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Genetics

Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in *Saccharomyces cerevisiae*

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ABSTRACT **The Snf1 protein kinase family has been conserved in eukaryotes. In the yeast *Saccharomyces cerevisiae*, Snf1 is essential for transcription of glucose-repressed genes in response to glucose starvation. The direct interaction between Snf1 and its activating subunit, Snf4, within the kinase complex is regulated by the glucose signal. Glucose inhibition of the Snf1-Snf4 interaction depends on protein phosphatase 1 and its targeting subunit, Reg1. Here we show that Reg1 interacts with the Snf1 catalytic domain in the two-hybrid system. This interaction increases in response to glucose limitation and requires the conserved threonine in the activation loop of the kinase, a putative phosphorylation site. The inhibitory effect of Reg1 appears to require the Snf1 regulatory domain because a *reg1*Δ mutation no longer relieves glucose repression of transcription when Snf1 function is provided by the isolated catalytic domain. Finally, we show that abolishing the Snf1 catalytic activity by mutation of the ATP-binding site causes elevated, constitutive interaction with Reg1, indicating that Snf1 negatively regulates its own interaction with Reg1. We propose a model in which protein phosphatase 1, targeted by Reg1, facilitates the conformational change of the kinase complex from its active state to the autoinhibited state.**

In the yeast *Saccharomyces cerevisiae*, the Snf1 (Cat1 and Ccr1) protein kinase is essential for the regulatory response to glucose starvation. When glucose is limiting, the Snf1 kinase activity is required for transcription of many glucose-repressed genes, including genes involved in alternate carbon source utilization, respiration, and gluconeogenesis (1, 2). Snf1 is also necessary for sporulation, glycogen storage, thermotolerance, and peroxisome biogenesis (3, 4).

The Snf1 protein kinase family has been widely conserved in eukaryotes. Many plants contain Snf1 homologs, and some have been shown to provide Snf1 function in yeast (5–7). The mammalian homolog, AMP-activated protein kinase (AMPK) (8, 9), is involved in cellular stress responses that cause ATP depletion (10). AMPK is activated by the elevated AMP:ATP ratio and inhibits biosynthetic pathways, including enzymes of lipid metabolism (11); Snf1 also inactivates acetyl-CoA carboxylase, suggesting conservation of this regulatory role in yeast (8, 12). Moreover, evidence that the AMP:ATP ratio in yeast correlates with Snf1 activity raises the possibility that signals have also been conserved (13).

In yeast, the Snf1 kinase is complexed with the Snf4 (Cat3) protein, which is required for Snf1 kinase activity both *in vivo* and *in vitro* (12, 14–16). The Snf1 kinase complex also contains a member of the Sip1/Sip2/Gal83 family (17–19), which interacts with Snf1 and Snf4 via distinct domains and serves a scaffolding function (20). All of these components of the kinase complex have counterparts in higher eukaryotes (21, 22).

The Snf1 kinase is regulated in response to the glucose signal and is activated when glucose is limiting (12). Genetic and biochemical evidence indicates that the C-terminal regulatory domain of Snf1 autoinhibits the catalytic domain in glucose-grown cells, whereas in glucose-deprived cells the Snf4 subunit binds to the Snf1 regulatory domain and counteracts this autoinhibition (23) (Fig. 1). In the two-hybrid system, the interaction between Snf1 and Snf4 is inhibited by high glucose (23), although biochemical evidence indicates that Snf4 remains associated with Snf1 regardless of glucose availability (8, 16, 20, 24). Thus, activation of the Snf1 kinase appears to be associated with a glucose-regulated conformational change of the kinase complex.

The molecular mechanism that causes these changes within the kinase complex in response to the glucose signal is not yet known. For many protein kinases, phosphorylation of the kinase or an associated subunit is a key regulatory event. Phosphorylation has been indirectly implicated in control of Snf1. (*i*) Snf1 contains a conserved threonine residue (T210) in the activation loop, or T-loop, near subdomain VIII, which is essential for Snf1 activity *in vitro* and *in vivo* (24); phosphorylation at this site is required for activation of other kinases (25). (*ii*) Partially purified Snf1 is inactivated by several mammalian protein phosphatases and reactivated by partially purified mammalian AMPK kinase and by a factor present in yeast extracts (12, 13); however, no Snf1 kinase kinase has yet been identified in yeast. (*iii*) Genetic evidence implicates protein phosphatase 1 (PP1), together with the Reg1 targeting subunit, in regulation of the Snf1 kinase (23, 26, 27).

In *S. cerevisiae*, the catalytic subunit of PP1 is encoded by the essential gene *GLC7* and has multiple roles in cellular processes (reviewed in ref. 28); the activity of PP1 is controlled by the association of Glc7 with different regulatory or targeting subunits (29). Genetic and biochemical evidence identified Reg1 as a regulatory subunit that directs the participation of PP1 in the glucose response mechanism (27), although Reg1 also affects other processes (30–33). The mutations *glc7-T152K* and *reg1*Δ relieve glucose repression of gene expression (26, 34). These mutations also relieve glucose inhibition of the two-hybrid interaction between Snf1 and Snf4, indicating that Reg1-PP1 modulates protein interactions within the Snf1 kinase complex in response to glucose (23). One possible mechanism of action is that Reg1-PP1 directly dephosphorylates Snf1 or another component of the kinase complex,

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Abbreviations: AMPK, AMP-activated protein kinase; β-gal, β-galactosidase; SC, synthetic complete medium; PP1, protein phosphatase 1; GAD, Gal4 activation domain.
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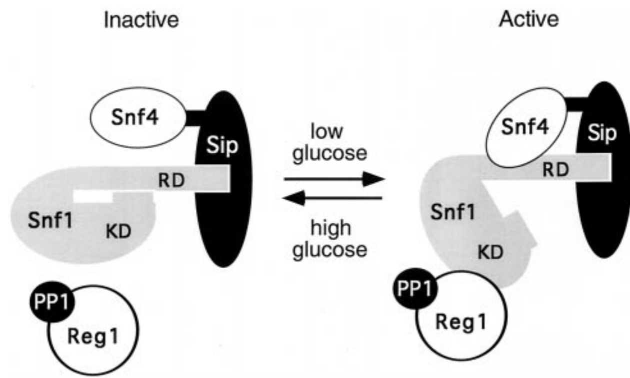

FIG. 1. Model for regulation of the Snf1 kinase complex. Previous studies (23) showed that when cells are grown in high glucose, the Snf1 kinase complex exists predominantly in an inactive state, in which Snf1 is autoinhibited by the binding of its kinase domain (KD) to its regulatory domain (RD). When cells are limited for glucose (low glucose), an active conformation of the kinase complex is favored; the kinase domain is freed from autoinhibition, and the regulatory domain now interacts with Snf4. See *Results* for evidence that Reg1-PP1 associates with Snf1 in low glucose. Sip, member of the Sip1/Sip2/Gal83 family of proteins, which bind to both Snf1 and Snf4 (20).

consistent with genetic interactions between mutations of *REG1* and *GAL83* (18). Alternatively, Reg1-PP1 could affect Snf1 by an indirect mode of action via a cascade of regulatory events.

In this study, we have used the two-hybrid system to show that Reg1 interacts with the Snf1 kinase domain and that the interaction is regulated by the glucose signal. We present evidence that the inhibitory effect of Reg1 requires the Snf1 regulatory domain. We also examine the roles of a conserved threonine in the activation loop and the Snf1 catalytic function in regulating interaction with Reg1. The findings suggest that Reg1 affects protein interactions within the kinase complex, and we propose a model in which Reg1-PP1 facilitates conformational change of the kinase complex from its active state to the autoinhibited state.

MATERIALS AND METHODS

Strains and Genetic Methods. The *S. cerevisiae* strains used were CTY10–5d (*MAT***a** *ade2 his3 leu2 trp1 gal4 gal80 URA3*::*lexAop-lacZ*) (gift of R. Sternglanz, State University of New York, Stony Brook); GGY1::171 (Δ*gal4* Δ*gal80 his3 leu2 ura3 URA3*::*GAL1-lacZ*) (35), FY250 (*MAT***a** *his3 leu2 trp1 ura3 SUC2*) (gift of F. Winston, Harvard Medical School, Boston); MCY3922 (*MAT*$\boldsymbol{\alpha}$ *snf1Δ10 URA3*::*GAL1-lacZ ade2 his3 leu2 lys2 ura3 SUC2*); and MCY3923 (MCY3922 *reg1Δ*::*HIS3*). The *reg1Δ*::*HIS3* allele was derived from pKL46, which was constructed by replacing *URA3* with *HIS3* in pUCsrn1Δ::URA3 (30). FY and MCY strains have the S288C genetic background. Standard methods for yeast genetic analysis and transformation (36) were used. Cells were grown in 1% yeast extract, 2% bacto-peptone or synthetic complete medium (SC) (36) lacking appropriate supplements to maintain selection for plasmids. The *Escherichia coli* strains used were XL1-Blue and SURE (Stratagene).

Construction of Plasmids. pRJ81 contains the 2.2-kb *Bam*HI fragment of pGAD-SNF1T210A (19) in the *Bam*HI site of pVP16 (37) (see Table 1). pRJ226 contains the 1.2-kb *Bam*HI/*Bcl*I fragment from pRJ81 in the *Bam*HI site of pACTII (38).

pRJ73 contains the 2.7-kb *Eag*I/*Bam*HI fragment from pCE110 (15) in the *Eag*I/*Bam*HI sites of pRS426. To construct pRJ80, we carried out a PCR reaction by using pRJ73 as template and the primers P5′ (5′-GGGGATCCACATGAGCAGTAACAAC-3′, *SNF1*) and T7 universal primer (Stratagene). The resulting 2.2-kb *Bam*HI PCR fragment containing the *snf1-K84R* sequence was cloned in the *Bam*HI site of pVP16. pRJ210 and pRJ224 contain the 2.2-kb *Bam*HI fragment and the 1.2-kb *Bam*HI/*Bcl*I fragment, respectively, from pRJ80 in the *Bam*HI site of pACTII.

Table 1. Plasmids

Plasmid	Expressed protein	Ref.
pRJ210	GAD-Snf1K84R	This study
pRJ90	GAD-Snf1KD	23
pRJ224	GAD-Snf1KDK84R	This study
pRJ226	GAD-Snf1KDT210A	This study
pRJ79	VP16-Snf1	23
pRJ80	VP16-Snf1K84R	This study
pRJ81	VP16-Snf1T210A	This study
pRJ208	VP16-Snf1KD	This study
pRJ209	VP16-Snf1RD	This study
pRJ65	LexA-Reg1	27

Snf1KD, residues 1–392. Snf1RD, residues 392–633. GAD and VP16 plasmids are derived from pACTII (38) and pVP16 (37). Other plasmids are described in *Materials and Methods*.

pRJ208 and pRJ209 contain the 1.2-kb *Bam*HI/*Bcl*I fragment (codons 1–392) and the 1.0-kb *Bcl*I/*Bam*HI fragment (codons 392–633), respectively, of pEE5 (39) in the *Bam*HI site of pVP16.

pRJ93 contains the 2.7-kb *Eag*I/*Bam*HI *SNF1* fragment of pCE101 (15) inserted into the corresponding sites of pRS424 (40). pKL3 was constructed in several steps. (*i*) The 1.0-kb *Eco*RI/*Hinc*II fragment from pCEsnf1Δ8 (15) containing the *SNF1* promoter region was cloned into the *Eco*RI/*Sma*I sites of pRS424 (40), giving pKL2. (*ii*) Using pSE1112 (41) as template, a PCR amplification was carried out with primers KL7 (5′-GCGCGGATCCATGAAGCTACTGTCTTCTATCGAAC-3′; *GAL4* specific) and KL8 (5′-GCGCGCGGCCGCTAATTAATCAGTCAACTTTGAACCAATCGTCTTG-3′; *SNF1* kinase domain specific) to generate a 1.4-kb fragment. (*iii*) The 0.9-kb *Bam*HI/*Not*I fragment derived from this PCR fragment was then cloned into the corresponding sites of pKL2. pKL3 expresses Snf1 residues 1–309 from the *SNF1* promoter; it contains seven nucleotides 5′ to the ATG not found in the native gene.

β-Galactosidase (β-Gal) and Invertase Assays. Cultures were grown to mid-log phase. β-Gal activity was assayed in permeabilized cells and expressed in Miller units, and invertase activity was assayed in whole cells as described (23).

RESULTS

Glucose-Regulated Interaction of Reg1 with the Kinase Domain of Snf1. We used the two-hybrid system (39) to test for interaction of Reg1 with the Snf1 protein kinase. Plasmids expressing LexA-Reg1 and VP16-Snf1 fusion proteins were used to transform strain CTY10–5d, which carries a *lexAop-lacZ* reporter. The transformants were blue in filter lift assays for β-gal activity, and quantitative assays confirmed a significant stimulation of activity relative to controls. To determine whether this interaction is regulated by the glucose signal, we assayed cells grown in 2% glucose and then starved by a shift to 0.05% glucose (Fig. 2). Transformants showed a 6.5-fold increase in β-gal activity after the shift from high to low glucose, indicating that interaction between Reg1 and Snf1 increases in response to glucose limitation.

To determine whether Reg1 interacts with the kinase domain of Snf1 (Snf1KD) or with the C-terminal regulatory domain (Snf1RD), we assayed two-hybrid interactions with fusions expressing the isolated domains (Fig. 2). LexA-Reg1 and VP16-Snf1KD showed glucose-regulated interaction, with a 28-fold increase in activity after a shift from high to low

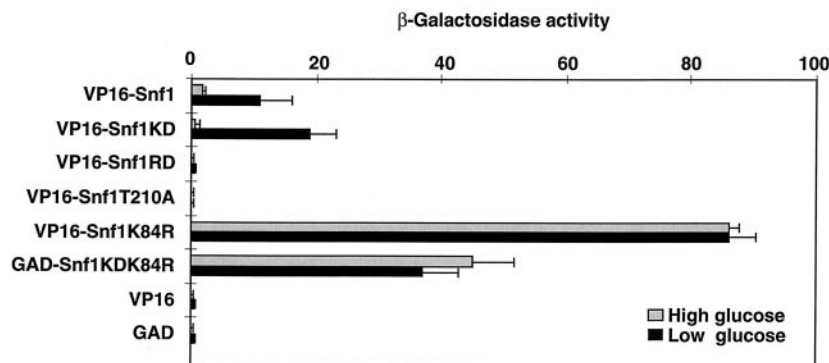

FIG. 2. Interaction of LexA-Reg1 with Snf1 fusion proteins in the two-hybrid system. LexA-Reg1 and the indicated activation domain fusion to Snf1 sequence were expressed from the *ADH1* promoter from plasmids listed in Table 1. Transformants of CTY10–5d were grown in selective SC + 4% glucose medium (high glucose, ▨). To obtain derepressed cells (low glucose, ■), transformants were shifted to SC + 0.05% glucose for 3 to 4 h, except that transformants expressing GAD-Snf1KDK84R and GAD were grown in SC + 2% raffinose/0.05% glucose. Values are the average β-gal activity of four to six transformants. In control experiments, values for VP16-Snf1K84R and GAD-Snf1KDK84R in combination with LexA were <1. The increased interaction detected in derepressed cells is not due to increased expression of LexA-Reg1 (27) or Snf1 hybrid proteins (ref. 23 and data not shown).

glucose, whereas no significant activity was detected with VP16-Snf1RD.

We also tested for interaction between LexA-Reg1 and a VP16 fusion to Snf4, the activating subunit for the Snf1 kinase. We did not detect blue color or significant β-gal activity. Moreover, Reg1 interacted with Snf1 in a *snf4*Δ mutant host (data not shown), indicating that Snf4 is not required for their association. These results are consistent with evidence that Snf4 contacts Snf1RD and not Snf1KD (23).

Reg1 acts with the Glc7 catalytic subunit of PP1 to affect Snf1 function, presumably by targeting Glc7 to the Snf1 complex (27). No interaction was detected between LexA-Glc7 (26) and any Gal4 activation domain (GAD) or VP16 fusion to Snf1, the mutant Snf1K84R (see below), or Snf4. However, we expect that such interaction must be bridged by Reg1, and Reg1 may not be a major partner for Glc7, which associates with many different proteins *in vivo* (28).

Inhibitory Effect of Reg1 Requires the Snf1 Regulatory Domain. The interaction of Reg1 with Snf1KD suggested that Reg1 exerts its regulatory effect through the catalytic domain. To test this hypothesis, we took advantage of the fact that Snf1KD provides partial Snf1 function (15, 23); a *snf1*Δ mutant transformed with a multicopy plasmid encoding Snf1KD shows low-level, glucose-regulated *SUC2* and *GAL1-lacZ* expression (Fig. 3). We therefore examined the regulation of *SUC2* and *GAL1-lacZ* expression in a *reg1*Δ *snf1*Δ mutant transformed with plasmids encoding Snf1 or Snf1KD. Transformants were assayed for invertase and β-gal activity after growth in glucose-repressing or inducing conditions (Fig. 3). The full-length Snf1 conferred glucose-insensitive gene expression, as predicted; activity was lower than in the *snf1*Δ host because these transformants were sick. Unexpectedly, Snf1KD behaved differently from the intact Snf1 and conferred glucose-regulated expression of both genes. We also noted that *reg1*Δ *snf1*Δ mutant cells expressing Snf1KD were much healthier than those expressing Snf1. Thus, the *reg1*Δ mutation relieves glucose repression only when the Snf1 protein kinase is intact, and removal of the Snf1 regulatory domain abrogates the mutant phenotype.

These results suggest that Reg1 promotes autoinhibition of the Snf1 kinase activity by the regulatory domain. Furthermore, the ability of Snf1KD to confer regulated gene expression in a *reg1*Δ mutant suggests that Snf1KD receives a glucose signal independent of Reg1.

Effect of Reg1 on the Interaction of Snf1 and Snf4 Within the Kinase Complex. The finding that the inhibitory function of Reg1 requires the Snf1 regulatory domain is consistent with evidence that Reg1 modulates protein interactions within the Snf1 kinase complex. A *reg1*Δ mutation relieves glucose inhibition of the two-hybrid interaction of Snf1 and Snf4, allowing interaction during growth in 2% glucose (23). To confirm that Reg1 negatively regulates the interaction of Snf1 and Snf4, we

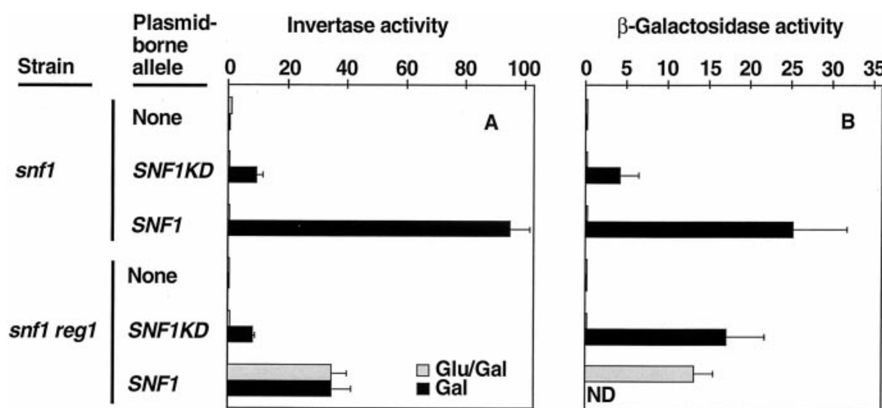

FIG. 3. Snf1KD regulates *SUC2* and *GAL1* independently of Reg1. Strains MCY3922 (*snf1*Δ *GAL1-lacZ*) and MCY3923 (*snf1*Δ *reg1*Δ *GAL1-lacZ*) were transformed with pKL3, pRJ93, or the vector pRS424. pKL3 and pRJ93 express Snf1 residues 1–309 (here referred to as Snf1KD; elsewhere Snf1KD comprises residues 1–392) and the entire Snf1, respectively, from the *SNF1* promoter. Transformants were grown to mid-log phase in selective SC + 2% glucose/2% galactose (Glu/Gal, ▨) and were shifted to SC + 0.05% glucose/2% galactose for 6 hr (Gal, ■). Values are average invertase and β-gal activities for four transformants. ND, not determined because these cultures grew poorly.

examined the effect of increased *REG1* gene dosage on this interaction in cells grown in low glucose (2% galactose/2% glycerol/2% ethanol/0.05% glucose). The presence of the multicopy *REG1* plasmid pRJ85 (27) caused a twofold decrease in β-gal activity for three pairs of hybrid proteins. Values for transformants carrying pRJ85 and the vector control were 123 and 248 units, respectively, for the pair $LexA_{87}$-Snf4/GAD-Snf1; 78 and 154 units for LexA-Snf4/GAD-Snf1; and 161 and 276 units for LexA-Snf1/Snf4-GAD (averages for three transformants; plasmids from ref. 23). These effects, while modest, correlate well with the effects on Snf1-dependent gene expression: *REG1* in multicopy reduces induction of *GAL1* 3-fold (42), and overexpression of LexA-Reg1 reduces derepression of *SUC2* 2- to 3-fold (27). Thus, these data support other evidence that Reg1 regulates protein interactions within the Snf1 kinase complex, promoting a conformation in which the Snf1 regulatory domain binds to the catalytic domain rather than to Snf4.

Because Reg1 and Snf4 appear to act antagonistically on Snf1, we asked whether the absence of Reg1 bypasses the requirement for Snf4 in activation of the Snf1 kinase. We constructed isogenic deletion mutant derivatives of FY250. The *snf4*Δ *reg1*Δ double mutant was indistinguishable from the *snf4*Δ mutant with respect to *SUC2* expression (data not shown), consistent with previous analysis of point mutants (43). Thus, constitutive *SUC2* expression in a *reg1*Δ mutant requires Snf4, suggesting that the state of the kinase complex in a glucose-grown *reg1*Δ mutant resembles the active state found in glucose-limited wild-type cells.

Mutation of the Conserved Activation Loop Threonine in Snf1 Impairs Interaction with Reg1 and Snf4. The Snf1 kinase domain contains a conserved threonine residue (T210) in the activation loop, or T-loop, that is phosphorylated during activation of many kinases (25). The T210 residue is essential for kinase activity, and mutation of T210 to alanine (*snf1-T210A*) abolishes Snf1 function *in vitro* and *in vivo* (24). T210 is also required for function of the isolated Snf1 kinase domain (24).

To assess the effect of the T210A mutation on the interaction of Snf1 with Reg1, we constructed VP16-Snf1T210A. The T210A mutation abolished interaction with LexA-Reg1 (Fig. 2). Immunoblot analysis confirmed that VP16-Snf1T210A is expressed at comparable levels (data not shown). Thus, residue T210 is required for Reg1 to bind to the Snf1 kinase. These findings are consistent with evidence that Reg1 interacts with Snf1 more strongly in glucose-deprived cells, where the kinase is activated.

We next examined the effects of the T210A mutation on the two-hybrid interaction between Snf1 and Snf4, which also increases in response to glucose limitation (23). In combination with each of several DNA-binding Snf4 fusion proteins, GAD-Snf1T210A produced much lower β-gal activity than did GAD-Snf1 (Fig. 4*A*). Western blot analysis confirmed that the levels of the mutant and wild-type proteins were comparable (Fig. 4*B*). Moreover, the T210A substitution does not simply disrupt the kinase complex because the mutant kinase coimmunoprecipitates with Sip1 and Sip2 (19). These results indicate that the two-hybrid interaction of Snf1 with Snf4 depends on the T210 residue and strongly suggest that phosphorylation of T210 is required for conformational changes in the kinase complex that allow the binding of Snf4 to the Snf1 regulatory domain.

Substitution of aspartate or glutamate at position 210 similarly abolished both Snf1 function *in vivo* and the two-hybrid interaction of Snf1 fusion proteins with Snf4 and Reg1 (ref. 24; I. Treich and M.C., unpublished results).

Mutation of the ATP-Binding Site of Snf1 Relieves Glucose Inhibition of the Interaction with Reg1. To determine whether the catalytic activity of Snf1 is required for its interaction with Reg1, we tested another catalytically deficient mutant kinase, Snf1K84R. The *snf1-K84R* allele alters the lysine that is invariant in the ATP-binding site of protein kinases (25), and Snf1K84R does not exhibit kinase activity *in vitro* or provide Snf1 function *in vivo* (15). In contrast to the results with T210A, the mutation K84R both enhanced the interaction with Reg1 and relieved glucose inhibition of this interaction. When VP16-Snf1K84R was paired with LexA-Reg1, β-gal activity was 8-fold higher than observed for wild-type VP16-Snf1 and was not inhibited by glucose (Fig. 2). The increased interaction in glucose-grown cells cannot be attributed to higher levels of VP16-Snf1K84R or to higher levels of LexA-Reg1 in the presence of VP16-Snf1K84R (data not shown).

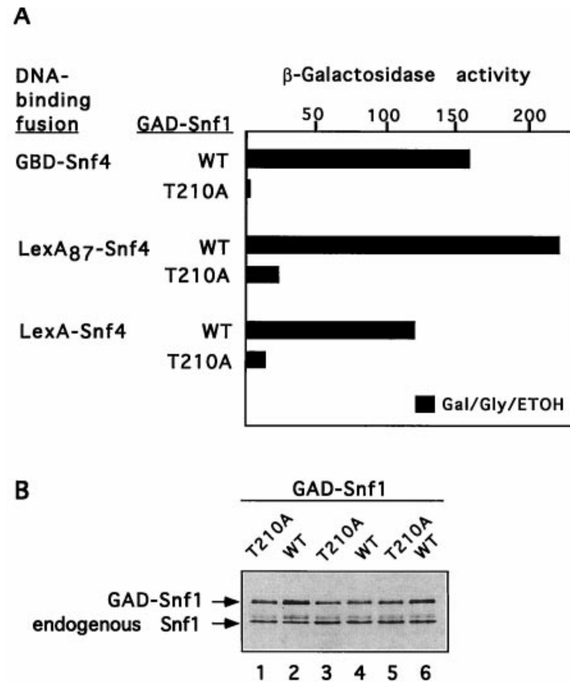

FIG. 4. Mutation T210A in Snf1 impairs interaction with Snf4 in the two-hybrid system. (*A*) Strains were GGY1::171 or CTY10–5d. Fusion proteins were expressed from pRJ57 and pRJ58 (23), pSD4 and pSG1 (gifts of Z. Xue & T. Melese, Columbia University, New York; see ref. 23), and pGAD-SNF1T210A (19). Transformants were grown in SC + 2% galactose/2% glycerol/2% ethanol/0.05% glucose. Values are the average β-gal activity of three to four transformants. GBD, GAL4 DNA-binding domain; $LexA_{87}$, LexA DNA-binding domain. (*B*) Immunoblot of GAD-Snf1 and GAD-Snf1T210A proteins. Protein extracts were prepared from representative transformants assayed in (*A*) carrying (lanes 1 and 2) GBD-Snf4, (lanes 3 and 4) $LexA_{87}$-Snf4, and (lanes 5 and 6) LexA-Snf4. Proteins (50 μg) were separated by an SDS/7.5% polyacrylamide gel and detected by immunoblotting by using affinity-purified anti-Snf1 antibodies (1) and enhanced chemiluminescence with ECL reagents (Amersham).

We also tested for interaction of Reg1 with a GAD fusion to the mutant form of the isolated kinase domain. Interaction between LexA-Reg1 and GAD-Snf1KDK84R was strong and was not inhibited by glucose (Fig. 2). In control experiments, no interaction was detected (<1 unit) with GAD-Snf1KDT210A in glucose-grown cells and both mutant GAD fusions were expressed at the same level; interaction with GAD-Snf1KD was similarly low in glucose (data not shown). Because the K84R mutation greatly increases the interaction of Reg1 with the isolated kinase domain, its effect on the interaction with the full-length Snf1 cannot be attributed simply to increased accessibility of the kinase domain within the complex. Thus, these findings suggest that Snf1 negatively regulates its own interaction with Reg1 via its catalytic function.

DISCUSSION

Previous studies implicated Reg1-PP1 in glucose repression of Snf1-dependent gene expression and in regulation of protein interactions within the Snf1 kinase complex. In glucose-grown cells, the Snf1 kinase complex exists predominantly in an inactive state, in which Snf1 is autoinhibited by the binding of its regulatory domain to its kinase domain; glucose-limiting conditions favor an active conformation of the complex, in which the Snf1 regulatory domain binds to the activating subunit Snf4 (23) (see Fig. 1). Mutation of *REG1* or *GLC7* leads to an active state of Snf1 and causes Snf4 to interact with Snf1 in both high and low glucose.

We have here examined the functional relationship of Reg1 to the Snf1 complex. We show first that Reg1 interacts with the Snf1 kinase domain in the two-hybrid system, indicating that Reg1 regulates the Snf1 complex by a direct mechanism, rather than through a cascade of phosphorylation/dephosphorylation events. Moreover, the two-hybrid interaction between Reg1 and Snf1 increases in response to glucose limitation, suggesting that Reg1 acts to inhibit the function of Snf1 subsequent to activation of the kinase.

Although Reg1 interacts with the isolated Snf1 kinase domain, Reg1 appears to exert its regulatory effects only on the intact Snf1 kinase complex. A *reg1*Δ mutation relieves glucose repression of *SUC2* and *GAL1-lacZ* gene expression when Snf1 is intact but not when the isolated kinase domain provides Snf1 function. This result is most simply interpreted to mean that Reg1 exerts an inhibitory effect only in the presence of the regulatory domain; however, we cannot exclude the possibility that the substitution of Snf1KD for Snf1 unmasks glucose regulation conferred by an otherwise cryptic regulatory pathway. With this caveat, the ability of Snf1KD to confer regulated gene expression in a *reg1*Δ mutant further suggests that Snf1KD receives a glucose signal by a pathway that is independent of Reg1.

Evidence indicates that Reg1 affects Snf1 function by modulating protein interactions within the kinase complex. Mutation of *REG1* promotes an active state of the complex, in which Snf4 binds to the Snf1 regulatory domain, even in glucose-grown cells (23). Conversely, we show here that increased *REG1* dosage reduces the interaction between Snf1 and Snf4 in glucose-deprived cells, in parallel with reduced Snf1-dependent gene expression. Together, the effects of absence or overexpression of Reg1 suggest that Reg1 promotes autoinhibition of the kinase activity by the regulatory domain and/or inhibits interaction of Snf1 with Snf4.

These findings lead us to propose the following model for the role of Reg1 in regulating the Snf1 kinase complex. When the Snf1 complex is activated, Reg1 binds to the kinase domain and targets the PP1 catalytic subunit to the complex. Reg1-PP1 facilitates return of the kinase complex to the inactive conformation, by dephosphorylating one or more sites on Snf1 or another component of the complex; it is not clear whether this function is stimulated by glucose. Reg1 then dissociates from the inactive complex. This model can account for the *reg1*Δ mutant phenotype if activation of the kinase occurs at some low level (rather than not at all) in glucose-grown cells. In a *reg1*Δ mutant, inactivation of the complex is impaired; thus, once activated, the kinase complex tends to remain in the active conformation, thereby causing a mutant phenotype in glucose-grown cells.

The interaction of Reg1 with Snf1 depends on T210, a conserved threonine in the T-loop. This residue is phosphorylated during activation of many kinases (25) and is essential for activity of both the intact Snf1 and the isolated kinase domain (24). We show here that the T210A mutation prevents interaction of Snf1 with Reg1. In contrast, the K84R mutation in the ATP-binding site of Snf1 did not do so, thereby excluding a simple requirement for Snf1 catalytic activity. The T210A mutation also abolished the two-hybrid interaction between Snf1 and Snf4 in response to glucose limitation. Together, these findings strongly suggest that phosphorylation of T210 is required to activate the Snf1 catalytic activity, promote interaction with Reg1, and induce conformational changes in the kinase complex.

We also report evidence that the Snf1 catalytic activity has a role in regulating the interaction between Snf1 and Reg1. The K84R mutation both enhanced the interaction with Reg1 and relieved inhibition by glucose, suggesting that Snf1 negatively regulates its own interaction with Reg1. The mechanism may entail phosphorylation of Reg1; however, the instability of Reg1 (27) hampers efforts to assess its phosphorylation state. The Snf1 catalytic activity is not required for Reg1 to affect protein interactions within the kinase complex because the K84R mutation did not mimic the effect of *reg1*Δ in relieving glucose inhibition of two-hybrid interactions between Snf1K84R and Snf4 (R.J., O. Vincent, and M.C., unpublished results).

These genetic findings implicate a Snf1 kinase kinase in the phosphorylation of T210. The different results obtained with the T210A and K84R mutants imply that T210 is not autophosphorylated. The K84R mutation eliminates Snf1 catalytic activity but does not cause the same phenotype as the T210A mutation, which prevents phosphorylation of this site. The corresponding threonine residue of the mammalian homolog AMPK is phosphorylated by AMPK kinase (44); moreover, both AMPK kinase and a factor present in yeast extracts are able to reactivate Snf1 after treatment with mammalian protein phosphatase 2A (12, 13). We therefore think it likely that a Snf1 kinase kinase exists in yeast and that its activity is elevated in response to glucose limitation. The observed regulation of the kinase complex could be achieved solely by modulating the activity of a Snf1 kinase kinase; however, it is also possible that dephosphorylation of T210, by Reg1-PP1 or by another phosphatase, is regulated by glucose.

It is interesting that Snf1KD alone is sufficient for partial Snf1 function, independent of Snf1RD, Snf4, or Reg1. Why is the native Snf1 complex so elaborate? Some proteins in the complex, in particular the Sip1/Sip2/Gal83 family, most likely mediate association of Snf1 with specific substrates or target the kinase to specific intracellular locations (17–20). We speculate that the regulated protein interactions within the complex serve to amplify the regulatory signals controlling kinase activity. The role of the Snf1 catalytic activity in controlling the association of Reg1 with Snf1 may represent a mechanism to fine tune the regulation.

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