

Isolation of Mutations in the Catalytic Domain of the Snf1 Kinase That Render Its Activity Independent of the Snf4 Subunit

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Activation of the Snf1 kinase requires at least two events, phosphorylation of the activation loop on threonine 210 and an Snf4-dependent process that is not completely defined. Snf4 directly interacts with a region of the regulatory domain of Snf1 that may otherwise act as an autoinhibitory domain. In order to gain insight into the regulation of Snf1 kinase by Snf4, deletions in the regulatory domain of the catalytic subunit were engineered and tested for their effect on Snf1 function in the absence of Snf4. Deletion of residues 381 to 488 from the Snf1 protein resulted in a kinase that was activated by glucose limitation even in the absence of the Snf4 protein. A larger deletion (amino acids 381 to 608) encompassing virtually the entire regulatory domain resulted in complete inactivation of the Snf1 kinase even in the presence of Snf4. A genetic screen for amino acid substitutions that conferred an Snf4-independent phenotype identified four point mutations in the Snf1 catalytic domain. One very conservative mutation, leucine 183 to isoleucine, conferred nearly wild-type levels of Snf1 kinase function in the absence of the Snf4 protein. Purified Snf1 kinase was inactive when isolated from *snf4* Δ cells, whereas the Snf1-L183I kinase exhibited significant activity in the absence of Snf4. Our data support the idea that Snf1 kinase activity is constrained in *cis* by an autoinhibitory domain and that the Snf4-mediated activation of Snf1 can be bypassed by subtle conformational changes in the catalytic domain of the Snf1 kinase.

The Snf1 kinase of *Saccharomyces cerevisiae* is a member of a highly conserved subfamily of serine-threonine protein kinases that includes the AMP-activated protein kinase (AMPK) found in mammalian cells (9). The Snf1 and AMPK enzymes function as heterotrimers, containing a catalytic alpha subunit and noncatalytic beta and gamma subunits (4, 23). Members of this kinase family are distinguished from other serine-threonine kinases by a high degree of sequence identity in the kinase domain and by the presence of a C-terminal regulatory domain that mediates interaction with the beta and gamma subunits (13). In mammalian cells, two genes have been identified for both the alpha and beta subunits (2, 26), while three gamma subunit genes have been found (2). The presence of multiple genes for each subunit combined with differences in tissue-specific expression results in the presence of numerous, distinct AMPK enzyme complexes that differ in subunit composition. Defining the exact role of these complexes remains a challenge.

In yeast, the subunit composition is less complex, with a single alpha subunit gene, *SNF1*, and a single gamma subunit gene, *SNF4*. However, yeast does carry three beta subunit genes, *SIP1*, *SIP2*, and *GAL83*, and therefore has the potential to express three distinct Snf1 enzyme complexes. The specific role of each complex is beginning to be understood. We have shown that the presence of a beta subunit is required for kinase function (19) and that yeast strains expressing a single beta subunit have distinct growth phenotypes as well as differing

abilities to phosphorylate the Sip4 protein (19). Work by Vincent et al. has shown that the beta subunits confer different subcellular localizations to the enzyme complex (25). Thus, the three forms of the Snf1 kinase present in yeast cells are likely to have specialized roles determined by different localization patterns and substrate specificities.

The focus of this study is on the regulatory role played by the gamma subunit of the Snf1 kinase complex. The gamma subunit, encoded by *SNF4*, is essential for the full activation of Snf1 kinase (1). Previous studies have found that the Snf1 and Snf4 proteins are held in the kinase complex through constitutive binding to the beta subunit (13). In addition, Snf4 makes direct contact with the regulatory domain of the catalytic alpha subunit, and this interaction is regulated by the availability of glucose (12). How binding of Snf4 to the regulatory domain controls the activity of the alpha subunit is not fully understood. One model for this regulation proposes the existence of an autoinhibitory domain present in the alpha subunit (3, 12). The gamma subunit and the kinase domain compete for binding to the autoinhibitory domain. Under conditions of glucose excess, the kinase domain binds the autoinhibitory domain, thereby forming an inactive complex. When glucose is limiting, the gamma subunit binds the autoinhibitory domain, displacing and thereby relieving the inhibition of the kinase domain. A second event, phosphorylation of a conserved threonine residue in the activation loop, is also required for the full activation of the Snf1 and AMPK enzymes (10, 14). However, these two events are not dependent on one another, since phosphorylation of Snf1 threonine 210 occurs normally in cells lacking the Snf4 protein (14).

In order to better understand the Snf4-mediated regulation of the Snf1 kinase complex, we used a combination of protein

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TABLE 1. Oligonucleotides

Oligonucleotide	Sequence ^a	Restriction change
SNF1-T390	GGTTATAGAGTACGCCGGAACG	NA ^b
SNF1-B1922	CATTCTTTTACGTTCCACCATC	NA
SNF1Δ381–415	gaagacactctgcattcaacgaaattaggACTTTTCAACAACAAAGCAAATCCC	Size
SNF1Δ381–488	gacactctgcattcaacgaaattaggGAAGCTTCTAAAATATCTCCTCTGTAAAC	Size
SNF1Δ381–608	gaagacactctgcattcaacgaaattaggAGTACTTTTTTCAGCCTACCCATTTTTAC	Size
SNF1-L1831	CATAGAGATCTGAAGCCTGAAAAACaTtCTACTAGATGAGCATCTGAATG	<i>Xmn</i> I
SNF1-K192R	CATCTGAATGTAcgtATTGCCGATTTTGGTTTGTCAAAC	<i>Bsa</i> AI
SNF1-1241N	GTGTGGTCATGTGGGGTTAaCCTTTATGTTATGCTTTGTCTG	<i>Hpa</i> I

^a Changes between upper- and lowercase signify boundaries between wild-type and altered sequences.

^b NA, not applicable.

engineering and random mutagenesis to isolate variants of the catalytic subunit that were no longer dependent on Snf4 for activation. Our results support the idea that the Snf4 subunit counteracts the effect of the autoinhibitory domain. Surprisingly, we also found that subtle changes in the catalytic domain are able to bypass the need for the gamma subunit.

MATERIALS AND METHODS

Yeast strains, media, and genetic techniques. The *S. cerevisiae* strains utilized in this study were FY1193 (*MATα ura3-52 leu2Δ1 his3Δ200 trp1Δ63 snf1Δ10*) and MSY563 (*MATα ura3-52 leu2Δ1 his3Δ200 trp1Δ63 snf1Δ10 snf4Δ1*). For carbon sources, glucose was present at 2% (grams/100 ml), while the glycerol-ethanol mixture was present at 3% (vol/vol) glycerol and 2% (vol/vol) ethanol. Raffinose medium contained 2% raffinose and 0.05% (grams/100 ml) glucose as the carbon source and antimycin A at 1 μg/ml. Plasmid transformations of yeast strains was by the lithium acetate procedure (7).

Site-directed mutagenesis. Deletions and point mutations in the *SNF1* gene were constructed by site-directed mutagenesis (5) with the oligonucleotides listed in Table 1. The integrity of all deletion junctions and point mutations was confirmed by DNA sequencing.

Random mutagenesis and genetic selection. Random mutations in the *SNF1* gene were introduced by PCR amplification of a DNA fragment encompassing the *SNF1* open reading frame from amino acid 130 through end of the frame, using primers Snf1-T390 and Snf1-B1922 (Table 1). *Taq* polymerase was used in reaction mixtures containing 2 mM MgCl₂ and a 0.2 mM concentration of each deoxynucleoside triphosphate. The amplified fragment was then cotransformed with the centromeric plasmid pRS314 (21) containing an epitope tagged *SNF1* gene that had been gapped at codons 175 and 628 by treatment with *Bgl*II and *Hpa*I (see Fig. 3). Transformants of the yeast strain MSY563 (*snf1Δ10 snf4Δ1*) were selected on SC medium (17) lacking uracil and replica plated to medium containing raffinose and antimycin A. Cells that were able to grow on raffinose medium (Snf⁺) were retained for further study. Plasmids isolated from Snf⁺ colonies were amplified in *Escherichia coli* and sequenced.

Western blots. Hemagglutinin (HA)-tagged Snf1 proteins were detected by Western blotting (20) with a mouse monoclonal antibody directed against the HA epitope (Santa Cruz Biotechnology).

Enzyme assays. Quantitative invertase assays were performed as previously described (20). Specific activity was defined in terms of milliunits of invertase activity (with 1 U being equal to the activity required to release 1 mmol of glucose per min) per unit of optical density at 600 nm of cells assayed. Snf1 kinase activity was assayed in reaction mixtures containing kinase buffer (20 mM HEPES [pH 7.0], 0.5 mM EDTA, 0.5 mM dithiothreitol, and 5 mM Mg-acetate), 0.2 mM [^γ-³²P]ATP (1,000 cpm/pmol), 10 μg of glutathione *S*-transferase (GST-Mig1 protein per ml, and approximately 2.5 ng of Snf1 kinase per ml. Reaction mixtures were incubated at 30°C for 20 min, and reactions were stopped by addition of 10 volumes of ice-cold 10% trichloroacetic acid. Samples were precipitated, washed in acetone, and resolved on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Dried gels were subjected to autoradiography.

Snf1 purification. The Snf1 protein was tagged and purified by the tandem affinity purification method (16) followed by an additional chromatography step on a 1-ml MonoQ column as described elsewhere (14a).

Structural model of the Snf1 kinase domain. A structural model of the Snf1 kinase domain was prepared by ProMod II as part of the Swiss-Model Automated Protein Modelling Server (8, 15). The Snf1 model used the Protein

Databank coordinates of the related kinases CHK1 and PKA (PDB files 1IA8, 1FOT, 1YDS, 1YDR, and 1YDT) and included Snf1 residues 48 to 317. Coordinates for the Snf1 structural model are available on request.

RESULTS

Mutants with deletions in the Snf1 regulatory domain.

Many proteins contain autoinhibitory domains that regulate enzyme activity in *cis*. Deletions in the regulatory domain of AMPK indicate that such an autoinhibitory mechanism plays a role in its regulation (3). Current models of the regulation of Snf1 kinase invoke a similar regulatory role for a region immediately C terminal of the Snf1 kinase domain (12, 13). Sequence alignments between the AMPK alpha subunits from yeast, *Drosophila*, and various mammalian species identified a conserved amino acid motif (amino acids 379 to 415 in Snf1) located directly after the kinase domain that was suggested to function as an autoinhibitory domain (3). To test this model directly, we engineered specific internal deletions in the regulatory domain of the Snf1 kinase alpha subunit by site-directed mutagenesis. The catalytic kinase domain, amino acids 1 to 380, was left intact. Three progressively larger deletion mutations were made starting at amino acid 381 and including amino acid 414, 488, or 608 (Fig. 1). The Δ381-414 deletion specifically removes the putative autoinhibitory motif identified by Crute et al. (3). The Δ381-488 deletion removes the majority of the regulatory domain that interacts with gamma subunit, Snf4 (12, 13). The largest deletion, Δ381-608, removes all but the final 30 amino acids of the C-terminal regulatory domain, including the entire Snf4-interacting region and most of the beta subunit-interacting region (13). The wild-type protein and all deletion constructs contained three copies of the HA epitope at the C terminus, allowing detection by Western blotting. The internal deletions did not reduce the accumulation of the Snf1 protein, since the wild type and all deletion mutants were detected at comparable levels (Fig. 1B). Indeed, the mutant with largest internal deletion, which removed amino acids 381 to 608, appeared to be more abundant than the wild-type protein when normalized to the loading control. An even greater increase in abundance was observed in mammalian cells when the regulatory domain was removed from the AMPK alpha subunit (3).

The ability of the internal deletion mutants to provide Snf1 kinase function in vivo was assayed by testing the ability of these constructs to complement an *snf1Δ10* mutant for growth on raffinose medium. Since the Snf4 subunit is thought to

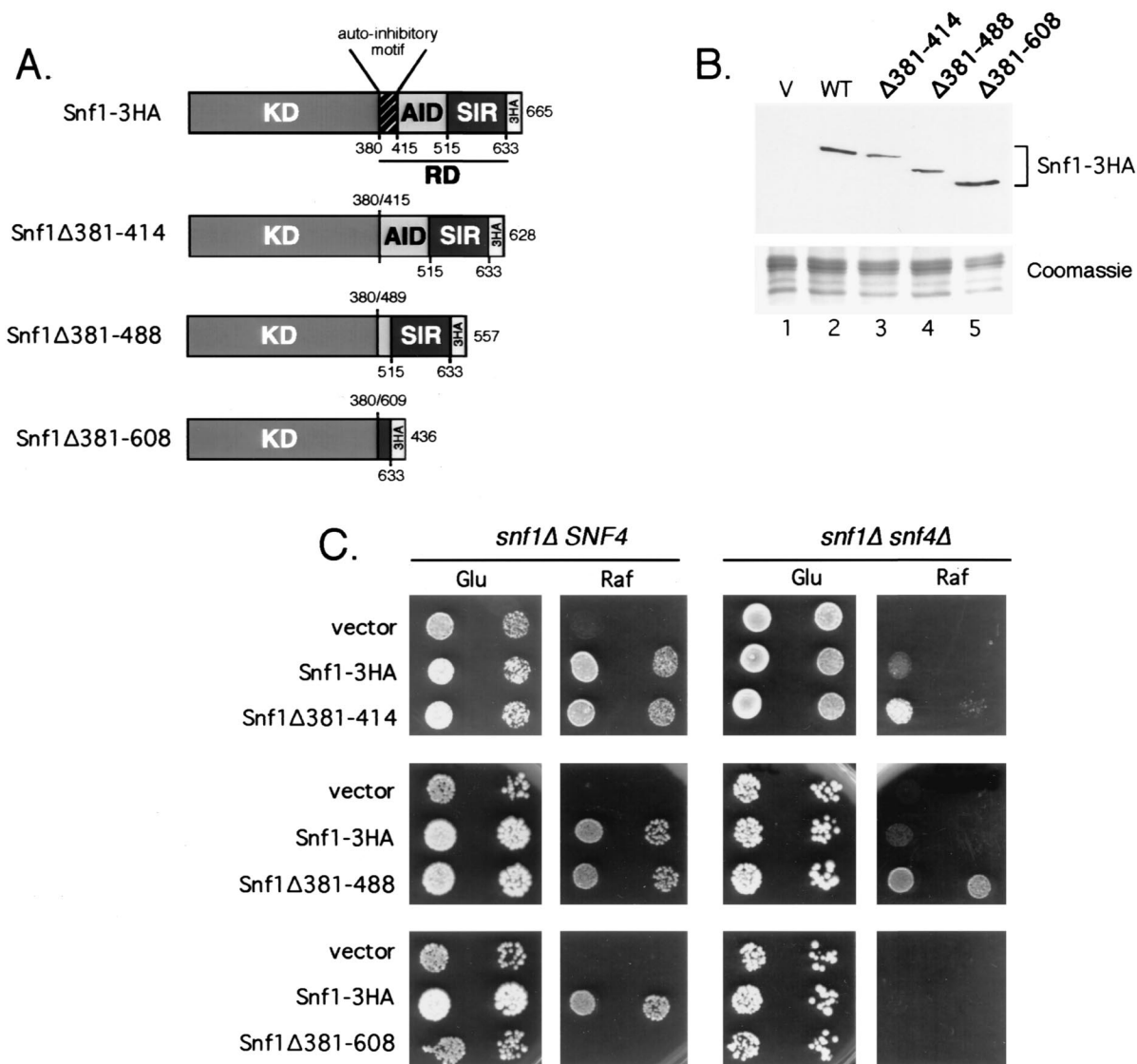


FIG. 1. Deletion of an internal inhibitory domain confers Snf4 independence. (A) Schematic representation of the Snf1 protein. Wild-type Snf1 and three internal deletion mutants are shown. Shown are the kinase domain (KD) (residues 1 to 380), the autoinhibitory domain (residues 380 to 415), the extended inhibitory domain (AID) (residues 380 to 515), the SIP-interacting region (SIR) (residues 515 to 633), the C-terminal epitope tag (3HA), and the regulatory domain (RD). (B) Western blot of Snf1 and internal deletion mutants. Equivalent aliquots of yeast whole-cell extracts (20 μ g of protein) were resolved by SDS-polyacrylamide gel electrophoresis and either transferred to a nylon membrane and probed with antibodies against the HA epitope or stained in Coomassie blue. Cells were transformed with vector (V) (lane 1), wild-type Snf1-3HA (WT) (lane 2), or internal deletion mutants (lanes 3 to 5). (C) Growth phenotypes of cells expressing no Snf1, wild-type Snf1, or internal deletion mutants. Cells (*snf1 Δ 10 SNF4* or *snf1 Δ 10 snf4 Δ 1*) transformed with the indicated plasmids were normalized to an optical density at 600 nm of 0.2, and 10-fold serial dilutions were spotted onto SC medium lacking uracil and containing either glucose (Glu) or raffinose (Raf) as the carbon source.

counteract the effect of the autoinhibitory domain, these constructs were tested in the presence and absence of the *SNF4* gene (Fig. 1C). In the presence of Snf4 protein, the *SNF1 Δ 381-414* and *SNF1 Δ 381-488* alleles were functional and indistinguishable from wild-type *SNF1*, indicating that these residues are not required for Snf1 function in this assay. In contrast, the *SNF1 Δ 381-608* allele was completely nonfunctional even though the protein is expressed at least as well as the wild type. In the absence of Snf4 protein, Snf1 kinase function is compromised and cells grow poorly on raffinose. Deletion of amino acids 381 to 414 and 381 to 488 from the Snf1 protein produces

a significant increase in the ability of the *snf4 Δ 1* cells to grow on raffinose relative to the cells expressing wild-type Snf1. The Δ 381-488 deletion consistently provides a greater degree of Snf4-independent function to the Snf1 kinase than the Δ 381-415 deletion.

Invertase activity assays provide an additional measure of Snf1 kinase function. To assess the activity of the internal deletion mutants in the absence of *SNF4*, plasmids carrying wild-type *SNF1*, the internal deletion mutations, or no insert (vector) were transformed into cells lacking chromosomal copies of *SNF1* and *SNF4*. Cells grown under glucose-repressing

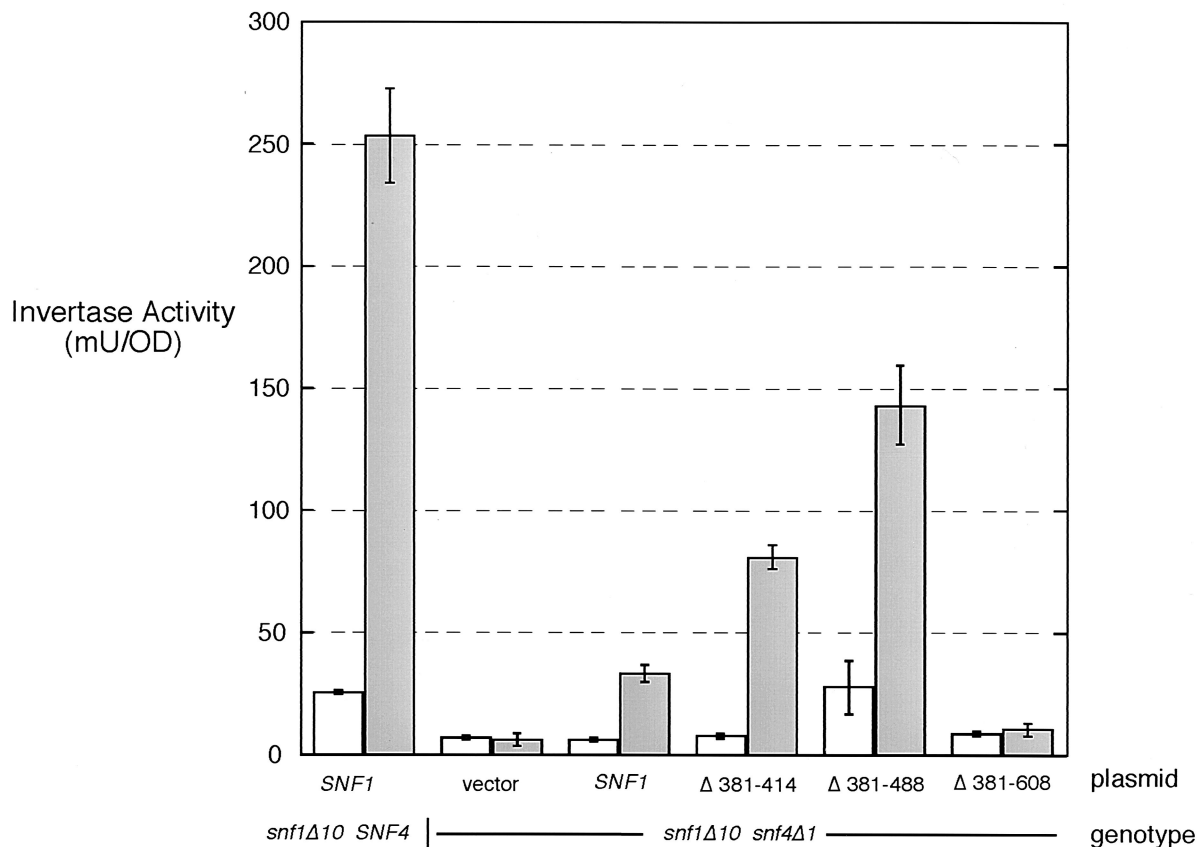


FIG. 2. Invertase activity of Snf1 deletion mutants. Centromeric plasmids expressing the indicated Snf1 protein were transformed into the indicated strains (*snf1Δ10 SNF4* or *snf1Δ10 snf4Δ1*). The mean invertase activity and the standard error from three independent transformants that were grown in 2% glucose (repressed) (open bars) or 0.05% glucose (derepressed) (shaded bars) are plotted. OD, optical density unit.

and -derepressing conditions were collected and assayed for their invertase activity levels (Fig. 2). The *SNF1Δ381-608* allele was not functional in this assay, since invertase expression was not induced more than with the vector control. Furthermore, the *SNF1Δ381-608* allele did not induce invertase in the presence of *SNF4* (not shown). In contrast, the *SNF1Δ381-415* and *SNF1Δ381-488* alleles both caused significant induction of invertase compared to the wild-type *SNF1* plasmid and vector control. The larger deletion ($\Delta 381-488$) was consistently found to provide greater levels of Snf4-independent activity. Also, the $\Delta 381-488$ deletion appeared to be weakly active under repressing conditions, although the effect is not large and variability in the measurement tempers this conclusion. Taken together, these data indicate that the autoinhibitory domain extends beyond the conserved motif present within amino acids 381 to 415. Second, amino acids 381 to 488 are not required for Snf1 function, and their absence confers Snf4-independent function to the Snf1 kinase. Third, amino acids 488 to 608 are required for Snf1 function even in the absence of the autoinhibitory domain.

Genetic selection for Snf4-independent alleles of SNF1. Additional Snf4-independent alleles of *SNF1* were identified in a genetic screen using PCR-mediated, random mutagenesis within the *SNF1* gene (Fig. 3). A centromeric plasmid carrying wild-type *SNF1* driven by its endogenous promoter was di-

gested by *Bgl*II and *Hpa*I, thus removing codons 175 through 628. The gapped plasmid and an overlapping PCR fragment amplified with *Taq* polymerase were used to transform an *snf1Δ10 snf4Δ1* strain to Ura⁺. Uracil prototrophs were recovered and screened for the ability to grow on raffinose medium. Plasmids conferring Snf4-independent growth following retransformation into naive *snf1Δ10 snf4Δ1* cells were screened by Western blotting for the ability to produce full-length Snf1 protein (data not shown). Nine clones that satisfied these criteria were sequenced. Each clone contained a unique collection of mutations that produced four to seven amino acid changes (Table 2) as well as several silent third-position codon changes (not shown). Two amino acid changes, L183I and K192R, were found in more than one independent clone, suggesting that these changes might confer the observed Snf4-independent phenotype. These mutations were introduced into *SNF1* by site-directed mutagenesis and were found to confer Snf4 independence comparable to that of the original isolates (data not shown). A combination of subcloning and site-directed mutagenesis was used to identify two additional point mutations, Y167H and I241N, that were able to confer Snf4 independence. Two alleles of *SNF1* contained multiple amino acid changes, none of which was able to confer an Snf4-independent phenotype on its own. These alleles, *SNF1-203* and *SNF1-214*, were not studied further.

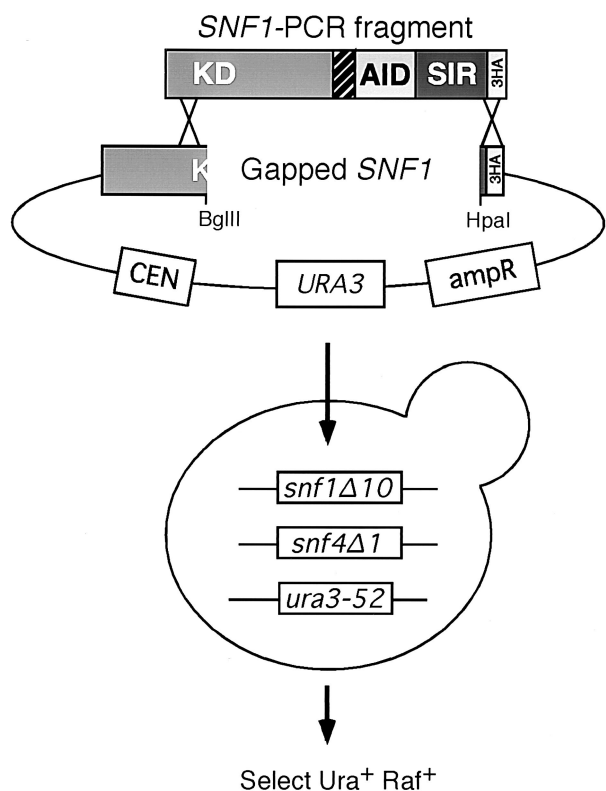


FIG. 3. PCR Mutagenesis strategy. Schematic representation of the pSNF1-316 plasmid gapped by digestion with *BglII*-*HpaI* and the PCR fragment used for gap repair are shown. The recipient strain (MSY563 [*snf1Δ10 snf4Δ1*]) is represented along with the selection strategy. Abbreviations are as described for Fig. 1.

Characterization of Snf4-independent point mutants. Random mutagenesis identified four point mutations in the kinase domain of the *SNF1* gene that each conferred some level of Snf4 independence (Fig. 4A). These alleles of *SNF1* were characterized further for their growth phenotypes on two carbon sources that require Snf1 kinase activity. An *snf1Δ10 snf4Δ1* strain was transformed with a centromeric plasmid expressing either wild-type Snf1 or one of the four point mutants, and serial dilutions of liquid cultures were spotted onto agar plates (Fig. 4B). All four point mutants confer enhanced growth compared to wild-type *SNF1* on both raffinose and glycerol-ethanol media. All four point mutants are expressed at levels

TABLE 2. Mutations conferring Snf4 independence

Clone	Amino acid changes ^a
SNF1-201.....	L183I , R248H, P374S, Q476L, V639A
SNF1-202.....	E187K, I241N , M244L, E311K, K319I
SNF1-203.....	D304N, I553F, F573Y, E607K
SNF1-205.....	L183I , D186E, E326K, N347I, A434T, L474V
SNF1-207.....	K192R , D304N, S353A, K400I, V402I, Q412L, M519L
SNF1-208.....	K192R , L227P, A382P, D407Y, Q453R, S513T, M624L
SNF1-211.....	Y167H , I193V, V468D, M624I
SNF1-213.....	L183I , E365K, N399I, S513L
SNF1-214.....	M286I, L314P, L319I, P339S, S370L, T446I

^a Mutations that by themselves confer Snf4 independence are boldfaced.

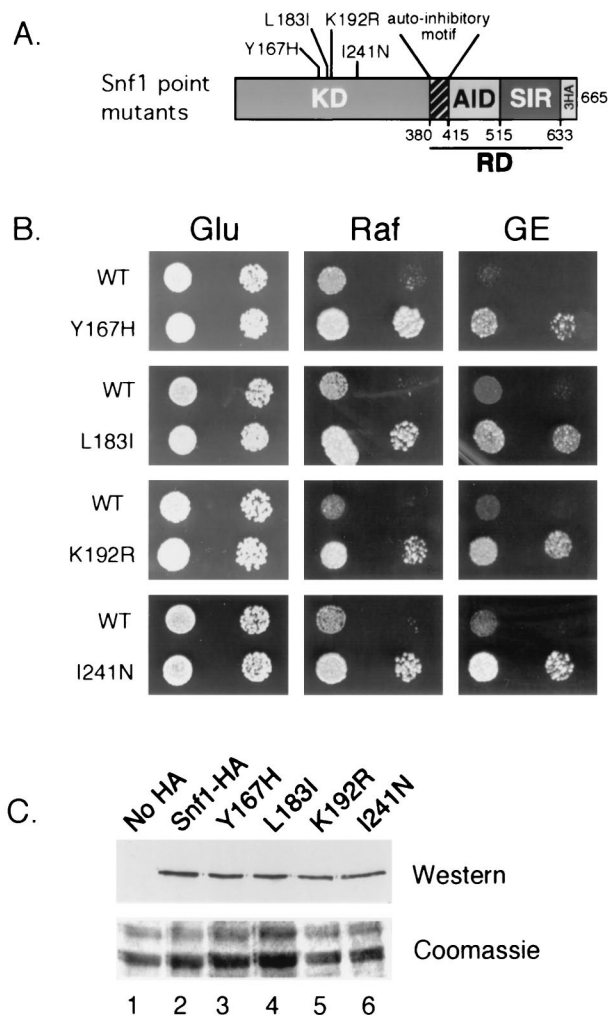


FIG. 4. Point mutations in *SNF1* that confer Snf4 independence. (A) The locations of four point mutations in the *SNF1* gene are drawn to scale. Abbreviations are as described for Fig. 1. (B) Growth phenotypes of wild-type Snf1 (WT) and point mutants on glucose (Glu), raffinose (Raf), and glycerol-ethanol (GE) media. All transformants were in the *snf1Δ10 snf4Δ1* strain. (C) Steady-state protein levels were analyzed by Western blotting (upper panel). Aliquots of protein extracts (20 μg) from MSY563 cells transformed with control vector (no HA) or Snf1-expressing plasmids as indicated were probed with monoclonal antibodies directed against the HA epitope. As a loading control, equivalent aliquots were analyzed in parallel by Coomassie blue staining (lower panel).

equivalent to that observed for wild-type protein when assayed by Western blot (Fig. 4C).

Invertase expression was also measured in cells expressing the four Snf1 point mutants in both *SNF4*⁺ and *snf4Δ1* backgrounds. In the presence of *SNF4*, all four point mutations were indistinguishable from wild-type *SNF1* (not shown). In the absence of *SNF4*, the four point mutations all confer levels of invertase expression significantly higher than that observed for wild-type *SNF1* (Fig. 5). The K192R allele is the weakest of the four Snf4-independent alleles. The L183I allele confers levels of invertase expression that are comparable to that observed in a wild-type *SNF4* background. None of the four point mutations are constitutively activating alleles of *SNF1*, since

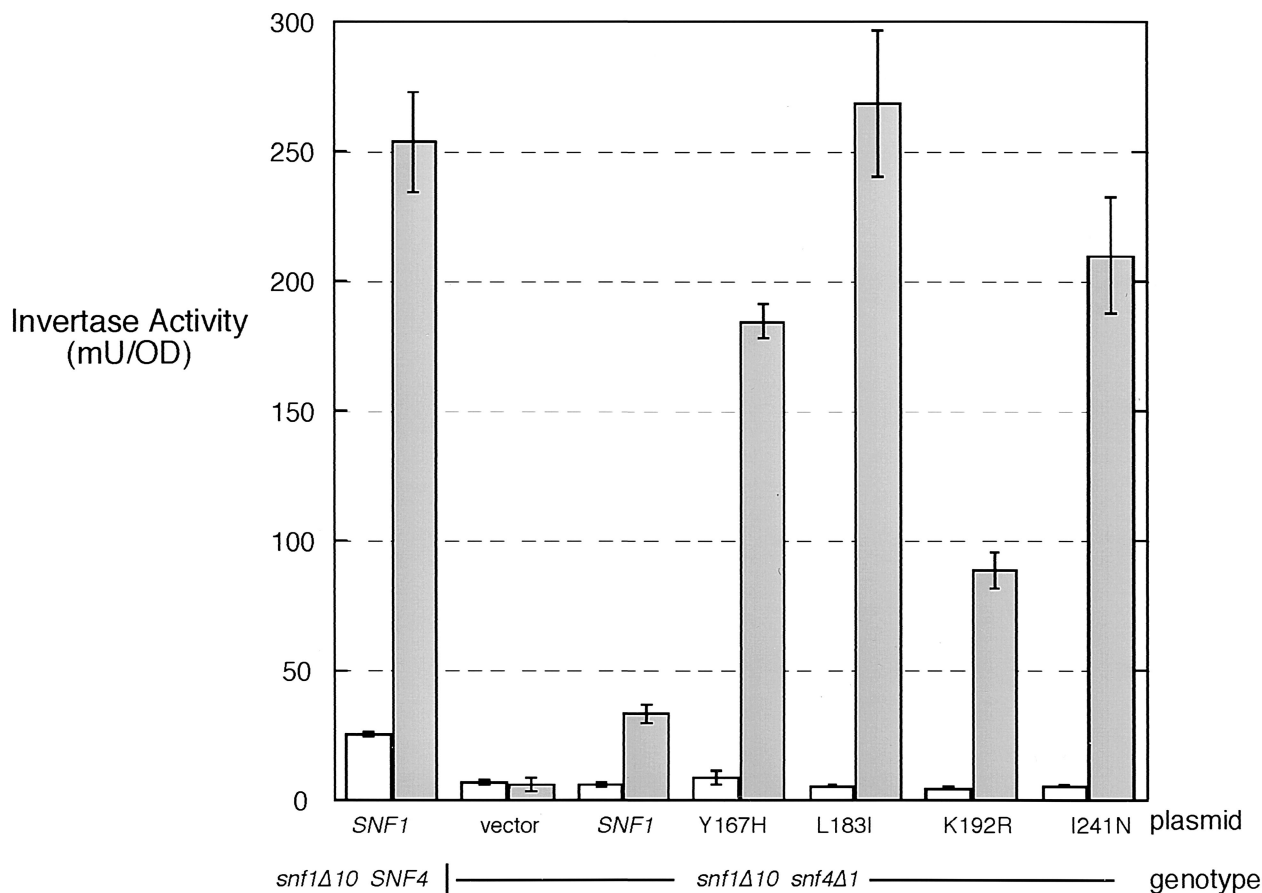


FIG. 5. Invertase activities of Snf1 point mutants. Centromeric plasmids expressing the indicated Snf1 protein were transformed into the indicated strains (*snf1Δ10 SNF4* or *snf1Δ10 snf4Δ1*). The mean invertase activity and the standard error from three independent transformants that were grown in 2% glucose (repressed) (open bars) or 0.05% glucose (derepressed) (shaded bars) are plotted. OD, optical density unit.

they do not cause invertase induction under high-glucose conditions. The regulation of invertase in response to glucose was normal; however, the need for *SNF4* was bypassed by these single amino acid changes.

In vitro kinase activity in the absence of Snf4 protein. Snf1 kinase complexes were purified by using a modification (14a) of the tandem affinity purification (TAP) protocol devised by Rigaut et al. (16). Wild-type *SNF1* and *SNF1-L183I* were TAP tagged at their C termini, and kinase complexes were purified from *SNF4* and *snf4Δ1* cells. Kinase activity was assayed in vitro for the ability to phosphorylate a recombinant GST fusion protein containing amino acids 202 to 414 from the yeast Mig1 protein, a known substrate of the Snf1 kinase (22, 24). Kinase complexes purified from cells expressing wild-type Snf1 and Snf4 proteins efficiently phosphorylated GST-Mig1, as well as a set of proteolytic breakdown products (Fig. 6A, lane 1). In the absence of the Snf4 protein, the purified Snf1 kinase exhibited greatly reduced activity (lane 2). When the L183I amino acid substitution was present in the Snf1 subunit, substantial kinase activity was restored to the complex lacking the Snf4 subunit (lane 3). In all reactions, equivalent levels of the catalytic subunit were used as judged by Western blotting (Fig. 6B). We conclude that the single amino acid substitution L183I is able to restore in vitro kinase activity to complexes lacking the Snf4 subunit.

DISCUSSION

The activities of the Snf1 kinase and its mammalian homologue, AMPK, are tightly regulated in vivo. Current models of the regulation of Snf1 kinase activity propose at least two steps

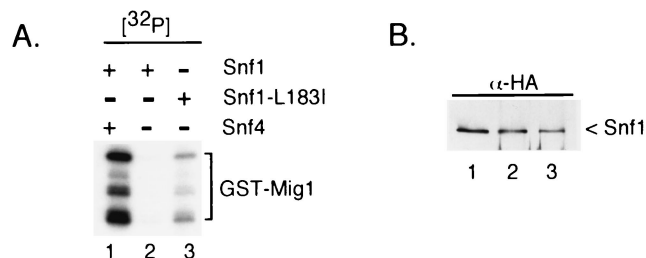


FIG. 6. In vitro kinase activity of Snf1-L183I. (A) Purified Snf1 kinase was assayed in reaction mixtures containing GST-Mig1 (amino acids 202 to 414) and [γ - 32 P]ATP. TAP-tagged wild-type Snf1 (lanes 1 and 2) and Snf1-L183I (lane 3) were purified from cells that lacked genomic copies of either *SNF1* (lane 1) or *SNF1* and *SNF4* (lane 2 and lane 3). Phosphorylated GST-Mig1 protein was resolved on an SDS-polyacrylamide gel and detected by autoradiography. (B) Levels of the catalytic Snf1 subunit were normalized by Western blotting with a monoclonal antibody against the HA epitope. Enzyme preparations were the same as in panel A.

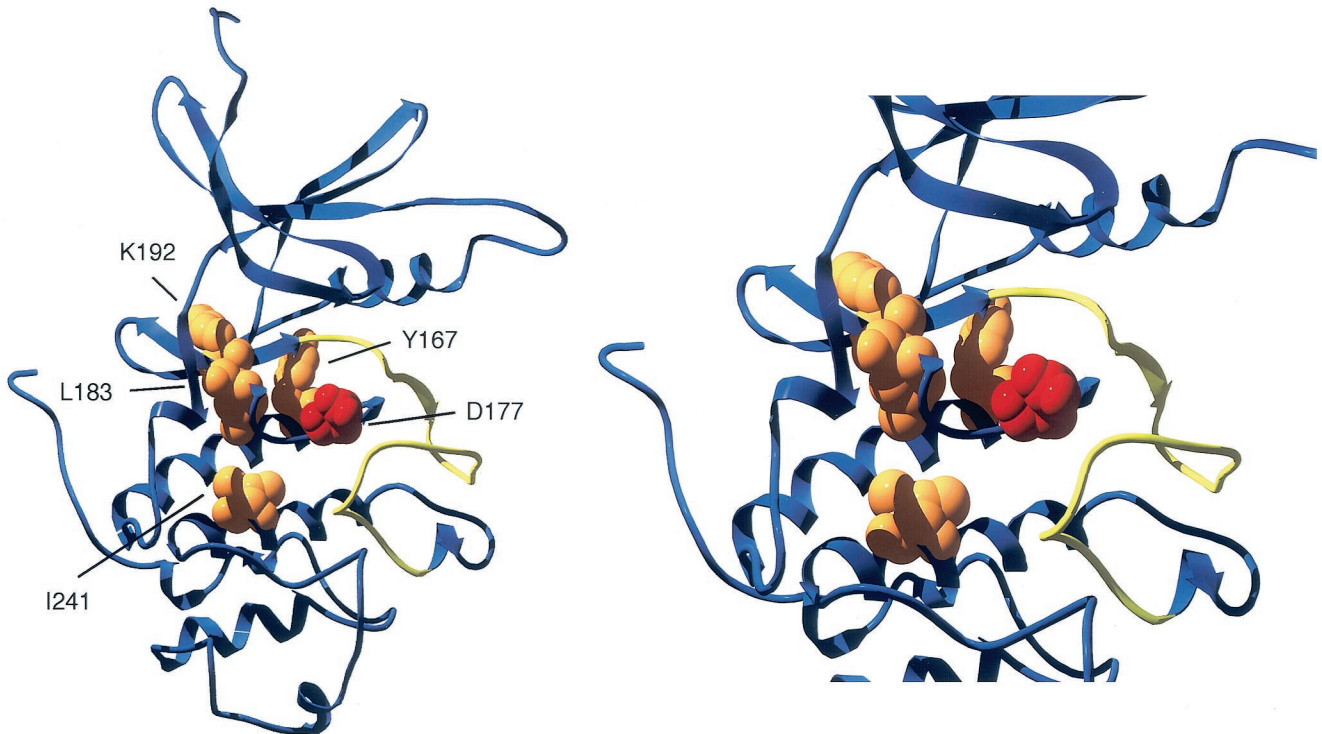


FIG. 8. Structural model of the Snf1 kinase domain. A ribbon diagram of the Snf1 kinase domain (residues 48 to 317) is shown in blue, and the activation loop (residues 202 to 219) is shown in yellow. The catalytic aspartate residue (D177) is represented in red. The four residues whose change can confer Snf4 independence are shown in orange.

nal and have been unable to find any evidence for activity using a truncated Snf1 kinase domain (data not shown). It is not clear to us why our results differ from those previously reported by Jiang and Carlson. Our data indicate that the catalytic domain of Snf1 by itself is not functional.

A second approach to examine the role of the gamma subunit was to screen for mutations in the *SNF1* gene that conferred Snf4 independence. The source of mutations was the PCR amplification of a large region of the *SNF1* gene by using *Taq* polymerase. Our expectation was that we would recover mutations in the autoinhibitory domain of the Snf1 subunit. Much to our surprise, the four point mutations that by themselves confer some degree of Snf4 independence were found in the catalytic domain and not in the autoinhibitory domain. We should note that our screen was not completely unbiased. The gapped plasmid used to generate our library of *SNF1* mutations encompassed codons 175 to 628. Our intended target was the Snf4 interaction domain (residues 392 to 495) mapped by Jiang and Carlson (12), which is entirely within the gap, while our unintended target, the catalytic domain (residues 1 to 360), was only partly covered by the gap. All four point mutations identified in this screen lie in a highly conserved region of the catalytic domain (Fig. 7). Three of the residues identified (Y167, K192, and I241) are invariant in orthologous enzymes from organisms as divergent as yeast, human, fly, nematode, and plant. The fourth residue identified in this screen, L183, is also highly conserved in its hydrophobic nature. Yeast, fly, nematode, and plant all have a leucine at this position, while the human enzyme contains a valine residue. The L183I mu-

tation is particularly intriguing, since it involves the shift of a single methyl group by no more than a few angstroms yet is sufficient to confer Snf4-independent activity to the Snf1 kinase. All four point mutations are located very close in primary sequence and in three-dimensional space to the catalytic aspartate residue (D177) in Snf1 kinase (Fig. 8).

How does the Snf1 autoinhibitory domain control the activ-

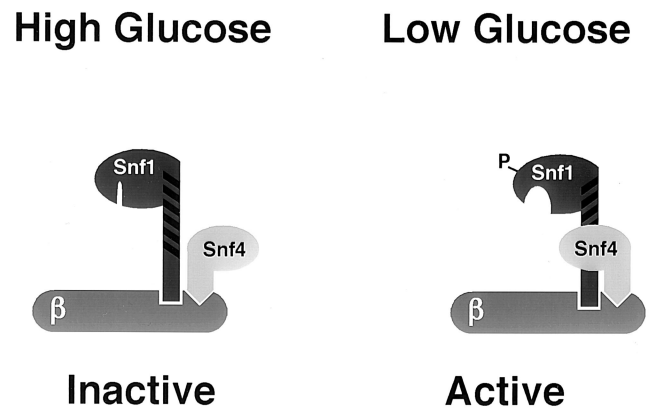


FIG. 9. Conformational regulation of Snf1 kinase. A model for the regulation of Snf1 kinase is proposed. Under high-glucose conditions, the Snf1 kinase autoinhibitory domain is unbound, but it holds the active site in a closed and inactive conformation. Under low-glucose conditions, the autoinhibitory domain is bound by Snf4 protein, which promotes an open and active conformation of the active site.

ity of the catalytic domain? Jiang and Carlson have proposed a competitive binding model in which the autoinhibitory domain is bound to the catalytic domain under high-glucose conditions and thereby blocks kinase activity directly (12). Under low-glucose conditions, the Snf4 subunit binds to the autoinhibitory domain, displacing and freeing the catalytic domain. It is possible that the four point mutations isolated in this study weaken the interaction between the kinase domain and the autoinhibitory domain, thereby bypassing the need for the Snf4 protein. We have attempted to measure the interaction between wild-type and mutant Snf1 kinase domains and the Snf1 autoinhibitory domain by using the two-hybrid system. However, we have been unable to detect this interaction even with the wild-type Snf1 kinase domain (data not shown). Consistent with earlier studies, we do detect strong interaction between the autoinhibitory domain and the Snf4 protein, and this interaction is strongly affected by carbon source. In the absence of a detectable interaction between the kinase domain and the autoinhibitory domain, we propose a model for the regulation of Snf1 kinase activity in which the catalytic activity of the Snf1 kinase is controlled by subtle conformational changes transmitted to the kinase domain by the autoinhibitory domain (Fig. 9). All four residues which confer Snf4-independent activity are located close to the active site of the kinase domain in both primary sequence (Fig. 7) and three-dimensional space, as predicted by a structural model of the Snf1 kinase catalytic domain (Fig. 8). Subtle changes in the packing of the central hydrophobic core of the catalytic domain may be sufficient to change the orientation of the catalytic residues, thereby controlling the activity of the kinase domain. The regulation of protein kinase activity by controlling the orientation of active-site residues is a common regulatory mechanism. For instance, the interactions between the Src kinase SH2 and SH3 domains with their respective ligands control the orientation of residues in the active site (11). We propose that the binding of the Snf4 protein to the autoinhibitory domain of the Snf1 protein promotes a favorable orientation of the active-site residues.

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