undergoing phosphorylation because of premature targeting to the vacuole.

We also produced Fur4SA-GFP in npi1/rsp5 mutant cells, which have very low levels of Rsp5p (Springael and Andre, 1998) and thus display impaired cell surface ubiquitylation. Under conditions resulting in the direct routing of uracil permease in the endosomal pathway, we observed fluorescence not only at the plasma membrane, but also in the vacuolar lumen, but also at the vacuolar membrane (Figure 3B). Fur4-GFP produced in the npi1 mutant strain gave very similar
pictures (unpublished data). This suggests that Rsp5p is required for the entry of Fur4p into the internal vesicles of the MVB and its subsequent delivery to the lumen of the vacuole but that residual ligase activity in npi1 cells may be sufficient for some MVB sorting. We investigated whether lysines 38 and 41, which are essential for the Rsp5p-dependent ubiquitylation of uracil permease at the cell surface (Marchal et al., 2000), were also involved in the diverted pathway. We looked at the subcellular distribution of fluorescence after production of the KR variant of Fur4-GFP in the presence of uracil in npi1 cells. The diverted variant permease was detected only at the vacuolar membrane and did not enter the vacuolar lumen (Figure 3B). This finding is consistent with the idea that Rsp5p exerts an effect on Fur4p directly by altering the ubiquitylation status of this protein. We also directly assessed the ubiquitylation state of the permease (see below). The behavior of the KR variant also indicated that, in addition to its involvement in internalization at the plasma membrane, lysines 38 and 41 are probably sites of ubiquitylation for MVB sorting. However, we observed that the KR variant was delivered to the vacuolar lumen in wild-type cells (Figure 3B). Hence, in addition to lysines 38 and 41, one or more other lysines probably act as sites of uracil permease ubiquitylation, ensuring the efficient entry of this protein into MVB, at least in cells with no major defect in the ubiquitylation system. This interpretation was confirmed using npi2/doa4 mutant cells (Springael et al., 1999), which have low ubiquitin levels and hence display impairment of various ubiquitin-dependent processes (Swaminathan et al., 1999). Fur4-GFP, when produced in npi2 cells, reached the vacuolar lumen (unpublished data) as it did in wild-type cells, whereas the KR variant was clearly present in the vacuolar membrane in addition to the lumen (Figure 3B). The use of doa4Δ cells rather than npi2 cells, which have a point mutation affecting a conserved region of the ubiquitin isopeptidase Doa4p (Springael et al., 1999), did not lead to more pronounced missorting of uracil permease (unpublished data). The decrease in the ubiquitin pool is therefore less crucial for the diverted pathway than for permease endocytosis, as wild-type uracil permease was strongly stabilized at the plasma membrane in npi2/doa4 mutant cells (Galan and Huguenauer-Tsapis, 1997). Overall, these results suggest that poorly ubiquitylated Fur4p, regardless of the reason for this defect, can be diverted from the Golgi apparatus toward the vacuole, but cannot efficiently enter the MVB and is therefore detected within the vacuolar membrane. Such a location has already been reported for the ABC transporter Ste6p and the tryptophan permease Tat2p in doa4 cells (Beck et al., 1999; Losko et al., 2001).

The Golgi-located ubiquitin ligase Tul1p has been shown to be required for the ubiquitylation and sorting of Cps1p or Phm5p to internal vesicles within MVB. In tul1Δ cells, Cps1p and Phm5p were found to be ultimately located on the vacuolar membrane rather than in the lumen (Reggiori and Pelham, 2002). We investigated whether this ligase was also involved in the intracellular trafficking of permeases. Production of the GFP-tagged KR variant in the tul1Δ strain in the presence of uracil revealed no accumulation in the limiting membrane of the vacuole (Figure 3B). Instead, this variant was found to be delivered to the vacuolar lumen, as for wild-type Fur4p in tul1Δ and the KR variant in parental cells (unpublished data). This indicates that the Tul1p ligase is not important for the sorting of permeases within the MVB. This is consistent with the role previously attributed to Tul1p in the recognition of polar transmembrane domains within abnormal proteins and biosynthetic precursors (Reggiori and Pelham, 2002). Proteins with many membrane-
Cup1 promoter (Ellison and Hochstrasser, 1991), and 

allowing normal ubiquitin under the control of the 

were cotransformed with plasmid Yep96, encoding 

normal ubiquitin under the control of the 

were cotransformed with plasmid Yep96, encoding 

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Uracil permease is ubiquitylated during 

trafficking from the Golgi apparatus to the 

vacuole, in a Rsp5p-dependent manner. The 

DOA4 gene had been deleted in the cells used 

here, which harbored either the wild-type or a 

mutant version (npil) of the RSP5 gene. The cells 

were first cultured with lactate as the carbon source, and CuSO4 (100 

µM) and galactose were then added and the cells 

were first cultured with lactate as the carbon source, and CuSO4 (100 

µM) and galactose were then added and the cells 

cultured for 2 h in the presence of uracil. Protein 

extracts were prepared and fractionated on equi- 

librium sucrose density gradients. (A) Aliquots of 

the various fractions from RSP5 cells producing 

the SA variant of Fur4-GFP were analyzed by 

Western immunoblotting for Fur4-GFP, GFP, and 

Pma1p. Sucrose gradient concentration increases 

from left to right in the figure. (B) The eight central 

fractions of the gradient shown in A were pooled 

and analyzed in parallel to equivalent fractions 

from the same cells that have produced HA- 

tagged ubiquitin (from the Yep112 plasmid; Ellis- 

son and Hochstrasser, 1991) instead of normal 

ubiquitin. A small line indicates the increase in the 

molecular weight of a ubiquitin conjugate. (C) As 

for A except that fractions of gradients from ex- 

tracts of npil mutant cells producing Fur4KR-GFP 

were analyzed by Western immunoblotting for 

Fur4-GFP, GFP, Pma1p, and Vat2p, a marker of 

the vacular membrane. (D) Pools of the eight 

central fractions of gradients from RSP5 and npil 

cells producing the SA or KR variant of Fur4-GFP 

were analyzed by immunoblotting with anti-GFP 

antibodies.

spanning domains, such as Fur4p, are thought to acquire a 

folding pattern in which the few charged residues present in 

the hydrophobic transmembrane domains are not exposed to 

the lipid environment.

We carried out experiments to determine directly the 

ubiquitylation status of Fur4p sorted to the endosomal pathway 

in cells harboring either wild-type or mutant RSP5 

genes. To facilitate the detection of Ub-permease conjugates, 

we used cells lacking the Doa4p ubiquitin isopeptidase, 

which is involved in the deubiquitylation of endocytic (Du- 

pre and Hagenauer-Tsapis, 2001) and biosynthetic sub- 

strates (Katzmann et al., 2001; Reggiori and Pelham, 2001). A 

normal pool of ubiquitin was restored by producing ubiqui- 

tin in a copper-regulated manner. We used the SA variant 

rather than the wild-type permease, to minimize ubiquityla- 

tion of the cell surface-located permease and its subse- 

quent endocytosis. Extracts were prepared from doa4Δ cells 

allowed to synthesize ubiquitin and Fur43ΔA-GFP for 2 h in 

the presence of uracil and fractionated by sedimentation on 

density gradient. In doa4Δ cells used in these conditions, 

permease trafficking to the vacuole was frequently retarded, 

possibly because of a toxic effect of copper, but this situation 
made it easier to detect potential ubiquitin conjugates. On 

immunoblots (Figure 4A), a smear was detected just above 

the main Fur4-GFP signal in intracellular fractions. To check 

whether this smear indeed correspond to ubiquitin conjugates, 

we carried out the same experiment in cells producing 

HA-tagged ubiquitin, and pools of the internal fractions 

from cells producing normal or HA-tagged ubiquitin 

were analyzed by immunoblotting (Figure 4B). We observed 
a difference in electrophoretic migration for bands above the 

main signal and the presence of HA-tagged ubiquitin re- 

sulted in an increase in the molecular weight of at least 

the first species that very likely corresponded to a mono-ubiq- 

uitylated permease. Therefore, permease was ubiquitylated 
during trafficking from the Golgi apparatus to the vacuole. 

The ubiquitylation status of the diverted permease was then 

analyzed in npil/doa4Δ cells producing Fur4KR-GFP and nor- 

mal ubiquitin (Figure 4C). Vacular fractions did not display 

any proteolytic GFP in agreement with the lack of fluo- 

rescence inside the vacuole (Figure 3B). A substantial amount 
of permease was again found in intermediate fractions; this 
distribution is consistent with the KR variant being able to 
reach the limiting membrane of the vacuole but not the 
lumen of npil cells (Figure 3B). Importantly, the extent of 
ubiquitylation of Fur4KR-GFP that has been diverted ap- 
peared to be very low, (Figure 4C), compared with that seen 
for the SA variant in RSP5 cells (Figure 4A), suggesting that 
the sorting to the endosomal pathway efficiently occurred 

Figure 4. Uracil permease is ubiquitylated during 

trafficking from the Golgi apparatus to the 

vacuole, in a Rsp5p-dependent manner. The 

DOA4 gene had been deleted in the cells used 

here, which harbored either the wild-type or a 

mutant version (npil) of the RSP5 gene. The cells 

were first cultured with lactate as the carbon source, and CuSO4 (100 

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Fur4-GFP, GFP, Pma1p, and Vat2p, a marker of 

the vacular membrane. (D) Pools of the eight 

central fractions of gradients from RSP5 and npil 
cells producing the SA or KR variant of Fur4-GFP 

were analyzed by immunoblotting with anti-GFP 

antibodies.
of alternative lysines (Figure 3B). In npi1/rsp5 cells, residual levels of ubiquitin conjugates were still present for the diverted KR variant (lane 4). This latter result is consistent with the added ubiquitin further, by replacing the KR variant (K272E) in which lysine 272, lying in a transmembrane segment, was replaced by a glutamic acid. This mutant has been shown to have a very low affinity for uracil and only residual transport activity (Urban-Grimal et al., 1995). A GFP-tagged version of this mutant protein was constructed and, as expected, Fur4KR-GFP was found to be located at the cell surface and did not transport uracil (our unpublished results). Because the chromosomally encoded uracil permease alone cannot increase uracil levels to such a point that the permease is sorted to the vacuole, another plasmid, encoding an active form of uracil permease, was introduced into cells. The presence of the two plasmids appeared to be very toxic to end3Δ cells. This difficulty was overcome by introducing the SA mutation into Fur4272Δ-GFP, to obtain a permease resistant to endocytosis, making it possible to use wild-type cells. Cells producing active Fur4p under the control of its own promoter from a multicopy plasmid were grown with lactate as the carbon source, with subsequent addition of uracil and galactose to induce synthesis of either Fur4Δ-GFP or Fur4SA-GFP, used as a control. The mutation of residue 272 almost entirely prevented diversion of the permease to the vacuolar pathway as the vacuoles showed only very faint labeling with this mutant permease (Figure 5B). This strongly suggests that the direct binding of intracellular uracil to its specific transporter is indeed the signal for sorting of the permease to the endosomal pathway for early degradation. However, an Ub-Fur4 chimera mutated for residue 272, Ub-Fur4272Δ-GFP, was diverted to the MVB pathway in the absence of added uracil, to the same extent as Ub-Fur4-GFP. This indicates that early sorting of the permease may also occur in the absence of uracil binding, at least for a protein with a fused ubiquitin moiety.

**Mono-ubiquitylation Is Not Sufficient for the Rapid Internalization of Uracil Permease at the Plasma Membrane or for the Efficient Sorting of This Protein to MVB**

We investigated whether the biosynthetic addition of ubiquitin restored normal turnover to a variant uracil permease lacking its ubiquitin acceptor lysines, resulting in stabilization of the permease at the plasma membrane. In wild-type cells growing exponentially on galactose, Fur4KR-GFP was located exclusively at the plasma membrane whereas Ub-Fur4KR-GFP was partitioned between the plasma membrane and vacuole (Figure 6A). The fluorescence observed within the vacuoles of cells producing the Ub-chimera can be accounted for, at least in part, by some diversion of this protein from the exocytic pathway, even in the absence of added uracil (see Figure 5). The binding of uracil is the main signal for the efficient sorting of Fur4p to the endosomal system. (A) End3Δ cells transformed with pFL38GalUB-FUR4-GFP or pFL38GalUB-FUR4KR-GFP were cultured with lactate, and the medium was then supplemented with galactose to induce the synthesis of GFP-tagged permease. After 2 h in the absence or presence of uracil, permease fluorescence (GFP) and vacuole staining (CMAC) were assessed. (B) WT cells cotransformed with Yep46FUR4 and either pFL38GalFUR4SA,272-GFP or pFL38GalFUR4SA-GFP were subjected to galactose induction in the presence of uracil and examined as in A.

**Exposure to Substrate Is the Main Signal for Sorting to the Vacuole**

We further analyzed the role of ubiquitin in marking permeases for direct sorting from the Golgi apparatus to the vacuole and investigated whether ubiquitylation was sufficient for such sorting by adding ubiquitin to the N-terminus of the GFP-tagged Fur4p. We fused to GFP-permeases a variant ubiquitin with all its relevant lysine residues mutated to arginine (Arnason and Ellison, 1994), to ensure that no additional ubiquitin was added to the ubiquitin moiety. We also modified the added ubiquitin further, by replacing glycine 96 with a valine, to prevent this molecule from being removed from the chimera. Ub-Fur4-GFP synthesized in the absence of uracil in end3Δ cells was partitioned between the cell surface and the vacuole (Figure 5A). We estimated the proportion of Ub-permease delivered to the plasma membrane in these conditions by measuring uracil uptake in end3Δ mutant cells producing either Ub-Fur4-GFP or Fur4-GFP and cultured in the absence of uracil. We obtained an activity ratio of 75–80% for cells producing the Ub-chimera with respect to cells producing the normal permease, indicating that there was 20–25% vacuolar diversion. Diversion to the vacuole, which was rather modest in the absence of uracil, became the predominant pathway in cells exposed to uracil (Figure 5A). Thus, a single ubiquitin can mediate some vacuolar sorting, but it is not sufficient for efficient targeting to the vacuole in the absence of the physiological signal. Nonubiquitylatable ubiquitin was also fused to the KR variant of Fur4-GFP and the chimeric protein produced in end3Δ cells. Newly synthesized Ub-Fur4KR-GFP behaved identically to Ub-Fur4-GFP: it was targeted to the vacuole in only moderate amounts in the absence of uracil but was very efficiently targeted to the vacuole after exposure to uracil (Figure 5A). The biosynthetic addition of ubiquitin to the N-terminus of Fui1-GFP resulted in similar behavior, with the Ub-chimeric permease efficiently directed to the VPS pathway only in uridine-treated cells. Thus, uracil itself, or uridine, seems to be the dominant signal in the secretory pathway for decreasing the amount of permease delivered to the cell surface.
Uracil uptake was measured various times after the addition of uracil, the wild-type and KR variant forms of Ub-permease chimeras by checking whether normal sorting to MVB was restored by biosynthetically added ubiquitin in conditions in which Rsp5p levels were very low. Figure 6B shows the distribution of Ub-Fur4-GFP and Ub-Fur4KR-GFP produced in nip1/rsp5 mutant cells. During exponential growth in the absence of uracil, these two proteins were partitioned between the cell surface and intracellular compartments. The vacuolar lumen contained both proteins but the KR variant was also detected at the vacuolar membrane, indicating impairment of its sorting at the MVB level. Blocking the synthesis of permease and allowing the cells to grow for a additional 2 h in the presence of uracil resulted in complete removal from the cell surface of the permease to which a ubiquitin had been fused, whether or not the target lysines were present (Figure 6B). The absence of target lysines in the Ub-chimeras indicated that multiubiquitylation increases the rate of internalization in endocytosis. Our results are consistent with conclusions drawn from studies with the yeast α-factor receptor, indicating that multivariate ubiquitylation increases the rate of internalization (Roth and Davis, 2000).

We further analyzed the intracellular trafficking of ubiquitin permease chimeras by checking whether normal sorting to MVB was restored by biosynthetically added ubiquitin in conditions in which Rsp5p levels were very low. Figure 6B shows the distribution of Ub-Fur4-GFP and Ub-Fur4KR-GFP produced in nip1/rsp5 mutant cells. During exponential growth in the absence of uracil, these two proteins were partitioned between the cell surface and intracellular compartments. The vacuolar lumen contained both proteins but the KR variant was also detected at the vacuolar membrane, indicating impairment of its sorting at the MVB level. Blocking the synthesis of permease and allowing the cells to grow for a additional 2 h in the presence of uracil resulted in the complete removal from the cell surface of the permease to which a ubiquitin had been fused, whether or not the target lysines were present (Figure 6B). The absence of target lysines in the Ub-chimera did not further delay the internalization step in nip1 cells (Table 2). However, the KR variant was clearly present at the vacuolar membrane, indicating that multiubiquitylation increases the rate of internalization (Roth and Davis, 2000).

We further analyzed the intracellular trafficking of ubiquitin permease chimeras by checking whether normal sorting to MVB was restored by biosynthetically added ubiquitin in conditions in which Rsp5p levels were very low. Figure 6B shows the distribution of Ub-Fur4-GFP and Ub-Fur4KR-GFP produced in nip1/rsp5 mutant cells. During exponential growth in the absence of uracil, these two proteins were partitioned between the cell surface and intracellular compartments. The vacuolar lumen contained both proteins but the KR variant was also detected at the vacuolar membrane, indicating impairment of its sorting at the MVB level. Blocking the synthesis of permease and allowing the cells to grow for a additional 2 h in the presence of uracil resulted in the complete removal from the cell surface of the permease to which a ubiquitin had been fused, whether or not the target lysines were present (Figure 6B). The absence of target lysines in the Ub-chimera did not further delay the internalization step in nip1 cells (Table 2). However, the KR variant was clearly present at the vacuolar membrane, indicating that its delivery to the vacuolar lumen was still largely impaired. One possible reason for this is that the ubiquitylation of some of the lysines in the permease is required for efficient sorting to the MVB. If Rsp5p levels were normal, it might be possible to overcome the absence of the normal target lysines, possibly by adding ubiquitin to alternative lysines, but this is not possible in mutants with low Rsp5p.

**Table 2.** Half-time (min) of the internalization step of endocytosis of GFP-tagged uracil permease derivatives

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<tr>
<td>WT</td>
<td>40</td>
<td>&gt;180</td>
<td>20</td>
<td>75</td>
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<td>nip1</td>
<td>&gt;180</td>
<td>&gt;180</td>
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Uracil uptake was measured various times after the addition of cycloheximide and the time corresponding to a 50% loss of activity is reported.
levels. Western blotting of membrane-enriched fractions from npi1 cells (Figure 6B) revealed that additional ubiquitin was probably added to Ub-Fur4-GFP but not to Ub-Fur4KR-GFP. Thus, in the absence of polyubiquitylation, the permease was not efficiently sorted to vesicles and became permanently resident in the vacuolar membrane after fusion of the MVB with the vacuole. Because some permease nonetheless reached the vacuolar lumen, the deficiency may lie in the sorting step being limiting, resulting in a fraction of the permease being trapped at the endosomal membrane and subsequently at the vacuolar membrane.

DISCUSSION

The plasma membrane transporters of S. cerevisiae play a critical role in controlling growth rate and the tight regulation of their removal from the cell surface is a key feature in the capacity of this organism to adapt rapidly to changing nutrient availability. We previously demonstrated the negative control exerted by uracil over its own transport. In particular, uracil decreases the stability of its specific permease, Fur4p, by promoting the Rsp5p-dependent ubiquitylation of this protein at the cell surface (Séron et al., 1999). This provides a signal for the internalization and delivery of Fur4p to the vacuole for degradation. We suggest that intracellular uracil from catabolic or external sources directly controls the fate of Fur4p, preventing the uptake of excess pyrimidine nucleotide precursors, which would be harmful to cells. The data reported here show that uracil also affects the exocytic trafficking of Fur4p by inducing the direct routing of this protein from the Golgi apparatus to the endosomal system for degradation without the necessity of passing via the plasma membrane. Similarly, exogenous uridine promotes the direct routing of its specific permease, Fui1p. A very efficient mechanism for responding to rapid changes in the nutritional state of nucleotide precursors is thus provided through the control of early protein sorting and cell surface protein stability. The metal transporter Smf1p provides another example of a transporter negatively regulated by its own substrate. Smf1p is not directed to the cell surface if manganese is plentiful but accumulates at the plasma membrane under conditions of manganese starvation (Liu and Culotta, 1999b). There are three closely related Smf metal transporters, operating at different locations within the cell, on a variety of metals, some of which are toxic. This system requires sophisticated regulation (Portnoy et al., 2000).

Our results show that ubiquitin plays an essential role in the entry into MVB of Fur4p diverted from the late secretory pathway to the vacuole. Direct analysis of the level of ubiquitylation of Fur4p prematurely targeted for degradation showed that the permease is ubiquitylated during direct routing to the vacuole. Npi1 mutation, which results in very low levels of the ubiquitin ligase Rsp5p, impaired the delivery of Fur4p, which accumulated at the vacuolar membrane rather than in the lumen. This impairment was even more marked if the npi1 mutation was combined with a cis-acting Fur4 KR mutation, and may be accounted for by a lack of ubiquitylation of the permease. Thus, the same cis- and trans-elements, Rsp5p and lysine residues at positions 38 and 41 are used for both the downregulation of uracil permease levels at the cell surface (Galan et al., 1996; Marchal et al., 2000) and the sorting of this protein from the biosynthetic pathway to the MVB. In contrast, phosphorylation of the serine residues within a PEST sequence of Fur4p that is important for ubiquitylation at the cell surface (Marchal et al., 1998) does not seem to be required for normal entry into MVB. Our data also show that alternative lysines can serve as ubiquitin acceptors for Fur4p in the diverted pathway, whereas this is not the case at the plasma membrane. Rsp5p may recognize relevant lysines for the ubiquitylation of intracellular permease in a less specific way, different from the process of recognition at the plasma membrane. The delivery of the general amino acid permease Gap1p to the cell surface appears to be controlled by the quality of the external nitrogen source. Rich nitrogen sources, such as NH4+ and glutamate, trigger the sorting of this permease exclusively to the vacuole, without passing via the plasma membrane (Roberg et al., 1997; Soetens et al., 2001). The features of Gap1p ubiquitylation important for the intracellular targeting of this molecule to the vacuole have been characterized. It is striking that the elements implicated in the intracellular ubiquitylation of Gap1p are identical to those involved in the ubiquitylation that signals its endocytosis. Both events require Rsp5p, the same Gap1 target lysines and the Bul proteins (Hellwell et al., 2001; Soetens et al., 2001). Bul1p and Bul2p were first identified as proteins that bind Rsp5p (Yashiroda et al., 1996). The overproduction of Bul proteins results in the sorting of Gap1p to the vacuole regardless of the nitrogen source and it has been suggested that the Bul proteins specify the intracellular polyubiquitylation of Gap1p, resulting in its efficient sorting to the vacuole (Hellwell et al., 2001). The double mutant bul1Δ bul2Δ has the inverse phenotype, causing Gap1p to be rerouted to the plasma membrane (Hellwell et al., 2001; Soetens et al., 2001). Similarly ubiquitylation of the tryptophan permease Tat2p is markedly decreased in bul1Δ cells and Tat2p appears to be targeted to the plasma membrane of these mutant cells even at high tryptophan concentrations, which promote the transport of Tat2p to the vacuole of wild-type cells (Umebayashi and Nakano, 2003). GFP-tagged Fur4p or its KR variant, newly synthesized in bul1Δ bul2Δ cells in the absence or presence of uracil, behaved similarly in these cells and in parental cells, and no difference was observed in the extent to which permeases were diverted (unpublished data). Although some of our data indicate that polyubiquitylation is more efficient than monoubiquitylation for internalization of uracil permease into MVB, we obtained no evidence that Bul proteins were involved in this process. Control of the cell surface delivery of transporters may involve different partners according to whether amino acids or pyrimidine nucleotide precursors are being transported, because of the different physiological significance of these molecules. The early degradation of Fur4p prevents the uptake of an excess of nucleotide precursors, which would be detrimental to the cells, and would be expected to be rapid and irreversible. Amino acid uptake involves many permeases classified according to their response to the source of nitrogen and regulation of the trafficking of these permeases makes it possible to optimize the use of all available nutrients. A pool of intracellular Gap1p exists under various growth conditions and it has been suggested that this pool is a type of physiological storage system (Hellwell et al., 2001). Conversely, Fur4p is located exclusively at the cell surface in the absence of endocytosis (see Figure 1A). Sorting decisions concerning the early degradation of Gap1p may therefore require additional factors that are not required for Fur4p disposal.

The precise location, between the Golgi apparatus and the endosomes, at which the intracellular sorting of uracil permease destined for the interior of the vacuole occurs is unclear. The identification of this site is hampered by the finding that a subset of exocytic cargoes do transit through an endosomal compartment before reaching the cell surface,
but can be rerouted to the classical exocytic pathway if this route is blocked, as in vps mutants (Gurunathan et al., 2002; Harsay and Schekman, 2002). It has been suggested that the plasma membrane H^+ATPase, Pma1p, reaches the cell surface directly from the Golgi apparatus (Gurunathan et al., 2002; Harsay and Schekman, 2002), but it is not yet determined whether plasma membrane transporters follow a route that passes through endosomes. Our results, obtained in pep12Δ cells, which display impaired vesicle fusion with late endosomes, must be considered within this context. Some permease newly synthesized in the presence of uracil was found in small vesicles in pep12 cells. This site would be expected for material blocked on its route from the Golgi apparatus to the endosomes. The sorting of Fur4p triggered by uracil occurred at the Golgi level in pep12 cells but it is impossible to determine from this observation whether such sorting occurs in the Golgi apparatus or in the endosomes in wild-type cells.

Another unanswered question concerns the site of Fur4p ubiquitylation in the direct route to the vacuole. Vacuolar proteins, such as the membrane precursor of Cps1p, receive ubiquitin before the Pep12 compartment (Katzmann et al., 2001). Tull1p, the ubiquitin ligase involved in this process, is a Golgi-resident transmembrane protein (Reggiori and Pelham, 2002). We showed that Tull1p was not involved in the sorting of Fur4p, and therefore probably not in its ubiquitylation, whereas Rsp5p-dependent ubiquitylation was clearly required for Fur4p sorting to the MVB. Staining for Rsp5p gives a punctate pattern at the plasma membrane and at perivacuolar sites, probably corresponding to late endosomal structures (Wang et al., 2001). Although this location is consistent with the ubiquitylation event occurring at the endosome rather than at the Golgi level, it is not definitive proof of this as a small amount of Rsp5p in the vicinity of the Golgi apparatus may have escaped detection. Our findings extend the list of known functions of Rsp5p in the intracellular trafficking of membrane-bound proteins. The role of ubiquitin ligases of the Rsp5/Nedd4 family may have been conserved throughout evolution. Recent data highlighted a role of Drosophila Nedd4 in the downregulation of a receptor of the nervous system (Murphey and Godenschwege, 2002).

Ubiquitin-dependent events may result in the sorting of the receptor to the endosomal pathway without passing via the plasma membrane (Keleman et al., 2002). We showed that Fur4p newly synthesized in the presence of uracil can reach the vacuole even if ubiquitylation is severely impaired. In such cases, it is present at the vacuolar membrane rather than in the vacuolar lumen. A vacuolar membrane location has also been demonstrated for the tryptophan permease Tat2p, in npt1 cells, after rapamycin treatment, which is known to induce the sorting of Tat2p for early degradation (Beck et al., 1999). These findings suggest that regardless of where ubiquitylation occurs, it is required for delivery into the vacuolar lumen. This is consistent with the crucial role assigned to ubiquitin in directing the sorting of proteins to MVB luminal vesicles through recognition by several Vps proteins carrying Ub-binding domains (Katzmann et al., 2002; Donaldson et al., 2003). In contrast, it is not consistent with ubiquitin being sufficient signal in itself to govern the routing of permeases to the plasma membrane or the degradative pathway. The presence of a nonubiquitylatable ubiquitin moiety fused to Fur4p indeed results in some diversion for early degradation, but most of this Ub-permease chimera is still delivered to the cell surface. Similarly, the fusion of a ubiquitin moiety to the C-terminus of both mutant α-factor receptor Sts2p (Terrell et al., 1998) and a-factor receptor Ste3p (Roth and Davis, 2000) was not pointed out to prevent the delivery of these proteins to the plasma membrane. In contrast, for certain other proteins, such as the plasma membrane SNARE protein Snclp, a single fused ubiquitin appears to be sufficient to route the protein to the endosomes (Reggiori and Pelham, 2002). In all these chimeras, the attached ubiquitin lacked one or several lysines preventing its extension into a polyubiquitin chain and whether the same results would be obtained with chimeric multispanning proteins containing wild-type ubiquitin remains unanswered. Anyhow, intrinsic features independent of the ability to accept ubiquitin seem to be required for the efficient targeting to the vacuole of proteins normally destined for the plasma membrane.

We previously suggested that intracellular uracil binds to a site on the cytoplasmic domain of the permease, inducing a conformational change (Séron et al., 1999). Such binding probably occurs in the biosynthetic pathway in cells exposed to uracil because a mutant permease strongly deficient in binding was not sorted to the endosomal pathway. Uracil binding may favor a particular folding pattern. Various mechanisms may take into account differences in the folding patterns of membrane proteins. Interaction with various lipids is undoubtedly one of the most important of these mechanisms, and sorting may be achieved by favoring the segregation or exclusion of proteins in particular lipid microdomains. The yeast plasma membrane contains lipid raft microdomains enriched in sphingolipids and ergosterol (Bagnat et al., 2000), and it has recently been shown that Fur4p at the cell surface is associated with rafts (Dupre and Hагuena в-Tsapis, 2003). Furthermore, Fur4p has been found to be associated with detergent-resistant membranes in sec18 mutant cells, suggesting that it enters lipid rafts early in the secretory pathway (Dupre and Hагuena в-Tsapis, 2003). It is possible that the binding of uracil to the permease disturbs the association of the permease with lipid rafts, preventing its packaging into post-Golgi vesicles destined for the cell surface. It was very recently reported that the association of Tat2p with the detergent-insoluble membrane rafts is required for plasma membrane delivery and if lipid rafts are altered, as in erg6Δ cells, Tat2p is missorted to the vacuole (Umebayashi and Nakano, 2003). This mechanism is similar to that proposed for Pma1p, an H^+ATPase mutant that is misfolded at nonpermissive temperature and targeted directly to the vacuole rather than the cell surface. Wild-type ATPase has indeed been shown to be incorporated into lipid rafts after leaving the endoplasmic reticulum whereas the mutant displays impaired raft association that may prevent its sorting to the plasma membrane (Bagnat et al., 2001).

We present evidence that a mutant form of Fur4p with a very low affinity for uracil is not targeted to the vacuole in the presence of uracil. In contrast, in cases of defective ubiquitylation, Fur4p reaches the vacuole as it is mostly missorted to the vacuolar membrane. Thus, uracil, acting as a ligand, seems to be the principal element signaling the sorting of its specific permease from the secretory pathway to the vacuole and ubiquitylation appears to occur subsequently. Mutant forms of the Smf1p metal transporter with probable deficiencies in ligand-binding were also found not to be sorted from the Golgi apparatus to the degradative pathway (Liu and Culotta, 1999a). However, the potential involvement of ubiquitin-dependent events in the trafficking of this transporter was not reported. Our conclusions concerning the role of ubiquitylation in regulating the exocytosis of Fur4p somehow differ from the mechanism proposed for Gap1p and Tat2p trafficking in which impaired ubiquitylation results in rerouting to the cell surface, preventing
Figure 7. Presumed sorting steps for newly synthesized uracil permease. At the Golgi level, free permease (oval) is targeted to the cell surface (1), whereas uracil-ligated permease (rectangle) is sorted to the VPS pathway (2). An induced change in the conformation of Fur4p may modify its association with lipid rafts, accounting for its sorting to either plasma membrane or endosomes. On delivery to the endosome, uracil-ligated Fur4p may be efficiently ubiquitylated (●) and sorted to inward budding vesicles of MVB (3), resulting in its degradation in the vacuolar lumen or it may be maintained at the surface of MVB due to defective ubiquitylation, resulting in its becoming a permanent resident of the vacuolar membrane (4). It may be retrieved by the recycling pathway to the plasma membrane (5) if uracil is removed or in cases of underubiquitylation (○). If the sorting decision governed by uracil takes place only at the endosome level, the various possible routes from that location remain valid.

sorting to the vacuole (Helliwell et al., 2001; Soetens et al., 2001). In contrast, a very residual ubiquitylation seems sufficient for uracil-induced sorting of Fur4p to the endosomal pathway, although we cannot completely exclude that a process of rerouting to the plasma membrane occurs, in a small extent, for poorly ubiquitylated Fur4p. Figure 7 depicts a model fitting our results including the fact that a fused ubiquitin, acting in part as a signal, did increase the extent of permease degradation. Fur4p that has bound its ligand is sorted at the Golgi level, possibly by being excluded from rafts domains. Once it reaches the endosomes, Fur4p may still have different fates. It is efficiently segregated in internal membranes of the MVB for lumen release if ubiquitylation proceeds rapidly and extensively. Alternatively, in cases of poor ubiquitylation, the permease is not recognized by the protein complexes that bind ubiquitylated cargoes and remains on the external membrane of endosomes. From there, depending on uracil concentration, the permease may be delivered to the limiting membrane of the vacuole after the fusion of mature MVB with vacuoles or packaged into budding vesicles, which are thought to ensure transport in an endosome-to-plasma membrane pathway. Vacular delivery and export to the cell surface may compete kinetically. It should be noted that the ubiquitylation of the permease, which is crucial in determination of its fate, is a reversible process and that Rsp5p and Doa4p, which are involved in the ubiquitylation/deubiquitylation of Fur4p (Galan et al., 1996; Dupre and Haguaener-Tsapis, 2001), are both found in the vicinity of endosomes (Amerik et al., 2000; Wang et al., 2001). In addition, ligand binding, the extent of which may change rapidly, may in turn modify the distribution of Fur4p in particular lipid microdomains and/or the ability of this protein to accept ubiquitin. In this model, a low level of ligand, including low intracellular levels of uracil of catalobic origin, might lead to efficient sorting of the permease to the endosomal system, but the final extent of permease degradation would be controlled by the level of ubiquitylation. Some permease reaching the endosomes might escape targeting to the vacuole and reach the plasma membrane from the endosomal compartment. Our preliminary data indicate that permease transported away from the cell surface via the endocytic pathway might also escape vacuolar degradation by being recycled to the plasma membrane. A second decision step at the level of the endosomes, common to permeases from both the exocytic and endocytic pathways, would add flexibility to control of the amount of permease reaching the cell surface, giving a higher quality control of plasma membrane content.

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REFERENCES


Fur4p Sorting to the Endosomal System


