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Characterization of the Glucose-Induced Inactivation of Maltose Permease in *Saccharomyces cerevisiae*

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The addition of glucose to maltose-fermenting *Saccharomyces cerevisiae* cells causes a rapid and irreversible loss of the ability to transport maltose, resulting both from the repression of transcription of the maltose permease gene and from the inactivation of maltose permease. The latter is referred to as glucose-induced inactivation or catabolite inactivation. We describe an analysis of this process in a maltose-fermenting strain expressing a hemagglutinin (HA)-tagged allele of *MAL61*, encoding maltose permease. The transfer of maltose-induced cells expressing the Mal61/HA protein to rich medium containing glucose produces a decrease in maltose transport rates which is paralleled by a decrease in Mal61/HA maltose permease protein levels. In nitrogen starvation medium, glucose produces a biphasic inactivation, i.e., an initial, rapid loss in transport activity (inhibition) followed by a slower decrease in transport activity, which correlates with a decrease in the amount of maltose permease protein (proteolysis). The inactivation in both rich and nitrogen-starved media results from a decrease in V_{max} with no apparent change in K_m. Using strains carrying mutations in *END3*, *REN1*(*VPS2*), *PEP4*, and *PRE1 PRE2*, we demonstrate that the proteolysis of Mal61/HAp is dependent on endocytosis and vacuolar proteolysis and is independent of the proteosome. Moreover, we show that the Mal61/HA maltose permease is present in differentially phosphorylated forms.

The addition of glucose to maltose-fermenting *Saccharomyces cerevisiae* cells causes an irreversible loss of the ability to transport maltose which is more rapid than can be explained by the combined effects of glucose repression of maltose permease gene transcription and cell growth (5, 8, 16). Similar irreversible inactivation of the high-affinity galactose and glucose transporters and of the gluconeogenic enzymes has been described, and the phenomenon is generally referred to as glucose-induced inactivation or catabolite inactivation (6, 13, 21, 33, 37, 39, 40).

Early studies of the mechanism of glucose-induced inactivation of the gluconeogenic enzymes demonstrated glucose-stimulated proteolysis of these cytoplasmic enzymes (35, 36, 47). The mechanism of fructose-1,6-biphosphatase inactivation has been investigated most extensively. Here, two distinct processes are stimulated in response to glucose: an initial, rapid, reversible phosphorylation, which is correlated with a decrease in enzyme activity but not protein levels, and a slower, irreversible proteolysis (10, 34, 47). The pathway of the proteolysis is as yet unresolved, with conflicting published reports of both vacuole-dependent and proteosome-dependent proteolysis (10, 11, 44).

MAL61 of the *MAL6* locus and its nearly identical homologs at the other *MAL* loci (*MAL11*, *MAL21*, *MAL31*, and *MAL41*) encode *Saccharomyces* maltose permease. Mal61p is a high-affinity (2 to 4 mM) proton/maltose symporter and is a member of the 12-transmembrane-domain family of sugar transporters (8, 9). Expression of *MAL61* is maltose induced and glucose repressed (8, 9, 22, 36). Maltose induction is mediated at the transcriptional level by the Mal activator, a sequence-specific DNA-binding transcription activator encoded by *MAL63* of the *MAL6* locus and its nearly identical homologs at the other *MAL* loci (*MAL13*, *MAL23*, *MAL33*, *MAL43*, and *mal64*). Glucose regulates maltose transport at two levels. First, glucose represses maltose permease gene transcription by mechanisms involving several gene products including Mig1p (the downstream target of the Snf1 protein kinase signal transduction pathway), Hxk2p (hexokinase 2), and Grr1p (unknown function) (22; reviewed in reference 25). Second, glucose induces posttranscriptional events resulting in the rapid, irreversible loss of maltose transport activity (16).

In this study, we used molecular genetic analysis to explore the mechanism of glucose-induced inactivation of maltose permease. Our results show that inactivation in both rich and nitrogen-starvation medium results from a decrease of apparent V_{max} with no change in apparent K_m; in rich medium, loss of maltose transport activity is paralleled by a loss in maltose permease protein; in nitrogen-starvation medium, maltose transport is inactivated by two independent mechanisms, a very rapid inhibition of transport activity and a slower proteolysis of maltose permease protein; and this proteolysis is dependent on endocytosis and vacuolar proteases and is independent of a functional proteosome. In this last regard, our results are consistent with those of a recent study of the glucose-induced inactivation of maltose permease, in which some of the same mutations used in the present study were employed (42). Additionally, our results suggest that differentially phosphorylated species of the Mal61p maltose permease are present in maltose-induced cells and that the distribution of the phosphorylated species varies with the carbon source.

MATERIALS AND METHODS

Strains and growth conditions. The *S. cerevisiae* strains used in this study and their relevant genotypes are listed in Table 1. Cells were grown either in rich medium (1% yeast extract, 2% peptone [YEP]) plus the indicated carbon source or in selection medium (0.67% yeast nitrogen base with ammonium [YNB]) plus the appropriate supplements and the indicated carbon source.

Strain construction. An epitope-tagged allele of the cloned *MAL61* containing

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
CMY1001[a]	*MAT***a** *MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200*	This study
CMY1002	*pep4*Δ (isogenic to CMY1001)	This study
CMY1003	*ren1*Δ::*LEU2* (isogenic to CMY1001)	This study
CMY1004	*end3-ts* (isogenic to CMY1001)	This study
CMY1006	*hxk2*Δ::*URA3* (isogenic to CMY1001)	This study
WCG4a	*MAT***a** *his3-11,15 leu2-3,112 ura3-Δ5 GAL*	16
WCG4-11/21a	*pre1-1 pre2-1* (isogenic to WCG4a)	16
WCG4-11/22a	*pre1-1 pre2-2* (isogenic to WCG4a)	16
YPH500	*MAT*α *leu2-Δ1 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 ura3-52 AGT1 MAL12 MAL31 MAL32*	38
600-1B	*MAT***a** *MAL11 MAL12 MAL13 leu2-3,112 ura3-52*	6
100-1A	*MAT***a** *mal11*Δ::*URA3 MAL12 MAL13 leu2-3,112 ura3-52*	6

[a] The *MAL61/HA* gene in CMY1001 is located at the *MAL1* locus position and replaces the *MAL11* sequence of the wild-type *MAL1* locus (see Materials and Methods).

the sequence encoding the hemagglutinin (HA) epitope at the 5′ end of the open reading frame was constructed by oligonucleotide-directed site-specific mutagenesis with the Bio-Rad Mutagene kit (27). The added sequence encodes 15 residues: a Met residue, the 12-residue epitope derived from the influenza virus HA protein, Pro and Gly residues to act as a hinge separating the epitope from the remainder of the protein, all followed by the Met residue at the start of the *MAL61* coding region. This cloned *MAL61/HA* gene was subcloned into a yeast *LEU2* integrating vector and targeted to the *MAL1* locus of strain 100-1A by using homology between the plasmid copy of *MAL61/HA* and the genomic *mal11*Δ::*URA3* gene, containing an internal deletion of *MAL11*. A single-copy integration strain was chosen by Southern analysis of the transformants, and uracil-negative recombinants were selected by using 5-fluoroorotic acid (4). Maltose fermentation was confirmed as in reference 7. These recombinants were then screened by Southern analysis to confirm the loop-out, and Western blot (immunoblot) analysis was done to confirm the presence of the epitope tag. This procedure replaced at least part, if not all, of the *mal11*Δ::*URA3* gene in strain 100-1A with *MAL61/HA* to produce strain 100-1A::MAL61/HA.

To facilitate the construction of isogenic mutant strains, we introduced a series of yeast selectable markers by crossing strain 100-1A::MAL61/HA to strain YPH500 (Table 1), a maltose-nonfermenting strain lacking a Mal activator gene but carrying *MAL1*-linked and *MAL3*-linked structural genes (*AGT1 MAL12* and *MAL31 MAL32*) (reference 45 and unpublished results). Strain CMY1001 is a maltose-fermenting random segregant containing solely the *MAL1* locus derived from strain 100-1A::MAL61/HA and was used in the following constructions (Table 1).

Strain CMY1002 carries a *pep4*Δ deletion/disruption but is otherwise isogenic to CMY1001. It was constructed by two-step gene replacement with plasmid pPLO2010 (obtained from Steven Nothwehr), which contains a *pep4*Δ allele lacking a *Hin*dIII fragment of the wild-type sequence carried on a *URA3* integrating vector. Plasmid DNA was linearized at a unique *Eco*RI site, which targets integration at the genomic *PEP4*, used to transform CMY1001, and uracil-positive transformants were selected. Uracil dependence and potential loop-out of the *URA3* gene and the adjacent *PEP4* were selected by using 5-fluoroorotic acid, and loss of vacuolar protease function was tested by the APNE (*N*-acetyl-phenylalanine-β-naphthyl-ester) plate assay (4, 50). Deletion of *PEP4* was confirmed by Southern blot analysis.

Strain CMY1003 is isogenic to CMY1001 but contains a *ren1*Δ deletion. Plasmid pSL1572 (obtained from George Sprague) contains a ren1Δ::LEU2 deletion/disruption. The plasmid was digested with *Bam*HI and *Sac*I and used to transform CMY1001 to leucine positive. Replacement of the genomic *REN1* was confirmed by Southern analysis.

Strain CMY1004 carries a temperature-sensitive allele of *END3* constructed by integrative disruption of the genomic copy with plasmid pLC2 (obtained from Howard Reizman). Plasmid pLC2 carries the *end3-ts* gene sequence with a small deletion of the 5′ end of the gene in a *LEU2* integrating vector. Plasmid DNA was digested at a unique *Xho*I site within the open reading frame, which targets integration to the genomic *END3* so as to create a deletion of the genomic copy plus integrate an *end3-ts* gene copy. Southern analysis was used to confirm that the event had occurred as expected (41).

Strains WCG4a, WCG4-11/21a, and WCG11/22a are members of an isogenic strain series containing *PRE1 PRE2*, *pre1-1 pre2-1*, and *pre1-1 pre2-2* alleles, respectively (18). *PRE1* and *PRE2* encode components of the proteasome. None of these strains ferment maltose, because they lack a Mal activator gene, although they carry the *MAL* structural genes. Therefore, to carry out our analyses, we introduced into these strains the *MAL63* Mal activator gene carried on a *URA3* CEN plasmid, pRS316MAL63 (36, 45). To be able to monitor maltose permease protein levels, we also introduced into these strains the *MAL61/HA* allele constructed for this study on a *LEU2* CEN plasmid, pRS315MAL61/HA (45).

Strain CMY1006 is isogenic to CMY1001 but contains a *hxk2*Δ null allele. Plasmid pRB528 (from David Botstein) carries a *hxk2*::*URA3* deletion/disruption. It was digested with *Eco*RI and used to transform CMY1001 to uracil positive. Replacement of the genomic copy of *HXK2* was confirmed by Southern analysis. CMY1006 was transformed with the constitutive Mal activator gene *MAL64-R10* carried on the episomal *HIS3* vector, pRS413 (14).

Inactivation protocol. Cells were grown at 30°C to early log phase (optical density at 600 nm [OD_{600}] 0.1 to 0.3) in rich medium containing 2% maltose, harvested by filtration with cellulose filters, and resuspended in either rich medium or nitrogen starvation medium (1.74 g/liter of yeast nitrogen base without amino acids and without ammonium sulfate) plus either 2% (vol/vol) ethanol or 2% (wt/vol) glucose. At selected time intervals, cells were harvested for Western analysis and maltose transport assays by using the cellulose filters. Growth dilution was calculated as the OD_{600} at time zero divided by the OD_{600} at time *x*.

Maltose transport assay. Maltose transport was measured by the uptake of [^{14}C]maltose as described by Cheng and Michels (9). Cells were harvested by filtration, washed, and resuspended in 0.1 M tartaric acid (pH 4.2) to an OD_{600} of 30. Samples (80 μl) of cells were taken and incubated with [^{14}C]maltose for 10 s at room temperature, washed four times with ice-cold water, and subjected to scintillation counting. With the exception of the results shown in Fig. 3, maltose transport rates were determined with 1 mM maltose. Assays were done in duplicate on at least duplicate cultures.

Western analysis and quantitation of relative protein levels. Cells were harvested, quick frozen in a dry ice-ethanol bath, and stored frozen at −70°C until used for preparation of protein extracts. Total-cell protein extracts were prepared by the method described by Davis et al. (12), with the addition of a protease inhibitor cocktail (2). Where noted, protein kinase and phosphatase inhibitors were also included (24). After assaying, equal amounts of protein (usually about 80 to 100 μg) were loaded into each lane of a standard 10 or 7.5% (where noted) acrylamide gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis by standard methods (29). Duplicate gels were stained with Coomassie blue to ensure even loading, and membranes were stained with amido black to ensure even transfer. Membranes were probed with mouse anti-hemagglutinin antibody (12CA5; Boehringer Mannheim) as the primary antibody and horseradish peroxidase-linked sheep anti-mouse immunoglobulin secondary antibody. Detection was visualized with the enhanced chemiluminescence (ECL) Western blotting kit (Amersham) on ECL-Hyperfilm.

The intensity of the signal was quantitated by scanning with a Beckman DU640 spectrophotometer, and relative Mal61/HA protein levels were determined by comparison of the area under the curve. Western blots were done in duplicate on all samples for duplicate experimental cultures, and relative protein levels were determined twice for each film.

Phosphatase treatment of Mal61/HA. CMY1001 was grown in rich medium under maltose-induced conditions to early log phase as described above. About 15 OD_{600} units of cells was harvested by filtration and quick frozen at −70°C. For the phosphatase-treated samples, the cells were thawed by being resuspended in 200 μl of 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES) buffer (pH 6.0)–1 mM dithiothreitol plus protease inhibitors, and an equal volume of glass beads was added. The mixture was vortexed for 10 min and centrifuged to recover the membrane fraction in the pellet. The pellet was resuspended in the PIPES buffer described above for the acid phosphatase treatment or in 0.1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) plus the protease inhibitors for the alkaline phosphatase treatment. The indicated numbers of units of acid phosphatase (Sigma) or alkaline phosphatase (Boehringer Mannheim) were added to the membrane preparations, and treatment was carried out at 37°C for 1 h. Following this, protein was extracted from the membranes by the same procedure as described above for whole cells and size separated by SDS-PAGE in 7.5% acrylamide gels run at constant amperage. Western blot analysis was done as described above. The sample receiving no treatment, labeled None in Fig. 7A, was extracted directly from whole cells as described above.

RESULTS

Construction, expression, and characterization of *MAL61/HA*, a hemagglutinin-tagged allele of *MAL61*. To monitor the level of maltose permease protein, we constructed an HA epitope-tagged allele of *MAL61*, referred to as *MAL61/HA* (described in Materials and Methods). This 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*

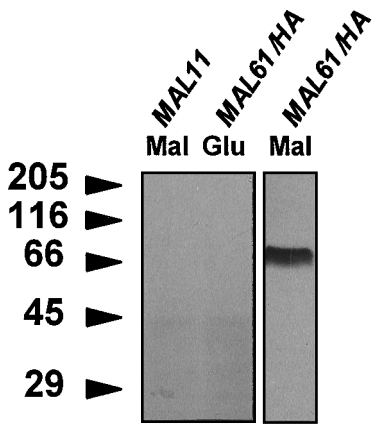

FIG. 1. Characterization and regulation of the Mal61/HA protein. Strains 600-1B and 100-1A(pMAL61/HA) were grown in rich medium plus 2% maltose or 2% glucose. Protein extracts were prepared and analyzed by Western blotting, as described in Materials and Methods.

sequence. This strain was crossed to YPH500 (Table 1), and strain CMY1001 (Table 1) is a maltose-fermenting segregant carrying only this reconstructed *MAL1* locus (*MAL61*/*HA*, *MAL12*, and *MAL13* encoding maltose permease, maltase, and the Mal activator respectively) and no other *MAL* genes. Thus, *MAL61*/*HA* is the sole maltose permease gene present in the strain.

Two isogenic *MAL1* strains, one containing the wild-type *MAL11* gene (strain 600-1B) and the other containing *mal11*Δ::*URA3* but transformed with a plasmid carrying *MAL61*/*HA* (strain 100-1A[pMAL61/HA]), were grown in selection medium on either 2% maltose or 2% glucose as the carbon source. As can be seen in Fig. 1, no cross-reacting species of protein is detected in the strain lacking the HA-tagged allele under these growth conditions. Additionally, the expression of *MAL61*/*HA* is maltose induced. The induced level of maltose transport activity is comparable to that in strains carrying the wild-type allele (data not shown). Moreover, the K_m of the Mal61/HA maltose permease (approximately 1 to 2 mM) is not significantly different from that reported for wild-type Mal61p (9).

Glucose-induced inactivation of maltose transport exhibits two components in nitrogen starvation medium but only one in rich medium. CMY1001 cells were grown to early log phase in rich medium containing 2% maltose, harvested, and transferred to either rich medium (YEP) or nitrogen starvation medium (YNB without amino acids or ammonium) with the indicated carbon source. At the times shown following the transfer, maltose transport activity was assayed and total-cell protein extracts were prepared. The relative amount of Mal61/HA protein in the total-cell extracts was determined by Western analysis of size-separated proteins with the anti-HA antibody as described in Materials and Methods. Each of these measurements (growth dilution, maltose transport rate, and maltose permease protein levels) was then plotted in a single graph relative to the value at the time of glucose addition. Semi-log plots are used because they demonstrate the kinetics of the glucose-induced responses more clearly than linear plots do.

Figure 2 compares the effects of glucose and ethanol on maltose transport and maltose permease protein levels in rich medium and nitrogen starvation medium. Several conclusions can be drawn. Glucose, but not ethanol, stimulates the inactivation of maltose transport and a decrease in level of maltose permease protein. In rich medium, the glucose-induced loss in transport activity parallels the loss in maltose permease protein levels in what appears to be a single process with simple kinetics. In contrast, in nitrogen starvation medium, the glucose-induced decrease in transport activity is more rapid than the decrease in protein levels at early time points. Ninety percent of the activity is lost within the first 60 min. On the other hand, loss of maltose permease protein occurs at the same rate as is seen in rich medium (half-life, approximately 45 min). These results indicate that when strain CMY1001 is transferred to nitrogen starvation medium plus glucose, two kinetically distinct processes contribute to the inactivation of maltose permease. The first is a rapid loss of the transport activity, which we refer to as the inhibition of transport activity, and the second is a slower loss of transport activity, which correlates with the proteolytic degradation of the maltose permease protein. The rapid inhibition of transport activity is not seen in rich medium, and Riballo et al. (42) did not see evidence of this inhibition in their studies, in which different strains were used. Moreover, in our analysis of strains carrying *PRE* mutations (described below), which was done with the same strains as those used by Riballo et al. (42), we also did not detect the rapid inhibition component.

Glucose affects the V_{max} but not the K_m of maltose transport. We carried out a kinetic analysis of the maltose transport activity remaining after 50% inactivation to determine whether the rapid inactivation seen in nitrogen starvation medium represents an increase in the K_m of the maltose transporter or a decrease in the V_{max}. CMY1001 cells were grown in rich medium plus maltose, harvested, and subjected to glucose inactivation in rich or nitrogen starvation medium. Samples were taken at time zero and at the indicated time at which 50% loss in transport activity was achieved in each medium. Lineweaver-Burk analysis of the results is shown in Fig. 3. As expected for the inactivation in rich medium, a 50% decrease in V_{max} was observed with no significant change in the K_m (approximately 1.25 mM). In nitrogen starvation medium, the V_{max} also is reduced approximately 50% with no apparent change in K_m. Thus, in both media, the amount of functional maltose permease is reduced with no change in kinetic characteristics, as had been suggested in a previous report (38).

Mechanism of proteolysis of maltose permease. We explored the pathway of proteolysis of the maltose permease protein by using mutations in genes known to control early steps in endocytosis (*END3*), vesicle targeting to the vacuole (*REN1*), vacuolar proteolysis (*PEP4*), and proteosome activity (*PRE1*, *PRE2*).

A temperature-sensitive mutation of *END3* was introduced into strain CMY1001 by targeted integrative disruption of the genomic copy of the gene by using a cloned copy of an *end3-ts* allele (34). Glucose-induced inactivation of maltose permease was monitored in the parental (CMY1001) and *end3-ts* (CMY1004) strains according to the inactivation protocol described in Materials and Methods, with the following exception. The strains were grown under induced conditions at the permissive temperature of 25°C and transferred to nitrogen starvation medium equilibrated to the nonpermissive temperature of 35°C.

Clearly, *END3* is required for the glucose-induced proteolysis of maltose permease protein (Fig. 4). The level of maltose permease protein in the *end3-ts* strain grown induced at the permissive temperature is approximately two- to threefold higher than that found in the isogenic parent strain (data not shown), and this increased protein level is reflected in an increased rate of maltose transport (Table 2). Interestingly, the rapid inhibition of maltose transport is still seen immediately after glucose addition at the nonpermissive temperature, sug-

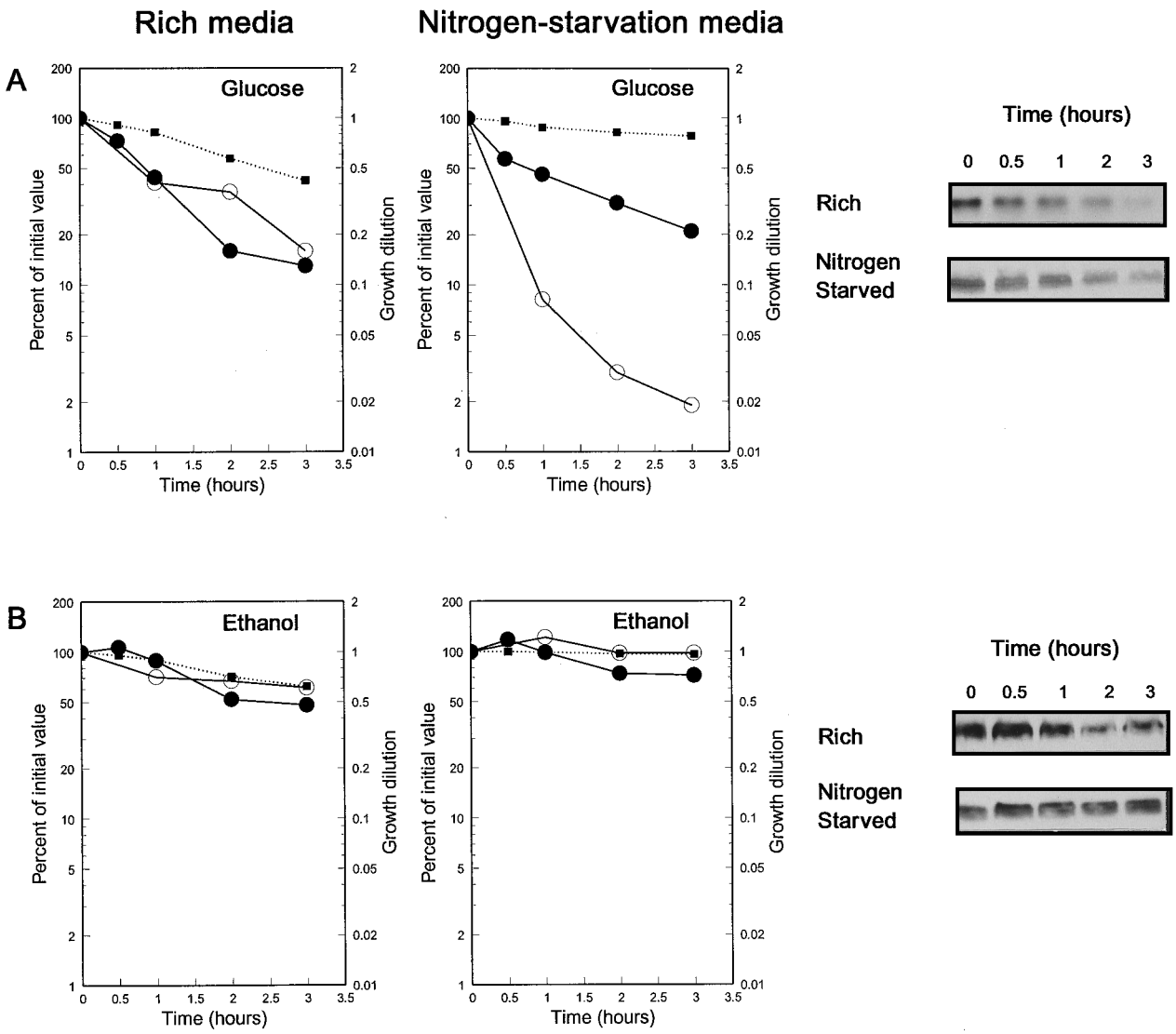

FIG. 2. Glucose-induced inactivation of maltose permease in rich medium and nitrogen-starvation medium. Strain CMY1001 was grown in rich medium plus 2% maltose, harvested, and transferred to either rich medium or nitrogen starvation medium plus either 2% glucose (A) or 2% ethanol (B). At the indicated times, the OD_{600} was determined and aliquots of the culture were removed for maltose transport assay and the preparation of total protein extracts for Western analysis of Mal61/HA protein levels, as described in Materials and Methods. Representative Western blots are shown, but the quantitation data used in the graph was obtained from the average of at least two experiments, each run on duplicate gels, and each scanned twice. The relative levels of Mal61/HA protein (●) and maltose transport activity (○) compared with the zero time sample are plotted along with the growth dilution (■). Growth dilution represents the growth of the culture during the course of the experiment and is calculated as the OD_{600} at time zero divided by the OD_{600} at time *x*.

gesting that the inhibition of transport activity is independent of the proteolysis process or is upstream of the End3p function.

The *ren1*Δ and *pep4*Δ mutations were created in CMY1001 by one-step gene replacement. *PEP4* encodes vacuolar proteinase A, which is required for vacuolar proteolysis and for the enzymatic activation of all of the vacuolar proteases including itself (1, 19). Ren1p (Vps2p) functions late in endocytosis and appears to be involved in vesicle transport from the endosome to the vacuole (12). Inactivation of maltose permease was monitored in these strains, and the results are shown in Fig. 5. No glucose-induced proteolysis of maltose permease is seen in either mutant strain. Quantitation of the relative amounts of maltose permease protein in these mutant strains compared with those in the isogenic wild-type strain shows approximately two- to threefold-higher levels in the *pep4*Δ strain but no comparable increase in maltose transport (Table 2) and two- to threefold lower levels of maltose permease protein in the *ren1*Δ strain with a coordinate decrease in transport rate (Table 2). The rapid inhibition of maltose transport is unaffected in both the *ren1*Δ and *pep4*Δ strains.

Finally, we tested the role of the proteosome in the glucose-induced proteolysis of maltose permease by using strains containing mutations in *PRE1* and *PRE2*, encoding components of the proteosome (18). An isogenic series of strains of the genotype *PRE1 PRE2* (WCG4a), *pre1-1 pre2-1* (WCG4-11/21a), and *pre1-1 pre2-2* (WCG4-11/22a) were transformed with CEN plasmids carrying the HA-tagged *MAL61/HA* allele and the inducible Mal activator gene *MAL63* (required because these strains lacked a Mal activator gene and an appropriately tagged maltose permease). Glucose-induced inactivation of maltose permease was monitored by the standard inactivation protocol, and the results are shown in Fig. 6.

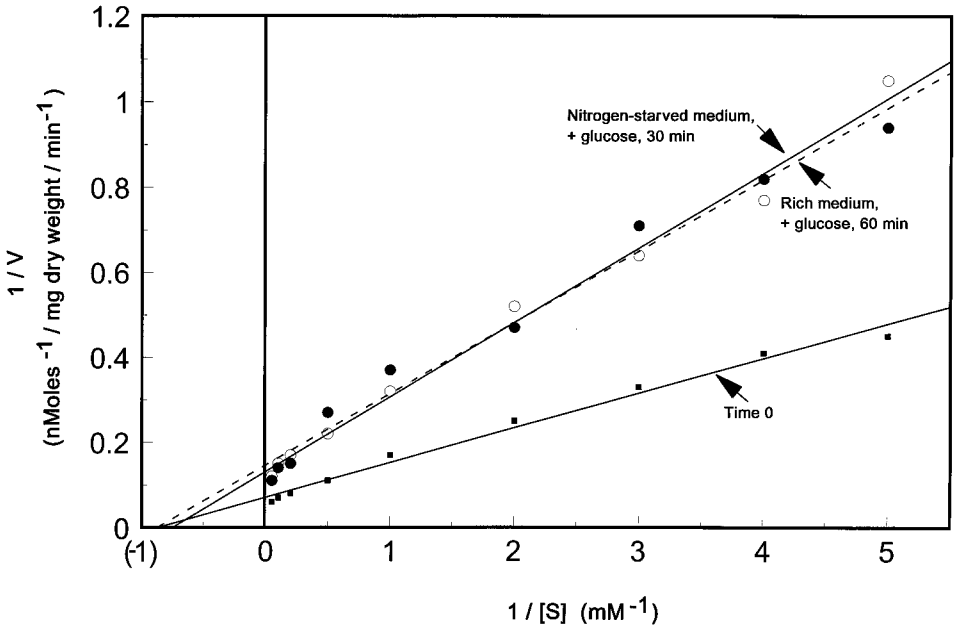

FIG. 3. Lineweaver-Burk plot of maltose transport in maltose-induced and glucose-inactivated cells. Strain CMY1001 was grown in rich medium and allowed to undergo a 50% glucose-induced inactivation in either rich (○) or nitrogen starvation (●) medium, as described for Fig. 2. At the indicated times, maltose transport was determined at a range of substrate concentrations. These values were compared with maltose transport in cells at time zero before inactivation (■).

No significant difference can be seen between the *pre1 pre2* mutant strains and the isogenic wild-type strain in the rate of glucose-induced proteolysis of maltose permease protein. Interestingly, the rapid glucose-induced inhibition of maltose transport seen in those strains isogenic to CMY1001 is not evident in these strains. Instead, the loss of maltose transport activity correlates with the loss of maltose permease protein, even under the nitrogen starvation conditions, as reported by Riballo et al. (42).

Differentially phosphorylated forms of Mal61/HA protein. The anti-HA antibody detects at least two species of Mal61/HA protein, which exhibit slightly different mobilities under the gel conditions used in the experiment in Fig. 7A. Treatment of total protein extracts with increasing amounts of acid phosphatase decreased the relative amount of the more slowly migrating form(s). With the maximal treatment, only the faster-migrating form was evident. Alkaline phosphatase treatment had no effect. This result suggests that the Mal61/HA maltose permease is differentially phosphorylated in maltose grown cells.

Preliminary studies were carried out to explore the possibility that the extent of phosphorylation of Mal61/HA protein varied in cells grown on different carbon sources. For this, we needed to express Mal61/HAp at detectable levels even under uninduced and glucose-repressed conditions. This was achieved by disrupting the *HXK2* gene of CMY1001 and introducing a copy of the *MAL64-R10*-constitutive Mal activator gene to produce strain CMY1006(pMAL64-R10) (see Materials and Methods) (Table 1). In work to be reported elsewhere, we showed that glucose repression of *MAL61/HA* transcription is almost fully relieved by disruption of *HXK2* but that the kinetics of both the glucose-induced inhibition of maltose transport and the proteolysis of maltose permease are unaffected in the *hxk2*Δ mutant strain when assayed using the inactivation protocol described here (23). Strain CMY1006(pMAL64-R10) was grown in selective medium with either 2% maltose, 2% glucose, or 2% ethanol as the carbon source. Total protein extracts were prepared with extraction buffer containing protease inhibitors and protein kinase and protein phosphatase inhibitors. Western analysis was carried out on different dilutions of protein extract to ensure that the intensity of the band was linearly related to protein levels. As can be seen in Fig. 7B, carbon sources affect the pattern of distribution of the phosphorylated species of Mal61/HAp and cells grown on glucose appear to accumulate the fully phosphorylated form.

DISCUSSION

Gorts (16) first described the glucose-induced inactivation of maltose transport. He noted that the inactivation took about 90 min and was irreversible in the absence of de novo protein synthesis under maltose-induced conditions. Kinetic analysis of maltose transport in *S. cerevisiae* strains identified both high-affinity (K_m, 2 to 4 mM) and low-affinity (K_m, about 70 mM) components (5, 9). Using genetically defined strains carrying a single maltose permease gene (*MAL61* or *MAL11*), Cheng and Michels (9) demonstrated that only the high-affinity component is the product of that gene. This report focuses on the glucose-induced inactivation of the high-affinity maltose permease encoded by *MAL61*. By using a strain expressing only a HA epitope-tagged allele of *MAL61*, we were able to monitor both maltose transport rates and levels of maltose permease protein and to directly compare the two.

We show that glucose but not ethanol stimulates the proteolysis of maltose permease in both rich medium and nitrogen starvation medium, a finding that is consistent with previous reports (31, 42). The half-life of Mal61/HAp measured under nitrogen starvation conditions, when little or no cell growth is occurring, is 8 h or greater in ethanol and 30 to 60 min in glucose. The half-life of several *Saccharomyces* membrane proteins has been determined and shown to vary from 13 min to 30 h depending on the protein, and for most of these proteins, the half-life is regulated by the physiological state of the cell and/or the presence or absence of ligand (3, 12, 26, 30, 39, 49). The

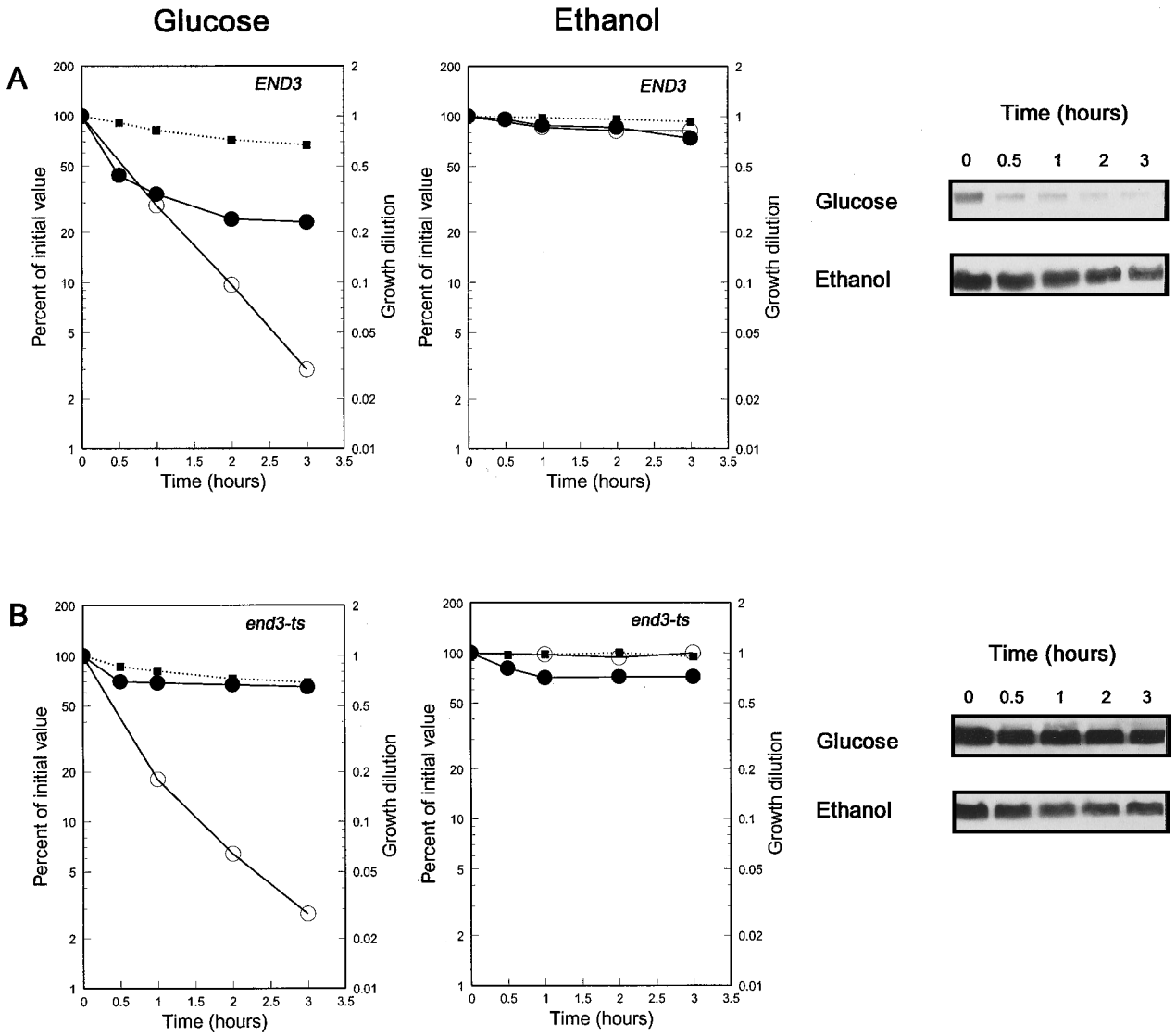

FIG. 4. Glucose-induced inactivation of maltose permease in an *end3-ts* strain defective in early endocytosis. Strains CMY1001 (A) and CMY1004 (*end3-ts*) (B) were grown as described in Materials and Methods except that growth took place at 25°C, harvested, and transferred to nitrogen starvation medium containing 2% glucose or 2% ethanol at 35°C. Samples were taken at the indicated times, and the growth dilution (■), maltose transport rate (○), and relative Mal61/HA protein levels (●) were determined, as described in Materials and Methods.

rate of degradation of the uracil permease is increased under a variety of stress conditions including starvation for carbon, phosphate, and nitrogen (49). On the other hand, degradation of the otherwise stable inositol permease is induced by the addition to the growth medium of inositol, which appears to stimulate a ligand-induced change in the protein targeting it to the degradation pathway (30). Recently reported studies of the α-factor receptor Ste2p showed that α-factor stimulated the already high constitutive rate of turnover (20).

Endocytosis and vacuolar proteolysis have been demonstrated to make up the pathway of degradation for all of the *Saccharomyces* membrane proteins studied to date, and maltose permease is no exception (3, 12, 20, 26, 30, 41, 42, 46, 49). Isogenic strains carrying mutations in *END3*, *REN1* (*VPS2*), or *PEP4* all lack glucose-induced proteolysis of the Mal61/HA maltose permease protein. Both the *end3-ts* (at the permissive temperature) and the *pep4*Δ strains accumulate two- to three-fold-higher levels of Mal61/HA protein than does the wild

TABLE 2. Maltose transport rates of strains mutated in genes required for endoytosis, vesicle trafficking, and proteolysis[a]

Strain	Relevant genotype	Transport rate (nmol/mg [dry wt]/min)
CMY1001	*END3 REN1 PEP4*	2.40, 2.47 (25°C)
CMY1002	*pep4*Δ	2.91
CMY1003	*ren1*Δ::*URA3*	1.49
CMY1004	*end3-ts*	4.84 (25°C)
WCG4a[pMAL61/HA][pMAL63]	*PRE1 PRE2*	3.23
WCG4-11/21a[pMAL61/HA][pMAL63]	*pre1-1 pre2-1*	2.63
WCG4-11/22a[pMAL61/HA][pMAL63]	*pre1-1 pre2-2*	3.77

[a] The strains were grown in rich medium with 2% maltose to early log phase at 30°C, unless otherwise indicated. Maltose transport rates were determined as described in Materials and Methods.

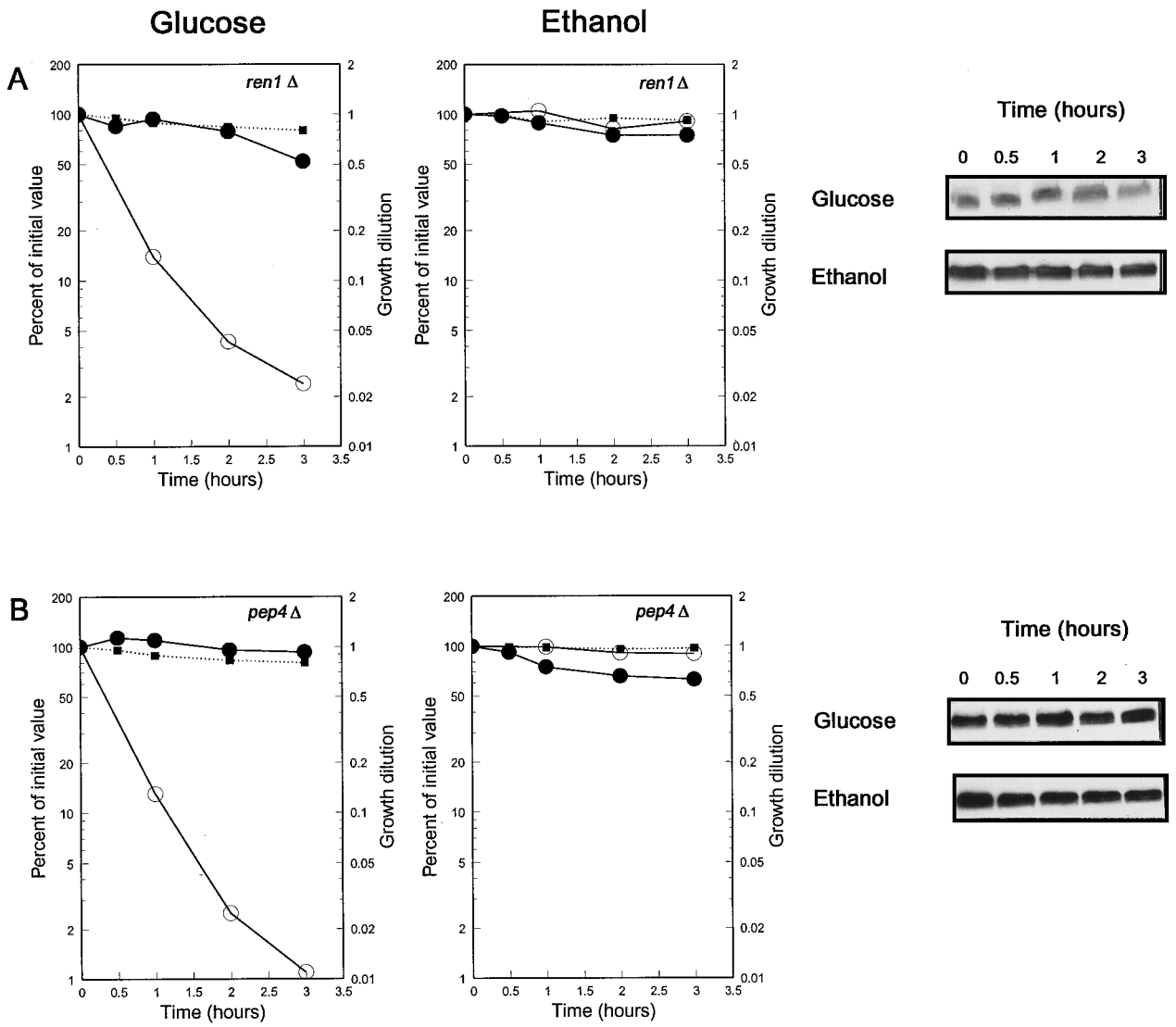

FIG. 5. Glucose-induced inactivation of maltose permease in a *pep4*Δ and a *ren1*Δ strain defective in vacuolar proteolysis and vesicle trafficking to the vacuole, respectively. Strains CMY1002 (*ren1*Δ) (A) and CMY1003 (*pep4*Δ) (B) were grown as described for Fig. 1, harvested, and transferred to nitrogen starvation medium containing 2% glucose or 2% ethanol, and at the indicated times, the growth dilution (■), maltose transport rate (○), and relative Mal61/HA protein levels (●) were determined, as described in Materials and Methods.

type, but only the *end3-ts* strain exhibits comparably higher rates of maltose transport. *END3* encodes a very early function in the endocytosis pathway, and this result suggests that the accumulated permease in the *end3-ts* strain is present at the plasma membrane in a functional form. Only a modest increase in maltose transport is seen in the *pep4*Δ strain, despite the abundant increase in levels of Mal61/HAp; this suggests that the permease is not at the plasma membrane but, instead, is in an internal compartment(s) such as the vacuole. The finding that the *ren1*Δ strain exhibits reduced levels of Mal61/HAp is somewhat surprising, considering that the *ren1-1* allele was selected for its ability to accumulate **a**-factor receptor at the cell surface as a result of a reduced rate of internalization of ligand-bound Ste3p (12). We had expected that the *ren1*Δ strain used in this study would accumulate Mal61/HAp and would exhibit higher levels of maltose transport if the maltose permease were able to recycle to the plasma membrane from the endosome, as suggested by Riballo et al. (42). Perhaps these unexpectedly low levels of maltose permease can be attributed to our use of the *ren1*Δ as opposed to the *ren1-1* allele. If so, this suggests a role for Ren1p in secretion as well as in targeting vesicles to the vacuole, at least for the maltose permease.

We found that in nitrogen starvation medium, the CMY1001 isogenic strains used in this study exhibited a loss of maltose transport activity that was more rapid than the loss of maltose permease protein, suggesting that inhibition of maltose permease specific activity, in addition to its proteolysis, is occurring. This inhibition was not reported by Riballo et al. (42), and we believe that it reflects differences in the strains used in the two studies. Consistent with the results of Riballo et al. (42), we also did not detect the inhibition component when we monitored the glucose-induced inactivation of Mal61/HA maltose permease in the WCG4a isogenic series of strains (Fig. 6).

Among the mechanisms one could propose for the initial glucose-induced inhibition of maltose transport activity are

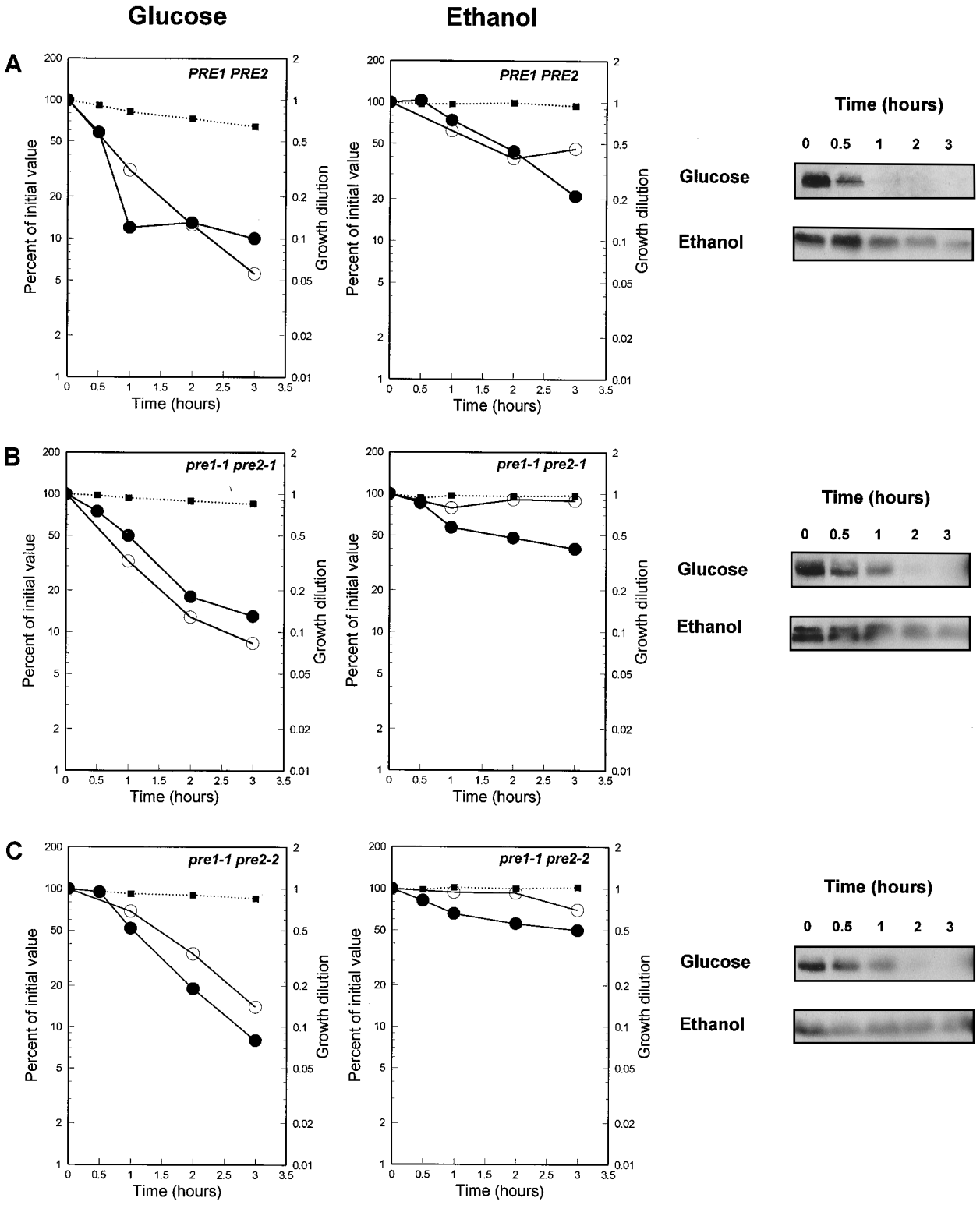

FIG. 6. Glucose-induced inactivation of maltose permease in *pre1 pre2* mutant strains with defects in proteosome function. Strains WCG4a (*PRE1 PRE2*) (A), WCG4-11/21a (*pre1-1 pre2-1*) (B), and WCG4-11/22a (*pre1-1 pre2-2*) (C) were all transformed with plasmids pMAL61/HA and pMAL63 to allow these host strains to express maltose-inducible *MAL61/HA*. The transformed strains were grown in selection medium plus 2% maltose and transferred to nitrogen starvation medium containing 2% glucose or 2% ethanol, and at the indicated times, the growth dilution (■), maltose transport rate (○), and relative Mal61/HA protein levels (●) were determined, as described in Materials and Methods.

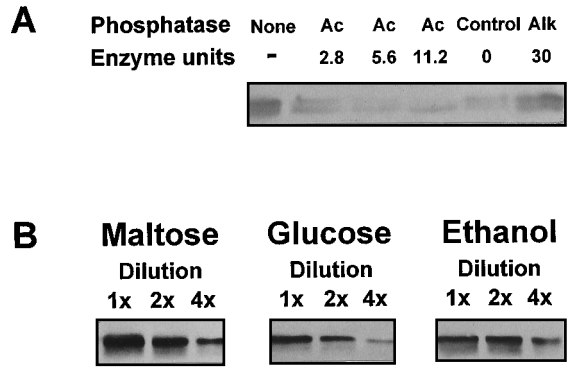

FIG. 7. Phosphorylation of Mal61/HAp. (A) Strain CMY1001 was grown in rich medium plus 2% maltose. A crude membrane preparation was made and treated with the indicated number of units of either alkaline or acid phosphatase for 1 h at 37°C. The lane labeled None is a sample extracted from whole cells with sample buffer and given no treatment at all, and the lane labelled Control was treated as were the acid phosphatase-treated samples but without added phosphatase enzyme. Differences in the total amounts of Mal61/HAp in the different lanes are the result of differences in the treatment procedures used for the acid versus alkaline phosphatase assays and the approximately threefold smaller amount of membrane preparation that was used for the acid phosphatase treatment. Protein extracts were prepared and analyzed by Western blotting, as described in Materials and Methods. The SDS-PAGE was done with 7.5% acrylamide gels at constant amperage to achieve the separation shown. (B) Strain CMY1006(pMal64-R10) was grown in rich medium plus either 2% maltose, 2% glucose, or 2% ethanol. Total protein extracts were prepared with extraction buffer containing a cocktail of protein kinase and phosphatase inhibitors (24). Three different dilutions of each extract were used for Western analysis.

posttranslational modification, alteration in subcellular compartment or structural conformation, and interaction with a negative regulatory factor. Phosphorylation of fructose-1,6-bisphosphatase at a site near the N-terminal end following glucose addition to the medium is responsible for the rapid inhibition of its enzyme activity, but the glucose-induced proteolysis of this enzyme does not appear to require phosphorylation at this site (32, 34, 43, 47). In contrast, the general amino acid permease, Gap1p, is phosphorylated in the active state, and dephosphorylation of Gap1p is correlated with its rapid inhibition in response to rich nitrogen sources (46). The finding that the inhibition of maltose permease occurs at wild-type rates in the *end3-ts* mutant strain, in which endocytosis is severely depressed, suggests that a change in compartment is not involved. The mechanism of this inhibition and the basis of these apparent strain differences are under investigation.

The rapid inhibition of maltose permease transport activity does not appear to be a prerequisite for the proteolysis of maltose permease, since the proteolysis occurs in strains and/or under growth conditions when inhibition is absent. Inhibition appears to occur early in inactivation, prior to the End3p-dependent step, and this is unlikely to represent a change in compartment. Moreover, both the inhibition of maltose transport and the proteolysis of maltose permease result in a decrease of V_{max} but not a change in K_m, which is consistent with results of studies reported by Busturia and Lagunas (5) but not with those of Peinado and Loureiro-Dias (38), who found that glucose induced an increase in K_m but no change in V_{max}. Taken together, these results suggest that the inhibition process occurs to maltose permease in the plasma membrane and could be a modification of the protein that fully inhibits function. Whatever the process, there appears to be strain-dependent variation of an as yet unidentified gene(s) and/or physiological conditions required for inhibition.

Two species of maltose permease with different mobilities in SDS-PAGE analysis were reported in studies of a maltose-fermenting strain of undefined *MAL* genotype (31, 48). Lucero et al. (31) detected maltose permease protein by using a polyclonal antibody, and it was therefore not clear whether the two species represented different modified forms of the permease or whether the antibody also detected Agt1p, a second maltose permease present in many laboratory strains (17). Analysis of the endopeptidase Lys-C peptides by Van den Broek et al. (48) suggested that the two forms were highly sequence homologous, indicating that Agt1p is an unlikely candidate for either species (17). We report here that the Mal61/HA maltose permease is present in differentially phosphorylated forms in maltose-grown cells and that the hyperphosphorylated species accumulate in glucose-grown cells. Phosphorylation has been implicated in the receptor-mediated endocytosis of the *Saccharomyces* α-factor receptor and in several mammalian membrane receptors, including the insulin receptor and the asialoglycoprotein receptor (15, 28, 51). The transport activity of the *Saccharomyces* general amino acid permease, Gap1p, is activated by phosphorylation, and the level of Gap1p phosphorylation is nitrogen source regulated (38). Similarly, the human insulin-responsive glucose transporter *GLUT4* is functionally activated in the phosphorylated state (24). The *Saccharomyces* uracil permease is also differentially phosphorylated, but the functional significance of this phosphorylation is unknown (49). Thus, phosphorylation of maltose permease might affect its transport activity and/or turnover. We are currently investigating Mal61/HAp phosphorylation in detail, with particular interest in its role, if any, in transport activity and glucose-induced inhibition and/or proteolysis.

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Igor Medintz and Hua Jiang contributed equally to this work.

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