DOI: 10.1534/genetics.105.051698

Glc7–Reg1 Phosphatase Signals to Yck1,2 Casein Kinase 1 to Regulate Transport Activity and Glucose-Induced Inactivation of Saccharomyces Maltose Permease

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Manuscript received September 28, 2005
Accepted for publication November 24, 2005

ABSTRACT

The Saccharomyces casein kinase 1 isoforms encoded by the essential gene pair *YCK1* and *YCK2* control cell growth and morphogenesis and are linked to the endocytosis of several membrane proteins. Here we define roles for the Yck1,2 kinases in Mal61p maltose permease activation and trafficking, using a *yck1*Δ *yck2-2*^{ts} (*yck*^{ts}) strain with conditional Yck activity. Moreover, we provide evidence that Glc7–Reg1 phosphatase acts as an upstream activator of Yck1,2 kinases in a novel signaling pathway that modulates kinase activity in response to carbon source availability. The *yck*^{ts} strain exhibits significantly reduced maltose transport activity despite apparently normal levels and cell surface localization of maltose permease protein. Glucose-induced internalization and rapid loss of maltose transport activity of Mal61/HAp-GFP are not observed in the *yck*^{ts} strain and maltose permease proteolysis is blocked. We show that a *reg1*Δ mutant exhibits a phenotype remarkably similar to that conferred by *yck*^{ts}. The *reg1*Δ phenotype is not enhanced in the *yck*^{ts} *reg1*Δ double mutant and is suppressed by increased Yck1,2p dosage. Further, although Yck2p localization and abundance do not change in the *reg1*Δ mutant, Yck1,2 kinase activity, as assayed by glucose-induced *HXT1* expression and Mth1 repressor stability, is substantially reduced in the *reg1*Δ strain.

IN *Saccharomyces cerevisiae*, the addition of glucose 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 requires endocytosis, vesicle trafficking, and vacuolar proteases. Inactivation is dependent on gene functions involved in these processes, including *END3*, which encodes an early function in endocytosis; *VPS2*, an ESCRT complex component needed for delivery of membrane proteins to the endosome and vacuole; and *PEP4*, encoding the major vacuolar protease (Medintz *et al.* 1996; Babst *et al.* 2002). Also, Medintz *et al.* (1998) demonstrated glucose-stimulated ubiquitination of maltose permease and found that degradation of maltose permease requires the ubiquitin conjugation enzymes, particularly Rsp5 ubiquitin ligase, and is blocked in *doa4*Δ mutants, which 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 nontransporting homolog of the Hxt glucose transporter family of sugar transporters (Ozcan *et al.* 1996). Pathway 2 measures glucose uptake by monitoring the rate of glucose metabolism through the early steps of glycolysis (Hu *et al.* 2000; Jiang *et al.* 2000a). Jiang *et al.* (2000b) found that deletion of *REG1*, encoding a targeting subunit of Glc7 protein phosphatase type 1, blocks signaling via pathway 2. Additional results from this study suggested that Glc7–Reg1 phosphatase does not act directly on maltose permease, since loss of Reg1p decreases rather than increases phosphorylation of the permease. Thus, Jiang *et al.* (2000b) proposed that an as-yet-unidentified kinase acts downstream of Glc7–Reg1 phosphatase in pathway 2 and could be directly or indirectly responsible for maltose permease phosphorylation and possibly for glucose-induced inactivation.

Plasma-membrane-localized casein kinase 1 encoded by *YCK1* and *YCK2* is a likely candidate for the downstream kinase activity of pathway 2. Saccharomyces encodes four casein kinase 1 isoforms. *YCK1* and *YCK2* encode plasma-membrane-localized isoforms with >90% similarity between their catalytic domains (Robinson *et al.* 1992; Wang *et al.* 1992; Vancura *et al.* 1993). *YCK1* and *YCK2* have an essential redundant function in cell growth and morphology, but both *YCK1* and *YCK2* act as multicopy suppressors of the sucrose-nonfermenting phenotype caused by loss of *SNF4* function (Robinson *et al.* 1992). *SNF4* encodes a positive effector of the Snf1 protein kinase, which is required for derepression under glucose growth conditions.

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The plasma membrane localization of the Yck1,2 kinases, and their identification as suppressors of the glucose derepression defect of the *snf4* mutant, support the possibility that Yck1,2 could provide the downstream kinase activity of pathway 2. Additional studies are also consistent with this proposal. Yck1,2 kinase activity stimulates the internalization of several Saccharomyces plasma membrane proteins, including the Ste2 α-factor receptor (Hicke 1999), the Ste3 **a**-factor receptor (Panek *et al.* 1997; Feng and Davis 2000), and Fur4 uracil permease (Marchal *et al.* 1998). Moreover, Feng and Davis (2000) report that the Yck1,2 kinases are required for Ste3p phosphorylation. In studies of *HXT* gene regulation by the Rgt2 glucose sensor, evidence was presented indicating that the Yck1,2 kinases promote the phosphorylation of Rgt2p-bound Mth1p and Std1p, leading to their degradation and to the inactivation of the Rgt1 repressor (Moriya and Johnston 2004). Therefore, we explored the role of Yck1,2 casein kinase 1 activity in the glucose-induced inactivation of maltose permease and investigated the possibility that the Yck1,2 kinases act with the Glc7–Reg1 phosphatase in this glucose-signaling pathway.

MATERIALS AND METHODS

Strains and plasmids: Strains LRB756, LRB906, and LRB1082 used here are closely related, differing at the *YCK* loci. Strains LRB906 (*MAT***a** *YCK1 YCK2 his3 leu2 ura3*) and LRB756 (*MAT***a** *his3 leu2 ura3-52 yck1-1Δ::ura3 yck2-2^{ts}*) have been described (Panek *et al.* 1997; Babu *et al.* 2002). Both strains carry defective copies of *MAL1* (*MAL11 MAL12 mal13*) and *MAL3* (*MAL31 MAL32 mal33*) loci, encoding functional copies of maltose permease and maltase and a nonfunctional copy of the *MAL*-activator gene. Thus, strains LRB906 and LRB756 do not ferment maltose and require a plasmid-borne copy of the *MAL*-activator gene for expression of the *MAL* structural genes. For this we used pUN90-MAL63 and YCp50-MAL63 carrying inducible *MAL63* in the CEN vectors pUN90 (Elledge and Davis 1988; Gibson *et al.* 1997) and YCp50 (Gibson *et al.* 1997), respectively.

PCR-based one-step gene replacement was used to construct CMY7000 (*MAT***a** *YCK1 YCK2 his3 leu2 ura3 reg1Δ::kan^R*) and LRB1082 (*MAT***a** *his3 leu2 ura3-52 yck1-1Δ yck2-2^{ts} reg1Δ::kan^R*) from strains LRB906 and LRB756, respectively (Longtine *et al.* 1998).

Strain KT1112 (*MAT***a** *leu2 ura3-52 his3 GLC7*) and the otherwise isogenic *glc7* mutant series KT1636 (*glc7-133*), KT1639 (*glc7-132*), KT1967 (*glc7-127*), KT1638 (*glc7-109*), and TW267 (*glc7-256*) are described in Baker *et al.* (1997) and Wu and Tatchell (2001) and were obtained from Kelly Tatchell, LSU Health Sciences Center, Shreveport, Louisiana.

Strain CMY1025 (*MAL1 doa4Δ::LEU2*) is a meiotic segregant from the cross of CMY1001 (*MAL1 DOA4*) and PMY270 (*mal1 doa4Δ::LEU2*) as described in Medintz *et al.* (1998).

Plasmids pYCK1 (pLJ721) and pYCK2 (pLS2.3) carry *YCK1* and *YCK2* on the high-copy vector YEp352 (Robinson *et al.* 1992). Plasmid DF041, a genomic *REG1* clone (Nasmyth and Tatchell 1980) in the 2μ high-copy vector YEp13, was obtained from Kelly Tatchell.

Plasmid pRS315-MAL61/HA carries an HA-tagged *MAL61* maltose permease allele under the control of its native promoter (Medintz *et al.* 1996). 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 (Niedenthal *et al.* 1996) by PCR into an *Xho*I site created at the 3′-end of the *MAL61/HA* ORF, to produce an in-frame *MAL61/HA-GFP* fusion. The construct was confirmed by the presence of a diagnostic *Nco*I site and by sequence analysis. A 4.4-kb *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 maltose with the same efficiency as does Mal61/HA permease (N. Gadura and C. A. Michels, unpublished results).

Plasmid pBM3212 carries the *HXT1* promoter-*lacZ* reporter gene in the multicopy *LEU2* vector YEp367R (Ozcan *et al.* 1996). pBM4560 (Moriya and Johnston 2004) carries an allele of *MTH1* in which a sequence encoding nine copies of the Myc-epitope (EQKLISEED) was inserted at the 3′-end of the ORF to encode a C-terminal 9xMyc-tagged Mth1 repressor. Both plasmids were obtained from Mark Johnston, Washington University Medical School.

Inactivation protocol: The standard maltose permease inactivation assay protocol (Medintz *et al.* 1996) was used for these studies with a few variations. Briefly, cells were grown at 30° to early log phase (OD_{600} 0.1–0.3) in selective media containing 2% maltose, harvested by filtration, and resuspended in nitrogen starvation media plus 2% glucose, referred to as YNSG. Cycloheximide (CHX) (30 μg/ml) was added to the cell suspension at time zero to inhibit protein synthesis. Three aliquots were taken at time zero and every hour to 3 hr. Cells of aliquot 1 were harvested by filtration and frozen immediately at −80° to be used for Western analysis. Cells of aliquot 2 were used to assay maltose transport activity. Cells of aliquot 3 were used to determine "growth dilution," which 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 is measured as the uptake of 1 mM ^{14}C-labeled maltose as described by Cheng and Michels (1991) and Medintz *et al.* (1996). Assays were done in duplicate on at least three independent transformants. The standard error was <15%.

Enzyme assays: Maltase activity was assayed as described in Dubin *et al.* (1985). Activity is expressed as nanomoles of *p*-nitrophenyl β-D-glucopyranoside (PNPG) hydrolyzed per milligram of total protein per minute. Assays were performed using total cell extracts prepared from at least duplicate cultures. The values reported are the average of duplicate assays and varied by ~15%.

β-Galactosidase was assayed in at least three independent transformants using total cell extracts (Hu *et al.* 1999). Activity is expressed as nanomoles of *o*-nitrophenyl β-D-galactopyranoside hydrolyzed per milligram of total protein per minute. The values reported are the average of duplicate assays of at least duplicate cultures and varied by ~20%.

Western blotting: At each time point 15 optical density (OD) units of cells (grown to an OD_{600} 0.3–0.5) were harvested by filtration on nitrocellulose filters (0.45 μm), washed with KPO_4 plus 2% NaAzide pH 7.4, and frozen immediately at −80° until used for preparation of protein extracts. Total cell extracts were prepared by thawing cells in HEPES buffer pH 7.5 supplemented with a protease inhibitor cocktail that contains AEBSF, pepstatinA, and E-64 and 1,10-phenanthroline (Sigma-Aldrich P8215) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich P5726 and P2850). The cells were harvested by centrifugation and resuspended in extraction buffer (40 mM TrisCl, pH 6.8, 8 M urea, 0.1 mM EDTA, 1% β-mercaptoethanol, and 5% SDS) plus the protease inhibitor and phosphatase inhibitor cocktails according to the

manufacturer's recommendations. The cell suspension was vortexed with glass beads (425–600 μm) for 15 min at 4° and solubilized for 15 min at 37° followed by 2 min of vortexing. Cell debris was removed by centrifugation for 5 min and the total protein extract was boiled for 3 min. Protein levels were assayed with a protein assay kit from Sigma-Aldrich (P5656). Equal amounts of protein were loaded per lane of 10% SDS–PAGE gels for monitoring protein levels or 7.5% SDS–PAGE gels for separation of differentially phosphorylated species. Mal61/HA protein was detected using anti-HA antibody (Boehringer Mannheim, Indianapolis), Mal61-GFP protein was detected using anti-GFP antibody (Santa Cruz), and Mth1-9xMyc protein was detected using anti-Myc antibody (Roche Diagnostic). Each membrane was also probed with anti-phosphoglycerate kinase (anti-PGK) antibody (Molecular Probes, Eugene, OR) as a loading control. Protein levels were visualized using the Vistra-ECF kit (Amersham, Buckinghamshire, UK) and a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Signal was quantified using software provided by the manufacturer. Loading variations were corrected for by normalizing to the PGK signal. Values presented are the average of results from experiments carried out on at least three independent transformants and the standard deviation varied from 5 to 10%. The immunoblots shown in Figures 1, 3, 4, 6, and 7 are representative of typical results.

The percentage of phosphorylated Mal61 protein was determined from the results of the Western blots quantified using the Storm 860 PhosphorImager. The percentage was calculated as the ratio of the amount of signal in the slowly migrating (highly phosphorylated) species divided by the total Mal61/HAp signal including the full range of migrating species. The results reported are from cell extracts prepared from at least duplicate cultures of three independent transformants.

Fluorescence microscopy: A Meridian/Olympus IMT-2 confocal microscope equipped with a ×100 oil, NA 1.40 lens, FITC filter set, and phase optics was used to visualize the GFP fluorescent signal. The imaging parameters were constant for all images, with the exception of *yck*^{ts} cells, for which the PMT value was increased by twofold. Images were processed using Adobe Photoshop. All images shown are representative of experiments done on three independent transformants.

RESULTS

Yck1,2 casein kinase 1 activity is required for maltose permease transport activity and glucose-induced inactivation: We first tested whether Yck1,2 kinase activity is required for any aspect of maltose permease expression or glucose-induced inactivation. HA-tagged Mal61/HA maltose permease was expressed in LRB906 (*YCK1 YCK2*) and LRB756 (*yck1*Δ *yck2*^{ts}), referred to here as *yck*^{ts}, by growth in maltose-induced conditions and subjected to the glucose inactivation protocol described in MATERIALS AND METHODS. The inactivation protocol used here differs slightly from that used previously by our laboratory (MEDINTZ *et al.* 1996, 1998, 2000; JIANG *et al.* 1997, 2000b) in that CHX is added when the cells are transferred to YNSG. In addition, the buffer used to prepare total cell extracts contains phosphatase inhibitors, which, in conjunction with the use of 7.5% PAGE gels, allowed us to evaluate maltose permease phosphorylation.

Figure 1A shows glucose-induced changes in both maltose transport activity and maltose permease protein levels in cells incubated at 24°, the permissive temperature for the *yck*^{ts} strain. In the parental strain, as was shown for other strain backgrounds (MEDINTZ *et al.* 1996), glucose induces a very rapid loss of maltose transport activity and a somewhat slower rate of Mal61/HA proteolysis. This difference is observed for most but not all strains and we believe that it results from a more rapid rate of permease internalization than vesicle delivery to the vacuole for degradation. While approximately the same level of maltose permease protein is detected in both the *YCK1 YCK2* and *yck*^{ts} strains at the start of the experiment (Figure 1, B and C), the level of maltose transport activity expressed by the *yck*^{ts} strain is only ~15% of that expressed in the wild-type strain (Figure 2). Glucose-induced proteolysis of maltose permease is essentially blocked in the *yck*^{ts} strain, such that Mal61/HA protein levels remain constant over the 3 hr following glucose addition (Figure 1A). However, the rapid glucose-induced loss of maltose transport activity is not observed in the *yck*^{ts} strain. Instead, glucose induces an increase of approximately sevenfold in maltose transport activity. Finally, consistent with previous reports that the *yck*^{ts} mutant shows defects even at the permissive temperature (PANEK *et al.* 1997), there was no obvious difference in the results shown here carried out at 24° and those obtained when the cells were shifted to 35° prior to glucose inactivation (data not shown).

The low level of maltose activity in the *yck*^{ts} strain could result from defective localization to the cell surface. We examined the localization of a functional GFP-tagged Mal61 protein in maltose-grown wild-type and *yck*^{ts} strains by confocal microscropy. Mal61/HA-GFP protein is expressed in wild-type and *yck*^{ts} cells at comparable levels (data not shown) and is present mainly in the plasma membrane in both strains (Figure 1B). In wild-type cells, a significant amount of fluorescence is observed in the vacuole, but only a faint vacuolar signal is present in the *yck*^{ts} cells. Thus, Yck1,2 kinase activity does not affect synthesis or plasma membrane localization of maltose permease protein but is required for its transport activity.

The dramatic glucose-induced increase in transport activity in the *yck*^{ts} strain, which occurs in the absence of new maltose permease synthesis, could result from changes in permease localization and/or specific activity. To distinguish between these possibilities, we followed localization of Mal61/HA-GFP after the addition of glucose. In the wild-type strain, the level of maltose permease at the cell surface is dramatically reduced by 1 hr after glucose addition, while fluorescence in the vacuole appears to be enhanced (Figure 1B). Over the course of 3 hr the vacuolar signal gradually declines, presumably due to degradation of the GFP-tagged protein. The results are quite different for the *yck*^{ts} strain. Both cell surface and vacuolar fluorescence remain constant during the 3-hr duration of the experiment. These results are consistent with the complete lack of glucose-induced proteolysis observed for Mal61-HA permease in

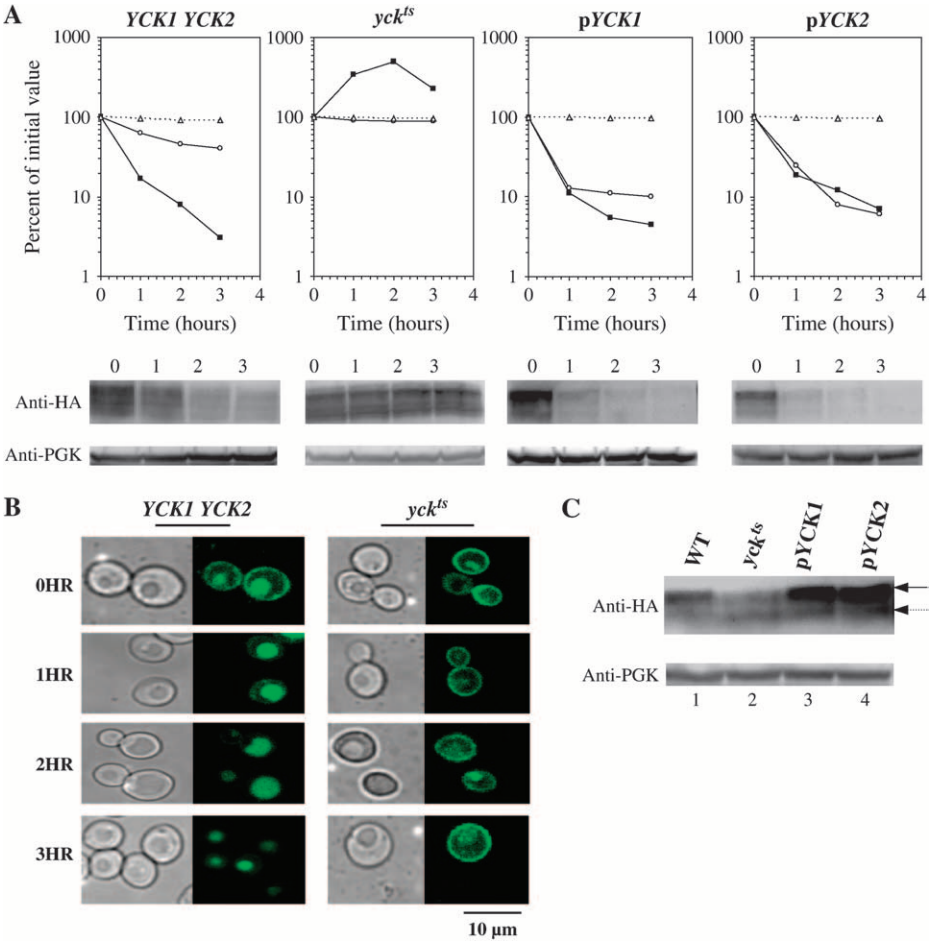

Figure 1.—Glucose-induced inactivation of maltose permease in *yck1*Δ *yck2*^{ts} and *YCK1,2* overexpressing strains. (A) Strains LRB906 (*YCK1 YCK2*) and LRB756 (*yck1*Δ *yck2*^{ts}), referred to as *yck*^{ts}, were transformed with plasmids pMAL61/HA, pMAL63, and YEp352, and strain LRB906 was transformed with plasmid YEp352-YCK1 or YEp352-YCK2. Transformants were grown at 24° in selective media plus 2% maltose, harvested by filtration, transferred to YNSG with CHX (30 μg/ml), and standard inactivation protocol followed at 24°. At the indicated times the growth dilution (△), maltose transport (■), and relative Mal61/HA protein levels (○) were determined as described in materials and methods. A representative Western blot probed with anti-HA antibody is shown. Western blotting of PGK is shown as the loading control. (B) Strains LRB906 (*YCK1 YCK2*) and LRB756 (*yck*^{ts}) were transformed with plasmids pMAL61/HA-GFP and pMAL63. Transformants were grown at 24° in synthetic medium with 2% maltose lacking histidine and uracil. Cells were harvested by filtration and transferred to YNSG containing CHX (30 μg/ml) and incubation at 24° with shaking was continued. Culture samples were taken at time zero and every hour over a 3-hr period, gently centrifuged, and GFP localization was visualized by confocal microscopy as described in materials and methods. The images represent a typical result from three independent transformants. (Left) Phase-contrast images. (Right) Fluorescent images. (C) Mal61/HAp expression levels and phosphorylation was determined by Western blotting using total cell extracts prepared from the strains described above in A grown to midlog phase in selective medium plus 2% maltose. Cell extracts were size separated on a 7.5% SDS–PAGE gel and probed with anti-HA and anti-PGK antibody. The arrows indicate the position of the phosphorylated (solid line) and hypophosphorylated (dotted line) species of Mal61/HAp.

the *yck*^{ts} strain, but fail to explain the dramatic glucose-induced increase in maltose transport activity observed for the *yck*^{ts} strain. We propose that this increased transport activity results from a glucose-induced stimulation of preexisting plasma-membrane-localized permease. It is interesting to note that glucose addition increases maltose transport activity to approximately the wild-type level, suggesting that the defect in permease activity in the *yck*^{ts} strain can be overcome by a glucose-stimulated event.

We have observed previously that Mal61/HA maltose permease migrates as a number of different mobility species on SDS–PAGE, of which two generally appear to predominate, and determined that these bands correspond to differentially phosphorylated species (Medintz *et al.* 1996). The slower-migrating (top band in Figure 1C) band is the phosphorylated species, since treatment with acid phosphatase causes the protein to migrate only at the position of the bottom band, presumably the hypophosphorylated species. We tested whether phosphorylation of Mal61p requires Yck1,2 kinase activity by comparing the migration of Mal61/HA protein isolated from wild-type and *yck*^{ts} strains. Approximately 80% of the Mal61/HA protein from the wild-type strain runs more slowly in the phosphorylated state, while from *yck*^{ts} cells only ~30% of the permease protein is phosphorylated (Figure 1C; compare lanes 1 and 2). Yck1p and Yck2p overproduction in the wild-type strain caused much higher relative amounts of the hyperphosphorylated species of maltose permease (Figure 1C, lanes 3 and 4) but no concomitant increase in maltose transport activity (Figure 2). The rate of glucose-induced loss of maltose transport activity does not change significantly upon Yck1,2 kinase overproduction, but the rate of Mal61/HA permease proteolysis increases dramatically to match the rate of rapid loss in transport activity (Figure 1A). These results are consistent with previous reports that protein phosphorylation, particularly by the Yck2 and Pkc1 kinases, regulates trafficking of plasma membrane proteins to the vacuole (D'Hondt *et al.* 2000; Friant *et al.* 2000; Marchal *et al.* 2000, 2002).

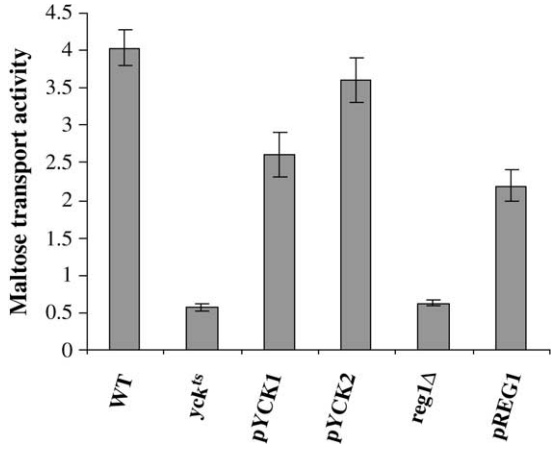

FIGURE 2.—Comparison of maltose transport activity of *yck*^{ts} and *reg1*Δ mutant strains. Strains LRB906 (*YCK1 YCK2 REG1*), LRB756 (*yck*^{ts}), and CMY7000 (*reg1*Δ) were transformed with plasmids pMAL61/HA and pMAL63 and plasmids YEp352, YEp352-YCK1, YEp352-YCK2, or plasmid DF041 (multi-copy *REG1*). Transformants were grown at 30° to midlog phase in selective minimal medium plus 2% maltose and maltose transport activity was assayed as described in MATERIALS AND METHODS. The error bars indicate the standard deviation from three independent transformants assayed in duplicate.

The Yck1,2 kinases require palmitoylation of a C-terminal di-cysteine motif for their plasma membrane localization and essential cellular function (ROTH *et al.* 2002; BABU *et al.* 2004). Akr1p is the palmitoyl transferase responsible for this modification (ROTH *et al.* 2002). *AKR1* mutants exhibit reduced phosphorylation of Ste3p, which presumably reflects at least in part the lack of membrane-associated Yck1,2 protein (FENG and DAVIS 2000). We found that the phenotype of an *akr1*Δ strain is nearly identical to that of the *yck*^{ts} (data not shown).

Together, these results indicate that plasma-membrane-associated casein kinase 1 encoded by *YCK1* and *YCK2* regulates the transport activity of Mal61 maltose permease, is required for an early step in the glucose-induced internalization of the permease, and regulates the rate of permease-containing vesicle trafficking to the vacuole and/or permease vacuolar degradation.

Yck1,2 kinase activity acts upstream of *DOA4*-mediated ubiquitination: Glucose-regulated degradation of maltose permease requires ubiquitin conjugation (MEDINTZ *et al.* 1998). Glucose stimulates the ubiquitination of Mal61/HA maltose permease and mutations in *RSP5*, encoding a HECT domain ubiquitin ligase, and in *DOA4*, encoding a ubiquitin hydrolase whose loss causes depletion of intracellular ubiquitin levels, leading to defects in glucose-induced proteolysis of Mal61/HAp. Ste2p and Ste3p require Yck1,2-dependent phosphorylation for the ubiquitination that stimulates internalization (HICKE *et al.* 1998; FENG and DAVIS 2000). Since Yck1,2 activity is required for Mal61 permease internalization, we tested genetically whether Yck1,2 kinase function is upstream of the ubiquitination step.

MAL61/HA-GFP was expressed in the *doa4* null strain CMY1025 with or without a high-copy plasmid carrying *YCK2*. Transformants were grown in maltose-induced conditions and subjected to the standard inactivation protocol and Mal61/HA-GFP localization was followed for 3 hr by confocal microscropy. The results are shown in Figure 3. Abundant Mal61/HA-GFP protein is observed at the cell surface of the *doa4*Δ mutant cells but little or no signal is visible in the vacuole, as expected. Functional levels of cell-surface-localized permease are not significantly altered in the *doa4*Δ mutant since maltose transport activity is only slightly lower than that observed in a congenic *DOA4* strain (4.6 *vs.* 3.6 nmol maltose/mg dry wt of cells/min). Glucose addition has little effect on maltose permease in *doa4*Δ cells. Maltose transport activity and cell surface localization remain approximately constant up to 3 hr after the addition of

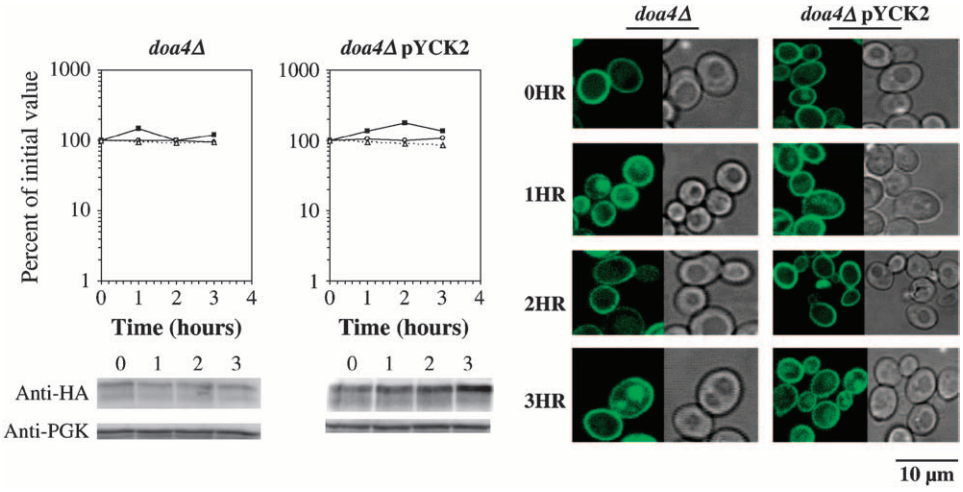

FIGURE 3.—Multicopy *YCK2* does not suppress the block in glucose-induced inactivation of maltose permease observed in a *doa4*Δ mutant. Strain CMY1025 (*MAL1 doa4*Δ*::HIS3*) was transformed with pUN30-MAL61/HA-GFP and the empty vector YEp352 or YEp352-YCK2. Transformants were grown at 30° to midlog phase in synthetic minimal medium lacking tryptophan and uracil plus 2% maltose as carbon source. Cells were harvested by filtration, transferred to YNSG with CHX, and standard inactivation protocol was carried out as described in the legend of Figure 1A. Alternately, cells were observed using confocal fluorescence and phase microscopy at the indicated times following the transfer to YNSG with CHX as described in the legend of Figure 1B. The photos to the left are fluorescent images and those to the right are phase-contrast images.

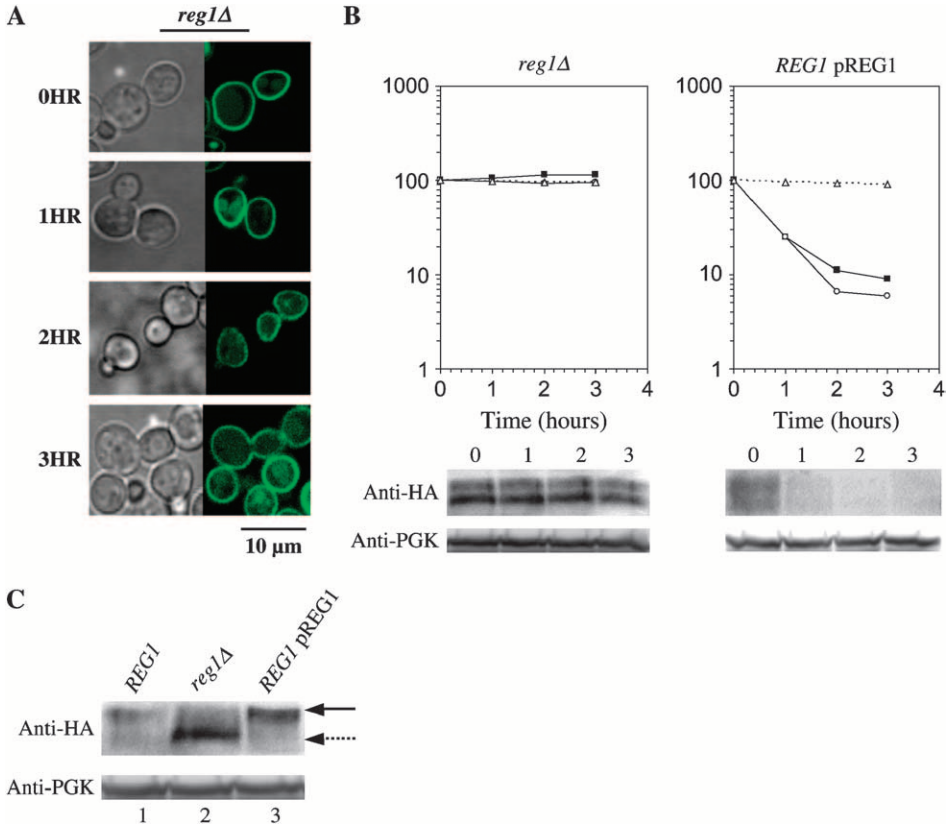

FIGURE 4.—Effects of *reg1* deletion and *REG1* overexpression on glucose-induced inactivation of maltose permease. (A) Strain CMY7000 (*reg1*Δ) was transformed with plasmids pUN70-MAL61/HA-GFP and pUN90-MAL63. Transformants were grown at 30° to midlog phase on synthetic medium lacking uracil and histidine plus 2% maltose, harvested by filtration, and transferred to YNSG media with CHX as described in the legend of Figure 1B. Subcellular localization of Mal61/HA-GFP was determined using confocal fluorescence microscopy at the indicated times following transfer to YNSG. (Left) Phase-contrast images. (Right) Fluorescent images. (B) Strain LRB906 (*REG1*) was transformed with plasmids pRS315-MAL61/HA, pUN90-MAL63, and either pUN70 (vector alone) or plasmid DF041 (multi-copy *REG1*). Strain CMY7000 (*reg1*Δ) was transformed with pRS315-MAL61/HA and pUN90-MAL63. Transformants were grown to midlog phase on synthetic medium lacking leucine, histidine, and uracil (for LRB906 transformants) with 2% maltose as the carbon source and glucose-induced inactivation assayed as described in the legend of Figure 1A. (C) The transformed strains described in B were grown in selective medium lacking leucine, histidine, and uracil (for LRB906 transformants) with 2% maltose as the carbon source, harvested, and total cell extracts were prepared and analyzed by Western blotting as described in the legend of Figure 1C. The arrows point to the phosphorylated (solid line) and hypophosphorylated (dotted line) species of Mal61/HA protein.

glucose and no proteolysis of maltose permease protein or vacuolar accumulation is evident after 3 hr.

Overexpression of *YCK2* causes a significant increase of the rate of maltose transport in the *doa4*Δ strain (6.0 *vs.* 3.6 nmol maltose/mg dry wt of cells/min) with little or no apparent increase in cell surface permease protein (Figure 3). Most important, multicopy *YCK2* does not suppress the block in glucose-induced inactivation in a *doa4*Δ mutant. Maltose transport activity is stimulated ~10% following glucose addition and remains above initial levels for up to 3 hr. It is important to note that despite higher levels of phosphorylated Mal61/HA-GFP protein in the *doa4*Δ [pYCK2] strain, no glucose-induced Mal61/HA-GFP permease proteolysis is observed and Mal61/HA-GFP permease remains at the plasma membrane. Thus, elevated levels of Yck2 kinase activity do not suppress the *doa4*Δ defects in maltose permease glucose-induced inactivation as predicted if Yck1,2-dependent phosphorylation is the upstream event.

Loss of *REG1* causes defects in maltose permease localization similar to those of the *yck*^{ts} mutant: We previously reported that deletion of *REG1* causes resistance to glucose-induced proteolysis of maltose permease, reduced rather than increased levels of maltose permease phosphorylation, and increased maltose transport activity upon glucose addition (JIANG *et al.* 2000b). Given the phenotypic similarity between the *reg1*Δ and *yck*^{ts} mutants, we investigated maltose permease localization in a *reg1*Δ strain. *MAL61/HA-GFP* was expressed in strain CMY7000 (*reg1*Δ) and localization of Mal61/HA-GFP permease was followed during the 3-hr course of glucose-induced inactivation. At the outset of the experiment, most GFP fluorescence is seen at the cell surface, with no significant accumulation in the vacuole (Figure 4A). This localization pattern is virtually identical to that observed for the otherwise isogenic *yck*^{ts} strain shown in Figure 1B. Moreover, addition of glucose to *reg1*Δ cells does not alter the pattern of localization. Mal61/HA-GFP fluorescence remains at the cell surface, with little or no localization to the vacuole. Thus, like *yck*^{ts}, *reg1*Δ does not appear to affect maltose permease protein synthesis or plasma membrane localization but does block vacuolar localization and glucose-induced internalization.

Since previous studies (JIANG *et al.* 2000b) were carried out using strains of a different genetic background, we examined the effect of *reg1*Δ on glucose inactivation of maltose permease in the background used in this study, comparing these effects with those of *yck*^{ts}. The results are reported in Figures 2 and 4.

TABLE 1

Glucose-repression sensitivity of maltase expression in *GLC7* mutants

	Maltase activity in nmol of PNPG hydrolyzed/min/mg protein			
GLC7 allele	Glycerol/lactate	Glycerol/lactate 2% maltose	Glycerol/lactate 2% glucose	Glycerol/lactate 2% maltose 2% glucose
GLC7	10	2146	35	44
glc7-109	37	660	25	71
glc7-127	52	3694	33	1234
glc7-132	58	1760	7	52
glc7-133	30	7138	24	1104
glc7-256	28	1636	58	471

Strain KT1112 (*GLC7*) and the otherwise isogenic *glc7* mutants KT1636 (*glc7-133*), KT1639 (*glc7-132*), KT1967 (*glc7-127*), KT1638 (*glc7-109*), and TW267 (*glc7-256*) were transformed with YCp50-MAL63 and grown to midlog on selective medium plus 2% glycerol, 3% lactate, and 2% maltose, and/or 2% glucose, as indicated. Maltase activity was determined as described in MATERIALS AND METHODS (DUBIN *et al.* 1985) on at least two independent transformants.

The level of transport activity in the *reg1*Δ strain on maltose is about eightfold lower than that observed in the parental strain and similar to that of *yck*^{ts}. This reduced transport activity is not reflected either in the level of maltose permease protein detected by immunoblot (Figure 4C) or in our estimate of the fluorescent signal in the *reg1*Δ and parental strains (Figures 1B and 4A). Therefore, the reduced transport activity observed in the *reg1*Δ mutant does not result from reduced permease protein expression or mislocalization, suggesting that maltose transport activity of maltose permease is dependent either directly or indirectly on *REG1*. JIANG *et al.* (2000b) reported only about a 40% decrease in maltose transport activity in a *reg1*Δ mutant of different genetic background. Nonetheless, the impact of *reg1*Δ on the maltose permease phenotypes reported here is qualitatively comparable in both strain backgrounds.

Approximately 70–75% of maltose permease protein is in the hypophosphorylated form in the *reg1*Δ strain (Figure 4C). This decrease in bulk phosphorylation is similar to that reported previously (JIANG *et al.* 2000b) for a *reg1*Δ mutant, as well as to the decrease shown here for the *yck*^{ts} strain. Other aspects of the inactivation phenotype of the *reg1*Δ strain also are similar to that of the *yck*^{ts} mutant. Glucose fails to stimulate proteolysis of maltose permease, but maltose transport activity increases slightly but reproducibly during the 3 hr following transfer to YNSG, although not to the same extent observed for *yck*^{ts} cells (Figures 1A and 4B).

In contrast, overexpression of *REG1* in an otherwise wild-type strain increases the rate of maltose permease proteolysis such that it now matches the rapid rate of loss of maltose transport activity (Figure 4B). This is similar to the effects of overexpressing *YCK1* or *YCK2*. Moreover, overexpression of *REG1* leads to accumulation of nearly all of the Mal61/HA protein as the phosphorylated species. These results are consistent with the idea that phosphorylation of the permease is positively regulated by Reg1p.

The requirement for Reg1p in glucose-induced inactivation of maltose permease likely reflects a requirement for PP1 activity: Reg1p is a regulatory subunit of protein phosphatase type 1, for which Glc7p is the catalytic component in yeast. Binding of Reg1p to Glc7p is enhanced in the presence of glucose and is required for glucose repression (TU and CARLSON 1995; SANZ *et al.* 2000). We used a group of previously characterized *glc7* mutant alleles (BAKER *et al.* 1997) to test whether Reg1 function in this pathway is as a Glc7p regulatory subunit by determining whether alleles conferring insensitivity to glucose repression, which correlates with loss of Reg1p–Glc7p binding, also cause resistance to glucose-induced inactivation of maltose permease. We selected *glc7-109*, *glc7-127*, *glc7-132*, and *glc7-133* strains (BAKER *et al.* 1997). Strains carrying *glc7-127* and *glc7-133* are resistant to 2-deoxyglucose, a nonmetabolized glucose analog that induces glucose repression (NEIGEBORN and CARLSON 1987). We also tested *glc7-256* in these assays. This allele alters a residue in the hydrophobic cleft on Glc7p, which is essential for binding to a common regulatory subunit motif, V/IXF (WU and TATCHELL 2001). Reg1p fails to interact with Glc7-256 mutant protein in the two-hybrid assay (WU and TATCHELL 2001).

The wild-type *GLC7* strain and strains carrying *glc7* mutant alleles were transformed with plasmid-borne *MAL61/HA* and *MAL63*, and transformants were assayed for glucose repression of maltase expression and glucose-induced inactivation of maltose permease. As shown in Table 1, maltose induction of maltase expression is blocked by the presence of glucose in strains carrying *glc7-109* and *glc7-132*. These mutants are sensitive to repression by 2-deoxyglucose and are presumed to carry alterations in binding sites of Glc7p-targeting subunits other than Reg1p (BAKER *et al.* 1997). On the other hand, strains carrying *glc7-127*, *glc7-133*, and *glc7-256*, those alleles shown to be resistant to 2-deoxyglucose or to prevent Reg1p binding (BAKER *et al.* 1997; WU and

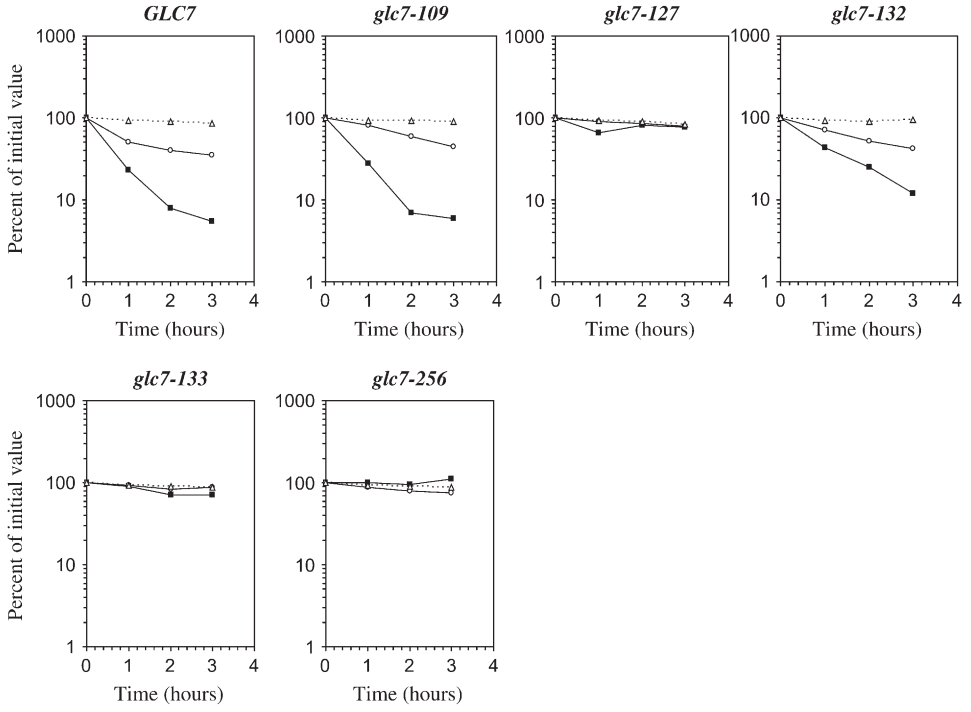

FIGURE 5.—Glucose-induced inactivation of maltose permease in *glc7* mutants encoding the catalytic subunit of protein phosphatase type 1. Strains KT1112 (*GLC7*), KT1636 (*glc7-133*), KT1639 (*glc7-132*), KT1967 (*glc7-127*), KT1638 (*glc7-109*), and TW267 (*glc7-256*) were transformed with pRS315-MAL61/HA and pUN90-MAL63. Transformants were grown at 30° to midlog phase on synthetic medium lacking leucine and histidine with 2% maltose. The standard inactivation protocol was followed as described in the legend of Figure 1A.

TATCHELL 2001), are resistant to glucose repression of maltase expression (Table 1).

Glucose effects on maltose permease in this same group of *glc7* mutant strains are presented in Figure 5. Strains carrying *GLC7* or mutant alleles *glc7-109* and *glc7-132*, which are sensitive to glucose repression, undergo similar glucose-induced inactivation of maltose permease. However, glucose-repression-insensitive *glc7-127*, *glc7-133*, and *glc7-256* mutants fail to exhibit glucose-induced inactivation of maltose permease. Thus, insensitivity to glucose-induced inactivation of maltose permease correlates with the glucose repression insensitivity of *GLC7* mutants. Consistent with the studies reported above for the *reg1*Δ strain, these results suggest that Glc7p–Reg1p interaction, and thus Reg1p-targeted PP1 phosphatase activity, is required for glucose to stimulate maltose permease endocytosis and proteolysis.

Epistasis analysis places *YCK* activity downstream of *REG1*: With the exception of the difference in the extent of the glucose-induced increase in maltose transport activity, *yck*^{ts} and *reg1*Δ have very similar effects on maltose permease subcellular localization, transport activity, phosphorylation, and resistance to glucose-induced inactivation. Therefore, we propose that the Yck1,2 kinase and Glc7–Reg1 phosphatase are components of a common glucose-signaling pathway and explore the relationship between these two regulators.

We constructed a *reg1*Δ *yck*^{ts} mutant, which is viable and grows on maltose with a doubling time comparable to that of the *yck*^{ts} mutant (data not shown). This strain was transformed with plasmid-borne *MAL61/HA* and glucose-induced inactivation was assayed. The phenotype of the *reg1*Δ *yck*^{ts} double mutant strain is similar to that of either single mutant with regard to maltose transport activity, maltose permease phosphorylation, and insensitivity to glucose-induced inactivation of maltose permease (Figure 6). Thus, the *reg1*Δ *yck*^{ts} mutant does not exhibit enhancement of the phenotype of the single-mutant strains, supporting our hypothesis that Yck1,2 kinase and Reg1–Glc7 phosphatase act in a common pathway. However, the sevenfold glucose-induced increase in maltose transport activity of the *yck*^{ts} strain is not observed in the *reg1*Δ *yck*^{ts} double mutant. Rather, we observed a slight increase similar to that of the *reg1*Δ mutant, suggesting that *reg1*Δ is epistatic to *yck*^{ts} in this glucose-signaling pathway.

To determine the order of function of these two activities, we examined the effect of high-copy *YCK1* on glucose-induced Mal61 inactivation in a *reg1*Δ strain and of high-copy *REG1* in the *yck*^{ts} strain. Overexpression of *REG1* in the *yck*^{ts} strain has little effect on the *yck*^{ts} phenotype (Figure 6A). Following glucose addition to the *yck*^{ts} [pREG1] strain, a modest increase in the rate of glucose-induced proteolysis of maltose permease is observed and maltose transport rates increase gradually and only about twofold. In contrast, overexpression of *YCK1* in the *reg1*Δ strain fully rescues the *reg1*Δ phenotype (Figure 6A). The rapid glucose-induced loss of maltose transport activity is restored and the rate of maltose permease degradation is significantly faster than that observed in the parental strain (Figure 1A). The rate of permease degradation is comparable to that observed in the wild-type strain carrying multi-copy *YCK1* (Figure 1A) and parallels the rate of loss of transport activity.

We compared the initial rates of maltose transport for these strains to those obtained for the wild-type and

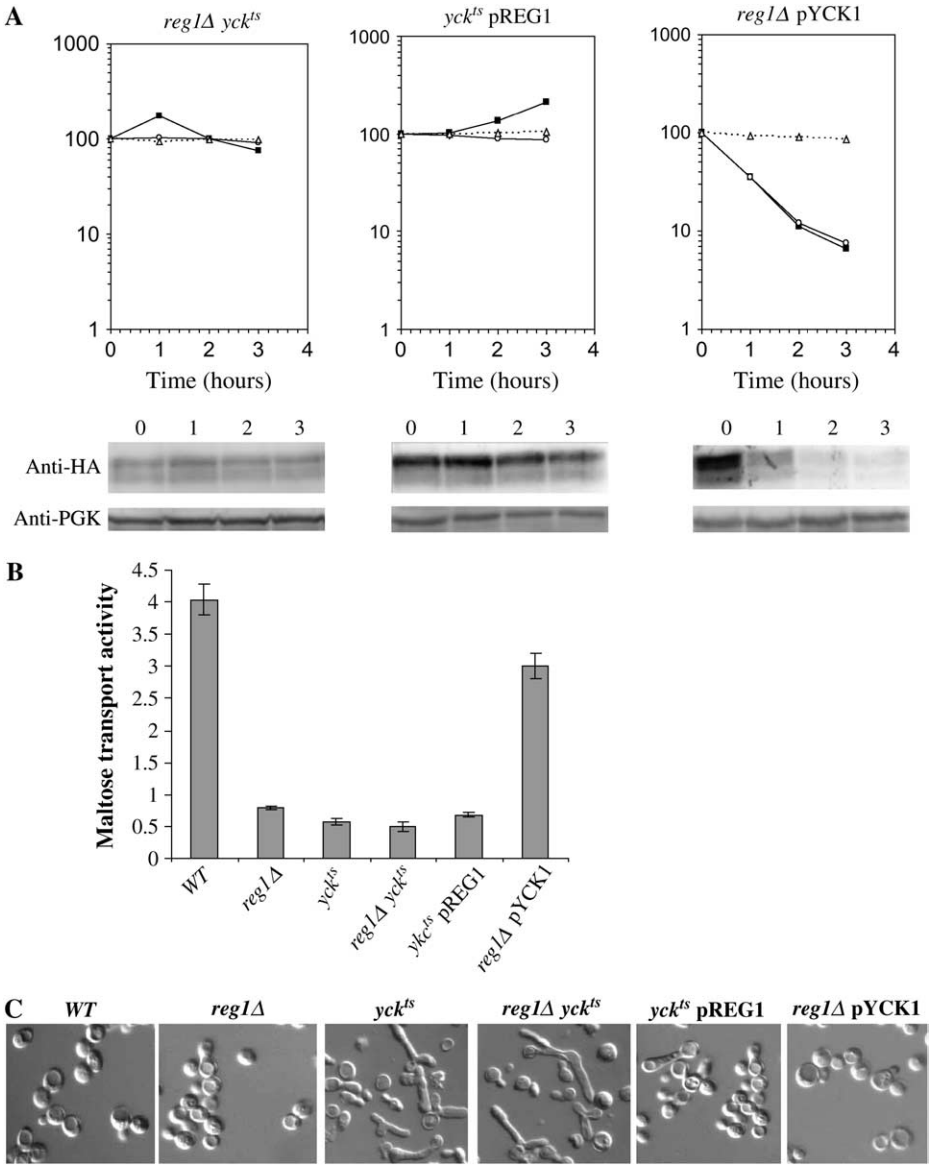

FIGURE 6.—Epistasis analysis places *GLC7-REG1* upstream of *YCK1* in glucose-induced inactivation of maltose permease. Strains CMY7000 (*reg1*Δ) and LRB756 (*yck*^{ts}) were transformed with pRS315-MAL61/HA, pUN90-MAL63, and YEp352, pYCK1, or DF041 (*REG1*). LRB1082 (*reg1*Δ *yck*^{ts}) was transformed with pRS315-MAL61/HA, pUN90-MAL63, and YEp352 vector. Transformants were grown to midlog phase at 30° on synthetic medium lacking histidine, uracil, and leucine with 2% maltose. The cells were harvested, transferred to YNSG medium with CHX, and the standard inactivation protocol was followed as described in the legend of Figure 1A. (B) Strains LRB906 (*YCK1 YCK2 REG1*) (WT), LRB756 (*yck*^{ts}), CMY7000 (*reg1*Δ), and LRB1082 (*reg1*Δ *yck*^{ts}) were transformed with plasmids pMAL61/HA, pMAL63, and plasmid YEp352 (vector only), pYCK1, or DF041 (*REG1*), as indicated. Transformants were grown to midlog phase at 30° on synthetic medium lacking histidine, uracil, and leucine with 2% maltose, harvested by filtration, and maltose transport activity was determined as described in MATERIALS AND METHODS. The error bars indicate the standard deviation from three independent transformants assayed in duplicate. (C) Phase-contrast images of the strains listed above for B grown to midlog at room temperature; images were taken with Meridian/Olympus IMT-2 confocal microscope using a ×40 lens.

parental mutant strains (Figure 6B). The *reg1*Δ, *yck*^{ts}, and *reg1*Δ *yck*^{ts} strains all show a similar very low rate of transport activity, and this rate is not enhanced when *REG1* is overexpressed in the *yck*^{ts} strain. However, overexpression of *YCK1* in the *reg1*Δ strain rescues maltose transport activity to almost wild-type levels. Together, these results are consistent with Reg1p acting upstream of the Yck1,2 kinases.

Figure 6C compares the morphology of wild-type, *reg1*Δ, *yck*^{ts}, *reg1*Δ *yck*^{ts}, *reg1*Δ [pYCK1], and *yck*^{ts} [pREG1] strains grown to midlog in 2% maltose at room temperature. A predominance of large-budded cells in the *reg1*Δ strain is clearly observed. JIANG *et al.* (2000b) reported that this is associated with a G_2 delay in a *reg1*Δ strain. Overexpression of *YCK1* restores an apparently normal bud-size distribution. The elongated bud morphology previously observed in *yck*^{ts} strains (ROBINSON *et al.* 1993) is evident under these growth conditions and is not significantly enhanced in the *reg1*Δ *yck*^{ts} double-mutant strain, although multiple-budded cells are slightly more frequent in this strain. Interestingly, although overexpression of *REG1* has no effect on the temperature-sensitive growth of the *yck*^{ts} strain (data not shown), it suppresses the morphology phenotype significantly. Dramatically fewer elongated buds and no multiple-budded cells are seen.

Yck1,2 kinase is inactive in a *reg1*Δ strain: The simplest explanation of our results so far is that Reg1–Glc7 phosphatase has a positive effect on Yck1,2 protein levels, localization to the plasma membrane, or activity. We did not observe any change in Yck2 protein levels or plasma membrane localization in the *reg1*Δ strain (L. C. ROBINSON, unpublished results). Therefore, we tested whether Yck1,2 kinase activity is compromised in the *reg1*Δ mutant. Mth1p and Std1p are repressors of *HXT1* expression (SCHMIDT *et al.* 1999; FLICK *et al.* 2003;

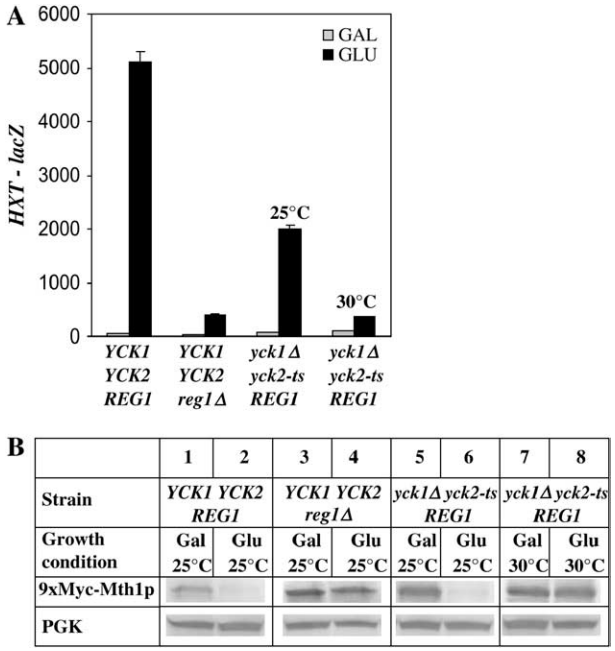

FIGURE 7.—*reg1*Δ blocks glucose-induced *HXT1* expression and Mth1 repressor degradation. Strains LRB906 (*YCK1 YCK2 REG1*), LRB756 (*yck*^{ts}), and CMY7000 (*reg1*Δ) were transformed with the *HXT1-lacZ* reporter plasmid pBM3212 (A) or plasmid pBM4560 carrying a 9xMyc-tagged allele of *MTH1* expressed from its native promoter (B) (OZCAN *et al.* 1996). Transformants were grown to midlog at 25° in selective minimal medium lacking leucine (A) or tryptophan (B) plus 2% galactose, harvested by filtration, resuspended in fresh medium plus 2% galactose at 25°, 4% glucose at 25°, or 4% glucose at 30°. (A) Cells were cultured in the indicated medium and temperature for 5 hr, harvested, total cell extracts were prepared, and β-galactosidase levels assayed as described in MATERIALS AND METHODS. (B) Cells were cultured in the indicated medium and temperature for 45 min, harvested by filtration, and total cell extracts were prepared for Western blot analysis as described in the legend of Figure 1B except that anti-Myc antibody (Roche Diagnostic) was used to detect the 9xMyc-tagged Mth1p. Equal loading of total-cell extract was confirmed using anti-PGK antibody.

LAKSHMANAN *et al.* 2003; KANIAK *et al.* 2004; MORIYA and JOHNSTON 2004) that are inactivated by degradation in the presence of glucose (MORIYA and JOHNSTON 2004). MORIYA and JOHNSTON (2004) report that glucose-induced degradation of Mth1 and Std1 repressors requires phosphorylation by the Yck1,2 kinases. As predicted from these data, they also report that glucose-stimulated *HXT1* expression and Mth1p and Std1p degradation are defective in the *yck*^{ts} strain. On the basis of these results, we used glucose-induced Mth1p degradation and *HXT1* expression to monitor Yck1,2 kinase activity *in vivo* in the *reg1*Δ strain.

The parental *YCK1,2 REG1* strain and the *yck*^{ts} *REG1* and *YCK1,2 reg1*Δ mutant strains were transformed with an *HXT1* promoter-*lacZ* reporter plasmid and β-galactosidase expression was assayed following growth in galactose and after 5 hr of growth in glucose (Figure 7A). Glucose induction of *HXT1* is clearly blocked in the *reg1*Δ mutant strain, indicating that the Yck1,2 kinases are inactive in the absence of this Glc7 phosphatase-targeting subunit. We note that glucose induction of *HXT1* is partially defective in the *yck*^{ts} strain at the permissive temperature of 25° but a more serious defect is observed at 30°.

These results are consistent with the difference in Mth1p stability shown in Figure 7B. Following 45 min of exposure to glucose, Mth1 protein levels are dramatically decreased in the wild-type strain but are relatively unchanged in the *reg1*Δ mutant strain and the *yck*^{ts} strain at 30°. In the *yck*^{ts} strain at the permissive temperature of 25°, we observe a very significant decrease in Mth1p levels, although a slight amount of protein remains. On the basis of these findings, we conclude that the activity of the Yck1,2 kinases cannot be stimulated by growth on glucose in a *reg1*Δ mutant.

DISCUSSION

Our results indicate that Yck1,2 kinase activity is a key player in multiple steps regulating maltose permease activity and trafficking. While not required for permease synthesis or cell surface localization, Yck1,2 kinase activity is required for activation of maltose transport activity. Further, decreased Yck1,2 kinase activity or lack of association of the Yck1,2 proteins with the plasma membrane blocks glucose-induced inactivation of maltose permease. The effects of *yck*^{ts} on maltose permease resemble those of *reg1*Δ (JIANG *et al.* 2000b) and those observed for glucose-repression-insensitive *glc7* mutants. On the basis of our results, we propose that the Yck1,2 kinases and Glc7–Reg1 PP1 phosphatase are components of a common signaling pathway in which Reg1-Glc7 phosphatase activates Yck1,2 kinase and modulates its activity level in response to carbon source availability.

The *yck*^{ts} mutant exhibits multiple defects specific to maltose permease activation and internalization: Despite the apparent normal levels of cell surface localization of maltose permease in the *yck*^{ts} strain, maltose transport activity is reduced approximately sevenfold. The basis for this low specific activity is unclear. It may be related to the significantly reduced level of permease phosphorylation observed in the *yck*^{ts} and *reg1*Δ mutants but some of our results argue against this possibility. For example, the unexpected increase in maltose transport activity observed upon addition of glucose to the *yck*^{ts} strain (Figure 1A) is not associated with a parallel increase in phosphorylated maltose permease protein. On the other hand, overexpression of *REG1* in the *yck*^{ts} strain (Figure 6) causes a significant increase in the level of phosphorylated permease with no concomitant increase in maltose transport activity. However, we are monitoring bulk phosphorylation of the permease, so we cannot exclude the possibility that activation of

maltose permease is regulated by phosphorylation of specific sites.

The very robust glucose-induced activation of maltose permease in the *yck*^{ts} strain is likely to provide clues as to the molecular basis of transport regulation by Yck1,2 kinase. We suggest that most of the cell-surface-localized maltose permease protein expressed in the *yck*^{ts} strain is inactive but is very rapidly activated by this glucose-stimulated event. Since it does not require *de novo* protein synthesis, the simplest explanation is a glucose-stimulated modification of the permease protein or its association with a preexisting regulatory cofactor that takes place via a pathway that does not require Yck1,2 kinase activity. It is possible that the membrane topology of maltose permease is altered in the *yck*^{ts} strain, but this seems unlikely to be so quickly remediated by glucose addition. The effects of this alternate pathway are normally obscured in the wild-type strain due to the rapid glucose-induced internalization of maltose permease. Medintz *et al.* (2000) reported a similar glucose-induced increase in maltose transport activity in cells expressing a mutant allele of Mal61 maltose permease lacking residues 49–78 of the N-terminal cytoplasmic domain. In this case, the stimulation was dependent on the presence of a wild-type allele of *RGT2* and occurred constitutively in cells expressing the dominant constitutive *RGT2-1* allele.

The *yck*^{ts} strain exhibits severe defects in glucose-induced internalization of maltose permease. It is unlikely that these internalization defects result from a generalized endocytic defect. Fluid-phase endocytosis, as assayed by Lucifer yellow uptake, does not require Yck1,2 casein kinase 1 activity (Friant *et al.* 2000). We observed that the rate of movement of the lipophilic dye FM4-64 (Fischer-Parton *et al.* 2000) from the plasma membrane to the vacuolar membrane occurs with approximately the same kinetics in wild-type and *yck*^{ts} strains during growth on maltose at permissive and nonpermissive temperatures (data not shown). Thus, the defects in maltose permease internalization and degradation are specific to glucose-induced turnover of Mal61 permease.

A requirement for phosphorylation for regulated internalization of plasma membrane proteins has been noted previously. Phosphorylation in these cases acts to target proteins for ubiquitination, which is required for recognition by endocytic machinery as well as for sorting in downstream compartments (Bonifacino and Traub 2003; Umebayashi 2003). The results reported here indicate that Yck1,2 kinase activity is upstream of maltose permease ubiquitination. 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 kinase activity is required for the phosphorylation of maltose permease that stimulates permease ubiquitination. Several putative Ser/Thr kinase target sites are present in the predicted cytoplasmic domains of Mal61p, four of which fit the casein kinase 1 target-site consensus (Kennelly and Krebs 1991). Additional studies will be required to determine whether maltose permease is the direct target of Yck1,2 kinase activity for these functions.

In summary, the Yck1,2 kinases play an important role in activating the maltose transport activity of maltose permease and in regulating the rate of movement of permease-containing transport vesicles and are essential for internalizing maltose permease from the plasma membrane in response to glucose. Which step or steps result from the direct phosphorylation of maltose permease by the Yck1,2 kinases remains to be determined.

The Yck1,2 kinases and Glc7–Reg1 phosphatase act in a common glucose-signaling pathway: Strains defective for Yck1,2 kinase activity or Glc7–Reg1 phosphatase activity share a strikingly similar maltose permease phenotype and the *yck*^{ts} *reg1*Δ double mutant resembles each single-mutant strain, suggesting that the Yck1,2 kinases and Glc7–Reg1 phosphatase act in a common pathway. Moreover, epistasis analysis places Yck1,2 kinase activity downstream of Glc7–Reg1 phosphatase in this pathway. These results are consistent with the hypothesis that Reg1–Gcl7 phosphatase is an activator of Yck1,2 kinase activity. Although there is no evidence that the Yck1,2 kinases are regulated by phosphorylation and dephosphorylation, at least one casein kinase 1 isoform is thought to be regulated by the phosphorylation state of a sequence in the C-terminal domain. For casein kinase 1δ, auto-phosphorylation of its carboxy-terminal domain was reported to be inhibitory, and its kinase activity is activated *in vitro* by phosphatase treatment (Graves and Roach 1995). In this regard, it is interesting to note that deficiency of protein phosphatase 2A or of the Yck1,2 kinases results in similar defects in bud morphogenesis and cytokinesis (Healy *et al.* 1991; Robinson *et al.* 1993), possibly due to similar effects on septin function at the bud neck. This similarity is not consistent with a relationship where opposing phosphatase and kinase activities act on a shared substrate, but is consistent with a scenario in which protein phosphatase 2A activates Yck1,2 kinase activity.

It should also be noted that while the observed defects in maltose permease activity and phosphorylation in the *yck*^{ts} and *reg1*Δ mutant strains grown in maltose are strikingly similar, their growth and morphology phenotypes are not (Figure 6C). Loss of *REG1* does not produce the elongated bud phenotype exhibited by *yck*^{ts} even at permissive temperatures nor does it cause a temperature-sensitive growth defect (data not shown). On the other hand, multicopy *REG1* does restore near-wild-type morphology to maltose-grown *yck*^{ts} cells and multicopy *YCK1* suppresses the large-budded phenotype of *reg1*Δ mutant strains (Figure 6C; Jiang *et al.* 2000b). These findings are consistent with our proposed Glc7–Reg1 phosphatase/Yck1,2 kinase regulatory pathway but suggest a nonessential role for Glc7–Reg1 phosphatase as a modulator of Yck1,2 kinase activity, perhaps coordinating

Yck1,2p activity with carbon source availability. We envision Glc7–Reg1 phosphatase participating in a priming event that increases Yck1,2p activity or responsiveness to signals. The Yck1,2p priming event could include changes in phosphorylation level, membrane association, localization to membrane subdomains, or some combination of these. Moreover, our results suggest that the variety of Yck1,2p cellular functions has differential requirements for this priming event.

Yck1,2 kinase is the keystone of two independent regulatory pathways controlling both glucose-induced inactivation and glucose induction: Moriya and Johnston (2004) recently added Yck1,2 kinase activity to a model for the Rgt2p-dependent high-glucose sensing pathway that regulates *HXT1* expression. They used the split-ubiquitin system to demonstrate that Rgt2p and Yck1p interact, showed that Yck1,2 kinase activity increases upon glucose stimulation, and demonstrated that activated Yck1,2 kinases phosphorylate Mth1p and Std1p when the latter proteins are tethered to the Rgt2p C-terminal cytoplasmic tail. Further, Yck1,2p-dependent phosphorylation marks these proteins for recognition and ubiquitination by SCF^{GRR1}. They propose that Rgt2p serves as the activator of Yck1,2 kinase in the presence of high extracellular glucose concentrations, although this is not demonstrated experimentally.

Our results extend the findings of Moriya and Johnston (2004) by proposing that a second signaling pathway, of which Glc7–Reg1 phosphatase is an essential component, contributes to the high-glucose signal at the plasma membrane and that both pathways are required to achieve the full high-glucose response. These pathways parallel those described by Jiang *et al.* (1997, 2000b) who demonstrated that two glucose-signaling pathways are involved in the glucose induction of maltose permease inactivation: pathway 1, the Rgt2p-dependent pathway, and pathway 2, the glucose transport-dependent pathway, of which Glc7–Reg1 phosphatase is an essential component. Glc7–Reg1 phosphatase is activated by growth on high concentrations of glucose via signals generated by the same pathway that inhibits Snf1 kinase in response to rapid glucose utilization (Tu and Carlson 1995; Sanz *et al.* 2000), but the nature of this glucose signal is unknown. Jiang *et al.* (1997, 2000b) showed that loss of *REG1* blocks glucose-induced inactivation of maltose permease (see also Figure 5) and that the constitutive *RGT2-1* allele causes glucose-induced proteolysis of maltose permease even in the absence of glucose (Jiang *et al.* 1997).

Our conclusion, on the basis of results reported in Figures 6 and 7, indicates that both pathways are required for these two glucose-induced processes. Glucose signaling via Rgt2p alone is not sufficient for glucose induction of *HXT1* expression and Mth1p degradation since these functions are defective in a *reg1*Δ mutant. Rgt2p-dependent signaling is also not sufficient to induce maltose permease inactivation in a *reg1*Δ mutant but is sufficient when *YCK1* is overexpressed, suggesting that higher levels of Yck1 kinase can compensate for signaling only via the Rgt2p pathway. Thus it appears that activation of Yck1,2 kinase to very high levels is required to induced maltose permease inactivation and *HXT1* expression and that this is achieved normally only by coordinated activation of both the Rgt2p-dependent and Glc7–Reg1 phosphatase glucose-signaling pathways, pathways 1 and 2, respectively. Once activated by both glucose-stimulated Rgt2p and Reg1–Glc7 phosphatase, Yck1,2 kinase then stimulates selective phosphorylation of targets such as Mth1p, Std1p, and possibly maltose permease, thereby marking them for ubiquitination and proteolysis.

In summary, our results connect two glucose-signaling pathways previously believed to represent independent responses to carbon source availability: the Rgt2 extracellular glucose sensor pathway and the Reg1–Glc7 phosphatase/Snf1 kinase pathway that sense intracellular glucose utilization rates (Carlson 1999; Johnston 1999; Sanz *et al.* 2000) and define a novel role for Reg1–Glc7 phosphatase as an activator of the Yck1,2 kinases.

We are grateful to Mark Johnston for providing plasmids and to Kelly Tatchell for providing *glc7* mutant strains and plasmids, for valuable discussions, and for critical reading of the manuscript. We thank Tara Williams-Hart for sharing her results prior to publication. This study utilized equipment of the Core Facility for Cell and Molecular Biology and we thank Areti Tsiola for her valuable assistance. This work was supported by grants to C.A.M. from the National Institutes of Health (GM28216) and to L.C.R. from the National Science Foundation (MCB-9974459). The results reported here were obtained in partial fulfillment of the requirements for the Ph.D. degree from the Graduate School of CUNY (N.G.).

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Communicating editor: A. P. Mitchell