

Yeast Pak1 Kinase Associates with and Activates Snf1

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Members of the Snf1/AMP-activated protein kinase family are activated under conditions of nutrient stress by a distinct upstream kinase. Here we present evidence that the yeast Pak1 kinase functions as a Snf1-activating kinase. Pak1 associates with the Snf1 kinase in vivo, and the association is greatly enhanced under glucose-limiting conditions when Snf1 is active. Snf1 kinase complexes isolated from *pak1Δ* mutant strains show reduced specific activity in vitro, and affinity-purified Pak1 kinase is able to activate the Snf1-dependent phosphorylation of Mig1 in vitro. Purified Pak1 kinase promotes the phosphorylation of the Snf1 polypeptide on threonine 210 within the activation loop in vitro, and an increased dosage of the *PAK1* gene causes increased Snf1 threonine 210 phosphorylation in vivo. Deletion of the *PAK1* gene does not produce a Snf1 phenotype, suggesting that one or more additional protein kinases is able to activate Snf1 in vivo. However, deletion of the *PAK1* gene suppresses many of the phenotypes associated with the deletion of the *REG1* gene, providing genetic evidence that Pak1 activates Snf1 in vivo. The closest mammalian homologue of yeast Pak1 kinase, calcium-calmodulin-dependent protein kinase beta, may play a similar role in mammalian nutrient stress signaling.

The Snf1 kinase of budding yeast and the mammalian AMP-activated protein kinase (AMPK) play critical roles in signaling nutrient stress (9). When activated, Snf1 and AMPK down-regulate metabolic pathways that consume ATP and stimulate pathways that promote ATP synthesis. Nutrient-sensing pathways, in particular, glucose-sensing pathways, are critical signaling pathways that are deregulated in type 2 diabetes. Recent studies of AMPK underscore its importance as a metabolic switch and suggest that activation of AMPK may provide a therapeutic benefit for patients with type 2 diabetes (38). The budding yeast Snf1 kinase and mammalian AMPK are orthologous proteins, and they play similar roles in controlling cellular metabolism. Snf1 kinase is needed for the proper response to nutrient stress conditions, such as growth on alternative carbon sources and sporulation (2). A full understanding of the Snf1/AMPK signaling pathways will first require identification of all of the components of the pathway. While several downstream targets of Snf1 and AMPK are both known and conserved between species (39), much less is known about components acting upstream of Snf1 and AMPK.

Biochemical and genetic experiments have shown that members of the Snf1/AMP-activated protein kinase family are regulated by phosphorylation of the conserved threonine residue in the kinase activation loop. This mechanism for controlling protein kinase activity is used both by members of the serine threonine protein kinase family and by members of the tyrosine protein kinase family. Biochemical fractionation of rat liver has shown that threonine 172 of the mammalian AMPK enzyme is phosphorylated by a distinct protein kinase called AMPK kinase (AMPKK) (11). In budding yeast, the analogous threonine is located at position 210. Carlson and coworkers first showed that replacement of this residue with alanine in-

activates Snf1 (5), suggesting that phosphorylation of Snf1 threonine 210 is required for Snf1 activation. Later, Hardie and colleagues demonstrated that Snf1 is regulated by phosphorylation of its activation loop threonine (37). Despite intensive study, the identity of the Snf1-activating kinase has not been determined. One possible explanation for this is that the Snf1 kinase catalyzes the autophosphorylation of threonine 210. An autophosphorylation event would explain the failure to identify a Snf1-activating kinase in genetic screens. More recently, we developed a phosphopeptide antibody that is specific for the Snf1 protein that has been phosphorylated on threonine 210 (21). By using this antibody, we have shown that phosphorylation of Snf1 threonine 210 correlates with glucose stress and Snf1 kinase activation. Further, phosphorylation of threonine 210 occurs normally in cells lacking a functional Snf1 kinase. Therefore, Snf1 kinase must be activated by an upstream kinase.

Recently, genomic studies of yeast protein complexes have used mass spectrometry to identify proteins in hundreds of affinity-purified complexes (6, 14). Affinity purification of protein complexes with tagged versions of the Snf1 and Snf4 proteins led to the identification of several other proteins that may be present in a complex with Snf1 kinase. Not surprisingly, the beta and gamma subunits of Snf1 kinase (Snf4, Sip1, Sip2, and Gal83) were identified as members of the Snf1 complex. More interesting was the finding that two protein kinases, Pak1 and Cka1, are also associated with either Snf1 or Snf4 (6). *PAK1* (polymerase alpha kinase 1) was isolated in a screen for multicopy suppressors of a temperature-sensitive mutation in *CDC17*, the gene encoding DNA polymerase alpha (15), however, a direct connection between Pak1 and DNA polymerase alpha was not demonstrated. Pak1 is likely to be a Snf1 kinase complex component since it was found independently in complexes isolated with both Snf1 and Snf4 as the tagged proteins (6) and since Pak1 was not found in association with any other proteins. The gene for protein kinase Cka1 is one of two yeast genes that encode orthologues of mammalian casein kinase II.

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The significance of the purported interaction between Cka1 and the Snf1 kinase complex is questionable since Cka1 was found to be present in several complexes of disparate functions (6, 14). Finally, analysis of proteins associated with protein kinase Tos3, a close homologue of Pak1, identified several proteins, including Snf4, the gamma subunit of the Snf1 kinase (14). The significance of this interaction is also questionable since no other Snf1 kinase components were detected in association with Tos3. In this study, we investigated the possibility that Pak1, Tos3, or Cka1 directly activates Snf1 by phosphorylation of the threonine residue in the Snf1 activation loop.

We focused on the Pak1 protein kinase since it was found in the Snf1 complex with both Snf1 and Snf4 as the tagged proteins. Furthermore, the closest mammalian homologue of Pak1 is calcium/calmodulin-dependent protein kinase kinase beta (CaMKK- β), a kinase known to phosphorylate and activate other protein kinases (1). The closest mammalian homologue of Tos3 is CaMKK- α . Pak1 and Tos3 are members of the calcium/calmodulin-dependent kinase family, which includes the Cmk1 and Snf1 kinases (16). The name *PAK1* can be somewhat confusing since a distinct and much larger body of literature uses the term PAK to refer to p21-activated kinases. Several mammalian kinases in the p21-activated kinase family go by the name PAK1, as does a PAK-encoding gene in *Schizosaccharomyces pombe* (17, 34). To make matters even more confusing, another yeast kinase gene, *PRK1* (YIL095w), was once annotated as *PAK1* (32). The net result of the presence of multiple names for the same genes and different genes with the same name is that the many databases contain incorrect annotations for these genes. Lest there be any confusion, this report concerns the product of *Saccharomyces cerevisiae* gene *PAK1* encoded by open reading frame YER129w.

MATERIALS AND METHODS

Strains and growth conditions. Growth of yeast was done with standard medium at 30°C (26). Glucose was present at 2 or 0.05% (grams per 100 ml), as indicated. Medium containing 2-deoxyglucose contained yeast extract (10 g/liter), peptone (20 g/liter), sucrose (20 g/liter), and 2-deoxyglucose (0.2 g/liter). The *S. cerevisiae* strains used in this study were MSY182 (*MATa ura3-52 leu2 Δ 1 trp1 Δ 63 his3 Δ 200*), FY1193 (*MAT α ura3-52 leu2 Δ 1 trp1 Δ 63 his3 Δ 200 snf1 Δ 10*), MSY608 (*MATa ura3 Δ 0 leu2 Δ 0 his3 Δ 1 met15 Δ 0 kin1::KAN*), MSY678 (*MAT α ura3 Δ 0 leu2 Δ 0 his3 Δ 1 met15 Δ 0 pak1::KAN*), MSY809 [*MATa ura3(52/0) leu2(1 Δ 1/0) his3(1 Δ 1/200) trp1 Δ 63 met15 Δ 0 snf1 Δ 10 pak1::KAN*], MSY839 [*MAT α ura3(52/0) leu2 Δ 0 lys2 Δ 0 trp1 Δ 63 reg1 Δ ::URA3 pak1 Δ ::KAN*], PY102 (*MATa his9-917 Δ lys2 leu2 Δ 1 ura3-52 trp1 Δ 63 reg1 Δ ::URA3*), and PY450 (*MAT α ura3-52 leu2 Δ 1 trp1 Δ 63 his3 Δ 200 reg1 Δ ::URA3 snf1 Δ 10*).

Plasmids. The *PAK1* gene was cloned from a plasmid library of yeast genomic DNA by screening pools of bacterial clones by PCR (28). A 5.7-kb fragment (*MunI-EagI*) encompassing the entire *PAK1* gene was subcloned into pRS424 (29) and designated pPAK1-424. The tandem affinity purification tag (25) was amplified by PCR and introduced into the 3' end of the *PAK1* gene in pPAK1-424 by homologous recombination. Pak1 was tagged at the C terminus with three copies of the myc epitope with plasmid pYM3 (36), PCR amplification, and homologous recombination. Clones of yeast cells expressing GST-Cka1 and GST-Tos3 (19) were isolated from pools of transformants by PCR prior to use. A plasmid expressing glutathione *S*-transferase (GST)-Mig1 in *Escherichia coli* has been described previously (22).

Western blotting. Tagged proteins were detected by Western blotting under conditions described previously (21). Mouse monoclonal antibodies against the hemagglutinin (HA; F7) and myc (9E10) epitopes were purchased from Santa Cruz Biotechnology. Antibodies against Snf1 protein phosphorylated on threonine 210 have been described previously (21). Antibodies were incubated with membranes for 2 h at a dilution of 1/1,000, except for the myc antibody, which was used at a dilution of 1/250 and incubated with membranes overnight.

Immunoprecipitation. Protein extracts were prepared from *snf1 Δ 10 pak1 Δ* (MSY809) cells transformed with empty plasmid vectors or with plasmids expressing Snf1-3HA or Pak1-3myc. Snf1 complexes were collected from 800 μ g of total protein by incubation with 2.5 μ l of undiluted monoclonal antibodies against HA for 1.5 h at 4°C and then incubated with 20 μ l of protein A beads (Sigma). The beads were washed three times with radioimmunoprecipitation assay buffer, and bound proteins were eluted by incubation in sodium dodecyl sulfate (SDS) sample buffer at 95°C.

In vitro kinase assays. Protein kinase assay mixtures contained 0.2 mM [γ -³²P]ATP (1,000 cpm/pmol) in 20 μ l of kinase buffer (20 mM HEPES [pH 7.0], 0.5 mM EDTA, 0.5 mM dithiothreitol, 5 mM Mg acetate) and were incubated at 30°C for 20 min. Substrate proteins (GST-Mig1) were present at approximately 10 μ g/ml. Snf1 kinase was added to autophosphorylation reaction mixtures at a final concentration of approximately 25 ng/ml. When GST-Mig1 was present as the phosphate acceptor, Snf1 kinase was added to a final concentration of 2.5 ng/ml. Proteins were precipitated on ice with 200 μ l of 10% trichloroacetic acid, washed in ice-cold acetone, and resolved by SDS-polyacrylamide gel electrophoresis. Incorporation of ³²P was detected by autoradiography of dried gels.

Protein purification. Snf1 kinase complexes were isolated from cultures grown on sucrose medium by tandem affinity purification (25), followed by Mono Q chromatography as previously described (22). Pak1 kinase was purified by tandem affinity purification (25). GST-Cka1 and GST-Tos3 were purified by glutathione agarose chromatography (24).

RESULTS

Pak1-dependent phosphorylation of Snf1 protein. Three protein kinases, Pak1, Cka1, and Tos3, were identified as potential members of the Snf1 kinase complex (6, 14). To test whether these kinases might also have the ability to phosphorylate and activate Snf1, we used affinity purification methods to isolate Pak1-TAP, GST-Cka1, and GST-Tos3 from yeast and assayed their ability to phosphorylate the Snf1 protein in vitro (Fig. 1A). Purified GST-Cka1 phosphorylated itself (lane 1) and GST-Mig1 (lane 3). When incubated with limiting quantities of purified Snf1, GST-Cka1 caused incorporation of ³²P into two additional proteins. On the basis of results reported in an earlier study (22), we have identified these bands as Sip2/Gal83 and Sip1. GST-Cka1 failed to promote any incorporation of ³²P into the Snf1 polypeptide. Similarly, the GST-Tos3 kinase phosphorylated itself (lane 4), GST-Mig1 (lane 6), and the beta subunits Sip2 and Gal83 (lane 5) but failed to phosphorylate the Snf1 polypeptide. With the same limiting quantities of purified Snf1 enzyme, purified Pak1 did promote phosphorylation of the Snf1 protein, as well as the Sip2 and Gal83 proteins and a 110-kDa polypeptide (lane 7). The ability of purified Pak1 to promote Snf1 phosphorylation was examined in more detail over a 10-fold range of Pak1 kinase concentration (Fig. 1B). Addition of increasing quantities of purified Pak1 kinase resulted in increased incorporation of ³²P into the Snf1 polypeptide. Three prominent phosphorylated species were detected in this reaction mixture. The Snf1 protein and the Sip2 and Gal83 proteins, subunits of the Snf1 kinase complex, are efficiently labeled with ³²P. Since a constant level of Snf1 kinase was present in these reaction mixtures (approximately 2.5 ng/ml) and the level of phosphorylation increased with the addition of Pak1 kinase, we concluded that the Pak1 protein kinase promotes phosphorylation of Snf1 kinase in vitro. A 110-kDa protein and the Sip2/Gal83 proteins are also phosphorylated in a Pak1-dependent and dose-responsive manner.

Pak1 associates with the Snf1 kinase complex in vivo. In order to provide independent verification that the Pak1 protein associates with the Snf1 kinase complex in vivo, we coex-

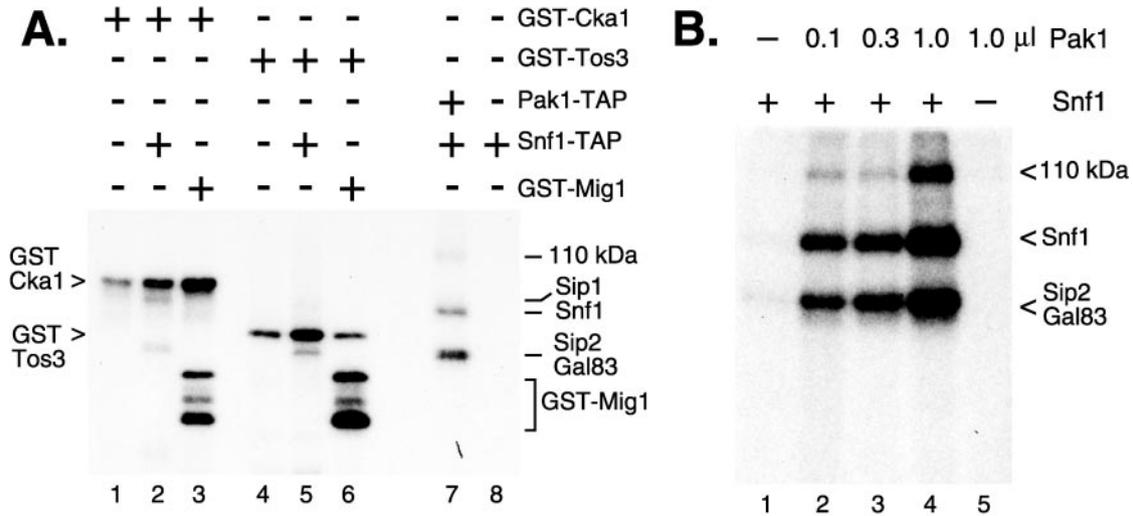


FIG. 1. Pak1 kinase, but not Cka1 or Tos3, promotes phosphorylation of Snf1 in vitro. (A) Reaction mixtures contained $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and additional purified proteins as indicated. Phosphorylated products were detected by autoradiography. (B) Pak1 kinase-dependent phosphorylation of Snf1 kinase components. A limiting concentration (2.5 ng/ml) of Snf1 kinase purified from *pak1Δ* mutant cells (lanes 1 to 4) was incubated with either no Pak1 or increasing concentrations of Pak1 kinase purified from *snf1Δ10* mutant cells. Snf1 kinase was omitted from the reaction mixture in lane 5.

pressed a Pak1 protein tagged with three copies of the myc epitope with Snf1 protein tagged with three copies of the HA epitope. Snf1 kinase complexes were immunoprecipitated with a monoclonal antibody directed against HA, and the bound proteins were then probed by Western blotting with antibodies directed against the myc epitope (Fig. 2). Since Snf1 activation is regulated in response to the glucose concentration, extracts were prepared from cells grown under high- and low-glucose conditions. Consistent levels of Snf1-HA and Pak1-myc were easily detected in the extracts by Western blotting (lower panels). Following immune precipitation with anti-HA antibodies, the Pak1 protein was detected in a complex with Snf1 from glucose-starved cells (lane 6) but only weakly detected in Snf1 complexes isolated from cells grown in glucose-rich medium (lane 3). We concluded that Pak1 associates with Snf1 in vivo and that the association is greatly enhanced under low-glucose conditions when Snf1 is activated.

Purification of Snf1 kinase from *PAK1* and *pak1Δ* mutant cells. To assess the impact of Pak1 on Snf1 kinase activity, the Snf1 kinase was affinity purified from *PAK1* and *pak1Δ* mutant cells. The *pak1Δ* mutant strain used in this experiment was a haploid segregant from the homozygous deletion strain set (7). Because of concerns about differences caused by different strain backgrounds, we used a related haploid *PAK1* mutant strain as our control (haploids MSY608 and MSY678 were derived from Research Genetics strains 31628 and 34056, respectively). Western blotting established that equivalent amounts of Snf1 protein were recovered from both preparations (Fig. 3A). When Mono Q fractions containing Snf1 kinase complexes were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, radioactivity was incorporated into three bands (Fig. 3B). On the basis of results reported previously (22), we have identified two of these bands as Sip2/Gal83 and Snf1. The beta subunits Sip2 and Gal83 were prominently labeled with ^{32}P . These proteins comigrated on SDS gels and appeared as a single band. The

third beta subunit, Sip1, was much less abundant (22) and was faintly visible in some reaction mixtures (Fig. 1, lane 2). The Snf1 polypeptide was also labeled with ^{32}P ; however, label incorporation was greatly reduced when Snf1 complexes were

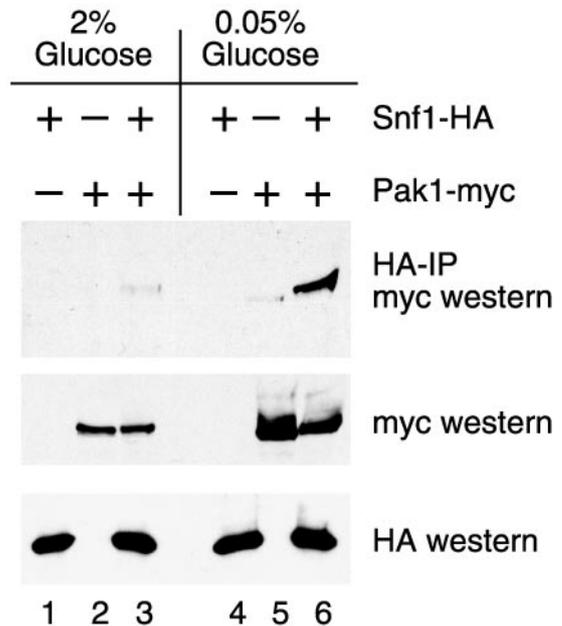


FIG. 2. Pak1 kinase associates with active Snf1 kinase in vivo. Yeast strain MSY809 (*snf1Δ pak1Δ*) was transformed simultaneously with two plasmids, either an empty vector (-) or a plasmid expressing Snf1-HA or Pak1-myc, as indicated above each lane. Protein extracts were prepared from cells grown in medium containing 2 or 0.05% glucose. Snf1 kinase complexes were immunoprecipitated (IP) with an anti-HA monoclonal antibody and probed by Western blot assay with anti-myc antibodies (top panel). Equivalent aliquots of each protein extract (20 μg) were analyzed directly by Western blotting with anti-myc (middle panel) and anti-HA (bottom panel) antibodies.

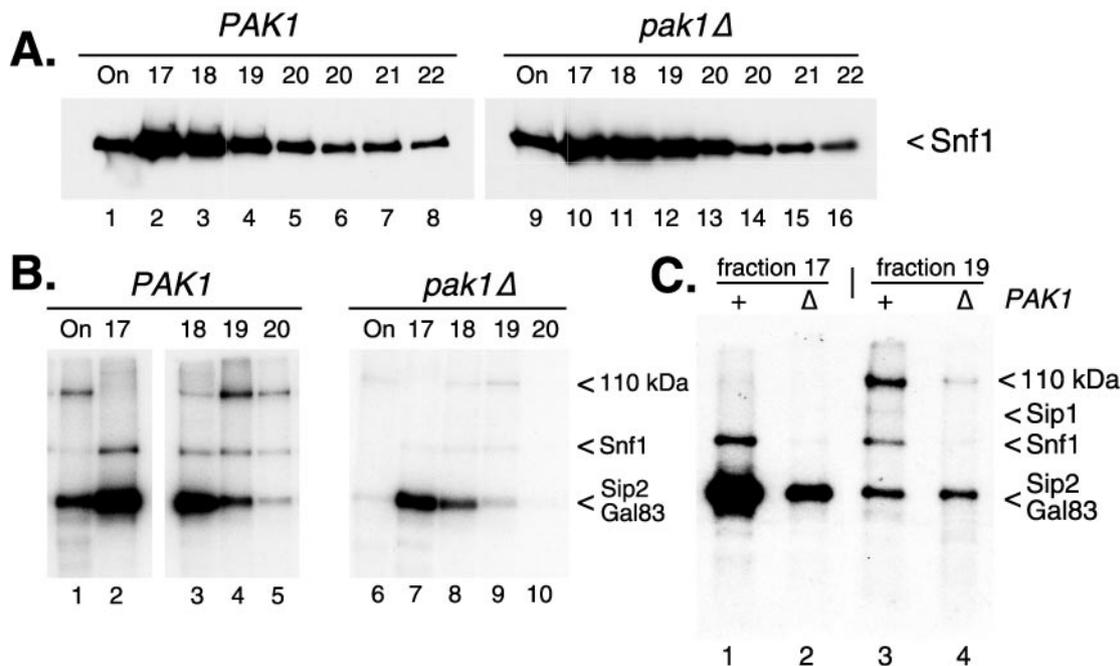


FIG. 3. Effect of *PAK1* on the activity of purified Snf1 kinase. (A) Western blot assay of Snf1 protein purified from *PAK1* and *pak1Δ* mutant cells (22). Input and peak fractions across the NaCl gradient of the final Mono Q column were probed with anti-HA antibodies. (B) Input and peak fractions across the NaCl gradient of the final Mono Q column were incubated with [γ - 32 P]ATP and resolved by SDS-polyacrylamide gel electrophoresis. Incorporation of radioactivity was detected by autoradiography. (C) Equivalent aliquots from fractions 17 and 19 prepared from *PAK1* and *pak1Δ* mutant cells were incubated with [γ - 32 P]ATP and resolved side by side on an SDS-polyacrylamide gel.

purified from *pak1Δ* mutant cells (Fig. 3B, compare lanes 2 and 7). Finally, a third band of approximately 110 kDa was also labeled. Putative members of the Snf1 kinase complex that are in this size range are Reg1 and Pak1 (14). The 110-kDa protein cannot be the Pak1 protein itself since it is detected in Snf1 complexes purified from *pak1Δ* mutant cells. The possibility that the 110-kDa protein is Reg1 is addressed below. Assuming that the abundance of the 110-kDa protein correlates with its 32 P incorporation, this protein was present in the Mono Q input (Fig. 3B, lanes 1 and 6) and in the later fractions from the Mono Q column but absent from early Mono Q column fractions (Fig. 3B, lanes 2 and 7). Thus, Snf1 kinase complexes of differing protein compositions may be separated by Mono Q chromatography. Two forms of the Snf1 kinase complex, those with abundant 110-kDa protein (fraction 19) and those lacking the 110-kDa protein (fraction 17), were incubated with [γ - 32 P]ATP, and the labeled proteins were analyzed side by side (Fig. 3C). First, Snf1 kinase complexes from both fractions showed an almost complete loss of incorporation of 32 P into the Snf1 polypeptide when the enzyme was purified from *pak1Δ* mutant cells. Second, a reduction in overall 32 P incorporation was observed in reaction mixtures containing the 110-kDa protein (fraction 17 versus fraction 19).

The Reg1 protein is present in Snf1 kinase complexes. Mass spectrometry analysis of proteins in the Snf1 kinase complex identified Reg1 as a component (6, 14). Furthermore, genetic and biochemical studies have shown that Reg1 associates with Snf1 and is phosphorylated in a Snf1-dependent manner (27). The Reg1 protein has a predicted molecular mass of 112 kDa. In vitro phosphorylation reactions were performed with Snf1

enzyme purified from *REG1* and *reg1Δ* mutant strains. The 110-kDa protein was not detected in reaction mixtures with Snf1 purified from *reg1Δ* mutant cells (Fig. 4A) even though equivalent levels of Snf1 enzyme were present (Fig. 4B). The simplest explanation for this is that the 110-kDa protein is the Reg1 protein. We have previously shown that the Reg1 protein is required to remove the activating phosphorylation of threonine 210 (21). Further support for the conclusion that Reg1 is the 110-kDa protein is the observation that fractions with an abundance of the 110-kDa band have lower Snf1 kinase specific activity (Fig. 5A, compare titrations of fractions 17 and 19). We noted also that very little 32 P was incorporated into the Snf1 protein purified from *reg1Δ* mutant cells (Fig. 4A). Failure to incorporate 32 P into Snf1 may be due to the fact that threonine 210 is already fully phosphorylated. Western blotting experiments demonstrated that the Snf1 protein was present in the complex purified from *reg1Δ* mutant cells (Fig. 4B) but was not efficiently phosphorylated in vitro.

Snf1 kinase requires Pak1 for full activity. The Snf1 kinase purified from *pak1Δ* mutant cells showed significantly lower levels of in vitro kinase activity than did an equivalent amount of Snf1 purified from *PAK1* cells (Fig. 5A). Snf1 kinase is much more active when assayed with GST-Mig1 as a phosphate acceptor, allowing the use of 10-fold-less Snf1 enzyme. Thus, the autophosphorylation products shown in earlier figures are not observed unless much longer exposures are shown. The Mig1 protein is a known Snf1 target, with four serine residues that are phosphorylated by Snf1 in vivo (33) and in vitro (30). Peak Snf1 complex fractions that lacked Reg1 (fraction 17) or contained abundant Reg1 (fraction 19) were assayed. Deletion of

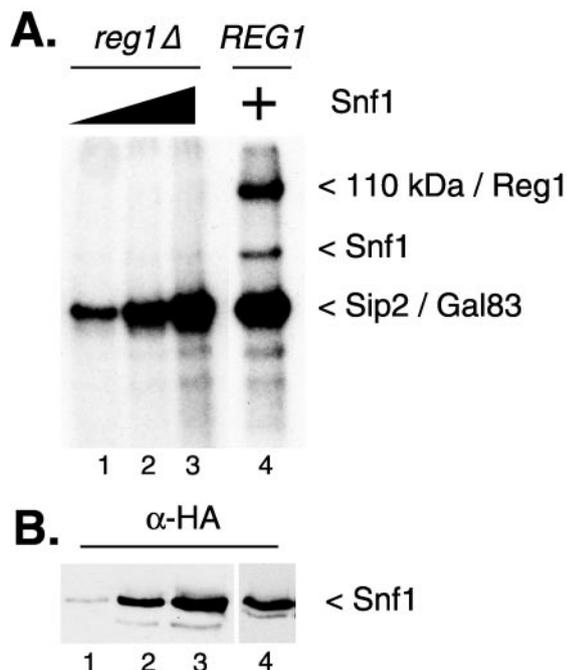


FIG. 4. Snf1 kinase complexes contain Reg1 protein. Snf1 kinase complexes prepared from *REG1* and *reg1Δ* mutant cells were assayed for autophosphorylation activity. Increasing concentrations of Snf1 kinase from the *reg1Δ* mutant cells were used (lanes 1 to 3). Lanes 2 and 4 contain comparable levels of Snf1 protein (approximately 25 ng/ml).

PAK1 led to greatly reduced Snf1 kinase specific activity (compare lanes 1 to 3 with lanes 4 to 6). Equivalent levels of Snf1 protein were included in these reaction mixture, as judged by Western blotting (Fig. 3A). We concluded that Snf1 kinase requires *PAK1* for maximal activity.

Purified Pak1 kinase activates Snf1 in vitro. To determine if the Pak1 kinase could activate Snf1 in vitro, Pak1 kinase was affinity tagged and purified from *snf1Δ10* mutant cells (25). The Snf1 kinase was purified from *pak1Δ* mutant cells and showed a low but detectable level of kinase activity in vitro (Fig. 5B). Addition of purified Pak1 kinase significantly increased Snf1 kinase activity, as judged by the level of GST-Mig1 phosphorylation (compare lanes 4 to 6 with lanes 1 to 3). In the absence of added Snf1 kinase, no phosphorylation of GST-Mig1 was observed, demonstrating that Pak1 itself does not phosphorylate GST-Mig1 (lane 7). Thus, Pak1 kinase is able to stimulate Snf1-dependent phosphorylation of GST-Mig1.

Genetic manipulation of *PAK1*. Our data support the idea that the Pak1 kinase is a Snf1-activating kinase. This hypothesis was examined genetically by phenotypic analysis of *pak1Δ* mutant strains. Our source for the *pak1Δ::KAN* allele was the homozygous diploid deletion collection (7). Haploid progeny of strain 36128 were crossed into our own strain background, and tetrads were analyzed for Snf phenotypes. None of our *pak1Δ* mutant strains exhibited even a weak Snf phenotype. All *pak1Δ* mutant strains grew as well as wild-type cells on raffinose-antimycin medium (Fig. 6A) and on glycerol-ethanol medium at 15, 30, and 37°C. We tested whether deletion of *PAK1*

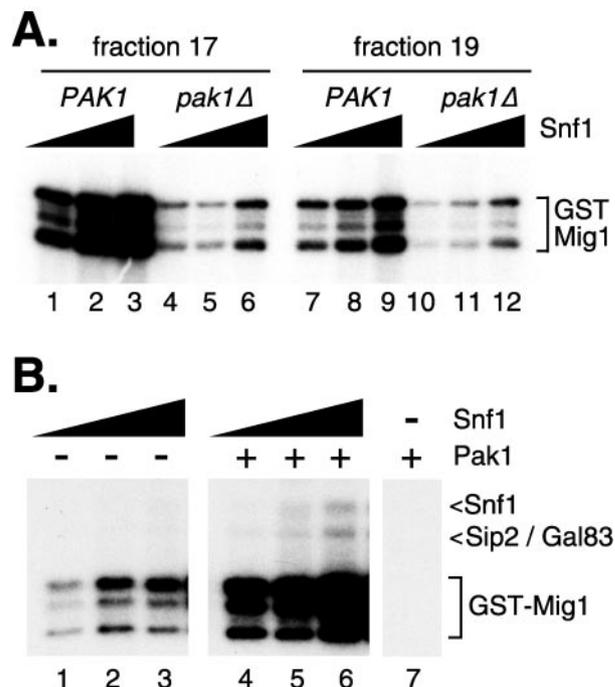


FIG. 5. Pak1-dependent activation of Snf1 kinase activity. (A) Increasing concentrations of Snf1 kinase complexes (fractions 17 and 19) were incubated with [γ - 32 P]ATP and recombinant GST-Mig1 protein. Incorporation of radioactivity into the GST-Mig1 protein (and its proteolytic fragments) was detected by autoradiography. Snf1 kinase had been purified from *PAK1* (lanes 1 to 3 and 7 to 9) and *pak1Δ* (lanes 4 to 6 and 10 to 12) mutant cells. (B) Increasing concentrations of Snf1 kinase purified from *pak1Δ* mutant cells (fraction 17) were incubated with [γ - 32 P]ATP, GST-Mig1 protein, and either buffer (lanes 1 to 3) or purified Pak1 kinase (lanes 4 to 6). Snf1 kinase was omitted from one reaction mixture (lane 7).

could exacerbate the weak Snf phenotype observed in the *sip2Δ gal83Δ* mutant strain. Deletion of *PAK1* did not affect the Snf phenotype of this strain (data not shown). All *pak1Δ* mutant strains were able to induce invertase expression with normal kinetics and to similar degrees (Fig. 6B). We also crossed our *pak1Δ* mutant strains with several other strains bearing deletions of other kinases that are candidates for being Snf1-activating kinases (*CKA1*, *CKA2*, *TOS3*, *PKH1*, *PKH2*, *KIN1*, *KIN2*, *NPR1*, et al.). None of the double mutants produced a synthetic Snf phenotype. We also examined the effect of an increased *PAK1* gene dosage. A wild-type allele of *PAK1* and a kinase-dead allele (D277A) were introduced into both wild-type and *pak1Δ* mutant cells on high-copy-number plasmids. In neither case was any change in growth on alternative carbon sources (data not shown) or invertase expression detected (Fig. 7C).

Suppression of *reg1Δ* by *pak1Δ*. We did detect genetic interaction between *pak1Δ* and *reg1Δ*. The Reg1 protein is one of the regulatory subunits of type 1 protein phosphatase Glc7 (35). The Reg1-Glc7 complex acts in opposition to the Snf1 signaling pathway by promoting the dephosphorylation of Snf1 threonine 210 (21). On glucose medium, *reg1Δ* mutant strains exhibit derepressed levels of invertase and galactokinase (20, 23) and form small colonies (4). Both phenotypes are Snf1 dependent. If Pak1 kinase is one of the Snf1-activating kinases,

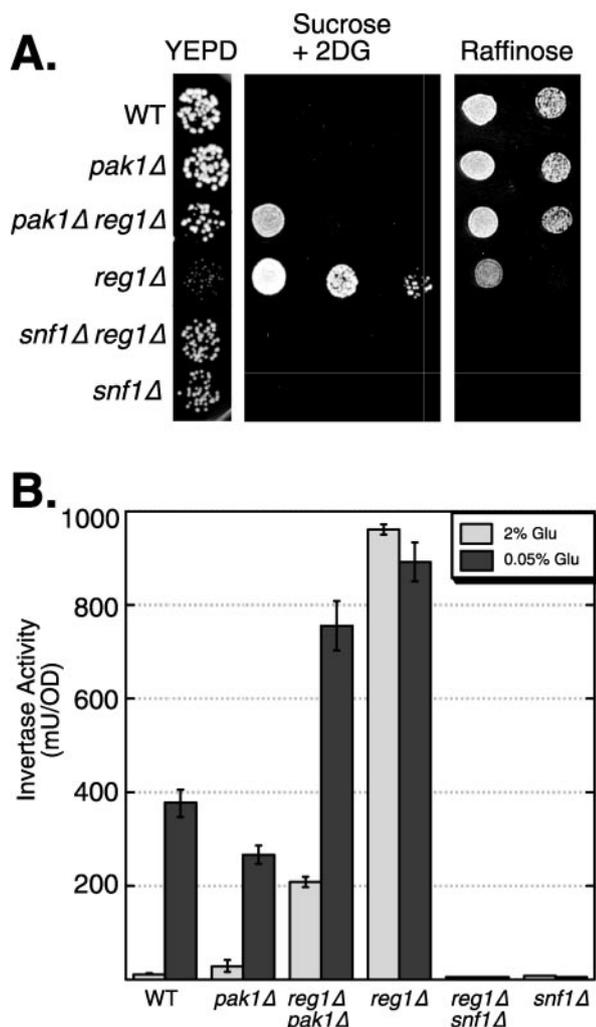


FIG. 6. Suppression of *reg1Δ* by deletion of *PAK1*. (A) Growth phenotypes were determined by spotting serial dilutions of yeast cultures on YEPD (26), sucrose medium containing 2-deoxyglucose (2DG), and raffinose medium. The relevant genotypes of the yeast strains are shown on the left. (B) Invertase expression of cells grown in 2 or 0.05% glucose (Glu). The relevant genotypes of the yeast strains are shown on the bottom. The mean invertase expression from three independent cultures is plotted. Error bars represent one standard error. WT, wild type; OD, optical density.

then deletion of the *PAK1* gene might suppress some or all of the Snf1-dependent phenotypes observed in *reg1Δ* mutant strains. To test this prediction, we crossed *pak1Δ* and *reg1Δ* mutant strains and characterized the phenotypes of the *pak1Δ reg1Δ* double mutant. We found that deletion of *PAK1* effectively suppressed a number of the phenotypes of *reg1Δ*. First, *reg1Δ* mutant cells grown on glucose medium formed small colonies and this phenotype was suppressed by deletion of *PAK1* (Fig. 6A). Wild-type cells are unable to grow on sucrose medium in the presence of 2-deoxyglucose (23). Deletion of *REG1* causes constitutive relief of glucose repression and allows cells to grow on sucrose medium in the presence of 2-deoxyglucose. Deletion of *PAK1* severely restricted the ability of *reg1Δ* mutant cells to grow on 2-deoxyglucose medium (Fig. 6A). In addition, the constitutive invertase expression observed

in *reg1Δ* mutant cells was partially suppressed in *reg1Δ pak1Δ* mutant cells (Fig. 6B). Therefore, *pak1Δ* suppresses many of the Snf1-dependent phenotypes observed in *reg1Δ* mutant cells.

Pak1 phosphorylates Snf1 on threonine 210. Previous studies have shown that the key regulatory phosphorylation site on the Snf1 protein is threonine 210 within the activation loop (5, 21). To determine whether Pak1 phosphorylates Snf1 on threonine 210, we used a phosphopeptide antibody that specifically recognizes Snf1 that has been phosphorylated on threonine 210 (21). Purified Snf1 kinase was incubated with or without ATP in the presence or absence of Pak1 kinase (Fig. 7A). Increased phosphorylation of threonine 210 was detected only when both Pak1 and ATP were present. Therefore, Pak1 kinase promotes the phosphorylation of threonine 210. We next tested whether manipulation of the *PAK1* gene copy number affects the phosphorylation of Snf1 threonine 210 in vivo (Fig. 7B). Strains expressing Snf1-HA were transformed with a high-copy-number plasmid with either no insert (2 μ m vector) or full-length *PAK1* (2 μ m *PAK1*). Protein extracts were prepared in duplicate from cells grown under high-glucose conditions (2% glucose), and the Snf1 polypeptide was collected by immune precipitation with anti-HA antibodies. Snf1 protein was then detected by parallel Western blot assays with antibodies directed against Snf1 phosphorylated on threonine 210 or against the HA epitope, as indicated. Increased *PAK1* gene dosage resulted in a significant increase in the level of phosphorylated threonine 210. Equivalent levels of total Snf1 polypeptide were present in all four extracts (lanes 1 to 4). To determine whether Pak1 kinase activity is required, a point mutation was introduced into the *PAK1* gene such that the catalytic aspartate residue was changed to alanine (D277A). Cells transformed with a high-copy-number *PAK1* plasmid with a wild-type (WT) kinase domain and cells transformed with a kinase-dead (D277A) high-copy-number *PAK1* plasmid were compared. Pak1-mediated stimulation of Snf1 threonine 210 phosphorylation required an intact Pak1 kinase domain (compare lanes 5 and 6). However, increased phosphorylation of Snf1 threonine 210 was not sufficient to activate Snf1, as judged by invertase assays (Fig. 7C). Taken together, our data indicate that the Pak1 kinase activates the Snf1 kinase by phosphorylation of the Snf1 activation loop in vitro and in vivo.

DISCUSSION

The Snf1 kinase is activated by an upstream kinase (21). However, genetic screens have failed to identify mutations in any protein kinase gene other than *SNF1* that produces a Snf⁻ phenotype. We have argued previously that the most likely explanation for these findings is that more than one kinase is capable of activating Snf1 in vivo. In this report, we present the following evidence that the Pak1 kinase is one of the Snf1-activating kinases. First, Pak1 associates with the Snf1 kinase complex in vivo and the association is greatly enhanced under growth conditions that promote Snf1 activation (Fig. 2.). This result puts Pak1 in the right place at the right time. Second, Pak1 kinase activates Snf1 kinase in vitro (Fig. 5B). Third, purified Pak1 is able to phosphorylate Snf1 protein on threonine 210 within the activation loop (Fig. 7A) and an increased *PAK1* gene dosage increases the level of threonine 210 phos-

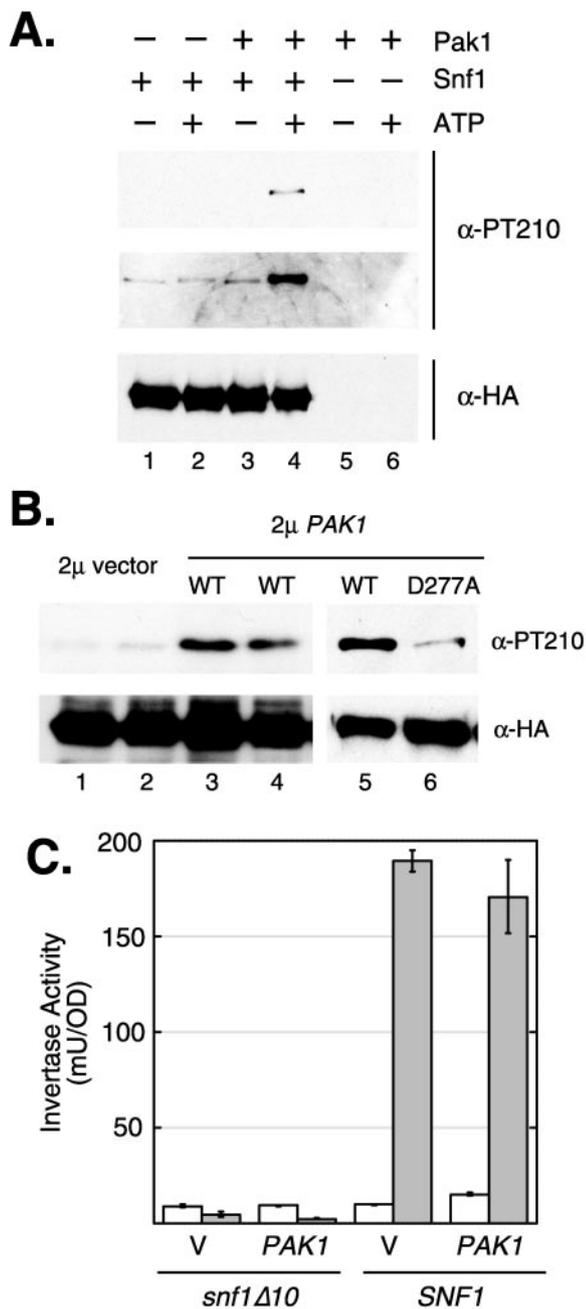


FIG. 7. Pak1 phosphorylates Snf1 on activation loop residue threonine 210. (A) Reaction mixtures contained Snf1 kinase purified from *pak1Δ* mutant cells (lanes 1 to 4), Pak1 kinase purified from *snf1Δ10* mutant cells (lanes 3 to 6), and ATP (lanes 2, 4, and 6). Products of the reaction were resolved on an SDS-polyacrylamide gel, transferred to a nylon filter, and probed first with an antibody specific for Snf1 protein containing phosphorylated threonine 210 (21). Both short (top panel) and long (middle panel) exposures of this Western blot are shown (α -PT210). Total Snf1 protein was detected by reprobing the same filter with an antibody directed against the HA epitope (α -HA). (B) Yeast strains expressing HA-tagged Snf1 were transformed with high-copy-number plasmid pRS424 (vector), pPAK1-424 (WT), or pPAK-D277A (D277A), as indicated. Snf1 kinase was immune precipitated from protein extracts prepared from cultures grown in high glucose (2%) and probed by Western blotting with antibodies specific for phosphorylated threonine 210 (α -PT210) or the HA epitope (α -HA). (C) Cells with and without *SNF1* were transformed with a high-copy-number plasmid containing either no insert (V) or full-length

phorylation in vivo. Finally, Snf1 kinase purified from *pak1Δ* mutant strains has a lower specific activity than Snf1 kinase purified from *PAK1* mutant strains (Fig. 5A). Taken together, these data demonstrate that Pak1 is a Snf1-activating kinase. Our current model for the phosphorylation events occurring in the Snf1 kinase complex is presented in Fig. 8.

The one troubling aspect of this work is that the *pak1Δ* allele does not produce any detectable Snf1 phenotype. We cannot formally rule out the possibility that Pak1 is a kinase whose in vivo function is to activate kinases other than Snf1. However, the findings that Pak1 associates with Snf1 in vivo under conditions in which Snf1 is active and that Pak1 promotes phosphorylation of Snf1 threonine 210 in vivo argue against this interpretation. Furthermore, we show that deletion of *PAK1* suppresses the phenotypes associated with *reg1Δ*. The Reg1-Glc7 PP1 phosphatase is known to act in opposition to Snf1 kinase. Deletion of *REG1* results in constitutively activated Snf1. The observation that the *pak1Δ* mutation suppresses the phenotypes caused by the *reg1Δ* mutation argues strongly that Pak1 is one of the Snf1-activating kinases in vivo. We favor the interpretation that activation of Snf1 kinase is a redundant function that is shared between Pak1 kinase and one or more other kinases. Hartwell and colleagues have pointed out that redundancy is an important source of buffering genetic variation (10). Many yeast genes have one or more paralogues that may mask phenotypes caused by mutations. In the case of Pak1, the closest relative is Tos3 (16); however, the *pak1Δ tos3Δ* mutant strain is also Snf1⁺ (data not shown). Identification of the other putative Snf1-activating kinase(s) would settle the question unambiguously and is a high priority for our future studies. An increased *PAK1* gene dosage leads to increased phosphorylation of Snf1 threonine 210 under glucose-repressing conditions but does not lead to activation of Snf1 kinase. Previously, we showed that activation of Snf1 kinase is a two-step process (21). We conclude that phosphorylation of Snf1 threonine 210 is required but not sufficient for Snf1 activation.

The *PAK1* gene was first isolated in a screen for high-copy suppressors of the temperature sensitivity phenotype caused by the *cdc17-1* mutation (15). *POL1* (also known as *CDC17* or *HPR3*) encodes one subunit of the alpha DNA polymerase-primase complex. An increased *PAK1* copy number partially suppresses the temperature sensitivity phenotype caused by several alleles of *POL1*. However, a direct connection between Pak1 and DNA polymerase alpha was not established. Furthermore, an increased *PAK1* gene dosage did not suppress mutations in other DNA polymerases or even other mutations affecting DNA polymerase alpha. The ability of *PAK1* to suppress the *cdc17-1* allele required a functional kinase domain. We show here that Pak1 kinase is able to phosphorylate the activation loop threonine of Snf1 kinase (Fig. 7). In addition, the mammalian homologues of Pak1 also activate downstream kinases by phosphorylation of activation loop residues. These

PAK1. Cells were collected from log-phase cultures grown in 2% (open bars) or 0.05% (filled bars) glucose and assayed for invertase activity. The mean invertase activity (milliunits per unit of optical density [OD]) from three independent transformants is plotted. Error bars represent one standard error.

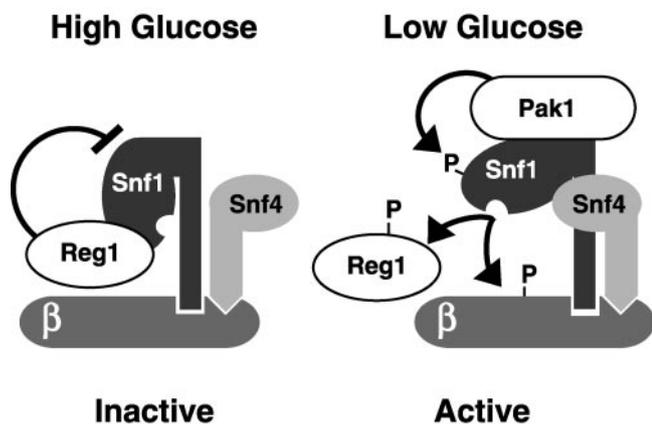


FIG. 8. Model of Pak1-dependent activation of Snf1 kinase. In the presence of a high glucose concentration, Snf1 kinase is in a complex with the Reg1 protein, which recruits protein phosphatase Glc7 (not shown). The Reg1/Glc7 complex dephosphorylates Snf1 threonine 210 (21). In the presence of a low glucose concentration, Pak1 associates with the Snf1 kinase complex and phosphorylates Snf1 on threonine 210. Snf4 also participates in activation of the Snf1 kinase through direct interaction with the Snf1 regulatory domain. Once activated, Snf1 kinase phosphorylates its beta subunit (Sip1, Sip2, or Gal83), the Reg1 protein, and additional substrates, such as Mig1 (not shown).

observations suggest that Pak1 suppressed the *cdc17-1* allele by activating a downstream kinase. It is unlikely that Pak1 is devoted to Snf1 activation. Some other *in vivo* target of Pak1 might provide an explanation of its ability to suppress mutations affecting the alpha DNA polymerase-primase complex.

Independent experiments demonstrate that Pak1 associates with the Snf1 kinase complex *in vivo*. In mass spectrometry studies of proteins associated with TAP-tagged Snf1 and TAP-tagged Snf4, both detected Pak1 as a complex member (6). We used myc-tagged Pak1 and HA-tagged Snf1 to demonstrate the association of these proteins by coimmunoprecipitation (Fig. 2). In the course of that experiment, we found that Pak1 accumulation increased under conditions of glucose limitation. A similar increase in Pak1 mRNA was not detected in microarray experiments (3, 18), suggesting that the increase in Pak1 protein abundance may reflect posttranscriptional regulation. Also, a shift in the SDS-polyacrylamide gel mobility of the Pak1 protein in response to glucose limitation was observed, suggesting that the activity or abundance of Pak1 may be regulated by a posttranslational modification.

The mammalian homologue of Snf1 kinase, AMPK, is the subject of intense study, in part because of its potential as a target for treatment of type II diabetes (12). Biochemical studies have shown that AMPK is activated by a distinct protein kinase designated AMPKK (11). The identity of AMPKK has not been determined. Our finding that Pak1 kinase is one of the Snf1-activating kinases in yeast suggests that the mammalian homologue of Pak1, CaMKK- β , may play a similar role in mammalian cells. Biochemical properties of AMPKK and CaMKK- β were used to argue that these enzymes are distinct (13). However, this study based its conclusion on efficiency of activation and relied on partially purified fractions containing AMPKK and CaMKK- β . In some studies, CaMKK- β has been used as a surrogate AMPKK since recombinant CaMKK- β is able to phosphorylate the activation loop threonine of AMPK

in vitro (8). Full activation of AMPK may involve more than one phosphorylation event (31). Perhaps efficient activation of AMPK requires the action of more than one kinase enzyme, a possibility that may explain the difficulty with the purification and identification of a single AMPKK. Our data suggest that CaMKK- β may participate in the activation of AMPK *in vivo* by phosphorylation of the activation loop threonine.

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