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Regulatory Domains of Snf1-Activating Kinases Determine Pathway Specificity

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In *Saccharomyces cerevisiae*, the Snf1 kinase can be activated by any one of three upstream kinases, Sak1, Tos3, or Elm1. All three Snf1-activating kinases contain serine/threonine kinase domains near their N termini and large C-terminal domains with little sequence conservation and previously unknown function. Deletion of the C-terminal domains of Sak1 and Tos3 greatly reduces their ability to activate the Snf1 pathway. In contrast, deletion of the Elm1 C-terminal domain has no effect on Snf1 signaling but abrogates the ability of Elm1 to participate in the morphogenetic-checkpoint signaling pathway. Thus, the C-terminal domains of Sak1, Tos3, and Elm1 help to determine pathway specificity. Additional deletion mutants of the Sak1 kinase revealed that the N terminus of the protein is essential for Snf1 signaling. The deletion of 43 amino acids from within the N terminus of Sak1 (residues 87 to 129) completely blocks Snf1 signaling and activation loop phosphorylation in vivo. The Sak1 kinase domain (lacking both N-terminal and C-terminal domains) is catalytically active and specific in vitro but is unable to promote Snf1 signaling in vivo when expressed at normal levels. Our studies indicate that the kinase domains of the Snf1-activating kinases are not sufficient by themselves for their proper function and that the nonconserved N-terminal and C-terminal domains are critical for the biological activities of these kinases.

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 (5). When activated, these kinases conserve cellular energy by promoting processes that generate cellular ATP while inhibiting those that consume ATP (19). 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 (18). 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 (4) by a distinct upstream kinase (11). Recently, we identified Sak1 as a Snf1-activating kinase (12). Sak1 associates with Snf1 and is capable of phosphorylating the activation loop threonine of Snf1 in vivo and in vitro (3, 12). 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 (7, 17). 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 (6, 10). 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 alpha mutations and was originally designated *PAK1* for *P*olymerase *A*lpha *K*inase (8). 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 (3). 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 (13). 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 (2). 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 (13). The C terminus of the yeast Sky1 kinase interacts with the

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TABLE 1. Plasmids used in this study

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

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

TABLE 2. Protein deletion constructs[a]

Kinase	AAs present	AAs deleted
Sak1	1–1142	
Sak1ΔC	1–500	501–1142
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	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

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

activation loop, holding the kinase in a constitutively active conformation (14). 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.

MATERIALS AND METHODS

Yeast strains and media. Yeast strains used in this study were MSY857 (*MAT*α *ura3*Δ*0 leu2*Δ*0 his3*Δ*1 met15*Δ*0 sak1*Δ::*KAN elm1*Δ::*KAN tos3*Δ::*KAN*) and MSY876 (*MAT*α *ura3 leu2 his3 sak1*Δ::*KAN elm1*Δ::*KAN tos3*Δ::*KAN snf1*Δ*10*). Yeast was grown at 30°C in standard media (15). Glucose was present at 2% or 0.05% (grams/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% (grams/100 ml) raffinose, 0.05% (grams/100 ml) glucose, and antimycin A (1 μg/ml).

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 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 2.

Protein extraction. Cultures of yeast cells (25 to 50 ml) were harvested in mid-log phase (optical density at 600 nm [OD_{600}], 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 (2a), with bovine serum albumin as a standard.

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 (11) 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.

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 (16). Three independent cultures were assayed, and the mean values are shown in the relevant figures.

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/pmol) 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.

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.

RESULTS

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 Fig. 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 (Fig. 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 1 and 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 (Fig. 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 (Fig. 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 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.

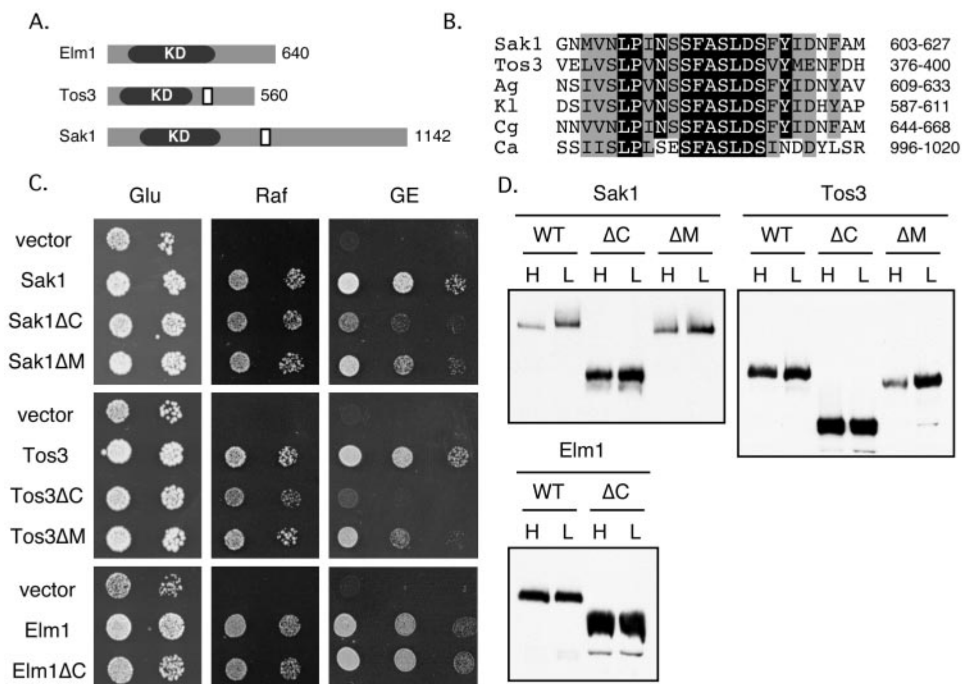

FIG. 1. C-terminal-deletion mutants of the Snf1-activating kinases. (A) Schematic representation of the Elm1, Tos3, and Sak1 kinases drawn to scale. The positions of the kinase domains (KD) and a conserved motif (open box) are indicated. (B) Sequence alignment of the conserved motif found in the C-terminal 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 glucose (H) or after shifting to low glucose (0.05%) for 3 hours (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.

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 (Fig. 2). Consistent with the growth phenotypes shown in Fig. 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 required for Snf1 function; however, those of Sak1 and Tos3 are needed for efficient Snf1 signaling.

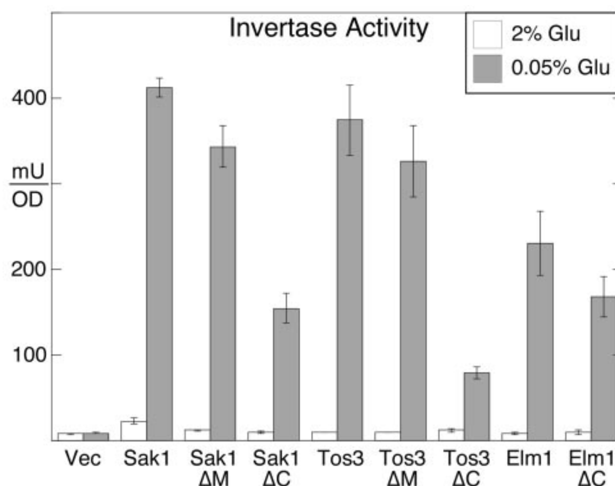

FIG. 2. Invertase expression in cells with Sak1, Tos3, and Elm1 mutations. Invertase activity was assayed with cells grown in 2% glucose (Glu) or after shifting to 0.05% glucose for 3 hours. Cells lacked all three Snf1-activating kinases but were transformed with low-copy-number plasmids expressing either 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 1 standard error. mU/OD, μmole glucose/min/OD of cells assayed.

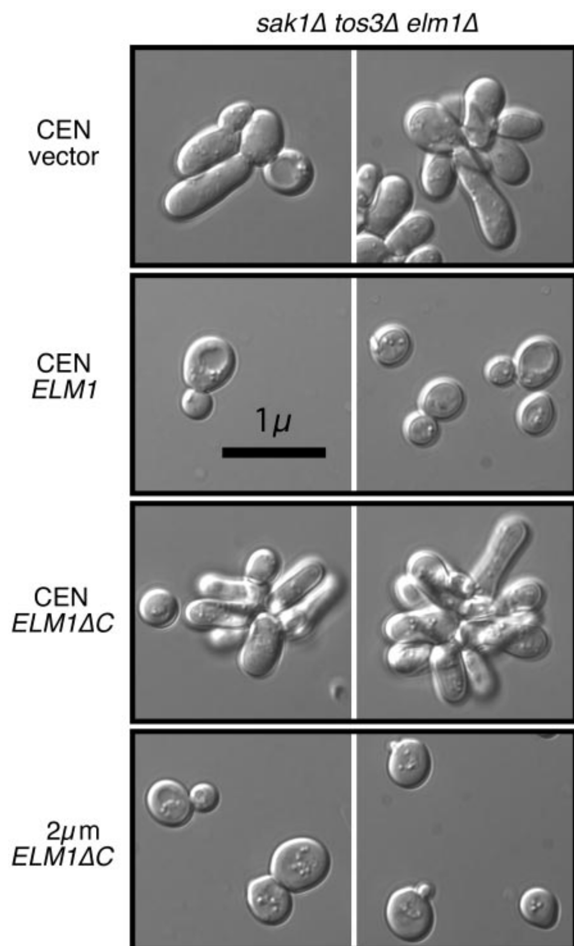

FIG. 3. Cell morphology of *elm1* mutants. Cell morphology was analyzed by differential interference contrast microscopy. Cells lacked all three Snf1-activating kinases but were transformed with either low-copy-number (CEN) or high-copy-number (2μm) plasmids expressing no kinase (vector), full-length Elm1 kinase (*ELM1*), or a C-terminal truncation of the Elm1 kinase (*ELM1ΔC*). Bar, 1 μm.

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 (Fig. 1C and 2). Indeed, by analysis of growth assays (Fig. 1C), Elm1ΔC activates Snf1 as well as full-length Elm1 does. Because Elm1 is also required for the morphogenetic checkpoint (1), 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 (Fig. 3, row 1). A low-copy-number plasmid (CEN) encoding full-length Elm1 rescues cells from this abnormal morphology (Fig. 3, row 2), while a low-copy-number plasmid expressing Elm1ΔC does not (Fig. 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 (Fig. 3, row 4), suggesting that the increased abundance of the kinase activity of Elm1 can compensate for the loss of its C terminus.

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 (3, 6, 10), 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 (Fig. 4A). Similarly, Sak1 constructs lacking their N termini are unable to induce invertase expression (Fig. 4B). All constructs were stably expressed at comparable levels, as judged by Western blotting (Fig. 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.

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 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 (3). Sak1ΔNΔC protein promotes phosphorylation of the GST-Snf1 kinase domain (Fig. 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 (Fig. 5, lane 4). Equivalent levels of Sak1ΔNΔC protein were present in the reactions, as judged by Western blotting of immune complexes run in parallel (Fig. 5B). We conclude that 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 (Fig. 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 (Fig. 3).

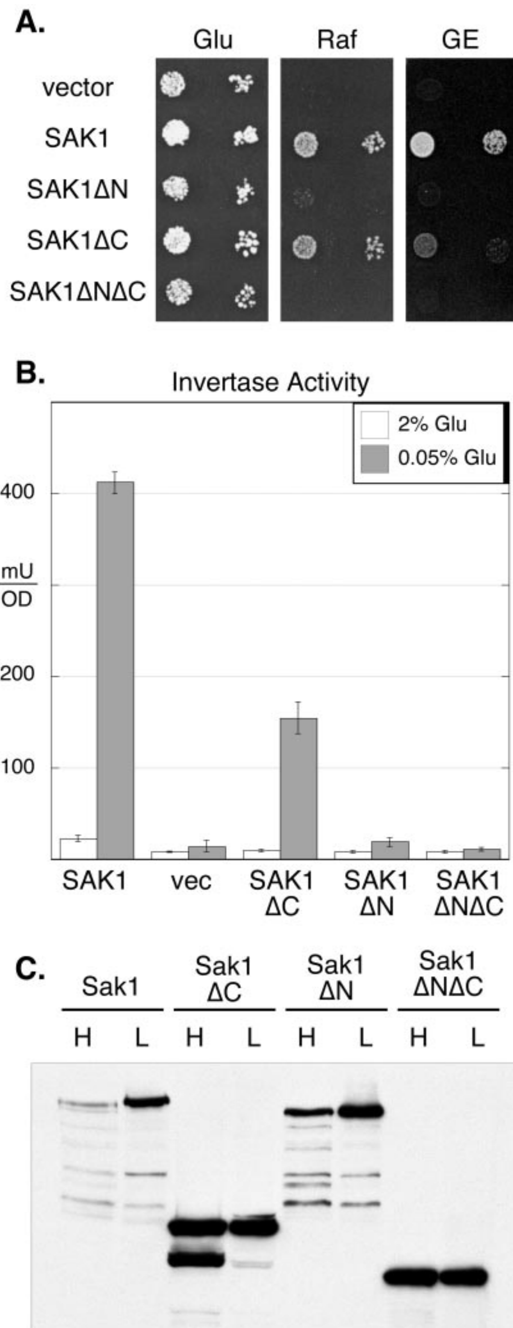

FIG. 4. Growth phenotypes and invertase expression in cells lacking the N or C terminus of Sak1. Cells lacking all three Snf1-activating kinases were transformed with low-copy-number plasmids expressing no kinase (vector), full-length Sak1 kinase, Sak1ΔN, Sak1ΔC, or Sak1ΔNΔC. (A) Serial dilutions of 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 hours. The mean invertase activity level for three independent transformants is plotted. Error bars represent 1 standard error. (C) Western blot of V5-tagged Sak1 proteins. Protein extracts were prepared from cells grown in high glucose (H) or after 3 hours 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.

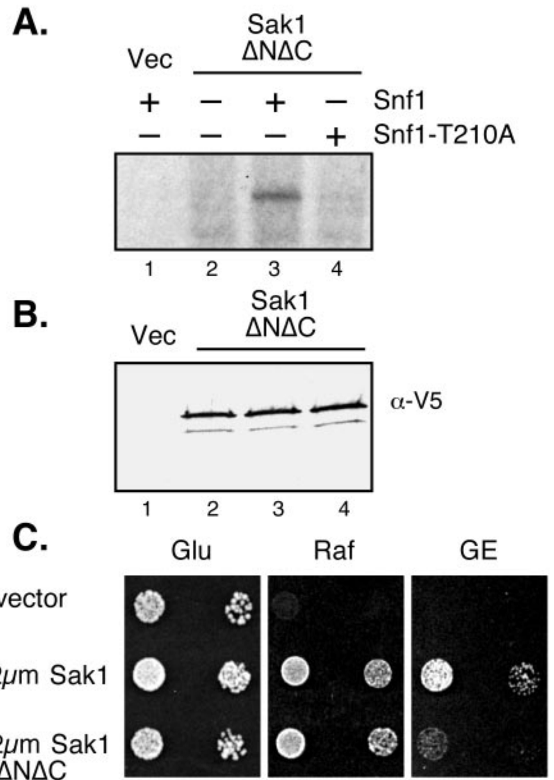

FIG. 5. The Sak1 kinase domain is catalytically active. (A) In vitro kinase assay. A *snf1*Δ *sak1*Δ *tos3*Δ *elm1*Δ yeast strain was transformed with a high-copy-number plasmid expressing V5-tagged Sak1ΔNΔC protein or with plasmid vector (Vec). Sak1ΔNΔC protein was immunoprecipitated with anti-V5 epitope 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 shown 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.

Localization of critical residues in the N terminus of Sak1. Since the amino terminus of Sak1 is required for Sak1 function (Fig. 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 (Fig. 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 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 (Fig. 7A) and induced invertase in

FIG. 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 Fig. 1B. The demarcations between ΔN1 and ΔN2 and between ΔN2 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.

low glucose (Fig. 7B). In contrast, Sak1ΔN2 failed to function in the Snf1 pathway, as demonstrated by the failure to induce invertase (Fig. 7B) or to promote growth on raffinose or glycerol and ethanol (Fig. 7A). All of the N-terminal-deletion constructs were expressed at equivalent levels in vivo, as judged by Western blotting (Fig. 7C). We conclude that Sak1 residues 87 to 129 are essential for Sak1 function in vivo.

Effect of deletions on Sak1's ability to phosphorylate the activation loop of Snf1. Upon glucose limitation, Sak1, Tos3, and Elm1 activate Snf1 by phosphorylation of threonine 210 (T210) in the activation loop (7, 17). 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 (11). When glucose is limiting, Snf1 threonine 210 is phosphorylated in cells expressing full-length Sak1 (Fig. 8A, lanes 1 and 2). When the carboxy terminus of Sak1 is deleted, Snf1 is still phosphorylated exclusively in low glucose, though the level of T210 phosphorylation is greatly reduced (Fig. 8, 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 (Fig. 8, lanes 7 and 8). Interestingly, this same construct was able to specifically phosphorylate the activation loop of Snf1 in vitro (Fig. 5). This analysis was extended to include the smaller deletions within the Sak1 N terminus. Deletion of the first half of the N terminus (ΔN1) 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 (ΔN2) completely blocked the ability of Sak1 to phosphorylate the activation loop of Snf1 (Fig. 8B). Equivalent levels of Snf1 protein are expressed in all the samples, as judged by Western blotting with anti-HA antibody (Fig. 8B, 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.

DISCUSSION

The Snf1-activating kinases of *S. cerevisiae* contain conserved kinase domains as well as large domains that contain only limited sequence conservation (Fig. 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 (Fig. 1C), impaired invertase induction (Fig. 2), and, in the case of Sak1, impaired phosphorylation of the Snf1 activation loop. 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 (17). In that study, the truncated Elm1 variant activated Snf1 more robustly than did full-length Elm1 but was unable to complement an *elm1* 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 Snf1, whereas Elm1ΔC associates better with Snf1 than with proteins at the bud neck.

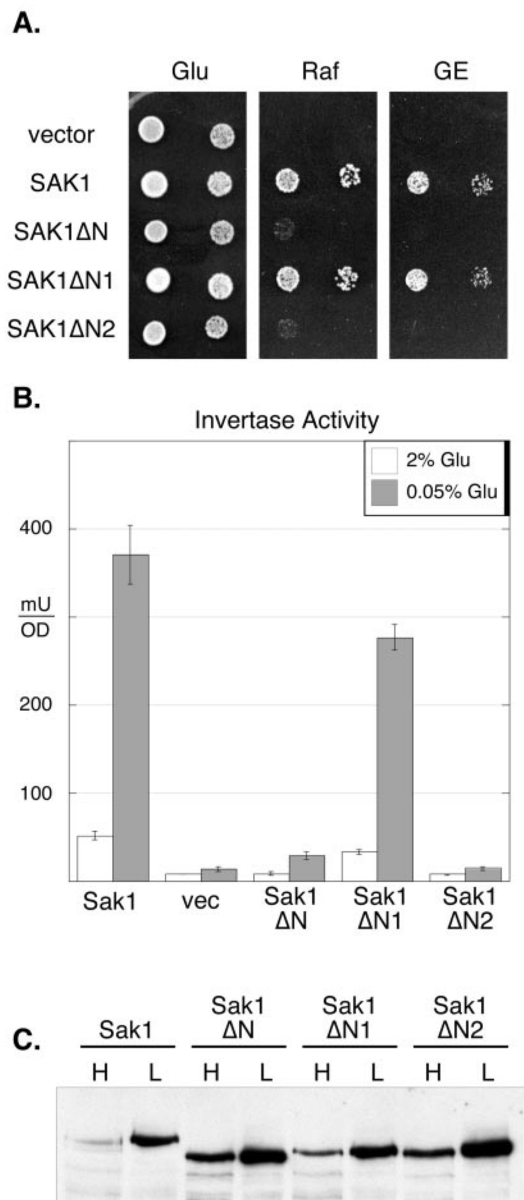

FIG. 7. Identification of critical residues in the Sak1 amino terminus. Cells lacking all three Snf1-activating kinases were transformed with a low-copy-number plasmid expressing an empty vector (vector), full-length Sak1, or Sak1 mutants lacking the entire N terminus (ΔN), the first half of the N terminus (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 hours. The mean invertase activity level for three independent transformants is plotted. Error bars represent 1 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 hours in low glucose (L) were analyzed directly by Western analysis with anti-V5 antibodies.

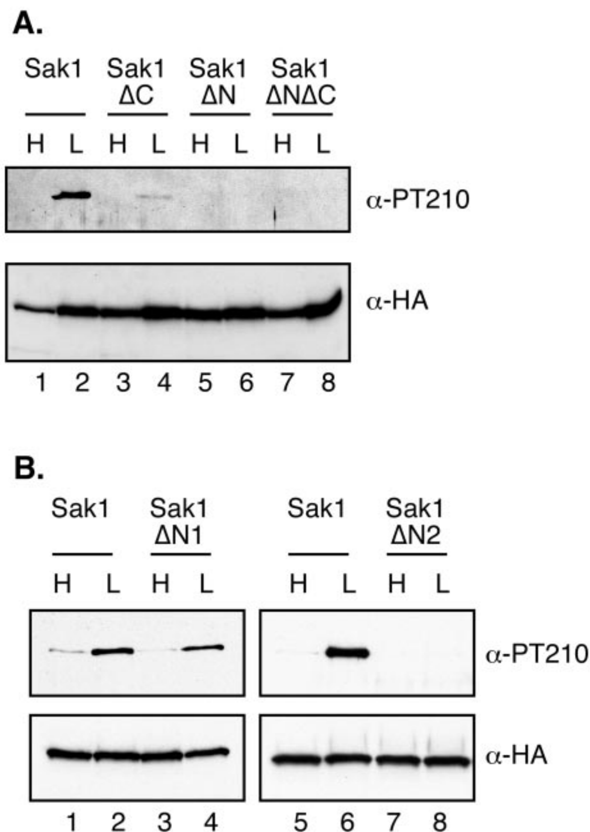

FIG. 8. Snf1 activation loop phosphorylation in Sak1 mutant strains. Cells lacking all three Snf1-activating kinases were cotransformed with two low-copy-number plasmids expressing Snf1-HA and full-length Sak1 or deletion mutants of Sak1, as indicated in the legend to Fig. 4. Protein extracts were prepared from cells grown in medium containing high glucose (H) or after 3 hours 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).

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* mutants (Fig. 3). The Tos3 protein kinase is localized in the cytoplasm in high and low glucose (9), while Sak1 appears to be localized in the cytoplasm in high glucose and at the vacuolar periphery in low glucose (6). 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 (Fig. 7A), invertase induction (Fig. 7B), and Snf1 activation loop phosphorylation (Fig. 8B). A small domain of Sak1 included in the ΔN2 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 (Fig. 5). Therefore, this small domain of Sak1 must play some critical regulatory role that remains to be elucidated by further studies.

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