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The Role of Yck1,2 Casein kinase 1 in the Trafficking and Glucose Induced Inactivation of Maltose Permease in *Saccharomyces cerevisiae*

by

NIDHI GADURA

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy,

The City University of New York.

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ABSTRACT

The role of Yck1,2 casein kinase 1 in the trafficking and glucose-induced inactivation of maltose permease in *Saccharomyces cerevisiae*

by

Nidhi Gadura

Advisor: Professor Corinne A. Michels

In *Saccharomyces*, glucose induces proteolysis of maltose permease that requires endocytosis, vesicle sorting and the vacuolar degradation enzymes, moreover, it is ubiquitin mediated. This dissertation continues the investigation of the mechanisms of this process and demonstrates the essential role of Yck1, 2 casein kinase 1.

Site-specific mutations of phosphorylatable Ser/Thr in the N-terminal PEST sequence dramatically slows the proteolysis but does not alter the ability of this protein to localize to the plasma membrane or its rapid glucose-induced loss of maltose transport activity. Altering a putative dileucine [D/EExxxLL/I] motif (involved in protein sorting) and a dilysine motif (putative ubiquitin conjugation sites) in the PEST sequence causes significant defects in maltose transport activity, mislocalization to a PVC and resistance to glucose-induced inactivation. Also in *doa4Δ* strain (depletes available ubiquitin) Mal61p localizes to the plasma membrane, remains hyperphosphorylated and transports actively. However, there is no glucose-induced inactivation, implying that ubiquitination at the plasma membrane is required for endocytosis of Mal61p.

Yeast casein kinase 1, encoded by *YCK1* and *YCK2,* is involved in endocytosis of several integral membrane proteins. We used strain *yck1Δ yck2-ts* and an *akr1Δ* (defective in the localization of Yck1,2 kinase to the plasma membrane), to explore the

role of Yck1,2 kinase in Mal61p glucose-induced inactivation. The results show that *yck-ts* strains exhibit significantly reduced ability to transport maltose and no impairment of its localization to the cell surface however glucose-induced inactivation is blocked. Overexpression of *YCK2* in *doa4Δ* does not restore glucose-induced inactivation of Mal61p indicating that *YCK1, 2* is upstream of *DOA4*.

Our previous study observed that glucose-induced inactivation in a *reg1*Δ strain is blocked and that phosphorylation of Mal61p decreases suggesting that Reg1p-Glc7p phosphatase acts indirectly on maltose permease and that there is a kinase intermediate that regulates phosphorylation. *reg1*Δ and *yck-ts* strains exhibit similar phenotypes regarding Mal61p localization, phosphorylation, and resistance to glucose-induced inactivation suggesting that these regulations are components of a common signaling pathway. Epistasis analysis is used to establish that *REG1* is upstream of *YCK1,2* in a novel Glc7-Reg1 phosphatase – Yck1,2 kinase in the signaling pathway controlling glucose-induced inactivation of maltose permease.

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Another two wonderful little people to thank are my sons Vineet and Nitin Gadura who spent numerous weekends with me in the lab (thanks to the internet that kept

them busy!). Last but not the least, my husband Vinod Gadura, who asked me every single day when I was going to finish college. Well guess what honey, I am finally done!

In their own special ways everyone has helped me through the years. I must say that their love and support is deeply appreciated and has made the work of this thesis possible.

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INTRODUCTION

Glucose is a global metabolic regulator in *Saccharomyces*. It controls the expression of many genes involved in carbohydrate utilization at the transcriptional and posttranslational levels (Johnston, 1999). The goal of these glucose-regulated processes is to insure that glucose is metabolized before other carbon sources and to provide a mechanism for cells to undergo rapid transition from using alternate carbon sources like maltose, galactose or sucrose to the fermentation of glucose.

In *Saccharomyces*, 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 (Jiang et al., 1997; Medintz et al., 1996). Maltose permease, encoded by *MAL61* of *Saccharomyces cerevisiae,* is an integral membrane protein required for the transport of maltose across the plasma membrane (Cheng and Michels, 1989). Addition of glucose to maltose fermenting cells causes a very rapid loss of maltose transport activity and also the proteolysis of maltose permease protein (Medintz et al., 1996). Medintz *et al.* (1996) showed that the degradation of maltose permease is ubiquitin mediated and occurs via endocytosis-dependent machinery. This study is focused on understanding not only the underlying mechanisms involved in glucose-stimulated endocytosis of maltose permease but also on identifying specific residues in maltose permease involved in its phosphorylation and which mark it for ubiquitin-dependent endocytosis and vacuolar proteolysis. Our results will provide insights into the mechanisms of down regulation of several mammalian receptors like PDGF, EGF, fibroblast growth receptor that appear to

require phosphorylation and ubiquitination for their ligand-stimulated degradation (Hicke, 1997).

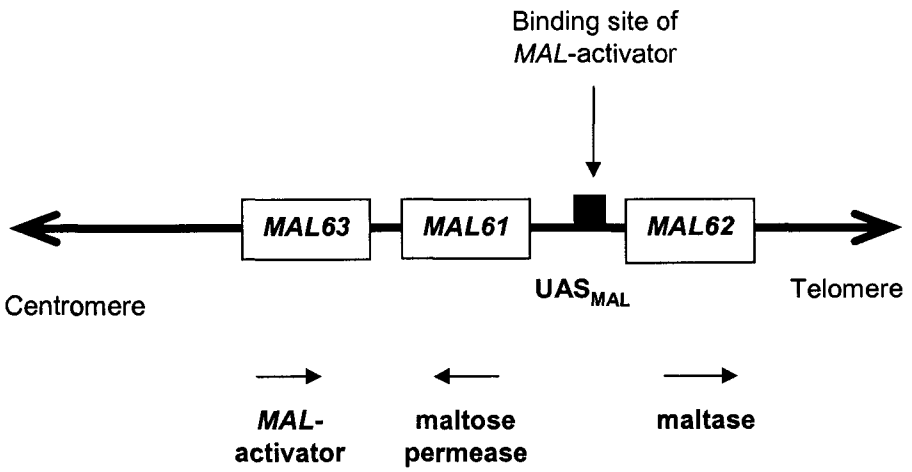
Part I. Maltose fermentation and *MAL* loci (brief review)

Maltose fermentation in *Saccharomyces* requires two enzymes, maltose permease and maltase reviewed in (Needleman, 1991). Maltose fermentation in *Saccharomyces* requires the presence of any one of the five unlinked complex loci: *MAL1, MAL2, MAL3, MAL4 or MAL6*. All of these loci are functionally equivalent (Needleman and Michels, 1983). Each *MAL* locus is a complex containing three genes required for maltose fermentation. The organization and structure of *MAL6* is shown in Figure 1. A numbering system was established to distinguish the gene functions mapping to the different *MAL* loci. Among the three genes required for maltose fermentation at each locus, GENE 1 encodes maltose permease, GENE 2 encodes maltase and GENE 3 encodes the DNA-binding *MAL*-activator (reviewed in (Needleman, 1991). Therefore, *MAL61* gene is GENE 1 function mapping to the *MAL6* locus (Needleman et al., 1984). The *MAL*-activator binds to the UAS_{MAL} upstream of GENE 1 and GENE 2 and is required for transcription of these genes (Chang et al., 1988; Levine et al., 1992).

Transcription of *MAL61* and *MAL62* is maltose induced and glucose repressed (Needleman et al., 1984). Induction is mediated by the *MAL*-activator but the mechanism by which the *MAL*-activator responds to maltose is not fully understood. Mal63 *MAL*-activator is an Hsp90 molecular chaperon client protein (Bali et al., 2003). It is found in complex with Hsp90 and stability of Mal63p is dependent on Hsp90 and other co-chaperons. It has been suggested that maltose binds directly to chaperon-bound Mal63p.

Figure 1. Organization of the *MAL6* locus

MAL61-MAL62 share a bi-directional promoter sequence that contains a *MAL*-activator binding site, UAS_{MAL}. Glucose repression of *MAL* gene transcription is also mediated when the Mig1, 2 repressor binds to sequences in this promoter sequence.



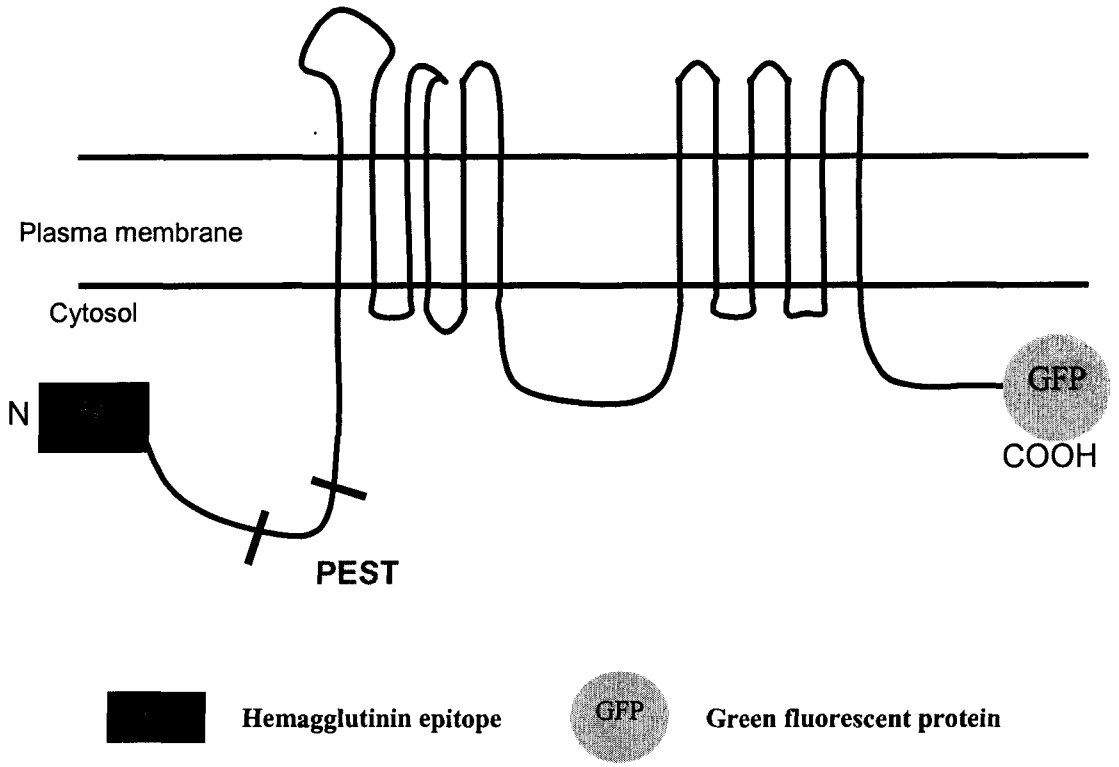
Maltose permease is also required for maltase induction (Charron et al., 1986). Results from our laboratory indicate that intracellular maltose is sufficient to induce *MAL* gene expression and acts via the *MAL*-activator (Wang et al., 2002).

(Hu et al., 1999) demonstrated that Mig1 repressor mediates glucose repression of the *MAL61* and *MAL62* structural genes as well as the *MAL63 MAL*-activator gene. Deletion of *MIG1* and its homologue *MIG2* fully relieves glucose repression of maltase expression yet maltose induction is still blocked by glucose even in a *mig1Δ mig2Δ* double mutant (Hu et al., 2000). Glucose-induced inactivation of maltose permease is believed to play a major role in this *MIG* repressor-independent glucose repression.

Maltose permease is a high-affinity maltose/proton symporter and a member of a 12 transmembrane domain (Figure 2) family of sugar transporters (Cheng and Michels, 1991). Hydropathy analysis shows significant sequence and structural homology of *Saccharomyces* maltose permease to other yeast, *E.coli* and human high-affinity glucose transporters (Cheng and Michels, 1989). The secondary structure of Mal61p consists of two blocks of six transmembrane domains separated by an approximately 71 residue intracellular region (Figure 2). The N-terminal and C-terminal domains of 100 and 67 residues in length, respectively, also are predicted to be intracellular. Maltose transport by maltose permease is independent of intracellular ATP levels but is coupled to the electrochemical gradient of protons, therefore Mal61p is a high-affinity proton symporter (Cheng and Michels, 1991; Han et al., 1995). Maltase is an α-glucosidase that hydrolyzes maltose to two molecules of glucose which can enter glycolysis and are metabolized to CO_2, ethanol, and ATP.

Figure 2. The structure of maltose permease

Mal61 maltose permease is a member of the 12 transmembrane domain family of sugar transporters. For our experiments, a HA-epitope tag has been placed at the N-terminus of the protein and GFP (green fluorescent protein) was fused in-frame to its C-terminus. The N-terminal cytoplasmic domain contains a PEST sequence.



Part II. Glucose-induced inactivation of maltose permease in *Saccharomyces*

The addition of glucose to maltose-induced fermenting cultures not only leads to cessation of maltose permease synthesis but also to a rapid decrease in maltose transport activity; this process is referred to as glucose-induced inactivation (Busturia and Lagunas, 1986; Gorts, 1969).

Glucose-induced inactivation of maltose permease is dependent on endocytosis and vacuolar proteolysis

Medintz *et al.* (1996) characterized the glucose-induced inactivation of maltose permease in *Saccharomyces*. For their studies, Medintz *et al.* (1996) used a genetically defined, maltose-fermenting strain containing a hemagglutinin (HA)-tagged allele of *MAL61*. This *MAL61/HA* allele was used to replace the *mal11::URA3* gene in strain 100-1A, a disruption of the maltose permease gene of *MAL1*, by a two-step gene replacement, thereby creating a *MAL1* locus containing the *MAL61/HA* sequence. Strain CMY1001 is a maltose-fermenting segregant carrying only this reconstructed *MAL1* locus (*MAL61/HA* encoding maltose permease, *MAL12* encoding maltase, *MAL13* encoding *MAL*-activator) and no other *MAL* genes. This construct is ideal for monitoring the levels of maltose permease protein and maltose transport activity because *MAL61/HA* is the sole maltose permease gene present in the strain.

Using this strain Medintz *et al.* (1996) showed that glucose stimulates the inactivation of maltose transport and a decrease in the level of maltose permease protein. Maltose permease degradation occurs via endocytosis and vacuolar proteolysis. This was demonstrated by using mutations in genes that control early steps in endocytosis (*END3*)

and vacuolar proteolysis (*PEP4*) (Medintz et al., 1996). They showed that in a *end3-ts* glucose-induced proteolysis of Mal61/HAp is blocked. Also, no glucose-induced proteolysis of maltose permease was seen in *pep4Δ* strain; *PEP4* encodes vacuolar proteinase A that is required for the activation of all vacuolar proteases. Moreover, glucose-induced proteolysis of maltose permease was unaffected by mutations in *PRE1* and *PRE2* that encode key proteosome components.

(Medintz et al., 1996) detected two species of Mal61/HA protein that differed slightly in gel mobility. Acid phosphatase treatment demonstrated that these represented differentially phosphorylated forms of the protein. Using *hxk2Δ* strain carrying a constitutive *MAL*-activator that expresses detectable levels of Mal61/HA they found that the level of phosphorylation varied in different culture conditions and the relative level of the hyperphosphorylated form (slower mobility) is greater in glucose-grown cells.

Glucose-induced inactivation of maltose permease is dependent on ubiquitination and the ubiquitin-conjugation enzymes

Ubiquitin, a 76 amino acid protein found in all eukaryotes, has long been known to play a key role in protein degradation of cytosolic proteins reviewed in (Hochstrasser, 1996a; Hochstrasser, 1996b). Covalent attachment of ubiquitin to the side chain of lysine residues in a protein targets these proteins for degradation by the 26S proteosomes, a multisubunit complex of proteases. Attachment is catalyzed by a series of enzyme complexes referred to as E1, ubiquitin activation enzyme; E2, ubiquitin conjugating enzyme; and E3, ubiquitin-protein ligase. In seminal work by (Hicke and Riezman, 1996) Ste2p, the *Saccharomyces* α-factor receptor and member of the seven

transmembrane domain class of integral membrane receptors, was shown to be ubiquitinated following ligand (α-factor) binding. This ligand-stimulated ubiquitination targeted Ste2p for endocytosis and degradation in the yeast vacuole. Since this report several yeast as well as mammalian receptors and permease, including maltose permease have been shown to undergo ubiquitination at the plasma membrane as a trigger to internalize and degrade that protein in a lysosome like vacuole (reviewed in (Hicke, 1999).

(Medintz et al., 1998) demonstrated that glucose-induced inactivation of maltose permease is dependent on ubiquitination. Moreover, ubiquitin conjugation required Npi1/Rsp5 ubiquitin-protein ligase. They show that deletion of *DOA4*, which depletes available ubiquitin levels, severely decreases the rate of glucose-induced proteolysis and overexpression of ubiquitin in *end3-ts* strain resulted in accumulation of slower moving species of maltose permease. This higher molecular weight species was shown to correspond to monoubiquitinated Mal61/HA permease (Medintz et al., 1998).

Recent studies reveal another role of ubiquitin at multiple protein sorting steps. Since ubiquitin conjugation is a cyclical process, its presence or lack thereof dictates the fate of protein as it exits from the TGN or from PM during endocytosis. Polyubiquitn is reported to serve as a signal for membrane proteins to be excluded from lipid rafts that are forming in the membranes of vesicles to be delivered to the plasma membrane (reviewed in (Umebayashi, 2003). Instead, these polyubiquitinated, newly synthesized proteins enter the MVB pathway enroute to the vacuole. This process utilizes the Rsp5 ubiquitin-protein ligase and Bul1,2 proteins (Magasanik and Kaiser, 2002; Umebayashi, 2003). Therefore, besides endocytosis from the plasma membrane, ubiquitin may play a

role in cargo protein sorting at Golgi or endosomal levels as well. These polyubiquitinated membrane proteins of endosomes are recognized by their ubiquitin moiety by ESCRT complexes of the MVB pathway. After ubiquinated cargo substrates are sorted into late endosomes, the cargo proteins are collected into invaginations by ESCRT complexes, the ubiquitin moiety is removed and recycled, and the cargo-containing vesicles are pinched off from the endosome membrane and accumulate in the lumen of this late endosome, referred to as a prevacuolar compartment (PVC) or the MVB (Horak, 2003; Umebayashi, 2003). The MVB then fuses with the vacuole dumping its contents into the lumen of the vacuole for degradation by the vacuolar enzymes.

Sequences in the N-terminal cytoplasmic domain of maltose permease are involved in glucose-induced inactivation

Cheng and Michels (1985) had reported the presence of a putative PEST sequence in the N-terminal cytoplasmic domain of Mal61p. (Rechsteiner, 1988) proposed that the polypeptide sequences rich in proline (P), glutamic acid (E), serine (S) and threonine (T) targets proteins for rapid degradation. By 1991, 43 out of 47 short-lived proteins were known to have PEST regions in them. There have been several studies since then linking this PEST sequence, especially when phosphorylated, as a sequence that marks the protein for degradation (Marchal et al., 1998; Rechsteiner and Rogers, 1996). Recognition of PEST sequences as ubiquitination target sites is proposed to be enhanced by phosphorylation by protein kinases and these protein kinases may themselves be regulated (reviewed in (Rechsteiner and Rogers, 1996).

Ample evidence in the literature suggests that PEST sequences are recognized by protein kinases that are responsible for phosphorylating these proteins. This also appears to be the case for several membrane proteins reviewed in (Hicke, 1999; Hochstrasser, 1996a; Hochstrasser, 1996b). Phosphorylation of the serines in the SINNDAKSS sequence of the C-terminal cytoplasmic domain of Ste2p of the α-factor receptor is required for ubiquitination (Hicke and Riezman, 1996). Uracil permease (Fur4p) also requires phosphorylation of a PEST-like sequence for ubiquitination and internalization (Volland et al., 1992). Phosphorylation has been implicated in the receptor-mediated endocytosis of several other *Saccharomyces* and mammalian receptors (Fallon et al., 1994; Hicke, 1997; Kublaoui et al., 1995; Zanolari et al., 1992). Therefore, phosphorylation of maltose permease is likely to play a role in regulating permease turnover.

(Medintz et al., 2000) carried out a deletion analysis of the N- and C-terminal domains of Mal61p maltose permease in an effort to identify sequences involved in marking the protein for degradation. They showed that the PEST-like sequence in the N-terminal cytoplasmic domain of *Saccharomyces* maltose permease is required for glucose-induced proteolysis and rapid inactivation of transport activity. Deletion of residues 49-78 from the N-terminal resulted in resistance to internalization and also a dramatic reduction in the relative amount of the ubiquitin-conjugated form of Mal61 protein. These results suggest that the presence of the PEST-like sequence within residues 49-78 is required for efficient glucose-induced ubiquitination and proteolysis of maltose permease. Medintz et al., (2000) also suggested that this sequence is the target of

the Rgt2p- and Glc7p-Reg1p-dependent glucose signaling pathways (to be discussed below).

(Medintz et al., 1998) had shown that L69, 70A mutation in the N-terminal cytoplasmic domain of maltose permease results in resistance to glucose-induced inactivation and causes an increase in transport activity after which they report a slow decrease due possibly to degradation of the protein. Human insulin-responsive glucose transporter, *GLUT4* also has a dileucine motif and so does yeast Gap1p, that seem to be required for the internalization of these proteins (Gabilondo et al., 1997; Springael and Andre, 1998). Mounting evidence implicates dileucine motifs in the internalization of several other plasma membrane proteins (reviewed in (Bonifacino and Traub, 2003). Not only is the dileucine motif required for sorting proteins at the plasma membrane but it has also been shown to be recognized by AP1 & 3 complexes at the TGN and serves as a signal for routing to early endosomes (Bonifacino and Traub, 2003; Darsow et al., 1998; Peden et al., 2001). Dileucine motifs are well conserved in membrane proteins found in a whole range of species from *C.elegans* to humans (Bonifacino and Traub, 2003). Several variations of dileucine motifs have been described and in Mal61/HA, maltose permease, leucines 69 and 70 are located in a putative [D/EExxxLL/I] class of dileucine motif. In addition to the two leucine/isoleucine residues of this class of dileucine motif the Glu and/or Asp residues at position -4 and -5 from the Leu/Ilu also are important in the recognition of this motif (Bonifacino and Traub, 2003).

Part III. Two glucose sensing/signaling pathways in *Saccharomyces*

The model of high glucose sensing and signaling pathways involved in glucose-induced proteolysis of maltose permease shown in Figure 3 is based on the work of

(Jiang et al., 1997; Jiang et al., 2000). Two glucose signaling pathways are involved in stimulating glucose-induced inactivation of maltose permease (Jiang et al., 1997). Pathway 1 utilizes Rgt2p, a sensor of high concentrations of extracellular glucose that also regulates *HXT1* expression encoding a low-affinity glucose transporter (Ozcan et al., 1996). *RGT2-1* is a dominant mutation that can suppress the high-affinity glucose-transport and growth defect of *snf3Δ* mutations (Marshall-Carlson et al., 1991). Snf3p and Rgt2p are glucose sensors that monitor extracellular glucose levels. *SNF3* protein sequence indicates that it encodes 12 transmembrane domain protein homologous to mammalian glucose transporters but plenty of evidence suggests that it is more likely a regulatory protein rather than a transporter (reviewed in(Bisson et al., 1993). Ozcan and Johnston, (1995) provide evidence that Snf3p functions as a glucose sensor responding to low levels of extracellular glucose and is required for the regulation of the *HXT* gene expression. Ozcan et al., (1996) characterized Rgt2p, showing that it encodes a putative hexose transporter with overall 60% sequence identity to Snf3p. Rgt2p is required for high glucose induction of *HXT1* gene expression and it seems to be involved in the glucose sensor monitoring high levels of extracellular glucose (Ozcan and Johnston, 1995).

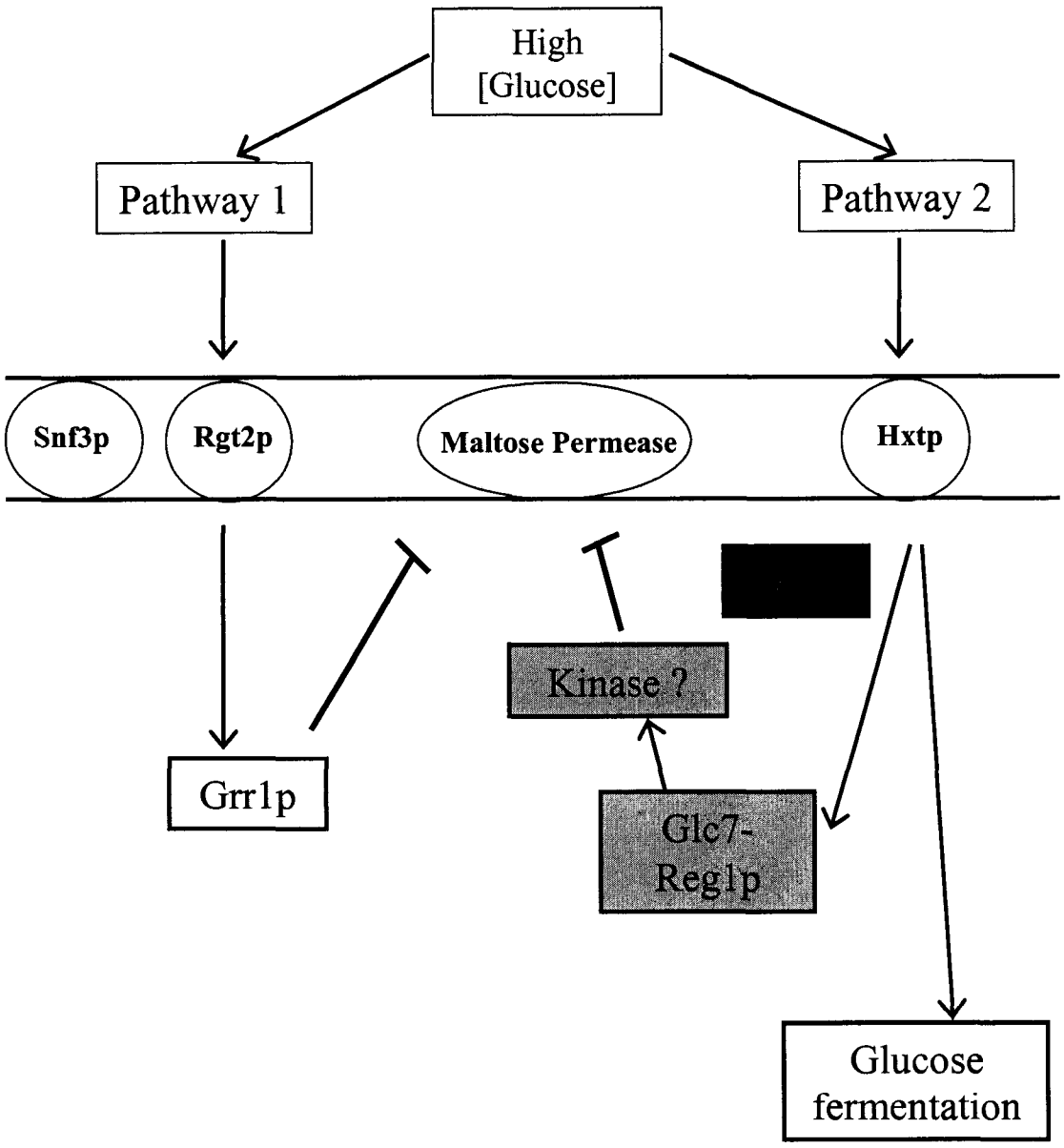
Ozcan and Johnston (1995) provide evidence that Grr1p is central in the signaling pathway regulating glucose induction of *HXT* gene expression. Grr1p is downstream of Rgt2p and Snf3p in the glucose signaling pathway used to monitor extracellular glucose concentrations (Ozcan et al., 1998; Ozcan et al., 1996). Grr1p is an F-box protein and is found in SCF^{GRR1} complex that functions as E3 ubiquitin-protein ligases (Li and Johnston, 1997). SCF complexes contain Skp2p, Cdc4p and an F-box protein like Grr1p,

identify substrates and catalyze the transfer of ubiquitin from E2-Ub to the target protein (Bai et al., 1996). Jiang et al. (1997) also show that Grr1p is a component of Pathway 1 and in response to extracellular glucose, is required for glucose-induced inactivation of maltose permease.

Pathway 2 uses high rates of glucose transport and phosphorylation to generate an intracellular signal (Jiang et al., 1997; Jiang et al., 2000). Reg1p is an essential component of pathway 2 (Jiang et al., 2000). Their results show that deletion of *REG1* blocks glucose-induced inactivation of maltose permease. Overexpression of *REG1* partially restores proteolysis of maltose permease in a *grr1Δ* strain. Reg1p is one of several targeting subunits of Glc7p, the sole protein phosphatase type-1 subunit encoded by *Saccharomyces.*

Glc7p was originally identified based on its role in glycogen metabolism where it acts directly to dephosphorylate and activate glycogen synthase (Anderson and Tatchell, 2001). *GLC7* is an essential gene that is involved in controlling diverse cellular processes, including glycogen accumulation (Feng et al., 1991), glucose repression (Tu and Carlson, 1995), and cell cycle progression (Zhang et al., 1995). It is proposed that different regulatory subunits of protein phosphatase direct the catalytic subunit to particular cellular locations thereby specifying its substrates (Cohen, 1989). Among the regulatory subunits that bind Glc7p are Gac1p, for targeting glycogen synthase (Stuart et al., 1994), Reg1 p and its homolog Reg2p, that direct the catalytic subunit to substrates including Snf1 kinase (Frederick and Tatchell, 1996), and Scd5p, a suppressor of clathrin deficiency, binding with PP1 is important for endocytosis and actin

Figure 3. Model for two glucose sensing/signaling pathways used for the glucose-induced inactivation of maltose permease



organization (Chang et al., 2002). These various targeting subunits bind to Glc7p at different but sometimes overlapping surface sites (Frederick and Tatchell, 1996). Binding of Reg1p and Glc7p is enhanced in the presence of glucose (Tu and Carlson 1995).

(Jiang et al., 2000) found that maltose permease phosphorylation is significantly reduced in *reg1Δ* strain suggesting that Glc7p-Reg1p phosphatase does not act directly on maltose permease. One can speculate that there must be a kinase downstream of Glc7p-Reg1p protein phosphatase (see Figure 3). Moreover, loss of N-terminal PEST sequence of Mal61p substantially reduces the glucose-induced rapid loss in transport activity in strains expressing only pathway 2, suggesting that residues 49-78 (PEST region) are the target of Pathway 2 (Medintz et al. 2000).

Part IV. Yeast casein kinase 1 encoded by *YCK1* and *YCK2*.

Casein kinases are protein kinases that are widely distributed in all eukaryotic cells and are defined by their ability to phosphorylate acidic substrates (reviewed in (Tuazon and Traugh, 1991). There are two classes of casein kinases. (Donella-Deana et al., 1985) showed that type-1 casein kinase is capable of phosphorylating both threonine and serine residues while type-2 is capable of phosphorylating only threonine. The two classes of casein kinases have different structure, nucleotide and peptide substrate specificity, and respond differently to various effectors like heparin (Agostinis et al., 1989). Further studies on sites that can be phosphorylated by casein kinase 1 have shown that clusters of 3 or 4 acidic residues like Asp, Glu, Ser (P) and Thr (P) are

considered as good substrates (Flotow et al., 1990). Yeast and mammalian casein kinases are homologous in function (Zhai et al., 1995).

Saccharomyces encodes several casein kinase 1 isoforms. *YCK1* and *YCK2* encode plasma membrane localized isoforms of CK1. *YCK3* encodes a vacuolar membrane associated isoform (Wang et al., 1996). Yet another isoform is the *HRR25* gene product, which is localized to the nucleus and appears to be involved in regulating DNA repair (DeMaggio et al., 1992). (Robinson et al., 1992) isolated *YCK1* (yeast casein kinase 1) as a suppressor of the requirement for *SNF4* function in *Saccharomyces*. Snf4 protein is a positive effector of the Snf1 protein kinase, which is required for derepression from glucose growth conditions. They showed that *YCK1* and *YCK2* genes have a redundant function in growth and loss of function of both genes results in inviability (Robinson et al., 1992).

YCK1 shares > 90% identity with *YCK2* in its catalytic domain (Vancura et al., 1993). *YCK1* and *YCK2* have a consensus sequence (Gly-Cys-Cys) for prenylation at their C-termini that allows them to localize to intracellular membranes. Vancura et al., (1994) showed that it is this dicysteinyl prenylation motif that is required for the plasma membrane localization because a mutation in this motif results in the mislocalization of Yck2p into the cytoplasm. Akr1p is a palmitoyl transferase and is required for the proper localization of Yck1,2p to the plasma membrane (Babu et al., 2002; Feng and Davis, 2000a; Roth and Davis, 1996). (Feng and Davis, 2000a) found that in *akr1Δ* strains Yck2p is distributed in the cytoplasm rather than localized to the plasma membrane. Additionally, Akr1p mutants exhibit reduced phosphorylation of Ste3p (Feng and Davis, 2000a). It was later identified that among the different isoforms of CKI, it is the C-

terminal region of these proteins that specify its localization into different subcellular compartments. Casein kinase 1 is itself regulated by its COOH-terminal. Carboxy terminal truncations of the enzyme resulted in the 3-fold activation in its catalytic rate. On the other hand, autophosphorylation results in a dramatic decrease in the affinity for protein substrate (Graves and Roach, 1995). This suggests that dephosphorylation of Yck1 by a phosphatase such as Glc7p-Reg1p might serve to activate its kinase activity.

Several lines of evidence indicate that Yck1,2 casein kinase plays a role in endocytosis and membrane protein trafficking. Panek et al. (1997) demonstrated a synthetic lethal growth defect in strains carrying temperature sensitive mutations in Yck1,2p (*yck-ts*) and in clathrin heavy chain (*chc1-ts*). They also suggest the role for this kinase in regulating polarized growth by modulating membrane remodeling. Yck2p is associated with the plasma membrane at all times but is transiently concentrated at areas of polarized growth and at site of cytokinesis (Robinson et al., 1999). Yck1,2 kinase has been implicated in stimulating the internalization of several yeast plasma membrane proteins. Marchal et al. (1998) showed that mutants defective in Yck1 and Yck2p show impaired internalization of the Fur4 uracil permease. Yck1p and Yck2p are required for Ste3p phosphorylation (Feng and Davis, 2000a; Feng and Davis, 2000b) as well as Ste2 α-factor receptor (Hicke, 1999). *yck1Δyck2-ts* cells showed a striking block to both constitutive and α-factor-induced levels of receptor phosphorylation. (Friant et al., 2000) found that overexpression of *YCK2* could bypass defects in endocytosis and actin cytoskeleton organization exhibited by an *lcb1-ts* mutant strain, which catalyzes the first step in sphingolipid biosynthesis.

Summary of thesis goals

The major goals of my thesis research are to explore the role of Yck1,2 casein kinase 1 in the glucose-induced inactivation of maltose permease and to determine whether the N-terminal PEST sequence of Mal61 maltose permease represents the target site of the glucose-signaling pathway that marks it for endocytosis and degradation. Medintz et al., (2000) carried out a deletion analysis of the PEST region in the N-terminal cytoplasmic domain and concluded that residues 49-78 play a major role in glucose-induced inactivation of maltose permease. Therefore, my first goal was to construct point mutations in the same region. I selectively mutated the serines and threonines in and around the PEST region to non-phosphorylatable alanines and monitored the impact of these changes on glucose-induced inactivation of maltose permease. In addition, I constructed mutations in the putative dileucine motif at residues 61-64 and a dilysine motif at residues 41 and 42. My results, described in Chapter 1, indicate that the N-terminal Ser/Thr that I mutated are not involved in the glucose induced internalization of maltose permease, rather they seem to be required for efficient delivery of Mal61p to the vacuole following endocytosis. The dileucine and the dilysine motifs seem to be involved in the trafficking of maltose permease to the plasma membrane as well as in the final steps of its delivery to the vacuole.

My second goal was to investigate the role of casein kinase 1 encoded by *YCK1* and *YCK2* in glucose-induced inactivation of maltose permease. Chapter 2 in the thesis will show that *YCK1 & YCK2* are essential for glucose-induced inactivation of maltose permease. I show that maltose permease localizes to the cell surface in a *yck-ts* strain but exhibits significantly reduced maltose transport activity and is mostly

hypophosphorylated. Moreover, I demonstrate that the ubiquitination of maltose permease is downstream of Yck1,2 kinase activity.

My third goal was to carry out an epistatsis analysis in order to establish the genetic relationship between *REG1-GLC7* and *YCK1,2.* Chapter 3 shows that Yck1-2p kinase is downstream of Reg1p-Glc7 phosphatase in the glucose-signaling pathway stimulating maltose permease inactivation.

MATERIAL AND METHODS

Strains

All yeast strains used in this study are listed in Table 1. Strain CMY1050 (genotype *MAT***a** *mal11Δ::HIS3 MAL12 MAL13 leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200*) is described in Medintz *et al.* 2000. *MAL1* is the sole *MAL* locus in CMY1050. It encodes an inducible allele of the *MAL*-activator (*MAL13*) and the gene encoding maltose permease was deleted by one-step gene replacement with *HIS3*. Strains CMY1025 (*MAL1 doa4Δ::HIS3*) and CMY1026 (*MAL1 DOA4*) are segregants from the cross of CMY1001 (*MAL1 DOA4*) and PMY270 (*mal1 doa4Δ::HIS3*) as described in Medintz *et al.* (1998). All the strains listed in Table 1 do not ferment maltose because they contain partially functional *MAL1* and *MAL3* loci that lack functional copies of the *MAL*-activator gene that are structurally similar to the *MAL1* and *MAL3* loci of S288C. Therefore, all of these strains require a plasmid-borne copy of the *MAL*-activator gene for expression of the *MAL* structural genes.

Plasmids and plasmid construction

All plasmids, including those previously described and those constructed during this study are listed in Table 2.

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*

Table 1. Yeast Strains

STRAIN	GENOTYPE	REFERENCE
CMY1050	*MAT***a** *mal11Δ::HIS3 MAL12 MAL13 leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200*	(Medintz et al., 2000)
CMY1025	*MAL1 doa4Δ::HIS3* Segregant from cross of CMY1001 *(MAL1 DOA4)* and PMY270 *(mal1 doa4Δ::HIS3)*	(Medintz et al., 1998)
CMY1026	*MAL1 DOA4* Segregant from cross of CMY1001 *(MAL1 DOA4)* and PMY270 *(mal1 doa4Δ::HIS3)*	(Medintz et al., 1998)
KT1357	*MAT***a** *MAL11 MAL12 mal13 MAL31 MAL32 mal33 ura3-52 leu2 his3 trp1*	(Frederick and Tatchell, 1996)
SL1463	*MATα leu2 ura3-52 trp1 his3Δ200*	(Panek et al., 1997)
SL3217	*MAT***a** *apl2Δ:TRP1 leu2 ura3-52 trp1 his3Δ200*	Sandy Lemmon University of Miami
SL3237	*MAT***a** *apl6Δ:HIS3 leu2 ura3-52 trp1 his3Δ200*	Sandy Lemmon University of Miami
SL3258	*MAT***a** *apl2Δ:TRP1 apl6Δ:HIS3 leu2 ura3-52 trp1 his3Δ200 bar1-1*	Sandy Lemmon University of Miami
SL3265	*MAT***a** *apl1Δ:LEU2 leu2 ura3-52 trp1 his3Δ200*	Sandy Lemmon University of Miami
LRB906	*MAT***a** *YCK1 YCK2 his3 leu2 ura3*	(Panek et al., 1997)

LRB756	*MAT***a** *his3 leu2 ura3-52 yck1-1Δ::ura3 yck2-2ts*	(Panek et al., 1997)
LRB1053	*MAT***a** *his3 leu2 ura3-52 akr1:: kan^R*	This study
CMY7000	Isogenic to LRB906 *MATa his3 leu2 ura3 reg1::kan^R*	This study
LRB1082	*MAT? his3 leu2 ura3-52 yck1-Δ1::ura3$^-$ yck2-2ts reg1::kan^R*	This study
KT1112	*MAT***a** *leu2 ura3-52 his3*	(Stuart et al., 1994)
KT1636	*MAT***a** *leu2 ura3-52 his3 glc7-133*	(Venturi et al., 2000)
KT1639	*MAT***a** *leu2 ura3-52 his3 glc7-132*	(Venturi et al., 2000)
KT 1967	*MAT***a** *leu2 ura3-52 his3 glc7-127*	(Venturi et al., 2000)
KT1638	*MAT***a** *leu2 ura3-52 his3 glc7-109*	(Venturi et al., 2000)
TW267	*MAT***a** *his3 leu2 ura3 glc7-256* a.k.a *glc7-147*	(Wu and Tatchell, 2001)

Table 2. Plasmids

Plasmid	Reference
pUN30-MAL61/HA	(Medintz et al., 2000)
pRS315-MAL61/HA	(Medintz et al., 1996)
YCp50-MAL43c	(Gibson et al., 1997)
pUN90-MAL63	(Elledge and Davis, 1988; Gibson et al., 1997)
pYCK1 a.k.a (pLJ721)	(Robinson et al., 1992)
pYCK2 a.k.a (pLS2.6)	(Robinson et al., 1992)
pUN30-MAL61/HA-GFP	This study
pUN70-MAL61/HA-GFP	This study
pUN30-MAL61/HA(T29A,S33A)	This study
pUN30-MAL61/HA-(T29A, S33A, S43A)	This study
pUN30-MAL61/HA-(T29A, S33A, S43A, S48A, S56A)	This study
pUN30-MAL61/HA-(T29A, S33A, S43A, S48A, S56A)-GFP	This study
pUN70-MAL61/HA-(T29A, S33A, S43A, S48A, S56A)-GFP	This study
pUN30-MAL61/HA-(T91A)	This study
pUN30-MAL61/HA-(T91V)	This study
pUN30-MAL61/HA-(T91E)	This study

pUN30-MAL61/HA-(L69,70A)	(Medintz et al. 2000)
pUN30-MAL61/HA-(L69,70A)-GFP	This study
pUN70-MAL61/HA-(L69,70A)-GFP	This study
pUN30-MAL61/HA-(E64,65R)	This study
pUN30-MAL61/HA-(K41,42R)	This study
pUN30-MAL61/HA-(K41,42R)-GFP	This study
pUN70-MAL61/HA-(K41,42R)-GFP	This study
DF041 (*REG1* overexpression 2μ plasmid)	Kelly Tatchell

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 same efficiency as strains transformed with plasmid pUN30 MAL61/HA expressing the HA-tagged allele.

A 4.4Kb *Sac*I/*Sal*I fragment containing the *MAL61/HA-GFP* gene was subcloned from plasmid pUN30-*MAL61/HA-GFP* into vector pUN70 to produce plasmid pUN70-MAL61/HA-GFP. GFP-tagged maltose permease is correctly delivered to the plasma membrane and transports with same efficiency as *MAL61/HA*.

The N-terminal *MAL61/HA* mutations constructed in pUN30-MAL61/HA were transferred into the *MAL61 /HA-GFP* gene as follows. Plasmid pUN30-MAL61/HA was digested with *Sac*I and *Pac*I and the fragment subcloned into the vector fragment of pUN30-MAL61/HA-GFP digested with *Sac*I and *Pac*I to produce an in-frame fusion *MAL61/HA-GFP*. The constructs were confirmed by the presence of a diagnostic *Nco*I site and by sequencing of the 5'end of the resulting *MAL61/HA-GFP*.

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 BIO-RAD Muta-Gene kit (BIO-RAD, Hercules, CA) according to the manufacturers protocols. Plasmid pUN30-MAL61/HA-L69,70A was obtained from (Medintz et al., 1998). The primers used for mutagenesis are listed in Table 1 of Chapter 1. All of the mutations were confirmed by full sequencing of the *MAL61/HA* ORF (PDTC, Rockefeller University, NY).

Gene disruption

In order to delete *REG1,* strain LRB906 was used to replace the open reading frame of *REG1* gene with *kan*^{R} by using a PCR-based one-step gene disruption. Plasmid pFA2-kanMX2 was used as a template to amplify G418 resistant marker (Wach et al., 1994). The 5'oligonucleotide contains 45bp upstream of the ATG of *REG1* followed by 19bp of *kan*^{R} 5' sequence (5'-AGCATGTAAATAGAAATGCGAGC CATGAAAGTAATAGCAAAAGTGCAGCTGAAGCTTCGT-3'). The 3' oligonucleotide contains 45 bp from *REG1* 3'sequence followed by 22bp of *kan*^{R} 3' sequence (5'-CTGGATTTTTATTTTCTCTT CATGTTGACTTCAAA ATTCTTTCTTGCATAGGCCACTAGT-3'). The PCR product was transformed directly into LRB906 and transformants were selected on YPD supplemented with 50mg/l Geneticin (G418 sulfate from Life Technologies, Gaithersburg, MD) (Guldener et al., 1996). Deletion of *REG1* was confirmed by PCR.

Dr. Lucy C. Robinson provided the following deletion strains. Strain LRB939 was used to delete *AKR1* gene by using Longtine deletion method with the following primers :

akr1F1: 5' CTA GAT AAA AAA ACA CTT CTT TGT TCA GAG TAG CTA ATT GCG GAT CCC CGG GTT AAT TAA 3'

akr1R1: 5' CTA AAA TAT ACA GTT TCT CCT AAT GAA AAC AAC AAA ATT TGA ATT CGA GCT CGT TTA AAC 3'

Strain LRB951 (Babu et al., 2002) was used to delete *REG1* in *yck1Δ yck2ts* strain. Longtine deletion method was used with the following primer set:

Regl-F1: 5' GCA AGC ATA TTG ACG AAG ACG AGA TAA GAA AAA TCC AAA ACG GAT CCC CGG GTT AAT TAA 3'

Regl-R1: 5' TTT TTA TTT TCT CTT CAT GTT GAC TTC AAA ATT CTT TCT TGA ATT CGA GCT CGT TTA AAC 3'

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 variations. 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, usually Gly/Lac (3% glycerol plus 2% lactate) or 2% maltose. The cells were harvested by filtration and, unless otherwise indicated, resuspended in nitrogen starvation medium plus 2% glucose, referred to as YNSG. Cyclohexamide (CHX) to a final concentration of 30μg/ml was added to the culture 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 1mM ^{14}C-labelled maltose as described by (Cheng and Michels, 1991; Medintz et al., 1996). 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 O.D. of cells (A_{600} 0.3 – 0.5) were harvested by filtration on nitrocellulose filters (.45μm), washed with KPO_4 plus 2% NaAzide 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 pH7.5 supplemented with Protease Inhibitor cocktail that contains AEBSF, pepstatinA, E-64 and 1,10-phenanthroline (Sigma-Aldrich P8215) and Phosphatase Inhibitor Cocktails 1and 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 & P2850). The cells were harvested by centrifugation and resuspended in extraction buffer (40mM TrisCl pH6.8, 8M Urea, 0.1mM EDTA, 1% β-mercaptoethanol and 5% SDS) plus the Protease Inhibitor and Phosphatase Inhibitor cocktails described above according to manufacturers recommended usage. The cell suspension was vortexed with glass beads (425-600 microns) for 15 minutes at 4^0, solubilized for 15 minutes at 37^0 C followed by another 2 minutes of vortexing. Cell debris was removed by centrifugation for 5 minutes and this total protein extract boiled for 3 minutes. Protein levels were assayed by Protein Assay Kit from Sigma-Aldrich (P5656). Equal amounts of protein were loaded per lane and size separated using longer 7.5% SDS-PAGE gels for all gels probed with anti-HA antibody (in order to show the range of differentially phosphorylated species) and 10% SDS-PAGE gels probed with anti-GFP antibody. Mal61/HA protein was detected using anti-HA antibody (Boehringer Mannheim) and Mal61/HA-GFP protein was detected using anti-GFP antibody (Santa Cruz). Each membrane was also probed with anti-PGK

(phosphoglycerol kinase) antibody (Molecular Probes) as a loading control. Protein levels were visualized by Vistra-ECF kit (Amersham) using a Storm 860 PhosphoImage analyzer (Molecular Dynamics) and the signal quantified using the manufacturer provided 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

Unless otherwise mentioned in the figure legends, for all the experiments in CMY1050 strain, cells were transformed with pUN30-HA/MAL61-GFP or the N-terminal mutant alleles and plasmid YCp50-MAL43c. Cells were grown to log phase in the appropriate medium with Gly/Lac 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. Cyclohexamide (CHX) (final concentration 30μg/ml) was added to the cell suspension at time zero. At appropriate time intervals cells were collected and viewed with a Meridian/Olympus IMT-2 confocal microscope using a 100X oil, NA 1.40 lens and a FITC filter or by phase optics. The imaging parameters were constant for all images.

FM4-64 dye (Molecular Probes) was used to stain membranes. Cells were incubated in medium containing 40 μM FM4-64 for 15 minutes and transferred to fresh medium lacking the dye. At appropriate times cells were viewed with a Meridian/Olympus IMT-2 confocal microscope using 100X 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. Experiments were carried out on three independent transformants.

OVERVIEW

This thesis is organized into three chapters. Chapter 1 describes the role of specific residues/motifs in and around the N-terminal PEST sequences in the trafficking of maltose permease and its glucose-induced inactivation. In Chapter 2 we explore the role of casein kinase 1 encoded by *YCK1,2* in glucose-induced inactivation of maltose permease. Chapter 3 is involved in establishing the genetic relationship between *REG1-GLC7* and *YCK1,2* in the glucose-signaling pathway stimulating maltose permease inactivation.

Chapter 1 is entitled "Multiple sequence signals in the N-terminal cytoplasmic domain of *Saccharomyces* maltose permease regulate trafficking and glucose-induced inactivation". We used site-specific mutation of the Ser/Thr residues to Ala in the PEST-like sequence of the N-terminal cytoplasmic domain of Mal61 maltose permease and identify sites of phosphorylation that potentially regulate Mal61p ubiquitination. However, alteration of even multiple Ser/Thr had no effect on the rapid loss of maltose transport activity. A putative dileucine [D/EExxxLL/I] internalization motif, located at residues 64 to 70 in Mal61p was found to be required for the proper delivery/localization of the permease to the plasma membrane. This motif is also involved in both the glucose-induced rapid loss of maltose transport activity and maltose permease degradation. Additionally, mutation of a pair of lysines at residues 41 and 42 to arginine similarly blocked both the rapid loss of transport activity and permease proteolysis. Lysines serve as conjugation residues for the ubiquitin. Ubiquitin is a small 76 amino acid polypeptide that when covalently attached to lysine side chains serves as a trigger for endocytosis of integral membrane proteins as well as sorting at the TGN. My

contribution includes making all the N-terminal mutations. The only exception was the dileucine mutant that was constructed by Igor Medintz. Xin Wang constructed pUN30-MAL61/HA-GFP. However, I subcloned all of the N-terminal mutants that I made as described in Chapter 1 into this plasmid and also constructed pUN70-MAL61-HA/GFP. All of the maltose transport assays, Western blot analyses, and confocal work was done by me.

Here we also show that deleting *DOA4* (which causes significantly reduced intracellular ubiquitin levels) has no effect on the delivery of maltose permease to the plasma membrane but has a major impact on endocytosis. Upon glucose induction in *doa4Δ* cells maltose permease continues to be hyperphosphorylated, transport actively and yet stay in the plasma membrane with little or no permease in the vacuole. *DOA4* and *doa4Δ* strains were taken from Igor Medintz's stock in the lab. We propose that the rapid loss of maltose transport activity results from the rapid ubiquitination-dependent relocalization of maltose permease to a compartment that restricts access to maltose in the medium. We also propose that this relocalization preceeds maltose permease degradation and is the first and essential step in the endocytosis and vacuolar degradation of maltose permease.

Chapter 2 is entitled "*YCK 1,2* encoded casein kinase 1 is required for transport activity and glucose-induced inactivation of *Saccharomyces* maltose permease". In order to investigate the possibility that Yck1, 2 casein kinase 1 is the kinase involved either directly or indirectly in the phosphorylation of Ser/Thr in the Mal61p PEST sequence, we used *yck1Δ yck2ts* strain. Defective Yck1,2 casein kinase 1 causes significant reduction in the rate of maltose permease proteolysis and blocks the loss in

maltose transport activity. Overexpression of *YCK1* or *YCK2* leads to a 2-fold increase in the relative level of the hyperphosphorylated species of maltose permease. We also show that deletion of *AKR1* significantly reduces the rate of maltose permease proteolysis and slightly increases the transport rate, similar to that seen in the *yck-ts* strain. y*ck-ts*, *akr1Δ* strains and overexpression plasmids were generously provided by Dr. Lucy C. Robinson. I did all the glucose-induced inactivation studies that include the transport assays, Western blot analyses and confocal microscopy.

Chapter 3 is entitled "A novel Glc7-Reg1 – Yck1 signaling pathway in glucose-induced inactivation of *Saccharomyces* maltose permease." In the *yck1Δ yck2-ts* strain we observed significantly reduced phosphorylation of maltose permease, little or no glucose-induced inactivation of maltose permease, and an increase in maltose transport activity comparable to results seen in a *reg1Δ* strain. Reg1p is a targeting subunit of protein phosphatase type 1 and is a central player in glucose sensing/signaling. Reg1p binds to the PP-type 1 catalytic subunit Glc7p in presence of glucose. In a *reg1Δ* strain phosphorylation and proteolysis of maltose permease is reduced whereas overexpression of *REG1* dramatically increases phosphorylation and degradation of maltose permease. *REG1* was deleted by me in LRB906 with one-step gene deletion method. *glc7* mutants were generously provided by Kelly Tatchell's lab. Two-hybrid analysis was done by Lucy Robinson and Kelly Tatchell's labs. Overexpression plasmids were provided by Kelly Tatchell and Lucy Robinson as well. I did all of the maltose transport assays, Western blot analyses and confocal microscopy. Epistasis analysis was done to determine a novel Glc7p-Reg1p -- YckI signaling pathway stimulating glucose-induced degradation of maltose permease. This study also revealed that *YCK1,2* functions downstream of the

GLC7-REG1 and infact this might be the kinase involved in phosphorylating maltose permease in the presence of glucose, thereby marking it for ubiquitin mediated endocytosis and vacuolar proteolysis.

CHAPTER 1

Multiple sequence signals in the N-terminal cytoplasmic domain of *Saccharomyces* maltose permease regulate trafficking and glucose-induced inactivation

ABSTRACT

In *Saccharomyces cerevisiae*, glucose addition to maltose fermenting cells causes a very rapid loss of maltose transport activity and the proteolysis of maltose permease protein. Proteolysis occurs in the vacuole and is dependent on ubiquitin conjugation and endocytosis. We show here that there is a slow constitutive turnover of Mal61p in glycerol/lactate medium in the absence of glucose. The rates of constitutive turnover and glucose-induced inactivation are comparable in nitrogen-rich and nitrogen-starved conditions. *doa4Δ* blocks constitutive and glucose-induced inactivation of Mal61p but does not alter its ability to localize to the plasma membrane and transport activity. Site-directed mutagenesis of specific sequences in the N-terminal cytoplasmic domain of Mal61 maltose permease was used to identify residues involved in sorting this integral membrane protein in the secretory and endocytosis pathways. Alteration of multiple Ser/Thr in the N-terminal PEST domain blocked Mal61p degradation but not the rapid glucose-induced loss of maltose transport activity. Alteration of a putative dileucine [D/EExxxLL/I] motif located at residues 64 to 70 of Mal61p causes significant defects in maltose transport activity, mislocalization to prevacuolar compartments, and resistance to glucose-induced inactivation. Mutation of a pair of lysines at residues 41 and 42 to arginine causes similar defects. Strains defective for the β subunit of adaptor protein complexes AP-1 and AP-3 exhibit significant defects in Mal61p localization to the plasma membrane but have little or no impact on its glucose-induced inactivation. We conclude that the N-terminal cytoplasmic domain of Mal61 maltose permease contains several sorting signals involved in its localization to the plasma membrane as an active maltose transporter.

Introduction

In *Saccharomyces*, 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, the maltose transporter essential for *MAL* gene induction and maltose fermentation (Jiang et al., 1997; Medintz et al., 1996; Wang et al., 2002). Addition of glucose to maltose fermenting cells causes a very rapid loss of maltose transport activity and also the proteolysis of maltose permease protein (Medintz et al., 1996). Our lab found that endocytosis, vesicle trafficking pathways, and vacuolar proteolysis are required for maltose permease proteolysis (Medintz et al., 1996; Medintz et al., 1998). We demonstrated glucose-stimulated ubiquitination of maltose permease and showed that degradation of maltose permease requires ubiquitin-conjugation enzymes. Finally, *doa4Δ*, which causes the depletion of cytoplasmic ubiquitin levels, blocks proteolysis of maltose permease and this is suppressed by overproduction of ubiquitin (Medintz et al., 1998).

Deletion of residues 48-79 of the N-terminal cytoplasmic domain of Mal61 maltose permease, containing a PEST-like sequence, blocks both its glucose-induced proteolysis and the rapid loss of maltose transport activity (Medintz et al., 2000). Regulated phosphorylation of so-called PEST sequences, sequences rich in proline, aspartate, glutamate, serine and threonine has been proposed to be involved in marking proteins for degradation (Marchal et al., 1998; Rechsteiner, 1988) and phosphorylation of serines in Ste2p (Hicke and Riezman, 1996), Fur4p (Volland et al., 1992), and several mammalian receptors (Hicke, 1997) is implicated in the receptor-mediated endocytosis of these integral plasma membrane proteins. In addition to the several potential

phosphorylation sites in the N-terminal domain of Mal61p, perusal of this region reveals other putative membrane protein sorting signals that could be involved in trafficking maltose permease through the secretory pathway and/or its endocytosis. Most interesting are residues 64 to 71 that contain a putative dileucine motif, a motif reported to be involved in sorting of transmembrane proteins to the endosome and internalization of membrane proteins (Bonifacino and Traub, 2003). Therefore, we undertook to tease apart the contributions to maltose permease membrane trafficking of various residues and sequence motifs located within the N-terminal cytoplasmic domain of Mal61 maltose permease using site-directed mutagenesis.

In several recent reports nitrogen availability was found to regulate the trafficking of *Saccharomyces* general amino acid permease, Gap1p, tryptophan permease Tat2p, via TOR nutrient signaling pathway. Nitrogen starvation has opposing effects on the trafficking of Gap1p and Tat2p to the plasma membrane (Roberg et al., 1997, Beck et al., 1999, Soetens et al., 2001). In view of these findings we decided to reinvestigate the impact of nitrogen starvation on the glucose-induced inactivation of maltose permease. We report here a previously unrecognized slow constitutive turnover of maltose permease that involves vacuole-mediated proteolysis. Also noteworthy, we find that neither the constitutive nor the glucose-induced inactivation of maltose permease requires nitrogen-starvation conditions.

DOA4 is not required for the localization of functionally active maltose permease to the plasma membrane but is required for its removal from the cell surface and the rapid glucose-induced loss of transport activity. We show that alteration of 3 to 5 serine and threonine residues in the PEST-sequence of the N-terminal cytoplasmic domain

significantly decreases the glucose-induced proteolysis of Mal61p but has no impact on the rapid inhibition of maltose transport activity. Mutation of the leucines at residues 69 and 70 or the dilysine pair at residues 41 and 42 causes defects in plasma membrane localization of Mal61 maltose permease. Strains expressing these mutant proteins exhibit significantly reduced maltose transport activity and are resistant to glucose-induced inactivation. Thus, the N-terminal cytoplasmic domain of Mal61 maltose permease contains a number of sequence motifs involved in its functional expression in the plasma membrane and regulation of its constitutive and glucose-induced turnover.

Results

Constitutive turnover of maltose permease is vacuole mediated and constitutive and glucose-induced inactivation occurs in both nitrogen-rich and nitrogen-starved medium

Our published studies of maltose permease glucose-induced inactivation reported that the rate of loss of maltose transport activity is significantly greater than can be explained by degradation of maltose permease protein (Medintz et al., 1996). To investigate the underlying mechanism for these findings, we constructed a C-terminally GFP-tagged allele of Mal61/HA maltose permease expressed from the *MAL61* native promoter to enable us to follow subcellular localization of the permease. A plasmid-borne copy was introduced into CMY1050, which contains a deletion of the full ORF of the maltose permease gene *MAL1*, the sole *MAL* locus in this strain, along with a plasmid carrying a constitutive *MAL*-activator gene, *MAL43-c*. When grown in glycerol/lactate medium Mal61/HA-GFP protein localizes to the plasma membrane but also is observed at significant levels in the vacuole (Figure 1A). This suggests the possibility that maltose is constitutively turned over even in the absence of glucose. (Medintz et al., 1996) had explored the possibility of constitutive permease turnover but had transferred the cells to medium containing ethanol, which later was found to inhibit maltose permease endocytosis (Lucero et al., 2000). We decided to reinvestigate the possibility of constitutive turnover of maltose permease.

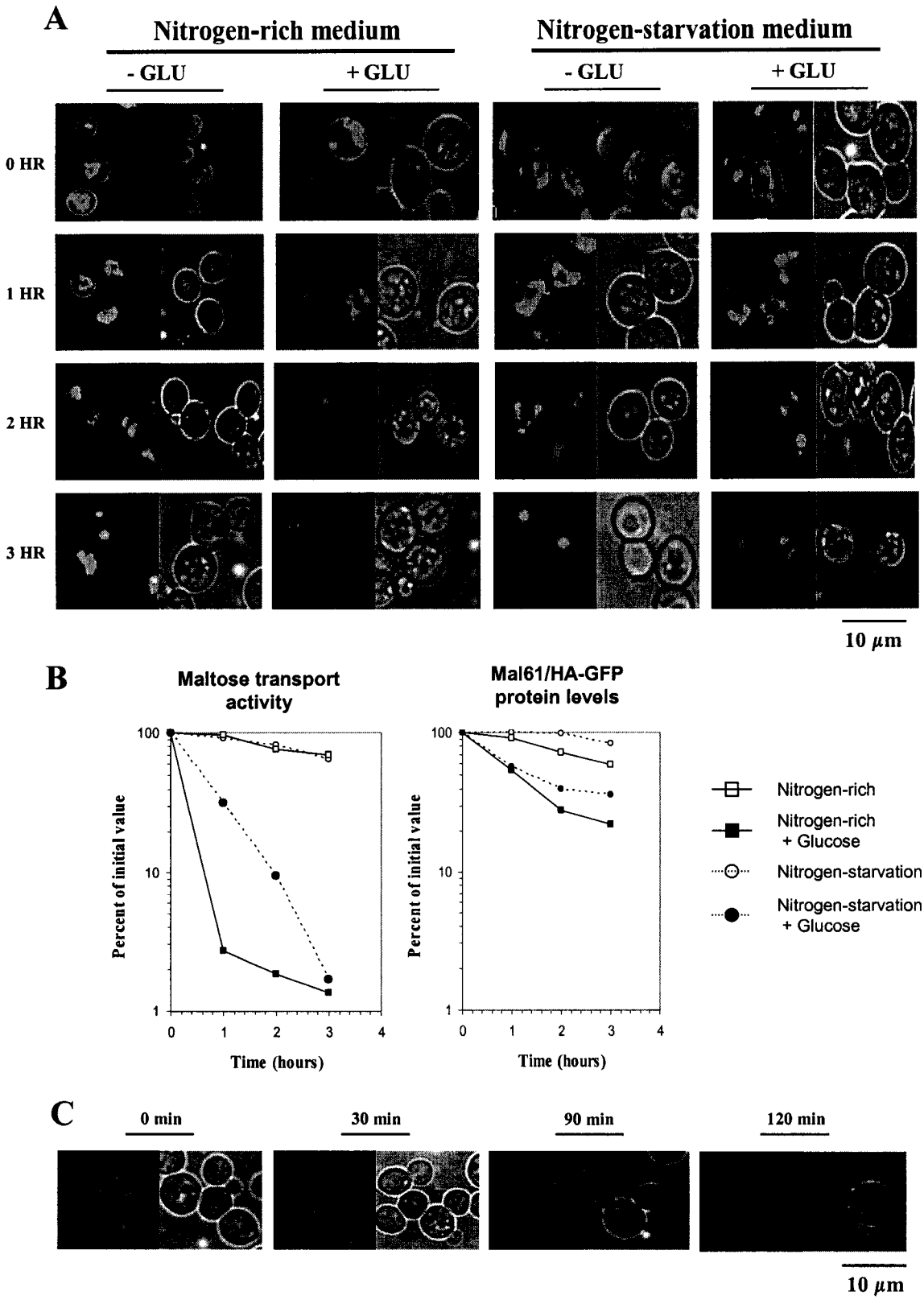
Strain CMY1050 transformed with plasmid borne copies of *MAL61/HA-GFP* and *MAL43-c* was grown in selective medium with glycerol/lactate as carbon source. At time

0, the cells were harvested and transferred to one of the following four media: nitrogen-rich medium containing cyclohexamide (CHX) with either glycerol/lactate or 2% glucose; or to nitrogen-starvation medium containing CHX with either glycerol/lactate or 2% glucose. Panel A follows the subcellular localization of Mal61/HA-GFP maltose permease in cells exposed to these conditions. Panel B compares the loss of maltose transport activity and degradation of Mal61/HA-GFP permease protein during 3 hours exposure to these four conditions. At time 0, Mal61/HA-GFPp is found at the cell surface and in the vacuole. It should be noted that the amount in the vacuole is quite substantial and probably represents about half of the total Mal61/HA-GFP protein. CHX-treated cells maintained in glycerol/lactate in either nitrogen-rich or nitrogen-starved conditions slowly loose Mal61/HA-GFPp from the cell surface. This loss is reflected in the slight decrease of maltose transport activity and Mal61/HA-GFP protein reported in Panel B. Thus, there is a slow constitutive rate of maltose permease internalization and vacuolar proteolysis in both nitrogen-rich and nitrogen-starved growth conditions.

The glucose-induced very rapid loss of maltose transport activity and maltose permease degradation occur in both nitrogen-rich and nitrogen-starvation conditions (Figure 1). In fact, not only are nitrogen-starvation conditions not required for glucose-induced inactivation of maltose permease, the results in Panel B indicate that inactivation may be somewhat attenuated by the nitrogen-starved conditions. Consistent with this, glucose causes a very rapid removal of Mal61/HA-GFPp from the cell surface (Figure 1, Panel A). By 1 hour the fluorescence signal is almost entirely intracellular and localized to what appears to be endocytic vesicles and the vacuole. Strain CMY1050 contains an abundance of intracellular vesicles that, in most cells, obscure the vacuole when imaged

Figure 1. Comparison of constitutive versus glucose-induced turnover of maltose permease

CMY1050 was transformed with pUN30-MAL61/HA-GFP and YCp50-MAL43c (encoding a constitutive *MAL*-activator allele). Transformants were grown to mid-log phase on YNB selective media lacking uracil and tryptophan and containing 3% glycerol and 2% lactate as carbon source. At time 0, the cells were harvested and transferred to the following media all containing cyclohexamide (CHX): Nitrogen-rich medium (YNB) lacking uracil and tryptophan with either 3% glycerol and 2% lactate (solid line, □) or with 2% glucose (solid line,■); Nitrogen-starvation medium (YNS) with either 3% glycerol and 2% lactate (dotted line, o) or with 2% glucose (dotted line, •). *Panel A*: Show subcellular localization of Mal61/HA-GFP maltose permease in cells exposed to the above mentioned conditions. Fluorescence and phase pictures of cells incubated in each of the conditions described above for the indicated times are shown. *Panel B*: Standard inactivation assays were carried out as described in Materials and Methods. The graphs compares the loss of maltose transport activity and degradation of Mal61/HA-GFPp during 3 hours exposure to the conditions mentioned above for the indicated times. *Panel C*: Strain CMY1050 cells were incubated in FM4-64 dye as described in the Methods and Materials. Images were taken at the indicated times.



by phase. The presence of the vacuole in this strain can be seen more clearly in Panel C that shows the movement of the fluid phase vacuolar membrane dye FM4-64 from the plasma membrane by endocytosis to intracellular membrane structures and the vacuolar membrane. It should be noted that the rapid glucose-induced removal of Mal61/HA-GFP from the cell surface occurs both in nitrogen-rich and nitrogen-starvation conditions and is significantly more rapid in nitrogen-rich conditions.

doa4Δ blocks glucose-induced inactivation of maltose permease but does not effect its localization to the plasma membrane

We previously reported that loss of *DOA4*, which causes severely decreased levels of available ubiquitin, decreases the rate of glucose-induced proteolysis (Medintz et al., 1998). Here, we use our GFP-tagged allele of Mal61p to investigate the trafficking and endocytosis of maltose permease in a *doa4Δ* mutant. Strains CMY1025 (*MAL1 doa4Δ*) and CMY1026 (*MAL1 DOA4*) were transformed with plasmid-borne *MAL61/HA-GFP* and *MAL43-c*, grown in selective medium with glycerol/lactate as carbon source, harvested, and transferred to nitrogen-starvation medium containing 2% glucose. The cells were observed by confocal fluorescence microscopy for 3 hours. The standard inactivation protocol was used to follow changes in maltose transport activity and Mal61/HA-GFP protein levels over the 3-hour time course. The results are shown in Figure 2.

Abundant levels of Mal61/HA-GFP protein are observed at the cell surface in the *doa4Δ* mutant strain, higher than seen in the *DOA4* strain. Most striking is the absence of any intracellular protein. The normally intense vacuole-localized fluorescent signal is not

observed. This result indicates that *doa4Δ* blocks all movement of Mal61/HA-GFP maltose permease to intracellular compartments and suggests that delivery to the plasma membrane may be slightly enhanced. This modest increase in plasma membrane localized Mal61/HA-GFP is not reflected in its functional activity since maltose transport rates are modestly decreased in the *doa4Δ* strain.

Glucose-induced inactivation of maltose permease is blocked by *doa4Δ*. Glucose addition does not stimulate removal of the permease from the cell surface, no significant accumulation of Mal61/HA-GFP is observed in intracellular vesicles or the vacuole over the time course of the experiment, although slight fluorescent signal is evident in the vacuole. Glucose does not cause a loss in maltose transport activity or permease proteolysis. This is despite the fact that the Mal61 protein is highly phosphorylated (the slower moving band in the Western blot). Taken together, these results clearly indicate that ubiquitination is required for glucose-induced internalization of maltose permease

Alteration of Ser/Thr residues in the N-terminal PEST sequence significantly decreases maltose permease phosphorylation and proteolysis

Phosphorylation of Ser/Thr residues of PEST sequences is associated with the regulated proteolysis of the protein and PEST sequences reportedly are attractive targets of the ubiquitination enzymes (Hicke, 1999; Hochstrasser, 1996b). To determine if the Ser and Thr residues in the N-terminal cytoplasmic domain of Mal61 maltose permease are involved in its glucose-induced proteolysis we mutated these residues, individually and cumulatively, to alanine by site-directed mutagenesis of a plasmid-borne copy of

Figure 2. Effects of *doa4Δ* on localization and glucose-induced inactivation of maltose permease

Strains CMY1025 (*MAL1 doa4Δ::HIS3*) and CMY1026 (*MAL1 DOA4*) were transformed with pUN30-MAL61/HA-GFP and YCp50-MAL43c. Transformants were grown on minimal selective media lacking uracil and tryptophan with 3% glycerol and 2% lactate to mid-log phase, harvested, and transferred to YNSG media for glucose-induced inactivation. Cells were observed using confocal fluorescence and phase microscopy at the indicated times following the transfer. Standard inactivation assays were carried out as described in Materials and Methods. The data represent the average of three independent experiments. The relative levels of Mal61/HA protein (o) and maltose transport activity (■) relative to the zero time sample are plotted along with growth dilution (Δ). Growth dilution is calculated as the OD_{600} at time zero divided by OD_{600} at time *x*. A representative Western blot is shown. The bottom panel shows AntiPGK as a loading control.

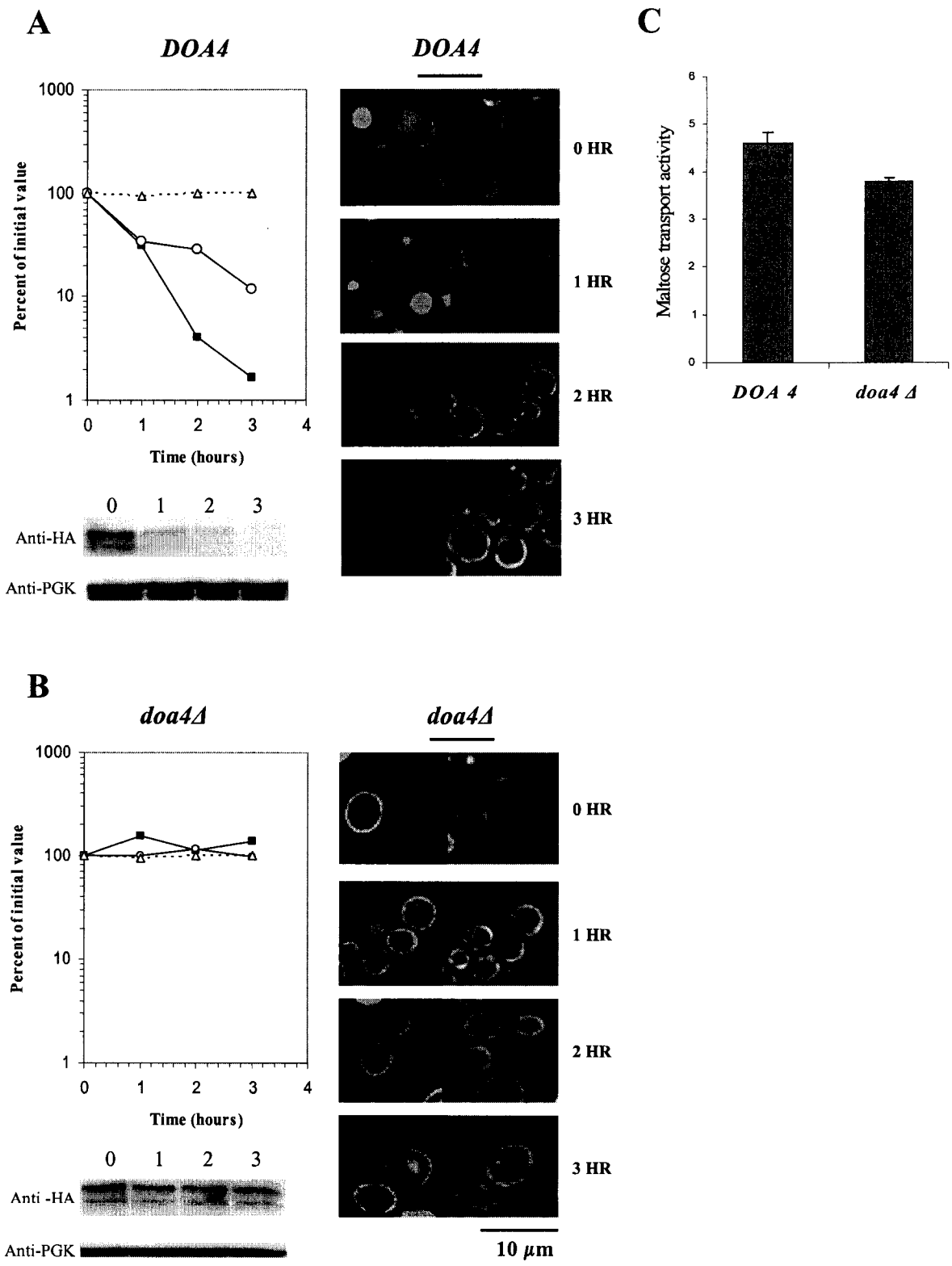


Figure 3. Targeted mutagenesis of the N-terminal cytoplasmic domain of Mal61/HA maltose permease

The residue numbers relate to the sequence of the wild type Mal61 maltose permease (Cheng and Michels 1989). The 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). Altered Ser/Thr residues that were mutated either singly or cumulatively are shown in bold face. The location of the putative dileucine motif (D/EExxxLL/I), dashed underline, and a pair of lysine residues, solid underline are indicated.

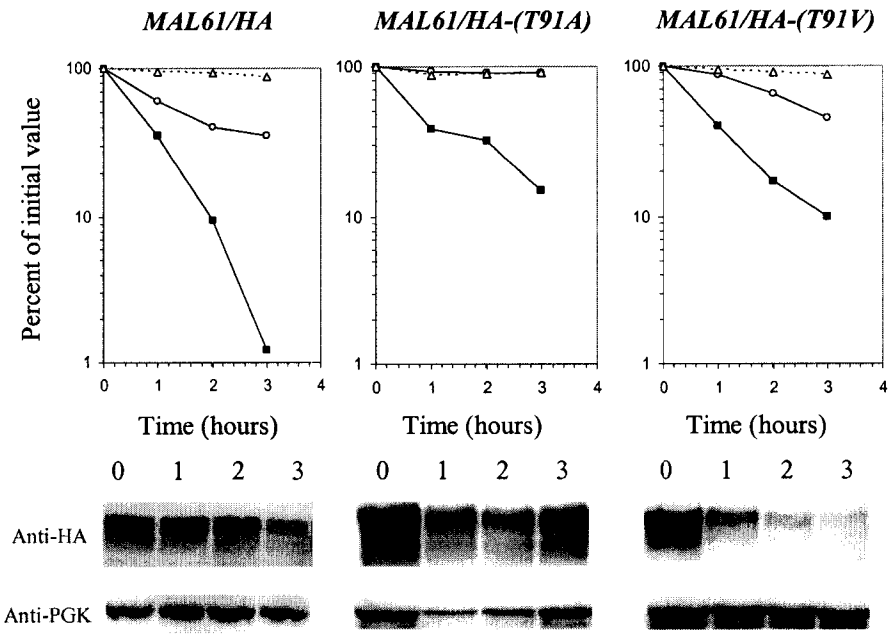
Table 1. Oligonucleotides used for *in vitro* mutagenesis of *MAL61/HA*

MAL61/HA allele	Sequence 5' → 3'*	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-(T91A)	TGTCTTCAAAGCTGCCATGAGTGGCAT	pUN30-MAL61/HA
MAL61/HA-(T91V)	TGTCTTCAAAGCTACCATGAGTGGCAT	pUN30-MAL61/HA
MAL61/HA-(T91E)	TGTCTTCAAAGCTTCCATGAGTGGCAT	pUN30-MAL61/HA
MAL61/HA-(L69,70A)	GGCGTCCTGCATAGCTTCATCGGCAGCGTCGGGGACTTCTTC	pUN30-MAL61/HA
MAL61/HA-(E64,65R)	AAGGTCGGGGACTCTTCTATTATTATCGTT	pUN30-MAL61/HA
MAL61/HA-(K41,42R)	ATCAAAATCACTTCTCCTACCTTGCTCCTC	pUN30-MAL61/HA

*Underline indicates site of alteration.

Figure 4. Effect of a single T91 mutants on the glucose induced inactivation of maltose permease

CMY1050 transformed with YCp50-MAL43c and pUN30-MAL61/HA carrying a single alteration in Threonine91 to A) alanine B) valine were grown in selective medium plus Gly/Lac and switched to YNSG with CHX. At the indicated times, the growth dilution (Δ), maltose transport (■) and relative Mal61/HA protein levels (o) were determined as described in Materials and Method. A representative Western blot is shown. Anti-PGK is used as a loading control.



MAL61/HA, plasmid pUN30-MAL61/HA. The specific residues altered are indicated in boldface in Figure 3. Plasmids carrying the mutant alleles were transformed into strain CMY1050 and the 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 Figures 4 and 5.

With the exception of Thr91, none of the single Ser/Thr substitutions to alanine were found to have significant impact on the glucose-induced inactivation of maltose permease (data not shown). The single residue mutation Thr91Ala in Mal61p blocked proteolysis completely (Figure 4). To explore this result further, we generated a Thr91Val mutation at this site thereby introducing an amino acid with a hydrophobic bulky side chain similar to that of threonine. Mal61/HA-(T91V) mutant protein 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.

Cumulative substitutions of 2, 3, or 5 Ser/Thr residues significantly slowed the rate of maltose permease proteolysis (Figure 5A) with the allele containing 5 altered residues exhibiting the slowest rate of degradation. Most importantly, we found that even in strains expressing the 5 Ser/Thr to Ala mutation, little or no impact was observed on the rapid glucose-induced loss of maltose transport activity (Figure 5A). Alteration of these five residues does not appear to seriously impair expression. Maltose transport activity expressed by transformants carrying the different mutant alleles is reported in

Figure 5. Glucose-induced inactivation of maltose permease in PEST region mutants

CMY1050 was transformed with plasmid-borne (pUN30) HA-tagged wild-type *MAL61/HA* allele, or *MAL61/HA (T29A, S33A)*, *MAL61/HA (T29A, S33A, S43A)*, *MAL61/HA (T29A, S33A, S48A, S56A)* mutant alleles and with YCp50-MAL43c, carrying the constitutive *MAL43-C MAL*-activator gene. Transformants were grown on selective medium lacking tryptophan and uracil plus 2% glycerol/3% lactate. *Panel A* shows standard inactivation assays were carried out as described in Materials and Methods. The data represent the average of three independent experiments. The relative levels of Mal61/HA protein (o) and maltose transport activity (■) compared with zero time sample are plotted along with growth dilution (Δ). Growth dilution is calculated as the OD_{600} at time zero divided by OD_{600} at time *x*. A representative Western blot is shown. Anti-PGK is used as a loading control. *Panel B* shows the maltose transport activity at time 0.

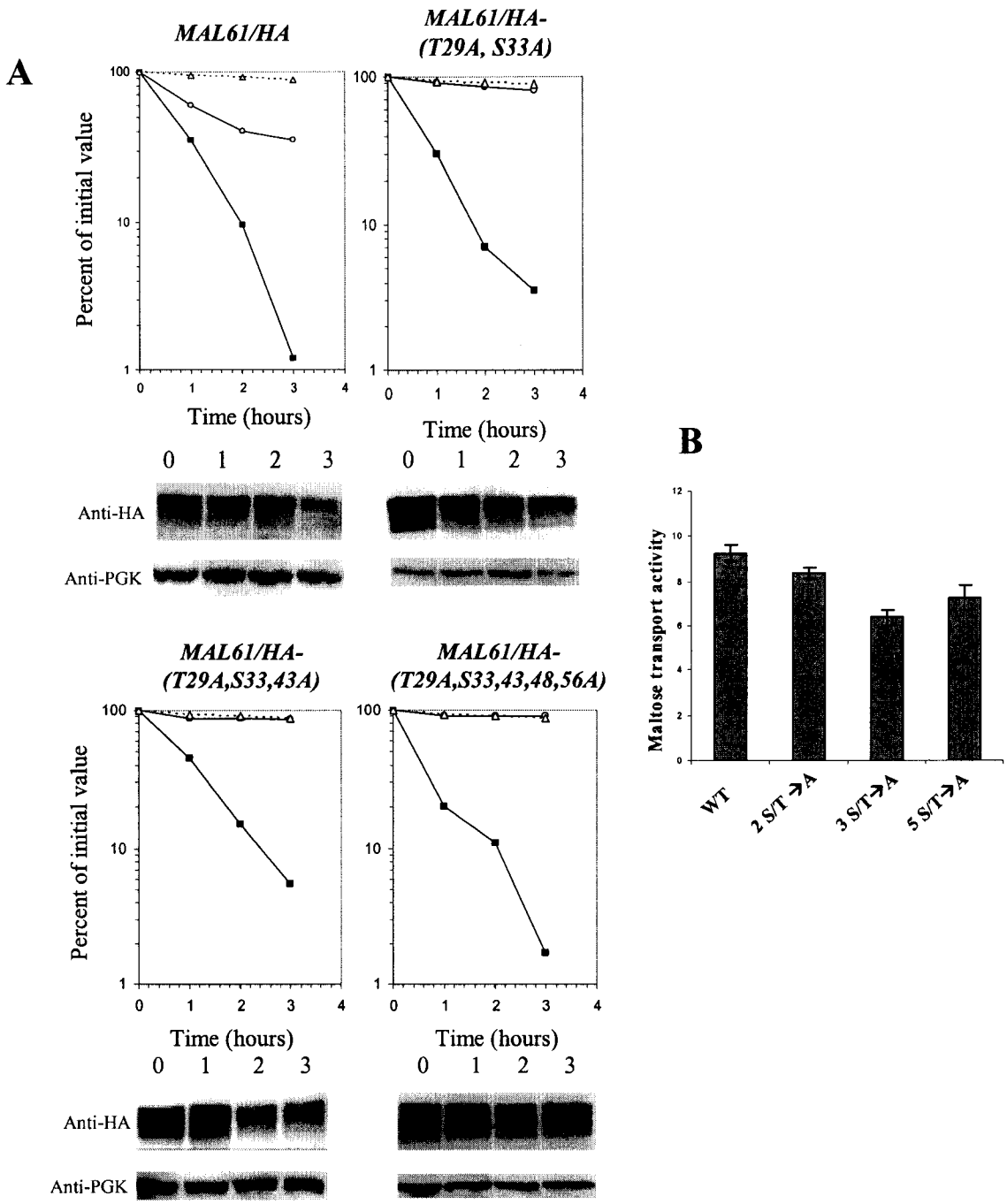


Figure 5B and these mutations cause at most a 25% decrease in activity. It also should be noted that alteration of these N-terminal Ser/Thr residues to the non-phosphorylated alanine reduces the overall phosphorylation level of the Mal61/HA protein. Nonetheless, even with all five residues mutated, we still observe a full range of phosphorylated species (Figure 5A). This suggests that Mal61/HA protein contains a significant number of phosphorylated residues, more than the ones altered here, that many of these residues become phosphorylated during synthesis and plasma membrane localization, and that particular phosphorylated species are too numerous to be distinguished by this gel separation method.

These results suggest that phosphorylation of Ser/Thr residues 29, 33, 43, 48, and 56 is not required for plasma membrane localization and transport activity of Mal61/HAp or for the rapid glucose-induced loss of maltose transport activity but is needed to target maltose permease for degradation. To explore this further a GFP-tagged allele of *MAL61/HA-(T29A, S33,43,48,56A)* was constructed and introduced into strains CMY1050 and KT1357 (Jiang et al., 2000) along with a plasmid-borne *MAL43-c*. Both strains were used because, while most of our analyses were carried out in CMY1050, the vacuole of CMY1050 is poorly defined and not as easily visualized by phase microscopy compared to KT1357. Glucose-induced inactivation of maltose permease in strain KT1357 show similar drop in the transport rate but the rate of proteolysis is significantly faster than in CMY1050 (data not shown). This increase in the rate of proteolysis in strain KT1357 can be clearly seen in Fig. 6 WT panels. In strain CMY1050 wild-type Mal61/HA-GFP protein localizes to the cell surface and quite abundantly to internal lobular structures (Figure 6, top panel). By comparison, in strain KT1357 Mal61/HA-

GFP can be seen at the cell surface and clearly in the vacuole. In both strains, glucose induces rapid removal of Mal61/HA-GFP from the cell surface and degradation of the protein in these intracellular compartments.

As expected, based on the nearly normal levels of maltose transport activity found in transformants expressing the GFP-tagged Mal61/HA-(T29A, S33,43,48,56A) mutant protein, the mutant permease localizes to the cell surface and to intracellular compartments but, as is clear by comparison of strains CMY1050 and KT1357, this intracellular compartment is not the vacuole. Glucose stimulates removal of the mutant permease from the cell surface but, consistent with the Western blot results reported in Figure 5, no loss of the fluorescence signal is observed. In CMY1050 the mutant protein remains stable in multiple vesicle-like structures while in KT1357 by 2 hours the GFP signal coalesces into one or two bright spots that look similar to what is described in the literature as an exaggerated prevacuolar compartment (PVC) (Raymond et al., 1992). It appears that the Mal61/HA-(T29A, S33,43,48,56A) mutant protein exhibits severe defects in the late stages of delivery to the vacuole but not during synthesis and delivery to the plasma membrane or in the initial steps of endocytosis.

Dileucine motif at residues 64 to 70 and lysines 41 and 42 are required for maltose permease plasma membrane localization and glucose-induced inactivation

(Medintz et al., 1998) showed that in strains expressing a mutant Mal61/HA maltose permease altered in pair of leucines at residues 69 and 70 of the N-terminal cytoplasmic domain glucose induces an increase in maltose transport activity followed by a slow decrease due possibly to degradation of the protein. Mounting evidence implicates dileucine motifs in the sorting and internalization of several plasma membrane proteins

(Bonifacino and Traub, 2003). Dileucine motifs are reported to be recognized at the *trans*-Golgi network (TGN) by 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] E X X X L L (Li and Johnston, 1997) class of dileucine motif (position marked by underline and boldface in Figure 3). In addition to the two leucines / isoleucine residues of 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 the recognition of this motif (Bonifacino and Traub, 2003; Pond et al., 1995; Sandoval et al., 2000). To test the possibility that residues 64 – 70 constitute a dileucine sorting motif we used the *MAL61/HA-(L69,70A)* allele constructed by (Medintz et al., 2000) and generated the Glu64,65Arg mutation altering residues located N-terminal to the dileucine pair at residues 69,70. 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 a constitutive *MAL*-activator allele *MAL43-c* and glucose-induced inactivation was assayed.

Unexpectedly, only about half of the Mal61/HA-L69,70A permease is phosphorylated compared to wild-type Mal61/HAp, where the bulk of the protein is in a highly phosphorylated form (Figure 6A). Moreover, transformants carrying *MAL61/HA-(L69,70A)* exhibit almost 10-fold less maltose transport activity than transformants carrying the wild-type allele. Based on Western blot analysis (data not shown) the level

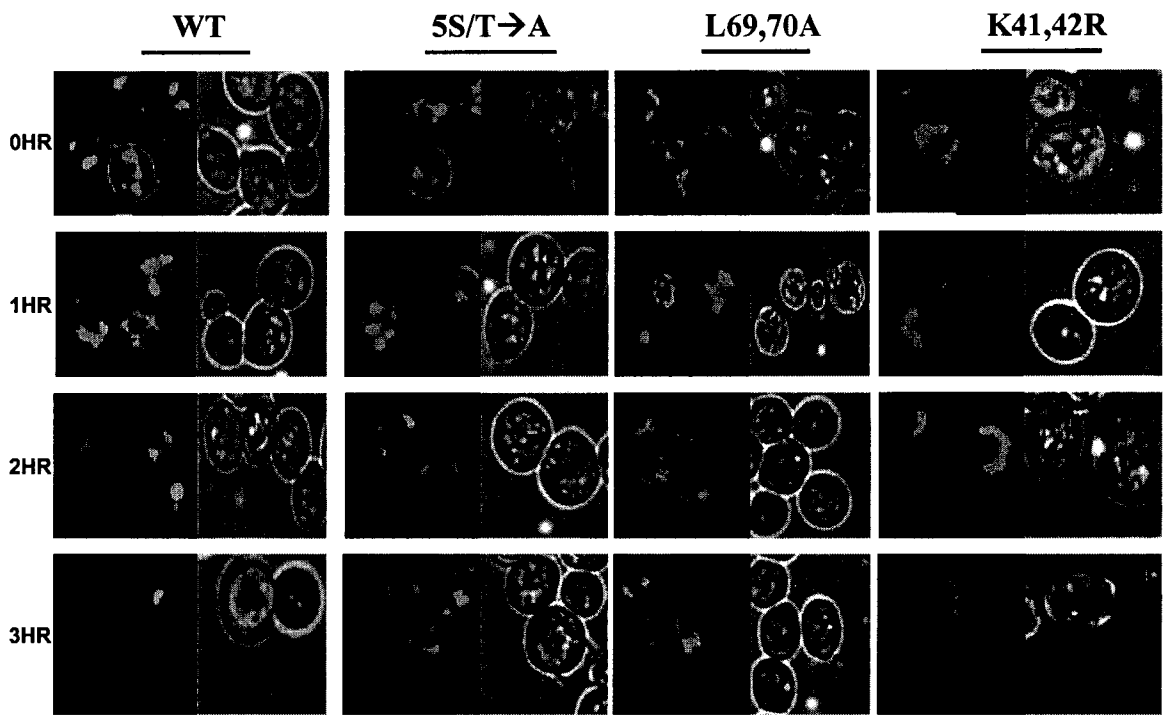
of the dileucine mutant maltose permease protein expressed by these transformants is comparable to that in cells expressing the wild-type allele. These conflicting findings are explained by Figure 7, which shows that localization to the cell surface of the mutant protein is dramatically reduced compared to wild-type. Most of the Mal61/HA-(L69,70A) mutant protein accumulates in cytoplasmic vesicles in CMY1050, possibly late endosomes or PVC, as evidenced by their appearance in strain KT1357. It is important to note the absence of vacuolar accumulation of this mutant protein in either strain CMY1050 or KT1357.

Also, alteration of the leucines at residues 69 and 70 fully blocks glucose-induced inactivation but does not appear to impact removal of this mutant protein from the plasma membrane. Figure 6A demonstrates that glucose stimulates only a very slight increase in maltose transport activity in strains expressing *MAL61/HA-(L69,70A)* instead of the rapid decrease in maltose transport activity observed in cells expressing the wild-type protein, although it must be kept in mind that the level of maltose transport activity in cells expressing this mutant permease is very low. Additionally, there is no evidence of glucose-induced proteolysis of the mutant protein. In contrast, in strain CMY1050 the low level of Mal61/HA-(L69,70A) mutant protein is removed from the cell surface within 1 hour of glucose addition although no change in the total level of the fluorescent signal is observed in cells expressing the GFP-tagged allele of *MAL61/HA-(L69,70A)* over the 3 hour time course (Figure 7). In strain KT1357, the fluorescent signal, which initially is dispersed in a number of cytoplasmic structures, becomes distinctly localized to one or two brightly fluorescent in prevacuolar compartments.

Figure 6. Effect of mutations in the dileucine (D/EExxxLL/I) and diLysine motif of MAL61/HA on the glucose-induced inactivation of maltose permease

Strain CMY1050 was transformed with plasmid pUN30-MAL61/HA-(L69,70A), pUN30-MAL61/HA-(E64,65R) or pUN30-MAL61/HA-(K41, 42R) along with YCp50-MAL43c. All transformants were grown as described in Figure 2, harvested, and transferred to YNSG with CHX. At the times indicated, *Panel A* shows the growth dilution (Δ), maltose transport activity (■), and the relative Mal61/HA protein levels (o) were determined as described in Materials and Method. The last panel shows Anti-PGK as a loading control. The results for *MAL61/HA* are taken from Figure 2. *Panel B* shows the maltose transport activity at time 0.

Strain CMY1050



Strain KT1357

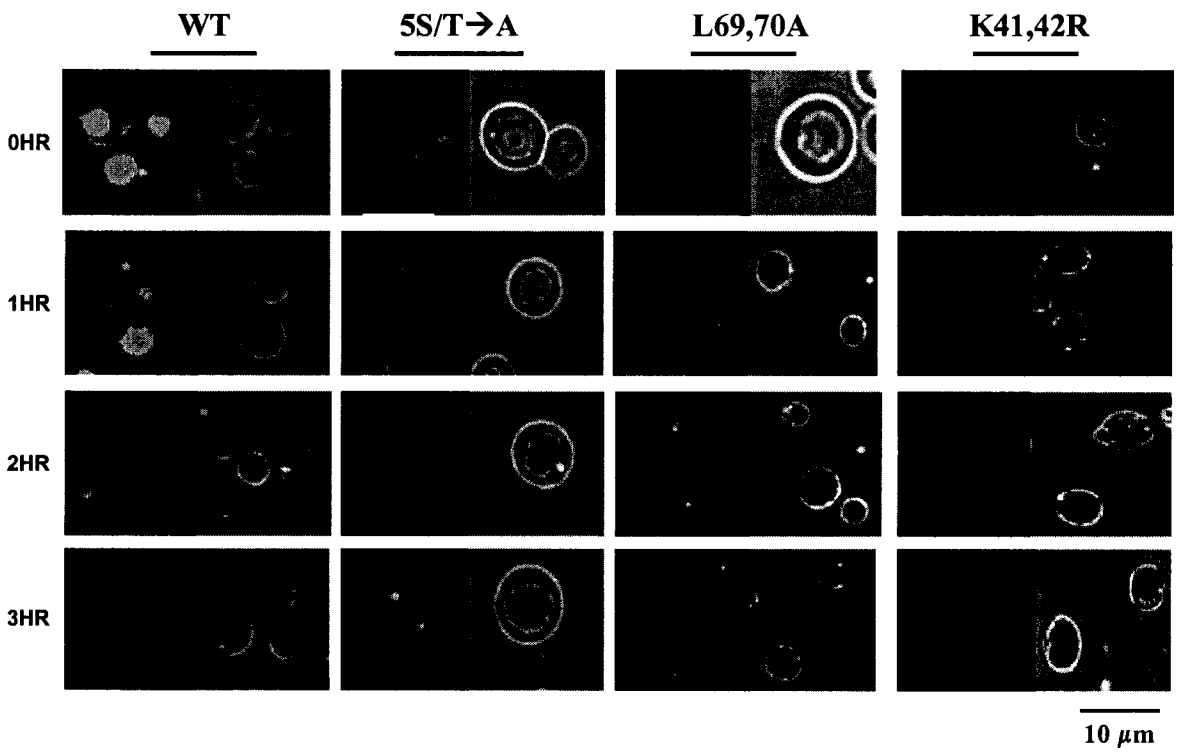
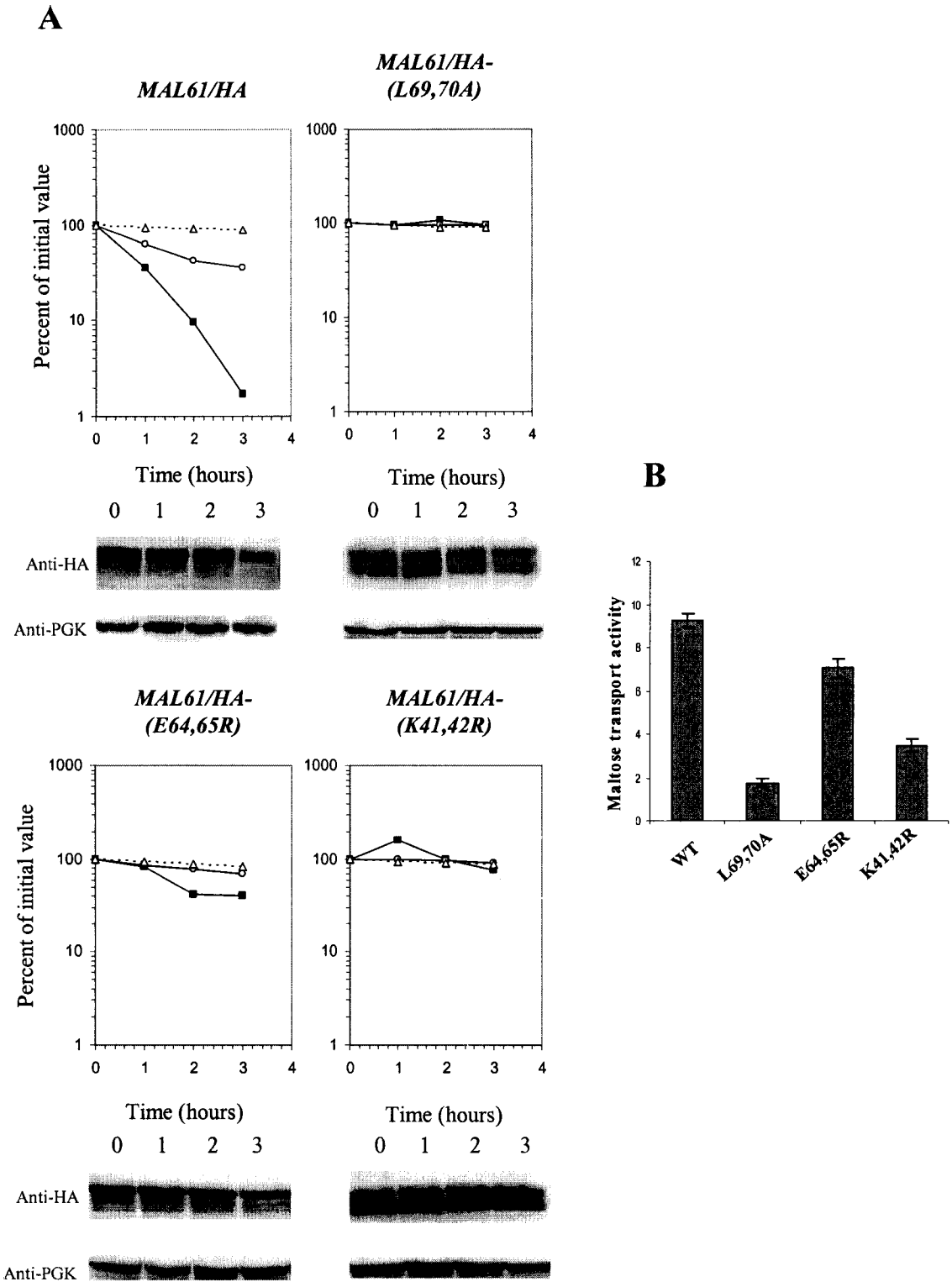


Figure 7. Subcellular localization of Mal61p in different N-terminal mutants

CMY1050 was transformed with pUN30 plasmid-borne *MAL61/HA-GFP* or the indicated mutant alleles and YCp50-MAL43c. Transformants were grown in media lacking tryptophan and uracil with 2% glycerol and 3% lactate. At time zero, cells were harvested by filtration and transferred to YNSG with CHX. *Panel* A shows the localization of Mal61-GFP or the mutant proteins at the indicated times after transfer.



The results in Figure 6 also support a role for the glutamates at residues 64 and 65 in the glucose-induced inactivation of Mal61/HA permease, but suggest that these residues are less important for maltose permease synthesis. Transformants carrying *MAL61/HA-(E64,65R)* express about 75% of the maltose transport activity found in strains carrying the wild-type gene (Figure 6B). Additionally, the phosphorylation level of Mal61/HA-E64,65R protein is decreased compared to wild-type but not to the extent seen for the Mal61/HA-L69,70A mutant protein (Figure 6A). We also observe a significant decrease in the rate of glucose-induced Mal61/HA-E64,65R permease proteolysis and loss of maltose transport activity but alteration of these glutamate residues does not completely block the processes.

A series of N-terminal deletions in and around the PEST sequence of Mal61 maltose permease led us to look for the putative ubiquitin conjugation sites within the same region of the protein. Medintz *et al.* (1998) showed that ubiquitin conjugation to Mal61/HAp is glucose-induced and required for glucose-induced proteolysis of maltose permease. Several lysine residues are found in the region the N-terminal PEST sequence of Mal61p and one or more could serve as potential ubiquitin conjugation sites. A pair of lysines at residues 41 and 42 are most interesting because of their close proximity to the PEST region and thus we decided to test their importance by generating the mutation *MAL61/HA-(K41,42R).*

Surprisingly, as is seen in Figure 6, cells expressing this dilysine mutation show significant defects in maltose transport activity. Protein levels appear to be comparable to wild-type (data not shown) but studies using the GFP-tagged dilysine mutant allele

shown in Figure 7 demonstrate that very little of the protein is observed at the cell surface and that predominantly this protein is seen in intracellular vesicles in strain CMY1050. More dispersed localization near the cell surface is seen in strain KT1357 as well as in what appears to be a prevacuolar compartment. Again, no significant vacuolar accumulation is observed in either strain CMY1050 or KT1357. Moreover, the Mal61/HA-K41,42R permease exhibits significantly less phosphorylation than is observed in the wild-type protein, similar to what is observed Mal61/HA-(L69,70A)p.

Transformants expressing *MAL61/HA-(K41,42R)* are resistant to glucose-induced inactivation. Glucose induces a slight but significant increase in maltose transport activity but no proteolysis of the mutant permease is observed. In Figure 7 one can see removal of the small amount of plasma membrane-localized GFP-tagged dilysine mutant protein but the overall amount of fluorescent signal remains stable and in intracellular vesicle compartments in CMY1050 or in an apparent prevacuolar compartment in KT1357 with little evidence of vacuole entry or degradation (Figure 7).

Taken together, we conclude that the putative dileucine motif found at residues 64 to 70 of Mal61/HA maltose permease, EEVPDLL, and the dilysine pair at residues 41 and 42 likely serve a role in proper delivery of Mal61/HA maltose permease to the plasma membrane. The late stages of maltose permease delivery to the vacuole also are controlled by these N-terminal sequences but, interestingly, early steps in endocytosis that regulate glucose-induced removal from the cell surface do not appear to be affected. Some aspect of these sequences appears to be important for achieving full phosphorylation of the protein and a possible correlation between transport activity and phosphorylation is suggested.

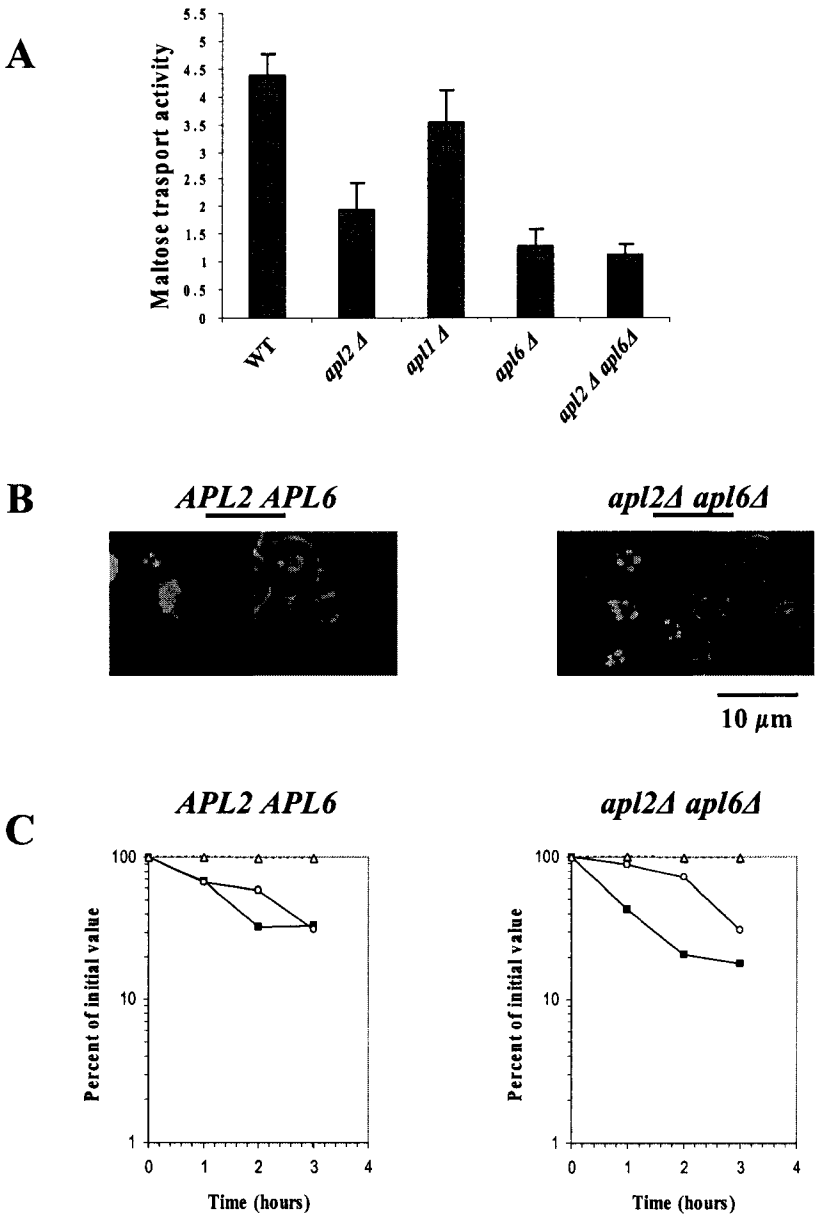
Adaptor complexes AP-1 and AP-3 play an important role in maltose permease plasma membrane localization

The [D/E] X X X L [L/I] class of sorting signal is recognized by adaptor protein complexes (AP complexes) that function in the selective recruitment of membrane proteins into transport vesicles for sorting through the secretory pathway and internalization from the plasma membrane (Bonifacino and Traub, 2003). In *Saccharomyces APL2, APL1*, and *APL6* encode the β subunit of AP-1, AP-2, and AP-3 complexes, respectively. Based on our results we suggest that the dileucine motif in the N-terminal cytoplasmic domain of Mal61/HAp functions as a sorting signal in the secretory pathway, we explored the role of the various AP complexes in maltose permease trafficking and endocytosis. An isogenic strain series carrying single gene disruptions or double gene disruptions in these genes obtained from Sandra Lemmon (University of Miami) were transformed with plasmid-borne *MAL61/HA* or *MAL61/HA-GFP* and *MAL43-c*, grown in selective medium with glycerol/lactate as carbon source, harvested, and transferred to nitrogen-starvation medium containing CHX and 2% glucose. The results are shown in Figure 8.

The rate of maltose transport activity in the single gene disruption strains *apl2Δ* and *apl6Δ,* but not in the *apl1Δ* strain, is approximately one-half and one-third, respectively, that found in the isogenic parental strain (Figure 8A). Moreover, the transport activity observed in the *apl2Δ apl6Δ* double mutant strain is significantly lower than that of the *apl2Δ* and *apl6Δ* single mutant strains. These results are consistent with the hypothesis that AP-1 and AP-3 are both involved in trafficking Mal61/HA protein to

Figure 8. Adaptor protein complexes AP-1 and AP-3 are involved in delivery of maltose permease to the plasma membrane

Strains SL1463 (*MATα leu2 ura3-52 trp1 his3Δ200*), SL3217 (*MATa apl2Δ:TRP1 leu2 ura3-52 trp1 his3Δ200*), SL3237 (*MATa apl6Δ:HIS3 leu2 ura3-52 trp1 his3Δ200*) and SL3258 (*MATa apl2Δ:TRP1 apl6Δ:HIS3 leu2 ura3-52 trp1 his3Δ200 bar1-1*) were transformed with plasmids pRS315MAL61/HA and YCp50/43c. Strains SL3265 (*MATa apl1Δ:LEU2 leu2 ura3-52 trp1 his3Δ200*) were transformed with plasmid pUN30MAL61/HA and YCp50-MAL43c. Transformants were grown in the selective dropout media with 3%Glycerol/2% Lactate as carbon source. Cells were harvested by filteration and transferred to YNSG with CHX and inactivation protocol was carried out as described in materials and methods. *Panel A* shows transport activity as described in materials and methods at t=0. The error bars indicate the standard deviation from experiments done with at least two independent transformants. *Panel B* shows the localization study; SL1463 and SL3258 were transformed with pUN30-HA/MAL61-GFP along with YCp50-MAL43c grown to log phase in selective media as indicated above and pictures were taken at t=0. *Panel C* shows the growth dilution (Δ), maltose transport activity (■), and the relative Mal61/HA protein levels (o) were determined as described in Materials and Method.



the plasma membrane and act in independent parallel pathways. Localization of Mal61/HA-GFP protein was studied in the *APL2 APL6* parental strain and the *apl2Δ apl6Δ* double mutant strain (Figure 8B). In the parental strain, Mal61/HA-GFP protein can be seen at the cell surface and in the vacuole, as is seen for strains CMY1050 and KT1357. Consistent with the results reported in Figure 8A, very little signal is observed at the cell surface in the *apl2Δ apl6Δ* double mutant strain but instead it is localized to multiple vesicles that appear to surround the vacuole.

The standard inactivation protocol was used to follow changes in maltose transport activity and Mal61/HA protein levels over the 3 hour time course in the *apl2Δ apl6Δ* double mutant strain and compared to the *APL2 APL6* parental strain (Figure 8C). The rate of glucose-induced inactivation is not significantly altered by loss of AP-1 and AP-3. We also tested the *apl1Δ apl6Δ* and *apl1Δ apl2Δ* double mutant strains and both exhibited apparently wild-type glucose-induced inactivation (data not shown).

Discussion

Constitutive turnover and glucose-induced inactivation of maltose permease are independent of nitrogen availability.

Our studies of glucose-induced inactivation of maltose permease, including most of the work reported here, were carried out in nitrogen-starvation medium (Jiang et al., 2000; Medintz et al., 1996; Medintz et al., 1998; Medintz et al., 2000; Wang et al., 2002). Medintz *et al.* (1996) demonstrated glucose-induced inactivation of Mal61/HA maltose permease in both nitrogen-rich (YP) and nitrogen-starvation (YNS) medium but selected nitrogen-starvation conditions for their inactivation protocol as a means of limiting cell growth during the course of the assay.

Recently, nitrogen availability was found to regulate the trafficking of the *Saccharomyces* general amino acid permease, Gap1p, and the high-affinity tryptophan permease, Tat2p, via the TOR nutrient signaling pathway (Beck et al., 1999; Magasanik and Kaiser, 2002; Roberg et al., 1997; Schmidt et al., 1998; Soetens et al., 2001). Nitrogen starvation has opposing effects on the trafficking of Gap1p and Tat2p. In cells grown in a nitrogen-poor medium Gap1p is delivered to the plasma membrane but in nitrogen-rich media newly synthesized Gap1p is instead directed to intracellular compartments, including the Golgi and endosomes, from which some reports suggest it can be reactivated (Nikko et al., 2003; Roberg et al., 1997; Soetens et al., 2001). On the other hand, in low tryptophan growth conditions Tat2p is found at the plasma membrane while growth on high tryptophan causes newly synthesized Tat2p to be localized to the vacuole for degradation (Schmidt et al., 1998). In both cases, the Ser/Thr protein kinase Npr1p, a component of the TOR pathway, is central to nitrogen-sensing but acts as an inhibitor of Tat2p and an activator of Gap1p plasma membrane expression (Schmidt et al., 1998; Soetens et al., 2001). Beck *et al.* (1999) also report that glucose starvation causes the degradation of Tat2p.

In view of these finding, we decided to reinvestigate the impact of nitrogen starvation on the glucose-induced inactivation of maltose permease. For this we used a slightly revised protocol that enables us to examine constitutive versus glucose-induced inactivation of maltose permease and whether nitrogen-starved conditions are required. Mal61/HA-GFP maltose permease is expressed constitutively from its native promoter using a constitutive *MAL*-activator allele and the cells are grown in glycerol/lactate to avoid the production of any intracellular glucose that could result from rapid maltose

utilization. To assay permease turnover, cyclohexamide is used to block protein synthesis. The results in Figure 1 clearly indicate that glucose alone is needed to stimulate internalization of Mal61/HA-GFP permease and Mal61/HA-GFP permease proteolysis. In fact, both responses to glucose, but particularly internalization, are somewhat attenuated by nitrogen starvation. Thus, nitrogen starvation conditions do not contribute to glucose-induced maltose permease internalization and vacuolar degradation.

This revised protocol also allowed us to reveal the existence of a slow constitutive turnover of maltose permease and show that this constitutive turnover of plasma membrane-localized maltose permease is also unaffected by nitrogen availability (Figures 1). Slow basal turnover rates are characteristic of most yeast membrane proteins. Ste3p is a noteworthy exception. Ste3p exhibits a high rate of constitutive turnover. Ste3p, the a-factor receptor, is internalized via two constitutive pathways, both of which are very rapid giving this protein an exceptionally short half-life under all conditions (Chen and Davis, 2000). The first is a constitutive, ligand-independent pathway that requires ubiquitination and targets Ste3p to the vacuole for degradation. The second pathway is ligand-dependent but ubiquitin-independent and Ste3p internalized via this pathway is able to recycle to the plasma membrane.

Doa4p is required for Mal61/HA-GFP vacuolar localization and glucose-induced inactivation of maltose permease

The function of ubiquitin as a trafficking signal is widely established (Hicke, 1999). Ubiquitin-conjugation is implicated as targeting signal in the endocytosis of Ste2p (Hicke, 1997), 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 yeasts and other organisms. During endocytosis from the plasma membrane, ubiquitinated protein is bound by epsins via their ubiquitin interacting motif (UIM) and perhaps other related membrane-associated proteins that act as cargo-dedicated intermediate adaptors for sorting to the vacuole via the late endosome – multvesiculated body (MVB) pathway (Bonifacino and Traub, 2003; Robinson, 2004; Wendland, 2002). Ubiquitin is also known as a key player in post Golgi trafficking where it is believed to divert newly synthesized proteins away from delivery to the plasma membrane and toward the endosome - MVB pathway and the vacuole (Beck et al., 1999; Bonifacino and Traub, 2003; Helliwell et al., 2001; Magasanik and Kaiser, 2002).

Previously, we demonstrated that glucose-induced proteolysis of maltose permease requires ubiquitin conjugation but some of our results suggested that the rapid loss of maltose transport activity that occurred almost immediately upon the addition of glucose was in some strains far more rapid than the rate of proteolysis and might result from an independent process (Medintz et al., 1996; Medintz et al., 1998). In this report, we follow the localization of GFP-tagged Mal61 maltose permease during glucose-induced inactivation in both a wild type and ubiquitination-defective *doa4Δ* strains. Our findings provide clear evidence that the rapid decrease in maltose transport activity and maltose permease proteolysis are interdependent processes in a common pathway and both are dependent on ubiquitin. Loss of *DOA4* completely blocks both the rapid loss of maltose transport activity and the rapid removal of maltose permease from the cell surface that is observed soon after the addition of glucose. Little or no significant intracellular or vacuolar accumulation of permease is observed in the *doa4Δ* strain and no proteolysis of maltose permease is detected. Clearly, from the results in Figure 2 the

precipitous drop in maltose transport activity correlates with rapid removal of Mal61/HA-GFP from the cell surface. Thus, these earliest steps in maltose permease internalization are ubiquitin-dependent and internalization is a precursor to endocytic vesicle formation and delivery to the vacuole for degradation. As such, the rate of internalization sets the maximal overall rate for glucose-induced inactivation and discrepancies between the rate of loss of maltose transport activity and maltose permease proteolysis most likely result from differences in the rate of delivery of endocytic vesicles to the vacuole. Such differences can be seen in Figure 6 that compares vesicle trafficking in strains CMY1050, which exhibits a slower rate of vesicle trafficking, to strain KT1357. Consistent with this, the rate of loss of transport activity and rate of permease proteolysis are similar in strain KT1357 (data not shown) while proteolysis is much slower in CMY1050 than the rate of loss of transport activity.

Subcellular localization of Mal61/HA-GFP maltose permease.

We show here that Mal61/HA-GFP permease is present at abundant levels in the vacuole even in cells grown in glycerol/lactate. This is an unusual finding. A review of the literature on *Saccharomyces* plasma membrane proteins such as Ste2p, the α-factor receptor (Hicke, 1997); Gal2p, the galactose transporter (Lau et al., 2000; Sherwood and Carlson, 1999); Pho86p, the phosphate transporter (Lau et al., 2000); Hxt1p, the low-affinity glucose transporter (Sherwood and Carlson, 1999); and Pma1p, the plasma membrane ATPase (Gong and Chang, 2001) indicates that under normal growth conditions these proteins localize to the plasma membrane with little intracellular accumulation. Only following an appropriate signal, such as ligand binding or phosphate addition, does one observe vacuolar accumulation of these proteins due to their selective

removal from the cell surface and delivery to the vacuole for degradation (Hicke, 1997; Hicke, 1999; Horak, 2003).

The abundant vacuolar localization of Mal61/HA-GFP does not result from overexpression since the GFP-tagged allele used in these studies is expressed from the native *MAL61* promoter. Moreover, this vacuolar localization is observed in different strain backgrounds (Figures 1, 2, and 5) and in both glycerol/lactate and maltose-grown cells (data not shown) indicating that it is unlikely to be the result of strain variations in vacuolar degradation rates or mislocalization resulting from physiological differences on different carbon sources. Similar subcellular distribution is reported for Fur4-GFP where it is attributed to the 2.5 hr basal turnover rate of Fur4p and resistance to vacuolar proteolysis (Dupre and Haguenauer-Tsapis, 2001; Volland et al., 1994). Given the slow rate of constitutive turnover of plasma membrane localized Mal61/HA-GFP maltose permease, over 12 hrs in nitrogen-starved conditions, it seems unlikely that this alone could be the source of such abundant levels of vacuolar Mal61/HA-GFP accumulation.

We propose that, like Gap1p and Tat2p, newly synthesized Mal61 maltose permease is sorted from the Golgi both to the plasma membrane and into the endosome-to-vacuole pathway, also referred to as the MVB pathway. Significant amounts of Gap1p and Tat2p permeases are observed in intracellular compartments and the vacuole as well as the plasma membrane under steady-state growth conditions. Under growth conditions where the genes encoding these permeases are constitutively transcribed, regulated expression of the transporter is controlled at the post-translational level by controlling sorting of these proteins to either the plasma membrane or the vacuole in response to nitrogen availability (Beck et al., 1999; Roberg et al., 1997; Schmidt et al., 1998; Soetens

et al., 2001). Comparable amounts of Gap1p are present in cells grown in ammonium, urea, and glutamate yet Gap1p transport activity is almost 100-fold higher in ammonium-grown cells and 10-fold higher in urea-grown cells compared to glutamate-grown cells and a large percentage of the protein is localized to the Golgi and endosomes (Roberg et al., 1997). Tat2p is localized to the plasma membrane in cells grown in low tryptophan but can be observed in intracellular compartments, including the vacuole, at significant levels in cells grown in both high and low tryptophan (Beck et al., 1999; Umebayashi, 2003).

Also, it is noteworthy that no Mal61/HA-GFP protein is observed in any intracellular compartments in the *doa4Δ* mutant strain even in glycerol/lactate grown cells. Similar results are observed for Tat2p localization in *doa4Δ* mutant cells grown in high and low concentrations of tryptophan (Beck et al., 1999; Umebayashi, 2003). Direct Golgi to vacuole sorting has been reported for Gap1p and Tat2p (Beck et al., 1999; Helliwell et al., 2001; Roberg et al., 1997; Umebayashi, 2003). This Golgi to vacuole pathway requires ubiquitination and utilizes the ubiquitin-protein ligase Rsp5p and Bul1,2 proteins (Helliwell et al., 2001; Umebayashi, 2003). We postulate that the results in Figure 2 suggest similar requirements for vacuolar localization of Mal61p and are currently investigating the sorting and trafficking patterns of newly synthesized Mal61p and the possible regulatory signals involved controlling intracellular distribution.

Phosphorylation of Ser/Thr residues in the maltose permease N-terminal cytoplasmic domain is required for glucose-induced proteolysis but not for plasma membrane localization or glucose-induced endocytosis

Downregulation of a number of plasma membrane proteins requires phosphorylation which reportedly makes them attractive targets for ubiquitination thereby triggering endocytosis (Hicke, 1999). The results reported here for Mal61p of *Saccharomyces* suggest a different role for the phosphorylation of the Ser/Thr residues in N-terminal cytoplasmic domain of the PEST sequence. 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 that correlates with a rapid removal from the cell surface of the GFP-tagged allele of this mutant protein. However, in contrast to wild-type permease, GFP-tagged Mal61/HA-(T29A, S33,43,48,56A) does not degrade in the vacuole. It persists in what appear to be vesicles, in strain CMY1050, or a prevacuolar compartment, in strain KT1357. These results suggest that normal glucose-induced ubiquitination of Mal61/HA-(T29A, S33,43,48,56A) protein occurs and is sufficient to internalize the mutant transporter. Nonetheless, the endocytic vesicles containing the mutant protein are defective and their cargo never reaches the vacuole.

Endocytic vesicles undergo a maturation process that results in the formation of a MVB capable of fusing with and dumping its contents into the vacuole for degradation (Bonifacino and Traub, 2003; Umebayashi, 2003). During this maturation process the internalized ubiquitin-conjugated cargo associates with a series of ESCRT (endosomal sorting complexes required for transport) complexes that promote invaginations of the vesicle membrane containing ubiquitinated cargo. Also during this maturation the conjugated ubiquitin is removed from the cargo protein. In the case of the Mal61/HA-(T29A, S33,43,48,56A) mutant protein we suggest that either deubiquitination does not occur properly or the protein is unable to associate productively with the ESCRT

complexes. As a result, vesicles containing Mal61/HA-(T29A, S33,43,48,56A) permease are unable to progress from endosomes to MVB and do not fuse with the vacuole. Instead, these vesicles mature into a prevacuolar compartment and the protein is not degraded.

A dileucine motif at residues 64 to 70 and a pair of lysines at residues 41 and 42 are involved in plasma membrane localization and glucose-induced inactivation of maltose permease.

Several distinct transmembrane protein sorting signals have been identified that include either tyrosine-based signals or two classes of dileucine motif based signals, so-called because an adjacent pair of leucines or isoleucine residues is an essential feature of the motif (Bonifacino and Traub, 2003). The [D/E]ExxxL[L/I] class of dileucine motif is found in the N-terminal domain of Mal61 maltose permease and results reported by Medintz et al. (1998) suggested a role for this sequence in the glucose-induced internalization of this protein. Instead, our results reveal that this sequence plays a very important role in the sorting of maltose permease to the plasma membrane and do not support a requirement for this motif in the internalization of Mal61p.

Extremely low levels of maltose transport activity are detected in strains expressing the Mal61/HA-L69,70A mutant permease. Localization data from the GFP-tagged allele revealed that very low levels of protein are localized to the cell surface and that the great abundance of the protein is in vesicles surrounding the vacuole in strain CMY1050 or in a prevacuolar compartment in strain KT1357. Vesicles carrying the Mal61/HA-L69,70A mutant permease exhibit a severe defect in entry into the vacuole for degradation. Taken together, the EEVPDLL sequen ce of Mal61p functions as a sorting

motif. It is required in the Golgi for proper delivery to the plasma membrane but it is also required for entry into the MVB pathway. The mutant protein seen in vesicles in strains CMY1050 or in the prevacuolar compartment in strain KT1357 is probably derived directly from the Golgi without passage through the plasma membrane, given the very low level of mutant transporter at the cell surface. Preliminary studies of this mutant permease in *doa4Δ* support this proposal (data not shown).

Our results for the dilysine mutant are rather surprising. The dilysine motif is most frequently associated with COPI-mediated retrieval of ER proteins from the Golgi and the motif is usually in a C-terminal location (Cosson et al., 1998). We had hypothesized that lysines 41 and 42 were used as sites for ubiquitin-conjugation and anticipated that mutation of these sites would have effects similar to those seen in *doa4Δ*. Instead, very low levels of maltose transport activity are seen for strains expressing the Mal61/HA-(K41,42R) mutant permease and very little of the GFP-tagged allele is observed at the cell surface. The GFP-tagged protein is seen scattered throughout the cytoplasm in small vesicles in strain CMY1050 or in a prevacuolar compartment in strain KT1357. Thus, Mal61/HA-(K41,42R) mutant permease exhibits sorting defects similar to Mal61/HA-L69,70A permease both in its inability to localize to the plasma membrane and in its inability to enter the MVB – vacuole pathway for degradation. In only one case, the MAL lipoprotein, has a dilysine-like motif been reported to function for sorting to the late secretory pathway (Puertollano et al., 2001). MAL is a chaperone in the integral protein sorting machinery of MDCK cells and is required for the delivery of influenza virus hemagglutinin to the apical cell surface. Mutations in the C-terminal RWKSS motif exhibit defects in MAL distribution into clathrin-coated vesicles at the TGN and in HA

delivery to the plasma membrane. The role of lysines 41,42 in Mal61p sorting remains to be determined.

Taken together, the results of our studies of mutations in the N-terminal cytoplasmic domain of Mal61 maltose permease indicate that this domain is largely used for sorting: sorting of newly synthesized protein from the Golgi into the late secretory pathway and sorting in the endosome into the MVB pathway.

Adaptor protein complexes AP-1 and AP-3 are important for the plasma membrane localization of Mal61 maltose permease

Three functionally distinct classes of AP complex have been identified in *Saccharomyces* that aide in the formation of coated vesicles at the plasma membrane, the TGN, and endosomes (Boehm and Bonifacino, 2001; Robinson, 2004). Each AP complex is a heterotetramer consisting of two large subunits (α or γ and β), a medium subunit (μ), and a small subunit (σ). Cargo proteins interact either directly or indirectly to different subunits of the AP complex. AP-2 is utilized for endocytosis at the plasma membrane while AP-1 and AP-3 are involved in membrane protein sorting at the TGN and endosomes.

AP-1 and AP-3 are localized to TGN and endosomal membranes and participate in coated vesicle formation for Golgi to plasma membrane sorting, Golgi to endosome sorting and back, and sorting from the Golgi and endosome to the lysosome/vacuole (Bonifacino and Traub, 2003; Robinson, 2004). Our results reported in Figure 8 indicate that both AP-1 and AP-3 are used for delivery of Mal61 maltose permease to the plasma membrane and that these complexes act independently in parallel pathways. Interestingly, loss of both AP-1 and AP-3 does not completely block Mal61 permease delivery to the

plasma membrane and about 15-20% residual transport activity is expressed in the AP-1 and AP-3 double null mutant strain (Figure 8). An alternate route to the plasma membrane appears to exist, possibly via the GGA proteins (Golgi-localized, γ-ear-containing, ARF-binding proteins) encoded by *GGA1,2*. These are a family of clathrin adaptors localized to TGN and endosomal membranes. It is not surprising that we find no significant impact of loss of AP-2 on maltose permease glucose-induced inactivation. Mutant strains that lack all functional AP complexes are nonetheless capable of endocytosis and the epsins Ent1p and Ent2p are believed to provide the necessary adaptor functions for clathrin-coated vesicle formation (Wendland et al., 1998).

CHAPTER 2

YCK 1,2 encoded casein kinase 1 is required for transport activity and glucose-induced inactivation of *Saccharomyces* maltose permease

ABSTRACT

Saccharomyces cerevisiae casein kinase 1, encoded by the essential gene pair *YCK1* and *YCK2,* controls cell growth and morphogenesis and has been linked to the endocytosis of several integral membrane proteins. We used strain *yck1Δ yck2-ts*, that expresses a temperature sensitive Yck1, 2 casein kinase 1, and an *akr1Δ* strain, that is defective in Yck1,2 kinase localization to the plasma membrane, to explore the role of Yck1,2 kinase in Mal61p maltose permease glucose-induced inactivation. The results show that *yck-ts* strains exhibit significantly reduced ability to transport maltose but only a slight decrease in maltose permease protein levels and no apparent impairment of its localization to the cell surface. Additionally, in the *yck-ts* strain glucose-induced internalization of Mal61/HAp-GFP and the rapid loss in maltose transport activity are not observed, indicating defects in the early steps of endocytosis, and maltose permease proteolysis is blocked. Loss of *AKR1* causes similar phenotypes as *yck-ts*. The resistance of maltose permease to glucose-induced inactivation observed in a *doa4Δ* mutant and is not rescued by overexpression of *YCK2*. Our results indicate that Yck1,2 casein kinase 1 is directly or indirectly required at multiple steps in the expression and glucose-induced inactivation of maltose permease and functions upstream of the ubiquitin conjugating enzymes that mark maltose permease for endocytosis and vacuolar proteolysis.

Introduction

In *Saccharomyces cerevisiae*, glucose addition to maltose fermenting cells causes a very rapid loss of maltose transport activity and proteolysis of maltose permease (Medintz et al., 1996). This glucose-induced inactivation of maltose permease was shown to be dependent on endocytosis, vesicle trafficking pathways, and vacuolar proteases. (Medintz et al., 1998) demonstrated glucose-stimulated ubiquitination of maltose permease and found that degradation of maltose permease requires ubiquitin-conjugation enzymes, particularly Rsp5 ubiquitin ligase, and is blocked in *doa4Δ* mutants that are depleted for available intracellular ubiquitin.

Two glucose sensing and signaling pathways stimulate glucose-induced inactivation of maltose permease (Jiang et al., 1997). Pathway 1 senses high extracellular glucose concentrations by means of the integral membrane protein Rgt2p, a non-transporting homologue of the Hxt glucose transporter family of sugar transporters (Ozcan et al., 1996). Pathway 2 measures extracellular glucose levels by monitoring the rate of glucose metabolism through the early steps of glycolysis (Jiang et al., 2000). Deletion of *REG1*, encoding a targeting subunit of Glc7 protein phosphatase type-1, blocks signaling via Pathway 2 (Jiang et al., 2000). Their results suggest that Glc7-Reg1 phosphatase does not act directly on maltose permease since loss of Reg1p causes decreased phosphorylation of the protein. (Jiang et al., 2000) propose that an as yet unidentified kinase is downstream of Glc7-Reg1 phosphatase in Pathway 2 and could be directly responsible for maltose permease phosphorylation thereby marking the protein for ubiquitination and endocytosis.

Plasma membrane localized casein kinase 1encoded by *YCK1* and *YCK2* is a likely candidate for this downstream kinase of Pathway 2. (Vancura et al., 1994) showed that Yck1,2 casein kinase 1 isoforms have a C-terminal prenylation motif that allows anchorage of the modified protein to the cytoplasmic face of the plasma membrane. Genetic interaction with functions involved in endocytosis has been reported. The growth and morphology defects exhibited by a *yck-ts* strain expressing only a temperature sensitive allele of *YCK2*, genotype *yck1Δ yck2-2ts*, are significantly enhanced when in combination with *chc1-ts*, a temperature sensitive allele of the clathrin heavy chain gene (Panek et al., 1997). Yck1,2 kinase has also been implicated in stimulating the internalization of several *Saccharomyces* plasma membrane proteins including Ste2 α-factor receptor (Hicke, 1999) , Ste3 **a**-factor receptor (Feng and Davis, 2000b), and Fur4 uracil permease (Marchal et al., 1998). (Feng and Davis, 2000a) also report that Yck1,2 kinase is required for Ste3p phosphorylation. Based on these reports we decided to explore the role of Yck1,2 casein kinase 1 in the glucose-induced inactivation of maltose permease.

In this report we provide evidence that plasma membrane-localized Yck1,2 kinase is required for activation of the transport activity of maltose permease but not for its synthesis or localization to the plasma membrane. In addition, glucose-induced inactivation of maltose permease is entirely blocked in the *yck-ts* strain. The *yck-ts* strain does not exhibit defects in fluid phase endocytosis, plasma membrane internalization, and vesicle transport to the vacuole even at the nonpermissive temperature, as monitored using the lipophillic dye FM4-64, suggesting that the function of Yck1,2 kinase in endocytosis is specific to maltose permease. We have previously reported that ubiquitin

conjugation is required for glucose-induced inactivation of maltose permease (Medintz et al., 1998). Here, we demonstrate that *YCK1,2* is upstream of *DOA4* in the signaling pathway stimulating glucose-induced inactivation of maltose permease.

Results

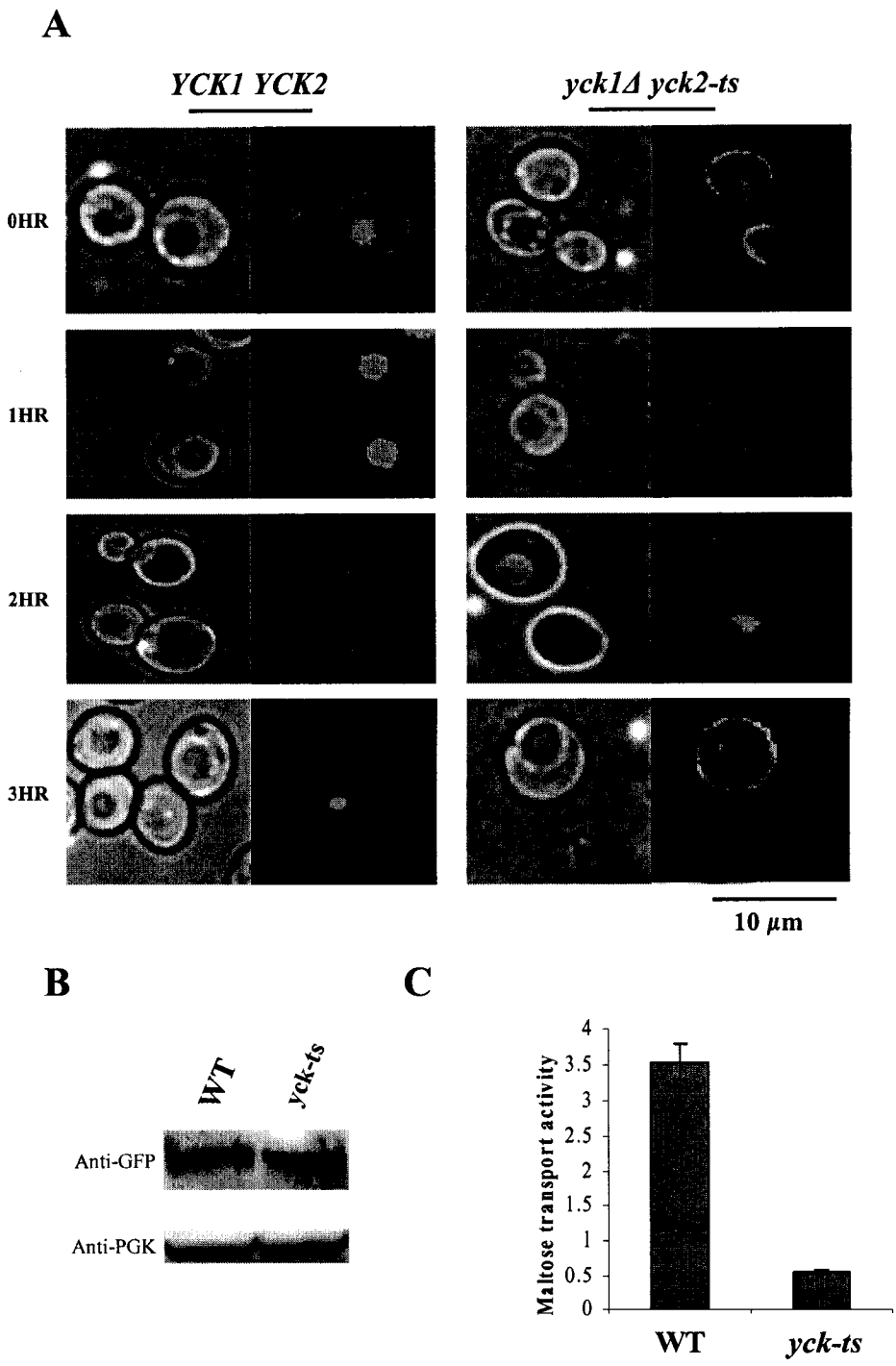
Yck1,2 casein kinase 1 is required for the localization of active maltose permease to the plasma membrane

To investigate a possible role for Yck1, 2 casein kinase 1 in maltose permease synthesis and trafficking we constructed a GFP-tagged allele by fusing the GFP gene in-frame at the 3'-end of *MAL61/HA* (Chapter 1). *MAL61/HA-GFP* is expressed from the native *MAL61* promoter and is carried by the pUN70 (referred to as plasmid pMAL61/HA-GFP). Strains LRB756, *yck1Δ yck2-ts*, and the isogenic parental strain LRB906, *YCK1 YCK2*, were transformed with plasmids pMAL61/HA-GFP and pMAL63, carrying the inducible *MAL63 MAL*-activator gene. Transformants were grown to log phase at 24°C in synthetic medium lacking leucine and histidine with 2% maltose as a carbon source, harvested by filtration, and resuspended in nitrogen starvation media plus 2% glucose, referred to as YNSG, with cyclohexamide to inhibit protein synthesis. At the indicated times cells were collected and viewed by confocal microscopy. The results are shown in Figure 1. It should be noted that the *yck1Δ yck2-ts* strain, to be referred to as *yck-ts*, is partially defective even at the permissive temperature (Panek et al., 1997).

In both the wild type and *yck-ts* strains pMAL61/HA-GFP protein is present at the cells surface (Figure 1A). Significant amounts of fluorescent signal is also present in the

Figure 1. Localization of GFP tagged Mal61p in *YCK1YCK2* and *yck-ts* strains

Strains LRB 906 (*YCK1 YCK2*) and LRB 756 (*yck-ts*) were transformed with plasmids pMAL61/HA-GFP and pMAL63. Transformants were grown at 24°C in synthetic medium with 2% maltose lacking histidine and uracil. Cells were harvested with filtration and transferred to YNSG containing CHX (30μg/ml) and incubation at 24°C with shaking was continued. *Panel A.* Culture samples were taken at time zero and every hour over a 3 hour period, gently centrifuged, and GFP localization visualized by confocal microscopy as described in Materials and Methods. The fluorescence signal in the *yck-ts* strain was enhanced by adjusting the laser voltage about 2-fold. All the other settings remain the same. *Panel B.* The level of Mal61/HA-GFPp was determined by Western blotting of total cell extracts separated on a 10% SDS-PAGE gel and probed with anti-GFP antibody. Western blotting of PGK is shown as the loading control. *Panel C.* Maltose transport activity was determined in the culture sample taken at time zero. The error bars indicate standard deviation from three independent experiments done in duplicate.



vacuole of wild-type cells but only a slight signal can be seen in the *yck-ts* cells. High levels of vacuolar localization of pMAL61/HA-GFP were reported previously in studies carried out on other strains and found to be dependent on ubiquitin conjugation, since no signal was observed in the vacuole of a *doa4Δ* mutant (Chapter 1). Despite the presence of significant amounts of pMAL61/HA-GFP at the cell surface, the *yck-ts* strain exhibits severe defects in maltose transport activity (Figure 1C). These results indicate that Yck1,2 kinase is required for activation of the transport activity of maltose permease but not for its synthesis or localization to the plasma membrane.

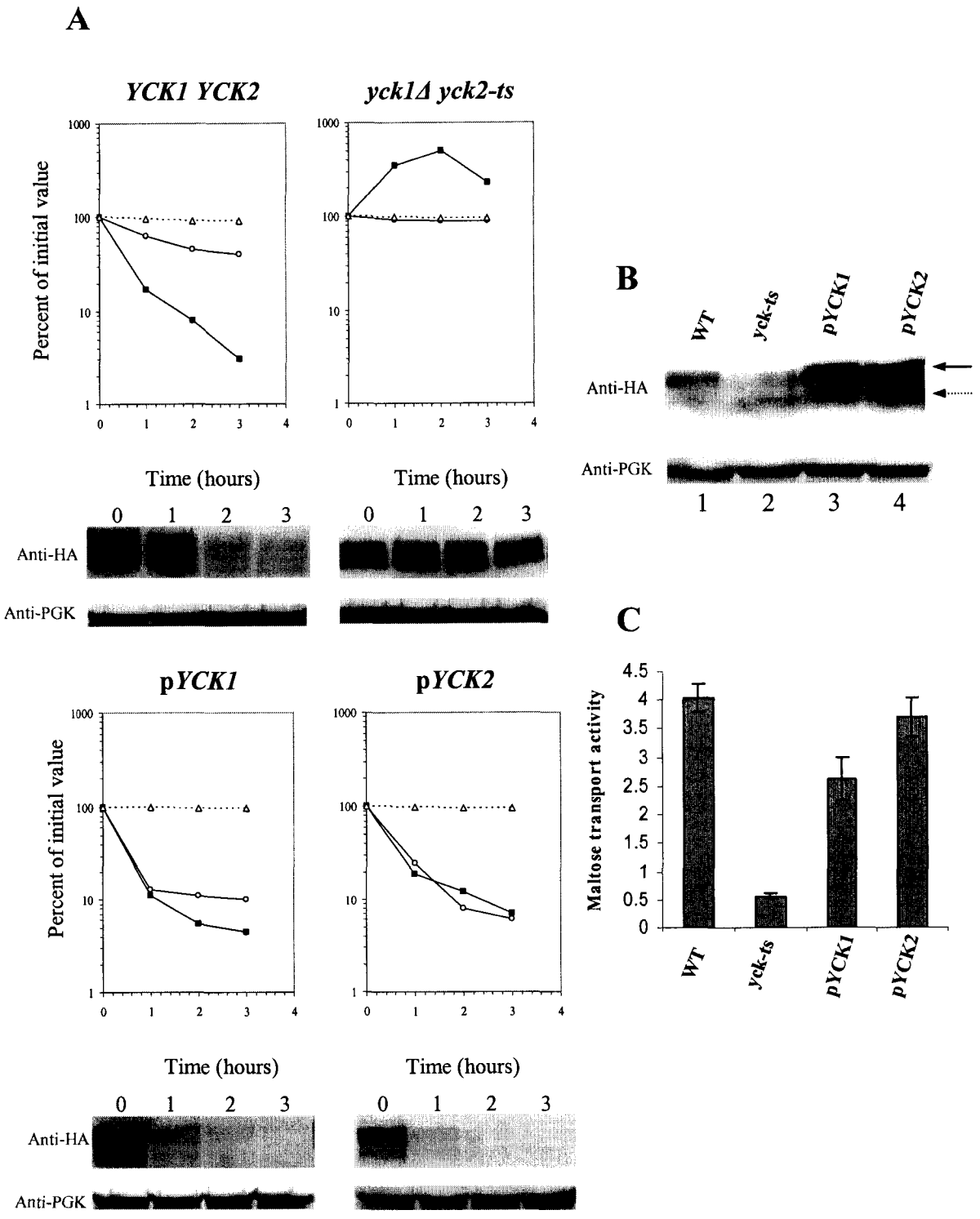
Yck1,2 casein kinase 1 is required for glucose-induced inactivation of maltose permease

Figure 1 follows changes in the localization of Mal61/HA-GFP maltose permease in strains LRB 906 (*YCK1 YCK2*) and LRB756 (*yck1Δ yck2-ts*) following the addition of glucose to maltose fermenting cultures. Within 1 hour in the wild-type strain the level of maltose permease at the cell surface is dramatically reduced and localization to the vacuole appears to be enhanced by 1 hour. Over the course of the 3 hours the vacuolar signal is gradually reduced, presumably by degradation. The results are quite different for the *yck-ts* strain. No glucose-induced loss of fluorescent signal from the cell surface is evident nor is there significant accumulation of Mal61/HA-GFP protein in the vacuole.

Strains LRB 906 (*YCK1 YCK2*) and LRB756 (*yck1Δ yck2-ts*) also were transformed with plasmids pMAL61/HA, pMAL63, and either YEp352, p*YCK1*, or p*YCK2* and subjected to the standard inactivation protocol as described in Materials and

Figure 2. Glucose-induced inactivation and phosphorylation of maltose permease in *yck1Δ yck2ts* and *YCK1,2* overexpressing strains

Strains LRB906 (*YCK1 YCK2*) (*Panel A*) and LRB756 (*yck1Δ yck2-ts*) were transformed with plasmids pMAL61/HA, pMAL63, and YEp352 or p*YCK1* or p*YCK2*. Transformants were grown at 24°C in selective media lacking leucine, histidine, and uracil plus 2% maltose, harvested by filtration, transferred to YNSG with CHX (30μg/ml) and inactivation followed at 24°C. At the indicated times the growth dilution (Δ), maltose transport (■) and relative Mal61/HA protein levels (o) were determined as described in Figure 1 and the Materials and Method. *Panel B* shows The level of Mal61/HAp was determined by Western blotting of total cell extracts separated on a 7.5% SDS-PAGE gel and probed with anti-HA antibody. Western blotting of PGK is shown as the loading control. The arrows indicate the position of the hyperphosphorylated (solid line) and hypophosphorylated (dotted line) species of Mal61/HAp. *Panel C* shows the maltose transport activity of the transformed strains determined in the culture sample taken at time zero of the inactivation protocol. The error bars indicate standard deviation from three independent experiments done in duplicate.



Methods. Transformants were grown to log phase in the selective medium lacking the appropriate supplements plus 2% maltose as a carbon source, harvested by filtration, and resuspended in nitrogen starvation medium plus 2% glucose with CHX. Figure 2A follows changes in maltose transport activity and the relative level of maltose permease protein. As has been shown for several other strain series (Medintz et al., 1996) we observe a rapid loss of maltose transport activity and Mal61/HA proteolysis. Also, the rate of Mal61/HAp proteolysis is significantly slower than the rate of loss of maltose transport activity. This discrepancy is observed in most strains and results in Chapter 1 suggest that is due to a slower rate of delivery of endocytic vesicles to the vacuole compared to a rapid rate of maltose permease internalization.

In contrast to the wild-type strain, the results in the *yck-ts* strain are novel. Glucose induces approximately a 7-fold increase in maltose transport activity (Figure 2). Given the apparent absence of increased accumulation of Mal61/HA-GFP protein at the cells surface in response to glucose (Figure 1 Panel A) it seems likely that this dramatic increase in maltose transport activity results from an increase in enzyme activity but the mechanism of the increased specific activity is not known. Glucose-induced proteolysis of maltose permease is essentially blocked in the *yck-ts* strain and, consistent with the results in Figure 1, no significant decrease in Mal61/HA protein levels is observed. Figure 2B also shows that the ratio of hyperphosphorylated (upper band) to hypophosphorylated (lower band) species of Mal61/HA maltose permease is lower in the *yck-ts* than in the isogenic wild-type strain (Medintz et al., 1996).

To further explore the role of Yck1,2 kinase in maltose permease synthesis and turnover, we overexpressed Yck1,2 casein kinase 1 in strain LRB 906 (*YCK1 YCK2*)

using multicopy plasmids carrying *YCK1* and *YCK2* (Figure 2A). We see a significant increase in the relative amount of the hyperphosphorylated species of maltose permease (Figure 2B) but no concomitant increase in maltose transport activity (Figure 2C). Glucose-induced inactivation of maltose permease was determined in these Yck1,2 kinase overexpressing strains (Figure 2A). We observed little or no increase in the rate of loss of maltose transport activity but the rate of Mal61/HA permease proteolysis increases dramatically to match the rate of rapid loss in transport activity.

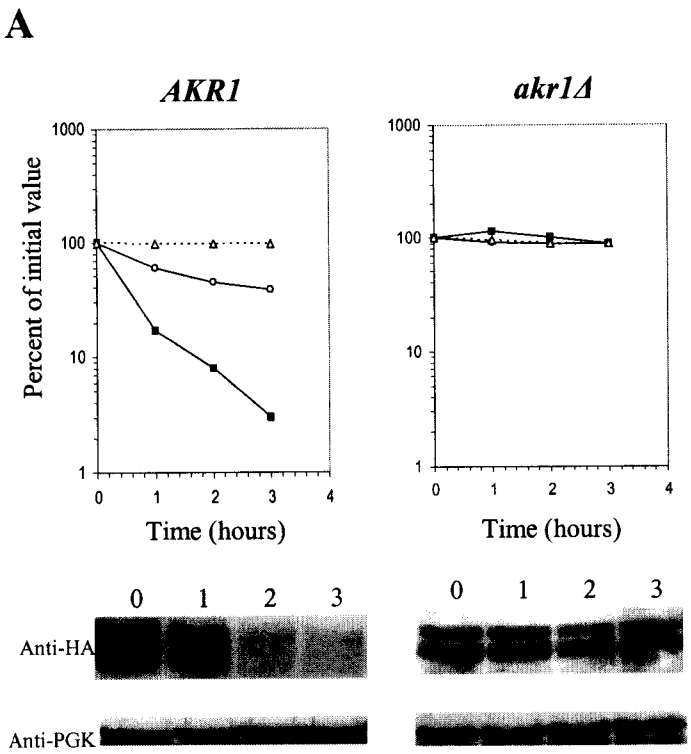
These results indicate that casein kinase 1 encoded by *YCK1* and *YCK2* is either directly or indirectly responsible for the phosphorylation of maltose permease, the expression of active maltose permease, the localization of maltose permease protein to the vacuole, as well as the glucose-induced internalization and proteolysis of maltose permease.

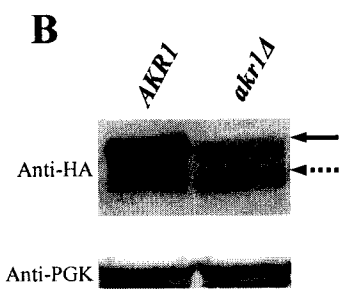
AKR1 deletion blocks glucose-induced inactivation of maltose permease

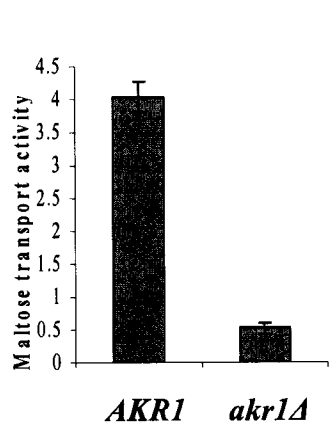
Yck1,2 kinase contains a prenylation motif at the C-terminus that is required for plasma membrane localization and biochemical function (Vancura et al., 1994). Akr1p is a palmitoyl transferase and is required for the proper localization of Yck1,2p to the plasma membrane (Babu et al., 2002; Feng and Davis, 2000a; Roth and Davis, 1996). (Feng and Davis, 2000a) found that in *akr1Δ* strains Yck2p is distributed in the cytoplasm rather than localized to the plasma membrane. Additionally, Akr1p mutants exhibit reduced phosphorylation of Ste3p (Feng and Davis, 2000a). Interestingly, *YCK1* and *YCK2* are an essential gene pair while a deletion of *AKR1* is viable (Babu et al., 2002). This allows us to test whether plasma membrane-localization of Yck1,2p is required.

Figure 3. Transport activity and glucose-induced inactivation of maltose permease in an *akr1Δ* strain

Panel A: Strain LRB906 (*YCK1 YCK2 AKR1*) and an isogenic *akr1Δ* strain were transformed with pMAL61/HA and YCp50-MAL63. Transformants were grown to log phase in selective medium lacking leucine and uracil plus 2% maltose, harvested by filtration, transferred to YNSG with CHX, and the standard inactivation protocol carried out. At the times indicated the growth dilution (Δ), maltose transport (■) and relative Mal61/HA protein levels (o) were determined as described in Materials and Method. *Panel B*: The transformed strains described in Panel A were grown in selective medium lacking leucine and uracil plus 2% maltose, harvested, total cell extracts prepared as described in Method and Materials, and analyzed by Western blotting using anti-HA antibody. The arrows point to the hyperphosphorylated (solid line) and hypophosphorylated (dotted line) species of Mal61/HA protein. Equal loading of total cell extract was confirmed using anti-PGK antibody. *Panel C*: Maltose transport activity was determined at time 0 for the strains shown in Panel A. Error bars signify the standard deviation from three independent experiments.







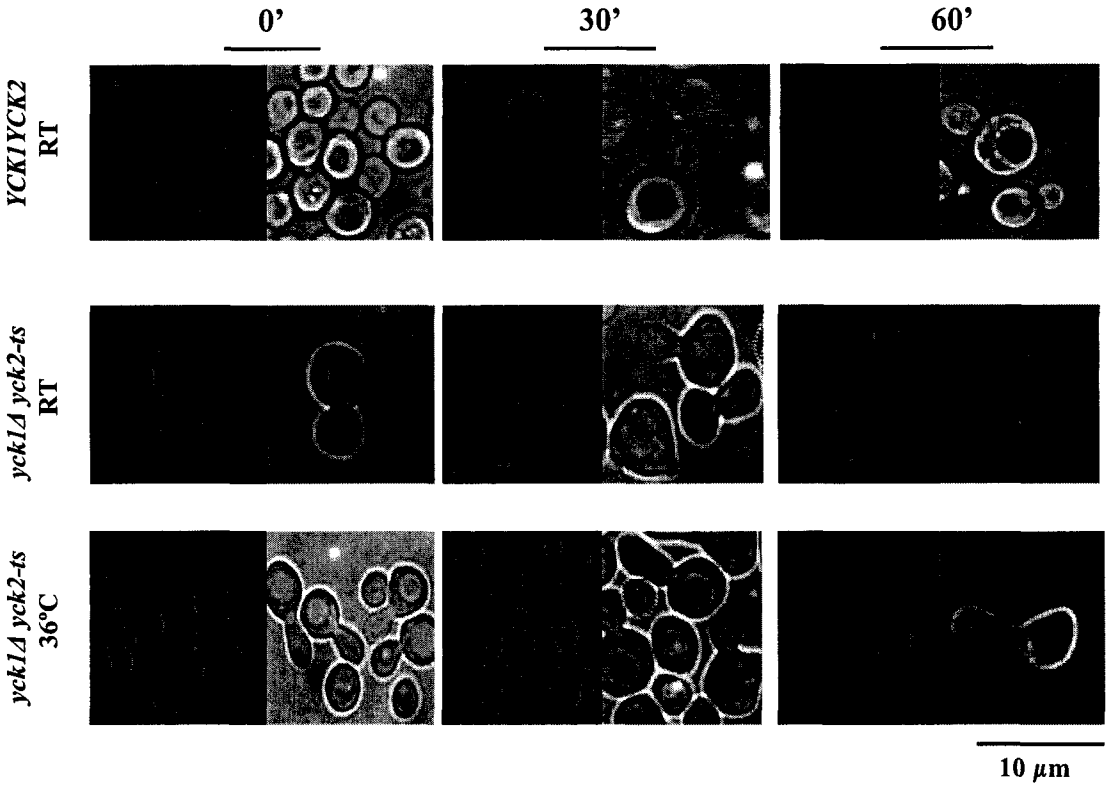
Strain LRB1053 was created by deleting *AKR1* in strain LRB939 (Babu et al., 2002). Yck1,2 kinase does not properly localize to the plasma membrane in this strain. Plasmids pMAL61/HA and YCp50-MAL63 were transformed into both LRB906 and LRB1053; transformants were grown in selective media with 2% maltose as carbon source, and subjected to standard glucose-induced inactivation protocol. The results are shown in Figure 3. As is found for the *yck-ts* strain, the *akr1Δ* strain expresses very low levels of maltose transport activity and there is a dramatic reduction in the relative level of hyperphosphorylated maltose permease. Glucose-induced inactivation of maltose permease is entirely blocked. No maltose permease proteolysis is observed in the *akr1Δ* strain and, in contrast to the more than 7-fold increase in transport activity seen in *yck-ts*, only a slight transient increase in maltose transport activity occurs.

yck-ts is not defective in fluid phase endocytosis

To determine if fluid phase endocytosis and vesicle formation is defective in the *yck-ts* strain or if the defects in these processes are specific to vesicles containing pMal61/HA-GFP we used FM4-64, a lipophillic membrane dye. FM4-64 is rapidly incorporated into the plasma membrane, internalized into intracellular transport vesicles, and soon appears in the vacuolar membrane (Fischer-Parton et al., 2000). Figure 4 compares the rate of movement of FM4-64 from the plasma membrane to the vacuolar membrane in wild type and the *yck-ts* strains. The results indicate that the dye reaches the vacuolar membrane in both strains at permissive as well as non-permissive temperatures with approximately the same kinetics. Thus, no generalized endocytic defect is exhibited by the *yck-ts* strain.

Figure 4. Strain comparison with FM4-64 vacuolar membrane dye

Strains LRB906 and LRB756 were grown at (RT) to mid-log and either maintained at RT or transferred to 36°C for 30 minutes before treatment with FM4-64. Cells were incubated for 15 minutes with FM4-64 as described in Materials and Methods, harvested by centrifugation and transferred to medium without the dye. Confocal microscopy images were taken at the indicated times.



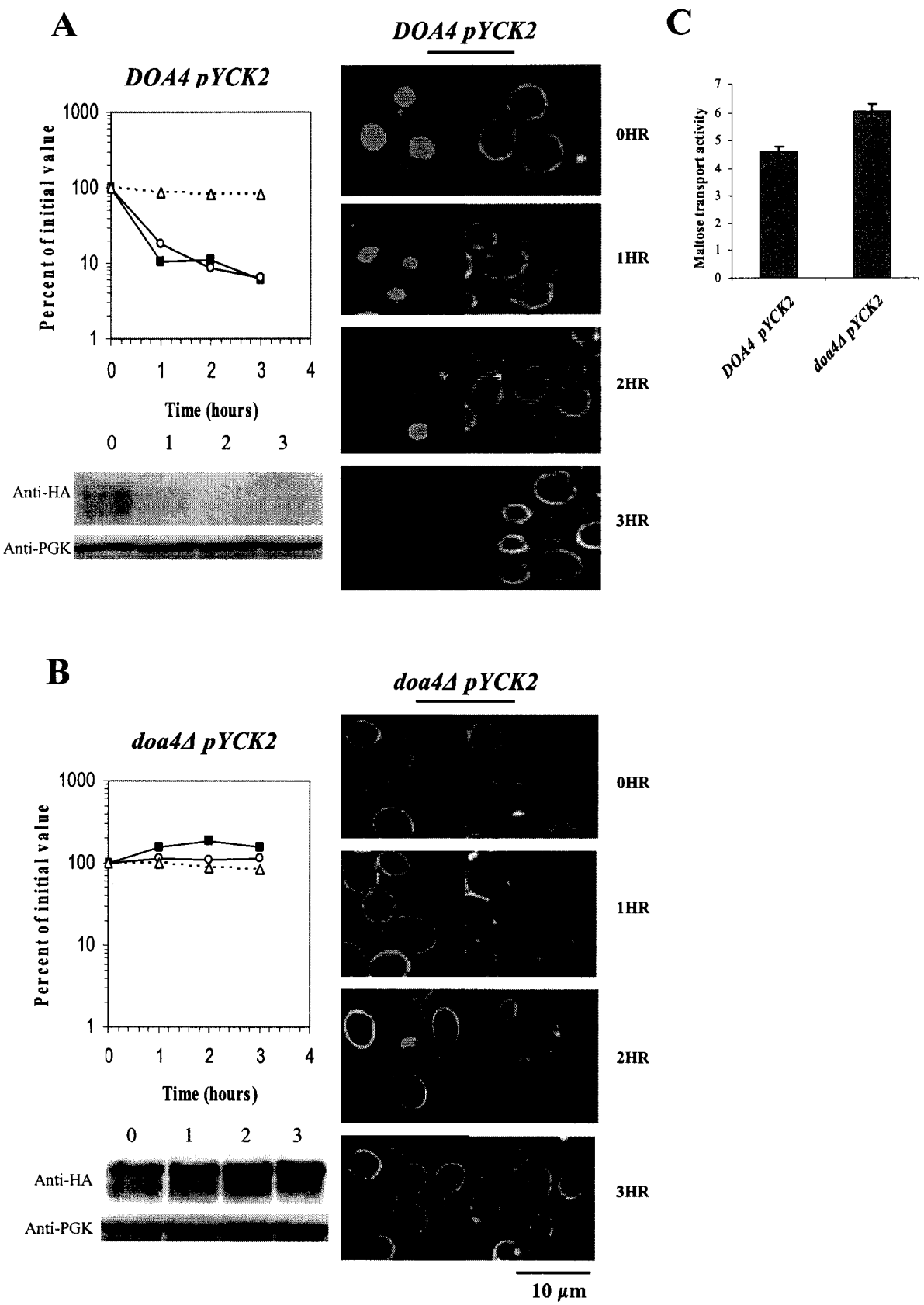
DOA4 mediated ubiquitination is downstream of YCK2 function

We previously reported that ubiquitin-conjugation is required for glucose-induced inactivation of maltose permease (Medintz et al., 1998). Glucose stimulates the ubiquitination of Mal61/HA maltose permease and mutations in *RSP5*, encoding a hect domain ubiquitin ligase, and *DOA4*, encoding a ubiquitin hydrolase whose loss causes depletion of intracellular ubiquitin levels, lead to defects in glucose-induced proteolysis of Mal61/HAp. Additionally, loss of *DOA4* blocks the rapid glucose-induced loss of maltose transport activity and maltose permease proteolysis (Medintz et al., 1998). We overexpressed *YCK2* in *DOA4* and *doa4Δ* strains to explore the genetic relationship of this ubiquitination step to the activity of Yck1,2 kinase. The results are shown in Figure 5.

In chapter 1 we found normal localization of Mal61/HA-GFP permease to the plasma membrane in *doa4Δ* mutant but the vacuolar localization of Mal61/HA-GFP permease observed in all strains studied was not seen in *doa4Δ*. As is quite evident from Figure 5, overexpression of *YCK2* does not restore vacuolar localization of Mal61/HA-GFP permease in the *doa4Δ* strain. Moreover, it should be noted that the Mal61/HA-GFPp synthesized is highly phosphorylated in the *doa4Δ* strain and overexpression of *YCK2* leads to a slight but significant increase in maltose transport activity. Consistent with the results reported in Figure 2, overexpression of *YCK2* increases the rate of glucose-induced proteolysis of Mal61/HA-GFPp in the *DOA4* strain so that the rate of proteolysis overlies the rate of loss of maltose transport activity. Most important, overexpression of *YCK2* does not suppress the block in glucose-induced inactivation in *doa4Δ* mutants (Medintz et al., 1998). As seen in Figure 5B in the *doa4Δ*

Figure 5. Overexpression of *YCK2* does not rescue the *doa4Δ* phenotype

Strains CMY1026 (*MAL1 DOA4*) (*Panel A*) and CMY1025 (*MAL1 doa4Δ::HIS3*) (*Panel B*) cells were transformed with pMal61/HA-GFP, pMal63, and p*YCK2*. Transformants were grown to log phase in selective medium lacking histidine, tryptophan and uracil plus 2% maltose, harvested by filtration, transferred to YNSG with CHX, and standard inactivation protocol carried out. At the times indicated the growth dilution (Δ), maltose transport activity (■) and relative Mal61/HA protein levels (o) were determined as described in Materials and Method. *Panel C*: Maltose transport activity was determined at time 0 of the inactivation protocol for the strains shown in Panels A and B. Error bars signify the standard deviation from three independent experiments.



mutant, maltose transport continues actively even 3 hours after glucose induction, no glucose-induced Mal61/HA-GFP permease proteolysis is observed, and Mal61/HA-GFP permease remains in the plasma membrane.

Discussion

Casein kinases are widely distributed in eukaryotic cells and are defined by their ability to phosphorylate acidic substrates (Tuazon and Traugh, 1991). There are two classes of casein kinases: type-1 is capable of phosphorylating both threonine and serine residues while type-2 acts only on threonine residues (Meggio et al., 1984). Further studies on casein kinase 1 target sites indicate that clusters of 3 or 4 acidic residues like Asp, Glu, Ser(P) and Thr(P) are considered the best substrates (Flotow et al., 1990).

Saccharomyces encodes several casein kinase 1 isoforms. *YCK1* and *YCK2* encode plasma membrane localized isoforms with better than 90% homology in their catalytic domain (Robinson et al., 1992; Vancura et al., 1993; Wang et al., 1992); *YCK3* encodes a vacuolar-membrane localized isoform (Sun et al., 2004); and *HRR25* encodes a nuclear isoform associated with DNA damage repair (DeMaggio et al., 1992; Ho et al., 1997). (Robinson et al., 1992) isolated *YCK1* as a suppressor of the requirement for *SNF4* function in *Saccharomyces*. Snf4 protein is a positive effector of Snf1 protein kinase that is required for derepression from glucose growth conditions. They showed that *YCK1* and *YCK2* genes have a redundant function in growth and strains deleted for both genes are not viable (Robinson et al., 1992). Yck1,2 kinase has a prenylation consensus sequence (Gly-Cys-Cys) at the C-terminus that allows association at the cytoplasmic face of intracellular membranes (Wang et al., 1992; Wang et al., 1996). *AKR1* encodes a

palmitoyl transferase required for prenylation of the carboxy terminus of Yck1,2 kinase (Feng and Davis, 2000a). Based on previous reports implicating Yck1,2 kinase in endocytosis and in the phosphorylation and degradation of several *Saccharomyces* plasma membrane proteins, we decided to explore the role of Yck1,2 kinase in glucose-induced inactivation of maltose permease.

Localization of Mal61/HA-GFP maltose permease is altered in the yck-ts mutant

Yck1,2 kinase is not required for synthesis and localization of Mal61/HA-GFP maltose permease to the cell surface. Approximately the same amount of maltose permease protein is detected by Western analysis in the wild type and *yck-ts* strains (Figures 1 and 2). Additionally, near normal fluorescence is observed at the cell surface in the *yck-ts* strain (Figure 1). Despite this, the level of maltose transport activity is dramatically reduced in the *yck-ts*. The basis for this low transport activity is not known. Given the methods utilized for this study it is not possible to confirm that the permease is positioned normally in the plasma membrane and exposed to extracellular maltose, its transport substrate.

Little or no Mal61/HA-GFP permease is observed in the vacuole in the *yck-ts* strain (Figure 1). Results in chapter1 show a very intense vacuolar fluorescent signal in several different strains expressing Mal61/HA-GFP permease even in glycerol/lactate grown cells. Their results suggest that newly synthesized Mal61/HA-GFP permease is trafficked from the trans-Golgi network (TGN) to both the plasma membrane through the secretory pathway and directly to the vacuole via endosomes and the multivesicular body (MVB) pathway. The MVB pathway requires polyubiquitination by Rsp5 ubiquitin ligase

and Bul1,2p (Bonifacino and Traub, 2003; Helliwell et al., 2001; Horak, 2004). Similar trafficking patterns have been reported for the general amino acid permease Gap1p and for one isoform of the tryptophan transporter Tat2p but in these cases trafficking is regulated by nitrogen availability (Magasanik and Kaiser, 2002; Schmidt et al., 1998; Umebayashi, 2003). The pattern of localization of Mal61/HA-GFP permease seen in the *yck-ts* strain in Figure 1 is similar to that observed in a *doa4Δ* mutant strain reported in Chapter 1. We suggest that the ubiquitination of maltose permease that appears to direct it from the Golgi to the MVB pathway and the vacuole is defective in *yck-ts*.

Yck1,2 casein kinase 1 is involved in Mal61/HAp phosphorylation and is required for expression of transport active maltose permease

Mal61/HA maltose permease is phosphorylated, probably at multiple sites (Medintz et al., 1996). Western analysis of Mal61/HA permease expressed in a variety of strains grown either in 2% maltose or in 3% glycerol/2% lactate exhibits a range of migration species due to the extent of phosphorylation but usually the bulk of the Mal61/HA is highly phosphorylated (Medintz et al., 1996; Jiang et al., 2000b). Phosphorylation of maltose permease is also observed for the wild-type strain used in these studies but is dramatically reduced in the *yck-ts* strain in which most of the Mal61/HA protein migrates as what appears to be an unphosphorylated form (Figure 2B; (Medintz et al., 1996). The *akr1Δ* mutant strain similarly expresses significantly reduced levels of the hyperphosphorylated species of maltose permease protein. Additionally, overexpression of *YCK1* or *YCK2* leads to an over two-fold increase in the abundance of hyperphosphorylated species of maltose permease (Figure 2B). These results suggest a

relationship between membrane-localized Yck1,2 casein kinase 1 and maltose permease phosphorylation but it appears that Yck1,2 kinase is not the sole kinase capable of phosphorylating Mal61/HA.

One possible explanation for the low level of maltose transport activity exhibited by the *yck-ts* mutant could be that Mal61/HA permease is poorly phosphorylated in the *yck-ts* and *akr1Δ* strains (Figures 2 and 3). Similar results, that is, reduced levels of Mal61/HA phosphorylation and low levels of maltose transport activity, were obtained with strains lacking Reg1p, a targeting subunit of Glc7 protein phosphatase type-1 (Jiang et al., 2000b). Further studies are required to determine whether the low transport activity of maltose permease in the *yck-ts* strain results from improper localization to the plasma membrane or that phosphorylation is required to activate the transport activity of maltose permease.

Specific Yck1,2 kinase target sites in maltose permease have yet to be identified. In chapter 1 we investigated the role of these putative target sites in glucose-induced inactivation. Using site-directed mutagenesis of Ser/Thr residues to alanine at sites in the N-terminal domain of Mal61p, they found that alteration of individual and multiple Ser/Thr residues had no impact on delivery of transport active maltose permease to the plasma membrane or glucose-induced internalization of maltose permease, despite decreases in protein phosphorylation levels. Thus, while our results reported here suggest that Mal61/HA maltose permease is a target of Yck1,2 kinase we have yet to identify the specific target residue(s).

Glucose-induced inactivation of maltose permease is blocked in the yck-ts mutant

The *yck-ts* strain exhibits severe defects in glucose-induced inactivation (Figures 1 and 2). Mal61/HA-GFP protein is not internalized in response to glucose, no vacuolar accumulation is observed, and maltose permease proteolysis is completely blocked. Clearly, Yck1,2 kinase is required at a very early step in the endocytosis of maltose permease. Similar defects in glucose-induced inactivation of maltose permease are observed in the *akr1Δ* strain indicating that proper membrane association of Yck1,2 kinase is also necessary. The rate of Mal61/HA proteolysis also increases dramatically in strains that overexpress *YCK1* or *YCK2* eliminating the discrepancy between the rate of loss of maltose transport activity and the slower rate of permease proteolysis that is commonly observed in different strains. Results in chapter 1 suggest that loss of transport activity reflects the rate of permease internalization and thus the initiating steps of endocytosis while the rate of proteolysis is a measure of the rate of delivery of the protein to the vacuole for degradation.

Unexpectedly, maltose transport activity increases dramatically upon glucose addition to the *yck-ts* strain (Figure 2). Similar results are seen in the *akr1Δ* mutant (Figure 3) and in a *reg1Δ* mutant (Jiang *et al.*, 2000b), although not to the same extent. As discussed above, all three strains, (*yck-ts*, *akr1Δ* and *reg1Δ*) also express very low levels of maltose transport activity in maltose-grown cells despite normal levels and localization of Mal61/HA-GFP permease to the cell surface (Jiang et al., 2000b). The reason for the low maltose transport activity and for the glucose-induced increase in transport activity in these strains is not known but is likely interconnected. The simplest explanation for the glucose-induced increase in maltose transport activity is that the lack

of endocytosis in the *yck-ts* strain causes an accumulation of permease protein at the plasma membrane. The results in Figure 1 do not support this. Nor do we observe a glucose-induced increase in permease phosphorylation (Figure 2). More detailed structural analysis is required to resolve this question but our best explanation is that maltose permease is not properly inserted into the plasma membrane.

Overexpression of *YCK2*, but not *YCK1*, suppresses defects in endocytosis and actin distribution observed in *lcb1* mutants (Friant et al., 2000). *LCB1* is required for sphingolipid biosynthesis (Nagiec et al., 1994). It plays an essential role in lipid raft formation that is needed for the delivery of certain proteins to the plasma membrane including the plasma membrane ATPase Pma1p (Wang and Chang, 2002). *LCB1* also is required for endocytosis and is involved in establishing polarized distribution of the actin cytoskeleton (D'Hondt et al., 2000; Friant et al., 2000; Wang and Chang, 2002). It has been proposed that sphingolipid acts as a signaling molecule to activate membrane-associated kinases, specifically Yck2p and Pck1p, and that protein phosphorylation by these kinases functions to regulate membrane trafficking (D'Hondt et al., 2000; Friant *et al.*, 2000). Our results are consistent with this role for Yck kinases. It is important to note that membrane internalization and delivery to the vacuole is normal in the *yck-ts* strain (Figure 4). Friant et al., (2000) also report that fluid phase endocytosis, which is followed using Lucifer Yellow, is normal in the *yck-ts* strain. This indicates that the defects in maltose permease endocytosis demonstrated here are not generalized defects but specific to maltose permease.

Ubiquitination is emerging as a key player in regulating not only endocytosis from the plasma membrane but also post-Golgi trafficking (Bonifacino and Traub, 2003;

Umebayashi, 2003). During endocytosis from the plasma membrane, ubiquitinated protein is bound by epsins via UIMs (ubiquitin interacting motifs), which act as cargo-dedicated intermediate adaptors for sorting cargo for its entry to vacuole via MVB. Ubiquitination also functions in the sorting newly synthesized protein in the TGN and endosomes. Here, polyubiquitinated proteins, which are produced by Rsp5p and Bul1,2p, are blocked from entry into lipid rafts and are instead delivered to the MVB pathway and the vacuole. We have shown that glucose-induced proteolysis of maltose permease requires ubiquitin conjugation for both glucose-induced endocytosis and for vacuolar delivery of the newly synthesized protein (Medintz et al., 1998). The results reported here indicate that Yck1,2 kinase is similarly required for both processes and we suggest that Yck1,2 kinase activity is needed at both the TGN and the plasma membrane to stimulate ubiquitination of maltose permease. Consistent with this, Figure 5 demonstrates that *DOA4* is downstream of *YCK2*. Most likely, as has been shown for Ste2p (Hicke et al., 1998), Ste3p (Feng and Davis, 2000), and Fur4p (Marchal et al., 1998), Yck1,2 casein kinase is also required for the phosphorylation of maltose permease and that this stimulates permease ubiquitination.

In summary, we propose that membrane-localized Yck1,2 casein kinase is responsible for the phosphorylation of maltose permease both of newly synthesized protein and of plasma membrane localized maltose permease in response to glucose. Phosphorylation appears to be required for the expression of functionally active plasma membrane localized maltose permease but also stimulates permease ubiquitination. Ubiquitination of newly synthesized permease, under as yet undefined physiological conditions, can lead to polyubiquitination and delivery of the protein to the vacuole for

degradation. Glucose-stimulated ubiquitination of plasma membrane localized maltose permease causes its rapid internalization and subsequent delivery to the vacuole for degradation. Thus, Yck1,2 kinase is an essential regulator of maltose permease transport activity, sorting in the secretory and MVB pathways, and glucose-induced endocytosis.

CHAPTER 3

A novel Glc7-Reg1 – Yck1 signaling pathway in glucose-induced inactivation of *Saccharomyces* maltose permease.

ABSTRACT

The addition of glucose to maltose fermenting *Saccharomyces* causes a rapid decrease in maltose transport activity and degradation of maltose permease, referred to as glucose-induced inactivation of maltose permease. Jiang *et al.* (2000) observed that glucose-induced inactivation of maltose permease is significantly reduced by loss of the Glc7 phosphatase type-1 targeting subunit encoded by *REG1*. Surprisingly, the extent of maltose permease phosphorylation decreases in a *reg1Δ* strain suggesting that Reg1p-Glc7p phosphatase acts indirectly on maltose permease and that a kinase lies downstream of Glc7p-Reg1p phosphatase in the pathway regulating maltose permease phosphorylation. In chapter 2 we demonstrate that casein kinase 1 encoded by *YCK1* and *YCK2* is also involved in the phosphorylation of maltose permease and that glucose-induced inactivation of maltose permease is blocked in a *yck-ts* strain. Here we report on the relationship between Yck1,2 kinase and Glc7-Reg1 phosphatase. We show that *reg1*Δ and *yck-ts* mutant strains exhibit very similar phenotypes with regard to maltose permease localization, phosphorylation, and resistance to glucose-induced inactivation. Additionally, the phenotype of the *reg1*Δ *yck-ts* double mutant strain is not significantly enhanced compared to the single mutant strains. Glucose repression insensitive *glc7* mutations that alter Reg1p-binding also cause insensitivity to maltose permease inactivation demonstrating that Glc7p - Reg1p interaction in the presence of glucose is required. Overexpression of *YCK1* suppresses *reg1*Δ while overexpression of *REG1* does not suppress *yck-ts*. These results establish that *REG1* is upstream of *YCK1,2* in a novel Glc7-Reg1 phosphatase – Yck1,2 kinase in the signaling pathway controlling glucose-induced inactivation of maltose permease.

Introduction

Three major glucose sensing and signaling pathways have been identified in *Saccharomyces*. The first utilizes two integral membrane proteins, Snf3p and Rgt2p, as sensors of low and high concentrations of extracellular glucose, respectively, and regulates the expression of the *HXT* glucose transporter genes (Johnston, 1999). Snf3p and Rgt2p are members of the 12 transmembrane domain family of sugar transporters with homology to the Hxt family of glucose transporters. They are distinguished from the Hxt transporters by the presence of a long, approximately 300-residue, C-terminal cytoplasmic domain and by the fact that they function solely as sensors and exhibit no transport activity. Downstream components of this pathway include Grr1p, an F-box protein and component of the SCF^{Grr1} ubiquitin-protein ligase, and Std1p and Mth1p, regulators of Rgt1 repressor (Lafuente et al., 2000; Lakshmanan et al., 2003; Schmidt et al., 1999; Tomas-Cobos and Sanz, 2002).

Key components of the second glucose signaling pathway are Snf1 protein kinase, a homologue of the mammalian AMP-activated kinase, and Glc7-Reg1 protein phosphatase type-1 (Carlson, 1999; Johnston, 1999; Sanz et al., 2000). Snf1 kinase is a multi-protein complex required for derepression from glucose growth conditions. It regulates transcription of a large number of genes, particularly those for metabolism of alternate carbon sources. Snf1p, the catalytic subunit of the kinase, is activated by phosphorylation by upstream kinases, Lkb1 kinase and others, in response to low rates of glucose metabolism (Woods et al., 2003). Glc7-Reg1 phosphatase inactivates Snf1 kinase activity by dephosphorylating Snf1p (Tomas-Cobos and Sanz, 2002). Glc7p, the catalytic subunit of protein phosphatase type-1, is directed to Snf1p by Reg1p, a targeting subunit

of Glc7p, and Reg1p binding to Glc7p is enhanced in the presence of glucose, reportedly in response to Reg1p phosphorylation by Hxk2 hexokinase (Tomas-Cobos and Sanz, 2002). In low glucose, Snf1 kinase phosphorylates Reg1p and inhibits interaction with Glc7p. Thus these two protein complexes antagonize each others activity in response to signals generated by the rate of glucose transport and catabolism.

The third glucose signaling pathway utilizes Gpr1p, a member of the 7-transmembrane domain family of G protein-coupled receptors, and a G protein alpha subunit Gpa2p, to regulate *FLO11* expression and pseudohyphal differentiation (Lorenz et al., 2000; Xue et al., 1998). Gpr1p is proposed to be the glucose sensor and the cyclic-AMP activated protein kinase Tpk2 is an essential downstream component of this signaling pathway (Pan and Heitman, 1999).

A major interest in our laboratory is the mechanism of glucose-induced inactivation of maltose permease, the maltose transporter required for the utilization of maltose by *Saccharomyces*. Addition of glucose to maltose fermenting cells causes a rapid decrease in maltose transport activity and the degradation of maltose permease protein that is dependent on endocytosis, vesicle trafficking, and the vacuolar proteases (Medintz et al., 1996). Ubiquitin and the ubiquitin-conjugation enzymes are essential for inactivation and in a *doa4Δ* strain, which is depleted of intracellular ubiquitin, internalization of plasma membrane localized maltose permease is blocked (Medintz et al., 1998). Glucose stimulates the ubiquitination of maltose permease and, as has been demonstrated for several other yeast and mammalian membrane proteins, this serves as a signal for the selective endocytosis and delivery to the vacuole for degradation (Hicke, 1999).

(Jiang et al., 1997) demonstrated that two glucose-signaling pathways regulate glucose-induced inactivation of maltose permease. Pathway 1 utilizes Rgt2p and Grr1p, two components of the extracellular glucose-sensing pathway discussed above. Pathway 2 responds to high rates of glucose transport and metabolism and is defective in a *reg1Δ* mutant (Jiang et al., 2000). (Jiang et al., 2000) found that a *reg1Δ* mutant is insensitive to glucose-induced inactivation of maltose permease and noted that maltose permease phosphorylation is dramatically reduced in the *reg1Δ* mutant indicating that Reg1p-Glc7p phosphatase does not act directly on maltose permease. Their results suggest the existence of a kinase downstream of Glc7p-Reg1p phosphatase that acts directly on maltose permease. Casein kinase 1, encoded by *YCK1* and *YCK2*, may be this kinase. A strain expressing only temperature sensitive Yck kinase, *yck1Δ yck2-ts*, exhibits defects in maltose permease similar to those of a *reg1Δ* mutant (Chapter 2). Maltose permease phosphorylation is dramatically decreased and glucose-induced inactivation is completely blocked.

In this study we report an investigation carried out to establish the relationship between Glc7-Reg1 phosphatase and Yck1,2 kinase in the glucose signaling pathway regulating maltose permease inactivation. We find that, as is seen in the *yck-ts* strain, maltose permease localizes to the cell surface in a *reg1Δ* mutant but in an inactive form. Glucose-induced inactivation of maltose permease is blocked in the *reg1Δ* mutant and permease is not internalized. Moreover, inactivation is dependent on physical interaction between Reg1p and Glc7p. Epistasis analysis using high-copy *REG1* and *YCK1* plasmids places Yck1,2 kinase downstream of Glc7-Reg1 phosphatase in a novel glucose signaling pathway controlling the downregulation of an integral membrane protein.

Results

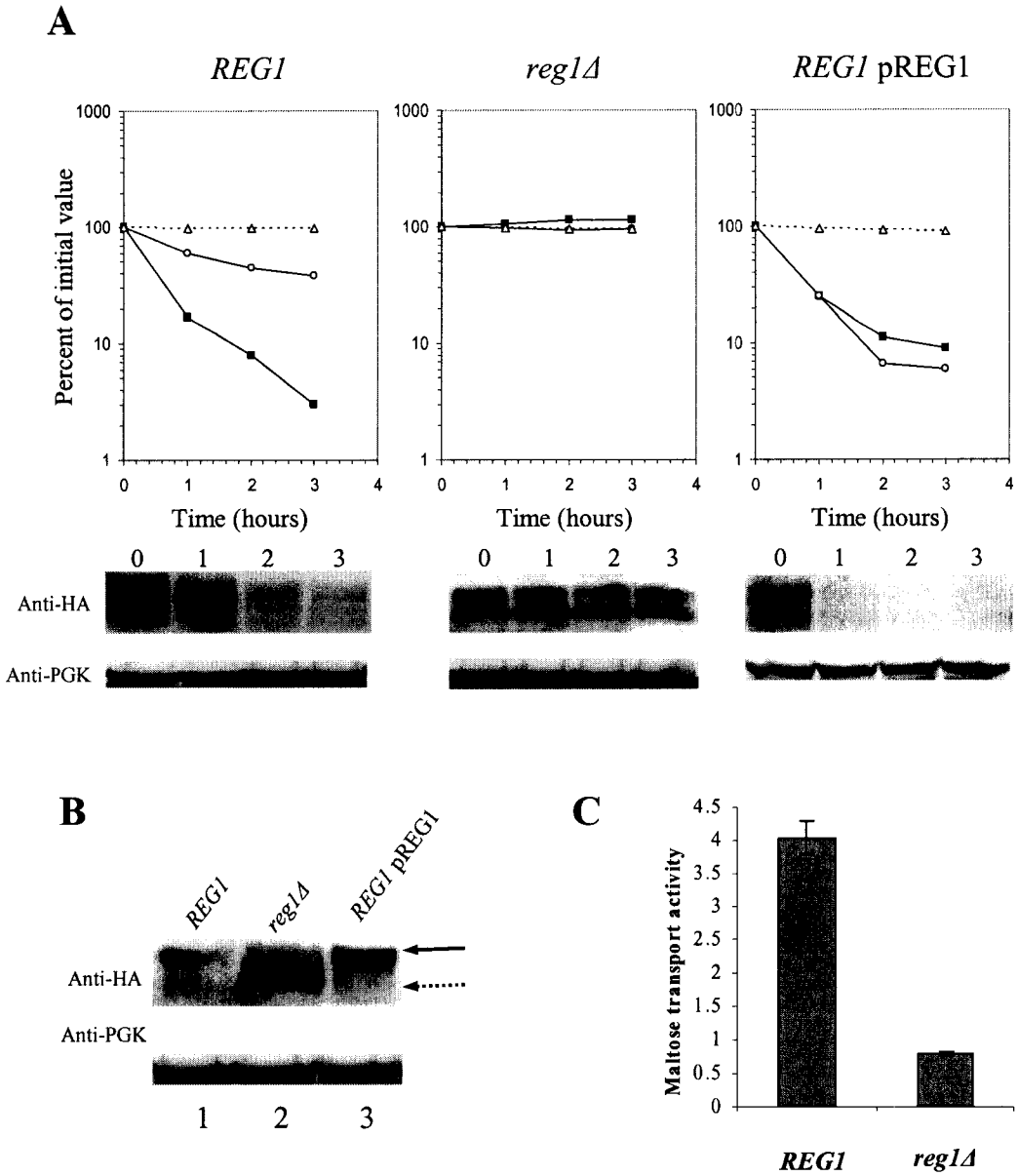
Reg1p was first identified as a negative regulator of glucose repression and *reg1Δ* fully relieves glucose repression of *SUC*, *GAL*, and *MAL* structural gene transcription (Carlson, 1999; Johnston, 1999). (Jiang et al., 2000) demonstrated that *REG1* is also a negative regulator of one of the two pathways found to be responsible for stimulating glucose-induced inactivation of maltose permease and *reg1Δ* mutants are resistant to inactivation. Recently, we found that casein kinase 1 encoded by *YCK1,2* is also required for glucose-induced inactivation (Chapter 2). A *yck-ts* mutant is resistant to inactivation but, in addition, other phenotypes of the *yck-ts* strain with regard to maltose permease are similar to those described for *reg1Δ* by (Jiang et al., 2000). Specifically, maltose permease phosphorylation is significantly reduced in the *yck-ts* strain and, surprisingly, glucose induces a greater than 5-fold increase in maltose transport activity. We decided to explore the relationship of Yck1,2 kinase and Glc7-Reg1 phosphatase in the glucose signaling pathways stimulating maltose permease inactivation.

Loss of REG1 causes defects in phosphorylation and glucose-induced inactivation of maltose permease similar to yck-ts

Strains LRB906 (*REG1*) and CMY7000 (*reg1Δ*) were transformed with plasmids pRS315-MAL61/HA, pUN90-MAL63, and pUN70 or p*REG1*. Transformants were grown in 2% maltose and the protocol described in Materials and Methods was carried out to follow glucose-induced inactivation. The protocol used here differs slightly from that used by (Jiang et al., 2000) in that cyclohexamide is added at time zero

Figure1. Effects of *reg1Δ* and *REG1* overexpression on glucose-induced inactivation of maltose permease

Panel A: Strain LRB906 (*REG1*) was transformed with plasmids pRS315-MAL61/HA, pUN90-MAL63, and either YEp24 or p*REG1*. Strain CMY7000 (*reg1Δ*) was transformed with pRS315-MAL61/HA and pUN90-MAL63. Transformants were grown to mid-log phase on YNB selective media lacking leucine, histidine and uracil with 2% maltose. At time 0, the cells were harvested and transferred to YNSG media with CHX for glucose-induced inactivation. The data represents the average of three independent experiments. The relative levels of Mal61/HA protein (o) and maltose transport activity (■) relative to the zero time sample are plotted along with growth dilution (Δ). Growth dilution is calculated as the OD_{600} at time zero divided by OD_{600} at time x. A representative Western blot is shown. PGK is shown as a loading control. *Panel B* shows a Western blot analysis of total cell extracts prepared from the time zero time point of the cultures in Panel A. The arrows indicate the hyper- (solid) and hypo-phosphorylated (dotted) species of maltose permease.



when the cells are transferred to nitrogen-starvation medium with glucose (YNSG). Additionally, the buffer used to prepare total cell extract contains phosphatase inhibitors. The latter addition, in conjunction with the use of 7.5% PAGE gels, allows us to more confidently evaluate the level of maltose permease phosphorylation. The results are reported in Figure 1.

In the *REG1* strain, glucose causes a very rapid loss of maltose transport activity and a somewhat slower degradation of maltose permease protein (Figure 1A). The difference in rate of loss has been reported previously for other strains and we believe that it reflects differences between the rate of internalization from the cell surface, which leads to the loss in transport activity, and the rate of vesicle delivery to the vacuole for degradation (Jiang et al., 1997; Medintz et al., 1996); Chapter 1). The *reg1Δ* strain is fully insensitive to glucose-induced inactivation. Glucose does not stimulate the proteolysis of maltose permease and maltose transport activity increases slightly and reproducibly during the three hours following transfer to YNSG, but not to the same extent observed in *yck-ts* (Chapter 2; Figure 4). In contrast, overexpression of *REG1* increases the rate of maltose permease proteolysis to the extent that it now matches the rapid rate of loss of maltose transport activity. In chapter 1 we report that loss of maltose transport activity results from the internalization of the permease from the plasma membrane while the rate of proteolysis is determined by the rates of vesicle trafficking, maturation of the prevacuolar compartments, and degradation by vacuolar proteases.

Also noteworthy is the relationship between Mal61/HA permease phosphorylation and *REG1* expression (Panel B). (Medintz et al., 1996) showed that Mal61/HAp is phosphorylated in maltose-grown cells and that the extent of phosphorylation increases in

the presence of glucose. The slower moving band in Panel B is the phosphorylated species and in acid phosphatase-treated extract all of the Mal61/HA protein is found in the faster moving band (Medintz et al., 1996). Compared to the *REG1* strain, considerably less Mal61/HA permease phosphorylation is observed in the *reg1Δ* strain. Moreover, overexpression of *REG1* leads to accumulation of nearly all of the Mal61/HA protein as the hyperphosphorylated species.

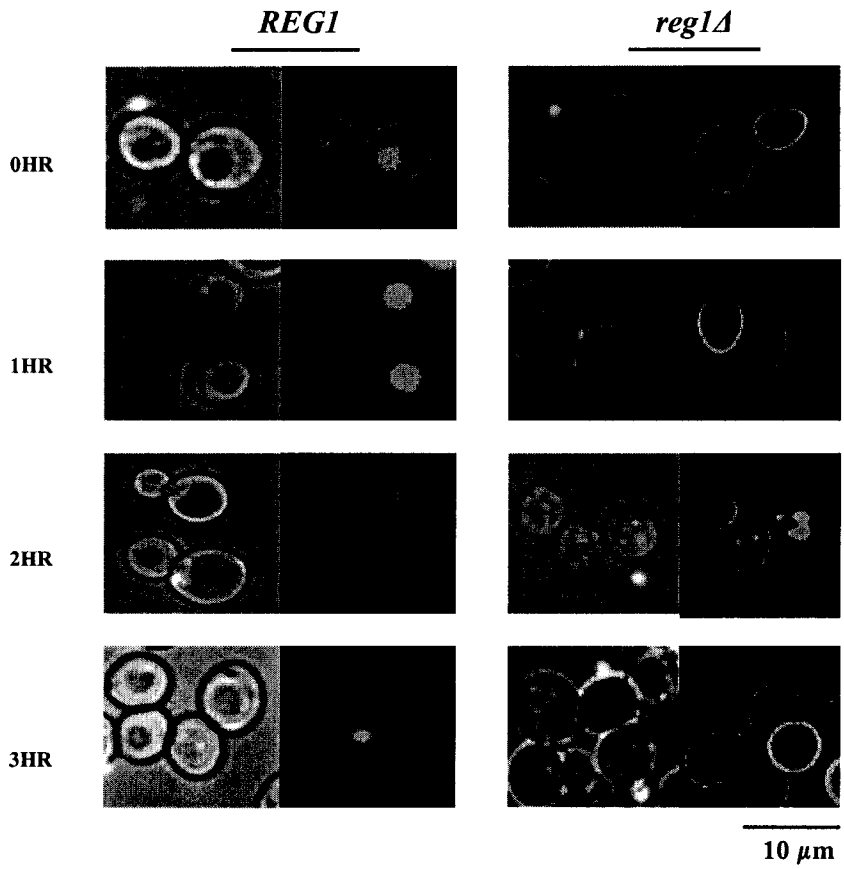
Panel C compares the maltose transport activity observed in the *REG1* and *reg1Δ* strains following growth in 2% maltose. In contrast to the observations of (Jiang et al., 2000) that report only a modestly lower transport rate, we report here that the level of transport activity in the *reg1Δ* strain is about 8-fold lower than that observed in the *REG1* strain. This reduced transport activity is not reflected in the level of maltose permease protein expressed in these cells (Figure 1B). The difference between our results and those of (Jiang et al., 2000) are most likely due to differences in the strain backgrounds. Similar, dramatically reduced rates of maltose transport activity are observed in the *yck-ts* strain (Chapter 2; Figure 4).

Localization of maltose permease in the reg1Δ

To explore the basis for the reduced maltose transport activity observed in the *reg1Δ* strain we used a GFP-tagged allele of Mal61 maltose permease, pUN70-MAL61/HA-GFP, to determine the subcellular localization the protein. Strains LRB906 (*REG1*) and CMY7000 (*reg1Δ*) were transformed with plasmids pUN70-MAL61/HA-GFP and pUN90-MAL63 and localization of Mal61/HA-GFP was followed during the 3 hour

Figure 2. Localization of maltose permease in a *reg1Δ* strain

Strains LRB906 and CMY7000 were transformed with pUN70-MAL61/HA-GFP and pUN90-MAL63. Transformants were grown to mid-log phase on minimal selective media lacking uracil and histidine plus 2% maltose, harvested, and transferred to YNSG media with CHX for glucose-induced inactivation. *Panel A* shows localization of Mal61/HA-GFP as observed using confocal fluorescence microscopy at the indicated times following transfer. The images represent a typical result from three independent transformants. *Panel B* reports maltose transport activity determined at time zero. The error bars indicate the standard deviation from experiments done with three independent transformants.



course of glucose-induced inactivation. Initially in the *REG1* strain Mal61/HA-GFP permease is localized to the cell surface and to the vacuole in these maltose-grown cells. This extensive vacuolar localization has been observed previously in other strains as well as in cells carrying a constitutive *MAL*-activator and grown in non-repressing glycerol/lactate medium (Chapter 1). The origin of this vacuolar Mal61/HA-GFP has not been determined but it is suggested that it might enter the vacuole directly from the trans-Golgi network and not via the plasma membrane (Chapter 1). Upon the addition of glucose to the *REG1* cells, one can observe that Mal61/HA-GFP protein is rapidly removed from the cell surface and is degraded in the vacuole.

In sharp contrast, the bulk of the fluorescent signal is found in the plasma membrane of *reg1Δ* cells with no significant accumulation in the vacuole. It should be noted that the fluorescent signal is comparable in the *REG1* and *reg1Δ* strains, consistent with the Western analysis in Figure 1 Panel B. Therefore, the reduced transport activity observed in *reg1Δ* does not result from reduced permease expression or mislocalization. Addition of glucose to the *reg1Δ* cells does not alter the pattern of localization. The fluorescent signal remains at the cell surface, little or no localization to the vacuole is observed.

Thus, overall, with the exception of the slight difference in the extent of the glucose-induced increase in maltose transport activity, the phenotype of the *yck-ts* and *reg1Δ* mutants with regard to maltose permease synthesis, subcellular localization, transport activity, and glucose inactivation is very similar. For these reasons, we suggest the possibility that Yck1,2 kinase and Glc7-Reg1 phosphatase are components in a

common glucose signaling pathway and this report explores the relationship of these two regulators.

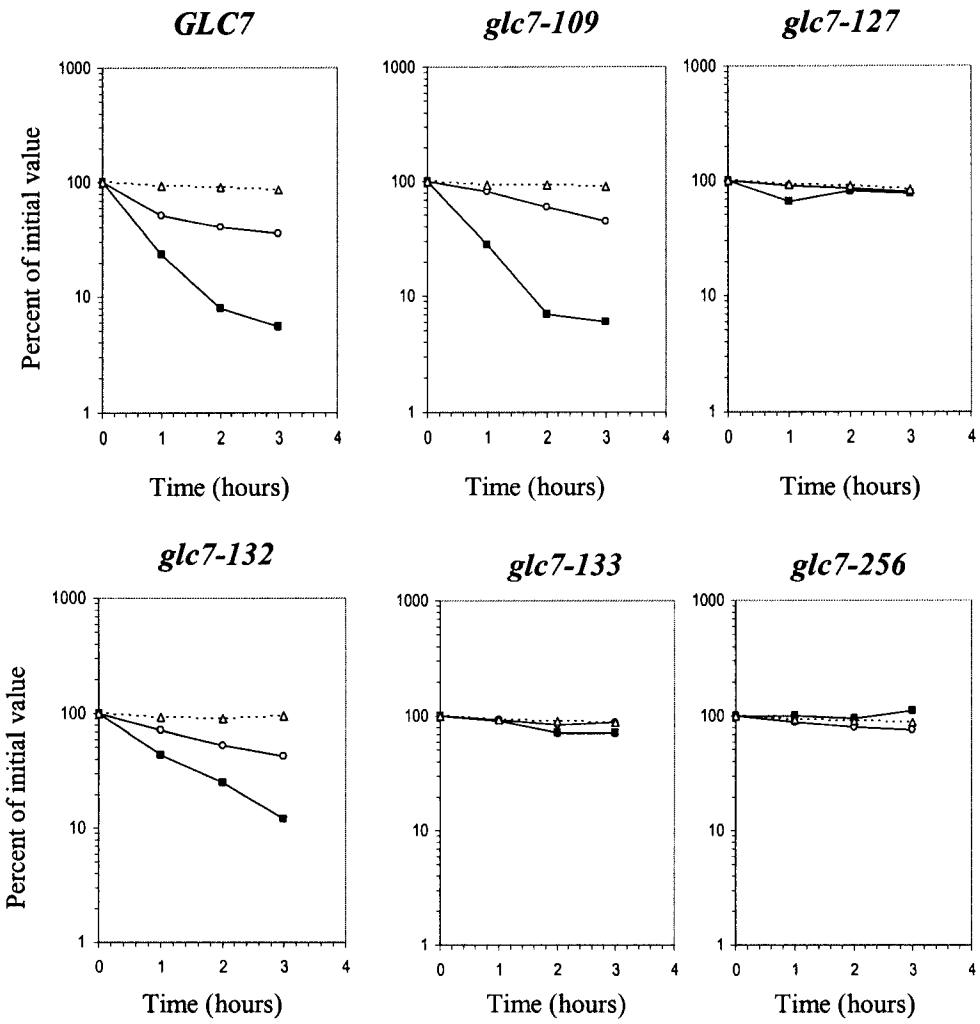
Physical interaction between Glc7p and Reg1p is required to stimulate glucose-induced inactivation of maltose permease.

Glc7p is the catalytic component and Reg1p is the regulatory component of protein phosphatase type-1. Binding is enhanced in the presence of glucose (Sanz et al., 2000; Tu and Carlson, 1995). We used various *glc7* mutant alleles altering specific phenotypes of this multi-functional regulatory phosphatase to determine whether lack of Reg1p binding to Glc7p is associated with resistance to glucose-induced inactivation of maltose permease (Frederick and Tatchell, 1996; Williams-Hart et al., 2002; Wu and Tatchell, 2001). We hypothesize that those *glc7* mutant alleles that are glucose repression insensitive and do not bind Reg1p should also be defective for glucose induced inactivation of maltose permease. Strain KT1112 (*GLC7*) and strains carrying the following *glc7* mutant alleles, KT 1636 (*glc7-133*), KT1638 (*glc7-109*), KT1639 (*glc7-132*), KT1967 (*glc7-127*) and TW267 (*glc7-256*) were transformed with pRS315-MAL61/HA and pUN90-MAL63 and sensitivity to glucose-induced inactivation of maltose permease assayed. Results are shown in Figure 3.

A direct measure of defective glucose repression pathway is the ability to grow on an alternate carbon source like sucrose or maltose in the presence of 2-deoxyglucose (2-DG). 2-DG is a non-metabolizable glucose analogue that inhibits the utilization of sucrose by repressing *SUC2* expression in glucose repression sensitive strains but not in

Figure 3. Reg1p-Glc7p interaction is required for glucose-induced inactivation of maltose permease

Strain KT1112 (*GLC7*) and strains KT1636 (*glc7-133*), KT1639 (*glc7-132*), KT1967 (*glc7-127*), KT1638 (*glc7-109*), and TW267 (*glc7-256*) carrying the indicated *glc7* mutant alleles were transformed with pRS315-MAL61/HA and pUN90-MAL63. Transformants were grown to mid-log phase on selective minimal media lacking leucine and histidine with 2% maltose, harvested and transferred to YNSG media for glucose-induced inactivation. Standard inactivation protocol was followed as described in Methods and Material. The data represent the average of three independent experiments. The relative levels of Mal61/HA protein (o) and maltose transport activity (■) relative to the zero time sample are plotted along with growth dilution (Δ). Growth dilution is calculated as the OD_{600} at time zero divided by OD_{600} at time x. The table lists the various *glc7* mutant alleles, the glucose-repression sensitivity phenotype of the *glc7* allele (Venturi et al., 2000; Wu and Tatchell, 2001), and the ability of the mutant glc7p to interaction with Reg1p, as determined by two-hybrid data analysis (Wu and Tatchell, 2001).



GLC7 – allele	Glucose repression	Reg1p interaction
GLC7	Sensitive	Yes
glc7-109	Sensitive	Yes
glc7-127	Insensitive	Not known
glc7-132	Sensitive	Yes
glc7-133	Insensitive	Not known
glc7-256	Insensitive	No

glucose repression insensitive mutants such as *reg1* strains (Neigeborn and Carlson, 1987). The interaction of Reg1p via its conserved V/IXF motif with Glc7p is required for glucose repression (Alms et al., 1999; Tu and Carlson, 1995). Of the five *glc7* mutants we tested for sensitivity to glucose-induced inactivation, *glc7-109* and *glc7-132* are sensitive to glucose repression and therefore are presumed to interact with Reg1p to mediate this repression (Venturi et al., 2000). The other three mutants tested, carrying *glc7-127, glc7-133* and *glc7-256*, are 2-DG resistant implying insensitivity to the glucose repression and an inability to bind Reg1p (Venturi et al., 2000; Wu and Tatchell, 2001). Two-hybrid analysis was done to correlate the association of Reg1p and Glc7p in a direct binding assay and the results confirm that glc7-256p fails to associate with Reg1p (Wu and Tatchell, 2001). Two-hybrid analysis is underway for *glc7-109*, *glc7-127*, *glc7-132*, and *glc7-133* to determine interaction, or lack thereof, with Reg1p. These results are summarized in the inserted table of Figure 3.

Strains carrying *GLC7* and mutant alleles *glc7-109* and *glc7-132*, which are glucose repression sensitive, are sensitive to glucose-induced inactivation of maltose permease (Figure 3). Strains carrying mutant alleles *glc7-127*, *glc7-133*, and *glc7-256* are insensitive to glucose repression and do not exhibit glucose-induced inactivation. Thus, insensitivity to glucose-induced inactivation of maltose permease correlates with the glucose repression insensitivity of *GLC7* mutations suggesting that Glc7p-Reg1p interaction is required for glucose to stimulate maltose permease endocytosis and proteolysis.

Epistasis analysis puts YCK1 downstream of REG1.

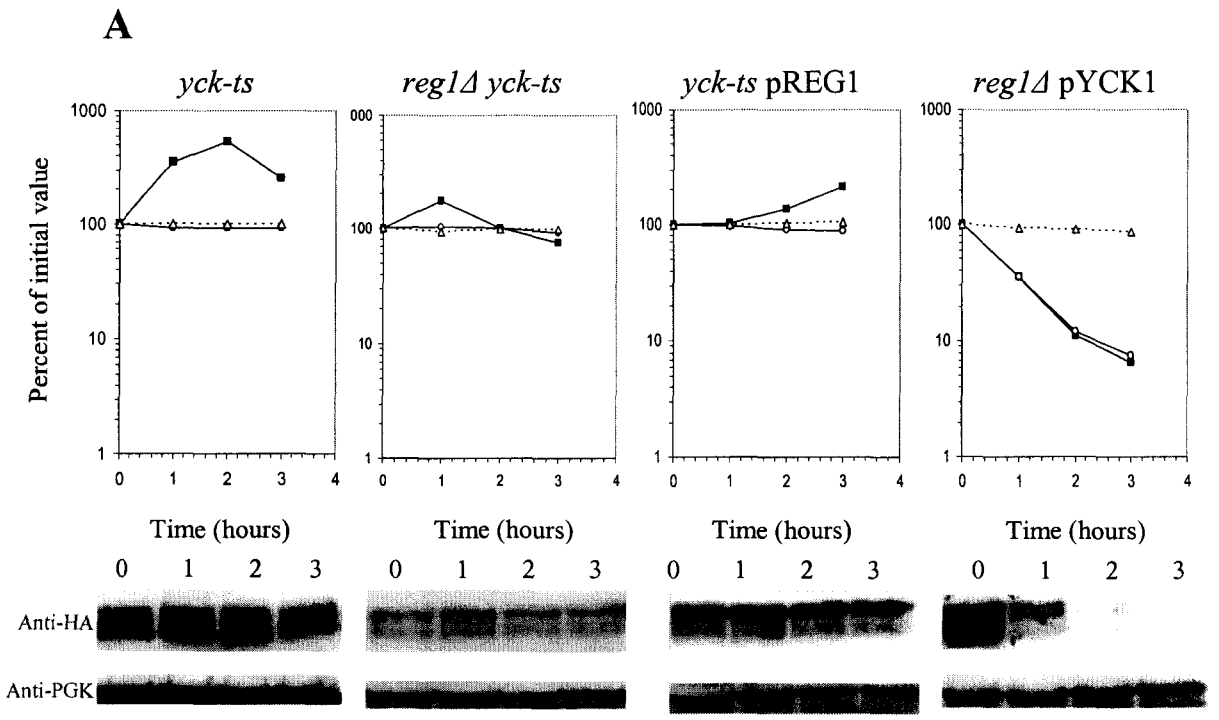
Both in *reg1Δ* (Figure 1A) and *yck-ts* (*yck1Δ yck2-ts)* (Figure 4A, taken from Chapter 2) glucose-induced inactivation is blocked and Mal61/HAp phosphorylation is defective, among other phenotypes described in detail above. It should be noted that the phenotypic defects *yck-ts* with regard to maltose permease activity and inactivation reported in Figure 4 (taken from chapter 2) are exhibited at 30°C, which is permissive for growth (Panek et al., 1997). The temperature sensitive mutant expressed in this *yck-ts* strain is partially defective at 30°C (Panek et al., 1997; Robinson et al., 1993). We also observe modest morphological defects in *yck-ts* cells grown at 30°C (data not shown).

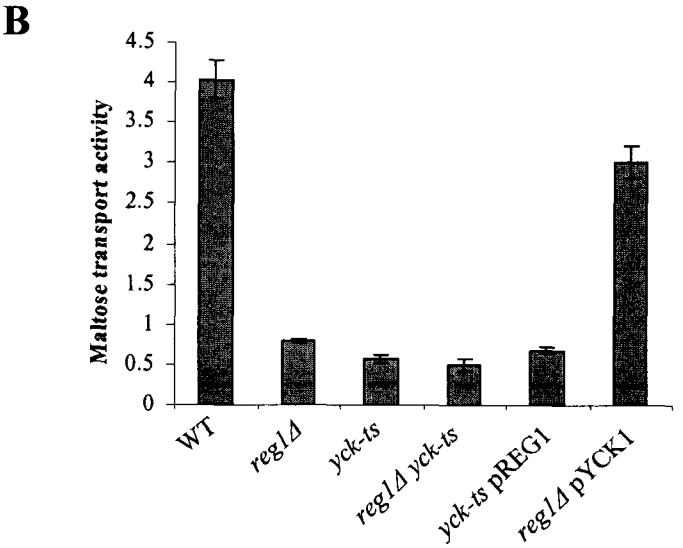
Strain LRB1082 (*reg1Δ yck-ts*) was made by deleting *REG1* in strain LRB951 (Babu et al., 2002). Strain LRB1082 (*reg1Δ yck-ts*) was transformed with plasmid-borne *MAL61/HA* and *MAL63* and glucose-induced inactivation assayed using standard protocol. The phenotype of this double mutant strain is similar to that of the single mutants with regard to maltose transport activity, maltose permease phosphorylation, and insensitivity to glucose-induced inactivation of maltose permease (Figure 4). Also, the 8-fold glucose-induced increase in maltose transport activity of the *yck-ts* strain is attenuated in the *reg1Δ yck-ts* double mutant but a slight increase is still observed. Thus, the *reg1Δ* and *yck-ts* double mutant does not exhibit enhancement supporting our hypothesis that the genes act in a common pathway.

Strains CMY7000 (*reg1Δ*) and LRB756 (*yck-ts*), were transformed with pRS315-MAL61/HA, pUN90-MAL63 and either vector (pUN70) or YEp352-YCK1 (Babu et al., 2002) or pREG1. Standard inactivation protocol was followed as described in Methods and Materials. Overexpression of *YCK1* in the *reg1Δ* strain fully rescues the

Figure 4. Epistasis analysis puts *GLC7-REG1* upstream of *YCK1* in glucose-induced inactivation of maltose permease

Strain CMY7000 (*reg1Δ*), LRB756 (*yck-ts*), were transformed with pRS315-MAL61/HA, pUN90-MAL63, and either YEp352 vector or pYCK1 or pREG1. LRB1082 (*reg1Δ yck-ts*) was transformed with pRS315-MAL61/HA, pUN90-MAL63, and YEp352 vector. Cells were grown on selective minimal media lacking histidine, uracil, and leucine with 2% maltose, harvested, and transferred to YNSG media for glucose-induced inactivation. Standard inactivation protocol was followed as described in Methods and Material. The data represent the average of three independent experiments. The relative levels of Mal61/HA protein (o) and maltose transport activity (■) relative to the zero time sample are plotted along with growth dilution (Δ). Growth dilution is calculated as the OD_{600} at time zero divided by OD_{600} at time x. A representative Western blot is shown. PGK is used as a loading control. *Panel B* reports the maltose transport activity at time 0 for the strains shown in Panel A.





reg1Δ phenotype (Figure 4 A) and restores both rapid glucose-induced loss of maltose transport activity and causes a very rapid rate of maltose permease degradation, even more rapid than in the parental *REG1 YCK1 YCK2* strain (Figure 1). Overexpression of *REG1* in the *yck-ts* strain barely suppresses the *yck-ts* phenotype. A very modest increase in the rate of glucose-induced inactivation of maltose permease is observed and maltose transport rates increase gradually and only about 2-fold. Panel B shows the rate of maltose transport at time 0 of the inactivation protocol for the strains shown in Panel A. While *reg1Δ, yck-ts* and *reg1Δ yck-ts* all show a similar very low rate of transport activity, this rate is not enhanced when *REG1* is overexpressed in *yck*-ts strain. However, overexpression of *YCK1* in the *reg1Δ* strain fully rescues the phenotype and maltose transport is increase significantly almost to wild-type levels. It is interesting to note that maltose permease is heavily phosphorylated in the *yck-ts* pREG1 strain yet very little proteolysis is observed. This might suggest that Glc7-Reg1 phosphatase is activating another kinase but that the sites of phosphorylation do not lead to glucose-stimulated ubiquitination and therefore do not restore sensitivity to glucose-induced inactivation.

Taken together, these results suggest that *REG1* is upstream of *YCK1* in the glucose signaling pathway stimulating inactivation pathway of maltose permease.

The effect of reg1Δ and REG1 overexpression the cell morphology and growth defects of yck-ts

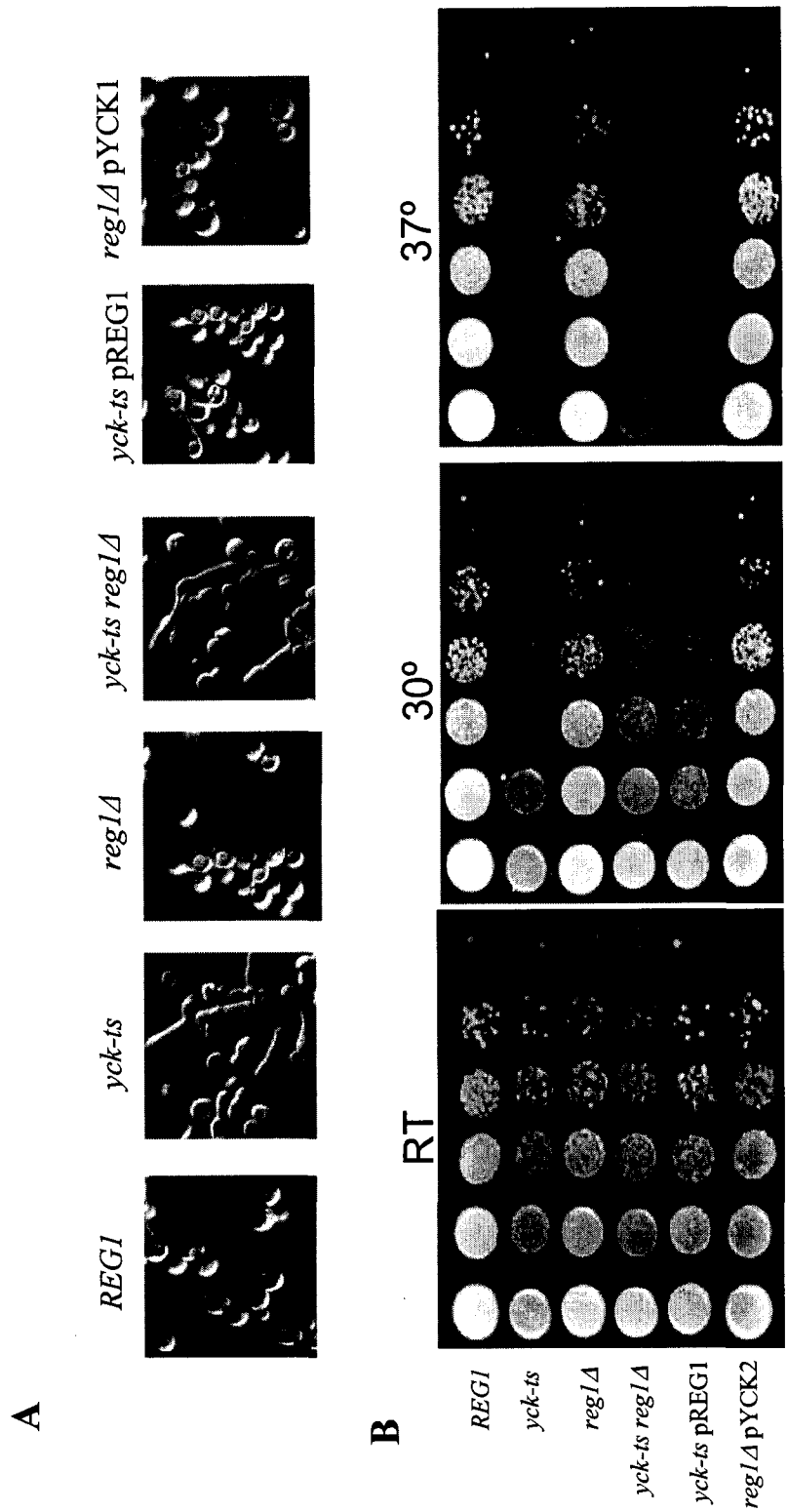
The *yck-ts* mutation is pleiotropic. At the nonpermissive temperature (37°C) growth is arrested with cells exhibiting severe morphological abnormalities including multiple, multinucleated and elongated buds (Panek et al., 1997; Robinson et al., 1993).

The results reported in Figure 4 note a very modest relief of the *yck-ts* phenotype of insensitivity to glucose-induced inactivation. We explored the possibility that *REG1* overexpression might affect the other phenotypes of *yck-ts*. *Panel A* of Figure 5 shows the morphology of cells of the strain series studied in Figure 4. Transformants were grown at 30°C and incubated at 37°C for 30 minutes before microscopic examination. In the *yck-ts* strain a significant percentage of the cells have elongated buds and many have multiple very long buds. The *reg1Δ* strain exhibits no morphological defects but, as was observed by (Jiang et al., 2000), the cells appear to be delayed in G2. The phenotype of the double mutant, *yck-ts reg1Δ*, is similar to *yck-ts* alone again indicating no enhancement. High copy *REG1* suppresses the aberrant morphology phenotype of *yck-ts*. No multiple budded cells are seen, far fewer cells with elongated buds are present, and the length of the buds is significantly reduced. Multicopy *YCK1* does not result in major morphological differences in the *reg1Δ* strain, although the cells appear larger with fewer larger-budded cells. In summary, *REG1* overexpression causes a modest suppression of the abnormal morphology phenotype of *yck-ts*.

Figure 5 *Panel B* compares the growth phenotype of this strain series at room temperature, 30°C (permissive for growth), and 37°C (nonpermissive for growth) (Robinson et al., 1993). Overexpression of *REG1* in the *yck-ts* does not suppress the no-growth phenotype at the nonpermissive temperature but a slight suppression is observed at 30°C. This result is consistent with the fact that *REG1* had not been identified as a multicopy suppressor of the *yck-ts* growth defect in previous studies (unpublished results).

Figure 5. Cell morphology and growth rate in WT vs. mutants

Strains LRB906 (*REG1*), CMY7000 (*reg1Δ*), LRB756 (*yck-ts*), and LRB1082 (*reg1Δ yck-ts*) were transformed with vector and YEp352 and strain LRB756 (*yck-ts*) was transformed with plasmid pREG1 or plasmid pYCK1. *Panel A:* Transformants were grown at room temperature, harvested by centrifugation, and viewed by phase microscropy with a Meridian/Olympus IMT-2 confocal microscope using a 40X lens. *Panel B*: Strains were grown to mid-log phase at room temperature in selective minimal media lacking uracil. The cells were harvested by gentle centrifugation and resuspended in sterile water to an OD600 of 1 for the dilution series, a series of 10-fold dilutions. Plates were incubated at room temperature (3 days) and at 30°C or 37°C (2 days).



Discussion

The results reported here suggest that Yck1,2 kinase and Glc7-Reg1 phosphatase are components of a glucose-signaling pathway stimulating glucose-induced inactivation of maltose permease and that Yck1,2 kinase lies downstream of Glc7-Reg1 phosphatase. First, *yck-ts* and *reg1Δ* mutants exhibit a similar phenotype. Both mutants express normal levels of maltose permease protein but phosphorylation levels are significantly reduced and maltose transport activity is about 8-fold lower in these mutants than in the wild-type strain despite normal localization to the cell surface (Figures 1, 2, 4; chapter 2). Moreover, no localization to the vacuole is observed in either the *yck-ts* or *reg1Δ* mutants, as is seen in the *REG1 YCK1,2* wild-type (Figure 2; chapter 2). Second, the phosphatase activity of Glc7p is required since we show that Glc7p - Reg1p interaction is required. Glc7p mutants that do not bind Reg1p are insensitive to glucose repression and also do not exhibit glucose-induced inactivation of maltose permease (Figure 3). Third, the *yck-ts reg1Δ* double mutant does not exhibit an enhanced phenotype compared to the single mutant strains (Figure 4). Fourth, *YCK1* is downstream of *REG1*. Overexpression of *YCK1* produces very rapid rates of glucose-induced inactivation in *reg1Δ* strain. On the other hand, overexpression of *REG1* has little impact on the insensitivity to glucose-induced inactivation exhibited by the *yck*-ts strain (Figure 4).

Also, we observed the mild suppression of the elongated bud phenotype of *yck-ts* strain. The underlying mechanism of this phenotype and the factors involved are not known but it is suggested to result from defects in organization of the actin cytoskeleton and in the localization to the bud neck of factors required for cytokinesis (Robinson et al., 1993). The no-growth phenotype of *yck-ts* at nonpermissive temperature is not

suppressed by overexpression of *REG1* suggesting that this phenotype results from different underlying mechanisms than the morphology defects and may not rely on signaling via the novel Glc7-Reg1 phosphatase – Yck1,2 kinase pathway identified here.

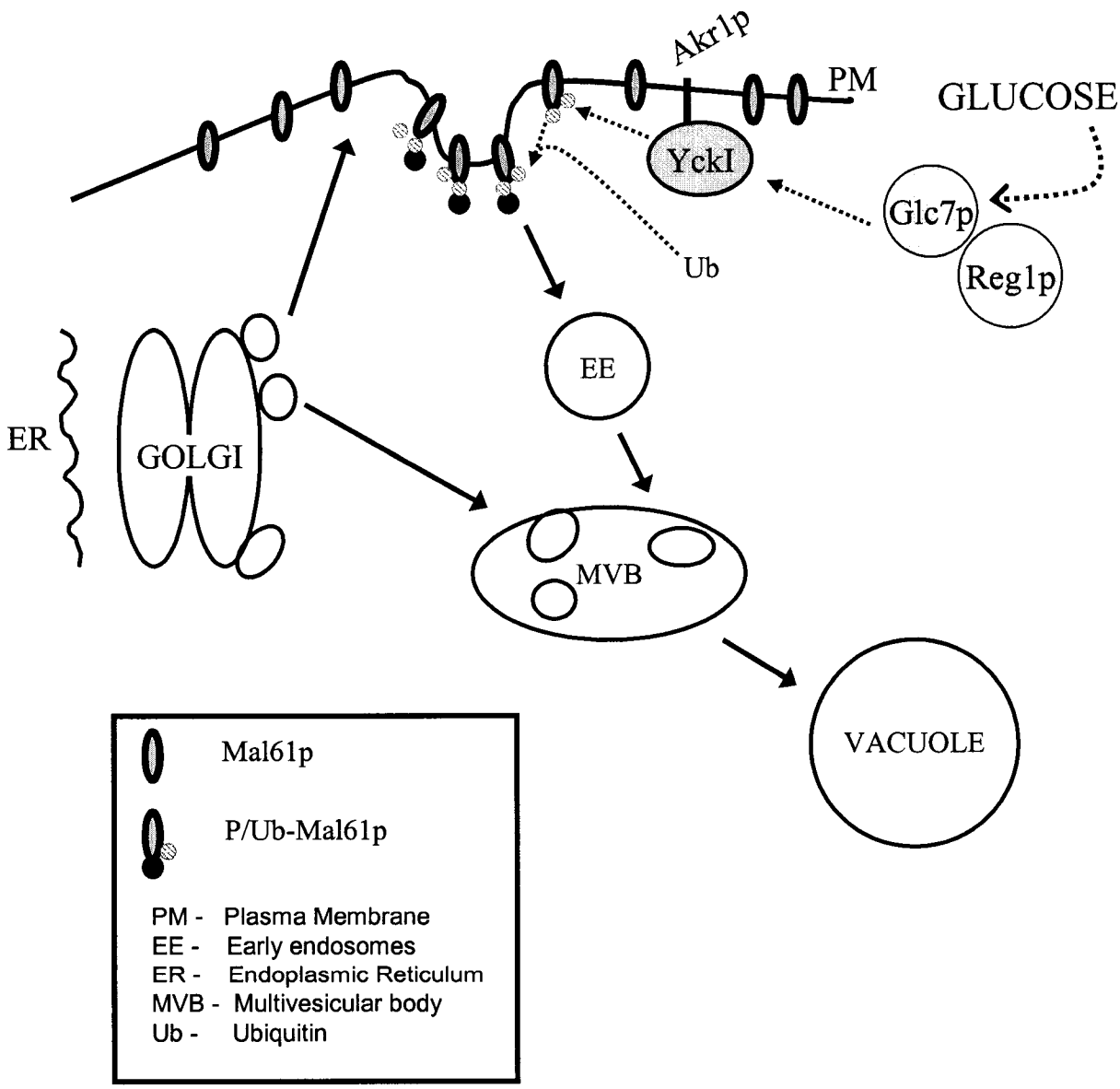
Our results as well as reports from other laboratories allow us to present a model for glucose signaling of glucose-induced inactivation of maltose permease via Pathway 2, the glucose-transport dependent pathway described by (Jiang et al., 1997). This is diagramed in Figure 6. Glc7-Reg1 phosphatase is activated by growth on high concentrations of glucose (Sanz et al., 2000; Tu and Carlson, 1995). The nature of this glucose signal is unknown. (Sanz et al., 2000) demonstrate that Snf1 kinase negatively regulates its own interaction with Reg1p. In low glucose conditions, Reg1p binds to Snf1 kinase and the N-terminal region of Reg1p is rapidly phosphorylated in a Snf1-dependent manner. Reg1p phosphorylation stimulates interaction with Glc7p and activates Glc7p catalytic activity, and leads to the dephosphorylation and inactivation of Snf1 kinase and release of Glc7-Reg1 complex from the Snf1p complex. Glc7p-dependent dephosphorylation of Reg1p occurs soon after the addition of glucose to the medium. The phosphorylated state of Reg1 is also affected by Hxk2 kinase. Once activated, we propose that Glc7-Reg1 phosphatase dephosphorylates and activates Yck1,2 casein kinase 1. Casein kinase 1 is believed to be regulated by the phosphorylation state of a sequence in the C-terminal domain. (Graves and Roach, 1995) propose that auto-phosphorylation of its carboxy-terminal domain inactivates casein kinase 1 and demonstrate that its kinase activity is activated *in vitro* by phosphatase treatment. We

propose that *in vivo* Yck1,2 kinase is activated by dephosphorylation by Glc7-Reg1 phosphatase which is itself activated by rapid glucose utilization.

The *Saccharomyces* 14-3-3 member proteins Bmh1 and Bmh2 physical interact with Reg1p (Mayordomo et al., 2003). Moreover, (Mayordomo et al., 2003) report that these two 14-3-3 proteins participate as positive effectors of glucose-induced inactivation of maltose permease and a *bmh1Δ bmh2Δ* double mutant exhibit reduced rates of permease proteolysis in response to glucose. Members of the 14-3-3 family of proteins are well-conserved in eukaryotes and function as regulatory molecules controlling several vital regulatory processes via their ability to bind kinases, phosphatases, and other signaling proteins (reviewed in Fu et al., 2000). Primarily, 14-3-3 proteins bind phosphorylated ligands. We propose that interaction of Bmh1,2p interact with phosphorylated Reg1p, and/or possibly phosphorylated Yck1,2 kinase, and facilitate the interaction between Reg1p and Yck1,2 kinase.

(Moriya and Johnston, 2004) present a model for glucose sensing and signaling in *Saccharomyces* via the Rgt2-dependent high-glucose sensing pathway regulating *HXT1* expression. (Jiang et al., 1997) reported that this same pathway also contributes to glucose-induced inactivation of maltose permease and that both Rgt2p and Grr1p are involved. (Moriya and Johnston, 2004) use the split-ubiquitin system to demonstrate that Rgt2p and Yck1p interact. They show that activated Yck1,2 kinase phosphorylates Mth1p and Std1p, when these are tethered to the Rgt2p C-terminal cytoplasmic tail, they mark these proteins for recognition and ubiquitination by SCF^{GRR1}. We suggest that the upstream events activating Yck1,2 kinase are addressed by our findings. Our model (Figure 6) proposes that Reg1-Glc7 phosphatase, activated via signals generated by the

Figure 6. Proposed glucose signaling pathway stimulating glucose-induced inactivation of maltose permease



same pathway that regulates Snf1 kinase in response to rapid glucose utilization, dephosphorylates and activates Yck1,2 kinase. It is this active kinase that contributes to the phosphorylation of maltose permease thereby making it an attractive target for ubiquitination (possibly mediated by SCF^{Grr1}), selective endocytosis, and vacuolar proteolysis.

CONCLUSION

Based on the results reported in this thesis we conclude the following regarding the role of Yck1,2 kinase and phosphorylation in the glucose-induced inactivation of maltose permease. The N-terminal cytoplasmic domain of Mal61 maltose permease contains sorting signals involved in trafficking of this integral membrane protein to the plasma membrane but phosphorylation of residues in a putative PEST sequence of this domain does not appear to be essential for glucose-induced inactivation of maltose permease. Our results in Chapter 1 show that mutation of residues Thr29 and Ser 32, 43, 48, and 56 significantly slows the rate of glucose-induced proteolysis of Mal61p but is not required for localization to the plasma membrane or for the rapid loss in the maltose transport activity, which is a measure of the rate of endocytosis of the permease from the cell surface. Putative dileucine and dilysine motifs located at residues 65-70 and 41,42, respectively, cause significant defects in maltose transport activity and mislocalization of permease to prevacuolar compartments instead of the plasma membrane and vacuole. Overall, the N-terminus seems to be involved in the trafficking of newly synthesized maltose permease. As yet, we have not identified the specific Ser/Thr residues whose phosphorylation marks maltose permease for inactivation. Several other Ser/Thr residues located outside the PEST region were not targeted in this study but could be important for glucose-induced inactivation.

In Chapter 2 we conclude that casein kinase encoded by *YCK1,2* is either directly or indirectly involved in the phosphorylation of maltose permease. This phosphorylation is required for activation of maltose transport, vacuolar localization, and glucose-induced

inactivation of maltose permease. In the *yck-ts* strain, which expresses defective Yck1,2 kinase, we do not observe glucose-induced inactivation of Mal61p. Moreover, maltose permease localizes to the plasma membrane but is either functionally inactive or not properly exposed to the extracelluar space. Also, compared to the wild type strain, maltose permease phosphorylation is significantly reduced in the *yck-ts* strain. We also demonstrate that *DOA4* mediated ubiquitination of maltose permease occurs downstream of *YCK1,2*- mediated phosphorylation. Ubiquitination of maltose permease is the crucial step for recognition by the endocytosis machinery. In a *doa4Δ* strain, maltose permease localizes normally to the plasma membrane and transports actively even after the addition of glucose. Overexpression of *YCK2* could not rescue the *doa4Δ* phenotype. While results reported here suggest that Mal61/HA maltose permease is a target of Yck1,2 kinase we have yet to identify the specific target residue(s).

The results described in Chapter 3 allow us to identify a novel Glc7-Reg1, phosphatase type-1 – Yck1,2 kinase signaling pathway utilized in glucose-induced inactivation of maltose permease. Our data suggests that it is the glucose-enhanced binding of Glc7p to Reg1p (a targeting subunit of Glc7p) that directs the phosphatase to Yck1,2 kinase which, when activated by dephosphorylation, phosphorylates maltose permease marking it for ubiquitination, ubiquitin-mediated endocytosis, and vacuolar degradation. A model consistent with the results of my research is presented at the end of Chapter 3.

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