The SNF1 kinase complex from *Saccharomyces cerevisiae* phosphorylates the transcriptional repressor protein Mig1p in vitro at four sites within or near regulatory domain 1

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Abstract Mig1p is a zinc finger protein required for repression of glucose-regulated genes in budding yeast. On removal of medium glucose, gene repression is relieved via a mechanism that requires the SNF1 protein kinase complex. We show that Mig1p expressed as a glutathione-*S*-transferase fusion in bacteria is readily phosphorylated by the SNF1 kinase in vitro. Four phosphorylation sites were identified, i.e. Ser-222, Ser-278, Ser-311 and Ser-381. The latter three are exact matches to the recognition motif we previously defined for SNF1 and lie within regions shown to be required for SNF1-dependent derepression and nuclear-to-cytoplasmic translocation.

Key words: SNF1; Mig1p; Glucose repression; Protein phosphorylation

1. Introduction

Glucose is the preferred carbon source for the budding yeast *Saccharomyces cerevisiae*. Glucose repression (catabolite repression) is the phenomenon whereby a battery of genes, e.g. those required for catabolism of alternative carbon sources, is switched off in the presence of high glucose. The *SNF1* (*CAT1, CCR1*) and *SNF4* (*CAT3*) genes are essential for derepression of these genes when glucose is removed [1,2]. The *SNF1* gene product (Snf1p) has a protein kinase domain at the N-terminus [3], while Snf4p is a regulatory subunit that forms a complex with Snf1p in vitro and in vivo [4–7]. The *SIP1*, *SIP2* and *GAL83* gene products represent alternate third subunits of the complex, which interact with Snf1p and Snf4p via conserved binding domains [8,9].

The mammalian homologue of the SNF1 complex is the AMP-activated protein kinase (AMPK) [10], a heterotrimer of α, β and γ subunits which are homologues of Snf1p, Sip1p/Sip2p/Gal83p and Snf4p, respectively [11–14]. AMPK is activated by elevation of AMP and depletion of ATP, via a complex multistep mechanism involving phosphorylation by an upstream kinase [15–19]. We have recently demonstrated that the kinase activity of the SNF1 complex is activated on removal of glucose from the medium [7,20], via a mechanism which also involves increased phosphorylation by an upstream kinase. The activating signal in yeast remains unclear, although activation is associated with large increases in AMP and decreases in ATP in vivo [7].

How the activated SNF1 complex derepresses glucose-repressed genes has also been unclear, although a number of genes which act downstream of SNF1 have been defined by genetic studies. The *MIG1* gene is of particular interest, in that mutations lead to partially constitutive expression of glucose-repressed genes, but not of other genes. Mig1p is a C_2H_2 zinc finger DNA-binding protein that binds to the promoter of several glucose-repressible genes [21–24]. Mig1p regulates repression of these genes by recruiting the co-repressors Ssn6p and Tup1p. The phosphorylation of Mig1p is increased under derepressing conditions [25] and is associated with its translocation from the nucleus to the cytoplasm [26]. Since both Snf1p and Snf4p have been reported to be partly nuclear [3,4], a simple hypothesis would be that on removal of glucose, Mig1p is directly phosphorylated by SNF1 within the nucleus, resulting in translocation to the cytoplasm and loss of its ability to repress transcription. In this paper, we show that the SNF1 complex phosphorylates Mig1p in vitro and identify four phosphorylation sites located at, or near, regions that have been shown to be required for SNF1-dependent relief from repression [27] or nuclear to cytoplasmic translocation [26].

2. Materials and methods

2.1. Materials

Anti-FLAG M2 resin was from Sigma. The SNF1 complex was purified from yeast as described previously [7].

2.2. Expression and purification of Mig1p in bacteria and yeast

Mig1 was amplified from *S. cerevisiae* cDNA (Clontech) using the polymerase chain reaction (PCR) and cloned into pGEX4T-3 (Amersham Pharmacia Biotech) for expression of glutathione-*S*-transferase (GST)-Mig1p in bacteria or pYX212 (R and D Systems) for expression of Mig1p in yeast. For Mig1p expressed in yeast, a sequence encoding the FLAG epitope tag (DYKDDDDK) was incorporated into the cDNA immediately following the initiating methionine. The serine residues identified as sites phosphorylated by SNF1 in vitro were altered to aspartic acid residues (222, 278, 381) or glutamic acid (311) by site-directed mutagenesis using the splicing by overlap

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Abbreviations: AMPK, AMP-activated protein kinase; GST, glutathione-*S*-transferase; PCR, polymerase chain reaction; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; VP16, viral protein 16

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extension PCR technique. GST-Mig1p was purified from 500 ml cultures by chromatography on glutathione-Sepharose 4B (Amersham Pharmacia Biotech), according to the manufacturer's instructions. FLAG epitope-tagged Mig1p was expressed in Δ*mig1* yeast (strain MYC3640) and immunoprecipitated from cell lysates (1 mg total protein) with 40 μl of a 50% slurry (v/v) of anti-FLAG resin (Sigma). Immune complexes were washed extensively with 25 mM HEPES, pH 7.4, 50 mM NaCl, 10% glycerol, 1% Triton X-100 and used for phosphorylation by SNF1.

2.3. Phosphorylation of Mig1p by the SNF1 complex

GST-Mig1p (1.3 mg/ml) was incubated for 30 min at 30°C with SNF1 (4.3 U/ml), $MgCl_2$ (4.7 mM) and [γ-^{32}P]ATP (357 000 dpm/nmol, 187 μM) in Na-HEPES (50 mM, pH 7.0), dithiothreitol (1 mM), Brij-35 (0.02% (w/v)). Mig1p expressed in yeast was phosphorylated in the immune complex with anti-FLAG resin using the same conditions. In some cases, the reaction was stopped by adding 5×sodium dodecyl sulfate (SDS) gel sample buffer followed by electrophoresis in 10% polyacrylamide gels [28]. The gel was stained with Coomassie blue, dried and subjected to autoradiography. In other cases, the reaction was stopped by adding ice-cold trichloroacetic acid (TCA) to 25% (w/v), the pellet was recovered by centrifugation (14 000×*g*, 2 min) and washed three times by addition of 1 ml of 25% TCA, vortexing and recentrifugation. The radioactivity in the pellet was determined by Cerenkov counting.

2.4. Analysis of phosphorylation sites on GST-Mig1p

TCA pellets containing ^{32}P-labelled GST-Mig1p were washed once with water and resuspended in Tris-HCl (50 mM, pH 8.0), dithiothreitol (1 mM), Brij-35 (0.02% (w/v). Trypsin was added (1:10 by weight) and the suspension incubated for 2 h at 37°C. The suspension was centrifuged (14 000×*g*, 2 min) and the supernatant (83% of total radioactivity) was applied to a Sep-Pak cartridge (Waters), eluted with 50% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) (86% recovery) and taken to near dryness in a Speedvac concentrator. The sample was applied to a Vydac Protein and Peptide C18 column (25×0.46 cm) equilibrated with 0.1% (v/v) TFA and eluted with a gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile as shown in Fig. 2A. Phosphopeptides were further purified by chromatography on Fe^{3+} bound to iminodiacetate-agarose gel [29] and by microbore HPLC on a Vydac Protein and Peptide C18 column (15×0.21 cm) in 0.09% TFA, eluting at 0.2 ml/min with a gradient from 0 to 70% acetonitrile over 140 min. Phosphopeptides were sequenced on an ABI 476A sequencer using a conventional pulsed liquid protocol to determine the PTH-amino acid released and by solid phase sequencing on an ABI 470A sequencer using peptides covalently attached to Sequelon membranes to determine the release of radioactivity.

3. Results

3.1. Expression of Mig1p and phosphorylation by SNF1

Mig1p was expressed as a fusion protein with GST and purified by affinity chromatography on glutathione-Sepharose. Despite a number of efforts, we were unable to prevent extensive degradation of the GST-Mig1p protein and the proportion of intact full length GST-Mig1p (predicted molecular mass 82 kDa) was below 1%, as judged by densitometric scanning of Coomassie-stained gels (data not shown). However, as shown in Fig. 1, SNF1, purified to near homogeneity as described previously [7,30], phosphorylated the 82 kDa polypeptide corresponding to full length GST-Mig1p and most of its major breakdown products. GST was not phosphorylated by SNF1 and there was no significant phosphorylation in controls containing SNF1 or GST-Mig1p alone. Due to the large spread of molecular masses of the phosphorylated polypeptide, we could not get an accurate estimate of the stoichiometry of phosphorylation. However, assuming an 'average' molecular mass of 40 kDa, the maximum labelling was around 0.63 mol phosphate per mole protein. The stoichiometry of

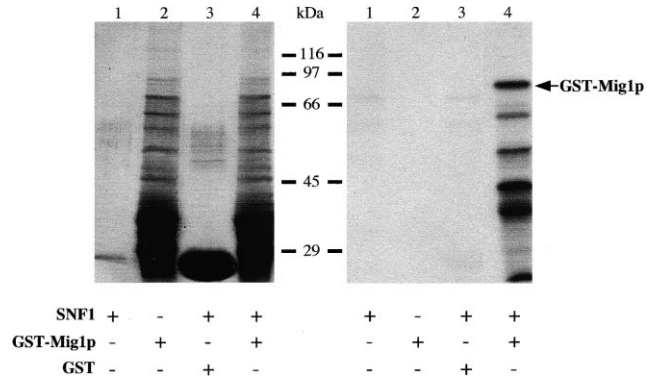

Fig. 1. Coomassie blue-stained gel (left panel) and autoradiogram (right panel) of GST (lane 3) and GST-Mig1p (lane 4), phosphorylated using [γ-^{32}P]ATP and purified SNF1. Control incubations lacked GST-Mig1p (lane 1) or SNF1 (lane 2). The polypeptide of the expected size for GST-Mig1p is indicated by the arrow on the right, polypeptides of a lower molecular mass are breakdown products. The migration of marker proteins of the indicated molecular mass is shown.

phosphorylation of the full length GST-Mig1p was certainly higher than this, because this polypeptide was more strongly labelled, although less abundant than the major breakdown products.

3.2. Identification of phosphorylation sites within Mig1p

Pilot experiments (not shown) showed that a very similar tryptic peptide map was obtained when we digested the total phosphorylated protein, rather than just the full length GST-Mig1p polypeptide excised from a gel. In further experiments, we therefore digested the total protein without prior gel separation. Fig. 2A shows a HPLC analysis of tryptic peptides, revealing the presence of four major peaks labelled 1–4 in order of elution. The on-line radioactivity monitor indicated that the second peak contained two closely eluting phosphopeptides (2a and 2b) but we were unable to resolve these preparatively. Although the proportion of radioactivity in peaks 1–3 was reproducible from digest to digest, the recovery of radioactivity in peak 4 was variable and on further analysis, it proved to contain a complex mixture of different labelled and unlabelled peptides. We suspect, therefore, that peak 4 represents incompletely digested material and it was not analyzed further. HPLC peptide mapping after different times of phosphorylation (not shown) revealed that peaks 1, 2a, 2b and 3 appeared at similar rates, although at all time points, peak 2a contained only about half of the radioactivity of 1, 2b and 3.

Peaks 1 and 3 were further purified on Fe^{3+}-chelating-Sepharose (which selectively binds phosphopeptides and some other acidic peptides), followed by microbore HPLC and subjected to sequence analysis (Fig. 2B). Pulsed liquid sequencing of peak 1 gave a single sequence corresponding to residues 278–292 of Mig1p. Solid phase sequencing showed that all of the [^{32}P]phosphate was released in the first cycle, corresponding to Ser-278. Pulsed liquid sequencing of peak 3 showed that it had a single sequence corresponding to residues 308–322 of Mig1p. Solid phase sequencing showed that all of the [^{32}P]phosphate was released in the fourth cycle, corresponding to Ser-311.

Purification of peaks 2a and 2b was more difficult. They could not be resolved from each other even after microbore

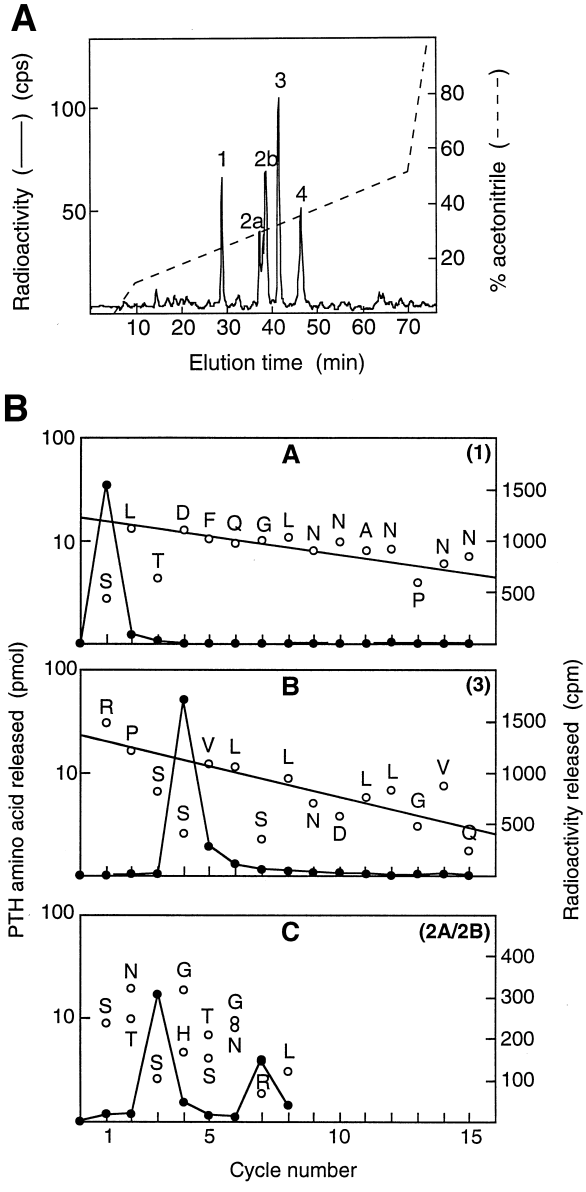

Fig. 2. (A) Reversed phase HPLC separation of tryptic phosphopeptides derived from Mig1p, phosphorylated using [γ-^{32}P]ATP and purified SNF1. Peptides were separated on a C18 column using the indicated gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile (dashed line). The continuous line shows the output from an on-line radioactivity monitor. (B) Amino acid sequencing of Mig1p phosphopeptides: (A) tryptic peptide 1; (B) tryptic peptide 3; (C) tryptic peptides 2a/2b after subdigestion with chymotrypsin and further purification. Conventional pulsed liquid sequencing was used to identify the PTH-amino acid(s) release at each cycle (open circles, note semi-logarithmic plot), whereas parallel solid phase sequencing runs using peptides covalently attached to Sequelon membranes (filled circles) were used to investigate the release of radioactivity. In some cycles in (C), two PTH-amino acids were observed. Note that the recoveries of PTH-serine and -threonine are always less than expected due to β elimination reactions, especially if the amino acid was phosphorylated.

HPLC on a C18 column. Pulsed liquid sequencing of this preparation gave a single major sequence commencing IPVSVKPPPSL, corresponding to the expected tryptic peptide from residues 186 to 225 (the bond following Lys-191 would not be expected to cleave efficiently because it is followed by proline). However, solid phase sequencing gave a major release of radioactivity at cycle 3, indicating that the 186–225 peptide was contaminated with another phosphopeptide. We therefore subdigested with chymotrypsin and purified the resulting digest on Fe^{3+}-chelating-Sepharose, followed by microbore HPLC. An apparently single radioactive peak was obtained, which was subjected to pulsed liquid sequencing. However, two amino acids were obtained in several cycles, indicating that two peptides were still present (Fig. 2B(C)). With the aid of the predicted Mig1p sequence, we could assign the two peptides as SNSHSGSRLK (residues 216–225, derived by chymotryptic digestion from the C-terminal end of the 186–225 peptide) and STSGTNL (residues 379–385, derived by chymotryptic digestion from the N-terminal end of the expected tryptic peptide from 379 to 413). On solid phase sequencing of this peptide, there was a major release of counts in cycle 3 and a minor peak in cycle 7. Since a release of radioactivity at cycle 3 was also observed when the tryptic peptide 186–225 was sequenced, the radioactivity in this cycle arose from the peptide STSGTNL (379–385) and not from SNSHSGSRLK (216–225). Taken together, these data are consistent with Ser-222 and Ser-381 being the two phosphorylated sites which generate tryptic peptides 2a/2b, with Ser-222 being a minor site.

3.3. Site-directed mutagenesis of phosphorylation sites

The phosphorylated serine residues we had identified within Mig1p were mutated to non-phosphorylatable acidic residues (aspartic acid or glutamic acid) by site-directed mutagenesis. Both wild-type Mig1p and the mutant Mig1p were expressed in Δ*mig1* yeast and immunoprecipitated using anti-FLAG resin. Wild-type Mig1p was readily phosphorylated by SNF1 whereas the $S^{222,\,278,\,381}D/S^{311}E$ mutant protein was not phosphorylated significantly under these conditions (Fig. 3). Mutation of the phosphorylation sites had no detectable effect on the level of expression of the proteins, as judged by Western blotting (data not shown). Similar results were obtained using GST-Mig1p constructs expressed in bacteria (results not shown). These results confirm that Ser-222, Ser-278, Ser-311

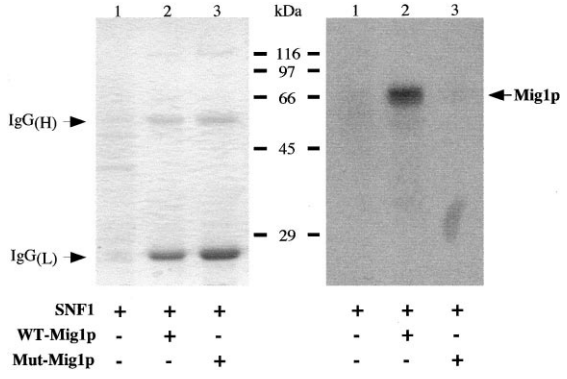

Fig. 3. Coomassie blue-stained gel (left panel) and autoradiogram (right panel) of wild-type (WT) Mig1p (lane 2) and $S^{222,\,278,\,381}D/S^{311}E$ (Mut) Mig1p (lane 3). Immune complexes isolated by immunoprecipitation from yeast cell lysates expressing either WT-Mig1p or Mut-Mig1p were incubated in the presence of [γ-^{32}P]ATP and purified SNF1. A control incubation containing SNF1 and [γ-^{32}P]-ATP was included (lane 1). Polypeptides corresponding to the immunoglobulin heavy and light chains present in the immune complex are indicated by arrows. The labelled polypeptide corresponding to Mig1p is also indicated by an arrow on the right. The migration of marker proteins of the indicated molecular mass is shown.

Table 1
The alignments of phosphorylated sites on *S. cerevisiae* Mig1p (ScMig1) with the homologous sites on *K. lactis* Mig1p (KlMig1) are shown

	P-5	P-3	P	P+4
Recognition motif:	***Hyd***-Xaa-Arg-Xaa-Xaa-***Ser***-Xaa-Xaa-Xaa-***Hyd***			
ScMig1 Ser222 :	-**Asn**-Ser-His-Ser-Gly-**Ser**-Arg-Leu-Lys-**Leu**-			
KlMig1 Leu238 :	-**Asn**-Ser-Ser-Ser-Gly-**Leu**-Leu-Leu-Pro-**Arg**-			
ScMig1 Ser278 :	-**Leu**-Pro-Arg-Pro-Arg-**Ser**-Leu-Thr-Asp-**Phe**-			
KlMig1 Ser305 :	-**Leu**-Pro-Arg-Thr-Arg-**Ser**-Trp-Thr-Asn-**Leu**-			
ScMig1 Ser311 :	-**Leu**-Lys-Arg-Pro-Ser-**Ser**-Val-Leu-Ser-**Leu**-			
KlMig1 Ser329 :	-**Asn**-Ser-Arg-Phe-Ser-**Ser**-Ser-Asn-Ser-**Leu**-			
ScMig1 Ser381 :	-**Leu**-Ser-Arg-Ser-Thr-**Ser**-Gly-Thr-Asn-**Leu**-			
KlMig1 Ser397 :	-**Pro**-Pro-Ser-Ser-Gly-**Ser**-Ala-Thr-Glu-**Gly**-			

The *S. cerevisiae* and *K. lactis* sequences were aligned by the GAP program, using default settings, within GCG. The phosphorylated serines in *S. cerevisiae* and the aligned residues in *K. lactis* are in an increased font size and bold type. The recognition motif for the SNF1 kinase [29] is shown in italics and residues thought to be important for phosphorylation are highlighted (in bold (P−5 and P+4) or underlined (P−3)). Residues in KlMig1 that do not match the recognition motif are boxed. Hyd represents a hydrophobic amino acid and Xaa any amino acid.

and Ser-381 are the only sites within Mig1p that are phosphorylated to a significant extent by SNF1 in vitro.

4. Discussion

Our results show that a GST-Mig1p fusion protein is an excellent substrate for the purified SNF1 complex. Four sites, i.e. Ser-222, Ser-278, Ser-311 and Ser-381 were identified as being phosphorylated by SNF1. The sequences around Ser-278, Ser-311 and Ser-381 match the consensus recognition motif for SNF1 (Table 1), which we have established from previous synthetic peptide studies [30]. All three sites contain hydrophobic residues (usually Leu, in one case Phe) at the P−5 and P+4 positions (bold type) and arginine at the P−3 position (underlined), where the phosphorylated serine is residue P (bold and increased font size). By contrast, the sequence around Ser-222 is not an optimal match to our recognition motif. It has histidine rather than arginine at the P−3 position and synthetic peptides with histidine at this position were phosphorylated 10-fold slower than those with arginine [30]. It also has asparagine at the P−5 position, a residue which has not yet been tested at this position in our peptide studies. Although not hydrophobic, asparagine is a reasonably bulky uncharged side chain and it is possible that it can be accommodated in the putative pocket on SNF1 [30] which binds the P−5 hydrophobic side chain.

Recently, Cassart et al. [31] have cloned a Mig1p homologue from the distantly related yeast *Kluyveromyces lactis* (KlMig1) and have shown that it restores glucose-repressible SUC2 expression when expressed in a *mig1* mutant of *S. cerevisiae*. Since the Mig1p function is therefore conserved between *S. cerevisiae* and *K. lactis*, it was of interest to see whether the phosphorylation sites identified in the *S. cerevisiae* protein (ScMig1) are conserved in *K. lactis*. Table 1 shows that the Ser-278 and Ser-311 sites from ScMig1 are conserved in KlMig1 (Ser-305 and Ser-329, respectively) and that the residues at P−5, P−3 and P+4 critical for recognition are also conserved, except that Ser-329 on KlMig1 has asparagine at the P−5 position. As noted above, *S. cerevisiae* SNF1 does phosphorylate at least one site with asparagine at the P−5 position (Ser-222 on ScMig1). The Ser-222 and Ser-381 sites are not conserved in KlMig1 (Table 1). A serine is present at position 397 (which aligns with Ser-381 in ScMig1) in KlMig1, but the critical residues at P−5, P−3 and P+4 are not conserved.

Based on the conservation with *K. lactis*, we would therefore argue that Ser-278 and Ser-311 on ScMig1 are the key sites, with Ser-222 and Ser-381 being secondary sites which may not have a critical function. Ronne's group [27] has recently defined a domain in ScMig1 (the R1 domain, residues 241–351) which is critical for SNF1-dependent gene regulation. This corresponds closely to the region (residues 261–400) required for SNF1-dependent nuclear to cytoplasmic translocation of Mig1p [26]. Intriguingly, Ser-278 and Ser-311 are both within the R1 domain, while Ser-222 and Ser-381 lie just outside it, on the N- and C-terminal sides, respectively. Using a Mig1-VP16 fusion in which the C-terminal effector domain of Mig1p was replaced by the activator domain of viral protein 16 (VP16), Ronne's group [27] created a factor which activated transcription from reporter genes containing Mig1p promoter elements. This activation was inhibited 5-fold in a *SNF1* strain compared to a Δ*snf1* strain, but only under derepressing conditions when the SNF1 complex would be active [7,20]. Deletion of the R1 domain completely abolished control by SNF1 and led to constitutive expression. Based on our previously published recognition motif for the SNF1 complex [30], Ronne's group independently identified Ser-278 and Ser-311 as potential SNF1 sites within the R1 domain and recently, they reported that mutation of both of these sites (together with Ser-310) to alanine in the Mig1-VP16 fusion resulted in an almost complete abolition of the effect of SNF1 (from 6.1-fold to 1.6-fold) [32]. Mutation of Ser-278 or Ser-310/Ser-311 alone reduced the effect of SNF1 to 2.0-fold and 2.6-fold, respectively. Our results now confirm that the SNF1 complex does indeed phosphorylate Ser-278 and Ser-311 efficiently in vitro. Using a mobility shift assay, Ronne's group also reported that the Mig1-Vp16 protein was phosphorylated on multiple sites and that mutation of Ser-278 and Ser-310/Ser-311 reduced, but did not abolish, the SNF1-dependent phosphorylation [32]. In an independent study, Carlson's group also used the recognition motif for SNF1 in order to identify potential phosphorylation sites. Mutation of Ser-222, Ser-278, Ser-311 and Ser-381 (together with Ser-310, Ser-379 and Thr-380) to alanine reduced most of the phosphorylation of a LexA-Mig1p fusion in response to low glucose, although not as substantially as did mutation of *SNF1* [33]. These results suggest that Mig1p contains additional sites for SNF1-dependent phosphorylation. Our data show that SNF1 phosphorylates Ser-222, Ser-278, Ser-311 and Ser-381 in vitro, but not at any other sites to any significant extent. It remains possible that Mig1p is phosphorylated at additional sites by a SNF1-dependent protein kinase. Consistent with this is the finding by Ronne's group that Mig1-Vp16 may

be phosphorylated at Ser-108, which, our results clearly show, is not phosphorylated by SNF1 in vitro.

Taken this together with the findings of Ronne's and Carlson's groups [27,32,33] and our own previous results showing that the SNF1 complex is activated by glucose removal [7,20], our current results strongly suggest that derepression of glucose-repressed genes is brought about in part by direct phosphorylation of Mig1p by SNF1.

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