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IDENTIFICATION AND CHARACTERIZATION OF TWO GLUCOSE SENSING /SIGNALING PATHWAYS STIMULATING GLUCOSE-INDUCED INACTIVATION OF MALTOSE PERMEASE IN *SACCHAROMYCES*

by

Hua Jiang

A dissertation submitted to the Graduate Faculty in Biology

in partially fulfillment of the requirements for the degree of Doctor of Philosophy,

The City University of New York.

1997

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF TWO GLUCOSE SENSING /SIGNALING PATHWAYS STIMULATING GLUCOSE-INDUCED INACTIVATION OF MALTOSE PERMEASE IN *SACCHAROMYCES*

by

Hua Jiang

Advisor: Professor Corinne A. Michels

Addition of glucose to maltose fermenting *Saccharomyces cerevisiae* cells causes a rapid and irreversible loss of maltose transport activity, resulting both from the repression of transcription of the maltose permease and from post-translational inactivation of maltose permease, which is referred to as glucose-induced inactivation of maltose permease. The inactivation process consists of two components: inhibition of maltose uptake, and proteolysis of maltose permease. The latter is dependent on endocytosis and vacuolar proteases, and is not dependent on the proteosome.

RGT2, GRR1 and *RGT1* are involved in glucose sensing and signal transduction pathways regulating gene expression. By using deletion and dominant point mutations in these genes, two glucose sensing/signaling pathways stimulating glucose-induced inactivation were identified and further investigation of each pathway was carried out.

Pathway 1 is predominantly responsible for the proteolysis of maltose permease. It is independent of glucose transport and uses a putative high-affinity glucose transporter, Rgt2p, as the sensor for high levels of extracellular glucose. The downstream components of this pathway include Grr1p and Reg1p. Loss of Grr1p appears to block the activity of Reg1p possibly by stimulating Reg1p degradation.

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Pathway 2 stimulates both the proteolysis of maltose permease and the rapid inhibition of maltose uptake. By using a series of sugar kinase mutations, I provide evidence that the initial steps of sugar metabolism, including transport and phosphorylation, are required for generating the inactivation signal. Any of the three hexokinases (hexokinase 1, 2 and glucokinase) is capable of serving as a sensor for intracellular glucose. Grr1p is indirectly involved in Pathway 2 because it is required for expression of high-affinity glucose transporters, encoded by the *HXT* genes. A number of other fermentable carbon sources, including fructose, mannose, galactose, and maltose, and the nonmetabolized glucose analog 2-deoxy-glucose also can stimulate inactivation of maltose permease to different extent through Pathway 2.

In conclusion, Pathway 1 and Pathway 2 are two distinct but interconnected pathways monitoring extracellular and intracellular glucose levels and transducing inactivation signals to different downstream components. These two pathways together with other known glucose signal transduction pathways ensure the rapid transition from utilization of alternative carbon sources to glucose fermentation by controlling gene expression and protein stability.

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I dedicate this thesis to my son Bobby, who is so special to me, and who has had to sacrifice for my work at such a young age. Hopefully, one day he will realize how grateful I am and that it is worthwhile.

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INTRODUCTION

The transport of sugar into a cell is the first, and perhaps most important, step of sugar metabolism, and often the rate limiting step in sugar utilization. In maltose fermenting strains, maltose uptake is carried out by maltose permease. The expression of the permease gene is induced by maltose and repressed by glucose. The activity of the permease is also regulated by an irreversible post-translational mechanism, which is stimulated by the presence of glucose in the medium. The goal of my project is to identify the glucose sensing/signal transduction pathways involved in glucose-induced inactivation of maltose permease.

Maltose permease and maltose fermentation by *Saccharomyces*

Maltose fermentation requires the presence of any one of the five *MAL* loci, *MAL1, 2, 3, 4, 6.* Each *MAL* locus contains three genes: Gene 1 encodes maltose permease, Gene 2 encodes maltase, and Gene 3 encodes a transcriptional activator. Maltose permease is responsible for high-affinity maltose uptake. Maltase is the α-glucosidase which hydrolyzes maltose into two molecules of glucose. The genetic nomenclature indicates both the locus position and the gene function. For example, *MAL61* encodes maltose permease (Gene 1) at *MAL6*. Genes 1 and 2 are divergently transcribed from a common promoter element and their expression is induced by maltose and repressed by glucose (Levine *et al.*, 1992; Hu *et al.*, 1995). The maltose-induced high level expression of Genes 1 and 2 requires the function of Gene 3 product, the *MAL*-activator protein, which binds to a sequence in the promoter region, referred to as the UAS_{MAL} (Levine *et al.*, 1992) (see Figure 1).

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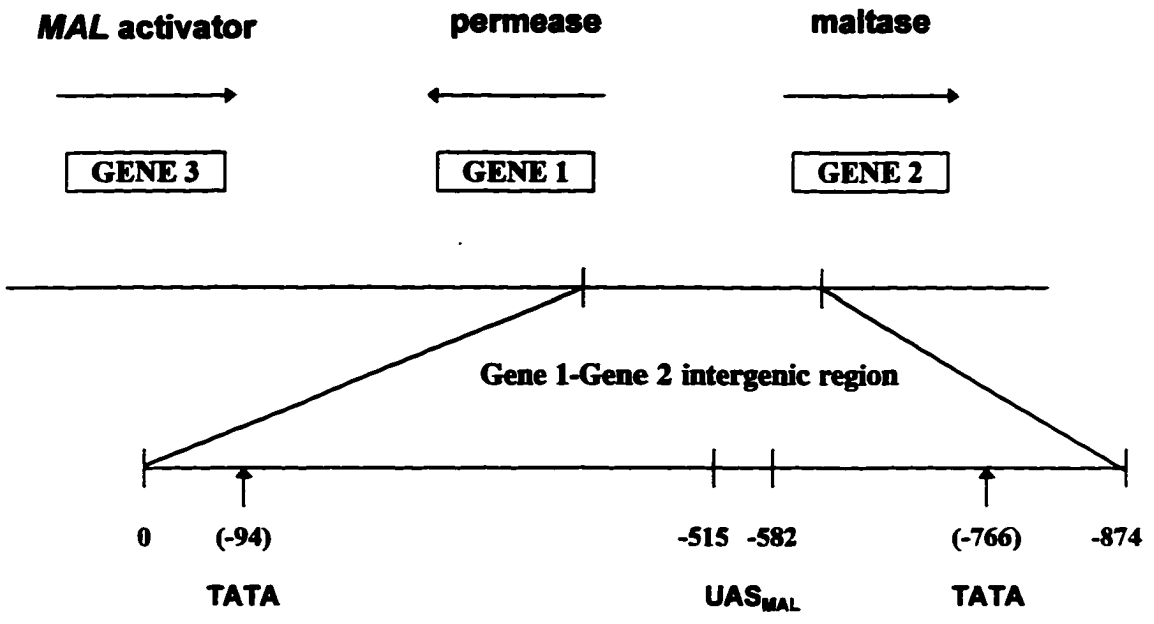

Figure 1. Organization of *MAL* locus.

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Maltose transport is an active process coupled to the electrochemical proton gradient, and independent of intracellular ATP levels (Serrano, 1977). Kinetic analysis of maltose uptake in induced cultures revealed both high-affinity (K_m of 4 mM) and low-affinity transport (K_m of 70 mM) (Busturia *et al.*, 1985; Cheng and Michels, 1991). There is evidence indicating that the low-affinity maltose uptake is an artifact caused by the trapping of maltose in the yeast periplasmic space (Benito *et al.*, 1992). Maltose permease, encoded by the Gene 1 homologs at each *MAL* locus, is a high-affinity maltose/proton symporter (Cheng and Michels, 1991), and is a member of the twelve-transmembrane-domain superfamily of sugar transporters. Han *et al.* (1995) identified a high-affinity α-glucoside transporter, called *AGT1,* which is 57% amino acid sequence identical to Mal61p. Mal61 maltose permease transports only maltose and turanose, whereas Agt1p has a broader substrate specificity (Cheng and Michels, 1991; Han *et al.*, 1995).

Glucose-induced inactivation in *Saccharomyces*

Glucose is a global metabolic regulator in *Saccharomyces* controlling expression of many genes at both transcriptional and post-translational levels (Table 1).

Glucose-induced inactivation (also referred to as catabolite inactivation) was first identified in *Saccharomyces* by Spiegelman and Reiner 50 years ago (reviewed in Holzer, 1984). Among the enzymes and sugar transport systems which are subject to glucose inactivation, the most extensively studied example is fructose-1,6-bisphosphatase. Two distinct processes have been demonstrated. First, the enzyme is reversibly inactivated by phosphorylation on a single serine residue, which causes a decrease of its catalytic activity (Muller and Holzer, 1981; Tortora *et al.*, 1981; Mazon *et al.*, 1982; Pohlig and Holzer,

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Glucose-induced regulatory processes
Post-translational activation of enzymes Trehalase 6-Phosphofructo-2-kinase Plasma membrane H^+-ATPase Glycogen synthase Glycogen phosphorylase
Post-translational activation of transport systems Potassium transport
Post-translational inactivation of enzymes Fructose-1,6-bisphosphatase Cytoplasmic malate dehydrogenase Isocitrate lyase Phosphoenolpyruvate carboxykinase
Post-translational inactivation of transport systems Galactose transport Maltose transport High-affinity glucose transport
Repression of transcription Genes involved in use of less-preferred carbon source (e.g. *GAL, SUC, MAL*) Genes involved in the glyoxylate shunt and gluconeogenesis Genes involved in the citric acid cycle and the respiratory chain *CTT1* (catalase), *UBI4* (polyubiquitin), *ADH2* (alcohol dehydrogenase), *SSA3* (Hsp70), *HSP12* (Hsp12) and others
Induction of transcription Several genes of the second part of glycolysis: *PDC1* (pyruvate decarboxylase), *PYK1* (pyruvate kinase), *ENO2* (enolase), *PGK1* (phosphoglycerate kinase), *ADH1* (alcohol dehydrogenase) Ribosomal RNA genes Ribosomal protein genes

Table 1. Glucose-induced regulatory processes (modified from Thevelein and Hohmann, 1995).

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1985; Rittenhouse *et al.*, 1986). Second, the enzyme is irreversibly inactivated by proteolytic degradation which leads to a decrease in the protein level (Tortora, *et al.*, 1981; Chiang and Schekman, 1991). Vacuolar proteases are involved in the proteolytic degradation of fructose-1,6-bisphosphatase. Work by Chiang and Schekman (1991) indicates that the degradation is *PEP4*-dependent and proteolysis takes place in the vacuole, the yeast equivalent of the lysosome. Transport of this soluble enzyme from the cytosol to the vacuole occurs in response to growth on glucose.

Early studies by Gorts (1969), using strains expressing genetically undefined maltose permease, showed that the maltose transport system was subject to glucose inactivation. Gorts (1969) reported that the addition of glucose to maltose-grown cells led to almost complete inactivation of maltose uptake within 60 minutes, whereas transfer of maltose-grown cells to ethanol containing medium did not cause a rapid loss of maltose uptake. Since *de novo* protein synthesis was required to recover maltose transport, the transporter was suggested to be proteolytic degraded. In contrast, Peinado and Loureiro-Dias (1986) reported that glucose-induced inactivation of maltose permease is the result of a change in substrate affinity due to a reversible modification process.

Chapter 1 describes work carried out by myself and Igor Medintz characterizing glucose-induced inactivation of maltose permease, using a genetically defined strain expressing only an hemagglutinin epitope-tagged allele of *MAL61* (Medintz *et al.*, 1996). We found that glucose, but not ethanol, inactivated maltose transport activity in two steps: it caused an initial very rapid loss of maltose transport activity associated with little decrease in protein levels, and a slower loss in maltose permease protein. Further, we demonstrated that this glucose-induced proteolysis of Mal61/HAp is dependent on

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endocytosis and vacuolar degradation, and is independent of proteosome function.

Glucose sensing/signaling pathways in *Saccharomyces*

Several sensing/signaling pathways involved in glucose triggered regulatory phenomena have been identified, these include the Snf1 protein kinase pathway, the glucose induction pathway of *HXT* gene expression, and the Ras-cAMP pathway.

The *Snf1* protein kinase signal transduction pathway

The *SNF1* pathway is the major signal transduction pathway regulating glucose repression. Through the isolation of nonrepressible and nonderepressible mutants and their suppressors, most of the components of this pathway and the mechanism of transcriptional regulation of glucose repressible genes have been fairly well characterized. The mechanism of glucose sensing for this pathway and the identity of the intracellular small molecule signal regulating Snf1 kinase activity remains poorly defined.

snf (sucrose-non-fermenting) mutations prevent expression of *SUC2* and other glucose repressible genes (Carlson, 1987). *SNF1-6* have been cloned, among them *SNF1* encodes a protein kinase (Ciriacy, 1977; Celenza and Carlson, 1986); *SNF4* encodes a protein which is physically associated with Snf1p and is required for maximal kinase activity of Snf1p (Entian and Zimmerman, 1982; Celenza *et al.*, 1989); *SNF2, 5, 6* gene products appear to be positive transcriptional activators for a large number of genes (Laurent *et al.*, 1991); and Snf3 protein is a putative high-affinity glucose transporter, which is not directly involved in regulation of *SUC2* expression (Marshal-Carlson *et al.*, 1990). The regulatory roles of *SNF3* and the suppressors of *snf3* mutations (*RGT1* and *RGT2*) are becoming elucidated, and will be discussed later.

The *SNF1* gene is essential for sucrose fermentation because it is required for

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derepression of the *SUC2* gene and many other glucose repressible genes including the *MAL* structural genes (Carlson, 1987). *SNF1* encodes a serine/threonine protein kinase which plays a central role in the regulation of glucose repression (Celenza *et al.*, 1986). The Snf1 kinase consists two domains: an N-terminal kinase domain (residues 1-391) and a C-terminal regulatory domain (residues 392-633) (Jiang and Carlson, 1996). Snf1p is associated with its activating subunit, Snf4p, and other proteins (e.g. Sip1p, Gal82p and Gal83p) in a complex (Celenza *et al.*, 1989; Yang *et al.*, 1992, 1994; Jiang and Carlson, 1996). The interaction between Snf1 and Snf4 proteins is regulated by the glucose levels. In high glucose, the regulatory domain of Snf1p binds to the kinase domain causing autoinhibition of the kinase activity, whereas in low (or no) glucose Snf4p binds to an overlapping site on the regulatory domain, and activates the kinase. Other components in the complex may serve as anchor or scaffold proteins, or may participate in sensing glucose concentration (Jiang and Carlson, 1996).

Genetic analysis identified a number of genes required for glucose repression including, *SSN6, TUP1, MIG1, SRG1, SIP1* and *SIP2* (reviewed by Johnston and Carlson, 1992 and Gancedo, 1992; Yang *et al.*, 1992, 1994). These genes encode global regulators controlling the expression of many glucose repressible genes. Other genes, such as *GAL82* and *GAL83,* are specifically required for glucose repression of a subset of genes. Mutations in the above genes relieve glucose repression of the transcription of the target gene. *SNF1* itself is required for derepression (release of glucose repression). Using suppressor analysis, Erickson and Johnston (1994), tested the order of action of these gene products and provide a working model for glucose repression of the *GAL* genes (Figure 2).

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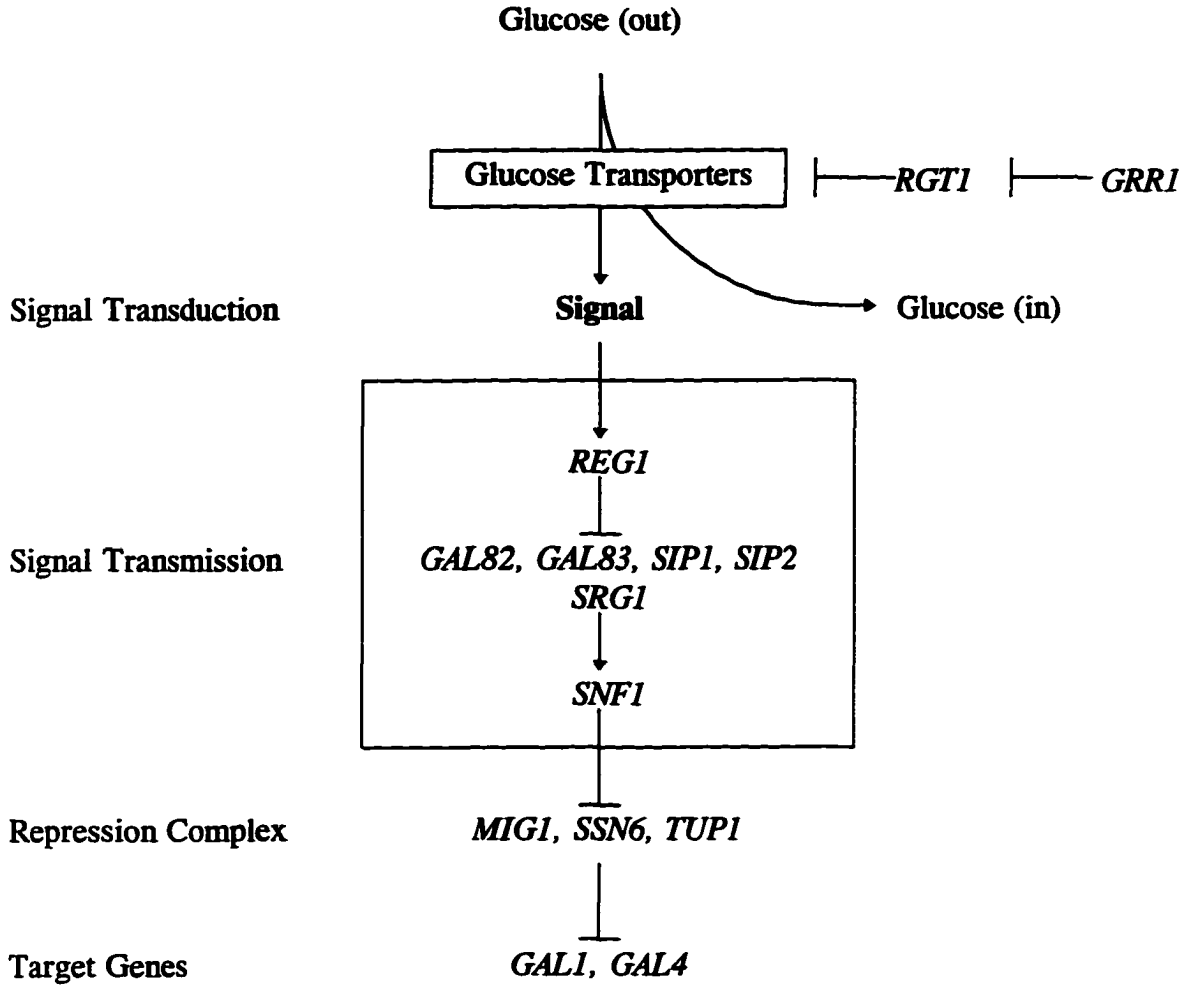

Figure 2. Working model for the functional organization of the genes required for glucose repression of the *GAL* genes. A line with an arrowhead implies positive regulation; a line with a bar denotes negative regulation (from Erickson and Johnston, 1994).

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Among these genes, *GRR1, RGT1, HXK2, REG1, GLC7* are of particular interest for this study. *GRR1* and *RGT1* genes are involved in regulation of the *HXT* gene expression (which encode the high-affinity glucose transporters). *HXK2* encodes yeast hexokinase 2. *GLC7* and *REG1* encode the catalytic subunit and a glucose-responsive regulatory subunit, respectively, of yeast protein phosphatase type-1 (PP1) (Feng *et al.*, 1991; Tu and Carlson, 1994, 1995). The regulatory role and mutant phenotype of each gene will be discussed separately below.

mig1, ssn6 and *tup1* mutations all suppress defects caused by loss of *SNF1* (Williams and Trumbly, 1990; Vallier and Carlson, 1994). *MIG1* encodes a zinc finger protein and binds to a GC-box, a motif present in the promoter of many glucose-repressed genes (Nehlin and Ronne, 1990; Nehlin *et al.*, 1991). The *SSN6* and *TUP1* gene products containing long stretches of polyglutamine, which are found in many transcriptional regulatory proteins (Schultz and Carlson, 1987; Williams and Trumbly, 1990). Ssn6 and Tup1 proteins are found physically associated in a large general repressor complex (Williams *et al.*, 1991). In addition, Mig1 and Ssn6 proteins interact in the two-hybrid system (Treitel and Carlson, 1995). It has been proposed that Mig1p represses transcription by recruiting a complex that contains the Tup1 and Ssn6 proteins to its target promoters (Keleher *et al.*, 1992; Trumbly, 1992). Mig1p is differentially phosphorylated in response to glucose availability (Treitel and Carlson, 1995). It is suggested that phosphorylation of Mig1p affects its binding to DNA, its association with the Ssn6p-Tup1p complex, or the ability of the complex to repress transcription (Treitel and Carlson, 1995).

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The glucose sensing and signal transduction pathways regulating *HXT* gene expression

HXT genes encodes a family of highly homologous putative hexose transporters. The mechanisms for the transcriptional regulation of *HXT* genes, especially *HXT1-4, HXT6* and *HXT7,* in response to glucose have been uncovered recently. Among these *HXT* genes, *HXT1* is induced by high levels of glucose; *HXT2* and *HXT4* are induced by low levels of glucose and repressed by high levels of glucose; *HXT3* is induced by glucose regardless of the sugar concentration; and *HXT6* (possibly also *HXT7*) has a high basal level of expression on nonfermentable carbon sources (Ozcan and Johnston, 1995, 1996; Ozcan *et al.,* 1996a, b; Liang and Gaber, 1996). Snf3p and Rgt2p are low and high glucose sensors, respectively, monitoring the extracellular glucose levels, whereas Rgt1p functions as a transcriptional regulator. A schematic model describing the regulatory mechanisms controlling *HXT* gene expression in response to glucose is shown in Figure 3.

By in large, the components in the glucose signaling pathways were revealed by two approaches, both of which focused solely on glucose repression, that is, glucose regulation of transcription. The first approach was the characterization of glucose repression resistant mutations using glucose analogues. The second approach was the characterization of *SNF* genes, suppressors of the *snf* genes (*SSN*), and the epistic analysis of these genes and other genes involved in glucose repression.

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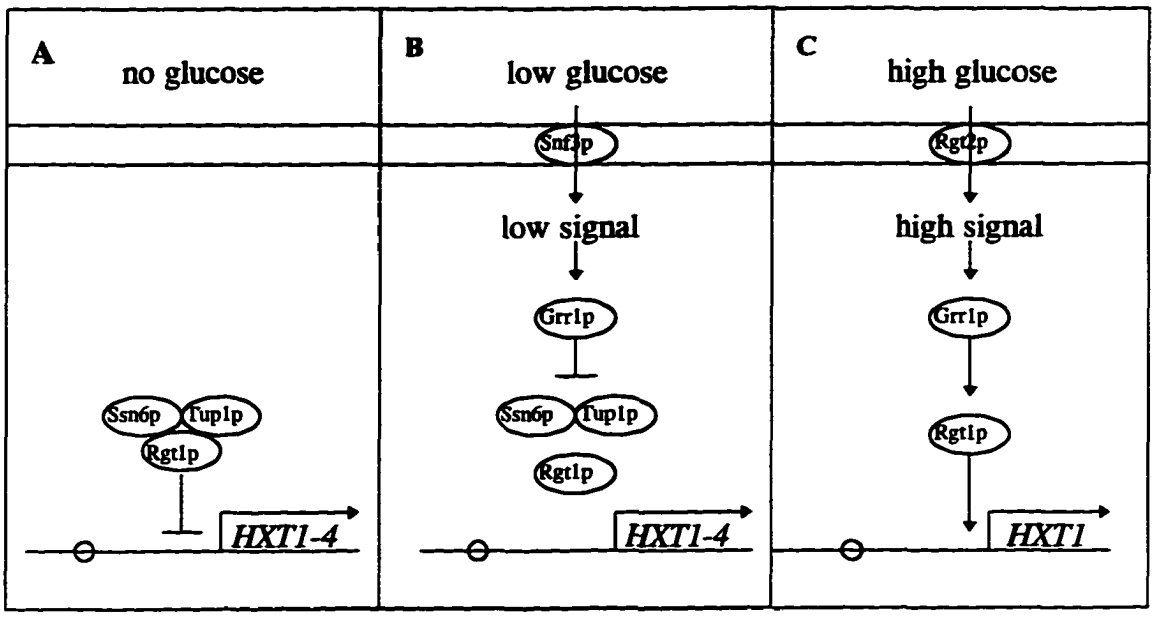

Figure 3. Schematic Model describing the three different modes of transcriptional activity of Rgt1p in response to glucose. In the absence of glucose, Rgt1p works as a transcriptional repressor (A); at low glucose, Rgt1p has no transcriptional activity (B); and at high concentrations of glucose, Rgt1p activates transcription (C) (from Ozcan *et al.*, 1996b).

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Glucose repression resistant mutations

Mutations in *GRR1, HXK2, GLC7* and *REG1* are able to relieve glucose repression. Mutation in the same genes were often isolated by different groups using different selection methods, thus, one gene may have several different names. Alternate nomenclature is indicated in parenthesis. The characteristics of these genes, and the regulatory roles of their gene products, especially their role in glucose sensing and signaling are discussed below.

GRR1(CAT80/COT2/TOC1). *GRR1* encodes a large hydrophobic, membrane associated protein of 1151 amino acids. It contains 12 leucine-rich repeats that are also found in other proteins mediating protein-protein interaction (Flick *et al.*, 1991).

Strains carrying *grr1* mutations have diverse phenotypes and defects in the transport of different molecules. The reduced growth rate on glucose is associated with a defect in high-affinity glucose transport, and a failure to express several of the *HXT* genes encoding high-affinity glucose transporters (Vallier *et al.*, 1994; Wendell *et al.*, 1994). *grr1* mutant strains show metal tolerance due to a defect in the transport of certain heavy metals (Conklin *et al.*, 1993). *grr1 trp1* double mutant strains have a very severe growth defect even in media containing tryptophan, suggesting that *grr1* mutants may be deficient for tryptophan transport (Flick *et al.*, 1991).

Deletion of *GRR1* relieves glucose repression of *SUC2, GAL,* and *MAL* genes (Bailey and Woodword, 1984). In the case of *SUC2, grr1Δ* mutations convert *SUC2* from glucose repressible to glucose inducible (Vallier and Carlson, 1994).

Recent studies provide evidence that Grr1p is a central component of the signaling pathway regulating glucose induction of *HXT* gene expression (Ozcan and Johnston,

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1995; Ozcan *et al.*, 1996b). This model places Grr1p downstream of Rgt2p and Snf3p in the glucose signaling pathway used to monitor extracellular glucose concentrations (see below), and is consistent with the previous result that *GRR1* does not affect glucose repression of *SNF3* gene (Vallier and Carlson, 1991).

Grr1p is an F-box protein. F-box is a novel structural motif, initially identified by sequence analysis of cyclin F, and later found to be shared by several proteins capable of binding Skp1p (Bai *et al.*, 1996). 50% of the F-box proteins have additional motifs involved in protein-protein interaction. Grr1p is most closely related to mammalian Skp2p, both of which have the leucine rich motifs (Bai *et al.*, 1996). Skp1p is an evolutionarily highly conserved protein. Yeast Skp1p is a subunit of CFB3, a multiprotein complex which binds to centromere DNA *in vitro* (Lechner and Carbon, 1991; Connelly and Hieter, 1996). Mammalian Skp1p is a subunit of a cyclin A-Cdk2 (cyclin-dependent kinase) complex, it is also capable of binding Skp2p, cyclin F and Cdc4p, through the F-box (Bai *et al.*, 1996). Skp1p is involved in ubiquitin-mediated proteolysis of Cln2p, Clb5p and the Cdk inhibitor, Sic1p (Bai *et al.*, 1996). It is proposed that Skp1p links a large number of proteins and the proteolysis machinery by two mechanisms. Firstly, it may directly bind to the proteolysis substrate, such as cyclin F. Secondly, it may bind to the adapter proteins, such as Cdc4p, Skp2p and Grr1p, which interact with the proteolysis substrate. *GRR1* is known to be involved in G_1 cyclin degradation. Barral *et al.* (1995) showed Cln1p and Cln2p were stabilized in *grr1Δ* mutants and *grr1Δ rgt1Δ* double mutants. Their data indicate that the proteolysis defect in *grr1Δ* mutants is not a result of a nutrient uptake defect.

***HXK2(HEX1/GLR1), HXK1,* and *GLK1*.** *HXK1*, *HXK2* and *GLK1* encodes

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hexokinase 1, hexokinase 2 and glucokinase, respectively. Their gene products are the phosphorylation enzymes which catalyze the initial step of glycolysis. All three kinases are capable of phosphorylating glucose; hexokinase 1 and 2 can phosphorylate fructose and mannose; glucokinase can phosphorylate mannose but not fructose (reviewed in Bisson *et al.*, 1993).

Previous studies show that the high-affinity glucose uptake in *Saccharomyces cerevisiae* is dependent upon the presence of glucose phosphorylation activity (Bisson and Fraenkel, 1983a, b). Wild-type cells exhibit both high- and low-affinity transport for glucose and fructose. *hxk1 hxk2* mutants lack high-affinity fructose transport, while the *hxk1 hxk2 glk1* triple mutants lack high-affinity transport for both glucose and fructose (Bisson *et al.*, 1983a). Conflicting results have been reported recently, which indicate that high-affinity glucose transport is not dependent on the phosphorylation of the substrate, but dependent on the intracellular concentration of glucose which is partially controlled by subsequent phosphorylation (Nevado *et al.*, 1994; Smits *et al.*, 1996).

Early models for the role of hexokinase 2 suggested that it is a bifunctional enzyme with a catalytic domain for hexose phosphorylation and a regulatory domain responsible for triggering the signal for glucose repression (Entian *et al.*, 1984). Later experiments involving a large number of mutant alleles of *HXK2* demonstrated a strong correlation between residual hexokinase activity and the level of glucose repression suggesting instead that the enzymatic activity itself is responsible for the signal (Ma *et al.*, 1989; Rose *et al.*, 1991).

It is reported that hexokinase 2 has protein kinase activity and this activity is regulated by glucose concentration in the culture (Herrero *et al.*, 1989). Hexokinase 2 is

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capable of autophosphorylation and also phosphorylates other proteins, such as α- and β-casein, thus its protein kinase activity may have a substrate specificity similar to casein kinases (Fernadez *et al.*, 1988; Herrero *et al.*, 1989). The high-affinity glucose transporter Snf3p contains several classical casein kinase-II-like consensus sites for protein phosphorylation (Bisson *et al.*, 1993). This raises the possibility that there is a direct interaction between the sugar transporter and the sugar kinase, but this has not been demonstrated experimentally.

Hxk2p is a phosphoprotein *in vivo*. In addition to autophosphorylation, its phosphorylation is negatively regulated by cAPK (Vojtek *et al.*, 1990). The function of this modification is not known, but there is a correlation between the extent of hexokinase 2 phosphorylation and the rate of hexokinase-dependent high-affinity glucose uptake.

HXK2 plays an important role in glucose repression (reviewed in Johnston and Carlson, 1992; Gancedo, 1992; Ronne, 1995). A number of *hxk2* point and null mutations result in constitutive expression of many glucose repressible gene. It is interesting that *hxk2* mutations, which cause complete derepression of the *GAL* and *SUC* genes, have little or no effect on glucose repression of the gluconeogenesis genes (Ronne, 1995). Overexpression of *HXK1* can restore glucose repression sensitivity of *hxk2* mutants, suggesting that hexokinase 1 and 2 have interchangeable functions in glucose repression, however, glucokinase is not able to substitute for hexokinase 2 in glucose repression (Rose *et al.*, 1991). Genetic evidence indicates that hexokinase 2 functions upstream of Snf1 kinase in the glucose regulatory pathway (reviewed in Johnston and Carlson, 1992). The fact that there is a substantial interaction between Snf1 and Snf4 proteins in *hxk2* mutants but not the wild-type under high glucose conditions also proves

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that *HXK2* is an upstream negative regulatory of the Snf1p pathway (Jiang and Carlson, 1996).

Hexokinase 2 is postulated to play a role in glucose sensing. Ozcan and Johnston (1995) suggest that *HXK2* is required for generating the high glucose signal for induction of *HXT1* gene expression. The finding that yeast hexokinase 2 can act as a glucose sensor in pancreatic β-ceils of transgenic mice supports the idea that *HXK2* is involved in glucose sensing (Epstein *et al.*, 1992; Voss-McCowan *et al.*, 1994).

GLC7(CID1/DIS2S1). *GLC7* codes for the catalytic subunit of yeast protein phosphatase type-1 (PP1), which is more than 80% identity to its mammalian counterpart (Feng *et al.*, 1991; Tu and Carlson, 1994). In *Saccharomyces, GLC7* is an essential gene and is involved in controlling diverse cellular processes, including glycogen accumulation (Feng *et al.*, 1991; Cannon *et al.*, 1994), glucose repression (Neigeborn and Carlson, 1987; Tu and Carlson, 1994), cell cycle progression (Hisamoto *et al.*, 1994; Zhang *et al.*, 1995), chromosome segregation (Francisco *et al.*, 1994), protein translation (Wek *et al.*, 1992), and sporulation (Neigeborn and Carlson, 1987; Feng *et al.*, 1991; Cannon *et al.*, 1994).

Protein phosphorylation and dephosphorylation is an universal post-translational regulatory mechanism. Although a large number of protein phosphatases have been identified (Walton *et al.*, 1993), there are still much fewer protein phosphatases than the corresponding protein kinases, and the substrate specificity of protein phosphatases is much lower than that of protein kinases (Cohen, 1989). Glc7 protein phosphatase is involved in many regulatory responses, however, little is known about its natural substrates and many of its regulatory subunits have not yet been identified. Glycogen

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synthase is the only known substrate of Glc7p protein phosphatase. Glc7p is responsible for the dephosphorylation (and hence activation) of glycogen synthase. *glc7-1* mutants are defective in glycogen accumulation (Feng *et al.*, 1991; Cannon *et al.*, 1994).

It is proposed that different regulatory subunits of protein phosphatase direct the catalytic subunit to particular cellular locations and thus direct phosphatase activity to specific substrates (Cohen and Cohen, 1989). Genetic studies suggest that Glc7 protein functions antagonistically to Snf1 protein kinase in glucose repression (Tu and Carlson, 1994). Early studies show that certain *glc7* mutations cause constitutive expression of invertase and maltase (Neigeborn *et al.*, 1987). Tu and Carlson (1994) demonstrate that *glc7-T152K* (Thr152 changed to Lys) mutants are resistant to glucose repression. Glc7 protein can act in opposition to Gcn2 kinase in regulating the expression of genes involved in amino acid biosynthesis. The Gcn2 protein kinase stimulates the expression of *GCN4*, a transcriptional activator of amino acid biosynthesis genes, in response to amino acid starvation. This is achieved by stimulating the phosphorylation of eIF-2α and thus increasing the translational efficiency of *GCN4* (Wek *et al.*, 1992). Overexpression of wild-type *GLC7* decreases the expression of *GCN4*, mimicking a *gcn2* mutation. A truncated allele of *GLC7* stimulates *GCN4* expression by increasing the phosphorylation of eIF-2α in *gcn2* mutants (Wek *et al.*, 1992). Glc7p also is required for cell cycle progression in G_2/M. $glc7^{Y170}$ mutants which are defective in the G_2/M phase exhibit a high level of *CDC28*-dependent protein kinase activity (Hisamoto *et al.*, 1994). *IPL1* gene encodes a protein kinase which is required for high-fidelity chromosome segregation during mitosis (Francisco *et al.*, 1994). $ipl1^{ts}$ mutants can not undergo proper chromosome segregation at 37°C, this can be partially suppressed by *glc7-1* mutations. In

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contrast, overexpression of the full length *GLC7* results in chromosome missegregation (Francisco, *et al.*, 1994). These results imply that Ipl1p and Glc7p may act on the same substrates.

Many proteins that are capable of interacting with Glc7p have been identified by using the two-hybrid system and co-immunoprecipitation, including its regulatory subunits, such as Gac1p, and Reg1p. *GAC1* encodes a protein similar to the regulatory subunit of PP1 from skeletal muscle that target PP1 to glycogen particles (Francois *et al.*, 1992; Stuart *et al.*, 1994), and *REG1* encodes a negative regulator of glucose repressible genes (Tu and Carlson, 1995; Huang *et al.*, 1996).

***REG1(HEX2/SRN1)* and *REG2*.** *REG1* encodes a regulatory subunit of Glc7 protein phosphatase type-1 (PP1) that targets the phosphatase to proteins involved in the glucose repression pathway (Tu and Carlson, 1995). Deletion of *REG1* completely abolishes glucose repression of all genes tested (reviewed in Johnston and Carlson, 1992). Overexpression of *REG1* suppresses glucose repression defects in *glc7-T152K* suggesting that this specific mutation may affect the interaction between the catalytic subunit of the phosphatase to its regulatory subunit (Tu and Carlson, 1995). The fact that a *reg1Δ* mutation completely relieves glucose repression of *SUC2* whereas *glc7-T152K* only partially relieves glucose repression supports the hypothesis that *glc7-T152K* reduces the interaction between Glc7p and Reg1p (Tu and Carlson, 1995). The interaction between Snf1p and Snf4p in *glc7-T152K* and *reg1Δ* has been examined, and the effects of these two mutations on Snf1p-Snf4p interaction are consistent with their effects on glucose repression. Under high glucose conditions, the regulatory domain of Snf1p binds to its kinase domain causing autoinhibition of the kinase activity, and the interaction between

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Snf1p and Snf4p is very weak. *glc7* mutation restores less than 10% Snf1p-Snf4p interaction, whereas *reg1* mutation restores 80% of the interaction (Jiang and Carlson, 1996).

REG1 is a negative regulator of *GAL* gene expression. *Reg1* mutants exhibit glucose repression resistance of galactokinase (*GAL1*). Overexpression of *REG1* results in 70% reduction of *GAL1* expression under induced conditions (Niederacher and Entian, 1991).

Dombek *et al.* (1993) showed that the expression of *ADH2* (encodes alcohol dehydrogenase) is negatively regulated by *REG1* via an *ADR1*-dependent pathway. *ADR1* is a transcription factor, which is required for the derepression of *ADH2* following growth on glucose. It is interesting that the apparent molecular weight of Adr1p is increased in a *reg1Δ* strain suggesting that Reg1p may be involved in post-translational modification, probably phosphorylation, of Adr1p.

Besides its role in regulating the transcription, *REG1* is also involved in RNA processing, including RNA splicing and export of RNA from the nucleus to the cytosol. In addition, it regulates the expression levels of two proteins of unknown function, p43 and p70. The expression of p70 is glucose repressible in the wild-type and constitutive in *reg1* mutants (Tung *et al.*, 1992).

The role of *REG1* in controlling glycogen accumulation is indirect. Huang *et al.* (1996) show that deletion of *REG1* results in overaccumulation of glycogen and suppression of glycogen deficient phenotype of *glc7-1* mutant strains. Moreover, they provide evidence that the stimulation of the glycogen accumulation in the *reg1Δ* deletion strains does not require the presence of Gac1p, the regulatory subunit involved in

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glycogen accumulation. They suggest that Reg1p and Gac1p compete for limiting amounts of Glc7p and thus loss or overproduction of one regulatory factor affect several other PP1-regulated processes indirectly.

Addition of maltose to a culture of certain *reg1* strains results in an inhibition of growth, glycolysis, *de novo* protein synthesis and a dramatic increase of intracellular glucose concentration, which is associated with uncontrolled, excessive maltose uptake (Entian, 1980; Entian *et al.*, 1990; Huang *et al.*, 1996).

REG2 was isolated on the basis of its interaction with *GLC7* in two-hybrid system, and the predicted protein product of *REG2* is homologous to Reg1p (Frederick and Tatchell, 1996). *reg1Δ* mutants exhibit a mild slow growth defect, *reg2Δ* mutants grow normally, and *reg1Δ reg2Δ* mutants display a severe growth defect which is partially suppressed by a loss-of-function mutation of *SNF1*. Based on these data, Frederick and Tatchell (1996) provide a model that Reg1p and Reg2p direct catalytic subunit of Glc7 protein phosphatase to substrates that are phosphorylated by the Snf1 kinase.

***SNF3* and its suppressors**

***SNF3*.** The *SNF3* gene was first identified in a series of mutant strains that are unable to use raffinose as a carbon source (Neigeborn and Carlson, 1984). Mutation in *SNF3* result in a severe defect in high-affinity glucose transport and a growth defect on low levels of glucose (Bisson *et al.*, 1987). Analysis of the predicted protein sequence of *SNF3* indicates that it encodes a 12 transmembrane domain protein homologous to mammalian glucose transporters, except it contains a very long carboxyl-terminal tail (approximately 303 amino acid in length) not seen in the mammalian transporters or in the yeast Hxt glucose transporters (Celenza *et al.*, 1988).

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Several lines of evidence suggest that Snf3p is more likely a regulatory protein than a high-affinity glucose transporter involved in glucose catabolism (reviewed in Bisson *et al.*, 1993). The expression level of *SNF3* is extremely low compared to those of the *HXT* genes, *SNF3* alone is not sufficient for the high-affinity glucose transport in a *HXT1-4* deletion strain (Celenza *et al.*, 1988; Bisson *et al.*, 1993; Ozcan *et al.*, 1995). Accumulating results suggest that Snf3p functions as a glucose sensor responding to low levels of extracellular glucose, and is required for regulation of the *HXT* gene expression (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996a, b; Liang and Gaber, 1996).

A multicopy suppressor of *snf3* mutation, *SKS1*, has been identified recently, and it encodes a putative ser/thr protein kinase. The putative kinase activity of Sks1p is required for the suppression of *snf3* mutation (Yang and Bisson, 1996). Overexpression of *SKS1* suppresses the growth defect of not only *snf3* but also *grr1* mutants on low glucose suggesting that *SKS1* functions downstream of *SNF3* and *GRR1* (Yang and Bisson, 1996).

RGT2. *RGT2-1* is a dominant mutation which suppresses the high-affinity glucose transport and growth defect of the *snf3Δ* mutations (Marshall-Carlson *et al.*, 1991). Ozcan *et al.* (1996a) recently characterized Rgt2p, showing that it encodes a putative hexose transporter with overall 60% sequence identity to Snf3p. Unlike *SNF3* whose expression is repressed by high glucose concentrations, *RGT2* is constitutively expressed at low levels (Ozcan *et al.*, 1996a). Rgt2p is required for high glucose induction of *HXT1* gene expression. It is proposed that Rgt2p is the glucose sensor monitoring high levels of extracellular glucose (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996a, b).

Suppression of *snf3* by RGT2-1 does not required the presence of *SKS1* implies

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that *SKS1* may not function in the same pathway as Rgt2p (Yang and Bisson, 1996).

RGT1. *rgt1* mutations were recessive and first isolated as suppressors of *snf3* mutations (Marshall-Carlson *et al.*, 1991). *rgt1* is also a suppressor of *grr1*, it not only suppresses growth and glucose transport defect of *grr1* mutants, but also restores glucose repression of *SUC2* in *grr1* mutants (Vallier *et al.*, 1994).

RGT1 gene plays a central role in *HXT* gene expression. *RGT1* encodes a bifunctional transcription factor regulating the *HXT* genes, and can serve as both an activator and a repressor of transcription in response of glucose (Ozcan *et al.*, 1996b). Rgt1p regulates the transcription of *HXT* genes by three different mechanisms: i) in the absence of glucose, it functions as a repressor; ii) in the presence of low levels of glucose, it has no regulatory function on *HXT* gene transcription; and iii) in the presence of high levels of glucose, it functions as an activator (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996b). The function of Rgt1p depends on the signals generated by two glucose sensors, Snf3p and Rgt2p, and transduced by Grr1p (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996a, b). The mechanism by which the signals from these two glucose sensors is distinguished and remains to be determined.

The Ras-mediated cAMP-dependent protein kinase pathway

Ras genes encode low molecular weight monomeric G-proteins that are highly conserved throughout all eukaryotic species. *Saccharomyces cerevisiae* contains two genes, *RAS1* and *RAS2*, that are both structurally and functionally homologous to human *Ras* genes (Powers *et al.*, 1984; DeFeo-Jones *et al.*, 1983). When bound with GTP, mature yeast *Ras* is capable of stimulating adenylate cyclase, the product of the *CYR1* gene (Toda *et al.*, 1985; Kataoka *et al.*, 1985). Activation of *Ras* is catalyzed by the

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product of the *CDC25* gene, a protein that promotes the change of *Ras* from the GDP- to the GTP-bound state (Robinson *et al.*, 1987; Broek *et al.*, 1987).

Ras genes are essential for cell viability in yeast. Strains lacking either *RAS1* or *RAS2* are viable, however strains lacking both genes are not viable unless they also contain other mutations, such as *bcy1* (encoding the regulatory subunit of cAMP-dependent protein kinase), to suppress the lethality of *RAS* deficiency (Toda *et al.*, 1985). In wild-type cells, the expression of *RAS1* and *RAS2* is regulated at both the transcriptional and translational levels via multiple regulatory mechanisms which appear to ensure that at least one of the two *RAS* gene products will be synthesized under different growth conditions (Breviario *et al.*, 1988). Yeast strains carrying *RAS2* disruptions fail to grow on nonfermentable carbon sources, including ethanol, glycerol, acetate and pyruvate, probably due to the low level expression of *RAS1* under such conditions (Tatchell *et al.*, 1985).

The yeast *RAS2*^{val19} allele contains a *RAS2* gene with a valine substituted for glycine at amino acid position 19, and is analogous to mammalian *ras*^{val12} oncogene. *RAS2*^{val19} is dominant to wild-type *RAS2* gene (Kataoka *et al.*, 1984). Compared to wild-type cells, the intracellular level of cAMP is elevated in *RAS2*^{val19} mutants (Toda *et al.*, 1985).

Addition of glucose to derepressed yeast cells causes a transient increase in cAMP level, the precise mechanism by which glucose influences *Ras* activity is currently not clear (Francois *et al.*, 1984).

The main function of cAMP in the cell appears to be to activate cAMP-dependent protein kinase (cAPK). This kinase is a heterotetramer, consisting of two regulatory

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subunits and two catalytic subunits. In yeast, there is only one gene, *BCY1*, which codes for the regulatory subunit of cAPK. The yeast cAPK catalytic subunit is encoded by: *TPK1*, *TPK2*, and *TPK3*. These three catalytic subunits display overlapping substrate specificity (Toda *et al.*, 1987a, b).

It has been proposed that cAPK is involved in the glucose inactivation of the galactose and high-affinity glucose transport systems (Ramos *et al.*, 1989). It was found that in *tpk1(w1) tpk2Δ tpk3Δ* mutants with a reduced cAPK activity, the galactose and glucose transporters were not sensitive to glucose inactivation, while in *bcy1* mutants with unregulated high levels of cAPK activity, the transport systems were either absent or expressed at a very low level. In that study, the role of cAPK was not clearly demonstrated. Additionally, only transport rates were measured. The galactose and glucose transport proteins levels themselves were not monitored, nor were transcription rates of the genes encoding these proteins. Contradictory results have been reported recently. Riballo *et al.* (1994) showed that glucose inactivation of sugar transport systems does not require cAPK activity. Further investigation is required to determine whether cAPK is involved in glucose-induced inactivation of sugar transporters.

cAPK can regulate gene expression at transcriptional level. It is involved in the phosphorylation (and hence partial inactivation) of the transcriptional activator *ADR1*, which regulates glucose repressible *ADH2* gene expression (Denis *et al.*,1991). Since cAPK plays a role in glucose inactivation of some sugar transporters, the cAPK pathway may indirectly affect transcription of inducible genes by reducing levels of inducer via inactivation of cognate sugar transporters.

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Summary of thesis goals

The major goal of my thesis project is to investigate the glucose sensing and signal transduction pathways involved in glucose-induced inactivation of maltose permease. I have shown that the glucose sensing and signal transduction pathways stimulating maltose permease inactivation partially overlap with the glucose sensing and signaling pathway regulating gene expression. So far, we have identified two glucose sensing/signaling pathways: Pathway 1 (Rgt2p-dependent pathway) is predominately responsible for proteolysis of maltose permease; and Pathway 2 (sugar transport and phosphorylation-dependent pathway) is responsible for both the proteolysis of maltose permease and the rapid inhibition of maltose transport. Rgt2p and sugar kinases (hexokinase 1, 2 and glucokinase) appear to act as sensors monitoring extracellular and intracellular glucose levels, respectively, in these two pathways. The identification of the two glucose sensing/signaling pathways is described in chapter 2, and the characterization of each pathway is described in chapter 3 and 4, respectively.

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OVERVIEW

This thesis is organized into four chapters. Chapter 1 describes the characterization of glucose-induced inactivation of maltose permease, especially the mechanism of the proteolysis of maltose permease. Chapters 2 through 4 describe the identification of two glucose sensing/signaling pathways stimulating glucose-induced inactivation and further investigation of each pathway.

Chapter 1 is entitled "Characterization of the glucose-induced inactivation of maltose permease in *Saccharomyces*". This paper is co-authored by I. Medintz, myself, E.-K. Han, W. Cui and C.A. Michels, and appeared in the Journal of Bacteriology (1996) **178**:2245-2254. In this study, we characterize the glucose-induced inactivation of maltose permease in a genetically defined maltose fermenting strain carrying only the *MAL61/HA* allele of maltose permease. 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 degradation, and is independent on the proteosome. In addition, we show that the Mal61/HAp maltose permease is present in differentially phosphorylated in maltose-grown cells. Igor Medintz and myself contributed equally to this work. My contribution includes construction of the parental strain CMY1001 and its isogenic *pep4Δ, end3-ts,* and *hxk2Δ* mutants strains, and performing of all the sugar transport assays. Igor Medintz's contribution to this work includes construction of the *ren1Δ* mutant strain, and carrying out most of the Western analyses. In addition, Eun-Kyoung Han constructed the HA-tagged allele of Mal61 maltose permease; Wen Cui demonstrated that maltose permease exists in two differentially phosphorylated forms.

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Chapter 2 is entitled "Two glucose sensing/signaling pathways stimulate glucose-induced inactivation of maltose permease in *Saccharomyces*". This manuscript is co-authored by myself, I. Medintz, and C.A. Michels, and will appear in the July, 1997 issue of the Molecular Biology of the Cell. This is our first paper addressing the question of how glucose is sensed and how the inactivation signals are transduced. A model of two glucose sensing/signaling pathways stimulating glucose-induced inactivation of maltose permease is presented in this paper. My contribution to this work includes construction of the mutant strains, carrying out all the sugar transport and maltase assays, and most of the Western analyses. Igor Medintz carried out some of the Western analyses which are presented in Figure 1A and 1B, Figure 2C and 2D, and Figure 4A.

Chapter 3 is entitled "Metabolic signals trigger glucose-induced inactivation of maltose permease in *Saccharomyces*". This manuscript is co-authored by myself, I. Medintz, B. Zhang, and C.A. Michels. It is submitted to Molecular and Cellular Biology. In this report, we undertake further investigation of Pathway 2 (glucose transport-dependent signaling pathway). We find that the initial steps of sugar metabolism, including transport and phosphorylation are essential to generate the inactivation signal. The nature of the inactivation signal in this pathway is also discussed. My contribution to this study includes carrying out all the sugar transport, maltase and β-galactosidase assays and most of the Western analyses. Igor Medintz carried out some of the Western analyses which are presented in Figure 2, and Figure 3A and 3B. Bin Zhang constructed a series of double and triple sugar kinase mutant strains.

Chapter 4 is entitled "Role of protein phosphatase type-1 regulatory subunits Reg1p and Reg2p in the glucose-induced proteolysis of maltose permease in

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Saccharomyces". It is submitted to Genes and Development. In this report, I provide evidence suggesting that Reg1p and Reg2p are downstream of Grr1p in Pathway 1 (Rgt2p-dependent signaling pathway) and are involved in stimulating glucose-induced proteolysis of maltose permease. In addition, the results suggest that Reg1/Glc7 is to be indirectly involved in phosphorylation of maltose permease. All the experiments were done by myself except that the construction the *reg1Δ rgt2Δ* mutant strain used in this study which was done by Bin Zhang.

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CHAPTER 1

Characterization of the Glucose-induced Inactivation of Maltose Permease in *Saccharomyces*

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ABSTRACT

The addition of glucose to maltose fermenting *Saccharomyces* cells causes a rapid and irreversible loss in 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-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, that is, 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 at least two differentially phosphorylated forms.

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INTRODUCTION

The addition of glucose to maltose fermenting *Saccharomyces* cells causes an irreversible loss in 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 (Baustruia and Lagunas, 1985; Cheng and Michels, 1989; Gort, 1969). 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 (Baustruia and Lagunas, 1986; DeJuan and Lagunas, 1986; Holzer, 1976; Matern and Holzer; Ramos and Cirillo, 1989; Ramos *et al.*, 1988).

Early studies of the mechanism of glucose-induced inactivation of the gluconeogenic enzymes demonstrated glucose-stimulated proteolysis of these cytoplasmic enzymes (Muller and Holzer, 1981; Needleman, 1991; Tortora *et al.*, 1981). The mechanism of fructose-1,6-bisphosphatase 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 (Chiang and Schekman, 1991; Mazon *et al.*, 1982; Tortora *et al.*, 1981). The pathway of the proteolysis is as yet unresolved with conflicting published reports of both vacuolar-dependent and proteosome-dependent proteolysis (Chiang and Schekman, 1991, 1994; Schork *et al.*, 1994).

MAL61 of the *MAL6* locus and its nearly identical homologues at the other *MAL* loci (*MAL11*, *MAL21*, *MAL31*, and *MAL41*) encode *Saccharomyces* maltose permease. Mal61p is a high-affinity (2-4 mM) proton/maltose symporter, and is a member of the

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twelve transmembrane domain family of sugar transporters (Cheng and Michels, 1989, 1991). Expression of *MAL61* is maltose-induced and glucose-repressed (Cheng and Michels, 1989, 1991; Needleman, 1991). 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 homologues 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 *grr1* (unknown function) reviewed in reference Johnston and Carlson (1992). Second, glucose induces post-transcriptional events resulting in the rapid, irreversible loss in maltose transport activity (Gort, 1969).

In this report we use 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 that 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 in a recent report of the glucose-induced inactivation of maltose permease which employed some of the same mutations used in the present study (Riballo *et al.*, 1995). Additionally, our results

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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 carbon-source.

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MATERIALS AND METHODS

Strains and growth conditions. The *Saccharomyces* 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) plus the indicated carbon source or selection medium (0.67% yeast nitrogen base with ammonium) plus the appropriate supplements and the indicated carbon source.

Strain construction. An epitope-tagged allele of the cloned *MAL61* containing the sequence encoding the HA-epitope at the 5' end of the ORF was constructed by oligonucleotide-directed site-specific mutagenesis using the BioRad Mutagene kit (Kolodziej and Young, 1991). The added sequence encodes 15 residues: a Met residue, the 12 residue epitope derived from the influenza virus hemagglutinin 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 using homology between the plasmid copy of *MAL61/HA* and the genomic *mal11Δ::URA3* gene, containing an internal deletion of *MAL11*. A single copy integration strain was chosen by Southern analysis of the transformants, and uracil$^-$ recombinants were selected using 5-fluoro-orotic acid (Boeke *et al.*, 1984). Maltose fermentation was confirmed as in reference Charron *et al.* (1986). These were then screened using Southern analysis to confirm the loop-out, and Western analysis was done to confirm the presence of the epitope-tag. This procedure replaced at least part if not all of the *mal11Δ::URA3* gene in strain 100-1A with *MAL61/HA* to produce strain

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Table 1. List of *Saccharomyces* strains

Strain	Genotype	Reference
CMY1001	*MATa MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200*	This study
CMY1002	*pep4Δ* (isogenic to CMY1001)	This study
CMY1003	*ren1Δ* (isogenic to CMY1001)	This study
CMY1004	*end3-ts* (isogenic to CMY1001)	This study
CMY1006	*hxk2Δ* (isogenic to CMY1001)	This study
WCG4a	*MATa his3-11,15 leu2-3,112 ura3-Δ5 GAL*	Heinemeyer *et al.*, 1993
WCG4-11/21a	*pre1-1 pre2-1* (isogenic to WCG4a)	Heinemeyer *et al.*, 1993
WCG4-11/22a	*pre1-1 pre2-2* (isogenic to WCG4a)	Heinemeyer *et al.*, 1993
YPH500	*MATα leu2-Δ1 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 ura3-52 AGT1 MAL12 MAL31 MAL32*	Sikorski and Hieter, 1989
600-1B	*MATa MAL11 MAL12 MAL13 leu2-3, 112 ura3-52*	Charron *et al.*, 1986
100-1A	*MATa mal11Δ::URA3 MAL12 MAL13 leu2-3, 112 ura3-52*	Charron *et al.*, 1986

The *Saccharomyces* strains used in this study are listed. It should be noted that 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).

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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 strain to YPH500 (see 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*) (unpublished results, Sikorski and Hieter, 1989). Strain CMY1001 is a maltose fermenting random segregant containing solely the *MAL1* locus derived from 100-1A::MAL61/HA, and was used in the following constructions (Table 1).

Strain CMY1002 carries a *pep4Δ* deletion/disruption and is otherwise isogenic to CMY1001. It was constructed by two-step gene replacement using plasmid pPLO2010 (obtained from Steven Nothwehr) which contains a *pep4Δ* allele lacking a *Hin*dIII fragment of the wild-type sequence carried on an *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$^+$ transformants selected. Uracil$^-$, potential loop-out of the *URA3* gene and the adjacent *PEP4*, were selected using 5-fluoro-orotic acid, and loss of vacuolar protease function was tested by the APNE plate assay (Boeke *et al.*, 1984; Wolf and Fink, 1975). Deletion of *PEP4* was confirmed by Southern analysis.

Strain CMY1003 is isogenic to CMY1001 but contains a *ren1Δ*. 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$^+$. 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

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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 ORF, 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 (Raths *et al.*, 1993).

Strains WCG4a, WCG4-11/21a, and WCG11/22a are an isogenic strain series containing *PRE1 PRE2*, *pre1-1 pre2-1*, and *pre1-1 pre2-2* alleles, respectively (Heinemeyer *et al.*, 1993). *PRE1* and *PRE2* encode components of the protease. None of these strains ferment maltose because they lack a Mal-activator gene, despite the fact that they carry the *MAL* structural genes. Thus, in order to carry out our analyses, we introduced into these strains the *MAL63* Mal-activator gene carried on a *URA3* CEN plasmid, pRS316MAL63 (Needleman, 1991; Sikorski and Hieter, 1989). In order to be able to follow 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 (Sikorski and Hieter, 1989).

Strain CMY1006 is isogenic to CMY1001 but contains a *hxk2Δ* null allele. Plasmid pRB528 (from David Botstein) carries a *hxk2Δ::URA3* deletion/disruption. It was digested with *Eco*RI and used to transform CMY1001 to uracil$^+$. 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 (Dubin *et al.*, 1989).

Inactivation protocol. Cells were grown at 30°C to early log phase (OD_{600} 0.1-0.3) in rich medium containing 2% maltose, harvested by filtration using cellulose filters,

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and resuspended in either rich medium or nitrogen-starvation medium (1.74 gm/liter yeast nitrogen base without amino acids and without ammonium sulfate) plus either 2% ethanol (vol/vol) or 2% glucose (wt/vol). At selected time intervals cells were harvested for Western analysis and maltose transport assays using cellulose filters. Growth dilution was calculated as the OD_{600} at time 0 divided by the OD_{600} at time X.

Maltose transport assay. Maltose transport was measured by the uptake of ^{14}C-maltose as described in Cheng and Michels (1991). Cells were harvested by filtration, washed and resuspended in 0.1 M tartaric acid (pH 4.2) to $O.D._{600}$ 30. Eighty microliters of cells were taken and incubated with ^{14}C-maltose for 10 sec. at room temp. then washed 4 times with ice cold water, followed by scintillation counting. With the exception of the results shown in Figure 3, maltose transport rates were determined using 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 the preparation of protein extracts. The preparation of total cell protein extracts is according to the method described by Davis *et al.* (1993) with the addition of a protease inhibitor cocktail (Ausubel *et al.*, 1995). Where noted, protein kinase and phosphatase inhibitors were also included (James *et al.*, 1989). After assaying, equal amounts of protein (usually about 80-100 mg) were loaded into each lane of a standard 10% or 7.5% (where noted) acrylamide gel for SDS-PAGE analysis using standard methods (Laemmli, 1970). Duplicate gels were stained with Commassie Blue to insure even loading and membranes were stained with amido black to insure even transfer. Membranes were probed with mouse anti-hemagglutinin anti-body (12CA5 Boehringer Mannheim) as the primary

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antibody and sheep anti-mouse Ig, HRP-linked secondary antibody. Detection was visualized using the 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 determined by comparison of the area under the curve. Westerns 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 were harvested by filtration, quick frozen at -70°C. For the phosphatase treated samples, the cells were thawed by resuspending in 200 ml of 40 mM PIPES buffer, pH 6.0, 1 mM DTT, plus protease inhibitors and an equal volume of glass beads was added. This was vortexed for 10 minutes, 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 HEPES buffer, pH 7.2, plus the protease inhibitors for the alkaline phosphatase treatment. The indicated number 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 hour. Following this, protein was extracted from the membranes using 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 analysis was done as described above. The sample receiving no treatment, labeled "None" in Figure 7B, was extracted directly from whole cells as described above.

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RESULTS

Construction, expression and characterization of *MAL61/HA*, a hemagglutinin-tagged allele of *MAL61*. In order to follow the level of maltose permease protein, we constructed a hemagglutinin epitope-tagged allele of *MAL61*, referred to as *MAL61/HA* (described in Materials and Methods). This allele was used to replace the disrupted *mal11::URA3* gene in strain 100-1A, a disruption of the maltose permease gene of *MAL1*, using two-step gene replacement thereby creating a *MAL1* locus containing the *MAL61/HA* sequence. This strain was crossed to YPH500 (see Table 1), and strain CMY1001 (see Table 1) is a maltose fermenting segregant carrying only this reconstructed *MAL1* locus (*MAL61/HA MAL12 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 *mal11Δ::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 Figure 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 (about 1-2 mM) is not significantly different from that reported for wild-type Mal61p (Cheng and Michels, 1991).

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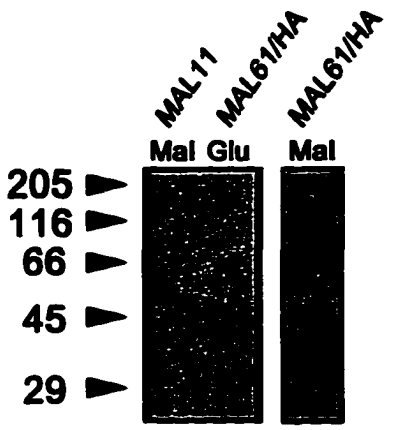
Figure 1. Characterization and regulation of the Mal61/HA protein. Strains 600-1B and 100-1A[pMAL61/HA] were grown in rich medium plus 2% maltose or 2% glucose. Protein extracts were propared and analyzed by Western blotting, as descried in Materials and Methods.



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Glucose-induced inactivation of maltose transport exhibits two components in nitrogen-starvation, but only one in rich media. CMY1001 cells were grown to early log phase in rich medium with 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 using the anti-HA antibody as described in Materials and Methods. Each of these measures (growth dilution, maltose transport rate, and maltose permease protein levels) were 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.

Figure 2 compares the effects of glucose and ethanol on maltose transport and maltose permease protein levels in rich media versus nitrogen-starved media. 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 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 minutes. On the other hand, loss of maltose permease protein occurs at the same rate as is seen in rich medium (half-life of about 45 minutes).

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Figure 2. Glucose-induced inactivation of maltose permease in rich versus nitrogen-starvation medium. Strain CMY1001 was grown in rich medium plus 2% maltose, harvested, transferred to either rich medium or nitrogen-starvation medium plus either 2% glucose (Panel A) or 2% ethanol (Panel 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 to the zero time sample are plotted along with the growth dilution (■,dotted line). 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.

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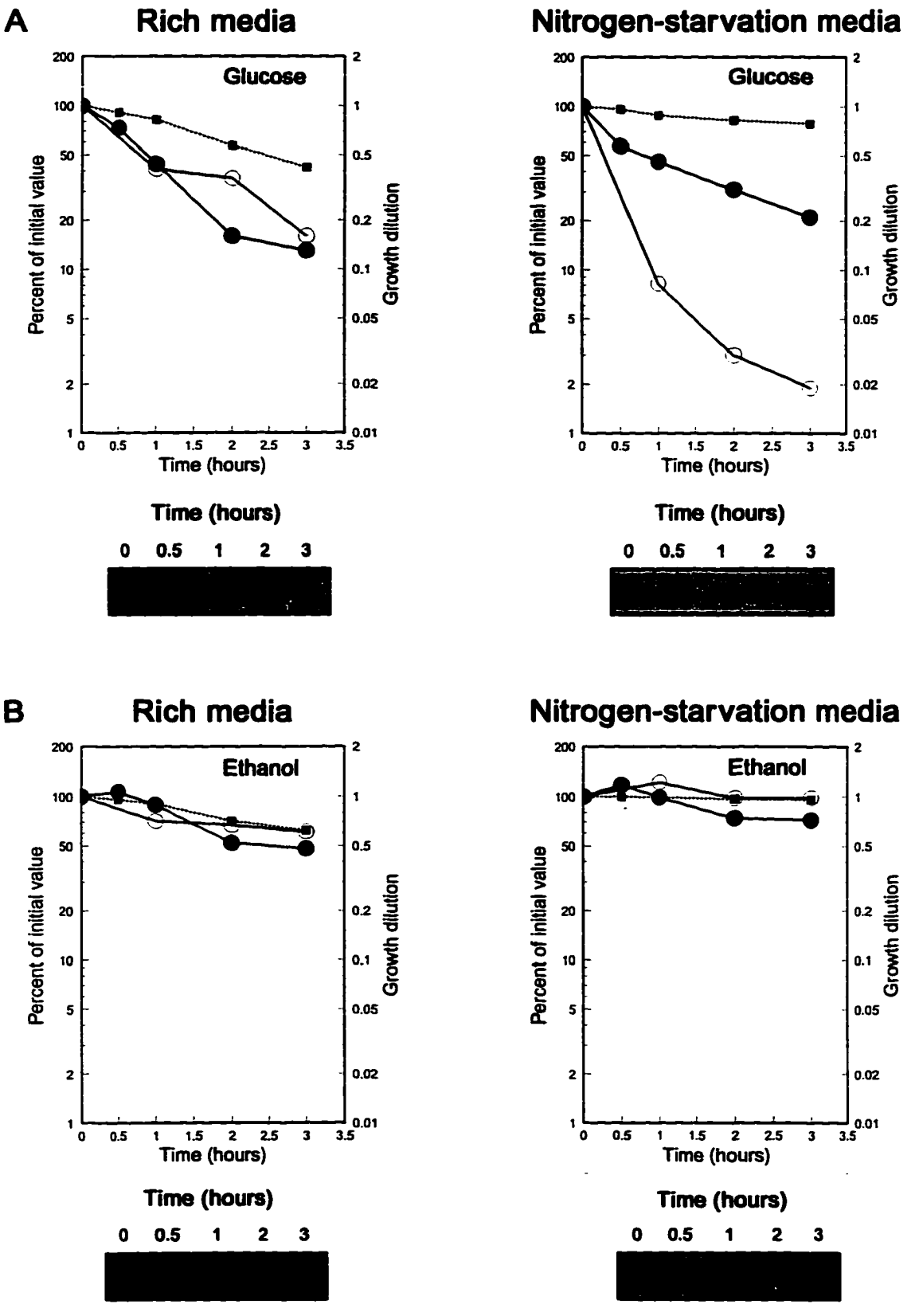

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These results indicate that, when strain CMY1001 is transferred to nitrogen-starvation medium plus glucose, two kinetically distinct processes contribute to the inactivation of maltose permease. The first is a rapid loss of the transport activity which we refer to as the inhibition of transport activity, and the second is a slower loss in 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, nor did Riballo *et al.* (1995) see evidence of this inhibition in their studies which utilized different strains. Moreover, in our analysis of strains carrying *PRE* mutations (described below), which utilized the same strains as did Riballo *et al.* (1995), 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 K_m of the maltose transporter or a decrease in V_{max}. CMY1001 cells were grown in rich medium plus maltose, harvested, and subjected to glucose inactivation in rich or nitrogen starved media. Samples were taken at time zero and at the indicated time where 50% loss in transport activity was achieved in each medium. Lineweaver-Burk analysis of the results is shown in Figure 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 (Peinado and Loureiro-Dias, 1986).

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Figure 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 Figure 2. At the indicated time, maltose transport was determined at a range of substrate concentrations. These were compared to maltose transport in cells at time zero before inactivation (■).

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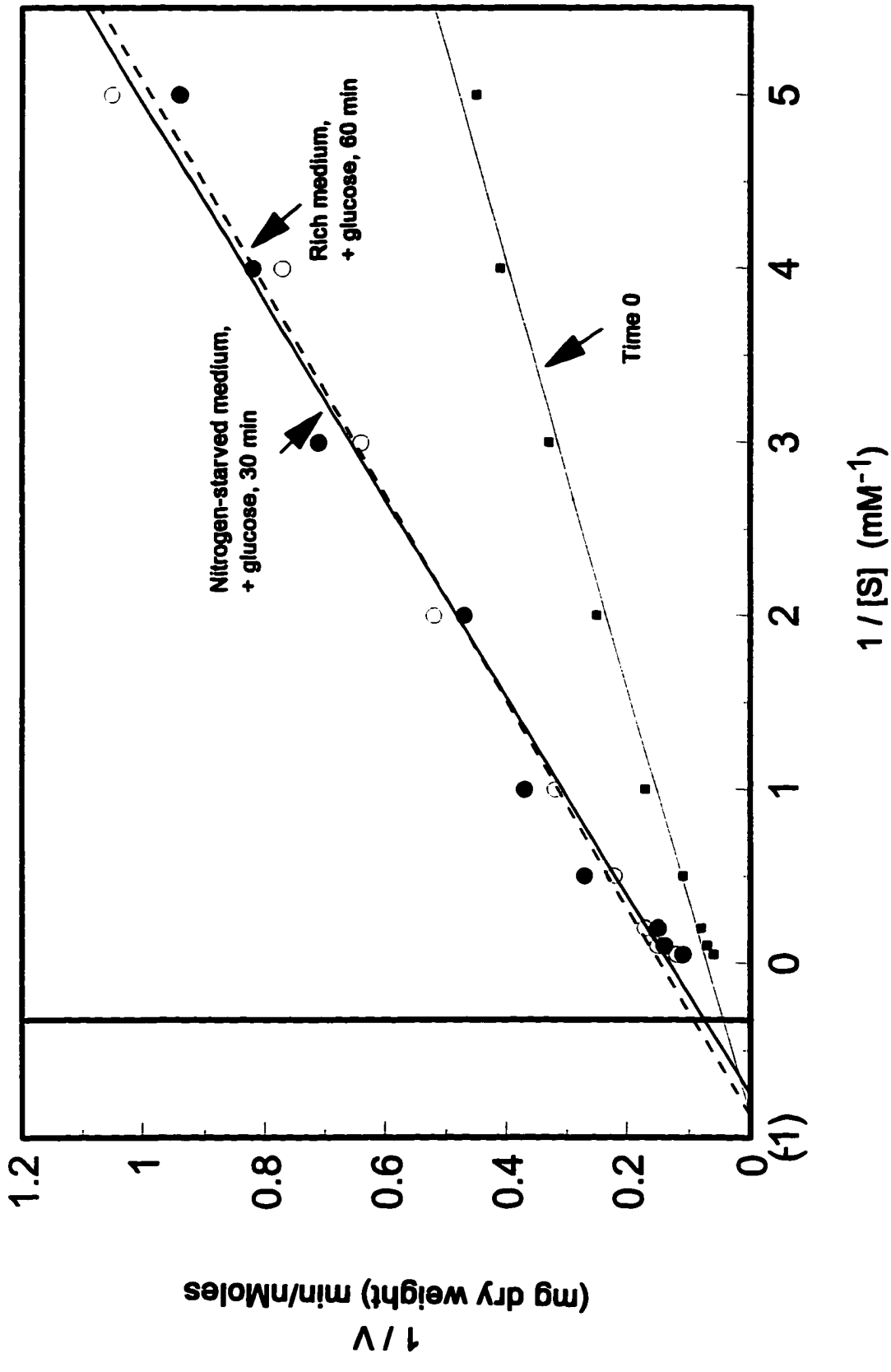

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Mechanism of proteolysis of maltose permease. We explored the pathway of proteolysis of the maltose permease protein using mutations in genes known to control early steps in endocytosis (*END3*), vesicle targeting to the vacuole (*REN1*), vacuolar 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 using a cloned copy of an *end3-ts* allele (Mazon *et al.*, 1982). Glucose-induced inactivation of maltose permease was followed in the parental (CMY1001) and *end3-ts* (CMY1004) strains with the following exception. The strains were grown in 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 (see Figure 4). The level of maltose permease protein in the *end3-ts* strain grown induced at the permissive temperature is approximately 2-3 fold 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, suggesting that the inhibition of transport activity is independent of the proteolysis process or is upstream of the End3p function.

The *ren1Δ* and *pep4Δ* 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 (Ammerer *et al.*, 1986; Hemmings *et al.*, 1981). Ren1p (Vps2p) functions late in

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Figure 4. Glucose-induced inactivation of maltose permease in an *end3-ts* strain defective in early endocytosis. Strains CMY1001 (Panel A) and CMY1004 (*end3-ts*) (Panel B) were grown as described for Figure 1 except 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.

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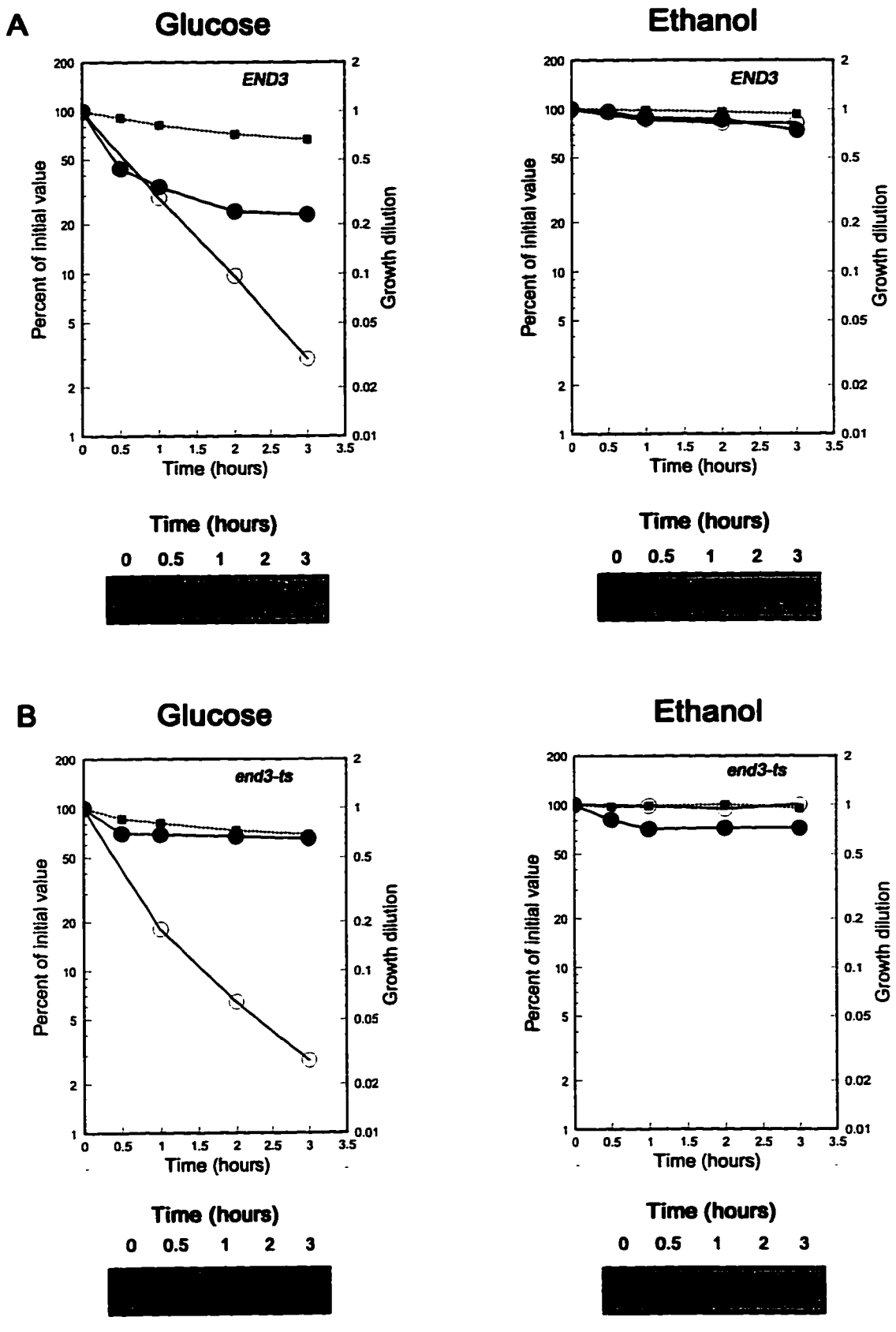

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Table 2. Maltose transport rates of strains mutated in genes required for endocytosis vesicle trafficking, and proteolysis

Strain	Relevant Genotype	Transport rate (nmol/mg[drywt]/min)
CMY1001	*END3 REN1 PEP4*	2.40, 2.47 (25°C)
CMY1002	*pep4Δ*	2.91
CMY1003	*ren1Δ::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

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.

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endocytosis, and appears to be involved in vesicle transport from the endosome to the vacuole (Davis *et al.*, 1993). Inactivation of maltose permease was followed in these strains, and the results are shown in Figure 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 to the isogenic wild-type shows approximately 2-3 fold higher levels in the *pep4Δ* strain but no comparable increase in maltose transport (Table 2), and 2-3 fold lower levels of maltose permease protein in the *ren1Δ* strain with a coordinate decrease in transport rate (Table 2). The rapid inhibition of maltose transport is unaffected in both the *ren1Δ* and *pep4Δ* 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 (Heinemeyer *et al.*, 1993). 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 a 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 followed using the standard inactivation protocol, and the results are shown in Figure 6.

No significant difference can be seen between the *pre1 pre2* mutant strains and the isogenic wild type 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 in maltose

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Figure 5. Glucose-induced inactivation of maltose permease in a *pep4Δ* and a *ren1Δ* strain defective in vacuolar proteolysis and vesicle trafficking to the vacuole. Strains CMY1002 (*ren1Δ*) (Panel A) and CMY1003 (*pep4Δ*) (Panel B) were grown as described in Figure 1, 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 (●) determined, as described in Materials and Methods.

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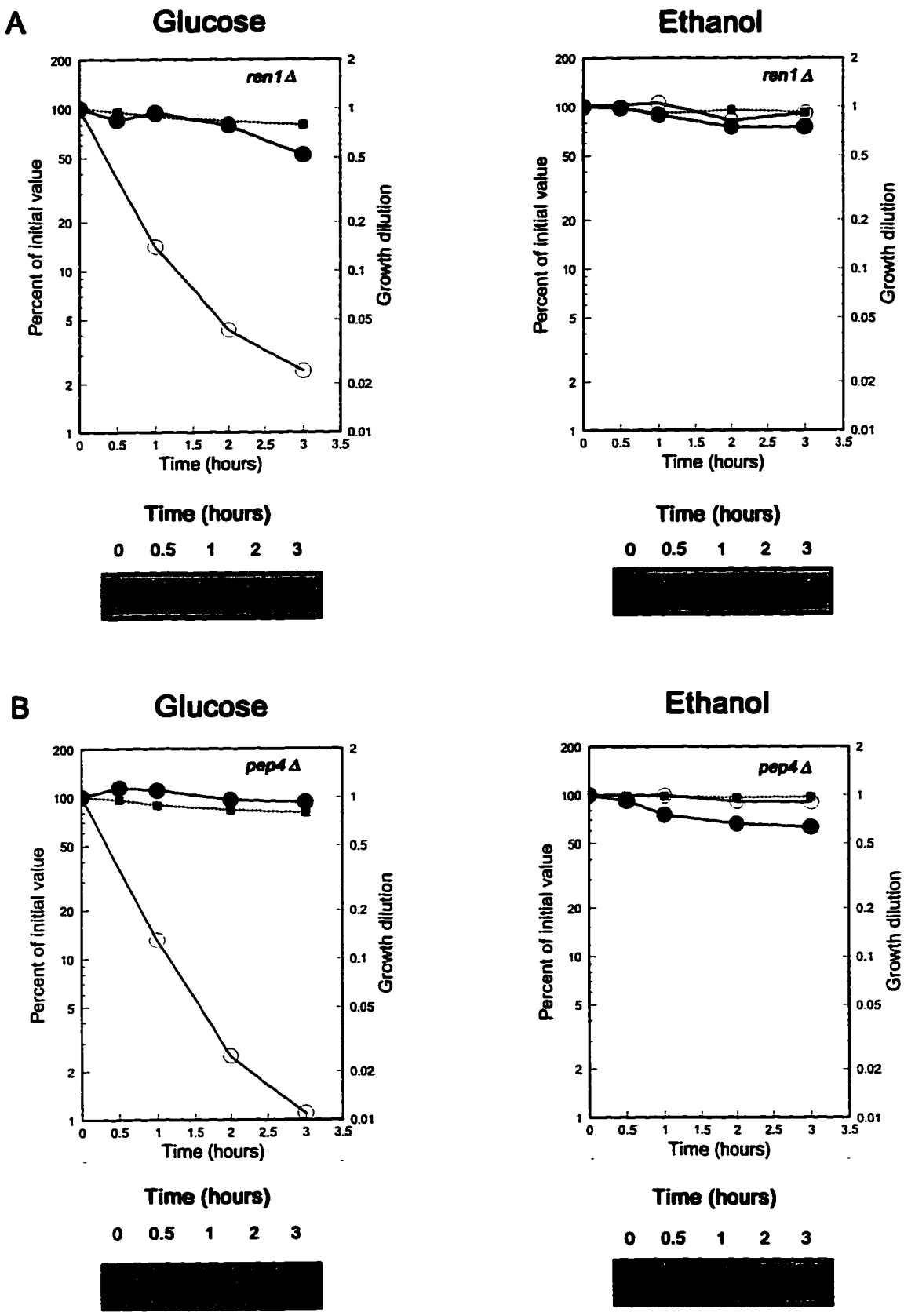

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Figure 6. Glucose-induced inactivation of maltose permease in *pre1 pre2* mutant strains with defects in proteosome function. Strains WCG4a (*PRE1 PRE2*) (Panel A), WCG4-11/21a (*pre1-1 pre2-1*) (Panel B), and WCG4-11/22a (*pre1-1 pre2-2*) (Panel C) 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, 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.

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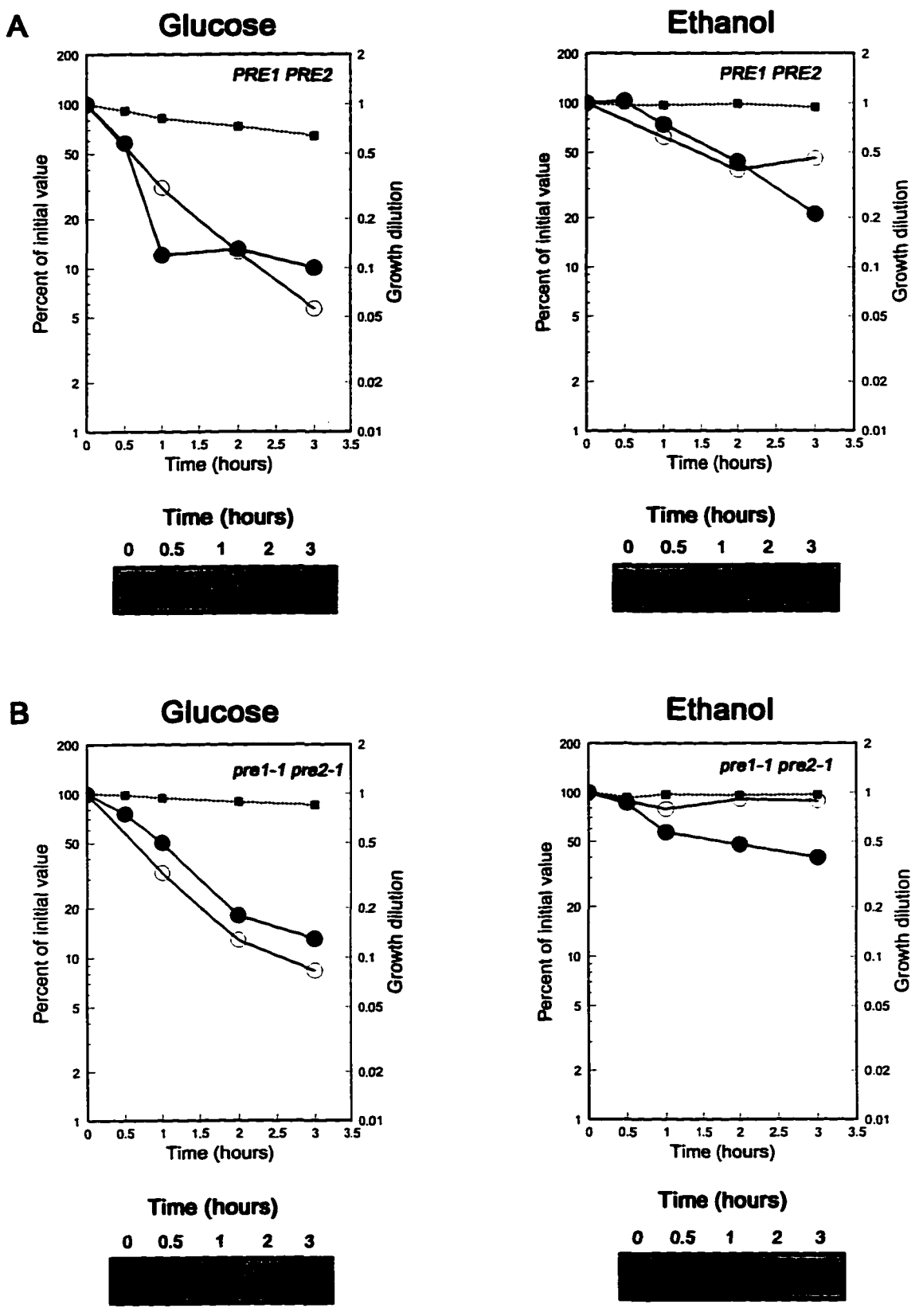

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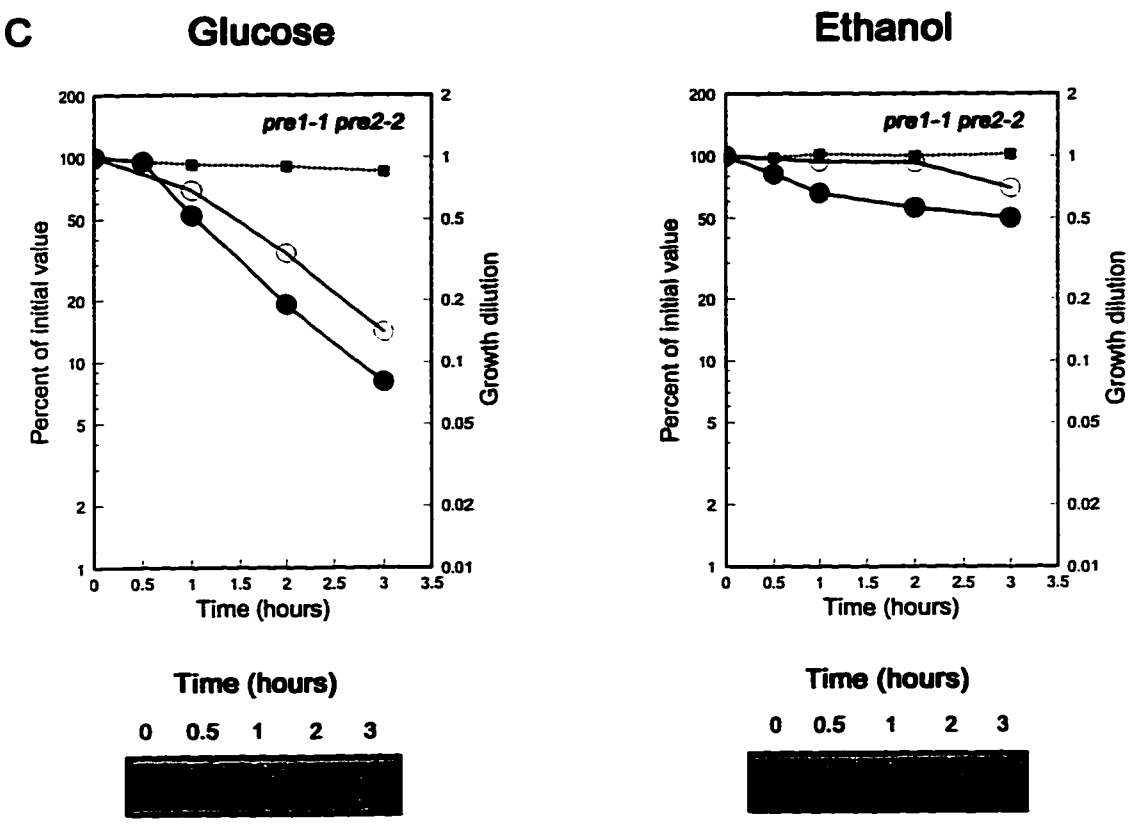
(Figure 6 continued)



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transport activity correlates with the loss in maltose permease protein even in the nitrogen-starvation conditions, as was reported by Riballo *et al.* (1995).

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 in the gel conditions used in Figure 7A. Treatment of total protein extracts with increasing amounts of acid phosphatase decreases the relative amount of the slower migrating form(s). At the maximal treatment, only the faster migrating form is 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 and 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 *hxk2Δ* mutation (Jiang *et al.,* submitted). 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 using extraction buffer containing protease inhibitors, and protein kinase and protein phosphatase inhibitors. Western analysis was carried out on different dilutions of protein

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extract to be sure that the intensity of the band was linearly related to protein levels. As can be seen in Figure 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.

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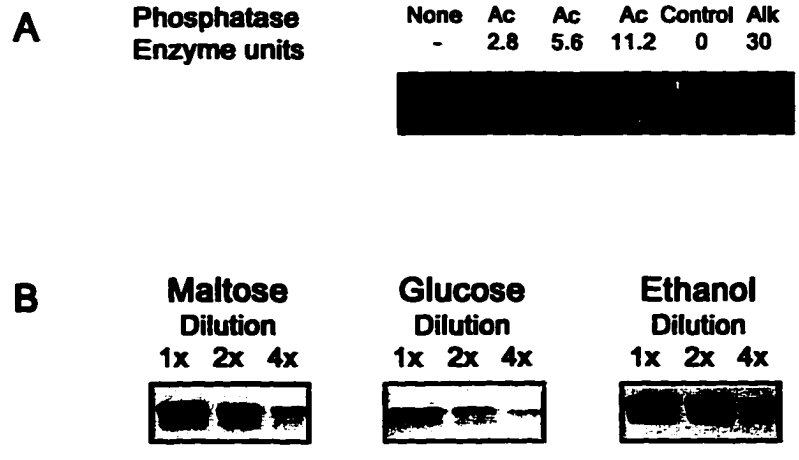
Figure 7. Phosphorylation of Mal61/HAp. Panel A. Strain CMY1001 was grown in rich medium plus 2% maltose. A crude membrane preparation was made, and treated with the indicated number of units of either alkaline or acid phosphatase for 1 hour at 37°C. The lane labeled "None" is a sample extracted from whole cells using Sample Buffer and given no treatment at all and the lane labeled "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 that approximately 3-times less membrane preparation 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 using 7.5% acrylamide gels at constant amperage to achieve the separation shown. Panel B. Strain CMY1006:pMal64-R10 was grown in rich media in either 2% maltose, 2% glucose, or 2% ethanol. Total protein extracts were prepared using the extraction buffer containing a cocktail of protein kinase and phosphatase inhibitors (James *et al.*, 1989). Three different dilutions of each extract were used for Western analysis.



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DISCUSSION

Gorts (1969) first described the glucose-induced inactivation of maltose transport. He noted that the inactivation took about 90 minutes, and was irreversible in the absence of *de novo* protein synthesis in maltose-induced conditions. Kinetic analysis of maltose transport in *Saccharomyces* strains identified both high-affinity (K_m 2-4 mM) and low-affinity (K_m about 70 mM) components (Busturia and Lagunas, 1985; Cheng and Michels, 1991). Using genetically defined strains carrying a single maltose permease gene (*MAL61* or *MAL11*), Cheng and Michels (1991) 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 hemagglutinin epitope-tagged allele of *MAL61*, we were able to follow both maltose transport rates and levels of maltose permease protein, and directly compare the two.

We show that glucose but not ethanol stimulates the proteolysis of maltose permease in both rich and nitrogen-starvation medium, a finding that is consistent with previous reports (Lucero *et al.*, 1993; Riballo *et al.*, 1995). The half-life of Mal61/HAp measured in nitrogen-starvation conditions, where little or no cell growth is occurring, is 8 hours or greater in ethanol and 30-60 minutes in glucose. The half-life of several *Saccharomyces* membrane proteins has been determined and shown to vary from 13 minutes to 30 hours depending on the protein, and, for most, the half-life is regulated by the physiological state of the cell and/or the presence or absence of ligand (Beino *et al.*, 1991; Davis *et al.*, 1993; Kolling and Hollenberg, 1994; Lai *et al.*, 1995; Ramos *et al.*, 1988; Volland *et al.*, 1994). The rate of degradation of the uracil permease is increased

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in a variety of stress conditions including starvation for carbon, phosphate and nitrogen (Volland *et al.*, 1994). On the other hand, degradation of the otherwise stable inositol permease is induced by the addition of inositol to the growth medium which appears to stimulate a ligand-induced change in the protein targeting it to the degradation pathway (Lai *et al.*, 1995). Recently reported studies of the a-factor receptor Ste2p showed that a-factor stimulated the already high constitutive rate of turnover (Hicke and Riezman, 1996).

Endocytosis and vacuolar proteolysis has been demonstrated as the pathway of degradation for all of the *Saccharomyces* membrane proteins studied to date, and maltose permease is no exception (Benito *et al.*, 1991; Davis *et al.*, 1993; Hieck and Riezman, 1996; Kolling and Hollenberg, 1994; Lai *et al.*, 1995; Raths *et al.*, 1993; Riballo *et al.*, 1995; Stanbrough and Magasanik, 1995; Volland *et al.*, 1994). 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 *pep4Δ* strains accumulate 2-3 fold higher levels of Mal61/HA protein 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 *pep4Δ* strain despite the abundant increase in levels of Mal61/HAp, and 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 *ren1Δ* strain exhibits reduced levels of Mal61/HAp is somewhat surprising considering that the *ren1-1* allele was selected for its ability to

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accumulate a-factor receptor at the cell surface as a result of a reduced rate of internalization of ligand-bound Ste3p (Davis *et al.*, 1993). We had expected that the *ren1Δ* 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.* (Riballo *et al.*, 1995). Perhaps these unexpectedly low levels of maltose permease can be attributed to our use of a *ren1Δ* as opposed to the *ren1-1* allele. If so, this suggests a role for Ren1p in secretion as well 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 is occurring in addition to its proteolysis. This inhibition was not reported by Riballo *et al.* (1995), and we believe that this reflects differences in the strains used in the two studies. Consistent with Riballo *et al.* (1995), we also did not detect the inhibition component when we followed the glucose-induced inactivation of Mal61/HA maltose permease in the WCG4a isogenic series of strains (Figure 6).

Among the mechanisms one could propose for the initial glucose-induced inhibition of maltose transport activity are post-translational 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 FBPase does not appear to require phosphorylation at this site (Marcus *et al.*, 1988; Mazon *et al.*, 1982;

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Rittenhouse *et al.*, 1986; Tortora *et al.*, 1981). 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 (Stanbrough and Magasanik, 1995). The finding that the inhibition of maltose permease occurs at wild-type rates in the *end3-ts* mutant strain where 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 is occurs in strains and/or growth conditions where 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} and not a change in K_m, which is consistent with studies reported by Busturia and Lagunas (Busturia and Lagunas, 1986) but not with those of Peinado and Loureiro-Dias (Peinado and Loureiro-Dias, 1986) 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 mobility in SDS-PAGE analysis were reported in studies of a maltose fermenting strain of undefined *MAL* genotype (Lucero *et al.*, 1993; Van den Broek *et al.*, 1994). Lucero *et al.* (1993) detected maltose

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permease protein using a polyclonal antibody, and thus it was not clear whether the two species represented different modified forms of the permease or if the antibody also detected Agt1p, a second maltose permease present in many laboratory strains (Han *et al.*, 1995). Analysis of the endopeptidase Lys-C peptides carried out by Van den Broek *et al.* (Van den Broek *et al.*, 1994) suggested that the two forms were highly sequence homologous indicating that Agt1p is an unlikely candidate for either species (Han *et al.*, 1995). 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* a-factor receptor and in several mammalian membrane receptors, including the insulin receptor and the asialoglycoprotein receptor (Fallon *et al.*, 1994; Kublaoui *et al.*, 1995; Zanolari *et al.*, 1992). 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 (Peinado and Loureiro-Dias, 1986). Similarly, the human insulin-responsive glucose transporter *GLUT4* is functionally activated in the phosphorylated state (James *et al.*, 1989). The *Saccharomyces* uracil permease is also differentially phosphorylated, but the functional significance of this phosphorylation is unknown (Volland *et al.*, 1994). Thus, phosphorylation of maltose permease might impact on 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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CHAPTER 2

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

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ABSTRACT

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 post-translational 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 permease. 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.

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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 post-translational levels (reviewed in Holtzer, 1976; Gancedo, 1992; Trumbly, 1992; and Johnston and Carlson, 1993). 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*) (Ozcan and Johnston, 1995; Ozcan *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 post-translational 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.*, 1996). Here we describe an investigation of the glucose sensing/signaling pathways utilized for the

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glucose-induced inactivation of maltose permease.

Maltose transport is subject to glucose-induced inactivation (Gorts, 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.

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MATERIALS AND METHODS

Strains, strain construction and plasmids. The *Saccharomyces* strains used in this study are derived from CMY1001 (Table 1) (*MATa 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 (*grr1Δ rgt1Δ*), and CMY1012 (*snf3Δ rgt2Δ*).

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 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 analysis.

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

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Table 1. List of *Saccharomyces* strains

Strain	Genotype	Reference
CMY1001	*MATa MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-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	*grr1Δ rgt1Δ* (isogenic to CMY1001)	This study
CMY1012	*snf3Δ rgt2Δ* (isogenic to CMY1001)	This study

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 Medintz, *et al.*, 1996 for full description).

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Replacement of *RGT1* was confirmed by Southern analysis.

In strains CMY1009 and CMY1012, the ORF of *RGT2* gene was replaced by kan^R using PCR based gene disruption. The kan^R gene from the *E.coli* transposon Tn903 when expressed in yeast results in aminoglycoside antibiotic G418 resistance of the transformants. For the PCR reaction, plasmid pBM3251 containing kan^R (obtained from Sabire Ozcan and Mark Johnston) was used as the template. The 5' oligo contains 45bp upstream of the ATG of *RGT2* followed by 19bp from kan^R 5' sequence (5'CAGAAACCACTATATATATATGGAAATATCTCGAATATTGCTTGTCAGCTG AAGCTTCGTACGC3'). The 3' oligo contains 45bp from *RGT2* 3' sequence followed by 22bp of kan^R 3' sequence (5'AAACGGTTTATAAGACCTCGAACGATCGTAAGA TGCTATTGGTTTGCATAGGCCACTAGTGGATCTG3'). The PCR product was transformed directly into CMY1001 and CMY1007, and transformants selected on YPD supplemented with 50 mg/l Geneticin (G418 sulfate from Gibco/BRL). 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 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 Ozcan and Mark Johnston.

Inactivation protocol. The protocol is described in detail in Medintz *et al.* (1996). Cells were grown at 30°C to very early log phase (OD_{600} of 0.1 to 0.3) in rich medium containing 2% maltose, harvested, and transferred to nitrogen-starvation medium (1.74 gm/liter yeast nitrogen base without amino acids and ammonium sulfate) plus 2%

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glucose (or 2% ethanol, or rich medium plus 2% glucose, as indicated). Cells were harvested at the indicated times over a 3 hour 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 analysis of SDS-PAGE gels using the anti-HA specific antibody, and the relative amount measured by densitometric comparison to the zero time sample. Western analysis was done in duplicate on duplicate samples 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 1mM ^{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 in order 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 using extracts from at least two separate cultures.

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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 hemagglutinin 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 log 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 hours immediately following 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).

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; or unpublished results). We have shown that the rapid inhibition of transport activity is not

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

Genotype	Maltose transport activity (nmol·mg dry wt^{-1}·min^{-1})	Maltase activity (nmol PNPG·mg protein^{-1}·min^{-1})
wild-type	**6.38**	**603**
snf3Δ	**7.47**	**581**
rgt2Δ	**6.72**	**667**
snf3Δ rgt2Δ	**7.81**	**702**
grr1Δ	**7.52**	**623**
rgt1Δ	**2.12**	**395**
grr1Δ rgt1Δ	**5.49**	**757**
***GRR1*[pRGT2-1]**	**1.89**	**525**
***grr1Δ*[pRGT2-1]**	**4.09**	**726**

Strains CMY1001 (parental strain), CMY1005 (*grr1Δ*), CMY1007 (*snf3Δ*), CMY1008 (*rgt1Δ*), CMY1009 (*rgt2Δ*), CMY1010 (*grr1Δ rgt1Δ*) and CMY1012 (*snf3Δ rgt2Δ*) were grown in rich medium plus 2% maltose. Strains CMY1001 and CMY1005 transformed with plasmid pRGT2-1 and were grown in selective medium lacking uracil and containing 2% maltose. Cells were grown to early log phase. For each strain, two aliquots of cells were harvested, one aliquot was used to measure maltose transport activity and the other one was used to determine maltase activity as described in Materials and Methods.

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Figure 1. Effect of *snf3Δ*, *rgt2Δ*, and *snf3Δ rgt2Δ* mutations on glucose-induced inactivation of maltose permease. Strains CMY1001 (parental strain), CMY1007 (*snf3Δ*), CMY1009 (*rgt2Δ*), and CMY1012 (*snf3Δ rgt2Δ*) 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 in the same panel. The relative protein level and transport activity at time X are compared to 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 underneath.

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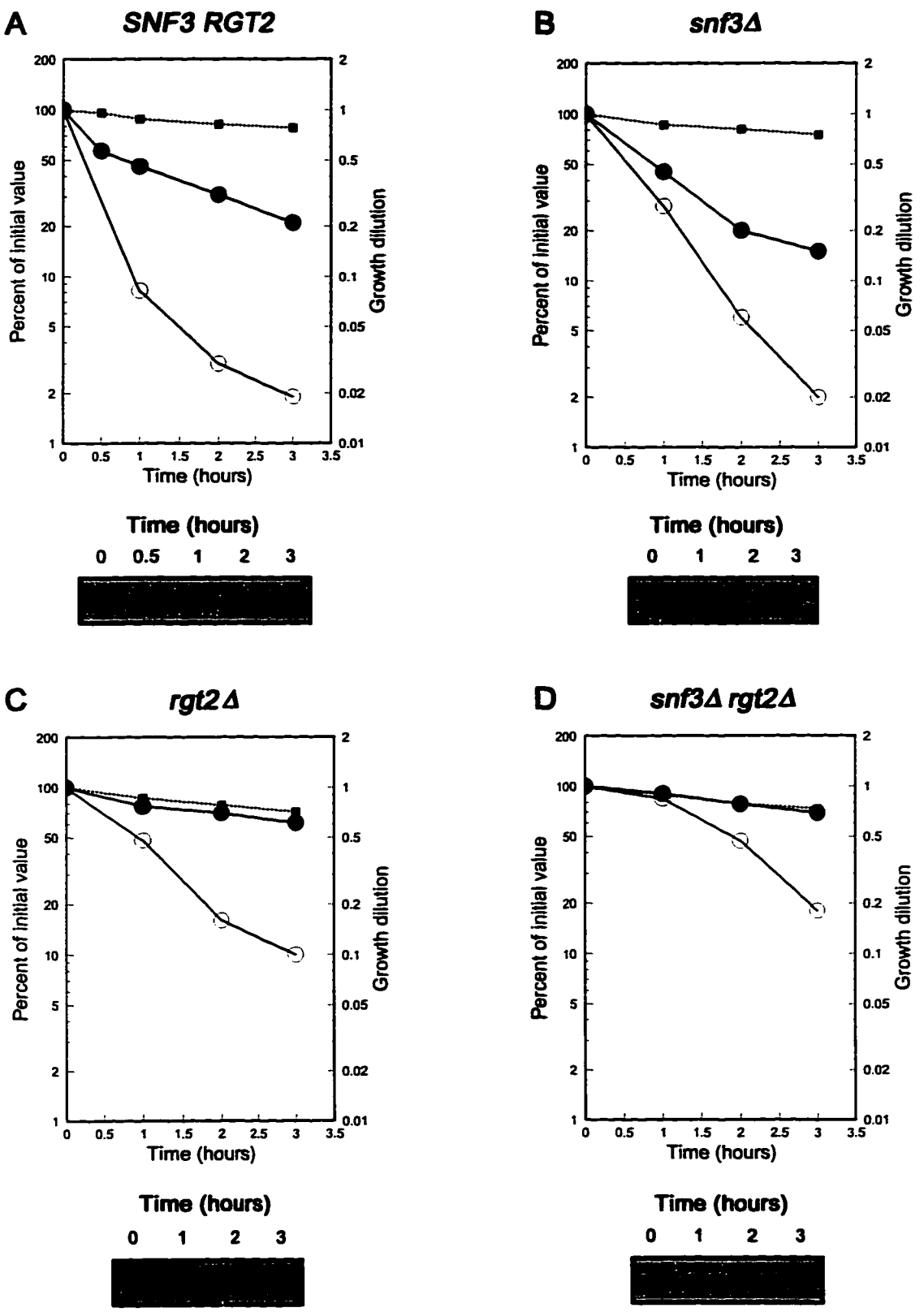

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blocked in *end3* or *ren1(vps2)* mutant strains suggesting that an initial, very rapid process is occurring which functionally inactivates the maltose permease prior to the rate limiting proteolysis (Medintz *et al.*, 1996). We have not yet determined 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 media containing low concentrations of glucose (Bisson *et al.*, 1987). The recently identified *RGT2* gene encodes a protein which is highly sequence homologous to Snf3p (Ozcan *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 (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996a, b). To test if *SNF3* or *RGT2* function as glucose sensors in glucose-induced inactivation of maltose permease, we constructed strains CMY1007 (*snf3Δ*), CMY1009 (*rgt2Δ*) and CMY1012 (*snf3Δ rgt2Δ*), which are all isogenic to strain CMY1001, and assayed their sensitivity to glucose-induced inactivation using 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 permease proteolysis and significantly reduces the rate of rapid inhibition of maltose transport. Deletion of *SNF3* enhances the *rgt2Δ* phenotype, and entirely blocks proteolysis, while the rapid inhibition of transport activity is reduced even further (Figure 1D).

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Results of kinetic analysis of glucose transport in the maltose-grown *snf3Δ, rgt2Δ, snf3Δ rgt2Δ* 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 to 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 *grr1Δ* cells. Thus, Grr1p is essential for glucose-induced inactivation and the glucose-insensitive phenotype of *grr1Δ* mutations is not an indirect result of the increased nitrogen starvation sensitivity of *grr1Δ* mutants.

The V_{max} of glucose transport in the maltose-grown *grr1Δ* strain is 15% of that seen in the parental strain (Table 4). This suggests the possibility 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.

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Table 3. Glucose transport activity in maltose-grown *snf3Δ*, *rgt2Δ*, and *snf3Δ rgt2Δ* mutant strains.

Genotype	Vmax (nmoles/mg dry wt · min)	Relative Vmax %	Km (mmoles)
Wild-type	**19.2**	**100**	**1.0**
snf3Δ	**7.7**	**40**	**0.8**
rgt2Δ	**37.0**	**193**	**0.6**
snf3Δ rgt2Δ	**4.5**	**23**	**0.9**

Strains CMY1001 (wild-type), CMY1007 (*snf3Δ*), CMY1009 (*rgt2Δ*), and CMY1012 (*snf3Δ rgt2Δ*) were grown in rich medium plus 2% maltose to early log phase, harvested, and glucose transport 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%.

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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 (Ozcan and Johnston, 1995). Mutations in *RGT1* suppress the poor glucose growth phenotype of *grr1Δ* mutations by restoring high-affinity glucose transport (Vallier *et al.*, 1994). We found that deletion of *RGT1* in the *grr1Δ* 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 *grr1Δ rgt1Δ* 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 *rgt1Δ* null strain, that is, no inactivation of maltose permease is seen in the *rgt1Δ* 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 *grr1Δ*. It is interesting to note that the *rgt1Δ* null strain exhibits reduced levels of maltose transport and maltase activity (Table 2), but a similar result is not seen in the *grr1Δ rgt1Δ*

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

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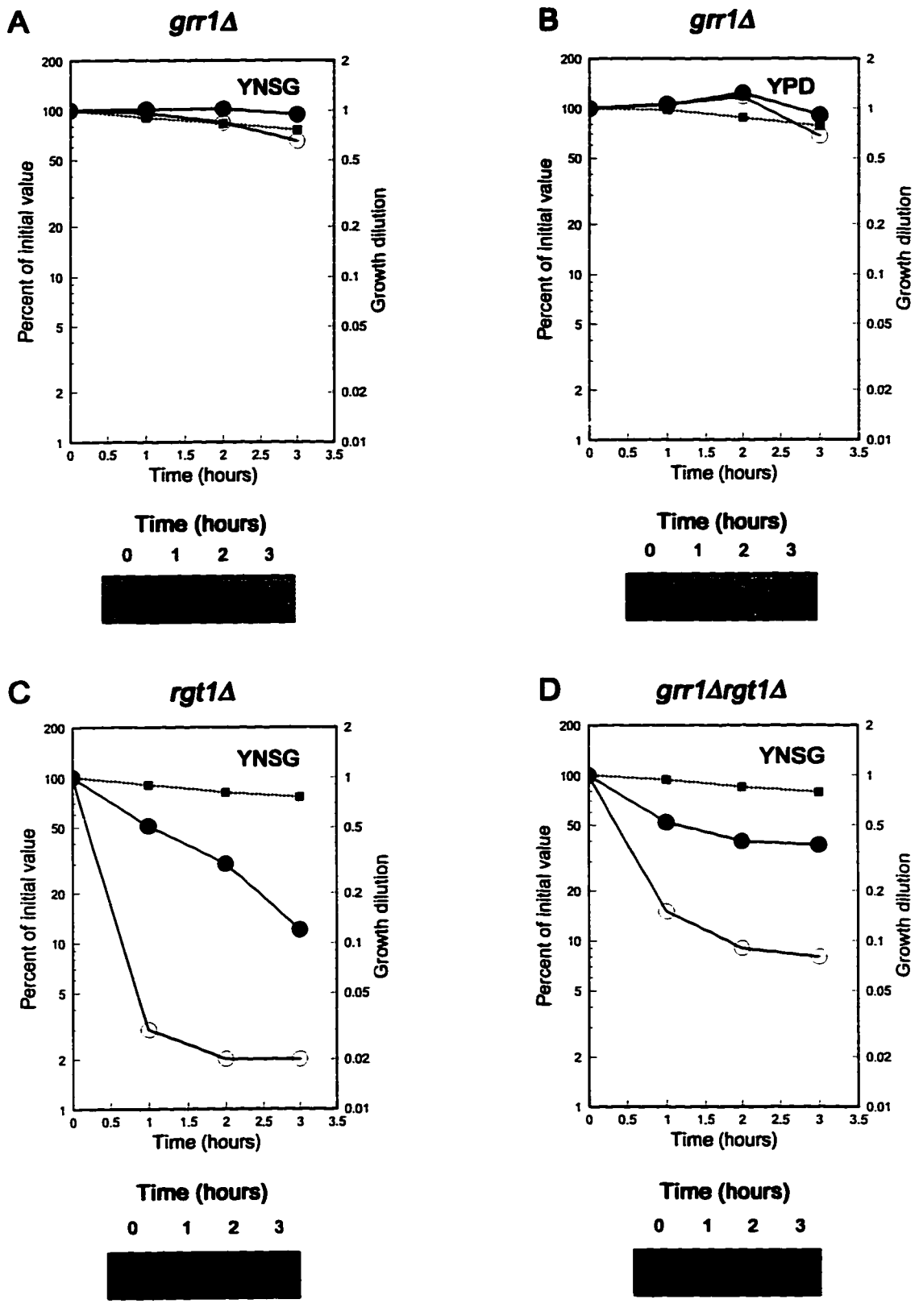

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Table 4. Glucose transport activity in maltose-grown *grr1Δ*, *rgt1Δ*, and *grr1Δ rgt1Δ* mutant strains and strains containing the *HXT1* overexpression plasmid.

Genotype	Vmax (nmoles/mg dry wt·min)	Relative Vmax %	Km (mmoles)
grr1Δ	2.9	15	1.3
rgt1Δ	28.8	150	1.1
grr1Δ rgt1Δ	20.0	104	1.9
GRR1[pADH1-HXT1]	21.7	113	1.6
grr1Δ[pADH1-HXT1]	5.0	26	1.8

Strains CMY1005 (*grr1Δ*), CMY1008 (*rgt1Δ*) and CMY1010 (*grr1Δ rgt1Δ*) were grown in rich medium plus 2% maltose. Strains CMY1001 and CMY1005 transformed with pADH1-HXT1 were grown in selective medium lacking uracil and containing 2% maltose. Glucose transport was assayed as described in Table 3. The relative activity of each strain is compared to that of CMY1001.

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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 *grr1Δ* 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 *grr1Δ* mutant strain, and inactivation assays were carried out. Results in Figure 3 show that 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; Ozcan 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),

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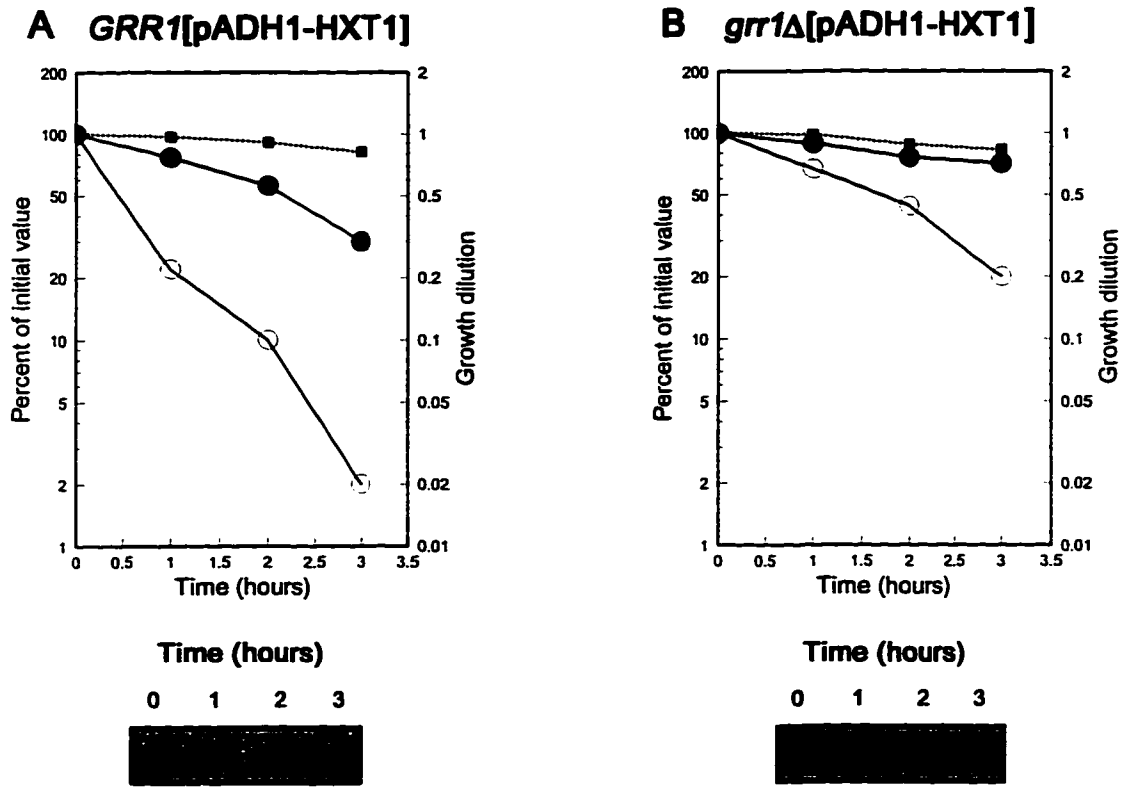
Figure 3. Effect of overexpression of *HXT1* on glucose-induced inactivation of maltose permease. Strains CMY1001 (parental strain) and CMY1005 (*grr1Δ*) were transformed with pADH1-HXT1. Both strains were grown in selective medium lacking uracil and containing 2% maltose. The standard inactivation assay protocol was used as described in Figure 1.



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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 hour. We do not know the basis of this delay but it could suggest that post-translational 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, 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.

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Figure 4. Effect of *RGT2-1* mutation on glucose-induced inactivation of maltose permease. Plasmid containing the *RGT2-1* dominant allele was introduced into strains CMY1001 (parental strain) and CMY1005 (*grr1Δ*). 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 (Panels A and B). The standard inactivation assays were carried out in strains CMY1001 and CMY1005 transformed with plasmid pRGT2-1 (Panels C and D).

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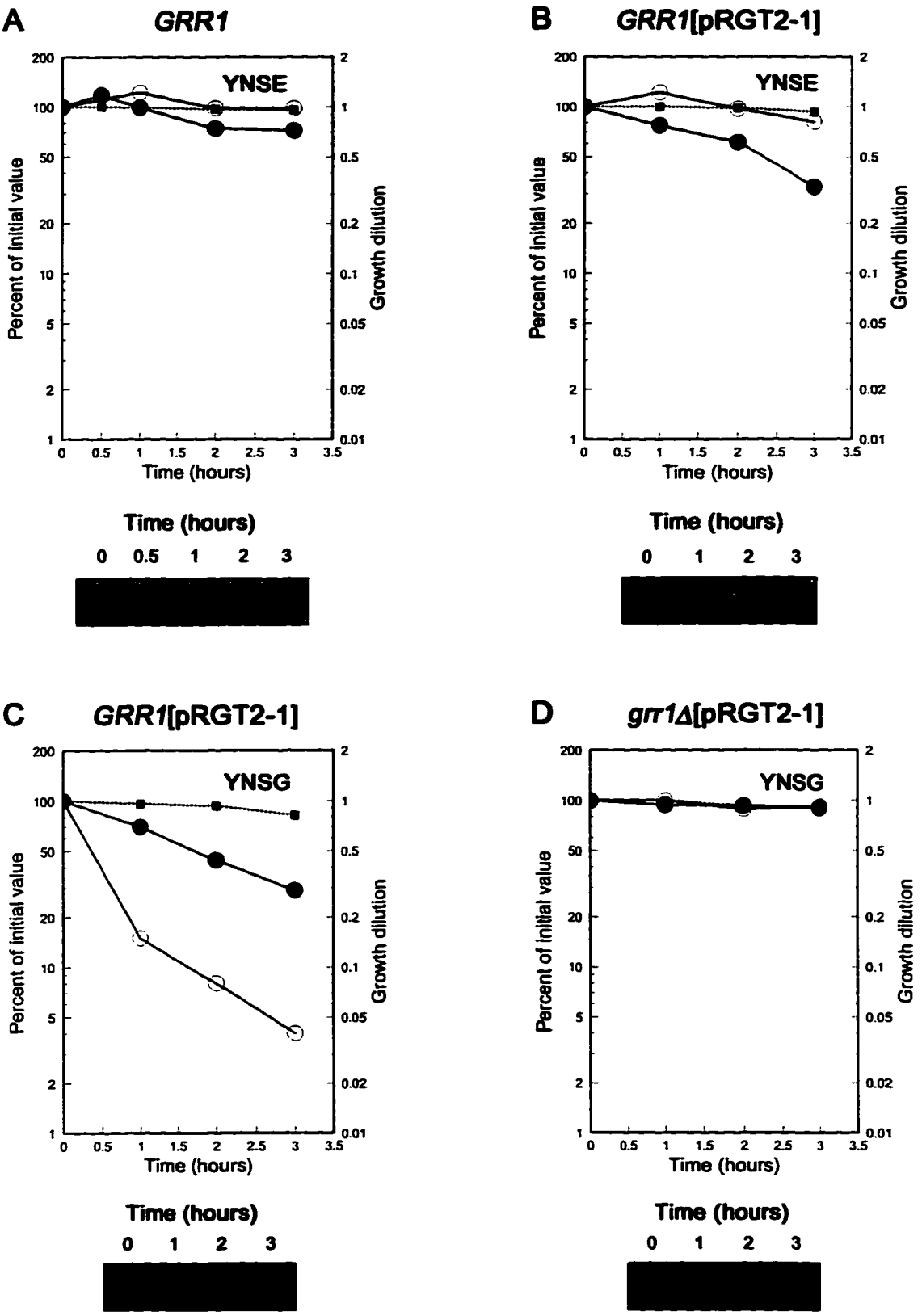

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Table 5. Glucose transport activity in maltose-grown *GRR1* and *grr1Δ* mutant strains containing the dominant *RGT2-1* allele.

Genotype	Vmax (nmoles/mg dry wt min)	Relative Vmax %	Km (mmoles)
GRR1[pRGT2-1]	12.5	65	1.6
grr1Δ[pRGT2-1]	2.5	13	1.8

Strains CMY1001 and CMY1005 (*grr1Δ*) transformed with plasmid pRGT2-1 were grown in selective medium lacking uracil and containing 2% maltose. Glucose transport was assayed as described for Table 3. The relative activities are compared to that of CMY1001.

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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 here that these same gene functions are utilized in a post-translational glucose-regulated process, glucose-induced inactivation of maltose permease (reviewed in Johnston and Carlson, 1993; Ozcan and Johnston, 1995, Ozcan *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 (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996a, b; Liang and Gaber, 1996). *RGT2-1* is a dominant suppressor of *snf3* mutations (Marshall-Carlson *et al.*, 1991). Ozcan *et al.* (1996a) recently characterized Rgt2p, showing that it encodes a

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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 which monitor low and high levels of extracellular glucose respectively, and transmit this signal to regulators of *HXT* gene expression, like Rgt1p, via Grr1p (Ozcan and Johnston, 1995; Ozcan *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 post-translational process also utilizes 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.

grr1Δ mutants 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 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, 1993; Ozcan and Johnston,

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1995; Vallier *et al.*, 1994). Null mutations in *RGT1* suppress the reduced hexose transport phenotype of *grr1* mutations (Marshall-Carlson *et al.*, 1991; Ozcan and Johnston, 1995; Vallier *et al.*, 1994). Rgt1p is a zinc cluster protein, and it has been suggested that Rgt1p is a DNA-binding repressor of the *HXT1-4* genes (Ozcan and Johnson, 1995; Ozcan *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 *grr1Δ rgt1Δ* strain is approximately half that in the parental strain. Restoration of glucose transport in the *grr1Δ* strain by overexpression of the *HXT* gene also partially restores glucose-induced inactivation in *grr1Δ* strain, both the proteolysis of maltose permease and the rapid inhibition of maltose transport activity. Taken together, these results along with the studies of the role of Rgt2p described above, clearly demonstrate that Grr1p plays an essential role in two inter-related 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 here 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; Ozcan and

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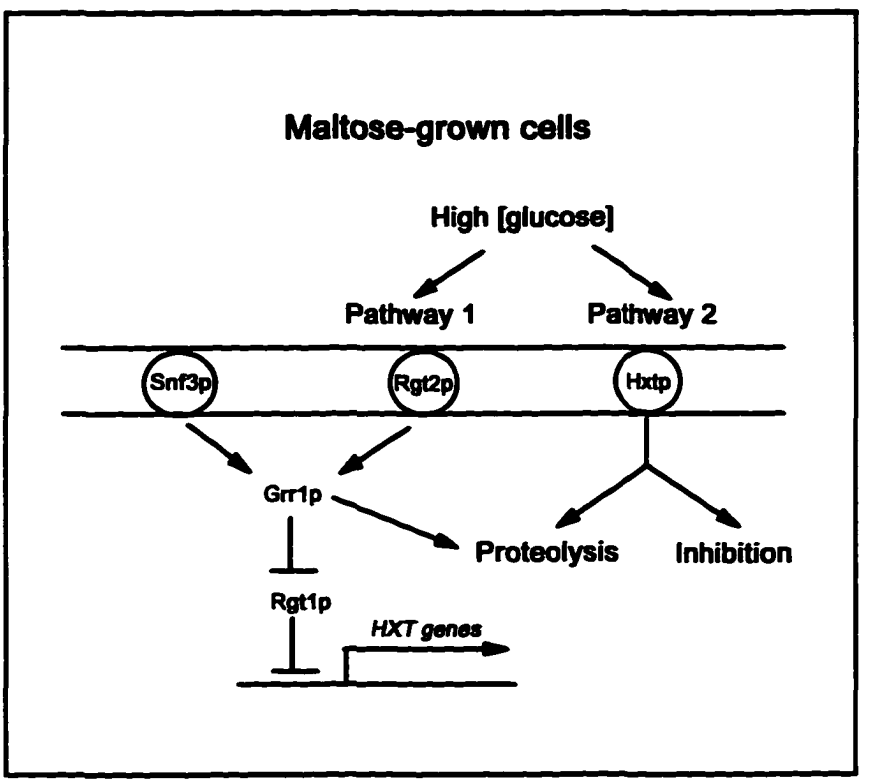
Figure 5. Model of the glucose sensing/signaling pathways used for glucose-induced inactivation of maltose permease.



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Johnston, 1995; Ozcan *et al.*, 1996a, b; Liang and Gaber, 1996). 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 while deletion of *GRR1* does, indicates that at least one other glucose sensing/signaling pathways is utilized for this system. Since restoration of glucose transport in the *grr1Δ* strain (by deletion of *RGT1* or overexpression 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 Ozcan *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 (Ozcan 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

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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 found 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 *grr1Δ* strains is not suppressed by *rgt1Δ* 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 G1 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

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Pathway 2. Instead, transcriptional regulation of *HXT* gene expression appears to serve this function. (These questions will be addressed in Chapter 3).

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-cyclic AMP 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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CHAPTER 3

Metabolic Signals Trigger Glucose-induced Inactivation of Maltose Permease in *Saccharomyces*

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ABSTRACT

Glucose is the preferred carbon source for most eukaryotic cells. Addition of glucose to *Saccharomyces cerevisiae* cells, causes downregulation of the expression of many genes involved in utilization of other carbon sources, by both transcriptional and post-translational mechanisms. Recently we identified two glucose sensing/signaling pathways capable of stimulating glucose-induced inactivation of maltose permease. One of these pathways appeares dependent on high-affinity glucose transport, and in this report, we undertake further characterization of this pathway. Our results provide evidence that high rates of metabolism of any of a number of fermentable sugars (including glucose, fructose, mannose, galactose and maltose) generate an inactivation signal. Sugar phosphorylation is essential. Moreover, all three hexose kinases (hexokinase 1, 2 and glucokinase) are capable of generating an inactivation signal in response to glucose, but the characteristics of this signal vary in strains expressing the different sugar kinases. The mechanism of glucose signal transduction by this pathway is discussed.

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INTRODUCTION

Glucose is a global metabolic regulator in *Saccharomyces* which controls the expression of many genes involved in carbohydrate utilization, gluconeogenesis, mitochondrial biogenesis, and cell cycle regulation (reviewed in Johnston and Carlson, 1992). In part, this regulation is achieved at the level of transcription by affecting the induction or repression of different sets of genes, and this is mediated by multi-gene transcription regulators such as Mig1p and Rgt1p (reviewed in Johnston and Carlson, 1992 and Ronne, 1995; Ozcan *et al.*, 1996b). Glucose also acts at the post-translational level by decreasing the enzyme activity and stability of certain target enzymes (reviewed in Thevelein and Hohmann, 1995). The overall effect of these glucose-regulated processes is to speed the transition from utilization of alternate carbon sources, such as maltose, galactose, sucrose, or ethanol, to glucose fermentation.

Maltose transport is subject to glucose-induced inactivation (Gorts, 1969; Alonso and Kotyk, 1978; Busturia and Lagunas, 1985; Cheng and Michels, 1991). Previous studies demonstrated that inactivation of maltose permease occurs in two steps: an initial very rapid loss of transport activity associated with little loss in permease protein levels (transport inhibition), and a slower decrease of transport activity associated with proteolysis of maltose permease protein (Riballo *et al.*, 1995; Medintz *et al.*, 1996). The rapid inhibition of maltose transport is evident only in nitrogen starvation conditions and is not seen in all strain backgrounds (Medintz *et al.*, 1996). Glucose-induced proteolysis of maltose permease requires endocytosis and vacuolar proteases, and is independent of the proteasome (Riballo *et al.*, 1995; Medintz *et al.*, 1996). Similarly, galactose permease, encoded by *GAL2*, also is subject to

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glucose-induced proteolysis (Horak and Wolf, 1997).

Several glucose sensing and signaling pathways involved in glucose regulated responses in yeast have been identified, including the *SNF1* pathway regulating glucose repression (reviewed in Gancedo, 1992; Johnston and Carlson, 1992; Erickson and Johnston, 1994); the Snf3p-Grr1p and Rgt2p-Grr1p pathways regulating glucose induction of *HXT* gene expression (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996a, b; Liang and Gaber, 1996); and the RAS-cAMP pathway (reviewed in Thevelein, 1992). Sugar transport and sugar phosphorylation also have been implicated in glucose sensing and signal transduction pathways in yeast (reviewed in Johnston and Carlson, 1992; Thevelein, 1992; Bisson *et al.*, 1993) as well as mammalian pancreatic α-, and β-cells (reviewed in Bisson *et al.*, 1993; Froguel *et al.*, 1993; Germen, 1993; Byrne *et al.*, 1995; Heimberg *et al.*, 1996). It is interesting to note that yeast hexokinase 2 appears to function as a glucose sensor in pancreatic β-cells in transgenic mice (Epstein *et al.*, 1992; Voss-McCowan *et al.*, 1994).

We recently revealed two glucose sensing/signaling pathways stimulating glucose-induced inactivation of maltose permease (Jiang *et al.*, 1997). One pathway is dependent on glucose transport, the other pathway is glucose transport-independent. We showed that Rgt2p, which was previously identified as the glucose sensor for induction of *HXT1* gene expression, functions as the glucose sensor in the glucose transport-independent pathway (Ozcan *et al.*, 1996a). In this report, we characterize the glucose transport-dependent pathway. Our results provide evidence that the initial steps of sugar fermentation, including transport and phosphorylation, function as metabolic gate-keepers for signaling the inactivation processes, and that sugar

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phosphorylation is essential. Moreover, in addition to glucose, other fermentable sugars such as fructose, mannose, galactose and even maltose are able to trigger the inactivation of maltose permease, but none of the nonfermentable carbon sources we tested appears to be sufficient.

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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 (*MATa mal11Δ::URA3 MAL12 MAL13 leu2-3,112 ura3-52*) (Charron *et al.*, 1986) by two-step gene replacement of the *mal11Δ::URA3* with the HA-tagged *MAL61/HA* maltose permease as described in Medintz *et al.* (1996) and Jiang *et al.* (1997). Strains CMY1001 (wild-type), CMY1005 (*grr1Δ*), and CMY1006 (*hxk2Δ*) are isogenic and are described in detail in Medintz *et al.* (1996) and Jiang *et al.* (1997).

Strains CMY1014 and CMY1015 are derived from CMY1006. In strain CMY1014 the ORF of *GLK1* was replaced by *HIS3*, and in strain CMY1015 the ORF of *HXK1* was replaced by *TRP1* using PCR-based gene disruption. The primers for disrupting *HXK1* are primer 1:5'TAAGAAACAATTGTGGCTTGCAATACTCAATTA GAATTCTTTTCTTTTAATCAAGCAGATTGTACTGAGAGTGC3' and primer 2: 5'CGTAATTGGATCTTTGCTTGCGTCACCAGTCCATCCATAGATATCTCTCAAT GCCGATTTCGGCCTATTGG3'. The primers for disrupting *GLK1* are primer 3: 5'CCGCTATCAACAGAACCCCAACCCCCCCATCAGTGCCAACTCAGCTTCCCT TGGTGAGCGCTAGGAG3' and primer 4: 5'TCAGCTCAACGCCACAGGCGG CACCACTCCGGAACCATCCTGGCCACACCGCATAGATCCGTCG3'. Strains CMY1013 and CMY1016 are derived from strains CMY1001 and CMY1006, respectively. In both strains, *HXK1* was disrupted using primers 1 and 2, and *GLK1* was disrupted using primer 3 and 4. Each gene disruption was verified by Southern analysis.

Strains 100-1B is isogenic to strain 100-1A, except at the *MAL1* locus, and has

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Table 1. List of *Saccharomyces* strains.

Strain	Genotype	Reference
CMY1001	*MATa MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200*	Medintz *et al.*, 1996
CMY1005	*grr1Δ* (isogenic to CMY1001)	Jiang *et al.*, 1997
CMY1009	*rgt2Δ* (isogenic to CMY1001)	Jiang *et al.*, 1997
CMY1013	*hxk1Δ HXK2 glk1Δ* (isogenic to CMY1001)	This study
CMY1014	*HXK1 hxk2Δ glk1Δ* (isogenic to CMY1001)	This study
CMY1015	*hxk1Δ hxk2Δ GLK1* (isogenic to CMY1001)	This study
CMY1016	*hxk1Δ hxk2Δ glk1Δ* (isogenic to CMY1001)	This study
100-1A	*MATa mal11Δ::URA3 MAL12 MAL13 leu2-3,112 ura3-52*	Charron *et al.*, 1986
100-1B	*MATa MAL11 mal12Δ::LEU2 MAL13 leu2-3,112 ura3-52*	Charron *et al.*, 1986

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genotype *MAL11 mal12Δ::LEU2 MAL13*.

Plasmids pADH1-GAL2 and pHXT1-LacZ were obtained from Sabire Ozcan and Mark Johnston. Plasmid pADH1-GAL2 is a *URA3* 2μ plasmid containing the *GAL2* coding region under the control of the constitutive *ADH1* promoter (S. Ozcan, personal communication). Plasmid pHXT1-LacZ carries a fusion of the *HXT1* promoter to *LacZ* on a *LEU2* CEN vector (Ozcan and Johnston, 1995). Plasmids pRS405-MAL61/HA and pMAL61/HA carry the HA-tagged *MAL61/HA* maltose permease gene (described in Medintz *et al.*, 1996) in plasmid vectors pRS405 and pRS416, respectively (Sikorsky and Hieter, 1989).

Inactivation assay protocol. The protocol is described in detail in Medintz *et al.* (1996). Strains were grown at 30°C to very early log phase (OD_{600} of 0.3) in either rich medium or selective medium lacking uracil, supplemented with 2% maltose or 2% maltose plus 2% galactose (as indicated) with the following exception. Strains CMY1016 and 100-1B[pMAL61/HA] do not grow in the presence of maltose. These strains were pregrown in rich or selective medium containing 2% galactose, respectively, and then 2% maltose was added to the medium in order to induce the expression of the *MAL* genes.

Cells were harvested, and transferred to nitrogen-starvation medium plus 2% glucose (or another sugar as indicated) or 0.5% 2-deoxy-glucose. Cell samples were taken at the indicated times over a 3 hour period, and for each sample, maltose transport rates were tested and total cell extracts were prepared for Western analysis. Growth dilution was calculated as the OD_{600} at time zero divided by OD_{600} at time X.

Western blotting. Western blotting analysis was carried out as described in

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Medintz *et al.* (1996). The Mal61/HA protein in the extracts was detected using anti-HA specific antibody and the ECL Western blotting kit (Amersham). The relative amount of each band on the ECL-Hyperfilm was measured by densitometric comparison to the zero time sample. Western analysis was done in duplicate from duplicate cell cultures.

Sugar transport assays. Maltose transport was measured as the uptake of 1 mM ^{14}C-maltose as described in Cheng and Michels (1991) and Medintz *et al.* (1996). Similar methods were used to measured 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 of glucose transport for the maltose- or maltose plus galactose-grown cells. Assays were done in duplicate cultures.

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

β-Galactosidase assays. β-Galactosidase activity was assayed in crude extracts as described by Ausubel *et al.* (1995). Cells were grown on selective medium lacking leucine and supplemented with 2% maltose plus 2% galactose, except that strain CMY1016 (*hxk1Δ hxk2Δ glk1Δ*) was pregrown on galactose containing medium to a low OD_{600} at which time 2% maltose was added, and the cells were grown for another 6 hours before being harvested. The specific activities are normalized to protein concentration, and are given as nmoles of o-nitrophenyl-β-galactoside/min/mg protein. The values presented are the average obtained from two or three independent transformants. Standard errors were less than 30%.

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RESULTS

Overexpression of *GAL2* suppresses the glucose-insensitive phenotype of *grr1Δ*. We wished to test whether glucose transport by the *HXT*-encoded transporters is essential to stimulate inactivation through signaling Pathway 2, the glucose transport-dependent pathway. *grr1Δ* mutant strains are resistant to glucose-induced inactivation of maltose permease (Figure 1B), because both glucose sensing/signaling pathways are absent. Grr1p is an essential component in the Rgt2p-dependent signaling Pathway 1, and loss of Grr1p causes a dramatic decrease in *HXT* gene expression and glucose transport (Table 2, Ozcan *et al.*, 1995; Jiang *et al.*, 1997). *GAL2* encodes the galactose transporter in *Saccharomyces cerevisiae* (Tschopp, *et al.*, 1986; Nehlin *et al.*, 1989; Ramos *et al.*, 1989). Recent studies demonstrate that Gal2p is able to transport glucose, albeit with lower affinity (Nishizawa *et al.*, 1995; Liang *et al.*, 1996). Plasmid pADH1-GAL2 carries the *GAL2* gene expressed from the constitutive *ADH1* promoter on a high-copy vector. This plasmid was introduced into wild-type strain CMY1001 as well as the isogenic *grr1Δ* mutant strain CMY1005.

Overexpression of *GAL2* partially restores glucose transport in the *grr1Δ* mutant strain (Table 2). The presence of plasmid pADH1-GAL2 increases the V_{max} of glucose transport 2 to 3-fold, but the K_m increases about 7-fold. The high K_m of glucose transport in the *grr1Δ*[pADH1-GAL2] strains clearly indicates that the increased level of glucose transport is mediated by Gal2p. As can be seen in Figure 1, overexpression of *GAL2* also partially restores both glucose-induced proteolysis of maltose permease and inhibition of maltose transport in *grr1Δ* strain. Thus, glucose transport by an *HXT* hexose transporter is not essential for stimulating inactivation of maltose permease.

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Figure 1. Effect of overexpression of *GAL2* on glucose-induced inactivation of maltose permease in maltose-grown *grr1Δ* strain. Strains CMY1001 (*GRR1*) and CMY1005 (*grr1Δ*) were transformed with either a *URA* vector (pRS416) or the *GAL2* overexpression plasmid pADH1-GAL2. Strains were grown in selective medium lacking uracil and containing 2% maltose, and the standard inactivation assay protocol was used (see Materials and Methods; Medintz *et al.*, 1996). The relative levels of Mal61/HAp protein (●, solid line), maltose transport (○, solid line) and growth dilution (■, dotted line) are plotted in the same panel. The relative protein level and transport activity at time X are compared to the corresponding values at time zero. Growth dilution is calculated as the OD_{600} at time zero divided by OD_{600} at time X.

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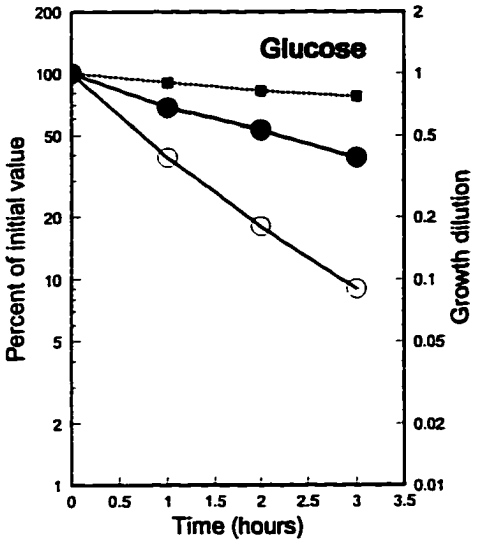
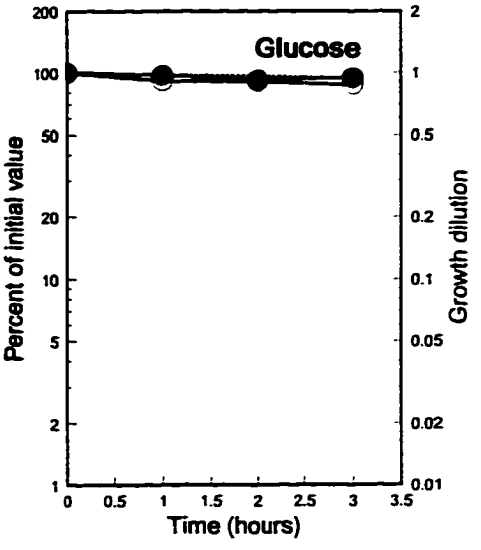
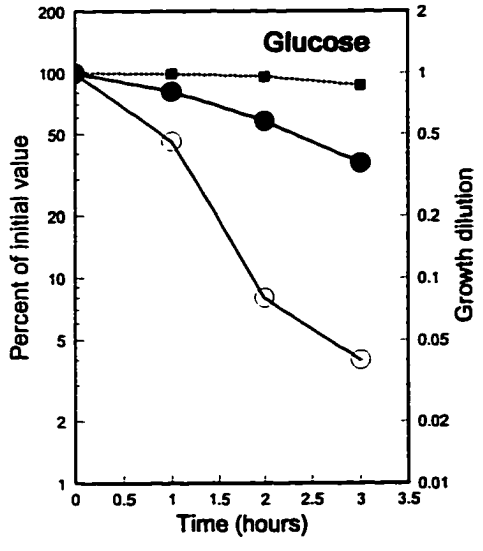
A *GRR1* [pRS416]



B *grr1Δ*[pRS416]



C *GRR1* [pADH1-GAL2]



D *grr1Δ*[pADH1-GAL2]

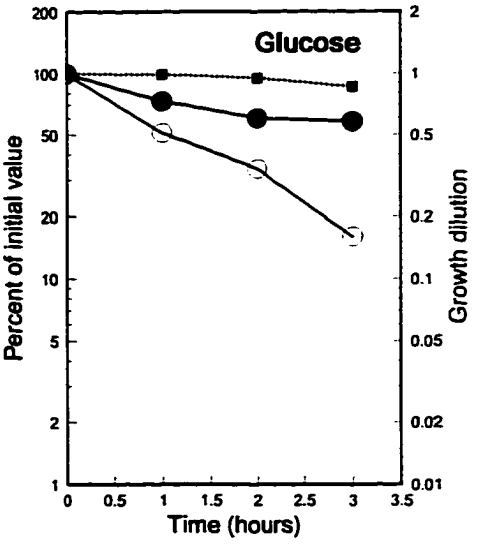


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Table 2. Glucose transport activity in maltose-grown *GRR1* and *grr1Δ* mutant strains containing the *GAL2* overexpression plasmid.

Genotype	Vmax (nmoles/mg dry wt min)	Relative Vmax %	Km (mmoles)
GRR1	19.2	100	1.0
grr1Δ	2.9	15	1.3
GRR1[pADH1-GAL2]	20.8	108	1.4
grr1Δ[pADH1-GAL2]	7.7	40	9.0

Strains CMY1001 (*GRR1*) and CMY1005 (*grr1Δ*) were grown in rich medium containing 2% maltose. Strains CMY1001 and CMY1005 transformed with plasmid pADH1-GAL2 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%.

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Overexpression of *GAL2* in strain CMY1001 results in only a modest increase in the V_{max} or K_m of glucose transport, indicating that glucose transport is still predominantly carried out by the *HXT*-encoded glucose transporters (Table 2), and the rate of glucose-induced inactivation in this strain also is only slightly potentiated.

Fermentable but not nonfermentable carbon sources stimulate inactivation of maltose permease. We undertook a comparative study of the ability of various fermentable and nonfermentable carbon sources to induce inactivation of maltose permease. Fructose and mannose also cause repression of glucose-sensitive genes, but these sugars are not as potent as glucose (reviewed in Johnston and Carlson, 1992). Both hexoses are transported by the *HXT*-encoded transporters and phosphorylated by the *HXK*-encoded hexokinases but with lower affinity than glucose (reviewed in Bisson *et al.*, 1993). Figure 2A and B show that both fructose and mannose stimulate the rapid inhibition of maltose transport activity but to a different extents. Fructose is as potent an inducer as glucose, but mannose is relatively ineffective. However, fructose is a poor inducer of maltose permease proteolysis, and mannose has no significant effect on maltose permease degradation. Their potency as inducers of inactivation compared to glucose correlates with their decreased affinity for the transporters and phosphorylation enzymes. Fructose and mannose inhibit maltose induction of *MAL* structural gene expression, but the effectiveness of these hexoses is significantly less than glucose and comparable to their ability to induce proteolysis in maltose-grown cells (Table 3).

Our previous results showed that ethanol is not able to stimulate inactivation of maltose permease (Medintz *et al.*, 1996). Several other nonfermentable carbon

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Figure 2. The ability of various fermentable and nonfermentable carbon sources to stimulate inactivation of maltose permease. Strain CMY1001 (wild-type) was grown in rich medium plus 2% maltose to early log phase, harvested and transferred to nitrogen starvation medium containing 2% fructose, 2% mannose, 3% glycerol, 2% lactate, and 2% acetate (as indicated).

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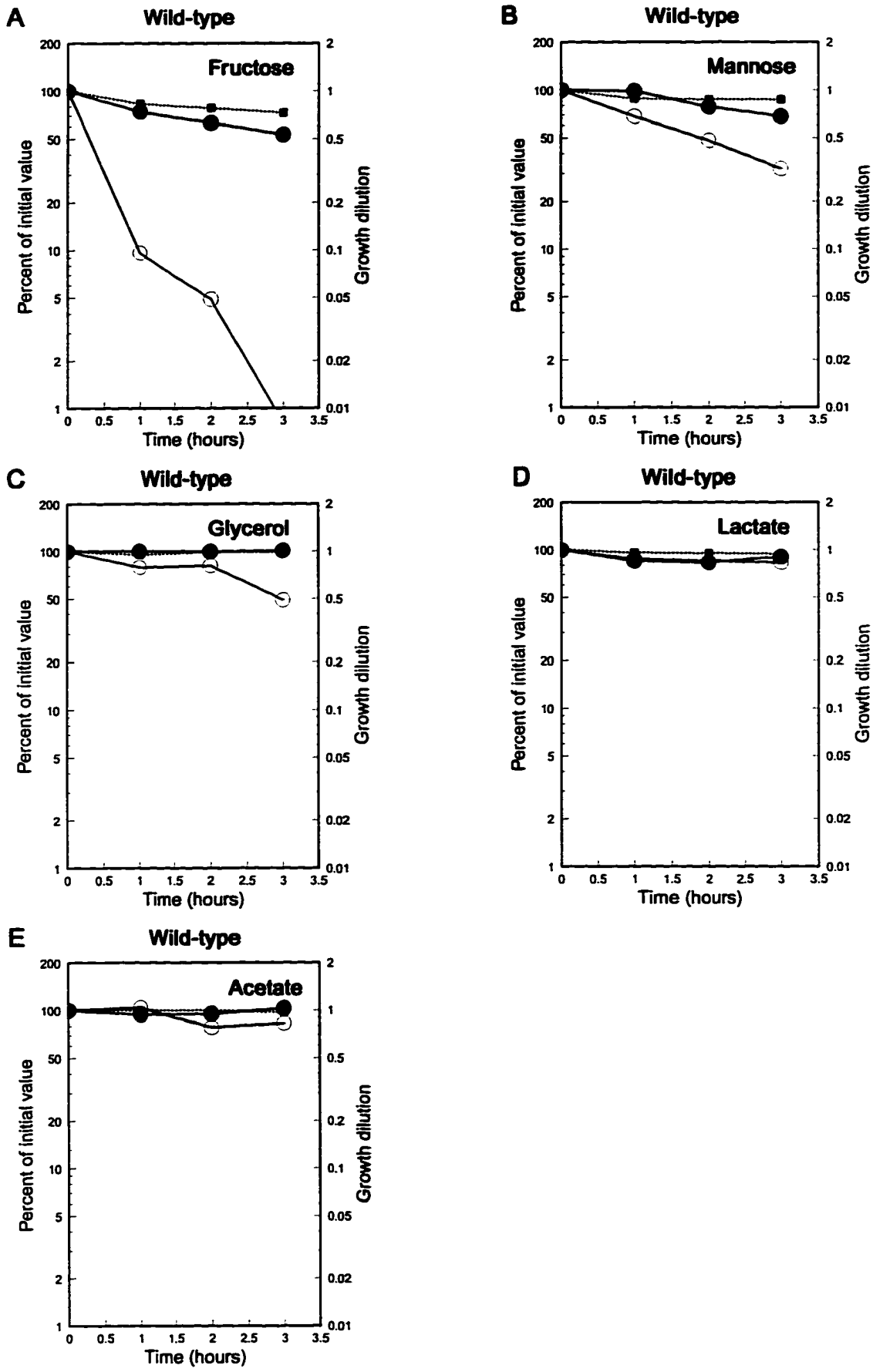

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Table 3. Effect of various fermentable carbon sources on the expression of maltose transport and maltase.

Strains	Carbon Source	Maltose transport activity (nmol·mg dry wt^{-1}·min^{-1})	Maltase Activity (nmol PNPG·mg ptn^{-1}·min^{-1})
CMY1001	Maltose	6.38	603
CMY1001	Glucose	0.04	<1
CMY1001	Galactose	0.07	8
CMY1001	Fructose	0.02	<1
CMY1001	Mannose	0.09	1
CMY1001	Maltose+Glucose	0.04	3
CMY1001	Maltose+Fructose	0.08	20
CMY1001	Maltose+Mannose	0.41	69
CMY1001	Maltose+Galactose	5.14	588
CMY1001 [pADH1-GAL2]	Maltose	2.36	313
CMY1001 [pADH1-GAL2]	Maltose+Galactose	0.75	146

Strain CMY1001 (wild-type) was grown on rich medium supplemented with 2% of various fermentable carbon sources (as indicated in the table). When two different carbon sources were used in combination, the concentration of each one is 2%). CMY1001 transformed with plasmid pADH1-GAL2 was grown in selective medium lacking uracil and containing 2% of indicated carbon sources. Cells were grown to early log phase (OD_{600} about 0.3), harvested, and the steady state maltose transport and maltase activities were determined as described in Materials and Methods.

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sources, including glycerol, lactate, and acetate were also tested, none were found to stimulate inactivation of maltose permease (Figure 2C to E), although glycerol does cause a modest inhibition of maltose transport activity.

Galactose is generally considered a nonrepressing sugar. We wished to test its ability to stimulate inactivation of maltose permease. CMY1001 grows poorly on galactose because *GAL2* expression and galactose transport rates are low (data not shown). When cells were pregrown in media containing only maltose or maltose plus galactose, galactose is not able to stimulate inactivation of maltose permease in strain CMY1001 (Figure 3A, B). Plasmid pADH1-GAL2 was introduced into the wild-type strain CMY1001 to allow high levels expression of galactose permease even in the absence of galactose. Strain CMY1001[pADH1-GAL2] was grown in media containing either maltose alone or maltose plus galactose to induce expression of the *GAL* genes (*GAL1, 7, 10*, encoding galactokinase, galactotransferase, and epimerase, respectively) (reviewed in Johnston and Carlson, 1992). In maltose-grown CMY1001 [pADH1-GAL2] cells, galactose still does not stimulate inactivation of maltose permease (Figure 3C). However, when strain CMY1001[pADH1-GAL2] is grown in galactose plus maltose, galactose induces both proteolysis of maltose permease and rapid inhibition of maltose transport (Figure 3D). The ability of galactose to repress maltose-induced expression of maltose transport and maltase is shown in Table 3. Consistent with the results reported above, galactose interferes with maltose induction of *MAL* gene expression only in maltose plus galactose-grown cells and only when *GAL2* is overexpressed.

Galactose-induced inactivation of maltose permease was assayed in *grr1Δ* and

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Figure 3. Induction of galactose utilization genes is required for galactose to stimulate inactivation of maltose permease. Strain CMY1001 was transformed with either a *URA* vector pRS416 or the *GAL2* overexpression plasmid pADH1-GAL2. The transformed strains were grown to early log phase in selective medium lacking uracil and supplemented with either 2% maltose or 2% maltose plus 2% galactose (as indicated), harvested, and transferred to the nitrogen starvation medium containing galactose.

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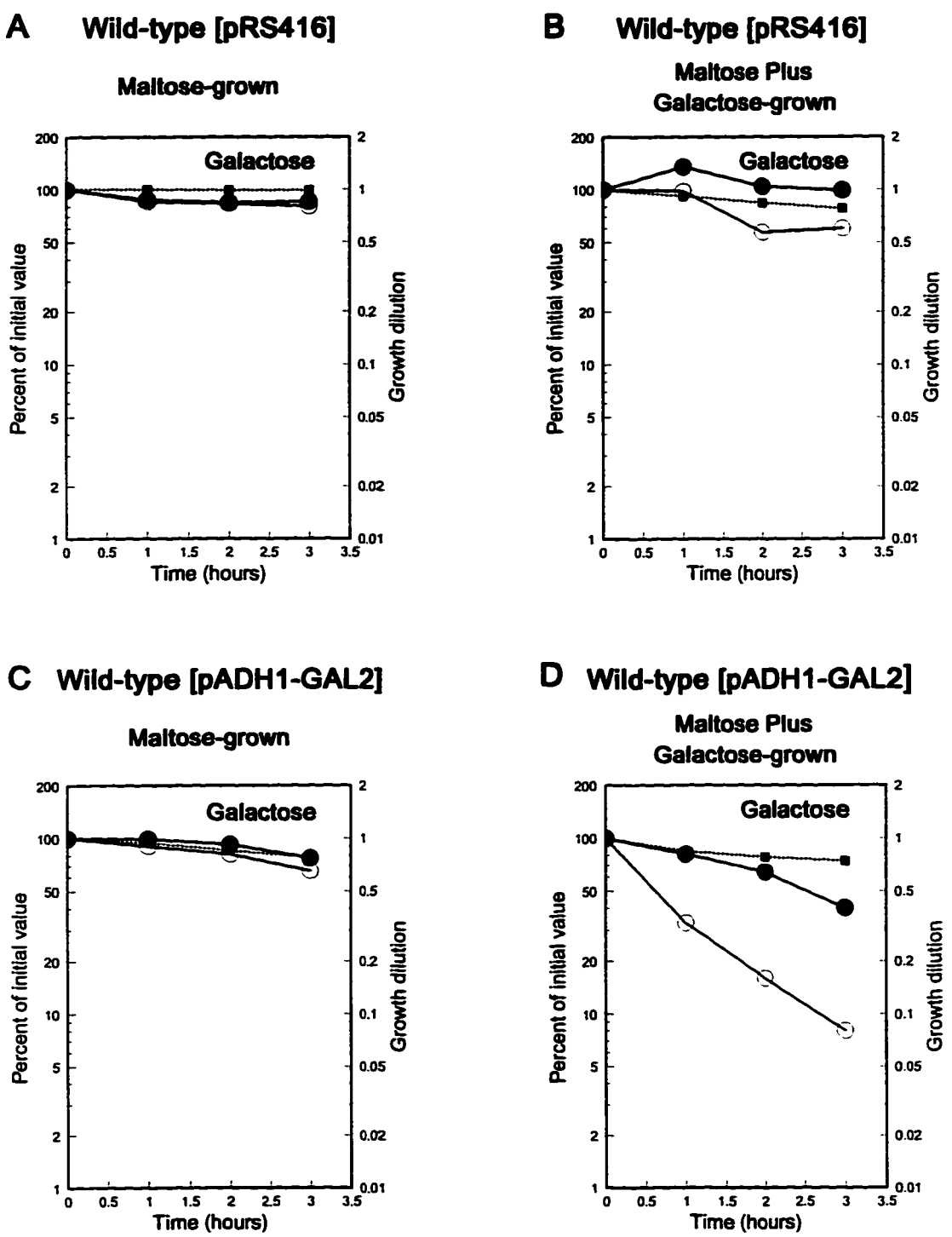

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grr1Δ[pADH1-GAL2] strains which lack the Rgt2-dependent pathway, and no significant effect of *GRR1* disruption was found (data not shown). That is, in maltose plus galactose-grown *grr1Δ*[pADH1-GAL2] strain galactose-induced inactivation of maltose permease was comparable to that seen in the wild-type strain carrying plasmid pADH1-GAL2 and grown in the same conditions (data not show). Taken together, these results indicate that rapid fermentation of galatose is sufficient to produce a potent signal for inactivation, but that galactose transport by itself is not sufficient to signal the inactivation of maltose permease.

Sugar phosphorylation is required for signaling inactivation. Sugar transport and sugar phosphorylation have been proposed to be involved in glucose signaling in yeast as well as in mammalian pancreatic α- and β-cells (reviewed in Thevelein, 1992; Bisson *et al.*, 1993 and references therein; Heimberg, *et al.*, 1996). We undertook the investigation of the role of sugar phosphorylation in the inactivation of maltose permease. Two different approaches were taken: the role of sugar kinases in inactivation of maltose permease was determined, and the ability of a nonmetabolizable glucose analog to induce inactivation was tested.

HXK1, HXK2 and *GLK1* encode *Saccharomyces cerevisiae* hexokinase 1, hexokinase 2 and glucokinase, respectively, which catalyze the first step in glycolysis and reportedly are involved in high-affinity glucose transport (reviewed in Johnston and Carlson, 1992). To explore the role of sugar kinases in the inactivation of maltose permease, we constructed a series of deletion mutations in these genes by PCR-based methods. The resulting mutant strains are all isogenic and each expresses only a single or none of the three kinase genes (see Table 1). Strains CMY1013, CMY1014 and

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CMY1015 were grown on maltose plus galactose. Strain CMY1016, the triple kinase disruption strain, does not grow on maltose plus galactose, probably as a result of the accumulation of high levels of intracellular glucose. Therefore, in order to assay the inactivation of maltose permease in this strain, it was necessary to pregrow strain CMY1016 in media containing only galactose, and induce maltose permease expression by the addition of maltose to the growth medium 6 hours prior to initiating the inactivation assay. The results shown in Figure 4A-D indicate that glucose phosphorylation is essential for signal production, and that any one of the kinases is sufficient to cause sensitivity to glucose-induced inactivation, but to various extents in strains expressing the different kinases. Strain CMY1013, expressing Hxk2 hexokinase, exhibits a level of glucose sensitivity similar to wild-type. Strains expressing only Hxk1 hexokinase or Glk1 glucokinase exhibit an attenuated response to glucose, and Glk1p is not sufficient to induce inhibition of maltose permease.

It has been suggested that the high-affinity glucose transport is dependent on sugar phosphorylation by any one of the kinases (Bisson and Fraenkel, 1983a, b), but the significance of this finding has been questioned recently (Nevado *et al.*, 1994; Smits *et al.*, 1996). Smits *et al.* (1996) provide evidence that the lack of high-affinity glucose transport in kinase-less strains is not a reflection of low levels of the transport protein. Instead, because the glucose transporters are facilitated transporters, they suggest that the kinases are needed to trap glucose inside the cell as glucose phosphate. We tested the rate of glucose transport in our kinase disruption strains. As reported in Table 4, strains expressing only *HXK2* exhibit a modest decrease in glucose uptake (to 69% of

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Figure 4. Effect of hexose kinase mutations on glucose-induced inactivation of maltose permease. Strains CMY1001 (*HXK1 HXK2 GLK1*), CMY1013 (*hxk1Δ HXK2 glk1Δ*), CMY1014 (*HXK1 hxk2Δ glk1Δ*), and CMY1015 (*hxk1Δ hxk2Δ GLK1*) were grown in rich medium supplemented with 2% maltose plus 2% galactose. Strain CMY1016 (*hxk1Δ hxk2Δ glk1Δ*) was grown in rich medium containing 2% galactose to early log phase (OD_{600} of 0.1), and then 2% maltose was added to the culture to induce *MAL* gene expression for another 6 hours before the inactivation assays were carried out.

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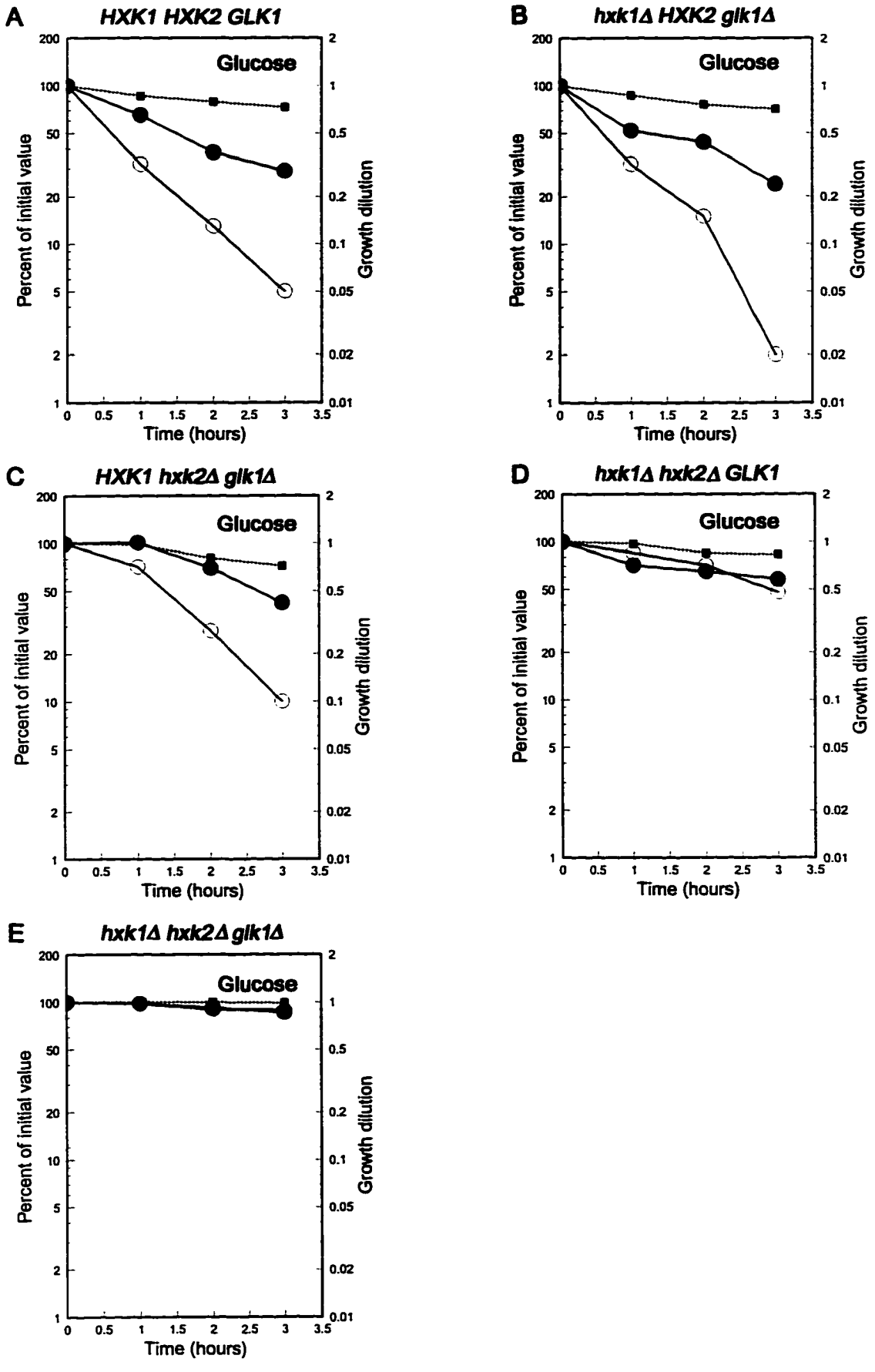

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Table 4. Glucose transport activity in *hxk1Δ glk1Δ*, *hxk2Δ glk1Δ*, *hxk1Δ hxk2Δ*, and *hxk1Δ hxk2Δ glk1Δ* mutant strains

Genotype	Velocity (nmoles/mg dry wt min)	Relative transport rate (%)	Km (mmoles)
HXK1 HXK2 GLK1	20.7	100	0.7
hxk1Δ HXK2 glk1Δ	14.2	69	0.8
HXK1 hxk2Δ glk1Δ	1.7	8	0.6
hxk1Δ hxk2Δ GLK1	2.2	11	1.0
hxk1Δ hxk2Δ glk1Δ	4.0	19	20.0

Strains CMY1013 (*hxk1Δ HXK2 glk1Δ*), CMY1014 (*HXK1 hxk2Δ glk1Δ*), and CMY1015 (*hxk1Δ hxk2Δ GLK1*) were grown in rich medium containing 2% maltose plus 2% galactose. Strain CMY1016 (*hxk1Δ hxk2Δ glk1Δ*) was grown on rich medium containing 2% galactose to early log phase, and then 2% maltose was added to the culture to induce *MAL* gene expression for another 6 hours before glucose transport assays were carried out. For each strain, the rate of glucose transport was determined at a substrate concentration of 1 mM. The K_m of glucose transport was calculated using a Lineweaver-Burk analysis

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the wild-type level), but mutant strains expressing only *HXK1* or *GLK1* show a dramatic decrease in glucose uptake (8% and 11%, respectively) compared to the wild-type strain. Strains with the triple kinase disruption exhibit low glucose uptake (19% of the wild-type level), and the K_m value of glucose transport is significantly higher than that of the wild-type or the double kinase disruption strains (Table 4).

We also measured *HXT* gene expression in the wild-type and each of our kinase mutant strains. Plasmids carrying *HXT1-LacZ* and *HXT2-LacZ* reporter genes were transformed into each strain, and the transformants were grown as described above for the inactivation assays. The results reported in Table 5 suggest that deletion of the kinase genes has only a modest effect on the expression of the *HXT* genes, particularly *HXT2*, and this effect is much less severe than the effect on glucose uptake. Our results are consistent with those of Smits *et al.* (1996) and suggest that the rate of glucose uptake is an indirect measurement of the rate of glucose phosphorylation and does not reflect the actual rate of glucose movement across the plasma membrane.

Fructose is capable of stimulating inactivation of maltose permease (see Figure 2A). It is not as potent as glucose for inducing proteolysis of maltose permease, but it is a very effective inducer of the rapid inhibition of maltose transport (Figure 5A). Only hexokinase 1 or 2, but not glucokinase is able to phosphorylate fructose. We found that in strains expressing either *HXK1* or *HXK2* alone, fructose stimulates inhibition of maltose transport, but not proteolysis (Figure 5B, C). Loss of function of both hexokinases, as in the strain expressing only *GLK1* completely abolishes fructose-stimulated inactivation of maltose permease (Figure 5D).

2-Deoxy-glucose is a glucose analog which can be phosphorylated but not

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Table 5. Regulation of *HXT* gene expression in mutant strains.

Genotype	β-galactosidase activity [a]	
	HXT1-LacZ	*HXT2-LacZ*
HXK1 HXK2 GLK1	106	7169
hxk1Δ HXK2 glk1Δ	98	6312
HXK1 hxk2Δ glk1Δ	61	4573
hxk1Δ hxk2Δ GLK1	49	3644
hxk1Δ hxk2Δ glk1Δ	17	3741

[a] Specific activity (nmol ONPG · mg protein^{-1} · min^{-1})

Strains CMY1013 (*hxk1Δ HXK2 glk1Δ*), CMY1014 (*HXK1 hxk2Δ glk1Δ*), CMY1015 (*hxk1Δ hxk2Δ GLK1*), and CMY1016 (*hxk1Δ hxk2Δ glk1Δ*) were each transformed with plasmids pHXT1-LacZ and pHXT2-LacZ separately. Strains CMY1013 (*hxk1Δ HXK2 glk1Δ*), CMY1014 (*HXK1 hxk2Δ glk1Δ*), and CMY1015 (*hxk1Δ hxk2Δ GLK1*) carrying the plasmids were grown in selective medium lacking leucine and containing 2% maltose plus 2% galactose. Strain CMY1016 (*hxk1Δ hxk2Δ glk1Δ*) transformants were grown in selective medium lacking leucine and containing 2% galactose to early log phase, and then 2% maltose was added to the culture to induce *MAL* gene expression for another 6 hours before β-galactosidase assays were carried out.

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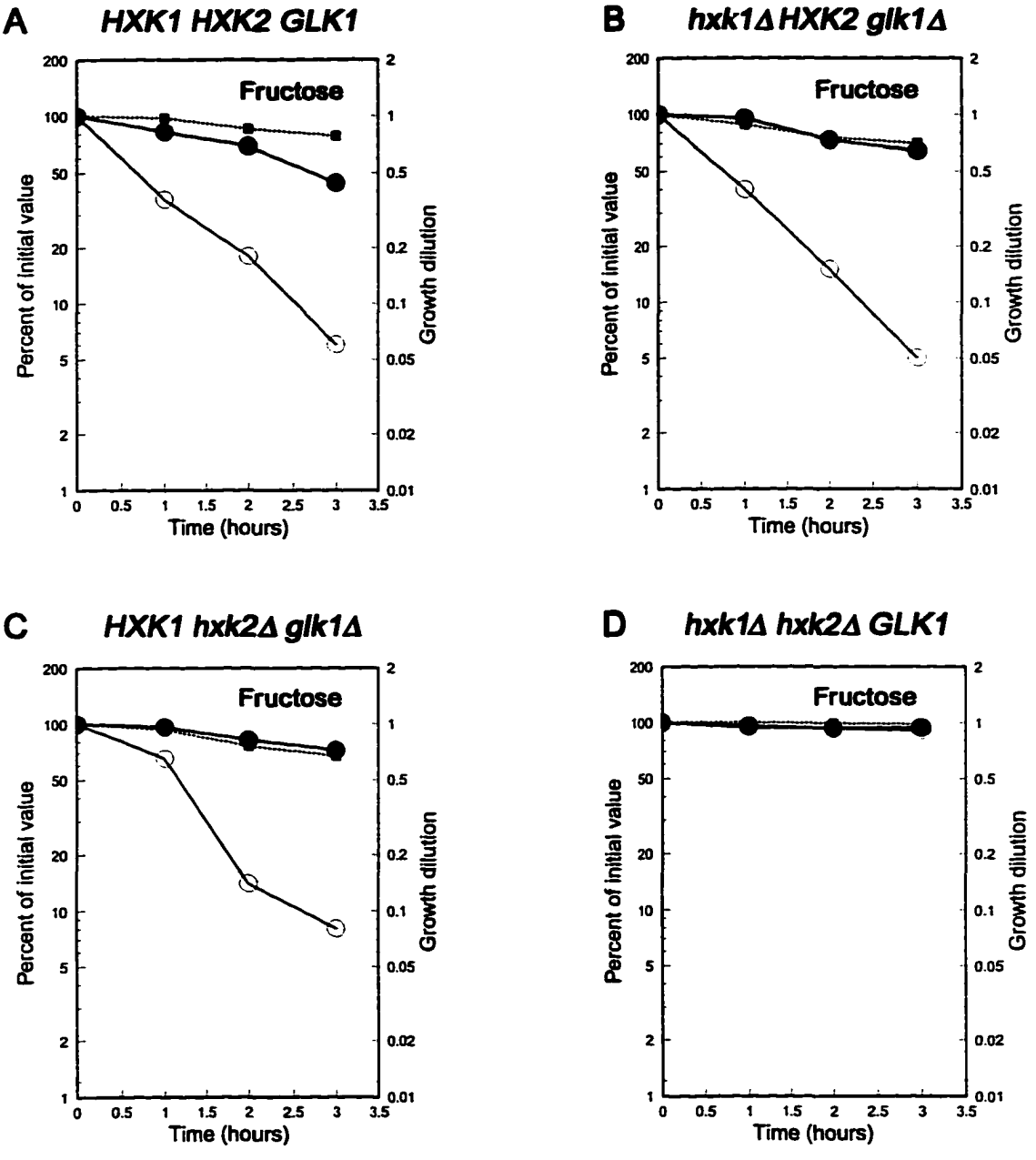
Figure 5. Effect of hexose kinase mutations on fructose-induced inactivation of maltose permease. Strains CMY1001 (*HXK1 HXK2 GLK1*), CMY1013 (*hxk1Δ HXK2 glk1Δ*), CMY1014 (*HXK1 hxk2Δ glk1Δ*), and CMY1015 (*hxk1Δ hxk2Δ GLK1*) were grown in rich medium supplemented with 2% maltose plus 2% galactose, harvested and transferred to nitrogen starvation medium containing 2% fructose.



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metabolized further. The ability of 2-deoxy-glucose to induce inactivation of maltose permease was examined in the wild-type strain and in an isogenic *rgt2Δ* strain. Previous studies indicated that the Rgt2p-dependent glucose signaling pathway is partially responsible for stimulating glucose-induced proteolysis of maltose permease but does not contribute to stimulating the rapid inhibition of maltose transport (Jiang *et al.*, 1997). Figure 6A shows that 2-deoxy-glucose is capable of causing rapid inhibition of maltose transport and proteolysis of maltose permease in the wild-type strain, however both rates are slower compared to those induced by glucose. The rate of inhibition of maltose transport is not affected by loss of Rgt2p, but proteolysis of maltose permease is reduced but not entirely blocked (Figure 6B). This result suggests that 2-deoxy-glucose is able to stimulate inactivation through both the Rgt2p-Grr1p-dependent pathway (Pathway 1) and the glucose transport-dependent pathway (Pathway 2) (Jiang *et al.*, 1997).

Maltose weakly signals inactivation of maltose permease. *MAL* gene expression in *MAL*-activator constitutive mutants is partially repressed by growth on maltose (Hu *et al.*, 1995). Since the inactivation assays are carried out in nitrogen-starvation medium which blocks protein synthesis (and therefore continued synthesis of maltose permease), we are able to test the possibility that maltose itself may stimulate inactivation of maltose permease. Maltose is capable of stimulating both proteolysis of maltose permease and the rapid inhibition of maltose transport in CMY1001 cells (Figure 7A). Deletion of *GRR1*, which completely blocks glucose-induced inactivation of maltose permease, has little effect on this maltose-stimulated inactivation processes (Figure 7B).

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Figure 6. The ability of 2-deoxy-glucose to stimulate inactivation of maltose permease in *RGT2* and *rgt2Δ* strains. Strains CMY1001 (*RGT2*) and CMY1009 (*rgt2Δ*) were grown in rich medium supplemented with 2% maltose harvested, and transferred to nitrogen starvation medium containing 0.5% 2-deoxy-glucose.

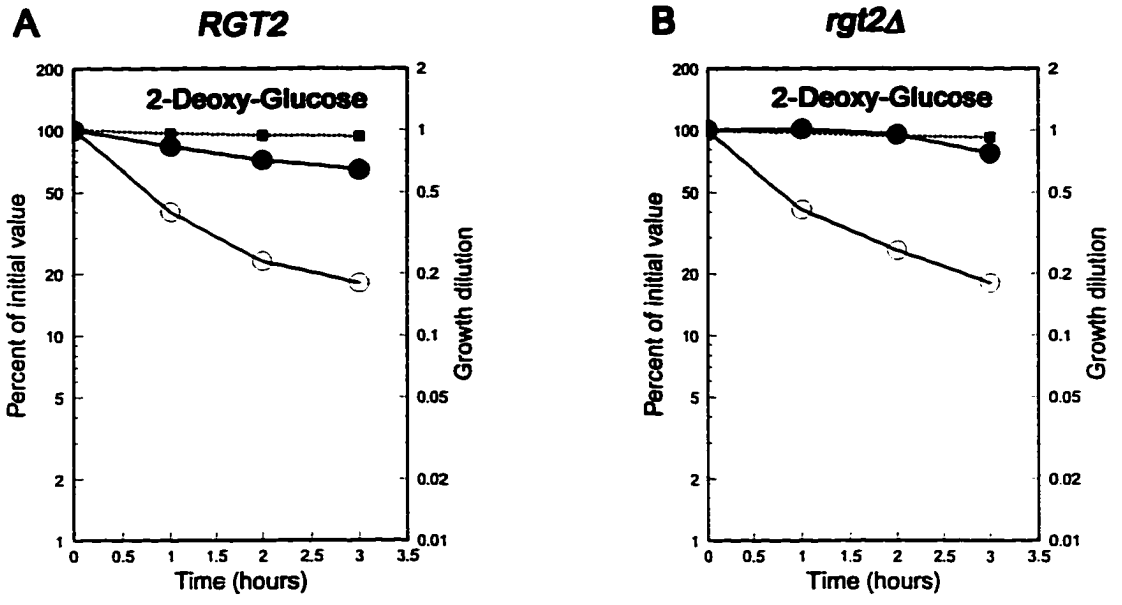

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Figure 7. Maltase function is required for maltose stimulated inactivation of maltose permease. Strains CMY1001(*GRR1*) and CMY1005 (*grr1Δ*) were grown in rich medium containing 2% maltose, harvested, and transferred to nitrogen starvation medium containing 2% maltose. Strains 100-1A[pRS405/MAL61/HA] (*MAL12*, see Materials and Methods for full description) and 100-1B[pMAL61/HA] (*mal12Δ*, see Materials and Methods for full description) were grown in selective medium lacking uracil and containing 2% galactose to early log phase (OD_{600} 0.1), and then 2% maltose was added to the culture to induce *MAL* gene expression for another 6 hours. Cells were harvested and transferred to nitrogen starvation medium containing 2% maltose.

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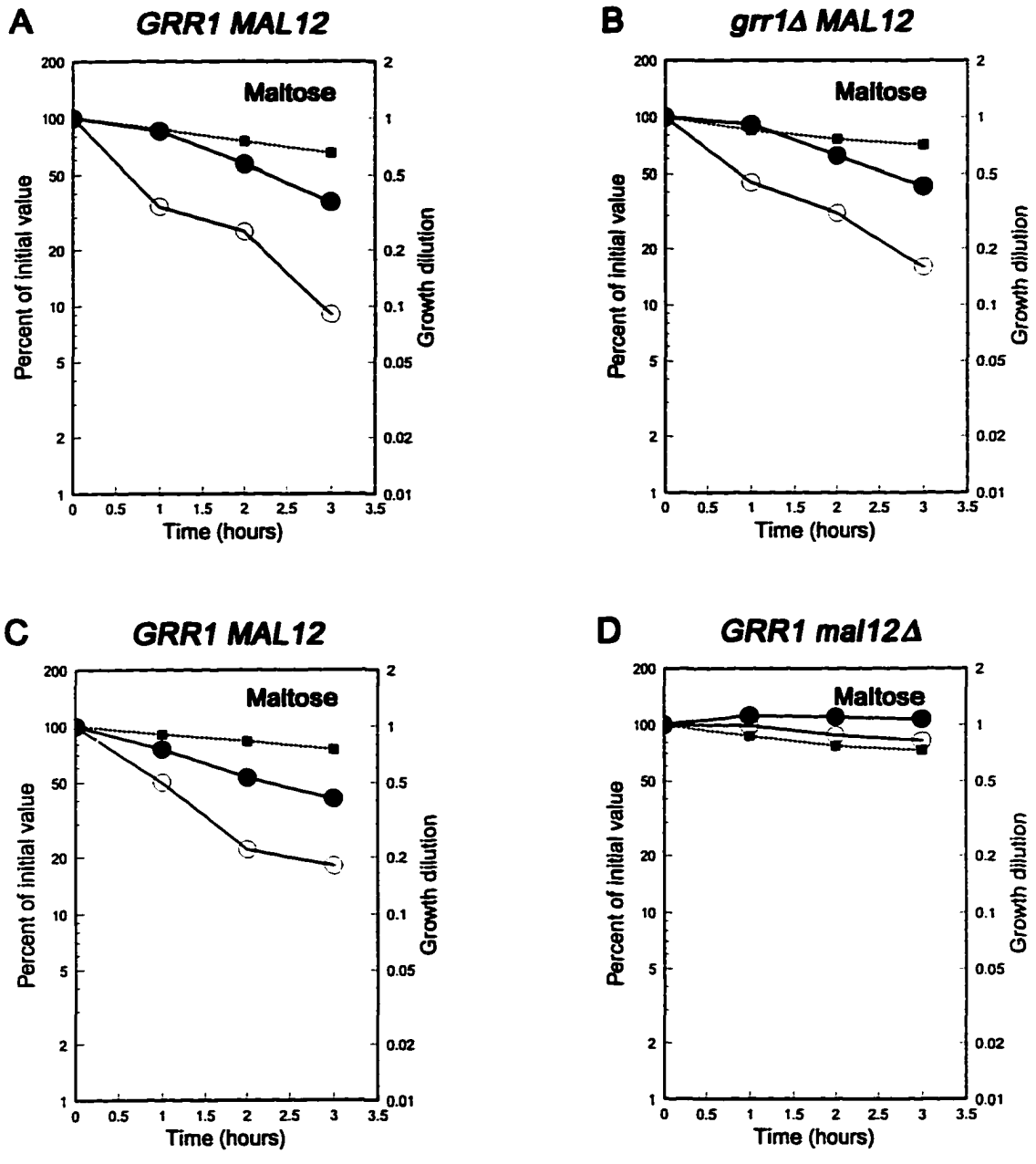

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Maltase, encoded by *MAL12* in strains carrying the *MAL1* locus, catalyzes the first step of maltose metabolism, that is, hydrolysis of maltose into two molecules of glucose. To determine if intracellular glucose production is required for this maltose-stimulated inactivation of its own transporter, we used strain 100-1A, which contains the *MAL1* locus (and no other *MAL* genes), and strain 100-1B, an isogenic strain containing a deletion of *MAL12* encoding maltase. We found that deletion of the maltase gene does not affect glucose-induced inactivation of maltose permease (data not shown), but does cause complete insensitivity to maltose-induced inactivation of maltose permease (Figure 7C and D). Thus, the ability of maltose to induce inactivation of maltose permease is dependent on the intracellular production of glucose by maltose hydrolysis, and maltose transport *per se* is not the inactivation signal.

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DISCUSSION

The glucose sensing and signal transduction pathways regulating *HXT* gene expression have been well characterized (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996a, b; Liang and Gaber, 1996). Two putative glucose transporters, Rgt2p and Snf3p, were identified as glucose sensors monitoring high and low extracellular glucose concentrations, respectively. Recently, we identified two glucose sensing/signaling pathways for the stimulation of glucose-induced inactivation of maltose permease (Jiang *et al.*, 1997). Pathway 1 is independent of glucose transport, utilizes Rgt2p as the glucose sensor of high levels of extracellular glucose, and is predominantly used to stimulate proteolysis of maltose permease. Our results indicate that Pathway 1 at least partially overlaps the signaling pathway described by Ozcan *et al.* (1996a). Pathway 2 is dependent on glucose transport, and in this report we undertake the further investigation of this glucose transport-dependent pathway.

Several lines of evidence presented here indicate that rapid sugar metabolism, at least to the point of glucose-6-phosphate production, is required to generate the Pathway 2 signal stimulating inactivation of maltose permease. We found that in *grr1Δ* mutant strains, which are deficient in both high-glucose signal production through Pathway 1 and Hxt-mediated high-affinity glucose transport, overproduction of galactose permease partially restores glucose transport and glucose-induced inactivation of maltose permease as well. Thus, glucose transport by the *HXT*-encoded high-affinity glucose transporters is not essential, and other transport proteins can substitute so long as the V_{max} of glucose transport is sufficiently high. We found that inactivation of maltose permease can be stimulated by galactose, usually considered as non-repressing

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sugar, but that galactose-induced inactivation requires not only overexpression of the galactose transporter but also induction of the other *GAL* genes. This suggests that galactose transport alone is not sufficient for signaling, and that utilization is required. More interestingly, since galactose utilization by-passes the hexokinases, phosphorylation by these enzymes is not essential (Figure 3C).

We found that sugar phosphorylation is required for the Pathway 2 signal. Hexokinase 1, hexokinase 2 and glucokinase catalyze the first step of glycolysis in *Saccharomyces*. All three enzyme can phosphorylate glucose; but only hexokinase 1 and 2, not glucokinase, phosphorylate fructose. Our results demonstrate that strains expressing at least one of the kinases are capable of generating the high glucose signal stimulating inactivation of maltose permease, and strains expressing at least one of the two hexokinases are capable of generating a high fructose signal, although the potency of the signal varies. These results are consistent with those reported by De Winde *et al.* (1996) who investigated the role of the hexokinases and glucokinase in regulating expression of several glucose-repressed genes. It is interesting to note that glucose-induced inactivation of maltose permease is completely blocked in the triple kinase deletion strain, and fructose-induced inactivation is completely blocked in the *hxk1Δ hxk2Δ GLK1* mutant strain, indicating that Pathway 1, the Rgt2-Grr1 extracellular glucose sensing pathway, is not active in the triple kinase-less mutant strain. This result is somewhat unexpected and suggests that the two signaling pathways defined by Jiang *et al.* (1997) may be interconnected at some key regulatory component. Finally, we found that 2-deoxy-glucose, a glucose analog that can be phosphorylated but not metabolized further, is capable of stimulating inactivation of maltose permease,

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implying that sugar phosphorylation is sufficient to generate the inactivation signal, and metabolism to other glycolytic intermediates is not required.

Taken together, our findings suggest that the signal generated by Pathway 2 results from a high rate of utilization of any of several fermentable sugars and does not specially require Hxt-dependent transport or Hxk- or Glk-dependent phosphorylation. Rather, metabolic flux appears to be the significant and controlling factor. Consistent with this is our finding that the potency of a particular sugar in generating a signal through this pathway, correlates with the affinity of these sugars for the hexose transporters and kinases: glucose>fructose>mannose. Thus, the hexokinases (and glucokinase) appear to function as glucose sensors in glucose-induced inactivation of maltose permease, because of their pivotal role in controlling the metabolic flux through the initial steps of glycolysis. This control is achieved by regulating the rate of glucose transport indirectly by regulating the rate of glucose phosphorylation.

Glucose is transported via facilitated diffusion. Kinetic studies report a low-affinity glucose transport (K_m =10 to 20 mM), and a high-affinity glucose transport (K_m =1 mM) (reviewed in Bisson *et al.*, 1993). High-affinity glucose transport is carried out by the gene products of a large family of 12 transmembrane domain proteins (encoded by the *HXT* genes) (Bisson *et al.*, 1993). Approximately 20 *HXT* homologues were identified by the *Saccharomyces* genome sequencing project, but, of those characterized, none appears to encode a low-affinity glucose transporter. High-affinity glucose uptake in *Saccharomyces cerevisiae* is dependent upon the presence of any one of the glucose phosphorylation enzymes (Bisson and Fraenkel, 1983a, b), and this dependence appears to be a consequence of the facilitated (as opposed to active)

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transport of glucose which requires phosphorylation to trap glucose in the cytosol (Nevado *et al.*, 1994; Smits, *et al.*, 1996). In support of this, Table 4 shows that the rate of glucose transport (at a substrate concentration of 1 mM) is decreased in all of our kinase disruption strains, but that the decrease in glucose transport rate is much more dramatic than can be explained simply by the decrease in *HXT* gene expression (Tables 4 and 5). Thus, the hexose kinases regulate glucose transport via their ability to remove glucose through the pool of intracellular free glucose through phosphorylation not via their ability to control the expression of the *HXT* genes or the activity of the Hxt transporters. Rapid sugar phosphorylation thus would lead to apparent rapid sugar transport. Taken together, these results indicate that the kinetic characteristics of each of the hexokinases and glucokinase and their expression patterns determine which of these is most important in glucose metabolism in different growth conditions, and put these enzymes in the role of glucose sensors.

A general glucose sensor (GGS) complex has been proposed by Thevelein and coworkers (Thevelein, 1992). In their model, the GGS complex contains (at least) a glucose transporter, a sugar kinase, and the *GGS1* gene product. The complex is proposed to have two functions: control of glucose influx into the yeast cell and activation of a number of signaling pathways responsible for various glucose-regulated processes. Unlike their mammalian counterparts, yeast hexokinases are not feed-back inhibited by glucose-6-phosphate, but instead are inhibited by trehalose-6-phosphate. Recent studies provide evidence that the influx of fermentable sugar into glycolysis may be controlled by the inhibition of the hexokinases by trehalose-6-phosphate (Blazquez *et al.*, 1993; Hohmann *et al.*, 1993). The *GGS1* gene encodes trehalose-6-phosphate

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synthase, and *ggs1Δ* mutants reportedly are unable to grow on glucose due to an uncontrolled influx of glucose and accumulation of sugar phosphates which eventually depletes the cytosol of free phosphate (Hohmann *et al.*, 1993). Deletion of *HXK2* reduces glucose phosphorylating activity and restores not only the growth on glucose but also all glucose-stimulated signaling pathways in *ggs1* mutants (Hohmann *et al.*, 1993). Since *HXK2* is the predominant sugar kinase expressed in glucose-grown cells, it plays an important role in glucose sensing, and therefore trehalose-6-phosphate synthase may also play an indirect role in glucose sensing via regulating *HXK2* kinase activity.

The exact nature of the signal of the glucose transport dependent pathway is not clear. Glucose, fructose, mannose, galactose, and 2-deoxy-glucose are able to induce inactivation of maltose permease, albeit with different potency. All of these hexoses are metabolized to glucose-6-phosphate suggesting that glucose-6-phosphate levels could be the intracellular inactivation signal. Alternatively, we would like to suggest that the high rate of sugar phosphorylation could alter the concentration of an intracellular second messenger. Recently, a working model for the mechanism of repression and derepression of genes in *Saccharomyces cerevisiae* in response to the availability of glucose has been proposed in which the AMP:ATP ratio might act as a signal for glucose repression in the Snf1 protein kinase signal transduction pathway (Wilson *et al.*, 1996). Wilson *et al.* (1996) suggest that high ATP levels (such as those which result from rapid fermentation of glucose, fructose, mannose, or galactose) could decrease AMP levels via an adenylate kinase reaction, a reversible reaction generate 2 ADP molecules from AMP and ATP. Changes in the AMP:ATP ratio might be an

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alternate candidate for this second messenger regulating Snf1 protein kinase.

Both glucose transport and glucose phosphorylation have been proposed as the glucose sensor in mammalian cells as well as in yeast. GLUT2 encodes a mammalian low-affinity glucose transporter, and is thought to play a permissive role in controlling glucose metabolism. In pancreatic β-cells, underexpression of GLUT2 results in decreased insulin secretion in response to increased glucose concentrations (reviewed in Unger, 1991; Bisson *et al.*, 1993). AtT-20ins cells are derived from anterior pituitary cells which express high-affinity glucose transporter GLUT1. These cells can secrete insulin but not in response to glucose. When transfected with GLUT2 cDNA, AtT-20ins cells become glucose-responsive (Hughes *et al.*, 1992). However, the role of GLUT2 in glucose sensing remains controversial. In normal β-cells, glucose transport capacity is in 100-fold excess compared to the capacity for glucose phosphorylation and catabolism, and phosphorylation, which is catalyzed predominantly by low-affinity glucokinase, appears to be the rate limiting step of glycolysis (reviewed in Efrat *et al.*, 1994). Accumulating evidence suggests an important role for pancreatic cell glucokinase in glucose sensing. There is a correlation between glucokinase levels and the ability to secrete insulin in response to glucose (reviewed in Bisson *et al.*, 1993). Glucokinase inhibitors also inhibit glucose-stimulated insulin secretion (Lenzen 1990), and mutations in glucokinase result in maturity onset diabetes of the young (MODY) (Froguel *et al.*, 1992; Vionnet *et al.*, 1992; Giah-Jain *et al.*, 1993; Devchand *et al.*, 1996). In glucagon producing pancreatic α-cells, glucokinase also may serve as a glucose sensor, and mediate glucagon release in response to extracellular glucose concentration (Heimberg *et al.*, 1996). Our results reported here suggest a homologous

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role for hexokinase in *Saccharomyces*, particularly hexokinase 2, in glucose sensing by Pathway 2 in the inactivation of maltose permease.

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CHAPTER 4

Role of Protein Phosphatase Type-1 Regulatory Subunits Reg1p and Reg2p in the Glucose-induced Proteolysis of Maltose Permease in *Saccharomyces*

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ABSTRACT

REG1 encodes a regulatory subunit of Glc7 protein phosphatase type-1 (PP1) that directs the phosphatase to its substrates in glucose repression signaling pathway. Deletion of *REG1* relieves glucose repression of many genes, including maltose permease and maltase. In this report, we explored the role of Reg1p in glucose-induced proteolysis of maltose permease. Deletion of *REG1* significantly reduces the rate of proteolysis of maltose permease suggesting that Reg1p is required for proteolysis of maltose permease. Using a *reg1Δ rgt2Δ* double deletion strain, we show that Reg1p is a component of the previously identified Rgt2p/Grr1p-dependent glucose signaling pathway. Moreover, overexpression of *REG1* partially restores the proteolysis in *grr1Δ* strains suggesting that Reg1p functions downstream of Grr1p. Loss of Grr1p appears to block the activity of Reg1p in the signaling pathway stimulating maltose permease proteolysis, possibly by stimulating Reg1p degradation. The role of recently identified *REG1* homolog, *REG2* in glucose-induced proteolysis of maltose permease is also discussed.

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INTRODUCTION

REG1 encodes a glucose-responsive regulatory subunit of Glc7 protein phosphatase type-1 (PP1) which targets the catalytic subunit to proteins involved in the glucose repression pathway. Binding of Reg1p and Glc7p *in vitro* is enhanced by the presence of glucose in the growth medium (Tu and Carlson, 1995). Deletion of *REG1* relieves glucose repression of many genes, including maltose permease and maltase (reviewed in Johnston and Carlson, 1992).

REG2 was identified on the basis of the interaction between Reg2p and Glc7p in the two-hybrid system. It encodes a smaller protein with sequence homology to Reg1p (Frederick and Tatchell, 1996). Overexpression *REG2* complements the mild slow-growth defect but not the glucose repression resistance of *reg1Δ* strains. The more severe slow-growth phenotype of *reg1Δ reg2Δ* strains is suppressed by *snf1* mutation (Frederick and Tatchell, 1996). These data suggest that Reg1p has a unique role in glucose repression, but that Reg1p and Reg2p play a similar role 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 *Saccharomyces cerevisiae* cells results in transcriptional repression of maltose permease and maltase genes and post-translational inactivation of maltose permease. Glucose-induced inactivation (also referred to as catabolite inactivation) consists of two independent processes: an initial very rapid inhibition of maltose transport activity associated with little decrease in protein levels (which occurs only in nitrogen deficient conditions), and a slower proteolysis of

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maltose permease protein (Medintz *et al.*, 1996). We previously identified two glucose sensing and signaling pathways stimulating glucose-induced inactivation of maltose permease. 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 (Jiang *et al.*, 1997). Pathway 2 is responsible for both the proteolysis of maltose permease and the rapid inhibition of maltose transport and uses the glucose phosphorylation enzymes as glucose sensors. Rapid glucose transport and phosphorylation are essential for generating the Pathway 2 inactivation signal (Jiang *et al.*, 1997; Jiang *et al.*, submitted).

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. Our results show that Reg1p is a component of the previously identified Rgt2p-dependent glucose signaling pathway (Pathway 1), and functions downstream of Grr1p. 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. We also provide evidence 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 glucose signal transduction.

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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 (Charron *et al.*, 1986) by two-step gene replacement of the *mal11Δ::URA3* with the HA-tagged *MAL61/HA* maltose permease as described in Medintz *et al.* (1996). Strain CMY1005 (*grr1Δ*) is otherwise isogenic to CMY1001, and is described in detail in Jiang *et al.* (1997). In strain CMY1017, the ORF of *RGT2* was replaced by *HIS3* using PCR-based gene disruption (Baudin *et al.*, 1993). The primers for disrupting *RGT2* are OM1031 and OM1032, which were gifts from Sabire Ozcan and Mark Johnston, and are described in Ozcan *et al.* (1996). The *RGT2* disruption was verified by Southern analysis.

Strains KT1357 (wild type), DF186 (*reg1Δ*), and DF184 (*reg2Δ*) were obtained from Kelly Tatchell. These strains are isogenic and are described in detail in Frederick and Tatchell (1996). None of these strains ferments maltose because of 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 (Danzi, 1997) or pUN90-MAL63, respectively. To be able to monitor maltose permease protein levels, we also introduced into these strains plasmid pRS315-MAL61HA (described in Medintz *et al.* 1996) carrying the HA-tagged maltose permease gene.

Strain CMY1018 (*rgt2Δ::HIS3 reg1Δ::URA3*) was obtained from a cross between strains CMY1017 (*rgt2Δ::HIS3*) and DF186 (*reg1Δ::URA3*). *URA3*$^+$ and *HIS3*$^+$ haploids were selected, and screened for their ability to ferment maltose. Western analysis was done on the maltose fermentors to confirm the presence of the

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Table 1. List of *Saccharomyces* strains

Strain	Genotype	Reference
CMY1001	*MATa MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200*	Medintz *et al.*, 1996
CMY1005	*grr1Δ* (isogenic to CMY1001)	Jiang *et al.*, 1997
CMY1017	*rgt2Δ* (isogenic to CMY1001)	This study
CMY1018	*reg1Δ rgt2Δ* (cogenic to CMY1017)	This study
KT1357	*MATa leu2 his3 trp1 ura3-52 AGT1 MAL12 MAL31 MAL32*	Frederick and Tatchell, 1996
DF186	*reg1Δ* (isogenic to KT1357)	Frederick and Tatchell, 1996
DF184	*reg2Δ* (isogenic to KT1357)	Frederick and Tatchell, 1996

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epitope tagged *MAL61/HA* gene integrated at *MAL1*.

Plasmid pREG1 (also called pDN70) was obtained from Mark Johnston. This plasmid carries *REG1* on a multicopy vector, YEp352 (Niederacher and Entian, 1991). Plasmids YCpIF15 and pREG2 (also called YCp-*Ha-REG2*) were obtained from Kelly Tatchell. YCpIF15 is a *TRP1* CEN plasmid, carrying the *GAL1* promoter. pREG2 is a YCpIF15 based plasmid, containing *REG2* ORF under the control of *GAL1* promoter (Frederick and Tatchell, 1996).

Inactivation assay protocol. 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-MAL61HA][pREG2] and DF186 [pUN30-MAL63][pRS315-MAL61HA][pREG2] were pregrown in selective medium supplemented with 2% galactose to early log phase, and then 2% maltose was added to the medium in order to induce the expression of the *MAL* genes for another 6 hours. The inactivation assay protocol is described in detail in Medintz *et al.* (1996). Cells were harvested, and transferred to nitrogen-starvation medium (yeast nitrogen base without amino acids and ammonium sulfate) plus 2% glucose. Aliquots were taken at the indicated times over a 3 hour period, and for each sample, 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 blotting. Western blot analysis was carried out as described in Medintz *et al.* (1996). The Mal61/HA protein in the extracts was detected by using anti-HA specific antibody and the ECL Western blotting kit (Amersham). The relative amount of each band on the ECL-Hyperfilm was measured by densitometric

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comparison to the zero time sample. Western analyses were done in duplicate from separate cell cultures.

Sugar transport assays. Maltose transport was measured as the uptake of 1 mM ^{14}C-maltose, as described in 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 for the maltose-grown cells. Assays were done in duplicate cultures.

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

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RESULTS

Published reports concerning 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 in maltose is suggested to result from the rapid generation of intracellular glucose (Entian, 1980; Entian and Loureiro-Dias). It is proposed that very high induced levels of maltose permease and maltase are expressed in *reg1* mutant strains grown on maltose, and that this leads to very high rates of maltose uptake and hydrolysis which produce concentrations of intracellular glucose so excessive that cell growth and/or division is severely repressed.

Other work in our laboratory had found that rates of induction and the fully induced levels of *MAL* gene expression are somewhat lower in strains expressing the *MAL63* *MAL*-activator compared to other *MAL*-activator genes. Based on this, we introduced plasmid pUN30-MAL63 into an isogenic series of strains carrying null mutations in *reg1Δ* (DF186) and *reg2Δ* (DF184), and the wild-type strain (KT1357). Growth on maltose was tested for each of these host strains lacks a *MAL*-activator gene. Even though copies of *MAL* structural genes can be found at the *MAL1* and *MAL3* locus positions, the host strains do not ferment maltose in the absence of the plasmid-borne *MAL*-activator gene.

Introduction of *MAL63* allowed these strains to ferment maltose, and express the *MAL* structural genes. The results in Table 2 demonstrate that both maltose permease and maltase are induced by maltose in all three strains, but the level of expression of maltose transport activity in the *reg1Δ* strain is slightly, but reproducibly, lower than that of the *reg2Δ* and wild-type strains. The basis of this is not known. Additionally,

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glucose repression is almost completely relieved by loss of Reg1p but is not affected by the loss of Reg2p. These results enabled us to proceed with an analysis of the role of Reg1 protein in glucose-induced inactivation of maltose permease.

Glucose-induced proteolysis of maltose permease in the wild-type, *reg1Δ*, and *reg2Δ* mutant strains. Both plasmids pUN30-MAL63, and pRS315-MAL61HA were introduced into the wild-type, *reg1Δ*, and *reg2Δ* strains described above to allow us to follow maltose permease protein Mal61/HAp using Western analysis. Strains were grown in selective medium containing 2% maltose, harvested, and transferred to nitrogen starvation medium containing 2% glucose. As seen in Figure 1A, glucose stimulates very rapid proteolysis in the wild-type strain; no HA-tagged maltose permease protein could be detected after 3 hours. In the *reg1Δ* strain, the rate of glucose-induced proteolysis is significantly slowed, and 3 hours after transfer to glucose, the level of HA-tagged maltose permease is still 40% compared to that at time zero (Figure 1B). Deletion of *REG2* has a more modest effect on glucose-induced proteolysis of maltose permease, reducing the rate of proteolysis about 2-fold (Figure 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 glucose signal stimulating proteolysis of maltose permease.

Overexpression of *REG2* suppresses the glucose inactivation phenotype but not the glucose repression phenotype of *reg1Δ*. The finding that deletion of *REG2* slightly reduces the rate of glucose-induced proteolysis of maltose permease suggests that Reg1p and Reg2p may have similar functions in regulating glucose-induced proteolysis of maltose permease. To explore this, plasmid pREG2, carrying the *REG2*

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

Genotype	Maltose transport activity (nmol/mg drywt/min)			
	Maltose	Mal+Glu	Glucose	Gly/Lac
REG1 REG2	2.55	0.04	0.01	0.17
reg1Δ REG2	1.93	1.02	0.23	0.20
REG1 reg2Δ	2.36	0.09	0.02	0.21
	Maltase activity (nmolPNPG/mg protein/min)			
	Maltose	Mal+Glu	Glucose	Gly/Lac
REG1 REG2	1022	11	4	35
reg1Δ REG2	1720	1255	35	33
REG1 reg2Δ	1580	14	6	33

Strains KT1357 (*REG1 REG2*), DF186 (*reg1Δ*) and DF184 (*reg2Δ*) transformed with plasmids pUN30-MAL63 and pRS315-MAL61HA were grown in selective medium lacking tryptophan and leucine, and containing 2% maltose, 2% maltose plus 2% glucose, 2% glucose, or 3% glycerol plus 2% lactate. Cells were harvested at early log phase (OD_{600} of 0.3) and maltase and maltose transport were assayed as described in Materials and Methods.

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Figure 1. Glucose-induced proteolysis of maltose permease in maltose-grown wild-type, *reg1Δ*, and *reg2Δ* strains. Strains KT1357 (*REG1 REG2*), DF186 (*reg1Δ*), and DF184 (*reg2Δ*) were transformed with plasmids pUN30-MAL63 and pRS315-MAL61HA. Strains were grown in selective medium lacking tryptophan and leucine, and containing 2% maltose. Time course of glucose-induced proteolysis of maltose permease was carried out as follows. Maltose-grown cells are harvested at early log phase (OD_{600} of 0.3), and transferred to nitrogen starvation medium containing 2% glucose. Cells are harvested at the indicated times over a 3 hour period, and for each sample, total cell extracts were prepared for Western analysis. The relative levels of Mal61/HAp protein (●, solid line), and growth dilution (■, dotted line) are plotted in the same panel. The relative protein levels at time X are compared to 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.

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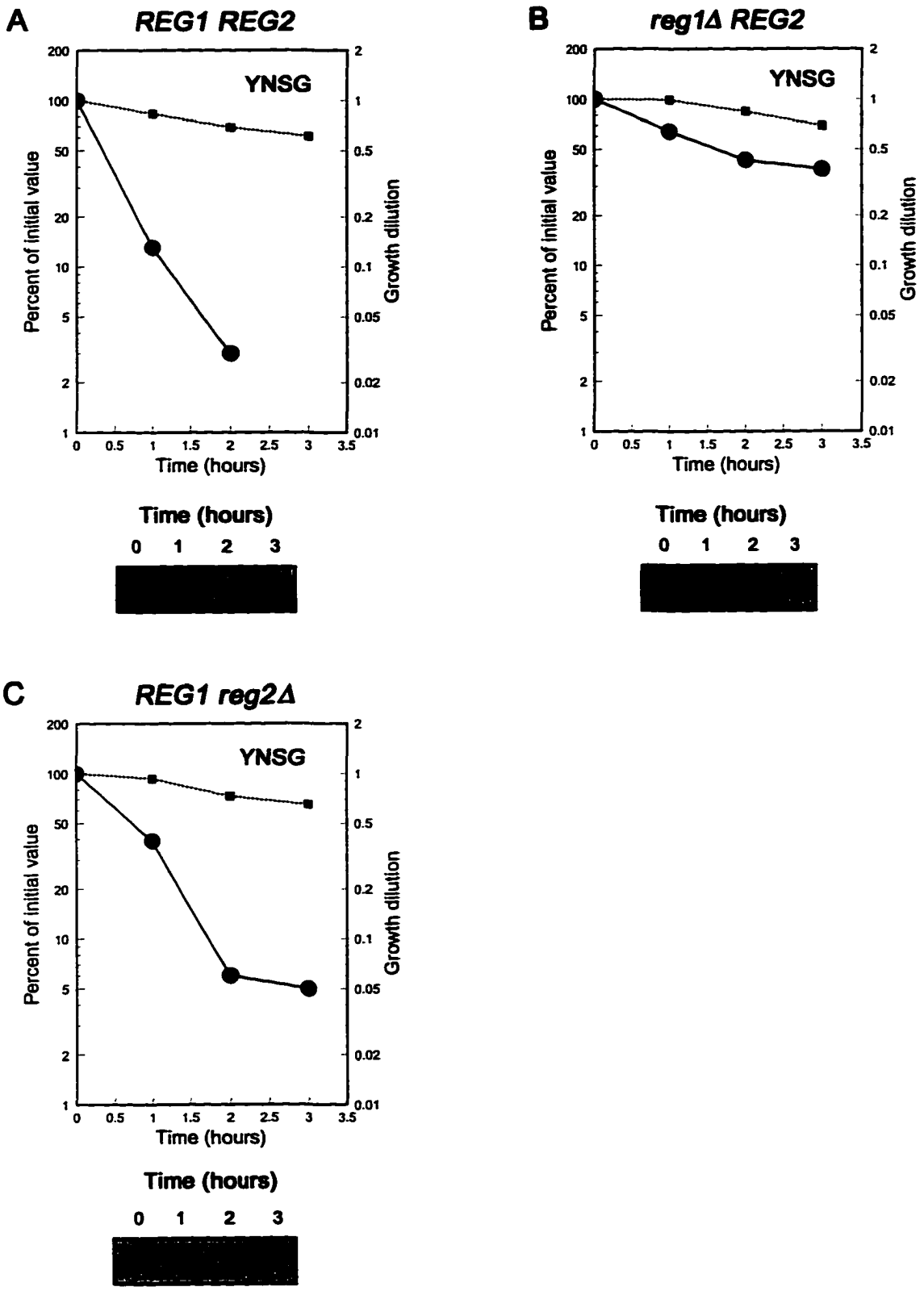

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Table 3. Glucose transport activity in maltose-grown *REG1* and *reg1Δ* mutant strains.

Genotype	Vmax (nmoles/mg dry wt · min)	Relative Vmax %	Km (mmoles)
REG1	28.6	100	1.2
reg1Δ	29.4	103	0.9

Strains KT1357[pUN30-MAL63][pRS315-MAL61HA] (*REG1*), and DF186 [pUN30-MAL63][pRS315-MAL61HA] (*reg1Δ*), 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%.

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gene under the control of *GAL1* promoter, and the control vector YCpIF15 were transformed into strains KT1357 (*REG1*) and DF186 (*reg1Δ*) separately and glucose-induced proteolysis of maltose permease was followed. The strains were pregrown in galactose containing medium to early log phase to allow the overexpression of *REG2*. Then maltose was added to the medium for an additional 6 hours prior to assaying glucose-induced proteolysis. The results are reported in Figure 2. Overproduction of Reg2p results in a more rapid rate of glucose-induced proteolysis of maltose permease (Figure 2C) compared to the *reg1Δ* (Figure 2D), but not to the level seen in the *REG1* strain (Figure 2A). No significant effect of overproduction of *REG2* on glucose-induced proteolysis is seen in *REG1* strain (Figure 2A compared to Figure 2B).

Interestingly, overexpression of *REG2* has no effect on the glucose repression insensitivity of *MAL* gene expression in *reg1Δ* strains (Table 4). For this analysis, the cells were pregrown in galactose to induce *REG2* expression, transferred to media containing the indicated carbon source for 6 hours, and maltose transport and maltase activity determined. Frederick and Tatchell (1996) also report that Reg2p overproduction does not suppress the glucose repression insensitive expression of invertase.

Role of Reg1p in glucose signal transduction. We previously identified two glucose signaling pathways stimulating glucose-induced inactivation of maltose permease (Jiang *et al.*, 1997). Pathway 1 requires the high glucose sensor Rgt2p and the signal transducer Grr1p, and predominantly stimulates proteolysis of maltose permease protein in response to glucose. Pathway 2 is dependent on rapid glucose transport and phosphorylation and stimulates both a rapid inhibition of maltose

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Figure 2. Effect of *REG2* overexpression on glucose-induced proteolysis of maltose permease in maltose-grown *REG1*, *reg1Δ* strains. Strains KT1357 (*REG1*), DF186 (*reg1Δ*) were each transformed with plasmids pUN90-MAL63 and pRS315-MAL61HA along with either a *REG2* overexpression plasmid pREG2 or a control vector YCpIF15. Strains were pregrown in galactose containing selective medium lacking histidine, leucine and tryptophan to early log phase, and then 2% maltose was added to the culture to induce maltose permease expression. At 6 hour after maltose induction, cells were harvested, transferred to nitrogen-starvation medium containing glucose and time course of glucose-induced proteolysis of maltose permease was carried out as described for Figure 2.

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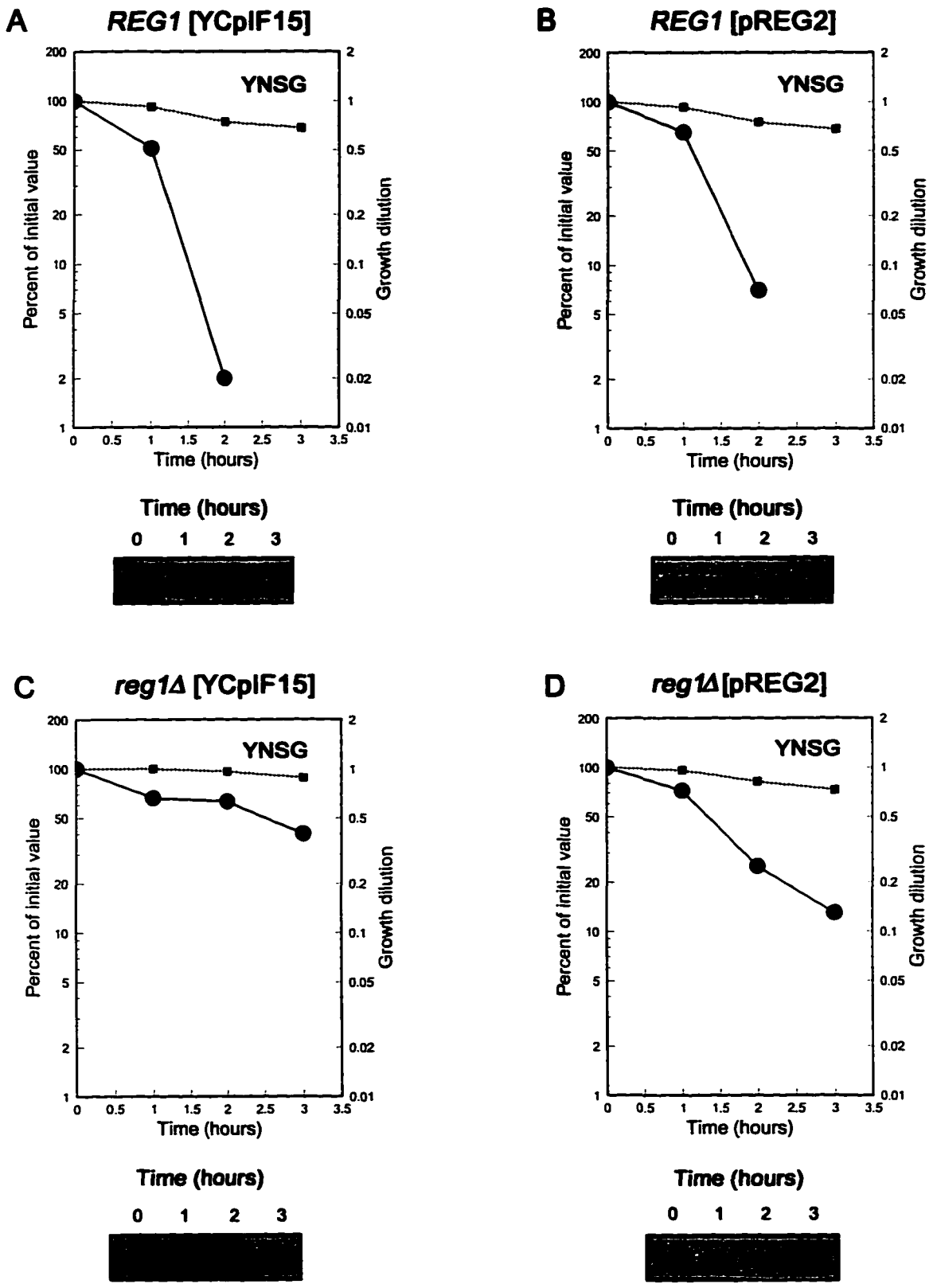

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Table 4. Effect of *REG2* overexpression on maltose permease and maltase expression.

Genotype	Maltose transport activity (nmol/mg drywt/min)			
	Maltose	Mal+Glu	Glucose	Gly/Lac
REG1 [YCpIF15]	3.67	0.16	0.04	0.20
REG1 [pREG2]	3.59	0.13	0.06	0.15
reg1Δ [YCpIF15]	2.29	1.75	0.16	0.16
reg1Δ [pREG2]	2.38	1.35	0.16	0.14
	Maltase activity (nmolPNPG/mg protein/min)			
	Maltose	Mal+Glu	Glucose	Gly/Lac
REG1 [YCpIF15]	648	31	4	43
REG1 [pREG2]	735	36	4	46
reg1Δ [YCpIF15]	1311	928	104	92
reg1Δ [pREG2]	1286	905	87	84

Strains KT1357 (*REG1*), DF186 (*reg1Δ*) transformed with plasmids pUN30-MAL63 and pRS315-MAL61HA along with either a *REG2* overexpression plasmid pREG2 or a control plasmid YCpIF15 were grown in selective medium lacking histidine, tryptophan and leucine. Different carbon sources supplemented in the medium and protocol for maltase assay are same as described for Table 2.

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transport activity and proteolysis of maltose permease protein. We wished to explore the relationship of Reg1p to these two signaling pathways.

A *reg1Δ rgt2Δ* strain (CMY1018) was constructed by crossing strains DF186 (*reg1Δ::URA3*) and strain CMY1017 (*rgt2Δ::HIS3*). Deletion of either *REG1* or *RGT2* partially inhibits glucose-induced proteolysis of maltose permease to similar extents, approximately 50% (Figures 1B and 3A). As shown in Figure 3, disruption of both *reg1Δ* and *rgt2Δ* provides no cumulative effect, and the phenotype of the double null strain is similar to that observed in either *reg1Δ* or *rgt2Δ* strain alone. This approximately 50% decrease in maltose permease proteolysis in these strains is not associated with a decrease in high-affinity glucose transport (Table 5). These results are consistent with the hypothesis that Rgt2p and Reg1p function in the same signaling pathway.

Grr1p plays a pivotal role in both Pathway 1 and Pathway 2, because it is a component of the Rgt2p-dependent signaling pathway, and because it regulates expression of glucose transporters in both high and low extracellular glucose (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996). Deletion of *GRR1* completely blocks glucose-induced rapid inhibition of maltose uptake as well as proteolysis of maltose permease (Jiang *et al.*, 1997). Moreover, Grr1p is downstream of Rgt2p in Pathway 1. Plasmid pREG1 carrying the *REG1* on a multicopy vector allows for the overexpression of *REG1* (Niederacher and Entian, 1990). When pREG1 is introduced into a *grr1Δ* strain, glucose-induced proteolysis of maltose permease is restored, but no rapid inhibition of maltose uptake is observed (Figure 4C and 4D). As can be seen in Figure 4D, the loss in transport activity parallels the loss in maltose permease protein. Additionally, the

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Figure 3. Glucose-induced proteolysis of maltose permease in maltose-grown *rgt2Δ* and *reg1Δ rgt2Δ* mutant strains. Strains CMY1017 (*REG1 reg2Δ*) and CMY1018 (*reg1Δ rgt2Δ*) were grown in rich medium containing 2% maltose to early log phase, harvested, transferred to nitrogen-starvation medium containing glucose and time course of glucose-induced proteolysis of maltose permease was carried out as described for Figure 2.

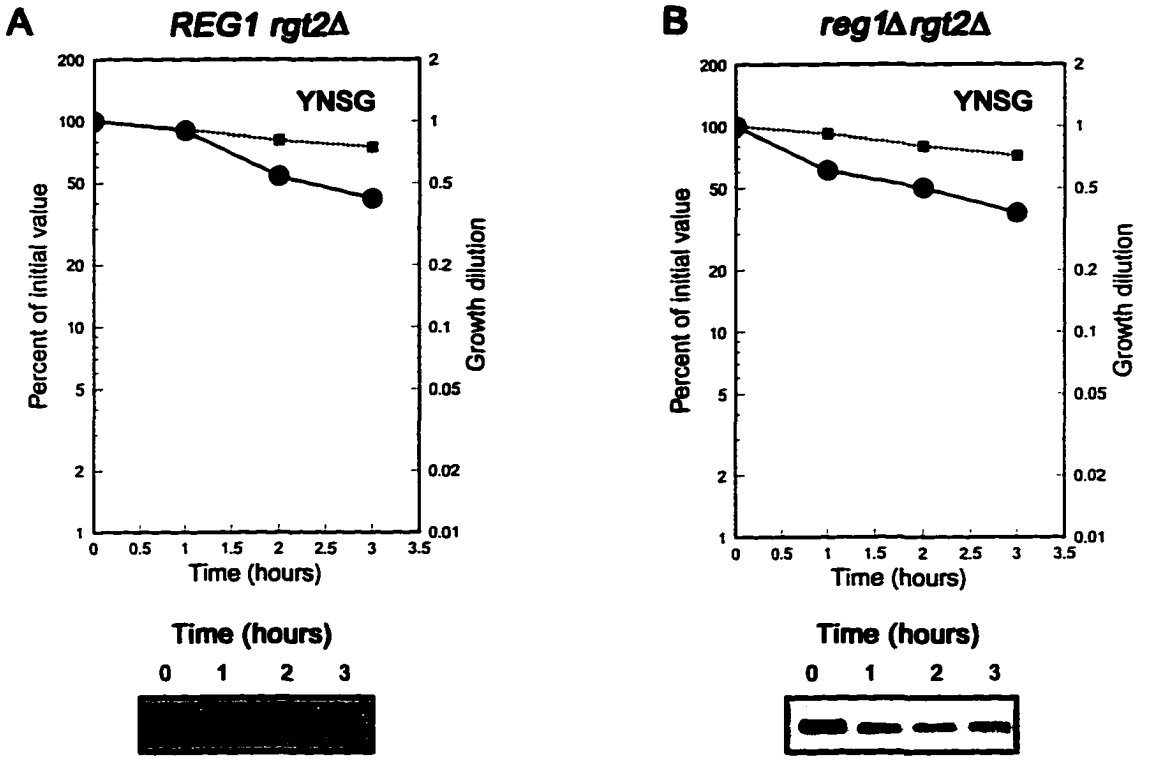

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Table 5. Glucose transport activity in maltose-grown *rgt2Δ*, and *reg1Δ rgt2Δ* mutant strains.

Genotype	Vmax (nmoles/mg dry wt · min)	Relative Vmax %	Km (mmoles)
REG1 RGT2	**19.2**	**100**	**1.0**
REG1 rgt2Δ	**37.0**	**193**	**0.7**
reg1Δ rgt2Δ	**20.0**	**104**	**1.1**

Strains CMY1001 (*REG1 RGT2*), CMY1017 (*REG1 rgt2Δ*), and CMY1018 (*reg1Δ rgt2Δ*) were grown in rich medium containing 2% maltose. Glucose transport was determined as described for Table 3. The relative activity of strain CMY1001 is given as 100%.

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Figure 4. Effect of *REG1* overexpression on glucose-induced inactivation of maltose permease in maltose-grown *GRR1* and *grr1Δ* strains. Strains CMY1001 (*GRR1*) and CMY1005 (*grr1Δ*) were transformed with either a *REG1* overexpression plasmid pREG1 or a control vector pRS416. Strains were grown in selective medium lacking uracil and containing 2% maltose. The standard inactivation assay protocol was used (see Materials and Methods; Medintz *et al.*, 1996). The relative levels of Mal61/HAp protein (●, solid line), maltose uptake (○, solid line) and growth dilution (■, dotted line) are plotted in the same panel.

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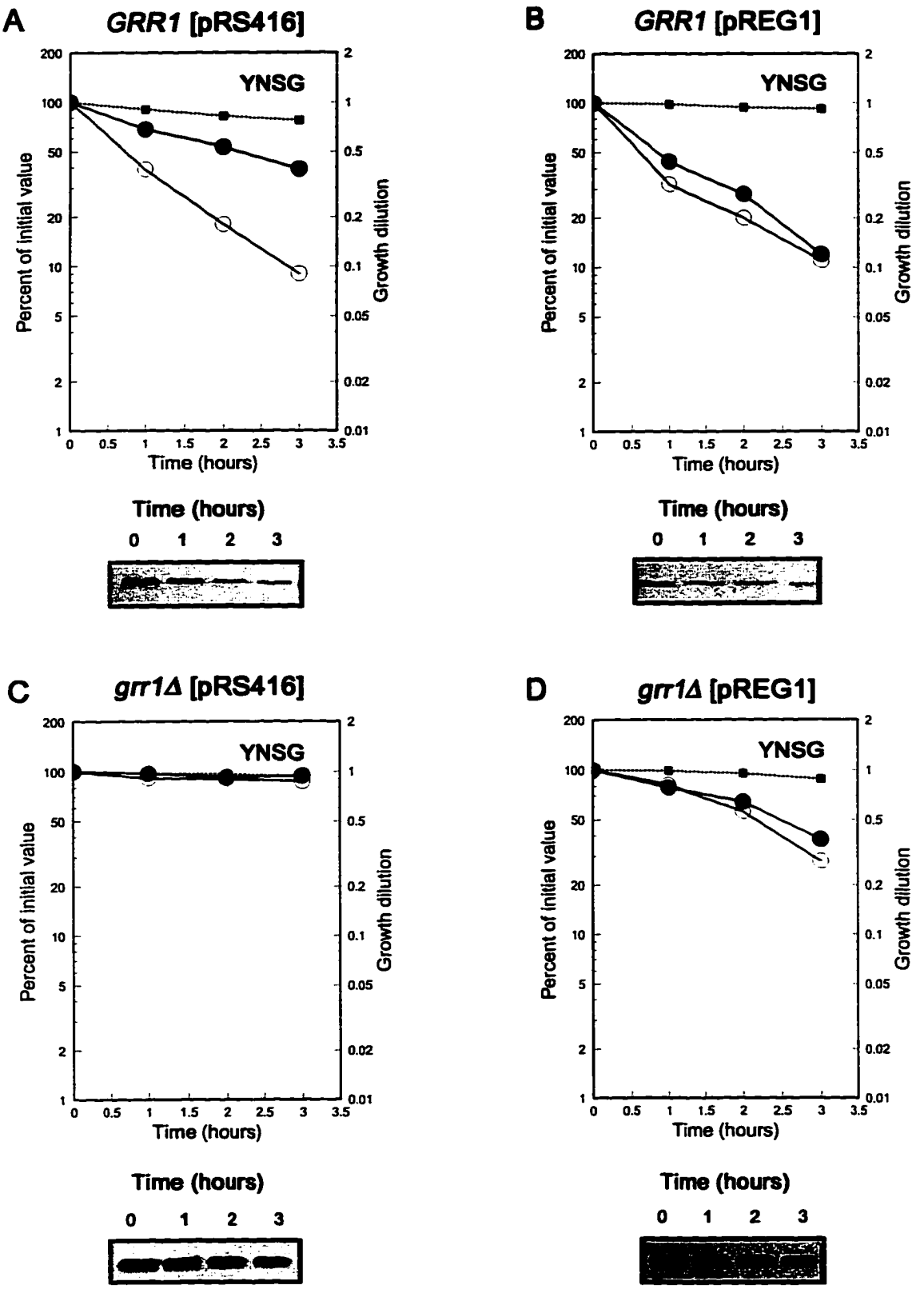

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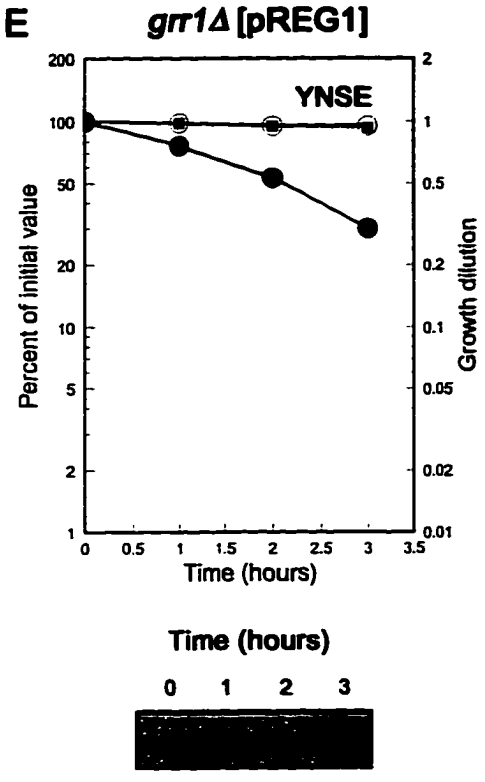
(Figure 4 continued)



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wild-type 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 (Figure 4B). Taken together, the results in Figure 4 strongly suggest that Reg1p overexpression compensates for the loss of glucose signaling through Pathway 1.

Grr1p is required for glucose induction of *HXT* gene expression (Ozcan and Johnston, 1995), 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). Interestingly, plasmid pREG1 does not suppress the loss of glucose transport in the *grr1Δ* mutant strain grown in maltose (Table 6). This suggests that the ability of plasmid pREG1 to restore proteolysis of maltose permease is constitutive, that is, does not require glucose induction. Figure 4E confirms this. Comparable rates of proteolysis are observed when ethanol (Figure 4E) as opposed to glucose (Figure 4D) is used in the inactivation medium.

Mutations in *GRR1* are pleiotropic. In addition to effects on high-affinity glucose transporter expression and glucose-induced inactivation of maltose permease, *grr1* mutant strains are resistant to glucose repression and exhibit an aberrant sausage-like cell morphology. Plasmid pREG1 is unable to suppress either phenotype (Table 7 and Figure 5).

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Table 6. Glucose transport activity in maltose-grown *GRR1* and *grr1Δ* mutant strains containing the *REG1* overexpression plasmid.

Genotype	Vmax (nmoles/mg dry wt min)	Relative Vmax %	Km (mmoles)
GRR1	19.2	100	1.0
grr1Δ	2.9	15	1.3
GRR1[pREG1]	20.0	108	1.4
grr1Δ[pREG1]	2.0	10	1.0

Strains CMY1001 (*GRR1*) and CMY1005 (*grr1Δ*) were grown in rich medium containing 2% maltose. Strains CMY1001 and CMY1005 transformed with a *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%.

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

Genotype	Maltose transport activity (nmol/mg drywt/min)			
	Maltose	Mal+Glu	Glucose	Gly/Lac
GRR1 [pRS416]	2.31	0.07	0.04	0.14
GRR1 [pREG1]	1.46	0.05	0.04	0.10
grr1Δ [pRS416]	4.12	3.15	0.30	0.25
grr1Δ [pREG1]	3.08	2.97	0.15	0.15
	Maltase activity (nmolPNPG/mg protein/min)			
	Maltose	Mal+Glu	Glucose	Gly/Lac
GRR1 [pRS416]	1356	7	<1	22
GRR1 [pREG1]	741	1	<1	17
grr1Δ [pRS416]	1783	1412	45	57
grr1Δ [pREG1]	1258	1064	34	48

Strains CMY1001 (*GRR1*), CMY1005 (*grr1Δ*) transformed with either a *REG1* overexpression plasmid pREG1 or a control plasmid pRS416 were grown in selective medium lacking uracil. Different carbon sources supplemented in the medium and protocol for maltase assay are same as described for Table 2.

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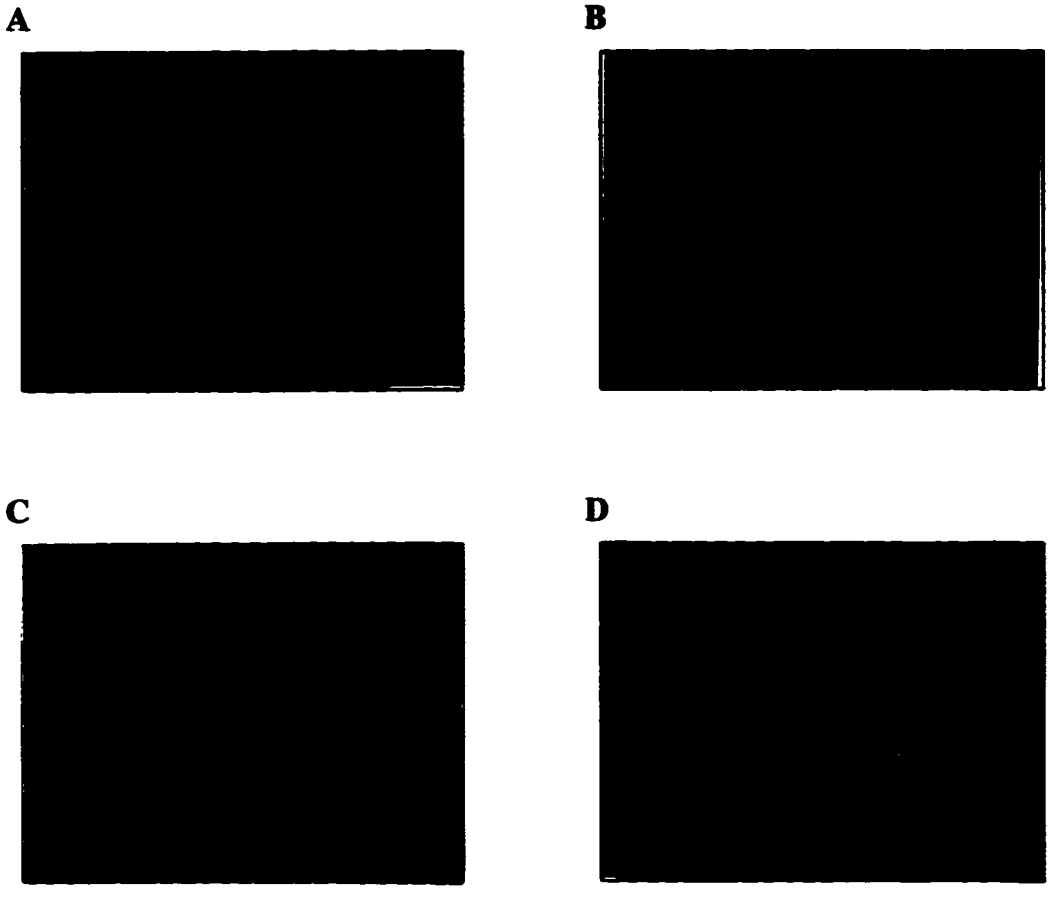
Figure 5. Morphology of wild-type, *grr1Δ*, *reg1Δ*, and *grr1Δ* [pREG1] cells. A. Wild-type (CMY1001); B. *reg1Δ* (DF186); C. *grr1Δ* (CMY1005); and D. *grr1Δ*[pREG1]. All strains were grown in glucose containing medium. All cells are shown at the same magnification.



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DISCUSSION

Early studies by Entian's group demonstrated that maltose causes inhibition of growth, glycolysis, and *de novo* protein synthesis in *reg1* strains because of rapid accumulation of intracellular glucose. The strains used in their studies carried the constitutive *MAL2-8C* allele which exhibits high levels of *MAL* gene expression even in the absence of maltose. 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). Strains KT1357 (*REG1*) and DF186 (*reg1Δ*) used in this study can not ferment maltose due to the lack of a functional *MAL*-activator gene. Plasmids carrying different *MAL*-activator genes were introduced into these strains. We found that the *reg1Δ* 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 *LexA-MAL63* fusions do not grow on maltose (our unpublished results). Thus, the inhibition effect of maltose in the *reg1Δ* strain appears to be *MAL*-activator dependent. By using *MAL63* to activate *MAL* gene transcription, we are able to carry out the analysis reported here.

Reg1p and Reg2p have overlapping functions and stimulate glucose-induced proteolysis of maltose permease. *REG1* encodes a regulatory subunit of Glc7 protein phosphatase (Tu and Carlson, 1995), and *REG2* encodes a Glc7p binding protein with significant homology to Reg1p (Frederick and Tatchell, 1996). Tu and Carlson (1995) demonstrated a physical interaction between Reg1p and Glc7p, and found that this interaction is strengthen in glucose medium. Frederick and Tatchell (1996) show that *reg1Δ* mutants exhibit a mild growth defect which is made more severe by disruption of

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REG2. The severe growth defect in *reg1Δ reg2Δ* strains is complemented by *REG2* overexpression and is alleviated by *snf1* mutation. They suggest that Reg1p and Reg2p may direct the Glc7 protein phosphatase catalytic subunit to substrates that are phosphorylated by the Snf1 protein kinase. Thus, Glc7 protein phosphatase may act in opposition to Snf1 protein kinase to regulate growth.

In this report, we provide evidence that *REG1* and *REG2* have redundant functions with regard to the stimulation of the 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 very modest (Figure 1). Moreover, overexpression of *REG2* partially restores proteolysis in a *reg1Δ* strain (Figure 2). This is the first identification of a specific phenotype for a *reg2Δ* mutant (Frederick and Tatchell, 1996).

Deletion of *REG1* completely relieves glucose repression, but *reg2Δ* strains are still subject to glucose repression (Table 3). Additionally, overexpression of *REG2* does not restore glucose repression sensitivity in a *reg1Δ* strains (Table 3). Therefore, although Reg1p and Reg2p have redundant functions with regard to glucose-induced proteolysis of maltose permease, Reg1p has a unique function in glucose repression. Substrates of the Reg1-Glc7 and Reg2-Glc7 protein phosphatases have not been identified, although Snf1 protein kinase is proposed as one possible target. Our results are consistent with those of Frederick and Tatchell (1996) indicating that Reg1p and Reg2p have distinct, but partially overlapping targeting activities.

Reg1p is downstream of Grr1p in the Rgt2p-dependent signaling pathway. Grr1p is a global regulator in *Saccharomyces* involved in numerous glucose-regulated

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processes, including *HXT* gene transcription, glucose repression of *GAL, SUC* and *MAL* genes, and cell cycle regulation. *GRR1* is involved in degradation of the G1 cyclins Cln1p and Cln2p (Barral *et al.*, 1995). G1 cyclins are short-lived proteins which contain PEST sequences, potential protein degradation signals, in their carboxy-terminal (Nash *et al.*, 1988; Hadwiger *et al.*, 1989). Cln1p and Cln2p are stabilized in *grr1Δ* mutants, or by deletion of the PEST sequence (Barral *et al.*, 1995). The elongated morphological defect in *grr1Δ* mutants can be mimicked by overexpressing *CLN1* (Barral *et al.*, 1995), suggesting that Grr1p plays a role in G1 cyclin degradation. Recent results indicate that Grr1p may be a ubiquitin-protein ligase, an E3, and function in the glucose regulated degradation of selected targets. Grr1p has two important structural motifs, the leucine rich repeats which mediates protein-protein interaction and the F-box, which appears to mediate its interaction with Skp1p (Bai *et al.*, 1996, Li and Johnston, submitted). Skp1p is a highly conserved protein known to be involved in ubiquitin-mediated proteolysis of Cln2p, Clb5p, and Sic1p (an inhibitor of Cdc28 cyclin-dependent protein kinase), and may link a large number of proteins to the proteolysis machinery (Bai *et al.*, 1996). It is interesting to note that the interaction between Grr1p and Skp1p is enhanced by high-levels of glucose (Li and Johnston, submitted). The results reported here suggest that Grr1p is required to stabilize Reg1 protein, and that Reg1p is downstream of Grr1p in the Rgt2p-dependent glucose signaling pathway.

Loss of Grr1p abolishes glucose-induced proteolysis of maltose permease (Jiang *et al.*, 1997). Full restoration of glucose transport in *grr1Δ* null mutations only partially restores glucose-induced proteolysis of maltose permease supporting the

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existence of a Grr1p-dependent Pathway 1. Jiang *et al.* (1997) went on to show that Grr1p is an essential downstream component of a signaling pathway which utilizes Rgt2p, a Snf3p homologue, to sense high extracellular glucose.

The results reported here are consistent with a model which places Reg1p downstream of Grr1p in the Rgt2p-dependent high glucose signaling pathway, Pathway 1. The model is diagrammed in Figure 6. We found that *rgt2Δ*, *reg1Δ*, and *reg1Δ rgt2Δ* strains all exhibit a similar decrease in the rate of maltose permease proteolysis. Had Reg1p been a component of Pathway 2, we would have expected a complete loss in glucose sensing/signaling and complete insensitivity to glucose-induced inactivation of maltose permease, similar to the phenotype seen in *grr1Δ* mutants, despite the fact that high-affinity glucose transport rates are normal. In fact, the phenotype of *reg1Δ* strains is similar to that of *grr1Δ rgt1Δ* double null strains where signaling through Pathway 1 is blocked by loss of Grr1p but signaling through Pathway 2, and glucose transport, is completely restored by loss of Rgt1p. Our results do not suggest a role for Reg1p in Pathway 2, but this possibility can not be excluded. Overexpression of *REG1* increases maltose permease proteolysis but does not appear to produce an increase in rapid inhibition of maltose transport (Figure 4B and 4D).

Reg1p overproduction suppresses glucose-induced proteolysis insensitivity in a *grr1Δ* disruption mutation. Thus, Reg1p is downstream of Grr1p. Since other phenotypes of *grr1Δ* mutations, such as loss of glucose transport and morphology defect are unaffected by *REG1* overexpression, or loss of Reg1p (Table 5 and 6; Figure 5). These Grr1p-dependent processes appear to be regulated independently of Reg1p.

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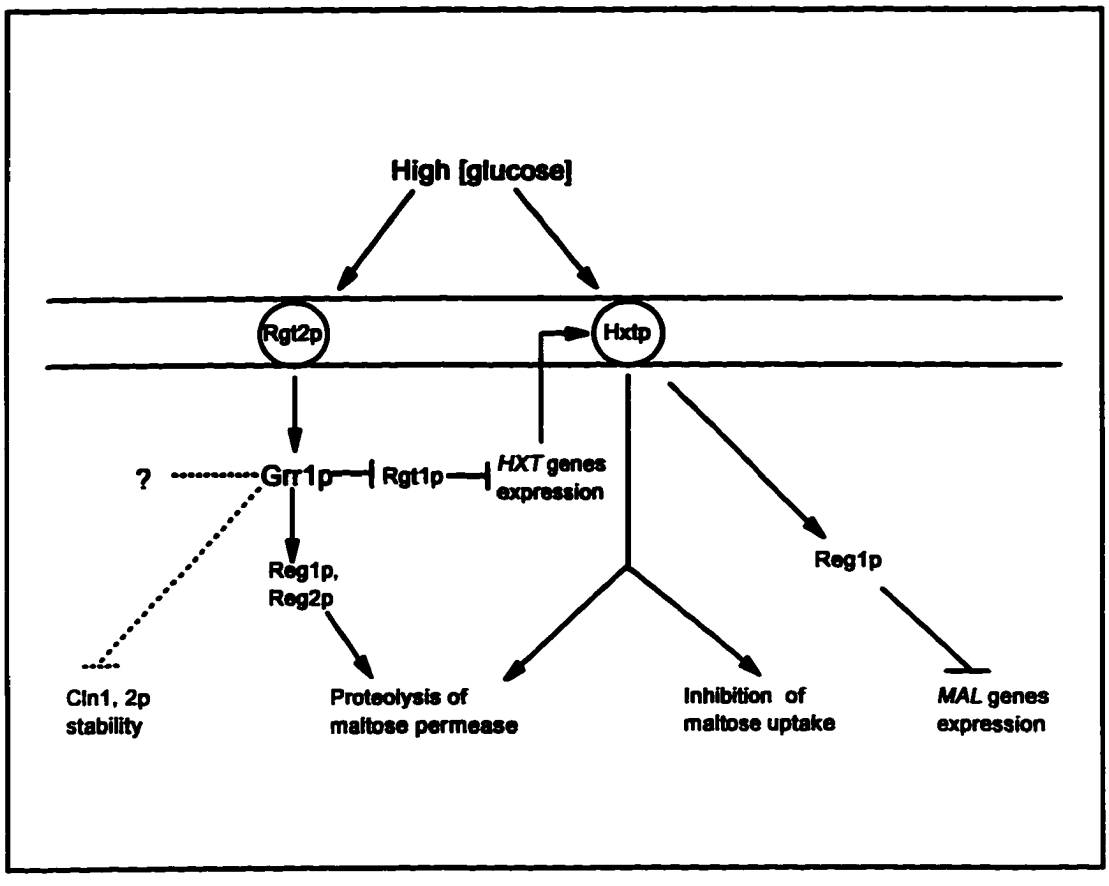
Figure 6. Model of glucose signal transdution pathways mediated by Grr1p



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Moreover, Grr1p appears to be a positive regulator of Reg1p although the mechanism of this is unclear, particularly in view of evidence indicating that Grr1p is a ubiquitin-protein ligase (Li *et al.*, submitted). In addition, Li and Johnston (1996) report an interaction between Grr1p and Reg1p using a two-hybrid system. Since Reg1p contains the PEST sequence, one might predict that Grr1p is involved in the degradation of Reg1p. However, they also find that the level of Reg1 protein is decreased in *grr1Δ* strains (M. Johnston, personal communication). Our result that overexpression of *REG1* partially restores proteolysis of maltose permease in a *grr1Δ* strain is consistent with the second finding. Deletion of *GRR1* could decrease the Reg1 protein levels either by stimulating Reg1p degradation, by inhibiting expression of *REG1*, or by both.

It should be noted that overproduction of Reg1p leads to constitutive proteolysis of maltose permease (Figure 4E). This suggests that the interaction between Reg1p and Glc7 is not entirely dependent on the effects of glucose, but may be controlled to some degree by the levels of the various regulatory subunits of Glc7p which compete for binding to limited amounts of the catalytic subunit. Such competition was suggested by Huang *et al.* (1996) who report the suppression of point mutations in *gac1*, another Glc7p regulatory subunit controlling glycogen synthase dephosphorylation, by *reg1* mutations.

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SUMMARY

The work described in this thesis focuses on an investigation of glucose sensing and signal transduction pathways stimulating a post-translational mechanism, glucose-induced inactivation of maltose permease. Two glucose sensing/signaling pathways were revealed, and several components of both pathways were identified.

Pathway 1 partially overlaps with Rgt2p-dependent glucose induction pathway described by Ozcan *et al.* (1996a). Both pathways utilize Rgt2p, as the glucose sensor for the detection of high levels of extracellular glucose and Grr1p, as the signal transducer. Grr1p is the branch point of the glucose inactivation and glucose induction pathways. The most downstream components of Pathway 1 that we have been able to identify are Reg1p and Reg2p, two regulatory subunits of Glc7 protein phosphatase type-1 (PP1). Grr1p may be involved in regulating the stability of Reg1p or the expression of *REG1*. Pathway 1 is predominantly responsible for proteolysis of maltose permease.

Pathway 2 is dependent on high rates of sugar metabolism. The initial steps of glucose metabolism, including transport and phosphorylation are essential for generating the glucose signal. Intracellular, not extracellular glucose is critical for Pathway 2. Hexokinase 1, 2 and glucokinase appear to function as glucose sensors monitoring the levels of intracellular glucose. Several other fermentable sugars, including fructose, mannose, galactose and maltose also are capable of stimulating inactivation of maltose permease through Pathway 2, although with different potency. Nonfermentable carbon sources are not able to induce inactivation. 2-Deoxy-glucose is a glucose analogue which can be phosphorylated but not further metabolized. The fact

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that 2-deoxy-glucose can cause inactivation of maltose permease suggests that phosphorylation is sufficient for generating the Pathway 2 signal. Pathway 2 is responsible for both proteolysis of maltose permease protein and rapid inhibition of maltose transport.

The triple kinase deletion strain is completely insensitive to glucose-induced inactivation of maltose permease indicating that Pathway 1 and 2 may be interconnected at some key components.

We proposed a working model for glucose sensing/signaling pathways stimulating glucose-induced inactivation of maltose permease, diagrammed in Figure 1. In our model, a line with an arrowhead indicates positive regulation, and a line with a bar indicates negative regulation. The question marks shown at the last steps of the pathways imply that the extreme downstream components which may directly interact with maltose permease have not been identified. Moreover, we are uncertain whether or not glucose-6-phosphate levels act as second messenger to signal high rates of sugar fermentation.

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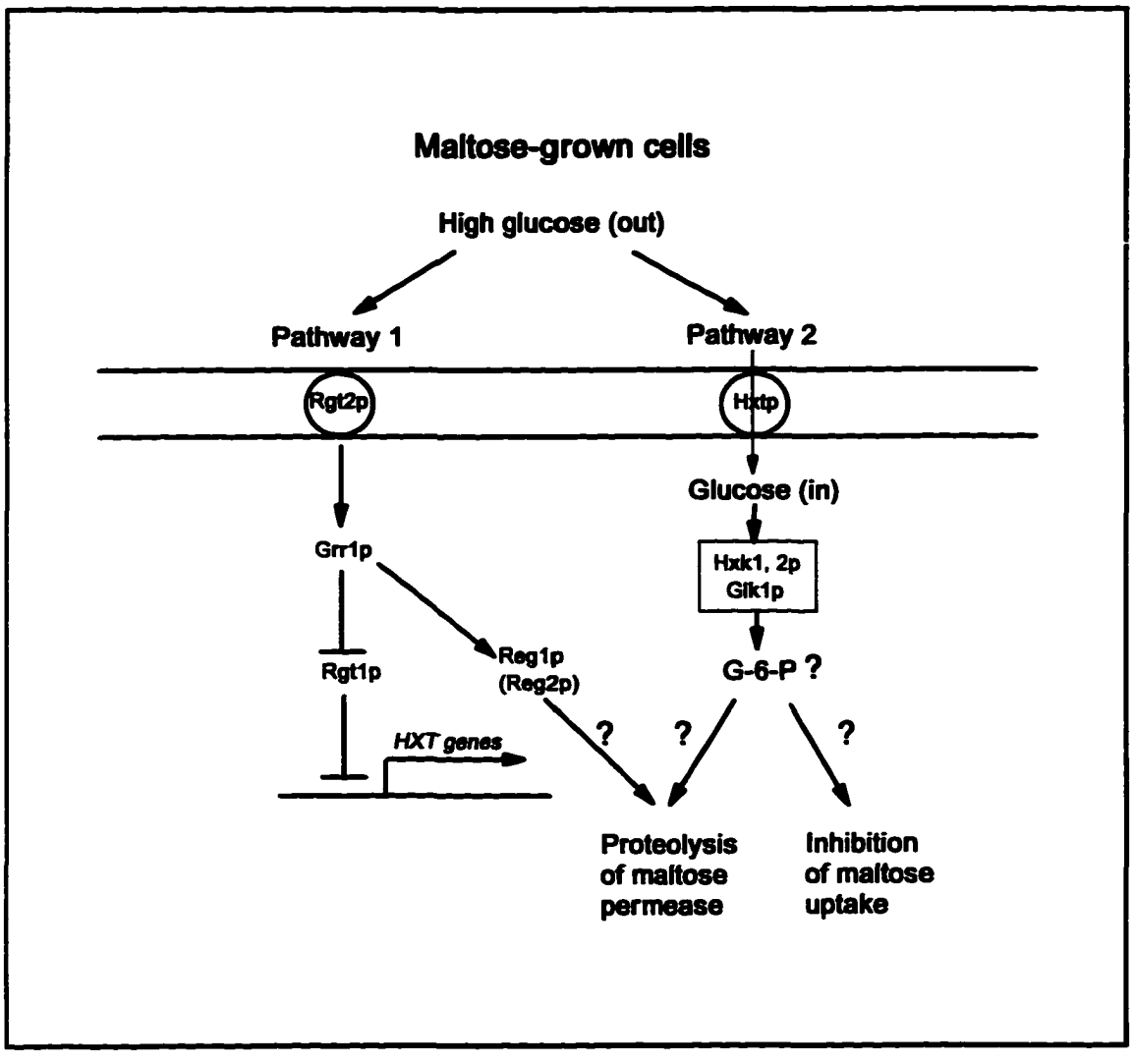
Figure 1. Working model of the glucose sensing/signaling pathways stimulating glucose-induced inactivation of maltose permease



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