GLUCOSE SENSING AND THE REGULATION OF THE AMP-ACTIVATED PROTEIN KINASE IN YEAST

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Snf1/AMPK family members mediate the nutrient stress response in eukaryotes. In yeast, Snf1 is required for survival during glucose deprivation. There are three different Snf1 isoforms; each contains the catalytic α subunit, one of three regulatory β subunits, and the regulatory γ subunit. In glucose limitation, Snf1 is stimulated by activation loop phosphorylation by three partially redundant Snf1-activating kinases (SAKs), Sak1, Tos3, and Elm1. In glucose abundance, the PP1 phosphatase Glc7 dephosphorylates Snf1. We investigated the relationships between the three SAKs and three Snf1 isoforms, the roles of the SAK nonkinase domains, and the mechanism(s) controlling glucose-regulated Snf1 phosphorylation.

Since Snf1 assembles into three complexes, the possibility existed that each SAK is dedicated to phosphorylating a single Snf1 isoform. To test this, we generated strains lacking different combinations of SAKs and β subunits. While our results indicate that the SAKs are not dedicated to specific Snf1 isoforms, each SAK exhibited distinct abilities to activate Snf1, depending on the β subunit and stress imposed on cells.

Domain deletion analysis was employed to characterize the SAKs' nonkinase domains. Deletion of Sak1 and Tos3 (but not Elm1) C-termini diminished the ability to activate Snf1. Deletion of the Elm1 C-terminal domain abrogated Elm1's participation in morphogenetic

signaling. Thus, the SAK C-terminal domains contribute to pathway specificity. Additional deletion mutants revealed an N-terminal Sak1 motif essential for Snf1 signaling. These data demonstrate that nonkinase N- and C-terminal domains are critical for SAK pathway specification.

Snf1 phosphorylation status is regulated by integration of the reaction rates of phosphorylation by the SAKs and dephosphorylation by Glc7. We sought to determine which reaction(s) is glucose-regulated. SAKs immunoprecipitated from extracts of cells grown in high- and low-glucose conditions exhibited similar activity toward Snf1, suggesting the SAKs exhibit constitutive activity. Snf1 dephosphorylation, however, is glucose-regulated. When *de novo* phosphorylation was inhibited, Snf1 phosphorylation was stable in low glucose but rapidly lost upon glucose addition. Glc7 catalytic activity is not glucose-regulated since another Glc7 substrate was dephosphorylated in both glucose conditions. Therefore, Snf1 phosphorylation status is controlled by Snf1's availability to serve as a Glc7 substrate.

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1.0 INTRODUCTION

The AMP-activated protein kinase (AMPK) is considered the "fuel gauge" of the eukaryotic cell (76). It is activated under conditions of energy stress, particularly when the AMP:ATP ratio is elevated. Once active, AMPK functions to modulate the metabolic program of the cell, phosphorylating both transcription factors and metabolic enzymes. The net effect of AMPK activation is the inhibition of ATP-consuming anabolic processes with the concomitant stimulation of ATP-generating catabolic processes (75).

AMPK homologues have been found in all higher eukaryotes with sequenced genomes (77). The Snf1 serine/threonine protein kinase is the *Saccharomyces cerevisiae* AMPK homologue (25, 130, 204). Like the mammalian enzyme, Snf1 is activated by energy stress. Glucose is the preferred carbon source of yeast, and a major function of Snf1 is the promotion of cellular survival under conditions in which glucose is scarce; in the absence of glucose, Snf1 is required for the utilization of alternative carbon sources (for example, fermentation of raffinose and sucrose or respiration of glycerol and ethanol) (77).

Snf1 is a basic-directed kinase with a loosely defined consensus substrate recognition sequence: ϕxRxxSxxxϕ (ϕ is a hydrophobic residue; x is any amino acid; R is a basic amino acid, usually arginine; and S is the phosphorylated serine or threonine) (172). When cells are grown in glucose-limiting conditions, Snf1 phosphorylates a number of protein substrates, including transcription factors, chromatin components, and metabolic enzymes. A non-exhaustive

schematic representation of the Snf1 signaling response to changes in glucose availability is depicted in Figure 1.1. Snf1 phosphorylates and thereby regulates the activity of both transcriptional activators and repressors. One well-characterized Snf1 substrate is Mig1, a Cys2-His2 zinc finger transcriptional repressor. Upon phosphorylation of four sites in the Mig1 protein by Snf1 (172, 186), the interaction of Mig1 with the co-repressors Ssn6 and Tup1 is abolished, phosphorylated Mig1 is exported from the nucleus by the exportin Msn5 (48), and glucose-associated transcriptional repression is relieved (145). Among the genes regulated by Mig1 (in conjunction with Ssn6 and Tup1) are those which promote the utilization of non-glucose carbon sources, including *SUC2*, the gene encoding invertase (122, 185). Invertase catalyzes the conversion of sucrose (to fructose and glucose) and raffinose (to fructose and melibiose) (67, 181). Snf1 phosphorylates and activates the transcriptional activators Sip4 and Cat8 (87, 151, 193). Additionally, when recruited to a promoter, Snf1 may directly stimulate transcription by its interaction with RNA polymerase II (111). A single laboratory has suggested that Snf1 may modulate chromatin structure by phosphorylating histone H3 serine 10 in a manner that affects subsequent histone modification and transcriptional activity (120).

Snf1 also phosphorylates and modulates the catalytic activity of metabolic enzymes, effectively downregulating biosynthetic pathways under conditions of glucose limitation. For example, Snf1 phosphorylates and inhibits the activity of Acc1 (204), acetyl coA carboxylase, the rate-limiting enzyme in fatty acid biosynthesis (163) and a regulator of *INO1* transcription (169). In addition to responding to glucose limitation, Snf1 is also involved in the adaptations to inositol limitation (167), sodium and lithium ionic stress (211), nitrogen limitation (112), alkaline pH (148), and genotoxic stress (53) and has roles in cellular aging (9), diploid pseudohyphal growth, haploid invasive growth (112), meiosis (94), and autophagy (197).

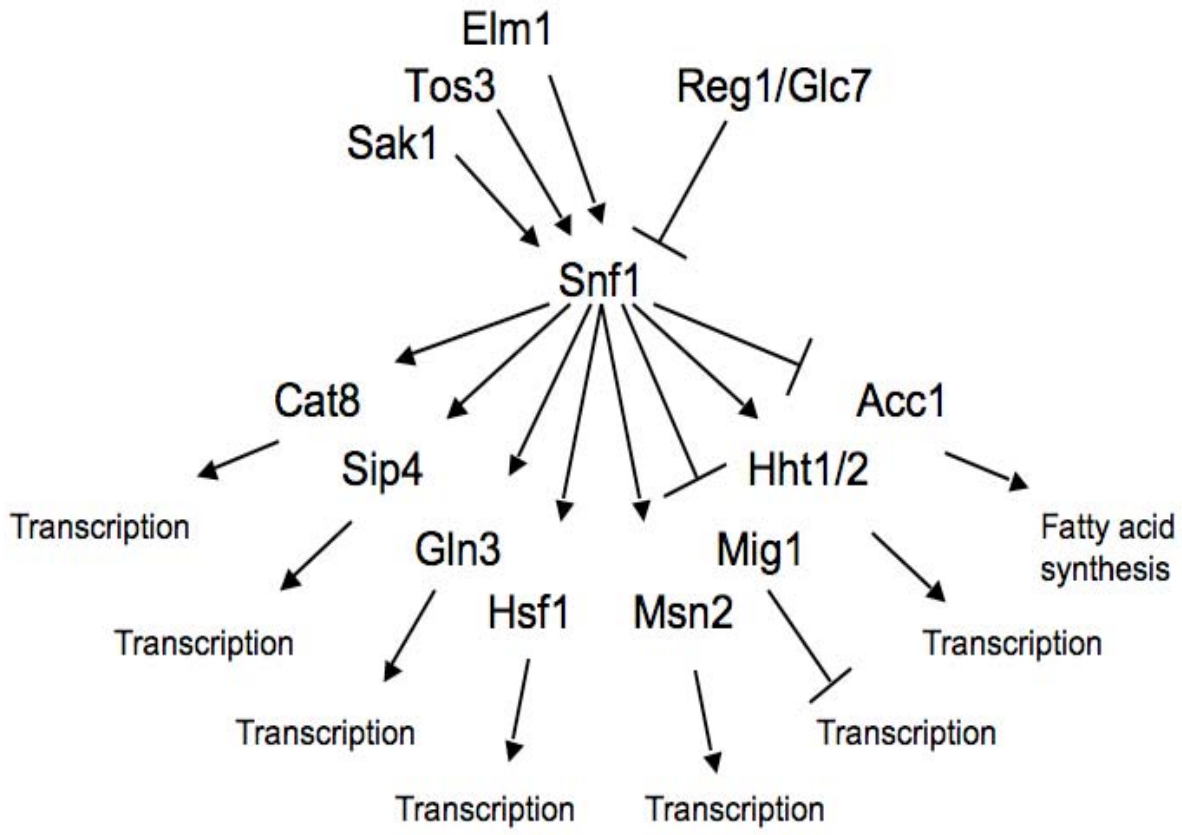

Figure 1.1 Snf1 signaling in response to changes in glucose availability.

Snf1 is phosphorylated and active in low-glucose conditions. Three partially redundant kinases, Sak1, Tos3, and Elm1, phosphorylate the Snf1 activation loop. Under conditions of glucose abundance, the Reg1/Glc7 PP1 dephosphorylates Snf1. When active, Snf1 phosphorylates and stimulates several transcriptional activators, including Cat8, Sip4, Gln3, Hsf1, and Msn2; phosphorylates and inhibits the transcriptional repressor Mig1; phosphorylates isoforms of histone 3 (Hht1 and Hht2), priming them for acetylation, thereby facilitating transcription; and phosphorylates and inhibits acetyl coA carboxylase (Acc1), a rate-limiting enzyme in fatty acid biosynthesis.

1.1 IDENTIFICATION AND INITIAL CHARACTERIZATION OF SNF1

SNF1 (sucrose non-fermenting 1) was originally identified in a genetic screen for yeast mutants which failed to ferment sucrose but were able to utilize glucose (27) and later shown to be allelic with *CCR1* (36) and *CAT1* (215). It was found to be distinct from the gene *SUC2*, encoding the enzyme invertase, which hydrolyzes sucrose to glucose and fructose. *SNF1* mutation did reduce the expression of *SUC2*, however, suggesting a role for *SNF1* in glucose derepression. Yeast with *SNF1* mutations grew more slowly than wild-type cells in low-glucose conditions and were defective in fermenting galactose and maltose as well as respiring nonfermentable carbon sources (27). Early studies indicated that *SNF1* RNA levels were not regulated by glucose abundance (31). Integration of cloned *SNF1* DNA in yeast by homologous recombination facilitated the mapping of the *SNF1* locus on the right arm of chromosome IV, not near other previously mapped mutations (30).

Subsequent to the identification and chromosomal mapping of *SNF1*, researchers sought to identify other genetically and biochemically interacting gene products by screening for yeast mutants which recovered the capacity to utilize alternative carbon sources in the absence of a functional *SNF1* gene. The revertants were termed *ssn* (supressor of *snf1*) mutations 1 through 8 (28). Several of the genes mutated in this study were later determined to be components of the general RNA polymerase II (RNAP II) transcriptional apparatus. *SSN2*, *SSN4*, *SSN5*, and *SSN7* (allelic to *SRB9*, *SIN4*, *SRB8*, and *ROX3*, respectively) encode subunits of the RNAP II mediator complex (173). *SSN3* and *SSN8* (allelic to *SRB10* and *SRB11*, respectively) encode an RNAP II-associated cyclin-dependent kinase and cyclin pair involved in RNAP II carboxy-terminal domain phosphorylation (118). In contrast to these general transcription factors, *SSN1* and *SSN6* were ultimately found to be allelic to the genes encoding the Mig1 zinc finger transcriptional

repressor and Cyc8 co-repressor (which functions in complex with Tup1), respectively, which work together to repress *SUC2* transcription in glucose abundance; relief of repression by these transcription factors was later shown to require Snf1 activity toward Mig1 (191). The implication of the *SSN* genes in transcriptional control highlights the important role of Snf1 in modulating gene expression. The Snf1 protein was found to be a kinase; this provided evidence that the signaling of glucose limitation would be mediated at least in part by protein phosphorylation (29).

1.2 YEAST: A MODEL SYSTEM FOR STUDYING AMPK/SNF1 SIGNALING

Yeast represents a good model system in which to study the AMPK/Snf1 signaling pathways. The AMPK/Snf1 pathway is highly conserved, and the study of AMPK in mammalian cells and Snf1 in yeast has been a mutually beneficial endeavor. In multiple cases, findings in one field have led directly to analogous discoveries in the other. For example, labs studying the mammalian AMPK purified the enzyme and demonstrated that it was a heterotrimer (43). When the molecular identities of the AMPK subunits were determined, it became clear that the previously identified yeast genes *SNF1* and *SNF4* encoded α and γ subunits of the yeast homologue of AMPK. Furthermore, yeast genetic analysis suggested that the AMPK γ subunit was an activator of the kinase complex (discussed below) (129) as has proven to be true (82). Another instance of the synergistic cooperation between research fields was the identification of the Snf1-activating kinases (SAKs) in the yeast system (92, 180), which led to the rapid identification of the mammalian AMPK kinases (AMPKKs) (81, 98, 203). Additionally, ease of

genetic manipulation and established biochemical assays make yeast an attractive organism for the investigation of conserved signaling pathways. By studying this pathway in both yeast and mammalian systems, progress in both fields has been greatly accelerated. Towards the end of better understanding the AMPK signaling pathway, determining how the Snf1 protein kinase is regulated in response to energy and nutrient stress is central to the work presented in this manuscript (Chapters 2-4). A brief review of the mechanisms by which the protein kinases in yeast are regulated follows.

1.3 MECHANISMS OF KINASE REGULATION

Reversible protein phosphorylation is a ubiquitous post-translational modification in prokaryotes and eukaryotes. It is critically involved in the regulation of nearly all cellular processes and signaling pathways. Protein kinases, the enzymes that catalyze the phosphotransfer reaction, constitute 2% of the genes of any given eukaryotic genome (125). Few of these kinases are constitutively active; unregulated activity would be deleterious or lethal to cells in the cases of most protein kinases. Cells have thus developed a variety of finely tuned mechanisms to precisely control the activities of these enzymes, including phosphorylation and dephosphorylation, protein binding, binding of nonprotein ligands, protein accumulation, and subcellular localization (as reviewed by Rubenstein and Schmidt in 2007, from which this section (1.2: Mechanisms of Kinase Regulation) was adapted) (155).

1.3.1 Phosphorylation and dephosphorylation

One of the most well-characterized mechanisms by which protein kinases are activated is phosphorylation of the activation loop (also called the T loop), the flexible polypeptide segment that connects the N and C lobes of the kinase domain (141). Activation loops are typically 20 to 35 residues long and bounded by the conserved residues DFG at the segment's N terminus and APE at the C terminus. The loop itself is less well conserved but often contains one or two conserved phosphorylatable residues (serine, threonine, or tyrosine). The conformation of the activation loop relative to the kinase domain changes with its phosphorylation status. The conformational shift controls the activity state of the kinase by either relieving the steric hindrance of the substrates to the active site, aligning the catalytic residues, or both (3, 141). Only 37 of 117 yeast protein kinases in the eukaryotic protein kinase family are currently known to be activated by phosphorylation of one or more critical residues within the activation loop. However, sequence conservation suggests that many more kinases may ultimately be added to this list. Additionally, many kinases are regulated by phosphorylation sites outside their activation loops. In contrast to activation loop phosphorylation, which is always activating, phosphorylation outside the activation loop can either stimulate or inhibit a kinase (155)

For those studying the regulation of protein kinases, it is not difficult to develop (even unwittingly) a kinase-centric worldview, whereby the phosphorylation events regulating pathways and enzymes are analyzed in terms of phosphate addition alone. In reality, the phosphorylation statuses of most substrates are a reflection of the equilibrium of phosphate addition by a kinase and phosphate removal by a phosphatase. At the moment, kinases are better studied than phosphatases. Indeed, in analysis of phosphorylation sites of protein kinases, the responsible kinase has been identified in many more cases than has the cognate phosphatase.

Nonetheless, the dephosphorylation of protein kinases has regulatory consequences and can be either activating or inactivating (155).

1.3.2 Binding proteins and nonprotein ligands

Protein-protein interaction is another major regulatory motif controlling the activities of protein kinases. Members of each major group of protein kinases are regulated by interaction of the kinase domain with other protein domains in either *cis* or *trans*. Association with protein binding partners can modulate the activation states of kinases in multiple ways and can lead to stimulation or inhibition of kinase activity. Elegant structural studies have elucidated the manner by which protein binding dictates the activities of several kinases (102, 115, 146). While many kinases are controlled by their interactions with other proteins or protein domains, a few are regulated by binding nonprotein ligands. In all documented cases in yeast, these interactions stimulate the activities of the respective kinases (155).

1.3.3 Accumulation and localization

Kinases are also regulated by management of protein accumulation. Hsp90 and its co-chaperone Cdc37 play an important role in the folding and accumulation of many if not most yeast protein kinases (124). The interaction of kinases with chaperones may also regulate kinase activity by controlling the transition between active and inactive conformations (1, 52, 70). The abundance of specific kinases may be modulated by changes in expression at the level of mRNA, protein, or both. mRNA and protein levels can be regulated at the stage of generation (transcription or translation) or stability (for example, protein degradation following ubiquitination (23, 196)).

Control of subcellular localization is another mechanism by which protein kinases may be regulated. Like protein accumulation, this mechanism does not necessarily involve a change in intrinsic catalytic activity but serves to position the enzyme at the right place and time to perform its respective function (155).

The primary goal of the work presented in the following chapters is to better understand the regulation of the Snf1 kinase in yeast. Prior research has demonstrated that Snf1 and its mammalian homologue AMPK are regulated by several of the mechanisms described in section 1.2 (as will be detailed below). Both are phosphorylated on their activation loops by distinct upstream activating kinases. Both seem to be controlled by regulated interactions with protein binding partners. Changes in subcellular localization may direct these kinases to their cognate substrates. Finally, AMPK activity is modulated by non-protein ligand interaction; whether this is the case for Snf1 remains an open question.

1.4 HETEROTRIMERIC STRUCTURE OF AMPK/SNF1

The mammalian AMPK and yeast Snf1 kinase exist as heterotrimers, composed of a catalytic α subunit, scaffolding and regulatory β subunit, and regulatory γ subunit (161) (see Table 1.1 for central components of the Snf1 and AMPK signaling pathways). The mammalian genome encodes two α subunits (α1 and α2), two β subunits (β1 and β2), and three γ subunits (γ1, γ2, and γ3), some of which are expressed in a tissue-specific manner (34, 123, 192). Several of these subunits also exist as multiple splice isoforms (154, 192). Thus, the mammalian AMPK enzyme can exist in over 12 alternative forms, as defined by the differential subunit identities. By contrast, yeast express only one catalytic α subunit (the Snf1 protein), three different β subunits

Table 1.1 Central components of the yeast Snf1 and mammalian AMPK signaling pathways.

Role		**Yeast: Snf1 pathway**	**Mammalian: AMPK pathway**
Heterotrimeric kinase complex	**Catalytic α subunit**	Snf1	α1 α2 α3
	Regulatory/ scaffolding β subunit	Sip1 Sip2 Gal83	β1 β2
	Regulatory γ subunit	Snf4	γ1 γ2 γ3
Upstream activating kinases		Sak1 Tos3 Elm1	LKB1 CaMKKα/β Tak1 (?)[a]
Inactivating phosphatase		Glc7	PP2C
Phosphatase targeting subunit		Reg1	*na*[b]

[a] Tak1 phosphorylates the Snf1 kinase activation loop in yeast cells and the AMPK activation loop *in vitro*, but its role as a legitimate *in vivo* AMPKK is yet to be established.

[b] *na*, not applicable. Orthologous component is not believed to be present in this system.

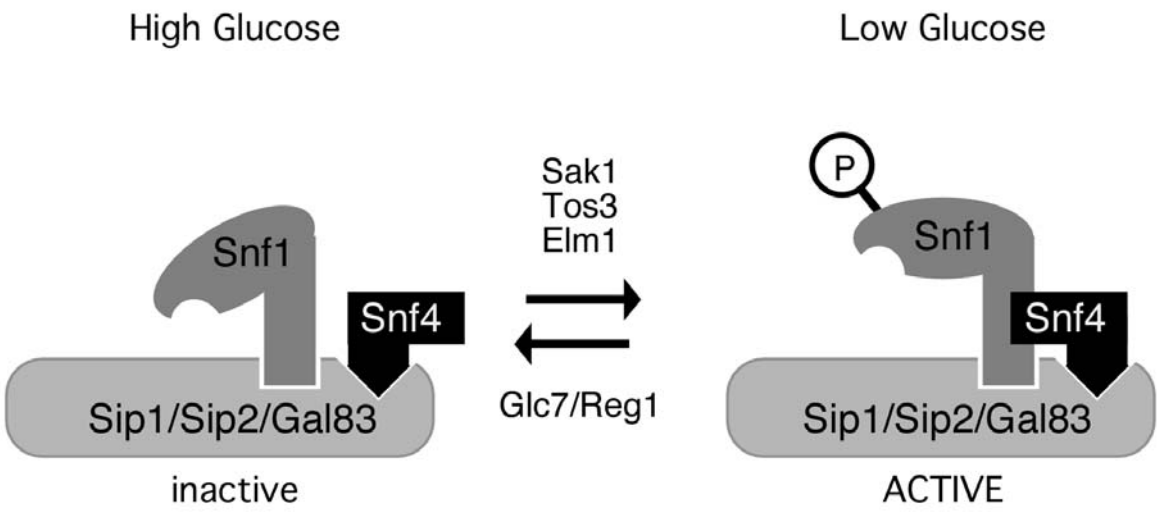

Figure 1.2 Snf1 structure and activation.

The active enzyme is a heterotrimer consisting of a catalytic α subunit (Snf1), one of three scaffolding and regulatory β subunits (Sip1, Sip2, or Gal83), and a regulatory γ subunit (Snf4). Snf1 is activated in low glucose by two distinct steps: phosphorylation of an activation loop threonine by one of three partially redundant Snf1-activating kinases (Sak1, Tos3, or Elm1) and interaction of Snf4 with the Snf1 autoinhibitory domain. In high glucose, Snf1 is inactivated by activation loop dephosphorylation by the PP1 Glc7 (targeted to Snf1 by Reg1) and abolition of the Snf1/Snf4 interaction.

(Sip1, Sip2, and Gal83), and one γ subunit (Snf4), yielding three potential heterotrimers defined by the β subunit identity (Figure 1.2).

The α subunit of Snf1 and AMPK consists of an N-terminal catalytic kinase domain, followed by a C-terminal regulatory domain. The regulatory domain contains sequences involved in interaction with the β and γ subunits. The α subunit autoinhibitory motif (discussed below), just C-terminal to the kinase domain, is believed to interact alternately with the α subunit catalytic domain within the same polypeptide (in the case of the autoinhibited, inactive enzyme) and γ subunit (in the case of the active enzyme) (103). The α subunit C-terminal region interacts with the β subunit (104).

Sip1 and Sip2 were identified in a yeast two-hybrid screen for Snf1-interacting proteins (209). Gal83, originally found to be involved in the regulation of galactose metabolism (127), was recognized as a Snf1-interacting protein based on homology with Sip2 (210). The C-terminal residues of the β subunits in yeast and mammals contain conserved sequences that mediate interaction with the α and γ subunits of the heterotrimer (104, 210). The β subunit N-terminal domains are more variable; indeed, it is these divergent sequences that direct the glucose-regulated subcellular localization of the Snf1 complexes of which they are constituents. In high glucose conditions, all three β-subunit-specific Snf1 heterotrimer isoforms are cytoplasmic. In glucose limitation, the Snf1-Gal83-Snf4 heterotrimer translocates to the nucleus, Snf1-Sip1-Snf4 relocalizes to the vacuolar membrane, and Snf1-Sip2-Snf4 remains cytoplasmic (194). Additional elements within the β subunit N termini may also confer specificity of Snf1 substrate selection (161, 193). Sip2 and Gal83 contain an N-terminal glycogen-binding domain, and Gal83 has been shown to bind glycogen *in vivo* (198). Sip1 and Sip2 contain myristoylation

sites at their N termini; the Sip1 myristoylation is required for its low-glucose vacuolar localization (85).

Early studies indicated that the β subunits were dispensable for Snf1 function *in vivo* (59, 210). However, these analyses relied upon data generated from yeast strains in which the β subunits were incompletely deleted. When the entire coding sequences for all three β subunits were eliminated, cells exhibited a Snf- phenotype: cells were unable to grow on alternative non-glucose carbon sources, did not induce invertase in response to glucose limitation, and Snf1 targets did not become phosphorylated (161). Therefore, the β subunits are required for Snf1 function.

Like Snf1, the γ subunit Snf4 (allelic to *CAT3*) was identified in a screen for mutants which failed to utilize sucrose (58, 140), and cells exhibit a Snf- phenotype in the absence of Snf4 (32, 129). Mammalian and yeast γ subunits contain two Bateman domains (Bateman 1 and Bateman 2), each composed of two CBS motifs (CBS1 through CBS4) (12). Each Bateman domain in the mammalian enzyme is capable of binding one molecule of AMP or ATP *in vitro* (164), and the binding of AMP has been shown to stimulate AMPK in multiple manners (82). Whether the homologous regions in yeast Snf4 bind AMP (or any other ligand) has long been a matter of speculation.

1.5 REGULATION OF AMPK/SNF1

1.5.1 AMPK/Snf1 activation loop phosphorylation

The activation of Snf1 and AMPK requires at least two distinct events: activation loop phosphorylation and changes in intermolecular interactions within the enzyme heterotrimer (129) (Figure 1.2). One critical step is phosphorylation of an α subunit activation loop threonine (threonine 210 for Snf1, threonine 172 for AMPK α1) by upstream activating kinases (79, 92, 129). Under conditions of energy limitation, Snf1 is phosphorylated by one of three partially redundant Snf1-activating kinases (SAKs), Sak1 (formerly known as Pak1), Tos3, and Elm1 (92, 180). The primary upstream activating AMPK kinase (AMPKK) in most mammalian cell types is the tumor suppressor kinase LKB1 (203). In some cells where AMPK activation may be regulated by conditions other than energy stress (for example, neural tissue), AMPK is phosphorylated by the calcium/calmodulin-dependent protein kinase kinases α and β (CaMKKα/β) in response to increases in cellular calcium levels (81, 98, 202). When LKB1 and CaMKKα/β are expressed in place of the three SAKs in yeast, Snf1 is phosphorylated in a glucose-regulated fashion (93). Finally, the mammalian Tak1 kinase (transforming growth factor-β activated protein kinase 1) has been shown to phosphorylate Snf1 when expressed in yeast and phosphorylate AMPK *in vitro* and may represent an additional physiological AMPKK in mammals (131).

The identification of the SAKs was a major breakthrough in the understanding of the Snf1 and AMPK signaling pathways. Sak1 was initially found to co-purify with Snf1 in proteomic studies of protein/protein interactions (68, 89). Subsequent studies demonstrated that Sak1 phosphorylated Snf1 *in vitro* at the critical Snf1 activation loop threonine. However, when

SAK1 was deleted, Snf1 was still found to be phosphorylated in a glucose-regulated fashion, suggesting that Sak1 was not the only SAK in the yeast cell (138). Further experiments with a GST-fusion kinase library demonstrated that Elm1, a related kinase could phosphorylate and activate AMPK and Snf1 *in vitro*. Yet, Snf1 was still phosphorylated in cells lacking *SAK1* and *ELM1*. Finally, Tos3, the third SAK, was identified by sequence homology to Sak1 and Elm1 and was also found to phosphorylate Snf1 *in vitro*. When the genes encoding all three SAKs (*SAK1*, *TOS3*, and *ELM1*) were deleted, Snf1 was no longer phosphorylated in conditions of glucose limitation, confirming that these three proteins represent the complete complement of SAKs (180). Cells that lack all three kinases exhibit a Snf- phenotype. Any one of the three SAKs is sufficient to confer a SNF+ phenotype (92, 180).

Sak1, Tos3, and Elm1 are members of the CaMKK family of enzymes. They each contain a highly conserved kinase domain that is flanked by highly variable and poorly functionally defined N- and C-terminal sequences. There exists an approximately 25-amino-acid sequence of homology of unknown significance in the C-termini of Sak1 and Tos3 (Figure 3.1b).

Sak1 (Snf1-activating kinase 1) was originally named Pak1 (polymerase alpha kinase) when it was identified in a screen for suppressors of mutations in DNA polymerase α (95); however, following its identification, no subsequent interaction of the kinase with polymerase α was established. To avoid confusion with p21-activated kinases (PAKs) (90), Pak1 was renamed Sak1. Tos3 (target of SBF 3) was originally identified in a screen for targets of the cell-cycle-regulated transcription factor SBF (101). However, no role for Tos3 in the control of cell cycle has been further elucidated.

Unlike Sak1 and Tos3, whose only characterized role in the cell is the activation of Snf1, Elm1 has another distinct function: maintaining normal cellular morphology. Cells that lack

Elm1 display an elongated morphology for which the kinase was named (17). Elm1 localizes to the yeast cell bud neck where it participates in the coordination of cell growth and cell division (20, 97, 132). It is involved in the organization of septins at the site of bud emergence during G1 (176).

1.5.2 Inactivation of AMPK/Snf1 by dephosphorylation

Upon a return to energy-rich conditions, AMPK and Snf1 are inactivated by dephosphorylation of their respective activation loop threonine residues. In contrast to the kinases catalyzing the phosphotransfer reaction in yeast and mammals, the AMPK- and Snf1-inactivating phosphatases do not seem to be in the same family of enzymes. The mammalian AMPK was shown to be dephosphorylated *in vitro* by the PP2Cα phosphatase (44). PP2Cα was subsequently found in a complex with AMPK purified from liver tissue and is believed to be the phosphatase responsible for AMPK activation loop dephosphorylation *in vivo* (159).

In *S. cerevisiae*, Glc7, a member of the protein phosphatase 1 (PP1) family in yeast, dephosphorylates Snf1 in high glucose (Figure 1.2). *GLC7* is an essential gene (61) and was genetically implicated in glucose signaling when a mutant variant (Glc7-T152K) resulted in the derepression of glucose-repressed transcription of the genes *SUC2* and *MAL3* (188). In addition to Snf1 inactivation, Glc7 is involved in a wide variety of cellular processes, including meiosis (10, 189) and sporulation (150), cell cycle and mitotic control (8, 62, 88), glycogen metabolism (61), ion homeostasis (199), vesicular membrane fusion (147), and cell wall integrity (8).

Like PP1 phosphatases of other species, the yeast Glc7 is targeted to its specific substrates by distinct targeting subunits. The accessory protein which targets Glc7 to Snf1 in glucose abundance is the Reg1 protein (187). Cells lacking Reg1 exhibit constitutively elevated

Snf1 activation loop phosphorylation and activity, regardless of glucose abundance (129). Similarly, mutations which disrupt the interaction between Reg1 and Glc7 (such as Glc7-T152K or Reg1-F468R) render Snf1 constitutively active without affecting other essential functions of Glc7 (160). Intriguingly, while Reg1 is believed to be required to mediate Glc7 dephosphorylation of Snf1 in high glucose conditions, yeast two-hybrid data has suggested that Reg1 associates preferentially with Snf1 in low glucose abundance (121). Additionally, Reg1 is phosphorylated in glucose limitation in a Snf1-dependent manner (160). The functional significance of this modification is unclear.

1.5.3 Regulation by the AMPK/Snf1 γ subunit

The second step in AMPK/Snf1 activation is mediated by the γ subunit. AMPK was so named because it was first defined as a kinase stimulated by AMP (212). Under conditions of energy stress (and elevated AMP:ATP ratio), AMP binds the mammalian γ subunit. AMP binding of the γ subunit of mammalian AMPK stimulates the enzyme in at least two manners: it stimulates the intrinsic activity of the enzyme and stabilizes the phosphorylation of the α subunit activation loop (82). The exact mechanism whereby AMP binding stimulates intrinsic AMPK activity is not clear, but it presumably involves relief of autoinhibition of the catalytic α subunit kinase domain by the C-terminal regulatory domain of the same subunit (40). The mammalian γ subunit can also bind ATP (under conditions of energy abundance and a lower AMP:ATP ratio), and this binding is inhibitory of kinase activity. The binding of AMP or ATP is mutually exclusive, and mammalian γ subunits exhibit a significantly greater affinity for AMP than ATP (164).

In contrast to the mammalian system, the yeast γ subunit, Snf4, has not been shown to bind AMP, ATP, or any ligand. In fact, purified Snf1 heterotrimer is not stimulated by AMP *in*

vitro (130, 204). Additionally, an amino acid required for mammalian γ subunit coordination of AMP has not been conserved in yeast. Mutation of histidine 151 in human γ1 to glycine largely abolishes AMP-dependence of AMPK activation; the corresponding residue in Snf4 is glycine 145 (2). However, several other residues critical for AMPK stimulation by AMP are conserved in Snf4, and Snf4 is critical for efficient activation of Snf1 in low glucose (Figure 1.2) (32, 129). Like the mammalian system, Snf1 contains an autoinhibitory sequence C-terminal to the kinase domain. This sequence alternately binds the Snf1 catalytic domain (in glucose abundance, presumably an inhibitory interaction) and Snf4 (in glucose limitation, presumably a stimulatory interaction) (103). Snf4 receives an as yet undefined low-glucose signal triggering its relief of Snf1 autoinhibition. Mutations within the Snf1 catalytic core have been identified which render regulated kinase activity independent of Snf4 (114).

1.6 AMPK AND HUMAN HEALTH

1.6.1 AMPK and Type II Diabetes

The AMPK signaling pathway is intricately connected with multiple aspects of human health, making it an important area of investigation. Of chief significance is the role of AMPK in regulating cellular and whole-organism energy homeostasis. AMPK in skeletal and cardiac muscle is normally activated by exercise and other conditions that deplete stores of ATP (135, 136). Over 15 million Americans suffer from Type II Diabetes; at least a quarter of these patients are undiagnosed (33, 73). AMPK mediates at least in part, and perhaps in whole, the therapeutic effects of the anti-diabetic medication metformin (Glucophage), the most widely prescribed

medication used to treat patients with Type II Diabetes (214). The exact mechanism by which metformin results in AMPK activation is not yet universally agreed upon. Initial reports suggested that AMPK activation in response to metformin was adenine-nucleotide-independent (80), potentially involving the generation of mitochondrially derived reactive nitrogen species and increased association of AMPK with its activating kinase, LKB1 (216). While more recent studies have confirmed a role for LKB1 in the mediation of metformin's therapeutic effects (166, 207), some investigators have posited that metformin functions as a mitochondrial poison, inhibiting respiratory complex I, with the ultimate effect of increasing the AMP:ATP ratio (22). Regardless of metformin's mechanism of action, it is clear that activation of the AMPK signaling pathway is beneficial to patients suffering from Type II Diabetes.

1.6.2 AMPK and Wolff-Parkinson-White Syndrome

Dominant mutations in the γ2 subunit of AMPK contribute to cardiac hypertrophy associated with aberrant excessive glycogen accumulation and ventricular pre-excitation in a condition known as Wolff-Parkinson-White Syndrome. It is not clear whether these mutations result in the gain or loss of AMPK function in cardiac tissue of afflicted patients (42). Ongoing investigation has failed to yield the mechanism(s) by which AMPK mutation results in cardiopathological conditions (49).

1.6.3 LKB1, AMPK, and cancer

The AMPK signaling pathway is also involved in cancer. Germline inactivating mutations in LKB1, the primary AMPK-activating kinase, have been implicated as causative of Peutz-Jeghers

Syndrome (PJS), a rare autosomal dominant cancer (86). Patients with PJS exhibit a predisposition to recurrent colon polyps and lesions as well as a high frequency of benign hamartomas and malignant intestinal tumors. LKB1 is a rare example of a kinase that functions as a tumor suppressor. More study is required to determine how mutations in LKB1 result in this condition (126). Sporadic mutations in LKB1 also appear in a small but significant number of cases of pancreatic and non-small-cell lung cancers (158).

Finally, pharmacological activation of AMPK has been investigated as a potential anti-cancer therapeutic (134). Recent studies have shown that AMPK activation inhibits the growth of multiple myeloma cell lines (13), colon cancer cell lines (24, 178), and glioma cell lines (100). Other investigators have demonstrated an anti-tumor effect of AMPK inhibition in certain cell types (107).

1.7 REGULATION OF GLUCOSE SIGNALING UPSTREAM OF SNF1

When the investigation described herein commenced, the Glc7-Reg1 PP1 had long been believed to be the phosphatase responsible for Snf1 dephosphorylation. By contrast, the identities of the SAKs had just recently been reported. Virtually nothing was known regarding the mechanisms controlling Snf1 phosphorylation in response to glucose availability. Thus, the purpose of this study was to better understand how Snf1 activation loop phosphorylation is regulated by glucose concentration. The primary objective was to determine if and how SAK and PP1 activity toward Snf1 is responsive to changes in environmental glucose levels.

Many questions surround the regulation of Snf1 activation loop phosphorylation. First, why are there three distinct SAKs? It seems plausible, if not likely, that each SAK might display

some specialization of function. Are the three SAKs completely interchangeable, or is each SAK dedicated to the activation of a distinct β-subunit specific isoform of Snf1? Are the SAKs differentially required for Snf1 activation under distinct stress conditions? The question of SAK specificity for Snf1 isoforms and stress conditions will be addressed in the second chapter.

Additionally, little is known about the nature and regulation of the SAKs. Each of the three SAKs shares a conserved kinase domain, but the sequences N- and C-terminal to the catalytic segment are highly divergent. Are these domains required for the activity or regulation of the SAKs? The functions of these potential regulatory domains will be discussed in the third chapter.

Importantly, the mechanism by which Snf1 activation loop phosphorylation regulation is controlled remains an unanswered question. Clearly, the phosphorylation state of Snf1 at any particular moment is a reflection of the integration of two reaction rates: the rate of phosphate addition by the SAKs and the rate of phosphate removal by the inactivating phosphatase, Glc7-Reg1. Initial investigation of the events upstream of Snf1 phosphorylation have focused on phosphate addition by the SAKs; however, it has not been demonstrated that SAK activity is in fact responsive to changes in glucose availability. Thus, the fourth chapter will explore whether the catalytic activities of the SAKs, the inactivating phosphatase, or both are regulated by fluctuations in glucose concentration.

It is hoped that clearer elucidation of the mechanisms of Snf1 signaling in *Saccharomyces cerevisiae* will lend themselves to analogous findings with respect to the human AMPK signaling pathway. Such discoveries may enable better therapeutic exploitation of the AMPK pathway in the development of drugs for the treatment of human conditions, including metabolic disorders, cancer, and heart disease.

2.0 SNF1 KINASE COMPLEXES WITH DIFFERENT BETA SUBUNITS DISPLAY STRESS-DEPENDENT PREFERENCES FOR THE THREE SNF1-ACTIVATING KINASES

Rhonda R. McCartney, **Eric M. Rubenstein**, and Martin C. Schmidt (2005).

Current Genetics 47, 335-44.

2.1 INTRODUCTION

Members of the Snf1/AMP-activated protein kinase family play important signaling roles under conditions of nutrient stress in diverse species of eukaryotes, ranging from unicellular yeast to plants, insects, and vertebrates. In *Saccharomyces cerevisiae*, fermentation of glucose is the preferred mode of growth and does not require Snf1 kinase. In contrast, Snf1 kinase is required for fermentation of alternative carbon sources and for aerobic growth on non-fermentable carbon sources (77). In laboratory strains grown in glucose medium, once the glucose is depleted, Snf1 kinase becomes active as cells prepare to switch to aerobic metabolism (26). Many of the genes whose expression is induced at the diauxic shift are not induced properly in cells lacking Snf1 kinase (213).

The Snf1 kinase complex is a heterotrimer composed of one α, one β, and one γ subunit. The α subunit contains the catalytic kinase domain and is encoded by the *SNF1* gene (29). The γ subunit, encoded by the *SNF4* gene, plays a regulatory role and is required for the full activation of Snf1 (32). The β subunit physically interacts with both the α and γ subunits and may act in part to hold the heterotrimer together (104). *S. cerevisiae* express three distinct β subunits encoded by the *SIP1*, *SIP2*, and *GAL83* genes. At least one of the β subunits must be expressed for Snf1 activity *in vivo* (161). The presence of three distinct isoforms of the Snf1 kinase suggests that the β subunits may provide for specialization of function. Indeed, the β subunits control the subcellular localization of the kinase (194) and play a role in substrate selection (161, 193).

Activation of Snf1 kinase is a complicated process involving at least two steps (129). Under conditions of glucose limitation, the Snf4 protein binds to and abrogates the effect of an inhibitory domain present in the C-terminus of the Snf1 protein (103). A second step required for Snf1 activation is the phosphorylation of a conserved threonine residue in the Snf1 activation loop by a distinct upstream kinase (129). We identified the Sak1 kinase of *S. cerevisiae* as one of the Snf1-activating kinases. Sak1 associates with Snf1 under conditions of glucose limitation and is capable of promoting the phosphorylation of the Snf1 activation loop *in vitro* and *in vivo* (138). However, deletion of the *SAK1* gene did not interfere with Snf1 activation, suggesting that additional Snf1-activating kinases were present. Recently, we and others determined that the full complement of Snf1-activating kinases was composed of three related kinases: Sak1, Tos3, and Elm1 (92, 180).

Relatively little is known about the Sak1 and Tos3 kinases. Sak1 was isolated as a high-copy suppressor of mutations in the DNA polymerase α gene and named polymerase α kinase (95). *TOS3* was identified in a genomic screen for promoters bound by the cell cycle-regulated transcription factor SBF (101). The name TOS stands for target of SBF. Numerous potential targets for SBF were identified, and many play roles in budding and cell wall biosynthesis. The *TOS3* gene promoter contains at least one potential binding site for SBF, although expression of *TOS3* mRNA does not appear to be cell cycle-regulated (35, 175). Elm1 kinase has been intensively studied by several groups, and its involvement in the Snf1 kinase pathway was unanticipated. Mutations in *ELM1* were first isolated by virtue of their elongated morphology (65, 110). Elm1 protein is localized at the bud neck (20), where it assembles in a complex with other proteins (182) and plays a role in controlling bud emergence and septin organization (176).

The finding that Elm1 also functions in carbon source-sensing pathways (92, 180) may reflect the integration of carbon source-response pathways with filamentous growth pathways (41).

In this study, we examine whether the three upstream kinases exhibit a specialization of function. The facts that there are three upstream kinases for Snf1 and that the Snf1 kinase associates with one of three different β subunits lead to the very simple hypothesis that each upstream kinase is dedicated to the activation of one isoform of the Snf1 kinase complex. Alternatively, the three upstream kinases may be promiscuous activators of all three isoforms of the Snf1 kinase. A similar study reported that the three Snf1-activating kinases were not dedicated to a specific isoform of Snf1 but that the Sak1 kinase played an important role in controlling the activity and localization of the Gal83 isoform in response to glucose stress (84). We report here that the different isoforms of Snf1 kinase can be activated by different upstream kinases and that the activating kinase preference varies, depending on the stress to which the cells are exposed.

2.2 MATERIALS AND METHODS

2.2.1 Yeast strains, media, and genetics methods

The *S. cerevisiae* strains used in this study are described in Table 2.1. Growth of cells utilized standard media at 30°C (153). Raffinose medium contained 2% raffinose, 0.01% glucose and 1 mg/ml antimycin A. Glycerol/ethanol medium contained a mixture of 3% (vol/vol) glycerol and 2% (vol/vol) ethanol. For testing inositol phenotypes, synthetic complete medium was made with yeast nitrogen base lacking inositol (Qbiogene, Carlsbad, CA). Strains with multiple gene

Table 2.1 *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype
FY1193	*MATα ura3Δ52 leu2Δ1 his3Δ200 trp1Δ63 snf1Δ10*
MSY182	*MATa ura3Δ52 leu2Δ1 trp1Δ63 his3Δ200*
MSY544	*MATα ura3Δ52 leu2Δ1 trp1Δ63 his3Δ200 sip1Δ::HIS3 sip2Δ::HIS3*
MSY549	*MATα ura3Δ52 trp1Δ63 his3Δ200 sip1Δ::HIS3 gal83Δ::HIS3*
MSY553	*MATα ura3Δ52 trp1Δ63 his3Δ200 sip2Δ::HIS3 gal83Δ::HIS3*
MSY676	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sak1Δ::KAN*
MSY683	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 tos3Δ::KAN*
MSY690	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 elm1Δ::KAN*
MSY694	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sak1Δ::KAN tos3Δ::KAN*
MSY697	*MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 tos3Δ::KAN elm1Δ::KAN*
MSY807	*MATa ura3Δ0 leu2Δ0 his3Δ1 snf1Δ10 sak1Δ::KAN*
MSY854	*MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 sak1Δ::KAN elm1Δ::KAN*
MSY858	*MATα ura3Δ0 leu2Δ0 his3Δ1 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN*
MSY863	*MATa ura3 leu2 his3 tos3Δ::KAN snf1Δ10*
MSY867	*MATa ura3 leu2 his3 trp1Δ63 snf1Δ10 sak1Δ::KAN elm1Δ::KAN*
MSY870	*MATα ura3 leu2 his3 lys2Δ0 snf1Δ10 tos3Δ::KAN elm1Δ::KAN*
MSY871	*MATa ura3 leu2 his3 trp1Δ63 lys2Δ0 met15Δ0 snf1Δ10 elm1Δ::KAN*
MSY874	*MATa ura3 leu2 his3 met15Δ0 snf1Δ10 sak1Δ::KAN tos3Δ::KAN*
MSY876	*MATα ura3 leu2 his3 snf1Δ10 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN*
MSY880	*MATa ura3 leu2Δ0 his3 lys2Δ0 sip1Δ::HIS3 gal83Δ::HIS3 sak1Δ::KAN tos3Δ::KAN*
MSY881	*MATα ura3 leu2Δ0 his3 lys2Δ0 sip1Δ::HIS3 gal83Δ::HIS3 tos3Δ::KAN elm1Δ::KAN*
MSY882	*MATa ura3 leu2Δ0 his3 sip2Δ::HIS3 gal83Δ::HIS3 sak1Δ::KAN elm1Δ::KAN*
MSY883	*MATα ura3 leu2 his3 sip1Δ::HIS3 sip2Δ::HIS3 sak1Δ::KAN elm1Δ::KAN*
MSY885	*MATα ura3 his3 lys2Δ0 sip1Δ::HIS3 gal83Δ::HIS3 sak1Δ::KAN elm1Δ::KAN*
MSY886	*MATα ura3 leu2Δ0 his3 lys2Δ0 sip1Δ::HIS3 gal83Δ::HIS3 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN*
MSY888	*MATa ura3 leu2Δ0 his3 lys2Δ0 met15Δ0 sip1Δ::HIS3 sip2Δ::HIS3 tos3Δ::KAN elm1Δ::KAN*
MSY889	*MATα ura3 leu2Δ0 his3 lys2Δ0 met15Δ0 sip1Δ::HIS3 sip2Δ::HIS3 sak1Δ::KAN tos3Δ::KAN*
MSY890	*MATα ura3 leu2Δ0 trp1Δ63 his3 met15Δ0 sip1Δ::HIS3 sip2Δ::HIS3 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN*
MSY891	*MATα ura3 leu2Δ0 his3 met15Δ0 sip2Δ::HIS3 gal83Δ::HIS3 sak1Δ::KAN tos3Δ::KAN*
MSY892	*MATα ura3 his3 lys2Δ0 sip2Δ::HIS3 gal83Δ::HIS3 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN*
MSY893	*MATa ura3 leu2Δ0 his3 sip2Δ::HIS3 gal83Δ::HIS3 tos3Δ::KAN elm1Δ::KAN*

deletions were produced by genetic crosses and sporulation. Gene deletions were confirmed by three primer PCR reactions that amplified different-sized products for the wild-type and null alleles.

2.2.2 Epitope tagging and plasmids

The Mig1 and Snf1 proteins were tagged with three copies of the hemagglutinin (HA) epitope at their C termini (129, 161) and expressed from their cognate promoters on low-copy-number plasmids. The Sak1, Tos3, and Elm1 proteins were all tagged with the V5 epitope (174) by amplifying their open reading frames and cloning them into pYES2.1 vectors (Invitrogen, UK). The open reading frames were then transferred by gap repair to pRS316 (171), such that expression was driven from each gene's native promoter.

2.2.3 Invertase assays

For invertase assays, cells were grown to the mid-log phase in medium containing 2% glucose and collected by centrifugation. Half of the cells were used to assay invertase activity (high glucose), and half were assayed after an additional 3 h in medium containing 0.05% glucose (low glucose). Quantitative invertase assays were performed as previously described (64, 162).

2.2.4 Western blotting

Tagged proteins were detected by Western blotting (162), using anti-HA antibodies from Santa Cruz Biotechnology (1:1,000 dilution) and peroxidase-conjugated sheep anti-mouse IgG

(1:5,000) from Jackson Immuno Research Laboratories. For analysis of the phosphorylation state of the Snf1 threonine 210 residue, the Snf1 protein was first immunoprecipitated from cell extracts (500 μg protein) with anti-HA antibodies prior to Western analysis with antisera specific for the Snf1 protein phosphorylated on threonine 210 (129).

2.3 RESULTS

2.3.1 Snf phenotypes of strains lacking Snf1-activating kinases

In order to characterize the activities of the Snf1-activating kinases, strains were constructed that lacked one, two, or all three of the Snf1-activating kinases, Sak1, Tos3, and Elm1 (Table 2.1). These strains were then tested for Snf phenotypes. Cells were grown in rich glucose media, and equivalent dilutions were spotted onto agar media with different carbon sources that required Snf1 function for growth (Figure 2.1). Strains lacking any one of these kinases were able to grow by fermentation of raffinose and by respiration of glycerol/ethanol, indicating that any two of the upstream kinases were fully capable of activating Snf1 kinase under these conditions. Strains expressing only one of the three Snf1-activating kinases were also tested. The *sak1*Δ *elm1*Δ, *tos3*Δ *elm1*Δ, and *sak1*Δ *tos3*Δ strains were able to grow on these carbon sources, indicating that Sak1, Tos3, and Elm1 kinases alone were able to activate sufficient Snf1 for growth under these conditions. Consistent with earlier reports (92, 180), strains lacking all three Snf1-activating kinases displayed a strong Snf- phenotype similar to that observed in *snf1*Δ*10* cells (Figure 2.1).

A more quantitative measure of Snf1 kinase activity is the induction of the enzyme invertase in response to glucose limitation. Invertase enzyme activity was assayed from cells

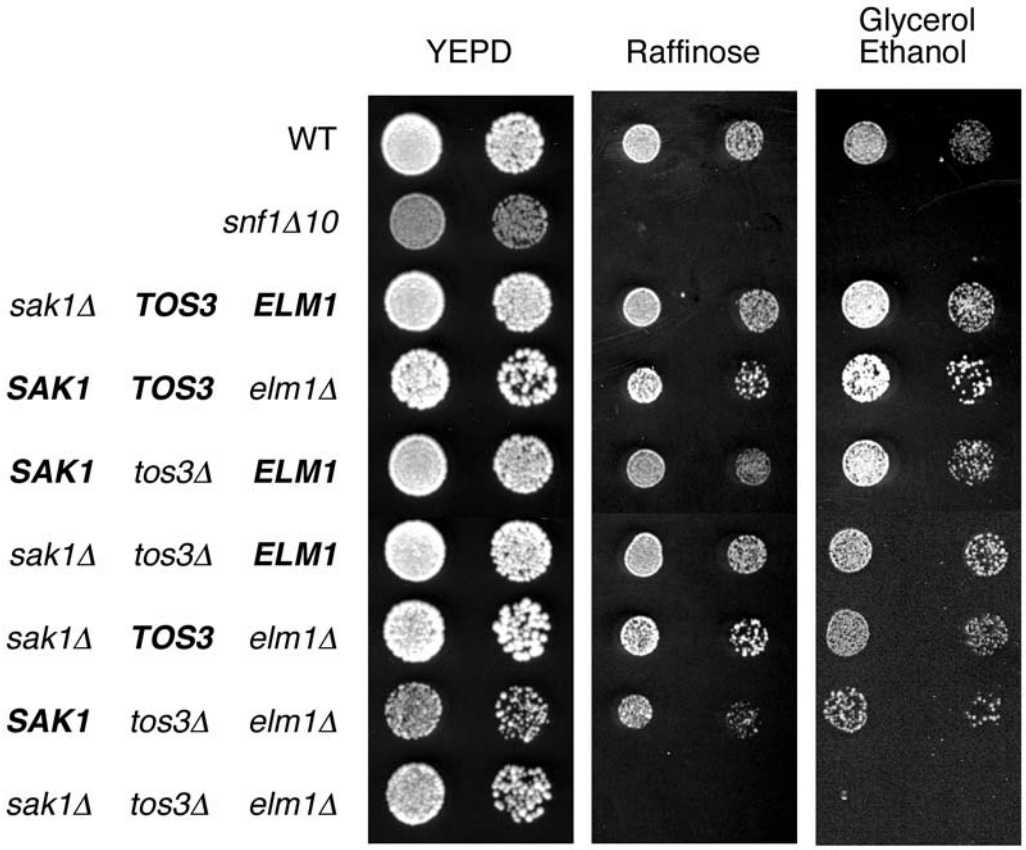

Figure 2.1 Growth phenotypes of Snf1-activating kinases on different carbon sources.

Serial dilutions of yeast cultures were spotted onto rich medium with glucose (YEPD) or synthetic complete medium with either raffinose plus antimycin A or a mixture of glycerol and ethanol as the carbon sources. The relevant genotypes of the yeast strains are shown on the left. WT, wild-type.

grown in the presence of abundant glucose (2%) or after 3 h in limiting glucose (0.05%). Strains lacking Snf1 kinase or all three Snf1-activating kinases were completely unable to induce invertase expression when shifted to 0.05% glucose (Figure 2.2). Strains lacking any one of the Snf1-activating kinases were able to induce invertase in response to glucose limitation. Indeed, cells lacking Elm1 kinase appear to over-express invertase. Strains expressing only one of the Snf1-activating kinases displayed a wide range of invertase expression patterns. Cells expressing Tos3 as the only Snf1-activating kinase repressed invertase effectively in 2% glucose and hyper-induced invertase when shifted to 0.05% glucose. Cells expressing Elm1 as the only Snf1-activating kinase repressed invertase effectively and induced its expression in a similar way to wild-type cells. Cells expressing Sak1 as the only Snf1-activating kinase showed relatively normal regulation of invertase expression. Thus, Sak1, Tos3, and Elm1 are each individually capable of promoting proper regulation of invertase in response to glucose limitation.

2.3.2 Mig1 phosphorylation in strains lacking Snf1-activating kinases

The Mig1 protein is a transcriptional repressor that is known to be a direct downstream target of the Snf1 kinase (172, 186). Mig1 phosphorylation can be detected by Western blotting as a reduction in its SDS gel mobility (185). We examined the ability of cells expressing a single Snf1-activating kinase to phosphorylate Mig1 protein in response to glucose limitation (Figure 2.3). As reported by Sutherland et al. (180), the phosphorylation of Mig1 is not observed in cells lacking Snf1 or all three of the Snf1-activating kinases (lanes 9 and 10), whereas in wild-type cells, Mig1 is phosphorylated when grown in low glucose (lanes 1 and 2). Cells expressing only

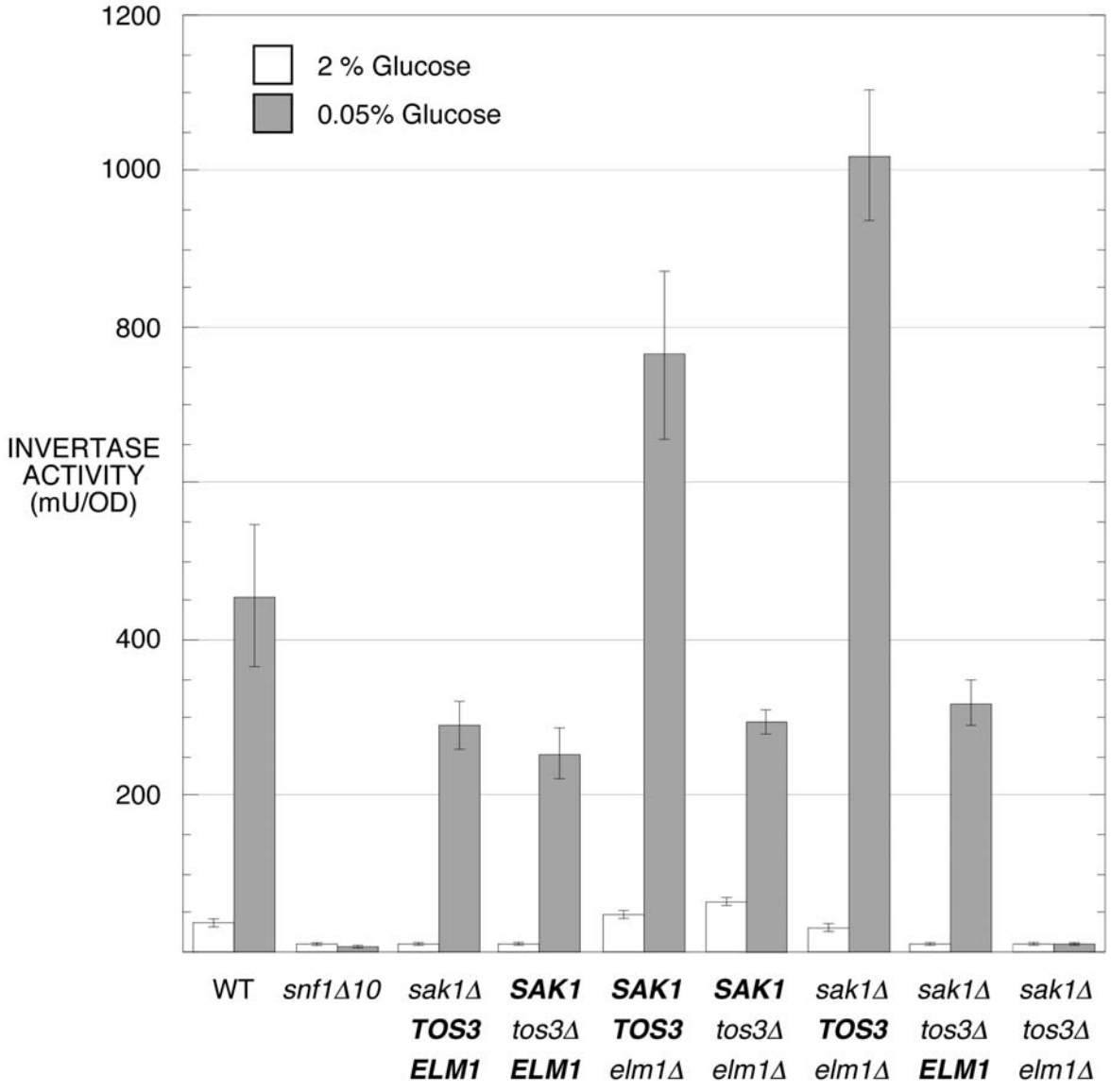

Figure 2.2 Invertase expression in Snf1-activating kinase mutants.

Invertase expression was assayed in cells grown in 2% glucose or after shifting to 0.05% glucose for 3 h. The relevant genotypes of the yeast strains are shown. The mean invertase expression from three independent cultures is plotted. Error bars represent one standard error. mU/OD, μmole glucose/min/OD of cells assayed.

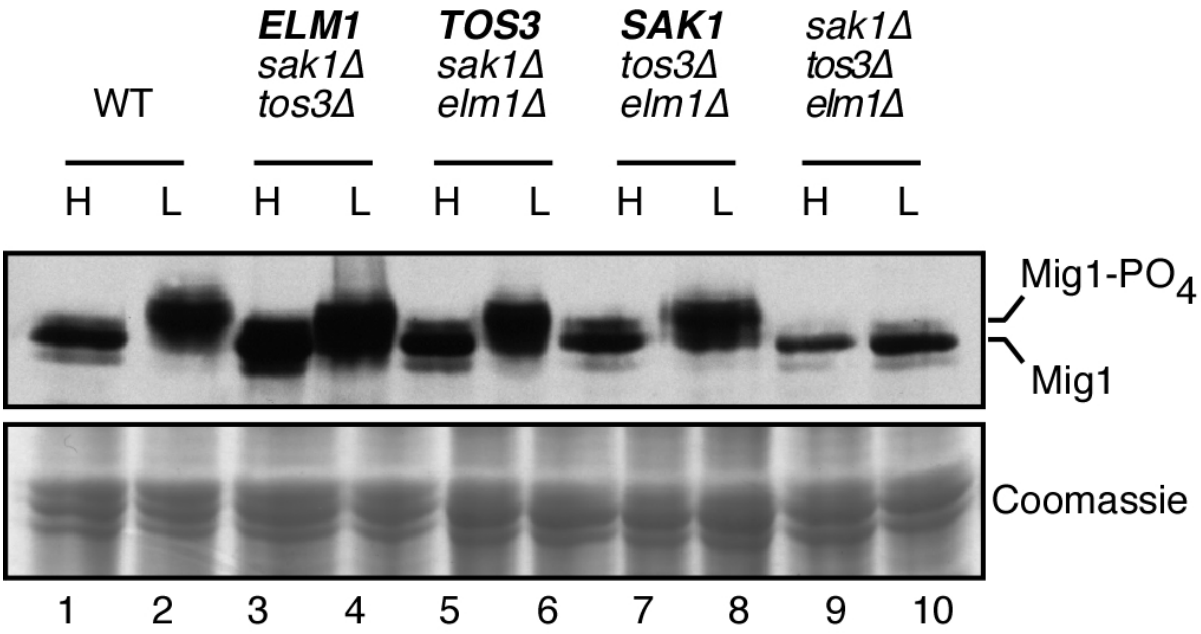

Figure 2.3 Phosphorylation of Mig1 by Snf1-activating kinase mutants.

Protein extracts were prepared from cells expressing HA-tagged Mig1 after growth in the presence of high (H) or low (L) glucose. A Western blot (upper panel) was performed with monoclonal antibodies directed against the HA epitope. The mobility of Mig1 and phosphorylated Mig1 is indicated on the right. As a control for equal loading of protein extracts, equivalent aliquots were run in parallel and visualized by Coomassie blue staining (lower panel).

a single Snf1-activating kinase were capable of phosphorylating Mig1 in response to glucose limitation (lanes 3–8). Although the absolute level of the Mig1 protein can vary between strains, we conclude that each of the Snf1-activating kinases was individually capable of promoting Mig1 phosphorylation.

2.3.3 Snf1-activating kinases are required for phosphorylation of the Snf1 activation loop

Previously, we showed that Sak1 kinase is able to phosphorylate the activation loop threonine of Snf1 (138). Here, we show that, in the absence of Sak1, Tos3, and Elm1 kinases, the Snf1 activation loop threonine shows absolutely no evidence of phosphorylation under low glucose conditions (Figure 2.4a). Therefore, Sak1, Tos3, and Elm1 are the complete complement of kinases that phosphorylate Snf1 threonine 210 in response to glucose limitation. We next examined the ability of cells lacking one or two of the Snf1-activating kinases to phosphorylate Snf1 threonine 210 (Figure 2.4b). All combinations of single and double mutants were able to phosphorylate Snf1 threonine 210 at least to some level in response to glucose limitation. However, some differences were observed. Most notably, the *elm1*Δ cells and the *elm1*Δ *tos3*Δ cells exhibited higher levels of threonine 210 phosphorylation under high glucose conditions (lanes 7 and 13). This finding is consistent with the higher levels of invertase expression observed in these cells in the presence of high glucose (Figure 2.2).

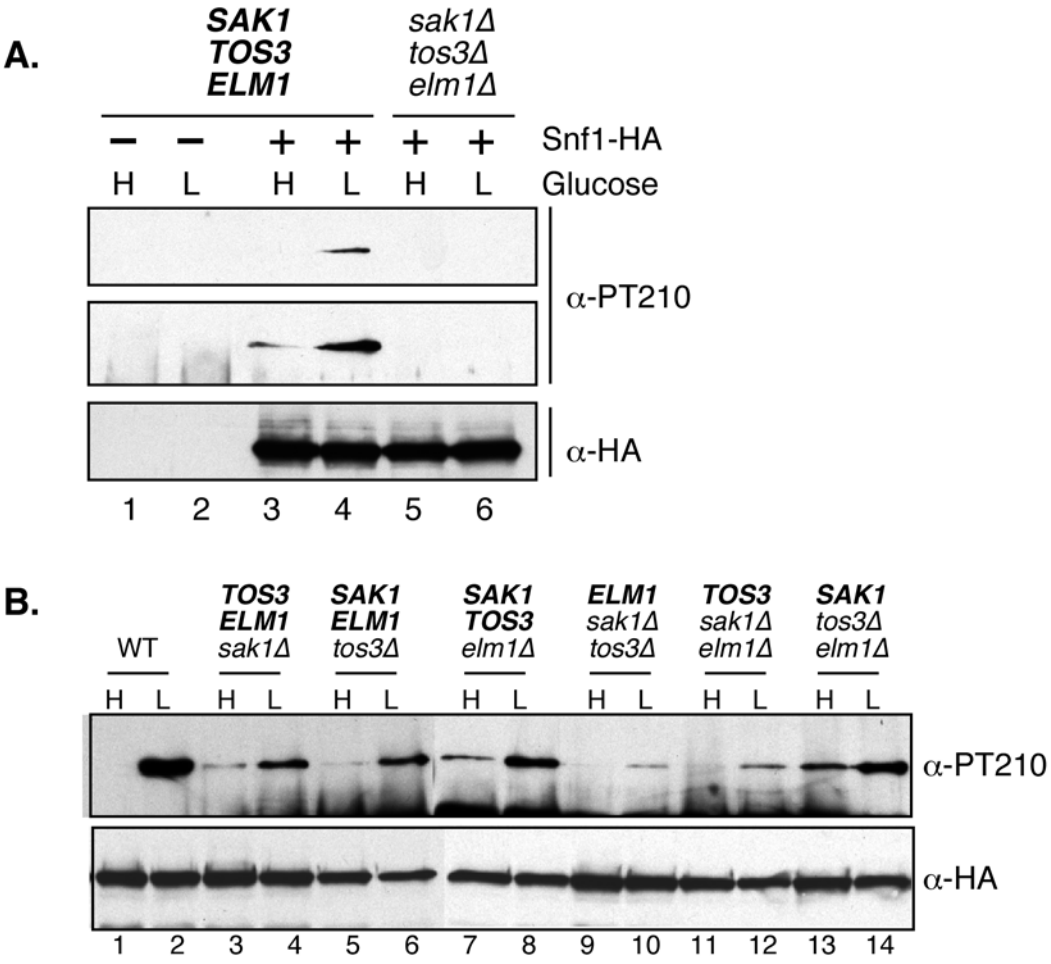

Figure 2.4 Phosphorylation of Snf1 threonine 210 requires Snf1-activating kinases.

Snf1 protein was immunoprecipitated with HA antibody from protein extracts of cells grown in either high (H) or low (L) glucose. Precipitates were analyzed by Western blotting using either anti-HA antibodies (α-HA) or antibodies specific for the Snf1 protein phosphorylated on threonine 210 (α-PT210). **A** Wild-type cells (lanes 1-4) or *sak1*Δ *tos3*Δ *elm1*Δ cells (lanes 5 and 6) were transformed with a plasmid expressing Snf1-HA (lanes 3-6) or an empty vector (lanes 1 and 2). A light and dark exposure of the α-PT210 Western is shown. **B** Wild-type cells (lanes 1 and 2) and cells lacking one or two of the Snf1-activating kinases (lanes 3-14) were transformed with a plasmid expressing Snf1-HA and analyzed as above.

2.3.4 β subunits of Snf1 kinase complexes confer strong upstream kinase preferences

Yeast strains were constructed such that they expressed a single β subunit in combination with a single upstream kinase. Each strain was tested for invertase expression under high- and low-glucose conditions (Figure 2.5). Cells expressing Sip1 as the only β subunit in combination with all three upstream kinases or with Sak1 or Tos3 showed relatively normal invertase regulation. However, the combination of Sip1 and Elm1 was much less effective at inducing invertase. When Sip2 was the only β subunit, only Sak1 kinase was able to induce invertase expression. The combinations of Sip2 with Tos3 or Elm1 were completely ineffective at invertase induction. When Gal83 was the only β subunit, a clear preference for Sak1 was observed, although Tos3 and Elm1 were individually able to function at a reduced level. We conclude that the β subunits do indeed confer upstream kinase specificity, although the situation is more complex than a simple one-to-one correspondence of upstream kinase to Snf1 complex isoform.

2.3.5 Snf1-activating kinase preference is stress-dependent

Cells expressing a single β subunit and a single upstream kinase were assayed for growth under conditions that required the Snf1 kinase. We tested growth by fermentation of raffinose, by respiration of a mixture of glycerol and ethanol, and by fermentation of glucose in the absence of inositol. The absence of inositol promotes transcriptional induction (derepression) of the *INO1* gene, a process which requires Snf1 kinase (167). Cells expressing Sip1 as their only β subunit were able to ferment raffinose in combination with either Sak1 or Tos3 but not Elm1 as the upstream kinase (Figure 2.6). As reported by Schmidt and McCartney (161), cells expressing

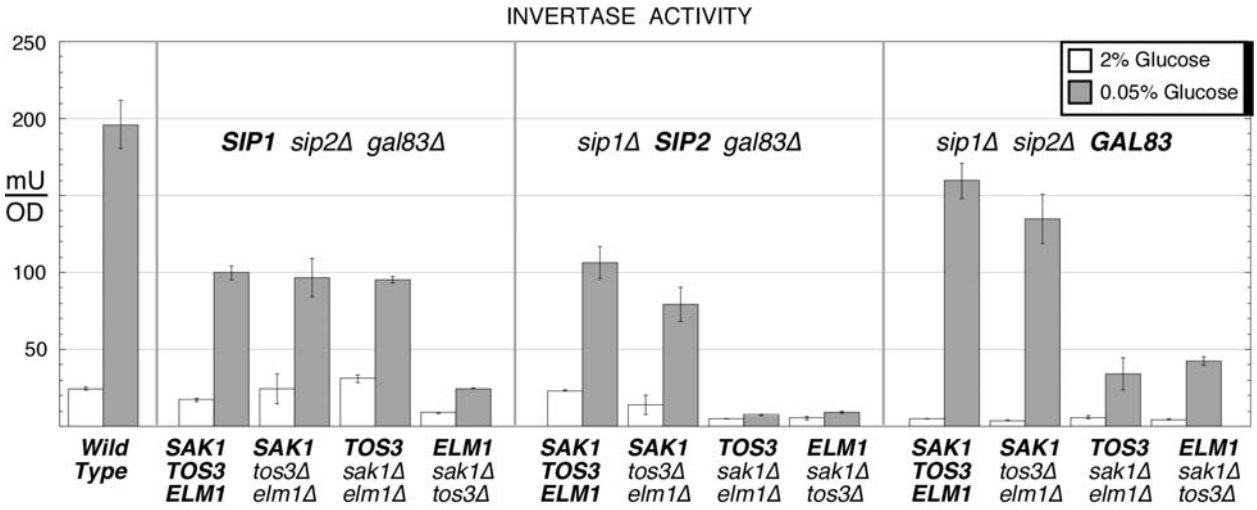

Figure 2.5 Invertase expression in cells expressing a single isoform of Snf1 kinase.

Invertase expression was assayed in cells grown in 2% glucose or after shifting to 0.05% glucose for 3 h. The genotypes of the yeast strains at the *SAK1*, *TOS3*, and *ELM1* loci are shown below, and the genotypes at the *SIP1*, *SIP2*, and *GAL83* loci are indicated above. The mean invertase expression from three independent cultures is plotted. Error bars represent one standard error.

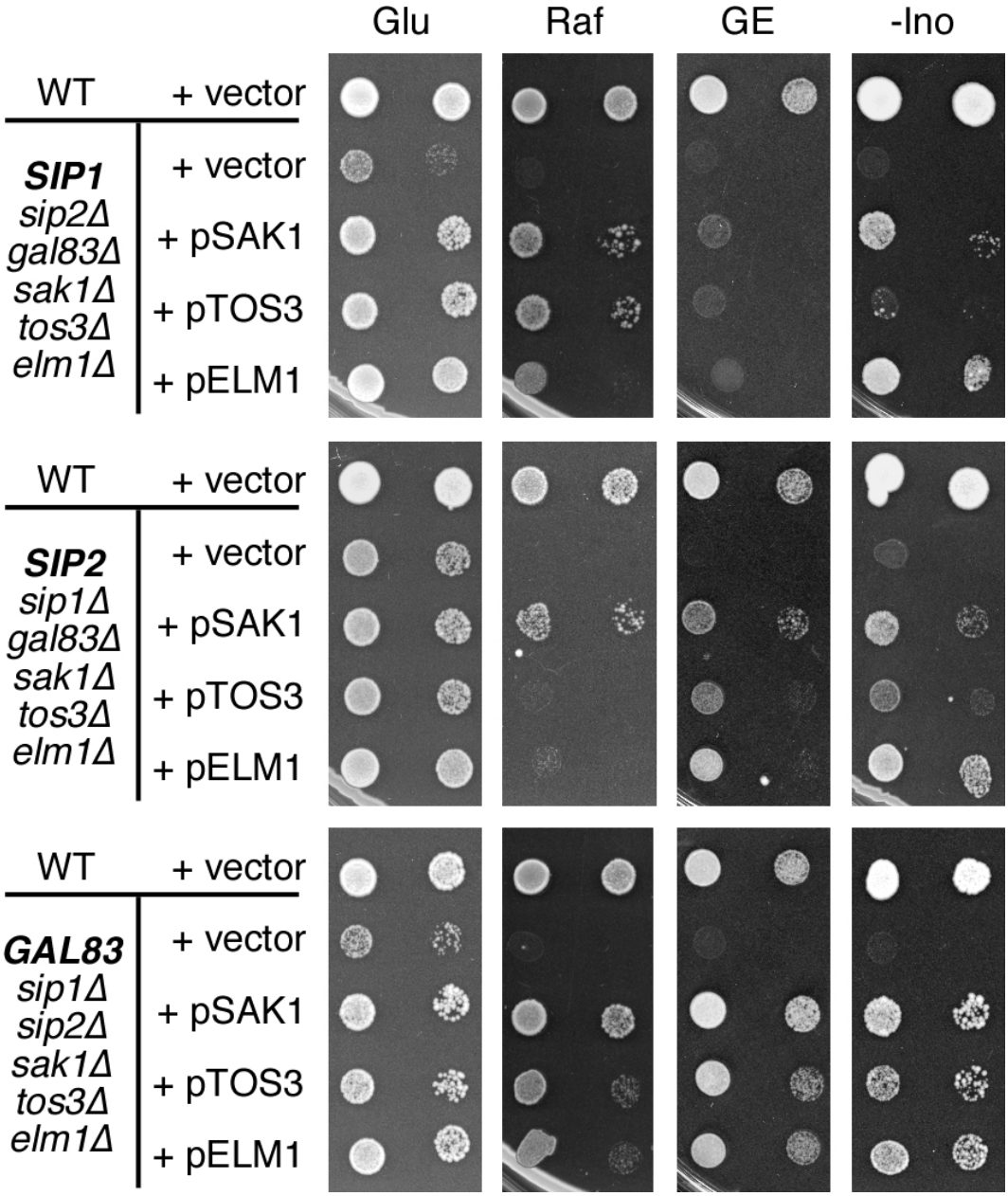

Figure 2.6 Growth phenotypes of cells expressing a single β subunit and Snf1-activating kinase.

Serial dilutions of yeast cultures were spotted onto synthetic complete medium lacking uracil, either with glucose (Glu), raffinose plus antimycin A (Raf), or a mixture of glycerol and ethanol as the carbon sources (GE), or with glucose as the carbon source and without inositol (-Ino). The relevant genotypes of the yeast strains are shown on the left. Each strain was transformed with a CEN *URA3* plasmid containing either no insert (vector) or the complete gene for the indicated Snf1-activating kinase.

Sip1 as the only β subunit were not able to grow aerobically and further deletion of upstream kinases did not suppress this growth defect. Sak1 and Elm1 but not Tos3 supported growth in the absence of inositol. Thus, the Sip1 isoform of the Snf1 kinase displays strong stress-dependent preferences for its upstream activating kinase. Cells expressing Sip2 as the only β subunit were able to ferment raffinose only when Sak1 was present. The combination of Sip2 with Tos3 or with Elm1 was not functional for growth on raffinose, and little or no invertase induction was observed in these cells (Figures 2.5 and 2.6). A different result was observed when cells were assayed for aerobic growth or for growth in the absence of inositol. Under those stresses, any one of the upstream kinases was sufficient for activation of the Sip2 isoform of Snf1 kinase. Finally, when Gal83 was the only β subunit, any one of the upstream kinases was able to provide Snf1 activation for growth on raffinose, although Sak1 was clearly the most efficient. Any one of the upstream kinases was able to activate the Gal83 isoform of Snf1 for growth on glycerol/ethanol or in the absence of inositol. We conclude that different isoforms of the Snf1 kinase display stress-dependent preferences for the Snf1-activating kinases.

2.3.6 Snf1-activating kinase expression in cells expressing different Snf1 isoforms

Different isoforms of Snf1 kinase are poorly activated by some of the upstream kinases. One explanation for this could be that the upstream kinases are poorly expressed or fail to accumulate in some mutant backgrounds. To determine whether deletion of genes encoding the Snf1 kinase β subunits might affect the abundance of the upstream kinases, we measured the abundance of the upstream kinases by Western blotting (Figure 2.7). Strains were constructed in which all three genes for the upstream kinases were deleted (*sak1*Δ *tos3*Δ *elm1*Δ). In addition, cells expressed either all three β subunits of the Snf1 kinase (denoted as WT) or only a single

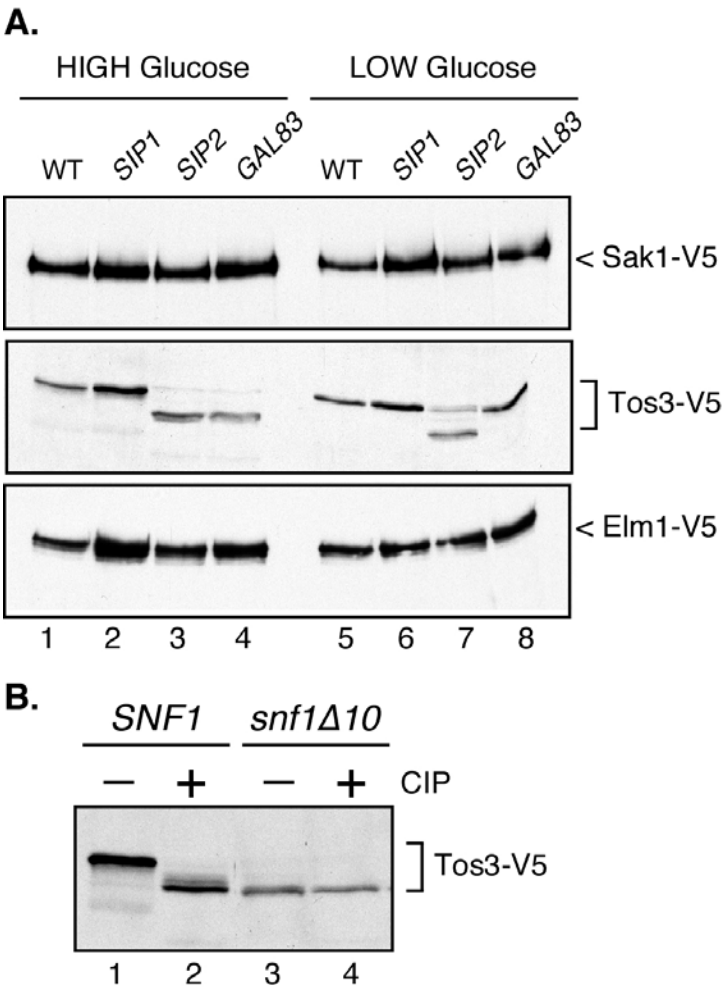

Figure 2.7 Accumulation of upstream kinases in cells expressing different isoforms of the Snf1 kinase.

A Strains with all three upstream kinases deleted (*sak1*Δ *tos3*Δ *elm1*Δ) were transformed with CEN plasmids expressing a single V5-tagged upstream kinase, as indicated on the right. In addition, cells contained genes either for all three β subunits (WT) or for a single β subunit, as indicated at the top of each lane. Protein extracts were prepared from cells grown in either high (H) or low (L) glucose. Tagged proteins from 500 μg extract were collected with anti-V5 antibodies. **B** Tos3-V5 mobility shift is due to Snf1-dependent phosphorylation. Tos3-V5 protein was expressed in cells containing (lanes 1 and 2) or lacking (lanes 3 and 4) Snf1. Protein extracts were incubated at 37°C for 1 h with (lanes 2 and 4) or without (lanes 1 and 3) 5 units of calf intestine alkaline phosphatase (CIP). Samples were analyzed by Western blotting with anti-V5 antibodies.

β subunit (shown above each lane). The upstream kinases tagged with the V5 epitope were expressed from their own promoters on low-copy-number plasmids and their abundance was measured by Western blotting. In all cases, equivalent aliquots were run in parallel and stained by Coomassie blue to verify that comparable protein levels were present in each sample (data not shown). Sak1 and Elm1 were both expressed at equivalent levels, regardless of the isoform of Snf1 present and independent of the glucose concentration. Tos3 kinase was present in all cell types at equivalent levels, but was found in both high- and low-mobility forms. The slower-migrating form is due to phosphorylation since it could be shifted to the faster-migrating species by treatment with phosphatase (Figure 2.4b, lane 2). The phosphorylation is Snf1-dependent, since it is not observed in *snf1Δ10* cells (lane 3). We conclude that the failure of some pairings of upstream kinases with certain isoforms of Snf1 kinase to function is not due to any problems with upstream kinase expression. For instance, the Sip2 isoform of Snf1 kinase was unable to induce invertase when Elm1 was the only upstream kinase present (Figure 2.5). This defect is not due to a failure to express Elm1 kinase (Figure 2.7a, lane 7).

2.3.7 Activation loop phosphorylation in strains expressing Sip2 as the only β subunit

Since the Sip2 isoform of Snf1 kinase exhibited the most stringent preference for Sak1 under low-glucose conditions, we tested whether the Snf1 activation loop threonine was phosphorylated in the Sip2 isoform of the enzyme in the presence of each of the upstream kinases. Cells expressing Sip2 as the only β subunit and either no upstream kinases or only a single upstream kinase were grown in the presence of high glucose (2%), low glucose (0.05%), or a non-fermentable carbon source (a mixture of glycerol and ethanol, GE). Snf1 protein was immunoprecipitated, and the level of phosphorylation on threonine 210 was assayed by Western

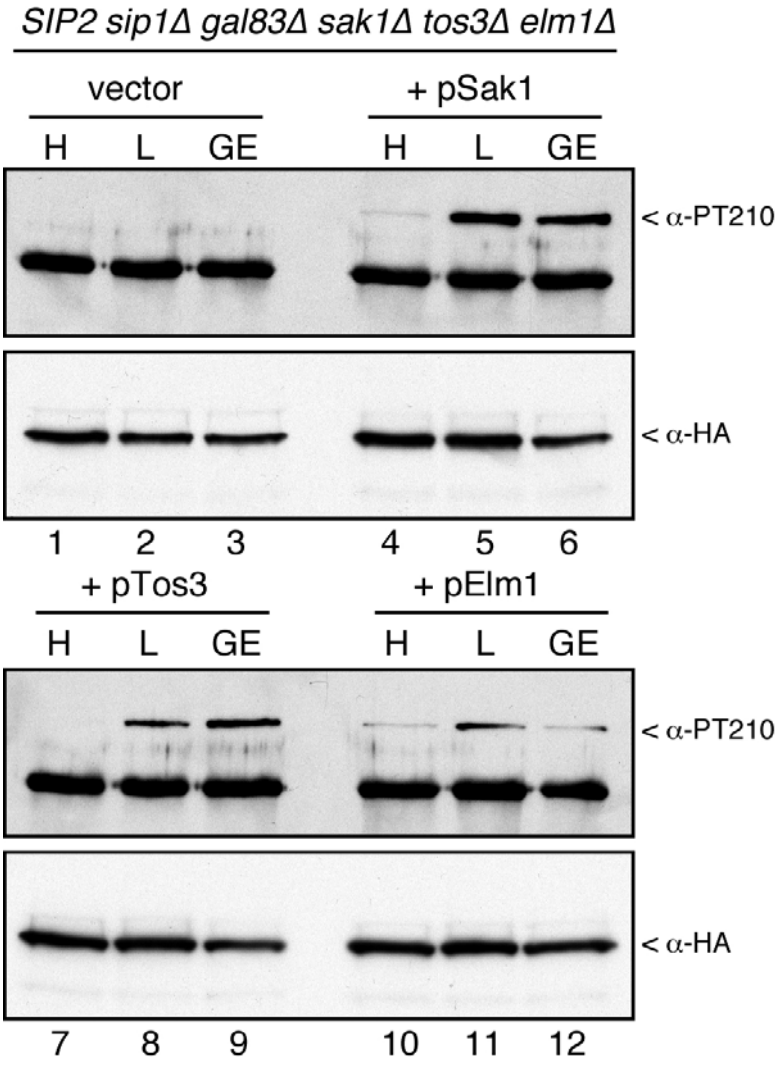

Figure 2.8 Phosphorylation of the Snf1 activation loop in cells expressing the Sip2 isoform of the Snf1 kinase. Proteins were immunoprecipitated with anti-HA antibody from protein extracts of cells grown in either high glucose (H), low glucose (L), or a mixture of glycerol and ethanol (GE). Precipitates were analyzed by Western blotting, using either anti-HA antibodies (α-HA) or antibodies specific for the Snf1 protein phosphorylated on threonine 210 (α-PT210). A single yeast strain (MSY886) was used in this experiment, and its relevant genotype is shown. Cells were transformed with a CEN plasmid with either no insert (vector; lanes 1-3) or full-length copies of the *SAK1* (lanes 4-6), *TOS3* (lanes 7-9), or *ELM1* (lanes 10-12) genes.

blot (Figure 2.8). In the absence of any upstream kinase (lanes 1–3), phosphorylation of threonine 210 was not detected under any growth condition. A Western blot against the HA epitope present at the C-terminus of the Snf1 protein indicated that equivalent levels of Snf1 were present in all the immunoprecipitates. When Sak1 was the only upstream kinase, the Sip2 isoform of Snf1 showed robust phosphorylation of threonine 210 when cells were grown on low glucose or a non-fermentable carbon source (lanes 5 and 6) but not when grown on high glucose (lane 4). When Tos3 (lanes 7–9) or Elm1 (lanes 10–12) was the only upstream kinase present, phosphorylation of threonine 210 was also detected, although the levels appeared lower than that observed with Sak1. We conclude that any one of the three upstream kinases is able to phosphorylate the Sip2 isoform of Snf1 kinase on threonine 210 in response to carbon source stress.

2.3.8 Mig1 phosphorylation in strains expressing Sip2 as the only β subunit

In growth and invertase assays that measured Snf1 activity, the Sip2 isoform of the Snf1 kinase displayed a distinct preference for the Sak1 kinase, and yet Tos3 and Elm1 were also able to phosphorylate Snf1 on threonine 210. Therefore, we next examined downstream signaling of the Sip2 isoform of Snf1. The phosphorylation state of the Mig1 protein in cells expressing Sip2 as the only β subunit was examined by Western blot (Figure 2.9). Hypo-phosphorylated Mig1 protein was detected in all cells examined when glucose was present at high concentrations (lanes 1, 4, 7, 10, 13). When shifted to low glucose, the hyper-phosphorylated form of Mig1 was detected in cells expressing all three upstream kinases (lane 2) or Sak1 only (lane 8). The Mig1 phosphorylation was completely dependent on the three Snf1-activating kinases, since no phosphorylation of Mig1 was observed when all three Snf1-activating kinases were deleted

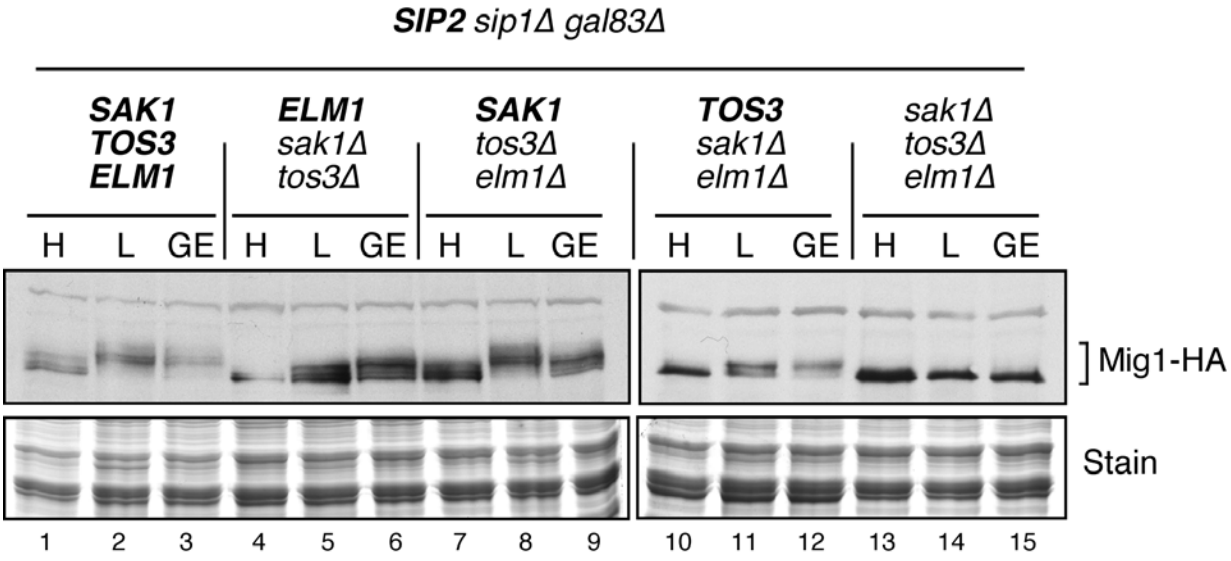

Figure 2.9 Mig1 phosphorylation in cells expressing only the Sip2 isoform of the Snf1 kinase.

Cells were transformed with a plasmid expressing HA-tagged Mig1 protein. Protein extracts were prepared from cells grown in synthetic complete medium containing either 2% glucose (H) or 3 h after shifting to 0.05% glucose (L) or glycerol/ethanol (GE), as indicated. Mig1 protein was detected with anti-HA antibodies. As a control for equal loading of protein, a gel run in parallel was stained with Coomassie blue and photographed (Stain). All strains in this experiment expressed the Sip2 protein as the only Snf1 kinase β subunit (*sip1*Δ *gal83*Δ). The different combinations of deletions in the Snf1-activating kinases are indicated.

(lanes 13–15). Interestingly, when Tos3 or Elm1 were the only upstream kinases present, significant levels of the hypo-phosphorylated Mig1 were detected in low glucose (lanes 5 and 11). This failure to effectively phosphorylate Mig1 in cells with only Sip2 and Tos3 or only Sip2 and Elm1 correlated with the poor growth properties of these cells on raffinose media (Figure 2.6) and with their inability to induce invertase expression (Figure 2.5). We also looked at the phosphorylation state of Mig1 after cells were shifted to a non-fermentable carbon source for 3 h. Cells expressing Elm1 as the only upstream kinase displayed increased ability to phosphorylate Mig1 relative to the low-glucose-grown cells (lanes 5 and 6), consistent with the observation that cells expressing only Sip2 and Elm1 were able to grow aerobically (Figure 2.6). The combination of Sip2 and Tos3 showed the lowest levels of Mig1 phosphorylation under both Snf1-activating conditions tested (lanes 11 and 12), and yet Tos3 was able to promote activation loop phosphorylation of the Sip2 isoform of Snf1 (Figure 2.7). These results suggest that additional levels of regulation of Snf1 kinase signaling must occur downstream of the activation loop phosphorylation step.

2.4 DISCUSSION

The underlying hypothesis being tested is that each of the three Snf1-activating kinases is dedicated to the activation of a single isoform of the Snf1 kinase. The data presented here and in an earlier report (84) disprove this very simplistic hypothesis. For instance, the Sak1 kinase is able to activate all three isoforms of the Snf1 kinase in response to low glucose, as judged by growth assays on raffinose (Figure 2.6) and invertase induction (Figure 2.5). Therefore, Sak1 is not dedicated to the activation of a single isoform of the Snf1 kinase. A similar conclusion was

reached by Hedbacker et al. (84). The converse of our hypothesis, that the three Snf1-activating kinases are promiscuous and equally capable of activating all three isoforms of the Snf1 kinase is also not supported by our data. For instance, the Sip2 isoform of the Snf1 enzyme was poorly activated if at all by Tos3 or Elm1 in response to low glucose, as judged by invertase induction and growth assays on raffinose (Figures 2.5 and 2.6). Therefore, the Sip2 isoform of Snf1 kinase exhibited a strong preference for the Sak1 kinase under low glucose conditions. Interestingly, the Sip2 isoform of the Snf1 kinase expanded its activating kinase preference to include Sak1, Tos3, and Elm1 during growth on ethanol or in the absence of inositol (Figure 2.6). Therefore, the upstream kinase preference was stress-dependent. We conclude that the dedicated activator hypothesis and its converse, the promiscuous activator hypothesis, are not valid. Our data support an intermediate model in which different isoforms of the Snf1 kinase do in fact exhibit strong and distinct preferences for specific Snf1-activating kinases and the preference varies, depending on both the β subunit identity and the stress response.

Yeast cells adapt to changes in glucose concentrations with both short-term and long-term changes in patterns of gene expression (56, 91). In this study, the invertase expression and the Mig1 and Snf1 protein phosphorylation states were measured 2–3 h after shifting to low glucose and are thus measurements of short-term responses. In contrast, the ability of cells to ferment raffinose or respire a mixture of glycerol and ethanol was assessed after several days of growth and reflect long-term responses. We noted a strong correlation between invertase induction, a short-term response and growth on raffinose, a long-term response. For instance, cells with Sip2 as the only Snf1 β subunit were able to induce invertase only in combination with Sak1 as the Snf1-activating kinase (Figure 2.5). Similarly, the combination of Sip2 with Sak1 showed much better growth on raffinose than the pairing of Sip2 with Tos3 or Elm1 (Figure 2.6).

Similar correlations were observed in Sip1 cells, where invertase induction with Sak1 and Tos3 also correlated well with growth on raffinose.

The observed preferences by the isoforms of Snf1 for the different upstream kinases might be explained by differences in subcellular localization. Studies using green fluorescent protein (GFP)-tagged proteins showed that Snf1, Snf4, and the β subunits are localized to distinct compartments (97, 194) and that their localization is dynamic, changing in response to the carbon source (85, 194). The localization of the upstream kinases is less well defined. Elm1 kinase displays a very restricted localization, limited to the bud neck (20, 97, 132). None of the Snf1 subunits have ever been found to be concentrated at this location. The localization of Tos3 has not been defined, since GFP fusion to Tos3 was not detected (97). The Sak1-GFP expressed from its genomic locus exhibits a cytosolic localization in high glucose (84). In low glucose, some of the Sak1-GFP fluorescence shifts to the outer vacuolar membrane. A similar shift is observed for Sip1 (85). Our data are consistent with the idea that the incompatibility of particular pairs of Snf1 isoforms and upstream kinases may be explained by differences in subcellular localization.

However, differences in the subcellular localization of Snf1 and its activating kinase cannot account for all of our observations. For instance, we found that the pairing of the Sip2 isoform of Snf1 with Tos3 kinase did not reconstitute efficient Snf1 signaling in response to low glucose (Figures 2.5 and 2.6). Yet, Tos3 phosphorylated the activation loop threonine of Snf1 when Sip2 was the only β subunit present (Figure 2.8). Since Tos3 was able to phosphorylate the activation loop threonine, the Snf1 and Tos3 proteins must co-localize at least transiently. The defect in Snf1 signaling appears to be downstream of the activation loop phosphorylation, since the Mig1 protein was not efficiently phosphorylated in response to low glucose in cells

expressing only Sip2 and Tos3 (Figure 2.9). It is important to note that, although we are able to detect activation loop phosphorylation, we are not able to assess what fraction of the total Snf1 pool is phosphorylated, nor can we determine from what subcellular compartment the activated Snf1 originates. The fact that Mig1 is poorly phosphorylated by the Sip2 isoform of Snf1 despite the fact that some fraction of the Snf1 is phosphorylated on threonine 210 may indicate that the activated Snf1 and Mig1 are not present in the same compartment. Alternatively, the Snf4-mediated activation step may be defective in cells expressing only Tos3 and Sip2.

Global studies of protein expression levels suggest that there are far fewer Snf1 molecules than β and γ subunits (71). All evidence for Snf1 kinase stoichiometry, including silver-stained gels of purified Snf1 complexes (137), suggests that the three subunits are associate in a 1:1:1 stoichiometry. Therefore, the three β subunits, possibly pre-bound to the abundant γ subunit (Snf4), must compete for binding to the far fewer molecules of Snf1. Of all the β subunits, the Sip2 protein may well be the most affected by a model in which Snf1 signaling is controlled by competitive binding to a limiting number of Snf1 proteins. Global expression analyses of the Snf1 kinase subunits did not detect any large changes in mRNA abundance for Snf1, Snf4, Sip1, or Gal83 in response to changes in carbon source. In contrast, microarray analysis of mRNA levels in response to glucose limitation (213) and growth on ethanol (66) detected large increases in the Sip2 message. Increased abundance of Sip2 protein in cells shifted to a non-fermentable carbon source was also shown by Western blots of a GFP-Sip2 fusion protein (194). In light of a model in which Snf1 signaling is defined by competition between paralogous proteins, we predict that increased levels of Sip2 protein leads to a greater proportion of Snf1 kinases complexes containing the Sip2 protein, leading to greater signaling through the Sip2 isoform of the Snf1 kinase complex. If Snf1 signaling pathways are defined in

part by competitive binding of subunits to the kinase, then caution is needed when interpreting studies which utilize gene deletions (such as this study). The absence of competing subunits would promote the association of the remaining subunits and affect the signaling pathways under investigation.

Finally, we ask which of the three Snf1-activating kinases activates Snf1 *in vivo*. The data presented here suggests that all three kinases play a role in Snf1 activation, depending on the stress and the β subunit identity. However, we must also consider the possibility that one (or two) of these kinases is the predominant activator(s) of Snf1 *in vivo* and that the other kinase(s) participates in Snf1 activation only when the cognate activator(s) is missing. Two lines of evidence suggest that Sak1 is the predominant activator of Snf1 *in vivo*. First, Sak1 is the most promiscuous, since it was able to activate all three isoforms of the Snf1 kinase complex under all stress conditions tested. Second, mass spectrometric experiments of proteins associated with Snf1 identified Sak1 but not Tos3 or Elm1 (68, 89). In addition, there is good reason to believe that Elm1 kinase participates in other signaling pathways, probably by activating the Snf1-related kinases, Hsl1, Kcc4, and Gin4 (20, 133, 182). Further experiments using techniques more subtle than gene deletions will be needed to determine whether Sak1 is the primary activator of Snf1 *in vivo*.

2.5 ACKNOWLEDGMENTS AND CONTRIBUTIONS

This work was supported by grant GM46443 from the National Institutes of Health. We thank Anna Leech for countless PCR reactions to confirm genotypes and thank Karen Arndt for critical reading of this manuscript.

RRM performed the experiments displayed in Figures 2.1-2.5 and 2.7-2.9. EMR performed the experiments displayed in Figure 2.6. EMR and MCS collaborated on research design and manuscript preparation.

3.0 REGULATORY DOMAINS OF SNF1-ACTIVATING KINASES DETERMINE PATHWAY SPECIFICITY

Eric M. Rubenstein, Rhonda R. McCartney, and Martin C. Schmidt (2006).

Eukaryotic Cell 5, 620-7.

3.1 INTRODUCTION

The serine/threonine protein kinase Snf1 in *Saccharomyces cerevisiae* and its mammalian orthologue, the AMP-activated protein kinase (AMPK), are central mediators of nutrient stress response. Members of this family are found in all eukaryotic organisms (77). When activated, these kinases conserve cellular energy by promoting processes that generate cellular ATP while inhibiting those that consume ATP (204). Interest in the AMPK signaling pathway has increased since the discovery that AMPK is activated by metformin, the most widely prescribed therapeutic used to treat type II diabetes (201). Understanding the molecular mechanisms that regulate this signaling pathway could lead to the discovery of new targets for the treatment of type II diabetes.

In yeast, many of the downstream signaling events of the Snf1 pathway are well understood. In contrast, the regulation of events upstream of Snf1 is just beginning to be elucidated. Activation of Snf1 requires the phosphorylation of threonine 210 in the Snf1 activation loop (60) by a distinct upstream kinase (129). Recently, we identified Sak1 as a Snf1-activating kinase (138). Sak1 associates with Snf1 and is capable of phosphorylating the activation loop threonine of Snf1 *in vivo* and *in vitro* (54, 138). However, Sak1 is not the only Snf1-activating kinase in yeast. In addition, two closely related kinases, Tos3 and Elm1, share this function with Sak1 (92, 180). Sak1, Tos3, and Elm1 exhibit the most similarity in the 300-residue kinase domain. Nonconserved N-terminal and C-terminal domains represent half of the protein for Tos3 and even more for Elm1 and Sak1. The three Snf1-activating kinases are not

functionally interchangeable but exhibit some specialization in function (84, 128). Since the N- and C-terminal domains are the most divergent, we hypothesized that these domains may define their different functional capacities.

SAK1 was first identified as a high-copy-number suppressor of temperature-sensitive DNA polymerase α mutations and was originally designated *PAK1* for *P*olymerase *A*lpha *K*inase (95). Subsequently, the term PAK has become widely used to refer to p21-activated kinases, a family of serine/threonine protein kinases that are regulated by small GTP-binding proteins. Yeast genes encode members of the PAK family (Ste20, Cla4, and Skm1), but the yeast *PAK1* product is not a member of the p21-activated kinase family. Thus, to avoid further confusion, the yeast *PAK1* gene (YER129W) has been renamed *SAK1*, for *S*nf1-*A*ctivating *K*inase (54). The Saccharomyces Genome Database has agreed to this name change.

Many protein kinases are regulated at least in part by the phosphorylation of one or more residues in their respective activation loops (141). This paradigm holds true for the Snf1 kinase. However, Sak1, Tos3, and Elm1 lack a conserved threonine residue in their activation loops, suggesting that the Snf1-activating kinases may be regulated by other means. Indeed, recent studies of LKB1, a functional mammalian orthologue of the Snf1-activating kinases, indicate that the phosphorylation of the LKB1 activation loop plays no role in its activation (19). Instead, LKB1 is activated by the binding of two accessory proteins, Mo25 and STRAD. Some kinases that do not require activation loop phosphorylation contain extensions of the activation loop or insertions elsewhere in the kinase domain. For instance, casein kinase 1 has an unusually long activation loop, and phosphorylase kinase has an insertion of a sequence that contacts and stabilizes the activation loop (141). The C terminus of the yeast Sky1 kinase interacts with the activation loop, holding the kinase in a constitutively active conformation (142). The

mechanisms that regulate the Snf1-activating kinases are not known. In this study, we examine the role(s) the N- and C-terminal nonkinase domains play in the regulation and function of the Snf1-activating kinases.

3.2 MATERIALS AND METHODS

3.2.1 Yeast strains and media

Yeast strains used in this study were MSY857 (*MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 sak1*Δ::*KAN elm1*Δ::*KAN tos3*Δ::*KAN*) and MSY876 (*MATα ura3 leu2 his3 sak1*Δ::*KAN elm1*Δ::*KAN tos3*Δ::*KAN snf1Δ10*). Yeast was grown at 30°C in standard media (153). Glucose was present at 2% or 0.05% (g/100 ml), as indicated in the relevant figures. The glycerol-ethanol medium contained a mixture of glycerol at 3% (vol/vol) and ethanol at 2% (vol/vol). The raffinose medium contained 2% (g/100 ml) raffinose, 0.05% (g/100 ml) glucose, and antimycin A (1 μg/ml).

3.2.2 Plasmids

Plasmids used in this study were generated using gap repair and standard subcloning protocols. Plasmids used in this study and their salient features are presented in Table 3.1. All Snf1-activating kinases were tagged with one or five copies of the V5 epitope at their C termini. Protein deletion constructs are summarized in Table 3.2.

Table 3.1 Plasmids used in this study.

Plasmid or Plasmid Type	Kinase expressed	Promoter	Epitope[a]	Marker	Replicon
pSnf1HA-315	*SNF1*	*SNF1*	3x HA	*LEU2*	CEN
High-copy number	*SAK1* or *ELM1*	*ADH1*	V5	*URA3*	2 μm
Low-copy number	*SAK1*, *TOS3*, or *ELM1*	Cognate	5x V5	*URA3*	CEN

[a] 3x or 5x, 3 or 5 tandem copies of the indicated epitope.

Table 3.2 Protein deletion constructs.

Kinase	AAs present[b]	AAs deleted
Sak1	1-1142	
Sak1ΔC	1-500	511-1142
Sak1ΔN	1, 130-1142	2-129
Sak1ΔNΔC	1, 130-500	2-129, 501-1142
Sak1ΔN1	1, 2, 87-1142	3-86
Sak1ΔN2	1-86, 130-1142	87-129
Sak1ΔM[c]	1-573, 628-1142	574-627
Tos3	1-560	
Tos3ΔC	1-350	351-560
Tos3ΔM	1-345, 399-560	346-398
Elm1	1-640	
Elm1ΔC	1-420	421-640

[b] AA, amino acid.
[c] ΔM, deletion of the conserved motif.

3.2.3 Protein extraction

Cultures of yeast cells (25 to 50 ml) were harvested in mid-log phase (optical density at 600 nm, 0.4 to 0.8), and protein extracts were prepared by vortexing them with glass beads in radioimmunoprecipitation assay buffer (50 mM Tris HCl [pH 8], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% NP-40, 0.5% deoxycholate) with protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 2 μg/ml chymostatin) and phosphatase inhibitors (50 mM NaF and 5 mM $NaPP_i$). For analysis of Snf1 activation loop threonine phosphorylation, cells were harvested following the addition of NaOH to 0.1 M, suspended in SDS sample buffer (62 mM Tris-Cl [pH 6.8], 10% glycerol, 5% β-mercaptoethanol, 3% SDS), and subjected to overnight dialysis against 2 liters of radioimmunoprecipitation buffer. Protein concentrations were determined using the Bradford method (21) with bovine serum albumin as a standard.

3.2.4 Western blotting

Horseradish peroxidase-conjugated mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) against the hemagglutinin (HA) epitope were used to detect HA-tagged Snf1. Rabbit polyclonal antibodies directed against phosphorylated Snf1 threonine 210 (129) were used to detect phosphorylated Snf1. Horseradish peroxidase-conjugated mouse monoclonal antibodies (Invitrogen, Carlsbad, CA) against the V5 epitope were used to detect Sak1, Tos3, and Elm1 variants that were epitope-tagged with V5.

3.2.5 Invertase assays

Invertase activity of log-phase cells grown in high- or low-glucose medium was quantitatively assayed using a colorimetric assay coupled to glucose oxidase (162). Three independent cultures were assayed, and the mean values are shown in the relevant figures.

3.2.6 Immune complex kinase assays

Cultures of yeast cells (50 ml) were harvested in mid-log phase (optical density at 600 nm, 0.4 to 0.8), and protein extracts were prepared by vortexing with glass beads in ice-cold lysis buffer (20 mM Tris-HCl [pH 7.2], 12.5 mM potassium acetate, 4 mM $MgCl_2$, 0.5 mM EDTA, 0.1% Tween 20, 12.5% glycerol, and 1 mM dithiothreitol) with protease inhibitors (2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM benzamidine, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, 2 μg/ml chymostatin, and 1 mM phenylmethylsulfonyl fluoride). Extracts were precleared by incubation with 20 μl 50% protein A-Sepharose beads (Sigma) at 4°C for 30 min. All protein A-Sepharose beads were prewashed twice with lysis buffer. V5-tagged Sak1 lacking the N and C termini (Sak1ΔNΔC) was immunoprecipitated from 200 μg protein with 0.75 μl anti-V5 antibody (Invitrogen) and 15 μl 50% protein A-conjugated beads at 4°C for 2 h. Immune complexes were collected by centrifugation, washed once with lysis buffer and twice with kinase assay buffer (20 mM HEPES [pH 7.0], 0.5 mM EDTA, 0.5 mM dithiothreitol, and 5 mM Mg acetate), and suspended in kinase buffer (20 μl final volume) containing 0.2 mM [γ-^{32}P]ATP (500 cpm/pmole) and the glutathione *S*-transferase (GST)-Snf1 kinase domain at approximately 50 μg/ml. Proteins were eluted from the beads by incubation in SDS sample buffer at 95°C for 5 min. Labeled proteins were resolved on an SDS-polyacrylamide gel and subjected to autoradiography.

3.2.7 Microscopy

Differential interference contrast images of yeast cells were collected with a Nikon 2000e microscope using a 1.45-numerical-aperture, 60× objective, a q-imaging Retiga EXI cooled charge-coupled-device camera, and Metamorph software.

3.3 RESULTS

3.3.1 Carboxy termini of Sak1 and Tos3 are required for efficient Snf1 pathway signaling

The locations of the kinase domains in the three Snf1-activating kinases (Sak1, Tos3, and Elm1) are displayed in Figure 3.1a. All three Snf1-activating kinases contain large C-terminal domains that share little homology outside of a small conserved motif found in Sak1 and Tos3 but not Elm1 (Figure 3.1a and b). We investigated whether this conserved motif or the entire carboxy-terminal domains of Sak1, Tos3, and Elm1 are required for Snf1 signaling. Cells lacking all three Snf1-activating kinases were transformed with low-copy-number plasmids expressing full-length or truncated versions of the Snf1-activating kinases. Tables 3.1 and 3.2 describe in more detail the plasmids and deletion constructs used in these studies. The truncated versions of Sak1, Tos3, and Elm1 were all stably expressed at comparable levels, as judged by Western blotting (Figure 3.1d). In the absence of all three Snf1-activating kinases, cells are unable to grow by fermentation of raffinose or by respiration of glycerol and ethanol (Figure 3.1c). Cells expressing full-length Sak1, Tos3, or Elm1 all recover the ability to grow on these alternative carbon sources, demonstrating that any one of the Snf1-activating kinases is sufficient to promote Snf1

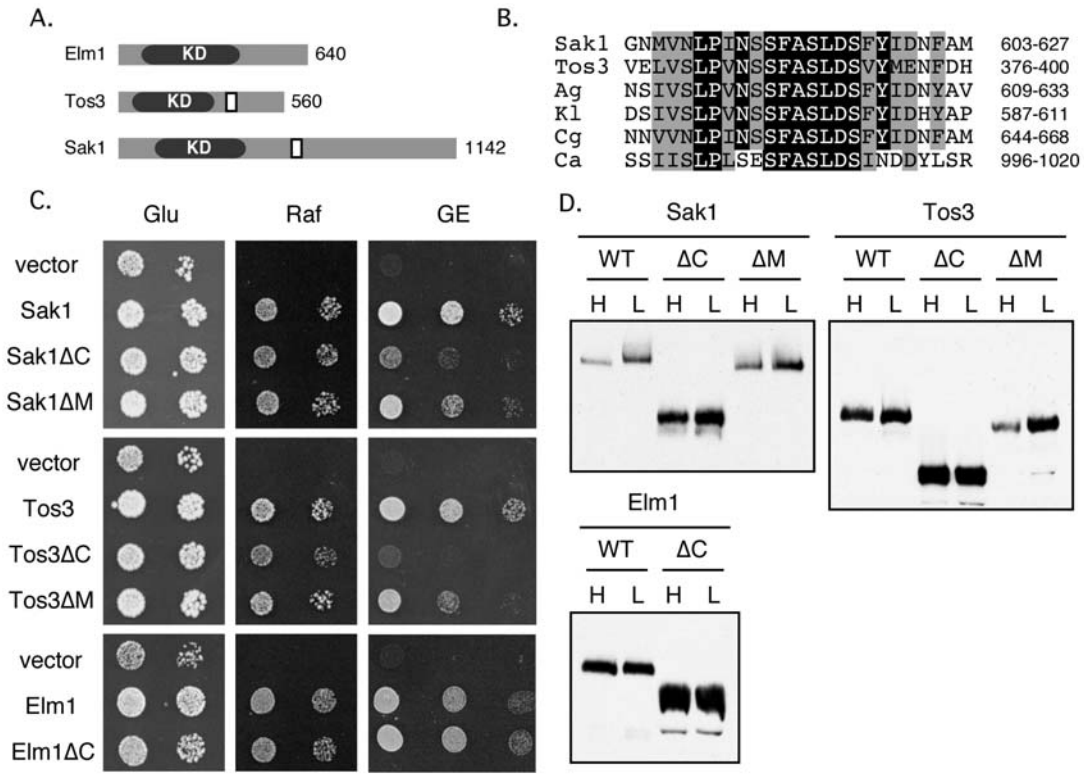

Figure 3.1 C-terminal-deletion mutants of the Snf1-activating kinases.

A Schematic representation of the Elm1, Tos3, and Sak1 kinases drawn to scale. The positions of the kinase domains (KD) and a conserved motif (open box) are indicated. **B** Sequence alignment of the conserved motif found in the C-terminal domains of Sak1 and Tos3 kinases from *S. cerevisiae* and of Sak1 homologues from *Ashbya gossypii* (Ag; GenBank accession no. AAS51175), *Kluyveromyces Lactis* (Kl; GenBank accession no. XP_453478), *Candida glabrata* (Cg; GenBank accession no. XP_448319), and *Candida Albicans* (Ca; GenBank accession no. EAK98348). **C** Serial dilutions of yeast cultures were spotted onto agar plates with glucose (Glu), raffinose (Raf), or a mixture of glycerol and ethanol (GE) as the carbon sources. Cells lacked all three Snf1-activating kinases but were transformed with low-copy-number plasmids expressing no kinase (vector), full-length kinase, a C-terminal truncation (ΔC), or a precise deletion of the conserved motif found in Sak1 and Tos3 (ΔM). **D** Western blot of V5-tagged Snf1-activating kinases. Protein extracts of the cells shown in panel **C** were prepared from cultures grown in high (H) glucose (2%) or after shifting to low (0.05%) glucose (L). Snf1-activating kinases were all tagged with five copies of the V5 epitope at the C terminus of each protein and were expressed from their cognate promoters on low-copy-number plasmids. WT, wild-type.

signaling. Deletion of the entire C-terminal domains from these kinases had no effect on their ability to grow by fermentation of raffinose. However, aerobic growth was markedly reduced in cells expressing Sak1ΔC or Tos3ΔC as the sole Snf1-activating kinase. Of the three C-terminal deletion constructs examined, the Tos3ΔC construct showed the most severe reduction in growth on glycerol-ethanol. In contrast, the Elm1ΔC construct appeared fully functional in these growth assays, suggesting that the Elm1 C terminus is not needed for its participation in the Snf1 signaling pathway. Deletion of the conserved motif found in Sak1 and Tos3 had no effect on the ability of cells to ferment raffinose but affected aerobic growth. Tos3 lacking the conserved motif displayed a 10-fold reduction in this spot dilution assay. While deletion of the conserved motif did impair functions, especially for Tos3, we conclude that the conserved motif is not essential for Sak1 or Tos3 participation in Snf1 signaling. Likewise, the entire C-terminal domains of Sak1 and Tos3 appear to be important but not entirely essential for Snf1 signaling. In contrast, the Elm1 C-terminal domain is entirely dispensable for growth on alternative carbon sources.

Invertase expression provides a more quantitative means by which to measure Snf1 signaling and thereby the function of these carboxy-terminal-truncation variants of the Snf1-activating kinases. When present as the only Snf1-activating kinase, either Sak1ΔC or Tos3ΔC was able to induce invertase, although less efficiently than the corresponding full-length proteins (Figure 3.2). Consistent with the growth phenotypes shown in Figure 3.1, the deletion of the C terminus had the largest effect on Tos3 function and the least effect on Elm1 function. Deletion of the conserved motif present in Sak1 and Tos3 had a small but detectable effect on invertase induction. We conclude that the conserved motif plays only a minor role in Snf1-mediated invertase induction. The C-terminal domains of Sak1, Tos3, and Elm1 are not absolutely

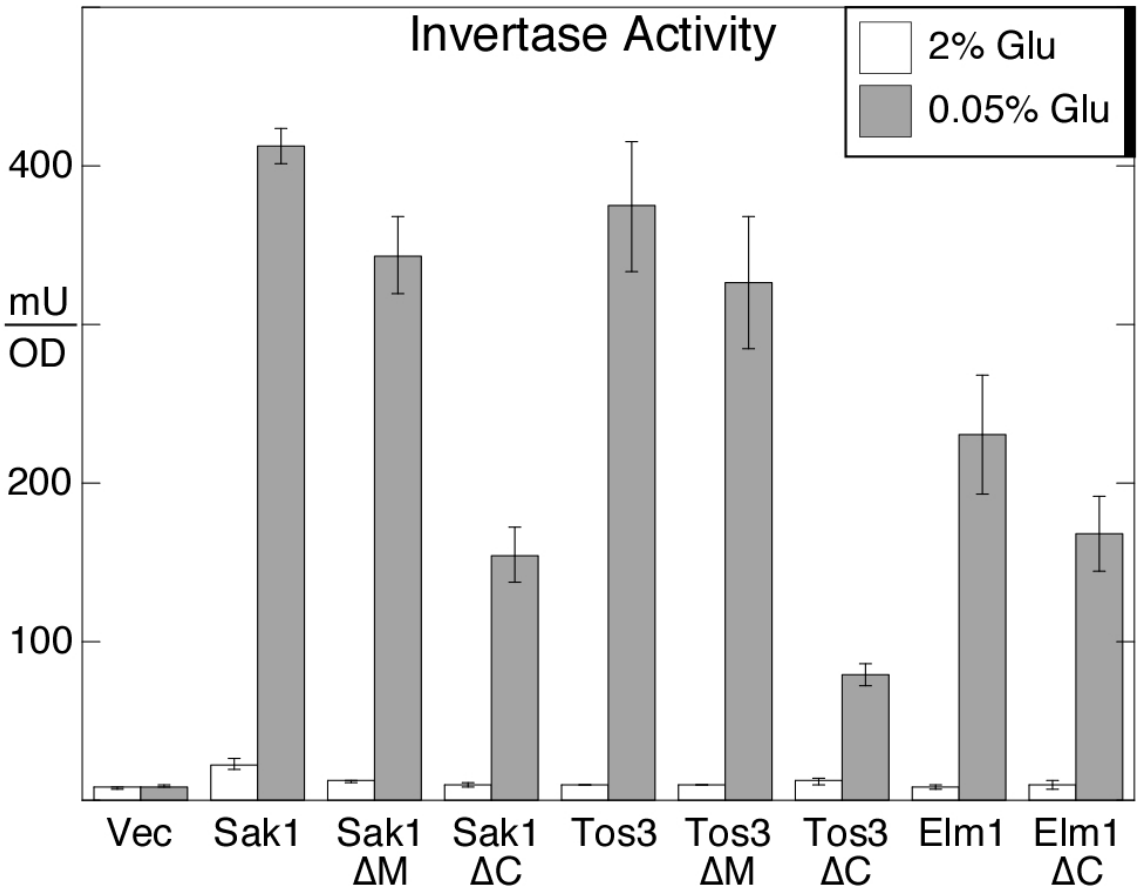

Figure 3.2 Invertase expression in cells with Sak1, Tos3, and Elm1 mutations.

Invertase activity was assayed with cells grown in 2% glucose (Glu) or after shifting to 0.05% glucose for 3 h. Cells lacked all three Snf1-activating kinases but were transformed with low-copy-number plasmids expressing either no kinase (Vec), full-length kinase, a C-terminal truncation (ΔC), or a precise deletion of the conserved motif (ΔM). The mean invertase activity level for three independent transformants is plotted. Error bars represent one standard error. mU/OD, μmole glucose/min/OD of cells assayed.

required for Snf1 function; however, those of Sak1 and Tos3 are needed for efficient Snf1 signaling.

3.3.2 The Elm1 carboxy terminus is required for normal morphology maintenance

The carboxy terminus of Elm1 is not required for efficient activation of the Snf1 signaling pathway (Figure 3.1c and 3.2). Indeed, by analysis of growth assays (Figure 3.1c), Elm1ΔC activates Snf1 as well as full-length Elm1 does. Because Elm1 is also required for the morphogenetic checkpoint (17), we examined the morphology of cells expressing wild-type Elm1 or Elm1ΔC. Cells lacking Sak1, Tos3, and Elm1 display elongated morphologies, and the cells are bunched together in clusters, a phenotype typically observed when *ELM1* is absent (Figure 3.3, row 1). A low-copy-number plasmid (CEN) encoding full-length Elm1 rescues cells from this abnormal morphology (Figure 3.3, row 2), while a low-copy-number plasmid expressing Elm1ΔC does not (Figure 3.3, row 3). Therefore, the carboxy terminus of Elm1 is required to specify its role in morphology maintenance. Intriguingly, however, when Elm1ΔC is present at a high copy number, normal morphology is restored (Figure 3.3, row 4), suggesting that the increased abundance of the kinase activity of Elm1 can compensate for the loss of its C terminus.

3.3.3 The Sak1 amino terminus is required for Snf1 signaling

Having established that the carboxy termini of Sak1 and Tos3 are required for efficient function in the Snf1 pathway, we next wished to assess what roles the amino termini might play in glucose signaling. Because Sak1 appears to be the primary Snf1-activating kinase (54, 84, 128),

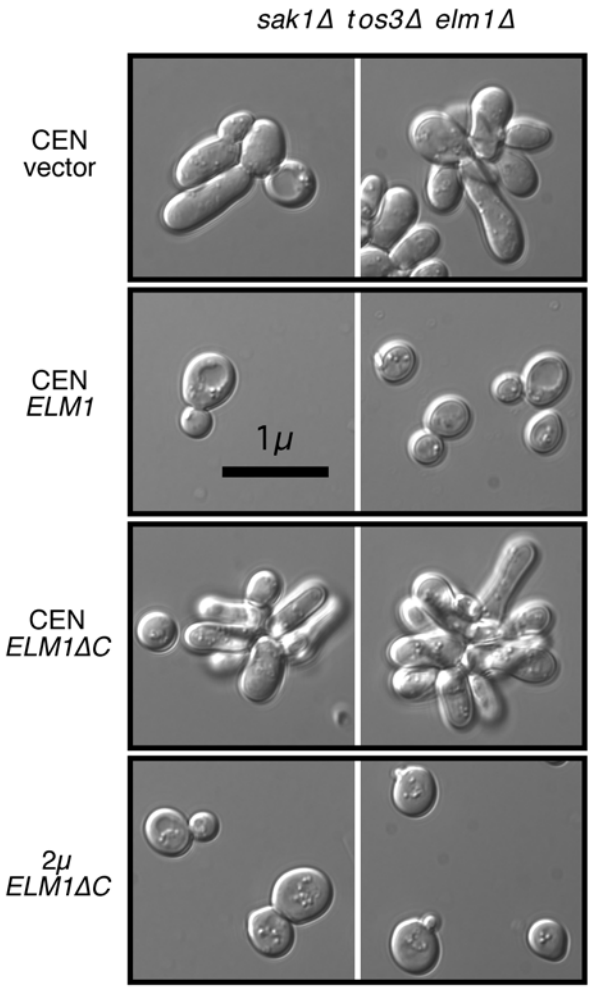

Figure 3.3 Cell morphology of *elm1*Δ mutants.

Cell morphology was analyzed by differential interference contrast microscopy. Cells lacked all three Snf1-activating kinases but were transformed with either low-copy-number (CEN) or high-copy-number (2μm) plasmids expressing no kinase (vector), full-length Elm1 kinase (*ELM1*), or a C-terminal truncation of the Elm1 kinase (*ELM1*Δ*C*). Bar, 1 μm.

we focused our attention on Sak1. We therefore engineered Sak1 constructs lacking only the amino terminus or both the amino- and carboxy-terminal domains (Sak1ΔN and Sak1ΔNΔC, respectively) and examined their abilities to activate Snf1 in response to glucose limitation. Cells expressing Sak1ΔN or Sak1ΔNΔC as the only Snf1-activating kinase are unable to grow on raffinose or glycerol-ethanol media (Figure 3.4a). Similarly, Sak1 constructs lacking their N termini are unable to induce invertase expression (Figure 3.4b). All constructs were stably expressed at comparable levels, as judged by Western blotting (Figure 3.4c). Thus, while the carboxy terminus of Sak1 is necessary for efficient transduction of Sak1-to-Snf1 signaling, the amino terminus is absolutely obligatory for Snf1 signaling outputs.

3.3.4 The Sak1 kinase domain is active *in vivo* and *in vitro*.

The deletion of the Sak1 N terminus completely blocks its ability to promote Snf1 signaling. One simple explanation for this would be that deletion of amino acids 2 to 129 inactivates the Sak1 kinase activity. This possibility was tested directly by immune complex kinase assays. The V5-tagged Sak1ΔNΔC protein was overexpressed and collected from yeast extracts with agarose beads conjugated with anti-V5 antibodies. Immune complexes were incubated with [γ-^{32}P]ATP and a recombinant substrate, the GST-Snf1 kinase domain, that has been used in previous studies (54). Sak1ΔNΔC protein promotes phosphorylation of the GST-Snf1 kinase domain (Figure 3.5a, lane 3). The reaction is specific for the activation loop of the Snf1 kinase domain since a single amino acid mutation in the substrate (threonine 210 to alanine) completely blocks the reaction (Figure 3.5a, lane 4). Equivalent levels of Sak1ΔNΔC protein were present in the reactions, as judged by Western blotting of immune complexes run in parallel (Figure 3.5b). We conclude that

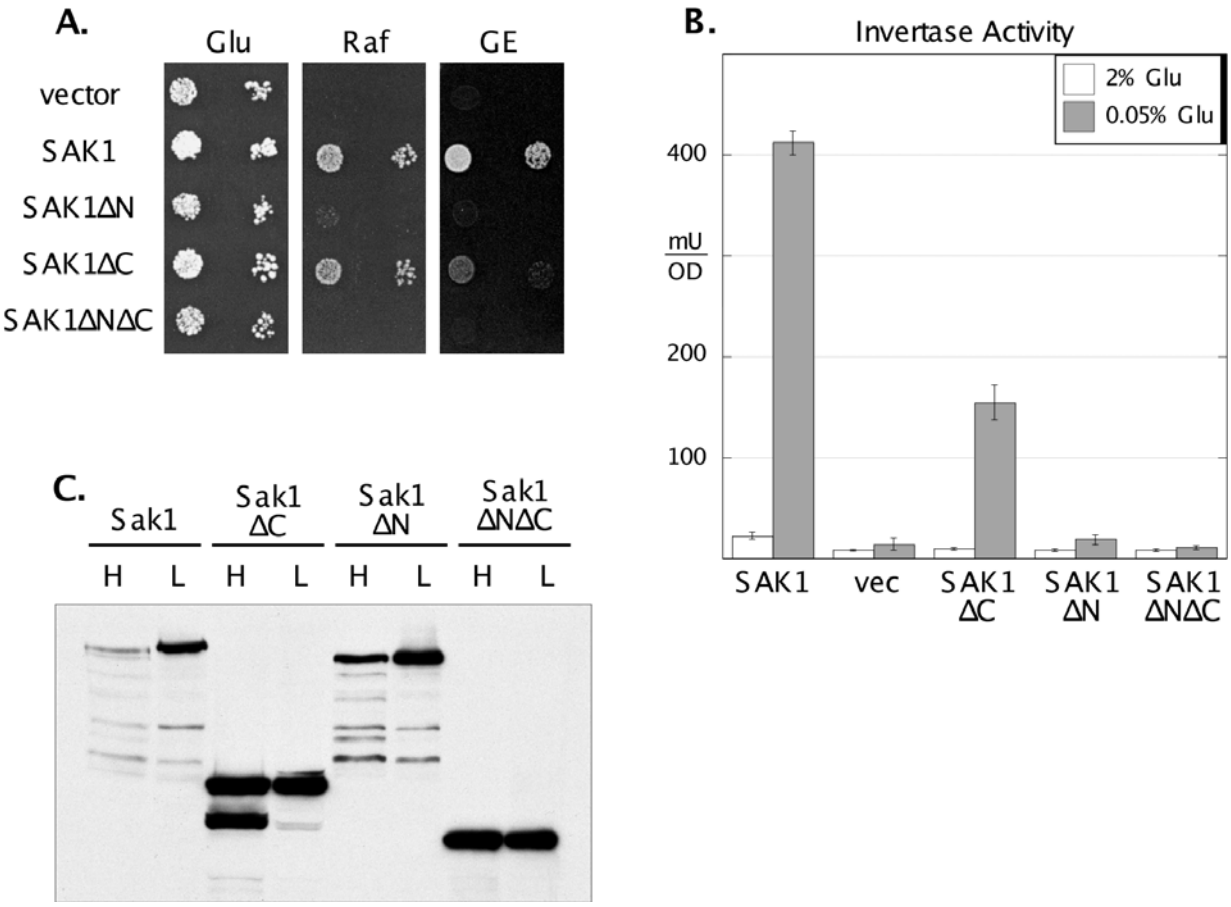

Figure 3.4 Growth phenotypes and invertase expression in cells lacking the N or C terminus of Sak1.

Cells lacking all three Snf1-activating kinases were transformed with low-copy-number plasmids expressing no kinase (vector), full-length Sak1 kinase, Sak1ΔN, Sak1ΔC, or Sak1ΔNΔC. **A** Serial dilutions of yeast cultures were spotted onto agar plates with glucose (Glu), raffinose (Raf), or a mixture of glycerol and ethanol (GE) as the carbon sources. **B** Invertase activity was assayed in cells grown in 2% glucose (Glu) or after shifting to 0.05% glucose for 3 h. The mean invertase activity level for three independent transformants is plotted. Error bars represent one standard error. **C** Western blot of V5-tagged Sak1 proteins. Protein extracts were prepared from cells grown in high glucose (H) or after 3 h in low glucose (L). Each construct contained five copies of the V5 epitope at its C terminus. vec, plasmid vector. mU/OD, μmole glucose/min/OD of cells assayed.

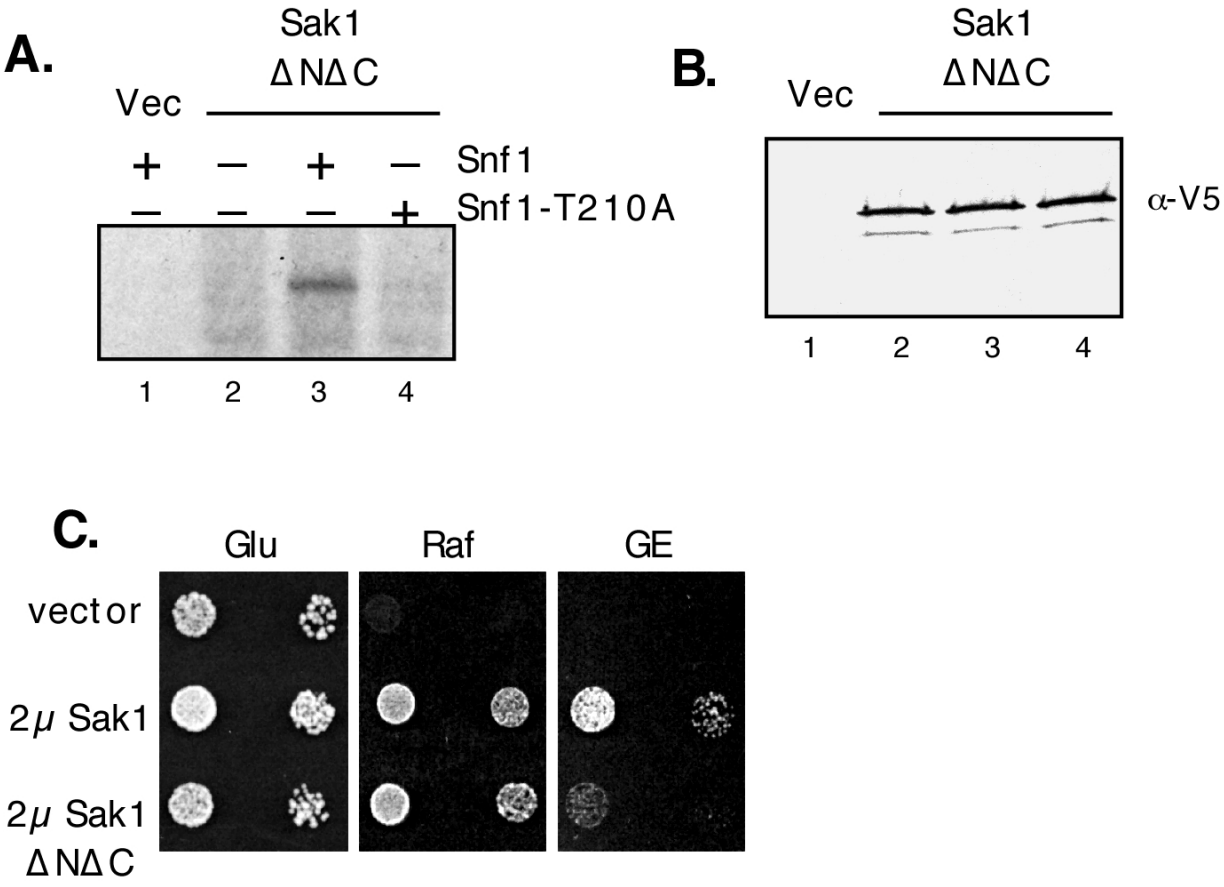

Figure 3.5 The Sak1 kinase domain is catalytically active.

A In vitro kinase assay. A *snf1*Δ *sak1*Δ *tos3*Δ *elm1*Δ yeast strain was transformed with a high-copy-number plasmid expressing V5-tagged Sak1ΔNΔC protein or with plasmid vector (Vec). Sak1ΔNΔC protein was immunoprecipitated with anti-V5 epitope beads and incubated with [γ-^{32}P]ATP either alone or with recombinant substrates purified from bacteria. The substrates tested were the GST-Snf1 kinase domain (lanes 1 and 3) and the GST-Snf1 kinase domain with the T210A mutation (lane 4). Labeled proteins were resolved on an SDS-polyacrylamide gel and detected by autoradiography. **B** Western blot of immunoprecipitated Sak1ΔNΔC. Protein extracts were immunoprecipitated as described for panel A and probed with anti-V5 antibody to detect levels of Sak1ΔNΔC protein. **C** A strain lacking all three Snf1-activating kinases (*sak1*Δ *tos3*Δ *elm1*Δ) was transformed with high-copy-number plasmids expressing no kinase (vector), full-length Sak1, or Sak1ΔNΔC. Serial dilutions of yeast cultures were spotted onto agar plates with glucose (Glu), raffinose (Raf), or a mixture of glycerol and ethanol (GE) as the carbon sources.

the Sak1 kinase domain is catalytically active and specific, despite the deletion of its N- and C-terminal domains.

Since the Sak1 kinase domain is active, we tested whether overexpression of the Sak1 kinase domain could activate Snf1 signaling. The Sak1 kinase domain (Sak1ΔNΔC) was overexpressed from the strong, constitutive *ADH1* promoter on a high-copy-number plasmid and assayed for its ability to activate Snf1 kinase. Serial dilutions of liquid cultures were spotted onto solid media containing glucose, raffinose, or a mixture of glycerol and ethanol as the carbon source. In spot dilution growth assays, we have found that aerobic growth requires a higher level of Snf1 signaling than does growth by fermentation of raffinose. Cells overexpressing full-length Sak1 were able to grow on raffinose and glycerol-ethanol, indicating robust Snf1 signaling (Figure 3.5c). In contrast, overexpression of the Sak1 kinase domain (Sak1ΔNΔC) provides a lesser degree of Snf1 signaling that is sufficient for fermentation of raffinose but not for efficient aerobic growth. Therefore, the Sak1 kinase domain is catalytically active *in vivo* but is partially defective. A similar result was obtained with Elm1, where deletion of the C terminus caused an impairment in the Elm1 signaling that could be suppressed by overexpression (Figure 3.3).

3.3.5 Localization of critical residues in the amino terminus of Sak1

Since the amino terminus of Sak1 is required for Sak1 function (Figure 3.4), we sought to identify the N-terminal residues critical for Sak1 function. Alignment of the N termini of the Snf1-activating kinases from *S. cerevisiae* with the N termini of homologous kinases from other fungal species identified two regions of partial sequence conservation (Figure 3.6). These two regions of conservation were deleted individually in two additional constructs and tested for their ability to promote Snf1 signaling. To accomplish this, Snf1 signaling outputs (invertase

Figure 3.6 Multiple sequence alignment of the N termini of the Snf1-activating kinases with fungal homologues.

The N termini of Snf1-activating kinases from *S. cerevisiae* (Sak1, Tos3, and Elm1) are aligned with the N termini of homologous proteins. Abbreviations and GenBank accession numbers of the other fungal sequences are given in the legend to Figure 3.1b. The demarcations between N1 and N2 and between N2 and the kinase domains are indicated. The yeast cyclin-dependent kinase Cdc28 lacks any N-terminal extension and is included in order to show the boundary of the conserved serine/threonine kinase domain.

induction and growth on alternative carbon sources) were assayed with cells expressing Sak1ΔN1 (lacking residues 3 to 86) or Sak1ΔN2 (lacking residues 87 to 129) as the only Snf1-activating kinase. When Sak1ΔN1 was expressed, the cells functioned normally by fermentation of raffinose and respiration of glycerol and ethanol (Figure 3.7a) and induced invertase in low glucose (Figure 3.7b). In contrast, Sak1ΔN2 failed to function in the Snf1 pathway, as demonstrated by the failure to induce invertase (Figure 3.7b) or to promote growth on raffinose or glycerol and ethanol (Figure 3.7a). All of the N-terminal-deletion constructs were expressed at equivalent levels *in vivo*, as judged by Western blotting (Figure 3.7c). We conclude that Sak1 residues 87 to 129 are essential for Sak1 function *in vivo*.

3.3.6 Effect of N- and C-terminal deletions on Sak1's ability to phosphorylate the Snf1 activation loop

Upon glucose limitation, Sak1, Tos3, and Elm1 activate Snf1 by phosphorylation of threonine 210 (T210) in the activation loop (92, 180). We wished to determine which of the Sak1 constructs developed for this study resulted in Snf1 T210 phosphorylation. Snf1-HA and the Sak1 variants were coexpressed in strains lacking the genomic copies of all three Snf1-activating kinases. Because these analyses are carried out in strains expressing Sak1 as the only upstream kinase, any observed phosphorylation of Snf1 can be confidently attributed to Sak1. Extracts were prepared from cells grown in high or low glucose. Snf1 was immunoprecipitated and probed for phosphorylation on threonine 210 by antibodies specific for the phosphorylated activation loop threonine (129). When glucose is limiting, Snf1 threonine 210 is phosphorylated in cells expressing full-length Sak1 (Figure 3.8a, lanes 1 and 2). When the carboxy terminus of

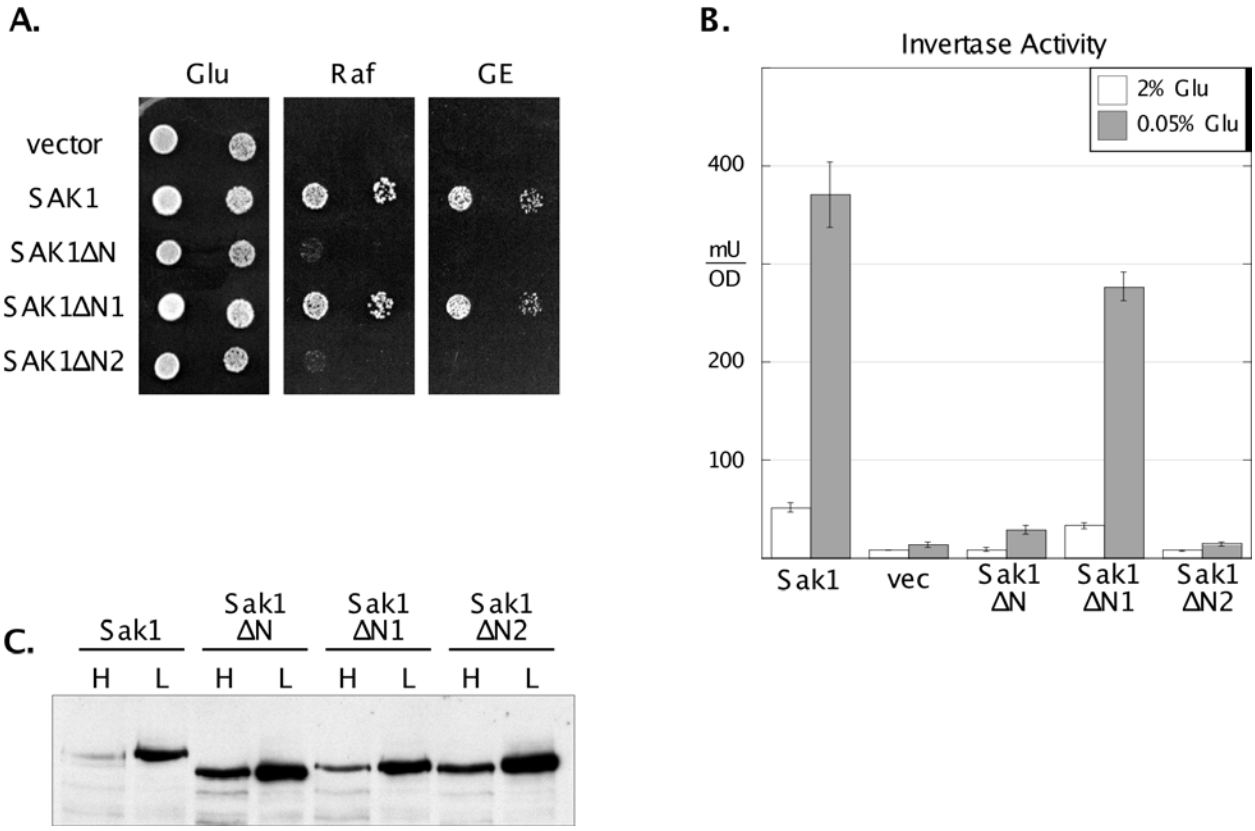

Figure 3.7 Identification of critical residues in the Sak1 amino terminus.

Cells lacking all three Snf1-activating kinases were transformed with a low-copy-number plasmid expressing an empty vector (vector), full-length Sak1, or Sak1 mutants lacking the entire N terminus (ΔN), the first half of the N terminus (ΔN1), or the second half of the N terminus (ΔN2). **A** Serial dilutions of liquid cultures were spotted onto media with glucose (Glu), raffinose (Raf), or a mixture of glycerol and ethanol (GE) as the carbon source. **B** Invertase activity was assayed in cells grown in 2% glucose or after shifting to 0.05% glucose for 3 h. The mean invertase activity level for three independent transformants is plotted. Error bars represent one standard error. mU/OD, μmole glucose/min/OD of cells assayed. **C** Western blot of V5-tagged Sak1 proteins. Extracts prepared from cells grown in high glucose (H) or after 3 h in low glucose (L) were analyzed directly by Western analysis with anti-V5 antibodies.

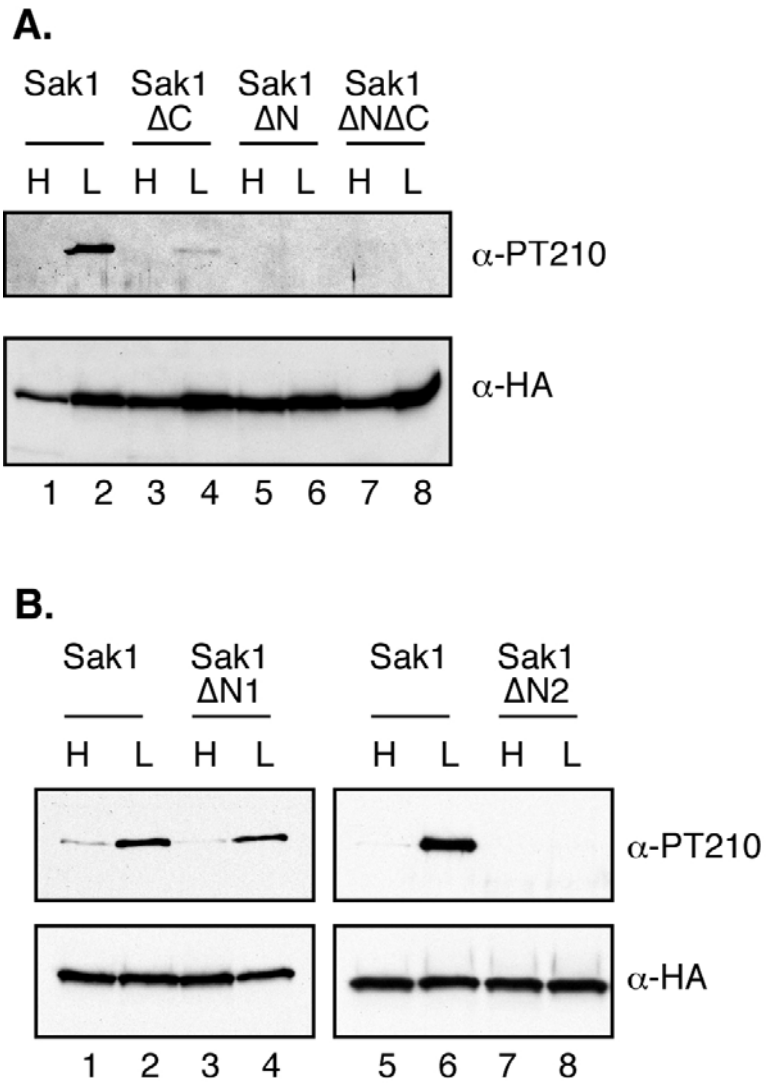

Figure 3.8 Snf1 activation loop phosphorylation in Sak1 mutant strains.

Cells lacking all three Snf1-activating kinases were cotransformed with two low-copy-number plasmids expressing Snf1-HA and full-length Sak1 or deletion mutants of Sak1, as indicated in the legend to Figure 3.4. Protein extracts were prepared from cells grown in medium containing high glucose (H) or after 3 h in low glucose (L). Snf1-HA was immunoprecipitated with anti-HA antibody and probed by Western blotting with antibodies directed against Snf1 phosphorylated on threonine 210 (α-PT210). Equivalent aliquots (20 μg) of each extract were also analyzed directly by Western analysis with anti-HA antibodies (α-HA).

Sak1 is deleted, Snf1 is still phosphorylated exclusively in low glucose, though the level of T210 phosphorylation is greatly reduced (lanes 3 and 4). Deletion of the N terminus of Sak1 completely blocks Snf1 phosphorylation in either high or low glucose (lanes 5 and 6). Similarly, in cells expressing the Sak1 kinase domain alone (Sak1ΔNΔC), Snf1 is not phosphorylated under either condition tested (lanes 7 and 8). Interestingly, this same construct was able to specifically phosphorylate the activation loop of Snf1 *in vitro* (Figure 3.5). This analysis was extended to include the smaller deletions within the Sak1 N terminus. Deletion of the first half of the N terminus (N1) did not adversely affect the ability of Sak1 to phosphorylate the activation loop threonine of Snf1, while deletion of the second half of the N terminus (N2) completely blocked the ability of Sak1 to phosphorylate the activation loop of Snf1 (Figure 3.8b). Equivalent levels of Snf1 protein are expressed in all the samples, as judged by Western blotting with anti-HA antibody (Figure 3.8, lower panels). We conclude that the ability of the Sak1 kinase domain to phosphorylate the activation loop of Snf1 *in vivo* requires regulatory domains in addition to its kinase domain.

3.4 DISCUSSION

The Snf1-activating kinases of *S. cerevisiae* contain conserved kinase domains as well as large domains that contain only limited sequence conservation (Figure 3.1a). In this report, we investigated whether these N-terminal and C-terminal domains of the Snf1-activating kinases are required for their biological function *in vivo*. Our results indicate that the C-terminal domains play an important role in determining pathway specificity. Deletion of the C termini of Sak1 and Tos3 greatly reduced their ability to activate Snf1 signaling. C-terminal deletions of Sak1 and

Tos3 showed impaired aerobic growth (Figure 3.1c), impaired invertase induction (Figure 3.2), and, in the case of Sak1, impaired phosphorylation of the Snf1 activation loop (Figure 3.8). All these results imply that the C termini of Sak1 and Tos3 are needed for efficient signaling in the Snf1 pathway. In contrast, deletion of the C terminus of Elm1 had no effect on aerobic growth, had only a modest effect on invertase induction, and caused a complete abrogation of its ability to function in the morphogenetic-checkpoint signaling pathway. Thus, the C termini of Sak1 and Tos3 direct them to the Snf1 pathway, while the C terminus of Elm1 directs it to a different signaling pathway.

In a screen to identify yeast proteins that phosphorylate mammalian AMPK *in vitro*, Sutherland et al. identified a carboxy-truncated Elm1 similar to what we have here designated Elm1ΔC (180). In that study, the truncated Elm1 variant activated Snf1 more robustly than did full-length Elm1 but was unable to complement an *elm1*Δ strain's morphological defect unless Snf1 was also deleted. These data support the notion that the C-terminal domains play an important role in pathway participation. Pathway selection could be specified by subcellular localization or various affinities for components of the signaling pathways in which the kinase participates. It is possible that full-length Elm1 might have a higher affinity for morphology signaling components than for Snf1, whereas Elm1ΔC associates better with Snf1 than with proteins at the bud neck. This may explain why a high-copy-number but not a low-copy-number plasmid expressing Elm1ΔC rescues cells from the elongated-morphology characteristic of *elm1*Δ mutants (Figure 3.3). The Tos3 protein kinase is localized in the cytoplasm in high and low glucose (108), while Sak1 appears to be localized in the cytoplasm in high glucose and at the vacuolar periphery in low glucose (84). Whether the C-terminal domains of Sak1 and Tos3

specify signaling through Snf1 by controlling its localization or its association with other proteins remains to be determined.

Finally, we identified a small region in the N terminus of Sak1 that is absolutely required for participation in the Snf1 signaling pathway. When Sak1 is expressed at normal levels, the deletion of amino acids 87 to 129 of Sak1 completely blocks its ability to activate Snf1, as judged by growth assays (Figure 3.7a), invertase induction (Figure 3.7b), and Snf1 activation loop phosphorylation (Figure 3.8b). A small domain of Sak1 included in the N2 deletion (residues 110 to 129) is conserved with other homologous fungal kinases, including Tos3, but not with Elm1. This region is not required for the catalytic activity of Sak1 since a construct consisting solely of the Sak1 kinase domain (residues 130 to 500) is catalytically active *in vitro* and *in vivo* (Figure 3.5). Therefore, this small domain of Sak1 must play some critical regulatory role that remains to be elucidated by further studies.

3.5 ACKNOWLEDGMENTS AND CONTRIBUTIONS

This work was supported by National Institutes of Health grant GM46443 (to MCS) and American Heart Association Predoctoral Fellowship 0615379U (to EMR). We thank Simon Watkins and the Center for Biological Imaging at the University of Pittsburgh School of Medicine for help with microscopy.

EMR performed the experiments displayed in Figures 3.1c, 3.2, 3.4a, 3.4b, 3.5, 3.7a, and 3.7b. RRM performed the experiments displayed in Figures 3.1d, 3.4c, 3.7c, and 3.8. MCS performed the experiment displayed in Figure 3.3. EMR and MCS collaborated on research design and manuscript preparation.

4.0 ACCESS DENIED: SNF1 ACTIVATION LOOP PHOSPHORYLATION IS CONTROLLED BY AVAILABILITY OF THE PHOSPHORYLATED THREONINE 210 TO THE PP1 PHOSPHATASE

Eric M. Rubenstein, Rhonda R. McCartney, Chao Zhang, Kevan M. Shokat, Margaret K. Shirra, Karen M. Arndt, and Martin C. Schmidt (2008).

Journal of Biological Chemistry, 283, 222-230.

4.1 INTRODUCTION

The Snf1 protein kinase of *Saccharomyces cerevisiae* is a founding member of a family of protein kinases that includes the mammalian AMP-activated protein kinase (AMPK) (77) and is present in all eukaryotes. Interest in this family has been increased by the finding that the medications used to treat type 2 diabetes activate AMPK (80). Indeed, the metabolic consequences of AMPK activation, which include increased glucose uptake and oxidation, increased fatty acid oxidation, inhibition of anabolic reactions, and stimulation of reactions that regenerate ATP (208), are beneficial to patients experiencing hyperglycemia. With the rise in the prevalence of obesity and type 2 diabetes in Western cultures, a more complete understanding of the mechanisms that regulate the activity of the AMPK/Snf1 enzymes is needed.

The AMPK/Snf1 enzymes function as heterotrimers with a catalytic α subunit associated with regulatory β and γ subunits. The α subunit contains a canonical kinase domain in its N terminus and an autoinhibitory domain in its C terminus (40). The catalytic activities of the AMPK/Snf1 enzymes are regulated in a complex manner. First, subunit interactions within the AMPK/Snf1 heterotrimer regulate enzymatic activity in response to the cellular energy status. Although not all agree on the mechanism, the γ subunit appears to play an important role in the regulation of AMPK/Snf1 catalytic activity. The mammalian γ subunit can bind AMP (165) and can interact with the α subunit autoinhibitory domain to abrogate its inhibitory potential (40, 103). An alternative but not necessarily exclusive model posits the presence of a pseudosubstrate sequence in the γ subunit (165). Second, AMPK/Snf1 kinases require phosphorylation of a

conserved threonine residue in their activation loops by a distinct upstream kinase (60, 79, 129). Concerted effort by several research groups led to the identification of the activating kinases for Snf1 and AMPK (78, 92, 138, 180, 203). Yeasts encode three Snf1-activating kinases (SAKs), Sak1, Tos3, and Elm1 (92, 138, 180), and all three SAKs must be deleted to block Snf1 signaling. In mammalian cells, LKB1 is the primary activating kinase of AMPK under conditions of energy stress (119, 203), although other kinases may contribute to AMPK activation in specific cell types or in response to other stimuli (81, 98, 131, 202).

It was hoped that the identification of the activating kinases for Snf1 and AMPK would rapidly lead to an understanding of the means by which cellular energy status controlled the activity of the AMPK/Snf1 pathway. However, several lines of evidence suggest that the AMPK/Snf1-activating kinases are not themselves regulated by energy status. First, LKB1, the primary activator of AMPK (78, 203), is the activation loop kinase for at least 12 other kinases that are responsive to different stimuli (119). If LKB1 were in fact regulated by cellular energy status, one would predict increased activity toward all of its substrates under conditions of nutrient limitation. This is not observed. Furthermore, when LKB1 was expressed in yeast in place of the SAKs, Snf1 activation loop phosphorylation responded normally to changes in glucose levels (93). Either LKB1 could sense energy status in both yeast and mammalian cells or the phosphorylation status of the Snf1 activation loop was not regulated at the level of phosphate addition. Third, reactions with purified components showed that the addition of AMP did not stimulate LKB1-mediated phosphorylation of AMPK but rather inhibited its dephosphorylation (159, 179). Fourth, mutations in the γ subunit of AMPK affect the ability of the PP2C phosphatase to dephosphorylate the AMPK activation loop (159). Finally, one of the three SAKs, Elm1, is required for normal cell morphology (20, 177). Cells lacking Elm1 display the

characteristic elongated morphology for which Elm1 was named. Since yeast cells display normal morphology in both glucose-rich and glucose-limited media, the Elm1 kinase must be active in both growth conditions. However, the Snf1 kinase activation loop shows a much greater degree of phosphorylation when cells are grown in limiting glucose conditions (129). The fact that Snf1 is not phosphorylated in glucose-rich media even when Elm1 is active suggests that the phosphorylation status of the Snf1 activation loop might not be regulated by phosphate addition, but rather by dephosphorylation. All of these data taken together suggest that the dephosphorylation reaction may be the regulated step in determining the phosphorylation status of the AMPK/Snf1 activation loop.

Genetic studies have long indicated that the dephosphorylation of the Snf1 activation loop was mediated by the yeast PP1 protein phosphatase Glc7 (188). Deletion of the *GLC7* gene is lethal, thus complicating study of the PP1 phosphatase in yeast. Glc7 can bind to several alternative regulatory subunits that are thought to direct the phosphatase to different substrates (205). The accessory subunit that directs Glc7 to the Snf1 signaling pathway is Reg1 (187). Deletion of the *REG1* gene results in constitutive activation of Snf1 and hyper-phosphorylation of its activation loop (129). Models for the AMPK/Snf1 pathway in which dephosphorylation is the key to regulation have been lacking, perhaps because the laboratories studying AMPK and Snf1 (ours included) tend to have a kinase-centric worldview. The experiments in this report are designed to directly test whether the SAKs, the deactivating phosphatase, or both are regulated by glucose.

4.2 MATERIALS AND METHODS

4.2.1 Yeast strains and media

Strains used in this study are presented in Table 4.1. Yeast were grown at 30°C in standard media. Glucose was present at 2 or 0.05% (g/100 ml), as indicated in the relevant figure legends. Raffinose medium contained 2% (g/100 ml) raffinose, 0.05% (g/100 ml) glucose, and 1 μg/ml antimycin A. In order to create a complete deletion of the *REG1* gene, a *reg1*Δ::*HIS3* allele was created using the *HIS3* gene from pRS403 (1622-bp SspI fragment) flanked by the sequences from the *REG1* locus. The upstream sequences from *REG1* were the 824-bp EcoRI-BsaAI fragment representing nucleotides –953 to –129 relative to the *REG1* ATG codon. The downstream sequences from *REG1* were the 237-bp BsaAI-XbaI fragment representing sequences 143–380 downstream from the *REG1* stop codon. *REG1* gene replacement was confirmed by PCR.

4.2.2 Plasmids and mutagenesis

All Snf1-activating kinases were expressed from their own cognate promoters, tagged with five copies of the V5 epitope at the C terminus, and introduced to cells on low-copy plasmids based on pRS315 (171). Point mutations in the ATP-binding pockets of Sak1, Tos3, and Elm1 were introduced with the Stratagene QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). TAP-tagged versions of the three SAKs have been described previously (54). A low-copy plasmid expressing the Mig1 protein tagged with the HA epitope at the C terminus has been previously described (161).

Table 4.1 *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype
FY1193	*MATa ura3Δ52 leu2Δ1 trp1Δ63 his3Δ200 snf1Δ10*
MSY857	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN*
MSY923	*MATa ura3 leu2 his3 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN snf1Δ10*
MSY951	*MATa ura3 leu2 his3 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN SNF1-3HA*
MSY955	*MATa ura3Δ52 leu2Δ1 his3Δ200 SNF1-3HA*
MSY956	*MATa ura3 leu2 trp1Δ63 his3 lys2Δ0 sak1Δ::KAN tos3Δ::KAN SNF1-3HA*
MSY958	*MATa ura3 leu2 trp1Δ63 his3 sak1Δ::KAN elm1Δ::KAN SNF1-3HA*
MSY961	*MATa ura3 leu2 his3 tos3Δ::KAN elm1Δ::KAN SNF1-3HA*
MSY963	*MATa ura3Δ52 leu2Δ1 his3Δ200 trp1Δ63 SNF1-3HA reg1Δ::HIS3*
MSY964	*MATa ura3 leu2 trp1Δ63 his3 lys2Δ0 sak1Δ::KAN tos3Δ::KAN SNF1-HA reg1Δ::HIS3*
MSY965	*MATa ura3 leu2 trp1Δ63 his3 sak1Δ::KAN elm1Δ::KAN SNF1-3HA reg1Δ::HIS3*
MSY966	*MATa ura3 leu2 his3 sak1Δ::KAN tos3Δ::KAN elm1Δ::KAN SNF1-3HA reg*1Δ*::HIS3*
MSY983	*MATa ura3 leu2 his3 tos3Δ::KAN elm1Δ::KAN SNF1-3HA reg1Δ::HIS3*
MSY987	*MATa ura3Δ52 leu2Δ1 trp1Δ63 his3Δ200 snf1Δ10 reg1Δ::HIS3*

4.2.3 Screen for altered-sensitivity alleles

Overnight saturated yeast cultures (200 µl) grown at 30°C were suspended in 3 ml of 47°C top agar (containing 0.07% (g/100 ml) Bacto Agar, synthetic complete media lacking uracil with 2% (g/100 ml) sucrose, 1 µg/ml antimycin A, and 0.01% (g/100 ml) glucose). The mixture was carefully mixed and poured onto agar plates containing synthetic complete media lacking uracil with 2% (g/100 ml) sucrose, 0.01% (g/100 ml) glucose, and 1 µg/ml antimycin A. The top agar mixture was allowed to cool and harden. Sterile filter discs were gently placed on the surface of the top agar. 3 µl of 2.5 or 25 µM candidate inhibitor drugs (in DMSO) were applied to the filter discs. DMSO alone was used as a negative control. Plates were incubated at 30°C overnight and

photographed. The design and use of the altered sensitivity allele of Snf1 will be described in detail elsewhere (168).

4.2.4 Invertase assays

Invertase activity of midlog cells grown in high and low glucose was determined quantitatively using a colorimetric assay coupled to glucose oxidase (72). Three independent cultures were assayed, and the mean and standard error values are plotted. The units of invertase activity used were milliunits/OD where one unit equals 1 μmole of glucose released/min.

4.2.5 Western blotting

Protein extracts were prepared using the NaOH cell lysis method as described previously (129). To detect Snf1 activation loop threonine 210 (T210) phosphorylation, 600–1200 μg of protein was immunoprecipitated in radioimmunoprecipitation buffer as previously described (129). Rabbit polyclonal antibody directed against phosphorylated Snf1 T210 was used as the primary antibody (1:1000) to detect phosphorylated Snf1. Horseradish peroxidase-conjugated goat anti-rabbit antibody was used as secondary antibody (1:15,000) (Santa Cruz Biotechnology, Santa Cruz, CA). For Snf1-HA detection, 20–40 μg of protein extract was used. Snf1-HA was detected by a horseradish peroxidase-conjugated antibody (1:1000) recognizing the HA epitope (Santa Cruz Biotechnology).

4.2.6 Immune complex kinase assay

Protein extracts were prepared from cells expressing a single SAK as a C-terminal TAP fusion (54) or without any SAK. SAKs were collected from 300 μg of protein extract by immunoprecipitation with IgG beads. Precipitates were washed five times in radioimmunoprecipitation buffer and two times in kinase assay buffer as described (54). Precipitates were then incubated in 20 μl of kinase assay buffer with 0.2 mM [γ-^{32}P]ATP (1000 cpm/pmole) and purified recombinant Snf1 kinase domain as the substrate. Reactions were incubated for 1 h at 30°C and then subjected to SDS-PAGE and autoradiography.

4.3 RESULTS

4.3.1 Snf1-activating kinases are active in high and low glucose

If the phosphorylation status of the Snf1 activation loop were regulated by phosphate addition, then one would predict that the SAKs would be inactive in high glucose and activated by low glucose. We have described above several lines of evidence that suggest the opposite, that the SAKs may be active in both high and low glucose. Three different experiments were conducted to assess whether glucose regulates the activity of one or more of the SAKs. First, we measured invertase activity in cells expressing a single SAK with and without the PP1 regulatory subunit Reg1. When Reg1 is absent, Snf1-dependent invertase expression is elevated in both high and low glucose (187), and the Snf1 activation loop becomes phosphorylated in high glucose (129). Consistent with prior observations, strains expressing Reg1 and all three SAKs exhibit normal,

glucose-regulated invertase induction (Figure 4.1). In the absence of Reg1, cells with all three SAKs display elevated invertase activity independent of glucose, indicating that one or more of the SAKs is active in high glucose. In the absence of all three SAKs, invertase activity is minimal regardless of the glucose concentration or the presence or absence of Reg1, confirming previous work indicating that Sak1, Tos3, and Elm1 represent the complete complement of SAKs (92, 180). In order to determine if any of the SAKs were responsive to changes in glucose levels, Sak1, Tos3, and Elm1 were each expressed as the only SAK in the presence and absence of Reg1. In the absence of Reg1, invertase is elevated in both high and low glucose regardless of which SAK is present (Figure 4.1), indicating that each of the three SAKs is capable of activating Snf1 in high glucose. When Reg1 is present, glucose regulation of invertase activity is restored. These results support the idea that the SAKs are active regardless of the glucose concentration.

Earlier work has suggested that Sak1 is the primary SAK. Of the three SAKs, only Sak1 is found in stable complex with Snf1 (54). Sak1 most efficiently activates all three β subunit-specific isoforms of Snf1 under multiple conditions (128) and is the only SAK that can promote nuclear localization of the Gal83 isoform of Snf1 (84). Consistent with these data, Sak1 most robustly induces invertase activity, particularly in the absence of Reg1 (Figure 4.1). The reduced induction of invertase in cells expressing Elm1 as the only SAK may be a reflection of sequestration of Elm1 at the bud-neck for its role in regulating cell cycle and morphogenesis (20).

A second measure of SAK activity is the measurement of activation loop phosphorylation using a phosphopeptide antibody specific for Snf1 phosphorylation on the activation loop threonine 210 (PT210) (129). In this experiment, cells were grown in high glucose in the presence or absence of Reg1, protein extracts were prepared, and total Snf1 and T210 phosphorylation were determined by Western blotting with either HA antibodies (Figure 4.2,

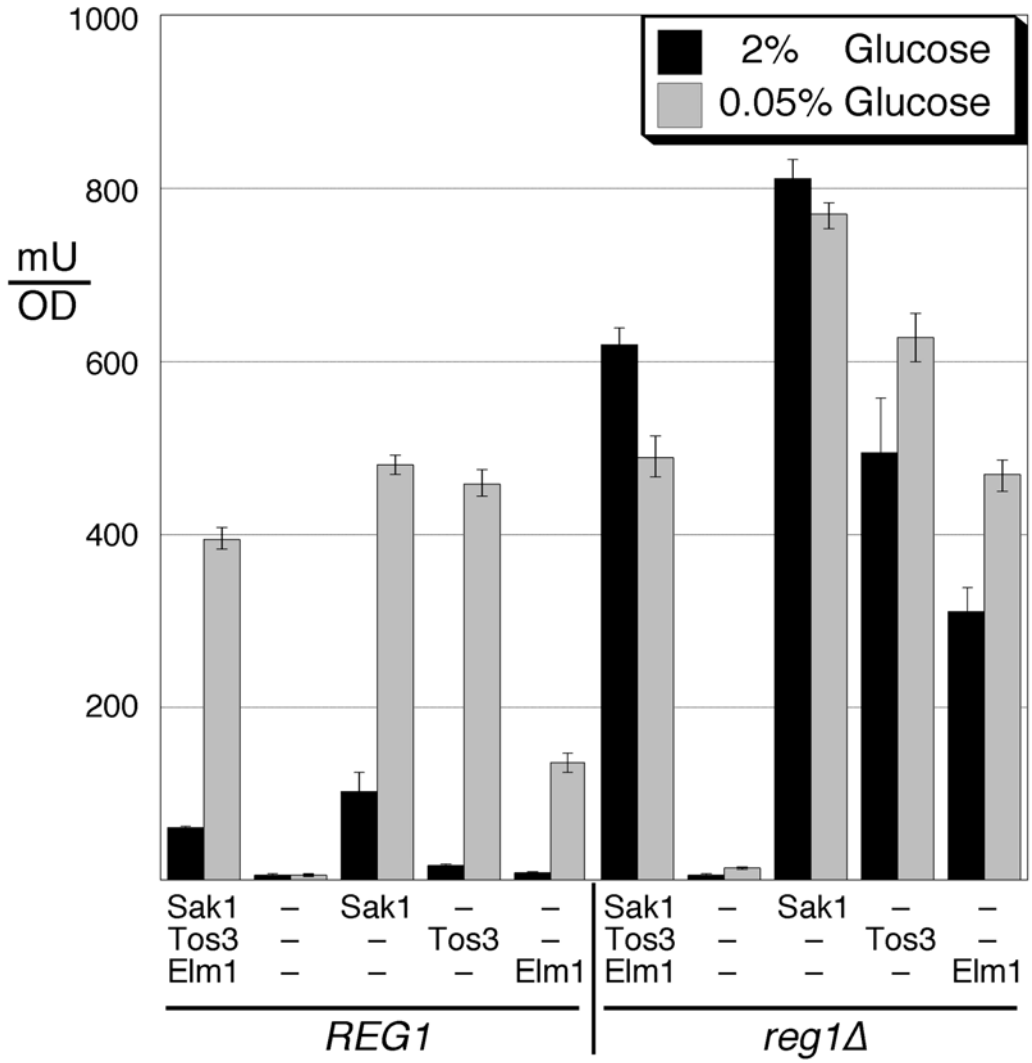

Figure 4.1 Snf1-activating kinases are active in high and low glucose.

Invertase activity was measured in cells containing all three, none, or only one of the SAKs in the presence or absence of *REG1* as indicated. Aliquots were assayed from cells grown in high glucose (2%) or after shifting to low glucose (0.05%) for 2 h. The mean value from three independent cultures is plotted with the error bars indicating one standard error.

bottom) or PT210 antibodies (top), respectively. In multiple independent experiments, the presence or absence of Reg1 affected the phosphorylation status of the Snf1 activation loop but did not affect the level of total Snf1 protein. Results from a representative experiment are shown in Figure 4.2. In wild-type cells, phosphorylation of the Snf1 activation loop is regulated in response to changes in glucose levels (lanes 1 and 2). In high glucose, cells expressing all three SAKs show low but detectable T210 phosphorylation when Reg1 is present and distinctly elevated phosphorylation when Reg1 is absent (lanes 3 and 4). In the absence of all three SAKs, no phosphorylation of T210 is detected, regardless of whether Reg1 is present or not (lanes 5 and 6). When the SAKs are expressed individually, the level of T210 phosphorylation is increased by the absence of Reg1, regardless of which SAK is present (lanes 7–12). Some variation is observed between the individual SAKs. Sak1 and Elm1 show higher basal phosphorylation (lanes 7 and 11) compared with Tos3 (lane 9). Nonetheless, the conclusion we reach is that the SAKs are capable of phosphorylating Snf1 in high glucose, supporting the idea that the SAKs are active in both high and low glucose conditions. However, these two experiments do not exclude the possibility that the Glc7-Reg1 complex regulates the SAKs. Therefore, our next experiment was designed to measure SAK activity without disrupting the Glc7-Reg1 complex.

Previously, we have used the tandem affinity purification protocol to isolate the three SAKs, Sak1, Tos3, and Elm1 (54). In that study, we also showed that the Sak1 complex constituents and activity were not affected by carbon source (glucose versus sucrose). The activity of Tos3 and Elm1 prepared from different carbon sources was not measured. In the third experiment to measure the activity of the SAKs, we conducted *in vitro* kinase assays with each SAK isolated from cells grown in high glucose or 2 h after a shift to low glucose. The SAKs were collected by immunoprecipitation and assayed for the ability to phosphorylate purified

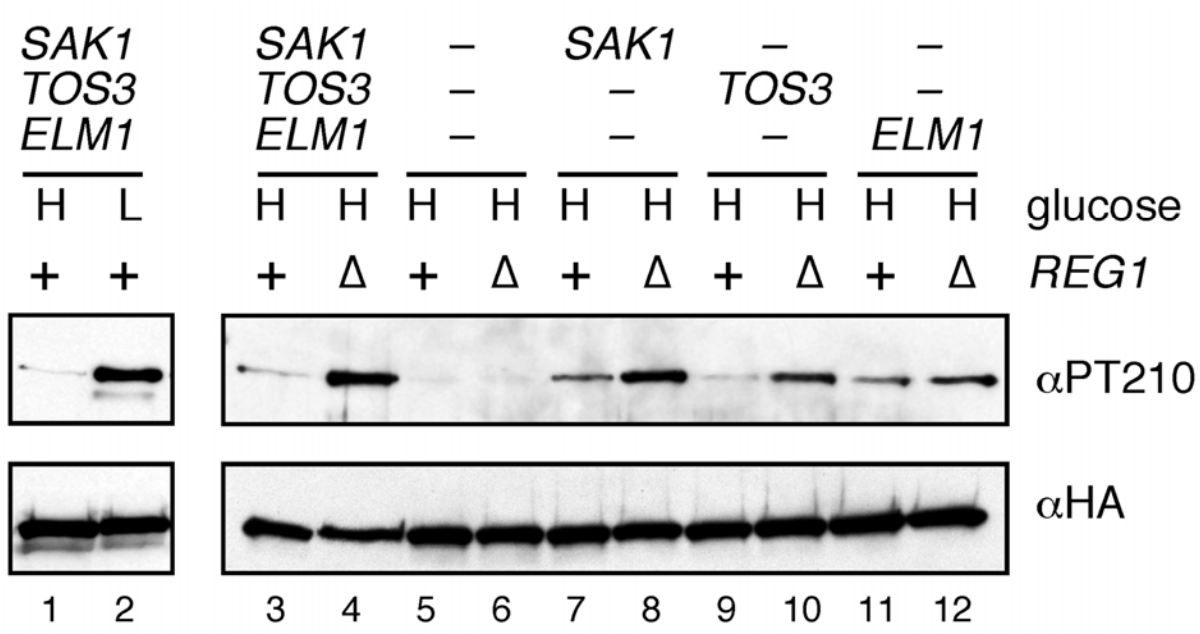

Figure 4.2 Phosphorylation of the Snf1 kinase activation loop in *reg1*Δ cells.

Western blot of Snf1 protein using antibodies directed against the phosphorylated form of the Snf1 activation loop (αPT210) or against the HA epitope. Cells expressing all three, one, or none of the SAKs (as shown) were grown in high (H) or low (L) glucose. Cells contained the wild-type *REG1* gene (+) or a complete deletion of the *REG1* (Δ) as indicated.

recombinant Snf1 kinase domain (Figure 4.3). In earlier experiments, we have shown that the SAKs phosphorylate the Snf1 kinase domain exclusively on threonine 210 (54). In order to be certain that we were in the linear range of the assay, immune complexes were collected from increasing quantities of extract (0–300 μg). The reaction depended on exogenous substrate, since no incorporation was observed when recombinant Snf1 kinase domain was omitted from the reaction. For each SAK, a similar level of activity was observed in reactions using extracts prepared from cells grown in high and low glucose. The first three experiments described here all support the idea that the SAKs are active in both high and low glucose. Since the phosphorylation status of the Snf1 activation loop is regulated by glucose, the simplest model remaining is that the dephosphorylation of Snf1 is regulated by glucose.

4.3.2 Identification of an altered-sensitivity allele of *TOS3*

To monitor the rate of Snf1 activation loop dephosphorylation, we sought to develop a means to rapidly and selectively inactivate the SAKs. In the absence of *de novo* phosphate addition, changes in Snf1 phosphorylation status may be attributed to the phosphatase, enabling us to determine if the activity of Glc7-Reg1 is regulated by changes in glucose concentration. A chemical genetic method has been developed for specifically inactivating protein kinases (16). By changing the "gatekeeper" residue in the ATP-binding pocket from a bulky hydrophobic residue to glycine or alanine, kinases can be engineered that are selectively inhibited by adenine analogues. An analogue-sensitive variant of the SAK Elm1 (threonine 200 mutated to glycine) has been reported (176). Because the primary role of Elm1 seems not to be Snf1 activation but rather participation in cell morphology signaling, we sought to identify analogue-sensitive alleles of the other SAKs, Sak1 and Tos3, whose only described role is activation of Snf1.

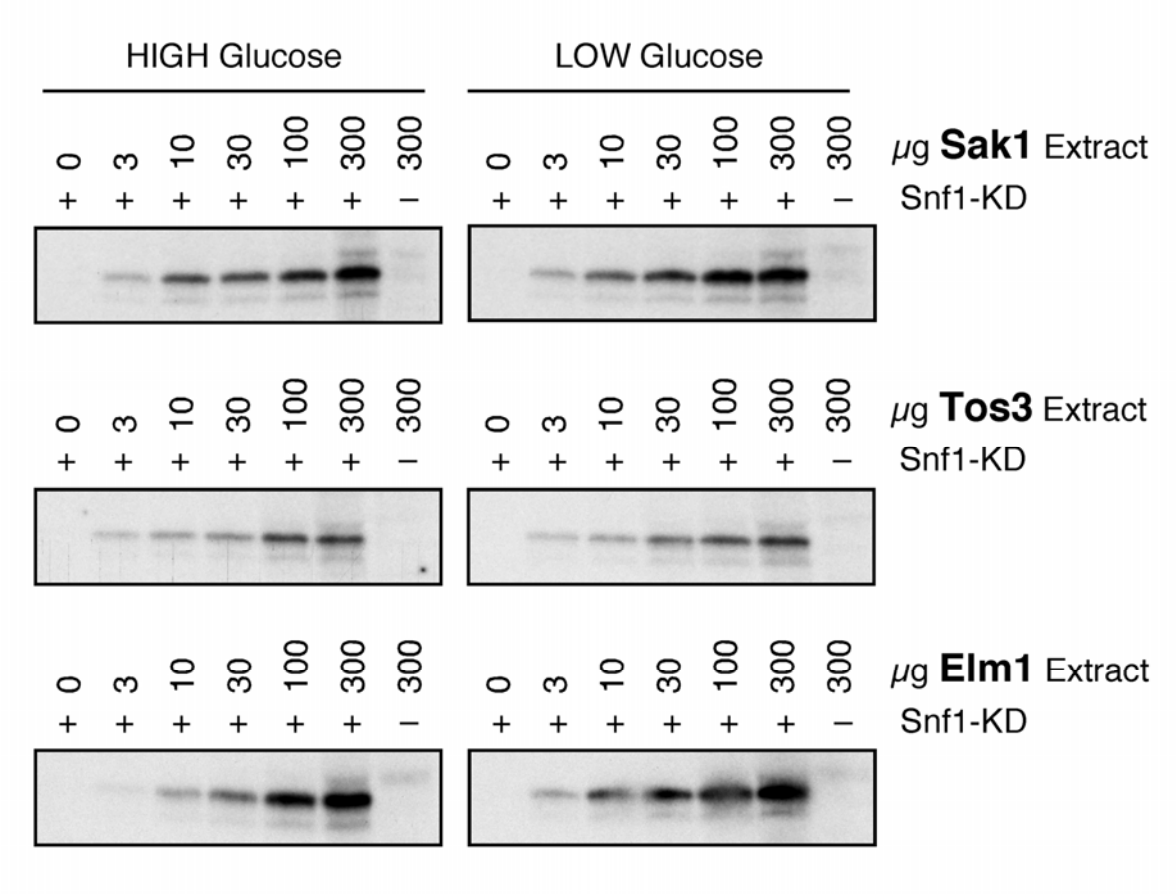

Figure 4.3 Activity of Snf1-activating kinases is independent of glucose.

In vitro kinase assays were performed with SAKs immunoprecipitated from protein extracts of cells grown in either high or low glucose. Cells expressed a single, TAP-tagged SAK (as shown). Precipitates were incubated in the presence of [γ-^{32}P]ATP with (+) or without (-) the addition of recombinant Snf1 kinase domain (Snf1-KD) as a substrate. Activity present in a titration from 0 to 300 μg of protein extract is shown.

The gatekeeper residues of Sak1 (leucine 223) and Tos3 (leucine 135) were changed to glycine and alanine. Plasmids encoding these kinase variants were introduced to cells that lacked the genes for all three SAKs. A library of adenine analogues was screened for the ability to block Snf1 signaling in cells expressing analogue-sensitive variants of the SAKs without affecting signaling in cells expressing the wild-type kinase. All attempts to identify a specific inhibitor for the altered sensitivity alleles of Sak1 were unsuccessful. However, we were successful with Tos3, using the Tos3-L135G allele in combination with the adenine analogue CZ22 (Figure 4.4). In a screen to identify specific inhibitors for our mutant kinases, cells were grown as a lawn with sucrose as the carbon source and antimycin A to prevent aerobic metabolism. Under these conditions, Snf1 signaling is required for growth. Adenine analogues suspended in DMSO were spotted onto filter discs. Formation of a halo around the disc was indicative of compromised Snf1 signaling, an outcome expected when the only SAK present in cells is inhibited. When cells were exposed to CZ22, a halo of growth inhibition was observed when Tos3-L135G was the only SAK. No halo was observed with cells expressing wild-type Tos3 (Figure 4.4a). Therefore, the growth inhibition by CZ22 is specific to the sensitized allele of *TOS3*.

In order to determine the effective concentration of CZ22, induction of the enzyme invertase was measured in cells grown in high glucose and shifted to low glucose as an indicator of Snf1 activation by Tos3-L135G or wild-type Tos3 (Figure 4.4b). Cells expressing the wild-type or sensitized Tos3 kinase as the only SAK were initially grown in glucose-rich media. CZ22 was included in the media at the shift to low glucose at concentrations of 0 (DMSO only), 1, 2.5, or 25 μM. In the absence of CZ22, cells expressing either the wild-type Tos3 or the sensitized Tos3-L135G show robust invertase induction upon shifting to low glucose, indicating that the Tos3-L135G allele is fully functional. When CZ22 is included in the reactions at 1 and 2.5 μM,

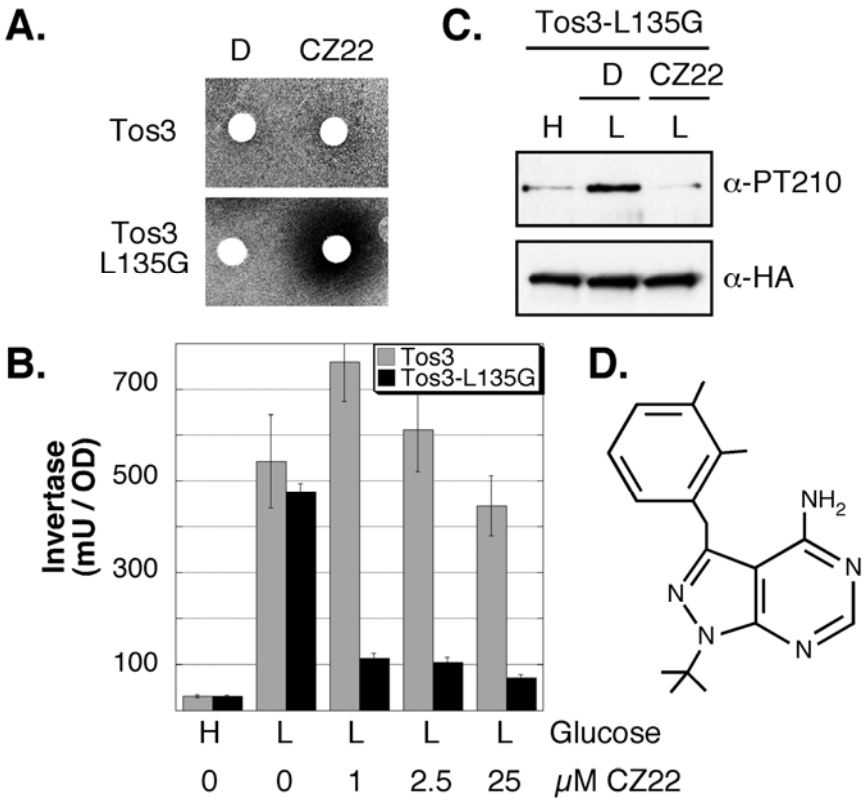

Figure 4.4 Analogue-sensitive allele of the Snf1-activating kinase Tos3.

A Cells expressing either Tos3 or Tos3-L135G as the only SAK were mixed with top agar and overlaid onto a plate containing sucrose as the carbon source. Sterile discs were spotted with either DMSO or the adenosine analogue CZ22 dissolved in DMSO. **B** Invertase assay. Cells expressing either the wild-type Tos3 or the analogue-sensitive Tos3-L135G as the only SAK were assayed for invertase induction after growth in high glucose (H) or following 2 h of incubation in media containing 0.05% glucose (L). The adenosine analogue CZ22 was added to the final concentration indicated. Mean values of invertase activity from three independent cultures are plotted with the error bars signifying one standard error. **C** CZ22 blocks Snf1 activation loop phosphorylation. Cells expressing Tos3-L135G as the only SAK were grown in high glucose (H) or shifted to low glucose (L) in the presence of added DMSO or 2.5μM CZ22 dissolved in DMSO. Total and activated Snf1 protein was assessed by Western blotting with anti-HA antibodies (α-HA) or the phosphopeptide antibody specific for the phosphorylated activation loop of Snf1 (α-PT210). Representative Western blots are shown. **D** Structure of the adenosine analogue, CZ22.

cells expressing wild-type Tos3 exhibited no defect in the induction of invertase. In contrast, significant inhibition (85%) of invertase induction was observed in cells expressing Tos3-L135G exposed to the same analogue concentration. Increasing the concentration of CZ22 did not produce a significant improvement in the degree of inhibition. All subsequent experiments with Tos3-L135G used 2.5 μM CZ22.

We observed a subtle but reproducible stimulation of invertase activity when cells expressing wild-type Tos3 were subjected to glucose limitation in the presence of 1 μM CZ22 (Figure 4.4b). The mechanism by which this occurs is not clear. However, the purpose of these experiments was to generate a tool with which we could rapidly and efficiently shut off SAK-to-Snf1 signaling. Here we have generated a variant of Tos3 that functions like the wild-type kinase but can be efficiently inhibited by the addition of low concentrations of CZ22.

We next directly analyzed the capacity of CZ22 to prevent Tos3-L135G-mediated phosphorylation of Snf1 (Figure 4.4c). Cells expressing analogue-sensitive Tos3 as the only SAK were grown in glucose-rich media and shifted to low glucose in the presence of DMSO or 2.5 μM CZ22. The phosphorylation state of the Snf1 activation loop as well as the level of total Snf1 protein were assessed by Western blotting. Glucose-grown cells exhibit low-level basal phosphorylation. As previously reported, Snf1 becomes phosphorylated when cells are shifted to low glucose media for 2 h in the absence of CZ22 (129). When CZ22 is included at the shift to low glucose, however, Snf1 never becomes phosphorylated beyond basal levels, clearly demonstrating that CZ22 inhibits the activity of Tos3-L135G toward the Snf1 activation loop. These data establish our analogue-sensitive allele of *TOS3* as a legitimate tool to study the regulation of Glc7-Reg1 protein phosphatase activity.

4.3.3 Snf1 dephosphorylation is regulated by glucose

To test whether dephosphorylation of the Snf1 activation loop is regulated by glucose, the rate of dephosphorylation was measured in cells with active (phosphorylated) Snf1. In one case, *de novo* phosphorylation of Snf1 was blocked by inhibiting the sole SAK, and the rate of dephosphorylation in low glucose was then measured. In the second case, *de novo* phosphorylation was blocked, glucose was added back to cultures, and the rate of dephosphorylation upon shifting to high glucose was measured. By comparing these two conditions, we were able to assess whether the dephosphorylation reaction is regulated by glucose. In multiple experiments, we found that the rate of Snf1 dephosphorylation was strongly regulated by glucose. A representative experiment is shown in Figure 4.5. Cells expressing the sensitized Tos3-L135G kinase as the only SAK were grown in high glucose (2%) and then shifted for 2 h to media containing only 0.05% glucose. Snf1 activation loop phosphorylation status and total Snf1 protein levels were again assessed by Western blotting. The phosphorylation status of the Snf1 activation loop is greatly increased when cells are shifted to low glucose (lanes 1 and 2). As a negative control, DMSO was added to the low glucose culture. When aliquots were removed after an additional 1, 3, or 5 min in low glucose plus DMSO, the activation loop phosphorylation remained stable and high (lanes 3–5). In contrast, when glucose was added to the cultures with or without CZ22, dephosphorylation of Snf1 to basal levels was observed after only 1 min of glucose addition (lanes 13 and 18). Therefore, the rate of dephosphorylation upon glucose addition is extremely rapid ($t_{1/2}$ < 0.5 min). Technical limitations prevent analysis of shorter time points. To assess the rate of dephosphorylation occurring in low glucose, *de novo* phosphorylation was inhibited by the addition of CZ22 to 2.5 μM. In the absence of new phosphorylation events, the activation loop of Snf1 retained its high level of phosphorylation

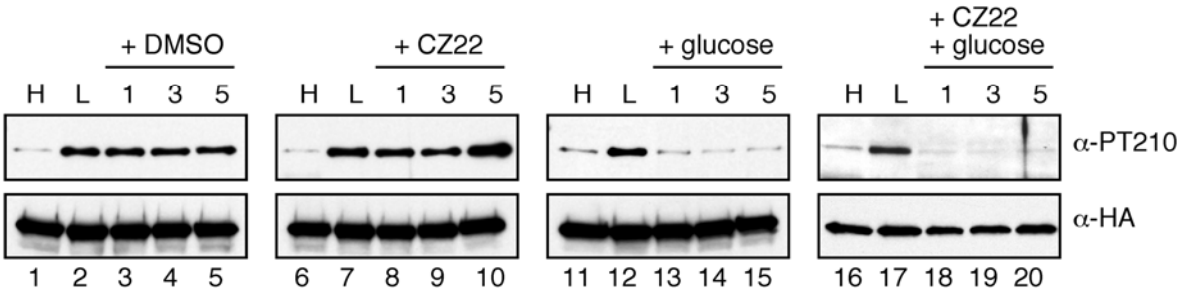

Figure 4.5 Activity of the Glc7-Reg1 phosphatase toward Snf1 is regulated by glucose.

Cells expressing the analogue-sensitive Tos3-L135G as the only SAK were grown in high glucose media (H) and then shifted to low glucose (L) for 30 min. DMSO, CZ22 dissolved in DMSO, or glucose was added, and aliquots were removed after 1, 3, or 5 min. Extracts were prepared, and the HA-tagged Snf1 protein was collected by immunoprecipitation and analyzed by Western blotting using antibodies directed against the phosphorylated form of the Snf1 activation loop (α-PT210). 20 μg of protein extracts were analyzed by Western blotting against total Snf1 protein (α-HA).

(lanes 8–10); therefore, the half-time for the dephosphorylation reaction in low glucose must be greater than 5 min. This indicates that the rate of Snf1 dephosphorylation is at least 10 times slower under glucose limitation than is observed under glucose abundance (compare $t_{1/2}$ of <0.5 min and >5 min). These results provide the first direct evidence that the rate of Snf1 dephosphorylation is regulated by glucose concentration *in vivo*. These experiments have also been undertaken with the analogue-sensitive Elm1-T200G (176) with similar results (data not shown).

4.3.4 Glc7-Reg1 phosphatase complex is active in low glucose

The glucose-regulated dephosphorylation of Snf1 could occur by one of two mechanisms. First, glucose concentration could control the phosphatase activity of the Glc7-Reg1 complex. Second, glucose levels might regulate the availability of phosphorylated Snf1 as a substrate to the inactivating phosphatase. Supporting the latter model, *in vitro* experiments with purified mammalian proteins have shown that AMP binding to the γ subunit of AMPK decreases the rate of AMPK activation loop dephosphorylation (159, 179). To test whether the regulation of Snf1 activation loop dephosphorylation is controlled by changes in Glc7-Reg1 activity or accessibility of the phosphatase to the Snf1 activation loop, we analyzed the glucose-regulated activity of Glc7-Reg1 toward another substrate, the transcription factor Mig1.

In order to examine the activity of Glc7-Reg1 phosphatase toward Mig1 in low glucose, we utilized an adenine analogue-sensitive variant of Snf1, Snf1-I132G, to be described elsewhere (168). In a manner similar to the inhibition of Tos3-L135G by CZ22, the I132G mutation in the ATP-binding pocket of Snf1 rendered it sensitive to the related compound, 2NM-PP1.

We first tested the capacity of 2NM-PP1 to completely inhibit Snf1-I132G phosphorylation of Mig1 tagged with the HA epitope (161). The phosphorylation status of Mig1-HA can be determined by anti-HA Western blotting, as phosphorylated isoforms exhibit a slower electrophoretic migration (185). Cells expressing Mig1-HA and either wild-type Snf1 or Snf1-I132G were grown in high glucose, where Mig1 is normally not phosphorylated. Aliquots were taken, and the remainder of the cells were divided and treated with either DMSO (as a negative control) or 25 μM 2NM-PP1 (suspended in DMSO) for 10 min prior to and throughout the shift to low glucose. In cells expressing either wild-type Snf1 or Snf1-I132G, Mig1-HA was seen as the dephosphorylated faster migrating isoform in high glucose (Figure 4.6a, lanes 1 and 4). The addition of 2NM-PP1 completely blocked the ability of Snf1-I132G to phosphorylate Mig1 (lane 6) but had no effect on the wild-type Snf1 (lane 3). The addition of DMSO did not inhibit either form of Snf1 kinase. Thus, the Snf1-I132G mutant is functional but can be selectively inhibited by 2NM-PP1.

We next wanted to analyze the activity of the Glc7-Reg1 phosphatase toward phosphorylated Mig1-HA in high and low glucose. In multiple experiments, we have found that Mig1 is rapidly dephosphorylated in low glucose when Snf1 kinase is inactivated by 2NM-PP1. A representative experiment is shown in Figure 4.6a. Cells expressing the analogue-sensitive Snf1-I132G and Mig1-HA were grown in high glucose, shifted to low glucose for 1 h such that Mig1-HA would be in the phosphorylated state. Cells were treated with either DMSO, 2NM-PP1 suspended in DMSO, or glucose for 5 min. Mig1-HA was found as the faster, unphosphorylated migrating form in high glucose (Figure 4.6a, lane 7) and the slower, phosphorylated migrating form upon shifting to low glucose (lane 8). The addition of DMSO had no effect on the Mig1-HA phosphorylation status (lane 9). Within 5 min of the addition of glucose to the media, Mig1-

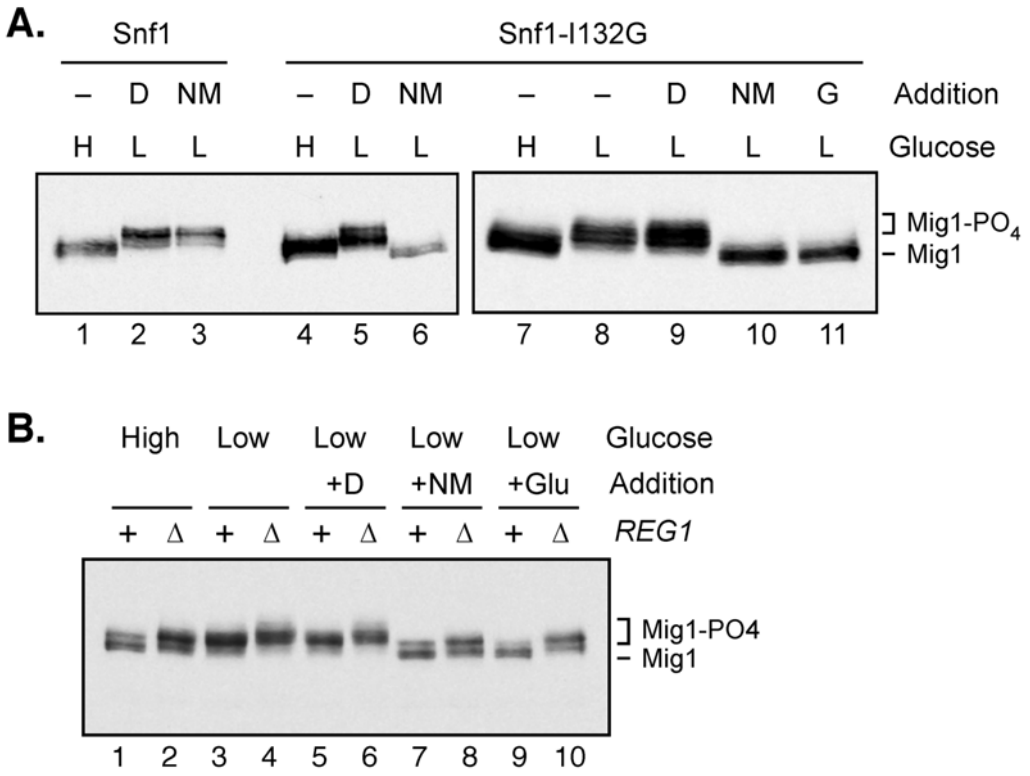

Figure 4.6 Glc7-Reg1 phosphatase is active toward Mig1 in high and low glucose.

A, Phosphorylation of Mig1 was measured by Western blotting of protein extracts prepared from cells grown in high glucose (H) or 1 h after shifting to low glucose (L). Cells expressed either wild-type Snf1 (lanes 1-3) or the analogue-sensitive Snf1-I132G (lanes 4-11). Cells were treated with DMSO (D) or the Snf1 inhibitor, 2NM-PP1 (NM), prior to and throughout the shift to low glucose as indicated (lanes 1-6). Cells were grown in high glucose (H) and shifted to low glucose (L) for 1 h (lanes 7 and 8, respectively). After 1 h in low glucose, cells were treated for 5 min with the addition of DMSO (D), the Snf1-I132G inhibitor 2NM-PP1 dissolved in DMSO (NM), or glucose (G) (lanes 9-11), as indicated. **B** Cells carrying a complete deletion of the *REG1* gene were transformed with low-copy plasmids expressing wild-type Reg1 protein (+) or empty vector (Δ). Cells also expressed HA-tagged Mig1 and the analogue-sensitive Snf1-L132G. Cells were grown in high glucose and shifted to low glucose to allow for phosphorylation of the Mig1 protein and were subsequently treated for 5 min with either DMSO (+D), 2NM-PP1 (+NM), or glucose (+Glu), as indicated. Extracts were prepared and phosphorylation of Mig1 determined by Western blotting.

HA was quantitatively returned to the faster, unphosphorylated form (lane 11). When the *de novo* phosphorylation of Mig1 was blocked with 2NM-PP1, Mig1-HA returned to the faster, unphosphorylated isoform within the same 5 min (lane 10). Therefore, the Glc7-Reg1 phosphatase is active toward Mig1 even in low glucose. These experiments demonstrate that Snf1 dephosphorylation by Glc7-Reg1 is regulated by glucose (Figure 4.5), whereas Mig1 dephosphorylation is not (Figure 4.6). Taken together, these results suggest that the catalytic activity of the Glc7-Reg1 phosphatase is not controlled by glucose availability; rather, it seems the availability of phosphorylated Snf1 as a substrate for dephosphorylation by Glc7-Reg1 is regulated by environmental glucose concentration.

The conclusion that the Glc7-Reg1 phosphatase is active toward one substrate (Mig1) under conditions where it is not active toward another (Snf1 activation loop) is based on the assumption that Mig1 is a direct substrate of the Glc7-Reg1 phosphatase. Although this conclusion is sometimes stated as if it were proven, an alternative interpretation for the effect of mutations in the *REG1* and *GLC7* genes on Mig1 phosphorylation has been noted (47). Mutations in *REG1* and *GLC7* cause hyperactivation of Snf1, which may in turn explain the increased phosphorylation state of Mig1. We used the analogue-sensitive allele of Snf1 to examine the effect of deletion of the *REG1* gene on the phosphorylation of Mig1. In this way, any changes in the phosphorylation state of Mig1 as a result of *REG1* deletion would be independent of the hyperactivation of Snf1. Cells expressing HA-tagged Mig1 and Snf1-I132G were shifted to low glucose for 1 h to allow for phosphorylation of Mig1. Cells were then treated for 5 min with 2NM-PP1 to inhibit the Snf1-I132G kinase activity. For controls, cells were either treated with DMSO or glucose (Figure 4.6b). When Snf1 kinase was inhibited by the addition of glucose, the dephosphorylation of Mig1 was rapid when Reg1 was present (lane 9). Deletion of

REG1 caused an increase in the phosphorylation state of Mig1 in both high and low glucose (lanes 2 and 4). When the activity of Snf1 was inhibited by the addition of 2NM-PP1, Mig1 was rapidly dephosphorylated (lane 7). Deletion of *REG1* inhibited the dephosphorylation of Mig1 (lane 8). Therefore, the effect of Reg1 on the phosphorylation state of Mig1 was not dependent on the hyperactivation of Snf1 kinase. This experiment is the first to separate the role of Glc7-Reg1 in the dephosphorylation of Mig1 from the hyperactivation of Snf1. These data provide strong evidence that Glc7-Reg1 is indeed active toward Mig1 in low glucose, although it is largely inactive toward the activation loop of Snf1.

4.4 DISCUSSION

Glucose abundance regulates the Snf1 signaling pathway. The identity of the glucose signal and the mechanism(s) by which it is transduced to the Snf1 kinase have remained elusive. The phosphorylation status of the Snf1 activation loop is determined by the relative rates of phosphorylation by the SAKs and dephosphorylation by the inactivating phosphatase PP1 (Glc7-Reg1). Until the present report, it has been unclear whether phosphate addition, phosphate removal, or both are regulated by changes in glucose availability.

We first asked if phosphorylation of Snf1 is regulated by glucose concentration. Snf1-dependent invertase activity was elevated in high and low glucose conditions in the absence of the PP1 regulatory subunit Reg1 when Sak1, Tos3, or Elm1 were present as the only SAK (Figure 4.1). Thus, each of the SAKs is capable of activating the Snf1 pathway in high glucose. Additionally, the fraction of total Snf1 that was phosphorylated was higher in glucose-rich conditions in *reg1*Δ strains than in *REG1*+ strains regardless of which SAK(s) was expressed

(Figure 4.2). These experiments support the idea that the SAKs are active independent of glucose, but two other possibilities existed. First, it was conceivable that the SAKs actually did exhibit glucose regulation but that the lower level of activity in high glucose was sufficient to activate Snf1 when PP1 function was disrupted. Second, the activity of the SAKs themselves might be regulated by PP1. These two possibilities were addressed by our third experiment; SAKs immunoprecipitated from extracts of cells grown in high and low glucose exhibited similar activity toward exogenous Snf1 kinase domain substrate (Figure 4.3). These results strongly suggest that the activities of the SAKs are glucose-independent. Additional lines of evidence supporting this idea were presented above.

If the SAKs are not regulated by glucose, then dephosphorylation of Snf1 by Glc7-Reg1 PP1 must be regulated by glucose. Using an adenine analogue-sensitive mutant of the SAK Tos3, we showed that the rate of Snf1 dephosphorylation is at least 10-fold greater in high glucose than in low glucose (Figure 4.5). This experiment did not distinguish between a direct effect of cellular energy status on the catalytic activity of the Glc7-Reg1 complex and an effect on the availability of the Snf1 activation loop to serve as a Glc7-Reg1 substrate. An adenine analogue-sensitive allele of Snf1 allowed us to determine that Glc7-Reg1 was active in low glucose, rapidly dephosphorylating another substrate, Mig1, in conditions under which Snf1 is resistant to dephosphorylation (Figure 4.6). This result suggests that glucose regulation of Snf1 dephosphorylation is achieved by controlling access of the phosphorylated Snf1 activation loop to PP1.

Our model of the regulation of Snf1 activation loop phosphorylation is presented in Figure 4.7. The three SAKs and the PP1 phosphatase are catalytically active regardless of glucose availability. In low glucose, however, the phosphorylated activation loop of Snf1 is

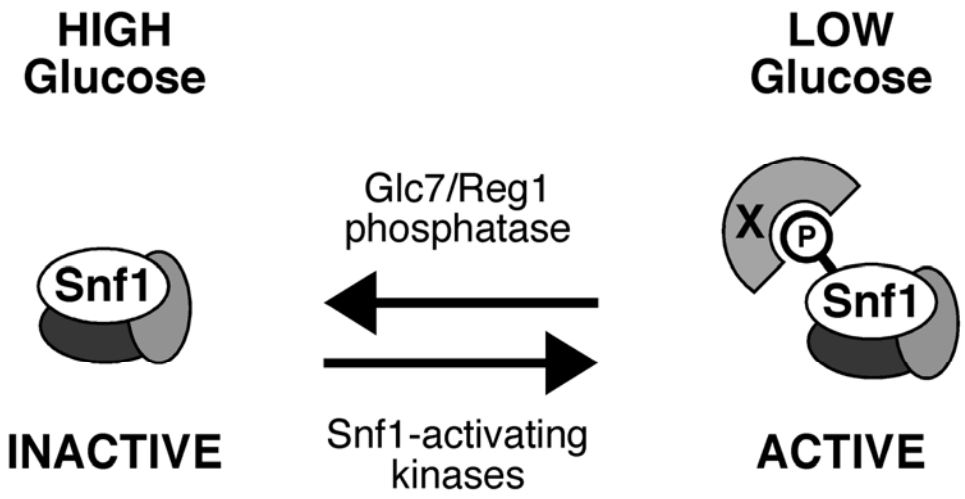

Figure 4.7 Model for the regulation of Snf1 kinase at the level of dephosphorylation.

In high glucose media, the Snf1 kinase is largely unphosphorylated and inactive due to the accessibility of the activation loop threonine 210 to the Glc7-Reg1 phosphatase. Under conditions of glucose limitation, the dephosphorylation of the Snf1 activation loop is inhibited by an unknown factor (X), leading to the accumulation of phosphorylated and active Snf1 kinase.

protected from dephosphorylation by an unknown factor (*X* in our schema), allowing the phosphorylated isoform of Snf1 to accumulate. How might the access of phosphorylated Snf1 to Glc7-Reg1 be controlled? We consider here two possibilities. The first is that, in low glucose, phosphorylated Snf1 and Glc7-Reg1 are maintained in distinct subcellular compartments, thus protecting phosphorylated Snf1 from PP1. Dombek *et al.* (51) have reported that Reg1-GFP fusions display constitutively cytoplasmic localization. Snf1, by contrast, has been shown to exhibit a complex, glucose-regulatedsubcellular localization pattern. In glucose abundance, all three β subunit-specific isoforms of Snf1 are cytoplasmic (194). Upon a shift to low glucose, however, the Snf1-Sip1-Snf4 complex becomes localized to the vacuolar periphery, the Snf1-Gal83-Snf4 complex becomes enriched in the nucleus, and the Snf1-Sip2-Snf4 complex seems to remain cytoplasmic under all conditions (83, 194). The idea that subcellular localization might control Snf1 dephosphorylation is challenged by the finding that the three isoforms of Snf1, each with a distinct localization pattern, all show similar regulation by glucose (128, 161). For this reason, we do not favor a model in which Snf1 dephosphorylation is controlled by subcellular localization.

A second possibility is that the phosphorylated threonine 210 residue is protected from dephosphorylation by Glc7 in low glucose by some protein factor (*X* in our model; Figure 7). The identity of this protein factor is unclear; however, two strong candidates come immediately to mind. One candidate is Snf4, the γ subunit of the Snf1 heterotrimer. In the mammalian enzyme, the γ subunit binds AMP and inhibits the dephosphorylation reaction (159, 179). In yeast, the γ subunit does not appear to bind AMP, and whether or not it binds some other small metabolite is a topic of great interest in the field. Two-hybrid interactions between Snf1 and Snf4 are reported to increase in response to glucose limitation (103). It is possible that interactions of Snf1 with the

γ subunit Snf4, possibly in conjunction with the β subunits, preclude dephosphorylation by PP1 in low glucose. It is clear, however, that although Snf4 and the β subunits are required for Snf1 function *in vivo*, at least some degree of glucose regulation of Snf1 phosphorylation is independent of the β and γ subunits, since cells lacking either all three β subunits, the γ subunit, or both retain glucose-regulated phosphorylation of threonine 210 (55). In fact, when Snf1 kinase lacking its C-terminal regulatory domain is expressed in the absence of β and γ subunits, the activation loop still exhibits glucose-regulated phosphorylation (55). Thus, some regulation must lie outside the Snf1 regulatory domain as well as the β and γ subunits.

The second strong candidate for X in our model is Reg1. Although Reg1 is thought to act as the targeting subunit for Glc7 that promotes Snf1 dephosphorylation in high glucose, it is possible that Reg1 maintains dual roles in Snf1 activation loop regulation. Reg1 is an abundant component of the Snf1 kinase complex as determined by two-hybrid analysis (121) and mass spectrometry (54). Furthermore, the interaction of Reg1 with the Snf1 kinase domain requires the activation loop threonine and is stronger in low glucose (121). In low glucose, Reg1 could function to protect the phosphorylated threonine 210. Some as yet unidentified signal would control whether Reg1 serves as protector of Snf1 activation loop phosphorylation or recruiter for the PP1 phosphatase.

Additional candidates for the identity of the X factor are other proteins found in the Snf1 complex, including chaperones and the phosphoprotein-binding 14-3-3 proteins, Bmh1 and Bmh2 (50, 54). Finally, the Snf1 kinase domain has been reported to dimerize in a manner that could make the activation loop inaccessible (139). Whether or not there is regulatory significance to the proposed Snf1 dimerization is unknown. Clearly, further experimentation will be needed to

determine precisely how glucose abundance controls Snf1 availability for dephosphorylation by PP1.

Finally, the glucose signal controlling Snf1 phosphorylation status is not known. Our data do not distinguish between a low-glucose signal that protects Snf1 from dephosphorylation and a high-glucose signal that promotes its dephosphorylation. Identifying the molecular cue(s) that regulates Snf1 activation loop phosphorylation status is of great interest. The primary consequence of this study will be to redirect the focus of future investigations of Snf1 activation loop phosphorylation away from the regulation of phosphate addition and toward the regulation of phosphate removal.

4.5 ACKNOWLEDGMENTS AND CONTRIBUTIONS

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EMR performed the experiments displayed in Figures 4.1, 4.3, 4.4a, 4.4b, 4.5 lanes 16-20, and 4.6. RRM performed the experiments displayed in Figures 4.2, 4.4c, and 4.5 lanes 1-15. EMR and RRM collaborated on Figure 4.6b. CZ and KMS assisted in the development of analogue-sensitive SAKs. MKS and KMA developed the analogue-sensitive Snf1 kinase. EMR and MCS collaborated on research design and manuscript preparation.

5.0 DISCUSSION AND FUTURE DIRECTIONS

Regulation of the *Saccharomyces cerevisiae* Snf1 kinase involves at least two critical components: phosphorylation of a conserved activation loop threonine residue at position 210 and a strong requirement for the γ subunit, Snf4. When threonine 210 is mutated to alanine, Snf1 is rendered catalytically inactive (60). The finding that the Snf1 activation loop is still phosphorylated when Snf1 has been rendered catalytically inactive by active site mutations indicated that one or more distinct upstream activating kinases exist that phosphorylate Snf1 (as opposed to Snf1 autophosphorylation) (129).

It was hoped that the identification of Sak1, Tos3, and Elm1 as the complete complement of Snf1-activating kinases (SAKs) (92, 180) would rapidly lead to a clearer understanding of the mechanism(s) by which changes in glucose availability control Snf1 activity. Multiple laboratories have expended significant effort and resources toward the determination of how glucose levels might regulate the activity of the SAKs. Indeed, Chapters 2 and 3 of this manuscript detail some of the work undertaken to elucidate the regulation of the SAKs. While much has been learned about the relationships between Sak1, Tos3, and Elm1 and their substrate, Snf1, initial investigation of the SAKs may have been naïvely clouded by a prevailing kinase-centric worldview (155).

Importantly, a recent pair of elegant biochemical studies of mammalian AMPK regulation has provided strong evidence that AMPK activation loop phosphorylation is regulated

by dephosphorylation by PP2C rather than by phosphate addition by the AMPKKs. AMP interaction with the AMPK γ subunit prevented dephosphorylation of the catalytic α subunit and stimulated intrinsic AMPK catalytic activity but had no effect on AMPK phosphorylation by the AMPKKs (159, 179). The regulated availability of AMPK/Snf1 kinases to serve as phospho-substrates for their cognate inactivating phosphatases is emerging as a shared mechanism of regulation. The data presented in Chapter 4 provides a broader perspective of the regulation of Snf1 activation loop phosphorylation in response to glucose limitation whereby the key glucose-sensitive component is shown to be the availability of phosphorylated Snf1 activation loop threonine 210 for dephosphorylation by the protein phosphatase I (PP1) Glc7 and its regulatory partner Reg1. These yeast and mammalian studies have supported a dramatic paradigm change in the understanding of the AMPK/Snf1 signaling pathways. Future work will be directed toward understanding the mechanism of regulated Snf1 dephosphorylation.

5.1 FUTURE DIRECTIONS: HOW MIGHT SNF1 DEPHOSPHORYLATION BE CONTROLLED?

In our current model of regulated Snf1 phosphorylation, neither the catalytic activity of the SAKs nor that of the Glc7-Reg1 PP1 is responsive to changes in glucose availability. Rather, glucose levels regulate access of the phosphorylated Snf1 activation loop threonine 210 to the inactivating phosphatase. It will be important to determine how this regulated access occurs. One possibility is that regulated Snf1 access to the phosphatase is a result of differential subcellular localization. We do not find this likely since mutations which disrupt the low-glucose redistribution of Snf1 (such as deletion of the Snf1 β subunits) do not disrupt regulated Snf1

phosphorylation (55). A second potential mechanism for controlled Snf1 access to the phosphatase is the existence of a protein factor (denoted as "X" in Figure 4.7) that prevents Glc7-Reg1-mediated dephosphorylation of Snf1 in low-glucose conditions. A few candidates for the identity of Factor X are discussed below.

5.1.1 Snf4

Based on homology with the mammalian AMPK enzyme, the Snf1 heterotrimer γ subunit Snf4 may seem the most likely candidate for managing access of Snf1 threonine 210 to the phosphatase. The mammalian γ subunit clearly plays such a role in regulating AMPK dephosphorylation. Mutations in the AMPK γ subunits that have been shown to cause disease in humans block *in vitro* interactions of the γ subunits with AMP (164) and compromise the AMP-dependence of enzyme activation (42). As mentioned above, AMP binding to the human AMPK γ subunit has been shown to inhibit dephosphorylation by PP2C (159, 179). Finally, dephosphorylation of AMPK heterotrimers incorporating γ subunits with mutated ligand-binding sites is rendered insensitive to AMP (159). Further characterization of the role of Snf4 in Snf1 regulation is merited.

The AMPK γ subunits display significant sequence and structural homology across species. Several (but not all) of the residues implicated as critical in human γ subunit AMP binding are conserved in Snf4, and the recent flurry of AMPK crystallographic data provides compelling evidence for structural and potentially regulatory conservation as well. The determined structures of portions of the mammalian (45, 149, 206), *S. cerevisiae* (7, 139, 156, 157), and *Schizosaccharomyces pombe* (105, 184) AMPK/Snf1 heterotrimers reveal striking conformational similarity. γ subunits of heterotrimers from mammalian AMPK, *S. cerevisiae*

Snf1, and *S. pombe* AMPK have all been co-crystallized with bound AMP. In the case of the *S. cerevisiae* Snf1 complex, though, it is not clear whether these interactions are physiologically significant or are crystallographic artifacts as AMP was included at very high concentrations in these crystal studies (7).

The Snf4 protein is required for efficient Snf1 function in glucose-limiting conditions (32, 129), and the kinase-stimulating interaction between Snf1 and Snf4 within the heterotrimer is believed to be enhanced in low-glucose conditions, as two-hybrid analyses have established a glucose-regulated association between the Snf1 regulatory domain and Snf4 (103). It is possible that these interactions play some role in regulating access of the Snf1 activation loop to PP1.

Other evidence indicates that there exists at least some Snf4-independent regulation of Snf1 phosphorylation. First, in cells lacking Snf4 or the β subunits (or the motifs in Snf1 required for interaction with Snf4 and the β subunits), Snf1 phosphorylation is normally glucose-responsive (55). Thus, in the absence of Snf4, some other mechanism exists which protects Snf1 activation loop phosphorylation in low glucose conditions. Second, mutations in Snf1 have been identified that render regulated Snf1 activity independent of Snf4 (114). Snf4 may thus account for some regulation of Snf1 phosphorylation status, but it clearly cannot account for the entirety of regulatory control of activation loop phosphorylation. Third, the two-hybrid interaction of Snf1 and Snf4 requires Snf1 threonine 210, as Snf1-T210A does not associate with Snf4 in this assay (121); conversely the Snf1/Snf4 association is rendered constitutive in cells lacking Reg1 (103). These data are consistent with Snf1 phosphorylation status regulating Snf1/Snf4 interaction rather than vice versa. However, interpretation of yeast two-hybrid data involving Snf1 is complicated by the finding that recruiting an active form of Snf1 to a promoter can stimulate RNA polymerase II-dependent transcription (111).

Thus, other strategies must be employed to determine what, if any, role Snf4 has in the regulation of Snf1 activation loop phosphorylation status. One option is to employ both a site-directed and randomized mutagenic approach to screen for *SNF4* alleles which alter Snf1 activity and phosphorylation. The availability of structural and sequence information will facilitate homology-based prediction of residues that may be critical for Snf4-mediated Snf1 regulation. Site-directed mutagenesis of amino acids predicted to be involved in ligand binding and interaction with Snf1 may illuminate mechanisms of Snf4-to-Snf1 signaling. Once these mutations have been introduced to Snf4, the ability of the mutant Snf4 variants to support regulated Snf1 activation loop phosphorylation and signaling can be directly assayed. Similarly, randomized mutagenesis of Snf4 may assist in furthering our understanding of the complex relationship between Snf1 and Snf4. A large-scale library of Snf4 mutants can be easily generated and screened for Snf1 gain-of-function or loss-of-function phenotypes by assessing the growth of yeast expressing candidate clones in a variety of growth conditions or measuring the expression of a Snf1-dependent reporter construct. Following the identification of candidate Snf4 mutants, Western blots against phosphorylated Snf1 activation loop could determine if mutant Snf4 proteins disregulated Snf1 phosphorylation status.

Our research group has purified each subunit of the Snf1 heterotrimer (55) and all three SAKs (54). In our continuing analysis of regulated Snf1 dephosphorylation, it is now essential that the PP1 complex components Glc7 and Reg1 be purified. Such efforts are currently underway. It will be important to be able to recapitulate the dephosphorylation of Snf1 by Glc7 in *in vitro* phosphatase assays. Whether Snf4 affects Glc7-mediated Snf1 dephosphorylation can be assayed by comparing the *in vitro* dephosphorylation rates of phosphorylated Snf1 in the presence and absence of Snf4.

Another open question regarding the yeast γ subunit Snf4 is whether Snf4, like its mammalian counterparts, binds AMP (or any ligand) *in vivo*. Thus far, the only biochemical evidence supporting AMP coordination by Snf4 are the structural studies described above in which AMP was included at high concentrations such that it is not clear whether these interactions might be reasonably predicted to occur in cells (7). Moreover, in contrast to what has been observed in mammalian cells, Snf1 is active under some conditions in which the AMP:ATP ratio is not elevated, such as when cells are forced to grow by respiration of ethanol and glycerol (18). It is possible that, with respect to the α/γ relationship, the yeast and mammalian enzymes are fundamentally different. Additional *in vitro* analyses have failed to confirm Snf4 interaction with or stimulation by AMP (130, 200). Researchers in our group have also been unable to observe any AMP-dependent stimulation of Snf1 holoenzyme activity *in vitro* (unpublished data), though the failure to detect AMP stimulation could conceivably be the result of Snf1 heterotrimer purified with the putative binding sites saturated with AMP. A recent study has suggested that LKB1-AMPK signaling could be stimulated by the glycolytic intermediate 3-phosphoglycerate (57). An expanded panel of potential stress-signaling ligands should be tested for interaction with Snf4, stimulation of Snf1 activity, and inhibition of Snf1 dephosphorylation by PP1.

It may be that, in contrast to the mammalian system, Snf4 serves primarily a structural rather than regulatory role, being required to stabilize the active conformation of a phosphorylated Snf1 catalytic α subunit. It would be of interest to determine if the mammalian γ subunits can complement the Snf- defect of *snf4*Δ yeast (like the mammalian AMPKKs for *sak1*Δ *tos3*Δ *elm1*Δ yeast (93)). In the event that a mammalian γ subunit did support regulated Snf1 signaling, inactivating mutations which block AMP binding in the context of the

mammalian enzyme could be introduced, and the resultant protein could be tested for *snf4*Δ complementation. If a human γ subunit which cannot bind AMP confers normal Snf1 signaling in yeast, this would strongly support a structural rather than regulatory requirement for the yeast γ subunit.

5.1.2 Reg1

An intriguing candidate for Factor X that might control access of Snf1 to the inactivating phosphatase is Reg1. This may seem counterintuitive, since Reg1 is required to target Glc7 to Snf1 for dephosphorylation in high-glucose conditions (121, 160). However, Reg1 is found in complex with Snf1 in both abundant and limiting glucose growth conditions. In fact, in their analysis of the regulatory interactions between Reg1 and Snf1, Ludin et al. found that two-hybrid Reg1/Snf1 interactions were strengthened in low glucose when Snf1 is normally phosphorylated and active (121). Co-immunoprecipitation experiments in our laboratory have confirmed these observations (unpublished data). Thus, we consider the possibility that Reg1 serves a dual role in controlling Snf1 activation loop phosphorylation status: targeting Glc7 to Snf1 in high glucose and protecting the phosphorylated Snf1 activation loop from dephosphorylation in low glucose.

To explore this hypothesis, a randomized mutagenic screen for Reg1 mutants that result in disregulated Reg1/Snf1 association and disregulated Snf1 activation loop phosphorylation should be undertaken. As described above in experiments proposed to identify Snf4 loss- or gain-of-function mutants, growth on defined carbon sources as well as expression of Snf1-dependent reporter constructs may facilitate the identification of such Reg1 variants. Mutant Reg1 candidates selected for additional analysis would then be assayed for association with Snf1 and for the aberrant regulation of Snf1 phosphorylation. The Reg1 dual-role model would be

supported, for instance, if cells expressing Reg1 mutants that interact more strongly with Snf1 in high glucose exhibit constitutive Snf1 activation loop phosphorylation. *In vitro* studies could also facilitate a better understanding of Reg1's role in controlling Snf1 phosphorylation. Once Glc7 and Reg1 have been purified, *in vitro* phosphatase assays of Snf1 by Glc7 can be performed in the presence or absence of Reg1 protein. Additionally, a panel of potential metabolic signaling molecules can be tested for their effect on these *in vitro* reactions.

Elevated Reg1/Snf1 two-hybrid interaction in low glucose remains intriguing in light of a Reg1 dual-role model. As was the case for Snf4, the association of Reg1 and Snf1 requires threonine 210 (121). It may be that the protective role of Reg1 is initiated when Reg1 recognizes and tightly binds the phosphorylated threonine. However, Reg1/Snf1 association is constitutive and glucose-independent when the Snf1 ATP-binding pocket is mutated (lysine 84 mutated to arginine) (121). Snf1-K84R is catalytically inactive, and this result suggests that Snf1 negatively regulates its own association with Reg1. The position of Reg1 in Snf1 signaling is not yet clear, and much work is likely to focus on this important protein.

5.1.3 Bmh1/Bmh2 and Ssb1/Ssb2

Other conceivable candidates for a protein factor which might play a role in the regulation of Snf1 activation loop phosphorylation are those which have been found in complex with purified Snf1 (54). Two such proteins are the yeast 14-3-3 proteins, Bmh1 and Bmh2. 14-3-3 family members bind phosphoproteins and have been shown to have diverse roles in other signal transduction pathways (63). It would be of interest to determine what, if any, role they might play in controlling Snf1 activation loop phosphorylation. One group has suggested these proteins may interact genetically and biochemically with Reg1 in maintaining glucose repression of Snf1-

dependent signaling (50). Analysis of these proteins is complicated by the fact that deletion of both Bmh proteins is lethal in yeast (69); thus, a more subtle analysis would be required to further explore the potential role they might play in regulating Snf1. Two other proteins found to interact with both Snf1 and Reg1 are the heat shock proteins Ssb1 and Ssb2 (50, 54). A minor role for these proteins in glucose repression has been observed (50), though it is unlikely that they account for a significant portion of the regulation of Snf1 phosphorylation.

5.1.4 Hxk2

The role of Hxk2 in glucose repression and Snf1 signaling has been controversial. Hxk2 is one of three hexose kinases (the others are Glk1 and Hxk1) that phosphorylate intracellular glucose in the first irreversible step of glycolysis following glucose uptake; of these three enzymes, Hxk2 provides the bulk of glucose kinase activity (15, 37, 195). Cells which lack Hxk2 display constitutive Snf1 signaling regardless of glucose levels (103). A simple interpretation of these data is that in the absence of Hxk2, a significant fraction of glucose phosphorylation is lost, drastically limiting the flux through glycolysis and hence causing cells to respond as if there were limited glucose present. One group, however, has described roles for Hxk2 in physically bridging the interaction between Snf1 and its substrate, Mig1, as a mechanism of regulating Snf1 signaling (5) and in the regulation of Mig1-dependent transcriptional repression of *SUC2* (4), leading them to propose that Hxk2 plays a role in signaling glucose abundance distinct from its glucose phosphorylation activity. It may be instructive to analyze whether Hxk2 can contribute to the regulation of Snf1 activation loop phosphorylation status.

5.1.5 Other nutrient-sensing pathways: TOR, HXT, and PKA pathways

The Snf1 signaling axis is not the only cellular network that is responsive to changes in nutrient availability. The TOR (Target of Rapamycin) signaling pathway is stimulated by nutrient abundance (46). While both yeast and mammalian TOR kinases are upregulated by high concentrations of glucose and nitrogen (11), the yeast TOR system is less well characterized than the mammalian network. The mammalian AMPK seems to function upstream of the TOR kinases, since, under conditions of energy limitation, AMPK phosphorylates and activates the TOR inhibitor, TSC2 (99, 109). Moreover, mammalian AMPK has recently been shown to phosphorylate the TOR-interacting protein, raptor. Phosphorylated raptor binds 14-3-3 protein in a manner that inhibits raptor-associated TOR kinase activity (74). These studies indicate that AMPK modulates mammalian TOR kinase activity by regulating the functions of multiple upstream targets.

The picture in yeast is not so clear. One study positioned Snf1 downstream of TOR in regulating filamentous growth in response to nitrogen availability (143). Another showed that Snf1 and TOR signaling independently converge in regulating the phosphorylation status of the transcriptional activator Gln3 (14). A third report further established a connection between the Snf1 and TOR pathways in regulating transcription of *INO1* in response to inositol limitation. Deletion of the *SNF1* gene resulted in decreased TBP binding of the *INO1* promoter and *INO1* transcription; deletion of the gene encoding the Sit4 phosphatase partially suppressed this *snf1*Δ phenotype. TOR signaling appeared to inhibit the repressive effect of Sit4 on *INO1* transcription (170). Whether the Snf1 and TOR signaling pathways directly regulate the same targets or if one is subject to direct regulation by the other is not clear. Given the ambiguity of data regarding the

relationship between Snf1 and TOR kinases in yeast (Tor1 and Tor2), the possibility that the TOR pathway might regulate Snf1 activation loop phosphorylation status should be investigated.

Glucose levels control expression of genes in the hexose transporter (HXT) family, which possess a range of affinities for glucose and allow for its uptake by facilitated diffusion (144). Two members of this family, Rgt2 and Snf3 exhibit the conserved 12-transmembrane domain structure but function as glucose sensors rather than sugar transporters (117). Rgt2 and Snf3 sense high and low glucose levels, respectively, and signal glucose abundance via their C-terminal cytoplasmic domains not present among the other HXT proteins (39, 144, 190). The proteins Std1 and Mth1 repress the transcription of genes encoding particular hexose transporters when bound to the cytoplasmic tails of Rgt2 and Snf3, respectively (162). Std1 and Mth1 inhibition of HXT expression is mediated by interaction with the transcription factor Rgt1 (113). The net effect is the expression of low-affinity glucose transporters under conditions of glucose abundance and high-affinity transporters when glucose is limiting. While there may be some interaction or cross-talk between this and the Snf1 signaling pathway, it is improbable that Rgt2, Snf3, or their downstream effectors control access of phosphorylated Snf1 to the Glc7 PP1, as Snf1 activity is normally glucose-regulated in cells lacking Rgt2, Snf3, Std1, and Mth1 (162).

The protein kinase A (PKA) signaling pathway also responds to glucose levels. In short, the glucose/sucrose-sensing G-protein coupled receptor Gpr1 signals via the G protein Gpa2 (116) to activate adenylate cyclase, which catalyzes the generation of cAMP (38). cAMP promotes PKA activation by binding the inhibitory PKA regulatory subunit Bcy1, resulting in its dissociation from and activation of one of three redundant PKA catalytic subunits, Tpk1, Tpk2, or Tpk3 (106, 183). While the PKA signaling pathway may regulate the subcellular localization of one β-subunit Snf1 isoform (Snf1-Sip1-Snf4) (85), cells with defective PKA signaling display

ordinary glucose regulation of Snf1-dependent invertase activity, indicating that neither PKA nor its pathway components are the key factor(s) controlling Snf1 activation loop phosphorylation status (96).

5.1.6 Snf1 dimerization

An unanticipated structural feature emerged in several of the published Snf1/AMPK crystal structures: dimerization. In a 2005 study, the non-phosphorylated Snf1 kinase domain crystallized in a homodimeric asymmetric unit with a helix of one molecule inserted into the active site cleft between the N- and C-terminal lobes of the other (156). A subsequent structural analysis of the Snf1 kinase domain revealed a homodimerization conformation which resulted in the activation loop threonine 210 being buried and presumably inaccessible to Snf1-activating kinases (139). Portions of the Snf1/AMPK complexes of both *S. cerevisiae* and *S. pombe* were crystallized as dimers of heterotrimers, though the interface of dimerization was different for the two species' enzymes: the dimerization interface of *S. cerevisiae* Snf1 heterotrimer was between the Snf4 γ subunits of each heterotrimer (7), while the *S. pombe* enzyme dimerized via reciprocal α/γ interactions (184). Unfortunately, the crystallized heterotrimers include the catalytic α subunit C-terminal regulatory domain but not the N-terminal kinase domain, making it more difficult to extrapolate biological and regulatory significance from these structures. Very recently, light scattering analysis of bacterially expressed and purified mammalian heterotrimeric AMPK provided evidence of homodimerization, but only at high concentrations. At concentrations closer to predicted physiological levels, AMPK existed as monomers of heterotrimers in solution (152).

We have not observed evidence of multimerization of the Snf1 heterotrimer. In size exclusion chromatography experiments, components of the Snf1 complex have eluted in fractions predicted to contain a complex of a molecular mass consistent with that of single heterotrimers (155 kDa) (55). It is possible that the Snf1 enzyme does in fact incorporate into dimers of heterotrimers in high-glucose conditions and that these dimers might dissociate into their constituent heterotrimers upon cell harvesting and/or protein extraction. Experiments in which cells are treated with cross-linking agents to preserve complex integrity before harvesting and protein extraction might enable more sensitive detection of higher order Snf1 complexes. If a dimer of heterotrimers is in fact observed *in vivo*, then its effect upon Snf1 phosphorylation status can be further investigated. Further, randomized mutagenic two-hybrid screens could then be designed to identify mutations in α, β, or γ subunits that alter dimerization dynamics. Other candidates (described above) for Factor X may be explored for roles in mediating or preventing glucose-regulated dimerization.

5.1.7 Snf1 ubiquitination

A very recent study has explored polyubiquitination as a novel regulatory mechanism for AMPK and AMPK-related kinases in mammalian cells. When the deubiquitinating enzyme USP9X was knocked down or inhibited, polyubiquitination of AMPK and two AMPK-related kinases (NUAK1 and MARK4) kinases was observed. The unusual lysine 29- and lysine 33-linked ubiquitin chains (lysine 48 and lysine 63 linkages are more commonly observed) did not affect protein stability; rather, the ubiquitin modifications inhibited LKB1-mediated phosphorylation of the activation loop threonine residues of NUAK1 and MARK4. While the residue(s) to which ubiquitin was conjugated was not determined, a conserved tryptophan within the kinase domains

of these proteins was determined to be required for the removal of the polyubiquitin chain(s) (6). This tryptophan is conserved in Snf1 (at position 305). To my knowledge, ubiquitination as a possible regulatory mechanism for Snf1 activation or activity has not been explored. Further, Snf1 mono- or polyubiquitination might not be detected by Western analysis if deubiquitinating enzymes are not inhibited during yeast protein extraction. We have mutated tryptophan 305 to alanine and have not observed a defect in Snf1-dependent growth on alternative carbon sources in preliminary analysis (unpublished data). It could be that Snf1 polyubiquitination is required for Snf1 repression in high-glucose conditions rather than for Snf1 activation in low-glucose. Ubiquitin conjugation may indeed represent an important component of signaling to the Snf1 activation loop, and more subtle functional and biochemical analysis is warranted.

5.2 SNF1 AND AMPK: COMPARED AND CONTRASTED

The work described herein and performed by others studying Snf1 and AMPK illuminate similarities and differences between the mammalian and yeast pathways. Several components and characteristics of these pathways have been highly conserved, justifying aggressive research into both pathways. Certainly, findings in each system have contributed to a broader understanding of the mechanisms regulating the response to energy limitation. On several occasions, findings in one field have facilitated discoveries in the other. Investigation of this pathway in a variety of model organisms will accelerate the elucidation of events upstream of AMPK/Snf1 activation.

The active form of both enzymes is heterotrimeric, consisting of a catalytic α subunit and regulatory β and γ subunits, and recent crystallographic information confirms a highly conserved

structural arrangement. Many of the mammalian AMPK and yeast Snf1 substrates have been conserved. Both kinases require at least two events for full activation: activation loop phosphorylation by related upstream kinases and a distinct step mediated by the γ subunit. While the nutrient-regulated activation loop phosphorylation of both AMPK and Snf1 were long presumed to be a reflection of the regulatory status of their respective upstream activating kinases, recent work in both fields has shown the opposite to be true. The AMPKKs and SAKs appear to be constitutively active, while dephosphorylation of AMPK and Snf1 by their respective inactivating phosphatases seems to be responsive to changes in energy status.

The differences between AMPK and Snf1 signaling have emerged over the past several years. For one, the existence of multiple genes encoding each AMPK subunit and the possibility of splicing variants therein renders AMPK inherently more complex than Snf1. Second, while each enzyme requires the γ subunit for efficient *in vivo* function, only AMPK γ subunits have been shown to bind and be activated by the ligand AMP. Little evidence is currently available to suggest that Snf4 is similarly responsive to AMP (or any other ligand). Third, the active form of the primary AMPKK, LKB1, is, like its substrate, a heterotrimer, composed of LKB1, and two auxiliary subunits, the pseudokinase STRAD and stabilizing interacting partner MO25. Yeast SAKs do not appear to function as oligomers. There are no known pseudokinases in yeast, and the yeast homologue of MO25, Hym1, is not found in complex with any of the SAKs (54). Fourth, while activation loop dephosphorylation is regulated by energy status in both systems, members of different phosphatase families dephosphorylate their respective substrates: PP2C is the major AMPK-inactivating phosphatase, while the Glc7-Reg1 PP1 is the primary Snf1-inactivating phosphatase. It remains to be seen, then, if the mechanisms controlling regulated activation loop dephosphorylation are conserved. If, as discussed above, the yeast γ subunit Snf4

is implicated in controlling threonine 210 phosphorylation status, this would indicate strong conservation of dephosphorylation regulation, since AMP binding to mammalian γ subunits inhibits dephosphorylation by PP2C. If, by contrast, Reg1 or an unrelated protein emerges as the key factor in inhibiting Snf1 activation loop dephosphorylation in nutrient limitation, this would represent a significant divergence in the regulation of this signaling module. The similarity or difference of other factors remains to be seen. For example, it is yet to be determined whether Snf1 is polyubiquitinated in a manner similar to the mammalian enzyme.

5.3 CONCLUDING REMARKS

The scientific process is an intellectually gratifying endeavor. It allows the curious individual to study phenomena, make observations, generate explanatory hypotheses, and design experimental strategies to test these hypotheses. Inevitably, the confirming or refuting of any particular hypothesis spawns novel observations, hypotheses, and experimentation. Such has clearly been the case in the study of the regulation of the Snf1 signaling pathway as detailed in the preceding pages. The discovery of three Snf1-activating kinases led to the generation of several hypotheses describing the mechanisms by which Sak1, Tos3, and Elm1 are regulated by glucose availability. The ensuing work led to the detailed analysis and characterization of these proteins and an unexpected discovery: these three SAKs displayed constitutive activity toward their substrate, Snf1. We then hypothesized and confirmed that Snf1 dephosphorylation represented the major regulatory node in the control of Snf1 activation loop phosphorylation status. We are now faced with a new question: How is Snf1 dephosphorylation controlled by glucose levels? This question will be followed by new hypotheses, a few of which are described above, and new experimental

designs. The process is naturally regenerative: the cycle repeats over and over, and the inquisitive mind will never exhaust the vast store of phenomena present in all of creation.

In addition to the personal satisfaction of scientific investigation, participating in basic biomedical research is a venture beneficial to humanity at large, elevating those who participate in and reap the benefits of this labor. Discoveries made at the laboratory bench contribute directly and indirectly to our understanding of human health and disease and pave the way for improved preventative behavior and treatments. The AMPK signaling pathway is emerging as an integral component of multiple areas of human health. It is hoped that the work presented in this dissertation will contribute to improved understanding and treatment of diabetes and other metabolic disorders, heart disease, and cancer.

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