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RESEARCH ARTICLE

Sequences in the N-terminal cytoplasmic domain of *Saccharomyces cerevisiae* maltose permease are required for vacuolar degradation but not glucose-induced internalization

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Abstract In *Saccharomyces cerevisiae*, glucose addition to maltose fermenting cells causes a rapid loss of maltose transport activity and ubiquitin-mediated vacuolar proteolysis of maltose permease. GFP-tagged Mal61 maltose permease was used to explore the role of the N-terminal cytoplasmic domain in glucose-induced inactivation. In maltose-grown cells, Mal61/HA-GFP localizes to the cell surface and, surprisingly, to the vacuole. Studies of *end3Δ* and *doa4Δ* mutants indicate that a slow constitutive internalization of Mal61/HA-GFP is required for its vacuolar localization. Site-specific mutagenesis of multiple serine/threonine residues in a putative PEST sequence of the N-terminal cytoplasmic domain of maltose permease blocks glucose-induced Mal61p degradation but does not affect the rapid loss of maltose transport activity associated with glucose-induced internalization. The internalized multiple Ser/Thr mutant protein co-localizes with Snf7p in a putative late endosome or E-compartment. Further, alteration of a putative dileucine [D/EExxxLL/I] motif at residues 64–70 causes a significant defect in maltose transport activity and mislocalization to an E-compartment but appears to have little impact on glucose-induced internalization. We conclude that the N-terminal cytoplasmic domain of maltose permease is not the target of the signaling pathways leading to glucose-induced internalization of Mal61 permease but is required for its subsequent delivery to the vacuole for degradation.

Keywords Maltose permease · Glucose-induced inactivation · *Saccharomyces cerevisiae* · Dileucine motif · PEST sequence

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Introduction

In *Saccharomyces cerevisiae*, *MAL61* encodes maltose permease, a high-affinity maltose/proton symporter required for the transport of maltose across the plasma membrane (Cheng and Michels 1991). Cheng and Michels (1989) showed that Mal61 permease exhibits both sequence and structural homology to other members of the family of 12 transmembrane domain superfamily of sugar transporters that includes the human glucose transporters GLUT1-12, the *S. cerevisiae* hexose transporters Hxt1-17 and Gal2, and plant sucrose transporters (Boles and Hollenberg 1997; Wieczorke et al. 1999; Williams et al. 2000; Joost and Thorens 2001). As for other members of this family, hydropathy analysis suggests that the N-terminal and the C-terminal domains, of approximately 100 and 67 residues in length, respectively, are cytoplasmic (Cheng and Michels 1989). Moreover, Cheng and Michels (1989) identified a putative PEST sequence encompassing approximately residues 49–78 of the N-terminal domain (see Fig. 1). PEST sequences, regions rich in proline, aspartate, glutamate, serine, and threonine, are commonly found in

proteins that are subject to rapid turnover, and regulated phosphorylation of PEST sequences is proposed to mark these proteins for degradation (Rechsteiner 1988; Marchal et al. 1998). Furthermore, phosphorylation of serines in Ste2p (Hicke and Riezman 1996), Fur4p (Volland et al. 1992), and several mammalian receptors (Hicke 1997) is required for the receptor-mediated endocytosis of these integral plasma membrane proteins.

In *S. cerevisiae*, glucose regulates *MAL* gene expression by multiple mechanisms (Hu et al. 2000). One of these mechanisms, referred to as glucose-induced inactivation, regulates the activity and protein level of maltose permease (Medintz et al. 1996; Jiang et al. 1997; Wang et al. 2002; Gadura et al. 2006). Addition of glucose to maltose fermenting cells causes both a very rapid loss of maltose transport activity and proteolysis of maltose permease (Medintz et al. 1996). Medintz et al. (1996, 1998) found that endocytosis, vesicle trafficking pathways, and vacuolar proteolysis are required for maltose permease proteolysis. They demonstrated that glucose stimulates ubiquitination of maltose permease and showed that degradation of maltose permease requires the ubiquitin-conjugation enzymes. Finally, *doa4Δ*, which causes depletion of cytoplasmic ubiquitin levels, blocks proteolysis of maltose permease but this could be suppressed by overproduction of ubiquitin (Medintz et al. 1998).

To localize sequences responsible for the glucose-regulated inactivation of maltose permease, Medintz et al. (2000) undertook a deletion analysis of the N- and C-terminal cytoplasmic domains of Mal61 maltose permease. Truncation or mutation of the C-terminal 50 residues of Mal61 permease had little impact on its activity or glucose-induced inactivation, but in-frame deletions involving residues 30–78, particularly of residues 48–78, within the N-terminal cytoplasmic domain of Mal61 maltose permease blocked both the glucose-induced rapid loss of maltose transport activity and permease proteolysis.

Here, we describe the results of site-directed mutagenesis of residues in the N-terminal cytoplasmic domain of Mal61 maltose permease to investigate in finer detail the contributions of the serine/threonine residues and other sequence motifs in this domain to its regulation via glucose-induced inactivation. Our results indicate that phosphorylation of the five serines and threonines in residues 29–56 is not required for glucose-induced internalization of Mal61p from the plasma membrane but is involved in the movement of the internalized permease to the vacuole for degradation. A similar role is proposed for a putative dileucine motif at residues 64–70, suggesting that the N-terminal cytoplasmic domain is not the target of the glucose-signaling pathway that results in inactivation of this integral membrane maltose transport protein.

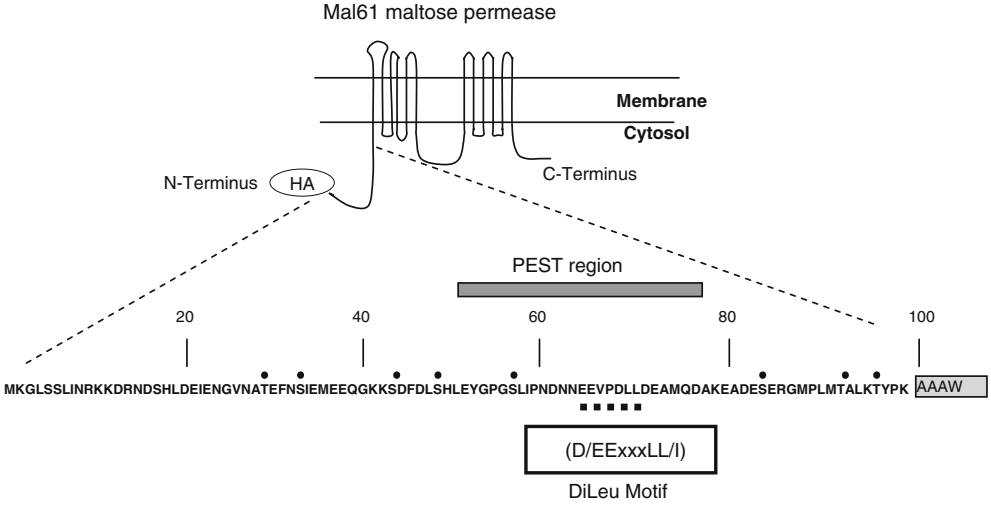

Fig. 1 Sequence of the N-terminal cytoplasmic domain of Mal61/HA maltose permease and proposed secondary structure of maltose permease. Secondary structure of maltose permease is based on hydropathy analysis (Cheng and Michels 1989). The residue numbers relate to the sequence of the wild-type Mal61 maltose permease (Cheng and Michels 1989). The proposed start of the first transmembrane domain is shown in a grey rectangle and the HA-tag is located at the N-terminus (Medintz et al. 1996). The Ser/Thr residues that were mutated either singly or cumulatively are indicated by a *filled circle* above the sequence. The location of the putative dileucine motif (D/EExxxLL/I) is indicated with *a dashed underline* and residues 48–79 containing the postulated PEST sequence are indicated with *a gray rectangle* above the sequence

Materials and methods

Strains and plasmids

The *S. cerevisiae* strains used in this study and their relevant genotypes are listed in Table 1. Briefly, strain CMY1050 is described in Medintz et al. (2000), and carries a mutant allele of *MAL1* as the sole *MAL* locus. This *MAL1* locus encodes maltase (*MAL12*), an inducible allele of the *MAL*-activator (*MAL13*), but the gene encoding maltose permease has been deleted by one-step gene replacement with *HIS3*. Strains CMY1025 and CMY1026 are congenic segregants from the cross of CMY1001 (*MAL1 DOA4*) and PMY270 (*mal1 doa4Δ::LEU2*) as described in Medintz et al. (1998).

PCR-based one-step gene replacement was used to construct CMY7001 (*MATα SNF7-RFP his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp1Δ::hyg*^{R}) from parent strain ATCC201389, BY4741 *SNF7-RFP* (generously provided by Erin K. O'Shea, UCSF), by deleting the *TRP1* open reading frame and replacing it with the hygromycin resistance marker gene, *hyg*^{R} (Longtine et al. 1998). The BY4741 series of strains, available from Invitrogen, are derivatives of S288C and thus contain partially functional alleles of *MAL1* and *MAL3*, as described in Charron and Michels (1988) and Michels et al. (1992). The transformants were selected on plates containing hygromycin and checked for disruption using the appropriate PCR primers. The same procedure was used to construct CMY7002 (*END3 trp1Δ::hyg*^{R}) and CMY7003 (*end3Δ trp1Δ::hyg*^{R}) in strains from the haploid deletion series isolated in BY4741 (purchased from ResGen, Invitrogen Corp., CA, USA).

Plasmids pUN30-MAL61/HA (Medintz et al. 2000) or pRS315-MAL61/HA (Medintz et al. 1996) contain the gene encoding an HA-tagged allele of Mal61 maltose permease expressed under the control of its native promoter. Plasmid YCp50-MAL43c contains the *MAL43-c* constitutive *MAL*-activator gene in the vector YCp50 (Gibson et al. 1997). Plasmid p413-GPD-MAL63/43c was constructed by inserting the constitutive *MAL63/43-c MAL*-activator gene into the expression vector p413-GPD (Danzi et al. 2000; Funk et al. 2002).

Plasmid pUN30-MAL61/HA-GFP was constructed by inserting a 0.8 kb *Sal*I fragment encoding the GFP ORF amplified from plasmid pGFP-C-FUS by PCR (Niedenthal et al. 1996) into an *Xho*I site created at the 3'-end of the *MAL61/HA* ORF to produce an in-frame fusion *MAL61/HA-GFP*. This C-terminal GFP maltose permease fusion protein is correctly delivered to the plasma membrane and strains carrying plasmid pUN30-MAL61/HA-GFP transport maltose with the same efficiency as strains transformed with plasmid pUN30-MAL61/HA expressing the HA-tagged allele (Gadura et al. 2006). To transfer the N-terminal *MAL61/HA* mutations constructed in pUN30-MAL61/HA into the *MAL61/HA-GFP* gene, the Mal61/HA Sac1-PacI fragment from plasmid pUN30-MAL61/HA was subcloned into *Sac*I-*Pac*I-digested pUN30-MAL61/HA-GFP. The constructions were confirmed by the presence of a diagnostic *Nco*I site and by sequencing of the 5'end of the resulting *MAL61/HA-GFP*.

Plasmid pWX307 contains *SNF7* cloned into the pYES-pro vector, where *SNF7* is tagged with V5 at the C-terminus and is expressed from its own promoter. Plasmid pWX307 was generously provided by Aaron Mitchell (Columbia University, NY, USA).

In vitro mutagenesis of *MAL61/HA*

Plasmid pUN30-MAL61/HA, or its derivatives carrying *MAL61/HA* mutant alleles, was used as template for in vitro mutagenesis using the BIO-RAD Muta-Gene kit (BIO-RAD, Hercules, CA, USA) according

Table 1 *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source
CMY 1050	*MAT***a** *mal11Δ::HIS3 MAL12 MAL13 leu2 ura3-52 lys2-801 ade2- 101 trp1-Δ63 his3-Δ200*	Medintz et al. (2000)
CMY1025	*doa4Δ1::LEU2 his3-Δ200 leu2 ura3-52 lys2-801 trp1MAL61/HA MAL12 MAL13 MAL31 MAL32*	Medintz et al. (1998)
CMY1026	*MAL1 DOA4* (congenic to CMY1025)	Medintz et al. (1998)
BY4741	*MATα MAL11 MAL12 mal13 MAL31 MAL32 mal33 his3Δ1 leu2Δ0 met15Δ ura3Δ0*	Invitrogen
BY4741 SNF7	Isogenic to BY4741 except *SNF7-RFP*	E. O'Shea
BY4741 end3Δ	Isogenic to BY4741 except *end3Δ*	This study
CMY7001	Isogenic to BY4741 except *trp1Δ::hyg*^{R}	This study
CMY7002	Isogenic to BY4741 *SNF7* except *trp1Δ::hyg*^{R}	This study
CMY7003	Isogenic to BY4741 end3Δ except *trp1Δ::hyg*^{R}	This study

to the manufacturer's protocol. Plasmid pUN30-MAL61/HA-L69, 70A was obtained from Medintz et al. (1998). The primers used for mutagenesis are listed in Table 2. The full sequence of each of the mutant alleles was confirmed (PDTC, Rockefeller University, NY, USA).

Inactivation protocol

The standard maltose permease inactivation assay protocol is described in detail in Medintz et al. (1996) and was used for these studies with a few modifications. Briefly, cells were grown at 30°C to early log phase (OD_{600} of 0.1–0.3) in selective media containing the indicated carbon source, Gly/Lac (3% glycerol plus 2% lactate). The cells were harvested by filtration and, unless otherwise indicated, resuspended in nitrogen starvation medium plus 2% glucose, referred to as YNSG. Cycloheximide (CHX) was added to the culture to a final concentration of 30 μg/ml at time zero to inhibit protein synthesis. Samples were taken at time zero and every hour thereafter over a 3-hour time period and used to determine maltose transport activity, maltose permease protein levels by Western analysis, and growth dilution. Growth dilution is calculated as the OD_{600} at time zero divided by OD_{600} at time *x*.

Maltose transport assay

Cells were harvested by filtration and resuspended in 0.1 M tartaric acid pH 4.2. Maltose transport was measured as the uptake of 1 mM ^{14}C-labelled maltose as described by Cheng and Michels (1991) and Medintz et al. (1996). Maltose transport units are expressed in nanoMoles maltose/min/mg dry weight of cells. Assays were done in duplicate on at least three independent transformants. The standard error is less than 15%.

Western blotting

At each time point 15 OD of cells (A_{600} 0.3–0.5) were harvested by filtration on nitrocellulose filters (0.45 μm), washed with KPO_4 plus 2% sodium azide pH 7.4, and frozen immediately at -80°C until used for the preparation of protein extracts. Total cell extracts were prepared by thawing the cells in HEPES buffer pH 7.5 supplemented with protease inhibitor cocktail that contains AEBSF, pepstatinA, E-64 and 1,10-phenanthroline (Sigma-Aldrich P8215, MO, USA) and phosphatase inhibitor cocktails 1 and 2 that contain a mixture of inhibitors of acid and alkaline phosphatases as well as tyrosine protein phosphatase and serine/threonine phosphatases (Sigma-Aldrich P5726 and P2850). The cells were harvested by centrifugation and resuspended in extraction buffer (40 mM TrisCl pH 6.8, 8 M Urea, 0.1 mM EDTA, 1% β-mercaptoethanol, and 5% SDS) plus the protease inhibitor and phosphatase inhibitor cocktails described above at concentrations recommended by the manufacturer. The cell suspension was vortexed with glass beads (425–600 μm) for 15 min at 4°C, solubilized for 15 min at 37°C followed by another 2 min of vortexing. Cell debris was removed by centrifugation for 5 min and this total protein extract was boiled for 3 min. Protein levels were assayed using the Protein Assay Kit from Sigma-Aldrich (P5656).

Equal amounts of protein were loaded per lane and separated using 7.5% SDS-PAGE gels to detect HA-tagged Mal61 (to show the range of differentially phosphorylated species) or 10% SDS-PAGE gels for Mal61/HA-GFP fusion protein. Separated protein was transferred to PVDF nitrocellulose membrane. Mal61/HA protein was detected using anti-HA antibody (Boehringer Mannheim, Germany) and Mal61/HA-GFP protein was detected using anti-GFP antibody (Santa Cruz, CA, USA). Each membrane was also

Table 2 Oligonucleotides used for in vitro mutagenesis of *MAL61/HA*

MAL61/HA allele	Sequence 5' → 3'[a]	Single-stranded DNA template
MAL61/HA-(T29A, S33A)	CATCTCTATCTCGTTGAATTCTTCAGCGTTCAC	pUN30-MAL61/HA
MAL61/HA-(T29A, S33A, S43A)	AAGATCAAAATCCTCTTTCTTACCT	pUN30-MAL61/HA (T29A, S33A)
MAL61/HA-*(*T29A, S33A, *S43A, S48A, S56A)*	TGGTATTAGTTCACCTGGACC	pUN30-MAL61/HA (T29A, S33A, S43A, S48A)
MAL61/HA-(L69,70A)	GGCGTCCTGCATAGCTTCATCGGCAGCGTCGGGGACTTCTTC	pUN30-MAL61/HA
MAL61/HA-(E64,65R)	AAGGTCGGGGACTCTTCTATTATTATCGTT	pUN30-MAL61/HA

[a] Underline indicates site of alteration

probed with anti-PGK (phosphoglycerol kinase) antibody (Molecular Probes) as a loading control. Protein levels were visualized by Vistra-ECF kit (Amersham, Freiburg, Germany) using a Storm 860 PhosphoImager analyzer (Molecular Dynamics, Piscataway, NJ) and the signal was quantified using the manufacturer's software. Loading variations were corrected by normalizing to the PGK signal. Values are the average of results from at least three independent transformants. The Western blots shown are representative of typical results.

Fluorescence microscopy

Cells were grown to log phase in the appropriate medium with Gly/Lac or 2% maltose as a carbon source. To follow glucose-induced inactivation, cells were harvested by filtration and resuspended in nitrogen starvation media plus 2% glucose, referred to as YNSG. The CHX (final concentration 30 μg/ml) was added to the cell suspension at time zero. At appropriate time intervals living cells were collected and viewed with a Meridian/Olympus IMT-2 confocal microscope using a 100× oil, NA 1.40 lens, and either FITC filter (GFP), propidium iodide filter (RFP) or phase optics. The imaging parameters were constant for all images.

The lipophilic dye FM4-64 (Molecular Probes, Eugene, OR) was used to stain membranes (Vida and Emr 1995). Cells were incubated in medium containing 40 μM FM4-64 for 15 min and transferred to fresh medium lacking the dye. At appropriate times cells were viewed with a Meridian/Olympus IMT-2 confocal microscope using a 100× oil, NA 1.40 lens, and a rhodamine filter set or by phase optics. The imaging parameters were constant for all images. All the images shown are representative of typical experimental results and based on observations of approximately 100 cells. Experiments were carried out on three independent transformants.

Results

Maltose permease localization

Our previous studies used maltose transport rates and permease protein levels to monitor glucose-induced inactivation of *S. cerevisiae* Mal61 maltose permease (Medintz et al. 1996; Jiang et al. 1997; Medintz et al. 1998; Jiang et al. 2000; Medintz et al. 2000). To enable us to follow subcellular localization of maltose permease, we constructed a C-terminally GFP-tagged allele of Mal61/HA maltose permease, as described in Materials and methods and Gadura et al. (2006). The fusion gene is carried on the CEN vector pUN30 and is expressed from the native *MAL61* promoter, thereby providing normal levels of expression under the control of the *MAL*-activator. Cells expressing the GFP tagged maltose permease show similar maltose transport activity and glucose-induced proteolysis rate when compared to the non-GFP tagged counterpart (data not shown). A plasmid-borne copy of MAL61*/HA-GFP* was introduced into strain CMY1050 (*mal11Δ MAL12 MAL13*), a *MAL1* strain that contains a deletion of the full chromosomal ORF of the maltose permease gene. No other *MAL* loci are present in CMY1050. Transformants were grown in 2% maltose to induce expression of *MAL61/HA-GFP* and localization of maltose permease was determined by confocal microscopy. The results are shown in Fig. 2.

Mal61/HA-GFPp fluorescence is observed at the cell surface and, surprisingly, substantial GFP signal is also evident in an intracellular compartment that appears to be the vacuole (Fig. 2a, left panels). Because in strains CMY1050 and CMY7002 (BY4741 background) the vacuole cannot be visualized by phase microscopy, vacuolar membrane in strain CMY1050 was visualized by staining with FM4-64 dye (Fig. 2b) (Vida and Emr 1995). The lipophilic dye FM4-64 moves rapidly from the plasma membrane to intracellular membrane structures and the vacuolar membrane. The merged image indicates that indeed maltose permease localizes to the vacuole. Whether the vacuolar fluorescence observed here represents full length Mal61/HA-GFP fusion protein or just a stable GFP moiety produced by partial degradation has not been determined.

Maltose utilization generates intracellular glucose that could stimulate maltose permease internalization and delivery to the vacuole sufficient to produce the observed vacuolar accumulation. To test this, a plasmid carrying the dominant constitutive *MAL*-activator allele *MAL63/43-c* was introduced into strain CMY 1050 carrying *MAL61/HA-GFP*. The presence of the constitutive *MAL*-activator allowed the transformants to be grown in glycerol/lactate rather than maltose as the carbon source, thereby avoiding the contribution of intracellular generated glucose that is formed during maltose fermentation. When grown in glycerol/lactate medium, Mal61/HA-GFP protein still localizes in the vacuole to abundant levels (Fig. 2a, right panels). Similar vacuolar localization of Mal61/HA-GFP maltose permease was observed in several other strain backgrounds (data not shown) following growth in maltose or when constitutively expressed and grown in glycerol/lactate. For example, strain CMY7002 (BY4741 series) grown in maltose and glycerol/lactate

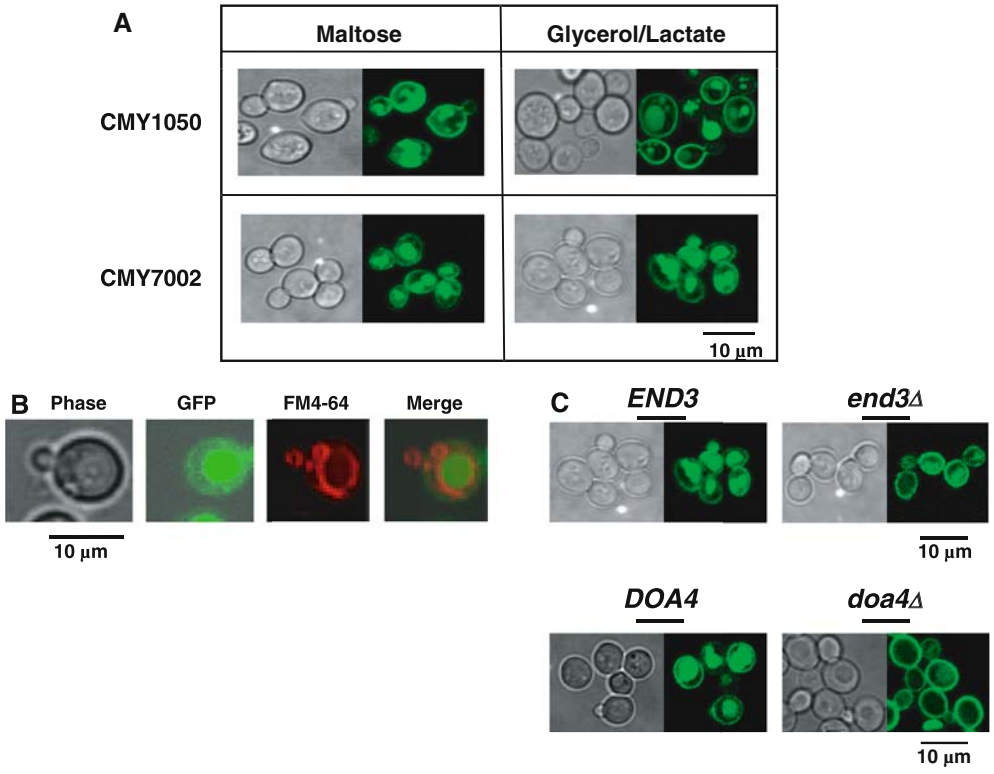

Fig. 2 Localization of maltose permease in different strains and growth conditions. **a** CMY1050 was transformed with pUN30-MAL61/HA-GFP and either YCp50-MAL63 (carrying an inducible *MAL*-activator gene) or YCp50-MAL43c (carrying a constitutive *MAL*-activator gene). CMY7002 (isogenic to BY4741 *trp1Δ::hyg*^{R}) was transformed with pUN30-MAL61/HA-GFP and either pUN90-MAL63 or p413-GPD-MAL63/43c (carrying a constitutive *MAL*-activator gene). Transformants were grown to mid-log phase on YNB selective media containing as carbon source either 2% maltose (for strains carrying an inducible activator) or 3% glycerol/2% lactate (for strains carrying a constitutive activator). Cells were collected in mid-log phase for observation by confocal and phase microscopy. **b** Strain CMY1050 cells transformed with pUN30-MAL61/HA-GFP and YCp50-MAL63 grown to mid-log phase and incubated in FM4-64 dye as described in Materials and methods. The images were taken after 120 min. **c** Strain CMY7002 (*END3*) and the isogenic strain CMY7003 (*end3Δ)* were transformed with pUN30-MAL61/HA-GFP and pUN90-MAL63. Strains CMY1025 (*MAL1 doa4Δ::HIS3*) and CMY1026 (*MAL1 DOA4*) were transformed with pUN30-MAL61/HA-GFP and YCp50-MAL63. Cells were collected in mid-log phase for observation by confocal and phase microscopy. Phase images are shown to the left of the fluorescence images

shows the same localization pattern (Fig. 2a) as CMY1050. Thus, the observed vacuolar localization of Mal61/HA-GFP permease is neither strain-specific nor carbon source-dependent but is characteristic of normal maltose permease trafficking.

GFP-tagged maltose permease localizes to the vacuole via the plasma membrane

To determine whether the GFP signal observed in the vacuole is dependent on permease internalization from the plasma membrane or is derived from direct trafficking from the Golgi, we investigated Mal61/HA-GFP permease localization in strains carrying *END3* and *DOA4* mutations. *END3* encodes a component of a protein complex associated with cortical actin and is required for an early step in endocytosis (Benedetti et al. 1994; D'Hondt et al. 2000). Null mutants in *END3* are viable but exhibit severe defects in plasma membrane protein internalization. Strains CMY7002 (*END3*) and CMY7003 (*end3Δ*), which are derived from BY4741, the parental strain of the yeast *MAT*α haploid deletion series (Table 1), were transformed with plasmid-borne *MAL61/HA-GFP* and *MAL63* and grown in selective medium with 2% maltose as a carbon source. As shown in Fig. 2c, the fluorescence signal in the vacuole is dramatically decreased in the *end3Δ* mutant indicating that vacuolar accumulation of Mal61/HA-GFP permease is largely dependent on *END3* and thus a constitutive internalization of Mal61/HA-GFP permease is most likely the source of the vacuolar-localized protein. The residual low-level fluorescent signal in the vacuole of the *end3Δ* mutant suggests the possibility that there may be a low level of direct Golgi to vacuole trafficking. However, direct trafficking is clearly not the major source of the vacuolar maltose permease under these growth conditions.

The *DOA4* encodes a ubiquitin hydrolase and *doa4Δ* mutations are severely depleted of free ubiquitin and exhibit defects in ubiquitin-dependent processes (reviewed in Hochstrasser 1996; Medintz et al. 1998). The function of ubiquitin as a membrane protein trafficking signal is widely established (Hicke 1999). Ubiquitin conjugation is thought to be a targeting signal for the endocytosis of Ste2p (Hicke and Riezman 1996), Ste3p (Roth and Davis 1996), Fur4p (Marchal et al. 2000), Gal2p (Horak and Wolf 2001), and Mal61p (Medintz et al. 1998), as well as many other integral membrane proteins in yeast and other organisms. During endocytosis from the plasma membrane, ubiquitinated proteins are sorted to the vacuole via the late endosome—multivesiculated body (MVB) pathway (Wendland 2002; Bonifacino and Traub 2003; Robinson 2004). Ubiquitin is also known as a key player in post-Golgi trafficking where it diverts newly synthesized proteins away from association with lipid rafts and delivery to the plasma membrane or toward the endosome—MVB pathway and the vacuole (Beck et al. 1999; Helliwell et al. 2001; Magasanik and Kaiser 2002; Bonifacino and Traub 2003; Umebayashi 2003).

Strains CMY1025 (*MAL1 doa4Δ*) and CMY1026 (*MAL1 DOA4*) were transformed with plasmid-borne *MAL61/HA-GFP* and *MAL63*, grown in selective medium with 2% maltose as carbon source, and the localization of Mal61/HA-GFP permease was determined by confocal microscopy (Fig. 2c, bottom panels). The absence of intracellular GFP signal is most striking. This result indicates that *DOA4* is required for movement of Mal61/HA-GFP maltose permease into intracellular compartments and is supportive of our hypothesis that a slow constitutive internalization of plasma membrane maltose permease is the major source of the vacuolar-localized protein. It is also consistent with our suggestion that the low residual vacuolar accumulation of Mal61/HA-GFP observed in the *end3Δ* mutant is derived from direct Golgi to vacuole trafficking via the MVB pathway.

Alteration of Ser/Thr residues and a dileucine motif of the N-terminal cytoplasmic domain significantly decreases maltose permease glucose-induced proteolysis but not internalization

The N-terminal cytoplasmic domain of Mal61 maltose permease, particularly residues 48–78, contains a putative PEST sequence rich in serine, threonine, proline, and acidic residues (Cheng and Michels 1989). Phosphorylation of Ser/Thr residues of PEST sequences is associated with regulated proteolysis reportedly by enhancing the recognition of nearby lysine residues by the ubiquitination enzymes (Hochstrasser 1996; Hicke 1999). Previously, we showed that deletion of residues 30–60, which are rich in serine and threonine residues, and residues 48–78 containing the putative PEST sequence blocks glucose-induced proteolysis of maltose permease suggesting that this region could be the target of the glucose signaling pathway marking maltose permease for inactivation (Medintz et al. 2000). Therefore, we undertook a detailed examination of specific residues in the N-terminal cytoplasmic domain of Mal61 maltose permease to determine their role in glucose-induced inactivation.

The Ser and Thr residues in the N-terminal cytoplasmic domain were changed individually and cumulatively to alanine by site-directed mutagenesis of a plasmid-borne copy of MAL61/HA, plasmid pUN30-MAL61/HA. The specific residues altered are marked by a filled circle in Fig. 1. Plasmids carrying the mutant alleles were transformed into strain CMY1050 along with the plasmid-borne constitutive *MAL*-activator MAL63/43-c. Standard inactivation protocol described in Materials and methods was used to determine the effect of the alteration on the glucose-induced inactivation of the mutant Mal61/HA permease. The results are reported in Fig. 3.

None of the single Ser/Thr substitutions had significant impact on the glucose-induced inactivation of maltose permease (data not shown). The mutation Thr91Ala blocked Mal61p proteolysis but a Thr91Val mutation at this site, which introduces an amino acid with a hydrophobic bulky side chain similar to that of threonine, undergoes glucose-induced proteolysis at a rate comparable to wild-type Mal61p. Thus, we suggest that the effects of the Thr91Ala mutation are not indicative of an essential role for threonine-91 in glucose-induced proteolysis but instead that this residue has some structural importance for the N-terminal cytoplasmic domain, perhaps because of its proximity to the first transmembrane domain.

Overall phosphorylation levels of the mutant Mal61/HA proteins are reduced, but even with all five residues mutated we still observe a full range of phosphorylated species (Fig. 3a) (Medintz et al. 1996). The Mal61/HA protein contains 12 potential kinase target sites in cytoplasmic domains. Although, the protein often appears to migrate as two mobility species, it is likely that multiple sites are phosphorylated and the results reported here are consistent with this.

Surprisingly, even cumulative substitution of two, three, or five Ser/Thr residues does not appear to significantly impair expression of the mutant maltose permease proteins (Fig. 3a). Maltose transport activity expressed by transformants carrying the different

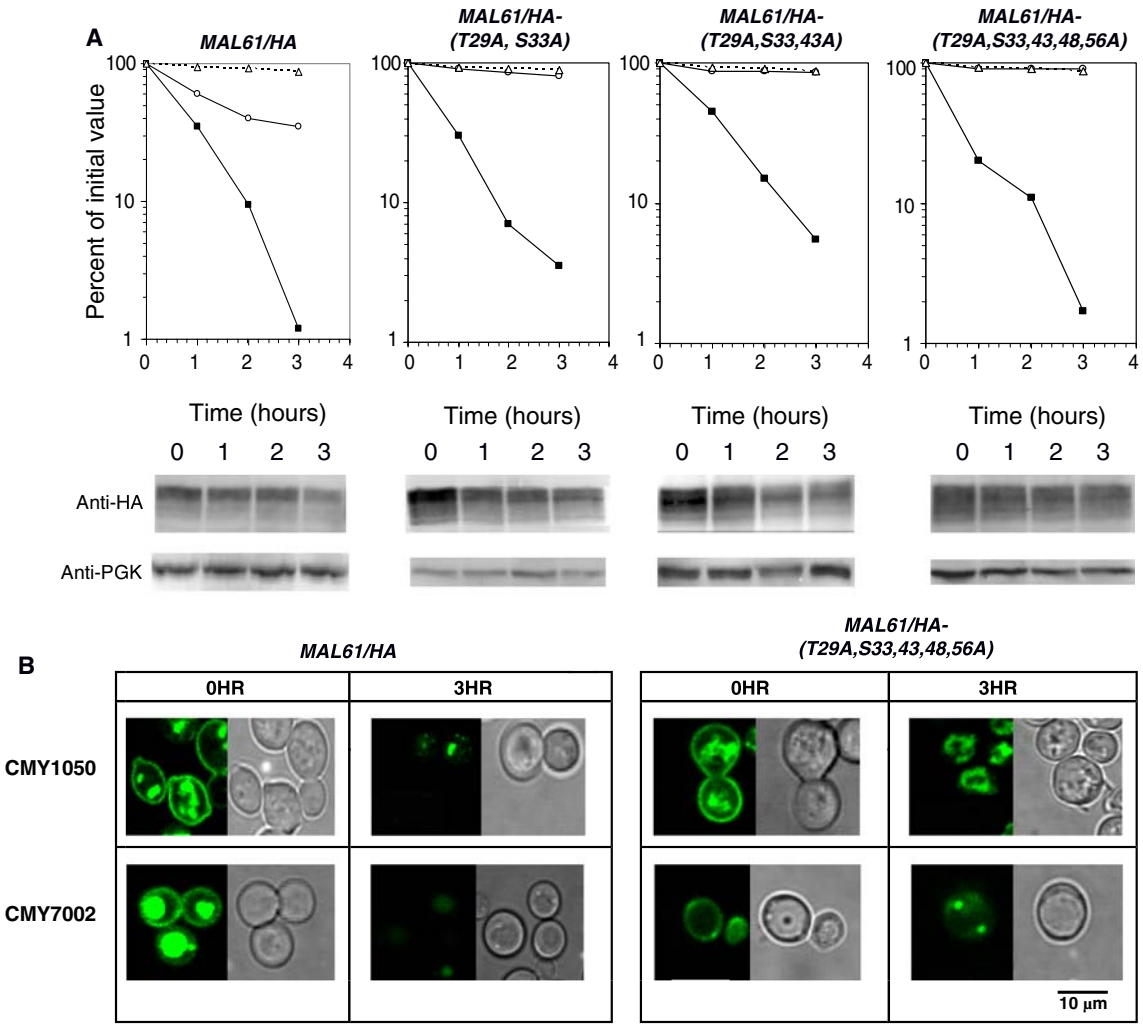

Fig. 3 Glucose-induced inactivation and subcellular localization of maltose permease mutants containing N-terminal Ser/Thr to Ala alterations. CMY1050 was transformed with HA-tagged wild-type *MAL61/HA* allele, or *MAL61/HA*-(*T29A, S33A*), *MAL61/HA*-(*T29A, S33A, S43A*), *MAL61/HA*-(*T29A, S33A, S43A, S48A, S56A*) mutant alleles each carried in the CEN vector pUN30, and with YCp50-MAL43c, carrying the constitutive *MAL43-c MAL*-activator gene. Transformants were grown in selective 3% glycerol/2% lactate medium lacking tryptophan and uracil. Standard inactivation assays were carried out as described in Materials and methods. The data represent the averages of three independent experiments. **a** The relative levels of Mal61/HA protein (*circle*) and maltose transport activity (*filled square*) compared with those of the zero time sample are plotted along with growth dilution (*triangle*). Growth dilution is calculated as the OD_{600} at time zero divided by OD_{600} at time *x*. Representative anti-HA immunoblots are shown along with the anti-PGK loading control. **b** Shows cells of CMY1050 and CMY7002 transformed with pUN30 plasmid-borne *MAL61/HA-GFP* or *MAL61/HA*-(*T29A, S33A, S43A, S48A, S56A*)-*GFP* and YCp50-MAL43c. Transformants were grown in 3% glycerol/2% lactate selective media. At time zero, cells were harvested by filtration and transferred to YNSG with CHX. Cells were collected at indicated times for observation by confocal and phase microscopy. The phase images are shown to the right

mutant alleles is reduced by only 10–30% compared to the transformant expressing wild-type *MAL61/HA* (data not shown). However, the rate of maltose permease proteolysis is reduced significantly when two or three Ser/Thr residues are altered together (Fig. 3a), with the allele containing five altered Ser/Thr residues exhibiting the lowest rate of degradation.

The results in Fig. 3 suggest that phosphorylation of Ser/Thr residues 29, 33, 43, 48, and 56 is not required for plasma membrane localization, transport activity, or the rapid glucose-induced internalization of maltose permease, but is needed to target maltose permease for degradation in the vacuole. To explore this further, a GFP-tagged allele of *MAL61/HA*-(*T29A, S33, 43, 48, 56A*) was constructed and introduced into parental strains CMY1050 and CMY7002 along with plasmid-borne *MAL43-c*. In strain CMY1050 wild-type Mal61/HA-GFP protein localizes to the cell surface and quite abundantly to internal lobular structures (Fig. 3b, top left panels). In strain

CMY7002, which has a more defined vacuole, Mal61/HA-GFP localizes at the cell surface and in the vacuole (Fig. 3b, bottom left panels). In both strains, glucose induces rapid removal of Mal61/HA-GFP from the cell surface and degradation of the protein in intracellular compartments.

Localization of the GFP-tagged Mal61/HA-(T29A, S33, 43, 48, 56A) mutant permease is shown in Fig. 3b (right panels). Consistent with the near normal levels of maltose transport activity, the mutant protein localizes well to the cell surface and to an intracellular compartment that, based on the fluorescence pattern in CMY7002, does not appear to be the vacuole. Glucose stimulates removal of the mutant permease from the cell surface but no significant loss of the fluorescence signal (Fig. 3b) or decrease in maltose permease protein levels (Fig. 3a) is observed. In strain CMY1050, the mutant protein remains stable in multiple vesicle-like structures while in CMY7002 the GFP signal coalesces into one or two bright spots (Fig. 3b, right panels). These intracellular structures are similar to structures described in the literature as class E compartments or exaggerated prevacuolar compartments (Raymond et al. 1992).

In summary, it appears that synthesis and plasma membrane delivery of Mal61/HA-(T29A, S33, 43, 48, 56A) mutant protein is unaffected by loss of these potential phosphorylation sites in the N-terminal cytoplasmic domain. The mutant protein also is internalized normally in response to glucose, but it exhibits a severe defect in glucose-induced degradation, possibly due to defects in the late stages of delivery to the vacuole.

Mal61 also contains in its N-terminal cytoplasmic domain a putative dileucine motif, a sequence motif implicated in the sorting and internalization of a number of plasma membrane proteins (Bonifacino and Traub 2003). Dileucine motifs are reported to be recognized at the *trans*-Golgi network (TGN) by the clathrin adaptor protein complexes AP-1 and AP-3 and may be responsible for routing some dileucine motif-containing integral membrane proteins to the basolateral membrane of polarized cells and to early and late endosomes (Bonifacino and Traub 2003; Traub and Apodaca 2003). In Mal61/HA maltose permease, leucines 69 and 70 are located in a putative [D/E]EXXXL[L/I] class of dileucine motif (position noted in Fig. 1). In addition to the two leucines/isoleucine residues in this class of dileucine motif, the glutamate (E) and/or aspartate (D) residues at positions -4 and -5 from the L/I pair of residues are also important in its recognition by adaptor complexes (Pond et al. 1995; Sandoval et al. 2000; Bonifacino and Traub 2003).

In strains expressing a mutant Mal61/HA maltose permease carrying a pair of Leu to Ala substitutions at residues 69 and 70 of the potential dileucine motif, glucose addition stimulates an increase in maltose transport activity that is followed by a slow decrease in transport activity, possibly due to degradation of the protein (Medintz et al. 2000). We tested the possibility that residues 64–70 constitute a dileucine sorting motif involved in Mal61 permease trafficking by following permease localization in strains expressing the *MAL61/HA-(L69, 70A)* allele (Medintz et al. 2000) or the *MAL61/HA-(E64, 65R)* allele, in which the upstream glutamate residues of the putative dileucine motif were substituted by arginine. Plasmids carrying the wild-type allele *MAL61/HA*, the dileucine mutation *MAL61/HA-(L69, 70A)*, and the di-glutamate mutation *MAL61/HA-(E64, 65R)* were introduced into CMY1050 (*mal11Δ*) along with a plasmid carrying the constitutive *MAL*-activator allele *MAL43-c.* Glucose-induced inactivation was assayed and the results are shown in Fig. 4.

Transformants expressing *MAL61/HA-(L69, 70A)* exhibit approximately 10-fold less maltose transport activity than transformants carrying the wild-type allele (1.73 units as compared to 9.26 units). Based on Western blot analysis (data not shown), the level of the dileucine mutant maltose permease protein expressed is comparable to that in cells expressing the wild-type allele. These conflicting findings are explained by the fact that cell surface localization of the mutant protein is dramatically reduced compared to wild-type, and most of the Mal61/HA-(L69, 70A)-GFP mutant protein accumulates in several cytoplasmic vesicles in CMY1050 and CMY7002 (Fig. 4b).

The defective localization of Mal61/HA-(L69, 70A)-GFP could indicate that the dileucine motif is required for sorting to the plasma membrane. This does not appear to be the case. *MAL61/HA-(L69, 70A)* was expressed in an *end3Δ* strain and, as shown in Fig. 5, the defects in maltose transport activity and plasma membrane localization of Mal61/HA-(L69, 70A)-GFP are significantly suppressed by the endocytosis defect. Thus, it appears that the decrease in plasma membrane localization of the mutant permease results from an increased rate of removal from the cell surface rather than mislocalization of newly synthesized protein.

Plasma membrane-localized Mal61/HA-(L69, 70A)-GFP is sensitive to glucose-induced internalization. Over the 3-hour time course following glucose addition to CMY1050 cells expressing the GFP-tagged allele of *MAL61/HA-(L69, 70A)*, the small amount of permease protein found at the cell surface is internalized but

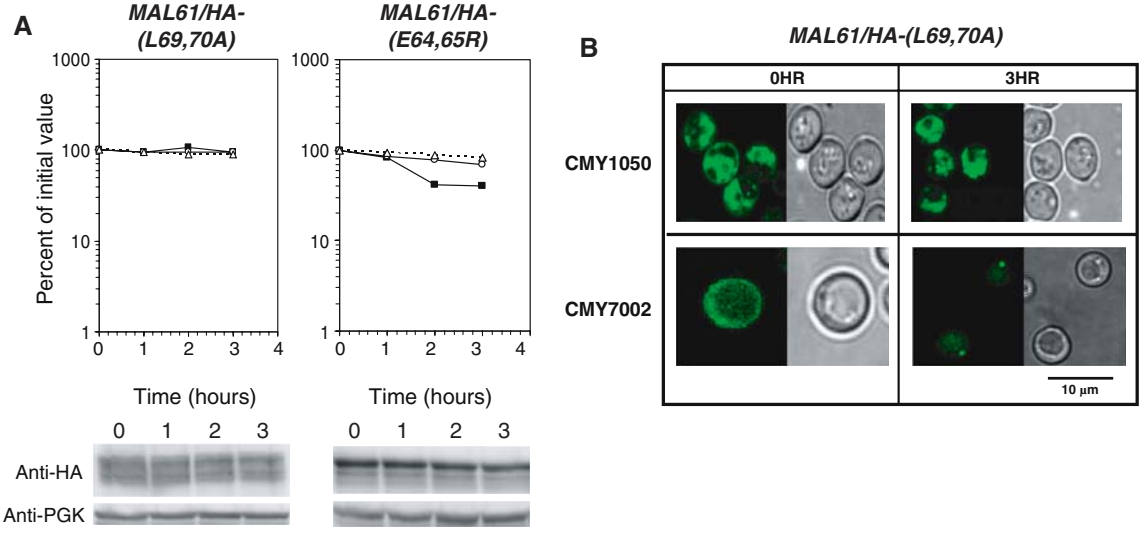

Fig. 4 Effects of mutations in the dileucine (D/EExxxLL/I) motif of Mal61 maltose permease on subcellular localization and glucose-induced inactivation. Strain CMY1050 was transformed with plasmid pUN30-MAL61/HA-(L69, 70A) or pUN30-MAL61/HA-(E64, 65R) and YCp50-MAL43c. All transformants were grown as described in Fig. 3, harvested, and transferred to YNSG with CHX. **a** Shows the growth dilution (*triangle*), maltose transport activity (*filled square*), and the relative Mal61/HA protein levels (*circle*) determined as described in Materials and methods. Anti-PGK is shown as a loading control. The results for *MAL61/HA* are taken from Fig. 3. **b** Shows strains CMY1050 and CMY7002 transformed with plasmid-borne (in vector pUN30) *MAL61/HA-GFP* or the indicated mutant allele and YCp50-MAL43c. Transformants were grown in 3% glycerol/2% lactate selective medium. At time zero, cells were harvested by filtration and transferred to YNSG with CHX. Immediately and after 3 h, cells were collected for observation by confocal and phase microscopy as described in Figure. The phase images are shown to the right

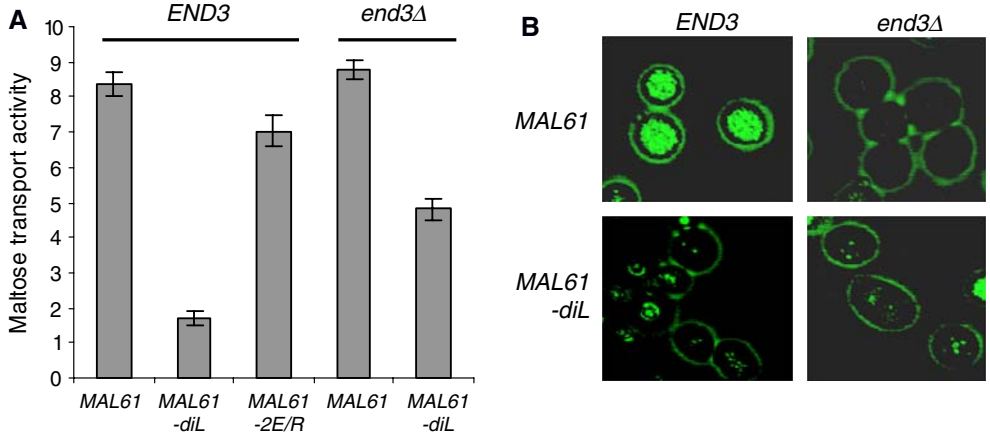

Fig. 5 Loss of *END3* suppresses defects in the maltose transport activity and plasma membrane localization of the dileucine mutant permease Mal61/HA-(L69, 70A). Strains CMY7001 (*END3*) and CMY7003 (*end3Δ*) were transformed with pUN90-MAL63 and either *MAL61/HA-GFP, MAL61/HA-(L69, 70A)-GFP*, or *MAL61/HA-(E64, 65R)-GFP* carried on the CEN vector pUN30. **a** Transformants were grown in selective 2% maltose media, harvested, and maltose transport activity was assayed as described in Materials and methods. **b** CMY7001 (*END3*) and CMY7003 (*end3Δ*) were transformed with pUN90-MAL63 and either *MAL61/HA-GFP* or *MAL61/HA-(L69, 70A)-GFP* and grown in selective 2% maltose media. Cells were collected at mid-log phase for confocal microscopy

is retained in intracellular vesicles (Fig. 4b). In strain CMY7002, GFP fluorescence, which initially is dispersed in a number of cytoplasmic structures, becomes localized to one or two distinct and brightly fluorescent subcellular compartments after 3 h (Fig. 4b). This loss of Mal61/HA-(L69, 70A)-GFP permease from the plasma membrane does not coincide with a decrease in maltose transport activity. The basis of this inconsistency is not clear but may be related to the very low maltose transport activity of the mutant permease.

Transformants carrying *MAL61/HA-(E64, 65R)* express near wild-type levels of maltose transport activity (7.04 maltose transport units compared to 9.26 units). Additionally, the phosphorylation level of Mal61/HA-E64, 65R protein is only slightly decreased compared to wild type, and clearly not to the extent seen for the Mal61/HA-L69, 70A mutant protein (Fig. 4a). However, alteration of glutamate resides 64 and 65 leads to a significant decrease in the rate of glucose-induced loss of maltose transport activity, and glucose-induced proteolysis of Mal61/HA-E64, 65R permease is nearly completely blocked.

In summary, the putative dileucine motif does not appear to play an important role in Mal61 permease sorting to the plasma membrane or in its glucose-induced internalization. Instead, dileucine motif mutant proteins accumulate in intracellular compartments and fail to progress to the vacuole for degradation.

Mal61 permease dileucine motif mutant proteins accumulate in a prevacuolar or E-compartment

Both the dileucine mutant protein Mal61/HA-L69, 70A and the di-glutamate mutant protein Mal61/HA-E64, 65R are resistant to glucose-induced proteolysis and localize to an intracellular compartment that does not appear to be the vacuole. To characterize this intracellular compartment, we tested whether the mutant permeases co-localize with Snf7p, a component of the ESCRT-III complex that is present in the membrane of the late endosome and functions in the internalization of ubiquitinated proteins from the late endosome membrane to form the MVB (Babst et al. 2002). Snf7p was visualized using a strain in which the sequence encoding red fluorescent protein (RFP) was fused to the 3'end of the SNF7 open reading frame (Huh et al. 2003). Strain CMY7001 is derived from this strain by the introduction of a *trp1Δ* mutation. Our initial studies of CMY7001 suggested that the SNF7-RFP fusion is not fully functional. When CMY7001 was transformed with plasmid-borne *MAL63* and *MAL61/HA-GFP*, we found that the GFP fluorescence does not localize to the vacuole as in wild-type strains but instead co-localized with Snf7-RFP protein (data not shown). We complemented this defect by transforming CMY7001 with plasmid pYES-pro-SNF7, which carries wild-type *SNF7* expressed from its native promoter on a high-copy plasmid (Fig. 5).

Strain CMY7001 [pYES-pro-SNF7] was transformed with plasmid-borne *MAL43-C* and either *MAL61/HA-GFP*, the dileucine motif mutant *MAL61/HA (L69, 70A)-GFP*, or the multiple Ser/Thr mutant allele *MAL61/HA-(T29A, S33A, S43A, S48A, S56A)-GFP*. Figure 6 shows the GFP-tagged permease proteins in green and Snf7-RFP in red. Snf7-RFP signal localizes to one or two small subcellular compartments, marking the location of the late endosome compartments. During growth on maltose, wild-type Mal61/HA-GFP protein is seen at the cell surface and in the vacuole with little or no overlap of the two fluorescence signals, indicating that the wild-type permease moves rapidly to the vacuole. In contrast, in strains expressing the multiple Ser/Thr-to-Ala or the dileucine mutant permeases the fluorescence signal is at the cell surface and in discrete subcellular compartments that overlap significantly with the endosomal RFP signal. These results suggest that the mutant proteins localize to the late endosome in a structure that is often referred to as the prevacuolar or E-compartment, and fail to progress from this compartment to the vacuole for degradation.

Discussion

Trafficking and subcellular localization of Mal61/HA-GFP maltose permease

The results reported here suggest that newly synthesized maltose permease localizes directly from the TGN to the plasma membrane, where it is relatively stable in the absence of glucose. We observed significant amounts of permease protein in the vacuole but this accumulation is dependent on endocytosis from the plasma membrane since only low levels of permease localize to intracellular compartments in an *end3Δ* strain (Fig. 2b). We uncovered a slow constitutive internalization of maltose permease, with a half-life of about 4–6 h as measured by loss of transport activity or protein levels (Gadura and Michels, unpublished results) that probably is the major source of the vacuolar permease. The modest level of vacuolar localization observed in the *end3Δ* mutant, which is absent in the *doa4Δ* (Fig. 2b) mutant, suggests that direct Golgi to vacuole trafficking via the MVB pathway may also be taking place but at a low rate. Vacuolar accumulation has also been reported for Fur4-GFP, which is attributed to the 2.5-hour basal turnover rate of Fur4p and resistance to vacuolar proteolysis (Volland et al. 1994; Dupre and Haguenauer-Tsapis 2001). Resistance to vacuolar proteolysis also appears to be true for Mal61/HA-GFP maltose permease and may reflect a stability of the GFP moiety alone, although this has not been tested.

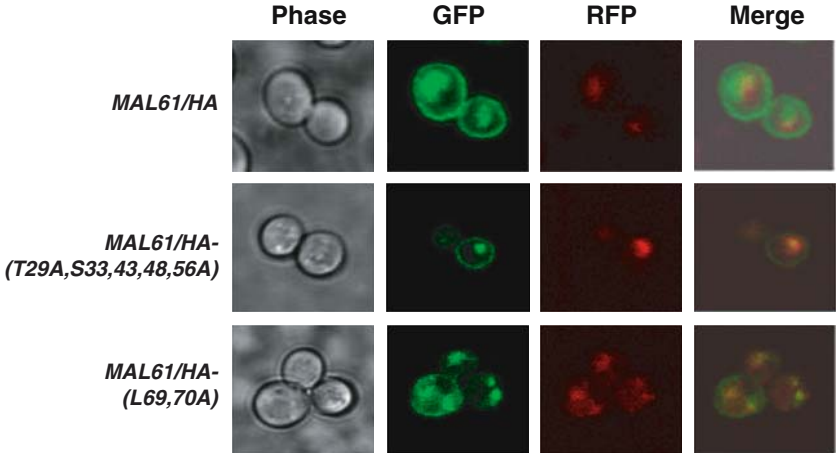

Fig. 6 Subcellular co-localization of Mal61/HA mutant permeases with the ESCRT-III complex component Snf7. Strain CMY7002 containing C-terminally tagged *SNF7-RFP* was transformed with pYES-pro-SNF7, pUN90-MAL63 and either *MAL61/HA-GFP*, *MAL61/HA* (*T29A, S33A, S43A, S48A, S56A*)-*GFP*, or *MAL61/HA*-(*L69, 70A*)-*GFP* carried on the CEN vector pUN30. Transformants were grown in selective media plus 2% maltose. Cells were collected at mid-log phase for observation by confocal and phase microscopy. Appropriate filter sets were used for GFP (*shown in green*) and RFP (*shown in red*) as indicated in Materials and methods. The images were merged using Adobe Photoshop

Phosphorylation of Ser/Thr residues in the Mal61 N-terminal cytoplasmic domain is required for glucose-induced proteolysis but not for glucose-induced endocytosis

Down regulation of a number of plasma membrane proteins requires phosphorylation, which reportedly makes them attractive targets for ubiquitination, thereby triggering their selective endocytosis (Marchal et al. 1998; Hicke 1999; Feng and Davis 2000). The results reported here for *S. cerevisiae* Mal61p suggest that phosphorylation of the Ser/Thr residues in the N-terminal cytoplasmic domain of the PEST sequence is not essential for internalization from the plasma membrane but may be required for delivery of the internalized protein to the vacuole. Mal61/HA-(T29A, S33, 43, 48, 56A) mutant permease localizes to the plasma membrane normally and glucose induces a rapid loss of maltose transport activity which correlates with the rapid removal of the GFP-tagged allele of this mutant protein from the cell surface (Fig. 3). However, in contrast to wild-type permease, GFP-tagged Mal61/HA-(T29A, S33, 43, 48, 56A) permease is not delivered to the vacuole and is not degraded. Instead, it persists in an intracellular compartment that we show is a late endosome, based on co-localization with the late endosomal membrane protein Snf7-RFP (Fig. 6). We suggest that these late endosomes containing Mal61/HA-(T29A, S33, 43, 48, 56A) permease are unable to progress from late endosomes to an MVB and do not fuse with the vacuole. Instead, they form a terminal compartment, sometimes referred to as an E-compartment, and the protein is not degraded.

Studies on Fur4p, the uracil permease, showed that cumulative mutations of Ser to Ala in the PEST-like sequence reduce the rate of turnover of this membrane protein whereas Ser to Glu mutations led to its constitutive turnover (Marchal et al. 1998; Marchal et al. 2000; Marchal et al. 2002). Phosphorylation of the Ser residues in the PEST-like sequence of Fur4p is dependent on Yck1,2 casein kinase 1 activity. In a yck^{ts} strain, Fur4-GFP is internalized, but accumulates in intracellular compartments consistent with prevacuolar compartments, based on colocalization with Pep12 protein (Marchal et al. 2002). These results suggested that phosphorylation of residues in the Fur4p PEST sequence is required for a late step in delivery of this protein to the vacuole but is not the initiating step in endocytosis. Our results for Mal61 permease suggest a similar scenario for its N-terminal PEST sequence.

The Yck1,2 kinase activity is required for glucose-induced inactivation of maltose permease (Gadura et al. 2006). In contrast to Fur4p, we found that in a yck^{ts} strain maltose permease localizes to the cell surface, albeit in an inactive state, but glucose-induced internalization is blocked at an early step in endocytosis. Our observation that Mal61/HA-(T29A, S33, 43, 48, 56A) mutant protein is still sensitive to glucose-induced internalization suggests that phosphorylation of these residues is not a prerequisite for internalization and thus, that these residues are not targets of Yck1,2 kinase activity. The Yck1,2 kinase target site(s) in Mal61 permease have not been identified, but three potential casein kinase 1 consensus sequences (S(P)-$X_{1\text{-}3}$-S/T) are located within the cytoplasmic loops

(Kennelly 1998). These sites are currently under investigation.

An N-terminal dileucine motif is involved in delivery of Mal61 to the vacuole

Several distinct signals for sorting proteins in the secretory pathway have been identified that include tyrosine-based signals and two classes of dileucine motif signals, so-called because an adjacent pair of leucine or isoleucine residues is an essential feature of the motif (Bonifacino and Traub 2003). A [D/E]ExxxL[L/I] class of dileucine motif is found in residues 64–70 the N-terminal domain of Mal61 maltose permease. Deletion of residues spanning this motif suggested a role for this region in the glucose-induced Mal61 proteolysis (Medintz et al. 1998). Our studies with GFP-tagged maltose permease here indicate that alterations in this motif block permease proteolysis by stalling the permease in an intracellular compartment, preventing delivery to the vacuole for degradation.

Strains expressing the Mal61/HA-L69, 70A mutant permease exhibit dramatically reduced levels of maltose transport activity and, consistent with this, very low levels of Mal61/HA-L69, 70A mutant permease localize to the cell surface. In the endocytosis-defective strain *end3Δ*, the level of cell surface GFP-tagged Mal61/HA-L69, 70A mutant permease is increased significantly, indicating that loss of the essential dileucine pair of this putative dileucine motif does not affect trafficking to the plasma membrane but instead causes reduced residency at the cell surface. Internalized Mal61/HA-L69, 70A mutant permease co-localizes with the late endosomal marker Snf7-RFP (Fig. 6), indicating that vesicles carrying the Mal61/HA-L69, 70A mutant permease exhibit a severe defect in entry into the vacuole for degradation.

Taken together, the results of our analysis of the N-terminal cytoplasmic domain of Mal61 maltose permease do not support a role for this region in the regulation of glucose-induced internalization. Nonetheless, alterations of residues in this domain have a significant impact on Mal61 permease proteolysis by affecting the late stages of delivery to the vacuole of internalized proteins. The mutant proteins tested in this study are diverted to a late endosome-like or prevacuolar compartment, based on the presence of Snf7p, and are resistant to degradation. Whether these sequences are required for formation of the MVB or simply cause a structural defect that inhibits late steps in delivery to the vacuole remains to be determined. Possible roles for dileucine-like motifs in late endosomal/lysosome sorting of other integral membrane proteins have recently been reported (Augustin et al. 2005; Tsacoumangos et al. 2005).

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