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RESEARCH ARTICLE

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Snf1 kinase complexes with different beta subunits display stress-dependent preferences for the three Snf1-activating kinases

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Abstract Three upstream kinases, Pak1, Tos3 and Elm1, are able to activate the Snf1 kinase. Since the Snf1 kinase itself assembles into three complexes that differ in their beta subunit identity, the possibility exists that each upstream kinase might be dedicated to a single isoform of the Snf1 kinase. To test this dedicated activator hypothesis, we generated a series of yeast strains that lacked different combinations of upstream kinases and beta subunits. Cells expressing only one of the three upstream kinases exhibited distinct abilities to activate Snf1, depending on the beta subunit present in the Snf1 kinase complex and the stress imposed on the cells. Pak1 and Gal83 were the most promiscuous. Pak1 was able to activate all three isoforms of the Snf1 kinase under all stress conditions tested. The Gal83 isoform of Snf1 was able to be activated by any of the three upstream kinases under aerobic growth conditions but showed a preference for Pak1 during growth on raffinose. Our results indicate that the three Snf1-activating kinases are not dedicated to specific isoforms of the Snf1 kinase. Instead, the different isoforms of the Snf1 kinase display stress-dependent preferences for the Pak1, Tos3 and Elm1 kinases.

Keywords Snf1 kinase · Snf1-activating kinase · Pak1 · Activation loop · Beta subunit

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 (Hardie et al. 1998). In laboratory strains grown in glucose medium, once the glucose is depleted Snf1 kinase becomes active as cells prepare to switch to aerobic metabolism (Carlson 1999). Many of the genes whose expression is induced at the diauxic shift are not induced properly in cells lacking Snf1 kinase (Young et al. 2003).

The Snf1 kinase complex is a heterotrimer composed of one alpha, one beta and one gamma subunit. The alpha subunit contains the catalytic kinase domain and is encoded by the *SNF1* gene (Celenza and Carlson 1986). The gamma subunit, encoded by the *SNF4* gene, plays a regulatory role and is required for the full activation of Snf1 (Celenza et al. 1989). The beta subunit physically interacts with both the alpha and gamma subunits and may act in part to hold the heterotrimer together (Jiang and Carlson 1997). *S. cerevisiae* express three distinct beta subunits encoded by the *SIP1*, *SIP2* and *GAL83* genes. At least one of the beta subunits must be expressed for Snf1 activity in vivo (Schmidt and McCartney 2000). The presence of three distinct isoforms of the Snf1 kinase suggests that the beta subunits may provide for specialization of function. Indeed, the beta subunits control the subcellular localization of the kinase (Vincent et al. 2001) and play a role in substrate selection (Vincent and Carlson 1999; Schmidt and McCartney 2000).

Activation of Snf1 kinase is a complicated process involving at least two steps (McCartney and Schmidt 2001). 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 (Jiang and Carlson 1996). A second step required for Snf1 activation is the phosphorylation of a

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conserved threonine residue in the Snf1 activation loop by a distinct upstream kinase (McCartney and Schmidt 2001). We identified the Pak1 kinase of *S. cerevisiae* as one of the Snf1-activating kinases. Pak1 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 (Nath et al. 2003). However, deletion of the *PAK1* 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: Pak1, Tos3 and Elm1 (Hong et al. 2003; Sutherland et al. 2003).

Relatively little is known about the Pak1 and Tos3 kinases. Pak1 was isolated as a high-copy suppressor of mutations in the DNA polymerase alpha gene and named polymerase alpha kinase (Hovland et al. 1997). *TOS3* was identified in a genomic screen for promoters bound by the cell cycle-regulated transcription factor SBF (Iyer et al. 2001). 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 (Cho et al. 1998; Spellman et al. 1998). 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 (Garrett 1997; Koehler and Myers 1997). Elm1 protein is localized at the bud neck (Bouquin et al. 2000), where it assembles in a complex with other proteins (Thomas et al. 2003) and plays a role in controlling bud emergence and septin organization (Sreenivasan et al. 2003). The finding that Elm1 also functions in carbon source-sensing pathways (Hong et al. 2003; Sutherland et al. 2003) may reflect the integration of carbon source-response pathways with filamentous growth pathways (Cullen and Sprague 2000).

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 beta 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 Pak1 kinase played an important role in controlling the activity and localization of the Gal83 isoform in response to glucose stress (Hedbacker et al. 2004a). 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.

Materials and methods

Yeast strains, media and genetics methods

The *S. cerevisiae* strains used in this study are described in Table 1. Growth of cells utilized standard media at 30°C (Rose et al. 1990). Raffinose medium contained 2% raffinose, 0.01% glucose and 1 mg/ml antimycin A. Glycerol/ethanol medium contained a mixture of 3% (v/v) glycerol and 2% (v/v) ethanol. For testing inositol phenotypes, synthetic complete medium was made with yeast nitrogen base lacking inositol (Qbiogene, Carlsbad, Calif.). Strains with multiple gene 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.

Epitope tagging and plasmid constructions

The Mig1 and Snf1 proteins were tagged with three copies of the hemagglutinin(HA) epitope at their C-termini (Schmidt and McCartney 2000; McCartney and Schmidt 2001) and expressed from their cognate promoters on low-copy-number plasmids. The Pak1, Tos3 and Elm1 proteins were all tagged with the V5 epitope (Southern et al. 1991) 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 (Sikorski and Hieter 1989), such that expression was driven from each gene's native promoter.

Enzyme 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 (Ganster et al. 1998; Schmidt et al. 1999).

Western analysis

Tagged proteins were detected by Western blotting (Schmidt et al. 1999), 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 mg protein) with anti-HA antibodies prior to Western analysis with antisera specific

Table 1 *S. cerevisiae* strains

Strain	Genotype
MSY182	*MAT*a *ura3-52 leu2Δ1 trp1Δ63 his3Δ200*
FY1193	*MAT*α *ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63 snf1-Δ10*
MSY676	*MAT*α *ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 pak1Δ::KAN*
MSY690	*MAT*a ura3Δ 0 leu2Δ 0 his3Δ 1 lys2Δ 0 elm1Δ ::KAN
MSY683	*MAT*α *ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 tos3Δ::KAN*
MSY694	*MAT*α*ura3Δ0 leu2Δ0 his3Δ1 lsy2Δ0 pak1Δ::KAN tos3Δ::KAN*
MSY854	*MAT*α*ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 pak1Δ::KAN elm1Δ::KAN*
MSY697	*MAT*α*ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 tos3Δ::KAN elm1Δ::KAN*
MSY858	*MAT*α *ura3Δ0 leu2Δ0 his3Δ1 pak1Δ::KAN elm1Δ::KAN tos3Δ::KAN*
MSY807	*MAT*a *ura3Δ0 leu2Δ0 his3Δ1 pak1Δ::KAN snf1Δ10*
MSY863	*MAT*a *ura3 leu2 his3 tos3Δ::KAN snf1Δ10*
MSY867	*MAT*a *ura3 leu2 his3 trp1Δ63 pak1Δ::KAN elm1Δ::KAN snf1Δ10*
MSY870	*MAT*α *ura3 leu2 his3 lys2Δ0 elm1Δ::KAN tos3::KAN snf1Δ10*
MSY871	*MAT*a *ura3 leu2 his3 trp1Δ63 lys2Δ0 met15Δ0 elm1Δ::KAN snf1Δ10*
MSY874	*MAT*a *ura3 leu2 his3 met15Δ0 pak1Δ::KAN tos3::KAN snf1Δ10*
MSY876	*MAT*α *ura3 leu2 his3 pak1*D*::KAN elm1Δ::KAN tos3::KAN snf1Δ10*
MSY553	*MAT*α *ura3-52 trp1Δ63 his3Δ200 sip2Δ::HIS3 gal83Δ::HIS3*
MSY893	*MAT*a *ura3 leu2Δ0 his3 sip2Δ::HIS3 gal83Δ::HIS3 tos3Δ::KAN elm1Δ::KAN*
MSY882	*MAT*a *ura3 leu2Δ0 his3 sip2Δ::HIS3 gal83Δ::HIS3 pak1Δ::KAN elm1Δ::KAN*
MSY891	*MAT*α *ura3 leu2Δ0 his3 met15Δ0 sip2Δ::HIS3 gal83Δ::HIS3 pak1Δ::KAN tos3Δ::KAN*
MSY549	*MAT*α *ura3-52 trp1Δ63 his3Δ200 sip1Δ::HIS3 gal83Δ::HIS3*
MSY881	*MAT*α *ura3 leu2Δ0 his3 lys2Δ0 sip1Δ::HIS3 gal83Δ::HIS3 elm1Δ::KAN tos3Δ::KAN*
MSY885	*MAT*α *ura3 his3 lys2Δ0 sip1Δ::HIS3 gal83Δ::HIS3 pak1Δ::KAN elm1Δ::KAN*
MSY880	*MAT*a *ura3 leu2Δ0 his3 lys2Δ0 sip1Δ::HIS3 gal83Δ::HIS3 pak1Δ::KAN tos3Δ::KAN*
MSY886	*MAT*α *ura3 leu2Δ0 his3 lys2Δ0 sip1Δ::HIS3 gal83Δ::HIS3 pak1Δ::KAN tos3Δ::KAN elm1Δ::KAN*
MSY544	*MAT*α *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 sip1Δ::HIS3 sip2Δ::HIS3*
MSY888	*MAT*a *ura3 leu2Δ0 his3 lys2Δ0 met15Δ0 sip1Δ::HIS3 sip2Δ::HIS3 tos3Δ::KAN elm1Δ::KAN*
MSY883	*MAT*α *ura3 leu2 his3 sip1Δ::HIS3 sip2Δ::HIS3 pak1Δ::KAN elm1Δ::KAN*
MSY889	*MAT*α *ura3 leu2Δ0 his3 lys2Δ0 met15Δ0 sip1Δ::HIS3 sip2Δ::HIS3 pak1Δ::KAN tos3Δ::KAN*
MSY892	*MAT*α *ura3 his3 lys2Δ0 sip2::HIS3 gal83::HIS3 pak1Δ::KAN tos3Δ::KAN elm1Δ::KAN*
MSY890	*MAT*α *ura3 leu2Δ0 trp1Δ63 his3 met15Δ0 sip1::HIS3 sip2::HIS3 pak1Δ::KAN tos3Δ::KAN elm1Δ::KAN*

for the Snf1 protein phosphorylated on threonine 210 (McCartney and Schmidt 2001).

Results

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, Pak1, Tos3 and Elm1 (Table 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 (Fig. 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 *pak1*Δ *elm1*Δ, *tos3*Δ *elm1*Δ and *pak1*Δ *tos3*Δ strains were able to grow on these carbon sources, indicating that Pak1, Tos3 and Elm1 kinases alone were able to activate sufficient Snf1 for growth under these conditions. Consistent with earlier reports (Hong et al. 2003; Sutherland et al. 2003), strains lacking all three Snf1-activating kinases displayed a strong Snf phenotype similar to that observed in *snf1Δ10* cells (Fig. 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 grown in the presence of abundant

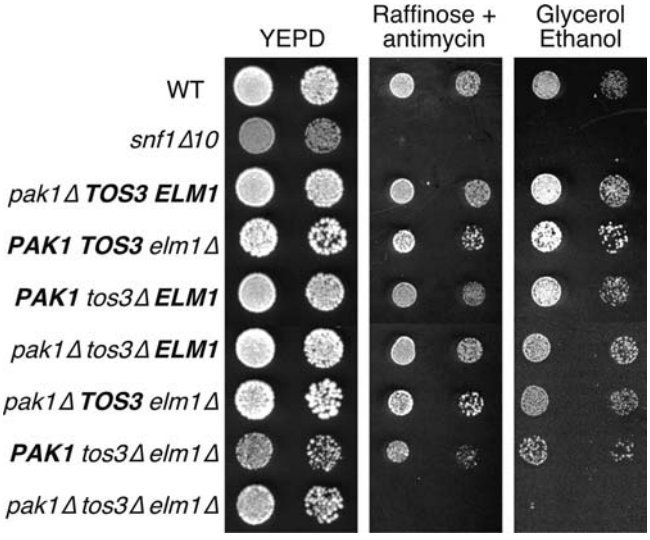


Fig. 1 Growth phenotypes of Snf1-activating kinase mutants on different carbon sources. Serial dilutions of yeast cultures were spotted onto rich medium with glucose (*YEPD*) or synthetic complete medium with either raffinose plus antimycin A, or a mixture of glycerol and ethanol as the carbon sources. The relevant genotypes of the yeast strains are shown on the *left*. *WT* Wild type

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 (Fig. 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 Pak1 as the only Snf1-activating kinase showed relatively normal regulation of invertase expression. Thus, Pak1, Tos3 and Elm1 are each individually capable of promoting proper regulation of invertase in response to glucose limitation.

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 (Treitel et al. 1998; Smith et al. 1999). Mig1 phosphorylation can be detected by Western blotting as a reduction in its SDS gel mobility (Treitel and Carlson 1995). We examined the ability of cells expressing a single Snf1-activating kinase to phosphorylate Mig1 protein in response to glucose limitation (Fig. 3). As reported by Sutherland et al. (2003), the phosphorylation of Mig1 is not observed in cells lacking Snf1 or all three of the Snf1-activating kinases, whereas in wild-type cells, Mig1 is phosphorylated when grown in low glucose (lanes 1, 2). Cells expressing only 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.

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

Previously, we showed that Pak1 kinase is able to phosphorylate the activation loop threonine of Snf1 (Nath et al. 2003). Here, we show that, in the absence of Pak1, Tos3 and Elm1 kinases, the Snf1 activation loop threonine shows absolutely no evidence of phosphorylation under low glucose conditions (Fig. 4a). Therefore, Pak1, 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 (Fig. 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, 13). This finding is

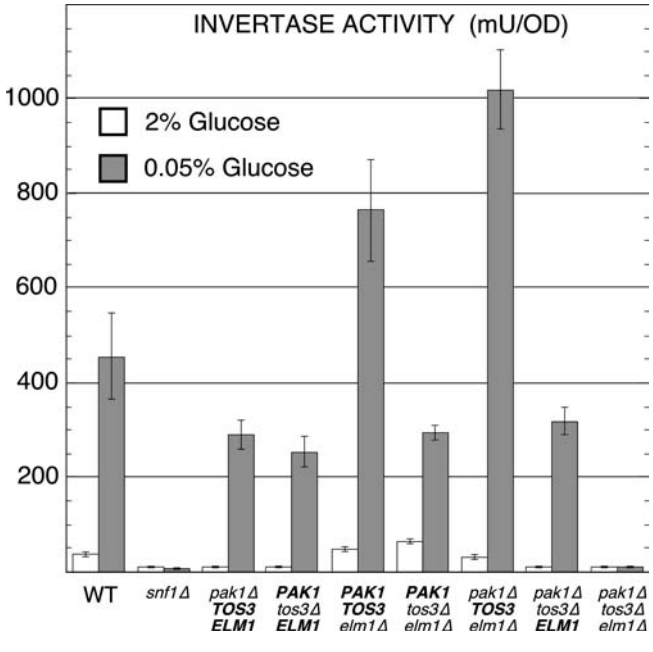


Fig. 2 Invertase expression in Snf1-activating kinase mutants. Invertase expression was assayed in cells grown in 2% glucose or after shifting to 0.05% glucose for 3 h. The relevant genotypes of the yeast strains are shown. The mean invertase expression from three independent cultures is plotted. *Error bars* represent one standard error. *OD* Optical density units, *U* units of enzyme activity

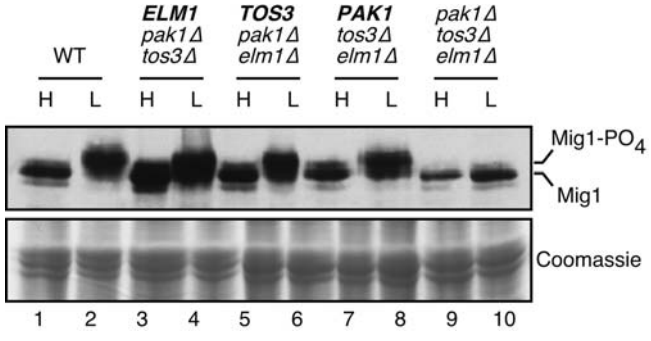


Fig. 3 Phosphorylation of Mig1 by Snf1-activating kinase mutants. Protein extracts were prepared from cells expressing HA-tagged Mig1 after growth in the presence of high (*H*) or low (*L*) glucose. A Western blot (*upper panel*) was performed with monoclonal antibodies directed against the HA epitope. 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*)

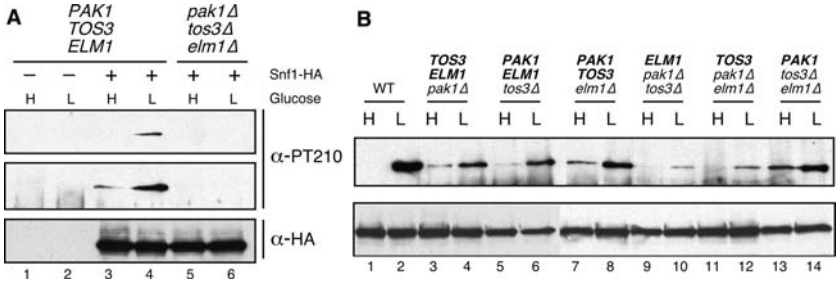


Fig. 4 Phosphorylation of Snf1 threonine 210 requires Snf1-activating kinases. Snf1 protein was immune precipitated with HA antibody from protein extracts of cells grown in either high (*H*) or low (*L*) glucose. Precipitates were analyzed by Western blotting, using either anti-HA antibodies (α-HA) or antibodies specific for the Snf1 protein phosphorylated on threonine 210 (α-PT210). **a** Wild-type cells (*lanes 1–4*) or *pak1*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*, *2*). A light and dark exposure of the α-PT210 Western is shown. **b** Wild-type cells (*lanes 1*, *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

consistent with the higher levels of invertase expression observed in these cells in the presence of high glucose (Fig. 2).

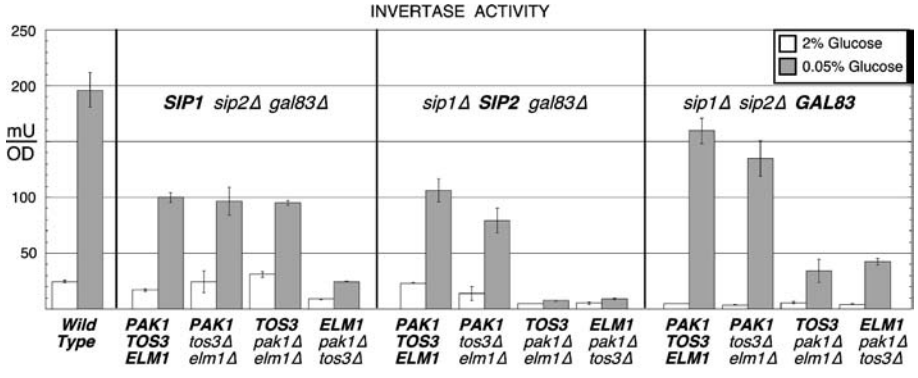
Beta subunits of Snf1 kinase complexes confer strong upstream kinase preferences

Yeast strains were constructed such that they expressed a single beta subunit in combination with a single upstream kinase. Each strain was tested for invertase expression under high- and low-glucose conditions (Fig. 5). Cells expressing Sip1 as the only beta subunit in combination with all three upstream kinases or with Pak1 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 beta subunit, only Pak1 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 beta subunit, a clear preference for Pak1 was observed, although Tos3 and Elm1 were individually able to function at a reduced level. We conclude that the beta 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.

Fig. 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 *PAK1*, *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

Upstream kinase preference is stress-dependent

Cells expressing a single beta 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 promoted transcriptional induction (derepression) of the *INO1* gene, a process which requires Snf1 kinase (Shirra and Arndt 1999). Cells expressing Sip1 as their only beta subunit were able to ferment raffinose in combination with either Pak1 or Tos3 but not Elm1 as the upstream kinase (Fig. 6). As reported by Schmidt and McCartney (2000), cells expressing Sip1 as the only beta subunit were not able to grow aerobically and further deletion of upstream kinases did not suppress this growth defect. Growth in the absence of inositol was supported by Pak1 and Elm1 but not Tos3. Thus, the Sip1 isoform of the Snf1 kinase displays strong stress-dependent preferences for its upstream activating kinase. Cells expressing Sip2 as the



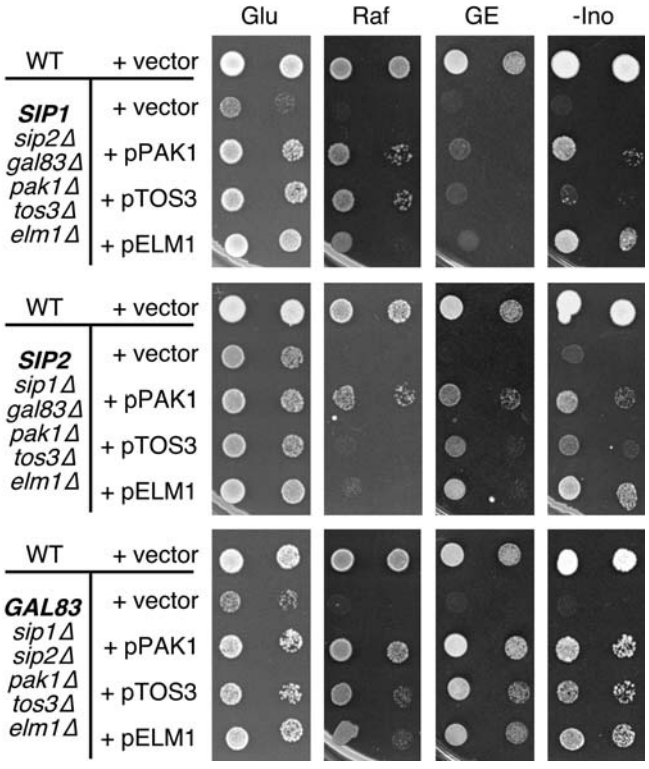


Fig. 6 Growth phenotypes of cells expressing a single beta subunit and upstream kinase. Serial dilutions of yeast cultures were spotted onto synthetic complete medium lacking uracil, either with glucose, raffinose plus antimycin A or a mixture of glycerol and ethanol as the carbon sources or with glucose as the carbon source and without inositol. 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

only beta subunit were able to ferment raffinose only when Pak1 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 (Figs. 5, 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 beta subunit, any one of the upstream kinases was able to provide Snf1 activation for growth on raffinose, although Pak1 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.

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

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 coding the Snf1 kinase beta subunits might affect the abundance of the upstream kinases, we measured the abundance of the upstream kinases by Western blotting (Fig. 7). Strains were constructed in which all three genes for the upstream kinases were deleted (*pak1*Δ *tos3*Δ *elm1*Δ). In addition, cells expressed either all three beta subunits of the Snf1 kinase (denoted as WT) or only a single beta 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 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). Pak1 and

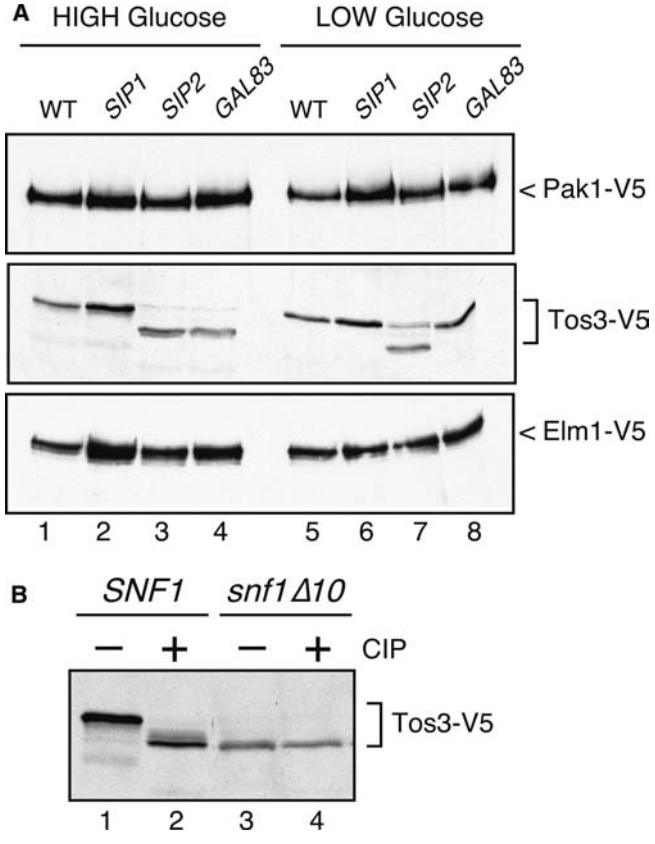


Fig. 7 Accumulation of upstream kinases in cells expressing different isoforms of the Snf1 kinase. **a** Strains with all three upstream kinases deleted (*pak1*Δ *tos3*Δ *elm1*Δ) were transformed with CEN plasmids expressing a single V5-tagged upstream kinase, as indicated on the *right*. In addition, cells contained genes either for all three beta subunits (*WT*) or for a single beta 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 beads and analyzed by Western blotting, using anti-V5 antibodies. **b** Tos3-V5 mobility shift is due to Snf1-dependent phosphorylation. Tos3-V5 protein was expressed in cells containing (*lanes 1*, *2*) or lacking (*lanes 3*, *4*) Snf1. Protein extracts were incubated at 37°C for 1 h with (*lanes 2*, *4*) or without (*lanes 1*, *3*) 5 units of calf intestine alkaline phosphatase (*CIP*). Samples were analyzed by Western blotting with anti-V5 antibodies

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 (Fig. 4b, lane 2). The phosphorylation is Snf1-dependent, since it is not observed in *snf1Δ10* cells (lane 3). We conclude that the failure to function of some pairings of upstream kinases with certain isoforms of Snf1 kinase 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 (Fig. 5). This defect is not due to a failure to express Elm1 kinase (Fig. 7a, lane 7).

Activation loop phosphorylation in strains expressing Sip2 as the only beta subunit

Since the Sip2 isoform of Snf1 kinase exhibited the most stringent preference for Pak1 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 beta 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. Snf1 protein was immunoprecipitated and the level of phosphorylation on threonine 210 was assayed by Western blot (Fig. 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 immune precipitates. When Pak1 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, 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 Pak1. 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.

Mig1 phosphorylation in strains expressing Sip2 as the only beta subunit

In growth and invertase assays that measured Snf1 activity, the Sip2 isoform of the Snf1 kinase displayed a distinct preference for the Pak1 kinase and yet Tos3 and Elm1 were also able to phosphorylate Snf1 on threonine

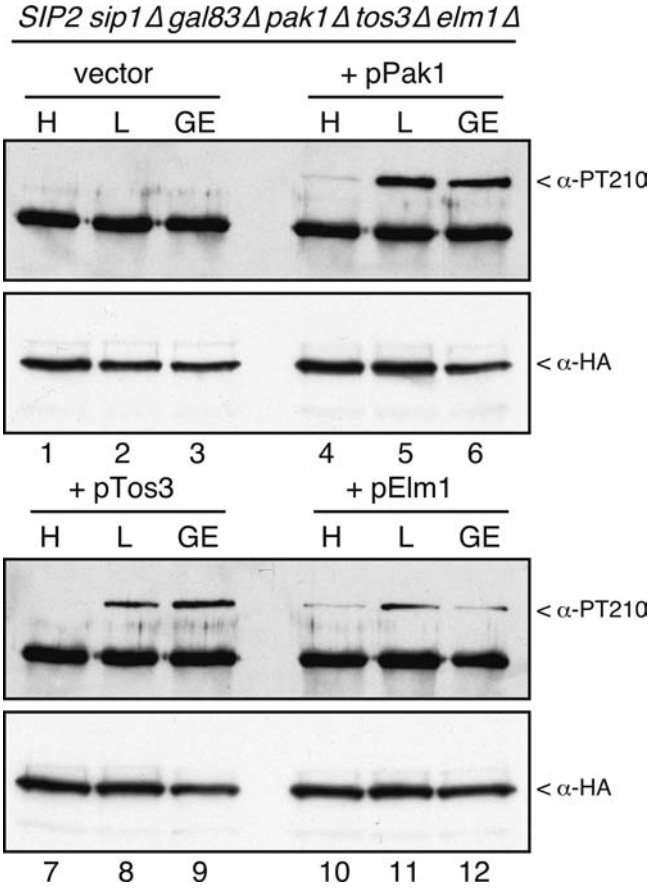


Fig. 8 Phosphorylation of the Snf1 activation loop in cells expressing the Sip2 isoform of the Snf1 kinase. Proteins were immunoprecipitated with HA antibody from protein extracts of cells grown in either high glucose (*H*), low glucose (*L*) or a mixture of glycerol and ethanol (*GE*). Precipitates 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 *PAK1* (*lanes 4–6*), *TOS3* (*lanes 7–9*) or *ELM1* (*lanes 10–12*) genes

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 beta subunit was examined by Western blot (Fig. 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 Pak1 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 (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, 11). This failure to effectively phosphorylate Mig1 in cells with only Sip2 and Tos3 or

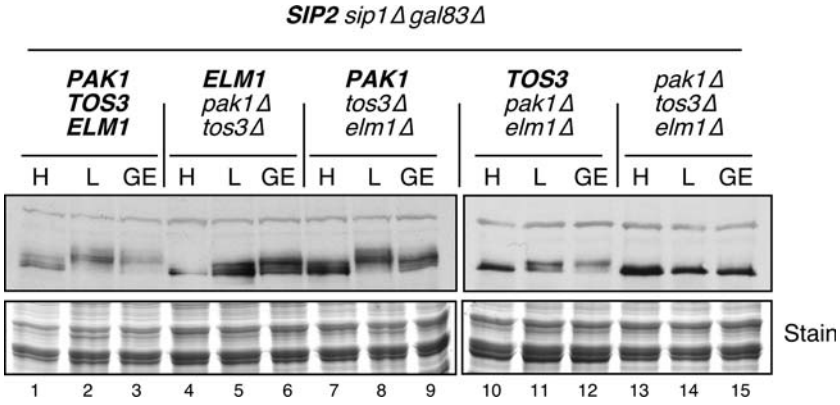


Fig. 9 Mig1 phosphorylation in cells expressing only the Sip2 isoform of the Snf1 kinase. Cells were transformed with a plasmid expressing HA-tagged Mig1 protein. Protein extracts were prepared from cells grown in synthetic complete medium containing either 2% glucose (*H*) or 3 h after shifting to 0.05% 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 beta subunit (*sip1Δ gal83Δ*). The different combinations of deletions in the Snf1-activating kinases are indicated

only Sip2 and Elm1 correlated with the poor growth properties of these cells on raffinose media (Fig. 6) and with their inability to induce invertase expression (Fig. 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, 6), consistent with the observation that cells expressing only Sip2 and Elm1 were able to grow aerobically (Fig. 6). The combination of Sip2 and Tos3 showed the lowest levels of Mig1 phosphorylation under both Snf1-activating conditions tested (lanes 11, 12) and yet Tos3 was able to promote activation loop phosphorylation of the Sip2 isoform of Snf1 (Fig. 7). These results suggest that additional levels of regulation of Snf1 kinase signaling must occur downstream of the activation loop phosphorylation step.

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 (Hedbacker et al. 2004a) disprove this very simplistic hypothesis. For instance, the Pak1 kinase is able to activate all three isoforms of the Snf1 kinase in response to low glucose, as judged by growth assays on raffinose (Fig. 6) and invertase induction (Fig. 5). Therefore, Pak1 is not dedicated to the activation of a single isoform of the Snf1 kinase. A similar conclusion was reached by Hedbacker et al. (2004a). 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 (Figs. 5, 6). Therefore, the Sip2 isoform of Snf1 kinase exhibited a strong preference for the Pak1 kinase under low glucose conditions. Interestingly, the Sip2 isoform of the Snf1 kinase expanded its activating kinase preference to include Pak1, Tos3 and Elm1 during growth on ethanol or in the absence of inositol (Fig. 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 upstream kinases and the preference varies, depending on both the beta 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 (Hohmann et al. 1999; Elbing et al. 2004). 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 beta subunit were able to induce invertase only in combination with Pak1 as the Snf1-activating kinase. Similarly, the combination of Sip2 with Pak1 showed much better growth on raffinose than the pairing of Sip2 with Tos3 or Elm1. Similar correlations were observed in Sip1 cells, where invertase induction with Pak1 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 beta subunits are localized to distinct compartments (Vincent et al. 2001; Huh et al. 2003) and that their localization is dynamic, changing in response to the carbon source (Vincent et al. 2001; Hedbacker et al. 2004b). The localization of the upstream kinases is less well defined. Elm1 kinase displays a very restricted localization, limited to the bud neck (Moriya and Isono 1999; Bouquin et al. 2000; Huh et al. 2003). 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 (Huh et al. 2003). The Pak1-GFP expressed from its genomic locus exhibits a cytosolic localization in high glucose (Hedbacker et al. 2004a). In low glucose, some of the Pak1-GFP fluorescence shifts to the outer vaculolar membrane. A similar shift is observed for Sip1 (Hedbacker et al. 2004b). 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 (Figs. 5, 6). Yet, the activation loop threonine of Snf1 was phosphorylated by Tos3 when Sip2 was the only beta subunit present (Fig. 7). 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 (Fig. 8). 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 beta and gamma subunits (Ghaemmaghami et al. 2003). All evidence for Snf1 kinase stoichiometry, including silver-stained gels of purified Snf1 complexes (Nath et al. 2002), suggests that the three subunits are associate in a 1:1:1 stoichiometry. Therefore, the three beta subunits, possibly pre-bound to the abundant gamma subunit (Snf4), must compete for binding to the far fewer molecules of Snf1. Of all the beta 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 (Young et al. 2003) and growth on ethanol (Gasch et al. 2000) 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 (Vincent et al. 2001). 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 beta 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 Pak1 is the predominant activator of Snf1 in vivo. First, Pak1 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 Pak1, but not Tos3 or Elm1 (Gavin et al. 2002; Ho et al. 2002). 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 (Bouquin et al. 2000; Mortensen et al. 2002; Thomas et al. 2003). Further experiments using techniques more subtle than gene deletions will be needed to determine whether Pak1 is the primary activator of Snf1 in vivo.

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