Snf1-Like Protein Kinase Ssp2 Regulates Glucose Derepression in Schizosaccharomyces pombe

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The function of two fission yeast genes, SPCC74.03c/ssp2+ and SPAC23H4.02/ppk9+, encoding an Snf1-like protein kinase were investigated. Deletion of ssp2+ caused a partial defect in glucose derepression of inv1+, fbp1+, and gld1+ and in assimilation of sucrose and glycerol, while a mutation in ppk9+ had no apparent effect. Scr1, a transcription factor involved in glucose repression, localized to the nucleus under glucose-rich conditions and to the cytoplasm during glucose starvation in wild-type cells. In contrast, in the ssp2Δ mutant, Scr1 localized to the nucleus in cells grown in glucose-rich medium as well as in glucose-starved cells. Immunoblot analysis showed that Ssp2 is required for the phosphorylation of Scr1 upon glucose deprivation. Mutation of five putative Ssp2 recognition sites in Scr1 prevented glucose derepression of invertase in glucose-starved cells. These results indicate that Ssp2 regulates phosphorylation and subcellular localization of Scr1 in response to glucose.

Most microorganisms, including yeasts, respond to environmental conditions to optimize use of available carbon sources by regulating the expression of many genes. Glucose, the carbon source preferred by most microorganisms, is recognized to be an important primary messenger molecule that mediates this adaptive response. In the presence of high levels of glucose, genes involved in the utilization of alternative carbon sources are repressed (subject to glucose repression), whereas genes required for glycolysis, glucose transport, etc., are expressed (subject to glucose induction). When glucose levels are limited, expression of glucose-repressed genes is derepressed (subject to glucose induction). When glucose levels are limited, expression of glucose-repressed genes is derepressed (subject to glucose induction). When glucose levels are limited, expression of glucose-repressed genes is derepressed (subject to glucose induction). When glucose levels are limited, expression of glucose-repressed genes is derepressed (subject to glucose induction).

In the budding yeast Saccharomyces cerevisiae, whose glucose-signaling pathway has been well studied, Snf1 protein kinase-mediated signal transduction plays a central role in glucose derepression of many genes (45). Under glucose-rich conditions, the Snf1 kinase, which forms a heterotrimeric enzyme complex with the γ subunit Snf4 and one of the three related β subunits, Gal83, Sip1, or Sip2, is inactivated via dephosphorylation by protein phosphatase 1 complex (Reg1-Glc7) (5, 20). Once cells are shifted to glucose-limiting conditions, the Snf1 kinase is activated by upstream kinases (Tos3, Sak1, and Elm1) and imported into the nucleus (17, 28, 38). Activated Snf1 kinase phosphorylates multiple transcription factors, causing the derepression/induction of many genes in the case of derepression of invertase-encoding Suc2, Snf1 kinase phosphorylates Mig1, a DNA-binding repressor that recruits the Ssn6-Tup1 corepressor to the promoters of many glucose-repressed genes, resulting in dissociation of Mig1 from the Suc2 promoter (reviewed in references 3 and 35).

In the fission yeast Schizosaccharomyces pombe, the function of the Snf1 pathway has not been characterized, although several factors involved in the glucose signaling pathway have been reported. We previously reported that a C2H2 Zn finger protein, Scr1, an S. pombe homologue of Mig1, represses the transcription of inv1+ (encoding invertase) and gld1+ (encoding glycerol dehydrogenase) under glucose-rich conditions (25, 41). In the presence of abundant glucose, transcription of fbp1+ encoding fructose-1,6-bisphosphatase is repressed by cooperative binding of Scr1 and Tup1-like Tup11 and Tup12 proteins to a cis-acting element in its promoter (13, 14, 19, 29). However, the possibility that the Snf1 kinase pathway is involved in glucose derepression of these genes has not been determined.

In the present study, we characterized the function of two S. pombe Snf1-like protein kinase genes and demonstrate that the Snf1 pathway plays important roles in glucose derepression.

MATERIALS AND METHODS

Strains and media. The parent S. pombe strain ARC039 (h− leu1-32 ura4-CI90T) (8) was provided by Asahi Glass Co., Ltd. (Yokohama, Japan). MM is synthetic minimal medium, and YES is standard rich medium (3% glucose, 0.5% yeast extract, and MM supplements). Both media were used to grow S. pombe as described elsewhere (27). 5′-Fluoro-orotic acid (FOA)-supplemented YNB medium (0.7% yeast nitrogen base, 2% glucose, 50 mg/ml uracil, 225 mg/ml each of leucine, adenine, histidine, and cysteine, and 0.1% FOA) was used to select for S. pombe ura4 mutants. YES-Suc medium is YES medium containing sucrose instead of glucose. YES medium containing 8% glucose was used for repressing conditions, and YES medium containing 0.05% glucose and 2% glucose was used for derepressed conditions. Transformation by electroporation was performed as described previously (37). Escherichia coli XL1-Blue (Stratagene, CA) was used for all cloning procedures.

Plasmid constructions. PAL-Ssp2-GFP and pAL-Ppk9-GFP were constructed as follows. A 2.7-kbp fragment containing the ssp2+ open reading frame (ORF) and the ssp2+ promoter was amplified by PCR using the following primers containing the indicated restriction sites: 5′-GTTTTGGCCCAAGTTCAGGCTCGTTATATTC-3′ and 5′-GTTTGTTCGACGCAGAAAAATAAACTTGTCAAC-3′ containing Apal and 5′-GTTTTGTTCGACGCAGAAAAATAAACTTGTCAAC-3′ containing SalI. A 2.4-kbp fragment containing the ppk9+ ORF and the ppk9+ promoter was amplified by PCR using the following primers containing the indicated restriction sites: 5′-GTTTTGGCCCAAGTTCAGGCTCGTTATATTC-3′ and 5′-GTTTGTTCGACGCAGAAAAATAAACTTGTCAAC-3′ containing Apal and 5′-GTTTTGTTCGACGCAGAAAAATAAACTTGTCAAC-3′ containing SalI.
GC-3' containing ApaI and 5'-GTTTTGTCGACGCAATTTTAAGGATC
AATTG-3' containing SalI. These fragments were digested with ApaI and
SalI and cloned into the corresponding sites of pAL (40). To construct
green fluorescent protein (GFP) fusion proteins, pAL-Ssp2 and pAL-Ppk9
were digested with SalI and NotI to facilitate cloning of SalI- and NotI-
digested GFP.

pJK148-Scr1 was constructed as follows. A 3.2-kbp fragment contain-
ing the scr1/H11001 ORF and the scr1/H11001 promoter was amplified by PCR using the
following primers: 5'-CGGTATCGATAAGCTCCTCGAGTATTTAGCT
AACCCACAC-3' and 5'-GGCGACCGGTGGATCGGGCTTGGTCATA
GGAGTTAACGGC-3'. The resultant PCR fragment was cloned into
pJK148 (22) digested with HindIII and BamHI using an In-Fusion PCR
cloning kit (TaKaRa Bio, Japan). pJK148-Scr1 S235A, pJK148-Scr1 S332A
S333A, and pJK148-Scr1 S408A S410A were constructed as described pre-
viously using pJK148-Scr1 as a template and the following primers with
serine-to-alanine conversions underlined (23): 5'-TTGGCTGCTGCGG
CTGCTAATCAATTGGATGCTGC-3' and 5'-AGCCGCAGCAGCCAA
CAATTGCATCTCGTTCATGG-3' for S235A, 5'-TCTAACGCTGCTAC
TAGCCTTCATTCAATTGG-3' and 5'-ATGCCGCCAGGTGTTAGATT
TGCTAGGCAGATAGGGAG-3' for S332A S333A, and 5'-AGGCTTT
TGCTCCCAACCCCAAGTGCTACACTC-3' and 5'-GGAGCCAAAG
CCTAGTGGAAAAAGTTAGGACTAC-3' for S408A S410A.

Fluorescence microscopy. Cells were collected by centrifugation and
suspended in 50 μl of culture medium, of which 5 μl was placed on a glass
slide. Fluorescent images of living cells were taken with a cooled charge-
coupled-device camera and stored digitally using MetaMorph software
(Universal Imaging, Downingtown, PA). For fixed samples, cells were
suspending in 70% ethanol, washed with phosphate-buffered saline, and
suspended in 5 μl of phosphate-buffered saline containing Hoechst 33342
dye (0.1 mg/ml).

GFP and 3 × FLAG tagging of Scr1. S. pombe strains expressing scr1/GFP
were constructed as follows. A 2.1-kbp fragment containing the scr1/H11001
ORF was amplified from S. pombe genomic DNA by PCR using the fol-
lowing primers: 5'-ATGTTCCGAAGCCACACCCAGTCCGAGATTTTAGCT
AACCCACAC-3' and 5'-GGCCACCGTGGATCGGGGCTGCTGATA
GAATTGACCC3'. The resultant PCR fragment was cloned into
pJK148-Scr1-H11001 as a template and the following primers with
serine-to-alanine conversions underlined (23): 5'-TTGGCTGCTGCGG
CTGCTAATCAATTGGATGCTGC-3' and 5'-AGCAGCACGCTGCTAC
TAGCCTTCATTCAATTGG-3'. The PCR product was then subcloned into pGEM-T Easy (Promega, WI). A 5.1-kbp fragment
was amplified by PCR using the following primers and pGEM-T Easy
scr1/H11001 as a template (fragment A): 5'-ATGCCGCCAGGTGTTAGATT
TGCTAGGCAGATAGGGAG-3' and 5'-AGGCTTT
TGCTCCCAACCCCAAGTGCTACACTC-3'. A 0.7-kbp fragment containing the GFP
gene was amplified using the following primers with pEGFP as a template
(fragment B): 5'-TCAGGCCACATGTGTTAGATT
TGCTAGGCAGATAGGGAG-3' and 5'-AGGCTTT
TGCTCCCAACCCCAAGTGCTACACTC-3'. A 2.3-kbp fragment containing the leu1/H11001 ORF was amplified from S. pombe genomic DNA by

FIG 1 Alignment of S. cerevisiae Snf1, S. pombe Ssp2, and S. pombe Ppk9. Amino acid residues identical to those in Snf1 are shaded. The threonine residue
essential for activation of the Snf1 kinase (210T) is indicated with an asterisk.
Ssp2 Is Necessary for Glucose Derepression

**Northern blot analysis.** Wild-type, ssps2Δ, and ppk9Δ cells were processed for Northern blot analysis as described above (see “Invertase assay”). Total RNA was extracted using hot phenol (34). Total RNA samples (20 μg each) were separated on a formaldehyde-agarose gel and transferred to a nylon membrane. Northern hybridization was performed at 55°C using 0.7- to 1.7-kb DNA probes for *inv1*+, *fbp1*+, *gld1*+, and *leu1*+. Labeling of probes and hybridization were performed using an AlkPhos Direct system according to the manufacturer’s instructions (GE Healthcare Co.). Primers used for amplifying probe fragments are listed below: for *inv1*+, 5′-TTTTCCAAAGACACACCTCTGTTAGAGGCGG-3′ and 5′-GAATGAGAAGTAAGGTTGGTCATGGG-3′; for *fbp1*+, 5′-TCTTATGCAGGAAGTTTGATTGTTTGGG-3′ and 5′-ATTCTCGGACAGCAAGGCAGG-3′; and for *gld1*+, 5′-GTTTTGAACTCGGCTTGACTGCGT-3′ and 5′-GGTTTGGACATCGGCTGACTGCG-3′. A fragment containing *gld1*+ was amplified using the following primers and pGEM-T Easy-FLAG-leu1+ as the template: 5′-ATGCGGAGCTGCAAGCTTGGATTTAATC-3′ and 5′-TCTGTCTGACGTATTTCTGAAAATGCATC-3′. A fragment containing *leu1*+ was amplified using the following primers and pGEM-T Easy-FLAG-leu1+ as the template: 5′-ATGCGGAGCTGCAAGCTTGGATTTAATC-3′ and 5′-TCTGTCTGACGTATTTCTGAAAATGCATC-3′.

**FIG 2** Localization of Ssp2 and Ppk9. (A) Wild-type cells expressing Ssp2-GFP were cultured on MM containing 8% glucose and were harvested during logarthmic growth (0 min). Cells were then shifted to MM containing 2% glycerol and 0.05% glucose as carbon sources and incubated for 30 or 60 min. (B) Wild-type cells expressing Ppk9-GFP were cultured on MM containing 8% glucose and were harvested during logarthmic growth (0 min). Cells were then shifted to MM containing 2% glycerol and 0.05% glucose as carbon sources and incubated for 30 or 60 min. Nomarski differential interference contrast micrographs (Nomarski), GFP fluorescence (GFP), and Hoechst 33342 staining (Hoechst) are shown.

**Immunoblot analysis.** Cells were collected by centrifugation for 2 min and were resuspended in 1× sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue, and 5% β-mercaptoethanol. Cells were boiled for 3 min and homogenized using glass beads. Cell extracts were centrifuged at 20,000 × g for 3 min, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and analyzed by immunoblotting.
RESULTS

Localization of *S. pombe* Snf1-like protein kinases. A search of the Sanger Centre Fission Yeast Genome Sequencing Project database (http://www.sanger.ac.uk/Projects/S_pombe/) for *S. pombe* homologues of *S. cerevisiae* SNF1 led to the discovery of SPCC74.03c/*ssp2*/H11001 and SPAC23H4.02/*ppk9*/H11001. *Ssp2* and *Ppk9* share 63% and 44% identities with *S. cerevisiae* Snf1, respectively, while the threonine residue essential for activation of the Snf1 kinase (7, 26) is conserved in both proteins (Fig. 1). The *ssp2* mutation was initially identified to be a suppressor of *ppe1* and *sts5* mutations. *Ppk9* has been designated a eukaryotic protein kinase catalytic domain-containing protein (1, 24). The *S. cerevisiae* Snf1 kinase forms a heterotrimeric enzyme complex with the 4-phosphate subunit Snf4 and one of three related β subunits, Gal83, Sip1, or Sip2 (20). It was reported that *S. pombe* Cbs2 is similar to the 4-phosphate subunit Snf4, that SPCC1919.03c is similar to β subunits, and that Cbs2 binds to two AMP-activated protein kinase (AMPK)-like catalytic subunits, Ssp2 and Ppk9 (12). However, it is not clear whether these Snf1-like protein kinases are involved in glucose derepression in *S. pombe*.

We constructed strains expressing Ssp2-GFP and Ppk9-GFP and analyzed the subcellular localization of these proteins. We found that Ssp2-GFP mainly localized in the nucleus both in glucose-starved cells and in cells grown in glucose-rich medium (Fig. 2A). On the other hand, Ppk9-GFP localized to the cytoplasm and nucleus both in glucose-starved cells and in cells grown in glucose-rich medium (Fig. 2B).

Disruption of *S. pombe* Snf1-like protein kinase genes. To characterize the function of these two Snf1-like protein kinase genes, we generated *ssp2*/H9004, *ppk9*/H9004, and *ssp2*/H9004 *ppk9*/H9004 mutants. Both *ssp2Δ* and *ppk9Δ* mutants exhibited a normal growth rate on MM containing 2% glucose (Fig. 3A), indicating that neither gene is essential for vegetative growth. However, deletion of *ssp2* prevented assimilation of glycerol (Fig. 3B) and failed to induce expression of *gld1*/H11001, encoding glycerol dehydrogenase (25), on glycerol medium (Fig. 3C). On the other hand, the *ppk9Δ* mutant grew at a rate similar to that of the parent and exhibited high expression of *gld1*/H11001 on glycerol medium. The growth rate and *gld1*/H11001 expression of the *ssp2Δ* *ppk9Δ* double mutant were found to be similar to those of the *ssp2Δ* single mutant. These results indicate that *ssp2Δ* is important for growth on glycerol and glucose derepression of *gld1*+ in *S. pombe*.

Invertase derepression in *ssp2Δ* and *ppk9Δ* mutants. To investigate the role of the *ssp2Δ* and *ppk9Δ* genes on glucose derepression of invertase, growth profiles of mutant strains on medium containing sucrose as the sole carbon source were examined (Fig. 4A). Because the *S. pombe inv1Δ* mutant exhibits a defect in sucrose fermentation only in the presence of antimycin (41), an antibiotic that inhibits cytochrome c reductase within the electron transport chain, the assay was performed in the presence of 10 μg/ml antimycin. The growth rate of the *ssp2Δ* mutant on sucrose medium was considerably lower than that of the parent strain, while the *ppk9Δ* mutant grew at a rate similar to that of the parent strain. Furthermore, the growth rate of the *ssp2Δ* *ppk9Δ* double
Invertase induction was similar to that observed in the mutant was complemented by overexpression of **assayed at 0, 1, 3, and 6 h after shifting to medium containing 2% glycerol and ute. Values are means 0.05% glucose as carbon sources. One unit of invertase activity is defined as the analysis. Expression of **mutants to sucrose availability. Identical vol-

FIG 4 Glucose derepression of invertase in **and **mutants. (A) Response of **and **mutants to sucrose availability. Identical volumes of 10-fold serial dilutions of exponentially growing wild-type parent strain, **, **, and ** cells were spotted onto YES (left) or amphotericin (Anti.; 10 μg/ml)-supplemented YES-sucrose medium (right) and incubated for 72 h at 30°C. (B) Glucose-derepression profiles of invertase in ** and **mutants. Activity of secreted invertase in each strain was assayed at 0, 1, 3, and 6 h after shifting to medium containing 2% glycerol and 0.05% glucose as carbon sources. One unit of invertase activity is defined as the amount of enzyme which catalyzes production of 1 nmol of glucose per minute. Values are means ± SEMs (n = 3). (C) Northern blot analysis of ** and ** in ** and ** mutants. Total RNA was harvested at 0, 3, and 6 h after glucose starvation (2% glycerol and 0.05% glucose) and subjected to analysis. Expression of ** was determined as an internal control.

**mutant was found to be similar to that of the ** single mutant (Fig. 4A). Next, we determined the amount of secreted invertase in wild-type and mutant cells. In the parent strain, secreted invertase was not detected when cells were exposed to high levels of glucose (time zero) but began to rise within 1 h after glucose depletion and reached a peak after 3 h (Fig. 4B, filled circles). Elevation of invertase activity after glucose depletion was suppressed in ** cells (open squares), while invertase activity in ** cells (open triangles) was found to increase to the same level observed in the parent cells. In the ** double mutant, the profile of invertase induction was similar to that observed in the ** mutant (open circles). The impaired invertase induction in the ** mutant was complemented by overexpression of ** but not by overexpression of ** (data not shown). To examine whether ** was derepressed in the ** and ** mutants by glucose depletion, Northern blot analysis was performed. In addition, the derepression profiles of ** were also analyzed. In the wild-type strain, expression of the two genes was completely repressed when cells were grown in glucose-rich medium (Fig. 4C, time zero), but expression of the two genes was significantly induced after glucose depletion. Induction of these genes by glucose depletion was partially defective in ** cells (Fig. 4C). In the ** mutant, the derepression profiles of the two genes were found to be similar to those in the parent strain (Fig. 4C). A more severe defect was not observed in the ** double mutant (data not shown).

During the course of these studies, we observed additional phenotypes associated with the ** strain, including heat sensitivity and a defect in sporulation, similar to that seen in the ** mutant (see Fig. S1 in the supplemental material) (4, 33, 42). In **, it has been demonstrated that the Snf1 kinase controls meiosis via transcriptional regulation of the meiotic regulators Ime1 and Ime2 (18), whereas a function for the Snf1 kinase in the heat-stress response is uncertain. Our results indicate that multiple biological processes, including the heat-stress response and sexual development, are both controlled by the Snf1-like protein kinase Ssp2 in **.

Localization and phosphorylation of ** in ** and ** mutants. Fission yeast Scr1, in cooperation with Tup11 and Tup12, which are involved in glucose-dependent transcriptional repression and chromatin alteration, represses transcription of ** and ** in ** and ** mutants. Total RNA was harvested at 0, 3, and 6 h after glucose starvation (2% glycerol and 0.05% glucose) and subjected to analysis. Expression of ** was determined as an internal control.

We next examined whether Scr1 is phosphorylated in response to glucose availability. Treitel et al. reported that Mig1, a DNA-binding repressor of ** that represses many glucose-repressed genes, is differentially phosphorylated in response to glucose availability (43). Immunoblot analysis showed that Mig1 is phosphorylated under both repressing and derepressing conditions and that Mig1 from derepressed cells migrates slower in SDS-PAGE than that from repressed cells. Hirota et al. detected no visible changes in Scr1 protein levels or mobility in response to glucose availability (13). However, we found that Scr1 mobility was affected by culture conditions. Although the estimated molecular mass of the Scr1-3×FLAG protein was about 62 kDa, the molecular mass of Scr1 from a wild-type strain grown under repressing conditions was approximately 80 kDa and the protein obtained under derepressing conditions was approximately 100 kDa (Fig. 5C). These bands corresponding to Scr1-3×FLAG were shifted to faster-migrating forms, approximately 62 kDa, after phosphatase treatment (Fig. 5D). These results show that Scr1 is phosphorylated both in glucose-starved cells and in cells grown in...
glucose-rich medium and is hyperphosphorylated upon depletion of glucose. The reason why phosphorylated Scr1 bands were not detected in the earlier study (13) remains uncertain. To test whether the Ssp2 and Ppk9 protein kinases are required for phosphorylation of Scr1, we constructed ssp2Δ/H9004, ppk9Δ/H9004, ssp2Δ/ppk9Δ/H9004, and cbs2Δ strains. Scr1-GFP-expressing cells were cultured on MM containing 8% glucose to an OD₆₀₀ of 0.8 (R). Cells were then shifted to MM containing 2% glycerol and 0.05% glucose as carbon sources and incubated for 1 h (DR). Nomarski differential interference contrast micrographs (Nomarski), GFP fluorescence (GFP), and Hoechst 33342 staining (Hoechst) are shown. (C) Immunoblot analysis of the Scr1-3×FLAG protein. Protein extracts were prepared from wild-type, ssp2Δ, ppk9Δ, ssp2Δ/ppk9Δ, and cbs2Δ strains expressing Scr1-3×FLAG grown in MM containing 8% glucose (R) or 2% glycerol and 0.05% glucose (DR) and subjected to SDS-PAGE. (D) Protein extracts prepared from wild-type cells grown in MM medium containing 8% glucose (R) or 2% glycerol and 0.05% glucose (DR) were preincubated with phage lambda protein phosphatase (A-PPase).

**FIG 5** Localization and phosphorylation of Scr1. (A) Constructs expressing Scr1-GFP and Scr1-3×FLAG. Scr1-GFP and Scr1-3×FLAG were integrated at the scr1+ locus using leu1* as a selectable marker. EGFP, enhanced GFP. (B) Localization of Scr1-GFP in wild-type, ssp2Δ, ppk9Δ, ssp2Δ/ppk9Δ, and cbs2Δ strains. Scr1-GFP-expressing cells were cultured on MM containing 8% glucose to an OD₆₀₀ of 0.8 (R). Cells were then shifted to MM containing 2% glycerol and 0.05% glucose as carbon sources and incubated for 1 h (DR). Nomarski differential interference contrast micrographs (Nomarski), GFP fluorescence (GFP), and Hoechst 33342 staining (Hoechst) are shown. (C) Immunoblot analysis of the Scr1-3×FLAG protein. Protein extracts were prepared from wild-type, ssp2Δ, ppk9Δ, ssp2Δ/ppk9Δ, and cbs2Δ strains expressing Scr1-3×FLAG grown in MM containing 8% glucose (R) or 2% glycerol and 0.05% glucose (DR) and subjected to SDS-PAGE. (D) Protein extracts prepared from wild-type cells grown in MM medium containing 8% glucose (R) or 2% glycerol and 0.05% glucose (DR) were preincubated with phage lambda protein phosphatase (A-PPase).

glucose-rich medium and is hyperphosphorylated upon depletion of glucose. The reason why phosphorylated Scr1 bands were not detected in the earlier study (13) remains uncertain. To test whether the Ssp2 and Ppk9 protein kinases are required for phosphorylation of Scr1, we constructed ssp2Δ, ppk9Δ, ssp2Δ/ppk9Δ, and cbs2Δ mutants expressing Scr1-3×FLAG. In the ppk9Δ mutant, Scr1 was modified as in wild-type cells. In the ssp2Δ, ssp2Δ/ppk9Δ, and cbs2Δ mutants, Scr1 was phosphorylated to the same level in cells grown in glucose-rich medium but was not hyperphosphorylated during glucose starvation. These results indicate that Ssp2 and Cbs2 are required for increased phosphorylation of Scr1 upon glucose deprivation but are not required for phosphorylation during growth in glucose-rich medium.

**Mutation of putative Ssp2 phosphorylation sites in Scr1.** Potential phosphorylation sites in Scr1 were analyzed by homology to budding yeast Mig1 on the basis of their similarity to the consensus substrate recognition sequence, which contains an arginine at position −3 and hydrophobic residues at positions −5 and +4 relative to the phosphorylated serine (6, 43). Mig1 possesses the following consensus serine residues: S278, S311, S312, S381, and S383. Treitel et al. reported that these serine residues appear to be phosphorylated in vivo, but mutation of these sites did not reduce phosphorylation of Mig1 as substantially as did mutation of SNF1, suggesting that Mig1 also contains additional serine residues that are substrates for Snf1 (44). S278, S311, S312, S381, and S383 of Mig1 are also conserved in Scr1 and correspond to residues S235, S332, S333, S408, and S410, respectively (Fig. 6A). We changed the phosphorylatable serine residues of Scr1 to alanine by site-directed mutagenesis (mutated sites are designated with upward-pointing arrowheads in Fig. 6A) and assayed for repression of invertase by the mutant proteins in response to glucose limitation. Under glucose-repressing conditions, the invertase activity of scr1Δ cells expressing the mutant scr1 alleles was repressed (data not shown). The derepressed invertase activity of scr1Δ cells expressing alleles harboring single or double mutations was as high as that in wild-type cells, but that of scr1Δ cells expressing the allele harboring five S-to-A substitutions (the quintuple allele) was significantly lower than that in wild-type cells (Fig. 6B). These results indicate that these five substitutions, S235A, S332A, S333A, S408A, and S410A, in Scr1 prevent glucose derepression of invertase in glucose-starved cells. While immunoblot analysis indicated that this mutated protein still displayed a glucose-dependent mobility shift, the shift was less pronounced (Fig. 6C). In addition,
fluorescence microscopy revealed that cells expressing the quintuple allele fused to GFP produced a protein that localized to the nucleus both in glucose-rich medium and during glucose starvation. In contrast, Scr1-GFP harboring single or double S-to-A substitutions rapidly translocated to the cytoplasm upon glucose removal (Fig. 6D). These results indicate that potential phosphorylation sites in Scr1, on the basis of similarity to Mig1, appear to be phosphorylated in vivo and are essential for export of Scr1 from the nucleus to the cytoplasm as well as for glucose derepression during glucose starvation. However, mutation of these serine residues did not reduce phosphorylation of Scr1 as substantially as did deletion of spo21, suggesting that Scr1 also contains additional sites for Ssp2-dependent phosphorylation.

**DISCUSSION**

In this report, the function of ssp2 encoding an Snf1-like protein kinase was characterized. Our results indicate that Ssp2 is involved in derepression of invertase, a glucose-repressible enzyme required for sucrose utilization, and glycerol dehydrogenase, required for glycerol utilization. In contrast, Ppk9 is not involved in glucose derepression, even though Ppk9 also closely resembles budding yeast Snf1 and forms a complex with Cbs2 in S. pombe cells (12).

It has been reported that transcription of fbp1 is repressed by glucose through activation of a cyclic AMP-dependent protein kinase A (PKA) pathway (2, 15, 16, 21) and is activated by glucose depletion through activation of a stress-activated protein kinase
(SAPK) pathway (29, 36, 39). In addition, regulation of fbp1+ involves activities of two mutually antagonistic transcription factors, Scr1 and Rat2, that modulate the function of Tup1-like Tup11 and Tup12 proteins (15). It is reasonable to speculate that Snf1-like protein kinases contribute to the regulation of fbp1+ because the budding yeast counterparts of the above-described transcription factors (Mig1 and Adr1, respectively) are modulated by the Snf1 kinase (reviewed in reference 32). To our knowledge, these are the first observations that indicate that the Snf1-like protein kinase is a component of the transcriptional machinery that regulates fbp1+. It is notable that the glucose–derepression profiles of inv1+, fbp1+, and gld1+ in the ssp2Δ mutant were very similar, suggesting a common regulatory mechanism.

Scr1 is phosphorylated both in glucose-starved cells and in cells grown in glucose-rich medium and is hyperphosphorylated upon glucose removal in an Ssp2–dependent manner. This hyperphosphorylation causes export of Scr1 from the nucleus to the cytoplasm. Analysis of Scr1 on the basis of similarity to Mig1 showed that mutation of serine residues 235, 332, 333, 408, and 410 affects its phosphorylation and reduces Ssp2–dependent glucose derepression. Although the extent of Scr1 phosphorylation during glucose starvation is largely dependent on the Ssp2 kinase, Ssp2 may not be directly responsible for all phosphorylation of Scr1. Scr1 is phosphorylated under glucose-rich conditions independently of the Ssp2 kinase because Scr1 is phosphorylated to the same extent both in the ssp2Δ mutant and in a wild-type strain under glucose-rich conditions. It had been expected that pppk9+ would have functional redundancy with ssp2+, because disruption of ssp2+ failed to completely block the glucose derepression of invertase at either the enzymatic or transcriptional level, nor did it completely block the phosphorylation of Scr1 under glucose-rich conditions. However, we found no experimental evidence that pppk9+ is involved in either the glucose derepression or phosphorylation of Scr1. Our results indicate that the mechanism of derepression of invertase in S. pombe is different from that in S. cerevisiae, where an SNF1 mutation completely blocks the glucose derepression of invertase (4, 30). This raises the possibility that S. pombe may possess an Snf1-like protein kinase–independent signal transduction pathway that upregulates invertase expression.

Collectively, our results indicate that the Snf1-like protein kinase Ssp2 functions in multiple biological processes in S. pombe, including glucose derepression. While the function of Ppk9 is still unclear, it may play a role in important biological processes because the pppk9Δ mutant has been reported to be sensitive to several toxins, including cytoskeletal poisons (1). While the current study is the first to our knowledge to describe a mechanism for Snf1-like protein kinase–dependent signaling in glucose repression/derepression in S. pombe, further work is clearly needed to identify additional components of the Snf1-like protein kinase pathway in order to better understand the machinery of glucose-mediated gene regulation in S. pombe.

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