THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 36, pp. 26170–26180, September 8, 2006

Subunits of the Snf1 Kinase Heterotrimer Show Interdependence for Association and Activity*

Received for publication, April 20, 2006, and in revised form, June 29, 2006 Published, JBC Papers in Press, July 17, 2006, DOI 10.1074/jbc.M603811200

Karin Elbing, Eric M. Rubenstein, Rhonda R. McCartney, and Martin C. Schmidt[1]

From the Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

The Snf1 kinase and its mammalian orthologue, the AMP-activated protein kinase (AMPK), function as heterotrimers composed of a catalytic α-subunit and two non-catalytic subunits, β and γ. The β-subunit is thought to hold the complex together and control subcellular localization whereas the γ-subunit plays a regulatory role by binding to and blocking the function of an auto-inhibitory domain (AID) present in the α-subunit. In addition, catalytic activity requires phosphorylation by a distinct upstream kinase. In yeast, any one of three Snf1-activating kinases, Sak1, Tos3, or Elm1, can fulfill this role. We have previously shown that Sak1 is the only Snf1-activating kinase that forms a stable complex with Snf1. Here we show that the formation of the Sak1·Snf1 complex requires the β- and γ-subunits *in vivo*. However, formation of the Sak1·Snf1 complex is not necessary for glucose-regulated phosphorylation of the Snf1 activation loop. Snf1 kinase purified from cells lacking the β-subunits do not contain any γ-subunit, indicating that the Snf1 kinase does not form a stable αγ dimer *in vivo*. *In vitro* kinase assays using purified full-length and truncated Snf1 proteins demonstrate that the kinase domain, which lacks the AID, is significantly more active than the full-length Snf1 protein. Addition of purified β- and γ-subunits could stimulate the kinase activity of the full-length α-subunit but only when all three subunits were present, suggesting an interdependence of all three subunits for assembly of a functional complex.

The sucrose non-fermenting-1 (Snf1)[2] kinase of *Saccharomyces cerevisiae* is a founding member of the Snf1/AMPK family of serine/threonine protein kinases. Members of this family are present in all eukaryotic cells where they play a central role in signaling nutrient limitation (1). In yeast, Snf1 is active under conditions when glucose is scarce and is absolutely required for aerobic growth and for fermentation of alternative carbon sources. Members of this family of protein kinases exist in cells as heterotrimers with a single catalytic subunit (α) that associates with two regulatory subunits (β and γ). Co-transfection experiments in mammalian cells demonstrated that each subunit is required for full reconstitution of AMPK activity (2). In yeast, it was initially thought that the β-subunits were not required for Snf1 function (3). However it is now clear that complete and precise deletion of all three β-subunit genes inactivates Snf1 signaling (4).

The interactions of the individual subunits within the Snf1/AMPK heterotrimer have been studied by a variety of methods including two-hybrid analysis, co-immunoprecipitation and *in vitro* binding assays. The β-subunits are thought to play a central role in heterotrimer formation because they contain two conserved sequence motifs that specify interaction with the α- and γ-subunits (5–7). In yeast, two hybrid analysis has mapped both the α-subunit interaction motif labeled KIS (kinase-interacting sequence) and the γ-subunit interaction motif labeled ASC (associates with the Snf1 complex) to the C termini of the β-subunits. These protein interaction motifs show a high degree of sequence conservation between β-subunits from species as diverse as yeast and human. At present, there is no evidence to suggest that the interactions between the α-subunit and the KIS motif and between the γ-subunit and the ASC motif are regulated. Current models of Snf1/AMPK heterotrimers posit that these interactions are constitutive and that the β-subunit holds α and γ together.

Whereas β may hold the Snf1/AMPK heterotrimer together, it is the reversible interactions of the α- and γ-subunits that regulate the enzymatic activity of the kinase domain. The α-subunit contains a kinase domain at its N terminus, followed by a regulatory domain that contains an AID and at its C terminus, a domain for interacting with the β-subunit (6). Deletion of the AID increases kinase activity (8) and creates a kinase that is no longer dependent on the γ-subunit for activation (9). Current models of Snf1/AMPK regulation posit that the kinase domain and the γ-subunit compete for binding to the α-subunit AID. When the AID is bound to the kinase domain, activity is inhibited. When the AID is bound to the γ-subunit, its ability to inhibit the kinase domain is abrogated. In yeast, the binding of γ to the AID is regulated by the availability of glucose (10), although the mechanism of this regulation is unclear. One significant difference between the yeast Snf1 and mammalian AMPK is the allosteric regulation by AMP (adenosine monophosphate). Whereas mammalian AMPK was so named because of its stimulation by AMP (11), Snf1 is not stimulated

* This work was supported by Grant GM46443 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dept. of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261. Tel.: 412-648-9243; E-mail: mcs2@pitt.edu.

[2] The abbreviations used are: Snf1, sucrose non-fermenting-1; AMPK, AMP-activated protein kinase; AID, auto-inhibitory domain; KIS, kinase-interacting sequence; ASC, associates with the Snf1 complex; CBS, cystathionine β-synthase; CaMKK-β, calcium-calmodulin-dependent protein kinase kinase β; HA, hemagglutinin; GST, glutathione *S*-transferase; TAP, tandem affinity purification; KD, kinase domain; UK, upstream kinase; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

directly by AMP (12). Structurally, the γ-subunit is composed almost entirely of four consecutive CBS (cystathionine β-synthase) domains. Recent studies have shown that tandem pairs of CBS domains form binding sites for AMP and other related nucleotides (13). Binding of AMP to the γ-subunit would then be predicted to promote either binding of γ to the AID in the α-subunit or to change the interaction with this domain in a way that abrogates its inhibition of the kinase domain. Further studies remain to fully document this model of regulation.

An additional layer of regulation of the Snf1/AMPK complex comes from the absolute requirement of the Snf1/AMPK kinases to be phosphorylated on their activation loops by a distinct upstream kinase. We identified Sak1 (formerly known as Pak1) as a yeast kinase that associates with and activates Snf1 *in vivo* and *in vitro* (14). Subsequently, we and others (15, 16) demonstrated that yeast express three related kinases, Sak1, Tos3, and Elm1, that are each capable of activating Snf1 by phosphorylation of its activation loop threonine. Of the three Snf1-activating kinases, Sak1 appears to be the primary activator of Snf1 *in vivo*. This conclusion is based on the findings that only Sak1 can activate all isoforms of Snf1 (17), only Sak1 forms a stable complex with Snf1 (18) and only Sak1 is capable of promoting nuclear localization of the Gal83 isoform of Snf1 (19). In mammalian cells, the primary activator of AMPK is the protein kinase LKB1 (19), whereas the calcium-calmodulin-dependent protein kinase kinase β (CaMKK-β) may be the AMPK activator in certain cell types (20, 21). Whereas it is well documented that the Snf1/AMPK enzymes require activation loop phosphorylation for activity, how this reaction is regulated is not currently known.

In this study, we purified Snf1 enzyme complexes and individual subunits to directly examine the interactions between the Snf1 kinase subunits with each other and with the Snf1-activating kinases. We particularly wanted to address the question of whether the active form of the Snf1 kinase is a dimer of the α- and γ-subunits. Other groups have reported that Snf1 purified by Ni^{2+}-Sepharose chromatography yields an active αγ dimer with little or no β-subunit present (12, 22). However, genetic data clearly show that Snf1 kinase function *in vivo* requires the β-subunits (4). Our results indicate that the active form of Snf1 is the αβγ heterotrimer.

EXPERIMENTAL PROCEDURES

Strains and Media—The yeast strains used in this study are shown in Table 1. Yeast were grown at 30 °C in synthetic complete media lacking the appropriate nutrient for plasmid selection. Glucose or sucrose was used as a carbon source at 2% (w/v). For induction of Snf1 signaling, cells were shifted to media containing 0.05% (w/v) glucose for 2 h.

Plasmid Constructions—pSAK1-TAP, pSAK1-D277A-TAP, and pSNF1-TAP have been described previously (18, 23). pSnf1-KD-TAP was constructed by cloning the TAP tag from pBS1479 (24) into pSNF1-Δ381–608 (9). Snf1 tagged with three copies of the HA (hemagglutinin) epitope at the C terminus (pSNF1–3HA) has been described previously (25). GST (glutathione *S*-transferase)-tagged Mig1 (pGEX-Mig1; residues 202–414) and Snf1 kinase domain (pGEX-SNF1-KD; residues 1–392) have been described previously (18, 23). GST-tagged Gal83 (pGEX-GAL83; residues 1–417) and Snf4 (pGEX-SNF4; residues 1–322) were constructed by PCR amplification of the full-length reading frames and insertion into pGEX2T (Amersham Biosciences). pYAD-Sak1 is a high copy number plasmid expressing a V5-tagged Sak1 from the *ADH1* promoter (26). pSNF4–3HA and pGAL83–3HA are based on the low copy number plasmid vector pRS316 (27). In both plasmids, three copies of the HA epitope were added by gap repair such that the epitopes were expressed at the C terminus of the protein.

TAP Purifications—For protein purification of tandem affinity purification (TAP)-tagged Sak1, Sak1-D277A, Snf1, and Snf1-KD, cells were grown in synthetic complete media containing sucrose (for Snf^+ strains) or glucose (for Snf^- strains) as the carbon source and harvested in the mid-logarithmic phase. Cells (2 liters) were suspended in 10 ml of IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40) containing protease inhibitors (1 mM benzamidine, 0.1 mM PMSF, 5 μg/ml aprotinin) and lysed by grinding in liquid nitrogen. Protein extracts were cleared by centrifugation twice for 10 min at 10,000 rpm at 4 °C. TAP-tagged proteins were bound and eluted from the IgG and calmodulin columns using the procedures developed by Rigaut *et al.* (28). Purified proteins were eluted in five 200-μl fractions. Fractions two, three, and four were pooled, and glycerol was added to a final concentration of 5% (v/v). Aliquots were stored at −80 °C.

GST Purifications—*Escherichia coli* (DH5α) was grown in 0.5 liters, and GST-tagged proteins were induced by addition of isopropyl-1-thio-β-D-galactopyranoside (0.5 mM final) for 3 h at 37 °C. Cells were lysed in 10 ml NETN (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing protease inhibitors (0.5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5 μg/ml chymostatin 0.5 μg/ml pepstatin A, 17 μg/ml PMSF) by sonication (3 bursts of 20 s). Cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4 °C. Lysates (2–10 ml) were mixed with 200 μl of glutathione beads (Amersham Biosciences; 50% slurry) for 1 h at 4 °C. Proteins were eluted by incubation of the beads in 0.5 ml of elution buffer (50 mM Tris-HCl, pH 8.0, 20 mM reduced glutathione) for 10 min at room temperature. Eluted proteins were dialyzed against the kinase assay buffer (20 mM HEPES pH 7.0, 0.5 mM EDTA, 0.5 mM dithiothreitol, 5 mM magnesium acetate). GST-Snf1-KD was further concentrated using an Amicon spin column (10 kDa cutoff).

Western Blotting—TAP-purified Snf1 was detected by the HRP-conjugated polyhistidine antibody (1:1000) which recognizes the naturally occurring repeat of histidine residues in Snf1. To detect Snf1 phosphorylation on threonine 210 (Thr^{210}), 300–500 μg of total protein was prepared and immunoprecipitated in radioimmune precipitation assay buffer as described previously (25). Thr^{210} phosphorylation was detected by the primary PT210 antibody (1:1000) and the secondary HRP-conjugated anti-rabbit monoclonal antibody (1:15 000) (Santa Cruz Biotechnology). Snf1 tagged with the HA epitope was also detected by an HA antibody (1:1000) (Santa Cruz Biotechnology) and a secondary HRP-conjugated mouse monoclonal antibody (1:15 000) (Santa Cruz Biotechnology). GST-tagged proteins were detected using a GST antibody (1:2000) (Upstate) and a secondary HRP-conjugated rabbit monoclonal

antibody (1:15 000) (Santa Cruz Biotechnology). The V5 epitope was detected by an HRP-conjugated monoclonal V5 antibody (1:5000) (Santa Cruz Biotechnology).

Kinase Assays—Kinase assays were incubated for 30 min at 30 °C. Reactions (20 μl) contained 0.2 mM [γ-^{32}P]ATP (1000 cpm/pmol), 20 mM HEPES pH 7.0, 0.5 mM EDTA, 0.5 mM dithiothreitol, 5 mM magnesium acetate, and substrates at ~50 μg/ml.

Protein Binding Assays—Lysates from *E. coli* expressing GST or GST-tagged Snf1-KD, Gal83 and Snf4 were bound to glutathione beads (20 μl) and washed once with 1 ml NETN and twice with 1 ml of PBS (135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) containing protease inhibitors (0.5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5 μg/ml chymostatin 0.5 μg/ml pepstatin A, 17 μg/ml PMSF). Yeast cells expressing V5-tagged Sak1 were lysed with glass beads in PBS buffer containing protease inhibitors (1 mM benzamidine, 0.1 mM PMSF, 5 μg/ml aprotinin). Yeast lysates were spun at 14,000 rpm for 10 min at 4 °C. 130 μg of the Sak1-V5 lysate was added to the beads, prebound with GST or GST-tagged protein, in a total volume of 500 μl of PBS buffer containing protease inhibitors. Reactions were rotated for 1 h at 4 °C. Beads were washed twice in PBS and once in PBS containing also 0.1% SDS, 0.5% deoxycholate, and 1% Nonidet P-40. Proteins were eluted by incubation in 25 μl of 2× SDS sample buffer for 5 min at 95 °C. Proteins were resolved on two separate 10% SDS-PAGE gels that were blotted onto nitrocellulose membrane and probed separately with antibodies directed against V5 and GST.

Gel Filtration Chromatography—Protein extracts were prepared from yeast cells lysed with glass beads in NHTG buffer (40 mM HEPES pH 7.3, 350 mM NaCl, 0.1% Tween, 10% glycerol). 200 μl of each extract (~1 mg of protein) was applied to a 20-ml Superose 12 column. The column was run in NHTG buffer without Tween at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and 50–100 μl aliquots of fractions 16–28 were trichloroacetic acid-precipitated and analyzed by Western blotting. Size standards used to calibrate the column were bovine thyroglobulin (670 kDa), rabbit muscle aldolase (158 kDa), and chicken ovalbumin (43 kDa).

RESULTS

Snf1 Subunit Stoichiometry—The Snf1 and AMPK complexes are believed to be heterotrimers composed of a single α-, β-, and γ-subunit (2, 5). Proteomic studies in yeast have been performed with the goal of quantifying the number of every protein in the cell (29). In these studies, the abundance of the Snf1 kinase α-, β-, and γ-subunits were found to be ~600, 4000, and 12,000, respectively. These results would suggest that the β- and γ-subunits are present in 6- and 20-fold molar excess over the α-subunit. If accurate, this finding predicts that the Snf1 kinase β- and γ-subunits exist in the cell either as free subunits or associated with other proteins. The yeast genome encodes several Snf1-like kinases, suggesting the possibility that the Snf1 β- and γ-subunits may exist in complexes with other Snf1-like kinases. To test this possibility, we examined the elution profiles of the β- and γ-subunits from gel filtration columns using protein extracts made from cells that did or did not express the Snf1 α-subunit (Fig. 1). Low copy number plasmid

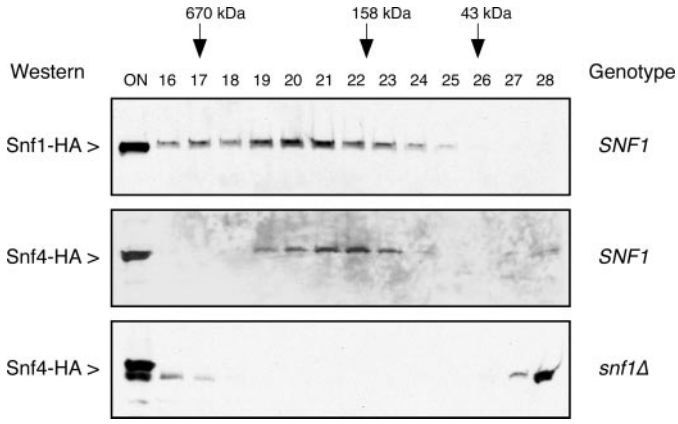

FIGURE 1. **Snf1 subunit stoichiometry.** Protein extracts were size-fractionated on a 20-ml Superose 12 column. HA-tagged Snf1 and Snf4 were detected by Western blotting as indicated on the *left*. The onput sample (*ON*), and fraction numbers of the samples are indicated. The relevant genotypes of the cells are shown on the *right*. The elution of size standards run separately on the same column are noted at the *top*.

constructs were engineered to express HA-tagged Snf4 (γ) or HA-tagged Gal83 (one of three alternative β-subunits in yeast) from their own cognate promoters. These plasmids were introduced to cells with a deletion of the gene being studied and protein extracts were fractionated on a Superose 12 column. In cells expressing Snf1, the Snf4 protein elutes primarily in fractions 19–23, a position in the elution profile consistent with incorporation in a complex the size of the Snf1 kinase heterotrimer (155 kDa). A trace amount of free Snf4 (36 kDa) is observed in fraction 28. In contrast, when the extract was prepared from cells lacking the Snf1 protein (*snf1*Δ), all of the Snf4 shifts to a later elution, consistent with free Snf4. These results are not consistent with the reported 20-fold molar excess of Snf4 over Snf1 (29). Instead, the great majority of Snf4 is associated with Snf1 *in vivo*. Similar experiments performed with Gal83, the most abundant β-subunit, were inconclusive and are not shown.

Sak1 Binds the α-Subunit of Snf1 Kinase Complex—The Snf1 kinase can be activated by any one of three upstream kinases. One of these, Sak1 (Snf1-activating kinase 1), forms a stable complex with Snf1 (18). Here we used bacterial-expressed GST fusions to the α-, β-, and γ-subunits to determine which subunit binds to the Sak1 kinase. In all cases, the GST protein was fused at the N terminus of the yeast protein. Expression of these isolated subunits in bacteria led to varying levels of recovery of full-length and proteolyzed fragments. Preparations of GST-Snf4 yielded primarily a protein with a size consistent with full-length GST-Snf4 (Fig. 2*B*, *lane 3*). Preparations of GST-Gal83 yielded a small amount of full-length protein and a mixture of truncated products (*lane 4*). Despite repeated attempts, we have been unable to express full-length GST-Snf1 in bacteria. However, we are able to express and purify a GST fusion to the kinase domain (KD; amino acids 1–392) of Snf1 (*lane 2*). These recombinant proteins were tested for their abilities to bind Sak1 protein present in a yeast extract prepared from cells lacking all components of the Snf1 kinase complex. The Sak1 protein was tagged with the V5 epitope to allow detection by Western blotting (Fig. 2*A*). The GST-Snf1-KD protein was able to efficiently bind Sak1 whereas neither GST-Snf4 nor GST-Gal83 showed

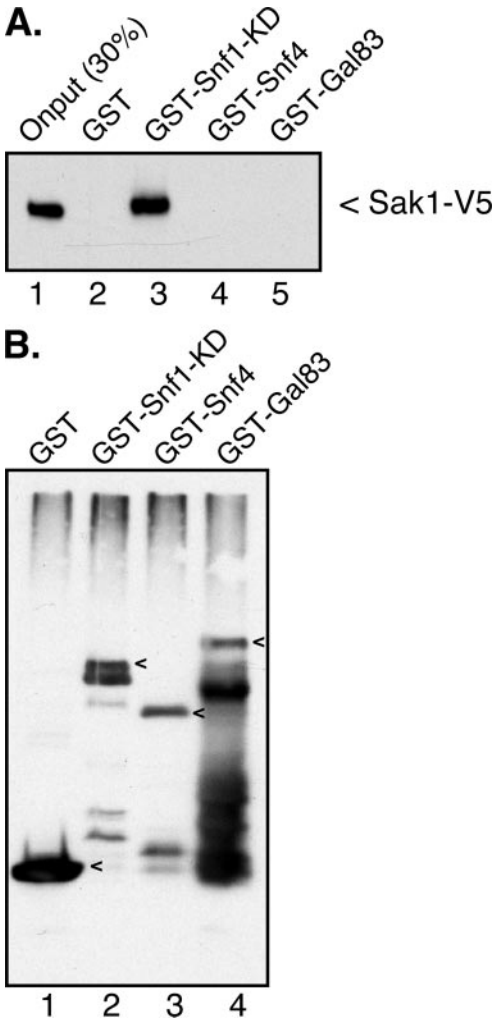

FIGURE 2. **Sak1 binds the α-subunit of Snf1 complex.** *A*, yeast extracts were prepared from cells that overexpressed the Snf1-activating kinase 1 (*Sak1*) with a V5 epitope tag on its C terminus and were passed over a glutathione column pre-bound with recombinant GST fusion proteins expressed in bacteria. Columns were washed extensively, and bound Sak1 protein was detected by Western blotting with antibodies directed against the V5 epitope. *B*, anti-GST Western blot shows the relative size and integrity of recombinant GST fusion proteins as indicated above each lane. Full-length proteins are indicated with an *arrow*.

any binding activity. We conclude that the Snf1 kinase domain is able to bind to Sak1 *in vitro* and that the primary site of interaction between Sak1 and the Snf1 kinase complex *in vivo* is most likely the kinase domain of Snf1.

β- and γ-Subunits Are Required for Stable Association of Sak1 with the Snf1 Kinase Complex in Vivo—Because we determined that the Sak1 protein binds to the Snf1 kinase domain *in vitro*, we decided to test the subunit requirements for Sak1 association *in vivo*. We have previously purified Sak1 using the TAP affinity protocol and identified associated proteins by mass spectrometry (18). Here, we TAP-purified Sak1 from cells that contained complete deletions of the genes for the α-, β-, or γ-subunits of the Snf1 kinase. The proteins associated with Sak1 were determined on SDS gels stained with silver nitrate (Fig. 3*A*). When Sak1 was purified from cells expressing all the Snf1 kinase subunits, the presence of Snf1, Gal83, and Snf4 are readily apparent (*lane 1*). In contrast, when Sak1 was purified from cells lacking either the α-subunit (*snf1Δ10*, *lane 2*), all three β-subunits (*sip1Δ sip2Δ gal83Δ*, *lane 3*), or the γ-subunit (*snf4Δ*, *lane 4*), all of the Snf1 kinase complex subunits are absent. A protein slightly larger than Snf4 is visible in all the Sak1-TAP fractions. This protein has been observed previously (18) but has not been identified by mass spectrometry. Because the Snf1 protein co-migrates with the chaperone proteins Ssa1/2, Ssb1/2, and Kar2 (18), we used Western blotting to test for the presence of the Snf1 protein (Fig. 3*B*). The Snf1 protein was only found associated with Sak1 when all Snf1 kinase complex subunits are expressed. Therefore, the Snf1 protein does not stably associate with the Sak1 kinase unless all subunits of the Snf1 heterotrimer are present. This finding is somewhat surprising since the Snf1 kinase domain can associate with Sak1 *in vitro* and in the absence of the β- and γ-subunits (Fig. 2). Nonetheless, the ability of Sak1 to form a stable complex with Snf1 *in vivo* requires the complete Snf1 heterotrimer be present.

Interdependence of Snf1 Heterotrimer Formation—The β-subunit is thought to play a key role in the assembly of the Snf1 heterotrimer. The C terminus of the β-subunit contains two conserved domains, termed KIS and ASC, that are thought to mediate interaction with the α- and γ-subunits, respectively (6). The abilities of the KIS and ASC domains to interact with the α- and γ-subunits *in vitro* do not require the presence of the other subunits (6), suggesting that these interactions can occur independently of each other. Here, we purified the Snf1 kinase complex using the TAP protocol and determined the subunit requirements for the association of the β- and γ-subunits with Snf1 (Fig. 4*A*). In previous experiments, we have purified Snf1-TAP and detected the Snf1, Snf4, and Gal83 subunits (18). Sip1 and Sip2 are much less abundant than Gal83 and were not detected by mass spectrometry (24). When Snf1 is purified from cells expressing all the Snf1 kinase complex subunits, the presence of Snf4 (γ) and Gal83 (β) are readily apparent on SDS gels stained with silver nitrate (*lanes 1* and *6*). When Snf1 was purified from cells lacking all three β-subunits (*sip1Δsip2Δgal83Δ*; *lane 2*), both the β- and γ-subunits are missing from the complex. In contrast to earlier reports (12, 22), we find no evidence for the existence of a stable αγ dimer. This suggests that the β-subunit is needed for the stable association of the α- and γ-subunits. Similarly, deletion of γ eliminates Snf4 from the complex and greatly reduces the amount of β-subunit found associated with Snf1 (*lane 3*). A trace amount of Gal83 could be detected but much less than is associated when both β and γ are present. For comparison, the Snf1 complex purified from wild-type cells was loaded twice on this gel at two different concentrations (*lanes 1* and *6*). These data suggest that there is an interdependence between the β- and γ-subunits for association with α. However, these findings could be easily explained if the abundance of one subunit was greatly reduced when the other was missing. This possibility was tested by Western blotting of protein extracts from cells that express the HA-tagged versions of the β- and γ-subunits with and without the other subunit (Fig. 4*B*). The presence or absence of the γ-subunit had no effect on the steady state level of the β-subunit, Gal83. Similarly, the presence or absence of the β-subunit Gal83 had no effect on the abundance of the γ-subunit Snf4. Therefore, the β- and γ-sub-

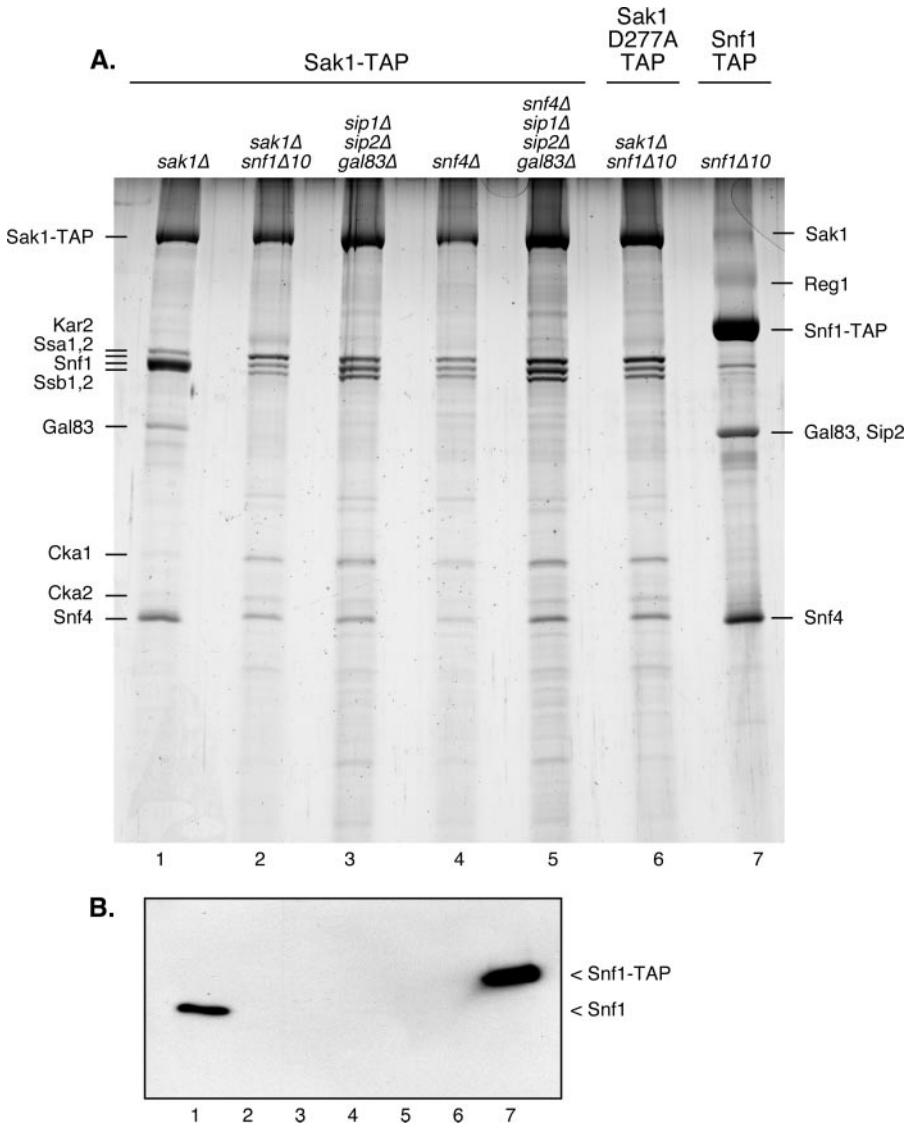

FIGURE 3. **Association of Sak1 with the Snf1 complex requires the β- and γ-subunits.** *A*, silver-stained gel of Sak1-TAP preparations from cells lacking different Snf1 kinase complex subunits. The genotypes of the strains from which the Sak1-TAP was purified is shown above each lane. *B*, Western blot using anti-His serum was used to detect the presence of the Snf1 protein. *Lanes 1–7* correspond to the samples shown in *A*.

units are present in the cell but fail to stably associate with the α-subunit when the γ- or β-subunit, respectively, is missing. These results demonstrate an interdependence of interaction with the α-subunit. Neither the β- or γ-subunit efficiently associates with the α-subunit in the absence of the other.

We also purified the Snf1 complex from cells lacking all three upstream kinases (*lane 5*) and found that the Snf1 heterotrimer remained intact. Therefore, the phosphorylation of the Snf1 activation loop is not required for the stable assembly of the Snf1 heterotrimer. Interestingly, a protein we have previously identified as Reg1 (14, 18) remains associated with Snf1 indicating that its association also does not require phosphorylation of threonine 210 (*lane 5*). Two hybrid studies indicated that Reg1 association with the Snf1 kinase domain was blocked by mutation of the activation loop threonine to alanine (30). Our results with TAP-purified Snf1 complex indicate that Thr^{210} phosphorylation is not required for the association of Reg1 with the Snf1 complex. However, the presence of the complete heterotrimer is necessary for both Reg1 and Sak1 association (*lanes 2–4*).

Subunit Requirements for Glucose-regulated Activation Loop Phosphorylation—A key regulatory step for the Snf1 kinase complex is the phosphorylation of the Snf1 activation loop on threonine 210 (25). The mechanisms by which these reactions are regulated are not known. One possibility is that access to the Snf1 activation loop for phosphorylation and dephosphorylation might be regulated. Such a mechanism has been proposed for AMPK (31). To test whether the β- and γ-subunits control accessibility of the activation loop, we measured the glucose-regulated phosphorylation of the Snf1 activation loop in cells lacking the β- and γ-subunits. Snf1 tagged with the HA epitope and expressed from its own promoter from a low copy number plasmid was immunoprecipitated with anti-HA antibodies (α-HA), and the phosphorylation status of its activation loop was assessed by Western blotting using a phosphopeptide antibody (α-PT210) specific for Snf1 protein phosphorylated on threonine 210 (25). Total Snf1 was assessed separately by Western blotting with anti-HA antibodies (Fig. 5*A*; *middle panel*). In cells expressing the β- and γ-subunits, the phosphorylation of Snf1 is readily detected and is increased when cells are shifted to low glucose (*lanes 1* and *2*). When the genes for all three β-subunits are deleted, the glucose-regulated phosphorylation of Snf1 activation loop is still observed (*lanes 3* and *4*). The same result is found when both the β- and γ-subunits are absent (*lanes 7* and *8*). In contrast, when the γ-subunit alone is absent, the Snf1 activation loop is still phosphorylated but its regulation by glucose appears disrupted (*lanes 5* and *6*). Interpretation of this result is complicated by the fact that the abundance of the Snf1 protein is reduced in cells lacking the γ-subunit and shifted to low glucose (*lane 6*). Equivalent quantities of total protein are present in all the samples as judged by Coomassie Blue staining (*lower panel*). We often detect low levels of Thr^{210} phosphorylation when cells are grown in high glucose. In this experiment, the phosphorylation of Thr^{210} is apparent in wild-type cells on high glucose (*lane 1*) but is lost when the β-subunits are absent, suggesting some disregulation of this step. However, cells lacking both the β- and γ-subunits show glucose-regulated phospho-

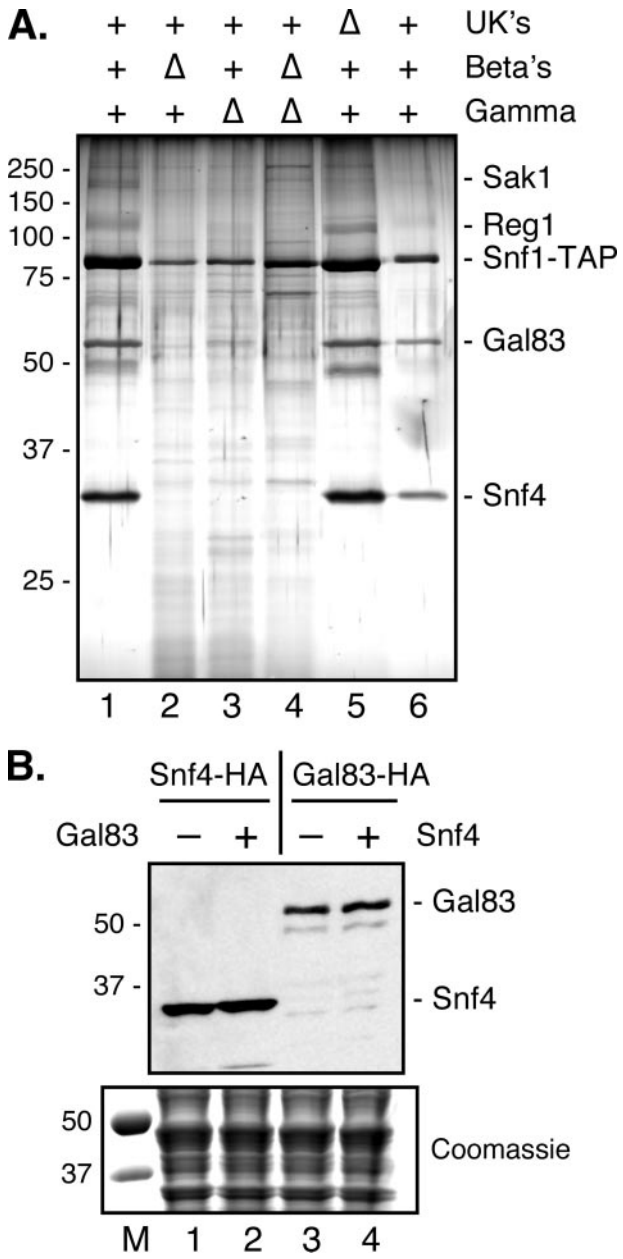

FIGURE 4. **Interdependence of Snf1 subunits for complex assembly.** *A*, Snf1-TAP was purified from strains lacking genes for all three Snf1-activating kinases (*UK*), for all three β-subunit genes, or for the γ-subunit as indicated *above* the lanes. The mobilities of protein size standards are indicated on the *left* whereas the mobilities of proteins identified previously by mass spectrometry are indicated on the *right*. Snf1 purified from wild-type cells in *lane 6* is the same sample as in *lane 1* but with 5-fold less protein loaded. *B*, Western blot of HA-tagged Snf4 and Gal83. A yeast strain lacking the genes for all three β-subunits as well as the γ-subunit of Snf1 was transformed with low copy number plasmids expressing Snf4-HA (*lanes 1* and *2*) or Gal83-HA (*lanes 3* and *4*). In addition, a second low copy number plasmid was introduced that was either empty vector (−) or expressed Gal83 or Snf4 as indicated (+). The *lower panel* shows the same samples stained with Coomassie Blue.

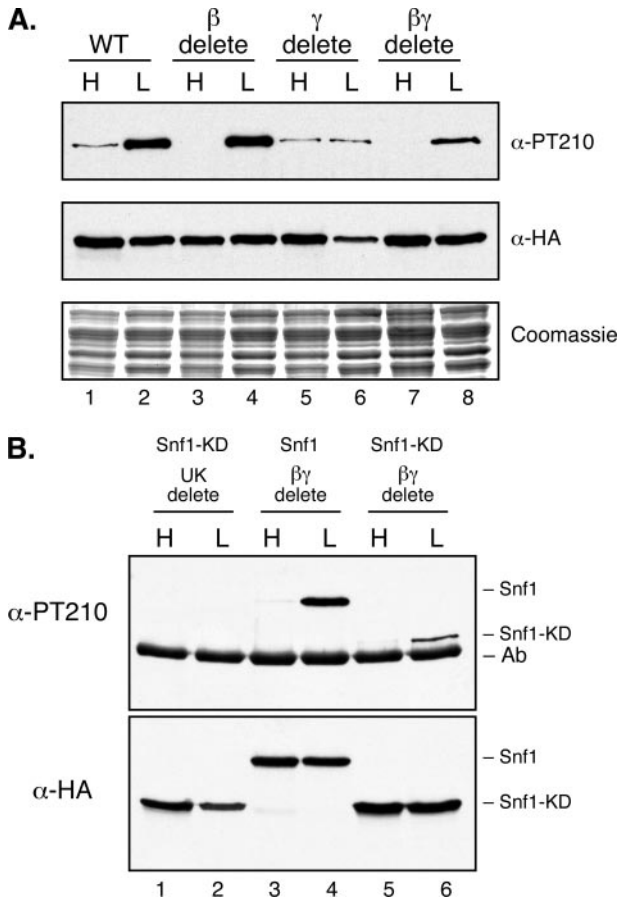

FIGURE 5. **Subunit requirements for Snf1 activation loop phosphorylation.** *A*, phosphorylation status of the Snf1 activation loop threonine was assessed by Western blotting with a phosphospecific antiserum directed against Snf1 threonine 210 (*α-PT210*). Total Snf1 protein was measured with antibodies directed against the HA epitope (*α-HA*). Equivalent aliquots of total protein were loaded as seen in a stained gel run in parallel (*Coomassie*). Extracts were prepared from strains lacking all three genes for the β-subunits (*β delete*), the gene for the γ-subunit (*γ delete*) or all four genes (*βγ delete*). *WT* indicates wild type. Cells were grown in high (*H*) or low (*L*) glucose as indicated *above* each lane. *B*, effect of the α-subunit regulatory domain on activation loop phosphorylation was assessed by Western blotting. Plasmids expressing either full-length Snf1 α-subunit or the Snf1 KD were introduced into strains lacking the genes for all three Snf1-activating kinases (*UK delete*) or the genes for the γ- and all three β-subunits (*βγ delete*).

rylation of the Snf1 activation loop (*lanes 7* and *8*). Therefore the glucose-mediated regulation of the Snf1 activation loop phosphorylation must include a mechanism that is independent of the β- and γ-subunits.

Role of the Regulatory Domain of the α-Subunit—The α-subunit (Snf1) of the Snf1 kinase complex contains a kinase domain in the N-terminal half of the protein (residues 1–390) and a regulatory domain in the C-terminal-half (residues 390–633). A portion of the α-subunit regulatory domain contains an AID that is counteracted by the γ-subunit (8, 10). We sought to determine whether this regulatory domain was required for the regulated phosphorylation of the Snf1 activation loop. Full-length Snf1 or the Snf1 KD was expressed in cells lacking the β- and γ-subunits. The level of Thr^{210} phosphorylation in cells grown in high and low glucose was measured as above. Both the full-length Snf1 and the Snf1 kinase domain displayed glucose-regulated phosphorylation of Thr^{210} (Fig. 5*B*, *lanes 3–6*). The level of phosphorylation was reduced in the Snf1 kinase domain but its regulation by glucose was still apparent. Thr^{210} phosphorylation was abolished when all three Snf1-activating kinases were absent (*lanes 1* and *2*). These data demonstrate that the regulation of the Thr^{210} phosphorylation can occur independently of both the β- and γ-subunits as well as the α-subunit regulatory domain.

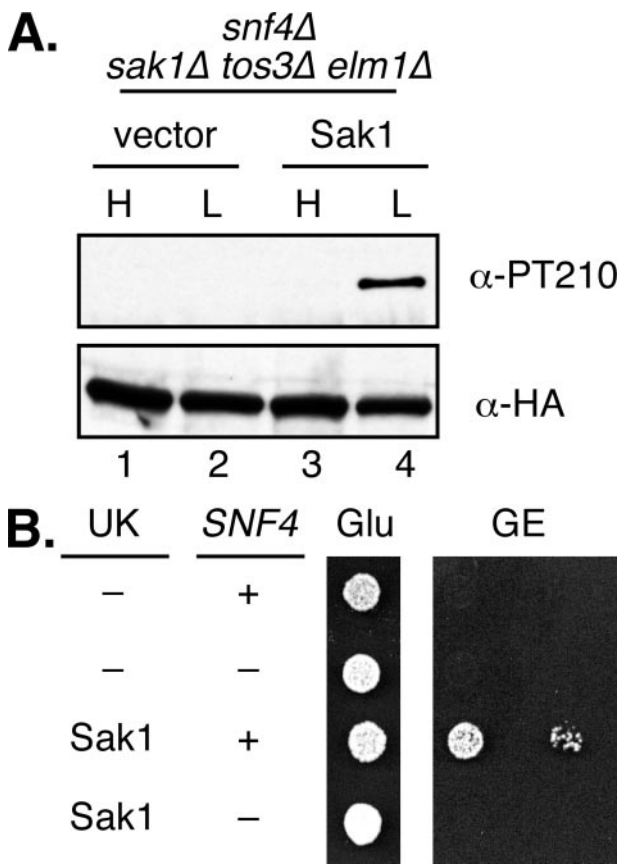

FIGURE 6. **Sak1 can phosphorylate Snf1 in the absence of complex formation.** *A,* phosphorylation status of the Snf1 activation loop threonine was assessed by Western blotting with a phosphospecific antiserum directed against Snf1 threonine 210 (*α-PT210*). Total Snf1 protein was measured with antibodies directed against the HA epitope (*α-HA*). Extracts were prepared from strains lacking the gene for the γ-subunit (*snf4Δ*) and all three genes for the Snf1-activating kinases. Plasmids expressing a single Snf1-activating kinase or empty vector were introduced as indicated. Cells were grown in high (*H*) or low (*L*) glucose as indicated above each lane. *B*, Snf1 signaling requires the γ-subunit. Cells lacking the γ-subunit and all three Snf1-activating kinases were transformed with two plasmids that were either empty vectors (−) or that expressed the γ-subunit Snf4 (+) or Sak1 as indicated. Cells were grown in glucose and spotted onto agar plates with either glucose (*Glu*) or a mixture of glycerol and ethanol (*GE*) as the carbon source.

Sak1·Snf1 Complex Formation Is Not Required for Regulated Phosphorylation of Snf1—Sak1 is unique among the three Snf1-activating kinases in that it forms a stable complex with Snf1 (18). Tos3 and Elm1 do not stably associate with Snf1 and yet they are able to promote sufficient Snf1 activation for aerobic growth. Therefore, the ability of Tos3 or Elm1 to form a stable complex with Snf1 is not a prerequisite for successful and glucose-regulated activation of Snf1. In this study we found that the ability of Sak1 to associate with Snf1 is abrogated when the β- or γ-subunits of the Snf1 complex are missing (Fig. 3). We next tested whether Sak1 retains its ability to activate Snf1 when it has lost the ability to form a stable complex with its target. Cells lacking the Snf1 kinase γ-subunit and all three Snf1-activating kinases were transformed with a low copy plasmid expressing Sak1. The Snf1 protein was then collected by immunoprecipitation and the phosphorylation status of the activation loop threonine was assessed by Western blotting (Fig. 6*A*). In the absence of any upstream kinase, phosphorylation of Snf1 activation loop is not detected (*lanes 1* and *2*). When Sak1 was present, glucose-regulated phosphorylation of Snf1 was observed (*lanes 3* and *4*). Therefore, the ability of Sak1 to phosphorylate Snf1 in response to glucose limitation does not require the γ-subunit and does not require the formation of a stable Sak1·Snf1 complex. Interestingly, the phosphorylation of Snf1 activation loop is not sufficient to activate Snf1 signaling in the absence of the γ-subunit because these cells are not able to grow aerobically (Fig. 6*B*). These results support our earlier contention that Snf1 activation requires two steps, activation loop phosphorylation and an activation step mediated by the γ-subunit (25). More relevant to this study, these data indicate that the ability of Sak1 to stably associate with Snf1 is not a requirement for the glucose-regulated phosphorylation of Snf1.

Purification and Characterization of the Snf1 α-Subunit—The α-subunit contains both catalytic and regulatory domains. We purified the α-subunit from yeast cells that lacked the genes for all the Snf1 kinase subunits or for all three Snf1-activating kinases. Examination of the proteins present in the α-subunit preparations by SDS gels stained with silver nitrate shows that when the full-length α and the truncated α kinase domain are TAP purified from strains lacking the β- and γ-subunits, the preparations also lack the proteins previously identified as Sak1 and Reg1 (Fig. 7*A*, *lanes 2* and *4*). The only proteins that consistently co-purify with the α-subunits from the βγ deletion and upstream kinase deletion strains are the chaperone proteins Ssa1 and Ssa2. When the α-subunits are purified from a strain lacking the Snf1-activating kinases (UK delete), the Gal83 and Snf4 proteins (βγ-subunits) are apparent in the full-length α preparation but lacking from the α-kinase domain preparation (*lanes 3* and *5*). These results are consistent with earlier studies which showed that the βγ interaction domain is present in the C terminus of the α-subunit (6). When Snf1 is purified from a strain lacking the Snf1-activating kinases, the protein identified as Sak1 is reassuringly missing but Reg1 remains associated as was noted in an earlier preparation (Fig. 4*A*). The Snf1-KD-TAP protein migrates slightly faster than Gal83. Neither Gal83 nor Snf4 are able to associate with the truncated α-subunit lacking the βγ interaction domain of the α-subunit. The kinase activity of the purified α-subunits was measured using GST-Mig1 as a substrate (Fig. 7*B*) with and without addition of the Snf1-activating kinase Sak1. Preparations of the α-subunits were diluted so the equivalent levels of α-subunit were present in each reaction. Comparison of kinase activity with and without Sak1 gives an indication of whether the activation loop was phosphorylated *in vivo* prior to purification. Full-length Snf1 purified from the βγ delete strain shows low but detectable kinase activity that can be stimulated by incubation with Sak1 (*lanes 2* and *3*). These data indicate that the full-length α is weakly phosphorylated *in vivo* and can be further activated *in vitro*. By comparison, when the full-length α-subunit is purified from cells lacking all three Snf1-activating kinases, no activity is detected unless Sak1 is included in the reaction (*lanes 4* and *5*). The level of activity of the complete heterotrimer is much greater than the isolated full-length α-subunit (compare *lanes 3* and *5*). The kinase domain of Snf1 purified from the βγ delete cells is active and can be activated further by Sak1 (*lanes 6* and *7*). When purified from cells expressing the Snf1-activating kinases, both the full-length and

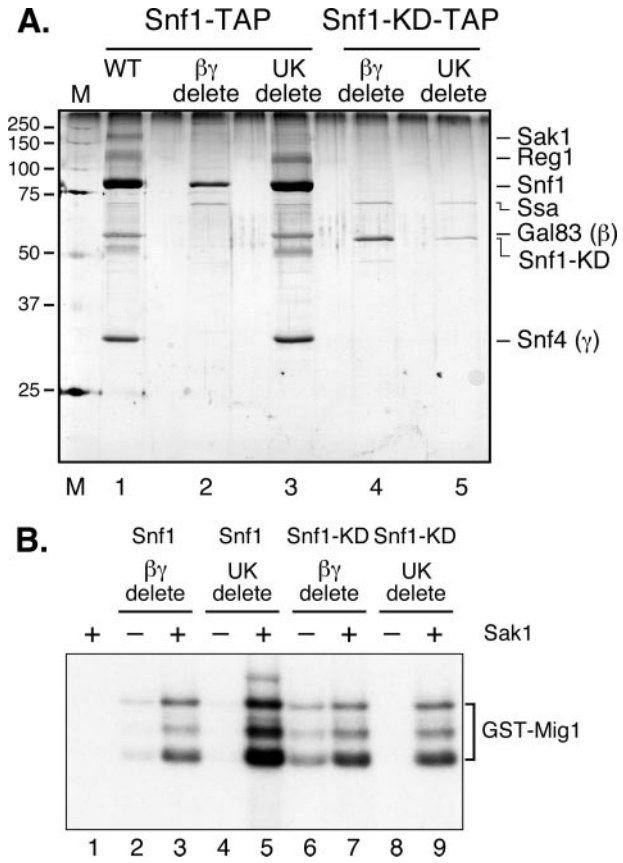

FIGURE 7. **Purification and activity of full-length and truncated α-subunits.** *A*, full-length Snf1 (*lanes 1–3*) and the Snf1 kinase domain (*lanes 4* and *5*) were TAP-purified from wild-type cells (*WT*; *lane 1*), cells lacking all of the genes for the Snf1 β- and γ-subunits (*βγ delete*; *lanes 2* and *4*) or all the genes for the Snf1-activating kinases (UK delete; *lanes 3* and *5*). Proteins were analyzed in an SDS protein gel stained with silver nitrate. Gel mobilities of molecular weight markers (*left*), and proteins identified previously by mass spectrometry (*right*) are shown. *B*, *in vitro* kinase assays were performed with the indicated proteins, [γ-^{32}P]ATP and GST-Mig1. Reactions contained either full-length Snf1 or the Snf1 kinase domain purified from cells lacking the genes for all the β- and γ-subunits (*βγ delete*; *lanes 2*, *3*, *6*, and *7*) or all the genes for the Snf1-activating kinases (UK *delete*; *lanes 4*, *5*, *8*, and *9*). Purified Sak1 was added as indicated (*odd lanes*). Snf1 was not added to the reaction in *lane 1*.

truncated α-subunit possess a low level of kinase activity that can be further stimulated by purified Sak1 *in vitro*. These findings indicate that the α-subunit can be recognized by the Snf1-activating kinases *in vivo* and *in vitro* independently of the β- and γ-subunits.

To further address the role of the regulatory domain of the α-subunit without the potential for trace contamination with other Snf1 subunits or the upstream kinases, we constructed the yeast strain MSY940 (Table 1) that lacked the genes for all the Snf1 kinase subunits and for all the Snf1-activating kinases (*snf1*Δ*snf4*Δ*gal83*Δ*sip1*Δ*sip2*Δ*sak1*Δ*tos3*Δ*elm1*Δ). Amazingly, this strain is viable and relatively healthy when grown on glucose. Plasmids expressing TAP-tagged full-length Snf1 or the Snf1 kinase domain were introduced to this strain. When purified from this deletion strain, the full-length and truncated α-subunits were apparent on silver-stained SDS gels (Fig. 8*B*). The purified α-subunits contained only one additional band that was identified previously by mass spectrometry to be a mixture of the Ssa1 and Ssa2 proteins (18). For comparison, the Snf1 heterotrimer purified from cells lacking all the genes for

TABLE 1
***S. cerevisiae* strains**

Strain	Genotype
FY1193	*MATα ura3–52 leu2Δ1 trp1Δ63 his3Δ200 snf1Δ10*
MSY557	*MATα ura3–52 leu2Δ1 trp1Δ63 his3Δ200 sip1Δ::HIS3 sip2Δ::HIS3 gal83Δ::HIS3*
MSY560	*MAT***a** *ura3–52 leu2Δ0 his3Δ200 sip1Δ::HIS3 sip2Δ::HIS3 gal83Δ::HIS3 snf1Δ10*
MSY568	*MATα ura3–52 leu2Δ1 snf4Δ1*
MSY573	*MAT***a** *ura3–52 leu2Δ1 his3 sip1Δ::HIS3 sip2Δ::HIS3 gal83Δ::HIS3 snf4Δ1*
MSY578	*MAT***a** *ura3–52 leu2Δ1 trp1Δ63 his3 snf1Δ10 snf4Δ1 sip1Δ::HIS3 sip2Δ::HIS3 gal83Δ::HIS3*
MSY676	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sak1Δ::KAN*
MSY913	*MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 sak1Δ::KAN snf1Δ10*
MSY921	*MAT***a** *ura3–52 leu2Δ1 his3Δ200 his4-912δ lys2-128δ snf1Δ10 snf4Δ1*
MSY923	*MATα ura3 leu2 his3 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN snf1Δ10*
MSY940	*MATα ura3 leu2Δ0 trp1Δ63 met15Δ0 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN snf1Δ10 snf4Δ1 sip1Δ::HIS3 sip2Δ::HIS3 gal83Δ::HIS3*

the Snf1-activating kinases is shown in *lane 1*. These preparations were diluted such that they contained equivalent levels of α-subunit as determined by Western blotting (Fig. 8*C*). Because these proteins were purified from strains lacking all three Snf1-activating kinases, all three preparations of α-subunit (full-length Snf1 with and without the β- and γ-subunits and the α-subunit kinase domain) lacked kinase activity (Fig. 8*A*, *lanes 2–4*). When purified Sak1 was added to the reactions, the kinase activity is greatly stimulated (*lanes 5–7*). The phosphorylation was specific for the Mig1 sequences in the GST-Mig1 fusion since the GST protein was not recognized (*lanes 8–10*). Of these three preparations, the full-length α-subunit has the lowest specific activity (*lane 6*) whereas the complete heterotrimer displayed the highest specific activity (*lane 5*). These results support the model in which the inhibition of the kinase domain by the AID can be reversed by either the presence of the β- and γ-subunits or by its deletion.

Interdependence of the β- and γ-Subunits for Stimulation of Kinase Activity—Earlier studies have reported that the Snf1 kinase can be active as an αγ dimer (7, 12, 22). To test whether the γ-subunit can stimulate the activity of the α-subunit in the absence of the β-subunit, we purified the individual Snf1 kinase subunits from the deletion strain MSY940. The kinase activity of the full-length α-subunit or the α-subunit kinase domain was then assayed in the presence of added β- and γ-subunits (Fig. 9A). When added alone, neither the β- nor the γ-subunits could stimulate kinase activity. However, when added together, the activity of the full-length α-subunit but not the kinase domain was stimulated (*lanes 4* and *8*). To more accurately assess the stimulation of the Snf1 kinase activity by the individual subunits, kinase reactions were run in triplicate and the incorporation of ^{32}P into the GST-Mig1 substrate was quantified with a phosphorimager (Fig. 9*B*). When added alone, neither the β- or γ-subunit could significantly stimulate the kinase activity of the purified α-subunit. However, when added together, the β- and γ-subunits stimulated the kinase activity of the α-subunit by 2.5-fold. Our data are not consistent with the idea that the Snf1 kinase complex is active as an αγ dimer. Instead, our data support the existence of Snf1 as a heterotrimer whose assembly and activity shows an interdependence on all three subunits.

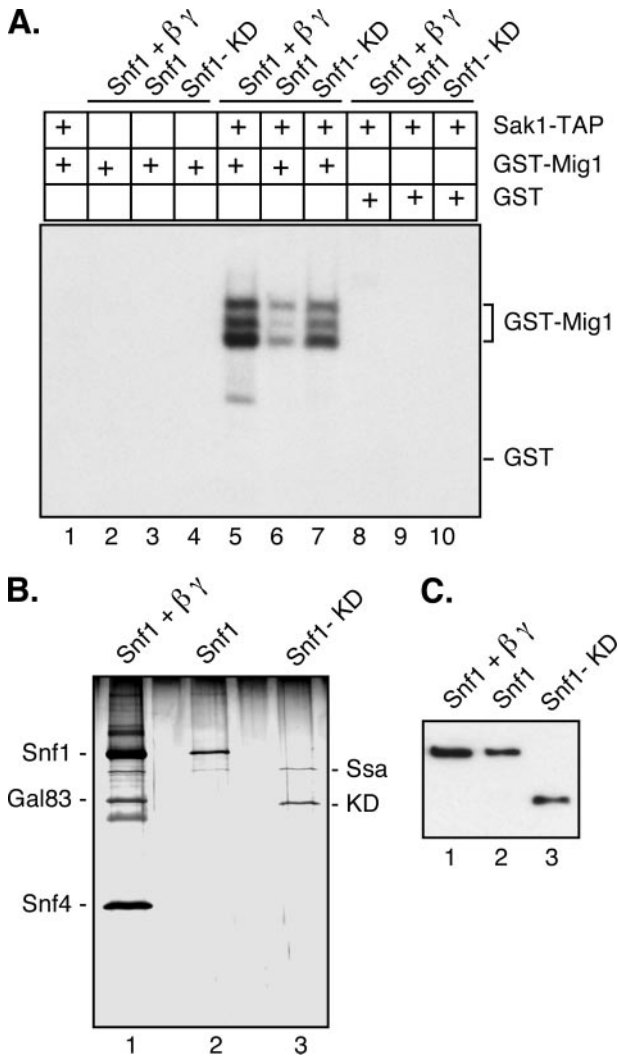

FIGURE 8. **α-Subunit regulatory domain inhibits kinase activity.** *A*, *in vitro* kinase assays were conducted with Snf1 kinases purified from cells lacking all the genes for the Snf1-activating kinases. Snf1 kinase preparations included the complete heterotrimer (*Snf1 + βγ*), full-length Snf1 lacking the β- and γ-subunits (*Snf1*) and the Snf1 kinase domain lacking the β- and γ-subunits (*Snf1-KD*). Reactions contained [γ-^{32}P]ATP and either GST-Mig1 or GST. Purified Sak1 was added to the reactions in *lanes 1*, and *5–10*. *B*, silver-stained protein gel of the Snf1 preparations used in the kinase reactions. *C*, Western blot using anti-His serum was used to detect the relative abundance of the Snf1 protein.

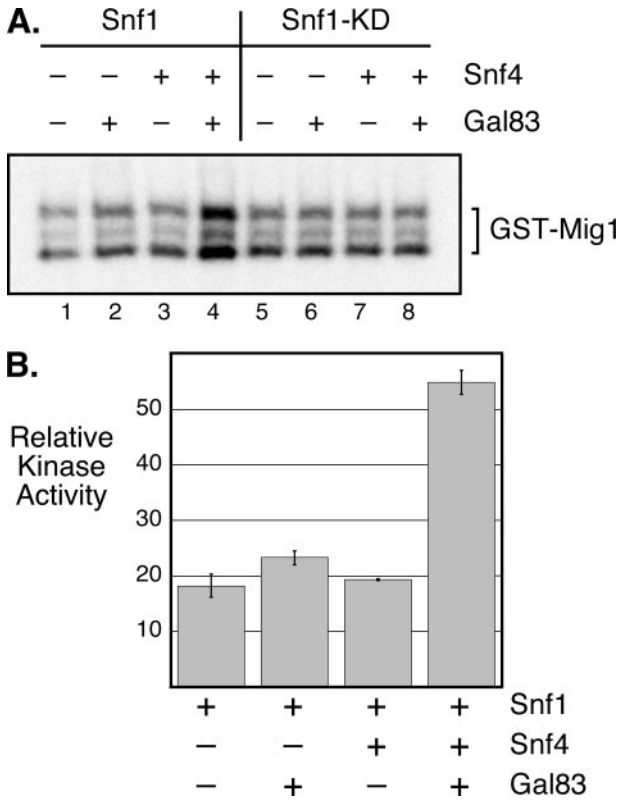

FIGURE 9. **Reconstitution of the Snf1 heterotrimer.** *A*, *in vitro* kinase assays (20 μl) were conducted with [γ-^{32}P]ATP, GST-Mig1 and either full-length α-subunit (*Snf1*) or the Snf1 kinase domain (*Snf1-KD*). Purified β-subunit (*Gal83*) and γ-subunit (*Snf4*) were added to the reactions as indicated. *B*, kinase reactions shown in *A* (*lanes 1–4*) were repeated in triplicate. The incorporation of ^{32}P into the GST-Mig1 proteins was quantified using a phosphorimager. The mean value from three reactions is plotted with the error bars representing one standard error.

DISCUSSION

In this study, we examined the oligomeric structure of the Snf1 kinase complex. Genetic data strongly support the idea that the functional unit of Snf1 protein kinase is a heterotrimer (4, 6). More recent studies of the yeast proteome suggested that the β- and γ-subunits of the Snf1 complex were present in molar excess over the α-subunit (29). These data laid open the possibility that the β- and γ-subunits might exist independently of the α-subunit, either as free subunits or alternatively, as subunits in other oligomeric complexes. Yeast encode a number of Snf1-like kinases whose oligomeric structures have not been determined and that might form complexes with Snf4 and Gal83. These possibilities were tested by gel filtration chromatography. The clearest results pertain to Snf4, a protein predicted to be present at a 20-fold molar excess over Snf1 (29). If free pools of Snf4 are really present *in vivo*, then they should be easily separated from the Snf1-Snf4-Gal83 heterotrimer by gel filtration. Our data indicate that the large majority of the cellular Snf4 is present in a complex with Snf1 and only a trace amount appears to be free (Fig. 1). Our data suggest that the numeric values of molecules per cell determined by quantitative Western blotting (29) are not very accurate for the Snf1 kinase subunits.

A second issue addressed in this report is the role of the β-subunit of the Snf1 kinase complex. The requirement of β-subunit for Snf1 kinase function *in vivo* has a confusing history with genetic and biochemical data initially suggesting they were not essential for Snf1 kinase function. First, genes for β-subunits were never identified in screens for Snf- (sucrose non-fermenting) mutants. Second, initial reports indicated that Snf1 kinase signaling was intact in cells in which all three β-subunit genes had been disrupted (3, 32). Finally, Snf1 kinase complexes purified by Ni^{2+} Sepharose chromatography appeared to be αγ dimers (12, 22). All these data combined supported the notion that, in contrast to the mammalian AMPK, the yeast Snf1 kinase complex did not require a β-subunit for function. However, this conclusion is erroneous. The β-subunit genes were never identified in loss of function screens because there are three redundant genes. Initial gene disruptions of the β-subunit genes were performed using an older methodology

that left small portions of the β-subunit genes intact (3, 32). More recent studies show that only a small region at the very C terminus of the β-subunit (85 residues) is sufficient for heterotrimer formation (7), and this region was present and expressed in the initial gene disruptions of *GAL83* and *SIP1*. Complete deletion of all three β-subunit genes completely abolishes Snf1 kinase signaling *in vivo* (4), demonstrating that the β-subunits are essential for Snf1 kinase function. When the Snf1 kinase complex is TAP purified, the complex contains the α-, β-, and γ-subunits as demonstrated by mass spectrometry (18). Why the β-subunit was not detected in Snf1 complexes purified by Ni^{2+} Sepharose chromatography is not clear. Gal83 is the most abundant of the β-subunits and we have noted that it migrates more slowly on SDS gels than predicted from its molecular weight. Furthermore, Gal83 stains poorly with silver nitrate. Perhaps the Gal83 protein was present in the Snf1 kinase preparations purified by Ni^{2+}-Sepharose chromatography but was below the level of detection.

The subunit requirements for the assembly of the Snf1 complex also supports the essential role played by the β-subunit. (Fig. 4). In the absence of the β-subunit, we find that γ no longer associates with the α-subunit. Similarly, in the absence of the γ-subunit, only a trace amount of the β-subunit is found associated with α. These findings indicate that the subunits of the Snf1 heterotrimer show an interdependence for assembly. They also argue strongly against the notion that the Snf1 complex exists as an αγ dimer *in vivo*. Similarly, the kinase activity of the Snf1 complex shows an interdependence on the three subunits. The kinase activity of the purified α-subunit can be stimulated *in vitro* by purified γ-subunit, but only when the β-subunit is also present. If Snf1 were active as an αγ dimer, then one would expect that stimulation of kinase activity by added γ-subunit would not depend on the presence of the β-subunit. This is not observed. Instead, we found that stimulation of the kinase activity by γ requires the β-subunit and that stimulation by β requires γ. Taken together, our data support a model in which the Snf1 kinase complex functions as a heterotrimer, and the assembly and function of the complex shows an interdependence on all three subunits.

The subunit requirements for Sak1 association with the Snf1 kinase complex was also examined. GST pull-down assays indicate that the Sak1 kinase interacts directly with the kinase domain of the α-subunit (Fig. 2) and in the absence of the β- and γ-subunits. However, stable association of Sak1 with the Snf1 kinase *in vivo* requires the presence of the β- and γ-subunits. This discordance between the *in vitro* and *in vivo* binding data is not easily explained. Of the three Snf1-activating kinases, only Sak1 forms a stable complex with Snf1. We show here that in a cell lacking the γ-subunit, Sak1 is unable to form a complex with Snf1 but is still able to phosphorylate the Snf1 activation loop (Fig. 5). At this point, we have been unable to determine the biological significance of the Sak1·Snf1 complex. Perhaps the association of Sak1 and Snf1 allows rapid signal transduction. It is worth noting that the Snf1 activation loop is phosphorylated when glucose-grown cells are collected by centrifugation (12). Presumably, cells in a pellet become glucose-starved, thereby activating the glucose signaling pathway. Further downstream events in this pathway, such as Mig1 phosphorylation, are not observed in cells collected by centrifugation. It is possible that the proximity of Sak1 and Snf1 accounts for this activation loop phosphorylation during centrifugation. In this study, we show that Sak1 is able to phosphorylate the Snf1 activation loop independent of complex formation, although the kinetics of the reaction were not determined. A second possible role of the Sak1·Snf1 complex relates to control of subcellular localization of the Snf1 complex. Sak1 is the only Snf1-activating kinase capable of promoting nuclear localization of the Gal83 isoform of the Snf1 complex (19). Perhaps the Sak1·Snf1 complex is required to promote nuclear localization.

Finally, we examined the role of the Snf1 AID domain and provide additional biochemical evidence that the AID inhibits the kinase activity. The α-subunit lacking the AID has a higher specific activity than the full-length α-subunit. The ability of the β- and γ-subunits to stimulate the activity of the Snf1 kinase depended on the presence of the C-terminal regulatory domain. Taken together, our data support the following model for the Snf1 kinase. The active form of the Snf1 kinase is a heterotrimer, and the effects of the AID can be mitigated by direct interaction with the β- and γ-subunits.

REFERENCES

1. Hardie, D. G., Carling, D., and Carlson, M. (1998) *Annu. Rev. Biochem.* **67,** 821–855
2. Dyck, J. R., Gao, G., Widmer, J., Stapleton, D., Fernandez, C. S., Kemp, B. E., and Witters, L. A. (1996) *J. Biol. Chem.* **271,** 17798–17803
3. Yang, X., Jiang, R., and Carlson, M. (1994) *EMBO J.* **13,** 5878–5886
4. Schmidt, M. C., and McCartney, R. R. (2000) *EMBO J.* **19,** 4936–4943
5. Woods, A., Cheung, P. C., Smith, F. C., Davison, M. D., Scott, J., Beri, R. K., and Carling, D. (1996) *J. Biol. Chem.* **271,** 10282–10290
6. Jiang, R., and Carlson, M. (1997) *Mol. Cell. Biol.* **17,** 2099–2106
7. Iseli, T. J., Walter, M., van Denderen, B. J., Katsis, F., Witters, L. A., Kemp, B. E., Michell, B. J., and Stapleton, D. (2005) *J. Biol. Chem.* **280,** 13395–13400
8. Crute, B. E., Seefeld, K., Gamble, J., Kemp, B. E., and Witters, L. A. (1998) *J. Biol. Chem.* **273,** 35347–35354
9. Leech, A., Nath, N., McCartney, R. R., and Schmidt, M. C. (2003) *Euk. Cell* **2,** 265–273
10. Jiang, R., and Carlson, M. (1996) *Genes Dev.* **10,** 3105–3115
11. Carling, D., Clarke, P. R., Zammit, V. A., and Hardie, D. G. (1989) *Eur. J. Biochem.* **186,** 129–136
12. Wilson, W. A., Hawley, S. A., and Hardie, D. G. (1996) *Curr. Biol.* **6,** 1426–1434
13. Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G., and Hardie, D. G. (2004) *J. Clin. Investig.* **113,** 274–284
14. Nath, N., McCartney, R. R., and Schmidt, M. C. (2003) *Mol. Cell. Biol.* **23,** 3909–3917
15. Hong, S. P., Leiper, F. C., Woods, A., Carling, D., and Carlson, M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 8839–8843
16. Sutherland, C. M., Hawley, S. A., McCartney, R. R., Leech, A., Stark, M. J., Schmidt, M. C., and Hardie, D. G. (2003) *Curr. Biol.* **13,** 1299–1305
17. McCartney, R. R., Rubenstein, E. M., and Schmidt, M. C. (2005) *Curr. Genet.* **47,** 335–344
18. Elbing, K., McCartney, R. R., and Schmidt, M. C. (2006) *Biochem. J.* **393,** 797–805
19. Hedbacker, K., Hong, S. P., and Carlson, M. (2004) *Mol. Cell. Biol.* **24,** 8255–8263
20. Woods, A., Dickerson, K., Heath, R., Hong, S. P., Momcilovic, M., Johnstone, S. R., Carlson, M., and Carling, D. (2005) *Cell Metab.* **2,** 21–33
21. Hurley, R. L., Anderson, K. A., Franzone, J. M., Kemp, B. E., Means, A. R., and Witters, L. A. (2005) *J. Biol. Chem.* **280,** 29060–29066

22. Mitchelhill, K. I., Stapleton, D., Gao, G., House, C., Michell, B., Katsis, F., Witters, L. A., and Kemp, B. E. (1994) *J. Biol. Chem.* **269,** 2361–2364
23. Nath, N., McCartney, R. R., and Schmidt, M. C. (2002) *J. Biol. Chem.* **277,** 50403–50408
24. Caspary, F., Shevchenko, A., Wilm, M., and Seraphin, B. (1999) *EMBO J.* **18,** 3463–3474
25. McCartney, R. R., and Schmidt, M. C. (2001) *J. Biol. Chem.* **276,** 36460–36466
26. Rubenstein, E. M., McCartney, R. R., and Schmidt, M. C. (2006) *Euk. Cell* **5,** 620–627
27. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
28. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999) *Nat. Biotech.* **17,** 1030–1032
29. Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K., and Weissman, J. S. (2003) *Nature* **425,** 737–741
30. Ludin, K., Jiang, R., and Carlson, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6245–6250
31. Hardie, D. G. (2004) *J. Cell Sci.* **117,** 5479–5487
32. Erickson, J. R., and Johnston, M. (1993) *Genetics* **135,** 655–664