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Two Glucose Sensing/Signaling Pathways Stimulate Glucose-induced Inactivation of Maltose Permease in *Saccharomyces*

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Glucose is a global metabolic regulator in *Saccharomyces*. It controls the expression of many genes involved in carbohydrate utilization at the level of transcription, and it induces the inactivation of several enzymes by a posttranslational mechanism. *SNF3*, *RGT2*, *GRR1* and *RGT1* are known to be involved in glucose regulation of transcription. We tested the roles of these genes in glucose-induced inactivation of maltose premease. Our results suggest that at least two signaling pathways are used to monitor glucose levels. One pathway requires glucose transport and the second pathway is independent of glucose transport. Rgt2p, which along with Snf3p monitors extracellular glucose levels, appears to be the glucose sensor for the glucose-transport–independent pathway. Transmission of the Rgt2p-dependent signal requires Grr1p. *RGT2* and *GRR1* also play a role in regulating the expression of the *HXT* genes, which appear to be the upstream components of the glucose-transport–dependent pathway regulating maltose permease inactivation. *RGT2–1*, which was identified as a dominant mutation causing constitutive expression of several *HXT* genes, causes constitutive proteolysis of maltose permease, that is, in the absence of glucose. A model of these glucose sensing/signaling pathways is presented.

INTRODUCTION

Glucose controls several aspects of cellular metabolism in *Saccharomyces*, particularly those involved in carbohydrate utilization. The expression of genes for the utilization of other carbon sources, such as maltose, galactose, sucrose, and ethanol, is regulated by glucose at both the transcriptional and posttranslational levels (reviewed in Holtzer, 1976; Gancedo, 1992; Johnston and Carlson, 1992; Trumbly, 1992). Analysis of glucose repression of transcription of the *SUC* and *GAL* genes has revealed several genes required for the response to glucose, including a DNA-binding repressor (*MIG1*), its inhibitor (*SNF1*), and a hexokinase (*HXK2*). Transcription of other genes is induced by glucose through a different regulatory mechanism that involves a DNA-binding repressor (*RGT1*), its inhibitor (*GRR1*), and glucose sensors (*SNF3* and *RGT2*); (Özcan and Johnston, 1995; Özcan *et al.*, 1996a,b). Interestingly, one of these regulators, *GRR1*, has been implicated in G_1 cyclin turnover as well, suggesting a role for glucose in the regulation the *Saccharomyces* cell cycle (Barral *et al.*, 1995).

In addition to its effects on transcription, glucose also speeds adaptation to glucose fermentation by a posttranslational regulatory mechanism referred to as glucose-induced inactivation. Glucose induces a rapid loss in activity of the enzymes of the gluconeogenic pathway (fructose 1,6-bisphosphatase, cytoplasmic malate dehydrogenase, isocitrate lyase, and phosphoenolpyruvate carboxykinase) and of several sugar transporters (the high-affinity glucose transporter, galactose permease, and maltose permease) by stimulating an increase in the rate of degradation of these enzymes (reviewed in Holtzer, 1976; Chiang and Schekman, 1991; Riballo *et al.*, 1995; Medintz *et al.*,

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Table 1. List of *Saccharomyces* strains

Strain	Genotype	Reference
CMY1001	*MAT***a** *MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101trp1-Δ63 his3-Δ200*	Medintz *et al.* (1996)
CMY1005	*grr1Δ* (isogenic to CMY1001)	This study
CMY1007	*snf3Δ* (isogenic to CMY1001)	This study
CMY1008	*rgt1Δ* (isogenic to CMY1001)	This study
CMY1009	*rgt2Δ* (isogenic to CMY1001)	This study
CMY1010	*grrlΔrgt1Δ* (isogenic to CMY1001)	This study
CMY1012	*snf3Δrgt2Δ* (isogenic to CMY1001)	This study

1996). Here, we describe an investigation of the glucose sensing/signaling pathways used for the glucose-induced inactivation of maltose permease.

Maltose transport is subject to glucose-induced inactivation (Görts, 1969; Alonso and Kotyk, 1978; Busturia and Lagunas, 1985; Cheng and Michels, 1991). We previously found that glucose, but not ethanol, inactivated maltose transport activity in two steps: it caused an initial very rapid loss of transport activity associated with little decrease in protein levels, and a slower loss of transport activity associated with a loss in maltose permease protein. We and others demonstrated that this glucose-induced proteolysis of Mal61/HAp is dependent on endocytosis and vacuolar degradation and is independent of proteasome function (Riballo *et al.*, 1995; Medintz *et al.*, 1996).

In this report, we explore the role of *SNF3*, *RGT2*, *GRR1*, *RGT1*, and high-affinity glucose transport in glucose sensing/signaling for glucose-induced inactivation of maltose permease. Our results suggest that Rgt2p and Grr1p play a central role in sensing/signaling the presence of extracellular glucose and stimulating glucose-induced inactivation of maltose permease. We propose that both glucose-transport–dependent and glucose-transport–independent signaling pathways contribute to the induction of the inactivation process.

MATERIALS AND METHODS

Strains, Strain Construction, and Plasmids

The *Saccharomyces* strains used in this study (Table 1) are derived from CMY1001 (*MAT***a** *MAL61/HA MAL12 MAL13 GAL*$^+$ *leu2 ura3–52 lys2–801 ade2–101 trp1-Δ63 his3–200*), which is described in detail in Medintz *et al.* (1996). A series of disruption mutations were made in CMY1001 as described below to produce the isogenic strains: CMY1005 (*grr1Δ*), CMY1007 (*snf3Δ*), CMY1008 (*rgt1Δ*), CMY1009 (*rgt2Δ*), CMY1010 (*grr1Δrgt1Δ*), and CMY1012 (*snf3Δrgt2Δ*).

Strain CMY1005 carries a *grr1* deletion/disruption constructed by one-step gene replacement. A *TRP1* CEN plasmid was introduced into CMY1001 before the *grr1Δ* mutation was introduced, because *grr1* mutant strains are unable to transport tryptophan. Plasmid pBM1829 was a gift from Mark Johnston (Flick and Johnston, 1991) and contains *grr1::LEU2*. The plasmid was digested with *Nde*I and *Sph*I prior to transformation. Deletion of *GRR1* was confirmed by Southern blot analysis.

In strain CMY1007, the *SNF3* gene is replaced by *snf3::HIS3* from pRR4. Plasmid pRR4 was obtained from Marian Carlson (Neigeborn *et al.*, 1986) and contains a *snf3::HIS3* deletion/disruption. This plasmid was digested with *Sal*I and *Eco*RI to facilitate the gene replacement. Histidine$^+$ transformants were selected and deletion of *SNF3* was confirmed by Southern blot analysis.

RGT1 was disrupted in CMY1001 and CMY1005 using plasmid pBM2861 provided by Mark Johnston (Özcan and Johnston, 1995). The plasmid was digested with *Bgl*II, transformed into each of the strains listed, and uracil$^+$ transformants were selected. Replacement of *RGT1* was confirmed by Southern blot analysis.

In strains CMY1009 and CMY1012, the open reading frame of *RGT2* gene was replaced by *kan*^{R} by using a polymerase chain reaction (PCR)-based gene disruption. The *kan*^{R} gene from the *Escherichia coli* transposon Tn*903* when expressed in yeast results in aminoglycoside antibiotic G418 resistance of the transformants. For the PCR, plasmid pBM3251 containing *kan*^{R} (obtained from Sabire Özcan and Mark Johnston) was used as the template. The 5′ oligonucleotide contains 45 bp upstream of the ATG of *RGT2* followed by 19 bp from *kan*^{R} 5′ sequence (5′-CAGAAACCACTATATATATATGGAAATATCTCGAATATTGCTTGTCAGCTGAAGCTTCGTACGC3′). The 3′ oligonucleotide contains 45 bp from *RGT2* 3′ sequence followed by 22 bp of *kan*^{R} 3′ sequence (5′-AAACGGTTTATAAGACCTCGAACGATCGTAAGATGCTATTGGTTTGCATAGGCCACTAGTGGATCTG-3′). The PCR product was transformed directly into CMY1001 and CMY1007, and transformants were selected on YPD supplemented with 50 mg/l Geneticin (G418 sulfate from Life Technologies, Gaithersburg, MD). The method of yeast transformation and selection for kanamycin-resistant transformants were described in Guldener *et al.* (1996). Deletion of the *RGT2* gene was verified by Southern blot analysis.

Plasmid pBM3270 (pRGT2–1) is a *URA3* CEN plasmid containing dominant *RGT2–1* allele. Plasmid BF307 (pADH1-HXT1) has the entire *HXT1* coding region under *ADH1* promoter on pRS316 vector. All of these plasmids were obtained from Sabire Özcan and Mark Johnston, Washington University School of Medicine, St. Louis.

Inactivation Protocol

The protocol is described in detail in Medintz *et al.* (1996). Cells were grown at 30°C to very-early logarithmic phase (OD_{600} of 0.1–0.3 U) in rich medium containing 2% maltose, harvested, and transferred to nitrogen-starvation medium (1.74 g/l yeast nitrogen base without amino acids and ammonium sulfate) plus 2% glucose (or 2% ethanol, or rich medium plus 2% glucose, as indicated). Cells were harvested at the indicated times over a 3-h period, and for each sample, maltose transport rates were determined and total cell extracts were prepared. The Mal61/HA protein in the extracts was visualized by Western blot analysis of SDS-PAGE gels using the anti-hemagglutinin (HA)-specific antibody, and the relative amount was measured by densitometric comparison to the zero time sample. Western blot analysis was done in duplicate on duplicate sam-

ples of cell extract from separate cell cultures. Growth dilution was calculated as the OD_{600} at time 0 divided by OD_{600} at time *X*.

Sugar Transport Assays

Maltose transport was measured as the uptake of 1 mM [^{14}C]maltose, as described in Cheng and Michels (1991). Similar methods were used to measure the uptake of [^{14}C]glucose, with the exception that the substrate concentration was varied to determine the K_m and V_{max} of glucose transport for the maltose-grown cells. Assays were done in duplicate on at least duplicate cultures.

Maltase Assay

Maltase activity was determined as described by Dubin *et al.* (1985). The values reported are the average of duplicate assays obtained with extracts from at least two separate cultures.

RESULTS

The parental strain used in this analysis (see Medintz *et al.*, 1996, for full description) contains only a single maltose permease gene that is tagged with the HA epitope. The HA epitope is located at the N terminus of the protein and has no measurable effect on the kinetic or regulatory properties of the transporter. Thus, in strain CMY1001 one can follow maltose transport activity of the maltose permease and the amount of maltose permease protein and can correlate the two.

The protocol used to assay sensitivity of maltose permease to glucose-induced inactivation is described in detail in Medintz *et al.* (1996). Briefly, cells are grown to early logarithmic phase in rich medium with 2% maltose (to induce maltose permease expression), harvested, and transferred to nitrogen-starvation medium plus 2% glucose. Maltose transport activity and maltose permease protein levels are measured at different time points during the 3 h immediately after the transfer to glucose. Maltose transport activity at time zero is given as 100%, although the absolute value differs from strain to strain, sometimes substantially (Table 2).

Table 2. Maltose-induced levels of maltose transport and maltase in mutant strains

Genotype	Maltose Transport Activity (nmol/mg [dry wt], min)	Maltase Activity (nmol PNPG/ mg protein/min)[a]
Wild-type	6.38	603
*snf3*Δ	7.47	581
*rgt2*Δ	6.72	667
*snf3*Δ*rgt2*Δ	7.81	702
*grr1*Δ	7.52	623
*rgt1*Δ	2.12	395
*grr1*Δ*rgt1*Δ	5.49	757
*GRR*1[pRGT2-1]	1.89	525
*grr1*Δ[pRGT2-1]	4.09	726

[a]PNPG, *p*-nitrophenyl α-D-glucopyranoside.

Figure 1A shows the results of an inactivation assay using the parental strain CMY1001. Glucose stimulates a loss in maltose permease protein at a rate that is faster than can be explained by the growth of the culture, and maltose transport activity is inhibited at an even faster rate. Both the rapid inhibition of maltose transport activity and the proteolysis of maltose permease are dependent on glucose and do not occur if ethanol or another nonfermentable carbon source is used in the assay (Medintz *et al.*, 1996; our unpublished results). Our previous results demonstrated that, in rich medium, the glucose-induced loss of maltose permease protein paralleles the loss in transport activity (Medintz *et al.*, 1996). Thus, glucose is both necessary and sufficient to induce proteolysis of maltose permease in either rich or nitrogen starvation medium, but both glucose and nitrogen starvation conditions are required for the very rapid loss of transport activity. Because growth dilution is minimized in nitrogen starvation conditions, it makes it possible to detect small changes in the rate of proteolysis, and for this reason, we have continued to use this condition for the inactivation assay. We have shown that the rapid inhibition of transport activity is not blocked in *end3* or *ren1(vps2)* mutant strains suggesting that an initial very rapid process is occurring that functionally inactivates the maltose permease prior to the rate-limiting proteolysis (Medintz *et al.*, 1996). We have not yet determined the mechanism of this rapid inhibition.

RGT2 But Not SNF3 Is Involved in Glucose-induced Inactivation of Maltose Permease

Strains carrying mutations in *SNF3* do not express high-affinity glucose transport and are unable to grow in medium containing low concentrations of glucose (Bisson *et al.*, 1987). The recently identified *RGT2* gene encodes a protein that has a high sequence homology to Snf3p (Özcan *et al.*, 1996a). Both Snf3p and Rgt2p are members of the putative glucose transporter family but instead appear to monitor extracellular glucose levels and signal via Grr1p to induce *HXT* (high-affinity glucose transporter) gene expression (Özcan and Johnston, 1995; Özcan *et al.*, 1996a,b). To test whether *SNF3* or *RGT2* function as glucose sensors in glucose-induced inactivation of maltose permease, we constructed strains CMY1007 (*snf3*Δ), CMY1009 (*rgt2*Δ), and CMY1012 (*snf3*Δ*rgt2*Δ), which are all isogenic to strain CMY1001, and assayed their sensitivity to glucose-induced inactivation with our standard inactivation protocol. The results in Figure 1B show that loss of Snf3p function has little or no obvious effect on the glucose-induced proteolysis of maltose permease and only slightly reduces the rate of inhibition of maltose transport. In contrast, loss of Rgt2p function (Figure 1C) dramatically slows the rate of maltose

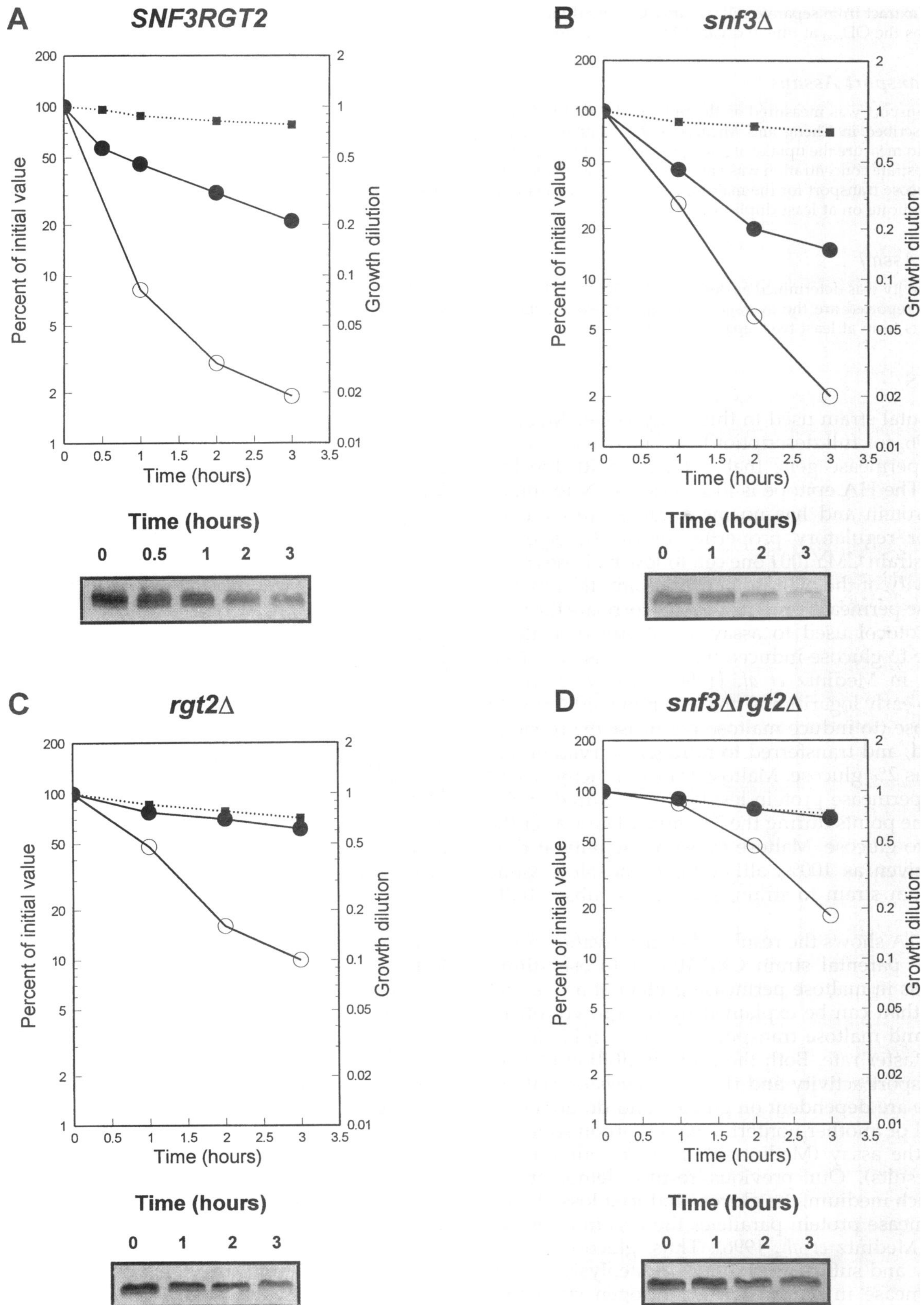

Figure 1.

Table 3. Glucose transport activity in maltose-grown *snf3*Δ, *rgt2*Δ, and *snf3*Δ*rgt2*Δ mutant strains

Genotype	V_{max} (nmol/mg [dry wt]/min)	Relative V_{max} (%)	K_m (mmol)
Wild-type	19.2	100	1.0
*snf3*Δ	7.7	40	0.8
*rgt2*Δ	37.0	193	0.6
*snf3*Δ*rgt2*Δ	4.5	23	0.9

permease proteolysis and significantly reduces the rate of rapid inhibition of maltose transport. Deletion of *SNF3* enhances the *rgt2*Δ phenotype and entirely blocks proteolysis, and the rapid inhibition of transport activity is reduced even further (Figure 1D).

Results of kinetic analysis of glucose transport in the maltose-grown *snf3*Δ, *rgt2*Δ, *snf3*Δ*rgt2*Δ cells are shown in Table 3. Disruption of *SNF3* reduces the V_{max} of glucose transport to less than half that in the parental strain. This is less severe than the effect of *SNF3* mutations in cells grown in low or no glucose where the V_{max} of glucose transport is reportedly reduced to 10–20% of the rate found in the parental strain (Bisson *et al.*, 1987). Nevertheless, it is clear from this result that Snf3p is expressed and functionally active in maltose-grown cells, despite its modest effect on glucose-induced inactivation. Disruption of *RGT2* on the other hand significantly increases the V_{max} of glucose transport (Table 3) in these maltose-grown cells.

The Role of GRR1 in Glucose-induced Inactivation of Maltose Permease

The *GRR1* gene product has been implicated in the regulation of glucose transporter gene expression, cell morphology, cell growth, and other processes (Flick and Johnston, 1991; Barral *et al.*, 1995). Strains with mutations in *GRR1* have a reduced rate of glucose transport due to reduced expression of several glucose transporters. Figure 2 shows that in both rich (Figure 2A) and nitrogen-starved (Figure 2B) media, glucose-induced inactivation of maltose permease is completely blocked in maltose-grown *grr1*Δ cells. Thus, Grr1p is essential for glucose-induced inactivation and the glucose-insensitive phenotype of *grr1*Δ mutations is not an indirect result of the increased nitrogen starvation sensitivity of *grr1*Δ mutants.

The V_{max} of glucose transport in the maltose-grown *grr1*Δ strain is 15% of that seen in the parental strain (Table 4). This suggests the possiblity that the reduced rate of glucose transport and/or the resulting reduced rate of glucose metabolism in the *grr1* mutant strain could be responsible for the insensitivity to glucose-induced inactivation. We tested this by restoring high rates of glucose transport to the *grr1* mutant by deleting *RGT1* and by overexpressing *HXT1*.

RGT1 encodes a DNA-binding repressor of several glucose transporter genes. Grr1p inhibits Rgt1p function in the presence of glucose, thereby derepressing *HXT* gene expression (Özcan and Johnston, 1995). Mutations in *RGT1* suppress the poor glucose growth phenotype of *grr1*Δ mutations by restoring high-affinity glucose transport (Vallier *et al.*, 1994). We found that deletion of *RGT1* in the *grr1*Δ strain fully restores high-affinity glucose transport in maltose-grown cells (Table 4) and partially restores glucose-induced inhibition of maltose transport and Mal61/HAp proteolysis (Figure 2D). Thus, Grr1p appears to affect glucose-induced inactivation, at least in part, because it is required for the expression of high-affinity glucose transport. But this does not appear to be the sole function of Grr1p because the rate of glucose-induced proteolysis of maltose permease remains slower in the *grr1*Δ*rgt1*Δ strain than in the wild-type strain despite the fact that the rate of glucose transport is essentially normal. Deletion of *RGT1* alone increases glucose transport rates (Table 4) and slightly increases the rate of glucose-induced inactivation of maltose permease. Inactivation of maltose permease remains dependent upon glucose induction in the *rgt1*Δ null strain, that is, no inactivation of maltose permease is seen in the *rgt1*Δ strain in the presence of ethanol (our unpublished results). Thus, these results indicate that the restoration of glucose transport is responsible for the suppression of the glucose inactivation insensitive phenotype of *grr1*Δ. It is interesting to note that the *rgt1*Δ null strain exhibits reduced levels of maltose transport and maltase activity (Table 2), but a similar result is not seen in the *grr1*Δ*rgt1*Δ double mutant strain. This has not been explored further.

In a second approach, we tested the ability of *HXT1* overexpression to suppress the resistance to glucose-induced inactivation of maltose permease exhibited by the *grr1*Δ strain. Plasmid pADH1-HXT1 containing *HXT1* gene under the control of the constitutive ADH1 promoter was introduced into the parental strain as well as the *grr1*Δ mutant strain, and inactivation assays were carried out. Results in Figure 3 show that

Figure 1 (facing page). Effect of *snf3*Δ, *rgt2*Δ, and *snf3*Δ*rgt2*Δ mutations on glucose-induced inactivation of maltose permease. Strains CMY1001 (parental strain), CMY1007 (*snf3*Δ), CMY1009 (*rgt2*Δ), and CMY1012 (*snf3*Δ*rgt2*Δ) were grown in rich medium containing 2% maltose, and the standard inactivation assay protocol was used (see MATERIALS AND METHODS; Medintz *et al.*, 1996). The relative levels of Mal61/HA protein (●), maltose transport activity (○), and growth dilution (■, dotted line) are plotted. The relative protein level and transport activity at time X are compared with the corresponding values at time zero. Growth dilution is calculated as the OD_{600} at time zero divided by OD_{600} at time X. Representative Western blots are shown at the bottom.

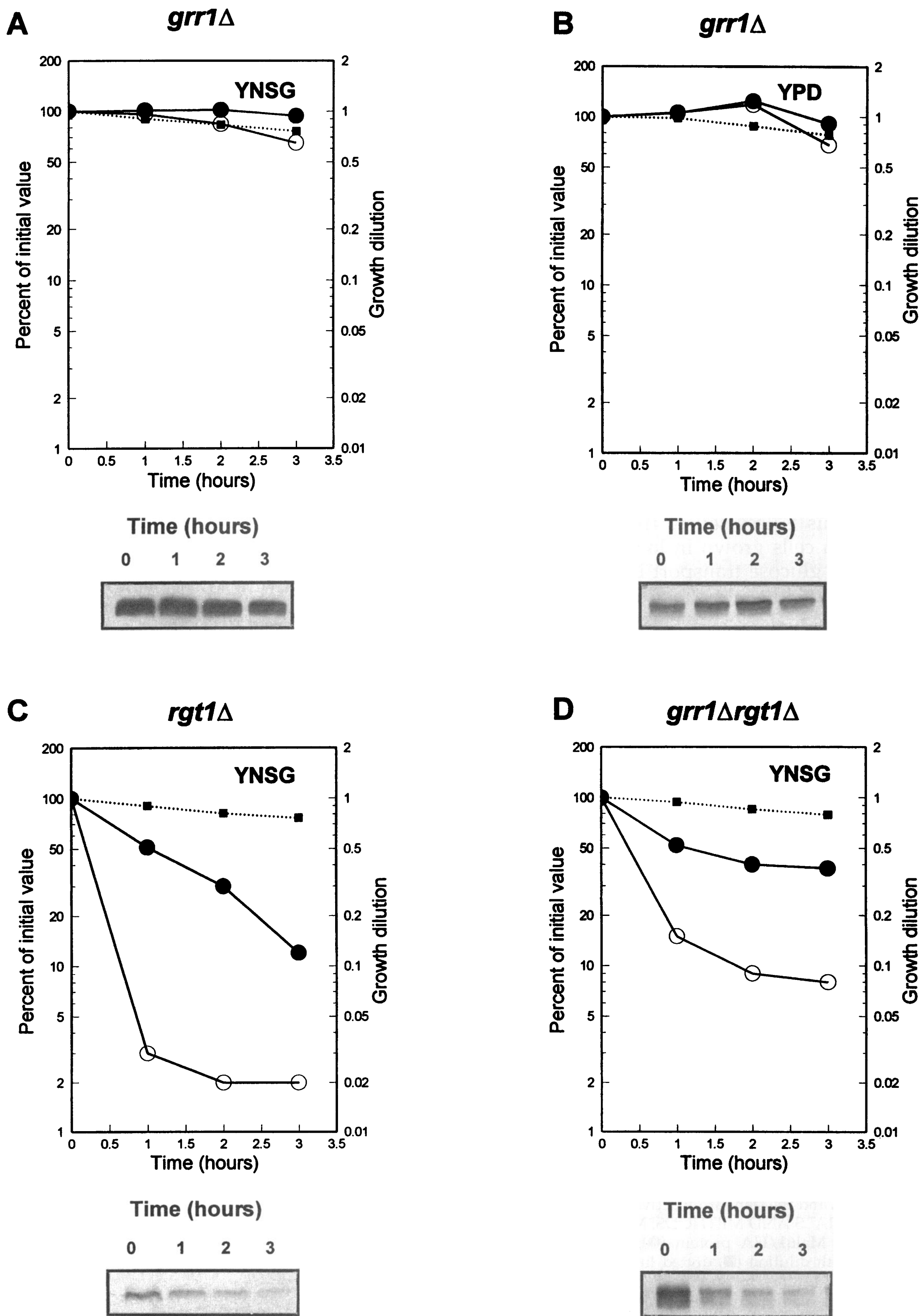

Figure 2.

Table 4. Glucose transport activity in maltose-grown *grr1Δ*, *rgt1Δ*, and *grr1Δrgt1Δ* mutant strains and strains containing the *HXT1* overexpression plasmid

Genotype	V_{max} (nmol/mg [dry wt]/min)	Relative V_{max} (%)	K_m (mmol)
grr1Δ	2.9	15	1.3
rgt1Δ	28.8	150	1.1
grr1Δrgt1Δ	20.0	104	1.9
GRR1[pADH1-HXT1]	21.7	113	1.6
grr1Δ[pADH1-HXT1]	5.0	26	1.8

overexpression of *HXT1* in a *grr1Δ* mutant strain partially restores the rapid inhibition of maltose transport activity but has only a minimal effect, at best, on the rate of proteolysis of maltose permease (Figure 3B). It should be noted that the rate of glucose transport in this maltose-grown *grr1Δ* strain is increased only about 10% by the presence of plasmid pADH1-HXT1 (Table 3), and this may not be sufficient to produce a significant increase in the rate of proteolysis. No significant effect of *HXT1* overexpression is seen in the parental *GRR1* strain (Figure 3A).

RGT2–1 Mutation Induces Inactivation of Maltose Permease in the Absence of Glucose

RGT2–1 was isolated as a dominant suppressor of *snf3*, and causes induction of *HXT1* expression even in the absence of glucose (Marshall-Carlson *et al.*, 1991; Özcan and Johnston, 1996a). To test whether *RGT2–1* can also produce a constitutive signal for inactivation of maltose permease, a plasmid containing this dominant allele was transformed into the parental strain CMY1001 as well as the *grr1Δ* mutant strain. Inactivation of maltose permease was assayed but in these experiments glucose was replaced by 2% ethanol in the inactivation medium. As previously reported, neither proteolysis of the permease protein nor rapid inhibition of the maltose transport activity is induced by ethanol in the wild-type CMY1001 strain (Figure 4A; Medintz *et al.*, 1996); however, when the *RGT2–1* allele is present, a slow but significant proteolysis of maltose permease is observed but no rapid inhibition (Figure 4B). Maltose transport activity also decreases but only slightly and only after a delay of about 1 h. We do not know the basis of this delay but it could suggest that posttranslational modifications are occurring in the ethanol-containing inactivation medium, which increase the specific activity of maltose permease. Glucose-induced proteolysis of maltose permease is not significantly changed by the presence of *RGT2–1* in the parental strain (Figure 4C), but rapid inhibition of maltose transport is slowed slightly and the V_{max} of glucose transport is reduced to 65% of the untransformed parental strain (Table 5). *RGT2–1* does not stimulate maltose permease proteolysis in a *grr1Δ* mutant (Figure 4D and Table 5).

Consistent with the constitutive proteolysis of maltose permease, strains carrying *RGT2–1* express reduced levels of maltose transport, approximately 30% of wild-type levels (Table 2). Interestingly, maltase activity is not significantly lowered by *RGT2–1*. Since maltase expression is an indirect measure of *MAL* gene transcription (maltase is insensitive to glucose-induced inactivation), the discrepancy between the effects of *RGT2-1* on maltose transport and maltase activity levels reported in Table 2 indicates that the constitutive *RGT2-1* signal only affects maltose permease turnover and not *MAL* gene transcription.

Figure 2 (facing page). Effect of *grr1Δ*, *rgt1Δ*, and *grr1Δrgt1Δ* on glucose-induced inactivation of maltose permease. Strains CMY1005 (*grr1Δ*), CMY1008 (*rgt1Δ*), and CMY1010 (*grr1Δrgt1Δ*) were grown in rich medium containing 2% maltose. The standard inactivation assay was carried out as described for Figure 1, except for B, where the cells were transferred to rich medium containing 2% glucose rather than nitrogen starvation medium.

DISCUSSION

SNF3, *RGT2*, *GRR1*, and *RGT1* have all been implicated in glucose sensing/signaling for glucose-regulated transcription in *Saccharomyces*, particularly in the regulation of the *HXT* genes. We show herein that these same gene functions are used in a posttranslational glucose-regulated process, glucose-induced inactivation of maltose permease (reviewed in Johnston and Carlson, 1992; Özcan and Johnston, 1995; Özcan *et al.*, 1996a,b and references therein). Three conclusions are clear from our results. First, the Rgt2p-dependent high glucose signal makes a significant contribution to the induction of maltose permease proteolysis. Second, transduction of the Rgt2p-dependent signal in glucose-induced inactivation of maltose permease also requires Grr1p and thus this signaling pathway partially overlaps the signaling pathway used to regulate *HXT1* glucose induction. Third, a second high glucose signal regulating inactivation of maltose permease is generated by high-affinity glucose transport and/or metabolism, and this signal induces both maltose permease proteolysis and rapid inhibition of maltose transport activity.

Originally, Snf3p was believed to function as a glucose transporter, but the accumulated findings now support a regulatory role for this protein (Marshall-Carlson *et al.*, 1991; Bisson *et al.*, 1993). *SNF3* is expressed at very low levels, and alone is insufficient to support growth on low glucose (Bisson *et al.*, 1993; Ko *et al.*, 1993). Recent studies show that *SNF3* is required for induction of *HXT* gene expression at low levels of glucose (Özcan and Johnston, 1995; Liang and Gaber,

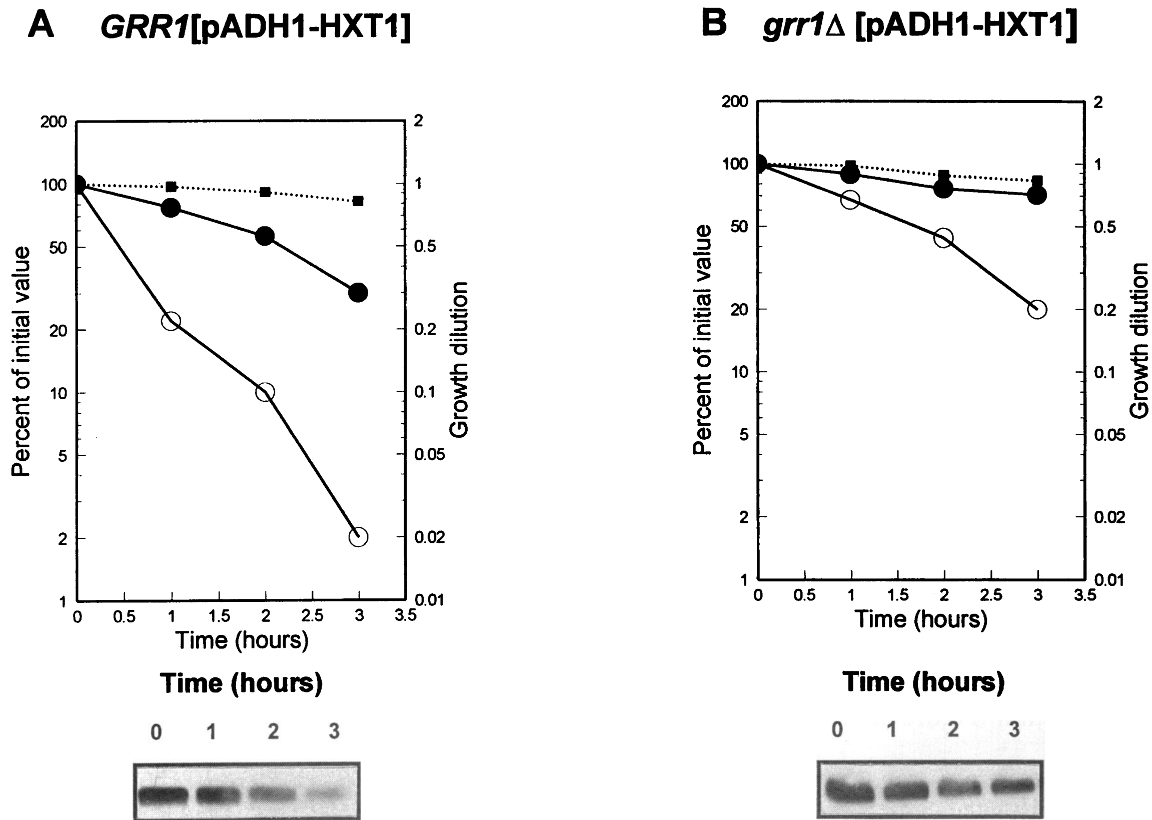

Figure 3. Effect of overexpression of *HXT1* on glucose-induced inactivation of maltose permease. Strains CMY1001 (parental strain) and CMY1005 (*grr1*Δ) were transformed with plasmid pADH1-HXT1. Both strains were grown in selective medium lacking uracil and containing 2% maltose. The standard inactivation assay protocol was used as described in Figure 1.

1996; Özcan *et al.*, 1996a,b). *RGT2–1* is a dominant suppressor of *snf3* mutations (Marshall-Carlson *et al.*, 1991). Özcan *et al.* (1996a) recently characterized Rgt2p, showing that it encodes a putative hexose transporter with overall 60% sequence identity to Snf3p. Rgt2p is required for high glucose induction of *HXT1* gene expression. It is proposed that Snf3p and Rgt2p are glucose sensors that monitor low and high levels of extracellular glucose, respectively, and transmit this signal to regulators of *HXT* gene expression, like Rgt1p, via Grr1p (Özcan and Johnston, 1995; Özcan *et al.*, 1996a,b).

Our results suggest that Snf3p is not involved in sensing the high glucose levels used to induce inactivation of maltose permease. Clearly, Snf3p is expressed in these maltose-grown cells where it is responsible for maintaining about half of the glucose transport levels (Table 3), but the impact of the loss of Snf3p on glucose-induced inactivation is minor and probably limited to its modest effect on glucose transport. Thus, Snf3p does not appear to be able to respond to high concentrations of extracellular glucose. Our results demonstrate that Rgt2p functions as a sensor of high extracellular glucose concentrations for the glucose-induced inactivation of maltose permease and that the Rgt2p-dependent signal transduction pathway for this posttranslational process also uses Grr1p. It is important to note that *RGT2–1* causes constitutive proteolysis of maltose permease protein but not the rapid inhibition of maltose transport normally seen immediately after transfer to the inactivation medium.

Mutations in *GRR1* exhibit an elongated cell morphology, very low rates of glucose transport, increased sensitivity to osmotic stress and nitrogen starvation, increased resistance to heavy metals, and loss of aromatic amino acid transport capacity including

Figure 4 (facing page). Effect of *RGT2–1* mutation on constitutive and glucose-induced inactivation of maltose permease. Plasmid containing the *RGT2–1* dominant allele was introduced into strains CMY1001 (parental strain) and CMY1005 (*grr1*Δ). Strains were grown in selective medium lacking uracil and containing 2% maltose. Strain CMY1001 with or without plasmid pRGT2–1 was transferred to nitrogen starvation medium containing ethanol (A and B). The standard inactivation assays were carried out in strains CMY1001 and CMY1005 transformed with plasmid pRGT2–1 (C and D).

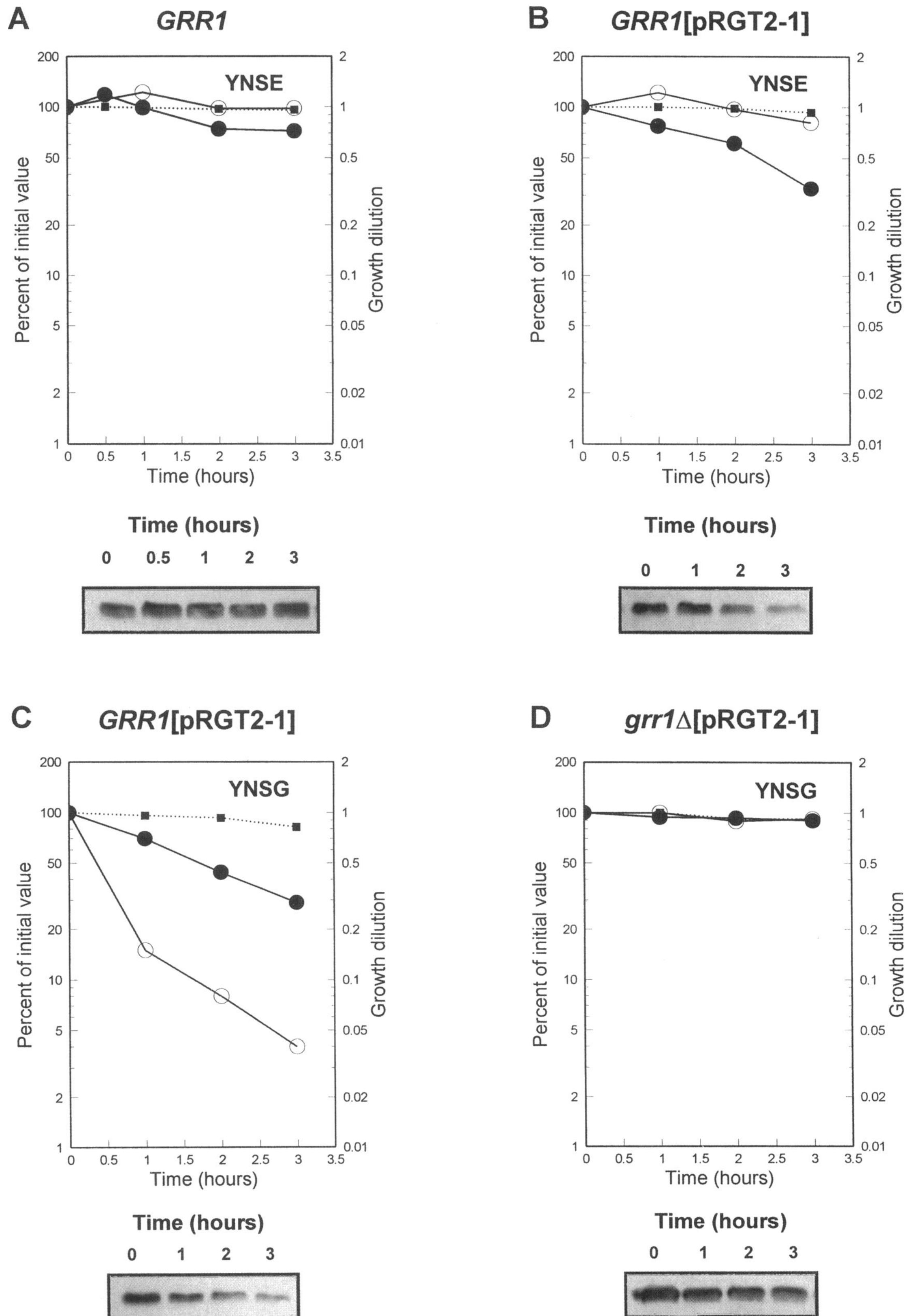

Figure 4.

Table 5. Glucose transport activity in maltose-grown *GRR1* and *grr1Δ* mutant strains containing the dominant *RGT2-1* allele

Genotype	V_{max} (nmol/mg [dry wt]/min)	Relative V_{max} (%)	K_m (mmol)
GRR1[pRGT2-1]	12.5	65	1.6
grr1Δ[pRGT2-1]	2.5	13	1.8

tryptophan transport (Flick and Johnston, 1991). The loss of glucose transport activity results from the inability of *grr1* mutants to express the high-affinity glucose transporter genes (Marshall-Carlson *et al.*, 1991; Erickson and Johnston, 1994; Vallier *et al.*, 1994; Özcan and Johnston, 1995). Null mutations in *RGT1* suppress the reduced hexose transport phenotype of *grr1* mutations (Marshall-Carlson *et al.*, 1991; Vallier *et al.*, 1994; Özcan and Johnston, 1995). Rgt1p is a zinc cluster protein, and it has been suggested that Rgt1p is a DNA-binding repressor of the *HXT1–4* genes (Özcan and Johnson, 1995; Özcan *et al.*, 1996b). Grr1p appears to be required to block the repressing action of Rgt1p.

We found that loss of Grr1p function completely blocked inactivation, and severely reduced the level of high-affinity glucose transport in maltose-grown cells. Deletion of *RGT1*, which fully restores high-affinity glucose transport to the maltose-grown *grr1* null strain, only partially suppresses the glucose insensitivity of inactivation, and the rate of maltose permease proteolysis of the *grr1Δrgt1Δ* strain is approximately half that in the parental strain. Restoration of glucose

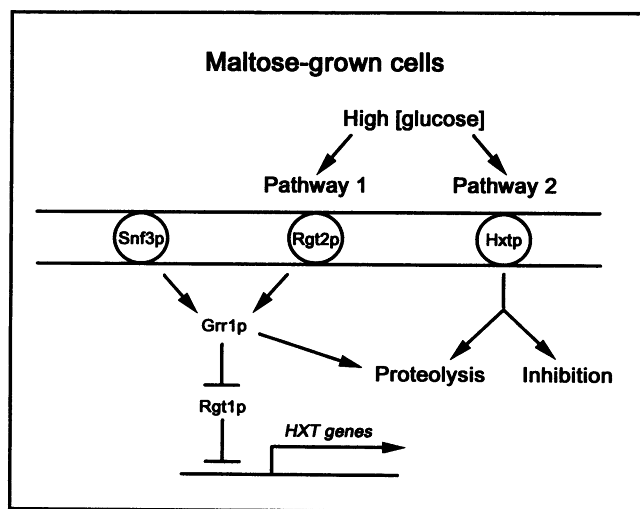

Figure 5. Model of the glucose sensing/signaling pathways used for glucose-induced inactivation of maltose permease.

transport in the *grr1Δ* strain by overexpression of the *HXT* gene also partially restores glucose-induced inactivation in the *grr1Δ* strain, both proteolysis of maltose permease and rapid inhibition of maltose transport activity. Thus, these results along with the studies of the role of Rgt2p described above, clearly demonstrate that Grr1p plays an essential role in two interrelated signaling pathways, both of which respond to high levels of glucose and induce inactivation of maltose permease.

Figure 5 shows our model for the glucose sensing/signaling mechanisms in maltose-grown cells. The model is derived from the results reported herein and from the results of previous studies on the genetic interactions among *SNF3*, *RGT2*, *GRR1*, and *RGT1* (reviewed in Johnston and Carlson, 1992; Erickson and Johnston, 1994; Özcan and Johnston, 1995; Liang and Gaber, 1996; Özcan *et al.*, 1996a,b). We propose that at least two glucose sensing/signaling pathways are present in maltose-grown cells, each with a different upstream glucose "sensor." Pathway 1 is independent of glucose transport per se and appears to cause only proteolysis of maltose permease and not rapid inhibition of maltose transport. Rgt2p is the glucose sensor for this pathway. It monitors glucose levels and transduces a positive signal via Grr1p in the presence of high extracellular glucose concentrations. Our finding that deletion of *RGT2* does not fully block glucose-induced inactivation of maltose permease, but that deletion of *GRR1* does, indicates that at least one other glucose sensing/signaling pathways is used for this system. Since restoration of glucose transport in the *grr1Δ* strain (by deletion of *RGT1* or overexpression of of *HXT1*) restores both proteolysis and inhibition, we propose that glucose transport and/or metabolism provides a second glucose sensing/signaling pathway for glucose-induced inactivation. Pathway 2 appears to stimulate both proteolysis of maltose permease and the rapid inhibition of maltose transport activity. Our suggestion that a second high glucose sensing/signaling pathway is present in yeast is consistent with the finding by Özcan *et al.* (1996a) that deletion of *RGT2* does not affect glucose repression of *GAL* gene expression in cells grown in galactose plus glucose.

Activation of Grr1p is required for expression of the various *HXT* genes at different concentrations of extracellular glucose (Özcan and Johnston, 1995). In low glucose conditions, Snf3p activates Grr1p, and in high glucose conditions, Rgt2p appears to serve this role. Thus, activation of Grr1p occurs over a wide range of glucose concentrations, and some mechanism must exist to distinguish Rgt2p-derived signals from those originating from Snf3p. Perhaps these same mechanisms are used to direct Grr1p to activate the proteolysis of maltose permease in high glucose.

The molecular mechanism of Grr1p function is unknown. Grr1p is a large protein containing several

leucine-rich repeats and is associated with other proteins in a large cytoplasmic complex (Flick and Johnston, 1991). Consistent with this, *grr1* mutations are very pleiotropic, and it is clear that this protein functions in many pathways in addition to the Rgt1p-dependent regulation of *HXT* genes. In this regard, it should be noted that the cell morphology phenotype of *grr1*Δ strains is not suppressed by *rgt1*Δ mutations, indicating that this phenotype as well is dependent upon another of the Grr1p functions (Vallier *et al.*, 1994). Most interestingly, Grr1p has been implicated in G_1 cyclin turnover (Barral *et al.*, 1995). It contains an F-box motif found in several proteins implicated in ubiquitin-mediated proteolysis and is homologous to Skp2 protein, which binds cyclins and may be involved in their ubiquitin-mediated degradation (Stewart *et al.*, 1994; Bai *et al.*, 1996). It is possible that the glucose-transport–independent pathway of glucose-induced inactivation similarly involves ubiquitin-mediated degradation, perhaps of a regulator of the inactivation process.

The nature of the high glucose signal and the identity of the glucose sensor and downstream components of Pathway 2 are not known. It is possible that glucose transport per se by the Hxt proteins is the glucose-sensing mechanism for Pathway 2, but other measures of the rate of glucose metabolism are equally likely. The high-affinity glucose transporters do appear to be the most upstream components of Pathway 2. It is not likely that *RGT2* and *SNF3* counterparts monitor the levels of extracellular glucose for Pathway 2. Instead, transcriptional regulation of *HXT* gene expression appears to serve this function.

In addition to the Rgt2p-dependent glucose signaling pathway, two other pathways have been identified in *Saccharomyces* that respond to increased availability of glucose: the Ras–cAMP pathway and the Snf1 protein kinase pathway (reviewed in Broach, 1991; Johnston and Carlson, 1992; Thevelein, 1992; Thevelein and Hohmann, 1995). A Ras-dependent transient burst of cAMP is produced in response to high levels of extracellular glucose, but its role in glucose signaling, if any, or that of the postulated increase in cAMP-dependent protein kinase A activity has not been demonstrated. The Snf1 protein kinase pathway plays a major role in the regulation of glucose repression of transcription. Components of this pathway include Glc7p, Reg1p, Snf1p, Snf4p, Sip1–4p, Gal82p, Gal83p, Ssn6p, Tup1p, and Mig1p, and the pathway appears to function as a protein kinase cascade with numerous downstream targets (Erickson and Johnston, 1994; Tu and Carlson, 1995; Lesage *et al.*, 1996). We are evaluating the contributions of both of these signaling pathways to glucose-induced inactivation.

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