Tpk3 and Snf1 protein kinases regulate Rgt1 association with *Saccharomyces cerevisiae* HXK2 promoter

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ABSTRACT

Hexokinase 2 is an essential factor for signalling repression through the *Saccharomyces cerevisiae* high-glucose sensing pathway. The main regulatory mechanism that controls the HXK2 gene expression in yeast is mediated by the Rgt1 and Med8 transcription factors, which repress HXK2 expression in low-glucose containing media. In this study, we show that the repression activity of Rgt1 is regulated by Snf1 and Tpk3 protein kinases. Binding of Rgt1 to the HXK2 promoter requires Rgt1 phosphorylation by Snf1 or by an Snf1-dependent protein kinase. Conversely, Rgt1 hyperphosphorylation by the Tpk3 or by a Tpk3-dependent protein kinase dissociates Rgt1 from the repressor complex. Two-hybrid and chromatin immunoprecipitation experiments indicate that an Snf1-dependent interaction between Rgt1 and Med8 in the repressor complex is also essential for Rgt1 repression. The repression of HXK2 transcription by Rgt1 likely occurs through the formation of a DNA loop in the HXK2 locus, spanning the promoter and coding regions. These results suggest that a novel silent-chromatin loop is responsible for Rgt1-dependent transcriptional regulation of the HXK2 gene.

INTRODUCTION

The *Saccharomyces cerevisiae* glucose signalling pathway is controlled by a series of transcription factors and auxiliary proteins (1–3). Among them, hexokinase 2 (Hxk2) is an important repressor in this pathway. Beyond its classic metabolic role in initiating glucose metabolism, Hxk2 plays an important role in controlling the expression of several genes (4,5). The HXK2 gene is highly expressed during growth in high-glucose and is sparsely expressed in low-glucose media (6). However, little is known about the molecular mechanisms that control the pattern of Hxk2 expression. In this study, we investigated whether Rgt1 regulates HXK2 gene expression during glucose metabolism under high- and low-glucose growth condition.

Recently, Rgt1 and Med8 have been identified as two essential factors involved in the repression of the HXK2 gene in low-glucose medium (7). The Med8 protein binds constitutively to the downstream regulatory sequence (DRS) of the HXK2 gene (7–9). Rgt1 binds to the RGT1 element of the HXK2 promoter in a carbon source-dependent manner (7). Additionally, the phosphorylation state of Rgt1 has been shown to be important during transcriptional regulation of HXT1 (10,11). Therefore, we postulated that glucose may shift the transcriptional activity of Rgt1 towards HXK2 expression by inducing changes in the phosphorylation state of the Rgt1 protein.

It is known that in *S. cerevisiae*, the cAMP-dependent protein kinase (PKA) is activated by extracellular glucose and inactivated during glucose deprivation (12). Structurally, the PKA holoenzyme is a heterotetramer composed of two regulatory subunits (Bcy1) and two catalytic subunits of three possible isoforms (Tpk1, Tpk2, Tpk3). These three catalytic subunits of PKA can have redundant functions in processes such as glycogen storage. However, these catalytic isoforms are not redundant in all cases. For example, Tpk2 is responsible for processes such as pseudohyphal growth, regulation of genes involved in trehalose degradation and iron uptake (13–15). Tpk1 is required for derepression of branched chain amino acid biosynthesis (14) and Tpk3 is specifically involved in the regulation of mitochondrial enzymatic content during growth (16). The subcellular localization of PKA is regulated by glucose. Indeed PKA holoenzymes in glucose-grown cells are localized to the nucleus (17). Since Rgt1 is always located in the nucleus (18), both PKA and Rgt1 localize to the same subcellular compartment during high glucose growth conditions. To elucidate the effects of PKA on Rgt1 during high glucose growth, we investigated each Tpk subunit in this process. Here, we show that Tpk3 is responsible for Rgt1 hyperphosphorylation during Rgt1 release from the HXK2 promoter.
Since the lack of phosphorylation abolishes the ability of Rgt1 to repress HXT1 transcription (10), we postulated that the absence of Rgt1 phosphorylation may affect HXK2 repression. In order to elucidate this mechanism, we investigated the effect of Snf1 kinase activity on Rgt1-dependent repression of HXK2. The subcellular distribution of Snf1 is also affected by glucose levels. However, unlike PKA, Snf1 kinase is directed to the nucleus in low glucose conditions in a Gal83-dependent manner (19,20). Therefore, both Snf1 and Rgt1 are located in the same subcellular compartment during low-glucose growth conditions. In this study, we show that the yeast protein kinase Snf1 is involved in the modification of Rgt1 required for HXK2 gene repression.

Finally, given previous data (18,21), and data derived from our current in vivo studies, we propose a model to explain how low-glucose levels can repress the HXK2 gene expression in yeast cells.

MATERIALS AND METHODS

Yeast strains and growth media

The S. cerevisiae strains used in these experiments are listed in Table 1 and are isogenic to the wild-type strain W303-1A, DBY1315 or BY4742. The rgt1Δ strain was obtained by transformation with an rgt1::LEU2 allele, which was produced by PCR using oligonucleotides complementary to RGT1 and LEU2 contained in pUG73 (26). The FMY152-t3 and FMY152-s1 strains were obtained by transformation of the FMY152 strain, respectively, with a tpk3::kanMX4 or snf1::kanMX4 alleles, which were obtained by PCR using oligonucleotides complementary to TPK3 or SNI1 and genomic DNA from the Y15016 or Y14311 strains. Yeast genomic DNA from the Y15016 or Y14311 strains. Yeast MAT a ade2-1 his3-11,15 Strain Genotype References

| Table 1. Yeast strains used in this study |
|----------------------------------------|
| Strain | Genotype | References |
|--------|----------|------------|
| W303-1A | MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 TPK1 TPK2 TPK3 | (22) |
| DLY1901 | Isogenic to W303-1A, except for rgt1::LEU2 | This work |
| MB23 | Isogenic to W303-1A, except for tpk2::HIS3 tpk3::URA3 | (23) |
| MB13 | Isogenic to W303-1A, except for tpk1::LEU2 tpk2::URA3 | (23) |
| MB12 | Isogenic to W303-1A, except for tpk1::LEU2 tpk2::HIS3 | (23) |
| DBY1315 | MATα ura3-1,2-212 lys2-801 gal2 | (24) |
| FMY152 | Isogenic to DBY1315, except with HXK2p::lacZ | (25) |
| FMY152-t3 | Isogenic to FMY152, except for tpk3::kanMX4 | This work |
| FMY152-s1 | Isogenic to FMY152, except for snf1::kanMX4 | This work |
| BY4742 | MATα: his3Δ1 leu2-0 lys2Δ0 ura3Δ0 | Euroscarf |
| Y15016 | Isogenic to BY4742, except for tpk3::kanMX4 | Euroscarf |
| Y13967 | Isogenic to BY4742, except for reg1::kanMX4 | Euroscarf |
| Y14311 | Isogenic to BY4742, except for snf1::kanMX4 | Euroscarf |

General DNA techniques

Restriction enzymes and T4 DNA ligase were from Roche; radioactively labelled isotopes were from Amersham International. Routine DNA manipulations were essentially performed as described previously (27).

Construction of plasmid DNAs

Rgt1 and Med8 fused to the hemaglutinin epitope (HA-Rgt1) were expressed in yeast from the ADH1 promoter in the plasmid pWS93 (28). The RGT1 insert was synthesized by PCR using genomic DNA as a template with the primer pair OL1+OL2 (OL1: 5'-AAGGATCCATGAAACGCCTGAA-CGTGT-3' and OL2: 5'-ATG GATCCTAAT ACCGCC-TAACTCGG-3'). Plasmid pWS93/RGT1 carried a 3512 bp BamHI fragment with the complete coding region of the RGT1 gene in pWS93. HA-Rgt1 was functional as it restores, in low-glucose media, HXK2 repression in an rgt1Δ strain. Plasmid pWS93/MED8 carried a 696 bp BamHI–SalI fragment with the complete coding region of the MED8 gene in pWS93. DNA sequencing verified these PCR-generated constructs.

The HXK2 reporter plasmid Ylpl357/HXK2+404 was constructed by placing sequences from –838 to +404 bp relative to the HXK2 translation start codon, upstream of a lacZ reporter gene on the integrative yeast vector YIp357 (29). Plasmid Ylpl357 is a yeast–Escherichia coli shuttle vector suitable for use as integrative vector with an URA3 yeast selectable marker (30).

Plasmid pGEX/RGT1 was constructed by subcloning a BamHI fragment, with the complete coding region of the RGT1 gene, from plasmid pWS93/RGT1 in frame into pGEX-4T (Amersham Biosciences).

Plasmid pGBK7/RGT1 carried the 3512 bp BamHI fragment from the pWS93/RGT1 plasmid, with the complete coding region of the RGT1 gene, in pGBK7 (Clontech). Plasmid pGADT7/MED8 carried a 669 bp EcoRI–BamHI fragment with the complete coding region of the MED8 gene in pGADT7 (Clontech). The MED8 insert was synthesized by PCR using genomic DNA as the template with the primer pairs OL3+OL4 (OL3, 5'-GCCGATCCATGTTACTAGATGAATCAG-3' and OL4, 5'-AAGGATCCGAT-TACTAGATGAATCAG-3'). Plasmid pACT2/HXK2-485 carried a 1455 bp NcoI/BamHI fragment with the complete
coding region of the HXX2 gene in pACT2 (31). The HXX2 insert was synthesized by PCR using plasmid pSP73/HXX2 (32) as the template with the primer pairs OL5+OL6 (OL5, 5'-TACCATGGTTTACGTATCGG-3'; OL6, 5'-ATGGATCCAAGACCG-3'). To make plasmid pGADT7/HXX2-416 a 408 bp EcoRI–HindIII fragment, obtained from pGADT7/HXX2, and carrying a 213 bp deletion in the 3' end of HXX2 DNA was first subcloned into the EcoRI and PstI sites of pUK21 and then isolated as an EcoRI–BamHI fragment and ligated into the EcoRI and BamHI sites of pGADT7 (Clontech). To make plasmid pGADT7/HXX2-136 a 408 bp EcoRI–BamHI fragment, obtained from pGADT7/HXX2, and carrying a 1050 bp deletion in the 3' end of HXX2 DNA was first subcloned into the EcoRI and HindIII sites of pUK21 and then isolated as an EcoRI–BamHI fragment and ligated into the EcoRI and BamHI sites of pGADT7. To make plasmid pGADT7/HXX2-87 a 261 bp EcoRI–Asp718 fragment, obtained from pGADT7/HXX2, and carrying a 1197 bp deletion in the 3' end of HXX2 DNA was first subcloned into the EcoRI and Asp718 sites of pSP73 and then isolated as an EcoRI–BamHI fragment and ligated into the EcoRI and BamHI sites of pGADT7.

Preparation of crude and nuclear protein extracts

Yeast protein extracts were prepared as follows: yeasts were grown in 10–20 ml of high or low-glucose medium at 28°C to an optical density at 600 nm of 1.0. Cells were collected, washed twice with 1 ml of 1 M sorbitol and suspended in 100 μl of 50 mM Tris–HCl (pH 7.5) buffer containing 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.42 M NaCl and 1.5 mM MgCl2. The cells were broken by vortexing (60 s) in the presence of glass beads (0.5 g), and 400 μl of the same buffer were added to the suspension. After centrifugation at 19 000 g (14 000 r.p.m.) for 15 min at 4°C, the supernatant was used as crude protein extract. Nuclear extracts were prepared by a method based on that of (33) with the modifications indicated in (34).

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 (27). Specific activity was calculated in relation to total protein in the crude extract, using BSA as the standard.

Gel retardation assays

To investigate interaction of Rgt1 with the sequence carrying the RGT1 element of HXX2 promoter we reconstituted the fragment from two complementary oligonucleotides (RGT1/HXX2 sense, 5'-tcgaGCAGTTTTTCCGTCTGAT-3'; RGT1/HXX2 antisense, 5'-tcgaATCGACCAGAAAATCTGC-3'). The complementary strands were annealed and either end was labelled with [α-32P]dCTP by fill-in, using the Klenow fragment of DNA polymerase I. The labelled double-stranded DNA was used as probe and the unlabelled was used as competitor in gel retardation assays. In all cases the oligonucleotides were synthesised with an added TCGA nucleotide overhang at the 5'-terminal end. Binding reactions contained 10 mM HEPES (pH 7.5), 1 mM DTT, 1–5 μg of poly(dI–dC) and 0.5 ng of end-labelled DNA in a volume of 25 μl. When unlabelled competitor DNA was added, its amount was 20 ng. The binding reaction mixtures included 20 μg (6 μl) of the corresponding nuclear extract and after 30 min of incubation at room temperature they were loaded onto 4% non-denaturing polyacrylamide gels. Electrophoresis was carried out at 10 V/cm of gel for 45 min to 1 h in 0.5× TBE buffer (45 mM Tris–borate, 1 mM EDTA). Gels were dried and autoradiographed at −70°C with an intensifying screen.

Yeast two-hybrid analysis

The yeast two-hybrid analysis (35) employed yeast vectors pGADT7, pACT2 and pGDBKT7 and host strains Y187, Y187-Dsnf1 and Y187-Dtpk3 (described above) in accordance with the Matchmaker two-hybrid system 3 from Clontech. The transformed yeasts were grown in SD or SE/Leu,Trp medium. Assays for β-galactosidase activity followed protocols described elsewhere (27). Qualitative assessment of expression from the lacZ reporter gene was made using X-gal as a chromogenic substrate for β-galactosidase in a colony-lift filter assay. The β-galactosidase activity was determined as indicated above. 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.

Immunological methods

Immunoprecipitation experiments were performed by using whole cell extracts, from a wild-type or hxx2A mutant yeast strains transformed with the pWS93/RGT1 plasmid. Extracts were incubated with anti-Hxk2 or anti-Pho4 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% deoxycholate), immunoprecipitated samples were boiled in SDS-loading buffer. The supernatant was subjected to 12% SDS–PAGE and detected by western blot using anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase (HRP)-conjugated protein-A by the ECL system (Amersham Biosciences). Western blot analysis was performed by using yeast-transformed cells containing appropriate plasmids. We found that it was important to rapidly denature proteins to ensure that the Snf1 kinase was not activated by glucose starvation during cell harvesting and protein extraction. Mutants or wild-type yeast cells were grown on 10 ml of high-glucose medium at 28°C until an optical density at 600 nm of 1.0. Cells were collected by centrifugation (3000 g, 4°C, 1 min), and crude extracts were prepared as follows: yeasts were suspended in 100 μl of 250 mM Tris–HCl (pH 6.8) buffer containing 8.0 mM EDTA, 2.5 mM β-mercaptoethanol, 8.0% SDS and 35% glycerol. The cells were broken by vortexing (60 s) in the presence of glass beads (0.5 g) and after adding 300 μl of phosphate-buffered saline (PBS) buffer the suspension was boiled for 3 min. After centrifugation at 19 000 g (14 000 r.p.m.) for 15 min at 4°C, the supernatant was used as crude protein extract. For western
GST pull-down experiments

GST fusion protein expression vectors (pGEX-HXK2 and pGEX-RGT1) were transformed into E.coli strain BL21(DE3) pLysS. Cells were grown to an OD600 of 0.5–0.8, induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 3 h, and collected by centrifugation. Cell pellets were resuspended in PBS buffer (150 mM NaCl, 100 mM Na2HPO4 and 18 mM NaH2PO4, pH 7.3) and sonicated. Insoluble material was removed by centrifugation (17000 g for 20 min at 4°C). Soluble extracts were incubated with glutathione-Sepharose 4B (Amersham Biosciences) for 1 h at 4°C, washed extensively with PBS buffer and resuspended in the same buffer. The GST-Hxk2 fusion protein coupled to glutathione-Sepharose beads was incubated with 2.5 U of thrombin (2 h at 4°C) for site-specific separation of the GST affinity tag from Hxk2 protein. Approximately, equal amounts of GST-Rgt1 coupled to glutathione-Sepharose beads were incubated with Hxk2 purified protein or yeast whole cell extracts from pWS93/MED8 transformed wild-type yeast strain, for 1 h at 4°C. The GST-Hxk2 fusion protein coupled to glutathione-Sepharose beads was incubated with 2.5 U of thrombin (2 h at 4°C) for site-specific separation of the GST affinity tag from Hxk2 protein. Approximately, equal amounts of GST-Rgt1 coupled to glutathione-Sepharose beads were incubated with Hxk2 purified protein or yeast whole cell extracts from pWS93/MED8 transformed wild-type yeast strain, for 1 h at 4°C in PBS buffer. Beads were gently washed five times with 2.5 ml of PBS buffer, boiled in 25 μl 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–PAGE followed by western blot using anti-HA antibodies and HRP-conjugated protein A. Bound antibodies were detected using the ECL system (Amersham Pharmacia Biotech).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described previously (36,37) with the following modifications. A 50 ml culture of yeast (OD600 = 1) was treated with formaldehyde (final concentration 1%) for 60 min at 20°C, and 2.5 ml of 2.5 M glycine was added to stop the cross-linking reaction. Cells were harvested and disrupted by vortexing 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 anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with the extract overnight at 4°C, then incubated anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with the extract overnight at 4°C, and the extract-antibody mixture then was incubated for an additional 3–4 h with protein A–Sepharose beads (Amersham Biosciences). Immunoprecipitates were washed 4-fold with 1 ml each of lysis buffer (50 mM HEPES, pH 7.5, 250 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin A). The DNA was eluted with elution buffer (100 mM sodium bicarbonate and 1% SDS). After reversal of the formaldehyde-induced cross-links, 1/5000 of input DNA and 1/45 of each immunoprecipitated DNA were used as templates for amplification by PCR. The sequences of primers for PCR were as follows: for the HXK2 promoter region containing the RGT1 element, OL7, 5’-ACTACGATTTTCTGAACCTCC-3’ and OL8, 5’-TAATTTCCGTGGAATCTCGAATC-3’ and for the HXK2 gene region containing the MED8 element, OL9, 5’-GGAATTGATGCAAACAAATTGAG-3’; and OL10, 5’-GATTGAGTGGTGCACAGGATAC-3’.

RESULTS

Glucose-regulated phosphorylation of Rgt1 in vivo

Previous results have indicated that in cells grown in low glucose, Rgt1 is dephosphorylated and binds to several HXTs regulatory promoter regions. Conversely, high glucose-induced phosphorylation of Rgt1 inhibits its DNA-binding activity (10,11). To determine whether the binding of Rgt1 to the RGT1 regulatory element within the HXK2 promoter entails dephosphorylation of Rgt1, we examined the phosphorylation state of Rgt1 in vivo during growth in high and low glucose (Figure 1). For this purpose, we prepared extracts from mutant rgt1Δ transformed cells with the HA-Rgt1 construct grown in the presence of high or low glucose. The extracts were analysed by western blot using a monoclonal HA antibody. As shown in Figure 1A (lane 1), Rgt1 from high-glucose grown cells display a slight shift in

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Figure 1. Rgt1 phosphorylation in response to glucose availability. (A) Cells, from an rgt1Δ mutant strain transformed with the HA-Rgt1 construct, were grown in high-glucose (H-Glc) or low-glucose (L-Glc) media until an OD600 of 1.0. Cell extracts were treated with or without λ-phosphatase for 30 min in the presence, or in the absence of phosphatase inhibitors. Rgt1 protein was detected from total cell extracts by SDS–PAGE followed by immunoblotting with anti-HA antibody. (B) The mutant rgt1Δ strain transformed with the HA-Rgt1 construct was grown in the presence of low-glucose until an OD600 of 1.0 and transferred to medium with high glucose for 30 min. Finally cells were transferred to a medium with low glucose for 60 min. Rgt1 was detected and identified as described in (A). The phosphorylated forms of Rgt1 are indicated as hP (hyperphosphorylated), P (phosphorylated) and dP (dephosphorylated).
molecular weight when compared with Rgt1 from low-glucose grown cells (Figure 1A, lane 4). To confirm that this decreased mobility is due to phosphorylation, we treated protein extracts from high-glucose grown cells expressing HA-Rgt1 with λ-phosphatase in the presence or absence of phosphatase inhibitors. The modified form of Rgt1 disappeared after treatment with λ-phosphatase but remained in the presence of phosphatase inhibitors. This indicated that the modification was due to phosphorylation (Figure 1A, lanes 2 and 3). Interestingly, Rgt1 in extracts from cells grown in the presence of low-glucose was also sensitive to λ-phosphatase, indicating that Rgt1 is also phosphorylated in low glucose (Figure 1A, lanes 5 and 6). Thus, the Rgt1 appeared to exist in three different phosphorylation states as resolved by its SDS-PAGE mobility: (i) a hyperphosphorylated state during growth in high glucose; (ii) an additional phosphorylated state during growth in low glucose medium, and (iii) a dephosphorylated state when the protein extracts were treated with λ-phosphatase.

Rgt1 hyperphosphorylation occurred rapidly (30 min) when the cells were shifted from low to high glucose medium (Figure 1B, lanes 2 and 5). Furthermore, this hyperphosphorylation was reversible when cells were reintroduced into low glucose media. During this event, the modified hyperphosphorylated form of Rgt1 rapidly disappeared (Figure 1B, lanes 6 and 7).

These results indicated that Rgt1 undergoes hyperphosphorylation in response to high-glucose in accordance with previous results (10). Our results also indicated that the basal phosphorylation state of Rgt1 is present in cells grown in low-glucose medium. The dephosphorylated state of Rgt1 was only detected after λ-phosphatase treatment of cell extracts and is likely not of significant physiological relevance.

**Tpk3 and Snf1 protein kinases regulate the Rgt1 phosphorylation state**

In cells grown in high glucose, PKA and Rgt1 proteins are localized to the nucleus (17,18) and PKA activity is stimulated by extracellular glucose. Thus, PKA is an intriguing candidate kinase that can directly hyperphosphorylate Rgt1. To determine whether PKA is responsible for Rgt1 modification, we used the double-disruption mutant strains of *tpk1 tpk2* (*tpk12*), *tpk1 tpk3* (*tpk13*) and *tpk2 tpk3* (*tpk23*), each lacking two of the three possible catalytic subunits of PKA. The utilization of these double-mutant strains avoids redundant functions of the catalytic subunits of PKA. Rgt1 hyperphosphorylation was detected in *tpk12* cells grown in high-glucose medium (Figure 2A, lane 3), but it did not take place in the *tpk13* and *tpk23* mutants (Figure 2A, lanes 4 and 5). This suggests that PKA complexes containing the Tpk3 subunit is directly or indirectly responsible for this Rgt1 modification (Figure 2A). To further confirm that the Tpk3 catalytic subunit of PKA is involved in Rgt1 hyperphosphorylation, we also used a *tpk3Δ* single-mutant strain. Rgt1 hyperphosphorylation was not detected in *tpk3Δ* mutant cells grown in high-glucose medium (Figure 2A, lane 9) indicating that Tpk3 is responsible for Rgt1 hyperphosphorylation.

Interestingly, the observed phosphorylation of Rgt1 under conditions of low-glucose growth was abolished in the *snf1* mutant strain (Figure 2B, lane 4). Furthermore, this phosphorylation was induced in *reg1Δ* mutants lacking the regulatory subunit of the Reg1-Glc7 protein phosphatase complex under conditions of high glucose (Figure 2C, lane 3). Since the Snf1 protein kinase is highly active in *reg1Δ* mutant cells during high-glucose growth, this suggests an essential role of Snf1 in the phosphorylation of Rgt1 under conditions of glucose limitation. Taken together, these results suggest that Rgt1 phosphorylation, in response to glucose limitation, is dependent on Snf1 protein kinase activity, and Rgt1 hyperphosphorylation, in response to high-glucose levels, is Tpk3-dependent.

**Snf1-dependent phosphorylation of Rgt1 promotes DNA binding to the HXK2 promoter and Tpk3-dependent hyperphosphorylation of Rgt1 promotes DNA release from the HXK2 promoter**

Previous reports have demonstrated that Rgt1 binds to the RGT1-binding site in the HXK2 promoter during low-glucose conditions (7). As shown above, in low glucose...
medium, Rgt1 is phosphorylated by the Snf1 protein kinase or by an Snf1-dependent kinase. Consequently, we investigated whether the phosphorylation state of Rgt1 determines the ability of this protein to bind to its target in the HXK2 promoter both in vivo and in vitro. To test this hypothesis, we used ChIP and gel electrophoretic mobility shift assays (EMSAs). Our results showed that in cells grown in low glucose medium, Rgt1 was recruited to the HXK2 promoter in the three *tpk* mutant strains (Figure 3A, a, lanes 2–4). Conversely, in high glucose medium, Rgt1 binding to the HXK2 promoter was abolished in the *tpk12* mutant, but not in *tpk13* and *tpk23* mutant cells (Figure 3A, b, lanes 2–4). From these results, we propose that Tpk3 protein kinase activity is necessary for in vivo Rgt1 release from the HXK2 promoter. In the *snf1* mutant strain, Rgt1 was recruited to the HXK2 promoter in the presence of high glucose, but no binding to the HXK2 promoter was detected in low-glucose medium (Figure 3A, a, lanes 5). This result suggests a direct correlation between in vivo Snf1-dependent phosphorylation of Rgt1, and its binding activity to the HXK2 promoter (Figure 3A, a, lane 5). The binding of Rgt1 to the HXK2 promoter in *snf1* mutant cells grown in the presence of high glucose (Figure 3A, b and a, lane 5) is not completely understood. One possible explanation could be that partial Rgt1 phosphorylation by an unknown protein kinase (Figure 2B, lane 2) could allow Rgt1 binding to the HXK2 promoter during high-glucose growth. No DNA amplification was observed when we used cells with untagged Rgt1. This result demonstrates that the observed signals are dependent on the HA-tagged protein.

Next, we determined whether the presence or absence of Snf1 and Tpk3 proteins could induce differential band shifting with nuclear extracts prepared from wild-type and *snf1Δ* and *tpk12Δ* and *tpk23Δ* mutant strains. To accomplish this, we used the RGT1 element of the HXK2 promoter in EMSAs. As shown in Figure 3B, we observed a retarded DNA–protein complex (CI) with nuclear extracts from wild-type cells grown in low-glucose medium, but not in high-glucose grown cells (Figure 3B, lanes 8 and 9). The CI complex was also detected by using nuclear extracts from both *tpk* mutant cells grown in low-glucose medium, and nuclear extracts from *tpk23* mutant cells grown in high-glucose medium (Figure 3B, lanes 5–7). However, the CI complex was not formed with extracts from *snf1Δ* mutant cells grown in low-glucose medium, or the *tpk12* mutant cells grown in high-glucose medium (Figure 3B, lanes 3 and 4). The specificity of this binding was demonstrated by competition assays with non-labelled oligonucleotides (data not shown). Together, these results suggest the involvement of Snf1 protein kinase in the control of Rgt1-binding activity to the HXK2 promoter. The Tpk3 protein kinase activity is also necessary for Rgt1 release from the HXK2 promoter in cells grown in the presence of high-glucose.

**Snf1 protein kinase regulates HXK2 gene expression**

To gain more insight into the relationship between the Rgt1 phosphorylation state, its DNA-binding activity, and HXK2 gene expression, we used *snf1Δ* and *tpk13Δ* mutant strains transformed with a single copy of the yeast reporter plasmid YEp357/HXK2+*ααA* (29) integrated at the *URA3* locus. Since the HXK2 promoter drives glucose-regulated expression of the reporter gene, we measured β-galactosidase activity in the presence of high and low levels of glucose. We found that, in low glucose-grown cells, *lacZ* expression increased 12-fold in *snf1* mutant cells with respect to the basal level detected in wild-type cells grown in low-glucose medium. The deletion of the TPK3 gene did not affect β-galactosidase activity. Conversely, in high glucose-grown cells, *SNF1* and *TPK3* gene deletions did not affect expression of the reporter gene (Figure 4). Thus, from these experiments, a clear relationship between Snf1-dependent phosphorylation state of Rgt1 and HXK2 gene expression can be deduced. Surprisingly, the absence of Tpk3 protein did not induce HXK2 gene repression in high glucose grown cells.
Rgt1 interacts with Med8 and Hxk2 in a glucose-dependent manner

As we have demonstrated previously, Rgt1 and Med8 form part of a DNA–protein complex that interacts with the RGT1 and DRS regulatory elements of the HXK2 gene promoter, respectively. Although Rgt1 only binds to DNA in a glucose-dependent manner, both Med8 and Rgt1 functions are required to repress HXK2 expression in low-glucose medium (7). However, a direct or indirect interaction between Rgt1 and Med8 proteins has not yet been demonstrated. Furthermore, Hxk2 is involved in a positive feedback loop that serves to amplify its own expression (25), and in the presence of high-glucose, the Med8 protein interacts with Hxk2 (9). Thus, it was plausible to postulate that Hxk2 could be directly or indirectly involved in the control of Rgt1 repression of the HXK2 gene. To test this hypothesis, we examined potential interactions of Rgt1 with Med8 and Hxk2 by yeast two-hybrid assays. The pGBKT7/RGT1 plasmid (Materials and Methods) was introduced together with a plasmid expressing a fusion of the Gal4 activation domain with Med8, or one with Hxk2 into an appropriate reporter strain. The interaction was monitored in low- and high-glucose conditions by determining β-galactosidase expression levels.

As shown in Figure 5B, full-length Rgt1 did not interact with Med8 in the presence of high glucose. However, a strong interaction occurred within 5 min after shifting the cells from medium containing high glucose to low glucose. On the other hand, full-length Rgt1 interacts with Hxk2 in the presence of high glucose, but does not interact at low glucose concentrations.

To identify the domains of Hxk2 that are important for interaction with Rgt1, sub-fragments of Hxk2 were similarly tested for its interaction with Rgt1. As shown in Figure 5B, Hxk2-416, Hxk2-136 and Hxk2-87 proteins were not able to interact with full-length Rgt1 fused to the Gal4 activation domain (GAD) were co-transformed into yeast strain Y187 with constructs encoding the Gal4 binding domain alone (GBD) or GBD fused to full-length (Rgt1, amino acids 1–1170) Rgt1. Full-length Rgt1 fused to the Gal4 DNA binding domain (GBD) was also co-transformed into yeast strain Y187 with the construct encoding the Gal4 activation domain alone (GAD), as indicated in (C) (dashed bar). The transformed yeast cells were grown in SD (high-glucose; H-Glc) or SE (low-glucose; L-Glc) media with selection for plasmid maintenance. Protein–protein interactions were examined in each transformant by the qualitative and quantitative assay methods for β-galactosidase activity. Strains from bars marked with asterisk gave a blue colony colour in the qualitative assay. The values are the averages of β-galactosidase activity for three transformants. Each measured value was within 12% of the average.
The interaction between Rgt1 and Hxk2 was confirmed by immunoprecipitation assays in cells expressing HA-Rgt1 and Hxk2. Cell extracts were immunoprecipitated with the antibodies indicated in Figure 6A. The resulting immunoprecipitates were assayed for the presence of Rgt1 by immunoblot analysis with anti-HA antibodies. As shown in Figure 6A, a signal for Rgt1 was observed only with samples immunoprecipitated with an anti-Hxk2 antibody (lane 3). In control reactions, no signals were obtained when the experiment was done without any antibodies, the anti-Pho4 antibody, or extracts from the hxk2Δ mutant strain (lanes 1, 2 and 4). Thus, this interaction is dependent on the production of HA-tagged Rgt1 and Hxk2. Since some unknown proteins in the extract could mediate the interaction between Hxk2 and Rgt1, we tested for the direct interaction between Rgt1 with Hxk2 using pull-down assays. We used purified Hxk2 protein and a bacterially produced GST-Rgt1 fusion protein. As shown in Figure 6B (lane 2), strong and specific retention was detected with the sample containing the purified GST-Rgt1 fusion protein. No signal was detected when purified GST was used in the experiment (lane 3). To confirm the Rgt1–Med8 interaction, we performed GST pull-down experiments with crude protein extracts and a purified GST-Rgt1 fusion protein. The extracts were obtained from yeast cells grown in SE (low-glucose) media with selection for plasmid maintenance. As shown in Figure 6C, a clear retention of Med8 protein was observed for the samples containing GST-Rgt1, and in crude extracts from the wild-type yeast strain W303-1A expressing a Med8 HA-tagged protein (lane 3). When a control with GST protein in the reaction mixture was used, no signal was observed (lane 2).

Snf1 protein kinase modulates Rgt1 interaction with Med8 and Hxk2

Our yeast two-hybrid data indicated that Rgt1 interacts with Med8 and Hxk2 in a glucose-dependent manner. This observation and the data presented above prompted us to investigate by yeast two-hybrid assays whether Snf1 and Tpk3-dependent phosphorylation of Rgt1 affects its interaction with Med8 and Hxk2.

As indicated in Figure 7A, in ∆snf1 mutant cells, full-length Rgt1 does not interact with Med8 and Hxk2 in the presence of high or low-glucose. Because the dephosphorylated form of Rgt1 was detected in the ∆snf1 cells grown in low-glucose medium (Figure 2B), its failure to interact with Med8 could be a consequence of the different phosphorylation states the two proteins. This suggests that Rgt1 interaction with Hxk2 may require its Snf1-dependent phosphorylation.

As shown in Figure 7B, in ∆tpk3 mutant cells, full-length Rgt1 does not interact with Med8 in the presence of high glucose. However, a strong interaction occurred within 5 min after shifting the cells from medium containing high glucose to low glucose medium. On the other hand, full-length Rgt1 interacted with Hxk2 in the presence of high glucose, but did not interact in low glucose concentration. As shown above, identical results were obtained with the wild-type strain (Figure 5B). Our data suggest that Rgt1 interaction with Med8 and Hxk2 does not require its Tpk3-dependent phosphorylation.

Rgt1 and Med8 forms loops in the HXK2 gene regulatory region via protein–protein interactions

Once we identified the in vivo and in vitro interaction of Rgt1 with Med8, and demonstrated that Snf1-dependent phosphorylation of Rgt1 regulates the binding activity between both proteins, a logical question to ask was whether in vivo
binding of Rgt1 and Med8 affects DNA secondary structure. This may include modifications such as looping, which could bring the Rgt1 and Med8 distal-binding sites into close proximity. To address this issue, we used a formaldehyde crosslinking-immunoprecipitation assay (ChIP) with cells grown in low-glucose containing medium. The results indicate that our chromatin sonication protocol breaks the DNA between the Med8 and Rgt1-binding sites, because no amplification was detected by using primer pair 7+10 for PCR (Figure 8B, lane 6). Interestingly, significant levels of the upstream HXK2 regulatory region containing the Rgt1-binding site were found when DNA–protein complexes were immunoprecipitated with the anti-Med8 antibody (Figure 8B, lane 7). Furthermore, significant levels of the downstream HXK2 regulatory region containing the Med8-binding site were found when DNA–protein complexes were immunoprecipitated with an anti-Rgt1 antibody (Figure 8B, lane 8). This co-immunoprecipitation of the DNA containing the MED8 element with anti-Rgt1 antibody, and the DNA containing the RGT1 element with anti-Med8 antibody, can only be explained if Rgt1 and Med8 proteins interact during the formaldehyde crosslinking step of the ChIP experiment.

However, no amplification was detected by using primer pair 7+10 for PCR in both anti-Med8 and anti-Rgt1 antibody for immunoprecipitations (Figure 8B, lane 9). Neither was a signal detected, when we used protein extracts from cells with untagged Rgt1 or Med8 proteins (Figure 8B, lanes 10 and 11). This control experiment suggests that the observed signals are HA-Rgt1 or HA-Med8 dependent.

**DISCUSSION**

These studies were undertaken to address the mechanisms of transcriptional control of the HXK2 gene. Hxk2 is the protein that initiates the intracellular metabolism of glucose by phosphorylation at C6, but Hxk2 also plays a vital role in glucose repression (4). A cellular fraction of this protein has a glucose-regulated nuclear localization and operates by interacting with Mig1 to generate a repressor complex in the nucleus of S.cerevisiae during growth in glucose (5). Similarly, recent studies in mammalian hepatic cells suggest that glucokinase (GK), the type IV isoenzyme of hexokinase (HKIV), has the capacity to enter the nucleus where it is essential to control the activity of transcriptional factors like ChREBP and SREBP (38). All these factors participate, by direct binding to DNA, and regulate the transcription of several genes involved in glucose metabolism and lipogenesis (39). An increasing amount of biochemical and genetic evidence points to the possibility that Hxk2 and HKIV could function through similar mechanisms in glucose signaling (40). Thus, experiments to elucidate the transcriptional control of the HXK2 gene are of maximal significance.

In this paper, we present a molecular analysis of Rgt1, a transcriptional regulator which together with the Med8 protein, regulates the HXK2 gene expression in a glucose dependent manner (7). The correlation between the Rgt1 phosphorylation state, DNA-binding activity to the HXT1 cis-acting elements, and HXT1 gene transcription is well established (10,11,37,41). However, the impact of Rgt1 on the HXK2 transcription has never been assessed properly. Here, we show through western analyses in different media and by using several null-mutant strains that Snf1 and Tpk3 protein kinases couple glucose levels to Rgt1 phosphorylation state. This affects the DNA-binding activity of Rgt1 and regulates the transcription of the HXK2 gene through its protein–protein interacting activity. The results presented here suggest that the Snf1 protein kinase is involved directly or indirectly in the
activation function of Rgt1 as a repressor. Deletion of the SNF1 gene results in a dephosphorylated Rgt1 protein with neither DNA-binding activity nor HXK2 repression in low-glucose medium. Moreover, in reg1 mutants lacking the regulatory subunit of the Reg1–Glc7 protein phosphatase complex and possessing constitutive Snf1 protein kinase activity Rgt1 phosphorylation is induced during glucose growth. These observations reaffirm previous observations on the regulation of HXTs genes (10,11,41) and implicate the Rgt1 mechanism to HXK2 transcriptional regulation.

Our results also suggest that the Tpk3 catalytic subunit of cAMP-dependent protein kinase is essential for Rgt1 release from the HXK2 promoter. Deletion of the TPK3 gene results in a loss of hyperphosphorylated Rgt1 and an increase of DNA binding activity in the presence of high-glucose.

HXK2 transcriptional repression is dependent on the binding of Rgt1 and Med8 to their target DNA elements within the HXK2 promoter (7). Interestingly, the data presented here suggests that an interaction between Rgt1 and Med8 is also essential for the repression of Rgt1 function. This idea is supported by the following findings: (i) deletion of SNF1 gene prevents Rgt1 binding to the HXK2 promoter in yeast cells growing in low-glucose medium and activates HXK2 gene expression, (ii) deletion of SNF1 gene prevents Rgt1 interaction with Med8 in low-glucose growing cells, (iii) deletion of the TPK3 gene prevents Rgt1 release from the HXK2 promoter in yeast cells growing in high-glucose medium, but does not affect HXK2 gene expression, (iv) Rgt1 does not interact with Med8 in the presence of high-glucose, and (v) proper HXK2 repression only occurs.

Figure 8. Med8 and Rgt1 proteins interact at the HXK2 promoter level. (A) Model showing the three DNA fragments that were analysed: the downstream HXK2 regulatory region containing the Med8-binding site (266 bp), the upstream HXK2 regulatory region which contains the Rgt1 binding site (286 bp), and as a control, a region of 854 bp which contains both Med8 and Rgt1-binding sites. (B) Wild-type cells expressing HA-tagged Rgt1 or HA-tagged Med8 proteins (a) and untagged Rgt1 or Med8 proteins (b) were grown in low-glucose medium until an OD₆₀₀ of 1.0, lysed and subjected to ChIP. Genomic DNA was used as positive PCR control (lanes 1–3). Crosslinked-sonicated DNA (lanes 4–6) and immunoprecipitated DNA (lanes 7–11) were amplified by PCR using primer pairs spanning the RGT1 (7+8) and MED8 (9+10) elements of the HXK2 gene. These primer pairs had been defined previously to characterize Rgt1 and Med8 DNA binding. Wild-type cells expressing untagged Rgt1 or Med8 proteins were used in the ChIP experiment as negative controls (lanes 10 and 11). PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.
Snf1-mediated phosphorylation is a key molecular switch that controls HXK2 repression. In cells grown in low glucose, the RGT1 element of the HXK2 promoter is occupied by the phosphorylated Rgt1, which interacts with Med8. This Rgt1 phosphorylation is important both for DNA and protein interaction. Under high-glucose conditions, Rgt1 is hyperphosphorylated in a Tpk3-dependent manner. Thus, Tpk3-mediated hyperphosphorylation of Rgt1 is a prerequisite for DNA release, but is not necessary to induce Rgt1 interaction with Hxk2.

A simplified diagram of how nutritional signals converge on Rgt1 through the Snf1 and Tpk3 protein kinases, according to our results, is presented in Figure 9. The glucose-regulated interaction of Rgt1 with Med8 and Hxk2 was proposed. This creates specific structures at the HXK2 promoter that would accommodate differences in the spacing of regulatory DNA elements and influence chromatin structure. Chromatin looping as a result of remote sequences being brought close together might be involved in gene activation or repression by the binding of components of the basal transcription machinery. This would facilitate or make difficult the formation of active transcriptional complexes (42). In fact, chromatin looping was detected recently in mammalian loci for β-globin (43), the Igf2 (44) and Dlx5 (45). Our results suggest that HXK2 regulation results from the formation of a loop in the DNA that includes the promoter and the coding region of the HXK2 gene between the Rgt1 and Med8 binding sites.

The interaction between Rgt1 and Med8 provides a new mechanism for modulating the transcriptional response of the HXK2 promoter to low glucose. We propose that this mechanism acts in concert with Snf1 or an Snf1-dependent protein kinase to initiate Rgt1 binding to the HXK2 promoter in low glucose. Conversely, the Tpk3 catalytic subunit of PKA initiates Rgt1 release from the HXK2 promoter in high-glucose conditions.

These results suggest that HXK2 transcription is repressed by the state of the chromatin over the promoter. The chromatin barrier over the HXK2 promoter could be released by binding the hyperphosphorylated Rgt1 to the Hxk2 nuclear protein. This Rgt1 sequestering results in the observed activation of the HXK2 expression, as it would make the promoter accessible to the RNA II polymerase complex, as well as to other mediator factors. Thus, our model also explains how Hxk2 is involved in a positive-feedback loop that serves to amplify its own expression (25).

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