Hxk2 Regulates the Phosphorylation State of Mig1 and Therefore Its Nucleocytoplasmic Distribution*

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Mig1 and Hxk2 are two major mediators of glucose repression in Saccharomyces cerevisiae. However, the mechanism by which Hxk2 participates in the glucose repression signaling pathway is not completely understood. Recently, it has been demonstrated that Hxk2 interacts with Mig1 to generate a repressor complex located in the nucleus of S. cerevisiae. However, the mechanism by which Mig1 favors the presence of Hxk2 in the nucleus is not clear, and the function of Hxk2 at the repressor complex level is still unknown. Here, we report that serine 311 of Mig1 is a critical residue for interaction with Hxk2 and that this interaction is regulated by glucose. Our findings suggest that Snf1 interacts constitutively with the Hxk2 component of the repressor complex at high and low glucose conditions. Furthermore, we show that Snf1 binds to Mig1 under low glucose conditions and that binding is largely abolished after a shift to high glucose medium. We found that phosphorylation of serine 311 of Mig1 by Snf1 kinase is essential for Mig1 protein nuclear export and derepression of the SUC2 gene in glucose-limited cells. These results allow postulating that the Hxk2 operates by interacting both with Mig1 and Snf1 to inhibit the Mig1 phosphorylation at serine 311 during high glucose grown.

The yeast Saccharomyces cerevisiae has developed mechanisms to adapt to fluctuations in food supply. This ability to grow in different media is a result of the capacity of S. cerevisiae to sense and respond to changes in the availability of nutrients. Although transcriptional control is the focus of the majority of investigated glucose responses, responses to environmental stimuli bring about changes in gene expression (1), mRNA stability (2), and post-translational modifications (3). Among sugars, glucose is the preferred carbon and energy source. The information regarding glucose availability is transduced to the nucleus by three main systems, the Mig1-Hxk2 glucose repression signaling pathway (4, 5), the Sfn3-Rgt2 sensing pathway (6), and the Gpr1-cAMP pathway (7). In the glucose repression signaling pathway, two major mediators are proteins, Mig1 and Hxk2. The functions of both proteins have been extensively studied, but there is limited information about possible interactions among them in the repression pathway. The Mig1-dependent aspect of the glucose repression pathway has been elucidated in great detail. Snf1 kinase, a homologue of mammalian AMP-activated kinase, plays a central role in regulating this part of the pathway. The Snf1 kinase, under low glucose conditions, is activated and modifies the phosphorylation state of Mig1 (8). Snf1 kinase forms a complex with an activating subunit Snf4 and one of the three Snf1-interacting proteins Sip1, Sip2, or Gal83, which target Snf1 to distinct subcellular locations (9). Regulation of Snf1 activity involves phosphorylation of the Snf1 catalytic subunit by three kinases (10, 11) and binding of Snf4 (12). In addition, a decreased Glc7 phosphatase activity, in a rreg1 mutant strain for example, or the absence of the Hxk2 protein may also contribute to Snf1 activation (13–15). The activated Snf1 protein kinase phosphorylates at least four serine residues of Mig1 protein in low glucose growth conditions (13). Mig1 phosphorylation causes translocation of the protein from the nucleus to the cytoplasm (16). Thus, the activity of Mig1 is regulated by phosphorylation and subcellular localization. In high glucose, Mig1 is dephosphorylated by the Glc7-Reg1 protein phosphatase complex (17) and is found primarily in the nucleus, where it can repress transcription.

In contrast to the fairly well defined Mig1 portion of the glucose repression signaling pathway, the mechanisms underlying the Hxk2 functions are not fully understood. Evidence demonstrates that the nuclear localization of both Hxk2 and Mig1 proteins are required for glucose repression signaling (18, 19). Current evidence suggests that Hxk2 nuclear localization is modulated by the availability of glucose, and Mig1 is required to sequester Hxk2 in the nucleus (20). Recent data revealed that the mechanism of Hxk2 sequestration in the nucleus is based on direct Hxk2 interaction with Mig1. This interaction is mediated by a 10-amino acid motif in Hxk2 located between Lys-6 and Met-15 (18, 20) and has been detected at the DNA level in vivo by chromatim immunoprecipitation experiments (21). Thus, these findings suggest that the main role of Hxk2 is produced by interacting with Mig1 to generate a repressor complex located in the nucleus of S. cerevisiae. However, the mechanism by which Mig1 favors the presence of Hxk2 in the nucleus is not
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| TABLE 1 | Recombinant plasmids and encoded proteins |
|---------|-----------------------------------------|
| Plasmid | Encoded product | Source |
| pGBKT7/MIG1–504 | Mig1 67 | This study |
| pGBKT7/MIG1–381 | Mig1 without amino acids from Gly-382 to Asp-504 | This study |
| pGBKT7/MIG1–352 | Mig1 without amino acids from Asn-353 to Asp-359 | This study |
| pGBKT7/MIG1–240/352 | Mig1 without amino acids from Ser-241 to Asp-359 | This study |
| pGBKT7/MIG1–123 | Mig1 without amino acids from Asn-123 to Asp-123 | This study |
| pGBKT7/MIG1–67 | Mig1 without amino acids from Cys-68 to Asp-454 | This study |
| pGBKT7/MIG1–4486 | Mig1 with Ser-278 changed to Ala 23 | This study |
| pACT2/HXK2–248 | Hxk2 248 | (20) |
| YEp352/MIG1::gfp | Mig1-GFP fusion protein | This study |
| YEp352/MIG1S10A::gfp | Mig1S10A-GFP fusion protein | This study |
| YEp352/MIG1S11A::gfp | Mig1S11A-GFP fusion protein | This study |
| pWS93/MIG1 | HA-tagged Mig1 expression controlled by the ADH1 promoter | This study |
| pWS93/MIG1 (S11A) | HA-tagged Mig1S11A expression controlled by the ADH1 promoter | This study |
| pGEX/HXK2 | GST-Hxk2 fusion protein | (42) |
| pGEX/MIG1 | GST-Mig1 fusion protein | This study |
| pGEX/MIG1 (S311A) | GST-Mig1S311A fusion protein | This study |
| pGBKT7/MIG1–504 | Mig1 with Ser-311 changed to Ala | This study |
| pGBKT7/MIG1–381 | Mig1 with Ser-311 changed to Ala | This study |
| pGBKT7/MIG1–240/352 | Mig1 with Ser-311 changed to Ala | This study |
| pGBKT7/MIG1–4486 | Mig1 with Ser-311 changed to Ala | This study |
| pACT2/HXK2–248 | Hxk2 248 | (20) |
| YEp352/MIG1::gfp | Mig1-GFP fusion protein | This study |
| YEp352/MIG1S10A::gfp | Mig1S10A-GFP fusion protein | This study |
| YEp352/MIG1S11A::gfp | Mig1S11A-GFP fusion protein | This study |
| pWS93/MIG1 | HA-tagged Mig1 expression controlled by the ADH1 promoter | This study |
| pWS93/MIG1 (S11A) | HA-tagged Mig1S11A expression controlled by the ADH1 promoter | This study |
| pGEX/HXK2 | GST-Hxk2 fusion protein | (42) |
| pGEX/MIG1 | GST-Mig1 fusion protein | This study |
| pGEX/MIG1 (S311A) | GST-Mig1S311A fusion protein | This study |
| pGBKT7/MIG1–504 | Mig1 with Ser-311 changed to Ala | This study |
| pGBKT7/MIG1–381 | Mig1 with Ser-311 changed to Ala | This study |
| pGBKT7/MIG1–240/352 | Mig1 with Ser-311 changed to Ala | This study |
| pGBKT7/MIG1–4486 | Mig1 with Ser-311 changed to Ala | This study |

* Fusion protein with the DNA-binding domain of Gal4p.

** Fusion protein with the activation domain of Gal4p.

clear, and the function of Hxk2 at the nuclear repressor complex level is still unknown.

In this study, the functions of Hxk2 in regulating the Mig1 phosphorylation state and its subcellular localization were analyzed. Here, we demonstrate that Hxk2 interacts in vivo with both Mig1 and the Snf1 catalytic subunit of the Snf1 protein kinase complex. We also show that serine 311 of Mig1 is a critical residue controlling Mig1 subcellular localization and derepression of genes regulated by the Hxx2-Mig1 glucose repression signaling pathway, such as the SUC2 gene.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Yeast two-hybrid experiments employed strain Y187 (MATa ura3–52 his3–200 ade2–101 trp1–901 leu2–3,112 gal4Δ gal80Δ URA3:: GALIask–GALIask.TATA–lacZ). Co-precipitation experiments utilized yeast strain W303–1A (MATa ade2–1 can1–100 his3–1 Δ leu2–3,112 trp1–289 ura3–52) (22) and DBY2184 (MATa hxx2–202 ura3–52 leu2–3,112 lys2–801 gal2) (23). GFP fluorescence experiments utilized yeast strain Y250 (MATa Suc2 ade2–1 can1–100 his3–1 Δ leu2–3,112 trp1–1 ura3–1 mig1–82::LEU2) (24), FMY301 (MATa mig1–1::LEU2 hxx2–202 ura3–52 leu2–3,112 lys2–801 gal2) (20), K112 (MATa ura3–52 leu2–3,112 his3–1 Δ trp1–289 MALBC Suc2 snf1::HIS3 HAP2) (25), and FMY302 (MATa hxx2::TRP1 ura3–52 leu2–3,112 his3–1 Δ trp1–289 MALBC Suc2 snf1::HIS3 HAP2). FMY302 strain was created by homologous recombination with the loxP-TRP1-loxP marker by using the method of PCR synthesis of marker cassettes (26). FMY303 strain was created by homologous recombination with the 3HA-TADHI–kanMX6 cassette obtained by PCR using as template plasmid pFA6a-3HA-kanMX6 (27). This strain has a modification of the SNF1 gene at the chromosomal level and produces a triple HA epitope-tagged Snf1 protein.

*Escherichia coli* DH5α (F O80lacZ ΔM15 recA1 endA1 gyrA96 thi-1 hsdR17(rk-rk-) supE44 relA1 deoR (lacZ YA-argF)U169) was the host bacterial strain for the recombinant plasmid constructions. Fusion protein expression were performed in *E. coli* BL21(DE3)pLysS (Promega, Madison, WI).

Yeast cells were grown in the following media: YEPD, high glucose (2% glucose, 2% peptone, and 1% yeast extract), YEPE, low glucose (0.05% glucose, 3% ethanol, 2% peptone, and 1% yeast extract), and synthetic media containing the appropriate carbon source and lacking appropriate supplements to maintain selection for plasmids (2% glucose (SD), or 3% ethanol and 0.05% glucose (SE), and 0.67% yeast nitrogen base without amino acids). Amino acids and other growth requirements were added at a final concentration of 20–150 μg/ml. The solid media contained 2% agar in addition to the components described above.

Plasmids—The plasmids used in this study are described in Table 1. For two-hybrid analysis, Mig1 is numbered from residues 1 to 504 (28), and the Hxx2 is numbered from residues 1 to 485 (29). In Mig1, residue 1 is the initiator methionine in the primary translation product. However, in Hxx2, residue 1 is a valine because the initiator methionine is cleaved off in the primary translation product. The plasmids carrying partial segments of MIG1 gene and the encoded products are named according to the codons/amino acids that are retained in the constructs. Plasmids pGBKT7/MIG1 and pACT2/HXK2 were constructed as indicated in a previous study (20). To make plasmids pGBKT7/MIG1 and pACT2/HXK2 were constructed as indicated in a previous study (20). To make plasmids pGBKT7/MIG1 and pACT2/HXK2 were constructed as indicated in a previous study (20). To make plasmids pGBKT7/MIG1 and pACT2/HXK2 were constructed as indicated in a previous study (20). To make plasmids pGBKT7/MIG1 and the linear DNAs were blunt-ended by filling and circulated by self-ligation. To make plasmid pGBKT7/MIG1 Δ240–352 a 423-bp SpeI-Nhel...
fragment was removed from plasmid pGBK77/MIG1, and the linear DNA was circulated by self-ligation.

To mutate sites in Mig1, the 1515-bp BamHI-BamHI fragment from plasmid pGBK77/MIG1, carrying the complete coding region of MIG1 gene, was cloned into pUC19 to give plasmid pUC19/MIG1. Site-directed mutagenesis was carried out by using plasmid pUC19/MIG1 as the template in the PCR reactions and the oligonucleotides described in Table 2. The mutations were confirmed by sequencing. Plasmids containing the corresponding mutations were digested with Spel-Nhel, and the resulting fragments were subcloned into the SpeI and Nhel sites of pGBK77/MIG1 to generate plasmids pGBK77/MIG1S278A, pGBK77/MIG1S310A, and pGBK77/MIG1S311A.

For fluorescence microscopy and glucose repression analysis, plasmids YEp352/MIG1::gfp, YEp352/MIG1S310A::gfp, and YEp352/MIG1S311::gfp were constructed as follows: a 969-bp BamHI-BglII fragment containing the gfp gene was subcloned, respectively, into YEp352/MIG1, YEp352/MIG1S310A, and YEp352/MIG1S311A plasmids first cleaved with BamHI. Plasmid YEp352/MIG1 was constructed as follows: a 2774-bp SacI-SacI fragment containing the complete coding region of MIG1 gene and 565 bp of the 5′ noncoding region promoter were subcloned into an SacI previously cleaved vector YEp352. The resulting plasmid, YEp352/MIG1, contained in a 2.77-kb SacI-SacI fragment the complete MIG1 gene under the control of its own promoter. Plasmids pGBK77/MIG1S278A, pGBK77/MIG1S310A, and pGBK77/MIG1S311A were digested with Spel-Nhel, and the resulting fragments were subcloned into the SpeI and Nhel sites of YEp352/MIG1 to generate plasmids YEp352/MIG1S278A, YEp352/MIG1S310A, and YEp352/MIG1S311A.

Plasmid pWS93/MIG1 was generated by generating a 1.5-kb PCR product containing the MIG1 gene into the BamHI site of vector pWS93, which expresses a triple HA epitope from the ADH1 promoter (a gift of P. Sanz, Valencia). The DNA sequence of all PCR-generated constructs was verified by sequencing. GST fusion vector pGEX/HXK2 was constructed as indicated previously (20).

**Fluorescence Microscopy**—Yeast strains expressing the Mig1-GFP, MIG1S310A-GFP, or MIG1S311A-GFP fusion proteins were grown to early log phase (A600 of <0.7) in synthetic media containing the appropriate carbon source and lacking the appropriate supplements to maintain selection plasmids. The cells (25 μl) were loaded onto poly-L-lysine-coated slides, and the remaining suspension was immediately withdrawn by aspiration. One microliter of DAPI (2.5 μg/ml in 80% glycerol) was added, and a covert slide was placed over the microscope slide. GFP and DAPI localization in live cultures was monitored by direct fluorescence using an Olympus BX61 microscope. Images were processed in Adobe Photoshop 6.0.

**Preparation of Crude Protein Extracts**—Yeast protein extracts were prepared as follows: yeast cells were grown in 10–20 ml of synthetic high glucose medium (SD-ura) at 28 °C to an optical density at 600 nm of 1.0. Half of the culture was shifted to synthetic low glucose medium (SE-ura) for 1 h. Cells were collected, washed twice with 1 ml of 1× sorbitol, and suspended in 100 μl of 50 mM Tris-HCl (pH 7.5) buffer containing 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.42 mM NaCl, and 1.5 mM MgCl2. The cells were broken in the presence of glass beads by one pulse of 20 s using a FastPrep (Thermo Electron Co.), and 400 μl of the same buffer was added to the suspension. After centrifugation at 19,000 × g (14,000 rpm) for 15 min at 4 °C, the supernatant was used as crude protein extract.

**Enzyme Assays**—For β-galactosidase activity determinations, crude extracts were prepared with glass beads as described above and o-nitrophenol-β-D-galactopyranoside (2 mg/ml) was used as a substrate (30). Specific activity was calculated in relation to total protein in the crude extract, using bovine serum albumin as the standard. Invertase activity was assayed in whole cells as previously described (31) and expressed as micromoles of glucose released per minute per 100 mg of cells (dry weight).

**Immunoblot Analysis**—Mutant or wild-type yeast cells were grown to an optical density at 600 nm of 1.0 in selective medium containing high glucose (2%) and shifted to 0.05% glucose for 1 h. Cells were collected by centrifugation (3000 × g, 4 °C, 1 min), and crude extracts were prepared as described above. For Western blotting, 20–40 μg of proteins was separated by 12% SDS-PAGE and transferred to enhanced chemiluminescence polyvinylidene difluoride transfer membrane (Amersham Biosciences) by electroblotting, which was then incubated with an anti-HA or anti-Hxk2 antibody. Horseradish peroxidase-conjugated protein-A was used as a secondary reactant. The complex was detected by the West Pico Chemiluminescent system (Pierce).

**Yeast Two-hybrid Analysis**—The yeast two-hybrid analysis (32) employed yeast vectors pGADT7 and pGBK77T7 and host strains Y187 (described above) in accordance with the Matchmaker two-hybrid system 3 from Clontech. The transformed yeasts were grown in high glucose (SD/-Leu,Trp) medium and shifted for 1 h to low glucose (SE/-Leu,Trp) medium. Assays for β-galactosidase activity followed protocols described elsewhere (30). Expression levels of the GAD and GBD fusion proteins were controlled by Western blot analysis. Experiments were performed a minimum of three times. Values shown are representative results from individual experiments.

**Coimmunoprecipitation Assays**—Immunoprecipitation experiments were performed by using whole cell extracts, from a wild-type yeast strain with a modified SNF1 gene, which codes for a
C-terminal Snf1 protein tagged with 3HA epitopes. Extracts were incubated with anti-HA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Pho4 polyclonal antibody for 1 h at 4°C. Protein A-Sepharose beads (Amersham Biosciences) were then added, and the mixture was incubated for 1 h at 4°C. After extensive washes with Staph A buffer (150 mM NaCl, 100 mM Na2HPO4, 18 mM NaH2PO4, pH 7.3, 20% Triton X-100, 1% SDS, 5% deoxycholate), immunoprecipitated samples were boiled in SDS-loading buffer. The supernatant was subjected to 12% SDS-PAGE and detected by Western blot using anti-Hxk2 polyclonal antibody and horseradish peroxidase-conjugated protein-A by the West Pico Chemiluminescent system (Pierce).

**RESULTS**

**Mig1 Undergoes Hxk2-dependent Nucleocytoplasmic Transport**—To gain insight into the molecular mechanism by which Hxk2 regulates Mig1 function, the glucose-dependent subcellular location of Mig1 was determined in Δhxk2 mutant cells. To detect Mig1 in live cells, GFP was fused to the C terminus of Mig1, and the fusion protein was expressed under control of the MIG1 promoter. It has been previously reported and our results confirm (Fig. 1) that, in high glucose, Mig1 is located in the nucleus and, upon glucose removal, Mig1 rapidly translocates to the cytoplasm (16, 33). Interestingly, fluorescence microscopy revealed that, in the absence of the Hxk2 protein, Mig1 is located in the cytoplasm both at high and low glucose conditions (Fig. 1). Previous results indicate that Hxk2 associates directly with Mig1 and that the 6KKPQARK15 motif of Hxk2 is required for interaction with Mig1 at high glucose-grown conditions (20). The most straightforward interpretation of this result could be that, in the absence of Hxk2, the protein kinase Snf1 is constitutively activated (15, 34) and, therefore, Mig1 should be constitutively localized in the cytoplasm. However, it could also be that the Mig1-Hxk2 complex is a carrier necessary for Mig1 translocation to the nucleus. This idea is supported by the fact that Hxk2 and Mig1 proteins are physically associated at the nuclear level (20, 21) and an Hxk2-Mig1-DNA complex seems to be required in vivo for glucose repression signaling (21).

Therefore, we checked Mig1 localization in the double Δhxk2Δsnf1 mutant strain to determine whether Snf1 protein kinase activity is epistatic to Hxk2 function. The results demonstrate that Mig1 is targeted to the nucleus in the absence of Hxk2 protein (Fig. 2), indicating that the Hxk2 protein function is dispensable for nuclear localization.

To determine whether the cytoplasmic localization of Mig1 in the Δhxk2 mutant cells growing in high glucose medium entails phosphorylation of Mig1, we examined Mig1 phosphorylation in vivo in response to high and low levels of glucose (Fig. 3). For this purpose, we prepared extracts from Δsnf1, Δhxk2, and Δhxk2Δsnf1 mutant strains transformed with the Mig1-3HA construct. The transformed yeast cells were grown in the presence of high or low glucose. The mobility of Mig1 on SDS-PAGE was examined by Western blotting using an anti-HA antibody. The mobility of Mig1 varies depending on its phosphorylation state (14). As shown in Fig. 3A (lanes 1 and 2), in the presence of Hxk2, Mig1 from low glucose grown cells displays a slight shift in molecular weight, resulting in decreased mobility, in comparison to Mig1 from high glucose grown cells. However, in the absence of Hxk2 the Snf1 protein kinase complex assumes an active conformation even in the presence of glucose (13, 35), and Mig1 protein from both high
Snf1 Interacts with Hxk2 and Mig1

Snf1 protein kinase activity is epistatic to Hxk2 function regulating Mig1 subcellular localization. Yeast strains K112 and FMY302 expressing Mig1-GFP from a plasmid (YEp352/Mig1::gfp) were grown as indicated in Fig. 1. Mig1-GFP is always localized to the nucleus both in Δsnf1 and Δsnf1/hxk2 mutant cells grown in high or low glucose conditions. The cells were stained with DAPI and imaged for GFP fluorescence and DAPI fluorescence, and then analyzed by phase-contrast optics. DNA was stained with DAPI.

A Fusion Protein with the Adequate Molecular Mass Was Detected

FIGURE 2. Snf1 protein kinase activity is epistatic to Hxk2 function regulating Mig1 subcellular localization. Yeast strains K112 and FMY302 expressing Mig1-GFP from a plasmid (YEp352/Mig1::gfp) were grown as indicated in Fig. 1. Mig1-GFP is always localized to the nucleus both in Δsnf1 and Δsnf1/hxk2 mutant cells grown in high or low glucose conditions. The cells were stained with DAPI and imaged for GFP fluorescence and DAPI fluorescence, and then analyzed by phase-contrast optics. DNA was stained with DAPI.

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FIGURE 3. Mig1 phosphorylation in response to Hxk2 and Snf1 availability. Cells, from wild-type and the Δhxk2 mutant strain (A) or wild-type, Δsnf1, and Δsnf1/Δhxk2 mutant strains (B) transformed with the HA-Mig1 construct (plasmid pWS93/MIG1), were grown in high glucose (H-Glc) or low glucose (L-Glc) media until an A_{595} of 1.0 was reached. The Mig1 protein was detected from total cell extracts by SDS-PAGE followed by immunoblotting with an anti-HA antibody. The phosphorylated forms of Mig1 are indicated as HA-Mig1-P (phosphorylated) and HA-Mig1 (dephosphorylated).

and low glucose-grown cells has decreased mobility (Fig. 3A, lanes 3 and 4) indicating that the protein is phosphorylated. In the absence of Snf1 or both Hxk2 and Snf1, Mig1 has an increased mobility pattern (Fig. 3B, lanes 3–6) indicating that the protein is dephosphorylated.

Mig1 Interacts with Hxk2 in a Glucose-regulated Manner and Requires Serine 311 for Interaction—Previous evidence has indicated that Snf1 is able to phosphorylate Mig1 at four sites (serines 222, 278, 311, and 381). These sites represent the differential phosphorylation of Mig1 in response to glucose (13, 36, 37). To get a more comprehensive picture of the mechanism by which Mig1 favors the presence of Hxk2 in the nucleus and how Hxk2 regulates the Snf1 activity coupled to the Mig1 phosphorylation, we tested whether the Snf1-dependent phosphorylation sites of Mig1 are important for its interaction with Hxk2.

With this aim, full-length, subfragments and single amino acid mutations of Mig1 were tested for their interaction with Hxk2 in a two-hybrid assay both at high and low glucose conditions (Fig. 4). As shown in Fig. 4B, full-length Mig1-504, Mig1-381, and Mig1-352 proteins were able to interact with the full-length Hxk2 in high glucose medium, but the interaction was not detected in cells grown in low glucose conditions. However, the Mig1-240, Mig1-123, and Mig1-67 truncated proteins did not interact with Hxk2. These results suggest that the interacting domain of Mig1 might include a motif containing serines 278, 310, 311, or 381. Because no significant binding of Hxk2 to an internal truncated Mig1-Δ240/352 protein was detected, we suppose that a motif containing serines 278, 310, or 311 could be critical to the Mig1-Hxk2 interaction. To address these possibilities, we converted the phosphorylatable serines 278, 310, or 311 to alanine by site-directed mutagenesis and examined the ability of Mig1-S278A, Mig1-S310A, and Mig1-S311A mutant proteins to interact with Hxk2 in a two-hybrid assay both at high and low glucose conditions. We found that Mig1-S311A is unable to interact with Hxk2 both in high glucose-grown cells and after a shift to low glucose, whereas Mig1-S278A and Mig1-S310A interact strongly in high glucose-grown cells but not after a shift to low glucose.

To test whether the mutant protein Mig1S311A was expressed, cell extracts were subjected to Western blot analysis. A fusion protein with the adequate molecular mass was detected (data not shown), which indicated that the point mutation was responsible for the absence of interaction shown by this Mig1 mutant protein.

The glucose-dependent interaction between Mig1 and Hxk2 was confirmed by GST pull-down experiments. We used crude protein extracts and a purified GST-Hxk2 fusion protein. The extracts were obtained from yeast cells grown in high and low glucose synthetic media with selection for plasmid maintenance. As shown in Fig. 4C, a clear retention of Mig1 protein was observed for the samples containing GST-Hxk2 and crude extracts from high glucose-grown wild-type cells expressing a Mig1 HA-tagged protein (lane 1). However, no signals were observed when the experiment was done using crude extracts from low glucose-grown cells expressing a Mig1 HA-tagged protein or the wild-type strain expressing the HA-Mig1S311A protein (Fig. 4C, lanes 2–4). The lack of interaction cannot be attributed to lack of expression, because the HA-Mig1 and the HA-Mig1S311A fusion proteins were detected both at high and low glucose conditions by Western blotting using anti-HA antibody (Fig. 4D). When a control with GST protein in the reaction mixture was used, no signal was observed (data not shown). Thus, taken together these results imply that serine 311 of the Mig1 protein is critical for Mig1 interaction with Hxk2.

Snf1 Interacts with Hxk2 and Mig1—Previous data suggest that Hxk2 and Mig1 are in a complex located in the nucleus (18, 20, 21), and Snf1 interacts with Mig1 in a glucose-regulated manner (13). Moreover, Snf1 activity is required for Mig1 phosphorylation in response to low glucose growth conditions (13,
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FIGURE 4. Interaction of Mig1 with Hxk2. A, schematic representation of the utilized constructs. ZN, zinc fingers; RD, repression domain. Serine residues that are phosphorylated by Snf1 are marked. B, yeast two-hybrid interaction of Mig1 with Hxk2. Mig1 interaction with Hxk2 protein during high glucose growth conditions (solid bars), Mig1 interaction with Hxk2 protein during low glucose growth conditions (empty bars) and with GAD alone (crossed bars). Partially (Mig1 1-381, aa 1–381; Mig1 1-352, aa 1–352; Mig1 1-240, aa 1–240; Mig1 1-123, aa 1–123; Mig1 1-67, aa 1–67; and Mig1 1-240/352, lacking aa 240–352), point mutations (Mig1 S278A, serine 278 has been changed to alanine; Mig1 S310A, serine 310 has been changed to alanine; and Mig1 S311A, serine 311 has been changed to alanine), and full-length (Mig1, aa 1–504) Mig1 fused to the Gal4 DNA binding domain (GBD) were individually co-transformed into yeast strain Y187 with constructs encoding the Gal4 activation domain (GAD) alone or GAD fused to full-length (Hxk2, aa 1–485). Hxk2. The full-length Hxk2 fused to the GAD was also co-transformed into yeast strain Y187 with construct encoding the GBD alone, as indicated in a previous study (20). The transformed yeast cells were grown in SD-media lacking appropriate supplements to maintain selection for plasmids and were harvested at early log-phase (A600 nm 0.6).

Protein-protein interactions were examined in each transformant by the qualitative and quantitative assay methods for β-galactosidase activity. The values are the averages of β-galactosidase activity for three transformants. Each measured value was within 12% of the average. C, GST pull-down assays of the interaction of Hxk2 with Mig1. GST pull-down assays of Mig1 with HA-Mig1 and HA-Mig1S311A. A GST-Hxk2 fusion protein was purified on glutathione-Sepharose columns. GST-Hxk2 was incubated with cell extracts from a wild-type strain transformed with pWS93/Mig1 (construct HA-Mig1) or pWS93/Mig1S311A (construct HA-Mig1S311A) plasmids. The transformed strains were grown in high glucose (H-Glc) (lanes 1 and 3) or low glucose (L-Glc) medium (lanes 2 and 4). For the control samples, GST protein was also incubated with the H-Glc and L-Glc cell extracts but no signals were detected (data not shown). D, Western blot analysis of HA-Mig1 and HA-Mig1S311A fusion proteins expression at high and low glucose conditions.

36). Thus, it is tempting to speculate that Snf1 interacts with Hxk2, and the Hxk2 functions in the Mig1 repressor complex is to inhibit Mig1 phosphorylation at serine 311 by Snf1, during high glucose conditions. To address these possibilities, we examined the ability of Snf1 to interact with Hxk2 by using an immunoprecipitation assay. Cell extracts from a strain with a modified SNF1 gene, which codes for a C-terminal Snf1 protein tagged with 3HA epitopes, were immunoprecipitated with the antibodies indicated in Fig. 5A. The resulting immunoprecipi-

tates were assayed for the presence of Hxk2 by immunoblot analysis with anti-Hxk2 antibodies. As shown in Fig. 5A (lanes 4 and 5), a strong and specific signal of Hxk2 was observed both with samples immunoprecipitated from high and low glucose-grown cultures. When an anti-Pho4 antibody or no antibody was used to detect nonspecific immunoprecipitation and nonspecific protein binding to anti-Hxk2 antibody, respectively, no signals were observed. Thus, this interaction is dependent on the production of HA-tagged Snf1 as well as anti-Hxk2 antibody. To confirm the interaction of Snf1 with Hxk2, we employed the GST pull-down technique. We used extracts from the Snf1-HA-producing strain and a bacterially produced GST-Hxk2 fusion protein. As shown in Fig. 5B (lanes 3 and 4), a strong and specific band was observed in extracts from either high or low glucose-grown cells.

To confirm that Snf1 interacts with Mig1 in a glucose-regulated manner and to address the influence of Mig1S311A mutation on this interaction, we have employed GST pull-down assays. We used extracts from the Snf1-HA-producing strain and bacterially produced GST-Mig1 and GST-Mig1S311A fusion proteins. As shown, in Fig. 5C (lanes 1 and 3), Mig1 or the Mig1S311A and Snf1 interact weakly in high glucose-grown cells, but after cells were shifted to low glucose medium for 5 min (lanes 2 and 4), a strong signal was observed. To confirm that the same amount of Snf1 protein is present in each sample used in these experiments, cell extracts from high and low glucose-grown cells were subjected to Western blot analysis (Fig. 5D).

These results, together with previous data, provide strong evidence that Snf1 interacts both with Hxk2 and Mig1 components of repressor complex. Because the interaction between Snf1 and Hxk2 was detected both at high and low glucose growth conditions, our data suggest that both proteins interact constitutively. However, the interaction between Snf1 and Mig1 was only detected at low glucose conditions indicating that the interaction is regulated by glucose concentration in the medium. Identical results were observed when the experiment
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FIGURE 5. Immunoprecipitation and GST pull-down assays of the interaction of Snf1 with Hxk2 and Mig1. A, in vivo co-immunoprecipitation of Hxk2 with Snf1. Cell extracts from the modified FMY303 strain grown in high glucose (H-Glc) or low glucose (L-Glc) medium (lanes 3 and 4), were immunoprecipitated with a monoclonal antibody to HA, or a polyvalent antibody to Pho4 (lane 2). Cell extracts from the wild-type strain W3031A grown in SD-high glucose medium, were used as control (lane 1). Immunoprecipitates were separated by 12% SDS-PAGE, and co-precipitated Hxk2 was visualized on a Western blot with an anti-Hxk2 antibody. B, GST pull-down assays of the interaction between Snf1-Hxk2. Purified Hxk2 interacts with Snf1. A GST-Hxk2 fusion protein was purified on glutathione-Sepharose columns. GST-Hxk2 was incubated with cell extracts from the modified FMY303 strain grown in H-Glc or L-Glc media (lanes 3 and 4). For the control samples, GST protein was also incubated with the H-Glc and L-Glc cell extracts (lanes 1 and 2). C, GST pull-down assays of the Snf1 interaction with Mig1 and Mig1S311A. GST-Mig1- or GST-Mig1S311A-purified proteins were incubated with cell extracts from the modified FMY303 strain. The transformed strain was grown in H-Glc (lanes 1 and 3) or L-Glc media (lanes 2 and 4). For the control samples, GST protein was also incubated with the L-Glc and H-Glc cell extracts, but no signals were detected (lanes 5 and 6). D, Western blot analysis of HA-Snf1 fusion protein expression at high- and low-glucose conditions.

Serine 311 of the Mig1 protein is essential for Mig1 export from the nucleus to the cytoplasm. Yeast strain H250 expressing Mig1S310A-GFP and Mig1S311A-GFP from plasmids (YEps352/Mig1S310A::gfp and YEps352/Mig1S311A::gfp) was grown on high glucose synthetic medium (SD-ura) until an A_{600nm} of 1.0 was reached and then transferred to synthetic medium with low glucose for 60 min. Mig1S310A-GFP has a nuclear and cytoplasmic location in Δmig1 mutant cells grown, respectively, in high glucose and low glucose conditions. However, Mig1S311A-GFP is always localized to the nucleus in Δmig1 mutant cells grown both in high glucose or low glucose conditions. The cells were stained with DAPI and imaged for GFP fluorescence and DAPI fluorescence, and then analyzed by phase-contrast optics. DNA was stained with DAPI.

was done using the GST-Mig1S311A fusion protein and extracts from the Snf1-HA-producing strain. Mig1S311A and Snf1 interact strongly in low glucose-grown cells but not after a shift to high glucose. These results suggest that the serine 311 of the Mig1 protein is not an essential residue for Mig1-Snf1 interaction.

Hxk2 Regulates the Snf1-dependent Phosphorylation of Mig1 at Serine 311—We tried to use Western blot analysis to determine whether Hxk2 binding to Mig1 affects serine 311 phosphorylation by the Snf1 kinase. However, our results indicated that the mutation of a single residue on Mig1 does not display any glucose-regulated shift in mobility of the protein (data not shown).

Because in high glucose, Mig1 is dephosphorylated by the Glc7-Reg1 protein phosphatase complex (17) and is located in the nucleus and upon glucose removal, Mig1 is rapidly phosphorylated by the Snf1 protein kinase complex (13, 16) and translocated into the cytoplasm (33). We utilized the correlation between location and phosphorylation of Mig1 to enhance our ability to detect whether Mig1 phosphorylation at serine 311 by the Snf1 kinase is a critical event responsible for the nucleocytoplasmic translocation of Mig1.

To detect Mig1 mutant proteins in live cells, GFP was fused to the C terminus of Mig1S310A and Mig1S311A, and the fusion proteins were expressed under the control of the MIG1 promoter (Fig. 6). The results indicate that Mig1S310A (used as control) had a glucose-regulated subcellular distribution identical to the wild-type Mig1 protein. However, Mig1S311A was detected in the nucleus both at high and low glucose conditions. The simple interpretation of this result is that serine 311 is critical for Mig1 translocation to the cytoplasm in a glucose-regulated manner. Moreover, translocation of Mig1 to the cytoplasm in response to low glucose conditions is due to Mig1 phosphorylation, and serine 311 is one of the differentially phosphorylated residues in response to glucose. These results strongly support the notion that serine 311 of Mig1 is the main phosphorylated residue responsible for determining the glucose-regulated location of Mig1. Since, a change in serine 311 to alanine in the Mig1 protein disrupted binding to Hxk2; our results also suggest that serine 311 phosphorylation mediated by the Snf1 kinase is inhibited by Hxk2 during high glucose growth conditions and results in targeting Mig1 to the nucleus.

Serine 311 of Mig1 Is Essential for Derepression of the SUC2 Gene—The MIG1 gene and the mutant alleles of Mig1 were subcloned into the S. cerevisiae expression plasmid YEps352 under the control of the MIG1 promoter and tested for the ability to restore glucose repression in a Δmig1 mutant strain. As depicted in Fig. 7, none of the mutated sites is essential for...
Mig1 repressor function. All of the Mig1 proteins analyzed containing these mutations restored glucose repression of \( SUC2 \) in the \( \Delta mig1 \) mutant cells grown under high glucose conditions. Moreover, the two Mig1 alleles coding for mutant proteins with serines 278 and 310 changed to alanine showed identical capacity of \( SUC2 \) gene derepression as a wild-type Mig1 protein. However, \( SUC2 \) gene derepression activity is lost in the Mig1S311A-expressing strain grown in low glucose medium. Therefore, we conclude that serine 311 of Mig1 is required for derepression of genes controlled by the Mig1-Hxk2 glucose repression signaling pathway. This derepression is mediated by serine 311 phosphorylation by the Snf1 kinase complex, which regulates the nucleocytoplasmic distribution of Mig1 protein.

**DISCUSSION**

Genetic evidence suggests that the Snf1 kinase inhibits repression by Mig1 during glucose limitation (37) and that Hxk2 is required to inhibit Snf1 activity in high glucose growth conditions (38). Current results favor a model in which, during high glucose levels, Hxk2 regulates the phosphorylation status of Reg1 and thus influences Glc7 phosphatase to negatively regulate Snf1 kinase activity (15). Because the Hxk2-Mig1 complex plays an essential role in glucose repression signaling of several genes (20), detailed knowledge of the interaction between these two proteins and the molecular mechanism by which Hxk2 regulates Mig1 function is required to understand the glucose repression mechanism.

Two-hybrid and GST pull-down experiments have shown that high glucose in the medium is required for Hxk2-Mig1 complex formation. To define the domains of Mig1 protein that are important for Hxk2 interaction, we screened several constructs containing deletions and site-directed mutations of the \( MIG1 \) gene for loss of interaction by the two-hybrid assay. Our results led us to identify a new Mig1 mutant (Mig1S311A) in which its interaction with Hxk2 is abolished. Thus, the first important observation described in this study is that serine 311 of the Mig1 protein is essential for Mig1 interaction with Hxk2.

The focus of this study has been on the regulation of Mig1 repressor function through its modification at serine 311. Because the Mig1S311A mutation confers a constitutive nuclear localization to Mig1 protein at high and low glucose conditions and inhibits derepression of the \( SUC2 \) gene, it is suggested that phosphorylation of Mig1 on serine 311 is essential for cytoplasmic Mig1 localization and derepression of the \( SUC2 \) gene. Thus, we have demonstrated that the interaction patterns of mutant proteins are correlated with their subcellular location and regulatory function in the yeast cell.

The correlation between Snf1 protein kinase activity, Mig1 phosphorylation state, and its subcellular location has been intensely studied (12, 15, 36). However, recently it was reported that, although both glucose stress and sodium ion stress cause a rapid increase in Snf1 activity by its phosphorylation at threonine 220, only glucose stress triggers the phosphorylation of...
Mig1 protein (14). Therefore, Snf1 activation by phosphorylation can be necessary but not sufficient to trigger the glucose derepression pathway. It is well documented that Snf1 phosphorylation by redundant Snf1-activating kinases and a step mediated by the Snf4 subunit is required for the metabolic changes allowing utilization of alternative carbon sources (14). However, our results suggest a role for the non-phosphorylated form of Snf1 during glucose growth, because Hxk2 is required to inhibit Mig1 phosphorylation by Snf1 kinase in high glucose growth conditions. Interestingly the non-phosphorylated form of Snf1 may have a direct role in controlling the expression of the TRK1 and TRK2 genes of the Trk high affinity potassium uptake system (39).

In conclusion, our findings allow us to propose a new mechanism by which Hxk2 participates in the repression process in yeast cells (Fig. 8). In high glucose growth conditions, nuclear Hxk2 interacts both with Mig1 and the Snf1 protein kinase complex to establish the repression state of several genes. Hxk2 interacts strongly with Mig1 in high glucose-grown cells and our findings implicate serine 311 of Mig1 as a critical residue for this interaction. However, the interaction between Snf1 and Hxk2 was detected both in high and low glucose growth conditions, and our data suggest that both proteins interact constitutively. Our results and previous data support that low glucose grown enhances interaction between Mig1 and Snf1, presumably transient because no interaction was detected in cells grown in raffinose (13) or ethanol for several hours (data not shown). Thus, our findings together with previous ones suggest that the Hxk2 protein functions as a bridge between Snf1 and Mig1 and is required to inhibit Mig1 phosphorylation at serine 311 by Snf1 kinase during high glucose growth conditions.

Sak1 kinase is the most important of the three kinases (Sak1, Tos3, and Elm1) for activating Snf1-Gal83 in response to glucose limitations (40). Snf1-Gal83 is the form that is responsible for most of the Snf1 nuclear activity in glucose-grown cells (41).

In low glucose-grown cells, the Hxk2 interaction with Mig1 is abolished and a transient increase in interaction between Snf1 and Mig1 was detected (Fig. 5C) (13). These interaction patterns potentially stimulate Mig1 phosphorylation by Snf1 kinase at serine 311. Although the differential phosphorylation of Mig1 in response to glucose involves four serines residues, our findings demonstrated that the only critical residue is serine 311. Phosphorylation of serine 311 results in Mig1 export from nucleus to cytoplasm and, ultimately, the derepression of genes regulated by the Hxk2-Mig1 glucose repression signaling pathway.

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