Nucleic Acids Research, 1994, Vol. 22, No. 4 597–603

Analysis of the SIP3 protein identified in a two-hybrid screen for interaction with the SNF1 protein kinase

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Received November 12, 1993; Revised and Accepted January 20, 1994 GenBank accession no. U03376

ABSTRACT

The *Saccharomyces cerevisiae SIP3* gene was identified in a two-hybrid screen for proteins that interact *in vivo* with the SNF1 protein kinase, which is necessary for release of glucose repression. We showed that the C-terminal part of SIP3, recovered through its ability to interact with SNF1, strongly activates transcription when tethered to DNA. We have cloned and sequenced the entire *SIP3* gene. The predicted 142-kD SIP3 protein contains a putative leucine zipper motif located in its C terminus. The native SIP3 protein also interacts with DNA-bound SNF1 and activates transcription of a target gene. A complete deletion of the *SIP3* gene did not confer phenotypes characteristic of *snf1* mutants. However, in a mutant deficient for the SNF1 kinase activity due to loss of the SNF4 stimulatory function, increased dosage of *SIP3* partially restored expression of the glucose-repressible *SUC2* gene. Overexpression of the C terminus of SIP3 caused defects in growth and *SUC2* expression which were remedied by overexpressing SNF1. Taken together, these genetic data suggest that SIP3 is functionally related to the SNF1 protein kinase pathway.

INTRODUCTION

The SNF1 serine–threonine protein kinase is required for the release of many genes from glucose repression, such as the *SUC* and *GAL* genes (1), and also affects other aspects of growth control in *S.cerevisiae* (2). Plant and mammalian homologs of the *SNF1* gene have been cloned, indicating that *SNF1* is conserved through evolution (3 and D.Carling, personal communication). In *S.cerevisiae*, it has been proven that the protein kinase activity of SNF1 is essential for its function *in vivo* (4). SNF1 is physically associated with SNF4, a protein which is necessary for maximal kinase activity (4, 5, 6). To identify regulators and targets of SNF1 kinase activity, we used the two-hybrid system, a genetic method for detecting protein–protein interactions *in vivo* (6). This system is based on the reconstitution of the yeast GAL4 transcriptional activator. GAL4 consists of two separable domains: a DNA binding domain (G_{BD}) and an activating domain (G_{AD}) (7, 8). The interaction of two hybrid proteins, one bearing G_{BD} and the other bearing G_{AD}, will bring together the two domains of GAL4, allowing transcriptional activation of a *GAL1-lacZ* reporter gene.

We screened plasmid libraries of yeast genomic DNA fragments fused in three different frames to G_{AD} (9), for interaction with a G_{BD}–SNF1 fusion (6), in the reporter strain GGY::171 carrying a *GAL1-lacZ* gene (10). By screening 300,000 colonies, we recovered 16 plasmids representing four new genes named *SIP1* through *SIP4* for SNF1 Interacting Protein (11). The SIP1 and SIP2 proteins were found to coimmunoprecipitate with SNF1 and to be phosphorylated *in vitro* in immune complex assays of SNF1 kinase activity (11, 12). *SIP1* was also recovered as a multicopy suppressor of growth defects due to reduced kinase activity (11). SIP1 and SIP2 share sequence homology with GAL83, which affects glucose repression of the *GAL* genes and shown to interact with SNF1 in the two-hybrid system (12, 13).

We report here the sequence and characterization of *SIP3*. The SIP3 protein is not homologous to the other SIP proteins. It contains a leucine zipper motif and can function as a transcriptional activator. The results presented in this paper suggest that SIP3 plays a role in the SNF1 kinase pathway.

MATERIALS AND METHODS

Yeast strains and genetic methods

S.cerevisiae strains MCY829 (*MATα his3-Δ200 lys2-801 ura3-52*), MCY2372 (*MATa his3-Δ200 leu2-3,112 ura3-52*) and GGY::171 (*Δgal4 Δgal80 his3 leu2 ura3::GAL1::lacZ*) (10) have been used in this study. The MCY strains have the S288C genetic background. Standard methods were used for genetic analysis and transformation (14). *E.coli* strain XL1-Blue (15) was used as the plasmid host. Growth on different carbon sources was scored by spotting cell suspensions on solid medium containing either 2% glucose, 2% sucrose, 2% raffinose, 2% galactose or 3% glycerol. Cells were grown anaerobically, except cells spotted on glycerol, by incubating plates in Biobag environmental Chamber type A (Becton–Dickinson).

Cloning of *SIP3* gene and sequencing

Bacteria harboring a yeast genomic DNA library cloned in YEp24 (16) were screened by colony hybridization (17). Labeled probes were prepared by the random priming procedure (Pharmacia).

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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 nucleotide −950 through +4201 by the dideoxy-chain termination method (18), using Sequenase (United State Biochemical) and either single-strand or double-strand plasmid DNA.

Construction of plasmids

To construct pPL7-1, we subcloned the 6.1-kb *Xho*I−*Sph*I fragment of pG_{AD}-$SIP3_{1104}$, in vector YEp366R (19) digested with *Sal*I and *Sph*I. pPL12-1 contains the *Sph*I−*Bam*HI fragment of pXY53 in pUC19 (the *Sph*I site is located in the YEp24 sequences, 0.6-kb 5′ to the *SIP3 Stu*I site). To construct pPL31-1, we introduced the 5-kb *Ban*II−*Bam*HI fragment in the *Xba*I−*Bam*HI fragment of the pRS316 centromeric vector (20). Then, we subcloned the 5-kb *Kpn*I−*Sac*II fragment of the resulting plasmid in the pRS426 2μ vector digested with *Kpn*I and *Sac*II (21).

Construction of gene fusions

To construct pLexA-$SIP3_{1107}$, pG_{BD}-$SIP3_{1107}$ and pG_{AD}-$SIP3_{1107}$, we used synthetic oligonucleotide primers to direct the synthesis of the C-terminal 122 codons of *SIP3* by the polymerase chain reaction (PCR) (22) (Perkin Elmer Cetus kit), with pPL12-1 as the template. To construct pLexA-$SIP3_{1107}$ and pG_{BD}-$SIP3_{1107}$, the primers were a 25-mer complementary to nucleotides 3310 through 3331, 5′CCCGAATTCGATAAGTT CACAATGC3′ designed to create an *Eco*RI site (underlined) and a 23-mer complementary to nucleotides 4001 through 3982, 5′CCCGGATCCTTCAGTAACTCGCG3′ overlapping the *Bam*HI site (underlined) located 309 bp 3′ to *SIP3* STOP codon. The amplified products were digested by *Eco*RI and *Bam*HI, purified and ligated to the *Eco*RI−*Bam*HI fragments of pEG202 (23), yielding pLexA-$SIP3_{1107}$, or pMA424 (24), yielding pG_{BD}-$SIP3_{1107}$. In pLexA-$SIP3_{1107}$, codons 1 through 202 of LexA and 3 codons derived from the pEG202 polylinker and the PCR primer are fused in frame to codon 1107 of SIP3. In pG_{BD}-$SIP3_{1107}$, codon 1 through 147 of G_{BD} and 3 codons derived from the pMA424 polylinker and the PCR primer are fused in frame to codon 1107 of SIP3. The remainder of *SIP3* ORF and the translational STOP codon are present in both fusions.

To construct pG_{AD}-$SIP3_{1107}$, the primers were a 25-mer complementary to nucleotides 3310 through 3342, 5′CCCGGATCCATAAGTTCACAATGC3′ designed to create a *Bam*HI site (underlined) and the 23-mer complementary to nucleotides 4001 through 3982. The amplified products were digested by *Bam*HI and inserted after purification in the *Bam*HI site of pGAD2F (9). In the resulting plasmid pPL19-5, codons 768 through 881 of G_{AD} and 6 codons derived from the the pGAD2F linker and the PCR primer are fused to codon 1107 of *SIP3*. In pPL19-5, the fusion gene is transcribed from the *ADH1* promoter. To decrease the level of G_{AD}-$SIP3_{1107}$ expression, we inverted the orientation of the 1.4-kb *Hin*DIII fragment containing the gene fusion, yielding pG_{AD}-$SIP3_{1107}$, in which the hybrid protein is expressed from the same unknown promoter used in the original pG_{AD} plasmid library (9).

To construct pLexA-SIP3, we used two primers to direct the synthesis of the N-terminal 409 codons of SIP3 by PCR, with pPL15-21 as the template. The primers were a 27-mer complementary to nucleotides −6 through 18, 5′CCCGAATTCATGTCCGTTCACGGGAGG3′, with an *Eco*RI site (underlined) immediately 5′ to the initiating ATG codon and a 22-mer complementary to nucleotides 1261 through 1240, 10 bp 3′ to the *Xba*I site, 5′GTCGCTTCAAAAGCTATTAACC3′. The amplified products digested by *Eco*RI and *Xba*I were subcloned in pUC19, yielding pPL23-1. The 1.2-kb *Eco*RI−*Xba*I fragment of pPL23-1 and the 2.7-kb *Xba*I−*Bam*HI fragment of pPL12-1 were ligated with the *Eco*RI−*Bam*HI fragment of pEG202. In the resulting pLexA-SIP3, codon 1 through 202 of LexA and 2 codons derived from pEG202 polylinker and the PCR primer are fused in frame to codon 1 of SIP3.

Disruption of the chromosomal *SIP3* locus

Plasmid pPL16-1 carrying the *sip3Δ1::HIS3* disruption was made by replacing the *Xba*I−*Xho*I fragment of pPL12-1 (the *Xho*I protruding ends were filled with the Klenow fragment of DNA polymerase I) with the 1.6-kb *Xba*I−*Sma*I fragment of pPL3-1, a pUC19 derivative which contains the *HIS3 Bam*HI fragment (Fig. 1).

The *sip3Δ2::HIS3* complete disruption was made in several steps. First, we eliminated the *Bam*HI site located 309 nucleotides 3′ to *SIP3* by subcloning the 2.7-kb *Xba*I−*Bam*HI fragment of pPL12-1, where the *Bam*HI protruding ends were filled with Klenow fragment, in the *Xba*I−*Hin*cII fragment of pUC19. The resulting plasmid cut by *Kpn*I and *Xba*I was ligated with the 4.6-kb *Eco*RI−*Xba*I fragment of pPL15-21, yielding plasmid pPL27-22, which contains the complete *SIP3* open reading frame (the *Eco*RI and *Kpn*I protruding ends were treated with Klenow fragment and the T4 DNA polymerase, respectively). *sip3Δ2::HIS3* was made by replacing the *Bam*HI−*Xho*I *SIP3* fragment in pPL27-22 by the *Bam*HI−*Xho*I *HIS3* fragment of pPL3-1, creating pPL28-1 (Fig. 1).

Diploid strain MCY829×MCY2372 was transformed to histidine prototrophy by either pPL16-1 cut with *Stu*I and *Eco*RI or pPL28-1 cut with *Sph*I and *Pvu*II. Southern blot analysis confirmed in both cases the presence of the disrupted allele at the *SIP3* locus on one chromosome homolog (data not shown).

Detection of protein interaction by filter assays

GGY::171 transformants were patched on selective synthetic complete plates containing glucose (2%) and incubated at 30°C for 2−3 days. β-galactosidase expression was determined qualitatively by replica-plating cells on nitrocellulose filters (Millipore, type HA 45 μM), which were incubated for 1 min in liquid nitrogen and transferred to plates containing two sheets of 3MM Whatman paper soaked with Z-buffer and chromogenic substrate X-gal (25). The plates were incubated at 30°C for 24 hours.

β-galactosidase assays

Cells were prepared from exponentially growing cultures. β-galactosidase activity was assayed in cells permeabilized with sodium dodecyl sulfate and chloroform and is expressed as described by Miller (26).

Invertase assays

Glucose-repressed and derepressed cells were prepared from exponentially growing cultures as described previously (27). Secreted invertase activity was assayed in whole cells and is expressed as micrograms of glucose released per minute per 100 mg (dry weight) of cells.

RESULTS

Isolation of *SIP3* sequences by the two-hybrid system

In the two-hybrid screen for SNF1 interacting proteins, the *SIP3* sequence was recovered once on a plasmid subsequently designated pG_{AD}-$SIP3_{1104}$ according to the SIP3 codon fused to G_{AD} (Fig. 1). This plasmid conferred blue color to the reporter strain only in the presence of G_{BD}-SNF1 (11). To confirm the specificity of the interaction with SNF1, we assayed β-galactosidase expression in strains containing pG_{AD}-$SIP3_{1104}$ in combination with plasmids expressing different G_{BD} fusion proteins. pG_{AD}-$SIP3_{1104}$ in combination with a plasmid expressing G_{BD}-SNF1 activated β-galactosidase expression from the *GAL1-lacZ* reporter gene (Table 1). This activation depended on the presence of the SNF1 protein fused to G_{BD} because no activation was detected in the presence of G_{BD} or a G_{BD}-lamin C hybrid protein (Table 1). Therefore, SIP3 satisfies the criteria of the two-hybrid system for a new protein that potentially interacts with SNF1 *in vivo*.

Cloning and sequencing of *SIP3*

To clone the entire *SIP3* gene, we probed a yeast genomic library (16) with the 6.6-kb *Sph*I insert from pG_{AD}-$SIP3_{1104}$ and obtained plasmid pXY53 (Fig.1). Sequence analysis indicated that the 5′ end of *SIP3* was missing from pXY53. Therefore, we reprobed the library with the 0.65-kb *Stu*I–*Xba*I fragment from the 5′ region of *SIP3* in pXY53 and recovered pPL15-21, which contains the complete *SIP3* gene (Fig.1). *SIP3* encodes an 1229 amino acid protein with a predicted molecular weight of 142 kD (Fig.2). *SIP3* is not homologous to any sequence in the GenBank database (release 79: October 15, 1993). Using the MOTIFS program (University of Wisconsin Genetics Computer Group), we identified near the C terminus of SIP3, beginning at residue 1156, 4 leucines spaced exactly seven residues apart that may constitute a leucine zipper (28). However, no basic DNA-binding domain or helix-loop-helix moti similar to those in the bZIP and bHLH-Zip families of proteins was identified N-terminal to this motif (29, 30). A second open reading frame located 3′ to *SIP3* was partially sequenced and does not correspond to any known gene.

The *SIP3* gene is located on chromosome XIV between *met2* and *rad 50*, as judged by hybridization to prime lambda-clone grid filters (L.Riles, personal communication).

The C-terminal region of SIP3 interacts with SNF1

Sequence analysis indicated that pG_{AD}-$SIP3_{1104}$ contains a G_{AD} domain fused to the last 125 amino acids of SIP3. We could not exclude that other sequences, located 3′ to *SIP3* in pG_{AD}-$SIP3_{1104}$, contributed to β-galactosidase activation in the two-hybrid experiments. Therefore, we constructed pG_{AD}-$SIP3_{1107}$, expressing only a fusion of G_{AD} to codon 1107 of SIP3, and pPL7-1, containing the remaining inserted sequences present in pG_{AD}-$SIP3_{1104}$ (Fig. 1). We co-transformed GGY::171 with each of these two plasmids and with a plasmid expressing G_{BD}-SNF1. No β-galactosidase activity was detected in transformants carrying pPL7-1, indicating that the sequences 3′ to *SIP3* do not contribute to activation (data not shown). The C-terminal 122

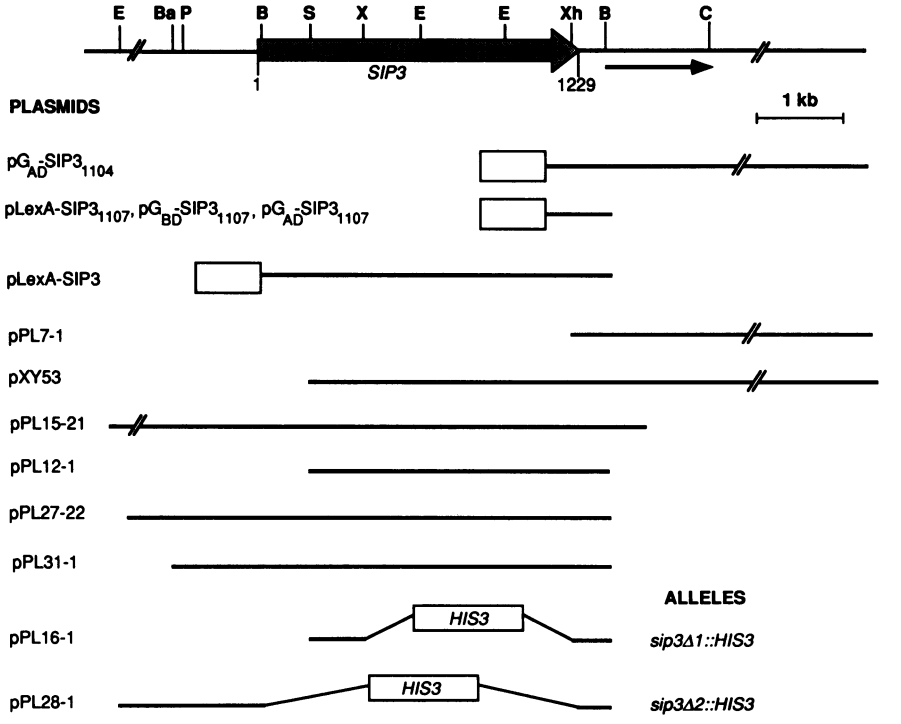

Figure 1. Maps of *SIP3* gene and plasmids. Plasmids are described in the text. Shaded box represents *SIP3* codong region. Open boxes represent LexA, G_{BD} and G_{AD}. Vector sequences are not shown. The *Eco*RI–*Pvu*II fragment is 2 kb. In pG_{AD}-$SIP3_{1104}$, two *Sph*I sites are located in the vector sequences at both extremities of the 6.6-kb insert. The distance between *Cla*I and *Sph*I site located downstream is 5.4 kb. Restrictions sites: B, *Bam*HI; Ba, *Ban*II; C, *Cla*I; E, *Eco*RI; P: *Pvu*II; S, *Stu*I; Xb, *Xba*I; X, *Xho*I are shown. The first and last amino acids of SIP3 are indicated. Part of an open reading frame identified by sequencing, is indicated by an arrow.

Table 1. Interaction of SIP3 and SNF1

DNA-binding hybrid	Activation hybrid	Color[a]	β-Galactosidase activity[b]
G_{BD}-SNF1	SNF4-G_{AD}	blue	204
G_{BD}-SNF1	G_{AD}-$SIP3_{1104}$	blue	86
G_{BD}	G_{AD}-$SIP3_{1104}$	white	ND
G_{BD}-lamin C	G_{AD}-$SIP3_{1104}$	white	ND
G_{BD}-SNF1	G_{AD}-$SIP3_{1107}$	blue	14
G_{BD}	G_{AD}-$SIP3_{1107}$	white	1
G_{BD}-lamin C	G_{AD}-$SIP3_{1107}$	white	ND
G_{BD}-$SIP3_{1107}$	G_{AD}	blue	428
G_{BD}-$SIP3_{1107}$	G_{AD}-SNF1	blue	464
G_{BD}-SNF1	SIP3	blue	4.6
G_{BD}	SIP3	white	0.4

Hybrid proteins are expressed from pEE5 (G_{BD}-SNF1) (6), pNI12 (SNF4-G_{AD}) (6), pG_{AD}-$SIP3_{1104}$, pLAM5 (G_{BD}-lamin C) (36), pG_{AD}-$SIP3_{1107}$, pG_{BD}-$SIP3_{1107}$ and pPL15-21 (SIP3). [a]Color was determined by filter assay. Transformants turned blue in a few hours, except those containing G_{BD}-SNF1 and SIP3 turned blue overnight. White transformants remained white after overnight incubation. White color always corresponded to ≤1 unit β-galactosidase activity. [b]Transformants of GGY::171 were grown to mid-log phase in selective synthetic complete medium containing galactose, ethanol and glycerol (2% of each). Values are the average of β-galactosidase activities in at least three, usually four, transformants. Standard errors were <15%. ND not determined.

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MSVHGRDPKK RQLRLISVAF KEASIDSPSF RASVNFFQTR VDALEDWIEK TVDFFDQKYK VSFEDFRRAK ETLLSQLLPP    80
PALLSNGFVS NQSFTPRLID SFNKDYYDFS MKLLQIVKGD DSSHSTALLE LMTTAIEPYR NVRKNFDFYQ GKYDSMLASY   160
QAIRISKTSL EPSSIKSDAL QLFEVQKNYL KASLDLISAI SAVKLSLDKF ILESMKVLKS RSIFITKDSG RKIDLSPCIN   240
EYLDNYAIWV ENSIEGSKVL DSDISNAKKQ AYRYTLKRIT PSSDTSDYNI RSIHSSKLLS KDTQVPPKSP EKSGWLYMKT   320
QVGKPTREIW VRRWCFLKNA VFGMFLLSPS KTYVEETDKF GVFLTNVRYD PEEDRKFCFE VKIFGNKVTE AHDNMSKDIT   400
LVFQTSNYLD LKSWLIAFEA TKKYVMSIQH DSLEYELAFK RFSPKFFEFA SSTTTSIDQL ITTFDKETES LYETLNCSIS   480
EYDILTLGEE KVFQFQMPTT PISTKMTQLA ILSNFLTKGS WFPNAVLANI WGTTDWSEYT ILPGKGKKPS SLLTIDGKRL   560
PIRNSTIYPQ YYSNELKVLD LQFKSLVFSP DQRLEKLPEE LLLFKFEALW CPNKKQKFSA TCFCTKDYIY CYMNSMEFIC   640
LTKISLSEIV SVEADRSSKK TLKLYDASGL QMKAIVLFSD YKLIASKLQY LLENKAIKNP NSNEEILVKF EQMEKESQEK   720
KQEELYKIEQ ENSFDRKATS VSKIIKSRVT FWEMSDDAST LLNRLKKLQT EYSITYNHEY EISSKGLAHI LFGDKSNAFP   800
KCLFLARKDG EEHGKRFWYK NKDINGKSQL VRKIPFRLDM TGNFLNTGKY HRDKESKMIF ATQRIVKIVD NKYYEVDLDP   880
FFVKVPFCHL LKLSIKFVIT ESYDVDNHLE IKLNMTASSS SLHVLYKLEY IDSRTGKTIE KLSLAEIICQ TWALKFAHSE   960
FLLIRRVLRY YLEKIGKHGK VIKAIKLCGI LGVLSNKSEE PATEKNGNSK ESESMQYDIR YSCTILFLVF IKLMVYRVTN  1040
LTFVFFRILI GILLLCAEKF SRINRMMVVG LLASIMINIL LSEKASVPYW SIKRAEKLFH DRLGSDKFTM QRAIYISDSD  1120
LLSSQLSVPS NNPIFEKFSE DNFNKDYQYS ETRKQLAMRR NELLIELRIL QDMEKQLVHD DYEKFLLEEV NKCSMVSIEM  1200
TDLWFNDTQL QNYCSICNEE LEKLRPPIT
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Figure 2. Predicted amino acid sequence of the *SIP3* product. Amino acids are numbered on the right. The glycine residue fused in frame with G_{AD} in the pG_{AD}-$SIP3_{1104}$ library clone is underlined. Bold letters mark the 4 leucine residues defining the leucine zipper motif. The GenBank accession number for *SIP3* is U03376.

amino acids of SIP3 fused to G_{AD} were sufficient for the interaction with G_{BD}-SNF1 (Table 1).

DNA bound LexA-$SIP3_{1107}$ fusion protein activates transcription

To test the interaction between SNF1 and the C-terminal 122 amino acids of SIP3 in the reciprocal combination, we constructed a G_{BD}-$SIP3_{1107}$ hybrid protein. Interestingly, this fusion activated *GAL1-lacZ* transcription in the absence of G_{AD}-SNF1, suggesting that the C-terminal region of SIP3 contains a transcriptional activation domain (Table 1).

To further characterize the transcriptional activating capacity of this region of SIP3, we created the LexA-$SIP3_{1107}$ fusion, which contains the same SIP3 sequences as the analogous G_{BD} fusion, and tested its ability to activate the transcription of target genes containing zero, one or six *lexA* operators located 5′ to the promoter of the *GAL1-lacZ* gene fusion (7). In this assay, transcription from the *GAL1* promoter is dependent on the binding of an activator protein to the *lexA* operator. We co-transformed a wild-type strain with pLexA-$SIP3_{1107}$ and each of the *lexAop-GAL1-lacZ* target plasmids. β-galactosidase activity from the target genes containing one and six *lexA* operators was increased 2000- and 5000-fold, respectively, confirming that the last 122 amino acids of SIP3 are involved in transcriptional activation (Table 2). High-level activation was also observed in a *snf1* mutant host (data not shown).

The native SIP3 protein interacts with SNF1 and activates transcription

The results obtained with the C-terminal domain of SIP3 suggest that the native SIP3 protein might both interact with G_{BD}-SNF1 and activate transcription, without requiring fusion to G_{AD}. To test this hypothesis, we co-transformed GGY::171 with pG_{BD}-SNF1 and the multicopy plasmid pPL15-21 expressing SIP3. Activation of β-galactosidase expression by SIP3 was detected and was dependent on the presence of G_{BD}-SNF1 (Table 1).

Table 2. Transcriptional activation by LexA-SIP3$_{1107}$ and LexA-SIP3

Expressed protein	β-galactosidase activity for plasmids containing indicated no. of *lexA* operators		
	0	1	6
LexA	0.4	1	1
LexA-SIP3$_{1107}$	0.4	930	2200
LexA-SIP3	0.4	ND	3.4

Proteins were expressed from pLexA-SIP3$_{1107}$, pLexA-SIP3 and pEG202 which expresses LexA protein (23). Target plasmids were pLRIΔ1 (no *lexA* operator), p1840 (one operator) and pSH18-18 (six operators) (7, 37, 38, S.Hanes and R.Brent, personal communication). MCY829 transformants were grown to mid-log phase in synthetic complete medium containing glucose (2%) and lacking histidine and uracil to select for both plasmids. Values are the average of β-galactosidase activities in at least three, usually four, transformants. Standard errors were <15%.

Table 3. Effect of *SIP3* gene dosage on invertase activity

Relevant genotype	Plasmid-borne gene	Temperature	Invertase activity
Wild type		30	273
sip3Δ1::HIS3		30	108
sip3Δ2::HIS3		30	272
Wild type (YEp24)	None	30	214
Wild type (pPL15-21)	*SIP3*	30	180
snf4-Δ2 (YEp24)	None	30	0.9
snf4-Δ2 (pPL15-21)	*SIP3*	30	5.3
snf4-Δ2 (pLN132)	*SNF4*	30	97
snf4-Δ2 (YEp24)	None	27	3.6
snf4-Δ2 (pPL31-1)	*SIP3*	27	15.5
snf4-Δ2 (pLN132)	*SNF4*	27	124

Strains carrying plasmids were grown in synthetic complete medium containing 2% glucose and lacking uracil to maintain plasmid selection and derepressed for 3 hours. Other strains were grown in rich medium (YEP) containing 2% glucose. Mid-log phase cultures were derepressed by shifting to the same medium containing 0.05% glucose for 3 hours (transformants) or 2.5 hours (other strains). Wild-type segregants from the tetrads were assayed. Values represent averages of derepressed invertase activity in two to four strains. Standard error is <15% except for *snf4Δ2* transformants containing pPL15-21, where the standard error was 22% due to variation in plasmid copy-number. For all strains values for glucose repressed cells were ≤1.

This activation was due to SIP3 and not other yeast sequences present in pPL15-21, because the pPL31-1 plasmid containing only *SIP3* also activated β-galactosidase expression (data not shown). The transformants expressing the G$_{BD}$-SIP3$_{1107}$ hybrid protein gave more β-galactosidase units than those co-expressing G$_{BD}$-SNF1 and SIP3. This difference could be due to a low level of *SIP3* expression from its native promoter in pPL15-21 or may suggest that SIP3 interacts weakly with SNF1.

We also tested the ability of a LexA-SIP3 fusion containing the entire SIP3 sequences to activate transcription. We detected significant β-galactosidase activity from the *GAL1-lacZ* target gene containing six *lexA* operators (Table 2). Immunoblot analysis using anti-LexA showed extensive degradation of the LexA-SIP3 hybrid protein (data not shown) which may account for the low level of β-galactosidase expression in this experiment. Nevertheless, these data confirm that LexA-SIP3 when bound to the LexA operator is capable of functioning as a transcriptional activator.

The entire SIP3 protein was also fused to G$_{BD}$ and G$_{AD}$. G$_{BD}$-SIP3 did not activate transcription by itself and neither G$_{BD}$-SIP3 nor G$_{AD}$-SIP3 interacted with G$_{AD}$-SNF1 or G$_{BD}$-SNF1, respectively (data not shown). These negative results probably reflect instability of the fusion proteins, as observed for LexA-SIP3.

Table 4. Invertase activity in strains overexpressing the SIP3 C terminus and SNF1

Plasmid-encoded SNF1 protein	Expressed G$_{BD}$ fusion protein	
	G$_{BD}$	G$_{BD}$-SIP3$_{1107}$
none	85 ± 11	6 ± 0.4
SNF1	68 ± 6	25 ± 5
SNF1-T210A	46 ± 1.5	3.5 ± 1

Wild-type MCY829 was transformed with plasmids pCE9 (SNF1) (39), YEpT210A (31), pG$_{BD}$-SIP3$_{1107}$ and vectors pMA424 (24) and YEp24. Transformants were grown at 30°C in synthetic complete medium containing 2% glucose and lacking histidine and uracil to select for both plasmids. Mid-log phase cultures were derepressed by shifting to the same medium containing 0.05% glucose for 3 hours. Values (± standard errors) represent averages of derepressed invertase activity in three transformants. For all strains, values for glucose repressed cells were ≤1.

Disruption of the *SIP3* chromosomal locus

To determine the phenotype caused by loss of *SIP3* gene function, we replaced the wild-type sequence on one chromosome of a diploid, either by the *sip3Δ1::HIS3* allele expressing only the first 409 amino acids of SIP3 or by the *sip3Δ2::HIS3* allele, a complete deletion of *SIP3* (see Materials and Methods and Fig. 1).

Upon sporulation of the diploid strain heterozygous for *sip3Δ2::HIS3*, all six asci analysed gave rise to two His$^+$ and two His$^-$ spores, showing that the *SIP3* gene is not essential for viability. *SNF1* is necessary for the expression of glucose-repressible genes in response to glucose deprivation, leading to defective growth of *snf1* mutants on various non-preferred carbon sources. Six tetrads were therefore tested for anaerobic growth on glucose, sucrose, raffinose or galactose or for aerobic growth on glycerol at 30°C, but no defects were observed in *sip3Δ2::HIS3* segregants. Because *snf1* mutants are unable to express *SUC2*, a glucose-repressible gene encoding invertase, we assayed invertase activity under repressing (2% glucose) and derepressing conditions (0.05% glucose) in four *sip3Δ2::HIS3* segregants. The mutants were indistinguishable from the wild type (Table 3). These data suggest either that *SIP3* is not involved in glucose repression or that *SIP3* function is redundant.

Two diploid transformants carrying the *sip3Δ1::HIS3* partially deleted allele were similarly characterized. Interestingly, *sip3Δ1::HIS3* segregants formed smaller spore clones than the wild-type segregants and upon further testing exhibited slower growth on rich medium (YEP) containing glucose but not raffinose, galactose or glycerol. The doubling time of a *sip3Δ1::HIS3* haploid strain on YEP−glucose was 2.3 times slower than wild type, but there was no difference between the two strains when they were grown on YEP−raffinose. This phenotype suggests that the expression of a truncated SIP3 protein, containing only the first 409 amino acids, is detrimental during growth on glucose. The amount of invertase produced in the *sip3Δ1::HIS3* strain under derepressing conditions was 2.5 times lower than in the wild type (Table 3). It is not yet clear whether this decrease in invertase activity reflects the involvement of *SIP3* in glucose repression or is an indirect result of the growth defect caused by *sip3Δ1::HIS3*.

To examine the relationship of SIP3 with SIP1 and SIP2, which were also identified as SNF1-interacting proteins in the two-

hybrid screen (11, 12), we constructed double mutants carrying *sip3Δ1::HIS3* allele and *sip1Δ3::URA3* or *sip2Δ3::LEU2*. *SIP1* and *SIP2* null mutations do not confer any growth defect (11, 12). Nevertheless, the growth defect of the *sip3Δ1::HIS3* mutant was exacerbated by *sip1Δ3::URA3*, but not by *sip2Δ3::LEU2*. The *sip3Δ1::HIS3 sip1Δ3::URA3* double mutant formed smaller spore clones and smaller colonies on YEP−glucose. No additional defects in growth on YEP−raffinose or invertase activity were detected. These results suggest a genetic interaction between *SIP1* and *SIP3*.

Increased *SIP3* gene dosage elevates invertase expression in *snf4* mutants

The more severe growth defect observed in the double mutant *sip3Δ1::HIS3 sip1Δ3::URA3* compared to the *sip3Δ1::HIS3* single mutant suggests that *SIP1* and *SIP3* share some related function. *SIP1* was also recovered as a multicopy suppressor of growth defects of a *snf4Δ2* mutant, which is deficient in SNF1 kinase activity, and increased gene dosage of *SIP1* restored invertase activity in this mutant (11). Therefore, *snf4D2* cells were transformed by the multicopy plasmid pPL31-1. Increased gene dosage of *SIP3* did not suppress the growth defects of the *snf4Δ2* mutant on sucrose, raffinose, galactose or glycerol. However, increased *SIP3* gene dosage partially restored the expression of invertase (Table 3). The suppression of invertase defect was also evident in assays of cells grown at 27°C, a temperature at which the requirement for SNF4 function is slightly relaxed (5). Increased *SIP3* gene dosage did not affect invertase expression in a wild-type strain (Table 3).

Overexpression of the C-terminal domain of SIP3 causes defects that are remedied by overexpression of SNF1

In the two-hybrid experiments, we noticed that GGY::171 cells containing only G_{BD}-$SIP3_{1107}$ or G_{AD}-$SIP3_{1107}$ hybrid proteins, expressed from the strong *ADH1* promoter, grew much more slowly than cells expressing these fusions from a weaker promoter. These findings suggested that overexpression of the last 122 amino acids of SIP3 is deleterious for the cell. The slow growth due to the overexpression of G_{BD}-$SIP3_{1107}$ was overcome by transforming GGY::171 simultaneously with pG_{BD}-$SIP3_{1107}$ and pG_{AD}-SNF1. These data are consistent with an interaction or a functional relation between SNF1 and SIP3 *in vivo*.

We examined *SUC2* expression in wild-type strain MCY829 transformed by pG_{BD}-$SIP3_{1107}$ alone or together with a multicopy plasmid expressing SNF1. Results showed a 13-fold decrease in derepression of invertase activity when G_{BD}-$SIP3_{1107}$ is expressed (Table 4). Co-expression of SNF1 with G_{BD}-$SIP3_{1107}$ partially restored the invertase activity, causing a 4-fold increase. In this assay, SNF1 was expressed from its native promoter and G_{BD}-$SIP3_{1107}$ from the strong *ADH1* promoter, so it is likely that SNF1 is expressed at a lower level than G_{BD}-$SIP3_{1107}$. Interestingly, increased dosage of the *snf1-T210A* allele encoding a defective SNF1 kinase (31) did not restore *SUC2* expression in strains expressing G_{BD}-$SIP3_{1107}$. This is not due to a dominant negative effect of *snf1-T210A* allele because SNF1-T210A mutant protein caused only a 2-fold decrease in invertase activity in G_{BD} transformants. Immunoblot analysis previously confirmed that the SNF1-T210A protein is expressed as the same level as SNF1 (31). These results indicate that the dominant negative effect of the C-terminal region of SIP3 on *SUC2* regulation can be suppressed by overexpressing a functional but not a deficient SNF1 protein kinase and further corroborate that SIP3 and SNF1 are functionally related.

SIP3 is not tightly associated with the SNF1 kinase complex

Immune complex assays of SNF1 protein kinase activity demonstrated *in vitro* phosphorylation of SNF1 and several coprecipitating proteins, including SIP1, SIP2 and GAL83 (12). To determine if SIP3 corresponds to one of the proteins that is phosphorylated in the immune complex, we immunoprecipitated SNF1 from both wild-type and *sip3D1::HIS3* extracts by using affinity purified polyclonal SNF1 antibody and incubated the immunoprecipitate with [γ-^{32}P]ATP (4, 5). We did not see any difference in the migration or the intensity of the labelled products between the two extracts, suggesting that SIP3 does not correspond to one of the proteins detected in this assay (data not shown).

To further characterize the interaction between SNF1 and the C-terminal 122 amino acids of SIP3 detected in the two-hybrid system, we immunoprecipitated SNF1 from a wild-type strain expressing LexA-$SIP3_{1107}$. Anti-LexA immunoblot analysis shows that LexA-$SIP3_{1107}$ hybrid protein is highly over-expressed in this strain but does not coimmunoprecipitate with SNF1 (data not shown). These results suggest that SIP3 is not tightly associated with the SNF1 protein kinase but do not exclude that SIP3 may be a substrate that interacts transiently with SNF1.

DISCUSSION

We report here the cloning and characterization of the *SIP3* gene, identified in a two-hybrid screen for proteins that interact with SNF1 protein kinase. The predicted 142-kD SIP3 protein contains at the C terminus a leucine repeat motif similar to a leucine zipper (28). Leucine zippers have been shown to promote formation of coiled coils (32) and to mediate dimer formation (33, 34, 35). Several proteins having a leucine zipper are transcriptional activators, for example GCN4, C/EBP, Jun and Fos (28). The native SIP3 protein and more specifically its C-terminal region containing the leucine zipper can activate transcription when tethered to DNA. It is possible that SIP3 activates transcription via dimerization with another protein.

Several lines of genetic evidence support the view that SIP3 functions in the SNF1 pathway. First, the isolation of SIP3 sequences by the two-hybrid system strongly suggests that SNF1 and SIP3 interact *in vivo*. In addition we showed that the native SIP3 protein interacts with DNA-bound G_{BD}-SNF1 and activates transcription of a target gene. Second, increased *SIP3* gene dosage partially restores derepression of *SUC2* in a *snf4Δ* mutant, which is deficient in SNF1 kinase activity due to loss of the SNF4 stimulatory function (5). Third, the overexpression of the G_{BD}-$SIP3_{1107}$ hybrid protein caused slow growth of strain GGY::171 on glucose and also impaired the derepression of *SUC2* in the wild-type strain MCY829. These defects were remedied by overexpression of G_{AD}-SNF1 or native SNF1 protein kinase, respectively. Interestingly, the invertase defect was not corrected by increased dosage of the *snf1-T210A* allele, which encodes a defective protein kinase activity (31). We can not exclude that the *snf1-T210A* mutation directly affects the interaction between SNF1 and G_{BD}-$SIP3_{1107}$ but it is more likely that a fully active SNF1 protein kinase is required to suppress the negative effect of G_{BD}-$SIP3_{1107}$ overexpression. Finally, the growth defect of a *sip3Δ1::HIS3* mutant is exacerbated by the *sip1Δ3::URA3* allele, suggesting a genetic interaction. *SIP1* and *SIP3* are also

similar in that increased dosage of either gene suppresses the *snf4Δ* defect in *SUC2* derepression. SIP1 physically associates with SNF1 and is phosphorylated in SNF1 immune complex assays (11). The relationship between SIP1 and SIP3 further strengthens the argument that SIP3 is functionally related to the SNF1 kinase pathway.

Some of the preceding experiments involve overexpression of SIP3 sequences, and it could be argued that SIP3, when expressed at its normal level, may not function in the SNF1 pathway. However, the final piece of evidence regarding the interaction of *sip1* and *sip3* does not depend on overexpression of SIP3. Taken together, our evidence supports a model in which SIP3 is involved in the SNF1 pathway.

The complete disruption of *SIP3* confers a phenotype indistinguishable from wild type suggesting that SIP3 is functionally redundant. Southern-blot analysis at low stringency conditions detected a sequence weakly homologous to the C-terminal half of *SIP3* (unpublished data). Further experiments will be required to determine whether this homology corresponds to a functionally redundant gene.

What is the role of SIP3 in the SNF1 pathway? In addition to its role in glucose repression, SNF1 has been shown to be involved in glycogen storage, thermotolerance, sporulation and general health (2). It is not clear yet, if SIP3 is specifically involved in one of this processes. The suppression of *snf4Δ* defect in invertase activity by increased *SIP3* gene dosage suggests that *SIP3* can contribute to regulation of *SUC2* expression. The observed growth defect for *SIP3Δ1::HIS3* mutants on glucose may indicate that SIP3 is involved in other aspects of growth controlled by the SNF1 pathway.

ACKNOWLEDGEMENTS

We thank Linda Riles for providing lambda clones grids and I.Treich, A.Rattray and K.Bowdish for comments on the manuscript. This work was supported by Public Health Service grant GM34095 from the National Institutes of Health (to M.C.). P.L. was supported by the Association pour la Recherche contre le Cancer and The Fondation Philippe.

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