Uracil uptake by *Saccharomyces cerevisiae* is mediated by the FUR4-encoded uracil permease. The modification of uracil permease by phosphorylation at the plasma membrane is a key mechanism for regulating endocytosis of this protein. This modification in turn facilitates its ubiquitination and internalization. Following endocytosis, the permease is targeted to the lysosome/vacuole for proteolysis. We have previously shown that uracil permease is phosphorylated at several serine residues within a well characterized N-terminal PEST sequence. In this report, we provide evidence that lysine residues 38 and 41, adjacent to the PEST sequence, are the target sites for ubiquitination of the permease. Conserved substitutions at both Lys38 and Lys41 give variant permeases that are phosphorylated but fail to internalize. The PEST sequence contains potential phosphorylation sites conforming to the consensus sequences for casein kinase 1. Casein kinase 1 (CK1) protein kinases, encoded by the redundant YCK1 and YCK2 genes, are located at the plasma membrane. Either alone supports growth, but loss of function of both is lethal. Here, we show that in CK1-deficient cells, the permease is poorly phosphorylated and poorly ubiquitinated. Moreover, CK1 overproduction rescued the defective endocytosis of a mutant permease in which the serine phosphoacceptors were replaced by threonine (a less effective phosphoacceptor), which suggests that Yck activity may play a direct role in phosphorylating the permease. Permease internalization was not greatly affected in CK1-deficient cells, despite the low level of ubiquitination of the protein. This may be due to CK1 having a second counteracting role in endocytosis as shown by the higher turnover of variant permeases with unphosphorylatable versions of the PEST sequence.

Phosphorylation of proteins at Ser, Thr, and Tyr residues is one of the most frequent forms of posttranslational modification in eukaryotic cells, and it is linked to the control of a multitude of cellular functions. The completion of the *Saccharomyces cerevisiae* genome sequencing project made it possible to determine that there are 113 conventional protein kinase genes, corresponding to 2% of the total number of genes. Casein kinase 1 (CK1) protein kinases are ubiquitous and abundant Ser/Thr-specific protein kinases. The activity of this protein kinase family relies upon upstream acidic and/or phosphorylated amino acids for substrate recognition (1). The members of each subfamily differ in substrate selectivity and subcellular location. There are four CK1 proteins in *S. cerevisiae*, forming two essential gene pairs (2–5). One class of CK1 isoforms is encoded by the duplicate genes YCK1 and YCK2, (yeast casein kinase 1) (3, 4). Cells with only one of these genes are able to grow, but the loss of function of both is lethal. The similar and functionally interchangeable kinases encoded by these two genes are peripheral plasma membrane proteins and are most probably anchored to the plasma membrane by a carboxy-terminal isoprenyl modification (6). The location of the Yck2 isoform at the membrane is dynamic, with this protein showing cell cycle-specific localization to sites of polarized growth (7). Wild-type levels of Yck activity are required for efficient constitutive endocytosis of the pheromone receptor Ste3p (8). Casein kinase 1 activity has also been shown to be required for efficient phosphorylation, which allows ubiquitination and the subsequent internalization of the other yeast pheromone receptor, Ste2p, in response to a-factor binding (9). It is still unknown whether casein kinase 1 directly phosphorylates these proteins.

The degradation of a growing number of permeases has been reported to require endocytosis and subsequent transport of the permeases to the vacuole, the site of degradation (10). Many permeases are internalized constitutively from the plasma membrane, and increasing the rate of internalization is a major mechanism in yeast cells for controlling the transport of nutrients in response to stress or nutritional changes (see Ref. 11 and references therein). Although there is substantial evidence that at least some of these plasma membrane proteins are phosphorylated (12–16), the effects of such a modification on their stability and function have been little studied except for the multidrug transporter Pdr5p (17) and uracil permease (18). Uracil permease (Fur4p) is a multispanning membrane protein encoded by the *FUR4* gene (19). Newly synthesized uracil permease is delivered to the plasma membrane via the secretory pathway, and several of its serine residues are phosphorylated at the cell surface (16). The turnover of uracil permease is constitutive, and the rate of turnover is increased by stress conditions such as nutrient starvation, heat shock, excess of uracil, and the inhibition of protein synthesis (11, 20). A crucial step in plasma membrane protein targeting for internalization involves the initial recognition of endocytic cargo proteins by the ubiquitination machinery. Phosphorylation of uracil permease regulates its cell surface ubiquitination (18), a process.

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that is required for subsequent internalization (21). After internalization, the permease is targeted to the vacuole for proteolysis (20, 21). Permease ubiquitination is mediated by the essential Npl1p/Rsp5p ubiquitin-protein ligase, which is also required for the ubiquitination of other yeast transporters: Gap1p, the maltose permease, Tat2p, and Zrt1p (22–26). Modification with ubiquitin occurs on two target lysines carrying short chains of ubiquitin extended by Lys63 (27). Recent analysis of the mode of ubiquitination of the Ste2p and Ste3p receptors (28, 29) and several transporters, Fur4p, Gap1p, Zrt1p, and Pdr5p (17, 26, 27, 30), revealed a similar situation, with in each case a small number of target lysines accepting short chains of ubiquitin. We have shown that Fur4p internalization is dependent upon the permease being phosphorylated at several serine residues within a proline/glutamic acid/serine/threonine-rich stretch of amino acids known as the PEST sequence (18). We report in this paper the critical role of lysines 38 and 41, N-terminal to the PEST sequence, in ubiquitin-mediated internalization of the permease. The ubiquitination of Fur4p and Ste2p is regulated by their prior phosphorylation (9, 18). The kinases that phosphorylate Fur4p have yet to be identified. In this study, we report that CK1 activity affects the phosphorylation status of the permease, which is important for its subsequent ubiquitination and internalization.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Growth Conditions—The wild type strain 27061b (MATa ura3 trp1) was derived from strain S1278b (31); the congeneric wild-type strain LRB341 (MATa his3 leu2 ura3–52) and CK1-defective strains LRB343 (MATa his3 leu2 ura3–52 yck1–1; his3) and LRB346 (MATa his3 leu2 ura3–52 yck1 yck2–1) have been described elsewhere (32). The chromosome-encoded uracil permease is produced in very small amounts, and cells that produce the permease from multicopy plasmids were used for accurate measurement of permease activity and for the immunodetection of the protein (16). The multicopy plasmid, p195GF (2p GAL3 GAL-FUR4) (20), carries the FUR4 gene under the control of the GAL10 promoter. The multicopy plasmid, p195GF (2p TRP1 p-GAL-PGK), and its derivative, p1164, containing the YCK2 gene under the control of the PGK promoter, were kindly provided by Sanofi Recherche (Labe`ge, France). Yeast strains were transformed as described by Gietz et al. (33). Cells were grown at 30 °C (or 24 °C for thermosensitive strains) in minimal medium (YNB) containing 0.67% yeast nitrogen base without amino acids (Difco) and supplemented with appropriate nutrients. The carbon source was 2% glucose, or 4% galactose plus 0.05% glucose. One A100 unit corresponds to approximately 2 × 107 cells/ml.

Mutagenesis—Site-directed mutagenesis of FUR4 was performed either using the Stratagene Chameleon double-stranded site-directed mutagenesis kit as recommended by the supplier (K38R and K38R/ K41R)) or by overlap extension using the polymerase chain reaction for the other mutations (34). Mutated constructs were identified by testing for restriction site polymorphism introduced by the mutagenic primers and by sequencing. For each mutagenesis, two independent mutant plasmids were used to transform yeast, and two yeast transformants were analyzed for each mutant plasmid.

Measurement of Uracil Uptake—Uracil uptake was measured in exponentially growing cells as described previously (16). Yeast culture (1 ml) was incubated with 5 μM [14C]uracil (ICN) for 20 min at 30 °C and then quickly filtered through Whatman GFC filters, which were then washed twice with ice-cold water and counted for radioactivity.

Yeast Cell Extracts and Western Immunoblotting—Cell extracts were prepared, and proteins were analyzed by immunoblotting as described previously (20) using an antiserum directed against the last 10 residues of Fur4p (gift from R. Jund and M. R. Chevallier, Institute of Molecular and Cellular Biology, Strasbourg, France). Primary antibodies were detected with a horseradish peroxidase-conjugated anti-rabbit antibody detected by ECL chemiluminescence (Amersham Pharmacia Biotech).

Membrane Preparation—Yeast cells (80 A100 units) in the exponential growth phase were harvested by centrifugation in the presence of 10 mM sodium azide and used to prepare plasma membrane-enriched fractions as described previously (21). Membrane-bound proteins were analyzed by Western blotting as described previously (20).

FIG. 1. yck− mutant cells are impaired in the phosphorylation and ubiquitination of Fur4p at restrictive temperature. A, the PEST sequence of the permease is indicated with one-letter amino acid code. For each mutant, the serines replaced by alanines or the 19-residue deletion are indicated. B, parental (YCK) and mutant (yck) cells were transformed with p195GF or its derivative carrying the 5SA variant permease gene. Cells were grown at 24 °C to logarithmic phase with galactose as the carbon source and were collected after incubation for 30 min at 37 °C. Protein extracts were prepared, resolved by SDS-polyacrylamide gel electrophoresis (using a 10% resolving gel), and analyzed for uracil permease by Western immunoblotting using antibodies directed against the last 10 residues of Fur4p. C, parental (YCK) and mutant (yck) cells were transformed with p195GF or derivatives carrying the variant 5SA and ΔPEST permease genes. Cells were grown at 24 °C to logarithmic phase with galactose as the carbon source and were collected before or after incubation for 30 min at 37 °C. Ubiquitinated forms of the permease were more readily detected in plasma membrane-enriched fractions. Plasma membrane-enriched fractions were prepared, and volumes chosen on the basis of equivalent permease signals were resolved by SDS-polyacrylamide gel electrophoresis (using a 11% resolving gel) and analyzed for uracil permease by Western immunoblotting. The bracket indicates ubiquitin-permease conjugates. The molecular masses of the markers are given in kDa.

RESULTS

Loss of Yck Kinase Activity Affects the Phosphorylation Status of Uracil Permease and Dramatically Reduces Its Ubiquitination—Using thermosensitive secretory mutants, it has been shown that uracil permease (Fur4p) is phosphorylated after its arrival at the cell surface (16). The permease is phosphorylated mostly at a PEST-like sequence extending from position 42 to 59 at the hydrophilic N terminus of Fur4p (Fig. 1A). This sequence has been shown to be essential for uracil permease turnover (18). The permease-PEST sequence contains potential phosphorylation sites conforming to consensus sequences for various kinases including CK1 (Ser45 if the preceding serines, Ser42 and/or Ser43, are phosphorylated and Ser55 and Ser56) (Fig. 1A). Two other potential sites for casein kinase 1 phosphorylation are present in the hydrophilic extremities of the permease. One is located in the extreme N terminus of the protein (Ser25), and the other is in the extreme C-terminal
domain (Ser622). Both termini of the permease extend into the cytoplasm (35). Point mutation analysis has been used to investigate the role of several serines in both extremities of the permease, including those belonging to all the potential casein kinase 1 phosphorylation sites (18). By replacement of the corresponding serines with alanines, it was shown that only serines located in a PEST region extending from position 42 to 59 in the permease were phosphoacceptors for turnover of the protein. We used a mutant lacking the YCK1 gene and carrying a temperature-sensitive allele of the YCK2 gene, yck2–2 (hereafter referred to as yck2ts), to investigate the role of Yck activity in controlling the phosphorylation status of the permease.

First, we followed the cell surface delivery of uracil permease in wild-type and yckts cells. Permease was produced under control of the inducible GAL10 promoter by adding galactose to the medium of cells grown on lactate at 24 °C and shifted for 30 min to 37 °C. Uracil uptake was monitored for 2 h. The level and kinetics of permease activity were similar in wild-type and mutant cells (data not shown). Thus, CK1 deficiency did not delay the delivery of uracil permease to the plasma membrane. We then compared the phosphorylation status of Fur4p produced in wild-type (YCK), and yckts cells after incubation at restrictive temperature (Fig. 1B). Phosphorylation of the permease produced in wild-type cells gave bands that migrated more slowly on immunoblots and which down-shifted upon alkaline phosphatase treatment (16, 18). We compared the electrophoretic mobilities of the wild type (YCK) and mutant (yck) cells. The loss of Yck1p and Yck2p activities altered the banding pattern of Fur4p, with the loss of slower migrating bands and the appearance of faster migrating bands. A significant change in phosphorylation pattern was also observed (but to a lesser extent) if either of the kinase isoforms were altered independently (i.e. if the Fur4p banding pattern was analyzed in either yck2ts cells or yckts cells incubated at 24 °C (data not shown). Changing all five serine residues of the PEST region to alanines (5SA variant permease) resulted in a much lower level of phosphorylation of the permease in wild-type cells (Ref. 18 and Fig. 1B). The banding pattern of the 5SA variant was more affected than that of Fur4p produced in yckts cells, and it was not affected further by production in yckts cells. These results show that Yck activity plays a direct or indirect role in phosphorylating the Fur4p-PEST sequence and that Yck1p and Yck2p contribute equally to the phosphorylation of the protein. Other kinases may also phosphorylate the permease because phosphorylation of specific serine residues by Yck activity within the PEST region is required for the correct ubiquitination of the permease.

The Loss of Yck Activity Extends the Half-life of the Permease—To determine whether mutations in the YCK1 and YCK2 genes affected Fur4p internalization, we studied permease activity after the inhibition of protein synthesis in wild-type and yckts cells grown at 24 °C and shifted to 37 °C for 30 min (Fig. 2A). The addition of cycloheximide caused a sharp decrease in uracil uptake in wild-type cells incubated at 37 °C. The decrease in uracil uptake was less severe in yckts cells shifted to the restrictive temperature. The relative protection (1.5 times) against loss of permease activity indicated that the defect in Yck activity stabilized the transporter at the plasma membrane. Extracts from cells withdrawn at various times after the addition of cycloheximide were analyzed by immunoblotting (Fig. 2B). Significant protection against degradation was observed in yckts cells. Therefore, internalization and the subsequent degradation of the permease depend directly or indirectly on Yck kinase activity.

The defective phosphorylation and ubiquitination of Fur4p in yckts cells is reminiscent of the profile of modifications observed for a variant permease with three alanine for serine substitutions in the PEST sequence (18). Internalization of this variant was strongly inhibited (relative protection, 2.7 times). Conversely, Fur4p internalization was slightly affected in yckts cells (relative protection, 1.5 times) (Fig. 2A) despite being severely underphosphorylated and underubiquitinated (Fig. 1, B and C). This suggests that Yck proteins may also be required for the regulation of some components of the endocytosis machinery. This possibility was investigated by examining the fate in yckts cells of variant permeases insensitive to phosphorylation within the PEST sequence (Fig. 2C). We first investigated the fate of the 5SE variant, in which all of the serines in the PEST sequence are replaced by glutamic acids, mimicking the negatively charged phosphoserines (18). Although the pattern of ubiquitination (data not shown) and internalization (Fig. 2C) of the 5SE variant was identical to that of Fur4p in wild-type cells shifted to 37 °C, this protein was internalized faster in yckts cells shifted to the restrictive temperature (1.4 times faster). We then assessed the turnover of the 5SA variant permease. Due to the lack of phosphoacceptors in the PEST sequence, the 5SA variant is very poorly ubiquitinated (Fig. 1C). Consequently, if protein synthesis was inhibited, almost no decrease in uracil uptake was detected throughout the experiment in wild-type cells. Upon the addition of cycloheximide to yckts cells, a significant decrease in uracil uptake was observed, such that the half-life of the 5SA variant could be measured within the duration of the experiment. With the first step of phosphorylation bypassed (5SE) or irrelevant (5SA), the
accelerated decay of permease activity became evident. This indicates that Yck activity may negatively regulate a trans-acting component involved in the internalization process and suggests that the limited stabilization of Fur4p at the plasma membrane observed in yckts cells may be due to the counter-acting effect of this possible second mode of regulation by YCK.

We investigated whether some of the serine residues within the PEST sequence are the true phosphoacceptors for casein kinase 1, using a 5ST variant in which all the serines in the PEST sequence had been replaced by threonines (5ST variant). Serine kinases can also use threonine, but somewhat less efficiently, and we have previously observed that the 5ST variant protein is indeed poorly phosphorylated (18). First, we compared the phosphorylation status of Fur4p and 5ST variant proteins in wild-type cells with and without overexpression of the YCK2 gene encoding one of the two homologous casein kinase 1 proteins (Fig. 3A). The overproduction of Yck2p altered the banding pattern of Fur4p, with the loss of faster migrating bands and the appearance of slower migrating bands corresponding to higher levels of phosphorylation of the protein. Upon overproduction of Yck2p, the 5ST protein also underwent a slight decrease in mobility but to a lesser extent, consistent with the poor ability of threonine residues to be phosphorylated. We then compared the level of internalization of Fur4p and 5ST variant proteins in wild-type cells and cells overproducing Yck2p. The addition of cycloheximide caused a decrease in the uracil uptake of cells producing wild-type permease (Fig. 3B). Permease immunoreactivity decreased, consistent with the decrease in uracil uptake (Fig. 3C). Uracil uptake decreased less rapidly in cells containing the 5ST

**YCK2 Overexpression Corrects the Defective Turnover of a 5ST Variant Permease**—We investigated whether some of the serine residues within the PEST sequence are the true phosphoacceptors for casein kinase 1, using a 5ST variant in which all the serines in the PEST sequence had been replaced by threonines (5ST variant). Serine kinases can also use threonine, but somewhat less efficiently, and we have previously observed that the 5ST variant protein is indeed poorly phosphorylated (18). First, we compared the phosphorylation status of Fur4p and 5ST variant proteins in wild-type cells with and without overexpression of the YCK2 gene encoding one of the two homologous casein kinase 1 proteins (Fig. 3A). The overproduction of Yck2p altered the banding pattern of Fur4p, with the loss of faster migrating bands and the appearance of slower migrating bands corresponding to higher levels of phosphorylation of the protein. Upon overproduction of Yck2p, the 5ST protein also underwent a slight decrease in mobility but to a lesser extent, consistent with the poor ability of threonine residues to be phosphorylated. We then compared the level of internalization of Fur4p and 5ST variant proteins in wild-type cells and cells overproducing Yck2p. The addition of cycloheximide caused a decrease in the uracil uptake of cells producing wild-type permease (Fig. 3B). Permease immunoreactivity decreased, consistent with the decrease in uracil uptake (Fig. 3C). Uracil uptake decreased less rapidly in cells containing the 5ST
variant. The relative protection was 1.5 times. The 5ST mutation also protected the permease against degradation. Upon overproduction of Yck2p, no change in the pattern of internalization and degradation of Fur4p was observed despite the protein being overphosphorylated, suggesting that a wild-type level of phosphorylation of Fur4p is sufficient for optimal internalization. In contrast, a more rapid decrease in uracil uptake was observed in cells containing the 5ST variant, indicating an almost complete reversion to the wild-type internalization phenotype. If Yck2p was overproduced, the 5ST variant protein was less resistant to degradation. The decrease in the amount of immunodetected 5ST paralleled the decrease in the amount of wild-type permease detected. Therefore, the rate of turnover of the 5ST variant was greater in cells overproducing Yck2p and was similar to that of Fur4p. These results suggest that Yckp may be able to recognize the PEST region of Fur4p directly in vivo.

Lys-to-Arg Substitutions at Lys38 and Lys41 Abolish Ubiquitination of the Permease and Extend Its Half-life—We previously found that the deletion of the PEST region prevents ubiquitination of Fur4p, whereas a variant of the PEST sequence with Ser-to-Ala substitutions bound ubiquitin but less efficiently than did the wild-type permease (Ref. 18; Fig. 1C). The PEST sequence is flanked by two lysine residues (Lys41 and Lys60) (Fig. 1A), one of which was removed when the PEST sequence was deleted. This deletion may affect directly the targets of ubiquitination. Fur4p ubiquitination involved the formation of mono- to tetrabiquitin-permease conjugates with chains of ubiquitin extended through the Lys63 of ubiquitin (27). Only mono- and diubiquitinated permease conjugates were observed in cells lacking all homosominal copies of ubiquitin genes and producing a mutant ubiquitin carrying a Lys-to-Arg mutation on Lys63 as their sole source of ubiquitin. This strongly suggests that two lysine residues in permease serve as ubiquitin acceptor sites, but these specific lysine residues are not known. Lys41 (N-terminal to the PEST region) is included in an EXKSS motif, similar to the DXKSS core of the Ste2p SINNDAKSS motif. Therefore, we focused our analysis on Lys41, Lys60, and Lys38, which is close to the region implicated. We used site-directed mutagenesis to replace these lysines with conservative but nonubiquitinable arginine residues. Since we knew that two lysines were required for ubiquitination in Fur4p, we converted them individually or in combinations. The corresponding variant proteins were tested for their degree of ubiquitination and their stability. First, we followed the cell surface delivery of the Lys-to-Arg variant permeases produced from the inducible GAL10 promoter by adding galactose to the medium of cells grown on lactate. Uracil uptake was monitored for 2 h. Permease activity appeared with the same kinetics whatever the permease produced (data not shown). Thus, the mutations did not delay the delivery of uracil permease to the plasma membrane. We then analyzed the ubiquitination status of the Lys-to-Arg variants (Fig. 4A). Western blot analysis of membrane extracts from wild-type cells expressing Fur4p showed a ladder of four minor bands with slower mobilities than the main permease signal and corresponding to mono-, di-, tri-, and tetrabiquitin-permease conjugates. The same profile was observed in cells producing the K60R variant, suggesting that the lysine residue C-terminal to PEST is not a target for ubiquitination. In contrast, the addition of a second substitution, K41R, resulted in a profile with two slower migrating bands corresponding to mono- and diubiquitin conjugates. The same profile was observed with substitution of Lys38 alone. Simultaneous substitutions of all three lysines resulted in the complete loss of detectable ubiquitin conjugates. A similar pattern with ubiquitin conjugates undetectable was observed with simultaneous substitutions at Lys38 and Lys41. These results suggest that Lys38 and Lys41 are the two target sites for ubiquitination of the permease. They also indicate that tetrabiquitination of the permease occurs via the attachment of ubiquitin chains each two subunits in length to each of the two target lysines. We then tested the stabilization of the permease after inhibition of protein synthesis (Fig. 4). The addition of cycloheximide caused a drop in permease activity (Fig. 4B). Permease immunoreactivity declined in parallel to the drop in uracil uptake (Fig. 4C). In contrast, the decrease in uracil uptake was less severe in cells producing variants able to bind up to two ubiquitin moieties on either Lys38 or Lys41. These results suggest that Yckp may be able to recognize the PEST region of Fur4p directly in vivo.
in uracil uptake was detected, and the amount of immunodetected permease remained constant throughout the 2 h of the experiment. Similar results were obtained if the PEST region was deleted (18). These results are consistent with the lack of detection of ubiquitin conjugates in cells producing the K38R/K41R mutant permease.

**DISCUSSION**

Our results show that Yck activity is involved in the phosphorylation of the PEST region of the permease, which, in turn, facilitates the ubiquitination and subsequent endocytosis of the permease. We also observed that the lack of casein kinase 1, even if phosphorylation and ubiquitination are strongly impaired, has only a modest effect on internalization of the wild-type permease. In this report, we also provide evidence that phosphorylation of Fur4p at PEST serines leads to ligation of ubiquitin at nearby Lys38 and Lys41.

Many proteins are in vitro substrates of casein kinase 1. However, a correlation between CK1-specific phosphorylation sites and sites phosphorylated in vivo has been shown for very few proteins. The plasma membrane H+-ATPase was the first substrate of casein kinase 1 to be described in yeast, and a loss of Yck function impairs the regulation of H+-ATPase activity by glucose (36). It has recently been shown that phosphorylation of the ATP-binding cassette transporter, Pdr5p, at serine residues (one at least is present in a consensus site for casein kinase 1) is abolished by mutations in the YCK1 and YCK2 genes (17). The Yck proteins are also required for phosphorylation of the α-factor receptor, Ste2p, and phosphorylation of this receptor promotes α-factor receptor internalization (9). It is unknown whether casein kinase 1 directly phosphorylates Ste2p. The PEST sequence of Fur4p contains potential phosphorylation sites conforming to consensus sequences for various kinases including casein kinase 1. Two other potential CK1 sites are present in the permease, in the extreme N and C termini of the protein. Replacement of the corresponding serines by alanines showed that only serines located in a PEST sequence replaced by alanines was found to be poorly ubiquitinated.

A variant permease with all of the serines in the PEST sequence replaced by alanines was found to be poorly ubiquiti- nated and consequently stabilized at the cell surface. Here we show that the wild-type permease was also poorly ubiquitinated when produced in yckΔ cells. However, its stabilization at the plasma membrane (as shown by the remaining permease activity after inhibition of protein synthesis) was weak and not correlated with its impaired ubiquitination. Moreover, the loss of stability of unphosphorylatable PEST variants of the permease upon production in yckΔ cells provides evidence for negative control by Yck proteins of the internalization of the permease. The formation of endocytic vesicles at the plasma membrane requires a cytoskeleton structure that contains actin patches. Several mutant strains with defects in actin or actin-related proteins, namely act1, arp2, end3, and end4, have been shown to display defective internalization of Fur4p at their restrictive temperature (20, 21, 38). An effect of YCK-mediated phosphorylation on actin cytoskeleton organization may account for the effects of Yck deficiency on endocytic processes. Recently, two novel protein kinases, Prk1p (39) and Ark1p (40), were shown to be associated with and to regulate the cortical actin cytoskeleton in yeast. It is possible that YCK-encoded CK1 negatively controls the formation of endocytic vesicles by regulating the actin cytoskeleton or that YCK-encoded CK1 regulates Prk1p and Ark1p kinases itself. Panek et al. (8) showed that normal clathrin function is required in a yckΔ strain, and they suggested that Yck activity may be required for a pathway that is parallel to or intersects a clathrin-dependent pathway. However, clathrin plays a minor role in budding at the plasma membrane in yeast. Strains carrying the conditional clathrin heavy chain allele chc1α are only partially affected in the endocytosis of Ste2p, Ste3p, and the maltose transporter (41, 42), and Fur4p internalization has been shown to be unaffected in the same mutant cells, indicating that endocytosis can take place via clathrin-independent mechanisms (43). Thus, it seems unlikely that Yck proteins are involved in the internalization of ubiquitinated Fur4p via a clathrin-mediated control mechanism.

The central role of ubiquitin in the down-regulation of many cell surface proteins including uracil permease has led to the general view that ubiquitin serves as a signal for endocytosis in vivo. It was recently demonstrated that the three-dimensional structure of ubiquitin is important in the endocytosis of Ste2p (45). These data suggest that a specific receptor that recognizes ubiquitin may be involved in the internalization process. The affinity of such a putative receptor for ubiquitinated proteins may be correlated to the number of ubiquitin moieties. This study demonstrates that the replacement of two adjacent lysines at positions 38 and 41 by arginine abolishes both the ubiquitination and internalization of Fur4p. This suggests that these two residues are the targets for ubiquitin attachment. With either one of the two lysine residues mutated, the tri- and tetraubiquitin-permease conjugates are not detected. These observations are consistent with earlier data suggesting that Fur4p is branched with short chains of ubiquitin (27) and demonstrate that these chains are built with no more than two ubiquitin moieties. Moreover, the less efficient internalization of the corresponding variant permeases suggests that monodiubiquitination of the permease is not sufficient for optimal internalization. The rate of internalization observed in variants with one of the two target lysines replaced with arginine (this study) was similar to that obtained in cells with UbK63R as the sole source of ubiquitin (27). Thus, the rate of internalization of the permease is similar whether it is conjugated with two ubiquitin moieties as a single chain of two ubiquitins or as two single ubiquitins attached at two different

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2 A. Gratias, personal communication.
lysines. This is consistent with the results obtained by L. Hicke indicating that monoubiquitination of the receptor, Ste2p, is sufficient to support its internalization (28).

Little is known about the sequence motifs responsible for the internalization of yeast cell surface proteins. A SINNDAKSS sequence was found to be necessary and sufficient for endocytosis of a C-terminal truncated Ste2p (46). Within this sequence, the Lys\textsuperscript{337} residue was found to be necessary for ubiquitination and to be one of the major ubiquitination sites in the full-length Ste2p receptor (28). A motif similar to the SINNDAKSS sequence, DAKTI, was identified in Ste6p (47). This motif is within a 52-amino acid-long acidic region required for ubiquitination of the protein. The DAKTI motif seems to be an important part of the signal, although a Lys-to-Arg mutation within it had only a minor effect on Ste6p turnover (suggesting the involvement of additional lysine residues). Two lysine residues included in similar sequences, ERKS and EYKSS, were shown to be essential, together with three other lysine residues included in similar sequences, ERKS and EYKSS, were shown to be essential, together with three other lysine residues included in similar sequences, ERKS and EYKSS, were shown to be essential, together with three other lysine residues included in similar sequences, ERKS and EYKSS, were shown to be essential, together with three other lysine residues included in similar sequences, ERKS and EYKSS, were shown to be essential, together with three other lysine residues included in similar sequences, ERKS and EYKSS, were shown to be essential, together with three other lysine residues included in similar sequences, ERKS and EYKSS, were shown to be essential, together with three other lysine residues included in similar sequences, ERKS and EYKSS, were shown to be essential, together with three other lysine residues included in similar sequences, ERKS and 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