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Regulation of Snf1 Kinase

ACTIVATION REQUIRES PHOSPHORYLATION OF THREONINE 210 BY AN UPSTREAM KINASE AS WELL AS A DISTINCT STEP MEDIATED BY THE Snf4 SUBUNIT*

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The yeast Snf1 kinase and its metazoan orthologues, the AMP-activated protein kinases, are activated in response to nutrient limitation. Activation requires the phosphorylation of a conserved threonine residue in the activation loop of the catalytic subunit. A phosphopeptide antibody was generated that specifically recognizes Snf1 protein that is phosphorylated in its activation loop on threonine 210. Using this reagent, we show that phosphorylation of threonine 210 correlates with Snf1 activity, since it is detected in cells subjected to glucose limitation but not in cells grown in abundant glucose. A Snf1 mutant completely lacking kinase activity was phosphorylated normally on threonine 210 in glucose-starved cells, eliminating the possibility that the threonine 210 modification is due to an autophosphorylation event. Cells lacking the Reg1 protein, a regulatory subunit for the Glc7 phosphatase, showed constitutive phosphorylation of Snf1 threonine 210. Exposure of cells to high concentrations of sodium chloride also induced phosphorylation of Snf1. Interestingly, Mig1, a downstream target of Snf1 kinase, is phosphorylated in glucose-stressed but not sodium-stressed cells. Finally, cells lacking the γ subunit of the Snf1 kinase complex encoded by the *SNF4* gene exhibited normal regulation of threonine 210 phosphorylation in response to glucose limitation but are unable to phosphorylate Mig1 efficiently. Our data indicate that activation of the Snf1 kinase complex involves two steps, one that requires a distinct upstream kinase and one that is mediated by the γ subunit of the kinase itself.

The Snf1 and AMP-activated protein kinases (AMPK)[1] define a highly conserved family of serine-threonine protein kinases found in fungi, plants, *Drosophila*, *Caenorhabditis elegans*, and mammals. Members of the Snf1-AMPK family are central components of signal transduction pathways that are activated in response to nutrient stress (1). The Snf1-AMPK enzymes are heterotrimers with a single catalytic α subunit and two regulatory subunits denoted β and γ (2–4). In the yeast *Saccharomyces cerevisiae*, the Snf1 kinase plays a critical role when glucose becomes limiting. The net effect of Snf1 kinase activation is to down-regulate certain enzymes to conserve ATP energy and to relieve glucose repression of gene expression. Downstream targets of Snf1 include metabolic enzymes such as acetyl-CoA carboxylase (5, 6) and transcription factors such as Mig1 and Sip4 (3, 7, 8). While there has been tremendous progress made in our understanding of glucose sensing and signal transduction (9), it is still unclear how glucose limitation is sensed and the information transduced to Snf1. Intracellular concentration of AMP, by analogy with the mammalian enzyme, is a good candidate for the signaling molecule, yet AMP has no effect on Snf1 kinase activity *in vitro* (6, 10). While AMP might not activate Snf1 directly, it is still possible that AMP might activate the Snf1 pathway by acting upstream of Snf1. To understand the regulation of Snf1 kinase, it is necessary to determine the number and identity of the regulators acting upstream of Snf1.

A common regulatory switch observed in protein kinases is the phosphorylation of one or more residues in the activation loop of the catalytic subunit. In the unphosphorylated state, the activation loop can block access of substrates to the active site (11). Phosphorylation of residues in the activation loop causes a large outward rotation of the activation loop, thus making the active site accessible to substrate and aligning active site residues for catalysis (12, 13). In many instances, the phosphorylation of one kinase is catalyzed by a distinct upstream kinase, thereby creating kinase cascades. The mitogen-activated protein kinases are held in a physical complex with their upstream activating kinases, thereby routing molecular signals through insulated cascades (14). Several members of the AGC group of protein kinases (protein kinase family including protein kinase A, protein kinase G, and protein kinase C), a group named after the protein kinases PKA, PKG, and PKC (15), are known to be phosphorylated by the phosphotidylinositol-dependent protein kinase PDK-1 (16, 17). An alternative mechanism for the modification of activation loop residues is autophosphorylation, either intermolecular or intramolecular. While PDK-1 phosphorylates the activation loop of other kinases, the phosphorylation of its own activation loop is most likely due to autophosphorylation, since it is observed with recombinant protein in the absence of all other mammalian kinases (18). Many of the Src family tyrosine kinases are activated by intermolecular autophosphorylation of residues in the activation loop (19, 20). In yet another variation on this theme, proteins can bind to the activation loop to exert control over kinase activity. In the case of TAK1, a mitogen-activated protein kinase kinase kinase (MAP3K), intramolecular autophosphorylation of the activation loop is stimulated by the binding of the TAB1 protein (21). In the case of the JAK family of kinases, binding of the activation loop by the JAB protein inhibits kinase activity (22). Thus, many different mechanisms of regulating protein kinase activity are mediated through events at the activation loop.

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[1] The abbreviations used are: AMPK, AMP-activated protein kinase; HA, hemagglutinin.

TABLE I
S. cerevisiae strains

Strain	Genotype	Source or reference
MSY182	*MAT**a** ura3–52 leu2Δ1 trp1Δ63 his3Δ200*	Ganster *et al.* (36)
FY1193	*MATα ura3–52 leu2Δ1 trp1Δ63 his3Δ200 snf1Δ10*	Fred Winston
MSY563	*MATα ura3–52 leu2Δ1 his3Δ200 lys2–128δ snf1Δ10 snf4Δ1*	This study
MSY568	*MATα ura3–52 leu2Δ1 snf4Δ1*	This study
PY102	*MAT**a** ura3–52 leu2Δ1 trp1Δ63 his4–917δ lys2–173R2 reg1Δ::URA3*	Karen Arndt

The activity of the Snf1 protein kinase, a member of the calcium/calmodulin-dependent protein kinase family of serine/threonine protein kinases (15), is also regulated by phosphorylation (10). The conserved threonine residue in the activation loop of Snf1 kinase is at position 210. The first suggestion that this residue was important for Snf1 regulation came from the finding that a threonine to alanine mutation at position 210 inactivated the Snf1 kinase (23). This study documented that threonine 210 was essential for Snf1 activity but did not address whether or not threonine 210 was modified. A subsequent study by Hardie and colleagues (10) showed that Snf1 kinase activity measured *in vitro* was greatly reduced by phosphatase treatment. This study indicated that the phosphorylation of Snf1 was essential for catalytic activity but did not identify the site(s) of modification. However, these studies and the numerous parallels with other protein kinases strongly suggested that the Snf1 kinase is activated by phosphorylation on threonine 210 and possibly other sites as well. The down-regulation of Snf1 kinase by dephosphorylation is almost certainly catalyzed by the PP1 phosphatase Glc7 in complex with the regulatory subunit Reg1 (24, 25).

Studies of the mammalian orthologue of Snf1, AMPK, have shown that its regulation is mediated by multiple phosphorylation events, the most important one being at the conserved threonine residue in the activation loop (26). Biochemical studies of rat liver extracts have identified an activity that is responsible for the phosphorylation of AMPK (27). The activating enzyme, called AMP-activated protein kinase kinase (AMPK kinase), is likely to be distinct from AMPK based on differences in chromatographic properties, response to allosteric regulation by AMP, and inactivation by phosphatase treatment. However, AMPK and AMPK kinase are very similar in size, and until the AMPK kinase is characterized at the molecular level, one cannot rule out the possibility that AMPK kinase is a modified form of AMPK itself.

The focus of this study is on the Snf1 signaling pathway in yeast. While genetic and biochemical evidence suggest that the Snf1 kinase is regulated by phosphorylation on threonine 210, genetic screens for Snf mutants have failed to detect any kinases that could act upstream of Snf1 kinase. One possible explanation for the failure to identify the Snf1-activating kinase genetically is that it may not exist. The Snf1 protein could be activated by an autophosphorylation mechanism. For instance, the Snf1 reactivating factor identified by Wilson *et al.* (10) could represent a protein that binds Snf1 and stimulates an autophosphorylation event similar to the effect of TAB1 protein on TAK1 kinase (21). An alternative explanation for the failure to identify a Snf1-activating kinase genetically is that more than one kinase may be capable of phosphorylating Snf1. Thus, in genetic screens for loss of function alleles, redundant Snf1-activating kinases would complement each other and never be detected. To further define the Snf1 signaling pathway, we have developed an assay to measure the phosphorylation of Snf1 kinase on threonine 210. The combination of genetics with an immunological assay for threonine 210 phosphorylation has allowed us to unambiguously determine that the Snf1 kinase is regulated by an upstream kinase.

EXPERIMENTAL PROCEDURES

Yeast Strains, Methods, and Genetic Techniques—*Saccharomyces cerevisiae* strains used in this study are described in Table I. Except where indicated, growth of yeast utilized standard medium at 30 °C (28). Glucose and raffinose were present at 2% (g/100 ml) except for derepressing medium, which contained 0.05% glucose (g/100 ml). Antimycin A was included at 1 μg/ml in all raffinose media. Transformation of yeast strains utilized the lithium acetate procedure (29). Repressed cultures were grown in 2% glucose and harvested in mid-log phase at an A_{600} of 0.4–0.8. Derepressed cultures were prepared by resuspending repressed cells in medium containing 0.05% glucose and continuing growth for an additional 2 h.

Epitope Tagging—Mig1 protein was modified to contain three copies of the HA epitope at its C terminus (3). The Snf1 protein was epitope-tagged by polymerase chain reaction-amplifying three copies of the HA epitope present in plasmid pMR2307 (30) with primers containing *Eco*RI sites at the termini. The tag was then inserted into the naturally occurring *Mun*I site, which partially overlaps the Snf1 stop codon. The resulting construct encodes full-length Snf1 protein with these additional amino acids added to the C terminus: SYPYDVPDYAGYPYDVPDYAGSYPYDVPDYAA.

Phosphothreonine 210 Antibodies—Antibodies directed against phosphorylated threonine 210 were purchased from Research Genetics (Huntsville, AL). A 13-mer peptide (DGNFLK[T-PO_4]SCGSPN) corresponding to amino acids 204–216 of Snf1 protein was synthesized such that the central threonine residue was phosphothreonine. The peptide was coupled to keyhole limpet hemacyanin and used to immunize two rabbits. Sera were pooled, and antibodies were purified by two sequential steps of affinity chromatography. First, antibodies that bound to a phosphopeptide column were purified. Next, antibodies that bound to a column containing the unphosphorylated peptide were removed. The collection of antibodies resulting from these affinity selections are referred to as the α-PT210 antibodies.

Protein Extracts—Protein extracts were prepared using a glass bead lysis procedure (31) in NHTG buffer (40 mM HEPES, pH 7.3, 350 mM NaCl, 0.1% Tween 20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of benzamidine, pepstatin A, leupeptin, and aprotinin). Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard. For analysis of Thr^{210} phosphorylation, protein extracts were prepared using a modification of the NaOH extraction procedure developed by Kushnirov (32). Cell cultures (25 ml) were grown to an A_{600} of 0.5–0.8. NaOH was added directly to the culture medium to a final concentration of 0.1 M, and cells were incubated for 5 min at room temperature. Cells were collected by centrifugation and proteins extracted by boiling in SDS sample buffer (10 μl per OD of cells) for 5 min. Debris was removed by centrifugation, and the supernatant fraction was recovered and extensively dialyzed against an excess of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 50 mM Tris-HCl, pH 8.0) supplemented with 50 mM sodium fluoride and 5 mM sodium pyrophosphate. Protein recovery following dialysis was typically 1.75 mg of protein from a 25-ml culture.

Western Blots and Immunoprecipitations—Western blots were performed using the method described previously (31). Monoclonal antibodies against the HA epitope were purchased from Santa Cruz Biotechnology and used at a dilution of 1:1000. Affinity-purified α-PT210 antibodies were used at a dilution of 1:1000. For immunoprecipitations, 300–500 μg of protein was incubated with 2 μl of anti-HA for 90 min at 4 °C with gentle agitation in RIPA buffer supplemented with protease and phosphatase inhibitors (50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1 mg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 2 μg/ml chymostatin). Immune complexes were collected by low speed centrifugation following incubation with 20 μl of protein A beads (Sigma) that had been washed in RIPA buffer. Bound proteins were eluted by boiling the beads in 15 μl of SDS sample buffer. Eluted proteins were resolved on an SDS 10% polyacrylamide gel and subjected to Western blotting with either

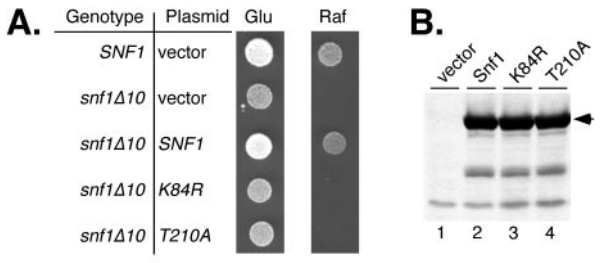

FIG. 1. **Point mutations affect Snf1 kinase function but not protein accumulation.** *A*, equal numbers of yeast cells were spotted onto solid medium containing either glucose (*Glu*) or raffinose (*Raf*) as the carbon source and grown for either 2 or 5 days, respectively. Yeast strains (*SNF1* or *snf1Δ10*) were transformed with low copy number plasmids containing either no insert (*vector*) or an epitope-tagged version of *SNF1.* The plasmid-encoded *SNF1* gene was otherwise wild type in sequence or contained the point mutations K84R or T210A as indicated. *B*, Western blot of HA-tagged Snf1 proteins. Twenty micrograms of protein from each yeast cell extract were resolved on an SDS gel and probed with anti-HA monoclonal antibody. Cells (*snf1Δ10*) were transformed with low copy number plasmids expressing either no Snf1 protein (vector; *lane 1*) or HA-tagged Snf1 proteins with otherwise wild type sequence (*lane 2*) or point mutations as indicated (*lanes 3* and *4*).

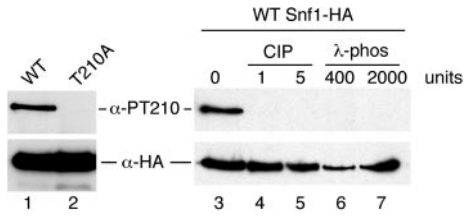

FIG. 2. **Specificity of the PT210 antibodies.** Protein extracts were prepared from cells expressing HA-tagged Snf1 (*lanes 1* and *3–7*) or HA-tagged Snf1 T210A (*lane 2*). Snf1 protein was collected by immunoprecipitation from 150 μg of protein with anti-HA monoclonal antibodies and resolved on SDS gels. One gel was probed with HA monoclonal antibody (*lower panel*) and the other with the α-PT210 antibodies (*upper panel*). Extracts were treated with calf intestine alkaline phosphatase (*lanes 4* and *5*) or λ-phosphatase (*lanes 6* and *7*) prior to immunoprecipitation.

anti-HA or α-PT210 antibodies (31). All Western blotting experiments have been repeated at least twice with extracts from independent transformants and have produced comparable results. A representative exposure of one blot is shown.

RESULTS

Point Mutations Inactivate Snf1 Kinase without Affecting Protein Accumulation—The Snf1 kinase is required by yeast for the fermentation of sucrose and the related trisaccharide raffinose (33). Strains that carry the deletion allele *snf1Δ10* manifest a pronounced growth defect on raffinose medium that can be complemented by a plasmid-encoded *SNF1* gene that bears three copies of the HA epitope at the C terminus (Fig. 1*A*). The point mutation K84R eliminates the catalytic activity of Snf1 kinase by disrupting the ATP binding domain (34). The K84R mutation was engineered in the epitope-tagged *SNF1* gene and tested for the ability to complement the *snf1Δ10* deletion for growth on raffinose. The K84R mutation completely blocks the ability of the plasmid-encoded gene to complement; however, the accumulation of the K84R-Snf1 protein, as judged by Western blot directed against the HA tag, is unaffected (Fig. 1*B*). In a similar manner, a mutation that converts the activation loop threonine to the nonphosphorylatable residue alanine was engineered and tested. The T210A allele of *SNF1* was also completely unable to complement the *snf1Δ10* deletion for the fermentation of raffinose, but this mutation had no effect on protein levels as judged by Western blot. The affects of these two point mutations on Snf1 kinase activity have been reported previously (23, 34). We show here that these two mutations inactivate the Snf1 kinase without affecting protein levels. Thus, these alleles are suitable for further studies to probe the regulation of Snf1 activity.

α-PT210 Antibodies Specifically Recognize Snf1 Phosphorylated on Threonine 210—To study the activation of the Snf1 kinase, we sought a reagent that could specifically detect Snf1 protein that had been phosphorylated on threonine 210. A 13-residue synthetic peptide, corresponding to amino acids 204–216 of Snf1 and containing phosphothreonine at position 210, was synthesized and used to immunize rabbits. Antibodies that recognized the phosphorylated peptide were affinity-purified and used in Western blotting experiments. A functional HA-tagged Snf1 protein was collected by immunoprecipitation and resolved on an SDS gel. When anti-HA monoclonal was used to probe the blot, equivalent levels of Snf1 protein were detected in extracts expressing wild type Snf1 or the T210A mutant (Fig. 2*B*, *lanes 1* and *2*). However, when the α-PT210 antibodies were used, the wild type, but not the T210A mutant, was detected. When the extracts were pretreated with either calf intestine alkaline phosphatase or λ-phosphatase, the α-PT210 antibodies were no longer able to detect the Snf1 protein (Fig. 2, *lanes 3–7*). Pretreatment with phosphatase did not alter Snf1 reactivity with the HA monoclonal antibody. Therefore the α-PT210 antibodies detected Snf1 protein only when threonine 210 was present and only when the endogenous phosphorylations were left intact. When combined with the knowledge of the immunogen, the simplest conclusion is that the α-PT210 antibodies specifically recognize Snf1 protein that is phosphorylated on threonine 210.

Threonine 210 Is Phosphorylated in Response to Glucose Limitation—The phosphorylation state of threonine 210 was assessed in cells that were grown under conditions of excess glucose (6%) or glucose limitation (0.05%) for 1 h. Since the mere act of centrifugation has been shown to be sufficient to activate Snf1 kinase (10), a procedure was devised to block cellular metabolism prior to harvest. Using a modification of a simple protein extraction procedure (32), cell metabolism was arrested first by the addition of sodium hydroxide to a concentration of 0.1 M for 5 min prior to harvesting cells by centrifugation. Protein extracts were then isolated from cultures expressing both wild type Snf1 protein from the chromosome and an epitope-tagged Snf1 protein from a centromere plasmid. When an extract from glucose-repressed cultures was probed directly by Western blot with the α-PT210 antibodies, very little reactivity was observed (Fig. 3*A*, *lane 1*). In contrast, extracts prepared from glucose-limited cultures readily detected a doublet with the α-PT210 antibodies. The *upper band* represents the epitope-tagged protein (75.6 kDa), and the *lower band* represents the chromosomal untagged Snf1 (72.0 kDa). Therefore, the phosphorylation state of the Snf1 threonine 210 correlates with glucose limitation. As a control, the same experiment was repeated using the T210A allele of *SNF1* as the plasmid-encoded gene. In this case, the wild type (*lower band*) Snf1 protein was phosphorylated normally in response to glucose limitation, but the T210A protein (*upper band*) shows no reactivity with the α-PT210 antibodies (Fig. 3*A*, *lanes 3* and *4*). Equivalent levels of the epitope-tagged Snf1 protein was detected in all the extracts (*lower panel*) confirming that the T210A protein was present, although not detected by the α-PT210 antibodies. In a similar experiment, extracts were prepared from cells expressing only the epitope-tagged Snf1 protein. The Snf1 protein was immunoprecipitated with anti-HA antibody prior to resolution by SDS gel and Western blot with both α-PT210 antibody and anti-HA antibody (Fig. 3*B*). The Snf1 protein is present in both extracts at comparable levels as judged by reactivity with the HA antibody. However,

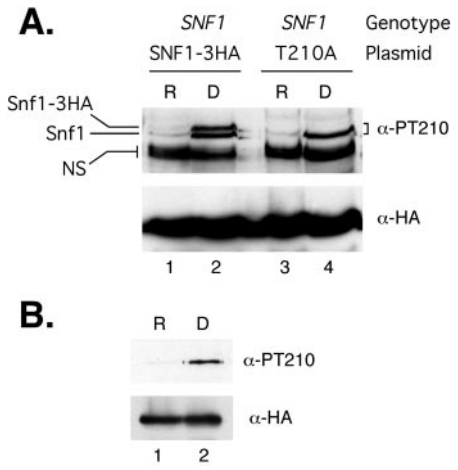

FIG. 3. **Phosphorylation of Thr^{210} is induced in response to glucose limitation.** *A*, protein extracts were prepared from cells expressing both wild type Snf1 protein from the chromosomal locus and epitope-tagged Snf1 protein from a plasmid. The epitope-tagged Snf1 was otherwise wild type in sequence (*lanes 1* and *2*) or contained the threonine to alanine mutation at position 210 (T210A). The Snf1 protein was collected from 150 μg of protein and was examined by Western blot with either anti-phosphothreonine antibody (*α-PT210*; *upper panel*) or anti-HA antibody (*lower panel*). Extracts were prepared from cells grown under repressing conditions (*R*; *lanes 1* and *3*) or derepressing conditions (*D*; *lanes 2* and *4*) using the NaOH extraction method. The mobilities of the untagged Snf1 protein (*Snf1*), the tagged Snf1 (*Snf1-HA*), and a nonspecific protein (*NS*) are indicated on the *left* of the anti-phosphothreonine (*α-PT210*) blot. *B*, protein extracts were prepared from repressed (*R*; *lane 1*) and derepressed (*D*; *lane 2*) cells, and Snf1-HA was immunoprecitated from 300 μg of protein with anti-HA prior to Western blot with anti-phosphothreonine 210 (*α-PT210*; *upper panel*) or anti-HA (*lower panel*).

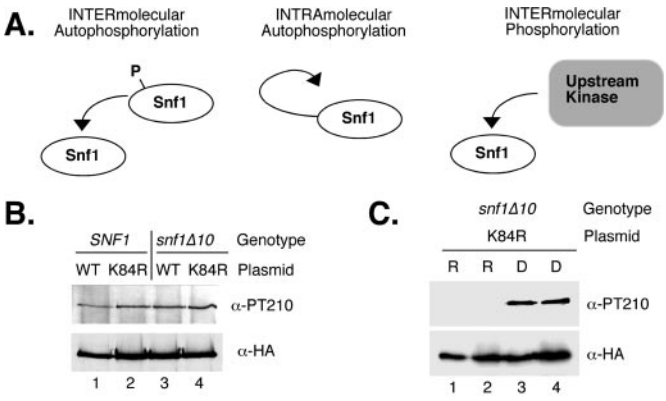

FIG. 4. **Phosphorylation of Snf1 T210 is catalyzed by an upstream kinase.** *A*, three possible mechanisms for the phosphorylation of Snf1 threonine 210. *B*, Western blot of Snf1 proteins that were immunoprecipitated with anti-HA antibodies from 150 μg of protein. *Upper panel* shows reactivity with α-PT210 antibodies, and the *lower panel* shows reactivity with anti-HA antibody. Extracts were prepared from derepressed cells that were either *SNF1*$^+$ (*lanes 1* and *2*) or *snf1Δ10* (*lanes 3* and *4*) on the chromosome and were transformed with plasmids expressing HA-tagged Snf1 that was otherwise wild type in sequence (*lanes 1* and *3*) or was catalytically inactive due to the K84R mutation (*lanes 2* and *4*). *C*, Western blot of Snf1 proteins that were immunoprecipitated from 300 μg of protein. Extracts were prepared from cells that were completely lacking in Snf1 kinase activity due to deletion of the chromosomal gene (*snf1Δ10*) and point mutation of the plasmid-encoded gene (K84R). Cells were grown in duplicate under repressing conditions (*lanes 1* and *2*) or derepressing conditions (*lanes 3* and *4*).

the reactivity with the α-PT210 antibodies is only found in derepressed cultures. Therefore, the phosphorylation of threonine 210 directly correlates with glucose stress and the activity of the Snf1 kinase.

Threonine 210 Is Phosphorylated by an Upstream Kinase—Several different mechanisms can be responsible for the phosphorylation of kinase activation loop residues. We sought to determine directly whether the Snf1 kinase is activated by an upstream kinase or by one of two possible modes of autophosphorylation (Fig. 4*A*). If Snf1 were activated by a distinct upstream kinase, then a mutation in the Snf1 active site should not affect the phosphorylation of the Thr^{210} site. However, if the Thr^{210} phosphorylation were the result of an autophosphorylation event, then an active site mutation in Snf1 would eliminate the Thr^{210} phosphorylation. Cells that contained either wild type *SNF1* or the *snf1Δ10* allele on the chromosome were transformed with HA-tagged Snf1 that was either catalytically active or inactivated by the K84R mutation in the ATP binding domain (34). Snf1 protein was harvested by immunoprecipitation with HA antibody and the phosphorylation state of threonine 210 assessed with the α-PT210 antibodies (Fig. 4*B*). The *SNF1* cells expressing the K84R kinase dead mutant are still able to phosphorylate Thr^{210}, thereby eliminating intermolecular autophosphorylation as a mechanism. The *snf1Δ10* cells transformed with a plasmid expressing the K84R kinase dead mutant have no Snf1 kinase activity, and yet they showed levels of phosphorylated threonine 210 equivalent to cells expressing active Snf1 kinase (Fig. 4*B*, *lanes 2* and *4*). This result eliminates intramolecular autophosphorylation as a mechanism. Cells lacking Snf1 kinase activity also exhibit regulated Thr^{210} phosphorylation in response to glucose limitation (Fig. 4*C*). These data demonstrate that the phosphorylation of Snf1 threonine 210 cannot be the result of autophosphorylation. The phosphorylation of Snf1 kinase on threonine 210 must be catalyzed by a distinct upstream kinase.

Sodium Stress Induces Phosphorylation of Snf1 Threonine 210 but Not Signaling of Glucose Derepression Pathway—Studies that examined the involvement of the Snf1 kinase in the response to sodium ion stress have arrived at different conclusions (35, 36). We tested whether sodium ion stress resulted in the phosphorylation of threonine 210 by treating cells for 1 h with 0.8 M NaCl. Protein extracts were prepared, and the epitope-tagged Snf1 protein was collected by immunoprecipitation. The level of threonine 210 phosphorylation measured by Western blot with the α-PT210 antibodies is consistently higher in cells that have been subjected to sodium ion stress (Fig. 5*A*). This experiment has been repeated three times with independent transformants, and in all cases the phosphorylation of Thr^{210} was detected in sodium-stressed cells but was less prominent than in glucose-stressed cells. We also tested whether sodium ion stress resulted in the phosphorylation of Mig1, a known target of Snf1 kinase in the glucose derepression pathway (7, 37). Earlier studies have shown that the mobility shift of Mig1 protein is due to phosphorylation (7, 38). Using the SDS gel mobility of HA-tagged Mig1 as an indicator of its phosphorylation state, we found that glucose limitation resulted in a large reduction in gel mobility, while sodium ion stress did not (Fig. 5*B*). Therefore, while both glucose stress and sodium ion stress caused a rapid increase in Snf1 phosphorylation on threonine 210, only glucose stress triggered the phosphorylation of Mig1 protein.

Dephosphorylation of Thr^{210} Requires Reg1—The Snf1 protein kinase directly interacts with Reg1, a regulatory subunit of the Glc7 protein phosphatase, and this interaction requires threonine 210 (24). In this experiment we test whether the Reg1 protein is required for the dephosphorylation of threonine 210. Cells with wild type *REG1* or with *reg1Δ* were transformed with a plasmid expressing epitope-tagged Snf1. Protein extracts were prepared, and the Snf1 protein was collected by immunoprecipitation. Cells bearing the *reg1Δ* allele showed phosphorylation of threonine 210 under repressing conditions (Fig. 6*A*). Therefore, Reg1 protein is required for the dephos-

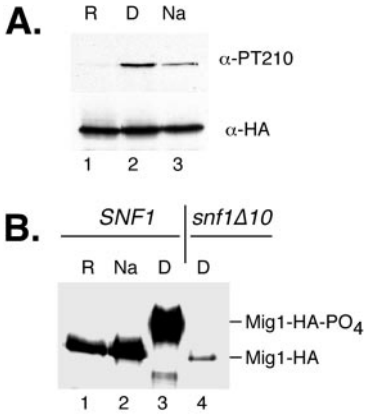

FIG. 5. **Phosphorylation of Thr^{210} in response to sodium ion stress.** *A*, Western blot of Snf1 protein collected by immunoprecipitation with anti-HA antibodies from 300 μg of protein. The *upper panel* shows reactivity with anti-threonine 210 antibodies (*α-PT210*), and the *lower panel* shows reactivity with anti-HA antibodies (*α-HA*). Extracts were prepared from cells grown under repressing conditions (*R*; *lane 1*), derepressing conditions (*D*; *lane 2*) or in repressing conditions supplemented with 0.8 M NaCl for 1 h (*Na*; *lane 3*). *B*, Western blot of Mig1 protein. Extracts were prepared from cells expressing HA-tagged Mig1 protein that had been grown under repressing conditions (*R*; *lane 1*), derepressing conditions (*D*; *lanes 3* and *4*), or repressing conditions supplemented with 0.8 M NaCl for 1 h (*Na*; *lane 2*). Cells were wild type *SNF1*$^+$ (*lanes 1–3*) or *snf1Δ10* (*lane 4*). Each lane contained 80 μg of protein.

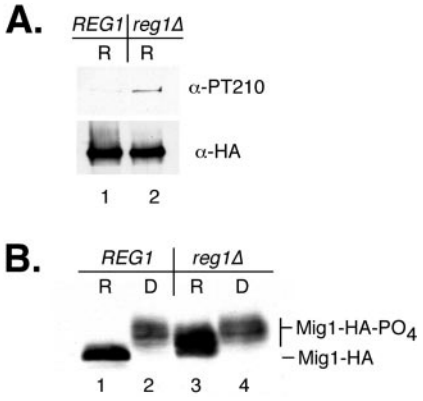

FIG. 6. **Dephosphorylation of Thr^{210} requires REG1.** *A*, Western blot of Snf1 protein harvested by immunoprecipitation from 400 μg of protein. Extracts were prepared from repressed cultures using the NaOH method. Cells were either *REG1*$^+$ or *reg1*Δ as indicated. The *upper panel* shows reactivity with the α-PT210 antibodies, and the *lower panel* shows reactivity with the anti-HA antibodies. *B*, Western blot of Mig1 protein. Protein extracts were prepared from repressed cells expressing HA-tagged Mig1 protein, and 20 μg of protein was loaded in each lane.

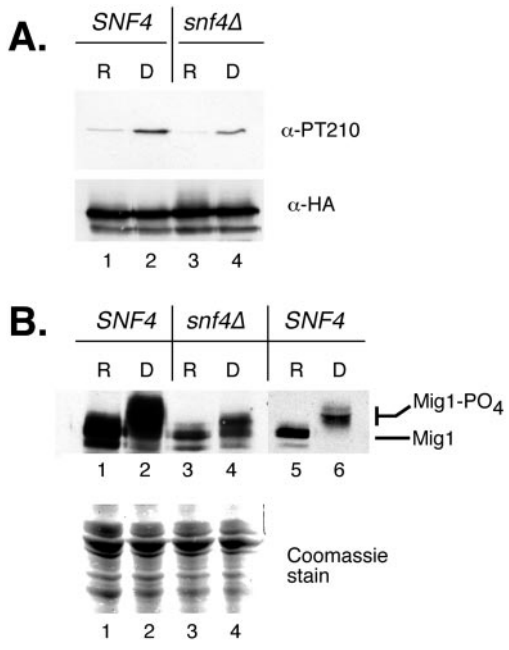

FIG. 7. **Snf4 is required for activation of Snf1 but not for Thr^{210} phosphorylation.** *A*, phosphorylation of Thr^{210} in response to glucose limitation was measured in cells with (*lanes 1* and *2*) and without (*lanes 3* and *4*) Snf4. Protein extracts were prepared from repressed (*R*; *lanes 1* and *3*) and derepressed (*D*; *lanes 2* and *4*) cells using the NaOH method, and Snf1 protein was collected by immunoprecipitation with anti-HA antibody from 500 μg of protein. Samples were divided in two and probed by Western blot with antibody to HA (*lower panel*) or phosphothreonine 210 (*upper panel*). *B*, Western blot of Mig1 protein. Protein extracts were prepared from cells expressing HA-tagged Mig1 protein and grown under repressing (*R*; *lanes 1*, *3*, and *5*) or derepressing conditions (*D*; *lanes 2*, *4*, and *6*) in the presence of wild type Snf4 protein (*lanes 1*, *2*, *5*, and *6*) or its absence (*lanes 3* and *4*). *Lanes 5* and *6* are a shorter exposure of *lanes 1* and *2*. 60 μg of protein was loaded in each lane. As a control for sample loading, equivalent aliquots from these extracts were examined by Coomassie staining (*lower panel*).

phorylation of Snf1 threonine 210. The phosphorylation state of Mig1 in *REG1*$^+$ and *reg1*Δ cells was also assessed by Western blot (Fig. 6*B*). The Mig1 protein in repressed cells bearing the *reg1*Δ allele shows a reduced SDS gel mobility that is consistent with partial phosphorylation. Mig1 protein in derepressed cells (either *REG1*$^+$ or *reg1*Δ) shows an even larger reduction in SDS gel mobility. Therefore, in repressed cells, the maintenance of Mig1 protein in unphosphorylated state also requires the Reg1 protein.

Phosphorylation of Snf1 Activation Loop in the Absence of γ Subunit Snf4—The γ subunit of the Snf1 kinase complex encoded by *SNF4* is required for the activation of the kinase by a mechanism that likely involves a conformational change and direct binding to the catalytic subunit (39). We asked whether the phosphorylation of threonine 210 required the presence of the γ subunit. Cells lacking the γ subunit were grown in abundant glucose and then transferred to medium with limiting glucose. Epitope-tagged Snf1 protein was collected by immunoprecipitation and then analyzed by Western blotting (Fig. 7*A*). The phosphorylation of threonine 210 was induced by glucose limitation regardless of the presence or absence of the Snf4 protein. Thus the γ subunit of the Snf1 kinase is not required for the phosphorylation of the activation loop of the catalytic subunit. However, the presence of the γ subunit is presumed to be necessary for Snf1 kinase activation, since *snf4*Δ cells grow poorly on alternative carbon sources and are defective in the derepression of invertase (40). The ability of the Snf1 kinase to phosphorylate Mig1 was assessed in the presence and absence of the Snf4 protein. The presence or absence of the Snf4 protein affects the abundance of the Mig1 protein, a result seen previously with cells lacking the Snf1 protein (3). Nonetheless, Mig1's gel mobility and hence its phosphorylation state can still be monitored. In the presence of Snf4 protein, all of the Mig1 protein is converted to the slower mobility species in derepressed cultures (Fig. 7*B*, *lanes 5* and *6*). In contrast, when the γ subunit is absent, the Mig1 protein is only partially converted to the slower mobility species in response to glucose limitation (*lane 4*). In the absence of Snf4 protein, a significant fraction of the Mig1 protein in derepressed cells is seen in the high mobility (unphosphorylated) form, whereas in Snf4-containing cells, only the slower mobility (phosphorylated) form is observed (compare *lanes 4* and *6*). Therefore, the Snf4 protein is required for efficient phosphorylation of Mig1 but not for the phosphorylation of Snf1 threonine 210.

DISCUSSION

The focus of this study has been on the regulation of Snf1 kinase through its modification at threonine 210. Phosphorylation of Snf1 on threonine 210 is essential for Snf1 kinase activity. This conclusion is based on the observation that a mutation of threonine 210 to alanine inactivates the kinase. The Snf1-T210A mutant is unable to grow by fermentation of raffinose (Fig. 1), unable to derepress invertase expression (23), and unable to direct the phosphorylation of Mig1.[2] Since this modification plays such a dominant role in the regulation of Snf1 activity, we sought to determine unambiguously whether the modification was catalyzed by an upstream kinase. By using an active site mutant of Snf1 and an immunological reagent that specifically recognized the phosphorylated threonine 210, we have now shown conclusively that the phosphorylation of threonine 210 is catalyzed by a distinct protein kinase. Understanding how the Snf1-activating kinase is regulated will shed light on the regulation of the Snf1 signaling pathway. For instance, is the Snf1-activating kinase subject to allosteric regulation by intracellular AMP concentrations? Since the Snf1-activating kinase has not been identified in genetic screens, it seems likely that there may be more than one kinase that is capable of modifying Snf1 on threonine 210. Of course, the next step is to identify the kinase(s) that modify the Snf1 on threonine 210.

While the modification of Snf1 on threonine 210 is necessary for kinase activity, it is not by itself sufficient. The Snf1 kinase is a heterotrimer. The β and γ subunits of the complex are also required for proper kinase function and regulation. We have shown previously that the β subunits play a role in substrate definition (3), and a recent report has shown that the different β subunits control the localization of the Snf1 kinase complex (41). The γ subunit encoded by *SNF4* plays an important regulatory role that involves direct protein-protein interaction with the regulatory domain of the catalytic subunit (39). We show here that the γ subunit's role in regulating Snf1 is distinct from the modification of threonine 210. Cells that are lacking the Snf4 protein are still able to phosphorylate Snf1 on threonine 210 in response to glucose limitation (Fig. 7). Therefore, the Snf4 protein is not required to recruit or stimulate the activity of the Snf1-activating kinase. Furthermore, whatever role Snf4 plays, it is not sufficient to activate the Snf1 kinase in the absence of phosphorylation on threonine 210. This conclusion is based on the finding that the T210A mutation that blocks phosphorylation of the activation loop inactivates the kinase despite the presence of wild type Snf4 (Fig. 1). These findings and work from other laboratories led us to a model for the regulation of Snf1 kinase activity (Fig. 8).

Full activation of the Snf1 kinase requires two distinct steps. In the presence of abundant glucose, the Snf1 kinase is inactive (Fig. 8*A*). Threonine 210 is unphosphorylated (Fig. 3), and the catalytic domain is bound to the N-terminal region of the regulatory domain (39, 42). In the presence of abundant glucose, the Snf4 protein is not bound to the regulatory domain of the catalytic subunit (39). Under conditions of glucose limitation, the Snf1 kinase becomes fully active (Fig. 8*B*). Threonine 210 is phosphorylated (Fig. 3), and the Snf4 protein is now bound to the regulatory domain of the α subunit (39). The phosphorylation of threonine 210 opens the active site, making it accessible to substrates (43). This change in active site accessibility is represented in our model by a larger cleft and the rotation of the catalytic domain away from the regulatory domain. Transition from inactive to active states can occur by two alternate routes. If the Snf1 kinase is phosphorylated on threonine 210 first (Fig. 8*C*), the active site may become more accessible. However, without the rearrangement of the Snf4 protein, the Snf1 kinase is only partially active. This conclusion is based on the finding that in *snf4*Δ cells, the Snf1 kinase is phosphorylated on threonine 210 and is at least partially able to phosphorylate Mig1 protein (Fig. 7). By contrast, the Snf1 kinase that has been the recipient of the Snf4 rearrangement but has yet been phosphorylated (Fig. 8D) is completely inactive. This conclusion is based on the finding that the Snf1-T210A mutant has no detectable Snf1 function as judged by fermentation of raffinose (Fig. 1) or phosphorylation of Mig1.[2]

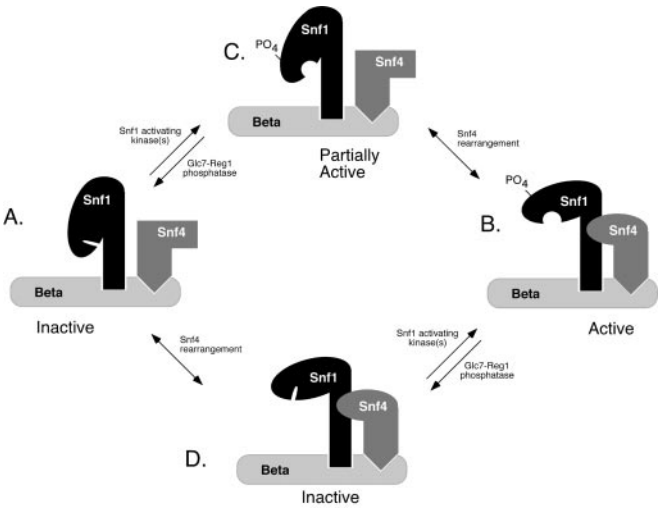

FIG. 8. **Two-step model for the regulation of Snf1 kinase.** This model is an extension of one first proposed by Jiang and Carlson (39). Under repressing conditions, the Snf1 kinase is catalytically inactive and is unphosphorylated on threonine 210 (*A*). Fully active Snf1 that is present in derepressed cells is phosphorylated on threonine 210 and has undergone a conformational rearrangement that is mediated by the Snf4 protein (*B*). Alternate intermediate forms of Snf1 are shown in *C* and *D*.

Two critical issues need to be resolved before we have a more complete understanding of Snf1 kinase regulation. First, the identity and regulation of the upstream kinase(s) need to be determined. Clearly this is a goal of both those studying the yeast Snf1 enzyme as well as those studying the mammalian AMPK enzyme. Second, what is the nature of the Snf4 rearrangement, and how is it controlled? Our data show here that the phosphorylation of threonine 210 can occur independently of the Snf4 step (Fig. 7). The converse, whether the Snf4 rearrangement occurs in the absence of Thr^{210} phosphorylation, was not tested in this study. However, two hybrid experiments have shown that the interaction of Snf4 with the regulatory domain can occur in constructs that lack the kinase domain including threonine 210 (39). Further examination of the nature of the Snf4 rearrangement and its control will shed further light on the mechanisms that regulate Snf1 kinase activity.

The regulation of Snf1 activity by two independent molecular switches provides for integration of multiple signals. This may be particularly useful for Snf1 kinase, since it is involved in multiple signaling pathways, including glucose stress, initiation of meiosis, heat shock, entry to stationary phase, and filamentous growth (44–47). In addition, it has been proposed that the Snf1 kinase plays a role in response to sodium ion stress (35), although our own studies failed to detect an effect of Snf1 kinase mutations on cell growth under sodium stress conditions (36). In this report, we show that sodium ion stress stimulates the phosphorylation of threonine 210 on Snf1 kinase; however, Mig1 phosphorylation was not observed (Fig. 5). This result could be interpreted in two ways. First, in the two-step model for Snf1 activation, phosphorylation of Snf1 is

[2] R. R. McCartney and M. C. Schmidt, unpublished results.

not by itself sufficient to activate the kinase. Snf1-dependent phosphorylation of Mig1 would not be observed if sodium ion stress failed to stimulate the Snf4-dependent step of Snf1 activation. Alternatively, it is possible that Snf1 kinase is activated by sodium ion stress but Mig1 is not a downstream target. Snf1 kinase exists in cells as three distinct complexes that are distinguished by the identity of the β subunits. It is possible that only one of the Snf1 kinase complexes responds to sodium ion stress, and its substrates are restricted by the β subunits directly or by their control of kinase localization (3, 41).

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