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Purification and Characterization of Snf1 Kinase Complexes Containing a Defined β Subunit Composition*

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The Snf1 kinase complex of *Saccharomyces cerevisiae* contains one of three possible β subunits encoded by either *SIP1*, *SIP2*, or *GAL83*. Snf1 kinase complexes were purified from cells expressing only one of the three β subunits using a tandem affinity purification tag on the C terminus of the Snf1 protein. The purified kinase complexes were enzymatically active as judged by their ability to phosphorylate a recombinant protein containing the Snf1-responsive domain of the Mig1 protein. The Snf1 kinase complexes containing Gal83 or Sip2 as the β subunit showed comparable and high levels of activity, whereas the Sip1-containing enzyme was significantly less active. Examination of the protein composition of the purified Snf1 enzyme complexes indicated that the Sip1 protein was present in substoichiometric levels. Increased gene dosage of *SIP1* rescued the ethanol growth defect observed in cells expressing Sip1 as their only β subunit and increased the *in vitro* activity of Snf1 kinase purified from these cells. Our studies indicate that the reduced activity of Snf1-Snf4-Sip1 kinase is due to low level of Sip1 accumulation rather than a limited ability of the Sip1 form of the enzyme to direct phosphorylation of specific substrates.

The Snf1 protein kinase of *Saccharomyces cerevisiae* is a member of a serine/threonine kinase subfamily that is highly conserved in all eukaryotes with orthologues identified in plants, insects, nematodes, and mammals (1). Members of Snf1 kinase subfamily function as metabolic sensors and are activated under conditions of nutrient stress. The mammalian orthologue of the Snf1 kinase, AMP-activated protein kinase (AMPK),[1] is activated by nutrient stress and by allosteric binding of AMP (2). In yeast, Snf1 kinase activity correlates with increased concentrations of AMP, but the enzyme does not exhibit allosteric regulation by AMP *in vitro* (3). Both Snf1 and mammalian AMPK phosphorylate and down-regulate enzymes that consume ATP such as acetyl-CoA carboxylase (4). In addition, Snf1 and AMPK phosphorylate transcription factors and thereby promote changes in gene expression patterns (5, 6). In yeast, the Snf1 kinase is required for numerous processes relating to stress responses including growth on alternative carbon sources, sporulation, glycogen synthesis, thermotolerance, and entry into stationary phase (5, 7, 8).

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[1] The abbreviations used are: AMPK, AMP-activated protein kinase; HA, hemagglutinin; 3HA, triple hemagglutinin epitope; TAP, tandem affinity purification.

Members of the Snf1 kinase family function as heterotrimeric enzymes. The biochemical roles of the α, β, and γ subunits have been characterized in some detail. The α subunits are generally 550–650 amino acids in primary sequence with the 300-amino acid serine/threonine kinase catalytic domain at the N terminus. The remaining 250–300 amino acids comprise a regulatory domain with two distinct parts. The first half of the regulatory domain contains an autoinhibitory sequence that down-regulates the kinase activity under nutrient-rich conditions (9). Under conditions of nutrient stress, the effect of the autoinhibitory domain is relieved through a regulated interaction with the γ subunit (10). The C-terminal half of the regulatory domain of the α subunit contains sequences required for association with the β subunit (11). The γ subunit, as noted above, plays an important regulatory role. It associates with the kinase complex through a constitutive interaction with the β subunit. In addition, the γ subunit associates with and activates the catalytic α subunit (10). The mechanism by which the association of the γ subunit with the α subunit is regulated is not currently understood. The β subunit plays an important role in the assembly of the heterotrimer. The C terminus of the β subunits contains a conserved 80-residue ASC domain that associates with the γ subunit and an internal KIS domain that associates with the α subunit (11). The role of the N-terminal domain of the β subunit is not certain but it has been implicated in both substrate selection and subcellular localization (12, 13).

The presence of multiple genes for each of the three subunits adds an additional layer of complexity. In mammals, two genes are found for the α subunit, three genes for the γ subunit, and two for the β subunit creating the potential to form 12 distinct AMPK heterotrimers. In yeast, the situation is simplified by the fact that a single α subunit gene, *SNF1*, and a single γ subunit gene, *SNF4,* are present. However, yeast do encode three β subunit genes, *SIP1*, *SIP2*, and *GAL83*, and immunoprecipitation data suggest that three distinct forms of the Snf1 kinase exist *in vivo* (14). The distinct roles played the three forms of the Snf1 kinase is not yet fully understood. We have reported previously that deletion of all three β subunit genes inactivates the Snf1 kinase complex (12). Furthermore, cells expressing only a single β subunit gene displayed distinct growth phenotypes and differing abilities to direct the phosphorylation of the transcription factor Sip4. Of particular interest was the finding that cells expressing Sip1 protein as their only β subunit were unable to grow aerobically on medium containing a mixture of glycerol and ethanol as the carbon sources. We hypothesized that the β subunits play a direct role in substrate selection and that the Snf1-Snf4-Sip1 enzyme complex was defective in phosphorylation of substrates required for aerobic growth. To test this hypothesis, we have purified and assayed the Snf1 kinase enzyme from cells that express all three or only a single β subunit.

TABLE I
S. cerevisiae strains

Strain	Genotype
FY1193	*MATα ura3–52 leu2-Δ1 his3-Δ200 trp1-Δ63 snf1-Δ10*
MSY182	*MATa ura3–52 leu2-Δ1 his3-Δ200 trp1-Δ63*
MSY541	*MATα ura3–52 leu2-Δ1 his3-Δ200 trp1-Δ63 sip2::HIS3 gal83::HIS3 snf1Δ10*
MSY543	*MATa ura3–52 leu2-Δ1 his3-Δ200 trp1-Δ63 sip2::HIS3 gal83::HIS3*
MSY548	*MATa ura3–52 leu2-Δ1 his3-Δ200 sip1::HIS3 gal83::HIS3 snf1Δ10*
MSY555	*MATα ura3–52 his3-Δ200 trp1-Δ63 sip1::HIS3 sip2::HIS3 snf1Δ10*
MSY818	*MATa ura3–52 leu2-Δ1 his3-Δ200 trp1-Δ63 sip2::HIS3 gal83::HIS3 SIP1–3HA::TRP1*

EXPERIMENTAL PROCEDURES

Yeast Strains, Methods, and Genetic Techniques—*S. cerevisiae* strains utilized in this study are described in Table I. Growth of yeast utilized standard media at 30 °C (15). The carbon source glucose and sucrose were used at 2% (w/v), and the glycerol-ethanol mixture was present at 3 and 2% (v/v), respectively. Transformation of yeast strains utilized the lithium acetate procedure (16).

TAP Tagging of SNF1—A 1.7-kb fragment containing the TAP tag and *Kluyveromyces lactis TRP1* gene was amplified from the plasmid pBS1479 (17) using primers 5′-CCCGGACTATGCAGGATCCTATCCATATGACGTTCCAGATTACGCTGCTTCCATGGAAAAGAGAAG-3′ and 5′-ATACATAAAAAAAAGGGAACTTCCATATCATTCTTTTAGGTTCCACCATTACGACTCACTCACTATAGGG-3′. Integration of the TAP tag was directed to a 3HA-tagged *SNF1* by transforming FY1193, a yeast strain lacking its genomic copy of *SNF1* but carrying SNF1-3HA on a plasmid (18). Plasmids from Trp+ transformants were amplified in *Escherichia coli* and confirmed by DNA sequencing.

Epitope Tagging of SIP1—The Sip1 protein was tagged with three copies of the HA epitope using PCR, the tagging plasmid pYM3 (19), and the primers Sip1-HA-Top-TAGTTTATGCACCTTGTTATTATAAAACACAAAAGTCTCAGATCAGTAATCGTACGCTGCAGGTCGAC and Sip1-HA-BotGAAAAAAAATTGAATTAATAGAGTTCGTGAGAATCATTGCGAATTGAGATTATCGATGAATTCGA GCTCG. Homologous recombination of the HA-tagging fragment into the *SIP1* chromosomal locus was confirmed by PCR using internal primers. Recombination into the 2μ plasmid pSIP1–151 was confirmed by restriction analysis following amplification of the plasmid in *E. coli*.

Snf1 Kinase Purification—TAP-tagged Snf1 kinase was purified by the method described by Rigaut *et al.* (20) with slight modifications as outlined in the legend for Fig. 1*B*. Yeast cells were lysed by extensive grinding in a mortar and pestle in the presence of liquid nitrogen in IPP150 buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40). All subsequent steps were performed at 4 °C. Protease inhibitors were included in buffers at concentrations of 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. Cell debris was removed by centrifugation at 10,000 rpm for 2 min, and the supernatant fraction was added to 200 μl of rabbit IgG-agarose (Sigma) and rotated for 2 h. Beads were collected at 1000 rpm for 10 min and washed with 20 ml of IPP150 buffer. The beads and bound proteins were washed with 5 ml of TEV protease buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, 1 mM dithiothreitol) and then incubated for 1 h with constant rotation in 1 ml of TEV protease buffer containing 40 units TEV protease (Invitrogen). Beads were removed by centrifugation, and the supernatant fraction was mixed with 3 volumes of IPP150 calmodulin-binding buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% Nonidet P-40, 10 mM β-mercaptoethanol). 1 M $CaCl_2$ was added to adjust the solution to a final concentration of 3 mM. The mixture was incubated with 200 μl of calmodulin beads (Stratagene) and rotated for 2 h. Beads were collected by centrifugation, transferred to a column, and washed with 30 ml of IPP150 calmodulin-binding buffer. Bound proteins were eluted from the column by 1 ml of IPP150 calmodulin elution buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM EGTA, 0.1% Nonidet P-40, 10 mM β-mercaptoethanol). Eluted proteins were loaded onto a 1-ml MonoQ column equilibrated in 10 mM Tris-Cl, pH 8.0, 0.1% Nonidet P-40, 10 mM β-mercaptoethanol, and 5% glycerol. Snf1 kinase was eluted with a 20-ml linear gradient of NaCl (150 mM to 1 M). Fractions (0.5 ml) were collected across the gradient and assayed for *in vitro* kinase activity. Active fractions were stored in small aliquots at −80 °C. To analyze the protein composition of the purified kinase fractions, protein samples were precipitated with 10% trichloroacetic acid, washed with chilled acetone, resolved in a 10% SDS-PAGE, and stained with silver nitrate using the Silver Staining Plus kit (Bio-Rad).

Kinase Assays—Snf1 kinase enzyme was incubated with 0.2 mM [γ-^{32}P]ATP (1000 cpm/pmol), ~10 μg/ml GST-Mig1 protein in 20 μl kinase buffer (20 mM Hepes, pH 7.0, 0.5 mM EDTA, 0.5 mM dithiothreitol and 5 mM Mg-acetate) at 30 °C for 20 min. Proteins were precipitated on ice with 200 μl 10% trichloroacetic acid, washed in ice cold acetone and resolved by SDS-PAGE.

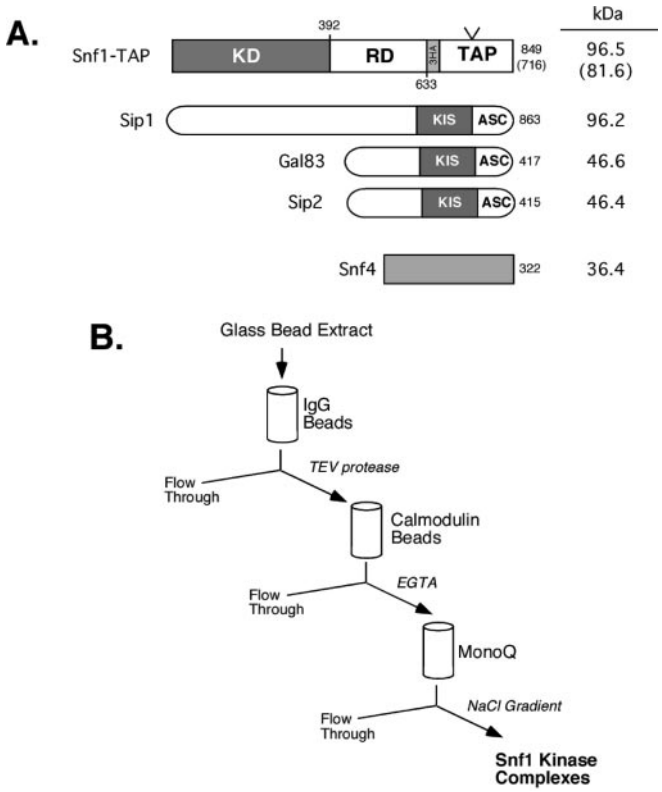

FIG. 1. **Snf1 tagging and purification strategy.** *A*, schematic diagram of the different subunits comprising the Snf1 kinase complex. The triple hemagglutinin epitope (3HA) and tandem affinity purification (*TAP*) tags were added to the 3′ end of the *SNF1* gene. The kinase and regulatory domains of the Snf1 protein are indicated by K_D and *RD*, respectively. The approximate site of TEV protease cleavage is indicated. The calculated molecular mass of the full-length unmodified proteins is indicated for each subunit. The molecular mass of the Snf1 protein after TEV protease cleavage is shown in *parentheses*. *B*, graphical representation of the purification strategy for the Snf1 kinase complexes. The TAP purification procedure was followed by fractionation on a 1-ml MonoQ column.

Western Blotting—HA-tagged Snf1 was detected by Western blotting (18) using mouse monoclonal antibody directed against the HA epitope (Santa Cruz Biotechnology).

RESULTS

Purification of Snf1 Kinase—The Snf1 kinase is a protein complex composed of three subunits. To purify the kinase in its active form, a tandem affinity purification tag (17) was added to the C terminus of a previously epitope-tagged version of the Snf1 subunit (18). The end result of these manipulations was a recombinant protein containing all 633 amino acids of Snf1 with an additional 216 amino acids at the C terminus (Fig. 1*A*). Despite the presence of the additional amino acids at the C

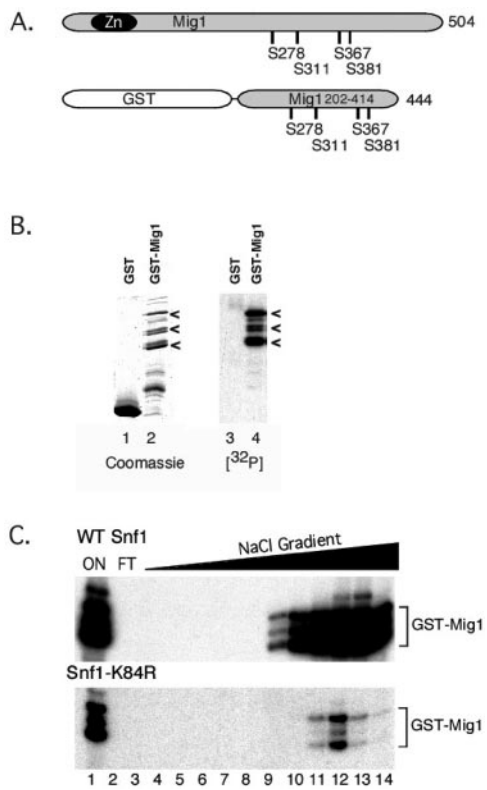

FIG. 2. **Snf1 kinase substrate specificity.** *A*, schematic representation of the Mig1 and GST-Mig1 proteins showing four serine residues that are target sites of the Snf1 kinase (23). *B*, fractions containing purified GST and GST-Mig1 proteins were resolved on an SDS-acrylamide gel and stained with Coomassie Blue (*lanes 1* and *2*, respectively). Purified GST (*lane 3*) and GST-Mig1 (*lane 4*) were incubated with purified Snf1 kinase and [γ-^{32}P]ATP. Labeled proteins were resolved on an SDS-acrylamide gel and subjected to autoradiography. The mobilities of full-length and proteolytic fragments of GST-Mig1 are indicated on the *right* (<). *C*, Snf1 kinase assay of MonoQ fractions. On-put (*ON*, *lane 1*), flow-through (*FT*, *lane 2*), and fractions from the NaCl gradient (*lanes 3–14*) were assayed for the ability to phosphorylate GST-Mig1 protein. Snf1 enzyme was purified from cells expressing wild type Snf1 (*WT Snf1*, *upper panel*) or a mutant enzyme containing the lysine to arginine substitution at position 84 (*Snf1-K84R, lower panel*).

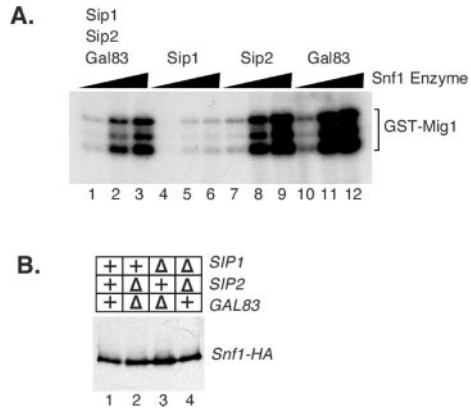

FIG. 3. ***In vitro* kinase activity of Snf1 kinase complexes with a defined β subunit composition.** *A*, Snf1 kinase complexes purified from cells expressing all three β subunits (*lanes 1–3*), only Sip1 (*lanes 4–6*), only Sip2 (*lanes 7–9*), or only Gal83 (*lanes 10–12*) were incubated with GST-Mig1 protein and [γ-^{32}P]ATP. A titration of increasing enzyme concentration for each kinase complex was performed. The mobilities of full-length and proteolytic fragments of GST-Mig1 are indicated on the *right*. *B*, Western blot analysis of Snf1 protein. The relative level of Snf1 protein in the four enzyme preparations was determined with antibodies directed against the 3HA tag. The presence (+) or absence (Δ) of each β subunit is indicated *above* the lanes. The yeast strains used in this experiment were FY1193, MSY541, MSY548, and MSY555.

terminus, the recombinant tagged *SNF1* gene was fully functional *in vivo* as judged by its ability to complement a *snf1Δ10* mutant for growth on raffinose and ethanol media (data not shown). The tagged Snf1 protein has a predicted molecular mass of 96.5 kDa, and its presence in extracts and column fractions was readily detected by Western blotting using antibodies directed against the three copies of the hemagglutinin tag. During the course of the TAP protocol, the C-terminal 133 amino acids are removed by TEV protease cleavage, reducing the predicted molecular mass of this recombinant protein to 81.6 kDa (Fig. 1*B*). Following TAP purification on IgG beads and calmodulin beads, the fractions containing Snf1 kinase were pooled and applied to a MonoQ column equilibrated in 150 mM NaCl. Bound proteins were eluted in a linear gradient of NaCl. The Snf1 kinase complex, detected by Western blotting (data not shown), was found in fractions containing ~350–400 mM NaCl.

Specificity of in Vitro Kinase Activity—The Mig1 protein, a zinc finger transcription factor, is a Snf1 substrate in which activity and nuclear localization are controlled by the phosphorylation of serine residues in the central regulatory domain (21–23). The *in vitro* kinase activity of the purified Snf1 kinase was measured in an assay containing [γ-^{32}P]ATP and purified recombinant GST-Mig1 protein containing the four target serine residues (Fig. 2*A*). GST and GST-Mig1 were purified from *E. coli* by glutathione affinity chromatography (24). Full-length GST-Mig1 as well as a series of proteolytic products were recovered (Fig. 2*B*, *lane 2*) and used as substrates for *in vitro* kinase assays. Purified Snf1 kinase efficiently phosphorylated GST-Mig1. The recognition and phosphorylation of GST-Mig1 was highly specific because the more abundant GST protein was not phosphorylated (compare *lanes 3* and *4*) even though it contains 16 serine and threonine residues. The phosphorylation activity in the Snf1 fraction was due to the Snf1 protein itself and not a contaminating kinase activity because the activity was greatly reduced (Fig. 2*C*) by a single amino acid substitution in the ATP binding domain of the catalytic subunit (25). Equivalent levels of the Snf1 subunit were present in both enzyme preparations as judged by Western blotting (data not shown).

Effect of β Subunit Identity on Kinase Activity—Yeast express three distinct forms of the Snf1 kinase that differ by the identity of the β subunit. To determine whether the β subunits contribute to substrate specificity, we purified Snf1 kinase from cells that expressed all three β subunits or only a single β subunit. In the former case, we expected the purified Snf1 kinase complexes to contain a mixture of all three forms, whereas in the latter cases, we expected to isolate a homogenous population of Snf1 kinase complexes containing a single β subunit. *In vitro* kinase assays with these four preparations of Snf1 kinase were performed using GST-Mig1 as the substrate. Snf1 kinase purified from cells expressing all three β subunits or from cells expressing only Sip2 or only Gal83 showed high levels of kinase activity (Fig 3*A*). In contrast, Snf1 kinase purified from cells expressing Sip1 as the only β subunit was significantly less active. A Western blot directed against the catalytic Snf1 subunit demonstrated that equivalent levels of Snf1 protein were present in the four preparations of Snf1 kinase (Fig 3*B*). Thus the abundance of the catalytic subunit could not account for the difference in the observed kinase activity.

Protein Composition of Purified Kinase Complexes—The subunit composition of the four preparations of Snf1 kinase was examined on an SDS protein gel stained with silver nitrate (Fig. 4*A*). Kinase purified from cells expressing all three β

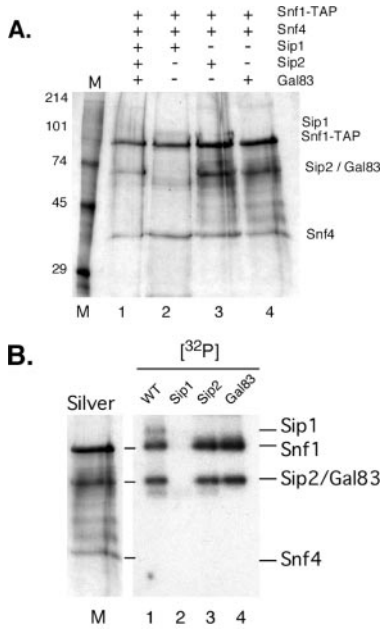

FIG. 4. **Subunit composition of Snf1 kinase complexes.** *A*, Snf1 kinase complexes purified from cells expressing all three (*lane 1*) or a single β subunit (*lanes 2–4*) were resolved on an SDS-acrylamide gel and stained with silver nitrate. Molecular mass markers (*M*) were loaded in *lane 1*. The mobilities of the Snf1 kinase subunits are indicated on the *right*. *B*, autophosphorylation reaction by purified Snf1 kinase complexes. Enzyme complexes containing all three or a single β subunit as indicated above each lane were incubated with [γ-^{32}P]ATP. Labeled proteins were resolved on an SDS-acrylamide gel and detected by autoradiography. The image of silver-stained proteins from the Gal83 form of the Snf1 kinase complex is shown on the *left* as a marker for gel mobility of the Snf1 subunits. The yeast strains used in this experiment were FY1193, MSY541, MSY548, and MSY555.

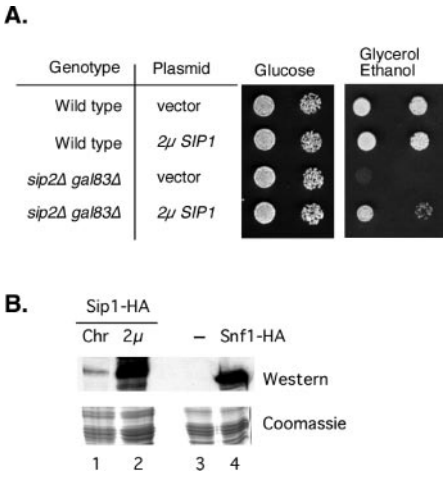

FIG. 5. **Increased gene dosage of *SIP1* rescues the growth defect in a *sip2Δ gal83Δ* strain.** *A*, serial dilutions of wild type cells and *sip2*Δ *gal83*Δ cells containing either a 2μ vector or 2μ *SIP1* were spotted onto agar plates containing either glucose or a mixture of glycerol and ethanol as the carbon source. The yeast strains used were MSY182 (wild type) and MSY543 (*sip2*Δ*gal83*Δ). *B*, increased gene dosage of *SIP1* causes increased accumulation of Sip1 protein. Protein extracts were prepared from *sip2*Δ *gal83*Δ cells containing an HA-tagged allele of *SIP1* integrated in the chromosome (*lane 1*) or into the 2μ *SIP1* plasmid (*lane 2*). HA-tagged proteins were detected by Western blotting (*upper panel*). Extracts with no HA tag (*lane 3*) or Snf1-HA (*lane 4*) are shown as controls. Equivalent aliquots of protein were loaded in each lane as judged by a Coomassie-stained gel of the same extracts run in parallel (*lower panel*). The yeast strains used in this experiment were MSY818 and MSY543.

subunits contained three major bands (*lane 1*). The mobilities of these bands when compared with molecular mass markers were consistent with the largest polypeptide corresponding to Snf1, the next largest being a mixture of Sip2 and Gal83 and the smallest of the three major bands being Snf4. A protein band corresponding to the predicted molecular weight of Sip1 was not apparent. The assignment of particular subunits to silver-stained bands was confirmed by examining protein composition of kinases purified from cells lacking the genes for specific subunits. The band migrating close to 65 kDa is assigned to the β subunits Sip2 and Gal83 because a band of the same mobility is present from cells expressing only Sip2 (*lane 3*) or only Gal83 (*lane 4*) but missing from cells expressing only Sip1 (*lane 2*). The band migrating close to 36 kDa is assigned to Snf4 because it is missing when the kinase is purified from a *snf4*Δ*2* mutant (data not shown). The band migrating at 80 kDa was identified as Snf1 by Western blotting. Identification of a band corresponding to the Sip1 protein was not possible. The faint band migrating at ~90 kDa in the kinase purified from Sip1-expressing cells (*lane 2*) could not be assigned unambiguously to Sip1 because a similar band is seen in the kinase preparation from cells that lack the *SIP1* gene (*lane 3*). We conclude that the subunits of the Snf1-Snf4-Sip2 and Snf1-Snf4-Gal83 kinases are present at stoichiometric levels. In contrast, the Sip1 protein is either poorly stained by silver nitrate, or more likely, it is present in substoichiometric ratios compared with the Snf1 and Snf4 subunits.

Autophosphorylation Activity—Immune complexes of the Snf1 kinase show significant autophosphorylation activity (26, 27). The ability of our purified Snf1 kinase complexes to catalyze autophosphorylation was examined (Fig. 4*B*). Purified Snf1 kinase complexes were incubated with [γ-^{32}P]ATP and resolved by SDS-PAGE. Incorporation of ^{32}P into the Snf1, Sip2, and Gal83 subunits was readily detected. The Snf4 subunit failed to incorporate any ^{32}P label under all conditions tested. Incorporation was also detected in a protein with an apparent molecular mass of 90 kDa (*lane 1*), consistent with the predicted molecular weight of Sip1. However, the labeling of this band was not consistently detected in the kinase purified from cells expressing Sip1 as the only β subunit. We conclude that Snf1 kinase or possibly some other protein kinase that co-purifies with Snf1 phosphorylates the Snf1, Sip2, and Gal83 proteins *in vitro*.

Increased Gene Dosage of SIP1 Restores Aerobic Growth to Cells Lacking Gal83 and Sip2—Cells expressing Sip1 as their only β subunit are unable to grow on nonfermentable carbon sources such as glycerol and ethanol (12). The finding that the Snf1 kinase purified from these cells contains less than stoichiometric levels of Sip1 suggests that the Snf1 defects observed in these cells may be due to the low level of Sip1 accumulation. To test this hypothesis, cells expressing Sip1 as their only β subunit were transformed with high copy number plasmids that contained either no insert or a fragment of genomic DNA spanning the entire *SIP1* gene. The ability to grow aerobically on media containing a mixture of glycerol and ethanol as the carbon source was assayed (Fig. 5*A*). Wild type cells grew aerobically when transformed with vector as well as the *SIP1* clone. Cells expressing Sip1 as the only β (*sip2*Δ *gal83*Δ) are unable to grow on glycerol-ethanol medium when transformed by plasmid vector. However, significant growth is restored when an increased gene dosage of *SIP1* is provided. The observed growth is not as robust as that observed in wild type cells, suggesting that increased gene dosage of *SIP1* does not completely restore Snf1 function to *sip2*Δ *gal83*Δ cells. Increased gene dosage of *SIP1* correlates with an increased accumulation of Sip1 protein (Fig. 5*B*), suggesting that low accumulation of the Sip1 protein limits Snf1 kinase activity in *sip2*Δ *gal83*Δ cells.

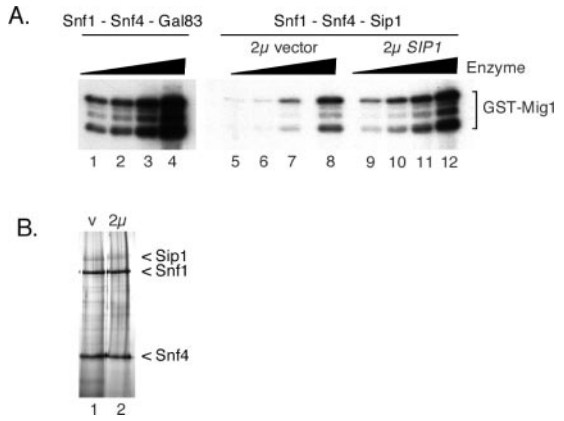

FIG. 6. **Increased gene dosage of *SIP1* increases *in vitro* activity of the purified Snf1 kinase.** Increasing concentrations of Snf1 kinase complexes purified from cells expressing Gal83 (*lanes 1–4*) or Sip1 (*lanes 5–12*) as the only β subunits were incubated with GST-Mig1 and [γ-^{32}P]ATP. Cells were transformed with a high copy number plasmid with either no insert (*lanes 5–8*) or with the full-length *SIP1* gene (*lanes 9–12*). The extent of GST-Mig1 phosphorylation was determined by autoradiography. *B*, silver-stained SDS-acrylamide gel of Snf1 kinase complexes purified from cells expressing Sip1 as the only β subunit and transformed with a high copy number plasmid with either no insert (*lane 1*) or with the full-length *SIP1* gene (*lane 2*). The mobilities of Sip1, Snf1, and Snf4 are indicated. The yeast strains used in this experiment were MSY555 and MSY541.

Increased Gene Dosage of SIP1 Increases in Vitro Kinase Activity of the Snf1 Kinase Complexes Containing Sip1 as the β Subunit—Snf1 kinase purified from cells expressing Sip1 as the only β subunit is significantly less active than Snf1 kinase purified from cells expressing only Sip2 or only Gal83. To test whether the low level of the Sip1 protein could account for the reduced activity, Snf1 kinase was purified from cells that contained extra copies of the *SIP1* gene on a high copy number plasmid. Snf1 kinase activity was assayed for the ability to phosphorylate GST-Mig1 protein using increasing concentrations of purified kinase (Fig. 6*A*). Increased gene dosage of *SIP1* resulted in significantly higher activity of the purified Snf1 kinase containing Sip1 as the only β subunit (compare *lanes 5–8* with *lanes 9–12*). However, increased gene dosage of *SIP1* does not fully restore activity to the Snf1 kinase because greater activity is observed when the kinase is purified from cells expressing only Gal83 (*lanes 1–4*). The protein composition of the Snf1 kinase purified from cells transformed with either empty vector or high copy number *SIP1* was examined on an SDS protein gel stained with silver nitrate. Protein bands corresponding to Snf1 and Snf4 are readily apparent. Detection of the Sip1 protein by silver staining is complicated by the presence of a contaminating protein of a similar mobility. However, it is clear that even increased dosage of the *SIP1* gene does not result in stoichiometric saturation of the Snf1 kinase complex with the Sip1 protein.

DISCUSSION

The chromatographic purification of the Snf1 kinase was first reported in 1994 by two independent research groups. Carling, Carlson, and co-workers (4) used DEAE chromatography and immunoprecipitation to isolate and assay a Snf1 kinase fraction. Mitchelhill *et al.* (29) took advantage of a naturally occurring run of 13 histidine residues near the N terminus of the Snf1 protein to purify the Snf1 enzyme complex using nickel-nitrilotriacetic acid agarose chromatography. The Snf1 kinase fraction produced by this method was highly purified and was able to phosphorylate a synthetic peptide substrate based on the sequence of rat acetyl-CoA carboxylase (28, 29). In the 8 years subsequent to these two papers, only a few studies (3, 23, 30) have reported any results using purified Snf1 kinase. The reason behind the paucity of studies on purified Snf1 kinase is due at least in part to the instability of the purified enzyme. In our experiments, Snf1 kinase preparations prepared by the nickel-agarose chromatography method described by Mitchelhill (29) contained highly active Snf1 kinase initially, but the activity was almost completely lost within 2 weeks of storage at −80 °C.[2] The lability of the Snf1 kinase activity presented a significant obstacle to its *in vitro* characterization. We report here a modified purification procedure based on the tandem affinity purification method developed by Seraphin's group (20). Snf1 kinase isolated by the TAP procedure is highly purified and active, but its activity is also unstable.[2] However, further purification of the Snf1 kinase complex by MonoQ chromatography produces an enzyme fraction with dramatically increased stability, showing only a modest decrease in activity following prolonged storage at −80 °C. These findings suggest that some contaminant that inactivates the enzyme co-purifies with Snf1 initially but is removed by MonoQ chromatography. The ability to isolate an active and stable fraction of Snf1 kinase will greatly facilitate its characterization.

The Snf1 enzyme is a heterotrimeric enzyme complex that exists *in vivo* in three distinct forms depending on the identity of the β subunit (14). Earlier studies have shown that the β subunits confer specialized functions to the Snf1 kinase and possible mechanisms for this include substrate selection (12), control of subcellular localization (13) or a combination of both. In previous studies (3, 4, 23, 29, 30), fractions containing purified Snf1 kinase were likely to contain a mixture of the three forms of the kinase in an unknown proportion. One goal of this study was to characterize the enzymatic activity of Snf1 kinase complexes with a defined β subunit composition. To accomplish this goal, the Snf1 kinase complex was purified from cells that express all three or only a single β subunit (12). Of particular interest to us was the activity of the Snf1 kinase complex, which contains Sip1 protein as the β subunit. Cells expressing Sip1 as the only β exhibit a partial Snf$^-$ phenotype (12) because they are able to grow on raffinose- but not on ethanol-containing media. We hypothesized that these cells were unable to grow on ethanol because the Snf1-Snf4-Sip1 enzyme complex was not able to recognize particular substrates that were critical for growth on ethanol. In this study, characterization of Snf1 enzyme complexes suggests that our initial hypothesis was not correct. Cells expressing Sip1 as the only β subunit exhibit a partial Snf$^-$ phenotype because the Sip1 protein is not present in the Snf1 complex at stoichiometric levels. The reasoning in support of this conclusion is presented below.

Snf1 enzymes were purified from cells expressing only one of the three possible β subunits. When assayed with a recombinant Mig1 protein as the phosphate acceptor, the Gal83 and Sip2 forms of the enzyme showed much higher levels of activity than the Sip1 form. In each assay, the same amount of catalytic subunit was used as judged by Western blot (Fig. 3*B*). The Gal83 form of the Snf1 kinase is slightly more active than the Sip2 form of the enzyme when assayed with GST-Mig1 protein as substrate (Fig. 3*A*). Whether the greater activity of the Gal83 form is due to a greater affinity for this particular substrate or to higher intrinsic activity is not known. An explanation for the reduced level of activity observed in the Snf1-Snf4-Sip1 enzyme was discovered when the subunit composition of the enzyme complexes was examined (Fig 4*A*). The predominant bands in our enzyme preparations were identified as the subunits of the Snf1 enzyme complex. The identity of the

[2] N. Nath, R. R. McCartney, and M. C. Schmidt, unpublished data.

Snf1 band was confirmed through its reactivity with antibodies directed against the HA epitope that was introduced at its C terminus. The Snf4 band was identified because it was specifically missing from enzymes purified from *snf4*Δ cells (data not shown). The Gal83 and Sip2 proteins co-migrate in this gel system as judged by the fact that the same band is visible in enzyme purified from cells expressing only Gal83 or only Sip2 and because this band is missing from the enzyme purified from cells expressing only Sip1 (Fig 4*A*). The calculated molecular mass for both Sip2 and Gal83 is ~46 kDa, and yet they migrate in an SDS gel with an apparent molecular mass of ~65 kDa (Fig. 4*A*). Both Sip2 and Gal83 have acidic isoelectric points, which may contribute to their reduced migration in SDS gels. The surprising result was that the Sip1 band, predicted to migrate more slowly than the Snf1 band, was not readily detectable. A faint band migrating more slowly than Snf1 is visible in the Sip1 enzyme (Fig 4*A*, *lane 2*); however, a similar species is also seen in the Gal83-containing enzyme (*lane 3*). Thus, we were not able to unambiguously identify the Sip1 protein in the purified Snf1 enzyme. The simplest conclusion is that the Sip1 protein is present in substoichiometric quantities, if it is present at all. The possibility that the Sip1 protein stains inefficiently with silver nitrate and Coomassie Blue (not shown) is unlikely because the activity of the Sip1 enzyme is also very low.

Earlier studies have reported that the Sip1 protein is a substrate of the Snf1 kinase (14, 31). We tested for whether the Sip1 protein could be detected by radioactive labeling (Fig. 4*B*). Snf1 enzyme preparations were incubated with [γ-^{32}P]ATP. Under these conditions, we readily detected labeling of the Snf1, Gal83, and Sip2 subunits. The Snf4 protein was not phosphorylated *in vitro* under any conditions tested. A band migrating at a position expected for Sip1 was faintly labeled in the enzyme prepared from wild type cells (Fig 4*B*, *lane 1*). This same band was never detected in enzyme preparations from *sip1*Δ cells. We conclude that the Sip1 protein is present in our purified fractions but that its abundance is much lower than that observed with the other β subunits and that the Snf1, Gal83, and Sip2 proteins are readily labeled *in vitro*. The kinase responsible for labeling the β subunits is most likely the Snf1 kinase itself and not some other co-purifying protein kinase. We note that other unidentified protein bands are visible in our most purified fractions. Ho *et al.* (32) identified proteins in the Snf1 complex by mass spectrometry including two other protein kinases. However, the K84R point mutation in the Snf1 catalytic domain greatly reduces the phosphorylation of Sip2 and Gal83 (data not shown), strongly suggesting that the Snf1 kinase is directly responsible for this activity.

The characterization of the Snf1-Snf4-Sip1 enzyme *in vitro* suggested that the low activity level of this enzyme was due to substoichiometric quantities of the Sip1 subunit. This result could be due to substoichiometric levels of the Sip1 protein *in vivo*, to loss of the Sip1 protein during purification, or to both. The fact that *sip2*Δ *gal83*Δ cells exhibit a partial Snf phenotype that can be suppressed by increased gene dosage of *SIP1* strongly argues that the Sip1 protein *in vivo* is unable to saturate the Snf1 enzyme complex. Furthermore, increased gene dosage of *SIP1* restores activity to the purified enzyme. This result also suggests that Sip1 protein is not preferentially lost during purification but is present in low levels *in vivo*. It is interesting to note that the Snf1 enzyme purified from *sip2*Δ *gal83*Δ cells does appear to contain stoichiometric levels of the Snf4 protein (Fig. 4*A*). Current models of the oligomeric structure of this enzyme maintain that the β subunit holds the α and γ subunits together in a complex and that the association of the γ and α subunit occurs only in the active conformation (5). Our preparation of Snf1-Snf4-Sip1 enzyme contains roughly stoichiometric levels of Snf1 and Snf4, reduced levels of Sip1 protein, and reduced levels of activity. If Snf4 associates with Snf1 only in its active conformation, why is the enzyme activity of the Snf1-Snf4 complex so low? Western blots that detect the phosphorylation state of the activation loop threonine indicate that all of our preparations have equivalent levels of threonine 210 phosphorylation (data not shown). Taken together, our data support the following ideas. First, Snf1 enzyme activity requires the presence of a β subunit for reasons other than the association of α and γ subunits. Second, the Snf1 enzyme is able to recognize and phosphorylate one substrate, the Mig1 protein, independent of the β subunit identity. Finally, the lower level of kinase activity in the Snf1-Snf4-Sip1 enzyme is due to limiting quantities of the Sip1 protein. The ability to purify active and stable Snf1 kinase complexes containing defined β subunit composition will allow a more detailed characterization of the substrate specificity of this important signaling molecule.

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