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REG1 binds to protein phosphatase type 1 and regulates glucose repression in *Saccharomyces cerevisiae*

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Protein phosphatase type 1 (PP1) is encoded by *GLC7*, an essential gene in *Saccharomyces cerevisiae*. The GLC7 phosphatase is required for glucose repression and appears to function antagonistically to the SNF1 protein kinase. Previously, we characterized a mutation, *glc7-T152K*, that relieves glucose repression but does not interfere with the function of GLC7 in glycogen metabolism. We proposed that the mutant GLC7^{T152K} phosphatase is defective in its interaction with a regulatory subunit that directs participation of PP1 in the glucose repression mechanism. Here, we present evidence that REG1, a protein required for glucose repression, is one such regulatory subunit. We show that REG1 is physically associated with GLC7. REG1 interacts with GLC7 strongly and specifically in the two-hybrid system, and REG1 and GLC7 fusion proteins co-immunoprecipitate from cell extracts. Moreover, overexpression of a REG1 fusion protein suppresses the *glc7-T152K* mutant defect in glucose repression. This and other genetic evidence indicate that the two proteins function together in regulating glucose repression. These results suggest that REG1 is a regulatory subunit of PP1 that targets its activity to proteins in the glucose repression regulatory pathway.
Keywords: glucose repression/protein phosphatase 1/regulatory subunit/*Saccharomyces cerevisiae*

Introduction

The reversible phosphorylation of proteins is a major post-translational regulatory mechanism in eukaryotic cells. The phosphorylation state of any protein depends on the relative activities of protein kinases and protein phosphatases. Both kinases and phosphatases have been shown to play critical roles in regulating diverse cellular processes (Hunter, 1987; Cohen, 1989, 1992; Hubbard and Cohen, 1993).

One of the major protein phosphatases found in eukaryotic cells is protein phosphatase type 1 (PP1). PP1 is highly conserved, with >80% identity between yeast and mammals (Ohkura *et al.*, 1989; Feng *et al.*, 1991) and participates in many different cellular regulatory mechanisms. Mammalian PP1 affects regulation of glycogen metabolism, protein synthesis and muscle contraction (Cohen, 1989) and has been implicated in control of cell cycle progression (Durfee *et al.*, 1993; Dohadwala *et al.*, 1994). Mitotic defects have also been associated with mutations in the PP1 genes of *Schizosaccharomyces pombe* (*dis2$^+$/bws1$^+$* and *sds21$^+$*; Booher and Beach, 1989; Ohkura *et al.*, 1989; Kinoshita *et al.*, 1990), *Aspergillus nidulans* (*bimG$^+$*; Doonan and Morris, 1989) and *Drosophila* (*PP1 87B*; Axton *et al.*, 1990). In the yeast *Saccharomyces cerevisiae*, protein phosphatase type 1 is encoded by *GLC7 (DIS2S1)*, an essential gene (Ohkura *et al.*, 1989; Feng *et al.*, 1991). Like its mammalian counterparts, the GLC7 protein phosphatase participates in regulation of diverse processes, including glycogen accumulation and sporulation (Feng *et al.*, 1991; Cannon *et al.*, 1994), cell cycle progression (Francisco *et al.*, 1994; Hisamoto *et al.*, 1994; Zhang *et al.*, 1995) and protein translation (Wek *et al.*, 1992). Previously, we have shown that the GLC7 protein phosphatase also has a role in the regulatory mechanism for glucose repression, acting antagonistically to the SNF1 protein kinase (Tu and Carlson, 1994). Here, we have further explored this role.

The SNF1 protein kinase is required for expression of glucose-repressed genes when glucose is limiting (Celenza and Carlson, 1986). SNF1 is physically associated in a large complex with several proteins, including the SNF4 activating subunit and members of a family of SNF1-interacting proteins (SIP1, SIP2, GAL83; Yang *et al.*, 1992). Genetic evidence suggests that for some glucose-repressed genes, SNF1 functions to relieve transcriptional repression mediated by MIG1, SSN6 and TUP1 (Nehlin and Ronne, 1990; Keleher *et al.*, 1992; Treitel and Carlson, 1995). The relationship of GLC7 to the SNF1 protein kinase became evident when we isolated the *GLC7* gene by complementation of a mutation (formerly *cid1-226*) that partially relieves glucose repression of *SUC2* (invertase) and *MAL3* (maltase) gene expression (Tu and Carlson, 1994). We showed that the mutation is an allele of *GLC7*, renamed *glc7-T152K*. Genetic analysis suggests that the GLC7 protein phosphatase acts antagonistically to the SNF1 protein kinase in glucose repression; various different relationships between PP1 and SNF1 in the glucose response mechanism are possible.

An interesting aspect of this genetic analysis is that the *glc7-T152K* allele confers a phenotype that is distinct from that of another well characterized mutation, *glc7-1* (Feng *et al.*, 1991; Cannon *et al.*, 1994; Stuart *et al.*, 1994). We showed that *glc7-T152K* does not impair glycogen accumulation, whereas *glc7-1* abolishes glycogen accumulation but does not affect glucose repression (Tu and Carlson, 1994). We proposed that *glc7-T152K* interferes with the interaction between the phosphatase catalytic subunit and a regulatory subunit that specifically directs the GLC7 activity to a target protein(s) involved in glucose repression of gene expression.

In mammalian systems, considerable evidence indicates

that regulatory subunits play major roles in controlling PP1 activity towards specific substrates (Cohen, 1989; Hubbard and Cohen, 1993). Specific regulatory or targeting subunits direct the PP1 catalytic subunit to different subcellular locations or substrates and/or alter regulatory properties. For example, in skeletal muscle, the glycogen-binding (G) regulatory subunit targets the catalytic subunit to glycogen particles (Cohen, 1989). Such regulatory subunits are conserved from mammals to yeast. In *S.cerevisiae*, the GAC1 protein is a sequence homolog of mammalian G subunit and is involved in activation of glycogen synthase and glycogen accumulation (François *et al.*, 1992). Moreover, the glycogen accumulation defect of *glc7-1* has been attributed to a defect in interaction of the mutant PP1 catalytic subunit with GAC1 (Stuart *et al.*, 1994). Other potential regulators of PP1 include GLC8, an inhibitor-2 homolog (Cannon *et al.*, 1994), and SHP1 (Zhang *et al.*, 1995). In *S.pombe*, the sds22 protein associates with and alters the substrate specificity of PP1 catalytic subunits encoded by $dis2^+$ and $sds21^+$ (Stone *et al.*, 1993) and genetic evidence supports the idea that sds22 regulates the mitotic function of PP1 (Ohkura and Yanagida, 1991). The *S.cerevisiae* homolog EGP1/SDS22 has similar properties (Hisamoto *et al.*, 1995; MacKelvie *et al.*, 1995).

We therefore sought to identify a regulatory subunit that controls GLC7 function in the glucose repression regulatory mechanism. The protein encoded by *REG1* (also called *HEX2* and *SRN1*) seemed a possible candidate. *REG1* was first identified as a negative regulator of glucose-repressed genes and a mutation of *REG1* relieves glucose repression of many genes (Entian and Zimmermann, 1980; Matsumoto *et al.*, 1983; for review see Johnston and Carlson, 1992). A *reg1* mutation (called *srn1*) was also identified as a suppressor of *rna1-1*, which affects pre-RNA processing and transport (Tung *et al.*, 1992); RNA1 is homologous to human Ran GTPase-activating protein (Bischoff *et al.*, 1995). A connection between *GLC7* and *REG1* was established because both *glc7* (called *cid1*) and *reg1* alleles were isolated in a search for mutants defective in glucose repression of *SUC2* (Neigeborn and Carlson, 1987; Tu and Carlson, 1994). The *glc7-T152K* and *reg1-69* alleles isolated in this search were shown to be functionally similar in that *snf1* was epistatic to both and the double mutant combination showed no synergy in relieving repression of *SUC2*. This genetic analysis suggests that these genes could function together in glucose repression of *SUC2*. Therefore, we considered REG1 as a candidate for a GLC7 regulatory subunit. REG1 is a 113 kDa protein containing putative nuclear localization signals (Niederacher and Entian, 1991; Tung *et al.*, 1992).

Here we have tested the model that the REG1 protein is associated with the GLC7 protein phosphatase. We first used the two-hybrid system to show that REG1 and GLC7 interact strongly and specifically. We then present biochemical evidence that the REG1 and GLC7 proteins interact physically. Finally, genetic evidence lends further support to the idea that the two proteins function together in regulating glucose repression. These data suggest that REG1 is a potential regulatory subunit of PP1 that targets its activity to proteins in the glucose repression regulatory pathway.

Table I. REG1 interacts with GLC7 in the two-hybrid system

DNA-binding hybrid	Activation hybrid	Color[a]	β-Gal activity[b]	
			Glu	Raf
LexA–REG1	GAD	white	<1	<1
LexA	GAD–GLC7	white	<1	<1
LexA–REG1	GAD–GLC7	blue	130	24
LexA–REG1	GAD–GLC7^{T152K}	blue	20	10
LexA–GLC7	GAD–GAC1	blue	1	10
LexA–GLC7^{T152K}	GAD–GAC1	blue	2	20
LexA–GLC7	GAD–SNF1	white	<1	<1
LexA–REG1	GAD–GAC1	white	ND	ND

[a]Four transformants were patched on selective SC-2% glucose plates, grown for 1 day and then the color of transformants was determined by filter assay.
[b]Transformants were grown in selective SC medium containing either 4% glucose (Glu, repressing condition) or 0.05% glucose plus 4% raffinose (Raf, derepressing condition). β-galactosidase activity is expressed in Miller Units. Values are averages of assays of four to six different transformants. Standard errors were <10%.
ND, not determined.

Results

REG1 interacts with GLC7 in the two-hybrid system

To test for interaction between the REG1 and GLC7 proteins, we first used the two-hybrid system (Fields and Song, 1989). We expressed hybrid proteins containing LexA fused to REG1 and the GAL4 activation domain (GAD) fused to GLC7. Interaction between the two fusion proteins *in vivo* was monitored by the activation of β-galactosidase expression from a *lexAop–GAL1–lacZ* target gene (Table I). Strains expressing both LexA–REG1 and GAD–GLC7 turned blue in a color assay, whereas control strains were white (Table I). In additional controls, we did not detect any blue color in transformants expressing LexA–REG1 in combination with either GAD–GAC1 (a fusion protein at codon 53; see Materials and methods) or GAD–SIP1 (Yang *et al.*, 1992). A GAD–REG1 fusion was also constructed and expressed but it did not complement the *reg1* mutation and no interaction with LexA–GLC7 was detected (data not shown).

Genetic evidence indicates that the REG1 function is required for repression of gene expression during growth in glucose. Expression of the *REG1* gene is not regulated by glucose availability (Niederacher and Entian, 1991). To test whether interaction between REG1 and GLC7 is modulated by glucose availability, we quantitatively assayed the β-galactosidase activity resulting from interaction of LexA–REG1 and GAD–GLC7 in the two-hybrid system under both glucose-repressing and -derepressing conditions (Table I). Both proteins are expressed from the strong *ADH1* promoter. In glucose-grown cells, β-galactosidase expression was stimulated >100-fold (130 U) relative to the controls. In raffinose-grown cells, β-galactosidase activity was still substantial (24 U), although 5-fold lower than in glucose-repressing conditions. In contrast, in cells expressing LexA–GLC7 (Tu and Carlson, 1994) and GAD–GAC1, β-galactosidase activity was 10-fold higher under derepressing conditions. These results indicate that the GLC7 phosphatase interacts strongly with REG1 under both glucose-repressing and derepressing conditions. In addition, it is possible that

Table II. Overexpression of LexA–REG1 restores glucose repression of *SUC2* expression in a *glc7-T152K* mutant

Relevant genotype	Expressed protein	Invertase activity	
		Repressed	Derepressed
glc7-T152K	LexA	26	250
	LexA–GLC7	2	220
	LexA–GLC7^{T152K}	21	230
	LexA–REG1	2	140
reg1Δ::URA3	LexA	240	230
	LexA–REG1	4	74
	LexA–GLC7	220	260

Invertase activity is expressed as μmol glucose released/min/100 mg cells (dry weight). Cultures were grown in selective SC-2% glucose (repressed) and shifted to SC-0.05% glucose medium for 3 h (derepressed). Values are averages for two to three transformants of MCY2616 (*glc7-T152K*) and MCY3278 (*reg1Δ::URA3*). LexA was expressed from pLexA(1–202)+PL (Ruden et *al.*, 1991). Standard errors were <20%.

the few-fold regulatory effect detected here reflects a physiologically significant regulation of the interaction between the native proteins. However, we have not determined whether protein levels are affected by glucose induction of the *ADH1* promoter.

REG1 and GLC7 fusion proteins co-immunoprecipitate

To provide biochemical evidence that REG1 and GLC7 protein phosphatase interact, we carried out co-immunoprecipitation experiments. Extracts were prepared from glucose-grown cells expressing both LexA–REG1 and HA–GLC7 (Sutton *et al.*, 1991), which carries a hemagglutinin (HA) epitope tag. LexA–REG1 complements a *reg1* mutation (Table II) and HA–GLC7 provides GLC7 function (Stuart et *al.*, 1994). The HA–GLC7 protein was immunoprecipitated with monoclonal HA antibody and the precipitate was analyzed by Western blotting for the presence of LexA–REG1 using LexA antibody (Figure 1A). In addition, LexA–REG1 was immunoprecipitated with LexA antibody and the precipitates were similarly analyzed for the presence of HA–GLC7 using HA antibody (Figure 1B). In each case, the REG1 and GLC7 fusion proteins co-immunoprecipitated. Control experiments with LexA–SIP1 (Yang *et al.*, 1992) and HA–SIP4 fusions ruled out artifactual interactions involving the LexA or HA moieties. In addition, we detected protein phosphatase activity (see Figure 3) coprecipitating with LexA–REG1 and not with LexA–SIP1 (data not shown). Thus, in glucose-repressed cells the GLC7 protein phosphatase is physically associated with REG1.

When extracts were prepared from derepressed cultures, we were not able to detect co-immunoprecipitation of LexA–REG1 and HA–GLC7 because amounts of LexA–REG1 protein were greatly decreased (>20-fold) relative to the levels found in glucose-repressed extracts (data not shown). However, we detected similar amounts of LexA–REG1 in repressed and derepressed cultures when cells were boiled directly in the gel loading buffer. These results, together with the positive two-hybrid results in derepressed cells, suggest that LexA–REG1 is simply not stable in the derepressed extracts. We do not know whether this apparent instability is related at a mechanistic level to the few-fold decreased two-hybrid interaction detected in derepressed cells.

The glc7-T152K mutation decreases interaction between GLC7 and REG1

We have proposed that the glucose repression defect of the *glc7-T152K* mutant results from a defect in interaction between the GLC7^{T152K} mutant protein and a regulatory subunit (Tu and Carlson, 1994). To test for a defect in interaction with REG1, we used the two-hybrid system to compare interaction of wild-type and mutant GLC7 proteins with LexA–REG1. We first expressed a derivative of GAD–GLC7 carrying the *glc7-T152K* mutation, called GAD–GLC7^{T152K}. Although we do not have reagents that detect the GAD–GLC7 fusion proteins, other evidence indicates that the T152K alteration does not affect stability of the protein: LexA fusions to the mutant and wild-type GLC7 are present at the same level (see below) and wild-type and mutant *GLC7–lacZ* fusions (Tu and Carlson, 1994) produced the same β-galactosidase activity (unpublished data).

In glucose-grown strains, LexA–REG1 in combination with the mutant GAD–GLC7^{T152K} fusion protein produced 6-fold less β-galactosidase activity than LexA–REG1 in combination with wild-type GAD–GLC7 (Table I). These findings suggest that the GLC7^{T152K} mutant protein is partially defective for interaction with REG1 *in vivo*. Moreover, the defect in interaction of the native proteins expressed from their chromosomal loci is probably more severe than indicated by the 6-fold decrease detected here because the overexpression of both fusion proteins compensates for the mutant defect (see below).

In a similar experiment, a mutant GLC7^{T152K} fusion protein showed no defect in interaction with GAC1, the glycogen regulatory subunit for PP1 (Table I). This is consistent with previous evidence that *glc7-T152K* mutant cells accumulate nearly normal levels of glycogen. In contrast, GAC1 shows a defect in interaction with the product of the *glc7-1* allele, which reduces glycogen accumulation (Stuart *et al.*, 1994). Thus, the specificity of the interaction defect correlates with phenotype.

Overexpression of LexA–REG1 suppresses the glc7-T152K mutant defect in glucose repression

If the glucose repression defect of the *glc7-T152K* mutant is due to a partial defect in interaction of PP1 and REG1, we reasoned that increased levels of REG1 expression might partially compensate for this defect and therefore suppress the *glc7-T152K* phenotype. To test this possibility we introduced pLexA–REG1, which expresses the fusion protein from the strong *ADH1* promoter, into a *glc7-T152K* mutant and assayed invertase activity. Glucose repression of *SUC2* expression was restored; in fact, the defect was suppressed as effectively by LexA–REG1 as by LexA–GLC7 (Table II). In contrast, overexpression of LexA–GLC7 did not suppress the *reg1Δ* defect in glucose repression. These data are consistent with the two-hybrid evidence that the *glc7-T152K* mutation reduces interaction between GLC7 and REG1, although suppression by LexA–REG1 overexpression can also be accounted for by other mechanisms. Moreover, these findings provide additional genetic evidence that GLC7 and REG1 function together in glucose repression.

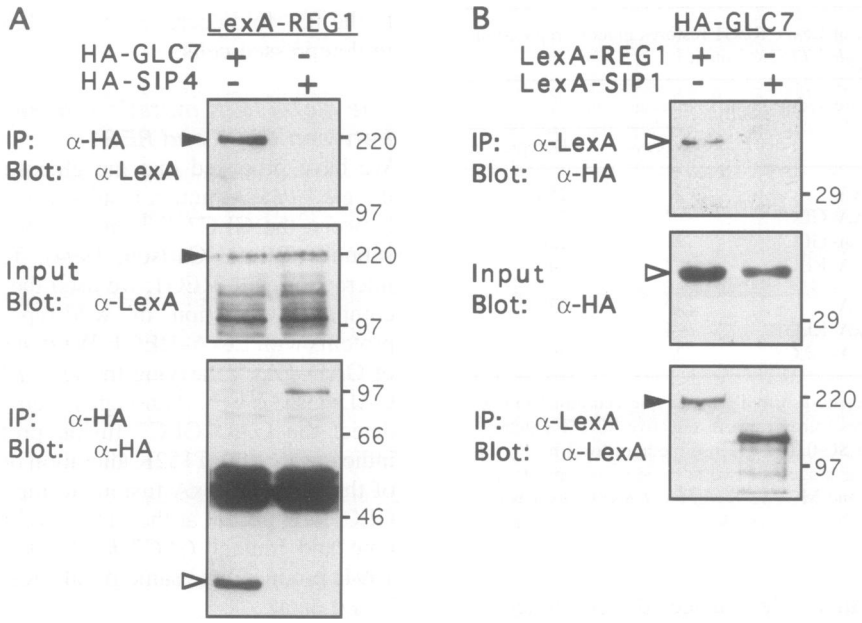

Fig. 1. Co-immunoprecipitation of LexA–REG1 and HA–GLC7. Protein extracts (250 μg) were prepared from glucose-grown wild-type strain MCY3237 expressing the indicated proteins. HA-tagged proteins (**A**) or LexA fusions (**B**) were immunoprecipitated (IP) with anti(α)-HA or anti(α)-LexA antibodies, respectively. Precipitated proteins were separated by SDS–PAGE and immunoblotted with α-HA or α-LexA, as indicated next to each panel. Upper panels show co-immunoprecipitation of LexA–REG1 (A) or HA–GLC7 (B). Lower panels show the same immunoblot reprobed with the primary antibody to confirm precipitation of either the HA-tagged protein (A) or the LexA fusion (B). Middle panels show immunoblots of the input proteins (25 μg). The smaller LexA–REG1 species are presumably degradation products. HA–SIP4 (98 kDa; gift from P.Lesage) and LexA–SIP1 (130 kDa; Yang et *al.*, 1992) were negative controls. Size standards are indicated in kDa. Positions of LexA–REG1 (closed arrows) and HA–GLC7 (open arrows) are marked.

We also assayed invertase expression in these strains under derepressing conditions (Table II). Overexpression of LexA–REG1 significantly reduced derepression of *SUC2* expression, suggesting that REG1 can function as a negative regulator of gene expression in both glucose-repressed and -derepressed cells.

Genetic analysis of reg1 and glc7-T152K

To compare the phenotypic effects of *glc7-T152K* and a *reg1* null allele, we constructed a set of strains in the same genetic background. We first introduced *reg1Δ::URA3*, a deletion of codons 52–480 (Tung *et al.*, 1992), into a wild-type diploid (see Materials and methods). Southern blot analysis confirmed the disruption. Tetrad analysis of diploids heterozygous for the *reg1* disruption showed 2+:2– segregations for growth on YEP-2% sucrose containing the glucose analog 2-DG, indicating that the disruptants are resistant to glucose repression of *SUC2*. We also disrupted the *REG1* locus in a heterozygous *+/glc7-T152K* diploid and recovered both single mutant and *glc7-T152K reg1Δ::URA3* double mutant segregants.

Mutant segregants were assayed for invertase activity (Table III). The *reg1Δ* mutation completely relieved glucose repression of *SUC2*, indicating that *REG1* is absolutely required for the glucose repression regulatory mechanism. In contrast, *glc7-T152K* caused only a partial release, consistent with the model that *glc7-T152K* reduces interaction between GLC7 and REG1, thereby partially impairing repression. The double mutant segregants showed quantitatively similar defects in glucose repression of *SUC2* expression as *reg1Δ* single mutants; no additive effect or synergy was observed (Table III). The similarity between the single and double mutant phenotypes further supports the view that *glc7-T152K* and *reg1Δ* relieve glucose repression via the same pathway.

Table III. *glc7-T152K* and *reg1Δ::URA3* relieve glucose repression

Relevant genotype	Invertase activity	
	Repressed	Derepressed
Wild-type	2	390
reg1Δ::URA3	320	410
glc7-T152K	53	360
glc7-T152K reg1Δ::URA3	280	370

Invertase activity is expressed as in Table II. Strains were grown to mid-log phase in YEP-2% glucose (repressed) and shifted to YEP-0.05% glucose (derepressed) for 2.5 h. Values are averages of assays of three to five different segregants. Standard errors were <21%.

We also found that *reg1Δ* and *glc7-T152K* cause distinct phenotypes with respect to various processes other than glucose repression. First, during the course of tetrad analysis, we noted a difference between the *reg1* and *glc7-T152K* growth phenotypes. After dissection, the *glc7-T152K* spore clones grew normally whereas *reg1* spores were slow to form visible spore clones and then subsequently showed normal growth.

Second, we assayed mutant strains for glycogen accumulation using the iodine staining method (Figure 2). The *glc7-T152K* mutant cells accumulated glycogen almost as well as wild-type, consistent with quantitative assays (Tu and Carlson, 1994) and *glc7-1* cells were completely deficient (François *et al.*, 1992; Cannon *et al.*, 1994). The *reg1Δ* mutant cells were not deficient, suggesting that *reg1Δ* does not cause any general defect in PP1 activity. Surprisingly, however, they accumulated

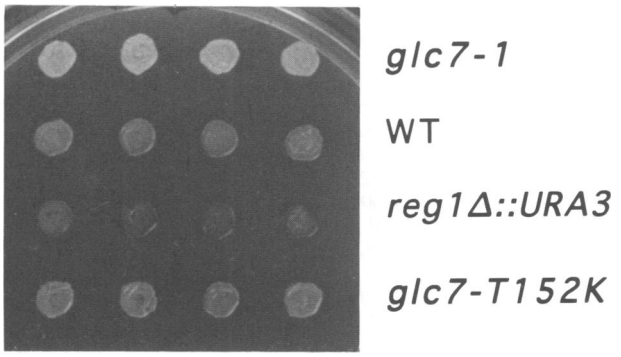

Fig. 2. Glycogen accumulation in mutants and wild-type. Yeast colonies were patched onto YEP-2% glucose plates, incubated at 30°C for 24 h and stained with iodine vapor. Strains were JC782.24D (*glc7-1*), FY250 (wild-type, WT), MCY3278 (*reg1Δ::URA3*) and MCY2616 (*glc7-T152K*).

glycogen to somewhat higher levels than wild-type. The basis for this enhanced accumulation is not clear (see Discussion).

Finally, diploids homozygous for *reg1Δ* sporulated efficiently while diploids homozygous for *glc7-T152K* were defective, as previously shown (Neigeborn and Carlson, 1987). This dramatic phenotypic difference suggests the possibility that *glc7-T152K* also affects some aspect of GLC7 function that is independent of REG1, for example, its interaction with another regulatory protein or its intrinsic enzymatic activity.

LexA–GLC7^{T152K} is partially defective for phosphatase activity in vitro

Genetic evidence argues against a drastic overall reduction in activity of the mutant GLC7^{T152K} phosphatase: *glc7-T152K* mutants show normal growth and glycogen accumulation, whereas null mutants are inviable and *glc7-1* mutants fail to accumulate glycogen. However, different cellular processes may have different thresholds of sensitivity to PP1 activity and sporulation could be particularly sensitive to impairment of PP1 activity. Therefore, we assayed protein phosphatase activity of the mutant and wild-type LexA fusion proteins. Immunoblot analysis showed that both fusions were expressed at the same levels (data not shown) and both displayed the expected functional behaviors *in vivo* (Table II). Proteins were immunoprecipitated with LexA antibody and the precipitates were assayed for protein phosphatase activity using ^{32}P-labeled phosphorylase as substrate (Figure 3A). Precipitates were also assayed after treatment with cobalt chloride plus trypsin, followed by addition of trypsin inhibitor which stimulates protein phosphatase activity (Feng *et al.*, 1991) (Figure 3B). Activity of the mutant LexA–GLC7^{T152K} was lower than that of LexA–GLC7 in these *in vitro* assays, suggesting that the unfused GLC7^{T152K} protein probably has reduced phosphatase activity relative to the wild-type protein *in vivo*.

Discussion

Here we present genetic and biochemical evidence that the REG1 protein is physically and functionally associated with the GLC7 type 1 protein phosphatase. Using the two-

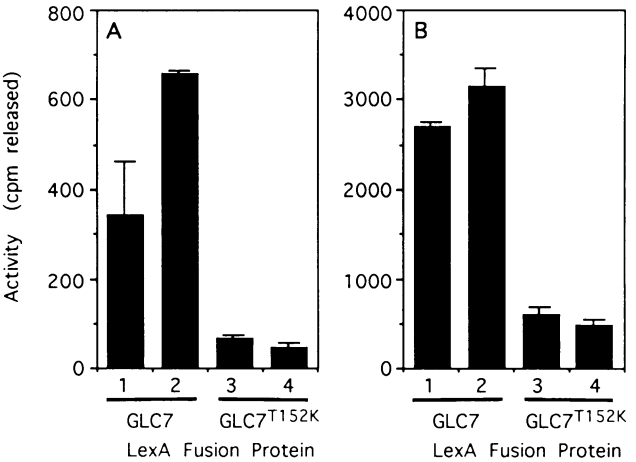

Fig. 3. Protein phosphatase activity of immunoprecipitated wild-type and mutant GLC7 fusion protein. Protein extracts (250 μg) were prepared from glucose-grown transformants of wild-type strain FY250 expressing LexA–GLC7 or LexA–GLC7^{T152K}, as indicated. Transformants also carried either pRS424 vector (bars 1 and 3) or pRJ85 (*REG1* in pRS424) (bars 2 and 4). LexA fusions were immunoprecipitated with anti-LexA antibody. (**A**) Phosphatase assays of the immunoprecipitate beads were performed using ^{32}P-phosphorylase as the substrate. (**B**) Immunoprecipitates were first treated with Co^{2+}/trypsin to stimulate phosphatase activity (see Materials and methods) and then assayed. Two transformants of each type were assayed. Immunoblot analysis of the four samples used for bars 1 and 3 showed that wild-type and mutant fusions were expressed at the same levels. In addition, transformants expressing LexA–GLC7 and GAD–GAC1 were assayed and values were similar to those for transformants carrying the multicopy REG1 plasmid. Bars represent phosphatase activity as ^{32}P c.p.m. released in the assay. Standard deviations are indicated.

hybrid system we showed that LexA–REG1 and GAD–GLC7 interact *in vivo*. We then demonstrated that LexA–REG1 and HA–GLC7 fusion proteins co-immunoprecipitate from cell extracts. Finally, genetic analysis indicates that REG1 and GLC7 function together to affect glucose repression. Mutations in both genes relieve glucose repression but the double mutant combination causes no synergy or additive effect. Morever, overexpression of a REG1 fusion protein effectively suppresses the glucose repression defect caused by *glc7-T152K*. These findings support the model that REG1 is a regulatory subunit of GLC7 that directs PP1 activity to targets in the glucose repression pathway.

Mutation of *REG1* relieves glucose repression of most genes that have been tested, including *SUC2*, *MAL3*, *GAL1*, *ADH2*, *HXT2*, *HXT4* and mitochondrial genes (Entian and Zimmermann, 1980; Matsumato *et al.*, 1983; Neigeborn and Carlson, 1987; Dombek *et al.*, 1993; Ulery *et al.*, 1994; Ozcan and Johnston, 1995); genes encoding gluconeogenic enzymes are exceptions (Entian and Zimmermann, 1980). This major loss of glucose repression can be explained by the absence of the regulatory subunit that mediates the effects of the GLC7 phosphatase on glucose repression. The *glc7-T152K* mutation causes more limited defects; for example, *glc7-T152K* does not relieve glucose repression of the *GAL1* promoter (Neigeborn and Carlson, 1987) or *ADH2* (Dombek *et al.*, 1993) and only partially relieves repression of *SUC2*. We suggest that *glc7-T152K* causes only a partial functional defect and that the requirement for GLC7–REG1 function for glucose repression is more stringent for some genes than for

others. Thus, *glc7-T152K* causes a phenotypic effect only for a subset of genes for which repression is more sensitive to GLC7–REG1 function.

Genetic evidence, from the two-hybrid system and overexpression studies, is consistent with the idea that the partial glucose repression defect in a *glc7-T152K* mutant results from decreased interaction between REG1 and $GLC7^{T152K}$. It is possible that Thr152 participates in specific protein–protein contact with REG1 which is disrupted by mutation to Lys. However, this mutation also has other effects on GLC7 function, as judged by the sporulation defect of *glc7-T152K* mutants, which is not shared by *reg1* mutants, and by the decreased phosphatase activity of the LexA–$GLC7^{T152K}$ protein *in vitro*. Thus, we cannot exclude the possibility that the presence of Lys at position 152 alters the conformation of GLC7 and thereby indirectly impairs interaction with REG1. Interestingly, the *glc7-1* mutation has analogous properties in that it impairs interaction with the GAC1 regulatory subunit and causes a major defect in glycogen metabolism but also has associated pleiotropic phenotypes (Feng *et al.*, 1991; Wek *et al.*, 1992; Cannon *et al.*, 1994; Francisco *et al.*, 1994).

Two key questions remain open regarding the role of GLC7–REG1 in glucose repression. First, what are the targets of phosphatase activity in the glucose repression mechanism? Previous genetic evidence suggests that GLC7 functions antagonistically to the SNF1 protein kinase and phosphorylation of SIP1 was altered in immune complex assays of SNF1 kinase activity from *glc7-T152K* mutants (Tu and Carlson, 1994). In addition, genetic evidence (Erickson and Johnston, 1993) suggested interaction between REG1 and GAL83, another protein associated with SNF1 kinase complexes (Yang *et al.*, 1994). Thus, regulators of SNF1, substrates of SNF1 or the SNF1 kinase itself are potential candidates for targets of GLC7–REG1 activity.

The second question is whether the function of REG1 or the GLC7–REG1 complex, is regulated in response to glucose availability. It has been shown that expression of the *REG1* gene is not glucose-regulated (Niederacher and Entian, 1991). Interaction between REG1 and PP1 fusion proteins in the two-hybrid system appeared to be regulated a few-fold but not dramatically; however, these fusion proteins are overexpressed and it remains possible that the interaction of the native proteins is regulated to a more significant extent. Other possible mechanisms include: regulation of the ability of REG1 to recognize substrates, differential localization of REG1 or GLC7–REG1 within the cell or control of phosphatase activity by an inhibitory subunit. Finally, it is conceivable that GLC7–REG1 activity is not regulated in response to glucose availability but its function is regulated by differential availability of phosphorylated substrate proteins.

In addition to their common effects on glucose repression, the *glc7-T152K* and *reg1Δ* mutations cause distinct phenotypes. The sporulation defect of *glc7-T152K* mutants may result from decreased enzymatic activity of $GLC7^{T152K}$ or from loss of interaction with another regulatory subunit that directs PP1 function in sporulation. The enhanced glycogen accumulation observed in *reg1* mutants could be accounted for by at least three possible mechanisms. First, REG1 and GAC1 may compete with each other and perhaps with other regulatory subunits for association with GLC7; when REG1 is absent, a greater proportion of the cellular pool of PP1 may be associated with GAC1. A second possibility is that mutation of *REG1* causes various physiological changes that indirectly result in enhanced glycogen accumulation. For example, the glucose repression defect of *reg1* mutants may cause glycogen accumulation to commence at an earlier stage of the growth curve, as seen for *glc7-T152K* mutant cultures (Tu and Carlson, 1994). Finally, enhanced accumulation could also reflect effects of *reg1* on *GAC1* expression.

REG1 has also been shown to affect processes that have not been examined in *glc7-T152K* mutants. For example, *reg1* impairs glucose induction of *HXT1* (glucose transporter) gene expression (Ozcan and Johnston, 1995). In addition, *reg1Δ* suppresses mutations that affect pre-RNA processing and transport (Tung *et al.*, 1992); in this case, it seems likely that GLC7 is also involved because mammalian PP1 has been implicated in mRNA processing (Mermoud *et al.*, 1994). Tung *et al.* (1992) have suggested that REG1 may serve to connect RNA processing and transport to carbon source regulation. Alternatively, REG1 may target PP1 activity to substrates involved in processes besides glucose repression.

Table IV. List of *Saccharomyces cerevisiae* strains

Strain[a]	Genotype
MCY835	*MATα glc7-T152K lys2-801 ura3-52*
MCY1093	*MATa his4-539 lys2-801 ura3-52*
MCY2616	*MATa glc7-T152K his3Δ200 lys2-801 trp1Δ1 ura3-52*
MCY2649	*MATα his3Δ200 leu2-3,112 trp1Δ1 ura3-52*
MCY2921	*MATa ade2-101 his3Δ200 trp1Δ1 ura3-52*
MCY3263	*MATα glc7-T152K reg1Δ::URA3 ade2-101 trp1Δ1 ura3-52*
MCY3268	*MATa reg1Δ::URA3 ade2-101 lys2-801 trp1Δ1 ura3-52*
MCY3278	*MATα reg1Δ::URA3 his3Δ200 leu2Δ1 trp1Δ63 ura3-52*
JC782.24D[b]	*MATa glc7-1 lys2 leu2 met4 ura3*
FY250[c]	*MATα reg1Δ::URA3 his3Δ200 leu2Δ1 trp1Δ63 ura3-52*
CTY10-5d[d]	*MATa gal4 gal80 URA3::lexAop-lacZ ade2-101 his3Δ200 leu2Δ1 trp1Δ901*

[a]MCY strains have S288C background and carry *SUC2*.
[b]Obtained from J.Cannon.
[c]Obtained from F.Winston; S288C genetic background.
[d]Constructed by R.Sternglanz (SUNY, Stony Brook).

Materials and methods

Strains and genetic methods

Saccharomyces cerevisiae strains are listed in Table IV. Standard methods for yeast genetic analysis (Rose *et al.*, 1990) and transformation (Ito *et al.*, 1983) were followed. 2-Deoxyglucose (2-DG) resistance was tested on YEP (1% yeast extract, 2% bacto-peptone) containing 2% sucrose and 200 μg/ml 2-DG (Sigma) under anaerobic conditions with Gas Paks (BBL). Control plates lacked 2-DG. Scoring was performed by spotting cell suspensions onto solid medium. Selective synthetic complete medium (SC) was used to grow strains carrying plasmids. To assess sporulation proficiency, diploid cells were grown on YEP-2% glucose plates overnight, patched on sporulation medium (Rose et *al.*, 1990), incubated at room temperature for 8 days and then examined microscopically for the presence of asci. *Escherichia coli* strain XL1-Blue was used as host for plasmids.

Construction of plasmids

To construct *GLC7* fusions, we designed two primers to amplify the entire *GLC7* coding region (codons 1–312) and to introduce *Bam*HI

sites 5′ and 3′ to the gene. The 5′ primer was 5′-CGGGATCCAA-GAAATGGACTCA-3′ and the 3′ primer was 5′-CGGGATCCTTTTTT-CTTTCTACCCCC-3′. Using the PCR, we amplified the *GLC7* sequence from pJT18 (Tu and Carlson, 1994). The *Bam*HI fragment from the amplified DNA was subcloned into the *Bam*HI site of pGADNOT (Luban *et al.*, 1993) to yield pGAD-GLC7. pGAD-GLC7^{T152K} was constructed by replacing the *Sal*I (one polylinker site) fragment of pGAD-GLC7 with the *Sal*I–*Xho*I fragment from pJTL18T152K (Tu and Carlson, 1994).

pRJ85, containing the *REG1* gene in pRS424 (Sikorski and Hieter, 1989), and pLexA-REG1 (pRJ65), containing the entire *REG1* coding region fused with LexA, were gifts of R.Jiang (this laboratory). To construct pLexA-REG1, sequence extending from codon 1 of *REG1* into the vector sequence 3′ to the cloned gene was amplified by PCR from pUCSRN1 (Tung *et al.*, 1992). An *Eco*RI site was introduced 5′ to the coding sequence in this reaction. The *Eco*RI–*Sal*I (pUC vector site) fragment from the amplified DNA was cloned into the cognate sites of pLexA(1–202)+PL (Ruden *et al.*, 1991). Next, the *Cla*I–*Sal*I fragment (*Cla*I site at codon 52) was replaced with the corresponding fragment from pUCSRN1, yielding pLexA-REG1. pHA-GLC7 (CB597), a YCp50 derivative expressing HA-tagged GLC7, was a gift of K.T.Arndt (Sutton *et al.*, 1991). pHA-SIP4 was a gift from P.Lesage (this laboratory). To disrupt the *REG1* gene, the *Eco*RI–*Xba*I fragment purified from pUCsrn1::URA3 (Tung et *al.*, 1992) was used to transform (Rothstein, 1983) the wild-type haploid strain FY250, wild-type diploid strain MCY1093 × MCY2649 and *+/glc7-T152K* heterozygous diploid strain MCY835 × MCY2921. The resulting allele was designated *reg1Δ::URA3*.

pGAD-GAC1 was isolated from a plasmid library of fusions between the GAL4 activation domain (GAD) and yeast genomic DNA fragments (Chien *et al.*, 1991) in a two-hybrid screen for GLC7-interacting proteins (unpublished data). Sequence analysis indicated that GAC1 is fused with GAD at residue 53.

β-Galactosidase and invertase assays

To test β-galactosidase expression, four transformants were patched on selective SC-2% glucose, grown for 1 day, replicated onto nitrocellulose filters, permeabilized (–70°C for 10 min) and incubated with X-Gal overnight (Breeden and Nasmyth, 1985). For quantitative assays, glucose-repressed cultures were grown to mid-log phase in YEP or selective SC medium containing 2% or 4% glucose; derepressed cultures were either grown in 4% raffinose plus 0.05% glucose or prepared by shifting to YEP or SC containing 0.05% glucose for 2.5 or 3 h, respectively. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (Miller, 1972). Invertase activity was assayed in whole cells (Tu *et al.*, 1993).

Immunoprecipitation assays

Protein extract and immunoprecipitation procedures were as described previously (Yang et *al.*, 1992), except the extraction buffer was 50 mM HEPES [4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid] pH 7.5, 100 mM NaCl, 1% Triton X-100, containing 2 mM phenylmethylsulfonyl fluoride and 1 mg/ml each of pepstatin, leupeptin and aprotinin and immobilized rProtein A (RepliGen) was used. Proteins were separated by SDS–PAGE and immunoblotted. Primary antibodies were anti-LexA (gift of C.Denis) and monoclonal HA antibody (12CA5, BabCo). Antibodies were detected by enhanced chemiluminescence with ECL reagents (Amersham International).

Protein phosphatase assay

Phosphatase activity in the immunoprecipitation was assayed by measuring the release of ^{32}P from labeled phosphorylase *a* as described by Feng *et al.* (1991) with the following modifications. The immunoprecipitates from 250 μg of protein extract were washed and resuspended with buffer (50 mM imidazole–HCl, pH 7.4, 5 mM EGTA, 2 mM dithiothreitol and 0.2 mg/ml BSA). To measure Co^{2+}/trypsin-stimulated phosphatase activity, immunoprecipitates were pre-incubated with 0.2 mM $CoCl_2$ for 5 min at 30°C and incubated with trypsin (0.02 mg/ml) for 10 min; soybean trypsin inhibitor was then added to 0.08 mg/ml (Feng et *al.*, 1991). Phosphatase reactions (25 μl) contained immunoprecipitate beads from 60 μg of protein extract (with or without Co^{2+}/trypsin treatment) in 40 mM imidazole–HCl, pH 7.4, 4 mM theophylline, 1.6 mM dithiothreitol, 2 mM EGTA, 0.08 mg/ml BSA. Phosphatase reactions were initiated by adding [^{32}P] phosphorylase (~5 μg, 50 000 c.p.m.). After incubation at 30°C for 10 min, the reaction was terminated by addition of 35 μl of 20% trichloroacetic acid and the ^{32}P released into the supernatant was measured.

Glycogen assay

Yeast colonies were patched onto YEP-2% glucose plates, incubated at 30°C overnight and stained with iodine vapor (Chester, 1968).

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