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.

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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 *SNF1* and genomic DNA from the Y15016 or Y14311 strains. Yeast

Table 1. Yeast strains used in this study

Strain	Genotype	References
W303-1A	*MATa ade2-1 his3-11,15 leu2 -3,112 trp 1-1 ura 3-1 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 tpk3::URA3*	(23)
MB12	Isogenic to W303-1A, except for *tpk1:: LEU2 tpk2::HIS3*	(23)
DBY1315	*MATα ura3 -52 leu2-3,2-112 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 Δ0 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

two-hybrid and co-precipitation experiments employed strains Y187 (*MATα ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4Δ gal80Δ URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*), Y187-Δ*snf1* and Y187-Δ*tpk3*. The strains Y187-Δ*snf1* and Y187-Δ*tpk3* were obtained by transformation of the Y187 strain, respectively, with an *snf1::kanMX4* or *tpk3::kanMX4* allele.

Yeast cells were grown in the following media: YEPD (4% glucose, 2% peptone and 1% yeast extract; high-glucose medium), YEPE (0.05% glucose, 3% ethanol, 2% peptone and 1% yeast extract; low-glucose medium) and synthetic media containing the appropriate carbon source and lacking appropriate supplements to maintain selection for plasmids [4% glucose, (SD); or 0.05% glucose and 3% ethanol, (SE); and 0.67% yeast nitrogen base without amino acids]. Amino acids and other growth requirements were added at a final concentration of 20–150 µg/ml. The solid media contained 2% agar in addition to the components described above.

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′-AAGGATCCATGAACGAGCTGAA-CACTGT-3′ and OL2: 5′-ATG GATCCTCAAT ACCAGCC-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 669 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 YIp357/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 YIp357 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 pGBKT7/RGT1 carried the 3512 bp BamHI fragment from the pWS93/RGT1 plasmid, with the complete coding region of the *RGT1* gene, in pGBKT7 (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′-GCGGAATTCATGTCA-CAATCTACTGCATC-3′; OL4, 5′-AAGGATCCGCAT-TACTAGATGATGTTGA-3′). Plasmid pACT2/HXK2-485 carried a 1455 bp NcoI/BamHI fragment with the complete

coding region of the *HXK2* gene in pACT2 (31). The *HXK2* insert was synthesized by PCR using plasmid pSP73/HXK2 (32) as the template with the primer pairs OL5+OL6 (OL5, 5′-TACCATGGTTCATTTAGGTCCAAAAA-3′; OL6, 5′-ATGGATCCAGCACCGA TGATACCAAC-3′). To make plasmid pGADT7/HXK2-416 a 1245 bp EcoRI–PstI fragment, obtained from pGADT7/HXK2, and carrying a 213 bp deletion in the 3′ end of *HXK2* 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/HXK2-136 a 408 bp EcoRI–HindIII fragment, obtained from pGADT7/HXK2, and carrying a 1050 bp deletion in the 3′ end of *HXK2* 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/HXK2-87 a 261 bp EcoRI–Asp718 fragment, obtained from pGADT7/HXK2, and carrying a 1197 bp deletion in the 3′ end of *HXK2* 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 $MgCl_2$. The cells were broken by vortexing (6× 20 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 *HXK2* promoter we reconstituted the fragment from two complementary oligonucleotides (RGT1$_{HXK2}$ sense, 5′-tcgaGCAGTTTTTCCGGTCGAT-3′; RGT1$_{HXK2}$ antisense, 5′-tcgaATCGACCGGAAAAACTGC-3′). The complementary strands were annealed and either end was labelled with [α-^{32}P]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 pGBDKT7 and host strains Y187, Y187-Δ*snf1* and Y187-Δ*tpk3* (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 *hxk2*Δ 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 Na_2HPO_4, 18 mM NaH_2PO_4, 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 (6× 20 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

blotting, 20–40 µg of proteins was separated by SDS–8% PAGE and transferred to enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences) by electro-blotting, which was then incubated with an anti-HA antibody or with anti-Hxk2 polyclonal antibody. HRP-conjugated protein A was used as secondary reactant. The complex was detected by the ECL detection system (Amersham Biosciences).

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 A_{600} 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 Na_2HPO_4 and 18 mM NaH_2PO_4, pH 7.3) and sonicated. Insoluble material was removed by centrifugation (17 000 *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 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 ($OD_{600} = 1$) 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, 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 leupeptine 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 immuno-precipitated 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′-ACTACGAGTTTTCTGAACCTCC-3′ and OL8, 5′-TAAT-TTCGTGGATCTCGAATC-3′ and for the *HXK2* gene region containing the MED8 element, OL9, 5′-GGAATTGATGCAA-CAAATTGAG-3′; and OL10, 5′-GATTGAGTGGTGTCAA-AGGTAC-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
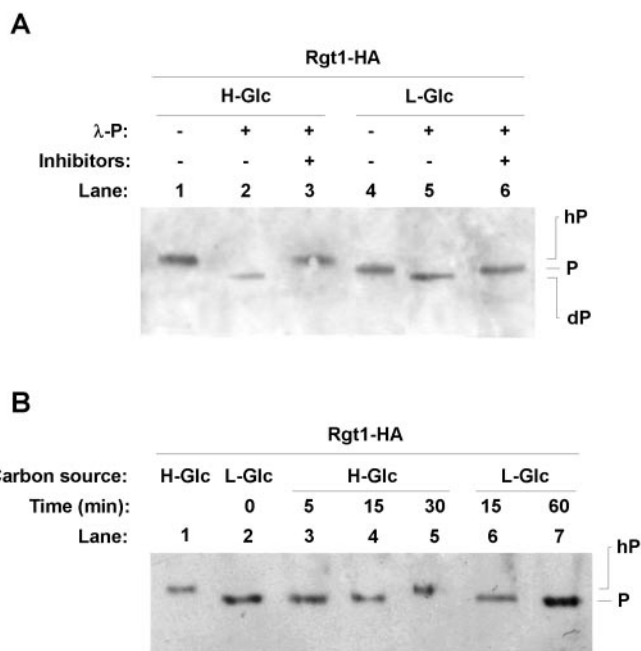

Figure 1. Rgt1 phosphorylation in response to glucose availability. (**A**) Cells, from an *rgt1Δ* mutant strain transformed with the HA-Rgt1 construct, were grown in high-glucose (H-Glc) or low-glucose (L-Glc) media until an OD_{600} of 1.0. Cell extracts were treated with or without λ-phosphatase for 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 OD_{600} 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*
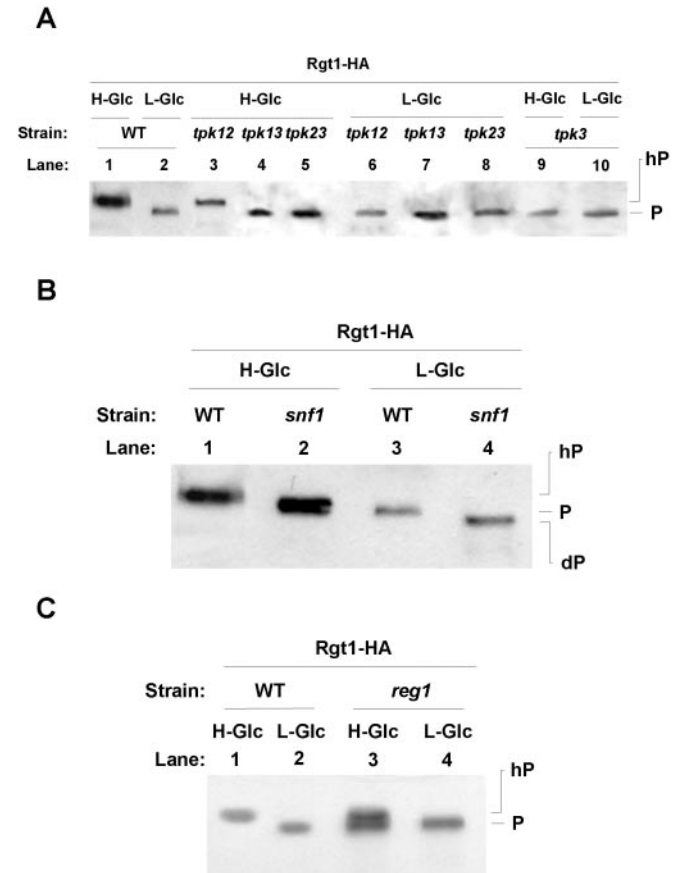

Figure 2. Tpk3 and Snf1 protein kinases are involved in *in vivo* Rgt1 phosphorylation. Cells from an *rgtΔ* mutant strain transformed with the HA-Rgt1 construct, were grown in high-glucose (H-Glc) medium until an OD_{600} of 1.0, and transferred to medium with low-glucose (L-Glc) for 60 min. HA-Rgt1 levels as well as electrophoretic mobility, were monitored in wild-type (W303-1A), *tpk12Δ,tpk13Δ, tpk23Δ* and *tpk3Δ* (**A**), *snf1Δ* (**B**) and *reg1Δ* (**C**) cells. The positions of hyperphosphorylated (hP), phosphorylated (P) and dephosphorylated (dP) Rgt1 are shown next to the immunoblots.

mutant strain (Figure 2B, lane 4). Furthermore, this phosphorylation was induced in *reg1* 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 *reg1* 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, b and a, lane 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, 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*, *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 *tpk3Δ* mutant strains transformed with a single copy of the yeast reporter plasmid YIp357/HXK2$_{+404}$ (29) integrated at the *URA3* locus. Since the *HXK2* promoter drives glucose-regulated expression of the
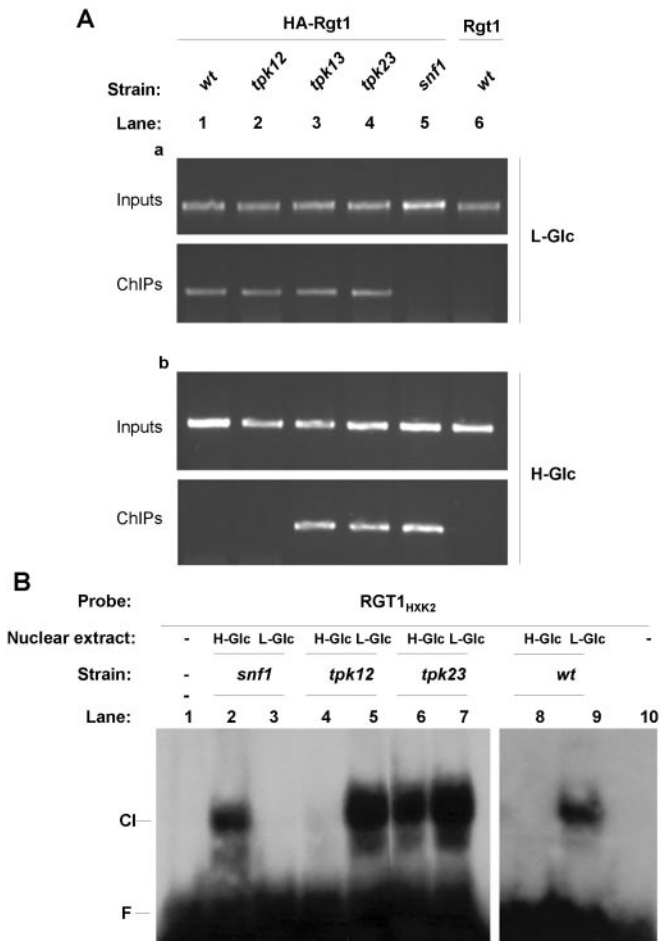

Figure 3. Rgt1 DNA-binding activity is regulated by Tpk3 and Snf1 *in vivo* and *in vitro*. (**A**) Association of Rgt1 with the RGT1 element of the *HXK2* promoter is detected by a ChIP assay. The W303-1A wild-type strain and the *tpk12, tpk13, tpk23* and *snf1* mutant strains, transformed with the HA-Rgt1 construct, were grown in high-glucose (H-Glc) medium (b) until an OD_{600} of 1.0 and then transferred to medium with low-glucose (L-Glc) for 60 min (a). The W303-1A wild-type strain was also transformed with an empty pWS93 plasmid to be used as a control with untagged Rgt1 (lane 6). The cells were treated with formaldehyde to cross-link proteins bound to DNA. ChIP was performed with anti-HA antibody. Input and immunoprecipitated DNA was amplified by PCR using primer pairs spanning the RGT1 element of the *HXK2* gene promoter. PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining. (**B**) EMSA was performed with probe RGT1$_{HXK2}$ by using nuclear extracts derived from exponentially growing wild-type cells, and *snf1, tpk1-2* and *tpk2-3* mutant cells as indicated above the lanes. Lanes 1 and 10 are probe alone controls. CI, marks the position of the Rgt1-dependent shifted complex. F, free DNA.

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.
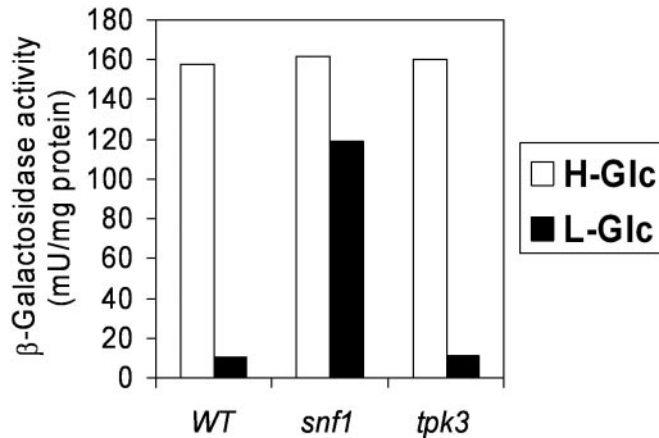
Figure 4. Influence of Snf1 and Tpk3 protein kinase activities on the regulation of *HXK2* gene expression. *HXK2* expression was measured by using the *lacZ* expression reporter gene. One copy of the $HXK2_{+404}::lacZ$ construct, containing the downstream Med8 binding and the upstream Rgt1-binding regulatory elements, was integrated into the chromosome at the *URA3* locus of the wild-type strain W303-1A, creating the FMY152 strain. The FMY152-t3 and FMY152-s1 strains were obtained by transformation of the FMY152 strain with a *tpk3::kanMX4* or *snf1::kanMX4* alleles, respectively. β-Galactosidase activities are the averages of results obtained from four to five independent experiments. Average values have SEs of 10% or less. Yeasts cultures were grown in high-glucose medium (open bars) until an OD_{600} of 1.0 and then transferred to medium with low-glucose (closed bars) for 60 min. β-Galactosidase activity was assayed in crude extracts.

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.
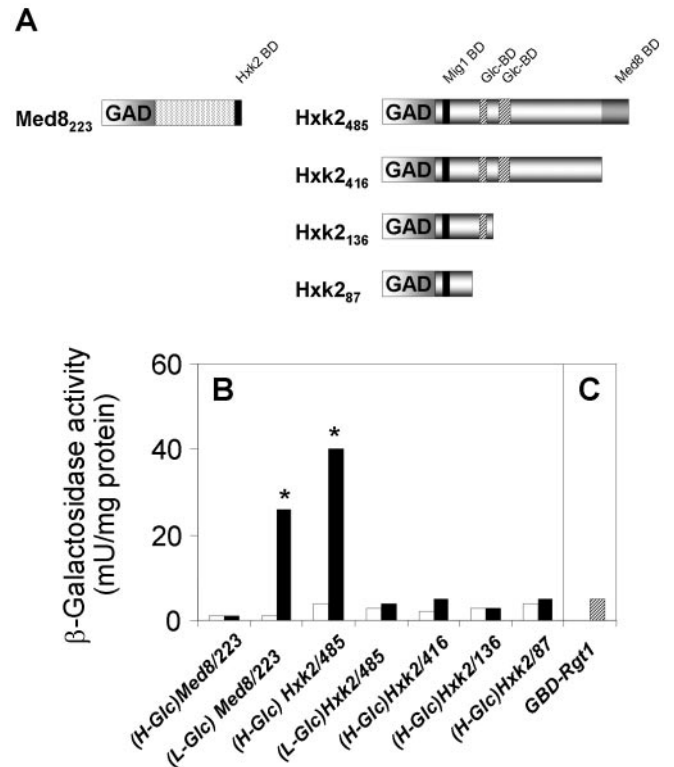

Figure 5. Interaction of Rgt1 with Hxk2 and Med8. (**A**) Schematic representation of the constructs employed. Hxk2 BD, Hxk2-binding domain; Mig1 BD, Mig1-binding domain; Glc-BD, glucose-binding domains; Med8 BD, Med8-binding domain. (**B**) Yeast two-hybrid assay of Med8 and Hxk2 interaction with Rgt1 protein (closed bars) and with GBD alone (open bars). Truncated (Hxk2–416, amino acids 1–416; Hxk2–136, amino acids 1–136 and Hxk2–87, amino acids 1–87) and full-length (Hxk2, amino acids 1–485) versions of Hxk2 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.

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 in the presence of high glucose. These results indicate that the interacting domain of Hxk2 might include a motif in its C-terminal region that is essential in this function. An alternative possibility is that the deletions introduced a structural modification, preventing the interaction, despite the presence of the interacting motif. The lack of interaction cannot be attributed to lack of expression, as the GAD-Hxk2-416, Hxk2-136 and Hxk2-87 fusion proteins were detected by western blotting using anti-Hxk2 antibody (data not shown).

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 *Δsnf1* 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 *Δsnf1* 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 *Δtpk3* 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.
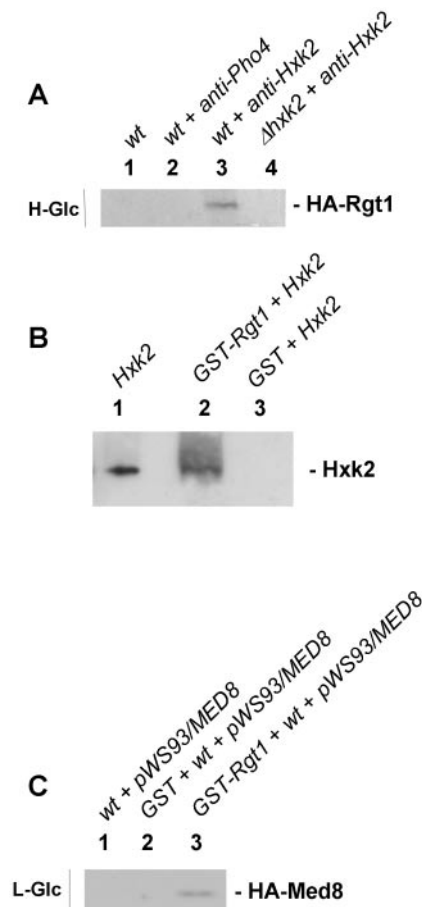

Figure 6. Immunoprecipitation and GST pull-down assays of the interaction of Rgt1 with Hxk2 and Med8. (**A**) *In vivo* co-immunoprecipitation of Rgt1 with Hxk2. *S.cerevisiae* Y187 and the mutant strain *hxk2Δ* were transformed with HA-tagged Rgt1 protein. Cell extracts from the transformed Y187 wild-type strain grown in SD (high-glucose) medium, with selection for plasmid maintenance (lane 1), were immunoprecipitated with a polyclonal antibody to Pho4 (lane 2), or a polyclonal antibody to Hxk2 (lane 3). Cell extracts from the transformed *hxk2Δ* cells grown in SD (high-glucose) medium, with selection for plasmid maintenance, were immunoprecipitated with a polyclonal antibody to Hxk2 (lane 4). Immunoprecipitates were separated by 10% SDS–PAGE and co-precipitated HA-Rgt1 was visualized on a western blot with an anti-HA antibody. (**B**) GST pull-down assays of the interaction between Rgt1-Hxk2. Purified Rgt1 interacts with purified Hxk2. A GST-Hxk2 fusion protein was purified on glutathione–Sepharose columns and incubated with thrombin to isolate native Hxk2 (lane 1). Hxk2 was incubated with purified GST-Rgt1 or with GST on glutathione beads and washed extensively. Co-precipitated proteins were resolved on 10% SDS–PAGE. The Hxk2 protein was visualized on a western blot with anti-Hxk2 antibody. (**C**) Med8 protein co-precipitates together with Rgt1 from cell extracts. Extract from the yeast strain W303-1A (transformed with the plasmid pWS93/MED8), expressing the Med8-HA fusion protein, was incubated with GST-Rgt1 or with GST purified from *E.coli* on glutathione beads. The yeast cells were grown in SE (low-glucose) medium with selection for plasmid maintenance. Co-precipitated proteins were resolved on 12% SDS–PAGE. The Med8 protein was visualized on a western blot with an anti-HA antibody.

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*
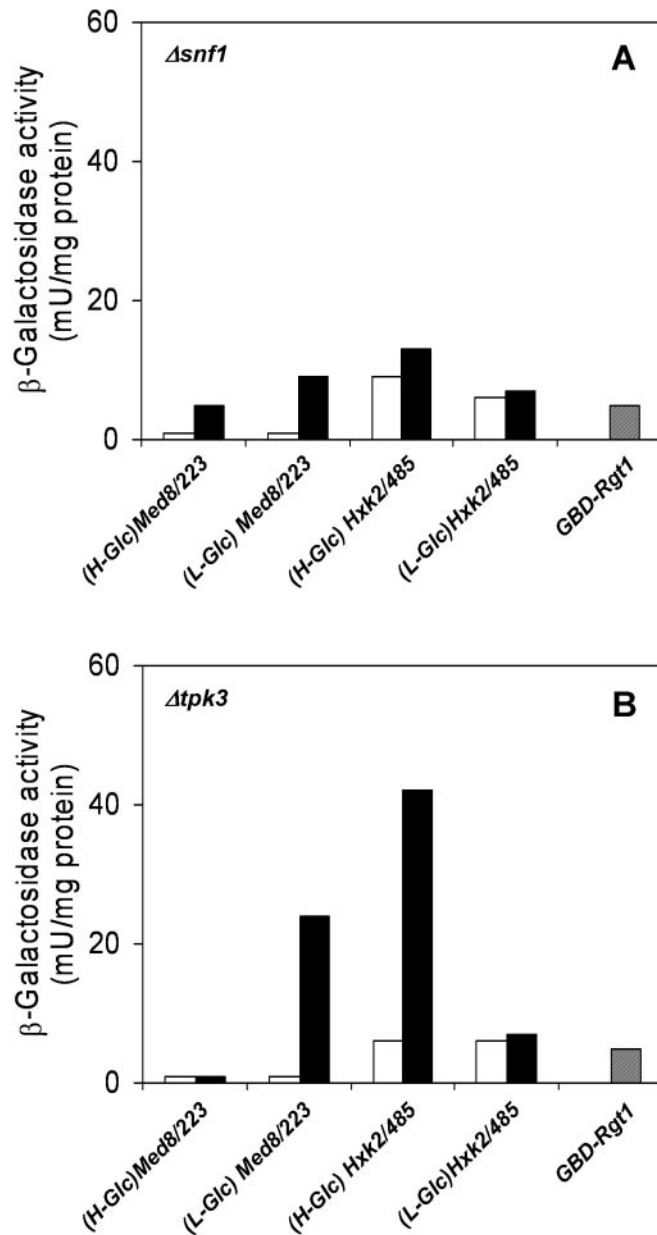
Figure 7. Interaction of Rgt1 with Hxk2 and Med8 in the absence of Snf1 and Tpk3 protein kinases. Yeast two-hybrid assay of Med8 and Hxk2 interaction with Rgt1 protein (solid bars) in the Δ*snf1* (**A**) and Δ*tpk3* (**B**) mutant strains. Control experiments with GBD alone (open bars) were also performed. The full-length Rgt1 fused to the Gal4 DNA-binding domain (GBD) was co-transformed into yeast strains Y187-Δ*snf1* and Y187-Δ*tpk3* with the construct encoding the Gal4 activation domain alone (GAD) (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 as indicated in the legend of Figure 5.

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 signalling (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
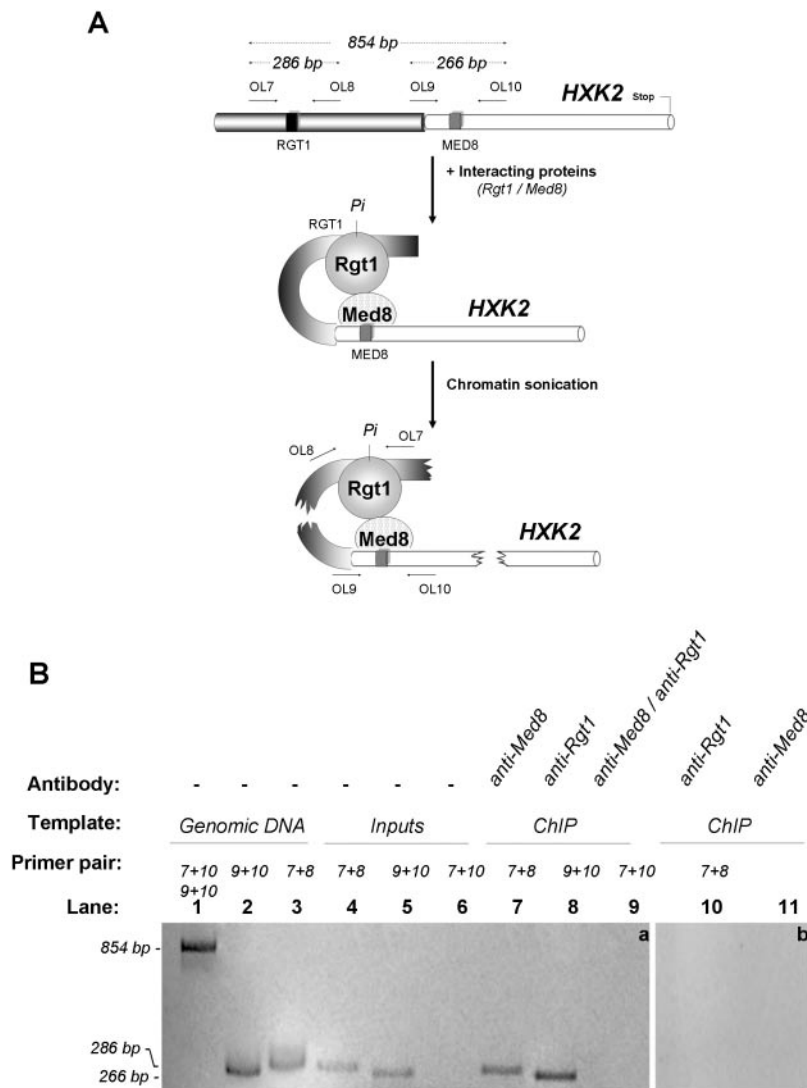
Figure 8. Med8 and Rgt1 proteins interact at the *HXK2* promoter level. (**A**) Model showing the three DNA fragments that were analysed: the downstream *HXK2* regulatory region containing the Med8-binding site (266 bp), the upstream *HXK2* regulatory region which contains the Rgt1 binding site (286 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 OD_{600} 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.

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
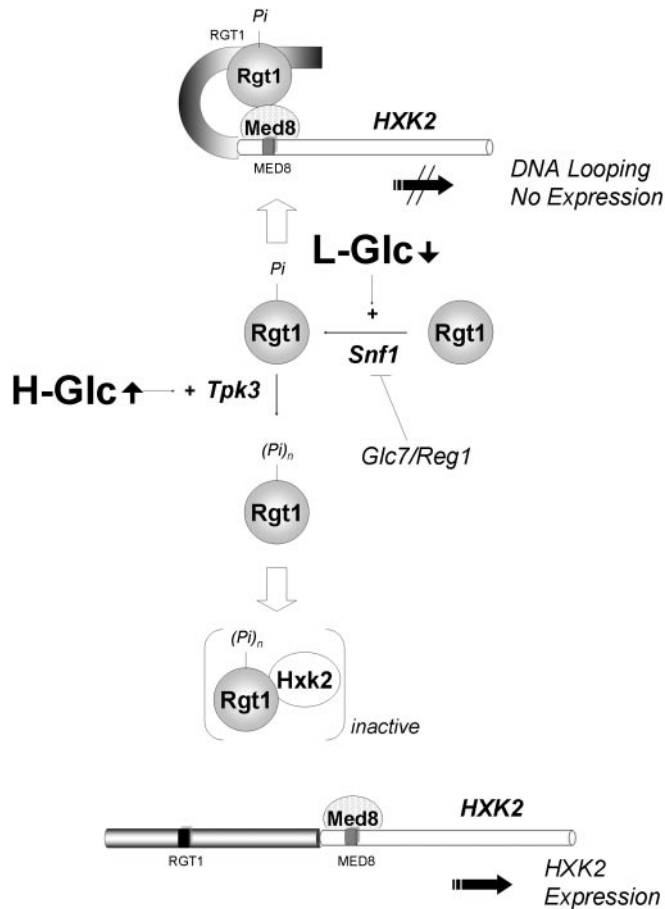
Figure 9. A model explaining the mechanism by which Snf1 and Tpk3 protein kinases regulate *HXK2* transcription. In low-glucose conditions (L-Glc(), Rgt1 is phosphorylated by Snf1 or an Snf1-dependent protein kinase. The phosphorylated Rgt1 binds to the RGT1 element of *HXK2* promoter to repress *HXK2* transcription by looping DNA and bringing the Rgt1 and Med8 distal binding sites into close proximity. In high-glucose conditions (H-Glc(), Rgt1 is hyperphosphorylated by Tpk3. The hyperphosphorylated form is sequestered outside its DNA-binding site by interacting with the nuclear Hxk2 protein, resulting in expression of the *HXK2* gene. An arrow denotes positive expression. An arrow marked with double bars denotes negative expression. Positive regulation is marked by plus.

in low-glucose, when Rgt1 is bound to the *HXK2* promoter and the interaction between Rgt1 and Med8 takes place.

Our results also demonstrate that Rgt1 interacts directly with Hxk2 and Med8 in a glucose-dependent manner, and that the C-terminal domain of the Hxk2 protein is required for the Hxk2–Rgt1 interaction. Since the C-terminal domain of the Hxk2 protein is also necessary for Med8 interaction in the presence of high glucose (9), a possible competition between the Med8 and Rgt1 proteins for Hxk2 binding could be established during growth on high glucose. However, an alternative possibility is that the Hxk2 C-terminal deletions have introduced structural modifications, preventing the interaction between Hxk2 and Rgt1 proteins despite the presence of the interacting motif.

The data presented above raises the issue about the influence of Snf1 and Tpk3-dependent phosphorylation of Rgt1 in its interaction with Med8 and Hxk2. Our results show that regulation of Rgt1 interaction with Med8 through

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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