Std1p (Msn3p) Positively Regulates the Snf1 Kinase in *Saccharomyces cerevisiae*

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ABSTRACT

The Snf1 protein kinase of the glucose signaling pathway in *Saccharomyces cerevisiae* is regulated by an autoinhibitory interaction between the regulatory and catalytic domains of Snf1p. Transitions between the autoinhibited and active states are controlled by an upstream kinase and the Reg1p-Glc7p protein phosphatase 1. Previous studies suggested that Snf1 kinase activity is also modulated by Std1p (Msn3p), which interacts physically with Snf1p and also interacts with glucose sensors. Here we address the relationship between Std1p and the Snf1 kinase. Two-hybrid assays showed that Std1p interacts with the catalytic domain of Snf1p, and analysis of mutant kinases suggested that this interaction is incompatible with the autoinhibitory interaction of the regulatory and catalytic domains. Overexpression of Std1p increased the two-hybrid interaction of Snf1p with its activating subunit Snf4p, which is diagnostic of an open, uninhibited conformation of the kinase complex. Overexpression of Std1p elevated Snf1 kinase activity in both *in vitro* and *in vivo* assays. These findings suggest that Std1p stimulates the Snf1 kinase by an interaction with the catalytic domain that antagonizes autoinhibition and promotes an active conformation of the kinase.

THE Snf1 kinase of the yeast *Saccharomyces cerevisiae* is a member of the Snf1/AMP-activated protein kinase family, which is highly conserved in eukaryotes and is important for responses to metabolic stress (Hardie *et al.* 1998; Kemp *et al.* 1998). In yeast, Snf1 has a primary role in the adaptation of cells to glucose limitation, regulating both the transcription of metabolic genes and the activity of metabolic enzymes (Gancedo 1998; Carlson 1999). Snf1 also affects developmental processes such as meiosis and sporulation, filamentation and invasive growth (Cullen and Sprague 2000; Kuchin *et al.* 2002), and life span and aging (Ashrafi *et al.* 2000).

The Snf1 kinase complex contains the catalytic (α) subunit Snf1p, the stimulatory (γ) subunit Snf4p, and one of the three β-subunits, Sip1p, Sip2p, or Gal83p; the β-subunit maintains the association of Snf1p and Snf4p in the complex, mediates interactions with downstream targets, and regulates the subcellular localization of the kinase (Yang *et al.* 1994; Jiang and Carlson 1997; Vincent and Carlson 1999; Schmidt and McCartney 2000; Vincent *et al.* 2001). The signals controlling Snf1 activity in response to glucose availability are not understood, although the AMP:ATP ratio may play a role under some conditions (Wilson *et al.* 1996; Gancedo 1998). An upstream kinase phosphorylates the conserved threonine residue in the activation loop (T210) and thereby activates Snf1 (Woods *et al.* 1994; Wilson *et al.* 1996; Ludin *et al.* 1998; McCartney and Schmidt 2001). The Reg1p-Glc7p protein phosphatase 1 (PP1) negatively regulates Snf1 function (Ludin *et al.* 1998; Sanz *et al.* 2000).

Previous studies showed that the Snf1 kinase complex undergoes glucose-regulated conformational changes (Jiang and Carlson 1996; Figure 1). Growth in abundant glucose favors a conformation in which the negative regulatory domain of Snf1p inhibits the catalytic domain and binding of Snf4p to Snf1p is excluded. Phosphorylation of T210 is required both to open the catalytic cleft and to relieve this autoinhibition (Estruch *et al.* 1992; Ludin *et al.* 1998). During conditions of glucose depletion or growth in alternate carbon sources, when T210 is predominantly phosphorylated (McCartney and Schmidt 2001), Snf4p binds to the regulatory domain and sequesters it from the catalytic domain (Celenza and Carlson 1989; Jiang and Carlson 1996). PP1 facilitates the transition back to an autoinhibited conformation (Ludin *et al.* 1998; Sanz *et al.* 2000).

In addition to the above mechanisms for regulation of Snf1 kinase activity, the homologous proteins encoded by the *STD1* (*MSN3*) and *MTH1* (*HTR1*, *DGT1*, *BPC1*, and *GSF1*) genes have been implicated in regulation of Snf1 function. *STD1* was identified as a multicopy suppressor of defects caused by overexpression of a truncated TATA-binding protein (TBP; Ganster *et al.* 1993), and further studies supported interaction between Std1p and TBP (Tillman *et al.* 1995). *STD1* was also recovered as a multicopy suppressor (*MSN3*) of defects caused by a deficiency in Snf1 kinase activity; overexpression of Std1p restored *SUC2* (invertase) expression in a *snf4*Δ mutant and also partially relieved glucose repression of *SUC2* in wild-type cells (Hubbard *et al.* 1994). Overexpression of the homolog Mth1p had minor effects. The

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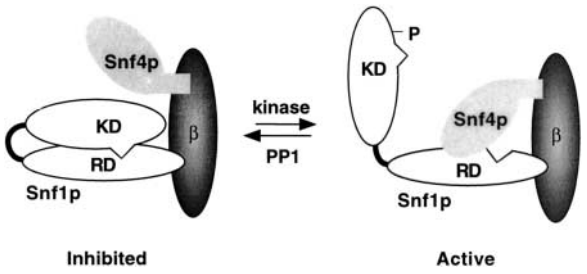


FIGURE 1.—Model of the Snf1 kinase. When the Snf1 kinase is in its inactive conformation, the catalytic domain of Snf1p (KD) is autoinhibited by the negative regulatory domain (RD). Phosphorylation of T210 by an unidentified upstream kinase promotes the opening of the catalytic cleft and also favors an active conformation of the complex in which the Snf4p activating subunit binds to the regulatory domain of Snf1p, thereby sequestering it from the catalytic domain. In cells grown in high concentrations of glucose, the Snf1 kinase is predominantly in an unphosphorylated, inactive conformation. Glucose depletion or growth in alternate carbon sources favors the active conformation of the kinase. PP1 (Reg1p-Gcl7p) has a role in dephosphorylation of Snf1. β, β-subunit Gal83, Sip1, or Sip2.

double deletion of *STD1* and *MTH1* reduced derepression of *SUC2* a fewfold, indicating that these proteins do not bear primary responsibility for control of Snf1 activity. Nonetheless, Std1p interacted with Snf1p by both two-hybrid and glutathione *S*-transferase (GST) pull-down assays (HUBBARD *et al.* 1994), and genetic evidence suggested that Std1p functions upstream of the Snf1 kinase, as effects of its overexpression require Snf1p (HUBBARD *et al.* 1994; SCHMIDT *et al.* 1999). Snf1p is also required for the relief of glucose repression caused by a mutant allele of *MTH1* (*gsf1-1*; SHERWOOD and CARLSON 1997; WIATROWSKI and CARLSON 2001) but not for other regulatory effects of Mth1p (SCHMIDT *et al.* 1999).

Std1p and Mth1p also have roles in one of the glucose signaling pathways. These proteins interact with the glucose sensors Snf3p and Rgt2p in the two-hybrid system and regulate the expression of *HXT* (hexose transporter) genes in response to glucose signals (OZCAN *et al.* 1993; GAMO *et al.* 1994; SCHMIDT *et al.* 1999; LAFUENTE *et al.* 2000; SCHULTE *et al.* 2000). This regulation of glucose transporters may account for some of the effects of Std1p overexpression on the Snf1 kinase pathway; however, the physical interaction of Std1p with Snf1p suggests that Std1p functions directly to elevate the kinase activity. Moreover, various defects of the *std1*Δ *mth1*Δ double mutant are not easily attributable to altered glucose transporter expression, such as impaired growth on galactose and glycerol, reduced derepression of invertase activity, and a defect in sporulation of the homozygous diploid (HUBBARD *et al.* 1994). Mutants lacking Snf1 kinase activity exhibit similar, but more severe, phenotypes, consistent with the possibility that the *std1*Δ *mth1*Δ mutant is partially impaired for Snf1 function.

Here we address the relationship between Std1p and the Snf1 kinase. We show that Std1p interacts directly with the catalytic domain of Snf1p and stimulates kinase activity. We present evidence that the regulatory mechanism involves antagonism of the autoinhibitory interaction of the Snf1p regulatory and catalytic domains.

MATERIALS AND METHODS

Strains: *S. cerevisiae* strains used here were CTY10-5d (*MAT***a** *ade2 his3 leu2 trp1 URA3::lexAop-lacZ gal4 gal80*), TAT7 (*MAT***a** *ade2 his3 leu2 trp1 ura3::lexAop-lacZ LYS2::lexAop-HIS3 gal80*), FY250 (*MAT*α *his3*Δ*200 leu2*Δ*1 trp1*Δ*63 ura3-52*) and MCY2635 (*MAT***a** *snf4-*Δ*2 ura3-52 his3*Δ*200*). Cultures were grown in synthetic complete (SC) medium (ROSE *et al.* 1990) lacking appropriate supplements to maintain selection for plasmids.

Plasmids: Plasmids used in this study are listed in Table 1. pSK79 contains the *STD1 Bam*HI fragment from pJH125 (HUBBARD *et al.* 1994) cloned into pACTII. pSK88, which expresses triple HA-tagged Mth1p (HA_3-Mth1) from the *ADH1* promoter, was constructed by cloning a *Bam*HI PCR fragment containing the *MTH1* coding sequence into vector pWS93 (SONG and CARLSON 1998). pSK89 is the same except that the *URA3* marker was replaced with *TRP1*. pSK92 was derived from pSK89 by replacing *MTH1* with the *STD1 Bam*HI fragment from pJH125. pSK93 is the self-ligated backbone of pSK89 and serves as the vector control.

β-Galactosidase assays: Filter lift assays for blue color were performed as described previously (JIANG and CARLSON 1997). At least five independent transformants were grown on selective SC medium containing 2% glucose for 1 day at 30°, and cells were then transferred to filters for assay of β-galactosidase activity. Color was allowed to develop for 80–150 min. For quantitative assays, cultures were grown to exponential phase in SC + 2% glucose; β-galactosidase activity was assayed in permeabilized cells and was expressed in Miller units (ROSE *et al.* 1990).

Immune complex kinase assays and immunoblots: Preparation of protein extracts, immunoprecipitation, and immune complex kinase assays were performed as described previously (TREITEL *et al.* 1998). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For immunoblot analysis, polyclonal anti-LexA antibodies (Invitrogen), monoclonal anti-HA (12CA5) antibodies, and ECL Plus reagents (Amersham) were used.

RESULTS

Std1p interacts with the catalytic domain of Snf1p: We first used the two-hybrid system to investigate the requirements for physical interaction between the Snf1 kinase and Std1p. Snf1p is the subunit of the kinase that interacts with Std1p, as no interaction was detected with Snf4p in two-hybrid or GST pull-down assays (HUBBARD *et al.* 1994) or with any of the three β-subunits, Gal83p, Sip1p, or Sip2p (data not shown). To determine whether the catalytic activity of Snf1p is required for interaction, we examined the interactions of mutant LexA-Snf1 proteins with a Gal4p activation domain (GAD) fusion to Std1p (Figure 2A). In filter lift assays for blue color, GAD-Std1 interacted well with the mutant LexA-Snf1-K84R, which has a substitution of the conserved lysine in the ATP-binding site (CELENZA and

TABLE 1

Plasmids used in this study

Plasmid	Expressed protein and vector	Reference
pIT469	LexA-Snf1; pEG202	Kuchin *et al.* (2000)
pRJ55	LexA-Snf1; pLexA(1-202)+PL	Jiang and Carlson (1996)
pRJ215	LexA-Snf1-K84R; pEG202	This lab
pRJ217	LexA-Snf1-T210A; pEG202	This lab
pRJ191	LexA-RD[a]; pLexA(1-202)+PL	This lab
pRJ189	LexA-KD[b]; pLexA(1-202)+PL	This lab
pRJ218	LexA-KD-K84R; pEG202	This lab
pRJ220	LexA-KD-T210A; pEG202	This lab
pRJ216	LexA-Snf1-G53R; pEG202	Kuchin *et al.* (2000)
pSK79	GAD-Std1; pACTII	This study
pSK89	HA_3-Mth1; pSK93	This study
pSK92	HA_3-Std1; pSK93	This study
pNI12	Snf4-GAD	Fields and Song (1989)

[a] RD, Snf1 regulatory domain (residues 392–633).
[b] KD, Snf1 catalytic domain (residues 1–392).

Carlson 1986), indicating that the interaction does not depend on Snf1p catalytic activity. However, no color appeared during the incubation period when GAD-Std1 was present in combination with LexA-Snf1-T210A, which is mutated at the conserved threonine residue in the activation loop, a site of phosphorylation that is necessary for kinase function (Estruch *et al.* 1992). Control experiments confirmed that LexA-Snf1-T210A is expressed and confers blue color in combination with a GAD fusion protein containing Gal83p sequences (data not shown). β-Galactosidase is stable, so the filter lift assay detects interactions that occur during the initial growth of the cells on high (2%) glucose or during the later stages of growth when cells become limited for glucose. Because overexpressed Std1p relieves glucose repression, we also assayed β-galactosidase activity quantitatively for key transformants during exponential growth in 2% glucose; activity was higher for LexA-Snf1 and LexA-Snf1-K84R than for LexA-Snf1-T210A (Figure 2A; see also Figure 2B).

The inactive Snf1-K84R and Snf1-T210A kinases differ with respect to their ability to undergo glucose-regulated conformational changes; the T210A mutation impairs the release of autoinhibition and the binding of Snf4 to the regulatory domain (Jiang and Carlson 1996; Ludin *et al.* 1998; Figure 1). Hence, the inability of GAD-Std1p to interact with LexA-Snf1-T210A suggests that Std1p does not bind to Snf1p in its autoinhibited state.

To map the region of Snf1p that interacts with Std1p, we used LexA fusions to the Snf1p catalytic domain (KD; residues 1–392) and the negative regulatory domain (RD; residues 392 to the end). In the filter assay, GAD-Std1 interacted with LexA-KD but not with LexA-RD (Figure 2A). GAD-Std1 also interacted with the mutant LexA-KD-K84R, confirming that catalytic activity is not required. Moreover, GAD-Std1 interacted with LexA-KD-T210A, indicating that in the absence of the Snf1p regulatory domain, interaction does not require T210 or its phosphorylation. In quantitative assays of cells growing in 2% glucose, β-galactosidase activity was not affected by the T210A mutation in LexA-KD (Figure 2B).

Because T210 is required for interaction of Std1p with the intact Snf1p, but not with the isolated catalytic domain, these results strongly suggest that Std1p interacts with the intact Snf1 kinase complex in its open conformation, in which the catalytic domain is not inhibited by the regulatory domain.

Std1p overexpression increases the two-hybrid interaction of Snf1p with its activating subunit Snf4p during growth in glucose: We next assayed the effects of Std1p on the two-hybrid interaction of Snf1p with Snf4p, which is diagnostic of the open conformation of the kinase complex (Figure 1). Although native Snf1p and Snf4p remain associated in a complex due to the β-subunit (Jiang and Carlson 1997), the two-hybrid interaction between Snf1p and Snf4p fusion proteins is inhibited by glucose and is strongly detected only under conditions that promote kinase activity, such as glucose depletion or growth in alternate carbon sources (Jiang and Carlson 1996). We first assayed the interaction of LexA-Snf1 and Snf4-GAD in strains overexpressing Std1p from the *ADH1* promoter (Table 2). In glucose-grown cells, overexpression of Std1p substantially increased the interaction of Snf1p and Snf4p, causing a 200-fold increase in β-galactosidase activity relative to control strains (110 *vs.* 0.5 units). Overexpression of Std1p had little effect on the strong Snf1p-Snf4p interaction observed in raffinose-grown cells. However, when cells were first grown in high glucose and then shifted to low (0.05%) glucose for 3 hr, values were higher in cells overexpressing Std1p (650 units compared to 170 for the vector control), suggesting that Std1p facilitates the increase that occurs in response to glucose limitation. These findings support the view that Std1p overexpres-

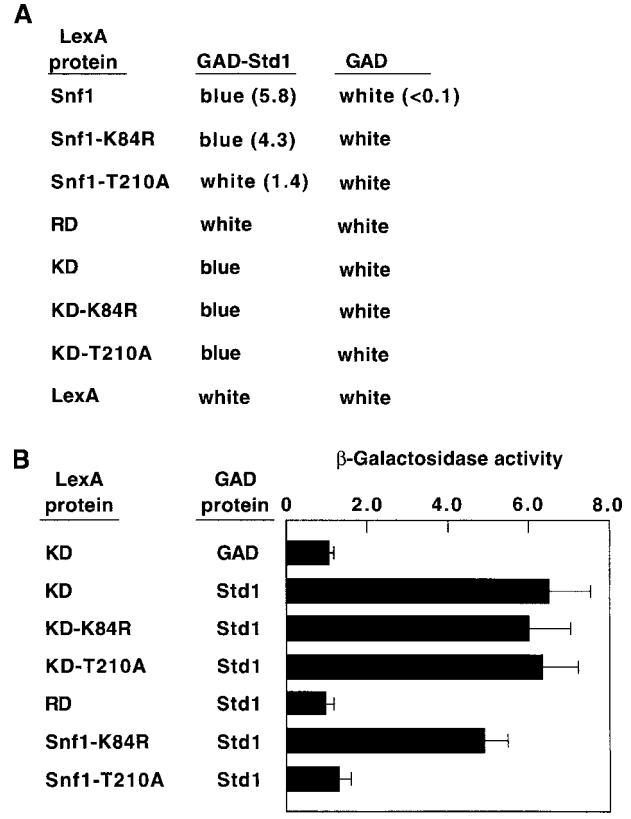


FIGURE 2.—Two-hybrid interactions of Std1p with wild-type and mutant Snf1p proteins. (A) Strain CTY10-5d was transformed with plasmids expressing the indicated proteins (see Table 1). LexA-Snf1 was expressed from pIT469. At least five independent transformants were assayed for β-galactosidase activity using the filter lift assay for blue color. Transformants were grown on selective SC medium containing 2% glucose for 1 day at 30°; during this time, cells in the patch become limited for glucose. Cells were then transferred to filters for assay. Since β-galactosidase is stable, two-hybrid interactions that occur during growth in either high or low glucose are detected. In further control experiments, LexA-RD and LexA-Snf1-T210A interacted with GAD-Gal83$_{198\text{-}417}$ (JIANG and CARLSON 1997), and LexA-RD interacted with Snf4-GAD, thereby confirming the expression of the LexA fusion proteins. Numerical values are average β-galactosidase activity for five transformants grown to mid-log phase in selective SC medium with 2% glucose. (B) Strain FY250 carrying the *lexAop-lacZ* reporter pSH18-18 (gift of S. Hanes, Wadsworth Center; KUCHIN *et al.* 2000) was transformed with plasmids expressing the indicated hybrid proteins. FY250 is a strain of the same S288C background that was used for analysis of *STD1*-related phenotypes (HUBBARD *et al.* 1994). Values are average activity for five transformants, grown as in A.

sion promotes a conformation of the kinase complex that is open rather than autoinhibited.

We also assayed the interaction of LexA-Snf1 and Snf4-GAD in *std1*Δ and *std1*Δ *mth1*Δ mutants, and interaction was glucose-regulated as in the wild type (data not shown), indicating that the presence of Std1p at its native levels is indistinguishable from the absence of Std1p. Thus, the effects of Std1p depend on its overexpression at levels comparable to those of Snf1p (both expressed from the *ADH1* promoter). This dosage sensitivity supports the idea that Std1p influences the Snf1p-Snf4p interaction by direct binding to Snf1p.

TABLE 2

Effects of overexpression of Std1p and Mth1p on the two-hybrid interaction of Snf1p and Snf4p

Hybrid proteins	Expressed protein	2% glucose	2% raffinose
LexA-Snf1 + Snf4-GAD	None	0.5	140
	Std1p	110	190
	Mth1p	5.7	220
LexA-Snf1 + GAD	None	0.1	6.0
	Std1	3.9	28
	Mth1p	0.4	36
LexA + Snf4-GAD	None	0.2	1.6
	Std1p	1.8	2.0
	Mth1p	2.0	1.9

Strain CTY10-5d was transformed with plasmids expressing the indicated hybrid proteins and plasmids expressing HA-Std1 or HA-Mth1 from the *ADH1* promoter or no protein (None; vector pSK93). LexA-Snf1 was expressed from pRJ55. Transformants were grown selectively to mid-log phase in SC medium containing 2% glucose or 2% raffinose. Values are average β-galactosidase activity for five transformants. Standard errors were 5–20%. Additional control transformants expressing LexA + GAD with Std1p or Mth1p gave values essentially the same as those shown for LexA + Snf4-GAD.

Std1p overexpression increases Snf1 activity in immune complex kinase assays: The effects of Std1p overexpression on the two-hybrid interaction of Snf1p and Snf4p suggested that Std1p correspondingly stimulates kinase activity. We used an immune complex kinase assay to assess the effects of overexpressed Std1p on Snf1 kinase activity *in vitro*; previous studies did not detect any effect of *STD1* in multicopy (HUBBARD *et al.* 1994), so in this experiment we overexpressed HA-Std1 from the *ADH1* promoter. Protein extracts were prepared from cells expressing LexA-Snf1 and either HA-Std1 or no protein (vector alone). LexA-Snf1 was immunoprecipitated with anti-LexA, and the precipitated proteins were incubated in kinase buffer in the presence of [γ-^{32}P]ATP. Overexpression of HA-Std1 increased the phosphorylation of the β-subunits Sip1 and Gal83 (Sip2 was not detected) and the phosphorylation of LexA-Snf1 (Figure 3). Control experiments with LexA-Snf1-K84R confirmed that Snf1 kinase activity was responsible for phosphorylation of the β-subunits; this mutant kinase has some residual autophosphorylation activity. Immunoblot analysis of the precipitated proteins also confirmed that amounts of the LexA protein were comparable in all lanes.

Std1p stimulates Snf1 kinase activity in an *in vivo* assay: We also used an *in vivo* assay to assess the effects of Std1p on Snf1 kinase activity. We previously showed that a catalytically hyperactive kinase, LexA-Snf1-G53R,

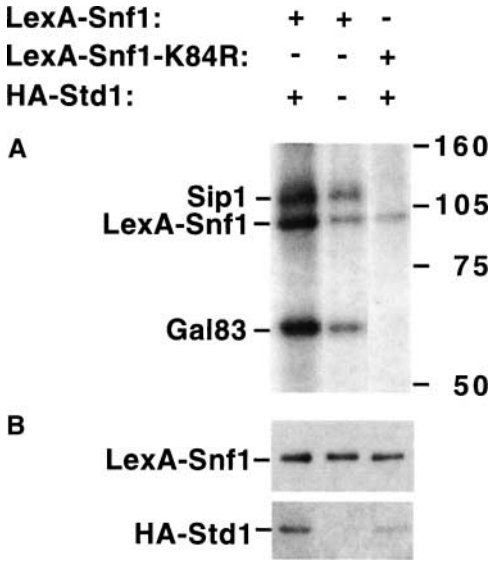
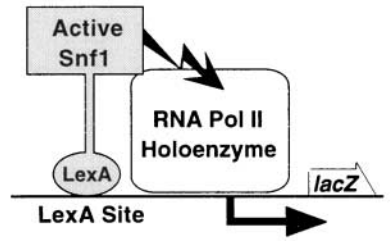


FIGURE 3.—Immune complex kinase assay. Strain FY250 expressed LexA-Snf1 or LexA-Snf1-K84R from pIT469 and pRJ215, respectively, and either expressed HA-Std1 from pSK92 or carried the vector pSK93. Transformants were grown in selective SC + 2% glucose, and protein extracts were prepared. Proteins were immunoprecipitated from extracts (100 μg) with polyclonal anti-LexA. (A) Half of the immunoprecipitate was incubated in kinase buffer containing [γ-^{32}P]ATP, and proteins were separated by 8% SDS-PAGE and subjected to autoradiography. Because Gal83p and HA-Std1 migrate close together, part of the kinase reaction was subjected to extended SDS-PAGE on a 10% gel, and proteins were transferred to a membrane. The membrane was exposed for autoradiography and then probed with anti-HA. This experiment verified that Gal83p, but not HA-Std1, is phosphorylated (data not shown). (B) Another portion of the immunoprecipitate was analyzed by immunoblotting with anti-LexA and anti-HA to detect LexA-Snf1 and HA-Std1, respectively. Molecular size standards are in kilodaltons.

A



B

LexA protein	Expressed protein	β-Galactosidase activity (U) 2% Glu	Shift to 0.05% Glu
LexA-Snf1	None	0.1	2.0
	Std1p	3.9	67
	Mth1p	0.4	43
LexA	None	0.3	1.7
	Std1p	0.9	2.3
	Mth1p	1.3	6.1
LexA-Snf1-G53R	None	5.2	120
	Std1p	29	110

FIGURE 4.—Stimulation of reporter transcription by LexA-Snf1 is enhanced by overexpression of Std1p and Mth1p. (A) The assay for kinase activity entails binding of LexA-Snf1 to LexA sites 5′ to the promoter of a *lacZ* reporter. Previous studies showed that when the kinase is sufficiently active, transcription of the reporter is stimulated, dependent on the LexA sites (KUCHIN *et al.* 2000). (B) Strains CTY10-5d and TAT7, carrying the reporter, were transformed with plasmids expressing the indicated proteins from the *ADH1* promoter. LexA-Snf1 was expressed from pIT469. TAT7 was used for experiments with LexA-Snf1-G53R. Control transformants carrying the vector pSK93 expressed no protein (None). CTY10-5d transformants also carried the vector pACTII. Transformants were grown selectively to mid-log phase in SC medium containing 2% glucose, and then half of each culture was shifted to 0.05% glucose for 3 hr. Values are average β-galactosidase activity for five transformants (four for LexA-Snf1-G53R), and standard errors were usually <20%.

activates transcription of a *lacZ* reporter containing LexA-binding sites in response to glucose limitation (KUCHIN *et al.* 2000; Figure 4A). Snf1-G53R carries a Gly-to-Arg substitution N-terminal to the kinase domain; this Arg residue, which is found in some plant Snf1 orthologs, increases the catalytic activity of the kinase but does not significantly affect its regulation by the glucose signal (ESTRUCH *et al.* 1992). Assays of the double mutant LexA-Snf1-G53R,K84R showed that transcription of the reporter depends on Snf1 catalytic activity, and the less active wild-type protein LexA-Snf1 exhibited little or no function in this assay (KUCHIN *et al.* 2000). Several lines of evidence suggested that the Snf1 kinase interacts directly with the RNA polymerase II holoenzyme.

We used this assay to assess the effects of Std1p overexpression on Snf1 activity *in vivo* (Figure 4B). LexA-Snf1, expressed from the *ADH1* promoter, did not significantly stimulate transcription of the reporter, as found previously. However, when Std1p was overexpressed, LexA-Snf1 caused low-level expression of the reporter in glucose-grown cells and substantial expression in response to glucose depletion (67 units of β-galactosidase activity). β-Galactosidase activity was similarly elevated in raffinose-grown cells (28 units; see Table 2). LexA-Std1 alone does not activate transcription of a reporter, indicating that recruitment of Std1p to the promoter is not sufficient. Assays of LexA-Snf1-K84R confirmed the dependence on kinase activity, and the Gal83p subunit of the kinase was required (data not shown), as found previously for LexA-Snf1-G53R (VINCENT *et al.* 2001). Thus, overexpression of Std1p causes LexA-Snf1 to behave like the hyperactive kinase LexA-Snf1-G53R.

Overexpression of Std1p also affected LexA-Snf1-G53R function. In glucose-grown cells, activation of the reporter was increased sixfold; however, activity in glu-

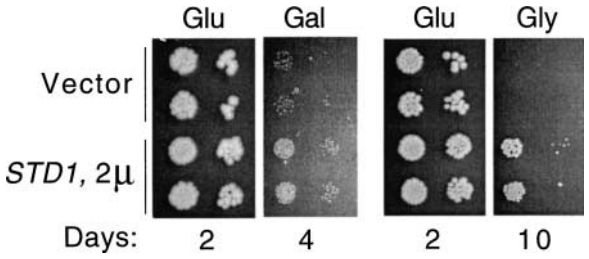


FIGURE 5.—Overexpression of Std1p improves growth of a *snf4*Δ mutant on galactose and glycerol. Strain MCY2635 (*snf4*Δ) was transformed with pJH68, a multicopy 2μ plasmid carrying *STD1* expressed from its native promoter (HUBBARD *et al.* 1994), and the vector Yep24. Two independent transformants were spotted, with 10-fold dilutions, onto rich medium (YEP) containing 2% glucose (Glu), 2% galactose (Gal), or 3% glycerol (Gly). Plates were photographed after incubation at 30° for the indicated number of days. For comparison of growth on glucose and galactose, the plates were incubated under anaerobic conditions.

cose-limited cells, already high, was not further elevated (Figure 4B). In an *std1*Δ *mth1*Δ double mutant, activation by LexA-Snf1-G53R was not impaired (data not shown), consistent with the preceding evidence that when Snf1p is overexpressed, the absence of Std1p is indistinguishable from its presence at native levels.

Overexpression of Std1p suppresses *snf4*Δ mutant defects in growth on galactose and glycerol: Previous studies showed that the *std1*Δ *mth1*Δ mutant exhibits defects in Snf1-dependent processes that are not related to glucose transport (HUBBARD *et al.* 1994). The evidence presented above for a direct role for Std1p in regulating the Snf1 kinase is based on assays using overexpressed Std1p. To confirm that overexpressed Std1p also affects phenotypes that are dependent on Snf1 kinase activity but independent of glucose transport, we examined growth on galactose and glycerol. A *snf4*Δ mutant, which lacks the activating subunit of the kinase, is defective in utilization of both carbon sources. Transformation of a *snf4*Δ mutant with a multicopy plasmid carrying *STD1* improved growth on both galactose and glycerol (Figure 5).

Std1p and Mth1p have distinct functional relationships to the Snf1 kinase: Std1p and Mth1p are very similar proteins (61% sequence identity) that have related but distinct functions in glucose signaling and control of *SUC2* and *HXT* expression (HUBBARD *et al.* 1994; SCHMIDT *et al.* 1999; LAFUENTE *et al.* 2000; SCHULTE *et al.* 2000). To assess the relationship of Mth1p to the Snf1 kinase, we tested Mth1p in the assays used above. No significant two-hybrid interaction between LexA-Snf1 and GAD-Mth1 was detected (data not shown). We assayed the interaction between LexA-Snf1 and Snf4-GAD in strains overexpressing Mth1p and found that Mth1p had a modest effect compared to that of Std1p (Table 2); β-galactosidase activity in glucose-grown cells was 5.7 units, which was 10-fold elevated relative to that in the vector control but was much lower than the activity in cells overexpressing Std1p (110 units). We also assayed the effect of overexpression of Mth1p on the ability of LexA-Snf1 to activate transcription of a reporter with LexA-binding sites. Again, in glucose-grown cells, the effect of Mth1p was modest compared to that of Std1p (0.4 and 3.9 units, respectively, relative to 0.1 units for the control; Table 2); however, the ability of LexA-Snf1 to activate the reporter in response to glucose limitation was enhanced to a similar extent by overexpression of Mth1p and Std1p (43 and 67 units, respectively). Thus, Std1p and Mth1p have somewhat different physical and functional relationships to the Snf1 kinase.

DISCUSSION

Previous studies have implicated Std1p in the Snf1 kinase glucose signaling pathway. In multicopy, the *STD1* gene both restored *SUC2* expression in a *snf4*Δ mutant, which is deficient in Snf1 kinase activity, and relieved glucose repression of *SUC2* in the wild type. Std1p and Snf1p interacted physically in two-hybrid and biochemical assays, suggesting that Std1p directly affects Snf1 function. Here we present evidence that Std1p stimulates Snf1 kinase activity by a mechanism involving an interaction with the catalytic domain that is antagonistic to the autoinhibitory interaction of the Snf1p regulatory and catalytic domains (Figure 6).

We first showed that Std1p interacts with the catalytic domain of Snf1p, independent of its catalytic activity. We also found that Std1p did not interact with Snf1-T210A, in which the catalytic domain is always inhibited by the regulatory domain, suggesting that the interactions of the Snf1p catalytic domain with its regulatory domain and with Std1p are mutually exclusive (Figure 6). These findings further suggest that Std1p promotes an active, uninhibited conformation of the kinase complex by binding to the catalytic domain. Several other assays supported the view that Std1p overexpression promotes an active conformation. In glucose-grown cells, overexpression of Std1p increased the two-hybrid interaction of Snf1p and Snf4p, which reflects the open conformation in which Snf4p binds the regulatory domain. Assays of Snf1 kinase activity *in vitro* and assays of the ability of LexA-Snf1 to stimulate reporter transcription *in vivo* both indicated that overexpression of Std1p elevates Snf1 kinase activity. Previous evidence that overexpression of Std1p relieves glucose repression of *SUC2* (HUBBARD *et al.* 1994) can also be accounted for by increased Snf1 activity.

The present findings are consistent with the isolation of *STD1* as a multicopy suppressor of *snf4*Δ (HUBBARD *et al.* 1994). Increased Std1p would compensate for the absence of Snf4p if the binding of Std1p to the catalytic domain excludes the regulatory domain. Also consistent is evidence that *std1*Δ exacerbates the leaky *snf4*Δ mu-

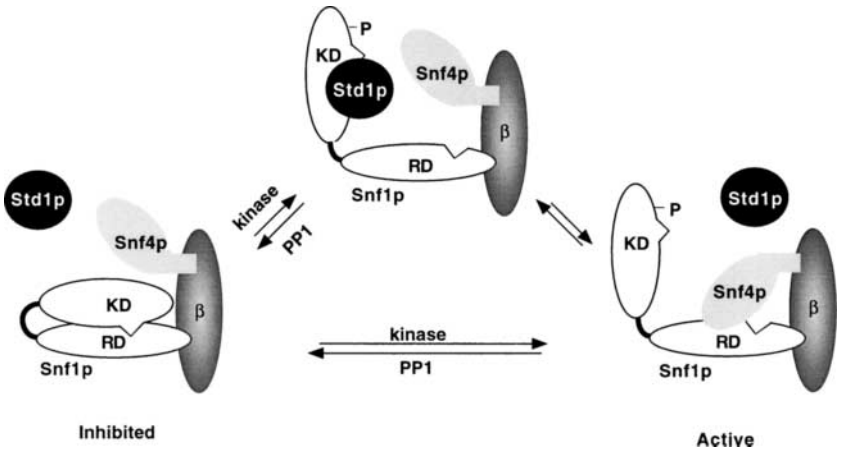


FIGURE 6.—Model for the interaction of Std1p with the Snf1 kinase. The kinase is represented as in Figure 1. Evidence presented in the text supports a model in which Std1p binds to the catalytic domain to facilitate formation of an active conformation. Std1p is not a stable, stoichiometric component of the complex. We have depicted Snf1p as bound to either Std1p or Snf4p, but it is possible that Snf1p can bind both Std1p and Snf4p at the same time.

tant phenotype observed at 23°, further reducing invertase activity 20-fold (HUBBARD *et al.* 1994).

What is the role of Std1p when it is expressed from its chromosomal locus? Because Std1p is not a stoichiometric component of the Snf1 kinase, it seems likely that Std1p promotes the establishment of an active conformation, while Snf4p has the primary role in maintenance (Figure 6). However, it remains possible that Std1p is stoichiometrically associated with certain subpopulations of the Snf1 kinase.

Genetic studies on the interactions of Std1p with glucose sensors are in accord with our findings (SCHMIDT *et al.* 1999; LAFUENTE *et al.* 2000). When cells are grown in high glucose, Std1p is thought to bind glucose sensors, primarily Rgt2p; when cells are grown in low or no glucose, Std1p is released and is free to interact with other proteins. Hence, when expressed from its native promoter, Std1p positively modulates Snf1 kinase activity only when cells are grown in low or no glucose. In contrast, overexpression of Std1p results in excess Std1p that is not sequestered by binding to glucose sensors and is therefore available to bind Snf1p even in glucose-grown cells.

The regulatory effects of Std1p in response to glucose signals are complex because Std1p not only modulates Snf1 kinase activity but also regulates the expression of hexose transporters (SCHMIDT *et al.* 1999; LAFUENTE *et al.* 2000). Hence, Std1p may also exert indirect effects on Snf1p activity as a consequence of its role in regulating glucose transport. Conversely, some effects of Std1p on glucose transport require Snf1p, such as the repression of *HXT1-lacZ* by overexpressed Std1p (SCHMIDT *et al.* 1999). Some effects of Std1p on Snf1-dependent phenotypes, however, are independent of glucose transport: overexpression of Std1p improves growth of a *snf4*Δ mutant on galactose and glycerol, whereas the *std1*Δ *mth1*Δ double mutation impairs growth on these carbon sources (HUBBARD *et al.* 1994).

The interaction of Std1p with Snf1 described here represents one of several mechanisms that contribute to the regulation of Snf1 kinase function. The kinase activity is regulated by autoinhibition (JIANG and CARLSON 1996), an upstream kinase (WOODS *et al.* 1994; WILSON *et al.* 1996; MCCARTNEY and SCHMIDT 2001), and the Reg1p-Glc7p PP1 (LUDIN *et al.* 1998; SANZ *et al.* 2000), and the β-subunits of the kinase regulate its subcellular localization (VINCENT *et al.* 2001). While the comparatively minor defects of the *std1*Δ *mth1*Δ double mutant in some Snf1-dependent processes indicate that Std1p does not have an essential regulatory role, it is important to note that the Snf1 kinase is a central player in very complex responses to nutrient stress. Regulatory mechanisms that are not essential under some growth conditions may be crucial under other conditions or during transitions to new conditions.

Finally, Std1p has been shown to interact not only with Snf1p but also with proteins as diverse as TBP and the glucose sensors Snf3p and Rgt2p. Genetic evidence connects Snf1p to TBP (SHIRRA and ARNDT 1999), and regulatory interactions between Snf1p and glucose sensors can be easily envisioned. We therefore consider it possible that Std1p also regulates a subset of Snf1 complexes by other mechanisms, perhaps by targeting Snf1 to particular substrates or promoters.

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