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Protein phosphatase type-1 regulatory subunits Reg1p and Reg2p act as signal transducers in the glucose-induced inactivation of maltose permease in *Saccharomyces cerevisiae*

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Abstract The *REG1* gene encodes a regulatory subunit of the type-1 protein phosphatase (PP1) Glc7 in *Saccharomyces cerevisiae*, which directs the catalytic subunit to substrates involved in glucose repression. Loss of *REG1* relieves glucose repression of many genes, including the *MAL* structural genes that encode the maltose fermentation enzymes. In this report, we explore the role of Reg1p and its homolog Reg2p in glucose-induced inactivation of maltose permease. Glucose stimulates the proteolysis of maltose permease and very rapid loss of maltose transport activity – more rapid than can be explained by loss of the permease protein alone. In a *reg1*Δ strain we observe a significantly reduced rate of glucose-induced proteolysis of maltose permease, and the rapid loss of maltose transport activity does not occur. Instead, surprisingly, the slow rate of proteolysis of maltose permease is accompanied by an increase in maltose transport activity. Loss of Reg2p modestly reduces the rates of both glucose-induced proteolysis of maltose permease and inactivation of maltose transport activity. Overexpression of Reg2p in a *reg1*Δ strain suppresses the effect on maltose permease proteolysis and partially restores the inactivation of maltose transport activity, but does not affect the insensitivity of *MAL* gene expression to repression by glucose observed in this strain. Thus, protein phosphatase type-1 (Glc7p-Reg1p and Glc7p-Reg2p) plays a role in transduction of the glucose signal during glucose-induced proteolysis of maltose permease, but only Glc7p-Reg1p is involved in glucose-induced inactivation of maltose transport activity and glucose repression of *MAL* gene expression. Overexpression of *REG1* partially restores proteolysis of maltose permease in a *grr1*Δ strain, which lacks glucose signaling, but does not rescue rapid inactivation of maltose transport activity or sensitivity to glucose repression. A model for the role of Reg1p and Reg2p in glucose signaling pathways is discussed. We also uncovered a previously unrecognized G2/M delay in the *grr1*Δ but not the *reg1*Δ strains, and this delay is suppressed by *REG1* overexpression. The G1/S delay seen in *grr1*Δ mutants is slightly suppressed as well, but *REG1* overexpression does not suppress other *grr1*Δ phenotypes such as insensitivity to glucose repression.

Key words Maltose permease · *REG1,2* · Glucose signaling · Yeast

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Introduction

REG1 encodes a regulatory subunit of type-1 protein phosphatase (PP1) that targets the catalytic subunit Glc7p to protein substrates involved in the repression by glucose of the transcription of several genes in *Saccharomyces cerevisiae*, particularly those involved in sugar metabolism (reviewed in Johnston 1999). Deletion of *REG1* relieves glucose repression. Binding of Reg1p to Glc7p is enhanced by glucose both in vivo and in vitro (Tu and Carlson 1995). Genetic and physical interactions between *REG1* and *SNF1* have been identified (Erickson and Johnston 1994; Frederick and Tatchell 1996; Li et al. 1997; Ludin et al. 1998; reviewed in Johnston 1999). It has been proposed that the phosphatase Glc7p-Reg1p inactivates Snf1 kinase in high-glucose conditions, making Reg1p an important component in the signal transduction pathway for glucose repression.

REG2 was identified on the basis of the interaction between its protein product and Glc7p in the two-hybrid system. It encodes a smaller protein with sequence homology to Reg1p (Frederick and Tatchell 1996). Overexpression of *REG2* complements the mild slow-growth defect of *reg1*Δ strains, but not their resistance to glucose repression. The more severe slow-growth phenotype of *reg1*Δ *reg2*Δ strains is suppressed by *snf1* mutations (Frederick and Tatchell 1996). These data suggest a unique role for Reg1p in glucose repression, but Reg1p and Reg2p appear to play similar roles in regulating cell growth. Frederick and Tatchell (1996) proposed a model in which Reg1p and Reg2p control the activity of PP1 towards substrates that are phosphorylated by the Snf1 protein kinase.

Addition of glucose to maltose-grown *S. cerevisiae* cells results in transcriptional repression of the *MAL* structural genes that encode maltose permease and maltase, and post-translational inactivation of maltose permease. This glucose-induced inactivation of maltose permease is caused by an initial, very rapid inhibition of maltose transport activity followed by slower proteolysis of the maltose permease protein (Medintz et al. 1996). We previously identified two glucose-sensing and signaling pathways that are involved in the stimulation of glucose-induced inactivation of maltose permease (Jiang et al. 1997). Pathway 1 is predominantly responsible for glucose-induced proteolysis of maltose permease, and requires the function of Rgt2p, a sensor of high extracellular glucose concentrations, and Grr1p, a glucose signal transducer (Ozcan et al. 1996). Recent studies of Grr1p have shown that it is one component of a multiprotein SCF complex with ubiquitin-protein ligase activity (Li and Johnston 1997). Grr1p is implicated in the proteolysis of G1 cyclins, and appears to be involved in regulatory pathways that control both the cell cycle and nutrient sensing (Barral et al. 1995; Bai et al. 1996; Jiang et al. 1997). Pathway 2 stimulates both rapid inhibition of maltose transport activity and proteolysis of maltose permease (Jiang et al. 1997). In work to be published elsewhere, we show that Pathway 2 shares several upstream components with the glucose repression pathway (Jiang et al. 2000) and with a newly identified glucose inhibition pathway that blocks induction of *MAL* genes by maltose in the presence of glucose (Hu et al. 2000). Rapid glucose transport and phosphorylation are essential for generating the Pathway 2 signal.

Using strains expressing the inducible *MAL*-activator gene *MAL63* we characterized the role of Reg1p, Reg2p, and type-1 protein phosphatase in glucose-induced proteolysis of maltose permease. We find that loss of Reg1p causes an approximately 50% decrease in the rate of glucose-induced proteolysis of maltose permease and nearly complete relief of glucose repression of *MAL* structural gene expression. Loss of maltose transport activity is also completely prevented – in fact, addition of glucose to a *reg1*Δ strain stimulates a transient increase in maltose transport activity. Reg2p appears to play a role similar to that of Reg1p in glucose-induced proteolysis of maltose permease, although loss of Reg2p has only a modest effect on the rate of proteolysis compared to that of Reg1p, and a similarly minor effect on maltose transport activity. We also provide evidence to show that *REG1* but not *REG2* is required for glucose repression of maltose permease and maltase, thereby distinguishing the function of these two sequence-homologous regulatory proteins in transmission of the glucose signal to different downstream targets. A novel G2/M cell cycle delay is observed in *grr1*Δ but not *reg1*Δ strains that is suppressed by overproduction of Reg1p.

Materials and methods

Strains and plasmids

The *Saccharomyces* strains used in this study are listed in Table 1. Strain CMY1001 was derived from strain 100-1A by two-step gene replacement of the *mal11*Δ::URA3 with the *MAL61/HA* gene, which encodes a hemagglutinin (HA)-tagged maltose permease, as described in Medintz et al. (1996). Strain CMY1005 (*grr1*Δ) is otherwise isogenic to CMY1001, and is described in detail by Jiang et al. (1997).

Strains KT1357 (wild type), DF186 (*reg1*Δ), and DF184 (*reg2*Δ) are isogenic and are described in detail by Frederick and Tatchell (1996). None of these strains ferments maltose because all lack a functional *MAL* activator gene. In order to carry out our analyses, we introduced into these strains the inducible *MAL63* (activator) gene carried on either a *TRP1* or a *HIS3* CEN plasmid, namely pUN30-MAL63 (Elledge and Davis 1988; Gibson et al. 1997) or pUN90-MAL63 (Elledge and Davis 1988), respectively. To allow us to monitor levels of maltose permease protein, we also introduced the plasmid pRS315-MAL61HA (described in Medintz et al. 1996), which carries the *MAL61/HA* gene described above.

Plasmid pREG1 (also called pDN70) was obtained from Mark Johnston. This plasmid carries *REG1* on the multicopy vector YEp352 (Ozcan and Johnston 1995). Plasmids YCpIF15 and pREG2 (also called YCp-*Ha-REG2*) are described by Frederick and Tatchell (1996). YCpIF15 is a *TRP1* CEN plasmid, carrying the *GAL1* promoter. pREG2 is a YCpIF15-based plasmid, encoding an HA-tagged form of Reg2 expressed under the control of the *GAL1* promoter.

Table 1 List of *Saccharomyces* strains used

Strain	Genotype	Reference
CMY1001	*MATa MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his 3-Δ200*	Medintz et al. (1996)
CMY1005	*grr1*Δ (otherwise isogenic to CMY1001)	Jiang et al. (1997)
KT1357	*MATa leu 2 his 3 trp1 ura3-52 AGT1 MAL12 MAL31 MAL32*	Frederick and Tatchell (1996)
DF186	*reg1*Δ (otherwise isogenic to KT1357)	Frederick and Tatchell (1996)
DF184	*reg2*Δ (otherwise isogenic to KT1357)	Federick and Tatchell (1996)

Assay of maltose permease inactivation

Unless otherwise specified, strains were grown at 30 °C to early log phase (OD_{600} of 0.3) in either rich medium or selective medium supplemented with 2% maltose. Strains KT1357 [pUN30-MAL63] [pRS315-MAL61/HA] [pREG2] and DF186 [pUN30-MAL63] [pRS315-MAL61/HA] [pREG2] were first grown in selective medium supplemented with 2% galactose to early log phase, then 2% maltose was added to the medium in order to induce expression of the *MAL* genes, and the cells were incubated for another 6 h. The inactivation assay is described in detail by Medintz et al. (1996). Cells were harvested, and transferred to nitrogen-starvation medium (yeast nitrogen base without amino acids or ammonium sulfate) plus 2% glucose unless otherwise indicated. Aliquots were taken at the indicated times over a 3-h period, and total cell extracts were prepared for Western analysis. Growth dilution is calculated as the OD_{600} at time zero divided by OD_{600} at time X.

Western analysis

Western analysis was carried out as described by Medintz et al. (1996). The Mal61/HA protein in the extracts was detected by using anti-HA antibody and either the ECL or ECF Western blotting kit (Amersham). The relative amount of each band on the ECL-Hyperfilm was measured by densitometric comparison with the zero-time sample. Western analyses were done in duplicate using extracts from at least two separate cell cultures. Experimental variation was about 15%.

Sugar transport assays

Maltose transport was measured as the uptake of 1 mM ^{14}C-labeled maltose, as described by Cheng and Michels (1991) and Medintz et al. (1996). Similar methods were used to measure the uptake of [^{14}C]glucose, with the exception that the substrate concentration was varied from 0.2 mM to 10 mM in order to determine the K_m and V_{max} of glucose transport in the maltose-grown cells. Assays were done on duplicate cultures.

Maltase assays

Maltase activity was determined as described by Dubin et al. (1985). The values reported are the averages of duplicate assays obtained using extracts from at least two separate cultures. Standard errors were less than 20%.

Results

Expression of the *MAL*-activator Mal63p allows *reg1*Δ mutants to grow and ferment maltose

Published reports on the phenotype of *reg1* mutants indicate that *reg1 MAL* strains are unable to grow on maltose (Entian 1980; Entian and Loureiro-Dias 1990; Huang et al. 1996). This growth inhibition by maltose is suggested to result from the rapid generation of intracellular glucose (Entian 1980; Entian and Loureiro-Dias 1990). It is proposed that very high levels of maltose permease and maltase are expressed in *reg1* mutant strains grown on maltose because of the absence of glucose repression. This in turn leads to very high rates of maltose uptake and hydrolysis that result in concentrations of intracellular glucose so excessive that cell growth and/or division is severely repressed. This maltose toxicity posed a potential problem for our analysis.

The *REG1 REG2* parental strain KT1357 is unable to grow on maltose because it lacks a functional *MAL*-activator gene, even though copies of the *MAL* structural genes can be found at the loci *MAL1* and *MAL3*. Introduction of a plasmid-borne *MAL*-activator gene is sufficient to allow maltose fermentation in this strain. Other work in our laboratory had found that both the rate of induction and the fully induced level of *MAL* gene expression attained are somewhat lower in strains expressing *MAL63* than other *MAL*-activator genes. Based on this, we introduced plasmid pUN30-MAL63, carrying *MAL63*, into strain KT1357 and an isogenic series of strains carrying null mutations in *reg1*Δ (DF186) and *reg2*Δ (DF184), and tested the ability of these transformant strains to grow in the presence of maltose.

Introduction of *MAL63* allowed all three strains to ferment maltose, and express the *MAL* structural genes (Table 2). The level of expression of maltose transport activity in the *reg1*Δ strain is slightly, but reproducibly, lower than that of the *reg2*Δ and *REG1 REG2* strains. The basis for this is not known but we believe that it could reflect a moderate toxic response to maltose, probably resulting from the accumulation of intracellular glucose. In addition, Table 2 shows that glucose repression of *MAL* gene expression is almost completely relieved by loss of Reg1p but is not affected by the loss of Reg2p. Most importantly, these findings enabled us to proceed with our analysis of the Reg1 protein and its role in glucose-induced inactivation of maltose permease.

Glucose-induced proteolysis of maltose permease in the *REG1 REG2*, *reg1*Δ, and *reg2*Δ mutant strains

Both pUN30-MAL63 and pRS315-MAL61/HA were introduced into the *REG1 REG2*, *reg1*Δ, and *reg2*Δ

Table 2 Effect of deletion of *REG1* or *REG2* on expression of maltose permease and maltase

Genotype[a]	Maltose transport activity (nmol/mg dry wt/min)				Maltase activity (nmol PNPG/mg protein/min)			
	Mal	Mal + Glu	Glu	Gly/Lac	Mal	Mal + Glu	Glu	Gly/Lac
REG1 REG2	2.55	0.04	0.01	0.17	1022	11	4	35
*reg1*Δ REG2	1.93	1.02	0.23	0.20	1720	1255	35	33
*REG1 reg2*Δ	2.36	0.09	0.02	0.21	1580	14	6	33

[a] Strains KT1357 (*REG1 REG2*), DF186 (*reg1*Δ) and DF184 (*reg2*Δ) transformed with plasmids pUN30-MAL63 and pRS315-MAL61/HA were grown in selective medium lacking tryptophan and leucine, and containing 2% maltose (Mal), 2% maltose plus 2% glucose (Mal + Glu), 2% glucose (Glu), or 3% glycerol plus 2% lactate (Gly/Lac). Cells were harvested at early log phase (OD_{600} of 0.3) and maltase and maltose transport activity were assayed as described in Materials and methods

strains described above, to allow us to follow the expression of maltose permease protein Mal61/HAp using Western analysis. Strains were grown in selective medium containing 2% maltose, harvested, transferred to nitrogen starvation medium containing 2% glucose, and both the level of maltose permease protein (monitored by Western analysis) and maltose transport activity were determined. As shown in Fig. 1A, glucose stimulates rapid proteolysis of maltose permease in the *REG1 REG2* strain and a corresponding loss of maltose transport activity. No HA-tagged maltose permease protein could be detected after 3 h. In the isogenic *reg1*Δ strain, the rate of glucose-induced proteolysis is significantly slowed, and 3 h after transfer to glucose the level of HA-tagged maltose permease is reduced only to 40% of the level at time zero (Fig. 1B). More notably, maltose transport activity increases almost 2-fold during the first hour after glucose addition and then slowly decreases to the original level by 4 h. Deletion of *REG2* has a more modest effect on glucose-induced proteolysis of maltose permease, reducing the rate of proteolysis by about twofold, and this decrease in permease protein levels is paralleled by a loss in transport activity (Fig. 1C). Loss of Reg1p has no apparent effect on expression of high-affinity glucose transport (Table 3). Thus, both Reg1p and Reg2p contribute to the generation of the glucose signal stimulating proteolysis of maltose permease, while Reg1p alone is involved in transmission of the glucose signal for the inactivation maltose transport activity.

Fig. 1A–C Glucose-induced proteolysis of maltose permease in maltose-grown wild-type (**A**), *reg1*Δ (**B**) and *reg2*Δ (**C**) strains. Strains KT1357 (*REG1 REG2*), DF186 (*reg1*Δ), and DF184 (*reg2*Δ) were transformed with the plasmids pUN30-MAL63 and pRS315-MAL61/HA, and grown in selective medium lacking tryptophan and leucine, and containing 2% maltose. The time course of glucose-induced proteolysis of maltose permease was determined as follows. Maltose-grown cells were harvested at early log phase (OD_{600} of 0.3), and transferred to inactivation medium (nitrogen starvation medium) containing 2% glucose. Cells were harvested at the indicated times over a 3-h, and total cell extracts were prepared for Western analysis. The relative levels of Mal61/HAp protein (*filled circles*), maltose uptake (*open circles*), and growth dilution (*filled squares*) are plotted in each panel. The relative protein levels at each time point are expressed as a percentage of the corresponding values at time 0. Growth dilution is calculated as the OD_{600} at time 0 divided by OD_{600} at time *x*. All experiments were done at least in duplicate using duplicate cultures. Duplicate gels were run for each extracts and densitometry readings were taken for each lane in duplicate. The results shown are the average of all of the trials and variation was generally less than about 15%. Representative Western blots are shown *below* each plot

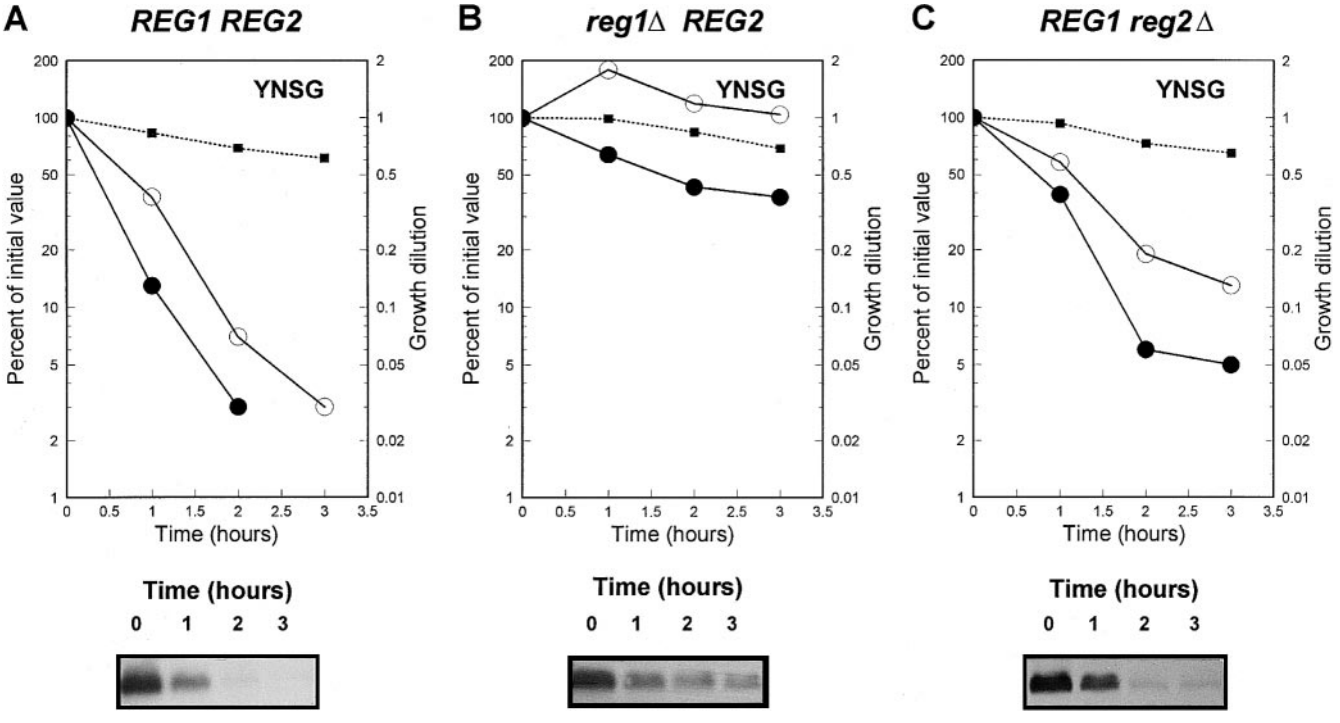
Overexpression of *REG2* suppresses the glucose inactivation phenotype but not the glucose repression phenotype of *reg1*Δ

The finding that deletion of *REG2* slightly reduces the rate of glucose-induced proteolysis of maltose permease

Table 3 Glucose transport activity in *REG1* and *reg1*Δ mutant strains

Genotype[a]	V_{max} (nmoles/mg dry wt/min)	K_m (mmoles)	Relative V_{max} (%)
REG1	28.6	1.2	100
*reg1*Δ	29.4	0.9	103

[a] Strains KT1357 [pUN30-MAL63] [pRS315-MAL61/HA] (*REG1*) and DF186 [pUN30-MAL63] [pRS315-MAL61/HA] (*reg1*Δ) were grown in selective medium lacking tryptophan and leucine, and containing 2% maltose. Glucose transport was determined at a range of substrate concentrations as described in Materials and methods. Lineweaver-Burk analysis was used to calculate the V_{max} and K_m of glucose transport in each strain. The relative activity of strain KT1357 is given as 100%



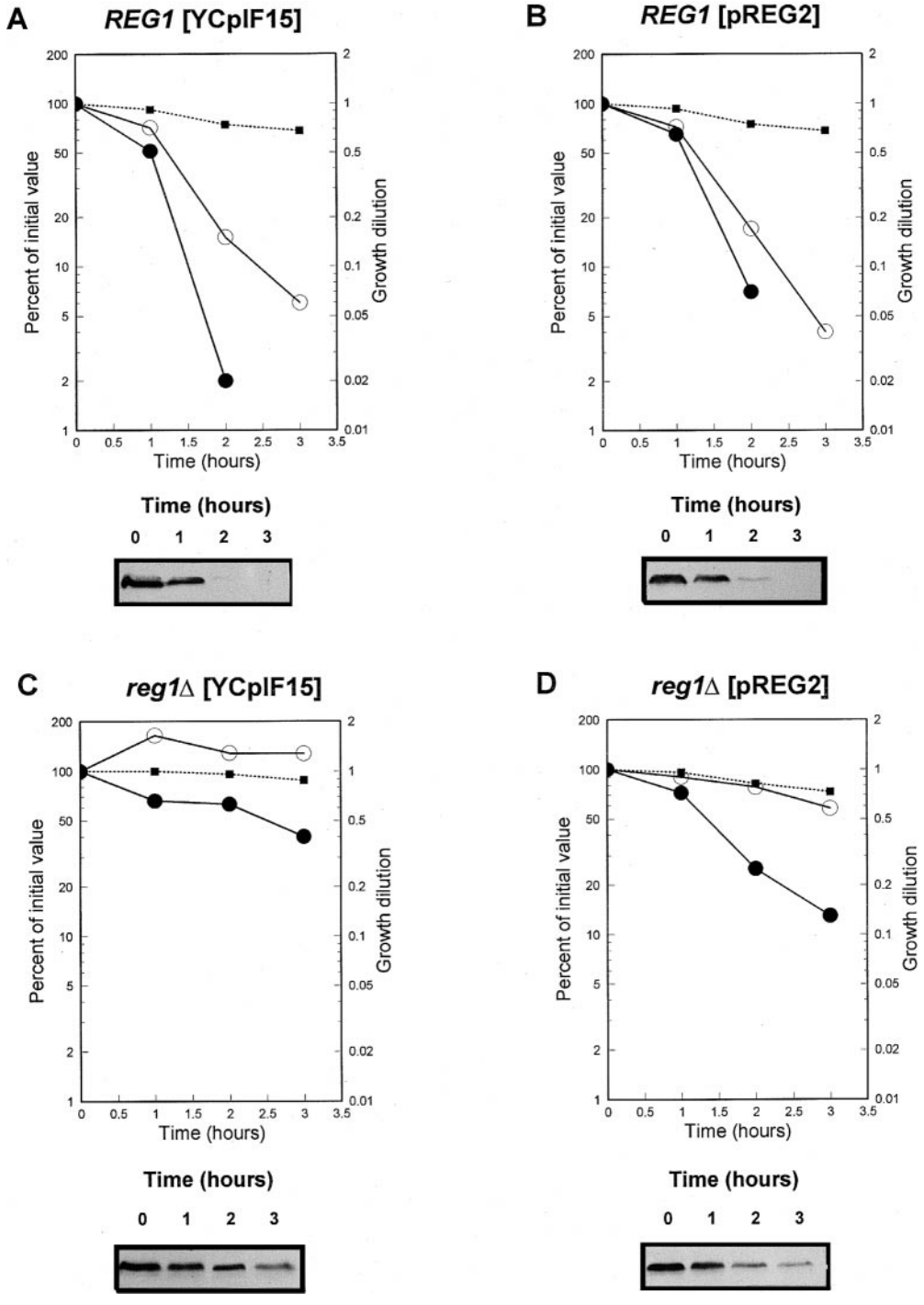


Fig. 2A–D Effect of *REG2* overexpression on glucose-induced proteolysis of maltose permease in maltose-grown *REG1* (**A**, **B**) and *reg1*Δ (**C**, **D**) strains. Strains KT1357 (*REG1*) and DF186 (*reg1*Δ) were each transformed with plasmids pUN90-MAL63 and pRS315-MAL61/HA, together with either a *REG2* overexpression plasmid (pREG2; **B**, **D**) or the control vector YCpIF15 (**A**, **C**). Strains were grown to early log phase in galactose-containing selective medium lacking histidine, leucine and tryptophan, and then 2% maltose was added to the culture to induce expression of maltose permease. At 6 h after maltose induction, cells were harvested, transferred to nitrogen-starvation medium containing glucose, and the time course of glucose-induced inactivation of maltose permease was assayed as described in the legend to Fig. 1

suggests that Reg1p and Reg2p may have similar functions in regulating glucose-induced proteolysis of the transport protein. To explore this, plasmid pREG2, carrying the *REG2* gene under the control of the *GAL1* promoter, and the control vector YCpIF15 were transformed separately into strains KT1357 (*REG1*) and DF186 (*reg1*Δ) and glucose-induced proteolysis of maltose permease was monitored. The strains were first grown in galactose-containing medium to early log phase to allow overexpression of *REG2*. Then maltose was added to the medium for an additional 6 h prior to as-

Table 4 Effect of *REG2* overexpression on expression of maltose permease and maltase

Genotype[a]	Maltose transport activity (nmol/mg dry wt/min)				Maltase activity (nmol PNPG/mg protein/min)			
	Mal	Mal + Glu	Glu	Gly/Lac	Mal	Mal + Glu	Glu	Gly/Lac
REG1 [YCpIF15]	3.67	0.16	0.04	0.20	648	31	4	43
REG1 [pREG2]	3.59	0.13	0.06	0.15	735	36	4	46
*reg1*Δ [YCpIF15]	2.29	1.75	0.16	0.16	1311	928	104	92
*reg1*Δ [pREG2]	2.38	1.35	0.16	0.14	1286	905	87	84

[a] Strains KT1357 (*REG1*) and DF186 (*reg1*Δ), transformed with plasmids pUN30-MAL63 and pRS315-MAL61/HA together with either a *REG2* overexpression plasmid (pREG2) or a control plasmid (YCpIF15) were grown in selective medium lacking histidine, tryptophan and leucine. The various carbon sources added to the medium and the assays for maltose transport and maltase activity were as described for Table 2

saying glucose-induced proteolysis. Figure 2 shows that in *reg1*Δ cells that overproduce Reg2p glucose-induced proteolysis of maltose permease occurs at a more rapid rate (Fig. 2D) than in the *reg1*Δ (Fig. 2C), but as fast as in the *REG1* strain (Fig. 2A). In addition, *REG2* overexpression restores glucose-induced inactivation of maltose transport activity but not to the rapid rate observed in the *REG1* strain. No significant effect of overproduction of the Reg2 protein on glucose-induced proteolysis is seen in the *REG1* strain (compare Fig. 2A and B).

In addition, overexpression of *REG2* has no effect on the insensitivity of *MAL* gene expression to glucose repression in *reg1*Δ strains (Table 4). For this analysis, the cells were grown in galactose to induce *REG2* expression, transferred to medium containing the indicated carbon source for 6 h, and maltose transport and maltase activity were determined. Similarly, Frederick and Tatchell (1996) report that Reg2p overproduction does not restore the sensitivity of invertase expression to glucose repression.

Reg1p overproduction suppresses some but not all aspects of the *grr1* phenotype

Loss of Grr1p blocks all glucose-induced inactivation of maltose permease (Jiang et al. 1997). Grr1p is an essential component of glucose inactivation signaling Pathway 1. Moreover, Grr1p is required for expression of the glucose transporters (Johnston 1999), which are essential for glucose signal generation by Pathway 2. Plasmid pREG1 carrying the *REG1* gene on a multicopy vector provides for the overexpression of *REG1* (Ozcan and Johnston 1995). When pREG1 is introduced into a *grr1*Δ strain, glucose-induced proteolysis of maltose permease is again observed and the loss in transport activity parallels the loss in maltose permease protein (Fig. 3C and D). The *GRR1* strain carrying pREG1 exhibits a significantly increased rate of glucose-induced proteolysis of maltose permease protein, but no increase in the rate of maltose transport (Fig. 3B). The results shown in Fig. 3 also indicate that the suppression of *grr1*Δ resistance to glucose-induced proteolysis by overproduction of Reg1p is constitutive, that is, it does not require glucose for stimulation. Grr1p is required for glucose induction of *HXT* gene expression (Ozcan et al. 1996), and deletion of *GRR1* leads to an approximately 85% decrease in the V_{max} of glucose transport in maltose-grown cells (Jiang et al. 1997). Plasmid pREG1 does not suppress the loss of glucose transport in the *grr1*Δ mutant strain grown in maltose (Table 5). Thus, the ability of plasmid pREG1 to restore proteolysis of maltose permease is independent of the glucose signal through Pathway 2, the glucose transport-dependent pathway, and should be constitutive. Figure 3E confirms this. Comparable rates of proteolysis are observed when ethanol (Fig. 3E) and glucose (Fig. 3D) are used in the inactivation medium.

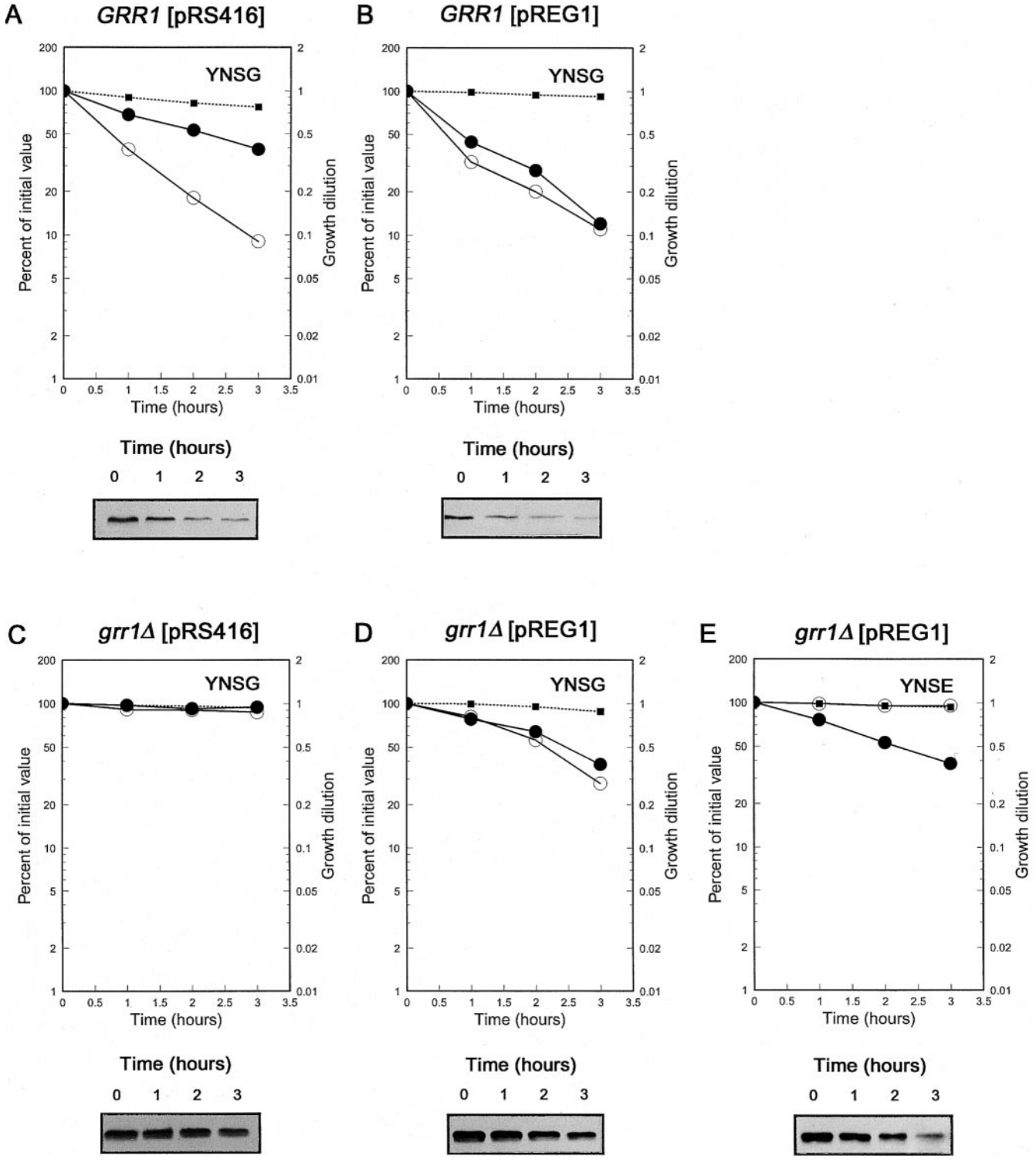
Table 5 Glucose transport activity in maltose-grown *GRR1* and *grr1*Δ mutant strains containing the *REG1* overexpression plasmid

Genotype[a]	V_{max} (nmoles/mg dry wt/min)	Relative V_{max} (%)	K_m (mmoles)
GRR1	19.2	100	1.0
*grr1*Δ	2.9	15	1.3
GRR1 [pREG1]	20.0	108	1.4
*grr1*Δ [pREG1]	2.0	10	1.0

[a] Strains CMY1001 (*GRR1*) and CMY1005 (*grr1*Δ) were grown in rich medium containing 2% maltose. Strains CMY1001 and CMY1005 transformed with the *REG1* overexpression plasmid pREG1 were grown in selective medium lacking uracil and containing 2% maltose. Glucose transport was determined at a range of substrate concentrations as described in Materials and methods. Lineweaver-Burk analysis was used to calculate the V_{max} and K_m of glucose transport in each strain. The relative activity of strain CMY1001 is given as 100%

Fig. 3A–E Effect of *REG1* overexpression on glucose-induced inactivation of maltose permease in maltose-grown *GRR1* and *grr1*Δ strains. Strains CMY1001 (*GRR1*; **A**, **B**) and CMY1005 (*grr1*Δ; **C–E**) were transformed with either a *REG1* overexpression plasmid (pREG1; **B**, **D**, **E**) or the control vector pRS416 (**A**, **C**). Strains were grown in selective medium lacking uracil and containing 2% maltose. The inactivation assay used is described for Fig. 1 but for the experiments shown in panels **A–D** the cells were transferred to inactivation medium containing 2% glucose and for **E** the cells were transferred to inactivation medium containing 3% ethanol. The relative levels of Mal61/HAp protein (*filled circles*), maltose uptake (*open circles*) and growth dilution (*filled squares*) are plotted in each panel. Representative Western blots are shown *below* each plot

grr1 mutations are pleiotropic. In addition to defects in glucose transporter expression and glucose-induced inactivation of maltose permease, *grr1* mutant strains exhibit an aberrant sausage-like cell morphology that appears to result from defects in G1 cyclin degradation (Li and Johnston 1997). In addition, we observed a novel *grr1*Δ phenotype in our strains not noted in previous reports. Glucose-grown *grr1*Δ cells (strain CMY1005) appear to be predominantly in the large-budded stage, suggesting a delay at the G2/M boundary (Fig. 4A, Table 6). FACS analysis confirmed that an unusually large percentage of the nuclei of these cells have 2C DNA content (Fig. 4B). Overproduction of Reg1p partially suppresses the G2/M transition defect and slightly decreases the abnormal, elongated morphology of *grr1*Δ mutants but their insensitivity to glucose repression is



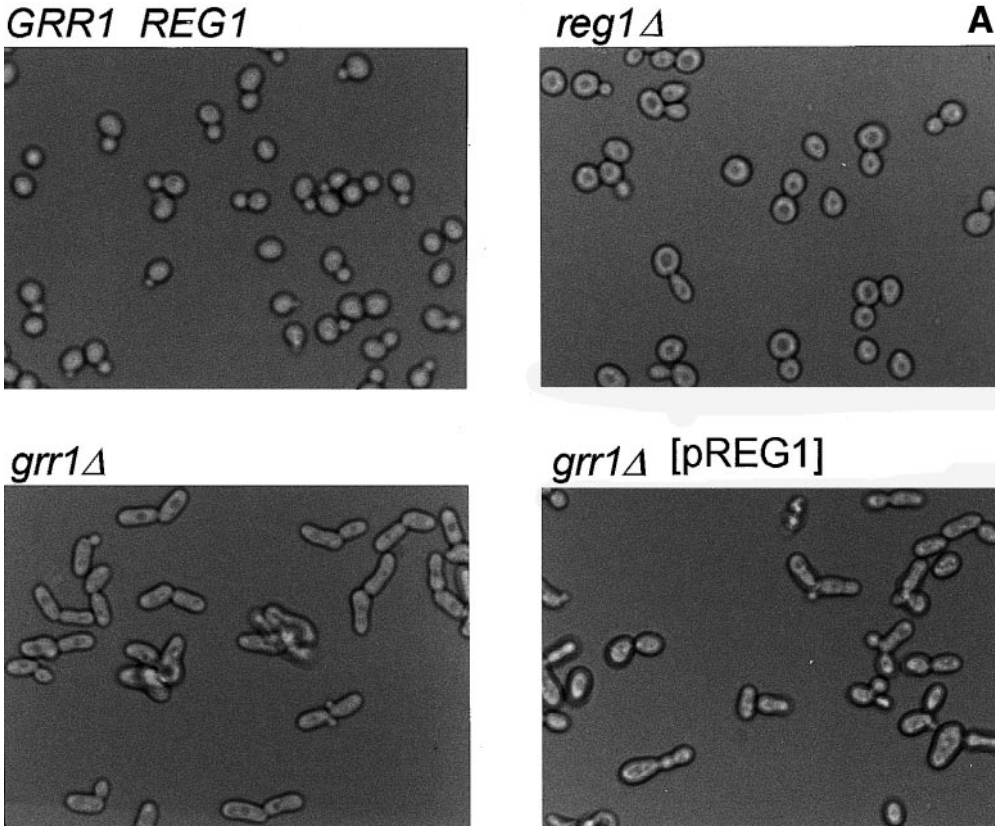
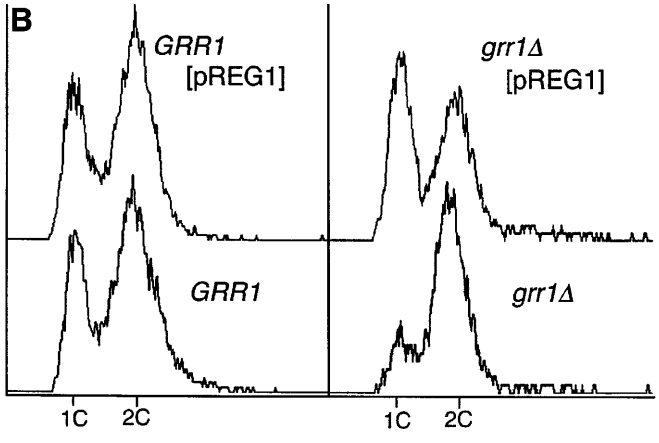


Fig. 4A Morphology of wild-type, *grr1*Δ, reg1Δ, and *grr1*Δ [pREG1] cells. Brightfield micrographs of *REG1 GRR1* (CMY1001), *GRR1 reg1*Δ (DF186), *grr1*Δ REG1 (CMY1005), and *grr1*Δ REG1 [pREG1] (CMY1005 [pREG1]) cells grown in selective medium with 2% glucose are shown. All strains were grown in glucose-containing medium, and are shown at the same magnification. **B** FACS analysis of wild-type (CMY1001) and *grr1*Δ (CMY1005) cells with and without the *REG1* overexpression plasmid pREG1 grown in selective medium with 2% glucose. The analysis was carried out as described by Baker et al. (1997)

unaffected (Fig. 4A, Table 7). Interestingly, the *reg1*Δ strain does not exhibit a similar G2/M delay, based on the normal morphology shown in Fig. 4A and the results of FACS analysis (data not shown). Thus, Reg1p is not directly involved in the observed G2/M delay. The ability of Reg2p overproduction to suppress *grr1*Δ cell cycle phenotypes was not tested.

Reg1p and phosphorylation of maltose permease

Medintz et al. (1996) reported that maltose permease is present in both phosphorylated and dephosphorylated forms in maltose-grown cells, and found that the phosphorylated species is more abundant in the presence of glucose. We compared the relative abundance of the phosphorylated versus the dephosphorylated species in *REG1* (KT1357) and *reg1*Δ (DF187) strains (Fig. 5). Medintz et al. (1996) used acid phosphatase treatment to demonstrate that the higher-mobility species is the dephosphorylated form. As is clearly demonstrated in Fig. 5, loss of Reg1p increases the relative level of the dephosphorylated form of maltose permease.



Discussion

Early studies by Entian and coworkers demonstrated that maltose causes inhibition of growth, glycolysis, and de novo protein synthesis in *reg1* strains; this phenotype was proposed to be due to rapid accumulation of intracellular glucose. The strains used in their studies carried the constitutive *MAL2-8C* allele, which causes high levels of *MAL* gene expression even in the absence of maltose (Gibson et al. 1997). Although very high levels of intracellular glucose are found in *reg1* cells, neither maltose permease nor maltase is overexpressed compared to the wild-type (Entian 1980; Entian and Loureiro-Dias 1990; Huang et al. 1996). The strains KT1357 (*REG1*) and DF186 (*reg1*Δ) used in this study cannot ferment maltose owing to the lack of a functional *MAL* activator gene, and plasmids carrying different

Table 6 Effect of deletion of *GRR1* on cell cycle kinetics

Genotype[a]	Unbudded	Small budded	Large budded
GRR1 REG1	41	29	30
*GRR1 reg1*Δ	47	19	34
grr1 Δ REG1	3	9	88
grr1 Δ REG1 [pREG1]	24	16	60

[a] All strains were grown in glucose medium to log phase. The numbers indicate the percentage of cells in different cell cycle stages

MAL-activator genes were introduced into these strains to induce expression of the *MAL* structural genes. We found that the *reg1*Δ strain is able to ferment maltose only in the presence of a single copy of the inducible *MAL*-activator *MAL63*. Strains carrying other *MAL*-activators or high-copy-number *LexA-MAL63* fusions do not grow on maltose (our unpublished results). Thus, the inhibitory effect of maltose in the *reg1*Δ strain appears to be *MAL*-activator dependent. By using *MAL63* to activate *MAL* gene transcription, we were able to carry out the analysis reported here.

Reg1p and Reg2p have overlapping and unique functions

REG1 encodes a regulator of the protein phosphatase Glc7 (Tu and Carlson 1995), and *REG2* encodes a Glc7p-binding protein with significant homology to Reg1p (Frederick and Tatchell 1996). Tu and Carlson (1995) have demonstrated a physical interaction between Reg1p and Glc7p, and found that this interaction is strengthened in glucose medium. Frederick and Tatchell (1996) found that *reg1*Δ mutants exhibit a mild growth defect that is made more severe by disruption of *REG2*. Moreover, the severe growth defect in *reg1*Δ reg2Δ strains is complemented by overexpression of *REG2* and is alleviated by *snf1* mutations. They suggest that Reg1p and Reg2p may direct the catalytic subunit of protein phosphatase, Glc7, to substrates that are phosphorylated by the protein kinase Snf1. Thus, Glc7 protein phosphatase may act in opposition to the protein kinase Snf1 to regulate growth.

In this report, we provide evidence that *REG1* and *REG2* have redundant functions with regard to the stimulation of glucose-induced proteolysis of maltose permease. We show that deletion of either *REG1* or *REG2* decreases the rate of glucose-induced proteolysis of maltose permease, although the effect of *REG2* disruption is modest (Fig. 1). Moreover, overexpression of *REG2* partially restores maltose permease proteolysis in a *reg1*Δ strain (Fig. 2). This is the first identification of a specific phenotype for a *reg2*Δ mutant (Frederick and Tatchell 1996). It should be noted that loss of Reg2p does not cause a glucose-induced increase in maltose transport activity (Fig. 1C) like that observed in the *reg1*Δ strain (Fig. 1B), nor does Reg2p overexpression restore rapid glucose-induced inactivation of maltose transport activity to a *reg1*Δ strain (Fig. 2D). Taken together, these results suggest that both Reg1p-Glc7p and Reg2p-Glc7p type-1 protein phosphatases are involved in stimulating glucose-induced proteolysis of maltose permease, but that the role of Reg1p-Glc7p in glucose-induced inactivation of maltose transport activity is unique to that form of the complex.

Reg1p also has a unique function in glucose-mediated repression of *MAL* gene expression. Deletion of *REG1* completely relieves glucose repression of *MAL* gene expression, but *reg2*Δ strains are still fully subject to glucose repression (Table 2). In addition, overexpression of *REG2* does not cause *reg1*Δ strains to become sensitive to glucose repression (Table 3). Our results are consistent with those of Frederick and Tatchell (1996), which indicated that Reg1p and Reg2p have distinct, but partially overlapping, targeting activities.

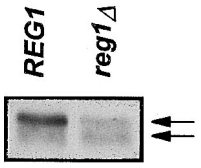


Fig. 5 Phosphorylation of maltose permease in strains expressing different levels of Reg1p. Strains KT1357 (*REG1*) and DF186 (*reg1*Δ) were each transformed with plasmids pUN90-MAL63 and pRS315-MAL61HA, and were grown in selective medium lacking tryptophan and leucine, and containing 2% maltose. SDS-PAGE was carried out using 7.5% polyacrylamide gels and 80 μg of total cell extract was loaded in each lane. The *arrows* indicate the positions of the two differentially phosphorylated species of maltose permease. The upper, more slowly migrating species is more highly phosphorylated (Medintz et al. 1996)

Table 7 Effect of *REG1* overexpression on maltose permease and maltase expression

Genotype[a]	Maltose transport activity (nmol/mg dry wt/min)				Maltase activity (nmol PNPG/mg protein/min)			
	Mal	Mal + Glu	Glu	Gly/Lac	Mal	Mal + Glu	Glu	Gly/Lac
GRR1 [pRS416]	2.31	0.07	0.04	0.14	1356	7	< 1	22
GRR1 [pREG1]	1.46	0.05	0.04	0.10	741	1	< 1	17
*grr1*Δ [pRS416]	4.12	3.15	0.30	0.25	1783	1412	45	57
*grr1*Δ [pREG1]	3.08	2.97	0.15	0.15	1258	1064	34	48

[a] Strains CMY1001 (*GRR1*) and CMY1005 (*grr1*Δ), transformed with either a *REG1* overexpression plasmid pREG1 or the control plasmid pRS416 were grown in selective medium lacking uracil. The various carbon sources added to the medium and protocols for assay of maltose uptake and maltase activity were same as described for Table 2

Roles of Reg1p and Reg2p in the regulation of maltose fermentation by glucose

Studies described elsewhere demonstrate that signal generation for stimulation of glucose-induced inactivation via Pathway 2 is dependent on the same upstream factors as the glucose repression signaling pathway, that is, rapid sugar transport and phosphorylation, particularly via the hexokinase Hxk2 (Jiang et al. 1997, 2000; reviewed in Johnston 1999). In another recent report, we identified a novel glucose-regulated process, referred to as glucose inhibition, whereby glucose acts to block maltose induction of *MAL* gene expression (Hu et al. 2000). The glucose inhibition signaling pathway also overlaps the glucose repression signaling pathway upstream, and includes the negative regulators Hxt2p and Reg1p (Hu et al. 2000). Thus, all three of these glucose signaling pathways (Pathway 2, the glucose inhibition pathway, and the glucose repression pathway) overlap upstream at least up to the signal transducer, type-1 protein phosphatase, which is common to all three pathways. The results described here indicate that type-1 protein phosphatase is the branch point at which the pathway stimulating proteolysis of maltose permease diverges from the others, including the pathway that stimulates inactivation of maltose transport activity. A model schematically illustrating these overlapping glucose signaling pathways and the divergent roles of Reg1p and Reg2p in glucose regulation of maltose permease is shown in Fig. 6.

Medintz et al. (1996) found that glucose-induced inactivation of maltose transport activity occurs via a process that is distinct from that which controls glucose-induced proteolysis of maltose permease. Mutations inhibiting endocytosis (*end3-ts*) or ubiquitination (*doa4*Δ) that block proteolysis do not affect inactivation (Medintz et al. 1996, 1998). Mutational analysis of the N-terminal cytoplasmic domain of Mal61/HA maltose permease localized the target site required for glucose-induced inactivation to a proline-, aspartate-, glutamate-, serine-, threonine-rich postulated PEST sequence located between residues 49 and 78, approximately (Medintz et al. in press). Deletion of residues 49–78 eliminates glucose-induced proteolysis of maltose permease. Moreover, strains expressing the ΔPEST mutant permease actually exhibit an increase in the amount of active maltose transporter present at the plasma membrane. This phenotype is very similar to that seen in the *reg1*Δ strain (Fig. 1B). In addition, mutation of a dileucine motif found at residues 69–70 had no effect on glucose-induced proteolysis of the mutant maltose permease but did cause a transient glucose-induced increase in maltose transport activity. Medintz et al. (in press) propose a novel inhibitory effect on the

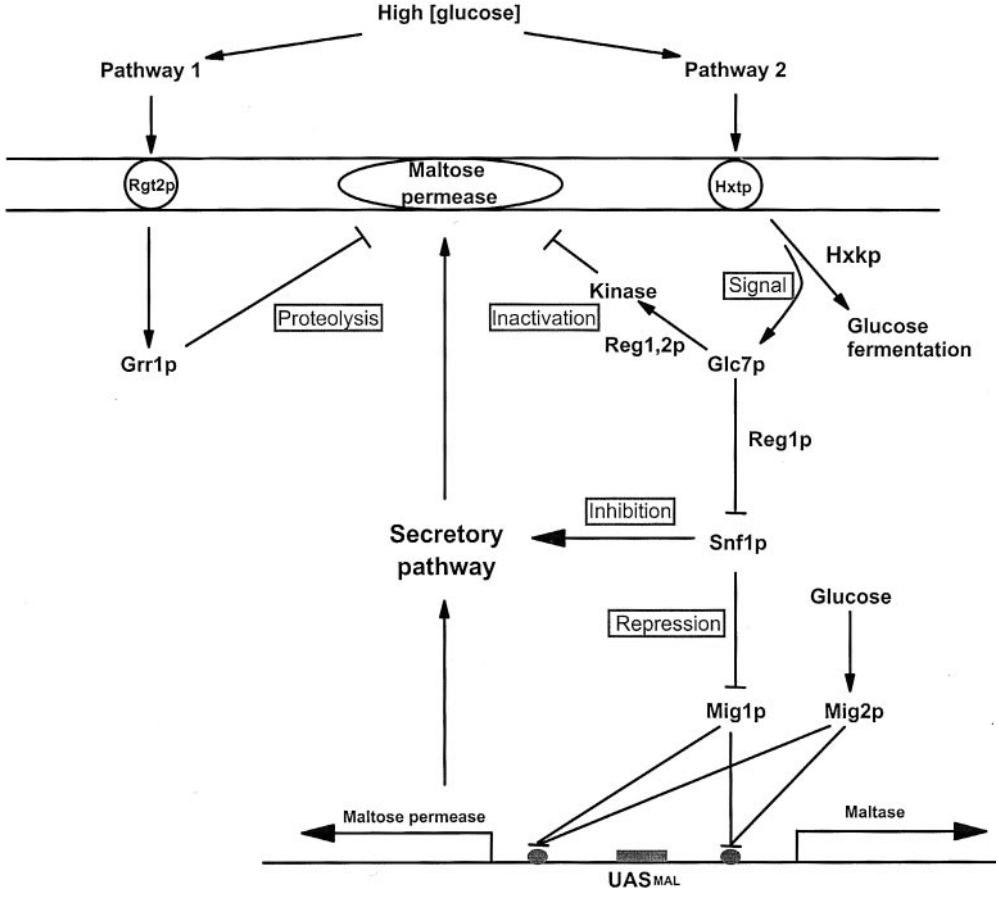


Fig. 6 Model for glucose signal transduction pathways that regulate glucose-induced inactivation of maltose permease, glucose inhibition of maltose induction, and glucose repression of *MAL* gene expression. Pathways 1 and 2 are described in Jiang et al. (1997). Pathway 1 stimulates only proteolysis of maltose permease. Pathway 2 stimulates both proteolysis of maltose permease and loss of maltose transport activity and this is referred to in the model as inactivation. Glucose inhibition is described by Hu et al. (2000)

trafficking of maltose permease through the secretory pathway that is mediated via this N-terminal target sequence. The results reported here indicate that this inhibition of maltose permease synthesis/secretion is mediated by the phosphatase Reg1p-Glc7p. Hu et al. (2000) found that Snf1 kinase is needed at the post-transcriptional level for the synthesis of maltose permease, either for its translation, trafficking through the secretory pathway or stability. Snf1p is a target of Glc7p-Reg1p (Johnston 1999). Thus, as we suggest in Fig. 6, the inhibition of maltose transport activity by Reg1p-Glc7p is most likely to be indirect and to operate via the inactivation of the kinase Snf1 (Ludin and Carlson 1998). We cannot exclude a more direct effect on maltose permease synthesis.

Overproduction of Reg1p in the *grr1*Δ null strain leads to constitutive proteolysis of maltose permease but does not cause constitutive inactivation of maltose transport activity (Fig. 3E) or restore glucose repression (Table 7). This is another distinction between the different downstream branches of this glucose signaling pathway. Thus, at least with regard to the pathway stimulating maltose permease proteolysis, the interaction between Reg1p and Glc7 is not purely dependent on glucose stimulation but may also be favored by increasing the intracellular concentration of this regulatory subunit. High levels of Reg1p may allow Reg1p to compete more successfully with the other PP1 regulatory subunits for binding to limited amounts of the catalytic subunit Glc7p. Such competition was suggested by Huang et al. (1996), who reported the suppression by *reg1* mutations of point mutations in *gac1*, a gene that encodes another regulatory subunit which controls dephosphorylation of glycogen synthase by Glc7.

Figure 6 suggests that the glucose signal is generated by rapid glucose metabolism. The exact nature of this signal is unknown, although many candidates have been postulated (Johnston 1999). Also unknown is the target of this signal. The results reported here and by Hu et al. (2000) are most simply explained if Reg1,2p-Glc7p type-1 protein phosphatase responds to the signal, but alternate explanations are possible.

Reg1p may act indirectly on maltose permease

We previously demonstrated that maltose permease synthesized by maltose-grown cells is present in two differently phosphorylated forms, and suggested that the more highly phosphorylated species is more abundant in glucose-grown cells (Medintz et al. 1996). In this report we demonstrate that maltose permease is present in two equally abundant species in the maltose-grown *reg1*Δ strain, but that the more phosphorylated form is more abundant in the *REG1* strain (Fig. 5). Since loss of *REG1* appears to cause decreased phosphorylation of maltose permease it is unlikely that the protein phosphatase Glc7p-Reg1p is directly involved in the post-translational modification of maltose permease. It is possible that the function of Glc7p-Reg1p is required for the activation of a protein kinase that phosphorylates maltose permease and thereby targets it for degradation. This is currently under investigation. Figure 6 includes this postulated kinase.

A role for Grr1p in G2/M progression

Progression through the eukaryotic cell cycle is dependent on the periodic degradation of regulators of cyclin-dependent kinase, such as the G1 and G2 cyclins, Sic1p, Par1p, and Pds1p, as well as on structural elements of mitotic chromosomes including Ace1p, a spindle component, and the proposed sister chromatid glue (reviewed in Hoyt 1997). As discussed above, Grr1p has been implicated in the degradation of the G1 cyclins Cln1p and Cln2p, and thereby in the regulation of the G1/S transition (Hadwiger et al. 1989; Li and Johnston 1997). The results in Fig. 4 suggest an additional positive function for Grr1p in G2/M progression. Glc7 protein phosphatase type-1 is known to be required for cell cycle progression from G2 to M (Hisamoto et al. 1994; Black et al. 1995; MacKelvie et al. 1995). Sds22p/Egp1p is an essential regulatory subunit for this mitotic function of Glc7p (Hisamoto et al. 1995; MacKelvie et al. 1995). It is unlikely that Reg1p at normal expression levels functions as a regulator of mitosis, since *reg1*Δ strains do not exhibit a G2/M delay. Thus, it appears that *REG1* overproduction suppresses the delay caused by loss of Grr1p by an indirect effect, possibly by reducing the availability of Glc7p via titration.

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