Casein kinase I controls a late step in the endocytic trafficking of yeast uracil permease

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Summary

The modification of yeast uracil permease by phosphorylation at the plasma membrane is a key mechanism for regulating transporter endocytosis. Uracil permease is phosphorylated at several serine residues within a well characterized PEST sequence. The phosphorylation of these residues facilitates the ubiquitination and internalization of the permease. Following endocytosis, the permease is targeted to the lysosome/vacuole for proteolysis. We have shown that in casein kinase 1 (CK1)-deficient cells, the permease is poorly phosphorylated, poorly ubiquitinated and that Yck activity may play a direct role in phosphorylating the permease. We show here that CK1-deficient cells accumulated permease that was subjected to endocytosis in an internal compartment on its way to the vacuole. Uracil permease, produced as a fusion protein with green fluorescent protein in CK1-deficient cells, was detected in dots adjacent to the vacuole. These dots probably correspond to the late endosome/prevacuolar compartment because they were partially colocalized with the Pep12p marker. This accumulation was abolished by mutations affecting the adaptor-related complex, AP-3. The CPY and ALP pathways to the vacuole were both unaffected in CK1-deficient cells. Our analysis provides the first evidence that CK1 is important for the delivery of proteins to the vacuole after endocytosis.

Key words: Endocytosis, *Saccharomyces cerevisiae*, Transporter, Late endosome, Vacuole, Kinase, CK1, AP-3

Introduction

Yeast are able to respond to changes in nutrient status or ligand binding by rapidly removing or downregulating plasma membrane proteins, such as permeases and receptors, by endocytosis, followed by degradation in the lysosome/vacuole. This process is an important mechanism in cellular regulation. Plasma membrane proteins targeted to the vacuole for degradation are first sorted to vesicles that deliver material to early endosomes. Over time, the early endosomes move towards the center of the cell to form late endosomes. The major transport route from the late Golgi compartment to the vacuole also delivers carboxypeptidase Y (CPY) and many other vacuolar proteins to the late endosome (Stack et al., 1995). In yeast, as in mammals, invagination of the limiting membrane of the late endosome leads to the formation of internal vesicles, giving rise to the multivesicular body (MVB). The mature MVB, loaded with internal vesicles, fuses directly with the lysosome/vacuole (Furtter et al., 1996; Odorizzi et al., 1998). In yeast, a clathrin adaptor (AP)-related complex, AP-3, also directs the vacuolar membrane protein alkaline phosphatase (ALP) and two vacuolar SNAREs, Vam3p and Nyv1p, to the vacuole via a pathway that is independent of the well studied CPY pathway (Cowles et al., 1997; Piper et al., 1997; Reggiori et al., 2000).

A crucial step in plasma membrane protein targeting for internalization and downregulation in yeast involves the initial recognition of endocytic cargo proteins by the ubiquitination machinery (Hicke, 1999). It has been suggested that phosphorylation is a prerequisite for the ubiquitination of uracil permease (Marchal et al., 1998), the α-factor pheromone receptor (Hicke et al., 1998) and the **a**-factor pheromone receptor (Feng and Davis, 2000), and therefore for the endocytosis of these molecules. Uracil permease is phosphorylated at the cell surface (Volland et al., 1992), principally at a PEST sequence extending from positions 42 to 59 at the hydrophilic N-terminus of the permease (Marchal et al., 1998). Phosphorylation of the uracil permease PEST sequence regulates the cell surface ubiquitination of this protein (Marchal et al., 1998), which is required for subsequent internalization (Galan et al., 1996). The two lysyl residues that map to the region N-terminal to the PEST sequence provide redundant acceptor sites for ubiquitination (Marchal et al., 2000). After internalization, the permease is targeted to the vacuole for proteolysis (Galan et al., 1996; Volland et al., 1994).

There are four CK1 proteins in *Saccharomyces cerevisiae*, corresponding to two essential gene pairs. One class of CK1 isoforms is encoded by the duplicate genes *YCK1* and *YCK2* (Yeast casein kinase 1) (Robinson et al., 1992; Wang et al., 1992). The similar and functionally interchangeable kinases encoded by these two genes, Yck1p and Yck2p appear to be required for phosphorylation of the Fur4p PEST-like sequence (Marchal et al., 2000), the α-factor receptor, Ste2p (Hicke et al., 1998), and the **a**-factor receptor, Ste3p PEST-like sequence (Feng and Davis, 2000). Low levels of Ste3p internalization are observed in cells defective for casein kinase 1 activity, but

internalization is partially restored by mutations in any of the genes encoding the subunits of AP-3 (Panek et al., 1997). The four AP-3 subunits (including Apm3p, the μ chain of the adaptor-related complex) were initially identified by loss-of-function suppressor mutations permitting the growth of *yck*^{ts} cells at restrictive temperature (Panek et al., 1997). Mutations in any of the four genes result in the same phenotype-suppression of loss of Yck activity. Yck1p-Yck2p are peripheral plasma membrane proteins and are most probably anchored to the plasma membrane by C-terminal isoprenyl modification (Vancura et al., 1994). Paneck et al. suggested that Yck-mediated phosphorylation may be required for some aspects of vesicle trafficking at the plasma membrane and that the AP-3 complex may be involved in this process (Panek et al., 1997). In this study, we identified a new function of the redundant type I casein kinases, Yck1p-Yck2p, in the Fur4p endocytic pathway. Yck defects conferred retention of uracil permease upon the late endosome/prevacuolar compartment but did not affect the sorting of CPY or ALP pathway cargoes to the vacuole. This accumulation was abolished in an AP-3-deficient background.

Materials and Methods

Yeast strains, plasmids and growth conditions

The congenic strains SLHR44 (*MAT**a** bar1 yck1-Δ::ura3 yck2-ts leu2 ura3-52 his3*) and SLHR46 (*MAT**a** bar1 yck1-Δ::ura3 yck2-ts apm3-delta::HIS3 leu2 ura3-52 his3*) and strains SL1463 (*MAT**a** leu2 ura3-52 his3-Δ200 trp1 GAL2*) and SL1653 (*MAT**a** leu2 ura3-52 his3-200 trp1 GAL2*) were kindly provided by Sandra Lemmon. RPY2 (*Matα vps27-ts leu2-3,112 ura3-52 his4-519 ade6 gal2*) and the parental strain RPY10 (*Matα leu2-3,112, ura3-52 his4-519 ade6 gal2*) were kindly provided by Tom Stevens. The other strains used are W303 (*Matα ura3-1 his3-11-15 leu2-3-112 trp1-1 can1-100*), IW-6A (*Matα ura3-1 his3-11-15 leu2-3-112 trp1-1 pep4-3*) (Volland et al., 1994) and SEY5076 (*Matα ura3-52 leu2-3-112 sec7-1*) (Emr et al., 1984). The chromosome-encoded uracil permease is produced in very small amounts and cells producing the permease from plasmids were used for accurate permease activity measurement and for immunodetection of the protein (Volland et al., 1992). The centromeric plasmid pFL38gF (ars-cen, *URA3 pGAL-FUR4*) (Séron et al., 1999), which carries the FUR4 gene under control of the GAL10 promoter, was used in this study. To determine the distribution of uracil permease in living cells, we constructed a plasmid encoding GFP fused to Fur4p. Fur4p was detected by tagging the 3′ end of the *FUR4* gene with a DNA fragment encoding the variant green fluorescent protein GFP (S65G, S72A), which displays stronger fluorescence than the original GFP (Cormack et al., 1997). Fusion PCR was used to generate a construct in which the part of the FUR4 coding sequence corresponding to the C-terminus, excluding the stop codon, was fused in-frame to the GFP coding sequence. The PCR product was digested with *Hpa*1 and *Bam*H1 and inserted into pFL38gF, giving pFL38gF-GFP. *FUR4-GFP* was introduced by subcloning into the multicopy plasmid p195gf (2μ, URA3 pGAL-FUR4) (Volland et al., 1994) in place of *FUR4* to give p195gF-GFP.

Yeast strains were transformed as described by Gietz et al. (Gietz et al., 1992). 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 2% galactose plus 0.05% glucose. For ALP experiments, exponentially growing cells were harvested, washed and resuspended at the same density in fresh medium depleted of inorganic phosphate (Rubin, 1973) for three hours before use. One A_{600} unit corresponded to approximately 2 x 10^7 cells per ml.

Uracil uptake

Uracil uptake was determined in exponentially growing cells as previously described (Volland et al., 1992). Yeast culture (1 ml) was incubated with 5 μM [^{14}C]uracil (I.C.N.) for 20 seconds at 30°C, then quickly filtered through Whatman GF/C filters. The filters were then washed twice with ice-cold water and their radioactivity was counted.

Yeast cell extracts and western immunoblotting

Cell extracts were prepared and proteins analyzed by immunoblotting as previously described (Volland et al., 1994) using antisera directed against peptides corresponding to amino acids 15-30 and the last 10 residues of Fur4p (gifts from R. Jund and M.R. Chevallier, IBMC, Strasbourg, France) or against GFP or ALP. Primary antibodies were detected with a horseradish-peroxidase-conjugated anti-rabbit (or anti-mouse for GFP and ALP primary antibodies) IgG secondary antibody and ECL (enhanced chemiluminescence; Amersham).

Pulse-chase labeling and immunoprecipitation

Cells were grown in YNB medium with glucose as a carbon source to an A_{600} of 1.0 (2 x 10^7 cells/ml). They were collected by centrifugation, resuspended in fresh medium at an A_{600} of 8.5 and incubated for 30 minutes at 37°C. Cells were labeled by incubation for 5 minutes with 150 μCi [^{35}S]-translabel (Amersham) per ml culture, chased with 10 mM unlabeled methionine plus cysteine. Aliquots of the culture (0.3 ml) were removed at various times during the chase, and cell extracts prepared by lysis with 0.2 M NaOH for 10 minutes on ice. Trichloroacetic acid was added to a final concentration of 5%, and the samples were incubated for an additional 10 minutes on ice. Proteins were processed for immunoprecipitation as previously described (Volland et al., 1992), except that they were heated for four minutes at 95°C. Immunoprecipitated proteins were separated by SDS-PAGE in 10% gels and were treated for fluorography as previously described (Volland et al., 1992).

Fluorescence and immunofluorescence microscopy

Cells were grown to mid-exponential growth phase in galactose minimal medium. To follow GFP fluorescence, 5×10^6 cells were collected by centrifugation in the presence of 10 mM sodium azide and resuspended in 50μl Citifluor plus sodium azide. Indirect immunofluorescence microscopy was performed on formaldehyde-fixed cells as follows: cells were collected and resuspended at the same density in spheroplast buffer (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4) and were fixed by incubation for 45 minutes at room temperature by adding formaldehyde directly to the medium to a final concentration of 3.7%. Cells were collected, resuspended at a final density of 2×10^7 cells per ml and incubated for eight minutes in 0.1 M Tris-HCl, pH 9.4 in the presence of 10mM DTT. They were then washed in spheroplast buffer. Cells were transferred to an Eppendorf tube and were converted to spheroplasts by incubation with 0.2 mg/ml Zymolyase 20 T (Seikagaku Corp., Tokyo, Japan) in spheroplast buffer. Spheroplasts were pelleted and washed twice with spheroplast buffer and were then resuspended in 150 μl of spheroplast buffer. Spheroplasts were spotted onto polylysine-coated slides and left in air to dry for five minutes. Slides were immersed in 0.1% Triton X-100 at 4°C for 10 minutes and then in PBS at 20°C for 30 seconds. The slides were rinsed with PBS and then incubated for one hour at room temperature with anti-Vat2p antibody or anti-Pep12p antibody diluted 1:50 in PBS supplemented with 1% BSA. The slides were washed three times for three minutes each with PBS and were then incubated with rhodamine-conjugated goat anti-mouse IgG at a dilution of 1:250 for 30 minutes at room temperature. The slides were mounted with Citifluor. Slides were examined under a Leitz microscope equipped with epifluorescence optics. Images were

acquired directly with a Princeton cooled CCD camera equipped with the Metaview Imaging System.

Antibodies

The two polyclonal anti-Fur4p antibodies were obtained from R. Jund and M. R. Chevallier (I.B.M.C., Strasbourg, France). Monoclonal anti-GFP antibody was purchased from Boehringer Mannheim. Polyclonal anti-CPY antibody was provided by H. Riezman. Monoclonal anti-Pep12p antibody was provided by T. Stevens. Monoclonal anti-Vat2p and anti-ALP were purchased from Molecular Probes. Rhodamine-conjugated goat anti-mouse IgG antibody was purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.

Results

Yck protein kinases are required for the degradation of internalized permease, and the loss of APM3 function overcomes this requirement

Uracil permease (Fur4p) is phosphorylated at the cell surface (Volland et al., 1992), principally at a PEST sequence located at the hydrophilic N-terminus of the permease (Marchal et al., 1998). This modification in turn facilitates the ubiquitination and subsequent internalization of the permease. In a previous study, we reported that CK1 activity affects the phosphorylation of the PEST region of the permease (Marchal et al., 2000). In CK1-deficient cells, the permease displays low levels of phosphorylation and ubiquitination. Constitutive internalization of the **a**-factor receptor, Ste3p, was also decreased in CK1-deficient cells (Panek et al., 1997). Mutation in any gene encoding a subunit of the adaptor-related complex AP-3 partially rescued this internalization defect. We used a mutant lacking the *YCK1* gene and carrying a temperature-sensitive allele of the *YCK2* gene *yck2-2* (hereafter referred to as *yck^{ts}*) to investigate further the role of Yck1p-Yck2p in endocytic trafficking of the permease (the presence of either of these kinases alone supports growth, but the loss of function of both is lethal). We investigated whether mutation of the AP-3 adaptor abolished the endocytic defect of Fur4p associated with mutations in the *YCK1* and *YCK2* genes by determining the rate of Fur4p endocytosis in *yck^{ts} apm3*-Δ double mutant cells. In the experiments presented here, we analyzed the endocytic trafficking of the permease, tagged at its C-terminus with the green fluorescent protein (GFP). First, we showed that the fusion protein was a functional transport protein with a uracil transport capacity similar to that of the wild-type protein (*Km* app: 9.9±1.7 μM versus 12.8±1.5 μM for the native protein). Second, we followed, under induction conditions, the appearance of uracil permease activity (Fig. 1A) and Fur4p-GFP fluorescence (Fig. 1B) in cells grown on lactate, then fed galactose at time zero of the experiment. Uracil transport was detectable 20-40 minutes after induction, whatever the gene expressed (*FUR4* or *FUR4-GFP*), indicating that the tag did not impair plasma membrane delivery of the permease. Thereafter, permease activity was stronger in the cells that produced the fusion protein. Addition of the tag resulted in an increase in permease activity soon after plasma membrane delivery of the protein. This probably resulted from partial stabilization of the chimera against basal endocytosis, which seemed to begin as soon as the protein was delivered to the plasma membrane (data not shown). Fluorescence was detected as punctuate staining, which was most intense in the buds after induction for a short period of time (t=40-80 minutes) (Fig. 1B). This staining probably corresponded to secretory vesicles, consistent with the known polarization of these vesicles to the bud. Cells with larger buds (t=100 minutes) displayed plasma membrane staining both in the buds and in the regions of the mother cells close to the buds. Hence, plasma membrane delivery can be followed by monitoring permease activity or GFP fluorescence. These data indicate that the addition of the tag did not impair plasma membrane delivery of the protein but may have delayed its internalization. It was therefore essential in subsequent experiments to follow the fate of either Fur4p or Fur4p-GFP in the different cells.

We investigated the turnover of plasma membrane uracil permease at a restrictive temperature in *yck^{ts}* and *yck^{ts} apm3*-Δ mutant cells, comparing it with that in wild-type and *apm3*-Δ cells (Fig. 2). We first studied permease activity after the inhibition of protein synthesis (Fig. 2A). Cells in the exponential growth phase, grown on galactose, were transferred to restrictive temperature and incubated for 20 minutes. *De novo* synthesis of Fur4p was prevented by incubation with 2% glucose for a further 10 minutes at 37°C before cycloheximide treatment. The addition of cycloheximide caused a sharp decrease in uracil uptake in wild-type cells incubated at 37°C. An identical sharp decrease in uracil uptake was observed in *apm3*-Δ cells. Thus, the deletion of *APM3* alone had no effect on Fur4p internalization. As previously described (Marchal et al., 2000), the decrease in uracil uptake was less severe in *yck^{ts}* cells shifted to restrictive temperature. The relative protection (a factor of 1.5) against

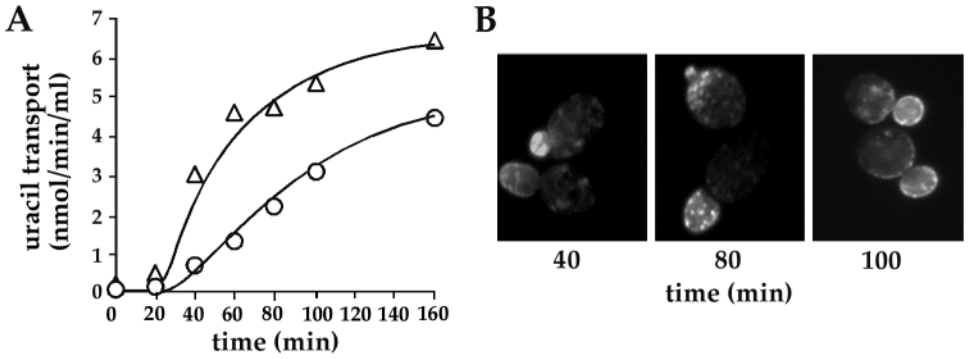

Fig. 1. The GFP-tagged uracil permease is correctly delivered to the plasma membrane. Wild-type cells transformed with p197gF or p195gF-GFP (A) or pFl38gF-GFP (B) and grown on lactate as a carbon source were induced by growth for two hours on galactose medium. (A) Uracil permease activity was followed at various times after addition of galactose. (O) and (Δ) corresponded to cells transformed with p197gF and p195gF-GFP respectively. (B) Cell fluorescence was followed at various times after addition of galactose. Fluorescence staining was only observed in budding cells. Cells were viewed with a FITC filter set.

loss of permease activity indicated that the defect in Yck activity stabilized the transporter at the plasma membrane. The decrease in uracil uptake of *yck*^{ts} *apm3*-Δ cells was similar to that of *yck*^{ts} cells. This suggests that the deletion of *APM3* in a *yck*^{ts} background does not abolish the internalization defect observed in *yck*^{ts} mutant cells. We also assessed permease endocytosis by following the internalization of untagged-Fur4p and obtained similar results (data not shown).

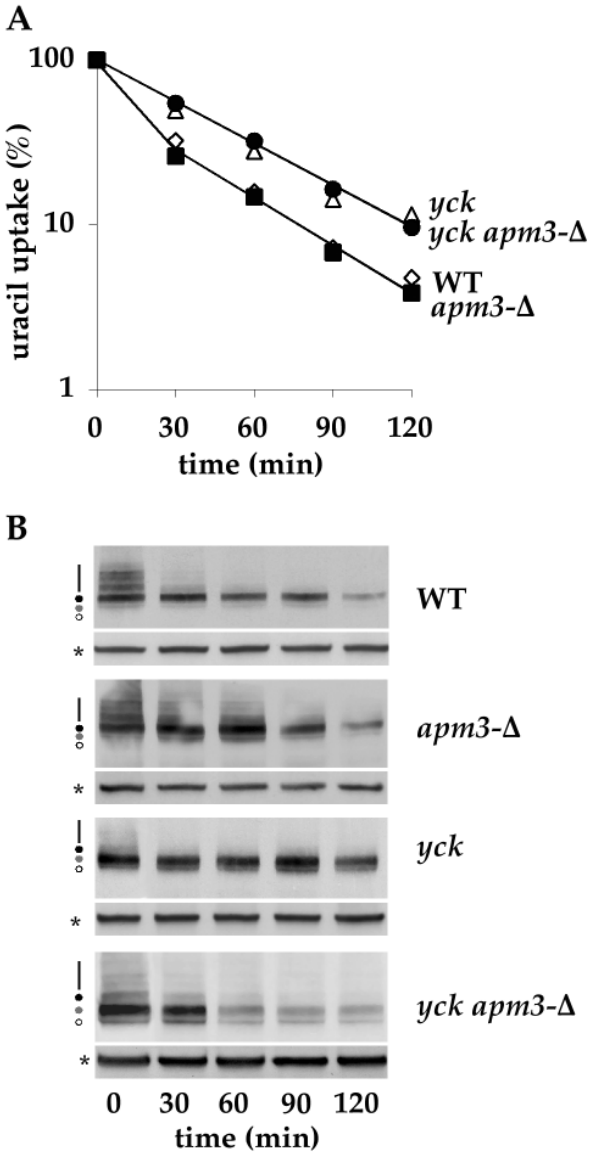

Fig. 2. Internalization and degradation of uracil permease are impaired in *yck*^{ts} cells. Wild-type, *apm3*-Δ, *yck*^{ts} and *yck*^{ts} *apm3*-Δ cells transformed with pFL38gF-GFP were grown to the exponential growth phase at 24°C with galactose as a carbon source. Cells were incubated at 37°C for 20 minutes. Adding glucose and incubating for 10 minutes at the same restrictive temperature stopped the synthesis of Fur4p-GFP. Cycloheximide (100 μg/ml) was then added. (A) Uracil uptake (permease activity) was measured at 37°C at various times after the addition of cycloheximide. The results are expressed as a percentage of the initial activity. (B) Protein extracts were prepared at the times indicated after the addition of cycloheximide. Aliquots were analyzed for uracil permease by western immunoblotting using anti-GFP antibody. *, blots were reprobed with anti-Pgk antibody to provide loading controls. **I**, mostly permease conjugated with ubiquitin. Open, gray and black circles corresponded to different levels of phosphorylation of the permease with the faster migrating bands corresponding to the lowest level of phosphorylation of the permease.

Extracts from cells withdrawn at various time points after the addition of cycloheximide were analyzed by immunoblotting (Fig. 2B). As previously described (Marchal et al., 2000), the loss of Yck activity altered the banding pattern of Fur4p, resulting in the loss of bands corresponding to proteins of low mobility and the appearance of bands corresponding to more mobile proteins, corresponding to a change in the phosphorylation pattern of the protein. Ubiquitination of the permease requires the PEST region of the permease to be phosphorylated (Marchal et al., 1998; Marchal et al., 2000), and the loss of Yck activity also reduced the level of permase ubiquitination. Significant protection against degradation was observed in *yck*^{ts} cells. Therefore, the internalization and subsequent degradation of the permease depended on Yck kinase activity. Comparison of the rate of internalization of the permease in the *yck* background with its rate of degradation indicated that turnover lagged substantially behind internalization. We found that 50% of the permease was internalized during the initial 30 minutes of cycloheximide treatment, but very little of the protein was degraded during the experimental time course. Therefore, degradation of the permease depends directly or indirectly on Yck protein kinases. In contrast, significantly more degradation of Fur4p occurred over the time period studied in *yck*^{ts} *apm3*-Δ cells than in *yck*^{ts} cells (or even in WT cells), whereas the deletion of *apm3* alone had no effect on Fur4p stability. Therefore, the loss of function of *APM3* rescued the degradation deficiency associated with the *yck*^{ts} mutation.

Internalized permease accumulates in perivacuolar structures in *yck*^{ts} mutant cells

We used fluorescence microscopy to identify the intracellular sites of permease accumulation in *yck*^{ts} mutant cells. We compared induced endocytosis of the GFP-tagged permease in WT, *apm3*-Δ, *yck*^{ts} and *yck*^{ts} *apm3*-Δ cells after the addition of cycloheximide at restrictive temperature (Fig. 3). Exponentially growing cells were transferred to restrictive temperature and incubated, as described above, before cycloheximide treatment. Therefore, most of the permease had at least reached the cell surface in all types of cell at time zero of the experiment. On the basis of our kinetic analysis of turnover (Fig. 2), we followed the fate of the internalized permease over two hours, testing every 30 minutes.

In the wild-type strain, Fur4p-GFP was detected as punctate staining at the cell periphery, as is often described for plasma membrane proteins (Hicke et al., 1997; Roberg et al., 1997). Punctate staining was also detected in the cytoplasm at time zero of the experiment (Fig. 3). This intracellular location was probably due to basal permease endocytosis, which was accelerated by shifting the cells to a non-permissive temperature. Cycloheximide treatment for 30 minutes led to the simultaneous disappearance of the fluorescent signal at the surface and the appearance of intracellular punctate staining. The strongest fluorescence was observed adjacent to the vacuole, in a punctate pattern, suggesting that this staining corresponded to a compartment previously described as the

pre-vacuolar compartment or the late endosome (Hicke et al., 1997; Piper et al., 1995). Punctate staining at the periphery of the cell, away from the vacuole, was less intense. These punctate structures may correspond to the previously described early endosome compartment (Hicke et al., 1997). Consistent with this designation of early and late endosomes, a shorter duration of cycloheximide exposure led to preferential staining at the periphery rather than around the vacuole (data not shown). After one hour of treatment, all the fluorescence colocalized with the vacuole. This staining became diffuse and less bright after two hours of treatment, indicating that the fusion protein was subject to degradation. Consistent with these observations, uracil uptake decreased to almost zero after two hours of treatment (Fig. 2A), and Fur4p-GFP was almost undetectable on immunoblots, whatever the antibody used to detect the fusion protein (antibodies directed against the hydrophilic N- or C-termini of the permease or against GFP) (Fig. 2B) (Data not shown). The *apm3* deletion alone had no effect on the targeting and delivery of Fur4p to the vacuole, and it neither delayed nor accelerated the process of degradation (Fig. 3).

In *yck*^{ts} mutant cells, the fluorescent permease was mostly detected as punctate staining at the cell periphery and as dots beneath the cell surface at time zero of the experiment (Fig. 3). The observed difference between *yck*^{ts} and *YCK* cells in intensity of Fur4p-GFP staining at the cell periphery is consistent with the slower permease internalization observed at restrictive temperature in *yck*^{ts} than in *YCK* cells (Fig. 2A). As expected from the kinetic analysis, 30 minutes after the addition of cycloheximide, the plasma membrane signal began to disappear gradually, and punctate staining, probably corresponding to endosomes, was transiently detected throughout the cytoplasm of *yck*^{ts} mutant cells. Over time, these punctuate structures moved into the area adjacent to the vacuole, suggesting that they were late endosomes. Punctate staining of lower intensity was observed in the cell periphery, away from the vacuole. After two hours of treatment, most of the fluorescent permease was still retained in compartments adjacent to the vacuole. No fluorescence was observed within the vacuole. Consistent with this observation, large amounts of Fur4p-GFP were still detected on immunoblots after two hours of cycloheximide treatment (Fig. 2B).

Fur4p-GFP was detected essentially as punctate staining at the cell periphery of *yck*^{ts} *apm3*-Δ mutant cells and as dots just beneath the surface at time zero of the experiment (Fig. 3). Thus, *apm3*-Δ, in combination with the *yck*^{ts} mutation, did not affect the rate of internalization of the permease. This is consistent with the results of the kinetic experiment presented in Fig. 2A. Treatment with cycloheximide for 30 minutes resulted in the simultaneous disappearance of the fluorescent signal at the surface and appearance of punctate staining within the cell. The most intense staining was observed adjacent to the vacuole. After treatment for one hour, all the fluorescence colocalized with the vacuole. This staining completely disappeared after two hours of treatment, indicating that the fusion protein was degraded in the vacuole. The disappearance

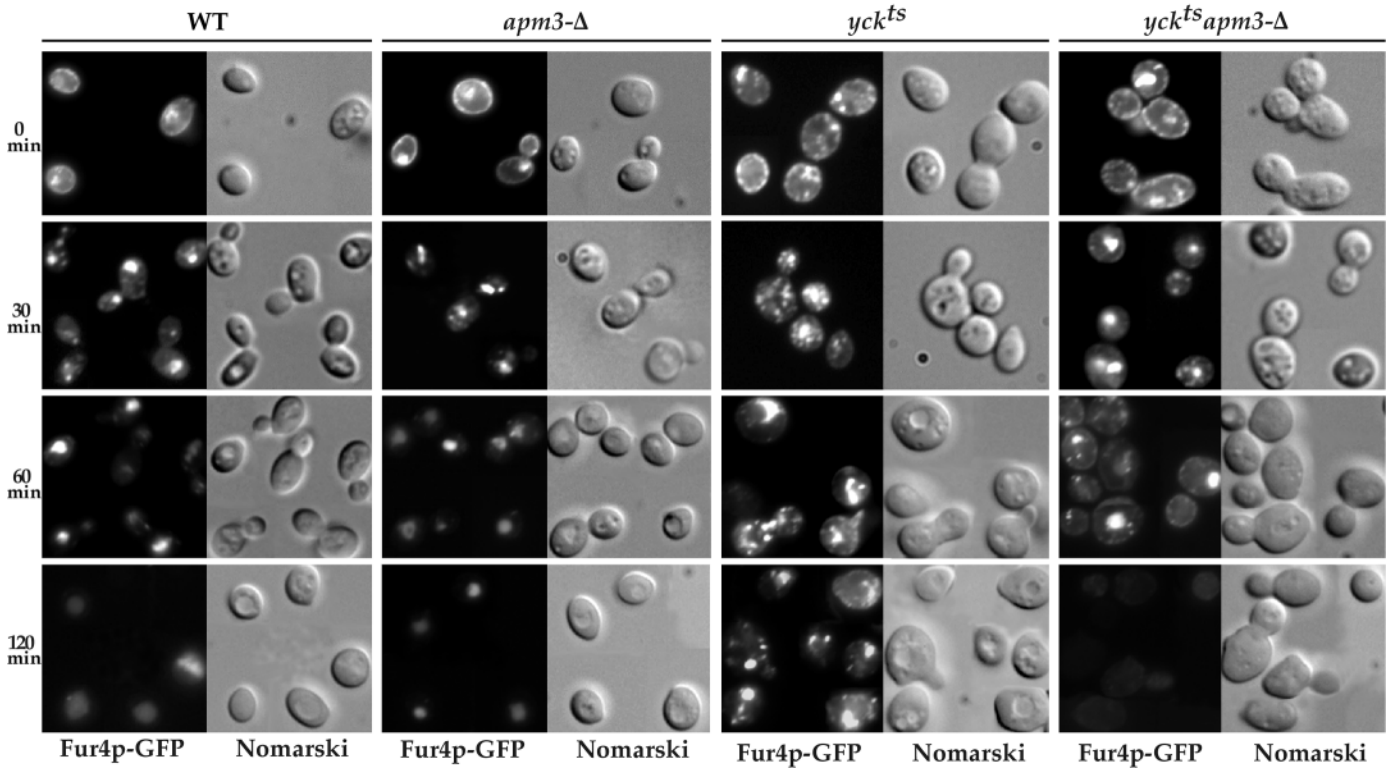

Fig. 3. Nomarski optics and distribution of Fur4p-GFP fluorescence in wild-type (WT), *apm3*Δ, *yck*^{ts} and *yck*^{ts} *apm3*-Δ mutant cells. Wild-type, *apm3*Δ, *yck*^{ts} and *yck*^{ts} *apm3*-Δ cells transformed with pFL38gF-GFP were grown to the exponential growth phase at 24°C in galactose medium. Cells were incubated at 37°C for 20 minutes. The synthesis of Fur4p-GFP was stopped by adding glucose and incubating for 10 minutes at the same restrictive temperature. Cells were observed at various times after the addition of cycloheximide at 37°C. Cells were viewed by Normarski optics or with a FITC filter set.

of staining seemed to be even more rapid than in the wild-type and *apm3*-Δ strains, possibly due to differences in genetic background. Consistent with this, Fur4p-GFP was not detected over the same time period on immunoblots of *yck*^{ts} *apm3*-Δ cells, regardless of the antibodies used to detect the fusion protein (antibodies directed against the hydrophilic N- or C-termini of the permease or against GFP) (Fig. 2B) (data not shown). So, the loss of the fluorescent signal in *yck*^{ts} *apm3*-Δ cells after two hours of treatment actually resulted from degradation of the permease fusion protein rather than from cleavage of the GFP moiety fused to the Fur4p C-terminal domain. Thus, after internalization, the pathway of Fur4p-GFP degradation in *yck*^{ts} *apm3*-Δ cells was very similar to that in wild-type cells. The deletion of *APM3* in cells with *yck* deficiency seems to abolish the *yck* phenotype.

To confirm the results obtained by fluorescence microscopy, we checked the location of Fur4p-GFP by density-gradient sedimentation of total cell extracts (data not shown). With this experimental approach, we were unable to discriminate between the permease resident in the endosomes and the permease delivered to the vacuolar compartment. The permeases from both these compartments fractionated with the endosomal fractions. Similar results were obtained in a experiments using cells deficient for the vacuolar hydrolase, Pep4p, which accumulated permease within the vacuole following endocytosis (Dupre and Haguenauer-Tsapis, 2001). Similar results were also reported for fractionation of the vacuolar hydrolase carboxypeptidase S (CPS) in *pep4* mutant cells (Odorizzi et al., 1998). The permease transported to the yeast vacuole probably enters the internal vesicles that form in late endosomes (multivesicular bodies or MVB) and are delivered to the vacuole. The density of the membranes of these vesicles is clearly different from that of the limiting vacuolar membrane.

Fur4p-GFP partially colocalized with Pep12p in *yck*^{ts} mutant cells

The vacuolar protein sorting (VPS) pathway merges with the endocytic pathway at the late endosome compartment. The distinguishing feature of the subset of *vps* mutants called class E mutants is the accumulation in the perivacuolar region of aberrant multilamellar structures known as the class E compartment, which is thought to be an exaggerated version of the physiological late endosome (Raymond et al., 1992). Cells carrying mutations in any one of the class E VPS genes accumulate vacuolar and Golgi proteins and proteins subjected to endocytosis in this class E compartment. The Ste3p receptor has been reported to accumulate in the class E compartment and to recycle from this compartment by a retrograde pathway to the cell surface in some class E *vps* mutants (Davis et al., 1993; Piper et al., 1995). One such mutant is *vps27*.

We followed the fate of Fur4p-GFP after protein synthesis inhibition in wild-type and *vps27*^{ts} cells grown on galactose at 24°C then shifted to restrictive temperature for two hours to establish the *vps27* deficiency. Permease expression was stopped by incubation with glucose for 10 minutes before cycloheximide treatment (Fig. 4). We monitored the rate of internalization of plasma-membrane-located Fur4p-GFP over two hours (Fig. 4A). The addition of cycloheximide caused identical sharp decreases in uracil uptake in both types of cell. Thus, the *vps27* mutation had no effect on Fur4p-GFP internalization. The lack of *vps27* activity did not stabilize the transporter at the plasma membrane. We further analyzed the distribution of Fur4p-GFP by fluorescence microscopy (Fig. 4B). At time zero of the experiment, Fur4p-GFP was barely detectable at the cell periphery and punctate staining was observed in the cytoplasm of wild-type and mutant cells. Fluorescent intracellular punctate staining was more intense in *vps27*^{ts} cells owing to the establishment of *vps27* deficiency. This intracellular staining was probably due to the basal endocytosis of permease, which was accelerated by shifting the cells to restrictive temperature for a long time period. Treatment for two hours resulted in an almost total loss of fluorescent signal in wild-type cells. In contrast, two hours of cycloheximide treatment resulted in loss of the fluorescent signal at the surface and intracellular staining, in the form of

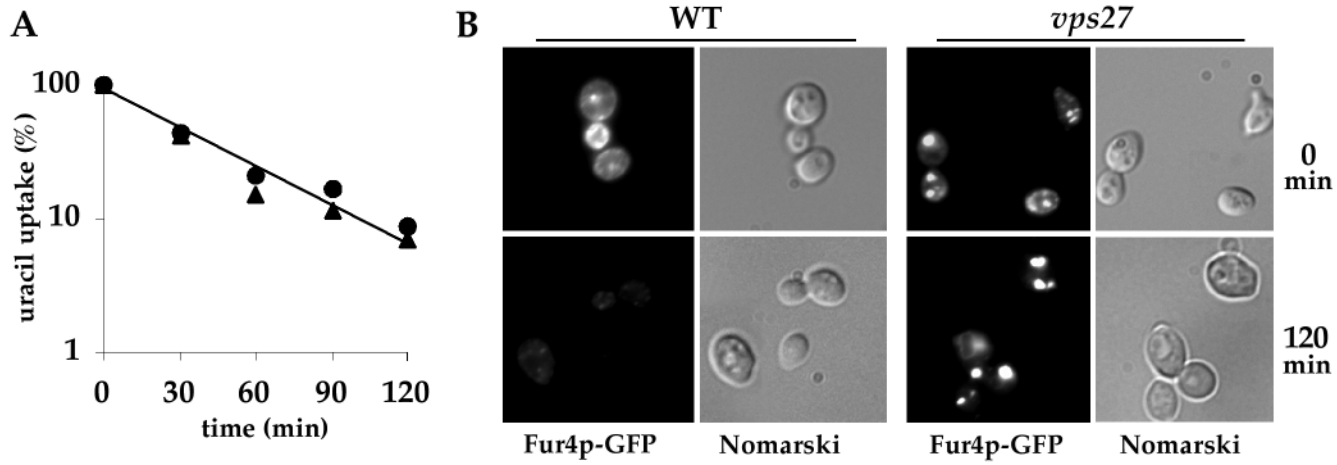

Fig. 4. Fur4p-GFP is retained in the class E compartment in *vps27*^{ts} mutant cells. Parental and *vps27*^{ts} mutant cells transformed with pFL38gF-GFP were grown to exponential growth phase at 24°C in galactose medium. Cells were transferred to 37°C for two hours. The synthesis of Fur4p-GFP was stopped by adding glucose and incubating for 10 minutes at the same restrictive temperature. Cycloheximide (100 μg/ml) was then added. (A) Uracil uptake (permease activity) was measured at 37°C at various times after the addition of cycloheximide. The results are expressed as a percentage of the initial activity. (B) Nomarski optics and distribution of Fur4p-GFP fluorescence in parental and *vps27*^{ts} mutant cells. Cells were observed two hours after adding cycloheximide at 37°C. Cells were viewed by Normarski optics or with a FITC filter set.

a few bright dots located adjacent to the vacuole in *vps27*^{ts} cells. These dots correspond to retention of the permease fusion protein in the class E compartment. This result is consistent with a general failure in late endosomal sorting in *vps27*^{ts} mutant cells as already described for other markers in these cells (Piper et al., 1995). The observed phenotype is reminiscent of the failure of the permease to exit from a late endocytic compartment in *yck*^{ts} mutant cells. The accumulation of Fur4p-GFP in *yck*-deficient cells closely resembles that observed in the class E compartment of *vps27*^{ts} cells.

Accumulation of Fur4p led us to investigate whether it colocalized with a marker of the late endosome, Pep12p (Becherer et al., 1996; Gerrard et al., 2000). We used fluorescence microscopy to determine the distribution of Fur4p-GFP and Pep12p in *yck*^{ts} mutant cells (Fig. 5). We studied the distribution of Fur4p-GFP and Pep12p 90 minutes after protein-synthesis inhibition in cells grown on galactose at 24°C then shifted for 30 minutes to the restrictive temperature to establish the Yck deficiency. Permease expression was stopped by incubation with glucose for 10 minutes before cycloheximide treatment. In wild-type cells, Pep12p displayed highly punctuate staining throughout the cytoplam, a pattern typical of endosomal proteins, using indirect immunofluorescence microscopy (Gerrard et al., 2000). In cells with a *yck* deficiency, essentially one to three large stained structures were observed (Fig. 5). Thus, Yck deficiency altered the distribution of Pep12p. This could be interpreted as a modification of endosome morphology. In the same cells, two to four large, stained structures were observed for Fur4p-GFP. Partial colocalization of Fur4p-GFP and Pep12p was observed in the same aberrant structures (Fig. 5). These structures may result from an increase in endosome fusion, reminiscent of the class E compartment.

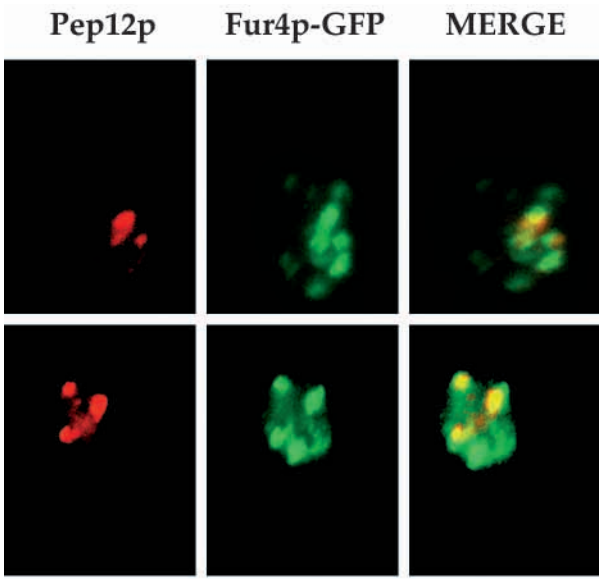

Fig. 5. Fur4p-GFP partially colocalizes with Pep12p in *yck*^{ts} cells. *yck*^{ts} cells transformed with pFL38gF-GFP were grown to exponential growth phase at 24°C in galactose medium. Cells were incubated at 37°C for 20 minutes. The synthesis of Fur4p-GFP was stopped by adding glucose and incubating for 10 minutes at the same restrictive temperature. Cells were observed 90 minutes after the addition of cycloheximide at 37°C. Cells were fixed and processed for immunofluorescence using the anti-Pep12p antibody followed by rhodamine-labeled goat anti-mouse IgG. GFP fluorescence was visualized with a FITC filter and Pep12p distribution was observed with a rhodamine filter. Regions of colocalization are shown in yellow on the overlay.

Vacuolar sorting of CPY, V-ATPase and ALP is normal in *yck*^{ts} mutant cells

Newly synthesized proteins are targeted to the vacuole via various pathways. The CPY pathway is the major route for transport of many resident vacuolar proteins, such as carboxypeptidase Y (CPY) and V-ATPase subunits, to the vacuole. It passes via an endosomal intermediate, the late endosome. Some membrane-bound proteins, such as ALP, travel from the late Golgi compartment to the vacuole by an alternative, vesicle-dependent route (Cowles et al., 1997; Stepp et al., 1997). Vesicles originating from the ALP and CPY pathways require similar components for final fusion to the vacuole. The retention of Fur4p in late endosomes in *yck*^{ts} mutant cells suggests that other cargoes destined for the vacuole may be retained in this compartment on their way. We investigated this possibility by studying the sorting of various vacuolar proteins in *yck*^{ts} mutant cells. We checked whether the biosynthetic delivery and processing of soluble vacuolar hydrolases was affected in *yck*^{ts} mutant cells, using carboxypeptidase Y (CPY) as a marker. CPY is synthesized as a precursor, which is then translocated into the lumen of the ER, where it is core-glycosylated. This ER form of CPY (p_1CPY) is 67 kDa. In the median Golgi compartment, CPY is further glycosylated, increasing its size to 69 kDa (p_2CPY). CPY is finally transported via late endosomes to the vacuole, where it is processed by proteinase A to produce the 61 kDa mature species (m CPY) (Graham and Emr, 1991).

We analyzed the intracellular fate of CPY by means of a pulse-chase experiment at restrictive temperature in wild-type, *apm3*-Δ, *yck*^{ts} and *yck*^{ts} *apm3*-Δ mutant cells (Fig. 6A). CPY was processed rapidly in the wild-type; the p_2 form appeared after only five minutes chase, and the protein was entirely processed within 20 minutes. As previously described (Panek et al., 1997), normal CPY processing and sorting were also observed in *apm3*-Δ cells. Normal CPY processing was also observed in *yck*^{ts} mutant cells. Thus, CPY trafficking from the ER to the vacuole appears to be normal in *yck*^{ts} mutant cells, suggesting that the loss of Yck function had no effect on the trafficking of CPY to the vacuole. We then studied the distribution of Vma2p/Vat2p, a subunit of the vacuolar H+ATPase in wild-type, *apm3*-Δ, *yck*^{ts} and *yck*^{ts} *apm3*-Δ mutant cells (Fig. 6B). Cells in the exponential growth phase were incubated for 30 minutes at non-permissive temperature and were treated with cycloheximide as described above (Fig. 2). We studied the distribution of Vma2p 90 minutes after protein-synthesis inhibition. In wild-type cells, Vma2p was detected in the vacuolar membrane by immunofluorescence with an anti-Vma2p antibody (Kane et al., 1992) (Fig. 6). The vacuolar staining pattern of the V-ATPase was normal in *apm3*-Δ cells (Stepp et al., 1997) (Fig. 6), consistent with the Vps pathway being intact in AP-3 mutants. As in wild-type cells, Vam2p was clearly present in the vacuolar membrane in *yck*^{ts}

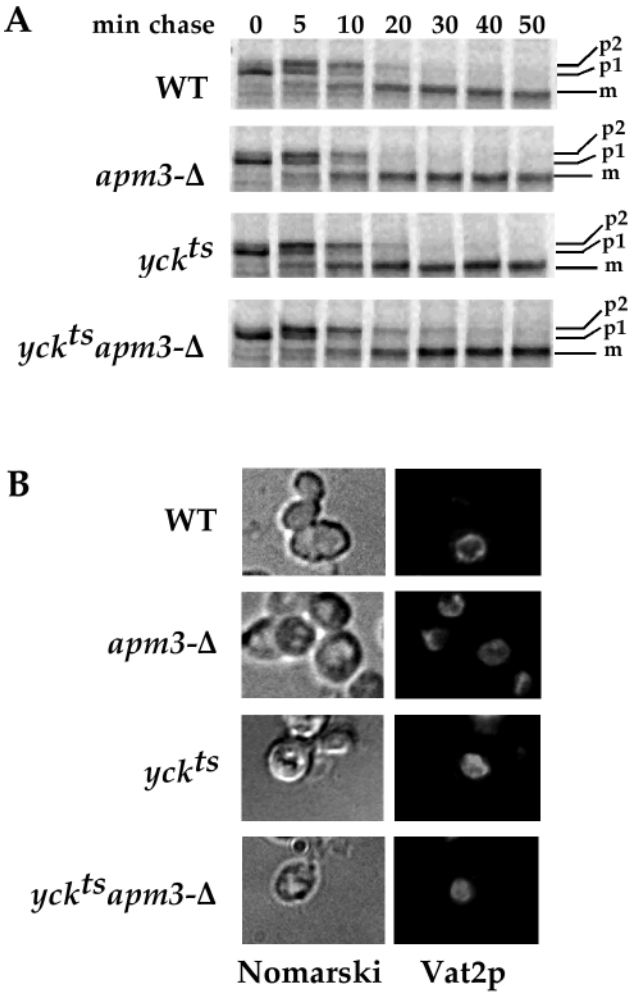

Fig. 6. *yck*^{ts} cells show wild-type processing of the soluble vacuolar protein CPY and wild-type localization of V-ATPase. (A) Processing of the soluble vacuolar protein CPY. Wild-type (WT), *apm3*Δ, *yck*^{ts} and *yck*^{ts} *apm3*-Δ cells were grown to exponential growth phase at 24°C in glucose medium, then incubated at 37°C for 30 minutes. Cells were pulse-labeled for 5 minutes with [^{35}S]-translabel, chased for the times indicated (min) and subjected to immunoprecipitation with CPY antiserum. The immunoprecipitates were analyzed by electrophoresis in 10% polyacrylamide-SDS gels, which were then subjected to fluorography. p1, ER form; p2, Golgi form; m, mature vacuolar form. (B) Immunolocalization of the vacuolar H^+ATPase subunit Vat2p. Cells were grown to exponential growth phase at 24°C in galactose medium, then incubated at 37°C for 90 minutes with cycloheximide as described in Fig. 2. Cells were prepared for immunofluorescence with the anti-Vma2p/Vat2p antibody. Cells were visualized by Normarski optics or with a FITC filter set.

and *yck*^{ts} *apm3*-Δ cells. This result is consistent with *yck* mutations causing no transport block in the Vps pathway via the late endosome compartment.

We further analyzed the sorting of ALP in Yck-deficient cells. ALP processing was observed at steady state in cells shifted for three hours to 37°C, as shown by immunoblotting of all cell extracts (Fig. 7). In wild-type cells, ALP exists predominantly as mature ALP (m-ALP) with a small amount of a smaller, aberrantly processed form (*ALP). *ALP is proposed to be produced by cleavage at the luminal

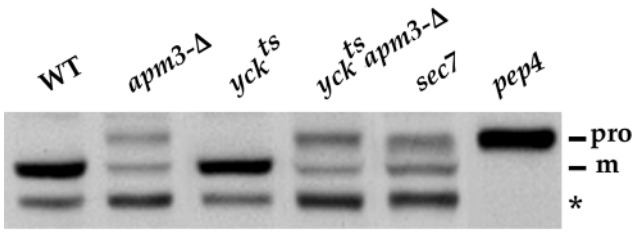

Fig. 7. *yck*^{ts} cells show wild-type processing of the vacuolar protein ALP. Wild-type (WT), *apm3*Δ, *yck*^{ts} and *yck*^{ts} *apm3*-Δ cells were grown to exponential growth phase at 24°C, then washed and resuspended at the same density in fresh medium depleted of inorganic phosphate for three hours at 37°C. Protein extracts prepared from wild-type and mutant cells were analyzed by SDS-PAGE and western immunoblotting for ALP. We used as a control strains *sec7*, defective in intra-Golgi trafficking at the non-permissive temperature, and *pep4*, defective in vacuolar protease processing. Pep4p-dependent cleavage of pro-ALP (pro) results in the formation of mature ALP (m) and a small amount of the aberrant form of ALP (*). The mature form of ALP is formed in *sec7* mutants during growth at the permissive temperature.

juxtamembrane region of the protein (Stepp et al., 1997). Only a Golgi-modified precursor form (pro-ALP) accumulated in *pep4* cells. In contrast, ALP in the pro-ALP form was detected, together with m-ALP in *apm3*-Δ cells. A pool of aberrantly processed *ALP was also observed. The accumulation of pro-ALP is consistent with the *amp3* product being required for the vacuolar localization of ALP. m-ALP and *ALP are still produced, owing to the re-routing of ALP via the CPY pathway (Stepp et al., 1997). Cells defective for Yck activity displayed a wild-type distribution of ALP. A *yck* and *apm3*-Δ double mutant gave an almost identical distribution of ALP to *apm3*-Δ alone, except that the pro-ALP precursor band was slightly more intense. These results are consistent with Yck deficiency causing no transport block in vacuolar protein sorting via the ALP pathway and no suppression of the ALP trafficking defect of *apm3*-Δ cells.

Discussion

We have previously shown that Yck activity is involved in phosphorylation of the PEST region of the permease, which, in turn, facilitates the ubiquitination and subsequent endocytosis of the permease (Marchal et al., 1998). We provide here evidence that Yck is required for the accurate delivery of internalized Fur4p to the vacuole. Fur4p-GFP accumulated in the late endosome/perivacuolar compartment of *yck*^{ts} cells reminiscent of the class E compartment, as shown by Fur4p-GFP staining in *vps27*^{ts} cells and by colocalization with Pep12p, a marker of the late endosome. However, Yck is not necessary for the biosynthetic transport of proteins from the Golgi to the vacuole via the CPY or ALP pathways. The observed accumulation of the fusion protein in *yck*^{ts} cells suggests that Yck1p-Yck2p is specifically involved in downstream endocytic trafficking before degradation in the vacuole. Loss of function of the AP-3 complex abolished the accumulation permease in perivacuolar compartments after endocytosis. However, the AP-3 complex is not itself necessary for the internalization of Fur4p, its transit through the endosomes or delivery to the vacuole.

Panek et al. provided evidence that the Ste3p receptor accumulates at the cell surface of *yck*^{ts} mutant cells and that this accumulation is reduced if any of the subunits of the AP-3 complex are altered (Panek et al., 1997). The location of the accumulation of the two membrane proteins Ste3p and Fur4p, both of which are susceptible to the suppressor effect of *apm3*-Δ, remains a matter of debate. The Ste3p receptor has been reported to accumulate in the prevacuolar compartment and at the cell surface of the *vps2*/*ren1* mutant (one of the class E *vps* mutants). Its accumulation at the cell surface is thought to reflect either the recycling of receptor forms from the late endosome compartment to the cell surface or accumulation resulting from the downstream block (Davis et al., 1993). A similar distribution of Ste3p was observed in *vps27* mutant cells (Piper et al., 1995). The existence of a transport route connecting the perivacuolar compartment and the cell surface would account for the accumulation of Ste3p at the cell surface in *yck*^{ts} mutant cells and abolition of the accumulation of the receptor at the cell surface by mutation of the genes encoding the AP-3 complex. Conversely, in *yck*^{ts} and *vps27*^{ts} mutant cells, uracil permease is trapped in an internal compartment with no obvious route back to the cell surface after its internalization. This was not due to the inhibition of protein synthesis in our experiments disrupting targeting to the vacuole or return to the cell surface. Indeed, the inhibition of protein synthesis and an excess of exogenous uracil in the medium are two conditions resulting in downregulation of the permease (Séron et al., 1999; Volland et al., 1994). In this study, we investigated the induced endocytosis of the permease by following loss of the permease in the absence of protein synthesis, which was blocked by adding cycloheximide. However, similar results were obtained when excess uracil was added to growing cells (data not shown). We suggest that the Yck1-Yck2p phosphorylation of Ste3p and Fur4p or of some endocytic component may be involved in a downstream trafficking event, increasing the endosome-to-vacuole transport of the two membrane proteins. The phosphorylation status of the permease retained in the class E compartment of *vps27*-deficient cells or in the vacuole of *pep4*-deficient cells was similar to that of the permease resident at the plasma membrane (data not shown). Thus, there seems to be no need for further phosphorylation of the permease for its sorting to the vacuole. Yck proteins may recruit or activate as yet uncharacterized effector proteins. Two gene products were recently characterized in a screening to identify mutations causing synthetic lethality with impairment of Yck functions: the t-SNARE protein Tlg2p and a previously uncharacterized protein, Rgp1p, which is involved in the recycling of proteins to the Golgi (Panek et al., 2000). Thus, Yck kinases, which regulate the internalization of several plasma membrane proteins (Feng and Davis, 2000; Hicke et al., 1998; Marchal et al., 2000), may enter the cell in endocytic vesicles, phosphorylate components of the endocytic pathway and be recycled back to the cell surface.

Yck1p and Yck2p have been reported to play multiple roles in protein trafficking at the plasma membrane. First, the Yck1-Yck2p-dependent phosphorylation of permeases and pheromone receptors at the plasma membrane triggers the ubiquitination of these proteins, which constitutes an internalization signal (Decottignies et al., 1999; Feng and Davis, 2000; Hicke et al., 1998; Marchal et al., 2000). Second, we have previously shown that Yck activity may negatively regulate a trans-acting component involved in internalization at the plasma membrane (Marchal et al., 2000). Third, the exocytic v-SNARE protein Snc1p, which is involved in the fusion of Golgi-derived secretory vesicles with the plasma membrane, has been shown to be phosphorylated in a Yck1-Yck2p-dependent manner (Galan et al., 2001). It has been suggested that Snc1p is recycled (Lewis et al., 2000) and that the phosphorylation state of Snc1p depends on its subcellular location. We suggest here that CKI may be involved in the vacuolar sorting of plasma membrane proteins after endocytosis.

Loss-of-function of the AP-3 complex abolishes the lethality of *yck* deficiency (Panek et al., 1997). We show here that loss of function of the AP-3 complex also abolishes the accumulation of permease in the perivacuolar compartment, in association with a loss of Yck functions. The relationship between Yck1-Yck2p and the AP-3 complex is unclear. Phosphorylation of the mammalian counterpart of the AP-3 adaptor complex is linked to synaptic vesicle coating. The mechanism of AP-3-mediated vesiculation from neuroendocrine endosomes requires phosphorylation of the adaptor complex at a step during or after AP-3 recruitment to membranes. The β3 subunit of the complex is phosphorylated by a kinase similar to casein kinase 1α (Faundez and Kelly, 2000). Activation of the AP-3 pathway in yeast may depend on phosphorylation by CK1 protein kinases. In cells lacking Yck, material transported from the cell surface by endocytic vesicular trafficking slows down for an unknown reason, and sorting to the vacuole should be restored upon elimination of the AP-3 pathway.

Many studies over a number of years have tried to define the intermediate compartments through which cargo molecules are transported and to identify the regulatory factors required for sorting in the endocytic pathway. The diverse levels of control exerted by Yck proteins in the endocytosis of Fur4p provide an illustration of the complexity of the endocytic process. Our results raise the possibility that there may be two different pathways from the late endosome to the vacuole, one CPY-specific and the other specific for endocytic cargo molecules. We now need to determine whether the new CK1 kinase functions can be demonstrated for other endocytic substrates and to find new targets of CK1 kinases.

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