

## Activation and inhibition of Snf1 kinase activity by phosphorylation within the activation loop



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### ABSTRACT

The AMP-activated protein kinase is a metabolic regulator that transduces information about energy and nutrient availability. In yeast, the AMP-activated protein kinase, called Snf1, is activated when energy and nutrients are scarce. Earlier studies have demonstrated that activation of Snf1 requires the phosphorylation of the activation loop on threonine 210. Here we examined the regulation of Snf1 kinase activity in response to phosphorylation at other sites. Phosphoproteomic studies have identified numerous phosphorylation sites within the Snf1 kinase enzyme. We made amino acid substitutions in the Snf1 protein that were either non-phosphorylatable (serine to alanine) or phospho-mimetic (serine to glutamate) and examined the effects of these changes on Snf1 kinase function *in vivo* and on its catalytic activity *in vitro*. We found that changes to most of the phosphorylation sites had no effect on Snf1 kinase function. However, changes to serine 214, a site within the kinase activation loop, inhibited Snf1 kinase activity. Snf1-activating kinase 1 still phosphorylates Snf1-S214E on threonine 210 but the S214E enzyme is non-functional *in vivo* and catalytically inactive *in vitro*. We conclude that yeast have developed two distinct pathways for down-regulating Snf1 activity. The first is through direct dephosphorylation of the conserved activation loop threonine. The second is through phosphorylation of serine 214.

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### 1. Introduction

The AMP-activated protein kinase (AMPK) is an ancient and highly conserved serine/threonine protein kinase that responds to nutrient and energy stress. When activated, AMPK restores energy balance by inhibiting processes that consume ATP and activating processes that produce ATP [1]. In yeast, the AMPK enzyme was identified by its requirement for growth by fermentation of sucrose [2] and was named Snf1 for Sucrose Non-Fermenting 1. The yeast enzyme is required for growth on many alternative carbon sources. The activity of AMPK is regulated primarily by the phosphorylation state of its activation loop threonine [T210 in yeast, T172 in mammals [3]]. Phosphorylation of the yeast activation loop threonine is catalyzed primarily by Sak1 (Snf1 Activating Kinase 1) and dephosphorylated by the PP1 phosphatase Glc7 in a complex with the Reg1 protein [4,5]. The phosphorylation status of Snf1 is

determined by the rate of dephosphorylation [6]. Under conditions of energy stress, AMPK enzymes bind low energy adenylate nucleotides (AMP and ADP) and adopt a phosphatase resistant conformation [7,8].

While phosphorylation of the AMPK activation loop is the primary site of regulation, additional phosphorylation sites in AMPK have been identified that act to fine tune its regulation. The serine residue adjacent to the conserved activation loop threonine, serine 173 (S173) in mammals, is phosphorylated by protein kinase A [9]. This modification impedes that activity of the LKB1 kinase, the mammalian kinase primarily responsible for activating AMPK by phosphorylation of T172. The down-regulation of AMPK by modification of S173 may play an important role in the release of free fatty acids from adipose tissue in response to PKA signaling. Another site of mammalian AMPK regulation is at S487. This site is phosphorylated by Akt and also inhibits the ability of LKB1 to activate AMPK [10]. In tumor cells with hyperactivated Akt, the down-regulation of AMPK may provide a selective growth advantage.

In yeast, little is known about the regulation of Snf1 kinase by phosphorylation at sites other than the activation loop threonine. The yeast Snf1 kinase is a heterotrimer composed of the catalytic  $\alpha$  subunit (Snf1), the nucleotide-binding  $\gamma$  subunit (Snf4) and one of three alternative  $\beta$  subunits (Gal83, Sip1 or Sip2). The different isoforms of the Snf1 are determined by which  $\beta$  subunit is incorporated into the heterotrimer. We and others have shown that the  $\beta$  subunits specify

*Abbreviations:* SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; MBP, myelin basic protein; PKA, protein kinase A; HA, hemagglutinin; GST, glutathione S-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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substrate specificity and subcellular localization [11,12]. The  $\beta$  subunits are themselves heavily phosphorylated by both Snf1 and other kinases. However, we have mapped the phosphorylation sites in Gal83 to the N-terminus (residues 12–141) and shown that deletion of this region has little effect on Snf1 kinase function in vivo [13]. In the present study, we turn our attention to phosphorylation sites within the Snf1 protein, the catalytic  $\alpha$  subunit.

## 2. Materials and methods

### 2.1. Yeast strains and genetic methods

The yeast strains used in this study were all derivatives of S228C. Yeast strains with specific gene deletions were generated by the *Saccharomyces* Genome Deletion project [14] and purchased from Thermo Scientific. Cells were grown at 30 °C using standard media lacking nutrients needed for plasmid selection [15]. Yeast growth assays were conducted by diluting overnight cultures to an OD<sub>600</sub> of 0.2 and spotting 3  $\mu$ l of 10-fold serial dilutions onto agar plates with the various carbon sources as indicated.

### 2.2. SNF1 mutations

The wild type *SNF1* allele and all derivatives used in this study contained 3 copies of the hemagglutinin tag (3HA) at the C-terminus [3]. Mutations in the *SNF1* gene were generated by oligonucleotide-directed mutagenesis with Pfu polymerase followed by *DpnI* digestion of the plasmid template [16]. All of the mutations were confirmed by DNA sequencing. Mutations resulting in single amino acid changes are denoted by the original residue, its position in the Snf1 primary sequence followed by the new residue. The single residue changes made were T210E, S211C, S211E, S214A, S214E, S214C, S214L, S214N, S214R, S214Y, S487A and S487E. Double mutations of nearby residues were made as follows: S411,413A; S411,413E; S630,632A; and S630,632E. A cluster of 5 phosphorylated sites (S401, S403, T408, S411 and S413) were all changed to alanine (S5A) or glutamate (S5E).

### 2.3. Proteins and purifications

Bovine protein kinase A (PKA) was purchased from Sigma (P2645). Yeast PKA was purified as a glutathione S-transferase (GST) fusion from yeast cells [17]. Mig1 protein (amino acids 202–414) and Snf1 kinase domain (amino acids 1–392) were fused to the C-terminus of GST and purified from bacteria [4,18]. Snf1 heterotrimers containing Snf1, Snf4 and Gal83 were co-expressed in bacteria and purified by nickel chromatography [7].

### 2.4. Western blotting

Snf1 tagged with three copies of the HA epitope was detected with a 1:3000 dilution of HA probe (Santa Cruz). Goat anti-mouse IgG DyLight 680 (Thermo) diluted 1:10,000 was used as the secondary antibody. For detection of phosphorylated Snf1, Phospho-AMPKalpha (Thr172) antibody (Cell Signaling) diluted 1:1000 was used. Goat anti-rabbit IRDye 800CW (Li-Cor) (1:10,000 dilution) was used as the secondary antibody. Blots were scanned by using an Odyssey scanner (Li-Cor). Integrated intensity values of bands were quantified by using Odyssey scanning software. Yeast extracts were prepared using the boiling method described by Kuchin and colleagues [19].

### 2.5. Kinase assays

In vitro kinase reactions (10  $\mu$ l) contained 0.2 mM [ $\gamma$ -<sup>32</sup>P]ATP (1000 cpm/pmol), 20 mM HEPES, pH 7.0, 0.1 M NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 5 mM magnesium acetate and substrate proteins at approximately 50  $\mu$ g/ml. The reactions were started by addition of

protein kinase enzymes, incubated at 30 °C for 30 min and stopped by the addition of SDS sample buffer. Proteins were resolved on an SDS-polyacrylamide gel. Gels were dried and examined by autoradiography. Quantitative kinase assays were conducted using the SAMS peptide [20].

### 2.6. Statistical analysis

For all bar plots, mean values using a minimum of three independent measurements are plotted with error bars representing one standard error. Statistical significance was determined using the student *t*-test for unpaired variables with equal variance unless otherwise indicated. Statistical significance is indicated as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; ns,  $p > 0.05$ .

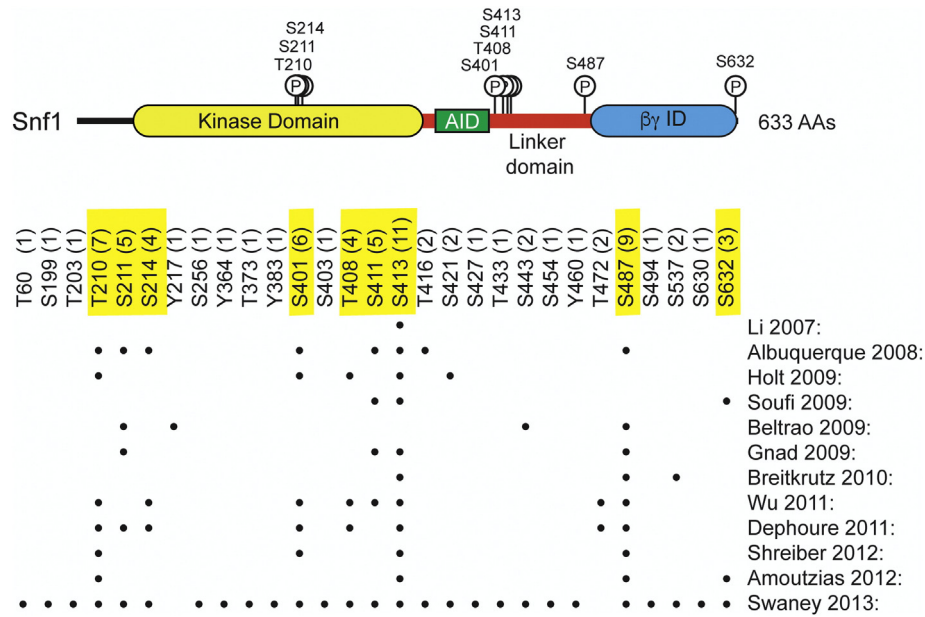
## 3. Results

### 3.1. Identification of phosphorylation sites within the Snf1 kinase complex

The advent of sophisticated methods of mass spectrometry and phosphopeptide purification has allowed the global analysis of phosphorylation sites in the yeast proteome [21–32]. We analyzed 12 datasets with a particular interest in the identification of phosphorylation events within the Snf1 protein (Fig. 1) as well as the other subunits of the Snf1 kinase complex (Appendix A). A total of 29 phosphorylation sites within the Snf1 protein were identified in these studies. Nine of the 29 sites observed in the Snf1 protein were detected in three or more of the studies. One site, the activation loop threonine 210 site, has been studied intensively and is known to be required for Snf1 kinase activity [3]. The other phosphorylation sites have not been studied nor have the regulatory consequences of phosphorylation at those residues.

### 3.2. Effect of mutations in Snf1 phosphorylation sites on growth

In order to determine the consequences of phosphorylation of the Snf1 protein, we engineered mutations in the *SNF1* gene that either blocked phosphorylation (serine/threonine to alanine) or that mimicked phosphorylation (serine/threonine to glutamate). Mutations were engineered in all nine of the sites in Snf1 that were detected in at least three phosphoproteomic studies (Fig. 1). Cells lacking Snf1, expressing wild type Snf1 or expressing a mutant Snf1 protein were assayed for the ability to grow on alternative carbon sources known to require Snf1 function (Fig. 2A). In the presence of a functional Snf1 kinase, cells are able to grow by fermentation of sucrose and raffinose and by aerobic metabolism on a mixture of glycerol and ethanol. In the absence of Snf1, cells can grow on glucose as the carbon source but are completely unable to grow on the other carbon sources. Most of the phosphorylation site mutations had no impact on the ability of the mutant Snf1 kinase to support growth on alternative carbon sources. The only mutations that had large effects on cell growth were mutations of threonine 210 and serine 214. Threonine 210 is the activation loop site known to be required for Snf1 activation [3]. Changing this residue to alanine completely inactivates the Snf1 kinase and produces a growth phenotype indistinguishable from the complete deletion of the *SNF1* gene. Changing threonine 210 to glutamate creates a hypomorphic allele with reduced but detectable function on sucrose media. Changing serine 211, the residue adjacent to threonine 210, had no effect on Snf1 function, regardless of whether the serine was changed to non-phosphorylatable residues (alanine or cysteine) or to the phosphomimetic (glutamate). This result was surprising given the fact that phosphorylation of the analogous residue inactivates the mammalian enzyme [9]. Changes in the serine 214 residue did have profound effects. The S214A mutation generated a hypomorphic allele with reduced activity. The S214E mutation completely inactivated the Snf1 kinase in this assay producing a growth phenotype indistinguishable from the complete deletion of the



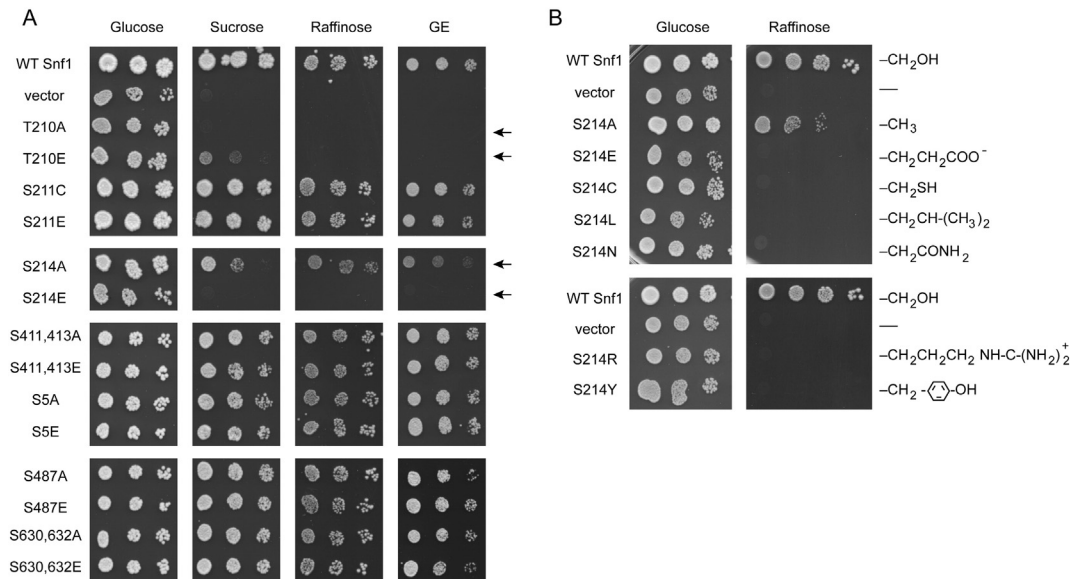
**Fig. 1.** Phosphorylation sites identified in the Snf1 kinase protein. A schematic representation of the Snf1 protein is shown with the location of functional domains as well as phosphorylation sites identified by mass spectrometry. AID, auto-inhibitory domain;  $\beta\gamma$  ID,  $\beta\gamma$  subunit interaction domain. Table below shows the number of times each phosphorylation site was identified in 12 phosphoproteomic studies [21–32]. Sites shaded in yellow were identified in at least three independent analyses.

*SNF1* gene. We made a series of amino acid substitutions to determine which chemical properties of the S214 side chain were required for kinase activity (Fig. 2B). Removing the hydroxyl group from the serine (S214A) was the only change at this position that was tolerated. S214A is a hypomorphic allele but growth on raffinose is clearly detectable. Five other changes tested at this position (C, L, N, R and Y) completely inactivated Snf1 function as judged by the lack of growth on raffinose media. These data suggest that any change at this position, including

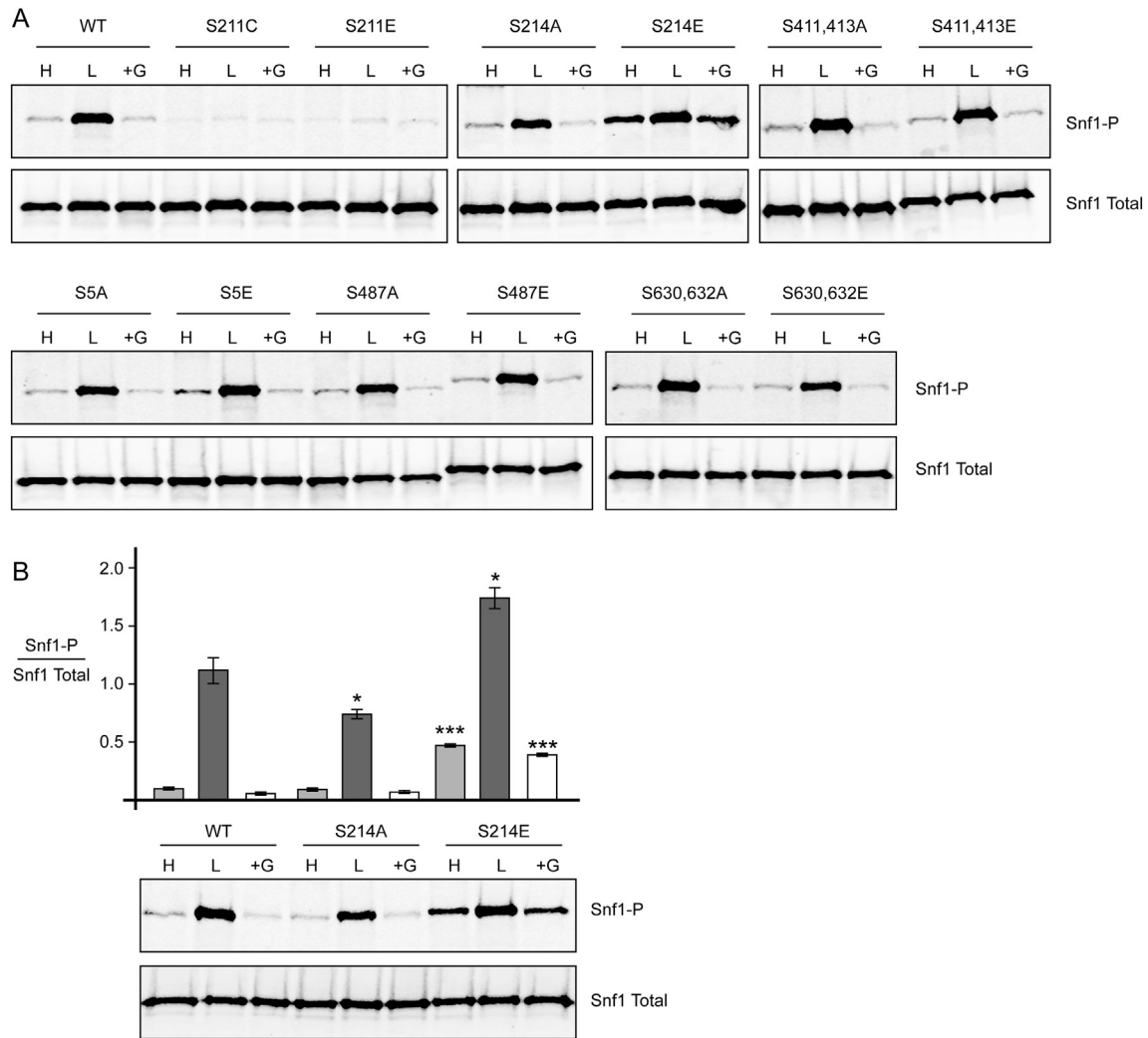
phosphorylation of the cognate serine residue, inactivates the Snf1 kinase.

### 3.3. Effect of Snf1 mutations on activation loop phosphorylation in vivo

One hallmark of Snf1 regulation is the phosphorylation status of the conserved activation loop threonine. We examined our panel of Snf1 phosphorylation site mutations for an effect on T210 phosphorylation



**Fig. 2.** Growth assays for cells expressing Snf1 with mutations in phosphorylation sites. (A) Cells lacking the *SNF1* gene were transformed with CEN plasmids expressing the Snf1 protein with the indicated amino acid substitutions. Sites were changed singly or in pairs (411 with 413 and 630 with 632). S5A and S5E mutants contained five amino acid changes to either Ala or Glu at residues S401, S403, T408, S411 and S413. A dilution series of cells was spotted onto media containing either glucose, sucrose, raffinose or glycerol/ethanol (GE) as the carbon source. Plates were incubated at 30 °C and photographed. Arrows indicate mutants with growth phenotypes distinguishable from wild type Snf1. (B) Cell growth was measured as in panel A using Snf1 proteins with different amino acid substitutions at S214. Chemical structures of the amino acid side chains are shown on the right.

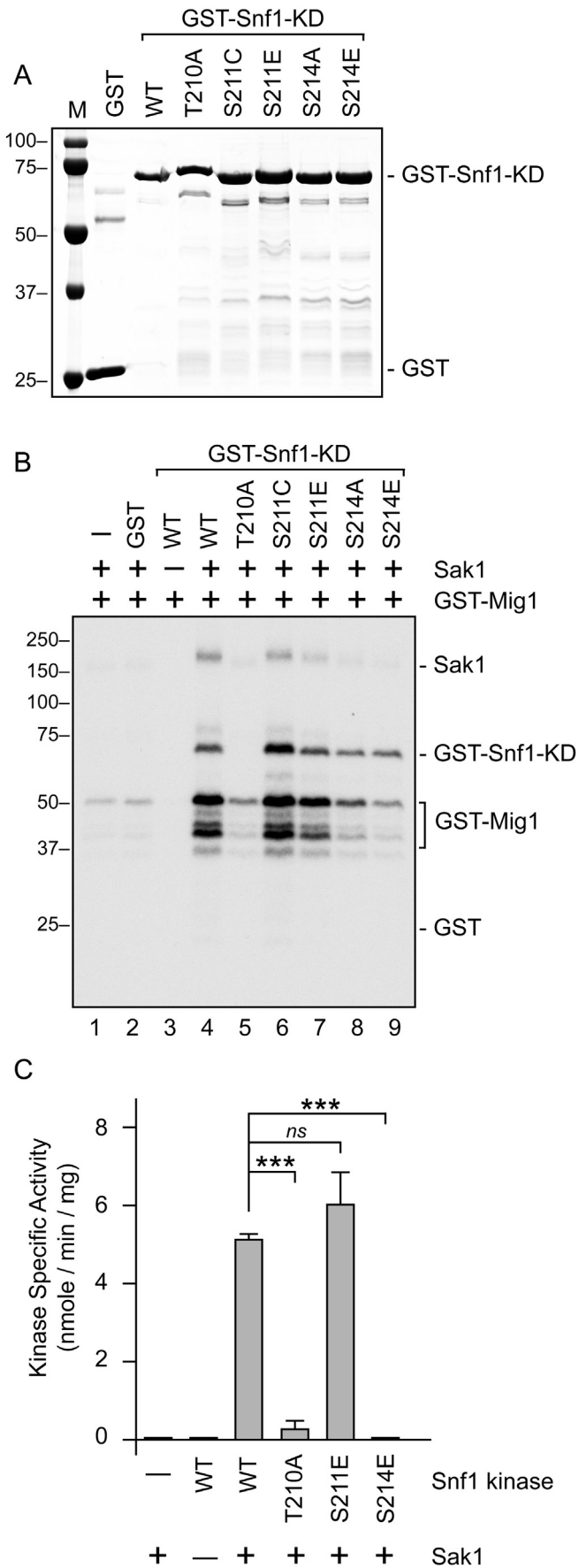


**Fig. 3.** Effect of phosphorylation site mutations on the phosphorylation of Snf1 activation loop threonine 210. (A) Cells expressing wild type (WT) Snf1 or a Snf1 allele with the amino acid changes shown were grown in high glucose (H), shifted to low glucose for 1 h (L) followed by addition of glucose (+G) for 15 min. Extracts were prepared and examined by western blotting using antibodies specific for Snf1 phosphorylated on threonine 210 (Snf1-P) or with HA antibodies (Snf1 Total). (B) Cells expressing wild type Snf1, Snf1-S214A or S214E were grown as above and extracts were prepared and assayed in triplicate. The mean value  $\pm$  SE of the ratio of phosphorylated Snf1 over total Snf1 is plotted. Representative blots are shown below. Values that show statistically significant differences from wild type values are indicated.

status. Normally, cells have low level of T210 phosphorylation when grown in high glucose, increased levels of T210 phosphorylation after shifting to low glucose and a rapid reduction in T210 phosphorylation when glucose is added back. We measured total Snf1 and phosphorylated Snf1 by quantitative western blotting (Fig. 3). None of the phosphorylation site mutants had any significant effect on the accumulation of total Snf1 protein, although some mutations (S411,413E and S487E) affected the gel mobility of the Snf1 protein. Most of the mutations had no effect on the glucose-regulated changes in T210 phosphorylation. The mutations that showed large effects on T210 phosphorylation were those that made changes in the activation loop (S211C, S211E, S214A and S214E). Changes in S211 to either cysteine or glutamate completely eliminated reactivity with the phospho-Snf1 antibody. Since the S211C and S211E mutations had no effect on Snf1 function in growth assays (Fig. 2), and since S211 is included in the peptide antigen used to generate the phospho-Snf1 antibody, we suspect that loss of detectable phosphorylation on T210 is due to alteration in the epitope and not a reduction in phosphorylation. Additional evidence to support this interpretation is presented below. Changes in the S214 residue had different effects on T210 phosphorylation. The S214A

mutation seemed to reduce phosphorylation at T210 while the S214E mutation seemed to increase phosphorylation on T210. We repeated this analysis by analyzing T210 phosphorylation from three independent cultures expressing wild type Snf1, S214A and S214E. Total Snf1 protein and phosphorylated on T210 was measured by quantitative western blotting (Fig. 3B). Changing serine 214 to alanine caused a small but reproducible reduction in T210 phosphorylation. Changing serine 214 to glutamate caused a significant increase in T210 phosphorylation under all growth conditions. Basal T210 phosphorylation of the S214E mutant in high glucose cultures were increased 4.7 fold relative to wild type Snf1 while after shifting to low glucose, the T210 phosphorylation of the S214E mutant was increased by 50%. Normally, phosphorylation of T210 correlates well with Snf1 activity. In the case of the S214E mutant, the level of T210 phosphorylation is higher than that observed with the wild type protein yet Snf1-S214E is completely inactive in growth assays (Fig. 2). We cannot rule out the possibility that the S214E change increases the avidity of the phospho-Snf1 antibody. However, we do present in vitro kinase data demonstrating that the Snf1-214E enzyme is phosphorylated on threonine 210 by Sak1 in vitro and yet is catalytically inactive.





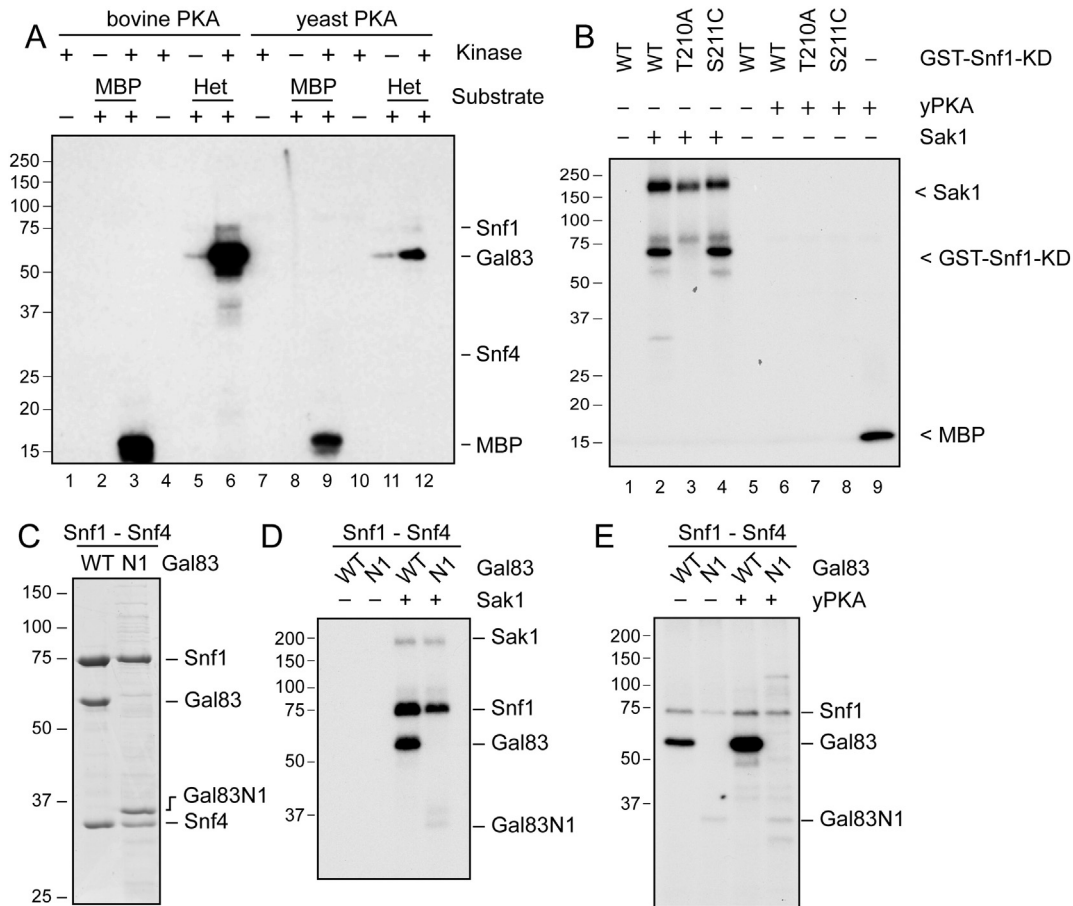
### 3.4. Effect of activation loop mutations on in vitro phosphorylation and activation of Snf1

In order to better characterize the effects of the activation loop mutations, we expressed each Snf1 kinase domain as a GST fusion. These proteins were expressed in bacteria and purified by affinity chromatography (Fig. 4A). The Snf1 kinase domains were then introduced to in vitro kinase assays with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , Snf1-activating kinase 1 (Sak1) and a recombinant substrate, GST-Mig1. In this assay we were able to determine whether the Snf1 kinase domain mutants were substrates for Sak1 and also whether they could be activated in vitro as measured by phosphorylation of the Mig1 protein. In the absence of Sak1, the bacterially expressed Snf1 kinase domain is inactive (Fig. 4B, lane 3). Incubation of the wild type Snf1 kinase domain with Sak1 results in the phosphorylation of the Snf1 kinase domain. This phosphorylation event occurs on threonine 210 since the T210A mutation completely abrogates incorporation of  $^{32}\text{P}$  into the Snf1 kinase domain (compare lanes 4 and 5). Sak1-mediated phosphorylation of Snf1 kinase domain on T210 results in Snf1 kinase activation as judged by the incorporation of  $^{32}\text{P}$  into the GST-Mig1 protein. Sak1 by itself has very little activity toward GST-Mig1 (lane 1) and no activity toward GST (lane 2). When wild type Snf1 kinase domain is incubated with Sak1, the incorporation of  $^{32}\text{P}$  into GST-Mig1 increases greatly and the increased incorporation relative to Sak1 alone is a measure of Snf1 kinase activation. When the activation loop mutant proteins were analyzed in this in vitro kinase assay, we found that the changes at serine 211 had little if any effect on the Sak1-mediated phosphorylation of Snf1 on T210 or on the activation of the Snf1 kinase as judged by the phosphorylation of GST-Mig1 (lanes 6 and 7). Therefore, we conclude that the Snf1-S211C and Snf1-S211E kinases are substrates for Sak1 and are themselves catalytically active and able to phosphorylate Mig1. These results are consistent with the growth assays that showed a Snf<sup>+</sup> phenotype for both S211C and S211E mutants (Fig. 2).

A very different result was observed with the mutants having alterations at serine 214. The S214A mutant was phosphorylated by Sak1 but showed greatly reduced kinase activity toward Mig1 (Fig. 4, lane 8). The S214E mutant was phosphorylated by Sak1 but showed no phosphorylation of Mig1 above what is seen catalyzed by Sak1 alone (lane 9). Therefore, serine 214 is not required for recognition by Sak1 and changes in serine 214 do not prevent phosphorylation at threonine 210. However, serine 214 is required for full activation of the Snf1 kinase domain. Changing serine 214 to alanine reduces activation potential while changing to glutamate completely inactivates the kinase, even though it is phosphorylated on threonine 210. These in vitro results are consistent with the in vivo results that showed the S214E mutant as inactive in growth assays (Fig. 2) but hyperphosphorylated at T210 (Fig. 3).

For a more quantitative measure of Snf1 kinase activity, we measured  $^{32}\text{P}$  incorporation into the SAMS peptide [20]. The wild type GST-Snf1 kinase domain readily phosphorylated the SAMS peptide with a specific activity of  $5.1 \pm 0.1$  nmol/min/mg (Fig. 4C). Snf1 activity was Sak1-dependent while the Sak1 enzyme showed no activity toward the SAMS peptide by itself. When the Snf1 variants were assayed, we found that the T210A and S214E mutations inactivated the enzyme while the S211E variant showed essentially wild

**Fig. 4.** Phosphorylation and activity of Snf1 activation loop mutants. (A) Snf1 kinase domains (KD) were expressed in bacteria and purified as GST fusion proteins. The wild type kinase domain (WT; residues 1–392) as well as proteins with the single amino acid changes indicated were resolved on an SDS gel and stained with Coomassie blue. (B) In vitro kinase assays with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and GST-Mig1 were incubated with Sak1 kinase purified from yeast (except lane 3) and the indicated GST-fusion protein. The gel mobility of molecular weight markers (left) and of the kinases and substrates (right) are indicated. (C) In vitro kinase assay with the SAMS peptide using the GST-Snf1 kinase domains and Sak1 as indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



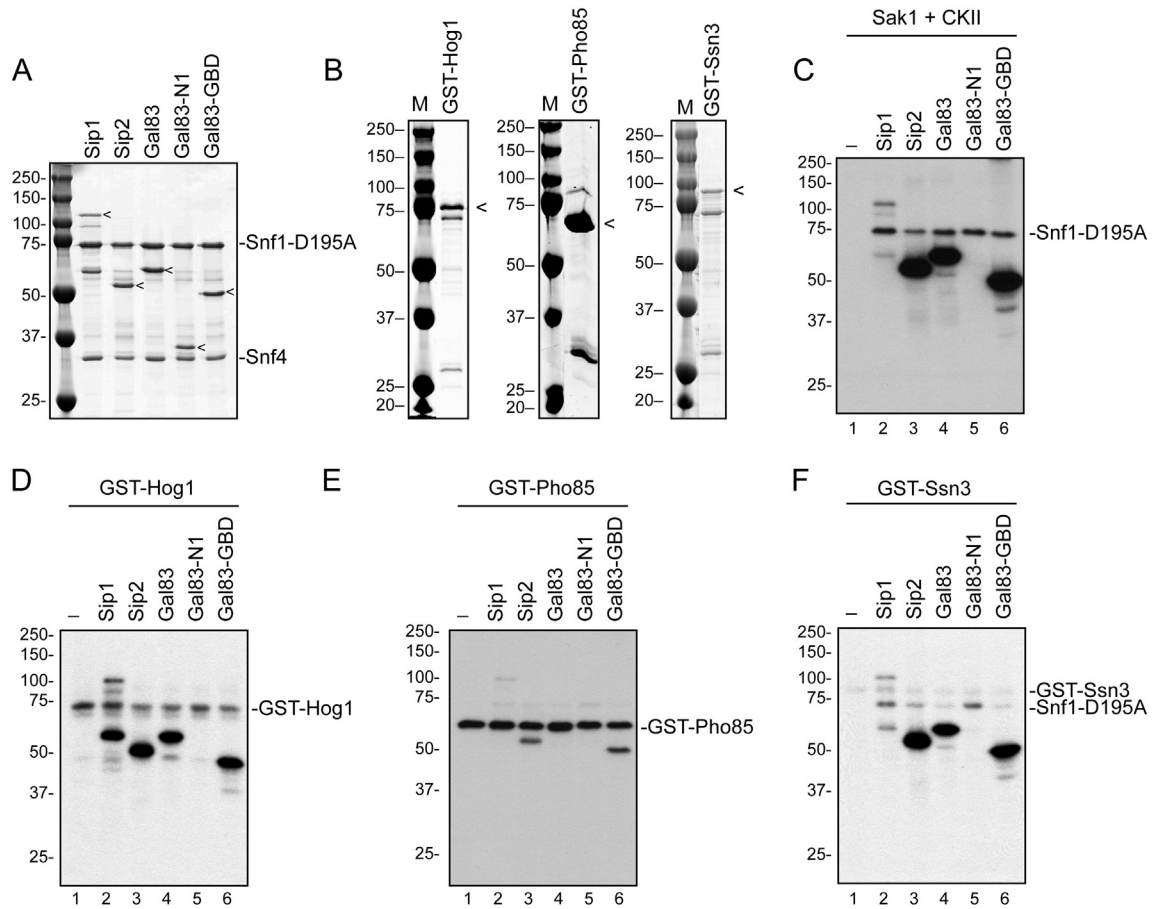
**Fig. 5.** Phosphorylation of Snf1 complex heterotrimer by protein kinase A. (A) In vitro kinase assays containing commercial bovine PKA or purified yeast PKA, [ $\gamma$ - $^{32}$ P]ATP and either myelin basic protein (MBP) or Snf1 kinase heterotrimers (Het) purified from bacteria as indicated. Phosphorylation of proteins was determined by autoradiography. (B) In vitro kinase assays containing [ $\gamma$ - $^{32}$ P]ATP and Sak1 or PKA kinases purified from yeast as indicated. Substrates were the GST-Snf1 kinase domain purified from bacteria (KD; residues 1–392) with the indicated amino acid substitutions or MBP (lane 9). (C) Coomassie stained gel of purified Snf1 heterotrimers containing Snf1, Snf4 and Gal83 or Gal83-N1 with a deletion of residues 12–140. (D) In vitro kinase assay with Snf1 heterotrimers incubated with and without Sak1. (E) In vitro kinase assay with Snf1 heterotrimers incubated with and without yeast PKA.

type levels of activity. Therefore, the T210 and S214 residues are essential for catalytic activity.

### 3.5. Phosphorylation of the Snf1 kinase heterotrimer by PKA

Studies with AMPK, the mammalian ortholog of Snf1, have indicated that PKA phosphorylates the AMPK activation loop and inactivates the enzyme [9]. The site that is targeted by mammalian PKA is the equivalent of the yeast serine 211, the serine residue adjacent to the conserved activation loop threonine (serine 173 in mammals). While our data suggest that inactivation of Snf1 may involve serine 214 and not 211, we wanted to test whether the Snf1 kinase was a substrate for PKA. We purified unphosphorylated Snf1-Snf4-Gal83 heterotrimer from bacteria and incubated it with [ $\gamma$ - $^{32}$ P]ATP and PKA, either commercial bovine PKA or our own affinity purified yeast PKA (Fig. 5A). Both PKA preparations were catalytically active as judged by their ability to phosphorylate myelin basic protein (MBP), a serine-rich, non-specific kinase substrate (lanes 3 and 9). When incubated with the Snf1 heterotrimer, both PKA preparations phosphorylated the Gal83 subunit with only very limited incorporation into the Snf1 subunit. The Snf4 subunit is not phosphorylated at all in these reactions. To determine if the Snf1 kinase domain was recognized by PKA, we incubated Sak1 and yeast PKA with the Snf1 kinase domain (residues 1–392) purified from bacteria as a GST fusion (Fig. 5B). The Sak1 kinase phosphorylates itself as well as the Snf1 kinase domain. The

site recognized by Sak1 is threonine 210 since the T210 A substitution blocks the phosphorylation by Sak1 (lane 3). Yeast PKA is active since it phosphorylates MBP (lane 9) but it fails to recognize the Snf1 kinase domain with or without the S211 site. Therefore, the weak activity of PKA toward the Snf1 subunit observed in the context of the Snf1 heterotrimer (Fig. 5A and E) must be localized to the C-terminal half of the Snf1 protein. The preferred PKA phosphorylation sites reside in the Gal83 subunit. We purified the Snf1 heterotrimer containing the Gal83-N1 protein (Fig. 5C) which lacks residues 12–141. In vitro kinase assays from our lab [13] as well as numerous mass spectrometry studies of the yeast phosphoproteome [21–26,28–32] have localized the primary phosphorylation sites to this N-terminal region of Gal83 (Appendix A). When incubated with Sak1 kinase, we detect incorporation of labeled phosphate into the Snf1 subunit and the full-length Gal83 subunit (Fig. 5D). The Gal83-N1 protein is not phosphorylated in this reaction indicating that the sites recognized by Sak1 and/or Snf1 lie in the Gal83 N-terminus. When the Snf1 heterotrimer is incubated with [ $\gamma$ - $^{32}$ P]ATP, we detect incorporation of labeled phosphate into the Snf1 subunit itself and into full-length Gal83 (Fig. 5E). This activity is due to autophosphorylation from the Snf1 kinase and requires a long exposure since the Snf1 has not been activated by Sak1. When yeast PKA is added into the reaction, we detect an increase in the incorporation of labeled phosphate into the full-length Gal83 protein but not Snf1 or Gal83-N1. Some variation in the levels of Snf1 autophosphorylation is observed and suggests that the Gal83 protein may affect this activity. The primary conclusions from



**Fig. 6.** Phosphorylation of Snf1 kinase by proline-directed kinases Hog1, Pho85 and Ssn3. (A) Coomassie stained gel of Snf1 heterotrimers purified from bacteria. The Snf1 kinase subunit contained the active site mutation D195A. The beta subunit was either Sip1, Sip2, Gal83, Gal83-N1 or Gal83-GBD as indicated. The mobility of the beta subunit is indicated. (B) Coomassie stained gels of proteins purified from yeast as GST fusions to the kinases Hog1, Pho85 and Ssn3. (C) In vitro kinase assay of the kinase dead Snf1 heterotrimers incubated with Sak1. TAP-purified Sak1 also contains CKII kinase [18]. (D, E and F) In vitro kinase assays with the kinase dead Snf1 heterotrimers incubated with the indicated yeast kinase.

these experiments are that PKA does phosphorylate the Snf1 kinase heterotrimers but the site(s) preferred by PKA are on the N-terminus of Gal83 and not the Snf1 kinase domain.

### 3.6. Phosphorylation of the Snf1 kinase heterotrimer yeast kinases Hog1, Pho85 and Ssn3

Three of the commonly detected phosphorylation sites on Snf1, S214, S413 and S487, are directly adjacent to proline residues (Fig. 8). S214 is in the activation loop of the Snf1 kinase, a region showing little amino acid variation even when Snf1 is compared to the alpha subunits from diverse species such as plants, vertebrates and nematodes. In contrast, the S413 and S487 sites are located in the highly variable linker domain (Fig. 1), a region that shows little primary sequence conservation with metazoans. However, multiple sequence alignment of this region with other fungal species shows that the S413 site is conserved within fungi (Fig. 8B) while the S487 site is not even conserved between fungal species (Fig. 8C). Analysis of protein kinase recognition motifs using peptide arrays found that most yeast kinases, including Snf1 and PKA, show a preference for sites near basic residues, especially arginine [33]. A smaller subset of kinases shows a strong preference for serine residues directly followed by proline. These proline-directed kinases are limited to members of the cyclin-dependent protein kinase and MAP kinase families. We took a candidate approach to attempt the identification of the S214, S413 and S487 kinases. We generated GST fusions to the Pho85 and Ssn3 cyclin dependent kinases and to the Hog1 MAP

kinase. We chose these three kinases since each had documented genetic and/or biochemical interactions with Snf1 [34–37]. For substrates, we purified from bacteria the unphosphorylated Snf1 heterotrimer with different beta subunits, including two deletions mutants of the Gal83 protein (Fig. 6A). The Snf1 subunit contained an amino acid substitution, D195A that greatly reduces its catalytic activity. In this way, we could use Snf1 as the substrate and not be concerned with its own catalytic activity. The Hog1, Pho85 and Ssn3 proteins were purified from yeast as GST fusions (Fig. 6B). As a control, we incubated the Snf1 heterotrimers with the Sak1 kinase TAP-purified from yeast. Earlier studies showed that the Sak1-TAP preparation also contains trace amounts of the casein kinase II [18]. The Sak1 kinase preparation most efficiently phosphorylated the Sip2, Gal83 and Gal83-GBD proteins. Detectable phosphorylation was also observed in the Snf1 subunit and to a lesser extent, the Sip1 protein (Fig. 6C). The Hog1 kinase autophosphorylates itself and efficiently phosphorylates the Sip2, Gal83 and Gal83-GBD proteins but not the Gal83-N1 protein, indicating that the site(s) in Gal83 recognized by Hog1 were in the N-terminal region missing from Gal83-N1 (Fig. 6D). Hog1 also phosphorylates a protein in the Sip1 heterotrimer preparation that we tentatively identify as a proteolytic fragment of the Sip1 protein. We cannot rule out the possibility that Hog1 weakly phosphorylates the Snf1 protein since the GST-Hog1 and Snf1 protein co-migrate on this gel. However, we do not detect an increase in the radioactivity incorporated in the GST-Hog1 band with and without Snf1 (Fig. 6D, lanes 1 and 2) suggesting that any phosphorylation of Snf1 would be minor compared to that observed for the  $\beta$  subunits. The Pho85 kinase autophosphorylates



itself and also clearly phosphorylates the Sip2 and Gal83-GBD protein (Fig. 6E). We suspect that the Gal83 protein is also recognized by Pho85 but it co-migrates with GST-Pho85 making it difficult to resolve the radioactive signals from these two proteins. The Ssn3 kinase efficiently phosphorylates the Sip2, Gal83 and Gal83-GBD proteins (Fig. 6F). Ssn3 also shows weak but unambiguous phosphorylation of the Snf1-D195A subunit.

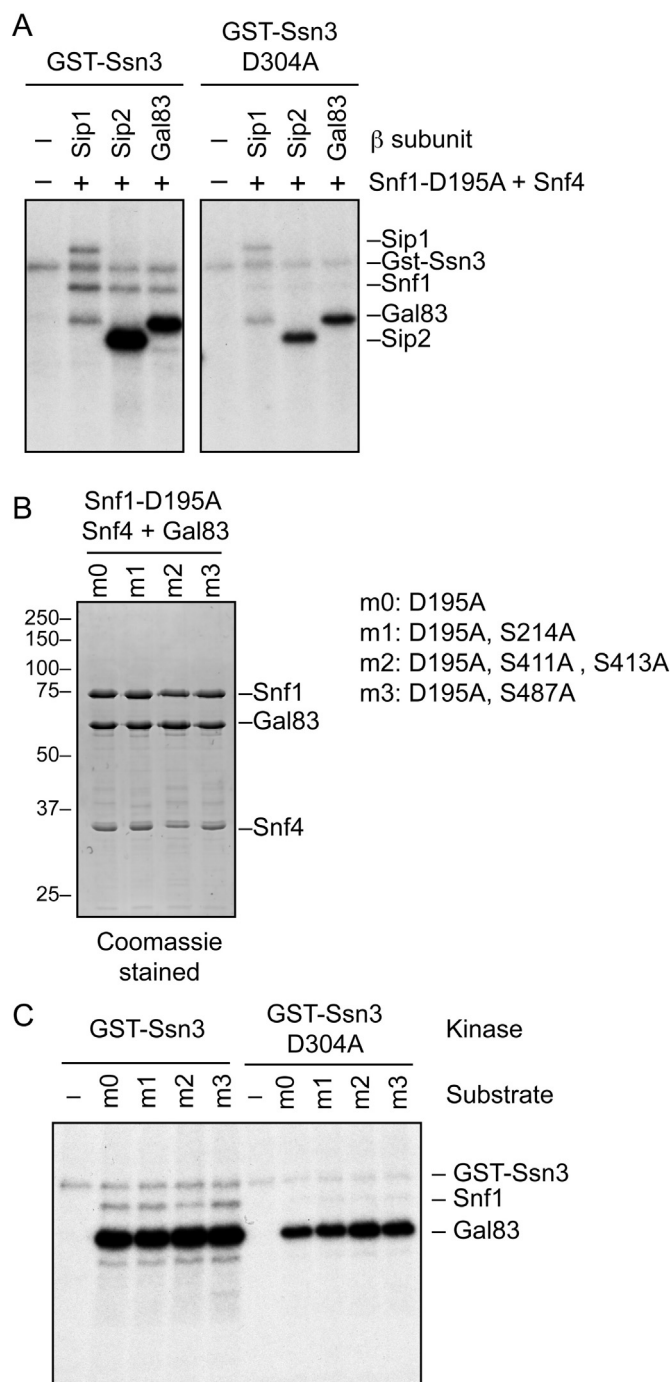
### 3.7. Phosphorylation of the Snf1 subunit by Ssn3

We next examined the catalytic activity and specificity of the Ssn3 kinase. Since we were using an Ssn3 kinase preparation that was purified from yeast, we were concerned that the weak but detectable activity toward the Snf1 subunit might be coming from some other kinase contaminating our Ssn3 preparation. To test this possibility, we introduced a point mutation (D304A) into the active site of the Ssn3 kinase domain. The wild type Ssn3 and the catalytically inactive Ssn3-D304A forms were purified from yeast and tested *in vitro* for the ability to phosphorylate the Snf1 subunit (Fig. 7A). Introduction of the D304A mutation greatly reduced the activity of the Ssn3 kinase toward the Snf1 subunit. We note that this mutation did not eliminate all activity of this kinase preparation. Since the aspartate 304 residue plays a direct role in the catalytic mechanism of phosphate transfer in protein kinases [38], we think it likely that some other kinase is in fact a contaminant in the GST-Ssn3 preparations. However, since the D304A mutation causes a large reduction in the phosphorylation of the Snf1 subunit, we conclude that Ssn3 directly phosphorylates the Snf1 protein *in vitro*. In an attempt to identify the site on Snf1 protein that is recognized by Ssn3, we purified inactive Snf1 kinase heterotrimers with different serine to alanine mutations at the proline-directed sites S214, S413 (along with the nearby S411) and S487 (Fig. 7B). When incubated with active and inactive Ssn3 preparations, all of the heterotrimers were phosphorylated by active Ssn3 but not the Ssn3-D304A enzyme (Fig. 7C). Therefore, Ssn3 does not utilize S214, S413 or S487 as the sole recognition sites.

## 4. Discussion

Most protein kinases are themselves subject to phosphorylation. In some cases, this is due to auto-phosphorylation while in other cases it is due to trans-phosphorylation catalyzed by distinct protein kinases. Phospho-proteomic studies in yeast have identified many phosphorylation sites in the Snf1 protein (Fig. 1). Here we used mutagenesis to assess whether these modification sites affected Snf1 kinase function *in vivo* (Fig. 2). The most commonly detected phosphorylation sites on the Snf1 protein, S413 and S487, could be changed to alanine or glutamate with no functional consequences. The only phosphorylation sites that had an effect on Snf1 function were in the activation loop of the kinase. One site in the activation loop, T210, has been studied intensively and is required for Snf1 kinase activity [3]. Commercial antibodies that detect phosphorylation at this site are routinely used to monitor the activation state of yeast Snf1 and mammalian AMPK.

We investigated the effects of alterations at two other activation loop phosphorylation sites, S211 and S214. The S211 site is adjacent to the conserved T210 phosphorylation site. In mammalian cells, phosphorylation of this site (S173 in mammals) by PKA inhibits AMPK activity by impeding the ability of LKB1 to phosphorylate AMPK on T172 [9]. Recognition of this site by PKA is somewhat surprising since the site does not fit to the consensus PKA site [RRXS [33]]. Our data suggest that this regulatory mechanism is not shared with yeast. First, substitution of S211 with phospho-mimetic and non-phosphorylatable residues had no effect on Snf1 kinase function *in vivo* as judged by growth on alternative carbon sources (Fig. 2). Second, Snf1-S211E was phosphorylated on T210 by Sak1 kinase *in vitro* (Fig. 4B) and was catalytically as



**Fig. 7.** Phosphorylation of Snf1 kinase by Ssn3. (A) *In vitro* kinase assay using GST-Ssn3 or the active site mutant Ssn3-D304A. Ssn3 was incubated with Snf1 heterotrimers containing the active site mutation D195A in the Snf1 subunit and the different beta subunits as indicated. (B) Coomassie stained gel of Snf1-D195A heterotrimers with different serine to alanine mutations as indicated. (C) *In vitro* kinase assay using Ssn3 kinases and Snf1-D195A heterotrimers with serine to alanine substitutions.

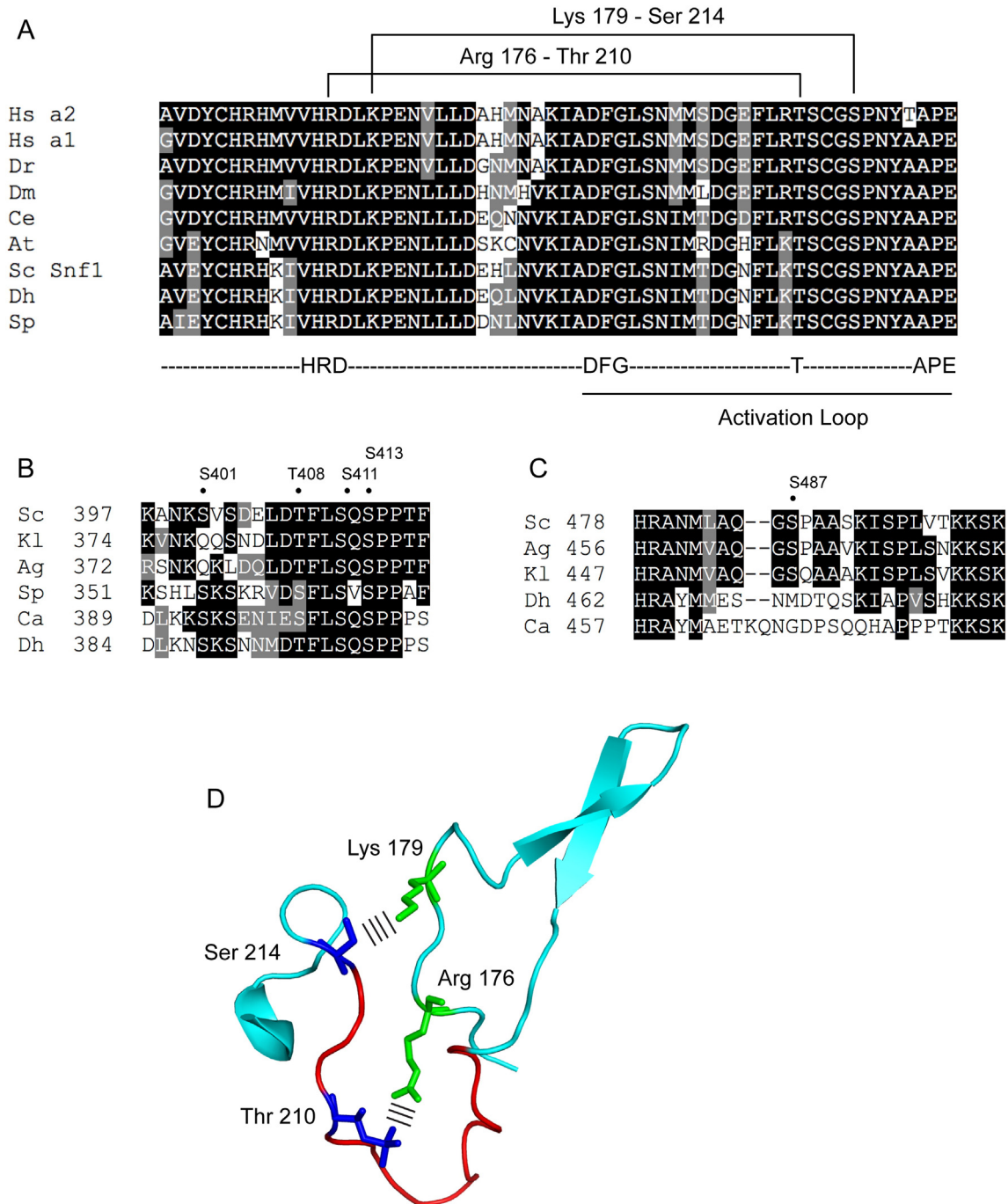
active as the wild type enzyme (Fig. 4C). Third, PKA phosphorylation of the Snf1 heterotrimer was primarily directed against the Gal83 subunit. Yeast PKA does not phosphorylate the Snf1 kinase domain with or without the S211 site (Fig. 5B). In contrast, substitutions of the S214 residue had large effects on Snf1 kinase. The Snf1-S214E is completely non-functional *in vivo* (Fig. 2) and is catalytically inactive *in vitro* (Fig. 4C) despite being phosphorylated on T210 *in vivo* and



in vitro (Figs. 3 and 4). Thus phosphorylation of Snf1 activation loop at one site, T210, is required for activity while at another site, S214, it inactivates the enzyme.

Analysis of sequence conservation and structural models of the Snf1 activation loop (Fig. 8) provide a possible explanation for the regulatory consequences of phosphorylation at T210 and S214. In many protein kinases, phosphorylation in the activation loop creates a strong hydrogen bond between the activation loop phosphate and the arginine in the conserved

HRD motif (R176 in Snf1). This interaction is necessary to properly position the adjacent aspartate residue (D177) for catalysis [39]. In structural models of active mammalian AMPK, the phosphorylated threonine and conserved arginine are close enough (2.6 Å) to form this strong hydrogen bond [40]. The position of Snf1 S214 is <4 Å from the conserved lysine residue at position 179. K179 is conserved throughout the serine/threonine protein kinase family and is important for catalysis by interacting with both the ATP and the carbonyl oxygen of the P-2 residue in the peptide



**Fig. 8.** Conservation of Snf1 phosphorylation sites. (A) Multiple sequence alignment of AMPK alpha subunits in the region near the activation loop showing highly conserved protein kinase motifs below. (B and C) Multiple sequence alignment of regions of the alpha subunit from fungal species. Species used in these alignments are as follows: Hs a2, human alpha-2; Hs a1, human alpha-1; Dr., *Danio rerio*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*; Sc, *Saccharomyces cerevisiae*; Dh, *Debaryomyces hansenii*; Sp, *Schizosaccharomyces pombe*; Kl, *Kluyveromyces lactis*; Ag, *Ashbya gossypii*; Ca, *Candida albicans*. (D) Three dimensional model of the Snf1 activation loop based on the PDB file 4CFF. Residue numbering is for the Snf1 protein.

substrate [39]. Phosphorylation of S214 would put a negative charge closer to K179 and would likely interfere with the ability of K179 to interact with the kinase substrates. Thus we propose that active Snf1 kinase can be down-regulated by two distinct mechanisms: 1) by dephosphorylation of T210 and 2) by phosphorylation of S214.

Identification of the kinase responsible for the phosphorylation of S214 and the conditions that favor its modification are of great interest. To answer these questions, we tried at first to develop a phosphopeptide antibody specific for the S214 site. However, these efforts were unsuccessful. Second, we took a candidate kinase approach. Since the S214 site is directly followed by a proline, we purified and tested three proline-directed kinases, Hog1, Pho85 and Ssn3, for the ability to phosphorylate Snf1 on S214. All three kinases tested (as well as PKA) showed catalytic activity in vitro and a strong preference for the Snf1 beta subunits. Ssn3 was the only kinase with a detectable ability to phosphorylate the Snf1 subunit, however that activity was not affected by the S214 A mutation. Therefore, further studies will be needed to identify the kinase responsible for the phosphorylation of Snf1 S214 and to determine the conditions under which this modification is used to regulate Snf1 activity.

## 5. Conclusions

Multiple phosphorylation sites within the Snf1 kinase complex have been reported. Here we identify one site, serine 214 within the kinase activation loop, as having the greatest potential for acting as a regulatory site in vivo. Changing this site to glutamate inactivates the catalytic activity of Snf1 kinase but does not block recognition of threonine 210 by the Snf1-activating kinase. These data suggest that yeast may utilize two distinct pathways to down regulate Snf1 activity: dephosphorylation of threonine 210 and phosphorylation of serine 214.

## Transparency document

The [Transparency document](#) associated with this article can be found, in online version.

## Acknowledgements

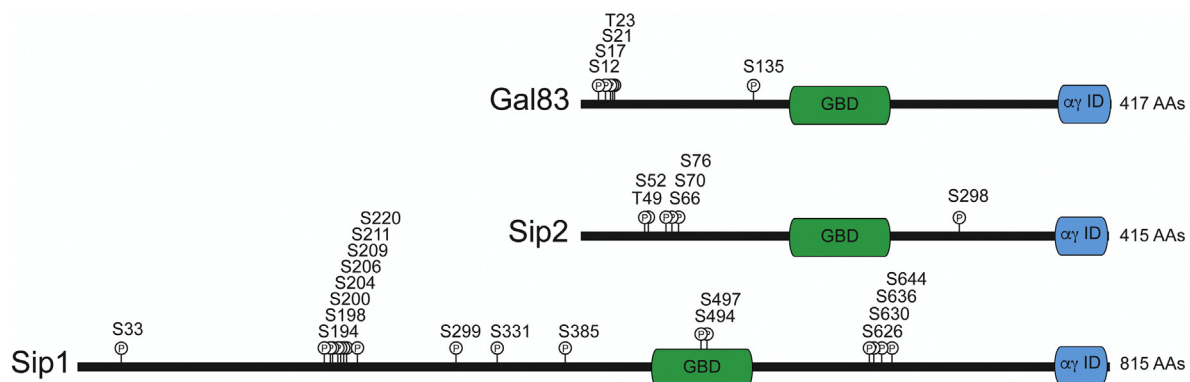
This work was supported by National Institutes of Health Grant GM46443.

## Appendix A

Phosphorylation sites in the beta subunits of Snf1. All sites detected in three or more of the 12 phosphoproteomic studies [18–29] are indicated. Proteins are drawn to scale to indicate position of sites relative to the glycogen binding domain (GBD) and the alpha-gamma interaction domain ( $\alpha\gamma$ -ID)

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