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Connection Between the Rag4 Glucose Sensor and the KlRgt1 Repressor in *Kluyveromyces lactis*

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ABSTRACT

The *RAG4* gene encodes for the sole transmembrane glucose sensor of *Kluyveromyces lactis*. A *rag4* mutation leads to a fermentation-deficient phenotype (Rag$^-$ phenotype) and to a severe defect in the expression of the major glucose transporter gene *RAG1*. A recessive extragenic suppressor of the *rag4* mutation has been identified. It encodes a protein (KlRgt1) 31% identical to the *Saccharomyces cerevisiae* Rgt1 regulator of the *HXT* genes (ScRgt1). The *Klrgt1* null mutant displays abnormally high levels of *RAG1* expression in the absence of glucose but still presents an induction of *RAG1* expression in the presence of glucose. KlRgt1 is therefore only a repressor of *RAG1*. As described for ScRgt1, the KlRgt1 repressor function is controlled by phosphorylation in response to high glucose concentration and this phosphorylation is dependent on the sensor Rag4 and the casein kinase Rag8. However, contrary to that observed with ScRgt1, KlRgt1 is always bound to the *RAG1* promoter. This article reveals that the key components of the glucose-signaling pathway are conserved between *S. cerevisiae* and *K. lactis*, but points out major differences in Rgt1 regulation and function that might reflect different carbon metabolism of these yeasts.

In contrast to *Saccharomyces cerevisiae*, *Kluyveromyces lactis* is a predominantly aerobic yeast that exhibits a distinct regulation pattern for glucose uptake. In the cells of *S. cerevisae*, the low-affinity glucose uptake appears to be constitutively expressed, whereas the high-affinity system is glucose repressed (Bisson and Fraenkel 1984). This situation results from the superimposition of multiple regulatory mechanisms of many different glucose transporter genes (Ozcan and Johnston 1999). In *K. lactis*, the low-affinity glucose uptake is glucose inducible and the high-affinity glucose uptake is constitutive (Wesolowski-Louvel *et al.* 1992a). The two systems are encoded by two single genes: *RAG1* encoding the low-affinity permease (Wesolowski-Louvel *et al.* 1992a) and *HGT1* encoding the high-affinity permease (Billard *et al.* 1996). While *HGT1* is constitutively expressed, *RAG1* expression is activated in the presence of high concentrations of glucose (Chen *et al.* 1992; Wesolowski-Louvel *et al.* 1992a). The Rag1 permease supports fermentative growth, which requires a high substrate flow. In the absence of Rag1, growth of the cell on high-glucose media depends on respiration. Consequently, *rag1* cells and mutants that do not express the Rag1 glucose transporter cannot grow on 5% glucose in the presence of antimycin A, which blocks respiration (the Rag$^-$ phenotype) (Goffrini *et al.* 1989; Wesolowski-Louvel *et al.* 1992b).

Several Rag$^-$ mutants have been isolated and at least four carry a mutation in genes that positively regulate the transcription of *RAG1*: *RAG4*, which encodes a glucose sensor (Betina *et al.* 2001); *RAG5*, which encodes a hexokinase (Prior *et al.* 1993); *RAG8*, which encodes an essential casein kinase 1 (Blaisonneau *et al.* 1997); and *RAG17*, which codes for enolase (Lemaire and Wesolowski-Louvel 2004). These genes are single-copy genes in *K. lactis*. These regulatory elements appear to define two different pathways: (i) a pathway involving the glucose sensor Rag4 that responds to extracellular glucose availability (pathway 1), and (ii) a pathway responding to an intracellular signal generated by glycolysis (pathway 2) in which Rag5 hexokinase and Rag17 enolase participate (Lemaire and Wesolowski-Louvel 2004). The participation of Rag8 casein kinase 1 in either of these two pathways has not yet been clearly established.

We looked for genetic suppressors of the *rag4* mutation to help in finding new elements of the Rag4-dependent glucose-sensing pathway. We found that when grown on glucose, a *rag4* null mutant spontaneously reverted to the Rag$^+$ phenotype at a very high rate. This allowed us to identify recessive mutations in the *KlRGT1* (*SRA1*) gene that bypass the need for Rag4

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TABLE 1

Strains used in this study

Strain	Relevant genotype	Source or reference
K. lactis		
MW270-7B	*MAT***a** *uraA1-1 leu2 metA1-1*	Billard *et al.* (1996)
MW159-1C	*MAT***a** *uraA1-1 adeT-600 lysA1-1 rag8-2*	Blaisonneau *et al.* (1997)
PM6-7A/VV30	*MAT***a** *uraA1-1 adeT-600 rag8-1*	Blaisonneau *et al.* (1997)
MW109-8C/FA49	*MATα lysA1-1 rag4-5*	Wesolowski-Louvel *et al.* (1992b)
MWK8/SRA	Isogenic to MWK8 except *sra1-6*	This study
MW360-5C	*MATα uraA1-1 leu2 metA1-1 rag4Δ2::LEU2 sra1-6*	This study
JLS4-11A	*MAT***a** *uraA1-1 lysA1-1 rag4-5 sra1-6*	This study
MWK6	Isogenic to MW270-7B except *rag4Δ2::LEU2*	This study
MWK7	Isogenic to MW270-7B except *rgt1Δ1::URA3*	This study
MWK7/F	Isogenic to MW270-7B except *rgt1Δ1::ura3*	This study
MWK8[a]	Isogenic to MW270-7B except *rag4Δ1::URA3*	Betina *et al.* (2001)
MWK9	Isogenic to MW270-7B except *rgt1Δ1::URA3 rag4Δ2::LEU2*	This study
MWK9/F	Isogenic to MW270-7B except *rgt1Δ1::ura3 rag4Δ2::LEU2*	This study
MWK21	Isogenic to MW159-1C except *rgt1Δ1::URA3*	This study
MWK22[b]	*MATα ura3 trp1 ade1-600 adeT-600 rag4Δ1::URA3*	Betina *et al.* (2001)
S. cerevisiae		
THY.AP4	*MAT***a** *ura3 leu2 lexA::lacZ::trp1 lexA::HIS3 lexA::ADE2*	Obrdlik *et al.* (2004)
THY.AP5	*MATα URA3 leu2 trp1 his3 loxP::ade2*	Obrdlik *et al.* (2004)
BY4741	*MAT***a** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0*	EUROSCARF

[a] Previously MW270-7B/Δ*rag4*.
[b] Previously JA6/Δ*rag4*.

for growth on high-glucose media in the presence of antimycin A. We show that *KlRGT1* encodes a repressor protein very similar to Rgt1 of *S. cerevisiae* (ScRgt1) that regulates *HXT* gene expression in this yeast (Ozcan *et al.* 1996). Our results show major differences between the two yeast systems: (i) KlRgt1 binds to the *RAG1* promoter in a glucose-independent manner, whereas ScRgt1 is removed from *HXT* promoters in response to glucose; and (ii) KlRgt1 is only a repressor of *RAG1* expression in *K. lactis*, whereas ScRgt1 is thought to both repress and activate *HXT1* expression in *S. cerevisiae* (Ozcan *et al.* 1996; Polish *et al.* 2005). Despite these differences, KlRgt1, like ScRgt1, seems to be regulated post-translationally through a glucose-dependent pathway involving the glucose sensor.

MATERIALS AND METHODS

Yeast strains, growth conditions, and yeast transformation: Yeast strains used are described in Table 1. Yeast cells were grown at 28° in a complete YP medium containing 1% Bacto yeast extract, 1% Bactopeptone (Difco, Detroit), supplemented with either 2% glucose (YPG) or a specified carbon source. Minimal medium contained 0.7% yeast nitrogen base without amino acids (Difco) and 2% glucose with auxotrophic supplements as required. The Rag phenotype was tested on GAA medium (YP medium containing 5% glucose and 5 μM antimycin A). For the G418 medium, YPG plates were supplemented with Geneticin (200 μg/ml; Life Technologies).

Genetics methods have been described previously (Wesolowski *et al.* 1982).

To monitor the behavior of KlRgt1 under repressing and inducing conditions, cells expressing KlRgt1-3HA inserted into the pCXJ22 vector (Chen 1996) bearing the *URA3* gene for complementation were pregrown to an OD_{600} of 2–3 in uracil-less minimal medium containing 2% glycerol and 2% galactose. The harvested cells were washed with water, divided into two fractions, and further incubated for 6 hr in uracil-less minimal medium containing 2% glycerol or 5% glucose. Yeast transformation was carried out as previously reported (Lemaire and Wesolowski-Louvel 2004).

Construction of deletion strains and plasmids: We constructed *Klrgt1* strains by inserting the 3.3-kb *Xho*I–*Bam*HI fragment containing the promoter region and two-thirds of the *KlRGT1* ORF into the *Xho*I and *Bam*HI sites of pBluescript KS phagemid (Stratagene, La Jolla, CA). The resulting plasmid was digested with *Bgl*II, which removed a 1.7-kb fragment. This internal fragment was replaced by a 0.9-kb *Bam*HI fragment containing the *URA3* marker from the pAF101 vector (Thierry *et al.* 1990). A 2.5-kb *Xho*I–*Bam*HI fragment containing the disrupted *KlRGT1* cassette was used to transform various host strains to disrupt the *KlRGT1* copy.

A 7.3-kb *Sac*I–*Eco*RV fragment containing the entire *KlRGT1* gene was inserted into the *Klori-ScARS-CEN*-based shuttle vector pCXJ22 (Chen 1996) using *Sac*I and *Sma*I sites. The resulting plasmid, pCXJ22-KlRGT1, was a source of *KlRGT1* gene used for further constructions and yeast transformations.

The *Klrgt1-6* allele was amplified by PCR using MWK8/SRA genomic DNA as a template and the primers P5′ RGT1/SacI (5′-ATCCGAGCTCCTTGAAACTGCCGTACAAACCA-3′) and P3′ RGT1/SmaI (5′-ATCCCCCGGGTTCCAACAGAAGGTG ATGGCTA-3′). The PCR product was digested with *Sma*I and *Sac*I and the fragment was inserted into the pCXJ22 vector, yielding pML203 plasmid.

We introduced the *Klrgt1-6* mutation in the wild-type *KlRGT1* gene by exchanging the 3′ region of the two alleles. A *Spe*I/*Sal*I digestion of pCXJ22-KlRGT1 removed a 2.8-kb fragment, which was replaced by a 0.54-kb *Spe*I–*Sal*I-digested fragment from pML203.

KlRgt1 was epitope tagged by *in vivo* recombination in *S. cerevisiae* using the BY4741 strain transformed with the pCXJ22-KlRGT1 plasmid. The resulting transformant was transformed with a 1898-bp DNA fragment made of the triple HA tag coding sequence followed by the *ADH1* gene terminator and the *kanMX6* module as a selectable marker. This DNA fragment (flanked by 40-bp sequences of the 3′ region of the *KlRGT1* locus to target recombination) was obtained by PCR with the pFA6a-3HA-kanMX6 plasmid as a template (Longtine *et al.* 1998) and the primers F2-3HA-RGT1 (5′-TGGTGCATTGCAGACACTGAAGAACTAGGATGGTTTAATC AGCGGATCCCCCGGGTTAATAA-3′) and R1-3HA-RGT1 (5′-GCTTGGTGACCTTTCCTTATTTAACTTTGTTTTATTCAGT TAGAATTCGAGCTCGTTTAAAC-3′). Plasmids contained in G418^R transformants were rescued in *Escherichia coli*. After molecular analysis, *K. lactis* strains were transformed with pCXJ22-KlRGT1-3HA plasmid.

Preparation of yeast RNA and probes: Total RNA was extracted from cells grown to an OD_{600} of ~2 by the acid phenol method. Northern blot analysis was carried out as described by Prior *et al.* (1993). Probes were obtained by PCR amplification using either *K. lactis* genomic DNA or the cloned genes as templates. The oligonucleotides used for *RAG1*, *RAG5*, *RAG17*, and *18S* have been previously described (Lemaire and Wesolowski-Louvel 2004). The *KlACT* probe was amplified by PCR using the following primers: Actin-up (5′-GAGGTATCTTGACTCTACGTTACC-3′) and Actin-down (5′-GACATGACGATGTTACCGTAC-3′).

Yeast cell extracts and immunoblotting: Crude cell extracts were prepared as previously described (Kushnirov 2000). For Western blotting analysis, equal quantities of proteins were loaded onto SDS–PAGE. Gels were transferred to a PVDF membrane (Qbiogene) and probed with 12CA5 anti-HA monoclonal antibodies (1:5000; Roche Diagnostics). Primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse secondary antibody (Upstate Biotechnology) and an enhanced chemiluminescence system (SuperSignal Western Femto; Pierce).

Chromatin immunoprecipitation : Chromatin immunoprecipitation (ChIP) experiments were carried out as previously reported (Kim *et al.* 2003) using 10^{10} cells cross-linked with formaldehyde (final concentration of 1%) by incubation for 15 min at room temperature. The reaction was quenched with glycine (final concentration of 125 mm). The cells were washed in TBS buffer and disrupted by vortexing six times for 1 min in 1 ml of lysis buffer (50 mm Tris–HCl pH 7.5, 100 mm NaCl, 1 mm EDTA) in the presence of protease inhibitor cocktail, PMSF, and 650 μl of glass beads. The lysate was supplemented with Triton X-100 (final concentration, 1%) and sodium deoxycholate (final concentration, 0.1%), sonicated, and then centrifuged at 15,000 × *g* at 4° for 10 min. The genomic DNA fragments, averaging 500–1500 bp and cross-linked to KlRgt1-3HA, were immunoprecipitated by incubation for 3 hr at 4° with 2 μl of 12CA5 anti-HA monoclonal antibodies. The immunocomplexes were recovered by further incubation for 1 hr with 50 μl of Protein A-conjugated agarose bead suspension (Sigma). The beads were then washed twice by 1 min incubation at room temperature in 1 ml of IP buffer (lysis buffer supplemented with Triton X-100 and Na-deoxycholate), twice with 1 ml of ChIP high salt lysis buffer (IP buffer containing 500 mm NaCl), twice with 1 ml of wash buffer (10 mm Tris–HCl pH 8, 250 mm LiCl, 1 mm EDTA, 0.5% NP-40 and 0.5% Na-deoxycholate), and then once with 1 ml of TBS buffer. Immunocomplexes were then eluted from the beads by incubation at 65° for 10 min in 150 μl of elution buffer (50 mm Tris–HCl pH 8, 1% SDS, 10 mm EDTA). The tubes were briefly vortexed during the incubation to help the elution. The cross-linking was reversed by incubating the elution fraction at 65° for 15 hr. The immunoprecipitated and input DNA were purified using a PCR purification kit (QIAGEN) and were used as templates in a 25-cycle PCR. PCR amplifications were carried out on 1/25 of the immunoprecipitation (IP) and 1/5000 of the chromatin before immunoprecipitation (input, IN). The reactions, in a 50-μl reaction volume, comprised 6 min at 95° followed by 25 cycles of 30 sec at 95°, 30 sec at 47°, 30 sec at 72°, and then 7 min at 72°. The primer sequences were as follows: for *pRAG1* (H51: 5′-GGAGAAACTTGTCCCTCTTCC-3′ and H52: 5′-TTTAGAG GGTCAAAGGCACCG-3′), for *pACT* (H1: 5′-TTTTTCTAGAG ATCCGCCTTTGAAGCTG-3′ and H15: 5′-AAAGAATTCAA GTCAGTAACCTGCGCATGG-3′), for *pRAG5* (RAG5CHIP5′: 5′-CATCTTCATTTTCGCAGCG-3′ and RAG5CHIP3′: 5′-CT GTGGTTGTATCTTATTC-3′), for *pENO* (ENOCHIP5′: 5′-CA CTCGTTCATCAGGTTTG-3′ and ENOCHIP3′: 5′-ATGTCT CATAATGTCTCGTC-3′).

Immobilized DNA binding assay: Immobilized DNA binding assay (IDBA) experiments were carried out as described by Kim (2004). Briefly, KlRgt1-3HA was immunoprecipitated from crude yeast cell extracts (3×10^9 cells) with anti-HA antibodies as reported above. The beads coated with KlRgt1-3HA were washed twice for 10 min at 4° in 500 μl of IP buffer and once in 500 μl of TGZD buffer (20 mm Tris–HCl pH 8.0, 75 mm KCl, 10 μM $ZnCl_2$, 5% glycerol). The beads were separated into two fractions and incubated under agitation at 25° for 30 min in 500 μl of TGZD buffer containing 1 μg of poly-dI-dC (Sigma-Aldrich) and 2×10^4 counts per minute of ^{32}P-labeled DNA fragments corresponding to either *pRAG1* or *pACT*. (^{32}P-labeled *pRAG1* or *pACT* DNA were prepared by PCR amplification using the primer sets indicated in ChIP experimental section, followed by end-labeling with [^{32}P]ATP and T4 polynucleotide kinase.) The beads were then washed at 25° for 5 min with 500 μl of TGZD buffer containing 250 mm NaCl. The bound DNA was eluted from the beads by incubation with 30 μl of Tris–EDTA buffer containing 1 m NaCl at room temperature for 5 min. KlRgt1-3HA was recovered by boiling the beads for 5 min in SDS–PAGE sample buffer. The eluted DNA and KlRgt1-3HA were analyzed by electrophoresis in polyacrylamide gels and visualized by autoradiography and Western blotting, respectively.

Phosphatase treatment of protein extracts: Immunoprecipitated KlRgt1 was treated with phosphatase. After washing with lysis buffer and phosphatase buffer, beads decorated with KlRgt1-3HA were incubated with 10 units of calf intestinal alkaline phosphatase (CIP) (Fermentas) at 37° for 30 min. After elution, the proteins were analyzed by Western blotting.

Split-ubiquitin interaction assays: The *RAG4* ORF was inserted *in vivo* into the pMetYCgate vector (*LEU2-CEN/ARS-kanMX*) (gift from P. Obrdlik) so that Cub-PLV was fused to the C-terminus of Rag4. The *RAG4* ORF was amplified by using B1-RAG4 (5′-acaagtttgtacaaaaaagcaggctctccaaccaccATGACTACT GATTCTGTTCCA-3′) and B2-RAG4 (5′-tccgccaccaccaaccactttg tacaagaaagctgggtaAGTGTTACTATTAATATCGGT-3′) primers (lowercase letters are the linkers B1 and B2 present in the pSUgate vectors) (Obrdlik *et al.* 2004) and MW270-7B strain genomic DNA (Table 1) as a template. The THY.AP4 strain was cotransformed with the 2.4-kb PCR product and the pMetYC-gate vector digested with *Pst*I and *Hin*dIII (Table 1). Transformants were selected as previously reported (Obrdlik *et al.* 2004). The *RAG8* ORF was inserted *in vivo* into the pNXgate vector (*TRP1-2-kanMX*) (gift from P. Obrdlik) so that NubG was fused to the N-terminus of Rag8. The *RAG8* ORF was amplified by using B1-RAG8 (5′-acaagtttgtacaaaaaagcaggctc tccaaccaccATGAGTATTACAGCGGGACCT-3′) and B2-RAG8 (5′-tccgccaccaccaaccactttgtacaagaaagctgggtaACAACAGCCAA GCTTGCTGAA-3′) primers and MW270-7B strain genomic DNA as a template. The THY.AP5 strain was cotransformed

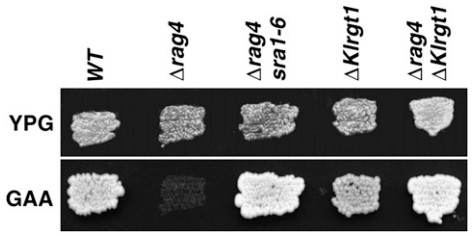

FIGURE 1.—Phenotypes of the revertants and various mutants. Strains grown on YPG plates were replicated on GAA plates. The photographs were taken after 24 hr incubation at 28°. MW270-7B (*WT*); MWK8 (Δ*rag4*); MWK8/SRA (Δ*rag4 sra1-6*); MWK7 (Δ*Klrgt1*); MWK9 (Δ*rag4* Δ*Klrgt1*).

with the 1.7-kb PCR product and the pNXgate vector digested with *Eco*RI and *Sma*I (Table 1). Transformants were selected as previously described (OBRDLIK *et al.* 2004).

For interaction assays, THY.AP4 and THY.AP5 transformants were mated on YPD plates by incubation for 6 hr at 28°. Diploids were selected by replica plating on tryptophan-less, uracil-less, leucine-less medium and then incubated at 28°. For growth assays (activation of *HIS3* and *ADE2* reporter genes), diploids were replicated on minimal media supplemented or not with 1 mM methionine. Growth was monitored for 3 days at 28°.

β-Galactosidase assays: β-Galactosidase activity assays were carried out with permeabilized yeast cells. Results are given in Miller units [1000 OD_{420} (ONPG)/OD_{600} (cells) volume (ml) time (min)]. Transformants were pregrown to log phase (OD_{600} 1–2) in minimal medium containing 2% glucose supplemented with adenine, histidine, and 1 mM methionine (repressing conditions). The harvested cells were washed with water, diluted 10-fold in minimal medium containing 2% glucose supplemented with adenine, histidine (inducing conditions), and further incubated to log phase (OD_{600} 1–2).

RESULTS

***sra* mutations restore *RAG1* expression in *rag4* mutant:** We observed that *rag4* null mutant cells (MWK8) reverted to the Rag^+ phenotype at a high rate (Figure 1; Δ*rag4 srat1-6*). We analyzed six of these revertants. When these strains were individually crossed with the *rag4* MWK22 strain (Table 1), the Rag^- phenotype of the diploids indicated that the suppressor mutations were recessive (*sra* for suppressor of *rag4* mutation). The 2:2 segregation of the Rag phenotype in the tetrads from meiosis of these diploids showed that they were single gene mutations. Moreover, when the revertant strains were crossed with each other, the resulting diploids had a Rag^+ phenotype, indicating that the six mutations affected a single locus, *SRA1*. We analyzed one revertant, *sra1-6* (MWK8/SRA strain, Table 1), further.

As the Rag^- phenotype of the Δ*rag4* mutant comes from a severe reduction in the expression of the low-affinity glucose transporter gene (CHEN *et al.* 1992; BETINA *et al.* 2001), we studied *RAG1* transcription in the revertant strain. Northern blot analysis showed that *RAG1* expression returned to high levels when the Δ*rag4 sra1-6* cells were grown on 2% glucose and was derepressed in the absence of glucose (Figure 2). The complete suppression of the Δ*rag4* mutation phenotypes by mutations in *SRA1* indicates that the *SRA1* gene product plays a negative role in *RAG1* expression and could act downstream from Rag4.

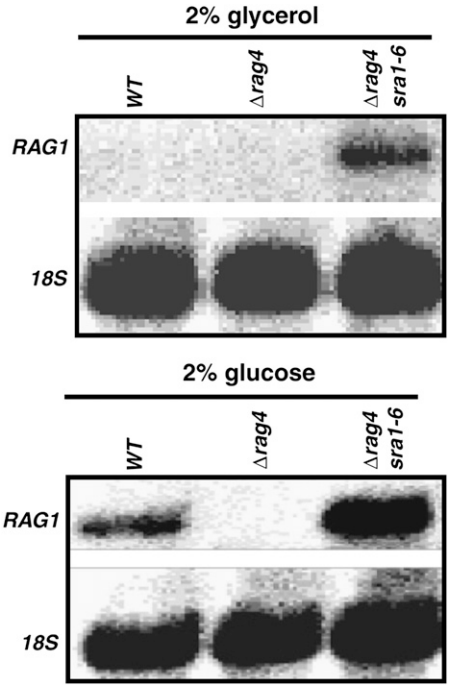

FIGURE 2.—Northern blot analysis of *RAG1* mRNA in the revertant cells. Total RNA was extracted from cells grown in the presence of either 2% glycerol or 2% glucose. Each slot was loaded with 5–10 μg of RNA and run on a 1.2% agarose-formaldehyde gel. The probes used are described in MATERIALS AND METHODS. rRNA (18s) was used as a standard. MW270-7B (*WT*); MWK8 (Δ*rag4*); MWK8/SRA (Δ*rag4 sra1-6*).

***SRA1* encodes a protein similar to the Rgt1 repressor of *S.cerevisiae*:** We cloned the *SRA1* gene by *in vivo* complementation by transforming *rag4 sra1-6* cells (Rag^+) with a wild-type genomic library of *K. lactis* made in the KEp6 vector (WESOLOWSKI-LOUVEL *et al.* 1988) and then screening Rag^- transformants. One Rag^- transformant contained an 8.7-kb complementing DNA. The nucleotide sequence of the extremities of this complementing DNA fragment showed that it spanned a region of chromosome VI (http://cbi.labri.fr/Genolevures/) containing two entire ORFs. The best candidate was KLLA0F25630g (EMBL accession no CR382126), which had some similarities with the ScRgt1 regulator of *S. cerevisiae HXT* genes (OZCAN and JOHNSTON 1995; OZCAN *et al.* 1996). The DNA region containing KLLA0F25630g was inserted into the pCXJ22 vector (MATERIALS AND METHODS) and was used to transform MWK8/SRA cells. Rag^- phenotype of the obtained transformants (data not shown) indicated that the *SRA1* locus did correspond to this gene encoding a protein similar to the ScRgt1 repressor.

The predicted protein (1007 amino acids) encoded by *SRA1* gene is 31% identical to ScRgt1 of *S. cerevisiae*

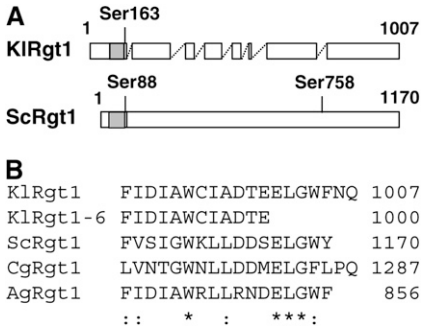

FIGURE 3.—Alignment of KlRgt1-orthologous sequences. (A) Alignment of ScRgt1 (*S. cerevisiae*) and KlRgt1 (*K. lactis*). Open and shaded boxes indicate aligned regions and the $Zn2Cys_6$ binuclear DNA binding domain, respectively. Gaps >20 amino acids are indicated by dashed lines. Conserved serine residues that are phosphorylated in ScRgt1 (POLISH *et al.* 2005) are also indicated. (B) Alignment of C-terminal region of KlRgt1, KlRgt1-6 (*K. lactis*), ScRgt1 (*S. cerevisiae*), CgRgt1 (*Candida glabrata*), and AgRgt1 (*Ashbya gossypii*). Asterisks and colons indicate identical or similar residue, respectively.

(1170 amino acids), 32% identical to the AGL083wp ORF (856 amino acids) of *Ashbya gossypii* (DIETRICH *et al.* 2004), and 29% identical to the CAGL0L01903g ORF (1287 amino acids) of *Candida glabrata* (DUJON *et al.* 2004). Alignment of these proteins showed that they have similar structural organizations, with a typical zinc-finger DNA binding motif consisting of six cysteine residues present in their N-terminal regions (Figure 3A shows the alignment of *S. cerevisiae* and *K. lactis* products). As the *SRA1* gene product is similar to ScRgt1 of *S. cerevisiae*, we have called it KlRgt1 and renamed the *sra1-6* mutation *Klrgt1-6*. By extension, we have called the *A. gossypii* and *C. glabrata* products AgRgt1 and CgRgt1, respectively.

We then amplified and sequenced the mutant allele *Klrgt1-6* (MATERIALS AND METHODS) to identify the suppressor mutation. We found that the mutation was a nonsense mutation resulting in a truncated KlRgt1 protein (1000 amino acids). Three of the 7 missing amino acids are highly conserved in Rgt1 orthologs (Figure 3B), suggesting that the C-terminal region of the protein may be essential for KlRgt1 function.

KlRgt1 is a repressor of *RAG1* expression: We constructed a Δ*Klrgt1* null mutant to determine more precisely the role of KlRgt1 in *RAG1* expression. This mutant displayed a Rag^+ phenotype (Figure 1) as the isogenic wild-type strain, indicating that the loss of KlRgt1 does not lead to a fermentation defect. Northern blot analysis showed that *RAG1* transcription was deregulated in Δ*Klrgt1* null mutant (Figure 4), as we observed in the revertant strain (Figure 2). *RAG1* was strongly transcribed in cells grown in glycerol-containing medium, suggesting that in the absence of glucose, KlRgt1 has a repressor function. In the presence of glucose, the level of *RAG1* expression was still strong and even higher than in the wild-type cells. This indicates

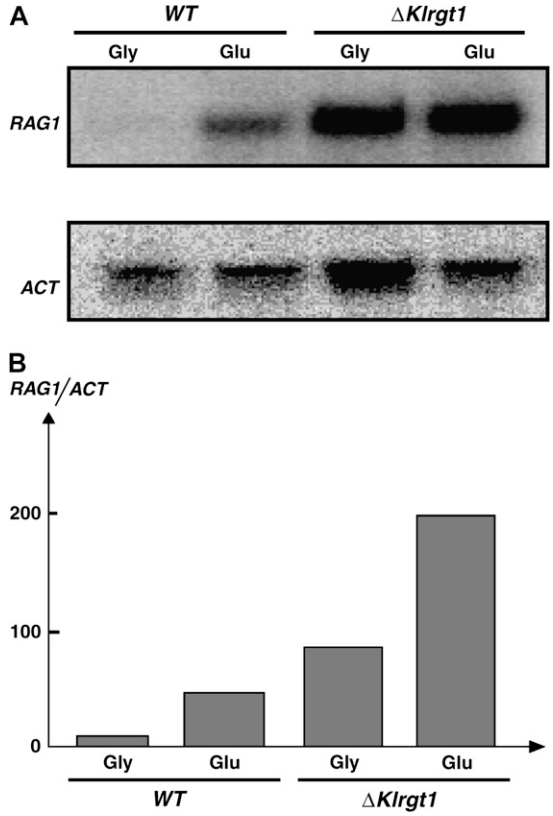

FIGURE 4.—*RAG1* transcription in *Klrgt1* cells. (A) Northern blot analysis was carried out as in Figure 2. MW270-7B (*WT*); MWK7 (Δ*Klrgt1*). (B) Densitometric scanning. The hybridization signals were quantified by means of a Cyclone PhosphoImager (Packard). The ratio between *RAG1* and *ACT* transcript is represented.

that the induction of *RAG1* expression in glucose mainly involves getting rid of KlRgt1 repression. However, *RAG1* expression could still be induced by glucose in the *Klrgt1* mutant (Figure 4B). Such a finding suggests that whether KlRgt1 has any activator function in *RAG1* expression, it is not the only one involved. Altogether, these data are consistent with KlRgt1 being a transcriptional repressor at the *RAG1* promoter in the absence of glucose.

KlRgt1 binds to the *RAG1* promoter *in vivo* and *in vitro*: We analyzed the interaction of KlRgt1 with the *RAG1* promoter *in vivo* by ChIP assay. We used a KlRgt1-3HA construct expressed from pCXJ22 vector (MATERIALS AND METHODS) transformed in *Klrgt1* mutant cells (MWK7/F strain, Table 1). The fusion protein was functional because *RAG1* transcription was repressed in the absence of glucose in the transformants (not shown). After KlRgt1-3HA immunoprecipitation, we used the immunoprecipitated and total input DNA as templates for PCR analysis using primers designed for the *RAG1* gene promoter. The amplified sequence contains six 5′-CGG ANNA-3′ putative binding sites (Figure 5A), corresponding to ScRgt1 target sites (KIM 2004). The promoter region of the actin gene was used as a control. We found

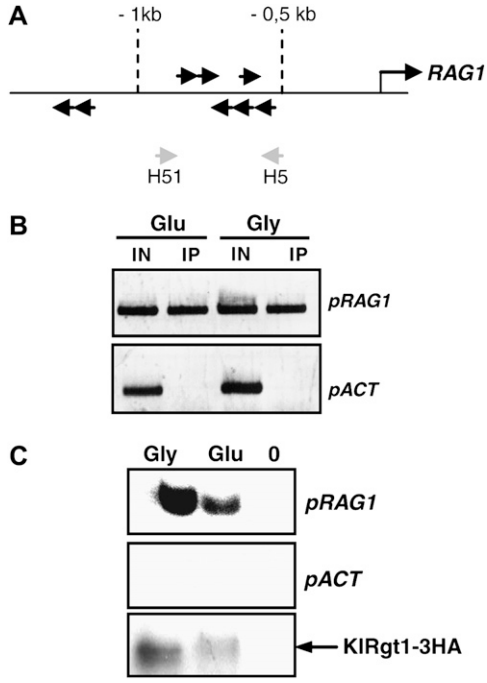

FIGURE 5.—Binding of KlRgt1 to the *RAG1* promoter. (A) Diagram of *RAG1* promoter (*pRAG1*). Location and orientation of putative KlRgt1 binding sites (5′-CGGANNA-3′) are indicated by solid arrows. The location of the H51 and H52 primers used to generate *pRAG1* DNA used for the ChIP and IDBA experiments are indicated by shaded arrows. (B) The association of KlRgt1 with the *RAG1* promoter was determined by ChIP experiments. Chromatin from cells expressing KlRgt1-3HA (MWK7/F) grown on media containing either 5% glucose (Glu) or 2% glycerol (Gly) was immunoprecipitated with anti-HA antibodies. Immunoprecipitated DNA (IP) and input DNA (IN) were amplified by PCR using the *pRAG1* primer set and an actin promoter (*pACT*) primer set as a negative control. PCR products were then analyzed by electrophoresis in 1.5% agarose gel. (C) *In vitro* DNA-binding activity of KlRgt1 was analyzed by IDBA. KlRgt1-3HA was immunoprecipitated as in B and incubated with ^{32}P-radiolabeled *pRAG1* or *pACT* DNA (both promoters were amplified using the same primer sets as above). The bound DNA was resolved in 6% polyacrylamide gel and visualized by autoradiography. KlRgt1 was analyzed by Western blotting using anti-HA antibodies (KlRgt1-3HA). A control (0) experiment was also carried out with cells containing an untagged KlRgt1 protein.

that KlRgt1-3HA was associated with the *RAG1* promoter in cells grown both in glycerol-containing medium and in glucose-containing medium (Figure 5B). We also used an *in vitro* DNA-binding assay, IDBA, to further determine the KlRgt1 DNA-binding activity (KIM 2004). In this experiment, the immunoprecipitated KlRgt1-3HA was used as an affinity matrix to retain radiolabeled promoter sequences. We found that KlRgt1 can bind to sequences upstream from *RAG1* in the absence of glucose and that this binding capacity remained in the presence of glucose (Figure 5C). The apparent weaker binding of KlRgt1 to the *RAG1* promoter in the presence of glucose could be explained by a weaker level of immunoprecipitated KlRgt1 (Figure 5C). However, we cannot exclude the existence of a modification of the affinity of KlRgt1 for the *RAG1* promoter in response to glucose. Nevertheless, in *K. lactis*, the derepression of *RAG1* gene expression is not due to the complete removal of the repressor protein from its promoter.

KlRgt1 is phosphorylated in response to glucose: As KlRgt1 binds to the *RAG1* promoter whatever the carbon source (glycerol or glucose) tested, we investigated whether it undergoes a post-translational modification in response to glucose. We found that KlRgt1-3HA from cells grown in the presence of glucose had a lower mobility in SDS–PAGE than KlRgt1-3HA from cells grown in the presence of glycerol (Figure 6A). This showed that KlRgt1 is modified in the presence of glucose. We tested whether phosphorylation caused the change in KlRgt1-3HA mobility in response to glucose by incubating with phosphatase (CIP) extracts from KlRgt1-3HA-containing cells grown in the presence of glucose. We found that phosphatase treatment increased the mobility of the protein (Figure 6B). This shows that glucose induces the phosphorylation of KlRgt1 and is consistent with the idea that glucose-induced phosphorylation inhibits the repressor function of KlRgt1. This glucose-induced phosphorylation of KlRgt1-3HA did not occur in Δ*rag4* mutant cells grown with high levels of glucose (Figure 6A). As *rag4* mutants, affected in the glucose sensor, exhibit a constitutive repression of *RAG1* gene expression, this suggests that the Rag4-dependent glucose pathway abolishes KlRgt1 repressor function through its phosphorylation.

Involvement of Rag8 casein kinase 1 in the glucose-signaling pathway: We have previously shown that the Rag8 casein kinase 1 positively regulates *RAG1* gene transcription (BLAISONNEAU *et al.* 1997). Therefore, we investigated whether it may be a component of the glucose-signaling pathway regulating the KlRgt1 repressor. Therefore, we constructed a *rag8* Δ*Klrgt1* double mutant. We found that the loss of KlRgt1 in the *rag8* mutant strain suppressed its Rag$^-$ phenotype (Figure 7A) and Northern blot analysis showed that *RAG1* expression was restored when the *rag8* Δ*Klrgt1* mutant was grown on glucose (Figure 7B). We also observed no KlRgt1 phosphorylation in response to glucose in *rag8* cells (Figure 7C). Thus, membrane-bound casein kinase Rag8 appears to be a component of the Rag4-dependent glucose-induction pathway acting upstream from KlRgt1.

This was further supported by showing that the Rag8 casein kinase interacts with the Rag4 glucose sensor. We tested for such an interaction by using the mating-based split ubiquitin system (mb SUS) developed to test interactions between membrane-associated proteins (OBRDLIK *et al.* 2004). In this system, the interaction between "prey" and "bait" can be detected through the reconstitution of a functional ubiquitin that leads to the release of an artificial transcription factor, PLV (A-LexA-VP16), that can activate the lexA-driven reporter genes *HIS3*, *ADE2*, and *lacZ*. In addition, one of the fusion

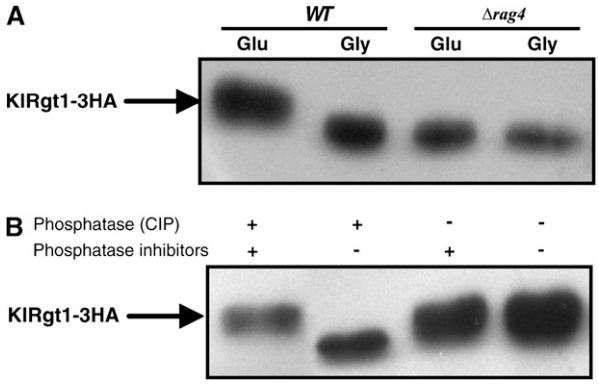

FIGURE 6.—KlRgt1 was phosphorylated in a Rag-4-dependent manner in response to glucose. (A) Cells transformed with the KlRgt1-3HA construct (MWK7/F, *WT* and MWK9, Δ*rag4*) were pregrown on 2% glycerol and the transferred to 5% glucose or 2% glycerol for 6 hr. After cell lysis, the protein extracts were separated in 6% SDS–PAGE and analyzed by Western blotting. (B) Phosphatase treatment of cell extracts from *WT* cells grown in the presence of glucose. KlRgt1-3HA was immunoprecipitated as in Figure 6 and incubated with (+) or without (−) 10 units of CIP in the presence (+) or absence (−) of phosphatase inhibitors at 37° for 30 min and then analyzed by Western blotting.

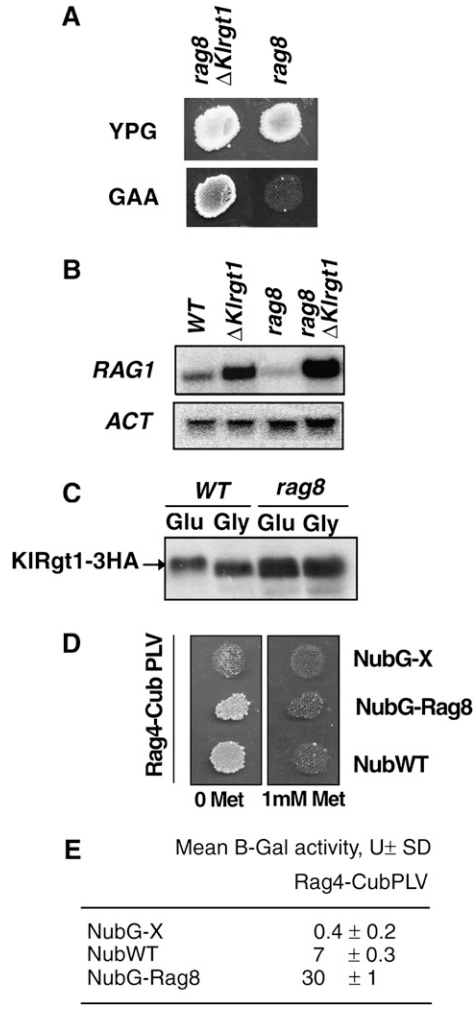

FIGURE 7.—Involvement of Rag8 casein kinase 1 in the glucose-signaling pathway. (A) The *rag8* mutation was suppressed by the *Klrgt1* mutation. The Rag phenotype of *rag8* (MW159-1C) and *rag8 Klrgt1* cells (MWK21) was determined as described in the legend of Figure 1. (B) *RAG1* expression was restored in a *rag8 Klrgt1* double mutant. Northern blot analysis of *RAG1* mRNA in cells grown on 2% glucose medium was carried out as indicated in Figure 2; an actin gene transcript (*ACT*) was used as an internal control. *WT* (MW270-7B); *Klrgt1* (MWK7); *rag8* (MW159-1C); *rag8 Klrgt1* cells (MWK21). (C) The *rag8* mutant was defective for KlRgt1 phosphorylation in response to glucose. Western blot analysis of the KlRgt1 modification in *WT* (MW270-7B) and *rag8* (MW159-1C) cells was carried out as described for Figure 6. (D) Interaction of Rag4-CubPLV with the NubG-Rag8 fusion protein. Replica plates showing diploid cells selected on minimal media with and without 1 mM methionine (Met) after 3 days incubation. NubWT and NubG are positive and negative controls, respectively. (E) Liquid β-galactosidase assay. Results are expressed in Miller units (U) and are the average of assays carried out in triplicate.

protein-encoding genes is under the control of *MET25*-regulated promoter, which can be repressed by methionine. We constructed *pMET25-RAG4-CubPLV* and *pADH1-NubG-RAG8* (MATERIALS AND METHODS). Diploids harboring Rag4 and Rag8 fusion proteins grew on adenine/histidine-free medium in the absence of methionine (Figure 7D), indicating a physical interaction between Rag8 and Rag4 proteins. We confirmed this interaction by β-galactosidase assays, which indicate the level of activation of the *lacZ* reporter gene (Figure 7E). Therefore, Rag4 (the *K. lactis* glucose sensor) and Rag8 casein kinase 1 physically interact, as previously reported for the glucose sensor Rgt2 and the casein kinase 1 Yck1 of *S. cerevisiae* (MORIYA and JOHNSTON 2004).

KlRgt1 controls hexokinase gene expression: Rag8 casein kinase, which is involved in *RAG1* expression regulation, is also required for the maximal expression of glycolytic genes (LEMAIRE *et al.* 2002) (our unpublished results). Therefore, the Rag$^-$ phenotype of *rag8* mutants is due to defects in both glucose transport and glycolysis. As the Rag$^-$ phenotype of *rag8* mutant is suppressed by *Klrgt1* mutation, we tested whether KlRgt1 is also involved in glycolytic gene expression. We measured the transcription of hexokinase- and enolase-encoding genes (*RAG5* and *RAG17*, respectively) in *WT* and *Klrgt1* cells grown in the absence or presence of 2% glucose (Figure 8A). As previously reported, the expression of both genes is induced by glucose (NEIL *et al.* 2004). The level of transcription of *RAG5* was clearly increased in the absence of glucose in *Klrgt1* cells, whereas that of *RAG17* was affected to a lesser extent. Therefore, we tested whether KlRgt1 could interact with the promoter sequence of the two genes. The regions of the *RAG5* gene promoter (−1052 to −640 bp) and the *RAG17* gene promoter (−933 to −512 bp) used as templates for PCR amplification contain three and one putative KlRgt1 binding sites, respectively (not shown). We found that KlRgt1 interacts with the *RAG5*, whereas no ChIP amplification of *RAG17* promoter sequence

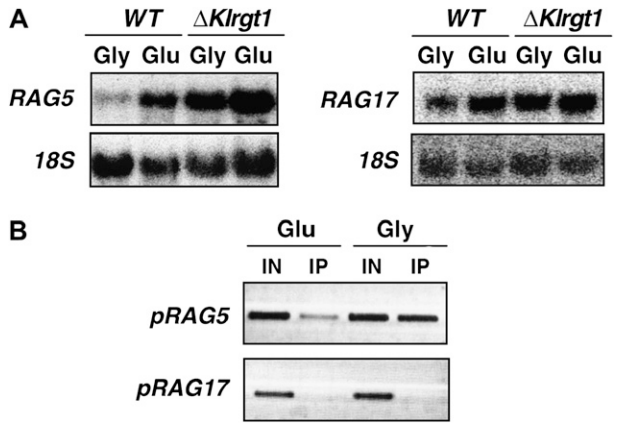

FIGURE 8.—Effect of KlRgt1 on the expression of glycolytic genes. (A) Northern blot analysis of *RAG5* (hexokinase encoding gene) and *RAG17* (enolase encoding gene) transcription in a *Klrgt1* mutant *vs.* wild-type cells. Yeast cell growth conditions and electrophoresis conditions were as indicated in Figure 2. *WT* (MW270-7B), *Klrgt1* (MWK7). (B) The interaction of KlRgt1 with the *pRAG5* and *pRAG17* promoters was analyzed by ChIP assay as for Figure 6. *pRAG5* and *pRAG17* DNA were amplified using sets of primers described in MATERIALS AND METHODS.

was detected (Figure 8B). These results clearly show that KlRgt1 represses hexokinase gene expression in the absence of glucose. Under the same conditions enolase gene appears indirectly affected by the repressor. However, we cannot exclude that the slight effect seen on *RAG17* expression in the *Klrgt1* mutant (Figure 8A) is due to the binding of KlRgt1 on its single site present in the *RAG17* promoter (but not detected in our ChIP experiments). As seen for the *RAG1* gene promoter, KlRgt1 binds to the *RAG5* promoter in both the absence and the presence of glucose. In this case the binding of KlRgt1 to DNA in the presence of glucose appears to be different than in the presence of glycerol (Figure 8B). This indicates that KlRgt1 gene regulation could occur through a differential binding affinity, modulated by its phosphorylation status.

DISCUSSION

We show that the Rag$^-$ phenotype and the transcriptional defect, seen in cells grown on glucose, of the glucose permease gene *RAG1* caused by a *rag4* null mutation can be suppressed by recessive mutations in the *KlRGT1* gene. We also show that *RAG1* is expressed at a high level in the absence of glucose in both the revertant and the *Klrgt1* mutant, demonstrating that *KlRGT1* encodes a negative regulator of the *RAG1* gene acting in the absence of glucose.

Amino acid sequence analysis of the *KlRGT1* gene product revealed a good match with the *S. cerevisiae* glucose transporter gene regulator ScRgt1 (OZCAN *et al.* 1996). In addition to a well-conserved DNA-binding domain consisting of a Zn_2-Cys6 cluster, there are several high-identity blocks in the two proteins. However, not all of these conserved regions correspond to functional domains identified in the ScRgt1 transcription factor of *S. cerevisiae* (POLISH *et al.* 2005). The loss-of-function mutation *Klrgt1-6* lies in a region (at the end of the C-terminal region of the protein) that escaped the functional analysis of ScRgt1 (POLISH *et al.* 2005). Also, because the *K. lactis* protein is shorter, there are several amino acid regions present in ScRgt1 that are missing in KlRgt1. These differences may explain the functional discrepancies observed between them (see below).

In the absence of glucose, ScRgt1 from *S. cerevisiae* binds to the *HXT* gene promoters and represses their transcription (OZCAN *et al.* 1996; FLICK *et al.* 2003; KIM *et al.* 2003; MOSLEY *et al.* 2003; POLISH *et al.* 2005). Some reports also suggest that in the presence of glucose, ScRgt1 behaves as a transcriptional activator of the *HXT* genes (OZCAN and JOHNSTON 1995; OZCAN *et al.* 1996; POLISH *et al.* 2005). Our data suggest that in *K. lactis*, KlRgt1 represses *RAG1* transcription only in the absence of glucose. This is supported, first, by the lack of Rag$^-$ phenotype of a *Klrgt1* mutant. Indeed, the disruption of an activator of *RAG1* gene is expected to generate the Rag$^-$ phenotype. For instance, even a twofold reduction of *RAG1* transcription in *sck1* mutant leads to a Rag$^-$ phenotype (LEMAIRE *et al.* 2002). Second, Northern blot analysis showed that *RAG1* transcription in the *Klrgt1* mutant grown on glucose was higher than that in wild-type cells. This last result also indicates that glucose induction of *RAG1* gene expression results mostly from inactivation of KlRgt1. Nevertheless, *RAG1* transcription was found to be still slightly glucose inducible. Therefore, additional activation mechanism of the *RAG1* gene in response to glucose remains to be determined.

We tested the ability of KlRgt1 to bind to the *RAG1* promoter *in vivo* and *in vitro* using ChIP and IDBA assays, respectively. We found that KlRgt1 binds continually to the *RAG1* promoter, even when *RAG1* is derepressed in the presence of glucose. This is unlike what is observed in *S. cerevisiae*, in which ScRgt1 dissociates from the *HXT* promoters in the presence of glucose. Under these conditions, glucose regulates the DNA-binding ability of ScRgt1 by inducing its phosphorylation (FLICK *et al.* 2003; KIM *et al.* 2003; MOSLEY *et al.* 2003). The difference between the two yeasts may be due to the absence in KlRgt1 of the amino acids 750–760 of ScRgt1. When this region is deleted, ScRgt1 constitutively binds DNA (POLISH *et al.* 2005). Consistent with this, we found that glucose regulates the repressor activity of KlRgt1 through post-translational modification: phosphorylated KlRgt1 does not repress *RAG1* expression but can still bind to the *RAG1* promoter. Furthermore, *rag4* mutant cells, in which KlRgt1 always functions as a transcriptional repressor independent of the presence of glucose, show no glucose-induced phosphorylation of KlRgt1. This suggests that the

Rag4-dependent-sensing pathway inhibits KlRgt1 transcriptional repression through the phosphorylation of KlRgt1. A similar situation has recently been reported for the yeast Mig1 repressor, which always binds to repressed promoters under both repression and activation conditions (Papamichos-Chronakis *et al.* 2004). However, as shown by the IDBA and ChIP assay performed with the promoter of *RAG1* and *RAG5*, respectively, we cannot exclude that its binding affinity can be modified, even if KlRgt1 is present on the *RAG1* promoter in the presence of glucose. In this case, KlRgt1 could either bind to its sites with a lower affinity or interact with different sites, thus allowing the binding of some activator protein to the promoter region.

Glycolytic genes, like the glucose transporter gene *RAG1*, are glucose inducible. Therefore, we tested whether their regulation involved the KlRgt1 repressor. Using hexokinase (*RAG5*)- and enolase (*KlENO/RAG17*)-encoding genes, we showed that only the hexokinase gene is repressed by the KlRgt1 repressor in the absence of glucose. This result indicates that yeast cells carefully regulate glucose use by tightly coregulating the first steps of glucose metabolism (its transport across the plasma membrane and its phosphorylation by hexokinase), glucose permease and hexokinase gene expression being repressed in the absence of glucose and induced in its presence. This finding could also be related to regulatory function of hexokinase, which is known to be involved in glucose induction of *RAG1* expression (Chen *et al.* 1992; Prior *et al.* 1993; Lemaire and Wesolowski-Louvel 2004). We previously showed that hexokinase together with other glycolytic enzymes (enolase and phosphoglycerate kinase) participate in a pathway responding to an intracellular signal generated by glycolysis to regulate *RAG1* expression (Lemaire and Wesolowski-Louvel 2004). As the first element of the intracellular glucose-sensing pathway, the hexokinase gene *RAG5* is regulated by KlRgt1; this indicates that the extracellular glucose-sensing pathway would trigger off the intracellular pathway. Therefore, in response to glucose, KlRgt1 will have a double control on glucose uptake by directly regulating the *RAG1* promoter and by controlling the intracellular signal mediated by glycolysis. Despite the fact that we found that the hexokinase gene *RAG5* itself is a target of KlRgt1, we cannot exclude its direct participation in the extracellular glucose-sensing pathway to regulate *RAG1*. These two possibilities are not exclusive. It should be noted that in *S. cerevisiae*, ScRgt1 has been shown to control the hexokinase gene *HXK2*, although in this case a connection to *HXT* gene regulation has not been established (Palomino *et al.* 2005; Palomino *et al.* 2006).

The regulation of ScRgt1 in *S. cerevisiae* also involves the paralogous proteins Mth1 and Std1, known to be negative regulators of *HXT* gene expression (Flick *et al.* 2003; Lakshmanan *et al.* 2003; Mosley *et al.* 2003). Mth1/Std1, which have been shown to interact with the Snf3 and Rgt2 glucose sensors (Schmidt *et al.* 1999; Lafuente *et al.* 2000), Yck 1 casein kinase 1 (Moriya and Johnston 2004), and the repressor ScRgt1 (Tomas-Cobos and Sanz 2002; Lakshmanan *et al.* 2003) are good candidates for relaying the glucose signal from the plasma membrane to the nucleus. The *K. lactis* genome (http://cbi.labri.fr/Genolevures/) contains a single orthologous gene for the *MTH1/STD1* paralogs.

Finally, the glucose-sensing and -signaling pathway appears to be well conserved between *S. cerevisiae* and *K. lactis*, although there are some important differences in the regulation of repressor function of KlRgt1 *vs.* ScRgt1. The most important difference is that KlRgt1 does not depart from the *RAG1* and *RAG5* promoters during growth in glucose to enable transcription. As a consequence the system would be less easily derepressed than in *S. cerevisiae* and is even not totally derepressed in the presence of high concentrations of glucose. This is especially shown by the higher level of *RAG1* transcription in the Δ*Klrgt1* mutant than in the wild type. This mechanism might provide a rapid reestablishment of repression under glucose limitation. In addition, *RAG1* induction seems to be complicated by the involvement of an additional activator that is not KlRgt1 itself, as in the case of ScRgt1 in *S. cerevisiae*.

Therefore, *RAG1* expression is tightly repressed in the absence or in the presence of low glucose. This regulation might reflect an adaptation of *K. lactis* to its natural environment, which is normally poor in glucose. Under these conditions, *K. lactis* metabolism would rely on respiration and the high-affinity glucose permease Hgt1. The presence of the regulated high-capacity glucose permease gene *RAG1* would allow *K. lactis* to adapt and to compete in a high-glucose environment. In this case the cells would be able to utilize glucose by both fermentation and oxidation, since respiration is not glucose repressed.

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