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The *REG2* Gene of *Saccharomyces cerevisiae* Encodes a Type 1 Protein Phosphatase-Binding Protein That Functions with Reg1p and the Snf1 Protein Kinase to Regulate Growth

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The *GLC7* gene of *Saccharomyces cerevisiae* encodes the catalytic subunit of type 1 protein phosphatase (PP1) and is essential for cell growth. We have isolated a previously uncharacterized gene, *REG2*, on the basis of its ability to interact with Glc7p in the two-hybrid system. Reg2p interacts with Glc7p in vivo, and epitope-tagged derivatives of Reg2p and Glc7p coimmunoprecipitate from cell extracts. The predicted protein product of the *REG2* gene is similar to Reg1p, a protein believed to direct PP1 activity in the glucose repression pathway. Mutants with a deletion of *reg1* display a mild slow-growth defect, while *reg2* mutants exhibit a wild-type phenotype. However, mutants with deletions of both *reg1* and *reg2* exhibit a severe growth defect. Overexpression of *REG2* complements the slow-growth defect of a *reg1* mutant but does not complement defects in glycogen accumulation or glucose repression, two traits also associated with a *reg1* deletion. These results indicate that *REG1* has a unique role in the glucose repression pathway but acts together with *REG2* to regulate some as yet uncharacterized function important for growth. The growth defect of a *reg1 reg2* double mutant is alleviated by a loss-of-function mutation in the *SNF1*-encoded protein kinase. The *snf1* mutation also suppresses the glucose repression defects of *reg1*. Together, our data are consistent with a model in which Reg1p and Reg2p control the activity of PP1 toward substrates that are phosphorylated by the Snf1p kinase.

The reversible phosphorylation of proteins has long been recognized as a widespread mechanism of posttranslational regulation among eukaryotes. The phosphorylation state of a given protein is dependent on the relative activities of protein kinases and protein phosphatases. Early biochemical studies suggested that protein phosphatases might represent a much smaller group of enzymes than protein kinases. Whereas most kinases recognize specific motifs of five or six amino acids (46), phosphatases generally exhibit a fairly broad substrate specificity (17, 64). These data contributed to the idea that cellular signaling responses were largely determined by the activities of specific protein kinases whereas phosphatases functioned at a low constitutive level (64). Recent advances have underscored the importance of protein phosphatases in controlling physiological processes and have demonstrated that protein phosphatases are in fact highly regulated.

The serine/threonine protein phosphatases are among the most conserved proteins throughout evolution. The type 1 protein phosphatase (PP1) is >80% identical in mammals and in yeasts (24, 55) and has been demonstrated to play key roles in a variety of cellular processes. In mammalian cells, PP1 regulates glycogen metabolism, muscle contractility, and protein synthesis (4, 17, 64) and has been shown to interact with the product of the retinoblastoma tumor suppressor gene (20). Studies of *S. cerevisiae* have likewise demonstrated multiple physiological roles for PP1, including glycogen metabolism (10, 24), sporulation (10), cell cycle progression (36, 84), chromosome segregation (27), protein synthesis (80), and glucose repression (74). The obvious question is, how does a single enzyme regulate so many diverse processes in so many different locations within the cell?

Several years ago, Cohen and colleagues introduced the idea of regulatory or targeting subunits in response to this question (18, 43). Their hypothesis proposed that both the subcellular location and the catalytic activity of PP1 were governed by a variety of regulatory subunits that directed the phosphatase to particular locations and/or altered its activity toward particular substrates. Several examples of PP1 regulatory subunits have now been described, and the list is likely to grow. PP1 isolated from mammalian skeletal muscle is found in association with a glycogen-targeting subunit that when bound to the catalytic subunit increases the activity of PP1 toward glycogen-associated substrates (41, 42). The smooth muscle form of PP1 is a heterotrimer composed of the catalytic subunit and a regulatory complex that enhances the rate at which the smooth muscle form of PP1 dephosphorylates smooth muscle myosin (1, 19).

Studies of *S. cerevisiae* by combined molecular and genetic approaches have led to the identification of new PP1 regulatory subunits. The *GAC1* gene is required for normal glycogen accumulation in *S. cerevisiae*. Gac1p is most similar to the mammalian glycogen-targeting subunit from skeletal muscle (28, 69), and it physically interacts with PP1 (67). A gene in the fission yeast *Schizosaccharomyces pombe* (*sds22*$^+$) and an *S. cerevisiae* homolog (*SDS22*/*EGP1*), encode essential PP1-binding proteins believed to be required for the metaphase-to-anaphase transition during mitosis (35, 48, 56, 66).

Most recently, the *REG1* gene product of *S. cerevisiae* has been demonstrated to bind PP1 and is proposed to direct the activity of PP1 in glucose repression (75). Glucose repression is the phenomenon in yeast cells whereby a large number of genes required for metabolism of alternate carbon sources are repressed in the presence of glucose. A previous study has reported that PP1, encoded by *GLC7*, is required for mainte-

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TABLE 1. *S. cerevisiae* strains used

Yeast strain[a]	Genotype	Source or reference
Y190	*MAT***a** *gal4 gal80 his3 trp1-90 ade2-101 ura3-52 leu2-3,112 met⁻ URA3*::*GAL*→*lacZ LYS2*::*GAL*(UAS[b])→*HIS3 cyh*[r]	S. J. Elledge
Y187	*MAT***a** *gal4 gal80 his3 trp-901 ade-101 ura3-52 leu2-3,112 met⁻ URA3*::*GAL*→*lacZ*	S. J. Elledge
KT1113	*MAT*α *leu2 ura3-52 his3*	K. Tatchell
KT1357	*MAT***a** *leu2 ura3-52 his3 trp1*	K. Tatchell
DF103	*MAT***a** *leu2 his3 ura3-52*	This study
DF104	*MAT***a**/α *leu2/leu2 his3/his3 ura3-52/ura3-52*	This study
DF118	*MAT*α *leu2 his3 ura3-52 reg2*::*URA3*	This study
DF119	*MAT***a** *leu2 his3 ura3-52 reg2*::*URA3*	This study
DF148	*MAT***a** *leu2 ura3-52 trp1 glc7*::*LEU2* [YCp-*mycGLC7*]	This study
DF173	*MAT***a** *leu2 his3 ura3-52 reg1*::*URA3*	This study
DF174	*MAT*α *leu2 his3 ura3-52 reg1*::*URA3*	This study
DF183	*MAT*α *leu2 his3 ura3-52 trp1 reg2*::*URA3*	This study
DF184	*MAT***a** *leu2 his3 ura3-52 trp1 reg2*::*URA3*	This study
DF185	*MAT*α *leu2 his3 ura3-52 trp1 reg1*::*URA3*	This study
DF186	*MAT***a** *leu2 his3 ura3-52 trp1 reg1*::*URA3*	This study
DF187	*MAT*α *leu2 his3 ura3-52 reg1*::*URA3 reg2*::*URA3*	This study
DF188	*MAT***a** *leu2 his3 ura3-52 reg1*::*URA3 reg2*::*URA3*	This study
DF189	*MAT***a** *leu2 his3 ura3-52 trp1 reg1*::*URA3 reg2*::*URA3*	This study
DF190	*MAT*α *leu2 his3 ura3-52 trp1 reg1*::*URA3 reg2*::*URA3*	This study
DF199	*MAT***a** *leu2 his3 ura3-52 rna1-2 rnh1*::*URA3*	This study
DF200	*MAT*α *leu2 his3 ura3-52 rna1-1 rnh1*::*URA3*	This study
DF201	*MAT***a** *leu2 his3 ura3-52 RNA1 rnh1*::*URA3*	This study
DF202	*MAT*α *leu2 his3 ura3-52 RNA1 rnh1*::*URA3*	This study
DF203	*MAT***a** *leu2 his3 ura3-52 reg1*::*LEU2*	This study
DF204	*MAT*α *leu2 his3 ura3-52 reg1*::*LEU2*	This study
DF205	*MAT***a** *leu2 his3 ura3-52 reg1*::*URA3 rna1-1 rnh1*::*URA3*	This study
DF206	*MAT*α *leu2 his3 ura3-52 reg1*::*URA3 rna1-1 rnh1*::*URA3*	This study
DF207	*MAT*α *leu2 his3 ura3-52 trp1 glc7*::*HIS3 reg1*::*URA3* [YCp-*mycGLC7*]	This study
ST172-15	*MAT***a** *leu2 ura3-52 his4 snf1-172*	70
EG649-14D	*MAT*α *leu2 ura3-52 reg2*::*URA3 snf1-172*	This study
LRA86	*MAT*α *leu2 ura3-52 his4 cdc35-13*	L. C. Robinson
EG622-2B	*MAT*α *leu2 ura3-52 bcy1-16*	This study
EG629-11A	*MAT*α *leu2 ura3 reg1*::*URA3 bcy1-16*	This study

[a] All strains excluding Y190 and Y187 are congenic to JC482 (11).
[b] UAS, upstream activation sequence.

nance of glucose repression (74). *reg1* mutants (21, 51, 53) as well as several mutant alleles of *glc7* (3, 74) are glucose derepressed. At least one of these PP1 glucose-derepressed alleles, *glc7-T152K*, is defective in its ability to interact with Reg1p while retaining the ability to bind the glycogen-targeting subunit (75).

In this report, we describe the identification of a gene encoding a fourth PP1 regulatory subunit in *S. cerevisiae*. This gene, termed *REG2*, encodes a product with homology to Reg1p. A cDNA encoding *REG2* was isolated on the basis of its interaction with PP1 in the yeast two-hybrid system. Biochemical evidence that Reg2p and PP1 interact physically is presented. Genetic data suggest that while some phenotypes associated with *reg1* are not exhibited by *reg2*, these two gene products may nonetheless perform redundant functions important for cell growth. Finally, we present genetic evidence that Reg1p, Reg2p, and the Snf1 protein kinase function together in the same signaling pathway to regulate cell growth.

MATERIALS AND METHODS

Strains, media, and growth conditions. The genotypes of the *S. cerevisiae* strains used in this work are listed in Table 1. New alleles were introduced into the JC482 background by yeast transformation or by performing a minimum of six serial backcrosses. Yeast transformation was performed by the lithium acetate method of Gietz et al. (29). Standard yeast genetic procedures for diploid construction, tetrad analysis, and medium preparation were as described by Rose et al. (61). *Escherichia coli* DH5αF′ was used for propagation of plasmids. Yeast strains were grown in rich YPD medium (1% yeast extract, 2% peptone, 2% glucose), or synthetic medium (0.67% yeast nitrogen base, 2% glucose) supplemented with amino acids. Diploid strains were induced to sporulate on medium containing 1% yeast extract, 2% peptone, and 2% acetate. For assays of galactose-inducible gene expression, strains were grown in minimal media containing 2% galactose and the appropriate amino acid supplements. 2-Deoxyglucose (2-DG) resistance was tested on synthetic media containing 2% sucrose and 200 μg of 2-DG (Sigma) per ml under anaerobic conditions with GasPaks (Difco Laboratories). Control plates lacked 2-DG.

Two-hybrid screen. Strains, plasmids, and cDNA library were a generous gift of Stephen Elledge. Strain Y190 was transformed with pAS-*GLC7* containing the *GLC7* gene fused to the Gal4p DNA binding domain in the pAS1-*CYH2* vector. The resulting Trp$^+$ prototrophic strain was transformed with 50 μg of a cDNA library fused to the Gal4p activation domain in plasmid pACT. Transformants were selected by plating cells on synthetic medium lacking tryptophan, leucine, and histidine and containing 25 mM 3-aminotriazole. Following growth at 30°C for 5 days, transformants were replica plated to synthetic medium containing 0.4% adenine and lacking tryptophan, leucine, and histidine. Colonies were screened for β-galactosidase activity by 5-bromo-4-chloro-3-indolylphosphate-β-D-galacto pyranoside (X-Gal; Diagnostic Chemicals Limited) filter lift assays (6). Of approximately 5×10^5 total transformants screened (as calculated from representative plates for which selection was not applied), 5 displayed various degrees of β-galactosidase activity. These five blue colonies were streaked onto selective media and retested for β-galactosidase activity with X-Gal. Of these, one turned a very dark blue, two turned a very light blue, and two showed no blue color at all. The three remaining positive colonies were streaked onto medium lacking uracil and containing 2.5 μg of cycloheximide per ml to select for loss of pAS1-*GLC7*. The resulting Trp$^-$ Leu$^+$ strains were then mated to strain Y187 containing pAS1-*X*, where *X* represents a variety of nonspecific fusions designed to serve as negative controls. Diploids were selected by growth on medium lacking tryptophan and leucine and then screened for β-galactosidase activity. Of the three pACT-cDNA clones tested, one showed strong induction of β-galactosidase when mated to Y187 harboring pAS1-*GLC7*. Induction of β-galactosidase was contingent on the presence of pAS1-*GLC7*, as the same strain showed

no color development when mated to Y187 harboring pAS1-*SNF1*, pAS1-Lamin, pAS1-p53, or pAS1-*CDK2*. Strains containing pACT-*GAC1* and pAS1-*GLC7* or pACT-*SNF4* and pAS1-*SNF1* served as positive controls for measuring β-galactosidase activity. Plasmids containing potential interacting cDNAs were rescued from strain Y190 and transformed into *E. coli* DH5αF′. Restriction enzyme analysis revealed two different plasmid species among 10 *E. coli* transformants analyzed. Following transformation into strain Y190 carrying pAS1-*GLC7*, clone 21-4 showed strong β-galactosidase activity. This plasmid was chosen for further analysis.

DNA sequencing of *REG2*. Restriction fragments from both the original *REG2* cDNA isolated in the two-hybrid screen (clone 21-4) and a genomic clone encoding *REG2* were subcloned into pBluescript SK (Stratagene Cloning Systems). Both the template and complementary strands of the *REG2* open reading frame were sequenced by using a double-stranded template and Sequenase enzyme and reagents (Sequenase version 2.0; United States Biochemical Corp.). Sequencing reactions used the M13 −20 and reverse primers. Custom-made oligonucleotides were used to fill internal gaps in the sequence. Synthesis of custom primers and some sequencing reactions were carried out by the Iowa State University DNA Sequencing and Synthesis Facility. A primer homologous to the Gal4p transcriptional activation domain in pACT was used to sequence through the *GAL4-REG2* cDNA gene fusion, confirming that the *REG2* coding sequence was in the correct reading frame.

Generation of *reg2*::*URA3*, *reg1*::*LEU2*, and *rna1-1* alleles. A *Hin*dIII-*Apa*I fragment containing the *REG2* coding sequence was excised from genomic clone QJ2, a gift of Jonathan Warner (45). This fragment was subcloned into the *Hin*dIII-*Apa*I sites of pBluescript SK to generate plasmid pBS-*REG2*. A blunt end was created by digestion of pBS-*REG2* with *Acc*I followed by treatment with the Klenow fragment of DNA polymerase I. Plasmid YEp24 (Stratagene), encoding the *URA3* selectable marker, was similarly blunt ended by digestion with *Bsp*D1 and subsequent treatment with Klenow enzyme. Both blunt-ended plasmids were then digested with *Nsi*I. The 1.5-kb *Nsi*I-*Bsp*DI *URA3* fragment was ligated into the *Nsi*I-*Acc*I sites of pBS-*REG2* to generate pBS-*reg2*::*URA3*, effectively replacing the entire *REG2* coding sequence with *URA3*. This construct was used to disrupt one of the wild-type alleles of *REG2* in the diploid strain DF104. One-step gene disruption (62) was carried out by using the linear *Bsp*HI-*Kpn*I fragment for transformation with selection on medium lacking uracil. Transformants heterozygous for the *reg2*::*URA3* disruption were verified by genomic Southern hybridization (data not shown).

To generate a *reg1*::*LEU2* null allele, plasmid pBM1966 (a gift from Mark Johnston) was digested with *Pst*I and then transformed into the diploid strain DF104. pBM1966 carries a *LEU2* disruption of the *reg1* coding sequence. Tetrad dissection of meiotic progeny from Leu^+ transformants yielded the expected 2:2 segregation of the *reg1* slow-growth phenotype. In subsequent studies, the *reg1*::*LEU2* allele behaved identically to a *reg1*::*URA3* disruption strain (a generous gift from Marian Carlson).

Isogenic *rna1-1* strains were generated by transforming strain DF104 with *Sst*I-linearized plasmid pRU6 (*rna1-1* allele) or pRU35 (*RNA1* allele). These plasmids were a gift of Anita Hopper and have been described previously (72).

Plasmid construction. The *Hin*dIII-*Xba*I fragment containing the *REG2* open reading frame was subcloned into the *Hin*dIII-*Xba*I sites of YEp351 (33) to generate YEp-*REG2*.

PCR amplification was used to place *REG2* under control of the *GAL1* promoter. Oligonucleotide primers were used to introduce *Bam*HI sites (underlined) at the 5′ (5′ AAGGATCCATGTGTGAGAATGACTTTGAGT 3′) and 3′ (5′ AATGGATCCT TATAGGAGCAGGTTGGGTAT3′) ends of the *REG2* open reading frame, using standard PCR conditions and *Pfu* polymerase (Stratagene). The *Bam*HI-*REG2* fragment was ligated into the *Bam*HI site of YCpIF15 (26) to produce YCp-*Ha-REG2*. In addition to being placed under *GAL1* control, protein coding sequences cloned into YCpIF15 are fused to the hemagglutinin (Ha) epitope which is recognized by monoclonal antibody 12CA5.

The *GLC7* coding sequence was inserted into the *Bam*HI site of pAS1-*CYH2* to create an in-frame fusion protein with the Gal4p DNA binding domain. This was accomplished by PCR amplifying *GLC7* with primers designed to introduce *Bam*HI sites (underlined) at the 5′ (5′-CGGGATCCGAATGGACTCAC-3′) and 3′ (5′-CGGGATCCCTAGGACGTG AATC-3′) ends of the gene. The *GAC1* coding sequence was similarly introduced into the *Bam*HI site of pACTII, generating an in-frame fusion with the Gal4p transcriptional activation domain. *GAC1* was amplified by using PCR primers designed to introduce *Bam*HI sites (underlined) at the 5′ (5′-CCGGGATCCTGGCCATGGTAATACAAACTGC TACT-3′) and 3′ ends (5′-CCGGGATCCCTCAAAGTCGCCATCGATC-3′) of the gene. PCR amplification was performed using *Pfu* DNA polymerase (Stratagene).

Immunoprecipitation. Procedures were as described by Stuart et al. (67) except that cellular extracts were prepared from early-log-phase cultures in breaking buffer containing 0.5% Triton X-100 and the 1,500 × *g* spin following cell breakage was omitted. Immunoprecipitation, electrophoresis, and immunoblotting were performed as described previously (67).

Invertase assays. Strains were grown to mid-log phase in selective synthetic medium containing 5% glucose. For measurements under derepressing conditions, cells were shifted to selective synthetic medium containing 0.05% glucose for 3 h. Invertase activity was assayed in whole cells as described previously (76).

Analytical procedures. Protein concentrations were determined by the procedure of Bradford (5), with bovine serum albumin as a standard. Qualitative glycogen assays were performed by inverting colonies growing on petri dishes over iodine vapor as previously described (15).

Nucleotide sequence accession number. The *REG2* nucleotide sequence is available through the EMBL database under accession number Z35919.

RESULTS

The *REG2* gene encodes a PP1-binding protein related to Reg1p. To identify new putative regulatory subunits of PP1 in *S. cerevisiae*, we used the yeast two-hybrid system devised by Fields and Song (25). A cDNA library fused to sequences encoding the Gal4p activation domain (amino acids 768 to 881) was coexpressed with a plasmid (pAS1-*GLC7*) containing the *GLC7* gene encoding PP1 fused to the Gal4p DNA binding domain (amino acids 1 to 147). Positive interacting clones were identified by the ability to induce transcription of the *GAL*-regulated *lacZ* reporter gene. Clone 21-4, which we have since renamed pACT-*REG2*, repeatedly showed high β-galactosidase activity. Induction of β-galactosidase was dependent on the presence of pAS1-*GLC7*, as no color development was observed in the presence of plasmids containing nonspecific gene fusions. The *GAC1*-encoded glycogen regulatory subunit interacts strongly with wild-type *GLC7* but is defective in its interaction with the glycogen-deficient allele, *glc7-1* (67). In contrast, pACT-*REG2* interacted strongly with both pAS1-*GLC7* and pAS1-*glc7-1*. Partial DNA sequence analysis followed by a BLAST search revealed that the cDNA insert in clone 21-4 was identical to a previously uncharacterized open reading frame located 366 nucleotides upstream of the *REB1* locus on chromosome II. The complete gene sequence was obtained from a genomic clone (a gift of Jonathan Warner) corresponding to a 5.8-kb region of chromosome II containing *REB1* (45). This gene, *REG2*, consists of an uninterrupted open reading frame 1,015 nucleotides in length encoding a protein product of 338 amino acids (Fig. 1). The original cDNA isolated in the two-hybrid screen was missing only the first 16 nucleotides of the *REG2* coding sequence. During the course of this work, our sequence data and location of *REG2* were confirmed in the published sequence of chromosome II (23) available in the databases.

A BLAST search revealed that the *REG2* coding sequence was most similar to that of the *REG1* gene from *S. cerevisiae*. *REG1* encodes a 1,014-amino-acid product reported to be a negative regulator of glucose-repressible genes (21, 51). In addition to its role in glucose repression, *REG1* was isolated as an extragenic suppressor of *rna1* mutants which are defective in both RNA processing and transport of RNA from the nucleus to the cytoplasm (57, 79). The predicted protein products of *REG1* and *REG2* are 29% identical and 48% similar. The similarity extends throughout the length of Reg2p but is limited to the central half of Reg1p (residues 217 to 763) (Fig. 1). Alignment by using the Bestfit program (Genetics Computer Group, Madison, Wis.) introduced 13 gaps into the Reg1p amino acid sequence (Fig. 1A) but only 3 small gaps in the Reg2p sequence. Despite the relatively low level of similarity, we were encouraged that Reg2p was in fact a regulator of PP1 activity by a recent report demonstrating that Reg1p also associates with PP1 and may direct its role in the glucose repression pathway (75).

Coimmunoprecipitation of Reg2p and PP1. To substantiate our hypothesis that *REG2* encodes a PP1-binding protein, we tested the association of Reg2p and PP1 in vitro with an immunoprecipitation assay. The *REG2* coding sequence was amplified by PCR and subcloned into the *Bam*HI site of plasmid YCpIF15 (Fig. 2). Insertion of a protein-coding sequence into

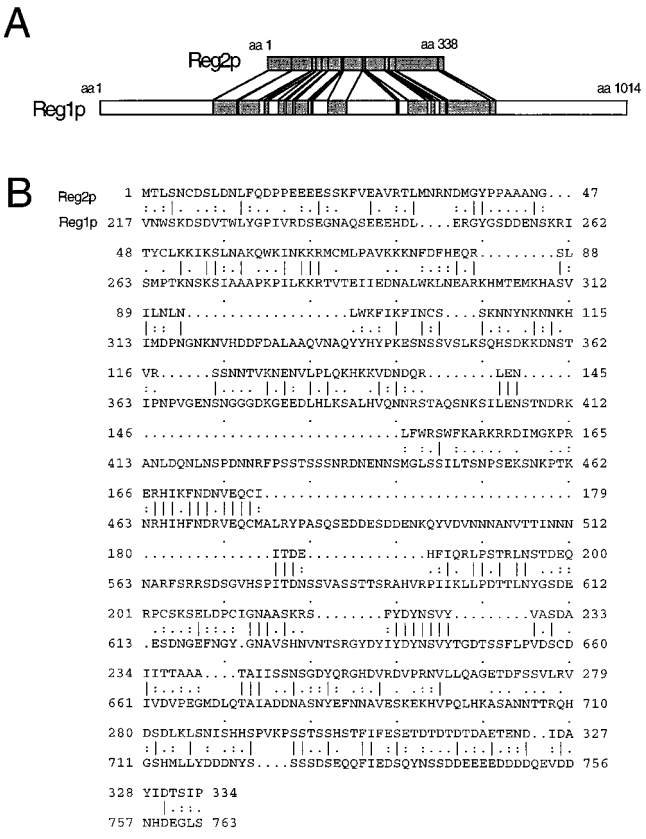

FIG. 1. The predicted *REG2* protein product is homologous to Reg1p. The BestFit program (Genetics Computer Group) was used to generate an alignment of the Reg1p and Reg2p amino acid (aa) sequences. Overall, the two proteins are 29% identical and 48% similar. (A) Reg1p and Reg2p are represented as bars. Regions of similarity are indicated by shading; black bars signify regions of identity. Gaps generated in the Reg1p sequence are indicated by open bars. (B) The Reg2p amino acid sequence is shown aligned to the central conserved region of Reg1p. Vertical lines are drawn between identical residues, and conservative changes are indicated by dots. Gaps inserted within Reg1p are represented by dotted lines.

the multiple cloning site of YCpIF15 places protein synthesis under control of the *GAL1* promoter, which drives transcription when cells are grown on galactose-containing medium but not when they are grown on glucose-containing medium. In addition, cloning of a protein-coding sequence into YCpIF15 results in the fusion of the Ha epitope tag to the amino terminus of the protein. Fusion of the Ha epitope to Reg2p did not impair function (see below). Cellular extracts from strains carrying YCp-*Ha-REG2* grown under inducing conditions were analyzed by immunoblotting. Figure 3A shows that Ha-Reg2p migrates at approximately 48 kDa. Ha-Reg2p is expressed at a level that is barely detectable in either a wild-type or a *reg2* strain but is up-regulated significantly in both the *reg1* and *reg1 reg2* mutants (Fig. 3A; compare lanes 2 and 3 with lanes 4 and 5). Because of this observation, immunoprecipitation experiments were performed in a *reg1*::*URA3* background. Epitope-tagged derivatives of Reg2p (Ha-Reg2p) and PP1 (Myc-Glc7p) were precipitated with monoclonal antibodies, 12CA5 and 9E10, respectively. Construction of epitope-tagged Glc7p has been described previously (68). Immunoprecipitates were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to nitrocellulose, and detected by immunoblotting. When Ha-Reg2p is directly precipitated with an anti-Ha antibody, a significant amount of Myc-Glc7p (migrating at 36 kDa) is coprecipitated (Fig. 3B, lane 3). Similarly, when anti-Myc antibody is used to precipitate Myc-Glc7p, a band corresponding to Ha-Reg2p is coprecipitated (Fig. 3B, lane 4). A band of 48 kDa is not observed in immunoprecipitations of Myc-Glc7p in the absence of YCp-*Ha-REG2* (data not shown) and cannot be precipitated with the anti-Myc antibody from strains lacking *myc-GLC7* (Fig. 3, lane 2). From these results, we conclude that Ha-Reg2p and Myc-Glc7p are physically associated in our cell extracts.

***REG1* and *REG2* are functionally redundant.** A *REG2* null allele was constructed to determine whether cells require *REG2* function. A diploid yeast strain was transformed with a linearized plasmid in which all but the carboxy-terminal 19 nucleotides of the *REG2* coding sequence were replaced with the *URA3* selectable marker (Fig. 2). The integrity of the *reg2*::*URA3* disruption was confirmed by genomic Southern

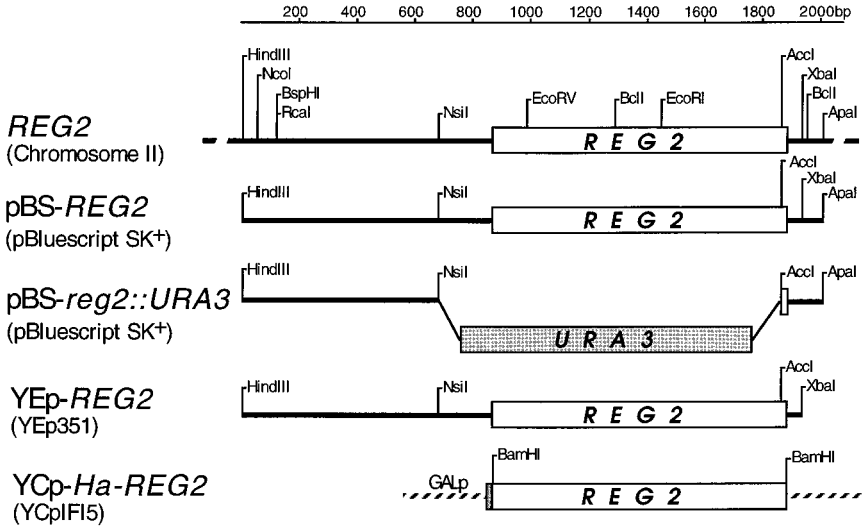

FIG. 2. *REG2* plasmid constructs. The restriction maps of genomic DNA contained in the various plasmids used for this study are depicted. The open bar corresponds to the *REG2* coding sequence, with surrounding chromosomal DNA represented by bold lines. The position of the *URA3*-tagged *reg2* deletion (pBS-*reg2*::*URA3*) is indicated by the shaded bar. The position of the Ha epitope tag in YCp-*Ha-REG2* is indicated by shading.

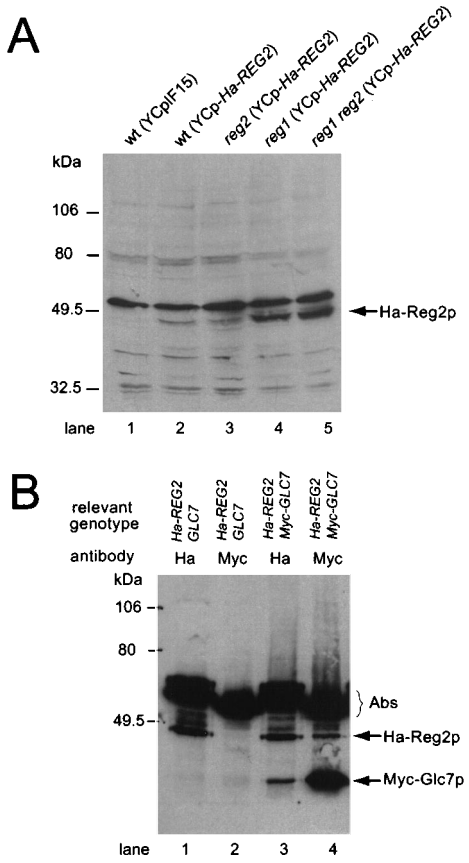

FIG. 3. Coimmunoprecipitation of Ha-Reg1p and Myc-Glc7p. (A) Cellular extracts were prepared from wild-type (KT1357), *reg1* (DF185), *reg2* (DF183), and *reg1 reg2* (DF189) strains carrying either the YCpIF15 vector (lane 1) or YCp-*Ha-REG2* (lanes 2 to 5). Proteins were separated by SDS-polyacrylamide gel electrophoresis, and Ha-Reg2p was detected by immunoblot analysis using an anti-Ha antibody. The position of Ha-Reg2p is indicated by the arrow. The band migrating at just over 50 kDa corresponds to a cross-reacting protein species that is present in strains lacking YCp-*Ha-REG2*. (B) Cellular extracts were prepared from a strain carrying either YCp-*Ha-REG2* alone (DF185) or both YCp-*Ha-REG2* and YCp-*mycGLC7* (DF207). Ha-Reg2p and Myc-Glc7p were immunoprecipitated with anti-Ha and anti-Myc antibodies, respectively. Precipitates were separated by SDS-polyacrylamide gel electrophoresis and then subjected to immunoblot analysis using both anti-Ha and anti-Myc antibodies. Positions of Ha-Reg2p and Myc-Glc7p are indicated by arrows.

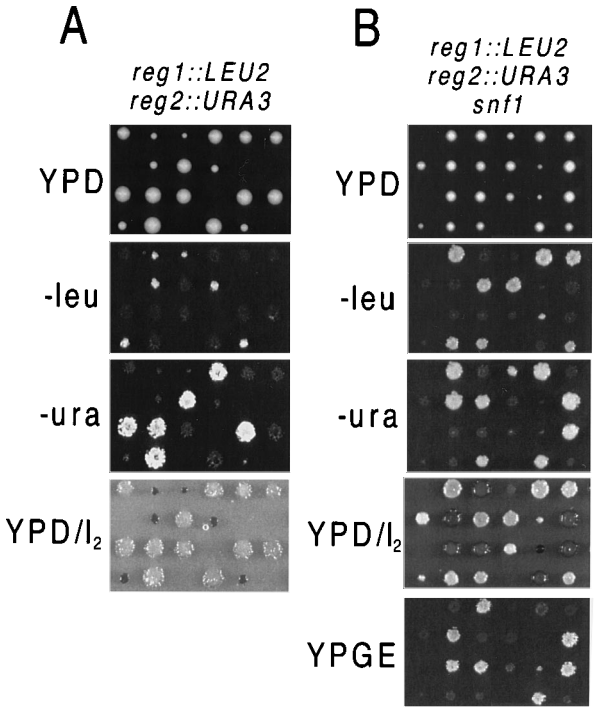

FIG. 4. *reg1 reg2* double mutants exhibit a severe growth defect that is suppressed by a mutation in the Snf1 protein kinase. (A) A *reg1*::*LEU2* disruption strain (DF203) was crossed to a *reg2*::*URA3* disruption strain (DF118). (B) A *reg2*::*URA3 snf1* double mutant (EG649-14D) was crossed to a *reg1*::*LEU2* disruption strain (DF203). Meiotic progeny from the resulting diploids were analyzed by tetrad dissection. Representative tetrads are pictured after growth at 24°C on YPD for 3 days and following subsequent replica plating to leucine-deficient (-leu), uracil-deficient (-ura), and YPEG (glycerol-ethanol) media to score the *reg1*::*LEU2*, *reg2*::*URA3*, and *snf1* genotypes, respectively. Tetrads that were replica plated to YPD and stained with iodine vapor are pictured in the bottom panel to illustrate the variation in glycogen accumulation exhibited by the different genotypes.

hybridization analysis (data not shown). Sporulation and tetrad analysis of meiotic progeny from the resulting *reg2*::*URA3*/*REG2* heterozygous diploid revealed no observable traits associated with loss of *REG2* function. The *reg2* null mutants grew normally at temperatures ranging from 11 to 37°C and on a variety of growth media, including 2% ethanol and glycerol, 0.9 M NaCl, 8 mM caffeine, or 10 μg of benomyl per ml. Additionally, the *reg2*::*URA3* mutants were normal with respect to glycogen accumulation, glucose repression, and sporulation. However, when a *reg2*::*URA3* haploid strain was mated to a strain carrying a *reg1*::*LEU2* null allele, the resulting Ura^+ Leu^+ double mutants exhibited a severe growth defect (Fig. 4A). While *reg1* single mutants have a slightly smaller colony size on plates in our strain background, the *reg1*::*URA3* *reg2*::*LEU2* double mutants formed pin-sized colonies not visible in Fig. 4A. The same result was seen in experiments in which both *reg1* and *reg2* were disrupted with *URA3*. Sporulation of a *reg1*::*URA3*/*REG1* *reg2*::*URA3*/*REG2* heterozygous diploid yielded tetratypes consisting of one normal-sized Ura^- colony (*REG1 REG2*), one normal-sized Ura^+ colony (*REG1 reg2*), one slowly growing Ura^+ colony (*reg1 REG2*), and one pin-sized Ura^+ colony (*reg1 reg2*). In every case in which there were two Ura^+ and two Ura^- colonies, the Ura^- (*REG1 REG2*) spores showed normal growth whereas the Ura^+ spores (*reg1 reg2*) exhibited a severe growth defect (data not shown). The *reg1 reg2* double mutants were not, however, growth arrested. These cells continued to grow and divide, albeit at a much lower rate than either wild-type or *reg1* single-mutant strains. Microscopic examination of *reg1 reg2* double mutants revealed no gross cell morphology defects and no accumulation of cells in a specific phase of the cell cycle.

To test whether overexpression of *REG2* could compensate for loss of *reg1*, plasmid YCp-*Ha-REG2* was transformed into both *reg1* and *reg1 reg2* mutant strains. This construct, containing *REG2* under control of the *GAL1* promoter, complemented the growth defect of *reg1*::*URA3* and *reg1*::*URA3* *reg2*::*URA3* strains when grown on galactose but not glucose (Fig. 5A). We conclude that overexpression of *REG2* complements the slow-growth phenotype of both *reg1*::*URA3* single mutants and *reg1*::*URA3* *reg2*::*URA3* double mutants, suggesting that *REG1* and *REG2* are functionally related.

Overexpression of *REG2* has no effect on the glucose derepression, glycogen hyperaccumulation, and *rna1-1* suppression phenotypes of a *reg1* mutant. *REG1* was originally identified on the basis of its role in carbohydrate metabolism and is thought to encode a negative regulator of glucose-repressible genes. Strains harboring a *reg1*::*URA3* disruption allele express

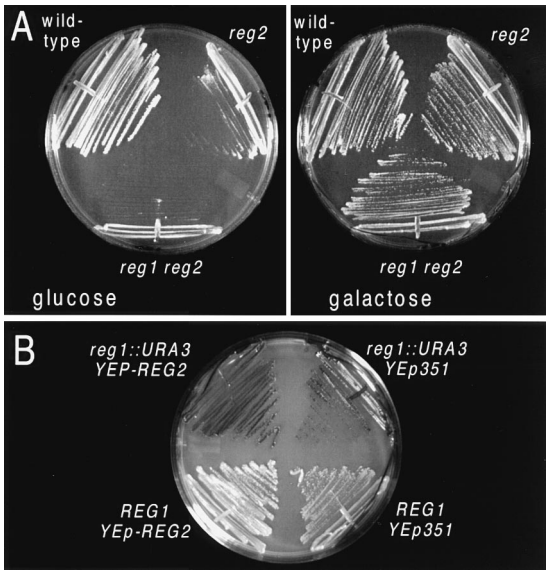

FIG. 5. Overexpression of *REG2* complements the growth defect of a *reg1* mutant but has no effect on the glycogen hyperaccumulation phenotype. (A) Plasmid YCp-*Ha-REG2*, containing the *REG2* coding sequence under control of the *GAL1* promoter, was transformed into a wild-type strain (KT1357), a *reg1* mutant (DF185), or a *reg1 reg2* double mutant (DF189). The resulting transformants are pictured following growth for 2 days at 37°C (a temperature at which the growth defect of a *reg1* mutant is exacerbated) on medium containing either 2% glucose or 2% galactose. (B) Both wild-type (DF103) and *reg1*::*URA3* (DF173) strains were transformed with either an empty vector (YEp351) or a high-copy-number *REG2* plasmid (YEp-*REG2*). To assay glycogen accumulation, transformants were streaked onto selective medium, grown for 2 days at 30°C, and then stained with iodine vapor.

invertase, a glucose-repressible enzyme, at a constitutively high level (54). Wild-type strains, on the other hand, express invertase poorly when grown on glucose. To determine if overexpression of *REG2* could complement the glucose-derepressed phenotype of *reg1* mutants, invertase activity was measured in a *reg1*::*URA3* strain carrying either a high-copy-number *REG2* plasmid (YEp-*REG2*) or a control vector (YEp351). Qualitative analysis of glucose derepression was also carried out by streaking the identical strains onto sucrose plates containing 2-DG. 2-DG is a glucose analog which stimulates glucose repression but cannot be metabolized. As a result, only strains that are glucose derepressed are able to utilize sucrose in the presence of 2-DG. From these experiments, we conclude that overexpression of *REG2* does not abrogate the glucose-derepressed phenotype of a *reg1* mutant. Even in the presence of a high-copy-number *REG2* plasmid, a *reg1* deletion strain maintains high invertase activity (Table 2) and remains 2-DG resistant (data not shown).

In addition to the slow-growth and glucose derepression phenotypes associated with *reg1* null mutants, we and others (39, 75) observed that these strains hyperaccumulate glycogen. Qualitative glycogen measurements are made by inverting a petri plate over iodine crystals. Wild-type colonies stain medium brown within 1 to 2 min, while strains deficient in glycogen accumulation remain yellow. We consistently observed that *reg1* disruption strains turn a dark brown color within 5 to 10 s after being inverted over iodine, while wild-type colonies remain unstained during the same time period. To test whether overexpression of *REG2* was able to complement the glycogen hyperaccumulation phenotype of a *reg1* null strain, a *reg1*::*URA3* strain was transformed with either YEp-*REG2* or a control vector (YEp351). Figure 5B shows the resulting transformants alongside wild-type strains carrying the identical plasmids following exposure to iodine crystals. The wild-type strain stains a yellow-light brown color irrespective of the *REG2* plasmid, whereas the *reg1*::*URA3* strain stains a very dark brown within the same time period (Fig. 5B). The *reg1*::*URA3* strain harboring plasmid YEp-*REG2* accumulates glycogen at levels comparable to those for the identical strain carrying an empty vector.

TABLE 2. Invertase activity

Strain	Relevant genotype	Invertase activity (U)[a]	
		Repressed	Derepressed
DF103	Wild type + YEp351	5	270
		6	242
DF103	Wild type + YEp-*REG2*	7	176
		4	312
DF118	*reg2* + YEp351	8	450
		5	237
DF118	*reg2* + YEp-*REG2*	7	219
		8	393
DF174	*reg1* + YEp351	297	292
		609	493
DF174	*reg1* + YEp-*REG2*	395	472
		315	315

[a] Two independent transformants from each strain were grown to mid-log phase in selective synthetic medium containing 5% glucose (repressing conditions). Aliquots were shifted to selective synthetic medium containing 0.05% glucose (derepressing conditions) and grown for an additional 3 h. One unit is defined as 1 μmol of glucose released per min per 100 mg (dry weight) of cells. The values shown represent invertase activity from each of two transformants assayed.

glc7-1 encodes an allele of the PP1 catalytic subunit, the distinguishing feature of which is a dramatic glycogen deficiency (10, 28, 58). The glycogen hyperaccumulation phenotype of *reg1*, which encodes a predicted subunit of PP1, prompted us to assay glycogen levels in a *reg1 glc7-1* double mutant. Crosses between *reg1* and *glc7-1* strains reveal that the double mutants accumulate an intermediate level of glycogen (data not shown). However, *glc7-1* does not suppress the colony size defect of *reg1*, and subsequent crosses to a *reg2* strain reveal that *glc7-1* does not suppress the severe growth defect of the *reg1 reg2* double mutants (data not shown).

Along with the proposed role of *REG1* as a negative regulator of glucose-repressible genes, *reg1* mutants have been demonstrated to suppress RNA processing defects and temperature-sensitive growth of *rna1-1* and *prp* mutants (49, 57, 79). The *RNA1* gene encodes a cytosolic protein (38) shown to affect pre-tRNA and pre-tRNA processing (37, 47) as well as transport of RNA from the nucleus to the cytoplasm (2, 44, 65). The *PRP* genes affect pre-mRNA processing, and several have been found to encode splicing factors or components of spliceosomes (63). Given that Reg1p and Reg2p may perform redundant functions, we tested whether overexpression of *REG2* might abolish *reg1* suppression of *rna1-1*. We reasoned that if defects associated with *rna1-1* mutants were suppressed by loss of *reg1* function, then overexpression of the functionally related *REG2* gene might prevent suppression. Nonetheless, a *reg1*::*URA3 rna1-1* double mutant (strain DF205) carrying *REG2* on a high-copy-number plasmid (YEp-*REG2*) was able to maintain growth at the nonpermissive temperature of 34°C (data not shown).

Together, the genetic data suggest that while *REG1* and *REG2* appear to have overlapping functions with respect to regulation of cell growth, important distinctions remain. Only *reg1* mutants are defective in glucose repression and glycogen

metabolism. Overexpression of *REG2* in a *reg1* deletion strain complements the slow-growth defect but does not compensate for defects in glucose repression or glycogen accumulation. Furthermore, overexpression of *REG2* has no effect on the ability of *reg1* to suppress *rna1-1*.

The severe growth defect of *reg1 reg2* double mutants is suppressed by a Snf1 protein kinase mutant. Mutants in the *SNF1*-encoded protein kinase are unable to activate glucose-repressible genes (13, 14). In previous investigations, it was reported that *snf1 reg1* double mutants exhibit the glucose-repressed phenotype of *snf1* rather than the derepressed phenotype of *reg1* (54, 78). To determine if the growth defect associated with *reg1 reg2* was also suppressed by *snf1*, we first crossed a *snf1* mutant to a *reg1*::*LEU2* mutant. For these experiments, we used *snf1-172*, an allele previously shown to behave very similar to a null allele (70). As previously reported, *snf1* was epistatic to *reg1*. The *snf1 reg1* double mutants had reduced glycogen levels and failed to grow on nonfermentable carbon sources, both hallmarks of a *snf1* null mutant (70). A *snf1 reg2*::*URA3* mutant was then crossed to a *reg1*::*LEU2* strain. Tetrad analysis of meiotic progeny from the resulting diploid are presented in Fig. 4B. Tetrads were replica plated to medium lacking uracil and medium lacking leucine as well as to YPEG to score the *snf1* genotype. Surprisingly, many of the Leu^+ Ura^+ progeny grew into normal-sized colonies. Tetrad analysis of 25 asci revealed that 14 *reg1*::*LEU2 reg2*::*URA3* spore clones grew into normal-sized colonies. All 14 were *snf1-172*, as determined by their failure to grow on YPEG and accumulate glycogen. Another 14 spore clones either failed to germinate or grew very slowly, and 12 of these were deduced unambiguously to be *reg1 reg2 SNF1* mutants.

Mutations in *snf1* as well as in components of the cyclic AMP (cAMP)-dependent protein kinase pathway have been found to affect some of the same physiological processes (31, 40, 70, 78). A *snf1* null mutant is unable to grow on nonfermentable carbon sources, is sensitive to heat stress and starvation, and fails to accumulate glycogen in response to nutrient depletion (31, 70). These phenotypes resemble those commonly associated with a hyperactive adenylate cyclase pathway. Mutations in adenylate cyclase (*CYR1*/*CDC35*), which decrease the level of cAMP in the cell, moderate the *snf1* phenotype. In contrast, mutations in the regulatory subunit of cAMP-dependent protein kinase (*BCY1*), which increase cAMP-dependent protein kinase levels, exacerbate the *snf1* phenotype (70). Together, the glucose repression and cAMP-adenylate cyclase pathways control the cell's response to glucose availability, ultimately affecting a large number of gene products involved in growth control (7, 60). Curiously, suppression of *rna1-1* by *reg1* is mediated by both of these signaling pathways. Mutations in either *snf1* or *bcy1* abolish suppression of *rna1-1* by *reg1* (78).

Given the relationship between *REG1*, *SNF1*, and the cAMP pathway, we were interested to see how mutations in either *cdc35* or *bcy1* might affect the growth of *reg1 reg2* double mutants. A *reg1*::*URA3* disruption strain was crossed to a temperature-sensitive *cdc35-13* mutant. The *cdc35-13* allele was chosen because it exhibits a partial growth defect at the semipermissive temperature of 30°C (59). Approximately one-quarter of the spores failed to grow into macroscopic colonies after 4 to 5 days of growth at 24°C. Tetrads were replica plated to synthetic medium and incubated at 37°C to score for temperature sensitivity and to medium lacking uracil to score the *reg1*::*URA3* genotype. From over 50 tetrads analyzed, 95% of wild-type, *cdc35-13*, or *reg1*::*URA3* spore clones grew into colonies. However, *cdc35-13 reg1*::*URA3* double-mutant spores either failed to germinate or germinated and divided a few times before arresting as microcolonies. No growth defect was observed when the *cdc35-13* mutant was crossed to a *reg2*::*URA3* strain.

The lethality of *reg1 cdc35-13* mutants indicates that *reg1* mutants are sensitive to reduced levels of intracellular cAMP. Since the major and possibly only physiological role of cAMP is to activate cAMP-dependent protein kinase (cAPK), it is likely that *reg1* mutants are sensitive to reduced cAPK activity. One explanation for the synthetic lethality of *cdc35-13* and *reg1* is that *reg1* and possibly *reg2* are positive regulators of adenylate cyclase or cAPK. If this is so, we reasoned that traits associated with a *reg1* and/or *reg2* deficiency might be ameliorated by increased activity of cAPK. *BCY1* encodes the regulatory subunit of cAPK, and loss-of-function mutations in *bcy1* bypass the requirement for adenylate cyclase and its activators, resulting in constitutively active cAPK (8, 50, 71, 81). We first crossed a *reg1*::*URA3* strain to a strain carrying *bcy1-16*, an ochre nonsense mutation that results in a truncated *BCY1* peptide after amino acid 344 (9). The resulting *reg1*::*URA3 bcy1-16* double mutant, which retained the slow-growth phenotype of the *reg1*::*URA3 BCY1* strain, was then crossed to a *reg2*::*URA3* mutant, and the resulting diploid was subjected to tetrad analysis. The *reg1 reg2* double mutants grew extremely slowly irrespective of the *bcy1* genotype (data not shown). Thus, the slow growth rate of the *reg1 reg2* strain is not suppressed by increased activity of cAPK. Together, these data indicate that the slow growth rate of the *reg1 reg2* strain is not due to a defect in activation of adenylate cyclase and suggest that *reg1* and cAPK act on the same physiological pathways but do so independently.

DISCUSSION

Here we describe a previously uncharacterized gene, *REG2*, whose product has 29% amino acid identity and 48% similarity with Reg1p. We present evidence that like Reg1p, Reg2p interacts with the *GLC7*-encoded PP1 both in vivo, as assayed by the two-hybrid system, and in vitro, as assayed by coimmunoprecipitation. Genetic analysis reveals that while disruption of either *reg1* or *reg2* alone results in a relatively mild slow-growth phenotype (*reg1*) or no defect at all (*reg2*), disruption of both *reg1* and *reg2* causes a severe growth defect. Both the slow-growth defect of a *reg1* mutant and the severe growth defect of a *reg1 reg2* double mutant are complemented by overexpression of *REG2*, indicating these two genes are functionally related. Finally, we demonstrate that the severe growth defect associated with a *reg1 reg2* double mutant can be rescued by a mutant allele of the Snf1 protein kinase.

The role of the *REG1* gene product as a negative regulator of glucose-repressible genes is well established (51, 54, 85). However, the pleiotropic phenotype of a *reg1* null mutant suggest that Reg1p may have other roles within the cell. This idea is consistent with our results suggesting that Reg1p and Reg2p perform redundant functions distinct from glucose repression. Overexpression of *REG2* suppresses the slow-growth defect of a *reg1* mutant but not the defect in glucose repression. Because *reg2* mutants themselves are not glucose derepressed, it seems improbable that the severe growth defect of the *reg1 reg2* double mutants is a direct result of defects in glucose repression. An alternative explanation is that Reg1p and Reg2p function together to direct PP1 activity in an as yet undetermined pathway controlling cell growth. Given that the sequence similarity between Reg1p and Reg2p is confined to central half (amino acids 217 to 763) of Reg1p, it seems likely that these sequences contain domains important for general growth regulation as well as the binding domain for PP1. The unconserved amino-

terminal and/or carboxy-terminal regions of Reg1p might comprise regulatory domains required for directing PP1 activity specifically to substrates in the glucose repression pathway.

If the growth defect of the *reg1 reg2* double mutant is the result of decreased PP1 activity, it seems logical that this defect could be compensated for by a mutation in an opposing protein kinase. Precedent for this type of antagonistic relationship has been reported for two other protein kinases and PP1 in *S. cerevisiae. GCN2* encodes a protein kinase that phosphorylates the α subunit of translation initiation factor 2 (eIF2α) in response to amino acid starvation. Phosphorylation of eIF2α leads to increased expression of Gcn4p, a transcription factor with a central role in the general amino acid control pathway (34). A dominant negative *glc7* mutant, caused by deletion of the C-terminal 104 amino acid residues of Glc7p, restores *GCN4* expression in strains containing a mutant *gcn2*-encoded kinase (80), consistent with the hypothesis that eIF2α is dephosphorylated by Glc7p. Another example involves the *IPL1*-encoded protein kinase that is required for chromosome segregation during mitosis. The temperature sensitivity of the *ipl1-1* allele can be partially suppressed by expression of another C-terminal deletion of *glc7*, by *glc7-1*, or by a mutation in *glc8*, a potential modulator of Glc7p (27, 77). In contrast, hyperexpression of Glc7p in wild-type strains leads to a loss in fidelity in chromosome segregation (27). Together, these results indicate that PP1 acts in opposition to Ip11p and suggests that the Ip11 kinase and PP1 may act on the same substrate(s).

A similar scenario can be proposed for Reg1p and Reg2p in which the growth defect of *reg1 reg2* mutants is caused by the failure of PP1 to dephosphorylate a substrate or substrates important for growth regulation. The hyperphosphorylation of these putative substrates in *reg1 reg2* mutants could be reversed by reducing the activity of the opposing protein kinase. Several lines of evidence implicate Snf1p as the kinase acting in opposition to Reg1p and Reg2p. First, *snf1* mutants exhibit traits that are opposite those observed for *reg1* mutants. *snf1* mutants are unable to induce glucose-repressed genes and fail to accumulate glycogen. In contrast, *reg1* mutants are constitutively glucose derepressed and hyperaccumulate glycogen. Second, a *snf1* mutation is epistatic to *reg1* and to the severe growth defect of *reg1 reg2*. The ability of increased expression of Reg2p to suppress the growth defect of *reg1* but not its glucose derepression phenotype suggests that distinct substrates are involved in growth-related and glucose repression defects. In our model, Snf1p would be responsible for phosphorylating the target substrates for both Reg1p- and Reg2p-associated PP1.

The importance of the *SNF1*-encoded protein kinase in relieving glucose repression is well established (53, 60, 73). However, not unlike *reg1* and *glc7* mutants, *snf1* mutants exhibit pleiotropic phenotypes including the inability to grow on nonglucose carbon sources, sensitivity to starvation and heat stress, failure to accumulate glycogen, and sporulation defects (70). These data are consistent with the idea that in addition to its role in glucose repression, Snf1p may regulate other cellular processes. Supporting this hypothesis are reports that Snf1p is present in both glucose-repressed and derepressed cells and appears to be distributed throughout the cell (14).

Recent evidence indicates that Snf1p and the related mammalian AMP-activated kinase (AMPK) may regulate a variety of metabolic pathways. Snf1p shows a remarkable degree of sequence identity (46%) to both mammalian AMPK and a protein kinase encoded by the RKIN1 gene from rye (12). Expression of cRKIN1 in yeast *snf1* mutants restores *SNF1* function (12), indicating Snf1p, AMPK, and RKIN1 form a conserved family of protein kinases. Mammalian AMPK functions in lipid metabolism, negatively regulating acetyl coenzyme A (acetyl-CoA) carboxylase (52), the enzyme catalyzing the first committed step in fatty acid synthesis. In a wild-type yeast strain, acetyl-CoA carboxylase undergoes a time-dependent decrease in activity when glucose is limiting (82). In *snf1* mutants, acetyl-CoA carboxylase activity remains high (82), suggesting that like its mammalian homolog, Snf1p may inactivate acetyl-CoA carboxylase in vivo. The proposed role of Snf1p in lipid metabolism is consistent with the idea that Snf1p may be involved in a wide range of metabolic pathways. It is of interest that mammalian acetyl-CoA carboxylase and hydroxymethylglutaryl-CoA reductase, both of which are in vivo substrates of AMPK (30, 52), are dephosphorylated by PP1 in vitro (16, 32).

Alternative models for the antagonistic behavior of *snf1* and *reg1/reg2* cannot be ruled out at this time. Reg1p and Reg2p might negatively regulate Snf1p directly or could regulate a component of a Snf1p-protein complex such that the growth defect of the double mutant is the result of increased Snf1p kinase activity. Among the potential substrates for this type of regulation are members of a family of functionally related proteins involved in glucose repression. This group includes Sip1p, Sip2p, and Gal83p, which associate with Snf1p and are phosphorylated in a *SNF1*-dependent manner (83). *gal83* mutants exhibit reduced Snf1p activity in vitro (83), and genetic evidence suggests that Reg1p and Gal83p act together (22). In this model, Reg1p and Reg2p would be predicted to have qualitatively different effects on Snf1p activity, since overexpression of Reg2p alleviates the growth defect of *reg1* without influencing its glucose derepression phenotype.

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