The Snf1 Protein Kinase and Its Activating Subunit, Snf4, Interact with Distinct Domains of the Sip1/Sip2/Gal83 Component in the Kinase Complex

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The Snf1 protein kinase plays a central role in the response to glucose starvation in the yeast *Saccharomyces cerevisiae*. Previously, we showed that two-hybrid interaction between Snf1 and its activating subunit, Snf4, is inhibited by high levels of glucose. These findings, together with biochemical evidence that Snf1 and Snf4 remain associated in cells grown in glucose, suggested that another protein (or proteins) anchors Snf1 and Snf4 into a complex. Here, we examine the possibility that a family of proteins, comprising Sip1, Sip2, and Gal83, serves this purpose. We first show that the fraction of cellular Snf4 protein that is complexed with Snf1 is reduced in a *sip1Δ sip2Δ gal83Δ* triple mutant. We then present evidence that Sip1, Sip2, and Gal83 each interact independently with both Snf1 and Snf4 via distinct domains. A conserved internal region binds to the Snf1 regulatory domain, and the conserved C-terminal ASC domain binds to Snf4. Interactions were mapped by using the two-hybrid system and were confirmed by *in vitro* binding studies. These findings indicate that the Sip1/Sip2/Gal83 family anchors Snf1 and Snf4 into a complex. Finally, the interaction of the yeast Sip2 protein with a plant Snf1 homolog suggests that this function is conserved in plants.

The Snf1 protein kinase family is widely conserved in eukaryotes and has been implicated in cellular responses to nutritional and environmental stresses. In the yeast *Saccharomyces cerevisiae*, the Snf1 (Cat1, Ccr1) serine/threonine kinase is essential for the response to glucose starvation and its activity is regulated by the glucose signal (41). Snf1 is required for the transcription of genes involved in alternate-carbon-source utilization, respiration, and gluconeogenesis and also plays roles in sporulation, glycogen storage, thermotolerance, and peroxisome biogenesis (5, 35, 37, 39).

The mammalian homolog, AMP-activated protein kinase (AMPK) (1, 3, 14, 28), is involved in cellular stress responses (8, 21) and regulates HMG-CoA reductase and acetyl-CoA carboxylase (20). Snf1 also inactivates yeast acetyl-CoA carboxylase, suggesting that the regulatory role in lipid metabolism is conserved (28, 41). Additional common targets seem likely since AMPK and Snf1 have similar substrate recognition motifs (9), but there is as yet no evidence implicating AMPK in transcriptional control.

Snf1 homologs have also been cloned from various plants (2, 17, 25, 29), and some of the plant kinases provide Snf1 function in yeast (2, 29). Other related protein kinases include PAR-1 of *C. elegans* (16), mouse Msk (33), and human p78, a protein implicated in pancreatic carcinoma (19).

In yeast cells, the Snf1 kinase is complexed with other proteins, including the activating subunit Snf4 (Cat3) (6, 36) and the Sip1, Sip2, and Gal83 proteins (44, 45). These components of the Snf1 kinase complex also have counterparts in higher eukaryotes (38, 40). The Snf4 protein activates the Snf1 kinase *in vivo* and *in vitro* (4, 6, 36, 41), and during glucose starvation Snf1 kinase activity increases in a Snf4-dependent manner (41). In the two-hybrid system, the interaction between Snf1 and Snf4 is regulated by glucose: high glucose levels inhibit interaction and glucose limitation induces interaction (23). Ge-

netic and biochemical evidence indicates that when glucose is limiting, Snf4 binds to the Snf1 regulatory domain and counteracts an autoinhibitory interaction between the Snf1 kinase domain and the regulatory domain (23).

Data indicating a regulated interaction between Snf1 and Snf4 in the two-hybrid system, however, is apparently contrary to coimmunoprecipitation and copurification data which suggests that Snf1 and Snf4 remain associated in cells grown in glucose (6, 11, 28, 45). A possible explanation is that previous biochemical experiments were not quantitative, and perhaps the two-hybrid results reflect the behavior of a fraction of the Snf1 and Snf4 proteins which are not associated in glucose-grown cells. In this report, we provide evidence that most, if not all, of the Snf4 protein in a cell extract is associated with Snf1 when glucose levels are high. A second possibility is that another protein(s) interacts with both Snf1 and Snf4, thereby anchoring them into a complex, within which their interaction varies in response to glucose. In this scenario, the two-hybrid system detects only direct interaction, which occurs in response to glucose starvation, but not indirect interaction bridged by the other protein(s).

The other known components of Snf1 kinase complexes, Sip1, Sip2, and Gal83, are candidates for the proposed bridging protein(s). Sip1, Sip2, and Gal83 constitute a family of related proteins. Sip1 and Sip2 were identified in a two-hybrid screen for proteins that interact with Snf1 and by other genetic approaches (30, 44, 45), and Gal83 was identified genetically by its effects on glucose regulation of *GAL* gene expression (10, 27). All three proteins interact with Snf1 in glucose-grown cells, as determined by assays using the two-hybrid system and by coimmunoprecipitation from cell extracts (45). A conserved C-terminal region of about 80 amino acids, designated the ASC domain, was shown to be sufficient for interaction with the Snf1 complex (45). In immune complex assays of Snf1 kinase activity, Sip1, Sip2, and Gal83 are phosphorylated; moreover, mutation of *GAL83* nearly abolishes Snf1 activity in such assays. Genetic evidence suggests that Sip1, Sip2, and Gal83 are alternate members of the kinase complex, possibly

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TABLE 1. Plasmids used in this study^a

Plasmid	Description	Source or reference
pRJ1	LexA ₈₇ -Sip1 ₄₃₂₋₈₆₃ in pSH2-1	45
pRJ2	LexA ₈₇ -ASC _{Sip1} (codons 771–863) in pSH2-1	45
pXY38	LexA ₈₇ -Sip2 in pSH2-1	45
pRJ10	LexA ₈₇ -Sip2ΔASC (codons 1–335) in pSH2-1	45
pRJ30	LexA ₈₇ -ASC _{Sip2} (codons 336–415) in pSH2-1	45
pRJ105	GAD-Sip2 in pGAD424	This study
pRJ106	GAD-Sip2ΔASC (codons 1–335) in pGAD424	This study
pRJ107	GAD-ASC _{Sip2} (codons 336–415) in pGAD424	This study
pRJ258	TRX-Sip2ΔASC (codons 1–335) in pET-32a(+)	This study
pRJ259	TRX-ASC _{Sip2} (codons 336–415) in pET-32a(+)	This study
pRJ324	GAD-Gal83-G235R in pGAD424	This study
pRJ325	GAD-Gal83 in pGAD424	This study
pNI12	Snf4-GAD fusion	12
pRJ31	GST-Snf4 in pGEX3X	23
pRJ58	LexA ₈₇ -Snf4 in pSH2-1	23
pRJ55	LexA-Snf1 in pLexA(1-202)+PL	23
pSG1	GAD-Snf1 in pGAD2F	42
pRJ206	GST-Snf1RD (codons 392–633) in pGEX3X	This study
pRJ101	GAD-Snf1 ₅₁₅₋₆₃₃ in pACTII	23
pRJ69	GAD-NPK5 in pGAD2F	This study

^a LexA₈₇, DNA-binding domain (residues 1 to 87). Vectors: pGAD424 (Clontech), pACTII (gift from S. Elledge) (24), pGAD2F (7), pSH2-1 (18), pLexA(1-202)+PL (32), pGEX3X (Pharmacia), and pET-32a(+) (Novagen).

serving as adaptors that mediate the interactions of Snf1 with specific targets (10, 44, 45). However, in vivo these proteins do not appear essential for function of the Snf1 pathway, as a *sip1Δ sip2Δ gal83Δ* triple deletion mutant does not exhibit defects associated with the Snf[−] phenotype (10, 45). No other mutant phenotypes were detected in the single or triple mutants (26, 45).

Here, we address the roles of Sip1, Sip2, and Gal83 in the Snf1 kinase complex. We show that the fraction of Snf4 that is complexed with Snf1 is reduced in a *sip1 sip2 gal83* triple mutant. Using the two-hybrid system, we show that Sip1, Sip2, and Gal83 interact independently with both Snf1 and Snf4. The ASC domain interacts directly with Snf4, and an internal region, conserved in all three proteins, interacts with the Snf1 regulatory domain (Snf1RD). These findings support the view that the Sip1/Sip2/Gal83 family of proteins anchors Snf1 and Snf4 into a complex.

MATERIALS AND METHODS

Strains, plasmids, and genetic methods. *S. cerevisiae* strains were CTY10-5d (*MATa ade2 his3-Δ200 leu2-3,112 trp1-901 gal80 gal4 lexAop-lacZ*), MCY829 (*MATα his3-Δ200 ura3-52 lys2-801*), MCY2649 (*MATa his3-Δ200 leu2-3,112 ura3-52*), MCY2634 (*MATa his3-Δ200 leu2-3,112 ura3-52 snf4Δ2*), MCY2916 (*MATa his3-Δ200 leu2-3,112 ura3-52 snf1Δ10*), YM3033 (*MATα ade2-101 his3-Δ200 ura3-52 lys2-801 gal80Δ-542 GAL83-2000 LEU2::pRY181*) (10), and MCY2728 (*MATa ade2-101 his3-Δ200 leu2-3,112 ura3-52 sip1Δ1::URA3 sip2Δ3::LEU2 gal83Δ::URA3*). Plasmids were constructed by standard methods (34) and are listed in Table 1. pRJ69 was constructed by PCR with an NPK5 clone (29) as template. pRJ324 and pRJ325 were made by PCR with genomic DNA from strains YM3033 and MCY2649, respectively, as templates. Plasmid pSH18-18 (gift from R. Brent) was used to transform strains to provide a *lexAop-lacZ* reporter. Standard genetic methods were followed, and yeast cells were grown in yeast extract-peptone (YEP) or selective synthetic complete (SC) medium containing 2% glucose (31), except where noted.

β-Galactosidase assay. Filter lift assays for blue color were performed as described previously (44). Transformants (6 to 8) were patched onto selective SC medium containing 2% glucose and grown for one day at 30°C; during this time cells in the patch become limited for glucose. Two-hybrid filter lift assays were then carried out and developed overnight. Since β-galactosidase is stable, two-hybrid interactions that occur in medium with either a high or low level of glucose are detected. For quantitative assays, transformants were grown to mid-

log phase in selective SC medium containing 2% glucose, and β-galactosidase activity was assayed in permeabilized cells and expressed in Miller units (31).

In vitro binding assay. Protein expression and in vitro binding assays were performed as described previously (22). Snf4 and the Snf1 regulatory domain were cloned into pGEX3X (Pharmacia) as glutathione S-transferase (GST) fusions (pRJ31 and pRJ206, respectively). The ASC_{Sip2} and Sip2ΔASC domains were cloned into pET-32a(+) (Novagen) to express a thioredoxin (TRX) fusion (pRJ259 and pRJ258, respectively). *Escherichia coli* XL-1 blue and GM48 (Stratagene) were used for expressing GST fusions, and *E. coli* BL21 (DE3) (Novagen) was used for expressing TRX fusions. The expression of fusion proteins was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside, 1 mM final concentration) to the culture. Bacterial pellets were resuspended in buffer ST (50 mM Tris-HCl [pH 8.0], 150 mM NaCl) with 100 μg of lysozyme per ml and were sonicated briefly on ice (13). Extracts were cleared by centrifugation at 12,000 rpm for 15 min in a Sorvall SS-34 rotor. Extracts containing equivalent amounts of GST-Snf4, GST-Snf1 regulatory domain, and GST proteins were incubated with glutathione-Sepharose 4B beads (Pharmacia) at 4°C for 1 h. After being washed, beads were split into several equal portions (each 100 μg of bound protein) for subsequent binding experiments. Extracts (350 μg) expressing TRX-ASC_{Sip2}, TRX-Sip2ΔASC, or TRX proteins were added to the above beads and allowed to bind at 4°C for 2 h in buffer ST (500 μl). After being extensively washed with cold ST (10 ml), beads were boiled in sodium dodecyl sulfate (SDS) sample buffer (2×) and loaded onto an SDS-10% polyacrylamide gel. Bound proteins were detected by Western analysis with a polyclonal antibody against TRX (Santa Cruz Biotechnology) and enhanced chemiluminescence (ECL) with ECL reagents (Amersham).

Nickel affinity chromatography. Yeast cells were grown to mid-log phase. Cells were harvested by centrifugation, washed once with 15 ml of cold ST buffer, and frozen at −80°C. Alternatively, cells were harvested by quick filtration through a 0.8-μm-pore-size filter (Micron Separations Inc.), washed once with 15 ml of cold ST buffer, and frozen immediately by immersion in dry ice-methanol at −80°C. Cells were broken by vortexing with glass beads in ST buffer with 2 mM phenylmethylsulfonyl fluoride. Protein extracts were clarified by centrifugation (2000 rpm, 10 min, Sorvall SS-34 rotor) at 4°C. Extract (500 μg) was incubated with 150 μl of Ni²⁺-nitrilotriacetic acid (NTA)-agarose beads (Qiagen) in 250 μl of ST buffer with 1% Triton X-100, 25 mM imidazole, and 0.5 mg of ovalbumin (Calbiochem) per ml for 2 h at 4°C on a nutator. Beads were then pelleted by centrifugation (500 rpm, 5 seconds, 4°C, Eppendorf microfuge), and the supernatant containing the unbound protein was removed. The beads were then washed 10 times with cold binding buffer (1 ml). To analyze the bound proteins, protein was eluted from the beads in ST plus 0.2 M EDTA (100 μl) or the beads were directly boiled in 2× sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. Antibodies were detected by ECL.

RESULTS

Snf4 protein is associated with Snf1 in cells grown in high and low levels of glucose. To examine the association of Snf1 and Snf4, we used nickel affinity chromatography to isolate Snf1, taking advantage of an intrinsic polyhistidine stretch (13 consecutive His) at the N terminus of Snf1. Extracts were first prepared from cells grown in high-concentration glucose (2%), and the Snf1 kinase complex was coordinated to Ni²⁺-NTA-agarose beads. All of the Snf1 protein present in the extract was bound to the beads (Fig. 1A). Binding was dependent on the presence of the polyhistidine stretch as the Snf1ΔH protein, which lacks these histidine residues (4), did not bind. We then prepared, in parallel, extracts from cells grown in either high-concentration glucose or galactose-glycerol-ethanol plus 0.05% glucose. The Snf1 protein was purified on the Ni²⁺-NTA-agarose resin, and the association of Snf4 with Snf1 was analyzed by immunoblotting with Snf4 antibody (11). In both cases, all of the Snf4 protein in the extract was bound with Snf1 to the beads (Fig. 1B). The results for controls with a *snf1Δ* strain (lanes 7 to 9) indicated that Snf4 does not bind to the beads in the absence of Snf1. Therefore, Snf4 protein is associated with Snf1 in cells grown in both high and low levels of glucose.

This finding, together with previous evidence (6, 11, 28, 45), supports the view that the glucose-regulated interaction between Snf1 and Snf4 in the two-hybrid system (23) reflects a conformational change in the kinase complex. A further implication is that in cells grown with a high glucose level another

FIG. 1. Copurification of Snf4 with Snf1 on Ni^{2+} -NTA affinity resin. Strains were MCY829 (wild type, WT), MCY2916 (*snf1Δ*), MCY2916 carrying pSNF1- Δ H, and MCY2728 (*sip1Δ sip2Δ gal83Δ*). pSNF1- Δ H is a *CEN* plasmid with the *SNF1-ΔH* allele, from which 12 adjacent His codons have been deleted (4). (A) Cells of the indicated genotype were grown to mid-log phase in YEP or SC medium containing 2% glucose, harvested by centrifugation, and used to prepare protein extracts. Proteins (500 μg) were incubated with Ni^{2+} -NTA-agarose beads, unbound protein (U) was removed, and one-fifth was analyzed. After being washed, beads were boiled in 2 $\times$ sample buffer, and one-fifth of the Ni^{2+} -bound protein (N) was analyzed. Proteins were separated on an SDS-10% polyacrylamide gel and were analyzed by immunoblotting with Snf1 antiserum (5). Lanes 1, 4, and 6 show 100 μg of total input protein (T). (B and C) Cells were grown in YEP medium containing either 2% glucose (Glu) or 2% galactose-glycerol-ethanol plus 0.05% glucose (Gal/Gly/ETOH) and were harvested by quick filtration. The binding assay was performed as described for panel A except that bound proteins were eluted from the beads and immunoblotting was performed with affinity-purified Snf1 and Snf4 antibodies (11, 45). (B) Lanes 1 and 4 show results for 50 μg of total protein, and lane 7 shows results for 100 μg . (C) Lanes 1 and 4 show results for 100 μg of total protein. In a previous study, the amount of Snf1 protein was reproducibly decreased in glucose-grown *gal83Δ* mutants relative to the wild type (45). Protein markers are in kilodaltons.

protein (or proteins) in the Snf1 complex maintains the association of Snf4 with Snf1.

Association of Snf4 with Snf1 is impaired in a *sip1Δ sip2Δ gal83Δ* triple mutant. The other known components of Snf1 complexes are Sip1, Sip2, and Gal83, which belong to a family of proteins whose members are thought to be alternate members of the kinase complex (10, 30, 44, 45). Previous studies demonstrated the interaction of these proteins with Snf1 in glucose-grown cells (44, 45). Thus, these three are candidates for proteins that could mediate the interaction between Snf1 and Snf4. To determine whether Sip1, Sip2, and Gal83 contribute to the association of Snf1 and Snf4 in complexes, we

tested for the copurification of Snf4 with Snf1 from a *sip1Δ sip2Δ gal83Δ* triple-deletion mutant using nickel affinity chromatography. In contrast to the results with the wild type, about half of the total Snf4 protein was in the unbound fraction, regardless of whether cells were grown in medium with a high level of glucose or in galactose-glycerol-ethanol plus 0.05% glucose (Fig. 1C). Thus, the association of Snf4 with Snf1 is impaired in the triple mutant, indicating that Sip1, Sip2, and Gal83 play a role in maintaining the structure of Snf1 kinase complexes. These results do not distinguish whether this family is partially responsible for the integrity of most Snf1 complexes or rather plays an essential role in about half of the (heterogeneous) Snf1 complexes.

Sip1, Sip2, and Gal83 interact independently with both Snf1 and Snf4. Sip1, Sip2, and Gal83 could contribute to the association of Snf4 with Snf1 either by directly interacting with each of these proteins, thereby anchoring them in a complex, or by indirectly contributing to the structural integrity of the complex. To address the possibility that Sip1, Sip2, and Gal83 interact independently with both Snf1 and Snf4, we used the two-hybrid system. We first tested for the two-hybrid interaction of LexA-Sip1₄₃₂₋₈₆₃, containing the C-terminal region that is homologous to Sip2 and Gal83, and LexA-Sip2 with Snf4-GAD (Fig. 2A). In each case, the interaction was readily detected in a wild-type host. Furthermore, the interaction was preserved in a *snf1Δ* host, indicating that Sip1 and Sip2 interact with SNF4 independently of Snf1. Sip1 and Sip2 also interacted with GAD-Snf1 in a *snf4Δ* mutant strain (Fig. 2A), consistent with previous coimmunoprecipitation results (45).

LexA-Gal83 activates transcription on its own, so interactions of Gal83 were assayed with a GAD-Gal83 fusion (Fig. 2C). GAD-Gal83 interacted with LexA-Snf1 and LexA₈₇-Snf4 in wild-type and also in *snf4Δ* and *snf1Δ* mutant hosts, respectively. Therefore, Sip1, Sip2, and Gal83 are each capable of interacting independently with both Snf1 and Snf4.

C-terminal ASC domain of Sip2 interacts with Snf4. Previous studies showed that Sip1, Sip2, and Gal83 contain a conserved C-terminal sequence of about 80 residues, designated the ASC domain (Fig. 3B), that mediates association with the Snf1 kinase complex in wild-type cells (45). Association was detected both by two-hybrid interaction and coimmunoprecipitation. From these data, however, it was not clear whether the ASC domain interacts directly with Snf1, Snf4, or yet another component of the complex. To determine whether the ASC domain interacts with Snf1 or Snf4, we tested two-hybrid interactions in mutant hosts. LexA fusions to the ASC_{Sip1} and ASC_{Sip2} domains interacted with Snf4-GAD in both wild-type and *snf1Δ* strains, and quantitative assays of β -galactosidase activity confirmed that interaction was strong in the *snf1Δ* mutant (Fig. 2A). Moreover, deletion of the ASC domain from Sip2 (Sip2 Δ ASC) abolished interaction with Snf4-GAD. Thus, the ASC domain is necessary and sufficient for Snf1-independent interaction with Snf4.

These findings suggested that the interaction detected between the ASC domain and Snf1 in wild-type cells (45) requires Snf4. Consistent with this model, interactions of LexA-ASC_{Sip1} and LexA-ASC_{Sip2} with GAD-Snf1 were abolished in a *snf4Δ* mutant (Fig. 2A). Control experiments confirmed that the loss of interaction cannot be attributed to the inability of the *snf4Δ* strain to respond to glucose starvation, as the ASC domains interacted with the catalytic mutant GAD-Snf1-K84R in a *snf1Δ* strain (data not shown). Further support for the idea that Snf4 mediates interaction of the ASC domain with Snf1 was provided by mapping the interacting regions of Snf1: the ASC_{Sip2} domain and Snf4 showed similar profiles of interaction with a panel of GAD-Snf1 fusions (23) (Fig. 4A).

FIG. 2. Two-hybrid interactions of Sip1, Sip2, and Gal83 sequences with Snf4 and Snf1. Strains were CTY10-5d (wild type, WT) and MCY2916 (*snf1Δ*) or MCY2634 (*snf4Δ*) carrying a *lexAop-lacZ* reporter on pSH18-18. Proteins were expressed from the plasmids listed in Table 1. Shaded bar, ASC domain. Interactions in filter lift assays are indicated by plus signs. (A) Values shown in parentheses are average β -galactosidase activities for two to six transformants (*, one transformant). Standard errors were <26% with one exception (40% for LexA-Sip2 plus Snf4-GAD in the *snf1Δ* mutant). LexA fusions did not interact with GAD or control GAD fusions. LexA₈₇-Sip2ΔASC plus GAD or Snf4-GAD gave a light blue color and low activity, so the interaction was scored as negative; in other experiments, GAD-Sip2ΔASC did not interact with LexA₈₇-Snf4 in the wild type. Immunoblotting confirmed that the ASC_{Sip1} and ASC_{Sip2} fusions were expressed in a *snf4Δ* mutant and that deletion of the ASC domain from Sip2ΔASC did not affect protein levels (43). The data for interaction with GAD-Snf1 in the wild type is from reference 45. (B) In controls, GAD-ASC_{Sip2} interacted with LexA-Snf1 and LexA₈₇-Snf4 in CTY10-5d. Dark bar, Snf1-interacting KIS region. (C) Values shown in parentheses are average β -galactosidase activities for four transformants grown in medium containing 4% glucose; GAD-Gal83 plus LexA yielded 0.7 U. Standard errors were <8%. GAD-Gal83₄₋₄₁₇ was used to test interaction with LexA-Snf1 in the *snf4Δ* strain. ND, not determined.

To confirm that the interaction between Snf4 and the ASC domain is direct, we tested their interaction in vitro using bacterially expressed GST-Snf4 and TRX-ASC_{Sip2}, a fusion between TRX and the ASC_{Sip2} domain. GST-Snf4 and GST proteins were immobilized on glutathione-Sepharose 4B beads and allowed to bind to bacterial extracts containing TRX-ASC_{Sip2} or TRX proteins. After being extensively washed, bound proteins were analyzed by Western blotting with an antibody against TRX. Specific interaction was detected between GST-Snf4 and TRX-ASC_{Sip2} (Fig. 5A).

A conserved region internal to Sip1, Sip2, and Gal83 interacts with Snf1. The preceding evidence shows that Sip1 and Sip2 interact with Snf4 in a Snf1-independent manner via the ASC domain. Evidence also indicates that Sip1, Sip2, and Gal83 interact with Snf1 in a Snf4-independent manner. Interactions were detected in the two-hybrid system in a *snf4Δ* strain (Fig. 2A and C), and Sip1 and Sip2 coimmunoprecipitated with Snf1 from a *snf4Δ* mutant extract (45). This Snf4-independent mechanism does not require the ASC domain, as LexA-Sip2ΔASC also interacted with GAD-Snf1 in a *snf4Δ* mutant (Fig. 2A).

To define the Sip2 sequence that interacts with Snf1, we tested a panel of GAD fusions to partial Sip2 sequences for interaction with LexA-Snf1. Assays were carried out in a *snf4Δ* strain to eliminate interaction mediated by Snf4. The Snf1-interacting region was mapped to residues 154 to 335 (Fig. 2B and 3A). An analysis of Gal83 further restricted the N terminus of the Snf1-interacting region, as codons 198 to 350 were sufficient (Fig. 2C). For Sip1, the region C-terminal to residues 511 was sufficient for interaction with Snf1 in a *snf4Δ* mutant, while a region C-terminal to codon 585 was not (Fig. 3A; data not shown). We will refer to the Snf1-interacting region in Sip1, Sip2, and Gal83 as KIS, for kinase-interacting sequence.

The KIS region of Gal83 includes the site of the semidominant mutation *GAL83-2000*, which confers partially glucose-insensitive *GAL* gene expression in mutants defective in inhibition of Gal4 by Gal80 (10, 27). The relevant mutation appears to be a change of Gly-235 to Arg, so we will refer to the mutation as *GAL83-G235R* here. The G235 residue is conserved in Sip1 and Sip2 and also in the *Kluyveromyces lactis* homolog Fog1 (15) and in mammalian Sip2 homologs (38, 40) (Fig. 3A). One possible model is that the mutant Gal83 protein competes better than the wild type with Sip1 and Sip2 for binding to Snf1 and thereby directs Snf1 more effectively to its targets in the galactose pathway, even allowing some phosphorylation in glucose. Another possibility is that the mutant Gal83 protein affects the regulation of Snf1 activity and that these kinase complexes are also directed by Gal83 to targets in the galactose pathway. To test the effect of this mutation on interaction with Snf1, we constructed a mutant GAD-Gal83-G235R fusion. Assays of interactions with LexA-Snf1 in a wild-type host, grown in medium containing glucose, showed that the mutation causes a very modest increase in β -galactosidase activity (Fig. 2C). Similarly, for cells grown in galactose-glycerol-ethanol plus 0.05% glucose, β -galactosidase activity values were 364 U for GAD-Gal83 and 736 U for the mutant protein (averages for four transformants; standard error, <12%). It is possible that these increases reflect improved interaction with Snf1, especially as overexpression of these fusion proteins may drive their association in vivo and thereby minimize the observed differences.

Sip2 interacts with the C terminus of the Snf1 regulatory domain. To delineate the Snf1 sequence that interacts with Sip2, we tested a series of GAD-Snf1 fusions (23) for interaction with LexA-Sip2ΔASC (Fig. 4A). The extreme C terminus of Snf1 (residues 515 to 633) was necessary and sufficient for interaction in wild-type and *snf4Δ* strains. This region is distinct from the binding site for Snf4 (23). LexA-Sip2 was also tested against the panel of GAD-Snf1 sequences in both wild-type and *snf4Δ* hosts, and as predicted, the results correspond to the sum of the interactions detected with Sip2ΔASC and ASC_{Sip2} (Fig. 4A).

To confirm that Snf1RD binds directly to Sip2ΔASC, we assayed for in vitro binding of bacterially expressed GST-Snf1RD and TRX-Sip2ΔASC fusions. Interaction was de-

FIG. 3. Conservation of the Snf1- and Snf4-interacting sequences in the Sip1 family. Sip2 sequences (45) are compared to those of the corresponding regions from Gal83 (10), Sip1 (44), *K. lactis* Fog1 (15), and rat AMPK β (40). Identical and conserved substitutions (D, E; K, R; I, L, V; S, T; F, Y) are shown in reverse contrast. Numbers indicate amino acid positions. Dashes indicate gaps. (A) KIS region, required for interaction with Snf1. The solid arrowhead marks residue 198 in Gal83; the sequence C-terminal to this position in GAD-Gal83₁₉₈₋₄₁₇ is sufficient for interaction with Snf1 in a *snf4* Δ mutant. The open arrowhead indicates position 585 of Sip1; sequences N-terminal to this point are required for interaction because GAD-Sip1₅₁₁₋₈₆₃ interacts with Snf1 in a *snf4* Δ mutant but GAD-Sip1₅₈₅₋₈₆₃ does not. (B) ASC domain, which binds to Snf4.

ected between these fusion proteins; GST and TRX served as controls (Fig. 5B).

A plant Snf1 homolog interacts with Sip2. Previously, we showed that the tobacco NPK5 kinase, a Snf1 functional homolog, interacts with the yeast Snf4 protein in a glucose-regulated manner (23). The Sip2-interacting region of Snf1 is conserved in NPK5 (Fig. 4B), raising the possibility that the ability to interact with the Sip1/Sip2/Gal83 family might also be conserved. To test this idea, we constructed a GAD-NPK5 fusion which complements the *snf1Δ* mutation in yeast for

growth on raffinose. GAD-NPK5 interacted with LexA-Sip2 in wild-type yeast cells (Fig. 6). Furthermore, this interaction also occurred in a *snf4Δ* mutant, indicating that it is not bridged by Snf4. Thus, interaction between the kinase and Sip2 is conserved from yeast to plants.

DISCUSSION

The Snf1 protein kinase and its activating subunit, Snf4, are complexed with members of a family of proteins comprising

FIG. 4. Mapping the Sip2-interacting region of Snf1. (A) Snf1 sequences were expressed as GAD fusions (23). Snf4, Sip2, Sip2 Δ ASC (Sip2₁₋₃₃₆), and ASC_{Sip2} (Sip2₃₃₂₋₄₁₅) were expressed as LexA₈₇ fusions (23, 45). Two-hybrid interactions were tested in strains CTY10-5d (wild type, WT) and MCY2634 (*snf4 Δ*) carrying pSH18-18. Filter lift assays were done for 8 to 12 transformants. Shaded bar, kinase domain (KD); open bar, KD-interacting region; hatched part of open bar, Snf4-interacting region; black bar, Sip2-interacting region. Data for interaction with LexA-Snf4 is from reference 23. (B) Snf1 sequence that interacts with Sip2 is compared to homologous sequences of *K. lactis* Fog2 (15), tobacco NPK5 (29), rye RKN1 (2), and rat AMPK α (3). Identities with Snf1 and conserved substitutions (D, E; K, R; I, L, V; S, T; F, Y) are shown in reverse contrast. Numbers indicate amino acid positions. Dashes indicate gaps.

FIG. 5. In vitro binding of Snf4 to ASC_{Sip2} and Snf1RD to Sip2ΔASC. Snf4 and Snf1RD were expressed as GST fusions. The indicated GST fusion or GST alone (100 μg) was immobilized on glutathione-Sepharose 4B beads (Pharmacia) and incubated with extracts (350 μg) expressing TRX or TRX fusions to ASC_{Sip2} (A) and Sip2ΔASC (B). After being washed, bound proteins were boiled in sample buffer, separated by SDS-10% polyacrylamide gel electrophoresis, and analyzed by immunoblotting with an antibody against TRX (Santa Cruz Biotechnology) and ECL. Protein extracts (350 μg) used for binding experiments (Input) were loaded in lanes 1 and 2. Protein markers are in kilodaltons.

Sip1, Sip2, and Gal83. Here, we present evidence that the loss of these proteins impairs the association of Snf4 with Snf1 in cell extracts. We then show that each of these three proteins interacts directly with both Snf1 and Snf4 via two distinct conserved domains. The C-terminal ASC domains of Sip1, Sip2, and Gal83 bind to Snf4 independently of Snf1, and the internal KIS region interacts with Snf1 independently of Snf4. We also show that Sip2 interacts with the regulatory domain of Snf1. These interactions were mapped by using the two-hybrid system and were confirmed by in vitro binding studies. Quantitative assays of key two-hybrid results, together with previous coimmunoprecipitation studies (45), showed that these inter-

FIG. 6. Conserved interaction between yeast Sip2 and tobacco NPK5. Two-hybrid interactions were tested between LexA₈₇-Sip2 and GAD-NPK5 in strains CTY10-5d (wild type) and MCY2634 (*snf4*Δ) with reporter pSH18-18. Filter lift assays of three transformants were developed overnight.

FIG. 7. Model for protein interactions in the Snf1 kinase complex. A kinase complex contains Snf1, Snf4, and one of the proteins Sip1, Sip2, and Gal83. Each of the latter interacts with Snf1 and Snf4 independently via the distinct ASC and KIS domains. Additional interactions have not been excluded; for example, it is possible that the ASC domain together with Snf4 interacts with the Snf1 regulatory domain. A kinase complex may also contain another component(s) not represented here. Previous studies provided evidence that in cells grown in medium containing a high level of glucose, Snf1RD (RD) binds to the Snf1 kinase domain (KD), causing autoinhibition of kinase activity; when glucose is limiting (little or no glucose), autoinhibition is relieved and Snf4 binds to an overlapping site in the regulatory domain (23).

actions occur in cells grown in high levels of glucose. Together, these findings indicate that one of the functions of the Sip1/Sip2/Gal83 family is to anchor Snf1 and Snf4 into a complex (Fig. 7).

These findings offer a resolution of a puzzling discrepancy between two-hybrid and biochemical evidence. In the two-hybrid system, the interaction between Snf1 and Snf4 is inhibited by glucose, whereas biochemical evidence indicates that Snf4 remains associated with Snf1 in glucose-grown cells. Previously, we proposed that other proteins could hold Snf1 and Snf4 together in a complex in medium containing glucose, despite their lack of direct interaction with one another (23). Evidence that Sip1, Sip2, and Gal83 serve such a function supports the view that the glucose-regulated interaction between Snf1 and Snf4 in the two-hybrid system (23) reflects a conformational change in the kinase complex (Fig. 7).

Genetic studies have addressed the role of Sip1, Sip2, and Gal83 in the Snf1 kinase pathway. Increased *SIP1* and *GAL83* gene dosage suppresses certain growth defects in *snf4* mutants (44, 45), and the *GAL83-2000(G235R)* allele relieves glucose repression of *GAL* genes in certain mutant backgrounds (10, 27). In these experiments, the differential effects on *SUC* and *GAL* gene expression suggested that Sip1, Sip2, and Gal83 serve as adaptors that mediate interaction of the kinase with specific intracellular targets; the extended N-terminal sequence which is divergent between Sip1, Sip2, and Gal83 may play this role. In addition, Gal83 is required for Snf1 kinase activity in vitro; kinase activity is dramatically reduced in immune complex assays of *gal83*Δ mutants, as is also the case for *snf4*Δ mutants (45). Nonetheless, the Sip1/Sip2/Gal83 family is not essential for many of the functions of the Snf1 kinase pathway in vivo, and a triple mutant lacking all three genes does not exhibit defects in carbon source utilization associated with a *Snf*⁻ phenotype (10, 45).

If the Sip1/Sip2/Gal83 family contributes to the association of Snf4 with the Snf1 kinase in a complex, how can we account for the nearly normal phenotype of the *sip1*Δ *sip2*Δ *gal83*Δ triple mutant? One simple explanation arises from the finding that in extracts from the triple mutant, half of the Snf4 protein still binds with Snf1 to nickel affinity beads. These results could indicate that Sip1, Sip2, and Gal83 are only partially responsible for maintaining the association of Snf4 with Snf1, with other unidentified proteins in the complex also contributing.

Alternatively, the kinase complexes in the cell may be heterogeneous, and only half of the complexes may require Sip1, Sip2, and Gal83 for their integrity. However, in either case, the remaining intact Snf1 kinase complexes could suffice to confer a normal phenotype. Another possibility is that the constitutive association of Snf4 with Snf1 optimizes the regulatory response to glucose starvation but is not required for adequate function of the kinase pathway, at least with respect to the phenotypes tested. Even if Snf4 and Snf1 are not complexed, their association in response to glucose deprivation may not be the limiting factor in vivo. These two possibilities are not mutually exclusive.

The composition of the Snf1-Snf4 complexes detected in the absence of Sip1, Sip2, and Gal83 is not clear. Other proteins may be functionally redundant with Sip1, Sip2, and Gal83. The Sip1/Sip2/Gal83 family does not appear to include other members; no other close sequence homologs are found in the yeast genome, and disruption of the more distant homolog YHR146 in a *sip1 sip2 gal83* triple mutant did not produce a Snf⁻ phenotype (26). However, nonhomologous proteins may serve a redundant function. A purified Snf1-Snf4 complex did not contain any other proteins in stoichiometric amounts (28, 38); however, this complex may have been purified from glucose-deprived cells. Under conditions of glucose limitation, Snf1 and Snf4 can interact directly, without assistance from other proteins (23).

Studies of Snf1-related kinase complexes in other organisms lend further support to the idea that the Sip1/Sip2/Gal83 family members are important components of the kinase complex. First, genetic evidence indicates a functional requirement in the yeast *K. lactis*. Both Fog1, a Sip1/Sip2/Gal83 homolog (Fig. 3), and Fog2, a Snf1 homolog, are required for expression of glucose-repressed genes, and *fog1* and *fog2* mutants have similar phenotypes (15). Second, the Sip2-interacting region in Snf1 is conserved in the Snf1 kinase family (Fig. 4B), and we show here that the tobacco homolog NPK5 interacts with Sip2. Finally, purified mammalian AMPK contains a catalytic subunit homologous to Snf1 (AMPK α) and homologs of Snf4 (AMPK γ) and Sip2 (AMPK β) (38, 40). AMPK β contains regions corresponding to the KIS and ASC domains (Fig. 3). Experiments with in vitro-translated proteins indicated that AMPK β interacts separately with AMPK α and AMPK γ and also mediates formation of a ternary complex (40).

Thus, Sip1, Sip2, and Gal83 represent a conserved family of proteins that may play dual roles in the function of the Snf1 kinase complex. Here, we present evidence that these proteins play a structural role in the complex, anchoring the Snf4 activating subunit together with the Snf1 kinase. This function employs two distinct domains that are conserved in each of the Sip1, Sip2, and Gal83 proteins. Previous genetic evidence suggested that these proteins have differing functions in the Snf1 pathway (10, 44, 45) and may serve as adaptors that mediate the interaction of the kinase with different substrate proteins or as targeting proteins that direct the kinase to different intracellular locations (45). Further study will be required to determine whether the divergent N-terminal sequences of Sip1, Sip2, and Gal83 play such a role.

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