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Yeast SNF1 Protein Kinase Interacts with SIP4, a C_6 Zinc Cluster Transcriptional Activator: a New Role for SNF1 in the Glucose Response

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The SNF1 protein kinase has been widely conserved in plants and mammals. In *Saccharomyces cerevisiae*, SNF1 is essential for expression of glucose-repressed genes in response to glucose deprivation. Previous studies supported a role for SNF1 in relieving transcriptional repression. Here, we report evidence that SNF1 modulates function of a transcriptional activator, SIP4, which was identified in a two-hybrid screen for interaction with SNF1. The N terminus of the predicted 96-kDa SIP4 protein is homologous to the DNA-binding domain of the GAL4 family of transcriptional activators, with a C_6 zinc cluster adjacent to a coiled-coil motif. The C terminus contains a leucine zipper motif and an acidic region. When bound to DNA, a LexA-SIP4 fusion activates transcription of a reporter gene. Transcriptional activation by SIP4 is regulated by glucose and depends on the SNF1 protein kinase. Moreover, SIP4 is differentially phosphorylated in response to glucose availability, and phosphorylation requires SNF1. These findings suggest that the SNF1 kinase interacts with a transcriptional activator to modulate its activity and provide the first direct evidence for a role of SNF1 in activating transcription in response to glucose limitation.

In *Saccharomyces cerevisiae*, glucose repression is an important regulatory mechanism that controls the expression of many genes involved in carbon metabolism (reviewed in reference 30). The SNF1 (CAT1, CCR1) serine-threonine protein kinase plays a central role in this regulatory mechanism and also in other aspects of cell growth and sporulation (10, 56, 60). The identification of SNF1 homologs in plants and mammals indicates that SNF1 has been conserved through evolution (1, 8, 24, 36, 45, 46).

In *S. cerevisiae*, mutation of *SNF1* causes failure to express glucose-repressed genes in response to glucose limitation and results in serious growth defects (10, 56). SNF1 kinase activity appears to be regulated in response to glucose because in vitro assays indicate that SNF1 activity toward a synthetic peptide substrate increases severalfold as glucose-grown cells are starved for glucose (66). Genetic evidence suggests that one role of SNF1 is to alleviate transcriptional repression by the MIG1-SSN6-TUP1 complex at the *SUC*, *GAL*, and other loci (31, 58, 61, 64). However, the SNF1 kinase pathway must also affect gene expression by other mechanisms, as some glucose-regulated genes (for example, the *ADH2* gene) are not subject to repression by MIG1-SSN6-TUP1 (15). One possibility is that SNF1 may activate positive regulators of gene expression, as suggested for the regulation of gluconeogenic genes (26, 55). As yet, there is no evidence that SNF1 directly phosphorylates a transcriptional regulator.

The SNF1 kinase is physically associated with other proteins in large complexes (19). The complexes contain an activating subunit, SNF4 (CAT3) (11, 12, 57), and the proteins SIP1, SIP2, and GAL83, which have sequence homology and are functionally related (17, 67, 68). SIP1, SIP2, and GAL83 coimmunoprecipitate with SNF1 and are phosphorylated in vitro (68). *GAL83* affects glucose repression of the *GAL* genes, and *SIP1* and *SIP2* in multicopy partially suppress a *gal83* mutation (17, 48). *SIP1* in multicopy also restores *SUC2* gene expression in mutants with reduced SNF1 kinase activity (67). It has been proposed that SIP1, SIP2, and GAL83 act as adaptors between SNF1 and specific targets of the kinase (68).

Previously, we undertook a two-hybrid screen (20) to identify proteins that interact with SNF1 in vivo, including proteins present in kinase complexes and, potentially, regulators and targets of the kinase. A library of fusions to the GAL4 transcription-activating domain (GAD) was tested for interaction with SNF1 fused to the GAL4 DNA-binding domain (GBD). Two proteins in the kinase complex, SIP1 and SIP2, were first identified in this screen (67). Also identified were two other SNF1-interacting proteins, designated SIP3 and SIP4. Genetic data suggest that SIP3 is functionally related to the SNF1 protein kinase pathway and may have a role in transcriptional activation (37). Here, we report the characterization of SIP4.

We present evidence that SIP4 is a transcriptional activator and a target of the SNF1 protein kinase. The predicted SIP4 protein is a C_6 zinc cluster protein homologous to the GAL4 family of transcriptional activators. The ability of SIP4 to activate transcription is regulated by glucose and requires a functional SNF1 protein kinase. We provide evidence that the SIP4 protein is phosphorylated in vivo in response to glucose starvation and that SNF1 is necessary for this phosphorylation. These findings directly implicate the SNF1 kinase in transcriptional activation as part of the cellular regulatory response to glucose.

MATERIALS AND METHODS

Strains and genetic methods. *S. cerevisiae* strains were GGY::171 (*gal4*Δ *gal80*Δ *GAL1-lacZ his3 leu2*) (22), CTY10-5d (*MAT***a** *gal4 gal80 URA3*::*lexAop-lacZ ade2 his3 leu2 trp1*) (constructed by R. Sternglanz, State University of New York, Stony Brook), MCY829 (*MAT*α *his3*-Δ*200 lys2-801 ura3-52 SUC2*),

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MCY2692 (*MAT***a** *snf1-K84R his3-Δ200 leu2-3,112 ura3-52 SUC2*), and MCY2693, which is isogenic to MCY2692 but of opposite mating type (*MAT*α). The MCY strains have the S288C genetic background. Standard genetic methods (51) were used. Yeast cells were grown in YEP (1% yeast extract, 2% Bacto Peptone) or synthetic complete (SC) medium lacking the appropriate supplements to maintain selection for plasmids. Growth on different carbon sources was scored by spotting cell suspensions or streaking for single colonies on medium containing either 2% glucose, sucrose, raffinose, or galactose or 3% glycerol, ethanol, or lactate. Cells were grown anaerobically, except on glycerol, ethanol, or lactate, by incubating plates in Biobag environmental chamber type A (Becton Dickinson). *Escherichia coli* XL1-Blue was used as the plasmid host.

Plasmids. The 3.4-kb *Sph*I insert in p2-38 (one *Sph*I site in the vector) was cloned in both orientations in pUC19, giving pXY54 and pXY55; *Xba*I digestion yields 1.2- and 2.2-kb fragments, respectively. Plasmids pXY56 and pXY57 were recovered by screening a yeast genomic DNA library cloned in YEp24 (9) by colony hybridization using the *Sph*I fragment in p2-38. pXY58 and pRJ6 contain the 3.3-kb *Bam*HI-*Xho*I fragment of pXY56 in the *Bam*HI and *Sal*I sites of pUC19 and pRS425 (13), respectively. pRJ7 was constructed by cloning the 2.2-kb *Xba*I-*Sph*I fragment of pXY54 into YEp366R (47). In pPL40, the 1.1-kb *Bam*HI-*Xba*I fragment of pXY54 has been replaced by the 2.4-kb *Bam*HI-*Xba*I fragment of pXY58. Plasmid pPL44 contains the 2.3-kb *Xba*I-*Sal*I fragment of pXY55 cloned into a pUC19 derivative lacking the *Pst*I site. pPL42 carrying the *sip4Δ1*::*HIS3* disruption was made by replacing the *Pst*I-*Xho*I fragment of pPL40 with the 1.8-kb *Pst*I-*Sal*I fragment of pPL3-1, a pUC19 derivative which contains the *HIS3 Bam*HI fragment.

pGAD-SIP4, which expresses the complete SIP4 sequence fused in frame to GAD, was constructed in several steps. We first performed a PCR using pXY58 as the template. Primers were O-PL24 (5′-CCGAATAGAGGATCCCCAAG AGG-3′), which is complementary to nucleotides −11 through 12 and creates a *Bam*HI site (underlined) at the 5′ terminus of *SIP4*, and O-PL25 (5′-GCCTG GGGATTTCTAGAACCG-3′), which is complementary to nucleotides 1219 through 1200 and overlaps the *Xba*I site in *SIP4*. The amplified product was digested with *Bam*HI plus *Xba*I and cloned into pPL44. Then, we swapped the *Pst*I-*Xba*I fragment with the sequence from pXY58 to eliminate possible mutations generated by the PCR. Finally, the 3.3-kb *Bam*HI-*Sal*I fragment of the resulting plasmid was cloned in pACTII (38) cut with *Bam*HI and *Xho*I, yielding pGAD-SIP4.

We cloned the 2.1-kb *Xba*I-*Sal*I fragment of pPL44 in pACTII cut with *Bam*HI and *Xho*I (both *Xba*I and *Bam*HI protruding ends were filled in with Klenow enzyme) to obtain pGAD-SIP4$_{402}$, which expresses the 427 C-terminal amino acids of SIP4 fused in frame to GAD.

To construct pLexA-SIP4, we performed a PCR using pXY58 as the template, primer O-XY (5′-CCCGAATTCACGATGGCCAAGAGG-3′), which is complementary to nucleotides −3 through 12 and creates an *Eco*RI site at the 5′ terminus of *SIP4*, and the universal reverse primer complementary to vector sequence 3′ to *SIP4*. The amplified product was cut with *Eco*RI plus *Xba*I and cloned in pPL44. We swapped the *Pst*I-*Xba*I region with the pXY58 sequence and then cloned the 3.3-kb *Eco*RI-*Sal*I fragment in pSH2-1 (25), yielding pLexA-SIP4.

pSIP4-lacZ contains the 1.2-kb *Bam*HI-*Pst*I fragment of pXY58 in YEp353 (47).

To generate the hemagglutinin (HA)-SIP4 protein, the *Bam*HI-*Xho*I and *Xho*I-*Kpn*I fragments of *SIP4* (*Kpn*I from pXY55) were ligated in two steps in pKB174, a derivative of pRS426 lacking the *Not*I site (6). The resulting plasmid was subjected to site-specific mutagenesis using O-PL29 (5′-CTGAATAGACG ATGGCCGGCGGCCGCAAGAGGAAATATGGC-3′), which is complementary to nucleotides −11 through 21 and generates a *Not*I site directly 3′ to the ATG codon. A *Not*I fragment from plasmid GTEP encoding an HA triple epitope tag (63) was inserted to create pHA-SIP4.

DNA sequencing and analysis. *SIP4* sequences in p2-34 and p2-38 were determined by extending a primer from the *GAL4* region (67). A set of nested deletions (Pharmacia), subcloned restriction fragments, and specific oligonucleotide primers were used to obtain overlapping sequence information for both strands of the region from nucleotides −642 through +2649 by the dideoxy-chain termination method (54), using Sequenase (United States Biochemical) and either single-strand or double-strand plasmid DNA. The protein sequence comparison was performed at the National Center for Biotechnology Information by using BLAST. Motifs were identified by using the MOTIFS program (University of Wisconsin Genetics Computer Group).

β-Galactosidase assays. GGY::171 or CTY10-5d transformants were patched on selective SC–2% glucose plates. After 2 to 3 days of incubation at 30°C, filter assays for detection of β-galactosidase, using the chromogen 5-bromo-4-chloro-3-β-D-galactopyranoside (X-Gal), were done as described previously (37). For quantitative assays, cells were prepared from exponentially growing cultures and permeabilized with sodium dodecyl sulfate (SDS) and chloroform. β-Galactosidase activity is expressed as described by Miller (44).

Immunoblot analysis and phosphatase treatment. Immunoblot analysis of total cell proteins was carried out as described previously (19) except that pepstatin, leupeptin, and aprotinin were each added to the extraction buffer to a final concentration of 1 μg/ml. Protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with a monoclonal HA (12CA5; BabCo) or polyclonal LexA antibody at a dilution of 1:10,000 or with anti-SNF1 antiserum at a dilution of 1:500, using the enhanced chemiluminescence procedure.

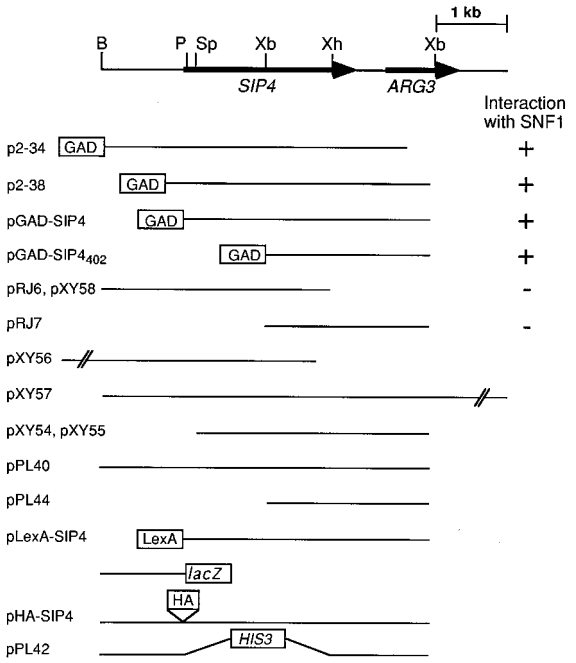

FIG. 1. Restriction maps of the *SIP4* gene and plasmids. Plasmids are described in the text. p2-38 contains *SIP4* sequence from nucleotide −237. Arrows designate coding regions and direction of transcription. LexA, LexA DNA-binding domain (residues 1 to 87); HA, triple HA epitope tag; GAD, GAL4 activation domain (residues 768 to 881). Restriction sites: B, *Bam*HI; P, *Pst*I; S, *Sph*I; Xb, *Xba*I; Xh, *Xho*I. Protein interactions were tested by the two-hybrid assay as in Table 1.

Samples to be treated with calf intestine phosphatase (CIP) were extracted in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (50 mM, pH 7.5) containing pepstatin, leupeptin, and aprotinin (each at 1 μg/ml) and phenylmethylsulfonyl fluoride (1 mM). The incubation mixture contained (in a final volume of 20 μl) 5 μg of protein extract, 50 mM Tris-HCl buffer (pH 7.9), 0.2% SDS, 1 mM each EDTA, dithiothreitol, and $MgCl_2$, 200 μM $ZnSO_4$, 1 μg each of pepstatin, leupeptin, and aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride. The reaction mixtures were incubated at 37°C for 1 h in the presence of 20 U of CIP (molecular biology grade; Boehringer Mannheim). Samples containing phosphatase inhibitors were supplemented with a cocktail of phosphatase inhibitors (final concentrations: 5 mM sodium fluoride, 5 mM sodium phosphate [pH 8.0], 10 mM sodium PP_i, 5 mM each EDTA and ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid [EGTA]). Mock samples were treated identically but without added CIP and phosphatase inhibitors.

Nucleotide sequence accession number. The GenBank accession number for *SIP4* is U17643.

RESULTS

Interaction of SIP4 and SNF1 protein kinase is detected only in glucose-starved cells. *SIP4* sequences were recovered on two plasmids, p2-34 and p2-38 (Fig. 1), in a two-hybrid screen for SNF1-interacting proteins (67). Both p2-34 and p2-38 in combination with a plasmid expressing GBD-SNF1 activated β-galactosidase expression from a *GAL1-lacZ* reporter, and expression depended on the SNF1 sequence (Table 1). In addition, p2-38 in combination with a plasmid expressing LexA-SNF1 activated expression of a *lexAop-lacZ* reporter (Table 1). Quantitative assays showed that β-galactosidase activity was not expressed in glucose-repressed cells but was expressed to high levels in cells grown in galactose, glycerol, and ethanol (Table 1).

Sequence analysis of p2-34 and p2-38 revealed no open reading frame at the junction between the GAD sequence and

TABLE 1. SIP4 interaction with SNF1 in the two-hybrid system[a]

DNA-binding hybrid	Activator protein	Color of transformants[b]	β-Galactosidase activity (U)[c]	
			R	D
GBD-SNF1	SIP4 (p2-34)	Light blue	<1	20
GBD-SNF1	SIP4 (p2-38)	Light blue	<1	16
GBD-lamin C	SIP4 (p2-38)	White	ND	<1
GBD	SIP4 (p2-38)	White	ND	<1
LexA-SNF1	SIP4 (p2-38)	Light blue	<1	220
LexA	SIP4 (p2-38)	White	<1	<1
LexA-SNF1	GAD-SIP4	Blue	160	138
LexA-SNF1	GAD-SIP4$_{402}$	Blue	ND	80
LexA-SNF1	GAD	White	<1	<1
LexA	GAD-SIP4	White	<1	<1

[a] Proteins were expressed from pEE5 (GBD-SNF1) (20), pLAM5 (GBD-lamin C) (3), pMA424 (GBD) (41), pRJ55 (LexA$_{202}$-SNF1) (28), pLexA(1-202)+PL (52), p2-34, and p2-38 (SIP4), pGAD-SIP4, and pACTII (GAD). The host was GGY::171 or CTY10-5d when interactions were tested with GBD or LexA fusions, respectively.

[b] Determined by filter assays on patches of cells. Transformants expressing SIP4 and GBD-SNF1 or LexA-SNF1 were light blue after overnight incubation because *SIP4* expression was partially derepressed as a result of exhaustion of glucose in the vicinity of the patch. Transformants expressing LexA-SNF1 and GAD-SIP4 turned blue in 1 h. White transformants remained white after overnight incubation.

[c] Transformants were grown in selective SC-His-Leu containing 2% glucose (repressing conditions [R]) or 2% each galactose, glycerol, and ethanol (derepressing conditions [D]). Overexpression of GAD-SIP4 was slightly deleterious for cells in liquid culture. Values are averages for at least four transformants. Values of <1 corresponded to white colony color. ND, not determined. Standard errors were <20%.

the yeast genomic inserts, suggesting that the SIP4 protein not only interacts with SNF1 but also activates transcription without being fused to GAD. Most of the region common to p2-34 and p2-38 appeared to be necessary for interaction with SNF1 and activation (Fig. 1). Sequence analysis of 3.3 kb spanning this region identified one long open reading frame of 829 codons. The 0.3-kb region at the 3′ extremity matched the 5′ noncoding sequence and the first 80 codons of *ARG3* on the left arm of chromosome X (Fig. 1).

SIP4 protein contains C_6 zinc cluster and leucine zipper motifs. The predicted 96-kDa SIP4 protein shows several noteworthy features common to transcriptional activators (Fig. 2). First, the N terminus contains a region (residues 45 through 76) homologous to the consensus sequence for the C_6 zinc cluster (Fig. 3). This domain, consisting of six conserved cysteines that chelate two zinc cations, is a zinc-dependent DNA-binding domain (49). The C_6 zinc cluster is characteristic of a family of transcriptional activators, including GAL4 (50) (Fig. 3). Like several other members of this family, including GAL4, LEU3, HAP1, PUT3, and PPR1, SIP4 contains a potential homodimerization domain C terminal to the C_6 zinc cluster (50). Five hydrophobic residues in this region (residues 110 through 124) form a 4-3 repeat characteristic of coiled coils. SIP4 also shares 65% identity with the CAT8 protein (26) over 20 amino acids located immediately C terminal to the C_6 zinc cluster motif (residues 77 through 96).

A putative leucine zipper, comprising one isoleucine and four leucines spaced seven residues apart, begins at residue 503. This motif, identified in many transcriptional regulators, has been shown to mediate dimer formation (34). SIP4 is the only member of the C_6 zinc cluster family with a leucine zipper motif.

The C-terminal half of SIP4 is sufficient for interaction with SNF1. We fused the 427 C-terminal amino acids of SIP4 to GAD, and the resulting GAD-SIP4$_{402}$ protein interacted with LexA-SNF1 in the two-hybrid system (Fig. 1; Table 1). In addition, the C terminus (residues 710 to 829) is highly charged and acidic (predicted pI of 4.5). Acidic regions have been identified in numerous activator proteins and implicated in transcriptional activation (27, 40).

Interaction between LexA-SNF1 and GAD-SIP4 is not glucose regulated. In the two-hybrid system, LexA-SNF1 and SIP4 together induced β-galactosidase expression only in glucose-starved cells (Table 1). Several processes could potentially be subject to inhibition by glucose: the interaction between SIP4 and SNF1, the expression of *SIP4*, or the ability of SIP4 to activate transcription. To test whether glucose inhibits the interaction between SNF1 and SIP4, we expressed a GAD-SIP4 protein (Fig. 1) from the *ADH1* promoter, which is slightly induced by glucose (14). In the two-hybrid system, LexA-SNF1 and GAD-SIP4 caused activation of the *lexAop-lacZ* reporter to similar levels in cells grown in glucose or in galactose, glycerol, and ethanol (Table 1). Thus, glucose availability does not modulate the interaction between LexA-SNF1 and GAD-SIP4. Because both fusions are overexpressed, it remains possible that interaction between the native SNF1 and SIP4 proteins is modulated.

Expression of SIP4 is glucose repressed. To determine if *SIP4* expression is repressed by glucose, we constructed on a 2μm plasmid a *SIP4-lacZ* fusion gene with 1.1 kb of 5′ noncoding sequence and the first eight codons of *SIP4* fused to *lacZ*. Expression of *SIP4-lacZ* increased 10-fold when glucose-grown cells were derepressed (Table 2). A similar effect of glucose was observed with the glucose-regulated reporter *CYC1-lacZ* but not with the negative control *GLC7-lacZ*. Derepression of *SIP4-lacZ* to a similar level was also observed in cells grown in galactose (data not shown).

The SNF1 protein kinase is essential for expression of many glucose-repressed genes (10). We therefore examined the expression of *SIP4-lacZ* in a *snf1-K84R* mutant, which lacks

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MAKRKYGRSY SLDDTDSCSN KVLIVPTGQS SSNAITDFSV RKAHACDRCR LKKIKCDGLK     60
PNCSNCAKID FPCKTSDKLS RRGLPKGYTE LLEKEVVRLT NMNASSSANA NSNLPFLNDT    120
FYCFDNYNTQ SENQRFLGHL TWNILTNTFP TQKAVVFTDD RNNIDLQLQL LTNFLNLNGD    180
FNHLPNFLLL KYDYNLQFLK NLLSVIIKDF FKRQNSLLLL LYPTNLWKNL LLDKINSTAM    240
TGEPITLLAL LYIIQFTWSC FDDFKLFKVT KLIVSLTTNS KLDLKVLQLV NLSIFYFMGA    300
SVDSCKSKSS LTEHSNVNSV IWTNDLLNLN FTNILNMGLY INPKNLIPIS GNNNNNKSNE    360
EDDRIVTFWC FQFLSSWWSL IQGLPKSNFL TEEFQPKSIS VLEIPRLKPF EILLNFIIYS    420
LDGCNLLNIS SLNVSDPNFQ FFQNELESFK KNLLLWNLYH NLSDHDNFRF LTSSSNKKLT    480
TNLLLKNLTG LNHKLNQPDF VEIQLTLFYL SLKLMTLKEG DQDLKKEDIS LEILSLYFLI    540
LTDDSNNDDN QQLQPQQLNL YHFTPFNSID IIDLCLNNLN NWSLSLKYES GQNQPHSSKI    600
KFEKFQNFLN HWCPIWYYDE FSTNPFLQIL KINFKLLPFE TIHYSQEEQR LLISLTKLRY    660
LDAVSSFNSS SVKSNFASKV NTQLNLLQHS SSNSNFLDAS PYDFNKIFMN NFENYDYETD    720
EGYAEDDDEE DSDSDNSLPL EIPFKKSKNK CKNRNKELSQ RLSLFENRDS NSVDFNTDTN    780
LNLNPDSPSV TSSKKKYLDH IILDNRDIVS NHDSSKQKFK IQNILNSTF              829
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FIG. 2. Predicted amino acid sequence of the *SIP4* product. Amino acids are numbered on the right. The C_6 zinc cluster motif is underlined (residues 45 to 76). Residues in reverse contrast letters define the coiled coil (residues 110 to 124) and the leucine zipper (residues 503 to 531) motifs.

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SIP4     45   ACDRCRLKKIKCD..GLKP.NCSNCAKIDFPCKTS
GAL4     10   ACDICRLKKLKCS..KEKP.KCAKCLKNNWECRYS
CAT8     69   ACDRCRSKKTRCD..GKRP.QCSQCAAVGFECRIS
PUT3     33   ACLSCRKRHIKCP..GGNP..CQKCVTSNAICEYL
MAL63     7   SCDCCRVRRVKCD..RNKP..CNRCIERNLNCTYL
PPR1     33   ACKRCRLKKIKCD..QEFP.SCKRCAKLEVPCTYR
LEU3     36   ACVECRQQKSKCDAHERAPEPCTKCAKKNVPCTLK
ARGRII   20   GCVTCRGRKVKCD..LRHP.HCQRCEKSNLPCGGY
PDR1     45   SCDNCRKRKIKCN..GKFP..CASCEIYSCECTFS
LAC9     95   ACDACRLKKWKCS..KTVP.TCTNCLKYNLDCVYS
QUTA     48   ACDSCRSKKDKCD..GAQP.ICSTCASLSRPCTYQ
QA-1F    75   ACDQCRAARERCD..GIQP.ACFPCVSQGRSCTYQ

CONSENSUS     ACD-CR-KK-KCD..---P.-C--C-K-N--CTY
                  RR
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FIG. 3. Comparison between the C_6 zinc clusters of SIP4 and other transcriptional regulators. Numbers refer to the position of the first amino acid listed for each protein. Dots indicate gaps in the sequence that were introduced for better alignment. This comparison does not include all proteins containing a C_6 zinc cluster. Proteins and references: GAL4 (29), CAT8 (26), PUT3 (42), MAL63 (33), PPR1 (32), LEU3 (21), ARGRII (43), PDR1 (2), LAC9 (53), QUTA (5), and QA-1F (4).

TABLE 3. SNF1-dependent glucose regulation of transcriptional activation by LexA-SIP4[a]

Hybrid protein	No. of *lexA* operators	Wild type			*snf1-K84R*		
		β-Galactosidase activity (U)		D/R	β-Galactosidase activity (U)		D/R
		R	D		R	D	
LexA-SIP4	0	<1	<1		<1	<1	
	1	57	430	7.6	3.3	7.3	2.2
	6	530	3,550	6.7	56	66	1.2
LexA-SNF6	0	<1	<1		<1	<1	
	1	610	1,260	2.1	ND	ND	
	6	2,050	2,710	1.3	1,520	2,060	1.3

[a] Proteins were expressed from pLexA-SIP4, pLexA-SNF6 (35), and pSH2-1 ($LexA_{1-87}$). Target plasmids were pLR1Δ1, p1840, and pSH18-18 (zero, one, and six *lexA* operators, respectively) (7, 16, 65). Wild-type MCY829 and mutant MCY2693 were grown in selective SC-His-Ura containing 2% glucose (repressed [R]). Mid-log-phase cultures were derepressed (D) by being shifted to SC-His-Ura containing 0.05% glucose for 3 h. Values are averages for at least four transformants. Standard errors were <20%. Values obtained for activation by $LexA_{1-87}$ of targets with one or six *lexA* operators were ≤1. ND, not determined.

SNF1 activity as a result of mutation of the conserved ATP-binding site (11). The absence of significant β-galactosidase activity indicates that SNF1 is required for derepression of *SIP4-lacZ* expression (Table 2).

Transcriptional activation by DNA-bound LexA-SIP4 is regulated by glucose. We next assessed the possibility that the function of SIP4 as a transcriptional activator is regulated by glucose. We expressed a LexA-SIP4 hybrid protein, containing the LexA DNA-binding domain (residues 1 through 87) fused to SIP4, from the *ADH1* promoter (Fig. 1). A wild-type strain was cotransformed with the LexA-SIP4 plasmid and reporter plasmids containing *GAL1-lacZ* with zero, one, or six *lexA* operators replacing UAS_{GAL} (7). When transformants were grown in glucose, LexA-SIP4 strongly activated β-galactosidase activity from the *lexAop-GAL1-lacZ* target genes (Table 3). The control activator was a LexA fusion to SNF6, a component of the SNF-SWI activator complex (35).

We next compared the abilities of LexA-SIP4 to activate transcription of these reporters under glucose-repressing and derepressing conditions (Table 3). β-Galactosidase activity was about sevenfold higher under derepressing conditions. Immunoblot analysis showed that the LexA-SIP4 protein is severalfold more abundant in glucose-repressed cells than in derepressed cells (Fig. 4). Therefore, sevenfold represents a minimum estimate of the increase in LexA-SIP4 activity in glucose-deprived cells.

SNF1 protein kinase modulates transcriptional activation by LexA-SIP4. To test whether transcriptional activation by LexA-SIP4 depends on SNF1 protein kinase activity, we examined the ability of LexA-SIP4 to activate *lexAop-GAL1-lacZ* transcription in a *snf1-K84R* mutant. β-Galactosidase activity was significantly lower in the mutant than in the wild type (17- and 9-fold for reporters with one and six *lexA* operators, respectively, in glucose-repressed cells; Table 3). Moreover, the increase in LexA-SIP4 activity in response to glucose starvation was abolished in *snf1-K84R* cells. In controls, LexA-SNF6 activity showed no dependence on SNF1 protein kinase activity. Immunoblot analysis indicated comparable levels of LexA-SIP4 in wild-type and mutant cells (Fig. 4). Thus, the SNF1 protein kinase is necessary both for basal transcriptional activation by LexA-SIP4 and for its regulation by glucose.

SNF1-dependent phosphorylation of HA-SIP4 in response to glucose deprivation. An HA epitope-tagged SIP4 protein was expressed from the *SIP4* promoter on a 2μm plasmid. The tagged protein, HA-SIP4, interacted with GBD-SNF1 and activated transcription in a two-hybrid experiment. To examine HA-SIP4 for differential modification in response to glucose availability, total proteins from glucose-repressed and derepressed wild-type cells were separated by SDS-PAGE and subjected to immunoblot analysis. Using an HA antibody, we detected HA-SIP4 in repressed samples as a major band mi-

TABLE 2. Glucose repression of a *SIP4-lacZ* gene fusion[a]

Fusion gene	Wild type		D/R	*snf1-K84R*		D/R
	β-Galactosidase activity (U)			β-Galactosidase activity (U)		
	R	D		R	D	
SIP4-lacZ	1.6	16	10	2.2	2.1	1.0
CYC1-lacZ	204	1,320	6.5	15	14	1.0
GLC7-lacZ	69	86	1.2	56	58	1.0

[a] Wild-type MCY829 and mutant MCY2692 were transformed with pSIP4-lacZ, pLGΔ-312 (*CYC1-lacZ*) (23), and pGLC7-lacZ (62). *SIP4-lacZ* and *CYC1-lacZ* are promoter fusions, and *GLC7-lacZ* expresses GLC7 fused to β-galactosidase. Transformants were grown in SC-Ura containing 2% glucose (repressed [R]). Mid-log-phase cultures were derepressed (D) by being shifted to SC-Ura containing 0.05% glucose for 3 h. Values are averages for three transformants. Standard errors were <20%.

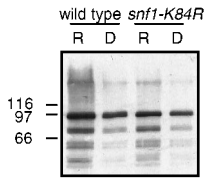

FIG. 4. Immunoblot analysis of LexA-SIP4 from glucose-repressed and derepressed cells. Wild-type MCY829 and mutant MCY2693 transformed with pSH18-18 (six *lexA* operators) and pLexA-SIP4 were grown and assayed for β-galactosidase activity as described in the footnote to Table 3. Protein extracts (5 μg) were separated by SDS-PAGE on a 7.5% gel and subjected to immunoblot analysis using anti-LexA. R, glucose repressed; D, derepressed. Protein size markers are indicated in kilodaltons. The predicted size of LexA-SIP4 is 106 kDa.

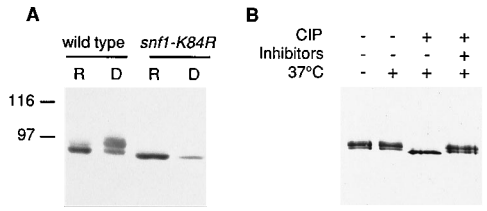

FIG. 5. Phosphorylation of HA-SIP4 in response to glucose starvation. Proteins were separated by SDS-PAGE in 7.5% (A) or 6% (B) polyacrylamide and subjected to immunoblot analysis using anti-HA. Size markers are indicated in kilodaltons. (A) Protein extracts (50 and 200 μg for wild type and *snf1-K84R* mutant, respectively) were prepared from strains MCY829 and MCY2693 transformed by pHA-SIP4. Cultures were grown in selective SC medium lacking uracil and containing 2% glucose (R [repressed]). Mid-log-phase cultures were derepressed by a shift to the same medium containing 0.05% glucose for 2 h (D [derepressed]). No band of 92 kDa was detected in cells transformed by a plasmid expressing the SIP4 protein. (B) Phosphatase treatment of HA-SIP4. Reactions were done on protein extracts (5 μg) of wild-type derepressed samples. The addition of CIP and phosphatase inhibitors is indicated above each lane. Reaction mixtures were incubated at 37°C for 1 h or kept on ice, as indicated. Dephosphorylation of HA-SIP4 occurred only in the presence of 0.2% SDS, suggesting that the phosphorylated residue(s) was not accessible to the phosphatase when HA-SIP4 was in its native conformation (data not shown).

grating at 92 kDa. We could also distinguish a minor band of lower mobility migrating at 96 kDa (Fig. 5A). Under derepressing conditions, the species of lower mobility became prominent, and the 92-kDa band decreased in intensity (Fig. 5A).

The functional association of SIP4 with the SNF1 protein kinase suggested that the modification could be phosphorylation. When derepressed protein extracts were treated with CIP, the lower-mobility species disappeared and concomitantly the 92-kDa band intensified (Fig. 5B). Addition of phosphatase inhibitors prevented the change in the pattern. Thus, the modification of HA-SIP4 under derepressing conditions corresponds to phosphorylation of the protein.

To test if phosphorylation of HA-SIP4 depends on the SNF1 protein kinase, we examined a *snf1-K84R* mutant. In both glucose-repressed and derepressed cells, immunoblot analysis detected a single protein of 92 kDa (Fig. 5A). This result indicates that SNF1 protein kinase activity is necessary for phosphorylation of HA-SIP4.

In the *snf1-K84R* mutant, the abundance of the HA-SIP4 protein dramatically decreased when the culture was shifted to low-glucose medium for 2 h, suggesting that the fusion protein is degraded. Instability of HA-SIP4 under derepressing conditions may explain why levels of HA-SIP4 protein did not increase in the wild type when cells were derepressed, whereas expression of the *SIP4-lacZ* promoter fusion increased 10-fold in the wild type.

LexA-GAL83 and GAD-SIP4 interact in the two-hybrid system. SNF1 is associated in a large complex with other proteins, including SNF4, SIP1, SIP2, and GAL83. SNF4 stimulates the kinase activity, and it has been proposed that SIP1, SIP2, and GAL83 differentially affect association of the kinase with different targets (68). To determine if one of these proteins mediates the interaction of SNF1 with SIP4, we tested the interaction between GAD-SIP4 and LexA-SNF4, LexA-SIP1, LexA-SIP2, or LexA-GAL83. No interaction with LexA-SNF4, LexA-SIP1, or LexA-SIP2 was detected by the filter color assay. LexA-GAL83 (68) in combination with GAD activated transcription (46 and 145 U of β-galactosidase activity in strain CTY10-5d during growth in glucose and galactose-glycerol-ethanol, respectively; values are averages for four transformants), but the presence of GAD-SIP4 significantly increased the β-galactosidase activity (660 and 990 U for repressed and derepressed conditions, respectively). Thus, the observed two-hybrid interaction between LexA-GAL83 and GAD-SIP4 was not glucose regulated, consistent with results for LexA-SNF1 and GAD-SIP4 (Table 1).

HA-SIP4 is not tightly associated with the SNF1 kinase complex. To further characterize the interaction between SIP4 and the SNF1 kinase complex, we tested for coimmunoprecipitation with SNF1 and GAL83. Protein extracts from wild-type transformants expressing HA-SIP4 and LexA-SNF1 or LexA-GAL83 were immunoprecipitated with a LexA antibody. Anti-HA immunoblot analysis showed that HA-SIP4 was expressed in both glucose-repressed and derepressed cells but did not coimmunoprecipitate with LexA-SNF1 or LexA-GAL83 (data not shown). We also incubated the immunoprecipitates with [γ-^{32}P]ATP to assay SNF1 protein kinase activity, but no labeled product corresponding to HA-SIP4 was identified. In a reciprocal experiment, an HA antibody was used for immunoprecipitation, but neither LexA-SNF1 nor LexA-GAL83 was detected in the immunoprecipitates (data not shown). These results suggest that HA-SIP4 is not tightly associated with SNF1 or GAL83 but are compatible with the idea that SIP4 is a substrate that interacts transiently with the SNF1 complex.

Genetic analysis of *SIP4* function. To determine the phenotype caused by deletion of *SIP4*, we introduced the mutation *sip4*Δ*1*::*HIS3* (Fig. 1) into the chromosomal locus of a diploid. Upon sporulation of two diploid transformants heterozygous for *sip4*Δ*1*::*HIS3*, all 11 asci gave rise to four viable spores. The mutant segregants grew well at 30°C on all carbon sources tested (glucose, sucrose, raffinose, galactose, lactate, ethanol, and glycerol), grew at 37°C on glucose, and showed wild-type regulation of *SUC2* expression. Diploids homozygous for *sip4*Δ*1*::*HIS3* sporulated normally. In addition, increased gene dosage of *SIP4* or *lexA-SIP4* did not suppress the growth defects or the invertase activity defects of a *snf4*Δ*2* mutant, which is deficient in SNF1 kinase activity (12). We also crossed the *sip4*Δ*1*::*HIS3* mutant with strains carrying *sip1*Δ, *sip2*Δ, or *sip3*Δ mutations but did not detect any growth defect in the double mutants.

Finally, we tested for autoregulation of *SIP4* expression. Proteins containing a C_6 zinc cluster DNA-binding motif typically recognize sites with the sequence $CGGN_xCCG$ (50). Examination of the *SIP4* promoter revealed one potential binding site, $CGGN_{23}CCG$ (positions −125 to −97). However, assays of *SIP4-lacZ* expression in a *sip4* mutant showed wild-type regulation and levels. Furthermore, a multicopy *SIP4* plasmid did not elevate *SIP4-lacZ* expression in the *sip4* mutant or wild type.

DISCUSSION

Identification of SIP4 as a transcriptional activator. SIP4 was identified as a SNF1-interacting protein in the two-hybrid system. Several lines of evidence implicate SIP4 in transcriptional activation. First, SIP4 was not fused to GAD in the plasmids recovered from the two-hybrid screen, suggesting that SIP4 contains a transcription-activating domain. Second, SIP4 has sequence homology with the GAL4 family of transcriptional activators, defined by a C_6 zinc cluster DNA-binding domain (50). Finally, SIP4 activates transcription when bound to DNA as a LexA-SIP4 hybrid protein.

Regulation of SIP4 expression and activity. In the two-hybrid system, induction of β-galactosidase activity by LexA-SNF1 and SIP4 was detected only in glucose-deprived cells. Our results indicate that glucose represses *SIP4* expression and

down regulates the ability of SIP4 to activate transcription. However, glucose did not appear to inhibit the interaction between overexpressed LexA-SNF1 and GAD-SIP4. The contribution of regulatory mechanisms at the level of *SIP4* expression and SIP4 activity may be sufficient to explain the regulated activation by LexA-SNF1 and SIP4 in the two-hybrid system. Alternatively, the interaction between SNF1 and SIP4 may also be modulated by glucose when SIP4 is expressed at normal levels from its own promoter.

SNF1 kinase activity is required for derepression of *SIP4-lacZ* expression in the absence of glucose. For several other glucose-repressed genes, genetic evidence indicates that SNF1 relieves transcriptional repression mediated by the MIG1-SSN6-TUP1 complex (31, 58, 61, 64); however, no sequence matching the MIG1 DNA-binding consensus (39) was found 5′ to *SIP4*.

Role for SIP4 in activation of glucose-repressed genes. The regulation by glucose of *SIP4* expression and activity strongly suggests a role for SIP4 in activation of glucose-regulated genes in response to glucose starvation. We did not detect any defect in carbon source utilization or sporulation associated with deletion of *SIP4*. The existence of a functional homolog of SIP4 is possible, although no sequence homolog was detected by Southern blotting at low stringency (data not shown). The SNF1 protein kinase is also involved in many other cellular processes, including peroxisome biogenesis, glycogen storage, and thermotolerance (59, 60). It seems likely that SIP4 is required for expression of one or more of the many genes that are controlled by the SNF1 pathway.

SNF1-dependent phosphorylation of SIP4 in glucose-deprived cells. Our results demonstrate that SIP4 is differentially phosphorylated in response to glucose availability, with increased phosphorylation in starved cells. Phosphorylation of SIP4 depends on SNF1 kinase activity, as no phosphorylation was detected in a *snf1-K84R* mutant. These findings, together with the interaction of SNF1 and SIP4 in the two-hybrid system, strongly suggest that SNF1 directly phosphorylates SIP4; however, it remains possible that SIP4 is phosphorylated by another kinase that is in turn dependent on SNF1.

Genetic evidence suggests that this differential phosphorylation is functionally significant. The ability of LexA-SIP4 to activate transcription increases in glucose-derepressed cells and requires the SNF1 kinase. Thus, the increased phosphorylation of SIP4 when glucose is limiting correlates with, and may be responsible for, the enhanced potency of SIP4 as a transcriptional activator.

It is worth noting that LexA-SIP4 showed significant ability to activate transcription in glucose-repressed cells and that this activation also depended on the SNF1 kinase. In in vitro assays, SNF1 kinase activity is detected in both glucose-repressed and derepressed extracts, although activity is elevated severalfold upon derepression (19, 66). The minor, slowly migrating species of HA-SIP4 detected in glucose-repressed cells may correspond to species that are phosphorylated by SNF1; consistent with this view, no slowly migrating band was detected in a *snf1-K84R* mutant. Thus, SIP4 most likely is phosphorylated to some degree in glucose-repressed cells, although clearly to a lesser extent than in derepressed cells. Glucose repression of SIP4 expression, together with differential phosphorylation, is probably required to achieve appropriate regulation of genes that are activated by SIP4.

A new regulatory role for the SNF1 protein kinase in the glucose response. Previous studies supported a role for the SNF1 protein kinase in relieving transcriptional repression mediated by the MIG1-SSN6-TUP1 complex. Here, we have presented direct evidence for a role of SNF1 in activating transcription in response to glucose limitation. The interaction between SIP4 and SNF1 in the two-hybrid system and the SNF1-dependent phosphorylation of SIP4 strongly suggest that SNF1 interacts with this transcriptional activator to modulate its activity.

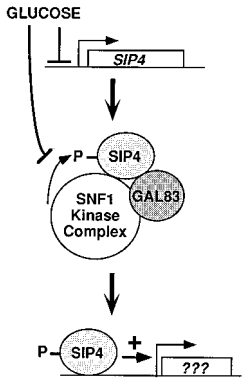

FIG. 6. Model for regulation of SIP4 activity by the SNF1 protein kinase. Expression of the *SIP4* gene is repressed by glucose, and the SNF1 protein kinase is required to relieve this transcriptional repression (not depicted here). The SIP4 protein interacts transiently with the SNF1 kinase complex and is phosphorylated, presumably by SNF1. GAL83 may mediate this interaction. Phosphorylation increases the potency of SIP4 as a transcriptional activator. SIP4 activates expression of specific glucose-regulated genes by binding to their promoters via its C_6 zinc cluster DNA-binding domain.

Genetic evidence also suggests interaction between SNF1 and other transcriptional activators. *MSN2* and its homolog *MSN4* encode zinc finger transcriptional activators and are multicopy suppressors of the *snf1-ts* defect in *SUC2* expression (18). Similarly, *CAT8* encodes a zinc cluster transcriptional activator necessary for derepression of gluconeogenic enzymes and is a multicopy suppressor of the growth defect of a *snf1* mutant on ethanol (26).

Previous data suggested that SIP1, SIP2, and GAL83 are present in different SNF1 complexes, and it was proposed that these proteins differentially affect the association of SNF1 protein kinase with specific targets (68). We present evidence that SIP4 interacts with GAL83, but not with SIP1 or SIP2, in the two-hybrid system. Further biochemical studies will be required to determine whether GAL83 mediates the interaction between SIP4 and SNF1.

The simplest model consistent with our data is diagrammed in Fig. 6. In response to glucose starvation, transcriptional repression of *SIP4* is relieved and expression of the SIP4 protein is elevated. SIP4 interacts transiently with the SNF1 protein kinase; GAL83 may facilitate this interaction. Phosphorylation of SIP4, presumably by SNF1, increases its potency as a transcriptional activator. The resulting elevated levels of phosphorylated SIP4 protein cause increased transcriptional activation of specific genes with promoters containing SIP4 binding sites. Thus, the SNF1 protein kinase functions not only to relieve transcriptional repression but also to enhance activation of gene expression as part of the regulatory response to glucose.

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