

# Gal83 mediates the interaction of the Snf1 kinase complex with the transcription activator Sip4

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**The Snf1/AMPK protein kinase family is widely conserved in eukaryotes. In *Saccharomyces cerevisiae*, the Snf1 kinase is an essential element of the glucose response pathway and has diverse regulatory roles. The Snf1 complex contains one of the related proteins Sip1, Sip2 and Gal83, which are also conserved in higher eukaryotes. Previous studies showed that the Sip1/Sip2/Gal83 component plays a structural role in the complex. We present evidence that this component also mediates the interaction of the Snf1 kinase complex with specific targets. We show that Gal83 mediates the association of the kinase with Sip4, a Snf1-regulated transcription activator of gluconeogenic genes. Gal83 interacts with Sip4 in two-hybrid assays *in vivo*, and bacterially expressed proteins bind *in vitro*. Moreover, Gal83 is required for the two-hybrid interaction of Sip4 with the Snf1 kinase. Gal83 also facilitates the rapid Snf1-dependent phosphorylation and activation of Sip4 in response to glucose limitation, indicating that Gal83 mediates the functional interaction of Snf1 with Sip4. Evidence indicates that Sip1 and Sip2 do not interact with Sip4. We propose that members of the Sip1/Sip2/Gal83 family confer specificity to the kinase complex in its interactions with target proteins.**  
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## Introduction

The Snf1/AMPK protein kinase family has been conserved through evolution, and members have been identified in organisms ranging from plants to mammals (for a review see Hardie *et al.*, 1998). In the yeast *Saccharomyces cerevisiae*, the Snf1 (Cat1, Ccr1) protein kinase has a major role in the glucose starvation signalling pathway (for reviews see Gancedo, 1998; Carlson, 1999; Johnston, 1999). The Snf1 kinase is activated when glucose is limiting (Woods *et al.*, 1994; Jiang and Carlson, 1996; Wilson *et al.*, 1996) and is required for the transcription of glucose-repressed genes involved in alternate carbon source utilization, respiration and gluconeogenesis, as well as for sporulation, glycogen accumulation, thermotolerance, peroxisome biogenesis and meiosis (Celenza and Carlson, 1986; Schuller and Entian, 1987; Thompson-Jaeger *et al.*, 1991; Simon *et al.*, 1992; Honigberg and Lee, 1998). The Snf1 homologues in plants also regulate

carbon metabolism via phosphorylation of metabolic enzymes and transcriptional regulation (Halford and Hardie, 1998; Purcell *et al.*, 1998); moreover, some of the plant homologues provide Snf1 function in yeast (Alderson *et al.*, 1991; Muranaka *et al.*, 1994; Bouly *et al.*, 1999). The mammalian homologue, AMPK, plays a similar role in cellular stress responses that cause ATP depletion (Carling *et al.*, 1994; Corton *et al.*, 1994; Mitchelhill *et al.*, 1994). AMPK regulates metabolic enzymes such as acetyl-CoA carboxylase, a role shared by Snf1 in yeast (Mitchelhill *et al.*, 1994; Woods *et al.*, 1994). Moreover, like Snf1, AMPK also plays a role in transcriptional regulation (Foretz *et al.*, 1998; Leclerc *et al.*, 1998).

The members of the Snf1 kinase family are found complexed with other proteins. In yeast, these proteins include the activating subunit Snf4 (Cat3) (Schuller and Entian, 1988; Celenza and Carlson, 1989; Celenza *et al.*, 1989) and Sip1, Sip2 and Gal83, which are related proteins and serve as alternate members of the kinase complex (Yang *et al.*, 1992, 1994; Erickson and Johnston, 1993). The Sip1/Sip2/Gal83 component plays a scaffolding function in the complex and anchors Snf1 and Snf4 via two distinct conserved domains (Jiang and Carlson, 1997). Although this component is not essential for Snf1 function (Erickson and Johnston, 1993; Yang *et al.*, 1994), *gal83Δ* greatly reduces the activity of Snf1 in immune complex assays (Yang *et al.*, 1994) and half of the cellular Snf4 protein no longer co-purifies with Snf1 from the *sip1Δ sip2Δ gal83Δ* triple mutant (Jiang and Carlson, 1997). Moreover, the Sip1/Sip2/Gal83 homologue in the yeast *Kluyveromyces lactis* (Fog1) is required for expression of glucose-repressed genes (Goffrini *et al.*, 1996). The Snf4 and Sip1/Sip2/Gal83 proteins are conserved in mammals and plants (Stapleton *et al.*, 1994; Woods *et al.*, 1996; Hardie *et al.*, 1998; Bouly *et al.*, 1999; Lakatos *et al.*, 1999), and cross-species interactions occur between the yeast and plant proteins (Jiang and Carlson, 1996, 1997; Bouly *et al.*, 1999; Lakatos *et al.*, 1999). In addition, at least two different members of the Sip1/Sip2/Gal83 family, designated the  $\beta$ 1 and  $\beta$ 2 subunits of the kinase complex, are also found in both plants and mammals and show different patterns of tissue-specific expression (Stapleton *et al.*, 1997; Thornton *et al.*, 1998; Bouly *et al.*, 1999).

Snf1 and Snf1-related kinases have multiple regulatory roles, and presumably multiple targets, in various cellular processes. An important question concerns the mechanism by which these kinases specifically recognize different targets. Here we address the possibility that in addition to its structural role in the kinase complex, the Sip1/Sip2/Gal83 component has a role in mediating the interaction of the kinase with specific targets. Genetic evidence suggests that Sip1, Sip2 and Gal83 have distinct functions in the Snf1 pathway. Dominant mutations in *GAL83* relieve glucose repression of the *GAL1* gene but do not affect

*SUC2* expression (Matsumoto *et al.*, 1981; Erickson and Johnston, 1993). Overexpression of Sip1, and to a lesser extent Gal83 but not Sip2, restores *SUC2* expression in *snf4* mutants (Yang *et al.*, 1992, 1994). These differential effects on *SUC2* and *GAL1* gene expression, and recent evidence that *SIP2* affects survival in stationary phase (Ashrafi *et al.*, 1998), suggest that Sip1, Sip2 and Gal83 act as adaptors between Snf1 and specific targets of the kinase.

In this work, we examine the interaction of the Snf1 kinase complex with Sip4, a Snf1-regulated, C6 zinc-cluster transcription activator which was identified in a two-hybrid screen for proteins that interact with Snf1 (Lesage *et al.*, 1996). Sip4 binds to the carbon source-responsive element (CSRE) in the promoters of the gluconeogenic genes (Vincent and Carlson, 1998). Transcription of the *SIP4* gene is glucose-repressed (Lesage *et al.*, 1996; Vincent and Carlson, 1998) and increases during the diauxic shift and sporulation (DeRisi *et al.*, 1997; Chu *et al.*, 1998). The phosphorylation of Sip4 is regulated by Snf1 and by glucose, and the ability of a LexA-Sip4 fusion protein to activate transcription of a reporter containing LexA binding sites is dependent on Snf1 and inhibited by glucose (Lesage *et al.*, 1996). These findings, together with the physical association of Snf1 and Sip4, strongly suggest that Snf1 directly modulates the activity of Sip4 in response to glucose.

Here, we present evidence that Gal83 mediates the physical and functional interaction of the Snf1 kinase complex with Sip4. We show that Gal83 interacts with Sip4 both *in vivo* and *in vitro* and that this interaction involves the conserved C-terminus of Gal83. We show that Gal83 is required for the rapid Snf1-dependent phosphorylation and activation of Sip4 in response to glucose limitation.

## Results

### C-terminus of Gal83 interacts with Sip4

Previous studies suggested that Gal83 interacts with Sip4 in the two-hybrid system, but interpretation was compromised because LexA-Gal83, when bound to DNA, activates reporter transcription (Lesage *et al.*, 1996). We therefore used a Gal4-activation domain (GAD) fusion to Gal83. LexA-Sip4 also activates transcription of a *lacZ* reporter with LexA binding sites, but this activation is strongly inhibited by glucose (only 1.5 U of  $\beta$ -galactosidase activity; see Figure 3). Hence, LexA-Sip4 and GAD-Gal83, both expressed from the *ADH1* promoter, were tested for two-hybrid interaction in cells grown on glucose (Figure 1). These two fusion proteins interacted well, yielding 22 U of activity. As reported previously, Sip4 did not interact with Sip1 or Sip2 (Lesage *et al.*, 1996; data not shown).

To identify the Gal83 sequence that binds to Sip4, we tested a series of GAD-Gal83 fusions for interaction (Figure 1). Previous work showed that Sip1, Sip2 and Gal83 share two conserved domains: the internal KIS (kinase-interacting sequence) region interacts with Snf1 and the C-terminal ASC (association with the Snf1 complex) domain binds to Snf4 (Yang *et al.*, 1994; Jiang and Carlson, 1997). The extreme C-terminus of Gal83 (residues 336–417), which corresponds to the ASC domain, was

GAD-Gal83 sequence	Interacts with: Snf1 KIS Snf4 ASC	Interaction with LexA-Sip4	
		WT	<i>snf1</i> $\Delta$
1-417		+	-
198-417		+	-
336-417		+	ND
1-350		-	ND

**Fig. 1.** Two-hybrid interaction of Sip4 with the C-terminus of Gal83. Strains were CTY10-5d (wild type, WT) and an isogenic derivative MCY4028 (*snf1* $\Delta$ ). Proteins were expressed from plasmids listed in Table I. GAD-Gal83 fusions contain the indicated Gal83 residues. Shaded bar, ASC domain; black bar, Snf1-interacting KIS region. Interactions in filter assays are indicated by + signs. GAD fusions did not interact with LexA or control LexA fusions. In control experiments, GAD-Gal83 $\Delta$ ASC (residues 1–350) interacted with LexA-Snf1. Two-hybrid interaction was also detected between LexA-ASC<sub>Gal83</sub> and GAD-Sip4. Immunoblotting confirmed that LexA-Sip4 is expressed in the *snf1* $\Delta$  mutant. ND, not determined.

necessary and sufficient for interaction with Sip4. Thus, this small region is bifunctional.

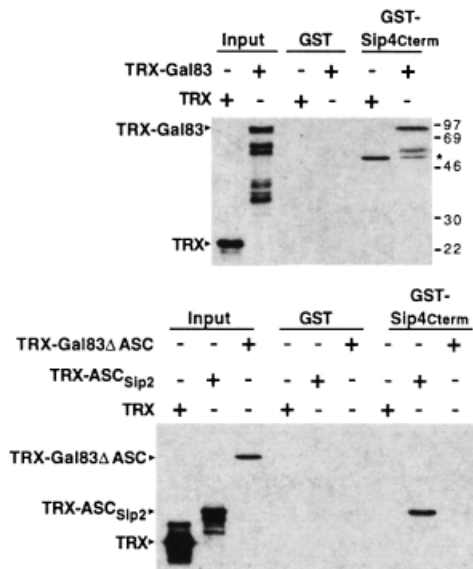
### Gal83 binds to the C-terminus of Sip4 *in vitro*

We showed that Gal83 binds directly to Sip4 by assaying bacterially expressed proteins for *in vitro* binding. We expressed a thioredoxin (TRX) fusion to Gal83, TRX-Gal83, and a glutathione *S*-transferase (GST) fusion to the C-terminal half of Sip4 (residues 402–829), designated GST-Sip4<sub>Cterm</sub>. GST-Sip4<sub>Cterm</sub> was used because the full-length GST-Sip4 proved unstable in bacteria, and this C-terminal region of Sip4 interacts with both Snf1 and Gal83<sub>198–417</sub> in the two-hybrid assay (Lesage *et al.*, 1996; data not shown), whereas the N-terminus of Sip4 (residues 1–402, containing the C6 zinc cluster) does not interact with either (data not shown). For the binding assay, GST-Sip4<sub>Cterm</sub> and GST proteins were immobilized on glutathione-Sepharose 4B beads and incubated with bacterial extracts containing TRX-Gal83 or TRX proteins. Beads were washed extensively, and the bound proteins were subjected to immunoblot analysis with a TRX antibody. TRX-Gal83 bound to GST-Sip4<sub>Cterm</sub>, and no binding was detected with the control proteins GST and TRX (Figure 2). To assess the requirement for the ASC domain for binding *in vitro*, we also expressed TRX-Gal83 $\Delta$ ASC, a truncated fusion of Gal83 lacking the ASC domain. No interaction was detected with GST-Sip4<sub>Cterm</sub> (Figure 2). These results indicate that the binding between Gal83 and Sip4 is direct and requires the ASC domain.

Sip2 and Gal83 are highly similar in the ASC domain (80% identity), and therefore we also assayed for binding of TRX-ASC<sub>Sip2</sub> to GST-Sip4<sub>Cterm</sub>. Binding was easily detected (Figure 2), as was two-hybrid interaction between LexA-ASC<sub>Sip2</sub> and GAD-Sip4 (data not shown). However, no two-hybrid interaction was detected between the full-length LexA-Sip2 and GAD-Sip4 or between LexA-Sip4 and GAD-Sip2 (Lesage *et al.*, 1996; data not shown), suggesting that in the context of the native Sip2 protein, the ASC<sub>Sip2</sub> domain does not bind Sip4.

### Integrity of the Snf1 complex is required for interaction with Sip4

To determine whether Gal83 is required for the interaction of Snf1 and Sip4, we assayed the two-hybrid interaction



**Fig. 2.** *In vitro* binding of Gal83 to the C-terminus of Sip4. GST-Sip4<sub>Cterm</sub> or GST alone were immobilized on glutathione-Sepharose beads and incubated with extracts containing TRX or TRX fusions to Gal83, Gal83 $\Delta$ ASC or ASC<sub>Sip2</sub>. Predicted molecular masses of the fusion proteins are 65, 57 and 28 kDa, respectively, but full-length Gal83 has a lower mobility than predicted, as described previously (Yang *et al.*, 1994). After being washed, bound proteins were boiled in sample buffer, separated by SDS-PAGE, and analysed by immunoblotting with antibody against TRX. A cross-reacting protein that bound to GST-Sip4<sub>Cterm</sub> in some experiments is indicated by an asterisk. The degradation product of TRX-Gal83, which bound to GST-Sip4<sub>Cterm</sub>, is predicted from its size to contain part of the ASC domain. Lanes marked 'Input' contain protein extracts used for binding experiments (2–8  $\mu$ g). Protein markers are in kilodaltons.

DNA-binding hybrid	Activator protein	$\beta$ -Galactosidase activity (U)	
		WT	<i>gal83</i> $\Delta$
LexA-Sip4	GAD-Snf1	74	3
LexA-Sip4	GAD	1.5	0.6
LexA-Snf1	GAD-Sip4	139	ND
LexA-Snf1K84R	GAD-Sip4	206	ND
LexA-Snf1T210A	GAD-Sip4	115	ND

**Fig. 3.** Two-hybrid interaction between Snf1 and Sip4 is impaired in a *gal83* $\Delta$  mutant. Strains were CTY10-5d (wild type, WT) and its isogenic derivative MCY4024 (*gal83* $\Delta$ ). Fusion proteins were expressed from plasmids listed in Table I. Values are the average  $\beta$ -galactosidase activity of four transformants. Standard errors were <26%. In control experiments, LexA fusions did not interact with GAD and GAD fusions did not interact with LexA or control LexA fusions (<0.4 U). In addition, the two-hybrid interaction between the cyclin-dependent kinase Srb10 and cyclin Srb11 [LexA-Srb10 and GAD-Srb11 (Kuchin *et al.*, 1995)] was not affected by the *gal83* $\Delta$  mutation. Immunoblotting confirmed that LexA-Sip4 and GAD-Snf1 are expressed at a similar level in wild-type and *gal83* $\Delta$  mutant strains. ND, not determined.

between LexA-Sip4 and GAD-Snf1 in a *gal83* $\Delta$  mutant.  $\beta$ -galactosidase activity was reduced 25-fold relative to the wild type (Figure 3), and immunoblot analysis confirmed that LexA-Sip4 and GAD-Snf1 were expressed at a similar level in both strains (data not shown). Thus, Gal83 is strongly required for this interaction. The combination LexA-Snf1 and GAD-Sip4 could not be tested because

GAD-Sip4 was not stable in the *gal83* $\Delta$  mutant (or in *snf1* $\Delta$  or *snf4* $\Delta$  mutants; data not shown).

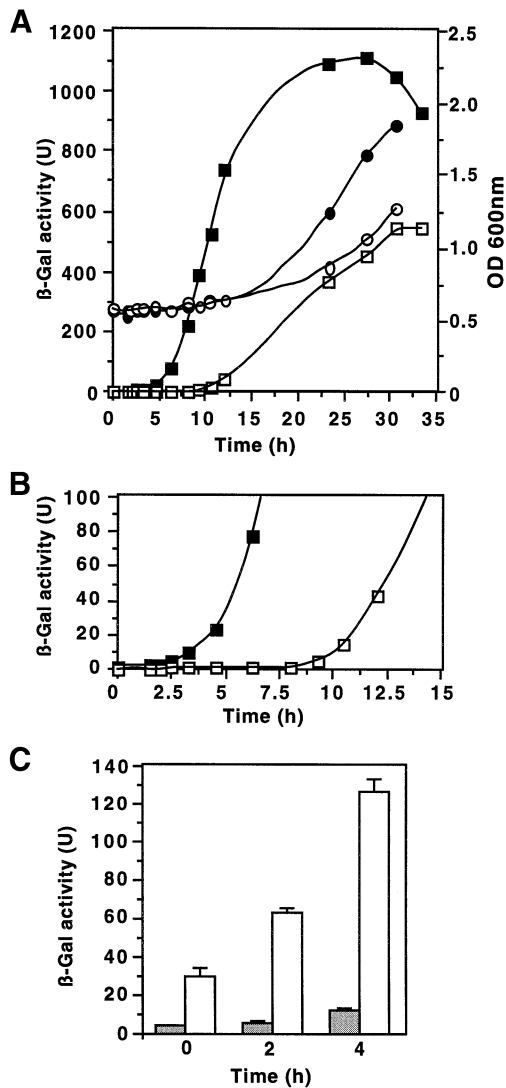
We next tested whether the interactions of Gal83 with Snf1 and Snf4 affect its association with Sip4. Assays of two-hybrid interaction between LexA-Sip4 and GAD-Gal83 or GAD-Gal83<sub>198–417</sub> in a *snf1* $\Delta$  mutant strain showed a requirement for Snf1 (Figure 1). A similar experiment in a *snf4* $\Delta$  mutant strain indicated that interactions between LexA-Sip4 and GAD-Gal83 or LexA-Sip4 and GAD-Snf1 also require Snf4 (data not shown). Immunoblot analysis confirmed that LexA-Sip4 is expressed in both mutant strains (data not shown). In contrast, GAD-Gal83 interacts with LexA-Snf1 in the absence of Snf4 and interacts with LexA-Snf4 in the absence of Snf1 (Jiang and Carlson, 1997; data not shown). Together, these results suggest that the binding of Sip4 to Gal83 requires the integrity of the Snf1 kinase complex.

To determine whether the association of Snf1 with Sip4 depends on the Snf1 catalytic activity, we examined the kinase-dead mutant proteins Snf1K84R and Snf1T210A, which have substitutions of the invariant lysine in the ATP-binding site and the conserved threonine residue in the activation loop, respectively (Celenza and Carlson, 1989; Estruch *et al.*, 1992). Both mutant kinases interacted well with Sip4 in a wild-type (Figure 3) or *snf1* $\Delta$  mutant host (data not shown). These results indicate that the Snf1 kinase activity is not required for the interaction between Snf1 and Sip4, which is consistent with evidence that this interaction is not glucose regulated (Lesage *et al.*, 1996).

### Mutations in GAL83 affect the glucose regulation of Sip4 activator function

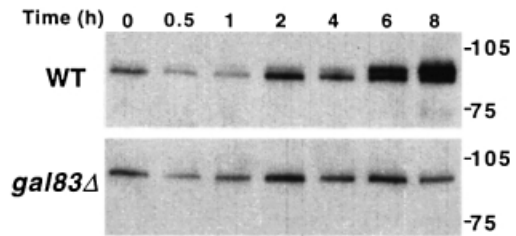
The transcription activation function of Sip4 is dependent on Snf1, and activation of a *lexAop-lacZ* reporter by LexA-Sip4 is abolished in a strain lacking Snf1 kinase activity (Lesage *et al.*, 1996). Evidence that Gal83 is required for the interaction of Sip4 and Snf1 predicts that a *gal83* $\Delta$  mutation should impair transcriptional activation by LexA-Sip4. To test this idea, we followed the kinetics of activation of a reporter by LexA-Sip4 in wild type and the isogenic *gal83* $\Delta$  deletion mutant after a shift from glucose to ethanol plus glycerol. Both strains were able to activate expression of the reporter, but activation was severely delayed (6 h) in the *gal83* $\Delta$  mutant strain (Figure 4A and B). Immunoblot analysis confirmed that LexA-Sip4 is expressed at the same level in both strains after 7 h (data not shown). The maximal level of  $\beta$ -galactosidase activity in the mutant strain was 50% of the level reached by the wild-type strain (Figure 4A). Moreover, the growth rate of the mutant strain was slower than that of wild type after the shift to ethanol plus glycerol (Figure 4A), consistent with the role of Sip4 in the transcription of the gluconeogenic genes (Vincent and Carlson, 1998). These results indicate that although Gal83 is not essential for Sip4-dependent activation, Gal83 is required for a rapid response to glucose limitation.

The dominant mutation *GAL83-2000* partially relieves glucose repression of *GAL1* in strains that do not require galactose induction (Matsumoto *et al.*, 1981). This mutation converts Gly235 to Arg (Erickson and Johnston, 1993) and is located in the Snf1-interacting domain (KIS) of Gal83, but does not significantly affect the interaction between LexA-Snf1 and GAD-Gal83 (Jiang and Carlson,



**Fig. 4.** Mutations in *GAL83* affect the regulation of Sip4 activator function by glucose. (A and B) Two different transformants of both wild-type CTY10-5d (filled symbols) and an isogenic *gal83* $\Delta$  mutant MCY4024 (open symbols) expressing LexA-Sip4 from the *ADH1* promoter were grown selectively to mid-log phase in 2% glucose and then shifted ( $t = 0$ ) to 2% ethanol + 2% glycerol. Samples were taken at the indicated times and  $\beta$ -galactosidase activity was assayed to monitor activation of the integrated *lexAop-lacZ* reporter. (A) Average  $\beta$ -galactosidase activity (squares) and OD<sub>600</sub> (circles) for two transformants of each strain; in each case the values were nearly identical for both transformants. (B)  $\beta$ -galactosidase activity at the initial time points on an expanded scale. Immunoblot analysis showed that LexA-Sip4 is expressed at the same level in both strains after 7 h. (C) Wild-type MCY2649 (solid bars) and isogenic derivative MCY4037 (*GAL83-2000*) (open bars) expressed LexA-Sip4 from the *ADH1* promoter and carried a *lexAop-lacZ* reporter (p1840, identical to 1145; Brent and Ptashne, 1985). Transformants were grown selectively to mid-log phase in 2% glucose ( $t = 0$ ) and then shifted to 2% ethanol + 2% glycerol for 2 or 4 h. Activation of the *lexAop-lacZ* reporter by LexA-Sip4 was measured by assaying  $\beta$ -galactosidase activity (average activity of four transformants). Values obtained for activation of *lexAop-lacZ* by LexA on glucose were  $<1$  in both strains.

1997; data not shown). We tested whether this mutation affects the glucose regulation of Sip4 activator function by measuring the activation of a *lexAop-lacZ* reporter by LexA-Sip4 in wild type and the isogenic *GAL83-2000* mutant (Figure 4C). Cultures were grown in 2% glucose and then shifted to ethanol plus glycerol.  $\beta$ -galactosidase



**Fig. 5.** Gal83 is required for phosphorylation of Sip4 in response to glucose limitation. Wild-type MCY2649 and an isogenic *gal83* $\Delta$  mutant MCY2720 were transformed with pOV64, which expresses HA-Sip4 from the native *SIP4* promoter. Transformants were grown selectively to mid-log phase in 2% glucose and then shifted ( $t = 0$ ) to 3% ethanol + 0.05% glucose. Samples were taken at the indicated times, and proteins prepared by the boiling method were analysed by immunoblotting with HA antibodies.

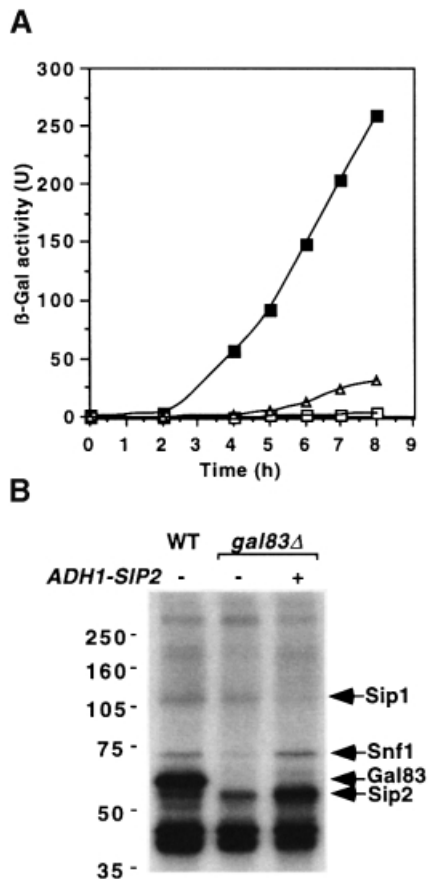
activity was 7.5-fold increased in glucose-grown mutant cells, indicating that the *GAL83-2000* mutation partially relieves the glucose inhibition of Sip4 activator function. Moreover, this increase was maintained (10-fold) at 2 and 4 h after the shift to ethanol plus glycerol. This mutant phenotype strengthens the case for a functional relationship between Gal83 and Sip4.

#### ***Gal83 is required for the phosphorylation of Sip4 in response to glucose limitation***

Sip4 is phosphorylated in response to glucose limitation, and this phosphorylation requires Snf1 (Lesage *et al.*, 1996). To test whether Gal83 is required for this phosphorylation of Sip4, we examined a haemagglutinin (HA) epitope-tagged Sip4, expressed from the native *SIP4* promoter, by immunoblot analysis. Wild-type and *gal83* $\Delta$  mutant strains were grown in glucose and then shifted to ethanol plus 0.05% glucose. Immunoblot analysis did not detect a form of HA-Sip4 with lower mobility, corresponding to the phosphorylated protein (Lesage *et al.*, 1996), in extracts from the *gal83* $\Delta$  mutant, even after 8 h (Figure 5). These results indicate that the phosphorylation of HA-Sip4 in response to glucose limitation depends on Gal83 and further indicate that Gal83 and Sip4 are functionally related. In addition, the Snf1-dependent increase in Sip4 expression that occurs in response to glucose limitation (Lesage *et al.*, 1996) was not observed in the *gal83* $\Delta$  mutant.

#### ***Overexpression of Sip2 does not effectively compensate for loss of Gal83***

Gal83 and Sip2 show strong sequence similarity; however, Sip2 does not interact with Sip4 in the two-hybrid assay, suggesting that Gal83 and Sip2 are functionally distinct. To address their functional specificity, we tested whether overexpression of Sip2 from the *ADH1* promoter compensates for the loss of Gal83 and restores rapid activation by LexA-Sip4 in a *gal83* $\Delta$  mutant. Transformants carrying pLexA-Sip4 and either pADH1-Sip2 or the parental vector were grown on glucose and shifted to ethanol plus glycerol. Activation of the *lexAop-lacZ* reporter was followed for 8 h after the shift. When Sip2 was overexpressed in the *gal83* $\Delta$  mutant,  $\beta$ -galactosidase activity increased slightly but remained 10-fold lower than the wild-type level (Figure 6A). In contrast, overexpression of Sip2 in the *gal83* $\Delta$  mutant restored the phosphorylation of Snf1 in



**Fig. 6.** Overexpression of Sip2 does not restore rapid activation of LexA-Sip4 in a *gal83Δ* mutant. Wild-type CTY10-5d expressed LexA-Sip4 and carried the vector pSK134 (filled squares). The isogenic *gal83Δ* mutant MCY4024 expressed LexA-Sip4 and carried either pSK134 (open squares) or its derivative pOV65, which carries an *ADH1-SIP2* fusion that expresses Sip2 from the *ADH1* promoter (open triangles). Transformants were grown selectively to mid-log phase in 2% glucose and then shifted ( $t = 0$ ) to 2% ethanol + 2% glycerol. (A) Samples were taken at the indicated times and activation of the integrated *lexAop-lacZ* reporter by LexA-Sip4 was measured by assaying  $\beta$ -galactosidase activity. Two *gal83Δ* mutant transformants expressing Sip2 produced nearly the same activity, and the average values are shown. (B) Protein extracts were prepared from samples of each culture at  $t = 0$ , and proteins were immunoprecipitated with anti-Snf1 serum. Immunoprecipitates were incubated with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  in kinase buffer and subjected to SDS-PAGE. Phosphoproteins were visualized by autoradiography. The phosphoproteins migrating faster than Sip2 are present because the anti-Snf1 was not affinity purified and serve as an internal control for loading.

immune complex kinase assays, as documented previously (Yang *et al.*, 1994), thereby confirming that Sip2 functions to replace Gal83 in the kinase complex (Figure 6B). Together, these results confirm the specificity of Gal83 in mediating the interaction between Snf1 and Sip4.

#### Another kinase besides Snf1 is associated with Sip4

The two-hybrid interaction of Snf1 and Sip4 suggested that the two proteins should co-immunoprecipitate from cell extracts. Protein extracts were prepared from a *snf1Δ* mutant strain overexpressing Snf1 and HA-Sip4 from plasmids. HA-Sip4 was immunoprecipitated with monoclonal HA antibody, and immunoblot analysis of the precipitate with Snf1 antibody showed that a fraction of

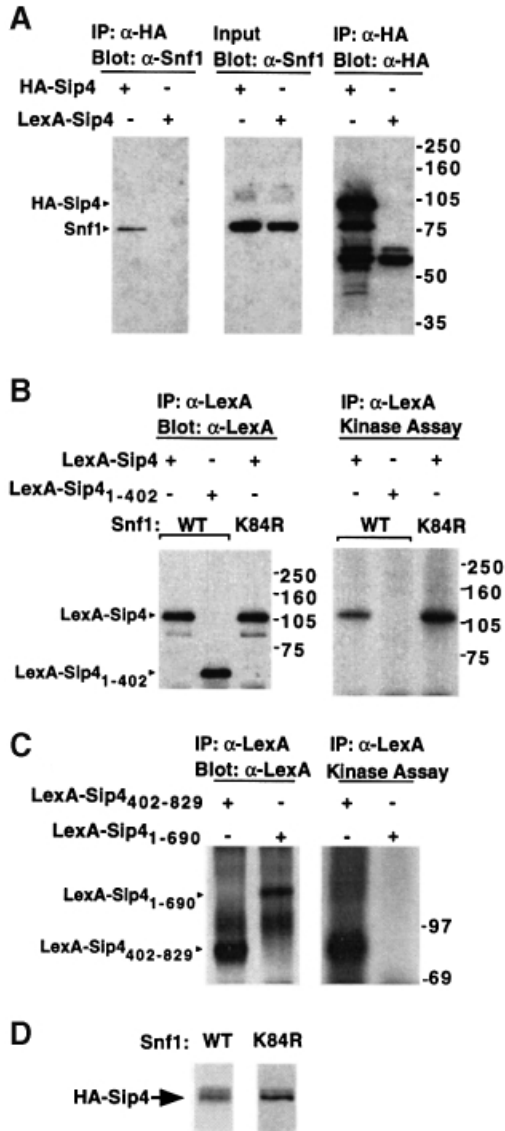
the Snf1 protein co-immunoprecipitated with HA-Sip4 (Figure 7A). In control experiments in which LexA-Sip4 was expressed, no Snf1 was precipitated by anti-HA.

Because the major phosphorylation of Sip4 in response to glucose limitation *in vivo* requires Snf1 (Lesage *et al.*, 1996; data not shown), we examined the kinase activity associated with immunoprecipitated Sip4. Protein extracts from wild-type and *snf1-K84R* mutant strains expressing LexA-Sip4 were immunoprecipitated with polyclonal LexA antibody and incubated with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Previous studies have shown that Snf1 kinase prepared from glucose-grown cells is activated during this assay (Estruch *et al.*, 1992). A labelled product corresponding to LexA-Sip4 was identified, but the same product was also detected when the kinase-dead mutant Snf1K84R was expressed (Figure 7B). Similar results were obtained in assays of HA-Sip4 immunoprecipitated from wild-type and *snf1-K84R* mutant strains (data not shown). Thus, Snf1 is not responsible for the major phosphorylation of Sip4 in these immune complex assays. To assess the specificity of the catalytic activity detected in these experiments, we also immunoprecipitated three truncated versions of Sip4 and carried out immune complex kinase assays. No labelled product corresponding to LexA-Sip4<sub>1-402</sub> or LexA-Sip4<sub>1-690</sub> was detected, whereas LexA-Sip4<sub>402-829</sub> was strongly phosphorylated (Figure 7B and C). Thus, a kinase other than Snf1 co-immunoprecipitates with and phosphorylates the C-terminus of Sip4 *in vitro*. Immunoblot analysis of HA-Sip4 weakly detected a species of lower mobility in glucose-grown *snf1-K84R* mutant and wild-type cells (Figure 7D), although this species was not reproducibly as prominent as shown here (see Figure 5). Together, these results indicate that Snf1 is not the only kinase associated with Sip4 and suggest that this other kinase phosphorylates Sip4 *in vivo*. It is possible that this kinase is stimulated by Snf1 and contributes to the Snf1-dependent phosphorylation of Sip4 observed in glucose-limited cells.

#### Discussion

Sip1, Sip2 and Gal83 are alternate members of the Snf1 kinase complex, an essential component of the glucose response pathway. Here we present genetic and biochemical evidence that Gal83 mediates the interaction of the Snf1 complex with Sip4, a Snf1-regulated transcription activator of gluconeogenic genes. The Snf1 kinase stimulates Sip4 activator function in response to glucose limitation, and we show that Gal83 functions to improve the kinetics of this process. These findings support a model in which the Sip1/Sip2/Gal83 proteins serve as adaptors that specifically mediate the interaction of the Snf1 kinase with different target proteins.

We present the following evidence that Sip4 and Gal83 interact both physically and functionally. First, Sip4 interacts with Gal83 *in vivo* in the two-hybrid assay; specifically, the C-terminus of Sip4 interacts with the ASC domain of Gal83. Secondly, bacterially expressed Sip4 and Gal83 proteins bind to one another *in vitro*. Thirdly, the *gal83Δ* mutation nearly abolishes the two-hybrid interaction between Sip4 and Snf1, indicating that Gal83 is required for interaction of Sip4 with the Snf1 kinase complex. Fourthly, *gal83Δ* delays the Snf1-dependent activation of



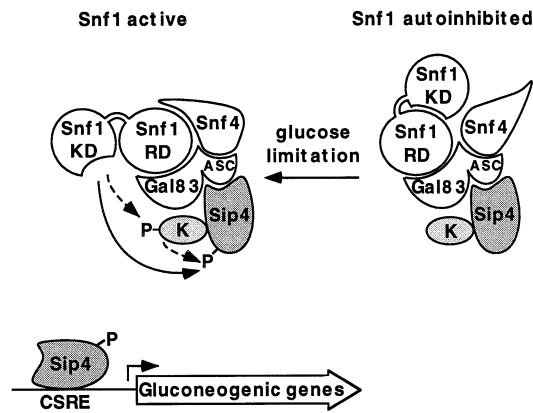
**Fig. 7.** Sip4 co-immunoprecipitates with Snf1 and another kinase. (A) MCY2916 (*snf1 $\Delta$ 10*) was transformed with plasmids expressing Snf1 and either HA-Sip4 or LexA<sub>87</sub>-Sip4, all from the *ADHI* promoter [pSK117, pOV29, and pPL49; called pLexA-Sip4 in Lesage *et al.* (1996)]. Protein extracts were prepared from cells grown in 2% glucose, and proteins (40  $\mu$ g) were immunoprecipitated (IP) with anti( $\alpha$ )-HA antibody, separated by SDS-PAGE and immunoblotted with affinity-purified SNF1 antibody (left panel). The input proteins (1  $\mu$ g) were analysed on the same gel (middle panel). The same immunoblot was reprobed with  $\alpha$ -HA to confirm the precipitation of HA-Sip4 (right panel). (B) Proteins were prepared from MCY3647 (WT) and MCY2693 (*snf1-K84R*) expressing LexA-Sip4 (full length, residues 1–829) or LexA-Sip4<sub>1-402</sub> (predicted molecular mass 118 and 68 kDa, respectively) and immunoprecipitated with  $\alpha$ -LexA antibody. Immunoprecipitates were incubated with [ $\gamma$ -<sup>32</sup>P]ATP in a kinase reaction and then subjected to SDS-PAGE. Phosphoproteins were visualized by autoradiography (right panel). The same immunoprecipitates were analysed by immunoblotting using  $\alpha$ -LexA to confirm the presence of LexA-Sip4<sub>1-402</sub> (left panel). (C) Strain W303 (WT) was transformed with plasmids expressing LexA-Sip4<sub>402-829</sub> and LexA-Sip4<sub>1-690</sub> (predicted molecular mass 72 and 102 kDa, respectively). Immune complex kinase assays (right panel) and immunoblot analysis (left panel) were carried out as in (B). Analysis of LexA-Sip4 and LexA-Sip4<sub>1-402</sub> expressed in this strain gave the same results as shown in (B). (D) MCY3647 (WT) and MCY2693 (*snf1-K84R*) expressing HA-Sip4 from the *SIP4* promoter (pHA-Sip4) were grown to mid-log phase in 2% glucose. Proteins were prepared by the boiling method and analysed by immunoblotting with HA antibodies.

Sip4 function in response to glucose limitation. Fifthly, *gal83 $\Delta$*  affects the Snf1-dependent phosphorylation of Sip4. Finally, the dominant mutation *GAL83-2000* partially relieves the glucose inhibition of Sip4 activator function. These findings indicate that Gal83 binds to Sip4 and mediates both the physical association of this transcription activator with the Snf1 complex and its functional interaction with the Snf1 kinase.

These results also indicate that Gal83 has a broader role in cellular regulatory processes than has heretofore been recognized. *GAL83* is so named because the gene was identified by mutations that partially relieve glucose repression of *GAL1* gene expression. However, this phenotype was only manifest in mutants already defective in the inhibition of Gal4 by Gal80, and a *gal83 $\Delta$*  mutation did not cause any defect in *GAL* gene regulation (Matsumoto *et al.*, 1981; Erickson and Johnston, 1993). Here, we report the first phenotype observed in a *gal83 $\Delta$*  mutant. We show that Gal83 is required for the association of Sip4 with the Snf1 kinase and for rapid transcription activation by Sip4 in response to glucose limitation. In addition, we show that the *GAL83-2000* mutation causes a defect in glucose inhibition of Sip4 activator function. Thus Gal83 is not, as previously thought, specific for the regulation of *GAL* genes, but rather has a broader function in directing the Snf1 kinase activity to different processes.

The region of Gal83 that binds to Sip4, in both two-hybrid and *in vitro* binding assays, is the C-terminal ASC domain. The ASC domain was previously shown to interact with Snf4, the activating subunit of the Snf1 kinase. Thus, this small region of 80 amino acids has two different functions: to anchor the Snf4 subunit to the kinase complex and to bind a target of the kinase. Evidence suggests that the conformation of the Gal83 protein is important for the binding to Sip4. First, the ASC domain of Sip2, which is 80% identical to the Gal83 sequence, also binds to Sip4, but the full-size Sip2 protein does not interact with Sip4 in the two-hybrid assay, despite interacting strongly with both Snf1 and Snf4 (this study; Jiang and Carlson, 1997). Secondly, the two-hybrid interaction of Gal83 with Sip4 requires both Snf1 and Snf4; this requirement is not simply for kinase activity, because Sip4 interacts with the dead kinases Snf1K84R and Snf1T210A. Thus, the integrity of the Snf1 kinase complex, but not the kinase activity, is required for its interaction with Sip4. These findings suggest that a specific conformation of the Gal83 protein in the kinase complex is important for the accessibility of the ASC domain to Sip4 *in vivo*.

Several lines of genetic evidence support the idea that the interaction of the kinase complex with Sip4 is mediated specifically by Gal83, and not by the other members of the Sip1/Sip2/Gal83 family. First, the phenotype of a *gal83 $\Delta$*  mutant indicates that Sip1 and Sip2 do not effectively substitute for Gal83 to mediate interaction of Sip4 with the Snf1 complex: *gal83 $\Delta$*  severely impairs the two-hybrid interaction of Sip4 with Snf1 and affects both the Snf1-dependent activation of Sip4 function and the Snf1-dependent phosphorylation of Sip4 in response to glucose limitation. Secondly, overexpression of Sip2 does not effectively compensate for the absence of Gal83; Sip2 overexpression in the *gal83 $\Delta$*  mutant restores the phosphorylation of Snf1 in immune complex kinase assays but



**Fig. 8.** Model for regulation of the Sip4 transcription activator in response to glucose limitation. Growth in the presence of glucose favours autoinhibition of the Snf1 kinase by the binding of its regulatory domain (RD) to the kinase domain (KD). When cells are limited for glucose, the Snf1 kinase complex undergoes a conformational change: autoinhibition is relieved and Snf4 binds to RD to stabilize an open conformation (Jiang and Carlson, 1996). Glucose repression of *SIP4* gene expression is relieved, and Sip4 protein levels increase. Sip4 associates with the Snf1 kinase complex through its interaction with the ASC domain of Gal83. The active Snf1 most likely phosphorylates Sip4 (arrow), thereby stimulating its ability to activate transcription. Sip4 is also associated with another protein kinase (K), which may weakly phosphorylate Sip4 in glucose-grown cells (not shown). It is possible that Snf1 stimulates the unidentified kinase, which then phosphorylates Sip4 (dashed arrows). The phosphorylated Sip4 activates transcription of the gluconeogenic genes. There is no evidence that binding of Sip4 to the CSRE or nuclear localization of Sip4 is glucose regulated.

does not substantially remedy the delay in activation of Sip4 when cells are deprived of glucose. These findings, together with the physical interaction of Sip4 with Gal83, but not Sip1 or Sip2, indicate that Gal83 is the member of this family that is responsible for mediating the interaction of the Snf1 kinase complex with Sip4.

We propose that the Gal83-mediated association of Sip4 with the kinase complex facilitates the rapid phosphorylation, and hence activation, of Sip4 when glucose becomes limiting in the cell's environment (Figure 8). Sip4 then functions as a transcription activator for gluconeogenic genes. Because Snf1 is physically associated with Sip4 and is required for the major glucose-regulated phosphorylation of Sip4, the simplest model is that Snf1 directly phosphorylates Sip4. However, evidence that another kinase is also associated with Sip4 raises the possibility that the regulation of Sip4 activity is somewhat more complex. Although the *in vitro* activity of this other kinase does not require Snf1, it remains possible that *in vivo* this kinase is stimulated by Snf1 and contributes to the Snf1-dependent phosphorylation of Sip4.

On the basis of our results showing that Gal83 mediates the interaction of the Snf1 complex with Sip4, we suggest that members of the Sip1/Sip2/Gal83 family serve as adaptors that specifically mediate the interaction of the kinase with a variety of different substrates. Gal83 most likely interacts not only with Sip4, but also with a regulatory factor for the *GAL* genes. In addition, Gal83 may function during sporulation, as transcription of *GAL83* is induced and its promoter contains an Ndt80 consensus site (Chu *et al.*, 1998); interestingly, this is not the case for *SIP1* or *SIP2*. Although there is less evidence regarding

the function of the other two members of the family, *SIP1* is a multicopy suppressor of the defect in *SUC2* expression in a *snf4* mutant (Yang *et al.*, 1992), and *SIP2* has recently been shown to affect cell survival during stationary phase (Ashrafi *et al.*, 1998).

Diverse members of the Sip1/Sip2/Gal83 family are also conserved in other eukaryotes. Two human homologues constitute the  $\beta 1$  and  $\beta 2$  subunits of AMPK and exhibit different patterns of tissue-specific expression (Thornton *et al.*, 1998). Interestingly, the AMPK  $\beta 1$  subunit is myristoylated, and Sip2 contains a site that can be myristoylated *in vitro* (Mitchell *et al.*, 1997; Ashrafi *et al.*, 1998). Two homologues have also been identified in *Arabidopsis thaliana*, and their expression is differentially regulated during development and under different growth conditions (Bouly *et al.*, 1999). These findings are consistent with evidence in yeast that members of the Sip1/Sip2/Gal83 family confer regulatory specificity to the Snf1 kinase complex.

## Materials and methods

### Strains and genetic methods

*Saccharomyces cerevisiae* strains were MCY2693 (*MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 ura3-52 snf1-K84R*), MCY2916 (*MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 ura3-52 snf1 $\Delta$ 10*), MCY3647 (*MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 lys2-801 ura3-52*), MCY2649 (*MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 ura3-52*) and its isogenic derivatives MCY2720 (*gal83 $\Delta$ ::URA3*) and MCY4037 (*GAL83-2000*), W303-1A (*MAT $\alpha$  trp1-1 leu2-3,112 his3-11,15 ura3-1 ade2-1 can1-100*), and CTY10-5d (*MAT $\alpha$  ade2-101 his3- $\Delta$ 200 leu2- $\Delta$ 1 trp1- $\Delta$ 901 gal4 gal80 URA3::lexAop-lacZ*) and its isogenic derivatives MCY4024 (*gal83 $\Delta$ ::TRP1*) and MCY4028 (*snf1 $\Delta$ ::TRP1*). The *GAL83::TRP1* allele was derived from pOV1, containing the *GAL83* gene with 269 bp of the promoter and the first 372 codons replaced by *TRP1*. *SNF1::TRP1* disruption was made using pOV66, in which 954 bp of the promoter and the first 628 codons of *SNF1* are replaced by *TRP1*. Standard genetic methods were followed, and yeast cells were grown in synthetic complete (SC) medium lacking appropriate supplements to maintain selection for plasmids (Rose *et al.*, 1990).

### Plasmids

Plasmids and vectors used in this study are listed in Table I. pOV11 and pOV12 are derivatives of pGEX3X and pLexA(1-202)+PL, respectively, containing a PCR fragment generated from pPL40 (Lesage *et al.*, 1996) with the primers OV12 and OV13. pOV16 and pOV18 are derivatives of pACTII or pET-32c(+), respectively, containing a PCR fragment generated from pRJ325 with the primers RJ#4 and RJ#6. To construct pOV35, we carried out a PCR by using pBM2439 (Erickson and Johnston, 1993) as template and the primers OV34 and OV38 and cloned the resulting fragment in the *Bam*HI site of pACTII. pOV36 was constructed by PCR with pBM2439 as a template and the primers OV34 and OV37. The resulting fragment was cloned in the *Eco*RI and *Bam*HI sites of pSH2-1. pOV64 was constructed by inserting the *Spe*I-*Sal*I fragment from pPL76 into the cognate sites of pRS425. pOV65 contains the *Eco*RI-*Sal*I fragment from pXY38 (Jiang and Carlson, 1997) cloned into the *Eco*RI and *Xho*I sites of pSK134.

### Oligonucleotide primers

Primers were as follows, with restriction sites underlined: OV12, 5'-CCCGATCCTAGAAATCCCCAGCGTGAAG-3'; OV13, 5'-GGGGATCCTTAGAAGGTCGAGTTCAAAATATTC-3'; RJ#4, 5'-GGGGGTCGACATATGGCTGGCGACAACCC-3'; RJ#6, 5'-GGGCGGTCGACGCAGTTGTGGAGGAGTCAACCAGGCC-3'; OV34, 5'-GGGGATCCTATTGCAATGGTGTATACAG-3'; OV37, 5'-GGGGAATTCAATAATCACCAAAAATATGGCC-3'; and OV38, 5'-GGGGATCCAAAATAATCACCAAAAATATGG-3'.

### $\beta$ -galactosidase assays

Transformants (6-8) were patched onto selective SC medium containing 2% glucose and grown for 1 day at 30°C. Filter lift assays for blue colour were performed as described previously (Yang *et al.*, 1992) and

**Table I.** Plasmids used in this study

Plasmid	Expressed protein and vector	Source or reference
pLexA <sub>202</sub> -Sip4 (pPL50)	LexA-Sip4; pLexA(1-202)+PL	Vincent and Carlson (1998)
pPL52	LexA-Sip4 <sub>1-402</sub> ; pLexA(1-202)+PL	this study
pPL54	LexA-Sip4 <sub>1-690</sub> ; pLexA(1-202)+PL	this study
pGAD-Sip4 (pPL69)	GAD-Sip4; pACTII	Lesage <i>et al.</i> (1996)
pHA-Sip4 (pPL76)	HA <sub>3</sub> -Sip4; pRS426	Lesage <i>et al.</i> (1996)
pSK117	Snf1; pSK37	Treitel <i>et al.</i> (1998)
pSK118	Snf1K84R; pSK37	S.Kuchin and M.Carlson (unpublished)
pSG1	GAD-Snf1; pGAD2F	gift from T.Melese
pACT-Gal83 (G198)	GAD-Gal83 <sub>198-417</sub> ; pACT	Jiang and Carlson (1997)
pRJ55	LexA-Snf1; pLexA(1-202)+PL	Jiang and Carlson (1996)
pRJ215	LexA-Snf1K84R; pEG202	Treitel <i>et al.</i> (1998)
pRJ217	LexA-Snf1T210A; pEG202	this study
pRJ259	TRX-ASC <sub>Sip2</sub> (codons 336-415); pET-32a(+)	Jiang and Carlson (1997)
pRJ260	TRX-Gal83; pET-32a(+)	this study
pRJ325	GAD-Gal83; pGAD424	Jiang and Carlson (1997)
pOV11	GST-Sip4 <sub>Cterm</sub> (codons 402-829); pGEX3X	this study
pOV12	LexA-Sip4 <sub>402-829</sub> ; pLexA(1-202)+PL	this study
pOV16	GAD-Gal83ΔASC (codons 1-350); pACTII	this study
pOV18	TRX-Gal83ΔASC (codons 1-350); pET-32c(+)	this study
pADH1-Sip4 (pOV29)	HA <sub>3</sub> -Sip4; pWS93	Vincent and Carlson (1998)
pOV35	GAD-ASC <sub>Gal83</sub> (codons 336-417); pACTII	this study
pOV36	LexA-ASC <sub>Gal83</sub> (codons 337-417); pSH2-1	this study
pOV64	HA <sub>3</sub> -Sip4; pRS425	this study
pOV65	Sip2; pSK134	this study

Vectors: pLexA(1-202)+PL (Ruden *et al.*, 1991); pSH2-1 (Hanes and Brent, 1989); pACTII (Li *et al.*, 1994); pGAD2F (Chien *et al.*, 1991); pGAD424 (Clontech); pRS425 (Christianson *et al.*, 1992); pET-32c(+) (Novagen); pGEX3X (Pharmacia); and pSK134 (derivative of pACTII lacking GAD; S.Kuchin and M.Carlson, unpublished).

developed for 30 min to 1 h. For quantitative assays, transformants were grown to mid-log phase in selective SC medium containing 2% glucose, unless otherwise indicated. β-galactosidase activity was assayed in permeabilized cells and expressed in Miller units (Miller, 1972).

#### Protein expression and in vitro binding assays

GST-Sip4<sub>Cterm</sub>, GST and TRX-Gal83ΔASC proteins were expressed from *Escherichia coli* DH5α transformed with pOV11, pGEX3X or pOV18, respectively. TRX-Gal83, TRX-ASC<sub>Sip2</sub> and TRX proteins were expressed from *E.coli* BL21 (DE3) (Novagen) transformed with pRJ260, pRJ259 or pET-32c(+), respectively. Bacterial protein extracts were prepared as described previously (Vincent and Carlson, 1998) except for GST fusions, where 0.5% Sarkosyl was added before sonication and 2% Triton X-100 was used. Extracts containing GST or GST-Sip4<sub>Cterm</sub> were incubated with glutathione-Sepharose 4B beads (Pharmacia) at 4°C for 1 h. After being washed three times with 1 ml of buffer STE (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) + 2% Triton X-100, beads with bound proteins (3-6 μg) were split into several equal portions. Extracts (500 μl) containing each of the TRX fusion proteins (0.5-2 mg) were added to the beads and allowed to bind at 4°C for 1 h in buffer STE + 1% Triton X-100. After being washed twice with buffer STE + 1% Triton X-100 and three times with buffer STE, beads were boiled in sample buffer and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide. Bound proteins were detected by Western analysis with a polyclonal antibody against TRX (Santa Cruz Biotechnology) and enhanced chemiluminescence with ECL reagents (Amersham).

#### Immunoblot analysis

The equivalent of 1 ml of culture at an OD<sub>600</sub> of 0.5 was collected by centrifugation for 1 min and resuspended in 50 μl of sample buffer plus 0.1 g of glass beads, and cells were broken immediately by two cycles of boiling for 3 min and vortexing for 30 s. After 1 min centrifugation, 5 μl of the supernatant were analysed by SDS-PAGE in 6% polyacrylamide and immunoblotting with monoclonal anti-HA antibody (12CA5). Antibody was detected by enhanced chemiluminescence with ECLplus reagents (Amersham).

#### Immunoprecipitation assays

Preparation of protein extracts and immunoprecipitation procedures were essentially as described previously (Celenza and Carlson, 1989). The

extraction buffer was 50 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 10% glycerol, containing 2 mM phenylmethylsulfonyl fluoride and Complete protease inhibitor cocktail (Boehringer Mannheim). rProteinA immobilized on Sepharose beads (RepliGen) was added to protein lysates, which were rotated for 1 h and then cleared by centrifugation at 20 000 g for 5 min. Monoclonal anti-HA (12CA5) or polyclonal anti-LexA (gift of C.Denis, University of New Hampshire, NH) antibodies were added, and samples were mixed for 1 h and cleared by centrifugation for 5 min at 20 000 g. The supernatant was mixed with immobilized rProtein A for 1 h. The beads were collected by brief centrifugation and washed four times with 1 ml of extraction buffer. The procedure was performed at 4°C or on ice. Proteins were separated by SDS-PAGE in 7.5% polyacrylamide and immunoblotted with anti-HA or affinity-purified anti-Snf1 (Yang *et al.*, 1994). Antibodies were detected by enhanced chemiluminescence with ECLplus reagents (Amersham).

#### Immune complex kinase assays

Preparation of protein extracts and immunoprecipitation were as described above. Beads were then washed in kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM DTT) and resuspended in 20 μl of kinase buffer. The kinase reaction was initiated by the addition of 20 μCi of [γ-<sup>32</sup>P]ATP (3000 Ci/mmol; NEN). Reaction mixtures were incubated at room temperature for 20 min, and reactions were terminated by the addition of 30 μl of sample buffer. Proteins were separated by SDS-PAGE in 7.5% polyacrylamide. After electrophoresis, the gel was stained, washed extensively in destaining solution containing 10 mM sodium pyrophosphate, dried and exposed to film at -70°C with an intensifying screen.

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