

Functional domains of yeast hexokinase 2

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Hxk2 (hexokinase 2) from *Saccharomyces cerevisiae* was one of the first metabolic enzymes described as a multifunctional protein. Hxk2 has a double subcellular localization: it functions as a glycolytic enzyme in the cytoplasm and as a regulator of gene transcription of several Mig1-regulated genes in the nucleus. To get more insights into the structure–function relationships of the Hxk2 protein, we followed two different approaches. In the first, we deleted the last eight amino acids of Hxk2 and replaced Ser³⁰⁴ with phenylalanine to generate Hxk2_{wca}. Analysis of this mutant demonstrated that these domains play an essential role in the catalytic activity of yeast Hxk2, but has no effect on the regulatory function of this protein. In the second, we analysed whether amino acids from Lys⁶ to Met¹⁵ of Hxk2 (Hxk2_{wrf}) are essential for the regulatory role of Hxk2 and whether there is an effect on the hexose kinase activity of this protein. In the present paper, we report that the Hxk2_{wca} mutant protein interacts

with the Mig1 transcriptional repressor and the Snf1 protein kinase in the nucleus at the level of the SUC2–Mig1 repressor complex. We have demonstrated that Hxk2_{wca} maintained full regulatory function because the glucose-repression signalling of the wild-type machinery is maintained. We also report that the Hxk2_{wrf} mutant allele is incapable of glucose repression signalling because it does not interact with Mig1 at the level of the SUC2–Mig1 repressor complex. The two mutants, Hxk2_{wca} and Hxk2_{wrf} retain single functions, as a transcriptional factor or as an enzyme with hexose-phosphorylating activity, but have lost the original bifunctionality of Hxk2.

Key words: catalytic domain, glucose repression, glucose signalling, hexokinase 2, regulatory domain, yeast.

INTRODUCTION

There are three glucose-kinase isoenzymes in *Saccharomyces cerevisiae* [1]: isoenzymes 1 and 2 phosphorylate both aldo- and keto-sugars, whereas glucokinase is specific for aldo-hexoses. Isoenzyme 2 of hexokinase (Hxk2) is the predominant glucose-kinase in *S. cerevisiae* grown on glucose media [2] and carries out at least two cellular functions. Classical structural studies of yeast Hxk2 reveal a well-defined catalytic domain that binds ATP and hexose (e.g. glucose), allowing transfer of a phosphoryl group from ATP to the C-6 of the sugar [3–5]. In addition to the well-known catalytic role of Hxk2, in the last few years, a new non-canonical function for this protein has been described. It has been suggested that the Hxk2 protein has special functions in transcriptional regulation [6]. Functional studies suggest that the main regulatory role of Hxk2 is produced by interaction with the transcriptional repressor Mig1 and the Snf1 protein kinase to generate a repressor complex in the nucleus [7,8]. Under high-glucose conditions, Hxk2 stabilizes the repressor complex, blocking Mig1 phosphorylation by Snf1 kinase [9]. Thus the complex is involved in the glucose-repression signalling of several Mig1-regulated genes. A determinant for the Hxk2–Mig1 interaction has been characterized as an internal sequence between Lys⁶ and Met¹⁵ of the Hxk2 protein [10]. Moreover, it has been suggested that a fraction of the Hxk2 protein is sequestered to the nucleus by interacting with Mig1 through this amino acid region.

Despite the large number of studies carried out to identify a correlation between catalytic activity and regulatory function in the Hxk2 protein, little is known about the existence of different

domains in the protein that may control each activity. Both point [11] and null [12] mutations in the *HXK2* gene have been found that blocked glucose repression of certain genes. Since the glucose-phosphorylating activity in the corresponding extracts was reduced, the idea that there was a correlation between the glucose-phosphorylating activity of Hxk2 and glucose repression appeared a very attractive one [1,13]. However, this idea can be challenged if we take into account the following results: (i) when the *GLK1* (glucokinase 1) gene is overexpressed in a $\Delta hxk1 \Delta hxk2$ double-null mutant the transformed strains are still insensitive to glucose repression, even though a 3-fold increase in phosphorylating activity is achieved [1], (ii) glucose repression is not linearly reduced with decreasing kinase activity [14], and (iii) mutant alleles with low catalytic activity are still fully functional in glucose signalling [15]. These results suggest that sugar kinase activity and sugar signalling are mediated at least in part through separate domains of Hxk2 [14–17]. Thus the correlation between the catalytic activity of Hxk2 and its ability to mediate glucose repression appears less likely at present.

In the present study, we have exploited the accumulated knowledge on the structure and transcriptional glucose-repression mechanism of Hxk2, to characterize the catalytic and regulatory domains of Hxk2. For the first time, we describe two mutant Hxk2 alleles, one without sugar kinase activity, but with almost full glucose-repression signalling capacity and the other without regulatory function, but with almost full catalytic activity. Our results offer strong evidence that there is no correlation between the regulatory functions of Hxk2 in glucose repression signalling and its catalytic activity.

Abbreviations used: ADH1, alcohol dehydrogenase 1; ChIP, chromatin immunoprecipitation; DTT, dithiothreitol; GAD, Gal4 activation domain; GBD, Gal4 DNA-binding domain; Glk1, glucokinase 1; GST, glutathione transferase; HA, haemagglutinin; Hxk2, hexokinase 2; Kan^R, kanamycin-resistance; SD, synthetic dextrose; SG, synthetic galactose; wca, without catalytic activity; wrf, without regulatory function; YEPD, yeast extract/peptone/dextrose; YEPG, yeast extract/peptone/galactose.

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EXPERIMENTAL

Strains

The strains used in the present study were derived from W303.1A (*MAT α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*) [18]. We created two loss-of-function alleles of *HXK2* by mutating the wild-type gene in plasmid *YEp352-HXK2* to generate *HXK2_{wca}* (encodes an Hxk2 protein without catalytic activity) and *HXK2_{wrf}* (encodes an Hxk2 protein without regulatory function) genes using a PCR-based mutagenesis protocol. The *Kan^R* (kanamycin-resistance) gene was inserted into the *XbaI* site, located in the yeast genome 371 bp from the *HXK2* stop codon, of plasmids *YEp352-HXK2_{wca}* and *YEp352-HXK2_{wrf}*. Then, by using these constructs, recombination cassettes were obtained by PCR; these cassettes contained the mutated *HXK2* locus and the *Kan^R* marker together with 515 bp from the 5' genomic region downstream of the *HXK2* gene. These linear DNAs were integrated into the *HXK2* locus of strains W303.1A and THG1 (*MAT α leu2-1 ura3-1 lys1-1 hck1::LEU2 hck2::LEU2 glk1::LEU2*) [19]. To confirm the correct insertion, we PCR-amplified the *HXK2* locus from strains W303.1A_{wca}, W303.1A_{wrf}, THG1_{wca} and THG1_{wrf} to detect the presence of the *HXK2_{wca}* and *HXK2_{wrf}* mutant alleles by sequencing analysis. The strain FMY20VP is derived from DBY2052 (*MAT α hck1::LEU2 hck2-202 ura3-52 leu2-3,2-112 lys2-801 gal2*) and contains the *GLK1* gene promoter replaced by the *ADH1* (alcohol dehydrogenase 1) constitutive promoter. Yeast two-hybrid experiments employed strain Y187 (*MAT α ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4 Δ gal80 Δ URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*). Immunoprecipitation experiments employed strain FMY303H2 (*MAT α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 hck2::kanMX4*) [20]. This strain has a modification of the *SNF1* gene at the chromosomal level and produces a triple-HA (haemagglutinin) epitope-tagged Snf1 protein. As a control, in ChIP (chromatin immunoprecipitation) experiments, strain DBY2052 was used [12].

Construction of Hxk2 mutants

To identify and understand the roles of the different domains of Hxk2, we designed various truncation, deletion and amino acid substitution mutants and tested their ability to confer function. The mutant design was based on hexokinase sequence alignments and structural data together with the published data on Hxk2 mutants [3,13,15,21].

To determine the functional role of several amino acids identified previously as involved in glucose and ATP binding to the catalytic site of the enzyme, single amino acid substitution mutants were constructed to yield the following: in Hxk2S157A, Ser¹⁵⁷, which is involved in glucose binding and is phosphorylated during γ -phosphate transfer from ATP to glucose [22–24], was replaced with alanine. In Hxk2T233A, Thr²³³, which may interact with the β -phosphate of ATP [3], was replaced with alanine. In Hxk2S304F, Ser³⁰⁴, which is localized in the active centre of the enzyme [13], was replaced with phenylalanine.

To assess the functional role of the C-terminal region of Hxk2, both a deletion mutant and a substitution mutant were constructed. The deletion mutant was constructed by insertion of a stop codon into a specific site within the coding region of the *HXK2* gene to yield *HXK2 Δ K⁴⁷⁸A⁴⁸⁵*, which encodes an Hxk2 protein without the last eight amino acids of the C-terminal end (Hxk2 Δ K⁴⁷⁸A⁴⁸⁵). The substitution mutant (Hxk2S479A) was constructed by changing Ser⁴⁷⁹ to alanine. Finally, from the *HXK2 Δ K⁴⁷⁸A⁴⁸⁵* and *HXK2S304F* genes we obtained the double mutant *HXK2 Δ K⁴⁷⁸A⁴⁸⁵-S304F* (*HXK2_{wca}*).

To analyse the functional role of the N-terminal region of the Hxk2, a truncation mutant and a substitution mutant were constructed. The truncation mutant was obtained by PCR-deletion of 30 bp, between nucleotides +19 and +48, the resulting gene *HXK2 Δ K⁶M¹⁵*, expressed a truncated Hxk2 protein without the amino acids from Lys⁶ to Met¹⁵ (Hxk2_{wrf}). The substitution mutant (Hxk2S14A) was obtained by changing Ser¹⁴ to alanine.

The constructs were cloned into the yeast YEp352 vector under the control of the native *HXK2* gene promoter. The resulting plasmids were transformed in DBY2052 (Δ *hck1* Δ *hck2* *GLK1*), a double-mutant strain in which the two hexokinase genes have been deleted.

Preparation of crude protein extracts

Yeast protein extracts were prepared as follows: yeasts were grown in 10–20 ml of synthetic or complex galactose medium [SG–ura (synthetic galactose lacking uracil); YEPG (yeast extract/peptone/galactose)] containing 2% galactose at 28°C to a *D*₆₀₀ of 1.0. Then, half of the culture was shifted to synthetic or complex high-glucose medium [SD–ura (synthetic dextrose lacking uracil); YEPD (yeast extract/peptone/dextrose)] containing 2% glucose for 1 h and the other half of the culture was shifted to synthetic or complex low-glucose medium [SE–ura (synthetic ethanol lacking uracil); YEPE (yeast extract/peptone/ethanol)] containing 0.05% glucose plus 3% ethanol for 1 h. Cells were collected, washed twice with 1 ml of 1 M sorbitol and suspended in 100 μ l of 50 mM Tris/HCl (pH 7.5) buffer containing 0.2 mM EDTA, 0.5 mM DTT (dithiothreitol), 0.5 mM PMSF, 0.42 M NaCl and 1.5 mM MgCl₂. The cells were broken in the presence of glass beads by one 20 s pulse using a FastPrep (Thermo Electron) and 400 μ l of the same buffer were added to the suspension. After centrifugation at 14000 rev./min for 15 min at 4°C using a Beckman F2402H rotor and Allegra 21R centrifuge (Beckman), 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 2 mg/ml ONPG (*o*-nitrophenyl β -D-galactopyranoside) was used as a substrate [25]. Specific activity was calculated in relation to total protein in the crude extract, using BSA as the standard. Invertase activity was assayed in whole cells as described previously [26] and expressed as μ mol of glucose released per min per 100 mg of cells (dry weight). Activities are the average of at least ten assays of at least five independent colonies.

Immunoblot analysis

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

Yeast two-hybrid analysis

The yeast two-hybrid analysis [27] employed yeast vectors pGADT7 and pGBDKT7 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 (synthetic dextrose lacking leucine and tryptophan)] medium until reaching a D_{600} of 0.8. Assays for β -galactosidase activity followed protocols described previously [25]. Activities are the average of at least ten assays of at least five independently isolated clones. Expression levels of the GAD (Gal4 activation domain) and GBD (Gal4 DNA-binding domain) fusion proteins were controlled by Western blot analysis. Strains lacking the reporter construct did not exhibit any significant activity.

Co-immunoprecipitation assays

Immunoprecipitation experiments were performed by using whole-cell extracts, from two wild-type yeast strains with modified *MIG1* and *SNF1* genes which code for C-terminal Mig1 and Snf1 proteins tagged with 3HA epitopes respectively. Extracts were incubated with anti-Hxk2 antibody (Santa Cruz Biotechnology) or anti-Pho4 antibody for 1 h at 4°C. Protein A–Sepharose beads (Amersham Biosciences) were then added and 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 and 5% sodium deoxycholate), immunoprecipitated samples were boiled in SDS-loading buffer. The supernatant was subjected to SDS/12% PAGE and detected by Western blot using anti-HA monoclonal antibody and horseradish-peroxidase-conjugated Protein A by the West Pico Chemiluminescent system.

GST (glutathione transferase) pull-down experiments

GST-fusion protein expression vector pGEX-MIG1 was transformed into *Escherichia coli* strain BL21(DE3) pLysS. Cells were grown to a D_{600} of 0.5–0.8, induced with 0.5 mM IPTG (isopropyl β -D-thiogalactopyranoside) at 37°C for 3 h, and collected by centrifugation at 12000 *g* for 5 min. Cell pellets were resuspended in PBS (150 mM NaCl, 100 mM Na₂HPO₄ and 18 mM NaH₂PO₄, pH 7.3) and sonicated. Insoluble material was removed by centrifugation at 17000 rev./min for 20 min at 4°C using a Beckman F2402H rotor and Allegra 21R centrifuge (Beckman). Soluble extracts were incubated with glutathione–Sepharose 4B (Amersham Biosciences) for 1 h at 4°C, washed extensively with PBS and resuspended in the same buffer. The GST–Mig1 fusion protein coupled to glutathione–Sepharose was incubated with yeast whole-cell extracts from W303.1A (wild-type) and the W303.1A_{wca} and W303.1A_{wrf} mutant strains. The cell extracts were obtained from yeast cells grown in YEPG and shifted to high- and low-glucose media for 1 h. Beads were gently washed five times with 2.5 ml of PBS, boiled in 25 μ l of sample-loading buffer (50 mM Tris/HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% Bromophenol Blue and 10% glycerol), and analysed by SDS/12% PAGE followed by Western blotting using anti-Hxk2 antibodies and horseradish-peroxidase-conjugated Protein A. Bound antibodies were detected using the West Pico Chemiluminescent system.

ChIP assay

ChIP assays were performed essentially as described previously [28]. Cells were harvested and disrupted by vortex-mixing in

the presence of glass beads, and the lysate was sonicated to generate DNA fragments that ranged in size from 200 to 400 bp. To immunoprecipitate HA-tagged proteins, we incubated the extract overnight at 4°C with anti-Hxk2 antibodies [29]. The sequence of primers used for the PCR amplification of the *SUC2* promoter region containing the MIG1 element were 5'-TTATTACTCTGAACAGGA-3' (sense) and 5'-AAGTCGTCAAATCTTTCT-3' (antisense). The sequence of primers used for the PCR amplification of the *ACT1* gene were 5'-GCCTTCTACGTTTCCATCCA-3' (sense) and 5'-GGCCAAATCGATTCTCAAAA-3' (antisense).

RESULTS

Identification of new loss-of-function Hxk2 mutants

To identify the domains of the Hxk2 protein involved in catalytic activity and glucose-signalling regulation, we generated several Hxk2 mutants containing one or more substitutions, truncations or deletions in the amino acid chain (Figure 1A). The different mutations were used to test their effect on fructose and glucose growth. The growth properties of each mutant were tested by spotting serial dilutions of galactose-grown cells on media containing fructose or glucose as the carbon source. As can be seen in Figure 1(A), all of the strains grew normally on both media except the double-mutant $\Delta h x k 1 \Delta h x k 2$ strain which had growth difficulties in fructose medium and the $\Delta h x k 1 \Delta h x k 2$ strain transformed with the YEp352-Hxk2_{wca} plasmid, which had clear growth problems in both fructose and glucose media.

The Hxk2_{wca} protein content of the $\Delta h x k 1 \Delta h x k 2$ strain transformed with the YEp352-Hxk2_{wca} plasmid was evaluated by Western blot analysis after SDS/PAGE, to determine whether impairment of growth in fructose medium could be related to a lower quantity of the Hxk2_{wca} protein. As can be seen in Figure 1(B), the Hxk2_{wca} mutant protein was expressed in this strain and migrated at its predicted molecular mass. Since impairment of Hxk2 function was not due to decreased protein expression, the growth defect in fructose medium suggests a lack of hexokinase activity. Moreover, since this strain has a native *GLK1* gene, which is not expressed in cells grown in glucose medium [2], but is fully expressed in cells grown in glucose medium in the absence of the Hxk2 protein [30], the absence of growth in glucose medium (Figure 1A) suggests that the Hxk2_{wca} protein maintains normal glucose-repression signalling.

In order to analyse the regulatory activity of the different mutants, we have replaced the *GLK1* promoter with the constitutive *ADH1* promoter in the $\Delta h x k 1 \Delta h x k 2$ strain, thus allowing the expression of Glk1 under high-glucose conditions in the presence of Hxk2. The strain obtained ($\Delta h x k 1 \Delta h x k 2$ *ADH1p::GLK1*) grew in glucose medium at the same rate as the corresponding wild-type yeast. This strain was transformed with multicopy plasmids containing different *HXK2* mutant genes under the control of the *HXK2* promoter and we measured the exocellular invertase activity under high- and low-glucose conditions. The expression of the *SUC2* gene for invertase is repressed in the presence of glucose in the growth medium, therefore we used the invertase level as a marker for glucose repression.

Figure 2 clearly shows that the wild-type phenotype (low invertase activity under high-glucose conditions) was restored after transformation of the double-mutant $\Delta h x k 1 \Delta h x k 2$ *ADH1p::GLK1* strain with the wild-type (*HXK2*) or the mutant (*HXK2*_{wca}) genes. When the double-mutant $\Delta h x k 1 \Delta h x k 2$ *ADH1p::GLK1* strain was transformed with the *HXK2*_{wrf} gene, the wild-type phenotype was not restored.

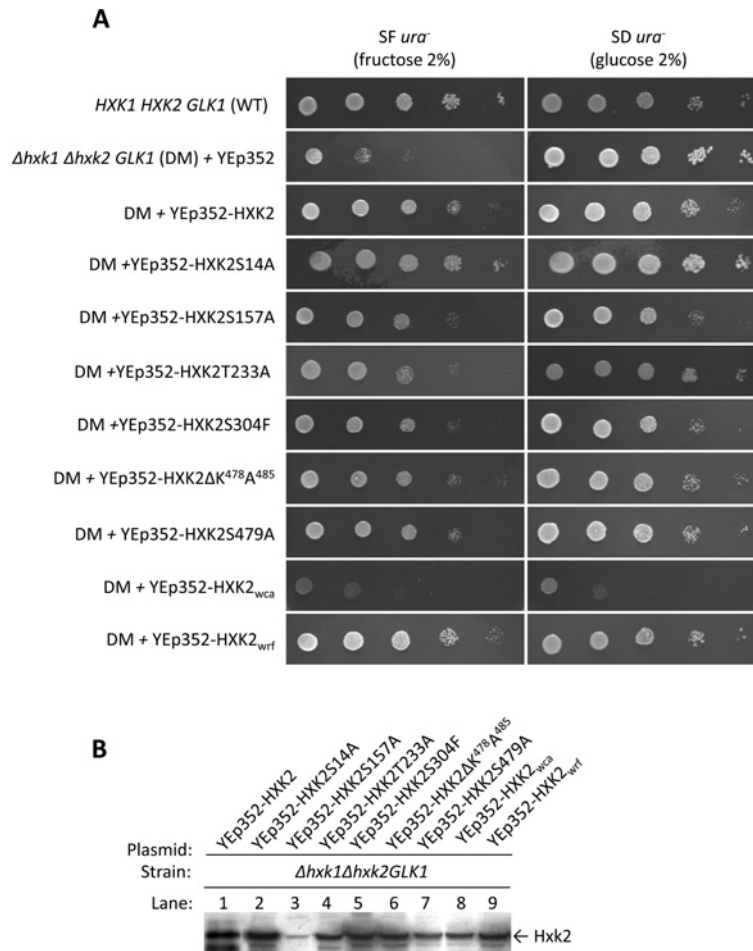


Figure 1 Growth assays and expression of Hxk2 mutants

(A) The double-mutant (DM) DBY2052 ($\Delta hxx1 \Delta hxx2$) strain was transformed with plasmids expressing the indicated *HXX2* mutant allele. The strain also contained both the empty vector and the wild-type *HXX2* allele as controls. Ten-fold serial dilutions of cultures grown in selective medium with galactose were spotted on to selective media containing fructose or glucose and incubated at 28 °C for 48 h. The mutants, listed on the left, are arranged in the order in which they occur in the primary sequence of Hxk2. The first two rows show wild-type (WT, W303.1A) and the double-mutant $\Delta hxx1 \Delta hxx2$ strain transformed with the empty vector (YEp352) as controls. The last two rows show the catalytic mutant allele *HXX2*_{wca} and the regulatory mutant allele *HXX2*_{wrf}. (B) Verification of expression of the wild-type and Hxk2 mutant proteins. The double-mutant strain DBY2052 ($\Delta hxx1 \Delta hxx2 GLK1$) lacking the two fructose-phosphorylating enzymes was transformed with the multicopy plasmids indicated. Yeasts were grown to the mid-exponential phase in selective medium with galactose and then shifted to high-glucose medium for 1 h, harvested and lysed in 1% SDS/PBS containing protease inhibitors. Aliquots of 20 μ g of protein/lane were resolved by SDS/12% PAGE. Hxk2 detection was performed using an anti-Hxk2 antibody.

However, this mutant that harbours a truncation between Lys⁶ and Met¹⁵ (Hxk2_{wrf}) [10] exhibited normal growth both in fructose and glucose media (Figure 1A), which suggests a normal catalytic activity. These results revealed that the Hxk2_{wca} protein exhibited a complete loss of catalytic function, but maintained a normal regulatory activity and the Hxk2_{wrf} protein exhibited complete loss of regulatory function, while maintaining wild-type catalytic activity.

These consistent results prompted us to obtain, for the first time, stable strains containing the two *HXX2* mutant alleles in the *HXX2* locus.

Replacement of the native *HXX2* gene by the loss-of-function *HXX2*_{wca} and *HXX2*_{wrf} mutant genes

To construct the loss-of-catalytic and regulatory function W303.1A_{wca} and W303.1A_{wrf} strains, we replaced the native *HXX2* gene with exogenous *HXX2*_{wca} and *HXX2*_{wrf} mutant sequences in the wild-type W303.1A strain.

The glucose-phosphorylating activity of the Hxk2_{wca} and Hxk2_{wrf} mutant proteins was first assessed by measuring the ability of strains containing these proteins to grow on medium with glucose as a carbon source (Figure 3A). All of the strains grew normally on galactose and glucose media, except the W303.1A_{wca} strain which had a clear growth problem in glucose medium. This growth problem may occur because the Hxk2_{wca} protein lacks catalytic activity and the *HXX1* and *GLK1* genes are not expressed under high-glucose conditions in the presence of a Hxk2 protein with normal regulatory function [2,30]. All of the strains grew well in galactose medium because, in the presence of galactose, the *HXX2* gene has a basal expression similar to that observed for non-fermentable carbon sources such as ethanol or glycerol [2]. Thus, under these conditions, the *GAL* genes are expressed and galactose is used for normal growth.

Although the studies described above measure the growth capacity on glucose medium of W303.1A_{wca} and W303.1A_{wrf} strains, they do not establish the Hxk2 catalytic activity of these strains. To address this issue, we constructed two new strains by integrating the *HXX2*_{wca} and *HXX2*_{wrf} genes

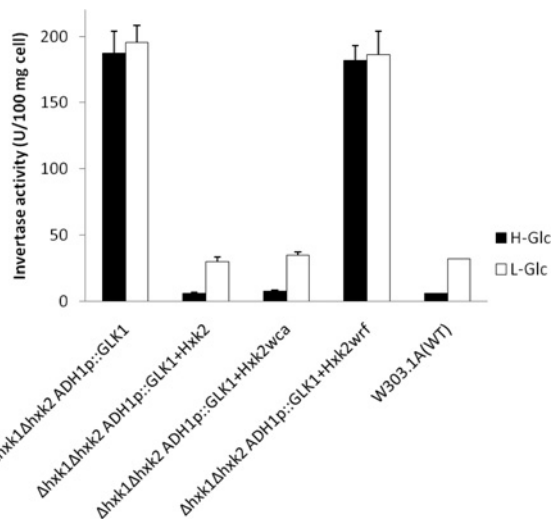


Figure 2 Effect of $Hxk2_{wca}$ and $Hxk2_{wrf}$ mutations on glucose repression signalling

The strain FMY20VP with the *GLK1* gene under the control of the *ADH1* constitutive promoter was transformed with plasmids containing *HXK2_{wca}* and *HXK2_{wrf}* mutant alleles. As controls, we used the FMY20VP strain transformed both with a plasmid containing the wild-type (WT) *HXK2* gene and the empty plasmid. We have also included invertase activity of wild-type strain W303.1A. The cells were grown on high-glucose medium (H-Glc, black bars) until a D_{600} of 1.0 was reached and then transferred to synthetic medium with low glucose for 60 min (L-Glc, white bars). Invertase activity was assayed in whole cells. Results are means + S.D. of ten measurements obtained with five independent isolated colonies.

into the *HXK2* locus of a triple mutant strain (THG1; $\Delta hxx1 \Delta hxx2 \Delta glk1$). The glucose-phosphorylating activity was determined in crude extracts from strains THG1_{wca} and THG1_{wrf} in a $\Delta hxx1 \Delta hxx2 \Delta glk1$ background. As can be seen in Figure 3(B), the glucose-phosphorylating activity of the THG1_{wca} strain was identical with that of the THG1 strain. Interestingly, the pattern of activity for the THG1_{wrf} strain was similar to that of the W303.1A strain. Thus our results indicate that the W303.1A_{wrf} strain maintained a normal (wild-type) Hxk2 catalytic activity, but the W303.1A_{wca} strain had lost its catalytic function.

To investigate the Hxk2-dependent glucose signalling function of the W303.1A_{wca} and W303.1A_{wrf} strains (Figure 3C), the cells were grown in galactose medium to a D_{600} of 1.0 and then shifted to high- and low-glucose media for 1 h. Finally the exocellular invertase activity was determined in the whole cells. The specific activity of invertase shows strong derepression on both high- and low-glucose media in the presence of the Hxk2_{wrf} mutant protein (W303.1A_{wrf} strain). On high- and low-glucose media, in the presence of the Hxk2_{wca} protein (W303.1A_{wca} strain), wild-type repression and derepression signalling was observed.

These results indicate that we had constructed two new strains of *S. cerevisiae* with loss-of-catalytic (W303.1A_{wca}) and regulatory (W303.1A_{wrf}) functions.

The Hxk2_{wca} mutant protein interacts with the Mig1 transcriptional repressor and the Snf1 protein kinase

Current evidence suggests that the main role of *S. cerevisiae* Hxk2 in the glucose signalling pathway is achieved through its interaction with both Mig1 and Snf1 proteins. It has been proposed that Hxk2 inhibits the phosphorylation of the Mig1 repressor when the cells are growing under high-glucose conditions, maintaining the transcriptional repression of target genes. To investigate the mechanism by which the *Hxk2_{wca}* mutant protein signals

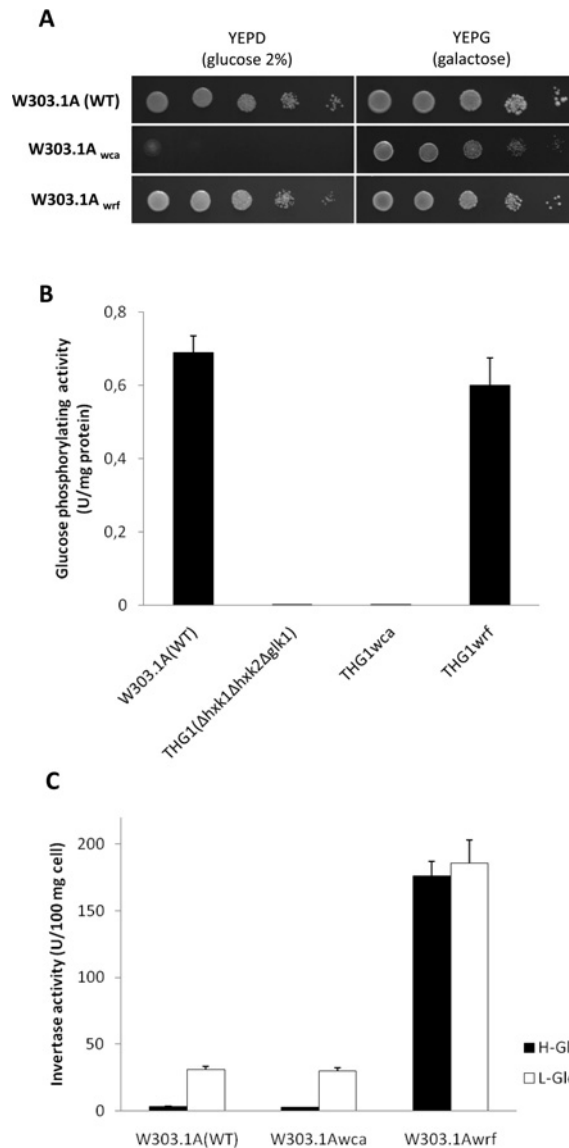


Figure 3 Growth assays, catalytic activity and regulatory function of W303.1A_{wca} and W303.1A_{wrf} strains

(A) The strains W303.1A_{wca} and W303.1A_{wrf} have the *HXK2_{wca}* and *HXK2_{wrf}* alleles integrated into the *HXK2* locus of strain W303.1A respectively. Ten-fold serial dilutions of cultures grown in YEPG were spotted on to complex media containing glucose or galactose and incubated at 28 °C for 48 h. The wild-type (WT) W303.1A strain was used as control. (B) The THG1_{wca} and THG1_{wrf} strains have the *HXK2_{wca}* and *HXK2_{wrf}* alleles integrated into the *HXK2* locus of strain THG1 respectively. The wild-type W303.1A and the triple mutant strain THG1 ($\Delta hxx1 \Delta hxx2 \Delta glk1$) were used as controls. The cells were grown on YEPG medium until a D_{600} of 1.0 was reached and then transferred to YEPD medium for 60 min. Glucose-phosphorylating activity was assayed in crude extracts. Results are means + S.D. of ten measurements obtained with five independent isolated colonies. (C) The strains W303.1A_{wca} and W303.1A_{wrf} were grown on YEPG medium until a D_{600} of 1.0 was reached and then transferred to high-glucose (H-Glc, black bars) and low-glucose (L-Glc, white bars) complex media for 60 min. Invertase activity was assayed in whole cells. Results are means + S.D. of ten measurements obtained with five independent isolated colonies.

glucose repression in the W303.1A_{wca} strain and the loss-of-repressing function in the W303.1A_{wrf} strain, we performed two-hybrid, co-immunoprecipitation and GST pull-down experiments.

First, we monitored the possible physical interaction between Mig1 and the Hxk2 mutant proteins Hxk2_{wca} and Hxk2_{wrf} using a yeast two-hybrid assay. Plasmid pGBKT7-Mig1 (expressing a GBD fusion to Mig1) was co-transformed with a plasmid

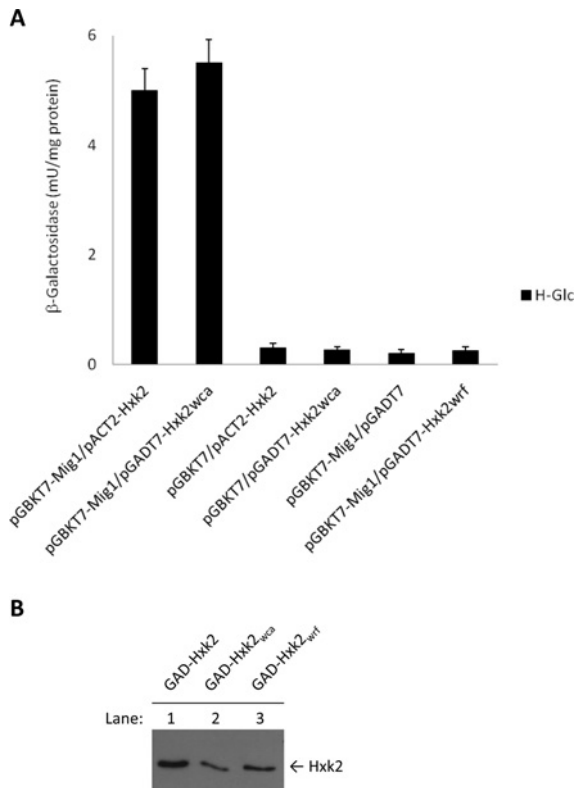


Figure 4 Yeast two-hybrid interaction of Mig1 with Hxk2_{wca} and Hxk2_{wrf} during high-glucose-grown conditions

(A) Mig1 fused to the GBD were individually co-transformed into yeast strain Y187 with constructs encoding GAD or GAD fused to wild-type Hxk2 and the mutant alleles Hxk2_{wca} and Hxk2_{wrf}. The wild-type Hxk2 fused to the GAD was also co-transformed into yeast strain Y187 with the construct encoding the GBD. The transformed yeast cells were grown in SD medium lacking appropriate supplements to maintain selection for plasmids and were harvested at early-exponential phase (D_{600} of 0.6) from high-glucose conditions (H-Glc). Protein-protein interactions were examined in each transformant by the quantitative assay method for β -galactosidase activity. Results are means \pm S.D. of ten measurements obtained with five independent isolated clones. Strains lacking the reporter constructs did not exhibit any significant activity. (B) Western blot analysis of the expression of different *HXK2* alleles under high-glucose conditions.

expressing a fusion of the GAD with Hxk2, Hxk2_{wca} or Hxk2_{wrf} into an appropriate reporter strain. The interaction between the selected proteins was monitored by measurement of the β -galactosidase activity under high-glucose conditions. As shown in Figure 4(A), the GAD-Hxk2 and GAD-Hxk2_{wca} fusion proteins produced a strong interaction with GBD-Mig1. However, this interaction was absent from a GAD-Hxk2_{wrf} mutant, indicating that the decapeptide between Lys⁶ and Met¹⁵ of Hxk2 is essential for Hxk2 interaction with Mig1, as observed previously [9]. Similar levels of all protein fusions were detected in all cases (Figure 4B). To confirm the interaction, we performed co-immunoprecipitation and GST pull-down assays. First, we examined the ability of Hxk2_{wca} and Hxk2_{wrf} to interact with Mig1 by using an immunoprecipitation assay. Cell extracts from a strain with a modified *MIG1* gene which codes for a C-terminal Mig1 protein tagged with 3HA epitopes, were immunoprecipitated with the antibodies indicated in Figure 5(A). The resulting immunoprecipitates were assayed for the presence of Mig1-HA by immunoblot analysis with anti-HA antibodies. As shown in Figure 5(A) (lanes 1 and 3), a strong and specific signal of Mig1 was observed both with samples immunoprecipitated from extracts of wild-type or Hxk2_{wca} strains grown under high-

glucose conditions. However, signals were not observed when the experiment was performed using extracts of wild-type or Hxk2_{wca} strains grown under low-glucose conditions, or when the experiment was performed using the strain expressing the Hxk2_{wrf} protein. The lack of interaction cannot be attributed to lack of expression, as the Hxk2_{wrf} protein was detected by Western blotting using an anti-Hxk2 antibody. When an anti-Pho4 antibody or no antibody was used to detect non-specific immunoprecipitation and non-specific protein binding to the anti-HA antibody respectively, no signals were observed. Thus this interaction is dependent on the production of HA-tagged Mig1 as well as an anti-Hxk2 antibody.

In order to determine the phosphorylation status of Mig1 in the Hxk2_{wca} and Hxk2_{wrf} mutant cells, we examined Mig1 phosphorylation *in vivo* in response to high and low levels of glucose (Figure 5B). For this purpose, we prepared extracts from wild-type, Hxk2_{wca} and Hxk2_{wrf} mutant strains transformed with the Mig1-HA 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 [31]. As shown in Figure 5(B), in the presence of either Hxk2 or Hxk2_{wca} proteins, Mig1 from low-glucose grown cells displays a slight shift in molecular mass, resulting in decreased mobility, in comparison with Mig1 from high-glucose-grown cells. However, in the presence of Hxk2_{wrf}, Mig1 from both high- and low-glucose-grown cells had decreased mobility (Figure 5B, lanes 5 and 6) indicating that the protein is phosphorylated. This result suggests that the Snf1 protein kinase complex assumes an active conformation even in the presence of glucose, a similar result to that obtained in the absence of Hxk2 [27,32].

To confirm the interaction of Mig1 with Hxk2_{wca}, we also employed the GST pull-down technique. We used extracts from the Hxk2_{wca}⁻ and Hxk2_{wrf}-producing strains and a bacterially produced GST-Mig1 fusion protein. As shown in Figure 5(C) (lanes 1 and 2), a strong and specific band was observed in extracts from wild-type and Hxk2_{wca} strains grown under high-glucose conditions. No retention was detected with the extracts obtained from low-glucose-grown cells or with extracts from the Hxk2_{wrf} strain grown under both high- and low-glucose conditions. Similar amounts of Hxk2, Hxk2_{wca} and Hxk2_{wrf} proteins were detected in these immunoprecipitates by immunoblot analysis with anti-Hxk2 antibody (Figure 5D).

Previous data indicate that Hxk2, Mig1 and Snf1 form a complex at the nuclear level and that Hxk2 functions as a bridge between Snf1 and Mig1, interacting with Snf1 to inhibit Mig1 phosphorylation at Ser³¹¹ under high-glucose conditions [9]. Thus it is tempting to speculate that the loss-of-function mutants of Hxk2 could have differences in their capacity to interact with the Snf1 protein. To address these possibilities, we examined the ability of Hxk2_{wca} and Hxk2_{wrf} proteins to interact with Snf1 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 Figure 6. The resulting immunoprecipitates were assayed for the presence of Snf1-HA by immunoblot analysis with anti-HA antibodies. As shown in Figure 6 (lanes 3 and 4, and 5 and 6), a strong signal of Snf1-HA protein 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 non-specific immunoprecipitation and non-specific protein binding to anti-HA antibody respectively, no signals were observed. Similar amounts of Hxk2, Hxk2_{wca} and Hxk2_{wrf} proteins were detected in

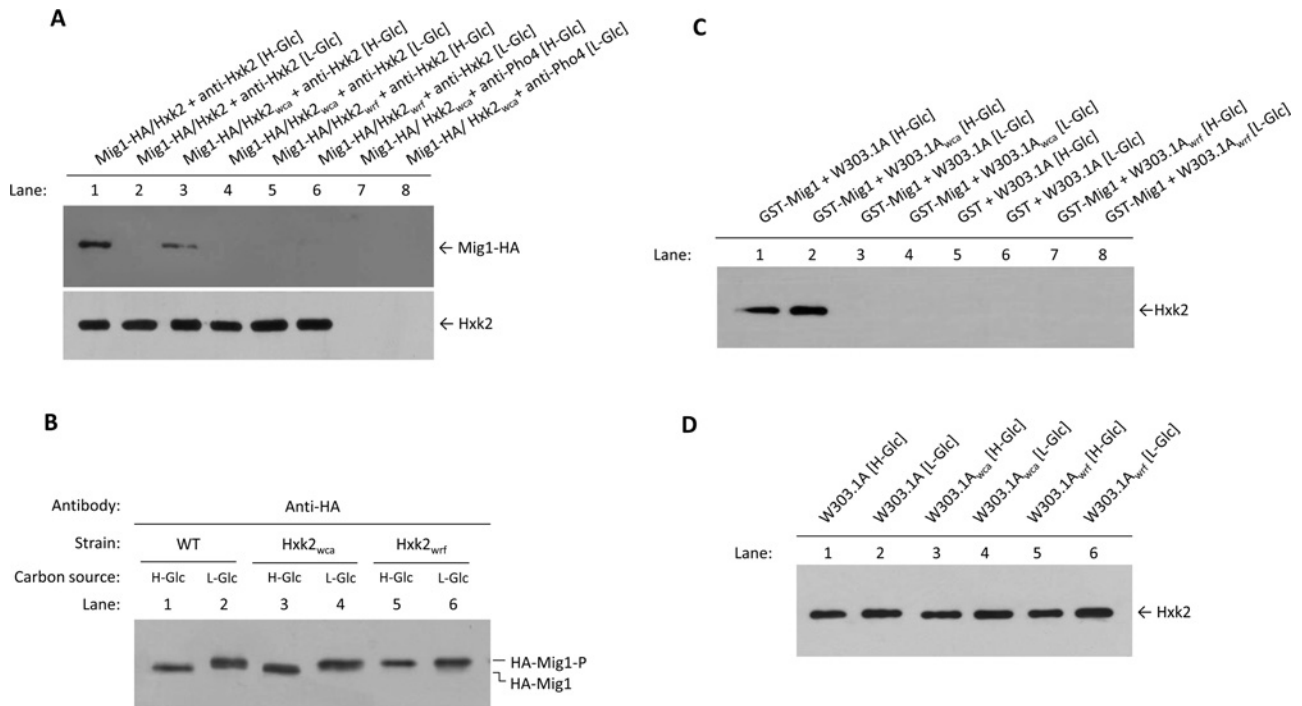


Figure 5 Interaction of Hxk2_{wca} and Hxk2_{wrf} with Mig1

(A) *In vivo* co-immunoprecipitation of HA-Mig1 with Hxk2_{wca} and Hxk2_{wrf}. The W303.1A, W303.1A_{wca} and W303.1A_{wrf} strains, transformed with plasmid pWS93/Mig1 which encoded an HA-tagged Mig1 protein, were grown in SG medium lacking appropriate supplements to maintain selection for plasmid, until a D_{600} of 1.0 was reached and then shifted to high- (H-Glc) and low- (L-Glc) glucose conditions for 1 h. The cell extracts were immunoprecipitated with a polyclonal anti-Hxk2 antibody (lanes 1–6) or a polyclonal antibody against Pho4 (lanes 7 and 8). Immunoprecipitates were separated by SDS/12% PAGE, and co-immunoprecipitated HA-Mig1 was visualized on a Western blot with a monoclonal anti-HA antibody. The level of immunoprecipitated Hxk2 in the blotted samples was determined by using an anti-Hxk2 antibody. (B) Mig1 phosphorylation in response to Hxk2_{wca} and Hxk2_{wrf} availability. Cells, from wild-type (WT), Hxk2_{wca} and Hxk2_{wrf} strains transformed with the HA-Mig1 construct (plasmid pWS93/Mig1), were grown in SG medium lacking appropriate supplements to maintain selection for plasmid, until a D_{600} of 1.0 was reached and then shifted to high- (H-Glc) and low- (L-Glc) glucose conditions for 1 h. The Mig1 protein was detected from total cell extracts by SDS/12% 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). (C) GST pull-down assays of the interaction of Hxk2_{wca} and Hxk2_{wrf} proteins with Mig1. A GST-Mig1 fusion protein was purified on glutathione-Sepharose columns. Equal amounts of GST-Mig1 were incubated with cell extracts from a wild-type strain (W303.1A) and the mutant strains W303.1A_{wca} and W303.1A_{wrf}. The yeast strains were grown in YEPG medium until a D_{600} of 0.6 was reached and then shifted to high- (H-Glc) and low- (L-Glc) glucose conditions for 1 h. For the control samples, GST protein was also incubated with the H-Glc and L-Glc cell extracts, but no signals were detected (lanes 5 and 6). (D) The level of Hxk2 present in the different extracts used in (C) was determined by Western blotting using an anti-Hxk2 antibody. The Western blots shown are representative of results obtained from four independent experiments.

these immunoprecipitates by immunoblot analysis with anti-Hxk2 antibody. Thus the interactions between Snf1 and both Hxk2_{wca} and Hxk2_{wrf} are dependent on the production of HA-tagged Snf1 as well as anti-Hxk2 antibody.

These results provide strong evidence that the Hxk2_{wca} mutant protein signals glucose repression in yeast by interacting with the Mig1 repressor. Moreover, the Hxk2_{wca} mutant protein probably plays a role identical with that of Hxk2 acting as a bridge between Snf1 and Mig1, to inhibit Mig1 phosphorylation by Snf1 at Ser³¹¹ under high-glucose conditions.

Furthermore, since the interaction between Mig1 and Hxk2_{wca} was detected only under high-glucose conditions, our data suggest that both proteins interact in a glucose-dependent manner. Our results also suggest that Hxk2_{wrf} is incapable of glucose-repression signalling because it does not interact with Mig1 either *in vivo* or *in vitro*. This result confirms that the ten-amino-acid motif between Lys⁶ and Met¹⁵, identified previously as essential for Hxk2 translocation to the nucleus [7], is required for the Hxk2–Mig1 interaction.

The Hxk2_{wca} mutant protein binds *in vivo* to the SUC2–Mig1 repressor complex

Previous reports have demonstrated that Hxk2 participates in the SUC2–Mig1 repressor complex through an interaction with

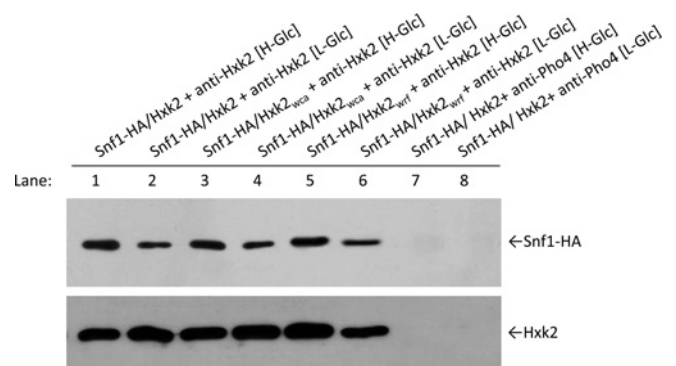


Figure 6 Interaction of Hxk2_{wca} and Hxk2_{wrf} with Snf1

Co-immunoprecipitation of HA-Snf1 with Hxk2_{wca} and Hxk2_{wrf}. The FMY303H2 strain, which encoded an HA-tagged Snf1 protein, was transformed with plasmids YEp352/Hxk2, YEp352/Hxk2_{wca} and YEp352/Hxk2_{wrf}. The cells were grown in SG medium lacking appropriate supplements to maintain selection for plasmid, until a D_{600} of 1.0 was reached and then shifted to high- (H-Glc) and low- (L-Glc) glucose conditions for 1 h. The cell extracts were immunoprecipitated with a polyclonal anti-Hxk2 antibody or a polyclonal antibody against Pho4 (lanes 7 and 8). Immunoprecipitates were separated by SDS/12% PAGE, and co-immunoprecipitated HA-Snf1 was visualized by Western blotting with a monoclonal anti-HA antibody. The level of immunoprecipitated Hxk2 in the blotted samples was determined by using an anti-Hxk2 antibody.

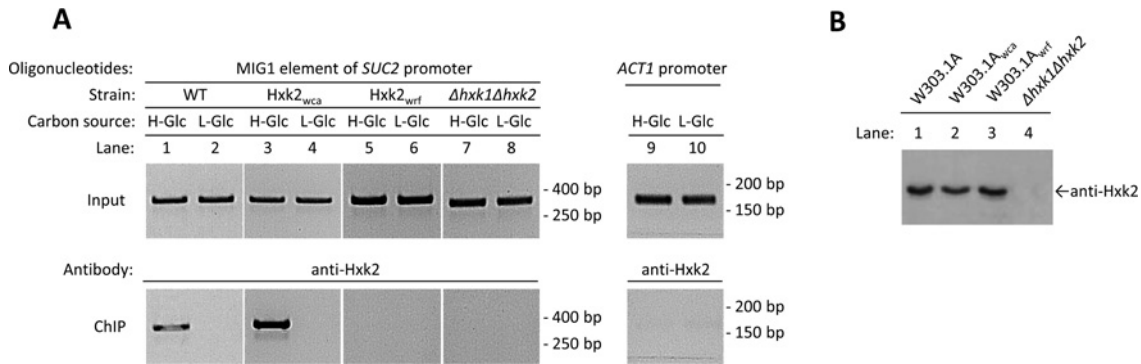


Figure 7 *In vivo* binding of Hxk2_{wca} to the MIG1 element of the *SUC2* promoter

(A) Wild-type (WT) W303.1A, the mutant strains W303.1A_{wca} and W303.1A_{wrf} and the double-mutant strain DBY2052 ($\Delta hxk1\Delta hxk2$) were grown to mid-exponential phase in YEPG medium and then shifted to high- (H-Glc) and low- (L-Glc) glucose conditions for 1 h. Cell extracts were prepared, immunoprecipitated with anti-Hxk2 antibodies and the DNA fragments were amplified by PCR using the combination of oligonucleotides indicated in the Materials and methods section. The amplified fragments were resolved by agarose gel electrophoresis. Migration of standard markers is indicated on the right. A ChIP assay representative of three independent experiments is shown. (B) Verification of expression of the wild-type and Hxk2 mutant proteins. The double-mutant strain DBY2052 ($\Delta hxk1\Delta hxk2GLK1$) lacking the two fructose-phosphorylating enzymes was used as control. Yeasts were grown to mid-exponential phase in complex medium with galactose, and then shifted to high-glucose medium for 1 h. The cells were harvested and lysed in 1% SDS/PBS containing protease inhibitors. Aliquots of 20 μ g of protein/lane were resolved by SDS/12% PAGE. Hxk2 detection was performed using an anti-Hxk2 antibody.

the Mig1 protein and not by direct binding to DNA [6–9]. As Hxk2_{wca} and Mig1 may form an *in vivo* complex (see above), we investigated the biological significance of this interaction by determining whether Hxk2_{wca} localized in the *SUC2*–Mig1 repressor complex in a glucose-dependent manner. To test this hypothesis, we used ChIP assays. Our results show that, in cells grown in high-glucose medium, both Hxk2 and Hxk2_{wca} proteins were recruited to a DNA fragment of the *SUC2* promoter containing the Mig1-binding site (Figure 7A, lanes 1 and 3). Conversely, in low-glucose medium, Hxk2 and Hxk2_{wca} binding to the *SUC2*–Mig1 complex was abolished (Figure 7A, lanes 2 and 4).

Interestingly, binding of the Hxk2 protein to the Mig1 site of the *SUC2* promoter was dependent on the presence of the decapeptide between Lys⁶ and Met¹⁵, since in W303.1A_{wrf} cells, no binding of Hxk2_{wrf} (lacks interaction with Mig1) to the *SUC2* promoter was observed under any growth condition (Figure 7A, lanes 5 and 6). The lack of DNA amplification cannot be attributed to lack of expression as the Hxk2_{wrf} protein because it was detected by Western blotting using an anti-Hxk2 antibody (Figure 7B). No DNA amplification was observed when we used cells without Hxk2 or oligonucleotides to amplify the *ACT1* promoter by PCR.

Taking all of these results together, we suggest that Hxk2_{wca} forms a complex with Mig1 and Snf1 similar to that of Hxk2, and that this complex is involved in the glucose-regulated expression of the *SUC2* gene. Thus Hxk2_{wca} maintains its regulatory function because it maintains the glucose signalling wild-type machinery, whereas Hxk2_{wrf} mutant protein lacks the regulatory function, because it is unable to form the repressor complex with Mig1 protein.

DISCUSSION

The role of the Hxk2 protein in glucose repression has been a matter of controversy for many years. Our data challenge the view that its role in glucose-repression signalling is directly related to its catalytic activity. This view was based on the inverse correlation between Hxk2 and invertase activity in a collection of random Hxk2 mutants [1,12]. Although previous results have shown Hxk2 mutants with low catalytic activity

that are still functional in glucose signalling [15], a complete separation of the two activities never has been achieved. We have used the accumulated knowledge about the structure of the catalytic and regulatory domains of the Hxk2 protein to design a set of new mutations. We probed the role of these Hxk2 mutations in the following Hxk2 functions: catalytic activity, regulatory activity and interactions with proteins essential for these functions. By screening for suppression of the catalytic activity while maintaining the regulatory function and suppression of the regulatory function and maintaining the catalytic activity in the Hxk2 protein, we have isolated novel alleles of the *HXK2* gene that have been long sought after. The results confirm what has been suspected for some time: that Hxk2 mediates catalytic and regulatory activities through different domains in the protein. The two separation-of-function mutants isolated, Hxk2_{wca} and Hxk2_{wrf}, are remarkable since they convert the Hxk2 from a bifunctional protein into a single-function protein with activity as a mediating factor in transcription (Hxk2_{wca}) or as an enzyme with hexose-phosphorylating activity (Hxk2_{wrf}).

The Hxk2_{wca} mutant protein encodes an Hxk2 protein without the last eight C-terminal amino acids and a replacement of Ser³⁰⁴, which is located in the active centre of the enzyme, with phenylalanine. The Hxk2_{wrf} mutant is an Hxk2 protein with an internal deletion between amino acids Lys⁶ and Met¹⁵. Since there is no overlap between the two domains controlling the Hxk2 catalytic and regulatory functions respectively, in the present study, we demonstrated that a correlation between catalytic activity and regulatory function in this protein does not exist.

In the glucose-repression signalling pathway, two major mediators are the proteins Mig1 and Hxk2. Under high-glucose conditions, nuclear Hxk2 interacts both with Mig1 and the Snf1 subunit of the Snf1 protein kinase complex to establish the repression state of several genes. Hxk2 interacts strongly with Mig1 in high-glucose-grown cells and previous findings implicate Ser³¹¹ of Mig1 as a critical residue for this interaction [9]. The interaction between Snf1 and Hxk2 was detected both under high- and low-glucose conditions and the data suggest that both proteins interact constitutively [9].

In low-glucose-grown cells, the Hxk2 interaction with Mig1 is abolished and a transient increase in interaction between Snf1 and Mig1 was detected [33]. This interaction pattern

potentially stimulates Mig1 phosphorylation by Snf1 kinase at Ser³¹¹. Phosphorylation of Ser³¹¹ results in most Mig1 being exported from the nucleus to the cytoplasm, and, ultimately, the derepression of genes regulated by the Hxk2–Mig1 glucose-repression signalling pathway.

To analyse the mechanism by which the loss-of-function mutants operate through the regulatory machinery, we have determined protein–protein interactions with Mig1 and Snf1 proteins, and protein–DNA interactions with the Mig1-binding site of the *SUC2* promoter. Since the Hxk2–Mig1 complex plays an essential role in glucose-repression signalling of several genes [7,9], detailed knowledge of the interaction between Hxk2_{wca} and Hxk2_{wrf} mutant proteins with Mig1 is required to understand how these two mutants operate in the glucose-repression mechanism. One of the important observations described in the present study is that two-hybrid assays, co-immunoprecipitation and GST pull-down experiments with purified Mig1 protein demonstrated that Hxk2_{wrf} does not interact with Mig1 under both high- and low-glucose conditions. The lack of interaction between Hxk2_{wrf} and Mig1 implies a loss of regulatory function. However, two-hybrid assays, co-immunoprecipitation and GST pull-down experiments with purified Mig1 protein demonstrated that Hxk2_{wca} interacted with Mig1 under high- but not low-glucose conditions, a similar behaviour to the wild-type Hxk2.

Although the interaction between Snf1 and Hxk2_{wrf} or Hxk2_{wca} was detected both under high- and low-glucose conditions and our data suggest that these proteins interact constitutively with Snf1, a loss of regulatory function was detected in the Hxk2_{wrf} mutant protein. Lack of the regulatory function of the Hxk2_{wrf} protein could be explained because the Hxk2 protein operates as a bridge between Snf1 and Mig1 and is required to inhibit Mig1 phosphorylation at Ser³¹¹ by Snf1 kinase under high-glucose conditions [9]. Since Hxk2_{wrf} has lost its interaction capacity with Mig1 under high-glucose conditions, the Mig1 protein could be phosphorylated by Snf1, and both Mig1 and Hxk2 are exported from the nucleus to the cytoplasm by the Msn5 [34] and Xpo1 (Crm1) exporters [35] respectively. Thus, under high-glucose conditions, several genes controlled by the Mig1 repressor complex are expressed because the Hxk2_{wrf} protein has lost its regulatory function.

This hypothesis is supported by the following facts: (i) although it has been reported that Snf1 is inactive under high-glucose conditions [31], it has also been described that 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 [36]; (ii) in the absence of Hxk2, the Snf1 protein kinase complex assumes an active conformation even in the presence of glucose [9,33], because the Mig1 protein from both high- and low-glucose-grown cells has decreased electrophoretic mobility, indicating that the protein is phosphorylated [9]; (iii) in the absence of Snf1 or both Hxk2 and Snf1, Mig1 has an increased mobility pattern, indicating that the protein is dephosphorylated [9,33,37]; (iv) in Hxk2_{wrf} mutants, lacking a domain required for Mig1–Hxk2 interaction, Mig1 is phosphorylated under both high- and low-glucose conditions (Figure 5A). Thus our data strongly suggest that the Hxk2 interaction with Mig1 under high-glucose conditions might prevent phosphorylation of Mig1 by Snf1.

In the present study, we have demonstrated that Hxk2_{wrf} is unable to signal glucose repression in *S. cerevisiae* because it was not present in the Mig1 repressor complex of the *SUC2* promoter. Furthermore, we have also demonstrated that Hxk2_{wca} is able to signal glucose repression in *S. cerevisiae* because it is physically associated with the Mig1 protein repressor, and CHIP assays confirmed that both Hxk2 and Hxk2_{wca} interacted with

Mig1 in DNA fragments containing the MIG1 site of the *SUC2* promoter. Therefore our results suggest that both Hxk2_{wca} and Hxk2 use similar mechanisms to regulate glucose signalling in yeast.

AUTHOR CONTRIBUTION

Rafael Peláez performed experiments and analysed data. Pilar Herrero designed and performed experiments. Fernando Moreno designed the study, analysed data and wrote the paper.

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REFERENCES

- Rose, M., Albig, W. and Entian, K. D. (1991) Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinases PI and PII. *Eur. J. Biochem.* **199**, 511–518
- Herrero, P., Galindez, J., Ruiz, N., Martínez-Campa, C. and Moreno, F. (1995) Transcriptional regulation of the *Saccharomyces cerevisiae* *HXK1*, *HXK2* and *GLK1* genes. *Yeast* **11**, 137–144
- Kuser, P. R., Krauchenco, S., Antunes, O. A. and Polikarpov, I. (2000) The high resolution crystal structure of yeast hexokinase PII with the correct primary sequence provides new insights into its mechanism of action. *J. Biol. Chem.* **275**, 20814–20821
- Anderson, C. M., Stenkamp, R. E., McDonald, R. C. and Steitz, T. A. (1978) A refined model of the sugar binding site of yeast hexokinase B. *J. Mol. Biol.* **123**, 207–219
- Anderson, C. M., Zucker, F. H. and Steitz, T. A. (1979) Space-filling models of kinase clefts and conformation changes. *Science* **204**, 375–380
- Moreno, F. and Herrero, P. (2002) The hexokinase 2-dependent glucose signal transduction pathway of *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **26**, 83–90
- Ahuatzi, D., Herrero, P., de la Cera, T. and Moreno, F. (2004) The glucose-regulated nuclear localization of hexokinase 2 in *Saccharomyces cerevisiae* is Mig1-dependent. *J. Biol. Chem.* **279**, 14440–14446
- Moreno, F., Ahuatzi, D., Riera, A., Palomino, C. A. and Herrero, P. (2005) Glucose sensing through the Hxk2-dependent signalling pathway. *Biochem. Soc. Trans.* **33**, 265–268
- Ahuatzi, D., Riera, A., Peláez, R., Herrero, P. and Moreno, F. (2007) Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. *J. Biol. Chem.* **282**, 4485–4493
- Herrero, P., Martínez-Campa, C. and Moreno, F. (1998) The hexokinase 2 protein participates in regulatory DNA–protein complexes necessary for glucose repression of the *SUC2* gene in *Saccharomyces cerevisiae*. *FEBS Lett.* **434**, 71–76
- Entian, K. D. (1980) Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in carbon catabolite repression in yeast. *Mol. Gen. Genet.* **178**, 633–637
- Ma, H. and Botstein, D. (1986) Effects of null mutations in the hexokinase genes of *Saccharomyces cerevisiae* on catabolite repression. *Mol. Cell. Biol.* **6**, 4046–4052
- Ma, H., Bloom, L. M., Walsh, C. T. and Botstein, D. (1989) The residual enzymatic phosphorylation activity of hexokinase II mutants is correlated with glucose repression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **9**, 5643–5649
- Yasuda, S., Arai, S., Mori, A., Isobe, N., Yang, W., Oe, H., Fujimoto, A., Yonenaga, Y., Sakashita, H. and Imamura, M. (2004) Hexokinase II and VEGF expression in liver tumors: correlation with hypoxia-inducible factor 1 α and its significance. *J. Hepatol.* **40**, 117–123
- Mayordomo, I. and Sanz, P. (2001) Hexokinase PII: structural analysis and glucose signalling in the yeast *Saccharomyces cerevisiae*. *Yeast* **18**, 923–930
- Hohmann, S., Winderickx, J., de Winde, J. H., Valckx, D., Cobbaert, P., Luyten, K., de Meersman, C., Ramos, J. and Thevelein, J. M. (1999) Novel alleles of yeast hexokinase PII with distinct effects on catalytic activity and catabolite repression of *SUC2*. *Microbiology* **145**, 703–714

- 17 Kraakman, L. S., Winderickx, J., Thevelein, J. M. and De Winde, J. H. (1999) Structure–function analysis of yeast hexokinase: structural requirements for triggering cAMP signalling and catabolite repression. *Biochem. J.* **343**, 159–168
- 18 Wallis, J. W., Chrebet, G., Brodsky, G., Rolfe, M. and Rothstein, R. (1989) A hyper-recombination mutation in *S. cerevisiae* identifies a novel eukaryotic topoisomerase. *Cell* **58**, 409–419
- 19 Belinchon, M. M. and Gancedo, J. M. (2007) Different signalling pathways mediate glucose induction of SUC2, HXT1 and pyruvate decarboxylase in yeast. *FEMS Yeast Res.* **7**, 40–47
- 20 Longtine, M. S., McKenzie, 3rd, A., Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P. and Pringle, J. R. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* **14**, 953–961
- 21 Kuser, P., Cupri, F., Bleicher, L. and Polikarpov, I. (2008) Crystal structure of yeast hexokinase PI in complex with glucose: a classical “induced fit” example revised. *Proteins* **72**, 731–740
- 22 Heidrich, K., Otto, A., Behlke, J., Rush, J., Wenzel, K. W. and Kriegel, T. (1997) Autophosphorylation-inactivation site of hexokinase 2 in *Saccharomyces cerevisiae*. *Biochemistry* **36**, 1960–1964
- 23 Behlke, J., Heidrich, K., Naumann, M., Muller, E. C., Otto, A., Reuter, R. and Kriegel, T. (1998) Hexokinase 2 from *Saccharomyces cerevisiae*: regulation of oligomeric structure by *in vivo* phosphorylation at serine-14. *Biochemistry* **37**, 11989–11995
- 24 Fernandez, R., Herrero, P., Fernandez, E., Fernandez, T., Lopez-Boado, Y. S. and Moreno, F. (1988) Autophosphorylation of yeast hexokinase PII. *J. Gen. Microbiol.* **134**, 2493–2498
- 25 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- 26 Gascon, S., Neumann, N. P. and Lampen, J. O. (1968) Comparative study of the properties of the purified internal and external invertases from yeast. *J. Biol. Chem.* **243**, 1573–1577
- 27 Fields, S. and Song, O. (1989) A novel genetic system to detect protein–protein interactions. *Nature* **340**, 245–246
- 28 Palomino, A., Herrero, P. and Moreno, F. (2006) Tpk3 and Snf1 protein kinases regulate Rgt1 association with *Saccharomyces cerevisiae* HXK2 promoter. *Nucleic Acids Res.* **34**, 1427–1438
- 29 Randez-Gil, F., Herrero, P., Sanz, P., Prieto, J. A. and Moreno, F. (1998) Hexokinase PII has a double cytosolic–nuclear localisation in *Saccharomyces cerevisiae*. *FEBS Lett.* **425**, 475–478
- 30 Rodriguez, A., De La Cera, T., Herrero, P. and Moreno, F. (2001) The hexokinase 2 protein regulates the expression of the *GLK1*, *HXK1* and *HXK2* genes of *Saccharomyces cerevisiae*. *Biochem. J.* **355**, 625–631
- 31 McCartney, R. R. and Schmidt, M. C. (2001) Regulation of Snf1 kinase: activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. *J. Biol. Chem.* **276**, 36460–36466
- 32 Mayordomo, I., Estruch, F. and Sanz, P. (2002) Convergence of the target of rapamycin and the Snf1 protein kinase pathways in the regulation of the subcellular localization of Msn2, a transcriptional activator of STRE (stress response element)-regulated genes. *J. Biol. Chem.* **277**, 35650–35656
- 33 Treitel, M. A., Kuchin, S. and Carlson, M. (1998) Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **18**, 6273–6280
- 34 De Vit, M. J., Waddle, J. A. and Johnston, M. (1997) Regulated nuclear translocation of the Mig1 glucose repressor. *Mol. Biol. Cell* **8**, 1603–1618
- 35 Peláez, R., Herrero, P. and Moreno, F. (2009) Nuclear export of the yeast hexokinase 2 protein requires the Xpo1 (Crm1)-dependent pathway. *J. Biol. Chem.* **284**, 20548–20555
- 36 Portillo, F., Mulet, J. M. and Serrano, R. (2005) A role for the non-phosphorylated form of yeast Snf1: tolerance to toxic cations and activation of potassium transport. *FEBS Lett.* **579**, 512–516
- 37 Ludin, K., Jiang, R. and Carlson, M. (1998) Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6245–6250

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