Sip5 Interacts With Both the Reg1/Glc7 Protein Phosphatase and the Snf1 Protein Kinase of *Saccharomyces cerevisiae*

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ABSTRACT

The Snf1 protein kinase is an essential component of the glucose starvation signalling pathway in *Saccharomyces cerevisiae*. We have used the two-hybrid system to identify a new protein, Sip5, that interacts with the Snf1 kinase complex in response to glucose limitation. Coimmunoprecipitation studies confirmed the association of Sip5 and Snf1 in cell extracts. We found that Sip5 also interacts strongly with Reg1, the regulatory subunit of the Reg1/Glc7 protein phosphatase 1 complex, in both two-hybrid and coimmunoprecipitation assays. Previous work showed that Reg1/Glc7 interacts with the Snf1 kinase under glucose-limiting conditions and negatively regulates its activity. Sip5 is the first protein that has been shown to interact with both Snf1 and Reg1/Glc7. Genetic analysis showed that the two-hybrid interaction between Reg1 and Snf1 is reduced threefold in a *sip5*Δ mutant. These findings suggest that Sip5 facilitates the interaction between the Reg1/Glc7 phosphatase and the Snf1 kinase.

THE Snf1 serine/threonine protein kinase is a member of a highly conserved family, including the mammalian AMP-activated protein kinase and various plant kinases (for review see Hardie *et al.* 1998). In the yeast *Saccharomyces cerevisiae* the Snf1 kinase is essential for regulating the transcription of genes involved in alternate carbon source utilization, respiration, and gluconeogenesis, and also plays important roles in sporulation, glycogen storage, thermotolerance, and peroxisomal biogenesis. The Snf1 kinase is found in complexes containing the activating subunit Snf4 and a member of the Sip1, Sip2, Gal83 family, which interacts with both Snf1 and Snf4. The Snf1 kinase includes two domains, catalytic and regulatory, which participate in the regulation of its activity (Jiang and Carlson 1996). When glucose is abundant, an autoinhibited conformation of the complex is favored in which the catalytic domain is bound to the regulatory domain. When glucose is limiting, the catalytic domain is phosphorylated by a putative upstream kinase or by alternative mechanisms (Woods *et al.* 1994; Wilson *et al.* 1996). Autoinhibition is relieved, and the activating subunit Snf4 binds to the regulatory domain, leading to an active conformation of the complex (Jiang and Carlson 1996).

The conformation of the Snf1 complex is also affected by the Reg1/Glc7 protein phosphatase 1 (PP1) complex (Tu and Carlson 1995; Jiang and Carlson 1996; Ludin *et al.* 1998). Reg1, the regulatory subunit of the complex, binds to the catalytic domain of Snf1 in low glucose and targets Glc7, the catalytic subunit, to the kinase complex. Glc7 dephosphorylates Snf1, or another component, and promotes the autoinhibited conformation of the Snf1 complex. In the absence of Reg1/Glc7 activity, the Snf1 complex, once activated, becomes trapped in the active conformation. The functional and physical interaction of the phosphatase and kinase complexes appears to be finely modulated. Reg1 is phosphorylated rapidly upon glucose depletion, dependent on Snf1, and this phosphorylation is required for the release of Reg1/Glc7 from the kinase complex; upon readdition of glucose, Reg1 is dephosphorylated, dependent on Glc7 (Ludin *et al.* 1998; P. Sanz and M. Carlson, unpublished results).

A detailed understanding of the regulation of Snf1 kinase activity will require the identification of all of the proteins that interact with the kinase. Previously, the two-hybrid system has proved useful in identifying such proteins. Sip1 and Sip2 (for *S*nf1-*i*nteracting-*p*rotein) were identified in a two-hybrid screen and shown to be components of the kinase complex (Yang *et al.* 1992, 1994; Jiang and Carlson 1997). Also identified in this screen were Sip3, a protein of unknown function involved in the Snf1 pathway (Lesage *et al.* 1994), and Sip4, a Snf1-dependent transcriptional activator of genes containing a carbon source-responsive element (CSRE; Lesage *et al.* 1996; Vincent and Carlson 1998). To identify other proteins that interact with Snf1, we performed a new two-hybrid screen. As the bait, we used a fusion of the DNA-binding domain of Gal4 (GBD) to the N-terminal catalytic domain of Snf1, with the regulatory domain truncated, to avoid the recovery of

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TABLE 1

Strains used in this study

Strain[a]	Genotype
MCY1854	*MAT*α *snf4*Δ*2 ade2-101 his4-539 ura3-52*
MCY2652	*MAT*α *snf1*Δ*10 ade2-101 his3*Δ*200 lys2-801 trp1*Δ*1 ura3-52*
MCY2728	*MAT*α *sip1*Δ*1::URA3 sip2*Δ*3::LEU2 gal83*Δ*::URA3 ade2-101 his3*Δ*200 leu2-3,112 ura3-52*
MCY2921	*MAT***a** *ade2-101 his3*Δ*200 trp1*Δ*1 ura3-52*
MCY3015	*MAT*α/**a** +/*ade2-101* +/*his3*Δ*200 leu2-3,112*/*leu2-3,112* +/*lys2-801 trp1*Δ*1*/*trp1*Δ*1 ura3-52*/*ura3-52*
MCY3278	FY250 *reg1*Δ*::URA3*
MCY3907	*MAT*α *sip5*Δ*::HIS3 sip1*Δ*1::URA3 sip2*Δ*3::LEU2 gal83*Δ*::URA3 ade2-101 his3*Δ*200 leu2-3,112 ura3-52*
MCY3910	*MAT*α *sip5*Δ*::hisG ade2-101 his3*Δ*200 leu2-3,112 lys2-801 trp1*Δ*1 ura3-52*
MCY3911	*MAT*α *sip5*Δ*::hisG snf1*Δ*10 ade2-101 his3*Δ*200 lys2-801 trp1*Δ*1 ura3-52*
MCY3914	*MAT***a** *sip5*Δ*::hisG ade2-101 his3*Δ*200 leu2-3,112 lys2-801 trp1*Δ*1 ura3-52*
MCY3916	CTY10-5d *sip5*Δ*::TRP1*
MCY3917	*MAT*α *sip5*Δ*::hisG snf4*Δ*2 ade2-101 his4-539 ura3-52*
MCY3918	*MAT***a** *sip5*Δ*::HIS3 ade2-101 his3*Δ*200 trp1*Δ*1 ura3-52*
MCY3919	FY250 *sip5*Δ*::HIS3 reg1*Δ*::URA3*
FY250	*MAT*α *his3*Δ*200 leu2*Δ*1 trp1*Δ*63 ura3-52*
PS5010-1B	FY250 *snf1*Δ*10*
CTY10-5d	*MAT***a** *ade2-101 his3*Δ*200 leu2*Δ*1 trp1-901 URA3::lexAop-lacZ gal4 gal80*
Y187	*MAT*α *ade2-101 his3*Δ*200 leu2-3,112 trp1-901 ura3-52 URA3::GAL1-lacZ gal4 gal80*
Y190	*MAT***a** *ade2-101 his3*Δ*200 leu2-3,112 trp1-901 ura3-52 LYS2::GAL1-HIS3 URA3::GAL1-lacZ gal4 gal80 cyh*^{r}

[a]MCY strains and FY250 (gift of F. Winston) have the S288C genetic background. CTY10-5d was a gift of R. Sternglanz, and Y187 and Y190 were kindly provided by S. Elledge.

previously identified Sip proteins. In this study, we characterize one of these new Snf1-interacting proteins, named Sip5. We show that Sip5 interacts with both the Snf1 kinase and the Reg1/Glc7 phosphatase complex.

MATERIALS AND METHODS

Strains and genetic methods: The *S. cerevisiae* strains used are listed in Table 1. Standard methods for yeast genetic analysis and transformation were used (Rose *et al.* 1990). Cells were grown in synthetic complete (SC) medium lacking appropriate supplements to maintain selection for plasmids.

Plasmids: Plasmids used in this study are listed in Table 2. pKL8, which expresses GBD-Snf1$_{1\text{-}309}$ from the *SNF1* promoter, was constructed in several steps. First, a 1.0-kb *Eco*RI/*Hin*cII fragment from pCEsnf1Δ8 (Celenza and Carlson 1989) containing the *SNF1* promoter was cloned into the *Eco*RI/*Sma*I sites of pRS424 (Christianson *et al.* 1992), giving pKL2. Using pSE1112 (GBD-Snf1 in pAS1; Durfee *et al.* 1993) as template, a PCR amplification was carried out with oligos KL7 (*GAL4* specific) and KL8 (*SNF1* kinase domain specific) to give a 1.4-kb fragment encoding GBD-HA-Snf1$_{1\text{-}309}$. This PCR fragment was cloned into the *Bam*HI/*Not*I sites of pKL2.

To construct pKL15 (GAD-Sip5), a *Bam*HI/*Xho*I fragment covering the entire coding region of *SIP5* was amplified by PCR from plasmid pKB111 (Bowdish *et al.* 1994) using oligos KL16 and KL17 and cloned into pACTII (Legrain *et al.* 1994). pKL30, expressing LexA-Sip5, contains the same fragment in pEG202 (Golemis *et al.* 1997).

pKL34, which expresses HA-Sip5 from the *SIP5* promoter, was constructed in several steps. First, a 5.0-kb *Sac*I fragment from pKB111 was subcloned into pRS424 (Christianson *et al.* 1992) to give pKL26. Second, using oligos KL22 and KL23 and plasmid GTEPI (Tyers *et al.* 1993) as template, we amplified by PCR a fragment containing three copies of the HA epitope flanked by *Nco*I sites. Tandem repeats of this fragment were inserted at the *Nco*I site at the first ATG of the *SIP5* coding sequence in pKL26.

Oligonucleotides: Oligonucleotides used as primers in PCR were the following: KL7: GCGCGGATCCATGAAGCTACTGT CTTCTATCGAAC (*Bam*HI site underlined), KL8: GCGCGCG GCCGCTAATTAATCAGTCAACTTTGAACCAATCGTCTG (*Not*I site underlined), KL16: GACGGATCCCCATGGGTAAT GTTCCAGGG (*Bam*HI site underlined), KL17: GACTCGAG TATGGTCTCAAAGAGGTGTTTCT (*Xho*I site underlined), KL22: CCATGGGCCGCATCTTTTACCCATACG (*Nco*I site underlined), KL23: CCATGGGGCGGCCGCACTGAGCAGCC (*Nco*I site underlined), KL24: TCACGACATAAGAACACCTTT GGTGG, KL28: CATAAAATGTAAGCTTTCGGGGC.

Two-hybrid screen: A two-hybrid screen (Fields and Song 1989) for proteins that interact with GBD-Snf1$_{1\text{-}309}$ was carried out in strain Y190, which contains two chromosomally located reporter genes, *GAL1-lacZ* and *GAL1-HIS3*. The strain was transformed with a library of *S. cerevisiae* cDNAs fused to the activating domain of Gal4 [GAD; generous gift of S. Elledge, Baylor University; see Elledge *et al.* (1991)]. To identify interacting proteins, transformants were selected in SC + 2% glucose plates for a His$^+$ phenotype in the presence of 30 or 60 mm 3-aminotriazole (3-AT) and were subsequently screened for β-galactosidase activity using a filter lift assay (Yang *et al.* 1992). Plasmids from 22 transformants that were both His$^+$ and blue were subjected to sequence analysis. A total of 7 clones encoded three ribosomal proteins and the remaining 15 clones encoded 10 different genes. To confirm the specificity of the interaction, Y190 cells were transformed again with these latter clones, and the resulting transformants were crossed with Y187 cells transformed with plasmids expressing GBD-Snf1$_{1\text{-}309}$ (pKL8) or GBD-Snf1 (pSE1112; Durfee *et al.* 1993). The resulting diploids were tested for blue color in the filter lift assay.

Disruption of chromosomal *SIP5* locus: To construct the *sip5*Δ*::hisG* mutation, we first subcloned a 1.8-kb *Sal*I/*Xba*I fragment from pKB111 (Bowdish *et al.* 1994) containing *SIP5* into pBS SK+/− (Stratagene, La Jolla, CA) to give plasmid

TABLE 2

Plasmids used in this study

Name	Expressed protein	Reference
pGAD-GLC7	GAD-Glc7	Tu and Carlson (1995)
pKL15	GAD-Sip5	This study
pNI12	Snf4-GAD	Fields and Song (1989)
pKL8	GBD-Snf1$_{1-309}$	This study
pSE1112	GBD-Snf1	Durfee *et al.* (1993)
pSB16	HA-Reg1	P. Sanz, unpublished results
pKL34	HA-Sip5	This study
pLexA-GLC7	LexA-Glc7	Tu and Carlson (1995)
pLexA-Mig1	LexA-Mig1	Treitel *et al.* (1998)
pRJ65	LexA-Reg1	Tu and Carlson (1995)
pLexA-Reg1F468R	LexA-Reg1F468R	Alms *et al.* (1999)
pKL30	LexA-Sip5	This study
pRJ55	LexA-Snf1	Jiang and Carlson (1996)
pRJ57	LexA-Snf4	Jiang and Carlson (1996)
pRJ79	VP16-Snf1	Ludin *et al.* (1998)
pRJ80	VP16-Snf1K84R	Ludin *et al.* (1998)

pKL16. A 3.8-kb *Spe*I/*Bam*HI fragment from pNKY51 (Alani and Kleckner 1987), carrying *URA3* flanked by two *hisG* genes from *Salmonella typhimurium*, was used to replace the *Spe*I/*Bgl*II fragment of pKL16, producing pKL17 (Figure 1). A 4.5-kb *Sal*I/*Xba*I fragment from the latter was used to transform the diploid strain MCY3015. A Ura$^+$ transformant was sporulated and dissected, and the Ura$^+$ segregants from two tetrads were streaked on SC + Ura plates containing 5-fluoroorotic acid (5-FOA; 0.5 mg/ml) to select for *URA3* pop-out events. PCR amplification of genomic DNA using oligos KL16 and KL17, and restriction site analysis of the resulting fragment, confirmed the presence of the *sip5*Δ*::hisG* allele. The same fragment was used to obtain *sip5*Δ*::hisG* derivatives of MCY2652 and MCY1854.

To construct the *sip5*Δ*::HIS3* allele, we cloned the *Bam*HI/*Afl*III *HIS3* fragment from pPL3.1 (Lesage *et al.* 1994) into the *Bgl*II/*Nco*I sites of pKL16, yielding pKL36 (Figure 1). A 1.7-kb *Sal*I/*Xba*I fragment from the latter was used to transform MCY2921, MCY3278, and MCY2728. Disruptants were shown to carry *sip5*Δ*::HIS3* by PCR amplification of genomic DNA with oligos KL16, KL17, and the *HIS3*-specific oligo KL24. *sip5*Δ*::HIS3* disruptants were tested for growth on YEP medium containing either 2% glucose, 3% glycerol, 3% ethanol, 2% raffinose plus antimycin A (100 μg/ml), 2% galactose plus antimycin A (100 μg/ml), or 2% sucrose plus the glucose analog 2-deoxyglucose (200 μg/ml).

The *sip5*Δ*::TRP1* allele was constructed by subcloning the *HIS3* fragment used above into the *Spe*I/*Nco*I sites of pKL16 to give pKL35, which then contained a *Pst*I site 14 bp 3′ to the *Nco*I/*Afl*III junction. A 0.7-kb *Pst*I/*Eco*RI fragment from YDp-W (Berben *et al.* 1991) containing *TRP1* was subcloned into the *Pst*I/*Eco*RI sites of pKL35, yielding pKL44 (Figure 1). The 2.0-kb *Xho*I/*Not*I fragment from pKL44 was used to transform strain CTY10-5d. Disruptants were confirmed by PCR amplification of genomic DNA using oligos KL16, KL17, and the *TRP1*-specific oligo KL28.

Invertase and β-galactosidase assays: Invertase activity was assayed in whole cells as previously described (Jiang and

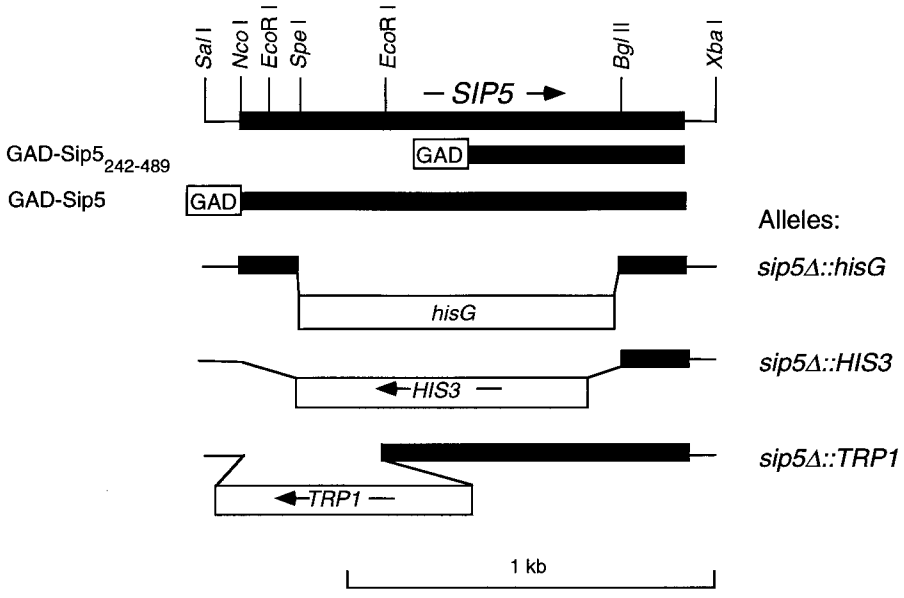

Figure 1.—Restriction map of the *SIP5* locus and mutant alleles. Black bars, *SIP5* coding region. GAD, Gal4 activating domain.

Carlson 1996). β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units (Miller 1972) as in Ludin *et al.* (1998).

Preparation of cell extracts by the fast boiling method: Cells corresponding to 1 unit A_{600} were collected by rapid centrifugation (14,000 rpm, 1 min), resuspended in 100 μl of Laemmli sample buffer, and boiled for 3 min. Glass beads (0.3 g, 450 μm diameter) were added to the suspension, and cells were vortexed at full speed for 30 sec. The suspension was boiled again for 3 min and centrifuged at 14,000 rpm for 1 min. A total of 10 μl of the supernatant was used for immunodetection.

Coimmunoprecipitation 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, 0.5% Triton X-100, 1 mm dithiothreitol, 10% glycerol, and contained 2 mm phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis). Anti(α)-LexA or α-HA monoclonal antibody (1 μl) was used in each immunoprecipitation reaction, and precipitates were analyzed by Western blotting.

Immunoblot analysis: Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting using polyclonal α-Snf1 (Celenza and Carlson 1986), monoclonal α-HA (Boehringer Mannheim), or monoclonal α-LexA (Clontech, Palo Alto, CA). Antibodies were detected by enhanced chemiluminescence with ECL or ECL Plus reagents (Amersham, Piscataway, NJ).

RESULTS

Identification of Sip5 in a two-hybrid screen for interaction with the kinase domain of Snf1: A two-hybrid screen for proteins that interact with the catalytic domain of Snf1 was carried out. A fusion between GBD and the kinase domain of Snf1, GBD-$Snf1_{1\text{-}309}$, was used as a bait to screen a library of cDNAs fused to GAD. Of the 22 clones recovered, 1 (clone 1-24) contained an in-frame fusion to codon 242 of an open reading frame (ORF) of unknown function on chromosome XIII (YMR140w) (Figure 1). The gene, designated *SIP5*, encodes a protein of 489 amino acids with a predicted molecular mass of 55.9 kD. Sip5 is not homologous to any other protein encoded by the *S. cerevisiae* genome and shows only a weak homology with a putative zinc-finger protein from *Schizosaccharomyces pombe* (AL031853) of unknown function.

To examine further the interaction between Sip5 and Snf1, we expressed the full-length Sip5 protein fused in frame to GAD and tested the resulting GAD-Sip5 protein in combination with a LexA fusion to Snf1. Interaction was monitored by assaying β-galactosidase expression from a *lacZ* reporter containing LexA-binding sites. GAD-Sip5 did not interact significantly with LexA-Snf1 in glucose-grown cells (Table 3). The interaction increased when cells were shifted to low (0.05%) glucose for 3 hr and was strong in cells growing in 2% galactose/2% glycerol/2% ethanol/0.05% glucose (gal/gly/EtOH) medium, indicating that the interaction between Sip5 and Snf1 is glucose regulated. No interaction was detected between GAD-Sip5 and a LexA fusion to Snf4, the activating subunit of the kinase complex. The two-hybrid assay does not always detect indirect interactions; however, this negative result also does not exclude direct interaction between Sip5 and Snf4.

Sip5 coimmunoprecipitates with Snf1: To confirm the interaction between Sip5 and Snf1 detected in the two-hybrid assay, we sought biochemical evidence for the association of these proteins. Protein extracts were prepared from wild-type cells expressing HA-Sip5 and LexA-Snf1. Immunoblot analysis detected two HA-Sip5 polypeptides migrating at 78 and 73 kD, the smaller species presumably corresponding to a degradation product (Figure 2A, middle). LexA-Snf1 was immunoprecipitated with α-LexA monoclonal antibodies, and the precipitates were analyzed by SDS-PAGE and immunoblotting with α-HA monoclonal antibodies. The 78-kD HA-Sip5 species coimmunoprecipitated with LexA-Snf1 (Figure 2A, top). In control experiments, HA-Sip5 did not coimmunoprecipitate with LexA or LexA-Mig1. Coimmunoprecipitation assays cannot be used to assess the glucose regulation of the Sip5-Snf1 interaction because even when cells are grown in high glucose, the Snf1 kinase is active in this assay (Estruch *et al.* 1992; Wilson *et al.* 1996; P. Sanz, unpublished results).

Because the Sip5 protein sequence contains a putative Snf1 phosphorylation consensus site (Dale *et al.* 1995) ($\underline{L}_{264}Y\underline{K}NG\underline{S}ECP\underline{I}$; the consensus residues are underlined), we examined the possibility that Snf1 phosphorylates Sip5. Wild-type (FY250) and *snf1*Δ mutant cells expressing HA-Sip5 were grown in high glucose and were then shifted to low glucose. Immunoblot analysis of proteins prepared under both growth conditions showed no differences in the mobility of HA-Sip5 (data not shown). In addition, *in vitro* kinase assays of Snf1 immune complexes in strains disrupted for *SIP5* (*sip5*Δ*::HIS3*, see below) or carrying a multicopy *SIP5* plasmid (pKL26) showed the wild-type pattern of phosphorylated products (data not shown). Thus, these experiments provided no evidence that Snf1 phosphorylates Sip5.

Sip5 interacts with the Reg1/Glc7 protein phosphatase complex: Previous studies showed that the Snf1 protein kinase interacts with Reg1, a regulatory subunit of the protein phosphatase complex (Ludin *et al.* 1998). Therefore, we tested Sip5 for interaction with Reg1 and Glc7, the catalytic subunit. GAD-Sip5 interacted strongly with LexA-Reg1 in cells growing in 4% glucose, and no increase was observed when cells were shifted to 0.05% glucose for 3 hr (Figure 3). In contrast, no significant interaction was detected between GAD-Sip5 and LexA-Glc7.

To determine whether the interaction of Sip5 with Reg1 requires the presence of Glc7 complexed to Reg1, we used a mutated form of Reg1, Reg1F468R, which has an alteration in the conserved Glc7-binding site (Alms *et al.* 1999). Unexpectedly, LexA-Reg1F468R in-

TABLE 3

Interaction between Sip5 and Snf1 protein kinase

LexA fusion	GAD fusion	β-Galactosidase (units)[a] R	S	D
LexA-Snf1	GAD-Sip5$_{242-489}$	17 ± 1.1	n.d.	28 ± 6.0
LexA-Snf1	GAD-Sip5	0.5 ± 0.1	3.1 ± 0.1	9.7 ± 3.2
LexA-Snf1	GAD	0.1 ± 0.05	0.4 ± 0.1	0.8 ± 0.1
LexA-Snf4	GAD-Sip5	0.5 ± 0.1	0.4 ± 0.1	1.0 ± 0.2

[a] CTY10-5d transformants expressing the indicated fusion proteins were grown in selective SC + 4% glucose medium (R) and shifted to SC + 0.05% glucose medium for 3 hr (S). The same transformants were also grown in selective SC + 2% galactose/2% glycerol/2% ethanol/0.05% glucose medium (D). Values are the average of β-galactosidase activity of four to six transformants ± standard deviation. n.d., not determined.

teracted more strongly (almost sevenfold) with GAD-Sip5 (Figure 3). Western blot analysis showed that the wild-type and mutant Reg1 fusion proteins were produced at similar levels (Figure 3). This result might suggest that Glc7 and Sip5 compete for binding to Reg1. However, overexpression of HA-Sip5 did not affect the interaction between LexA-Reg1 and GAD-Glc7 (141 ± 13 units in CTY10-5d cells expressing HA-Sip5 from multicopy plasmid pKL34, and 136 ± 16 units in cells expressing the vector pRS424; values are the average β-galactosidase activity for four different transformants grown in 4% glucose ± standard deviation), and a *sip5*Δ mutation (see below) also did not affect this interaction. These experiments do not exclude the possibility of competition for binding, because the two-hybrid partners are overexpressed, but provide no support for this idea.

Sip5 coimmunoprecipitates with Reg1: The interaction between Sip5 and the Reg1/Glc7 phosphatase complex was confirmed by coimmunoprecipitation. Protein extracts were prepared from a *sip5*Δ*::hisG* strain (MCY3914; see below) expressing HA-Reg1 and LexA-

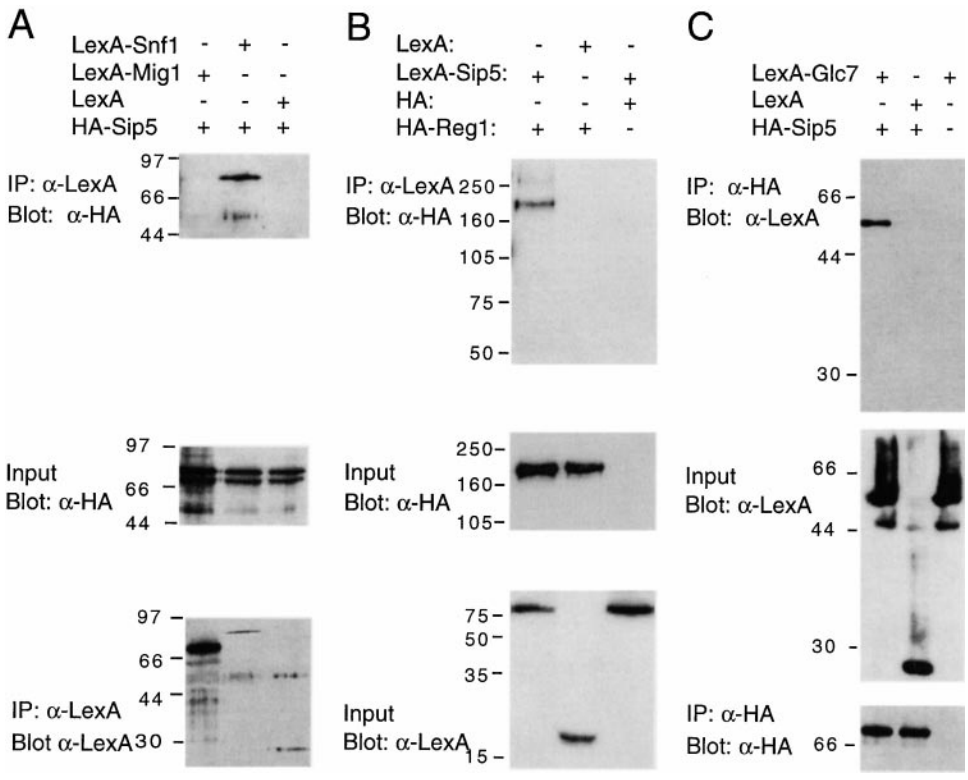

Figure 2.—Sip5 coimmunoprecipitates with Snf1 and Reg1/Glc7. (A) Protein extracts (500 μg) from wild-type cells expressing HA-Sip5 and LexA fusion proteins and grown in 4% glucose were immunoprecipitated (IP) with α-LexA antibodies. The precipitates were resolved by 10% SDS-PAGE, blotted, and immunodetected with α-HA (top). Input extracts (25 μg) were also immunodetected with α-HA (middle). The filter shown at the top was then stripped and reprobed with α-LexA to detect the immunoprecipitated LexA fusion protein (bottom). (B) Protein extracts (250 μg) from *sip5*Δ*::hisG* (MCY3914) cells expressing HA-Reg1 and LexA-Sip5 were immunoprecipitated with α-LexA. The precipitates were resolved by 7% SDS-PAGE and immunoblotted with α-HA (top). Input proteins (1 μg) were also immunoblotted with α-HA (middle) or α-LexA (bottom; 12% SDS-PAGE was used in this case). Control strains expressed LexA or the triple HA epitope from the vectors pLexA(1-202+PL) (Ruden *et al.* 1991) or pWS93 (Song and Carlson 1998), respectively. (C) Protein extracts (250 μg) from wild-type cells expressing LexA-Glc7 and HA-Sip5 were immunoprecipitated with α-HA, and the precipitates were resolved by 10% SDS-PAGE and immunodetected with α-LexA (top). Input extracts (25 μg) were also immunoblotted with α-LexA (middle). The filter shown at the top was reprobed with α-HA to detect immunoprecipitated HA-Sip5 (bottom). Size standards are indicated in kilodaltons.

LexA-fusion	β-Galactosidase (Units) GAD-Sip5 R	GAD-Sip5 S	GAD R	GAD S
LexA-Reg1	62	76	0.7	0.6
LexA-Reg1F468R	427	393	3.3	3.2
LexA-Glc7	0.3	0.1	0.2	0.1
LexA	0.9	4.7	n.d.	n.d.

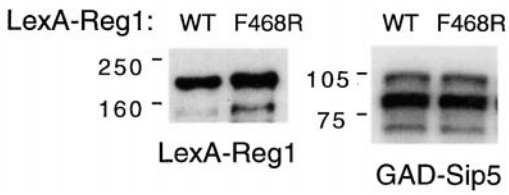

Figure 3.—Interaction between Sip5 and the Reg1/Glc7 complex. CTY10-5d transformants expressing the indicated fusion proteins were grown in selective SC + 4% glucose medium (R) and then shifted to SC + 0.05% glucose medium for 3 hr (S). Values are the average β-galactosidase activity of four transformants; standard deviations were <10%. Extracts were prepared from transformants expressing GAD-Sip5 and wild-type (WT) LexA-Reg1 or LexA-Reg1F468R by the fast boiling method (see materials and methods), and 10-μl samples were immunoblotted with α-LexA (left) or α-HA (right). GAD-Sip5 is expressed from the vector pACTII and contains one copy of the HA epitope.

LexA-fusion	Activating domain	β-Galactosidase (Units) Wild type R	Wild type S	*sip5Δ::TRP1* R	*sip5Δ::TRP1* S
LexA-Reg1	VP16-Snf1	4.3	21	2.3	6.7
LexA-Reg1	VP16-Snf1K84R	297	n.d.	167	n.d.
LexA-Reg1	GAD-Glc7	150	162	148	128
LexA-Snf1	Snf4-GAD	1.2	80	1.7	126

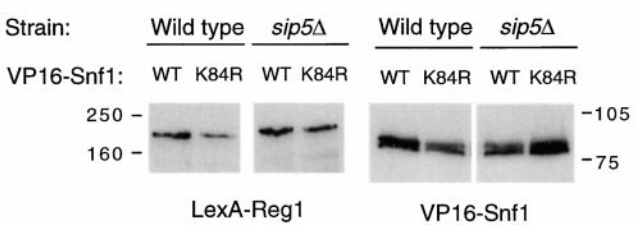

Figure 4.—Protein interactions in a *sip5*Δ mutant background. CTY10-5d and a *sip5Δ::TRP1* mutant derivative expressing the indicated proteins were grown as in Figure 3. Values are the average β-galactosidase activity of four transformants; standard deviations were <15% in all cases. Extracts were prepared by the fast boiling method (see materials and methods) from the corresponding transformants expressing LexA-Reg1 and wild-type (WT) VP16-Snf1 or VP16-Snf1K84R. Extracts (10 μl) were immunoblotted with α-LexA (left) or α-Snf1 polyclonal antibodies (right). n.d., not determined.

Sip5. Proteins were immunoprecipitated with α-LexA, and the precipitates were analyzed by SDS-PAGE and immunoblotting with α-HA. HA-Reg1 coimmunoprecipitated with LexA-Sip5 (Figure 2B). In addition, protein extracts from wild-type transformants expressing LexA-Glc7 and HA-Sip5 were immunoprecipitated with α-HA antibodies, and the precipitated proteins were subjected to immunoblot analysis with α-LexA. LexA-Glc7 coimmunoprecipitated with HA-Sip5 (Figure 2C). This coimmunoprecipitation may be an indirect result of the association between Reg1 and Sip5, as no significant two-hybrid interaction was detected between Glc7 and Sip5, but it also remains possible that Glc7 and Sip5 interact directly.

Disruption of the *SIP5* gene: To examine the phenotype of a *sip5* null mutant, the *sip5*Δ*::hisG* allele (see materials and methods and Figure 1) was introduced into a diploid strain (MCY3015). Tetrad analysis of the heterozygous diploid yielded viable *sip5* mutant segregants, which showed wild-type growth on glucose and raffinose and were unable to grow on sucrose in the presence of the glucose analog 2-deoxyglucose, indicating that both glucose repression and derepression of *SUC2* expression occur normally. The mutation *sip5*Δ*::HIS3* (see Figure 1) was also introduced into a haploid strain (MCY2921). The disruptant showed the same phenotype as the parent strain with respect to growth on different carbon sources, tolerance to high salt (1 m NaCl or 1 m KCl), 2 m ethylene glycol, 6% ethanol, or 3% formamide, and regulation of invertase synthesis. In addition, a diploid homozygous for *sip5*Δ (MCY3910 × MCY3918) was able to sporulate and yielded viable spores.

We also constructed the double mutants *snf1*Δ*10 sip5*Δ*::hisG*, *snf4*Δ*2 sip5*Δ*::hisG*, and *reg1*Δ*::URA3 sip5*Δ*::HIS3*, which behaved like the corresponding single mutant parents with respect to all the phenotypes tested above. Finally, we disrupted *SIP5* in a *sip1*Δ *sip2*Δ *gal83*Δ triple mutant (MCY2728). One member of the Sip1/Sip2/Gal83 family is present in a kinase complex and interacts with both Snf1 and Snf4, and in the triple mutant about half of the cellular Snf4 protein is no longer associated with Snf1; this is not sufficient to cause any pronounced Snf1-related growth defect (Jiang and Carlson 1997). The *sip5*Δ*::HIS3* quadruple mutant (MCY3907) resembled the parent triple mutant with respect to growth on different carbon sources, but the derepressed invertase activity was elevated 1.5-fold in comparison to the parental strain: 465 ± 54 *vs.* 307 ±

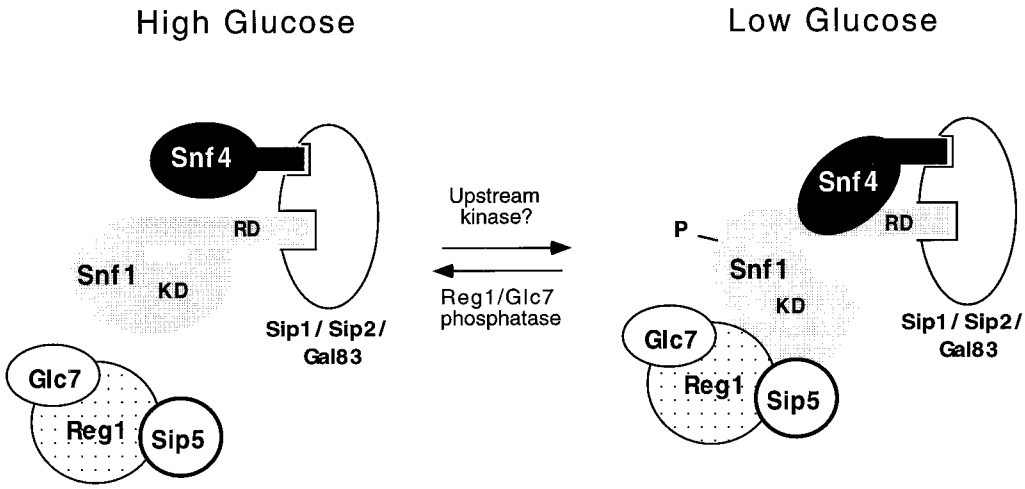

Figure 5.—Model of interaction of Sip5 with the Snf1 kinase and Reg1/Glc7 phosphatase complexes. When cells are grown in high glucose, an autoinhibited conformation of the Snf1 complex is favored in which the kinase domain is bound to the regulatory domain (RD). When glucose is limiting, the catalytic domain is phosphorylated, possibly by an upstream kinase; autoinhibition is relieved and the activating subunit Snf4 binds to the regulatory domain, leading to an active conformation of the complex. Reg1 and Sip5 both interact with Snf1 in response to glucose limitation, and both interact with the kinase domain (KD). Sip5 interacts with the Reg1/Glc7 complex regardless of glucose availability. Genetic evidence presented here suggests that Sip5 facilitates the interaction of Reg1/Glc7 with the Snf1 complex. Reg1/Glc7 functions to promote the transition back to the autoinhibited conformation of the kinase complex. This model depicts Sip5 in direct contact with those proteins for which a two-hybrid signal was detected. It is possible that other unidentified proteins are required for these interactions. The data also do not exclude the possibility that Sip5 interacts directly with other components of the Snf1 complex or with Glc7.

35 units in cells shifted to 0.05% glucose for 3 hr (values are the average of 10 quadruple mutants and 4 triple mutants, which carried the empty vector pEG202 so that the auxotrophic markers would be the same in both strains).

To assess the effects of Sip5 on protein-protein interactions within and between the Snf1 and Reg1/Glc7 complexes we introduced *sip5Δ::TRP1* into strain CTY10-5d and assayed β-galactosidase activity resulting from two-hybrid interactions (Figure 4). In the *sip5Δ* mutant, the interaction between LexA-Snf1 and Snf4-GAD in low glucose increased 1.5-fold, suggesting that the absence of Sip5 slightly favors the active conformation of the kinase complex. The *sip5Δ* mutation did not affect the interaction between LexA-Reg1 and GAD-Glc7. However, the interaction between LexA-Reg1 and VP16-Snf1 in low glucose was reduced 3-fold. We also assayed the interaction between LexA-Reg1 and VP16-Snf1K84R, an inactive mutant with a substitution of the invariant lysine in the ATP-binding site (Celenza and Carlson 1986). This interaction, which is not inhibited by glucose (Ludin *et al.* 1998), was reduced ~2-fold. Western blot analysis showed that differences in fusion protein levels are unlikely to account for these results (Figure 4). These findings suggest that Sip5 functions to promote the association of Reg1 with Snf1. Because Reg1/Glc7 is a negative regulator of the kinase complex, the reduced association of Reg1 with Snf1 in the mutant could also account for the enhanced two-hybrid interaction between Snf1 and Snf4.

Various other assays revealed no phenotype. The N terminus of Reg1 (LexA-Reg1$_{1\text{-}400}$) is rapidly phosphorylated, dependent on Snf1, when cells are shifted to medium containing low glucose, and then it is dephosphorylated when glucose is added back (P. Sanz and M. Carlson, unpublished results). In a *sip5Δ* mutant, this phosphorylation and dephosphorylation followed the same kinetics as in wild type (data not shown). Finally, we detected no mutant phenotype in cells overexpressing Sip5.

DISCUSSION

We have used the two-hybrid system to identify a new protein, Sip5, that interacts with the Snf1 protein kinase complex in response to glucose limitation. Coimmunoprecipitation studies confirmed the association of Sip5 with Snf1. Previously, Snf1 was shown to interact with Reg1, the regulatory subunit of the Reg1/Glc7 protein phosphatase complex, under glucose-limiting conditions, and we therefore tested for interaction between Sip5 and Reg1. We show that Sip5 interacts strongly with Reg1 in the two-hybrid assay and that this interaction is not inhibited by glucose. Coimmunoprecipitation studies confirmed the association of Sip5 with Reg1/Glc7. Thus, Sip5 is the first protein that has been shown to interact with both the Snf1 kinase complex and the Reg1/Glc7 phosphatase complex.

Two lines of evidence suggest that Sip5 interacts primarily with the Reg1 component of the phosphatase complex. Sip5 did not interact with the catalytic subunit Glc7 in the two-hybrid system, and Sip5 interacted better with the Reg1F468R mutant protein, which is defective in binding Glc7 (Alms *et al.* 1999), than with wild-type Reg1. Thus, the binding of Sip5 to Reg1 does not require Glc7. It remains unclear whether the improved binding to the F468R mutant reflects competition between Sip5 and Glc7 or a somewhat altered conforma-

tion of the mutant protein that enhances binding to Sip5. These data suggest that Reg1 mediates the coimmunoprecipitation of Sip5 and Glc7 but do not exclude the possibility that Sip5 and Glc7 interact directly.

Genetic analysis of *sip5*Δ mutants revealed no striking phenotypic differences from the wild type with respect to growth on different carbon sources, tolerance to different stress conditions, sporulation, or germination. No synergy nor synthetic lethal phenotypes were observed in mutants carrying *sip5*Δ in combination with *snf1*Δ, *snf4*Δ, or *reg1*Δ. However, evidence suggests that Sip5 has a modest role as a negative regulator of the Snf1 kinase. First, the introduction of *sip5*Δ into the *sip1*Δ *sip2*Δ *gal83*Δ triple mutant caused a 1.5-fold increase in derepression of invertase activity; the absence of the Sip1/Sip2/Gal83 component may make the Snf1 kinase complex more sensitive to loss of Sip5. The two-hybrid interaction between Snf1 and Snf4 in glucose-limited cells, which is an indicator of an open, active conformation of the kinase complex, was also 1.5-fold higher in a *sip5*Δ mutant than in the wild type. Finally, the most significant phenotype detected was an effect of *sip5*Δ on the interaction of Reg1 and Snf1. The two-hybrid interaction between Reg1 and Snf1 was reduced 3-fold in a *sip5*Δ mutant relative to the wild type.

These genetic findings suggest that Sip5 negatively regulates the Snf1 kinase by promoting the interaction of Reg1/Glc7 with the kinase. When taken together with the physical interaction of Sip5 with both Snf1 and Reg1, these results suggest that Sip5 directly facilitates the interaction between the Reg1/Glc7 phosphatase and the Snf1 kinase when glucose is limiting (Figure 5). Reg1/Glc7 functions to promote the transition of the active complex back to the autoinhibited state. The Snf1 kinase plays a central role in a regulatory response that is crucial to the yeast cell, and evidence indicates that the activity of this kinase is highly regulated, by multiple regulatory mechanisms. Sip5 appears to contribute to one of these mechanisms.

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