Characterization of KlGRR1 and SMS1 Genes, Two New Elements of the Glucose Signaling Pathway of Kluyveromyces lactis

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Received 17 December 2007/Accepted 22 May 2008

The expression of the major glucose transporter gene, RAG1, is induced by glucose in Kluyveromyces lactis. This regulation involves several pathways, including one that is similar to Snf3/Rgt2-ScRgt1 in Saccharomyces cerevisiae. We have identified two new yeast genes, SMS1 and SMS2, which encode a protein showing an average of 50% identity with Mth1 and Std1, regulators of the ScRgt1 repressor. The expression of SMS1 (casein kinase I) is regulated by glucose, both at the transcriptional and the posttranslational level. Two-hybrid interactions of Sms1 with the glucose sensor and KlRgt1 repressor suggest that Sms1 mediates the glucose signal from the plasma membrane to the nucleus. All of these data demonstrated that Sms1 was the Kl. lactis homolog of MTH1 and STD1 of S. cerevisiae. Interestingly, MTH1 and STD1 were unable to complement the Δsms1 mutation.

The availability of glucose in the extracellular environment leads to the transcriptional regulation of many genes, enabling cells to adapt their metabolism to the variation of its concentration. This glucose response, which requires glucose-sensing systems and signal transduction pathways, is a complex process and has been extensively studied in the fermentative yeast Saccharomyces cerevisiae (for a review, see reference 34). In this yeast, a subset of the many genes induced by glucose is a family of hexose transporters encoded by the HXT gene, but they remain to be identified (28).

In the last decade, the comprehension of yeast biology has been enriched by the genomic exploration of other hemiascomycetes yeasts, like Kluyveromyces lactis (6). One of major differences between K. lactis and S. cerevisiae is the aerobic fermentative-respiratory metabolism of K. lactis, a lifestyle more typical of eukaryotic organisms than the S. cerevisiae fermentative metabolism. K. lactis is evolutionarily close to S. cerevisiae, despite the latter having diverged after a whole-genome duplication (WGD) event (45). As a consequence, in contrast to the gene redundancy in S. cerevisiae, K. lactis has a simplified glucose uptake system that relies on the proteins encoded by two genes, HGT1 and RAG1. HGT1 encodes a constitutive high-affinity glucose permease (2). RAG1 encodes a low-affinity permease, which is activated in the presence of high concentrations of glucose (5, 43). The Rag1 permease is necessary for supporting fermentative growth, which requires a high flow of substrate. In the absence of Rag1, the cell becomes respiration dependent for growth on high-glucose media. Thus, rag1 mutants are unable to grow on 5% glucose when respiration is blocked by antitrycin A (Rag− phenotype) (9, 44). Many Rag− mutants defective in the key genes of the fermentation pathway have been isolated (9, 44), and their study has iden-
tified genes coding for proteins that positively control RAG1 expression. In fact, as in S. cerevisiae, several pathways seems to be involved in RAG1 regulation (Fig. 1) (20, 25, 33). One pathway is similar to the Rgt2-Yck-ScRgt1 pathway in S. cerevisiae, which controls HXT1 expression (23, 33). In K. lactis, extracellular glucose causes the Rag4 sensor (1), in cooperation with the Rag8 casein kinase I (3), to generate an intracellular signal inducing the expression of the RAG1 gene. The glucose signal ultimately inhibits the function of KlRgt1, a transcriptional repressor of RAG1 (33). A main difference between the two yeast systems is that KlRgt1 acts solely as a repressor (33), while ScRgt1 is thought to be turned into an activator in the presence of glucose (29).

Our studies of the K. lactis system allowed us to show that a transcriptional activator of the Myc family, encoded by the SCK1 (RAG16) gene, couples glucose transport and glycolysis by participating in the glucose induction of RAG1 and glycolytic genes through an interconnected pathway (19, 25). Indeed, Rag8 casein kinase I controls this transcriptional factor at the transcriptional and posttranslational levels (25). Interestingly, we also demonstrated that enolase and a functional glycolysis also are required for the glucose induction of the RAG1 gene (20), suggesting the existence of a metabolic intracellular glucose signaling in addition to the Rag4 pathway, which is solely dependent on extracellular glucose.

There are several pieces of evidence that suggest that glucose signaling pathways are conserved in both S. cerevisiae and K. lactis. In particular, it has been shown that RAG1 and HXT1 have similar expression profiles (5, 28) and that the expression of RAG1 is correctly induced in response to glucose when it is introduced in S. cerevisiae (27). But the involvement of an Sck1-Rag8-like pathway in HXT1 regulation has not been reported yet for S. cerevisiae, despite the presence of the corresponding functional homologs (3, 19). In return, the orthologs of the S. cerevisiae GPR1/GPA2 genes are present in the K. lactis genome, but their participation in RAG1 regulation is not known. Thus, K. lactis appears to be an alternative and complementary model to study yeast glucose responses. Nevertheless, to study a whole system in detail and to be able to estab-

**FIG. 1.** Comparison of glucose signaling pathways in S. cerevisiae and K. lactis. A simplified model of the Snf3/Rgt2/Rag4-dependent pathway in the two yeasts is presented. Phosphorylation events on Std1/Mth1, Rgt1, and Sck1 are indicated (P). The Gpr1/Gpa2-dependent signaling pathway, known to participate in ScRgt1 regulation (phosphorylation) in S. cerevisiae, is not represented.

**TABLE 1.** Known factors of the glucose signaling pathway and their corresponding functions

| Function          | Factor in:       | S. cerevisiae | K. lactis | Reference or source |
|-------------------|------------------|---------------|-----------|---------------------|
| Glucose permease  | Hxt1, Hxt2, Hxt3 | Rag1          |          | 27, 44              |
| Glucose sensor    | Snf3, Rgt2       | Rag4          |          | 1, 27               |
| Casein kinase I   | Yck1, Yck2       | Rag8          | 3, 23     |                     |
| F-box protein     | Grr1             | Rag19         | 21, this study |                     |
| Corepressor       | Mth1, Std1       | Sms1          | 35, this study |                     |
| Repressor         | ScRgt1           | KlRgt1        | 29, 33    |                     |

* The expression of glucose permease is controlled by glucose and Rgt1.
lish the connections between the various pathways, it is necessary to identify all the participating elements. Compared to S. cerevisiae, several factors of the glucose sensor signaling pathway still were missing from the K. lactis model (Fig. 1). In this study, we identified missing key components in the Rag4-dependent signaling pathway of K. lactis: RAG19, coding for the homolog of the F-box protein Grr1 of S. cerevisiae, and SMS1, which codes for a Klrg1 corepressor of RAG1.

**MATERIALS AND METHODS**

**Yeast strains and growth conditions.** The yeast strains used in this study are listed in Table 2. Yeast cells were grown at 28°C in a complete yeast peptone medium containing 1% Bacto yeast extract, 1% Bactopeptone (YP; Difco, Detroit, MI), 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 (YPG plates were supplemented with Geneticin (200 μg/ml; Life Technologies). For the G418 medium, YPG plates were supplemented with (Difco) and 2% glucose with auxotrophic supplements as required. The G418 medium was also introduced into the URA3-Klort-CEN-based vector pCXJ18 by inserting a 4.9-kb KpnI-Sall fragment of pML217, resulting in the pMH15 plasmid. SMS1-3HA was introduced into the LEU2-Klort-CEN-based

**TABLE 2. Strains used in this study**

| Strain              | Relevant genotype | Source or reference |
|---------------------|-------------------|---------------------|
| MW270-7B            | MATa ura1-1 leu2 met15-1 | This study         |
| PM6-7A              | MATa ura1-1 ade6-T600 | This study         |
| PM6-7A/VV30         | MATa ura1-1 ade6-T600 rag8-1 | This study         |
| PM6-7A/VV32         | MATa ura1-1 ade6-T600 rag4-1 | This study         |
| PM6-7A/VV60         | MATa ura1-1 ade6-T600 rag19-1 | This study         |
| MWK6                | Isogenic to MW270-7B, except rag4Δ2::LEU2 | This study         |
| MWK7                | Isogenic to MW270-7B, except Klrg1Δ1::URA3 | This study         |
| MWK7/F              | Isogenic to MW270-7B, except Klrg1Δ1::ura3 | This study         |
| MWK9/F              | Isogenic to MW270-7B, except Klrg1Δ1::ura3 rag4Δ2::LEU2 | This study         |
| MWK18               | Isogenic to MW270-7B, except rag19Δ1::URA3 | This study         |
| MLK54               | Isogenic to MW270-7B, except sms1Δ1::kanMX4 | This study         |
| MLK56               | Isogenic to PM6-7A, except sms1Δ1::kanMX4 | This study         |
| MW355-2D            | MATa ura1-1 ade2-1 rag19-4 | This study         |
| MW358-4A            | MATa ura1-1 leu2 met1-1 ragΔ2::LEU2 | This study         |
| MW383-9B            | MATa lys1-1 rag8-5 sms1Δ1::kanMX4 | This study         |
| MW384-2B            | MATa ura1-1 leu2 met1-1 rag19-1 sms1Δ1::kanMX4 | This study         |
| MW385-2B            | MATa ura1-1 leu2 met1-1 ragΔ2::LEU2  sms1Δ1::kanMX4 | This study         |
| **S. cerevisiae**   |                    |                     |
| THY-AP4             | MATa ura3 leu2 lexA::lacZ::trp1 lexA::HI3 lexA::ADE2 | 26               |
| THY-AP5             | MATa URA3 leu2 trp1 his3 lexA::ade2 | 26               |
| W303-1B             | MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 | 42               |
| BY4741              | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 | Euroscarf         |
| Y16902              | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 Δgrr1::kanMX4 | Euroscarf         |
| EGY48               | MATa ura3 trp1 his3 leu2::pLexAOp1-LEU2 + pSH18-34 (pLexAOp-lacZ) | 40               |

**TABLE 3. Plasmids used in this study**

| Plasmid | Description | Source or reference |
|---------|-------------|---------------------|
| pCXJ18  | K. lactis URA3 low-copy vector | 4          |
| pCXJ20  | K. lactis LEU2 low-copy vector | 4          |
| pCXJ12  | K. lactis URA3 multicopy vector | 4          |
| pCXJ24  | K. lactis URA3 multicopy vector | 32         |
| KCP491  | K. lactis URA3 low-copy vector | 32         |
| pRS416  | S. cerevisiae URA3 low-copy vector | 36         |
| pGD60   | KCP491 carrying RAG19 | This study         |
| pGD160  | pRS416 carrying RAG19 | This study         |
| pMH12   | pCXJ20 carrying SMS1-3HA | This study         |
| pMH15   | pCXJ18 carrying SMS1-3HA | This study         |
| pML214  | pCXJ22 carrying SMS1 | This study         |
| pML217  | pCXJ22 carrying SMS1-3HA | This study         |
| pML237  | pCXJ22 carrying MTH1 | This study         |
| pML238  | pCXJ22 carrying STD1 | This study         |
| pML258  | pCXJ22 carrying ScrgT1-3HA | This study         |
| pML259  | pCXJ22 carrying KlrtG71-3HA | This study         |
| pJG4-5  | S. cerevisiae TRP1 vector carrying B42 fusions from pGAL1 | 48         |
| pLex202 | S. cerevisiae HIS3 vector carrying LexA fusions from pADH1 | 48         |
| pMetYCgate | S. cerevisiae LEU2 vector carrying Cub-PLV fusions from pMET25 | 26         |
| pNXgate | S. cerevisiae TRP1 vector expressing NubG fusions | 26         |
vector pCXJ20, resulting in the pMH12 plasmid; a 4.9-kb Splh-KpnI fragment that had been made blunt using T4 DNA polymerase was inserted into the SmaI site of the vector.

SeMTH1, ScSTD1, and ScRGT1 were amplified by PCR using genomic DNA of the W303-1B strain (Table 2). The 2,814-bp (SeMTH1) and 2,847-bp (ScSTD1) PCR fragments, flanked by BamHI and SalI sites, were inserted in pCXJ22 that had been digested with BamHI and SalI, resulting in pML237 and pML238, respectively. The 5,025-bp PCR fragment containing ScRGT1 was cloned in the same way in pCXJ24, yielding pML258.

RNA preparation and Northern blot analysis. Total RNA extraction and Northern blotting conditions were performed as previously reported (25, 33). Primers used to obtain RGG1, RAG5, and K4ACT probes have been described previously (20, 33). SMS1 primers are available upon request.

Yeast cell extracts and immunoblotting. For Western blot analysis, cells containing the appropriate plasmid were grown to mid-exponential phase in selective medium, harvested, and washed with ice-cold water. Protein extracts were prepared according to Yaffe and Schatz (47). Immunodetection conditions were as previously described (33).

ChIP analysis. Chromatin immunoprecipitation (ChIP) experiments were carried out as previously reported (33). The primers used to amplify the RAG1 and K4ACT promoter sequences were those previously used (33).

Yeast two-hybrid analysis. The S. cerevisiae EGY48 strain (Table 2) was cotransformed with pIG4-5 (Table 3), expressing the entire SMS1, STD1, or MTH1 ORF fused to the B42-activating domain from the GAL1 promoter, and pLex202-RGT1, expressing the entire K4RT1 ORF fused to the LexA DNA binding domain. The transformed yeast cells were grown on glucose selective medium lacking histidine and tryptophan. LEU2 and lacZ reporter gene expression was assessed on galactose-selective plates, either without leucine or medium lacking histidine and tryptophan.

RESULTS

**K. lactis RAG19 is a functional homolog of the S. cerevisiae GRR1 gene.** In our laboratory rag mutant collection, the rag19-1 mutant showed an impaired transcription of the RAG1 gene when cells were grown in the presence of glucose (Fig. 2A). This suggested that the RAG1 gene product was a positive regulator of RAG1 expression. RAG19 has been cloned by the in vivo complementation of the rag19-1 mutation (Fig. 2B), as described in Materials and Methods. We constructed a Δrag19 null mutant that displayed a Rag phenotype as the original mutant. The absence of the complementation of the rag19 null mutation (strain MWK18) by the rag19-1 mutation (strain MW355-2D) in a diploid constructed by crossing the two mutants demonstrated that the cloned gene indeed corresponded to the Rag19 locus.

The deduced Rag19 protein is 1,239 amino acids long and has significant identity with several members of the F-box proteins of the SCF ubiquitination complexes. The highest identity (43%) is shared with Grr1 of *S. cerevisiae* (7), which defines the SCF<sup>Grr1</sup> complex, which targets a subset of proteins for ubiquitin-mediated proteolysis via proteasome (37). A protein alignment of Grr1 with Rag19 indicates that the latter protein is related to the former through a conserved domain of 30 amino acids. The identity (43%) is shared with Grr1 of *S. cerevisiae* (7, 21). In that case, glucose derepresses HXT gene expression via a cascade of events that are dependent upon Grr1, leading to the inhibition of the ScRgt1 repressor function (29, 31). Pleiotropic defects are associated with *grr1* mutation, which is consistent with the diversity of the pathways in which Grr1 is involved. Thus, in addition to impaired growth on glucose, *grr1*
mutants have abnormally elongated cell morphology (7). A similar situation also was observed in the K. lactis rag19-1 mutant, which displayed severe changes in cell morphology as well as the Rag phenotype (Fig. 2B).

We tested whether K. lactis RAG19 complements the S. cerevisiae grr1 mutation. Strain Y16902 (Table 2) was transformed with plasmid pGD160 (Table 3), a low-copy plasmid containing RAG19. The results showed that RAG19 complements the growth defect on glucose (Fig. 2D) and the cell morphology defect of the grr1 null mutant (data not shown).

Taken together, these data demonstrate that K. lactis RAG19, termed KIRGRI, is a functional homolog of S. cerevisiae GRR1. This also shows that key components of the glucose signaling pathway regulating ScRgt1 repressor function are conserved between S. cerevisiae and K. lactis. We also found that the expression of the hexokinase-encoding gene RAG5 was impaired in the rag19-1 mutant (Fig. 2A); this reinforced our previous data that showed the hexokinase gene is a target for KIRGRI (33).

Characterization of K. lactis SMS1. In S. cerevisiae, the regulation of ScRgt1 also involves the paralogous proteins Mth1 and Std1, known to be negative regulators of HXT gene expression and proposed to be targets of SCFGT1 (8, 13, 18, 24). Therefore, we searched the K. lactis genome (http://cbi.labri.fr/Genolevures) for the MTH1 and STD1 paralogs and found a single gene, the KLLA0F15928g ORF (EMBL accession number IGI00226036), which is a syntenic homolog to STD1. This gene product (457 amino acids long) shares 52 and 48% identity with Mth1 and Std1, respectively. Thus, the gene was named SMS1 (for similar to Mth1 and Std1).

We tested whether K. lactis Sms1 also is required in the repression of RAG1 and RAG5 gene transcription by KIRGRI (33), using the null mutant Δsms1 that we had constructed. Northern blot analysis (Fig. 3A) showed that, as in the case of ΔKIRGRI, the transcription of both genes was derepressed in the Δsms1 mutant in the absence of glucose. This indicates that Sms1 acts as a repressor in the K. lactis glucose signaling pathway, as do Mth1 and Std1 in S. cerevisiae. This functional similarity prompted us to investigate whether the Δsms1 mutation could be complemented by the two S. cerevisiae genes. In fact, although they were expressed (data not shown), neither MTH1 nor STD1 individually, nor both together, were able to repress RAG1 transcription in Δsms1 cells grown in the absence of glucose (Fig. 3B and C). As ScRGT1 could complement a ΔKIRGRI mutation (our unpublished results), each gene (MTH1 or STD1) was coexpressed together with ScRGT1 in the Δsms1 strain (Fig. 3C). Again, in this case, the S. cerevisiae genes failed to repress the RAG1 expression in the absence of glucose.

Sms1 is involved in the glucose signaling pathway regulating the KIRGRI repressor. We further investigated the role of Sms1 in the glucose signaling pathway that regulates KIRGRI function through genetic and physical interactions with the other components of the pathway. Genetic crosses were performed between the Δsms1::kanMX4 mutant strain, MLK54 (Rag−), and rag4 (glucose sensor), rag8 (casein kinase I), and

FIG. 3. Sms1 controls RAG1/RAG5 genes via the RAG4-dependent signaling pathway. (A) Control of RAG1 and RAG5 transcription by Sms1. Total RNA was extracted from wild-type (WT) (MW270-7B), Δsms1 (MLK54), and ΔKIRGRI (MWK7) cells grown in YP medium containing either 2% glucose (+) or 2% glycerol (−). (B) MTH1 and STD1 genes fail to complement the Δsms1 mutation. Δsms1 (MLK54) cells were transformed either with pCXJ18, pMH15 (SMS1-3HA), or pCXJ22 expressing either MTH1 or STD1. Total RNA was extracted from transformants grown on a selective synthetic medium containing 2% glycerol. As MTH1 and STD1 genes were cloned using PCR amplification, two plasmids (a and b) were purified from independent E. coli transformants and used to transform Δsms1 cells. (C) Expression of ScRGT1 does not help STD1 and MTH1 genes to complement the Δsms1 mutation. The MLK54 (Δsms1) strain was transformed either with pCXJ18 or pMH15 (SMS1-3HA) or cotransformed with pML258 (carrying ScRGT1) and pML237/pML238 plasmids (carrying either MTH1 or STD1). Total RNA was extracted from transformants grown on a selective synthetic medium containing 2% glycerol. (D) RAG1 expression is restored in several Δsms1 rag double mutants. Total RNA was extracted from cells grown on YPG medium: WT (MW270-7B), Δsms1 (MLK54), Δrag4 (MWK6), Δsms1 Δrag4 (MW385-2B), rag8 (PM6-7A/VV30), Δsms1 rag8 (MW383-9B), rag19 (PM6-7A/VV60), and Δsms1 rag19 (MW384-2B). Transcripts were analyzed by Northern blotting using 32P-labeled RAG1-, RAG5-, and KIACT-specific probes (see Materials and Methods). KIACT was used as an internal standard. When necessary, the hybridization signals were quantified with a phosphorimager. Numbers indicate the ratio between RAG1 and KIACT transcript levels (RAG1/KIACT).
FIG. 4. Sms1/Std1/Mth1 physical interactions. (A) Two-hybrid interaction with KlRgt1. The interaction of the fusion proteins LexA-KlRgt1 and B42 fused to Sms1, Std1, or Mth1 coexpressed in strain EGY48 was tested through the activation of the reporter genes LEU2 and lacZ on glucose (glu) or galactose (gal) plates (see Materials and Methods). The colored panel shows a filter lift assay for β-galactosidase activity. (B) Two-hybrid interaction with Rag4. The interaction of Rag4-CubPLV with NubG fused to Sms1, Std1, or Mth1 was tested through the three reporter genes ADE2, HIS3, and lacZ. Diploid transformants were streaked on minimal medium supplemented with adenine and histidine (left) and replicated after growth on minimal medium (right). NubG is a negative control. The adjacent numbers are the averages of three to seven assays of liquid β-galactosidase activities (Miller units).

rag19 (KlGrr1) mutant strains (Rag−), strains in which RAG1 expression is affected by glucose (1, 3, 5). Meiosis analysis revealed that the loss of Sms1 suppressed the Rag− phenotype of the three mutants (data not shown). RAG1 expression that was analyzed in the Rag− double mutant spores showed that RAG1 transcript was restored to high levels when the double mutant cells were grown on 2% glucose (Fig. 3D). These data emphasize the negative role played by the S. cerevisiae SMS1 gene product in RAG1 expression and place it downstream of Rag4, Rag8, and KlGrr1 in the cascade.

In S. cerevisiae, Std1 and Mth1 interact with both the ScRgt1 repressor (18, 39) and the Smf3 and Rgt2 glucose sensors (17, 35). Therefore, we tested for similar protein interactions involving Sms1 in K. lactis. First, by using a yeast two-hybrid assay, we investigated Sms1 (fused to the B42 activation domain) interactions with KlRgt1 (fused to the LexA DNA binding domain) in the EGY48 strain (Table 2). The Sms1 interaction with KlRgt1 was performed by assaying the two reporter genes (LEU2 and lacZ) that are available in EGY48 (Fig. 4A). As can be seen, the Sms1-KlRgt1 interaction could be established only by the lacZ assay. In fact, the growth phenotype depending on the LEU2 reporter was delayed, because the high-level expression of the B42-Sms1 protein was toxic to the cells (not shown).

We then used the mating-based split-ubiquitin system, adapted to membrane proteins (26), to test the interaction between Sms1 and the Rag4 glucose sensor. Cub-PLV was fused to the C terminus of Rag4, and NubG was fused to the N terminus of Sms1 (see Materials and Methods). Physical interaction between Rag4 and Sms1 fusion proteins was assessed by the expression of the three reporter genes (ADE2, HIS3, and lacZ) of the system (Fig. 4B). The interactions of Sms1 with both the membrane-bound glucose sensor and the nuclear KlRgt1 repressor suggest that Sms1 participates in mediating the glucose signal from the membrane to the nucleus.

Mth1 and Std1 proteins interact with both the Rag4 glucose sensor and the KilRgt1 repressor. We made the assumption that the absence of the complementation of Δsms1 mutation by MTH1 and STD1 could be due to the failure of the S. cerevisiae proteins to interact with the K. lactis glucose sensor and/or repressor. To test for this, we employed the same two-hybrid assays used for Sms1 interactions. Surprisingly, Std1 and Mth1 were able to interact with Rag4 and KlRgt1 (Fig. 4). It should be noted that, in contrast to the B42-Sms1 protein, neither B42-Std1 nor B42-Mth1 fusion proteins were toxic to the cells (Fig. 4A).

Sms1 prevents KlRgt1 phosphorylation in the absence of glucose. The negative role played by Sms1 in the glucose regulation of RAG1, as well as its physical interaction with KlRgt1, suggests its participation in the repressor function of KlRgt1. We recently showed that KlRgt1 repression is abolished by phosphorylation in response to high glucose concentrations (33): phosphorylated KlRgt1 in glucose-grown cells displays, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, decreased electrophoretic mobility (Fig. 5A). Therefore, we determined whether Sms1 is involved in this glucose-mediated phosphorylation of KlRgt1. Western blot analysis showed that KlRgt1 was always phosphorylated in Δsms1 cells independently of the carbon source used (Fig. 5A). In contrast, in rag4 cells and in Klgrr1 cells, only the KlRgt1 slow-migrating hypo-
phosphorylated form was detected (Fig. 5A and data not shown). Thus, Sms1 and Rag4, like KlGrr1, have opposite effects on KlRgt1 phosphorylation.

Apparently, KlRgt1 does not completely dissociate from the RAG1 promoter subsequently to its phosphorylation (33), but phosphorylated KlRgt1 has a modified binding affinity to DNA (Fig. 5B). Therefore, we tested whether Sms1 could participate in the binding of KlRgt1 to DNA using ChiP. The results showed that, in Δsms1 cells grown in the absence of glucose, the binding of KlRgt1 to DNA was as weak as that in the wild-type cells grown on glucose (Fig. 5B).

Taken together, these data suggest that Sms1 and KlGrr1 participate in the Rag4 pathway that regulates KlRgt1 activity through its phosphorylation. The disruption of the interaction between Sms1 and KlRgt1 causes the constitutive phosphorylation of KlRgt1 and leads to its almost complete dissociation from the RAG1 promoter. Therefore, Sms1 and, presumably, its interaction with KlRgt1, is required for regulating KlRgt1 repressor function mainly by preventing its phosphorylation. This finding is in accord with data from S. cerevisiae showing that Mth1 inhibits the phosphorylation (inactivation) of ScRgt1 (29, 31).

KlRgt1 regulates SMS1 expression. We tested the potential effect of glucose on the regulation of SMS1. For this purpose, we determined the relative abundance of SMS1 mRNA. Although SMS1 mRNA was barely detectable in cells grown on glycerol, significantly greater amounts were detected in cells cultivated in glucose (Fig. 6A, panel 1). The repression of SMS1 gene expression in the absence of glucose (Fig. 6A, panel 1), together with the presence of seven putative KlRgt1 binding sequences (5′-CCGGANNA-3′), according to Kim et al. (15), in the SMS1 promoter (1.2 kb), prompted us to establish whether KlRgt1 regulates SMS1 transcription. We measured SMS1 mRNA amounts in Δklrgt1 cells grown in the absence or presence of 2% glucose (Fig. 6A, panel 1). SMS1 mRNA levels in mutant cells in the absence of glucose were significantly increased, indicating a negative control exerted by KlRgt1.

This result was confirmed by ChiP assays in Δklrgt1 cells carrying an episomal copy of the KlRGT1-3HA fusion (33) and grown in glycerol medium. Under these conditions, KlRgt1 was found to interact with the SMS1 promoter (Fig. 6A, panel 2), whereas no amplification was detected from the KlACT promoter or in the immunoprecipitation fraction of cells transformed with empty vector.

SMS1 gene product is regulated by glucose. All of the results presented above strongly suggest that Sms1 of K. lactis plays a role that is similar to that of Mth1 and Std1 of S. cerevisiae in the signal transduction pathway, i.e., components that are inhibited by the glucose signal. Thus, we examined whether glucose affects the Sms1 protein. We used an SMS1-3HA construct expressed from the low-copy-number pCXJ20 or pCXJ18 vector (see Materials and Methods). The fusion protein was functional, because RAG1 repression was restored in the Δsms1 cells transformed with the construct and grown in the absence of glucose (data not shown). Conversely to SMS1 expression (Fig. 6A, panel 1), Sms1 protein, which was detectable in glycerol-grown cells, was absent when cells were grown on glucose (Fig. 6B, panel 1). There are two possibilities: either SMS1 mRNA translation is blocked, or Sms1 protein is lost posttranslationally in glucose-grown cells. To test for this, we examined the effect of glucose on Sms1 protein in cells transferred from medium containing glycerol to medium containing glucose. Cells growing in glycerol medium were transferred to glucose medium, and samples were taken during a 60-min time course (Fig. 6B, panel 2). It appeared that Sms1 was rapidly depleted (after 1 min) from the cells transferred into glucose medium, suggesting a rapid degradation of the protein rather than a translational defect of SMS1 mRNA in glucose-grown cells. This rapid degradation of Sms1 would allow a prompt
induction of RAG1 transcription. We also found that Sms1 was
no longer degraded in the rag4, rag8, and rag19 cells grown in
glucose (Fig. 6B, panel 1). This indicated that Sms1 degrada-
tion requires the glucose signal delivered by the upstream
components of the cascade: the glucose sensor Rag4, the ca-
sein kinase Rag8, and KlGrr1.

We tested whether the glucose-induced expression of SMS1
allowed the replenishment of Sms1, and therefore the prompt
establishment of RAG1 repression, after glucose exhaustion.
Cells growing in glucose medium were transferred to glycerol
medium, and samples were taken over a 1-h time course (Fig.
6C). Indeed, the rapid synthesis of Sms1 (after 5 min) (Fig. 6B,
panel 1) was correlated with a decrease in the RAG1 transcript
level, which was fully depleted within 15 to 30 min (Fig. 6C,
panel 2).

Thus, the glucose regulation of SMS1 and its gene product
contributes to the regulation of RAG1 expression upon glucose
availability.

DISCUSSION

The aerobic yeast K. lactis is a suitable model for studying
glucose sensing through the regulation of RAG1 glucose trans-
porter gene expression. All of the positive regulators of RAG1
expression identified so far have been characterized by means
of a powerful genetic screening for fermentation-deficient rag
mutants (44). The novel RAG gene described in this study, RAG19,
which is the homolog of GRR1 of S. cerevisiae (coding for
the F-box protein ScGrr1) (8, 21), also is required for the
induction of RAG1 by glucose. The KlGrr1 gene coding for
the repressor of RAG1 expression previously was identified in
a genetic screen for suppressors of the rag4 mutation (33).
In this study, we identified another negative regulator of the
RAG1 gene, the SMS1 gene encoding a KlRgt1 corepressor,
through its putative product, which shares similarities with
both Std1 and Mth1 of S. cerevisiae.

Glucose signaling is conserved between K. lactis and S. cer-
evisiae, and this study confirmed that the general structure of
the Snf3/Rgt2/Rag4 signaling pathway, which senses extracel-
ulous glucose, is very similar between the two yeasts. The Δrag4
(glucose sensor), rag8 (casein kinase 1), and rag19 (KlGrr1
F-box protein) mutations are suppressed by the introduction of
the sms1 deletion. Sms1 is undetectable in glucose-grown cells,
and its degradation is prevented in rag4, rag8, and rag19 mu-
tants. These data confirm that all of these components belong
to the same pathway and that Sms1 acts downstream of Rag4,
Rag8, and KlGrr1. In agreement with the S. cerevisiae model
(Fig. 1) (23), we found that Sms1 interacts with the glucose
sensor Rag4, which may promote its phosphorylation by Rag8,
its subsequent ubiquitylation by the KISCgrg1 complex, and its
proteosomal degradation. In Mth1 and Std1 of S. cerevisiae,
several serines, potentially phosphorylated by Yck1 and Yck2,
have been localized, and their mutation/deletion prevents Std1
and Mth1 degradation and leads to HXT1 derepression (23,
30). In our studies, we did not demonstrate that Rag8 phos-
phorylates Sms1. However, a sequence alignment between
Sms1, Std1, and Mth1 shows that all of these potential casein
kinase I phosphorylation sites are strictly conserved in Sms1
(not shown). Consistently, we found that the B42-Sms1 protein
was able to interact with a LexA-Rag8 fusion in a two-hybrid
assay (data not shown). In the present study, we demonstrated
the participation of Sms1 with KlRgt1 in gene repression. In
the Δsms1 null mutant, RAG1 and RAG5 (hexokinase) genes
were no longer repressed in the absence of glucose, indicating
that Sms1, like KlRgt1 (33), acts as a negative regulator of both
glucose transport and glycolysis. Thus, both S. cerevisiae and K.
lactis exhibit similar pathways for regulating the Rgt1 repres-
sor, in which the cornerstone appears to be the regulation of
the Rgt1 corepressors’ (Mth1, Std1, and Sms1) cellular abun-
dance in response to glucose. We previously showed that, in K.
lactis, additional transcription factors, such as the Sck1 activa-
tor, also contribute to the glucose-induced expression of the
KIRgt1-regulated genes, RAG1 and RAG5 (25). Interestingly,
the Rag4-KIRgt1 pathway also controls SCK1, emphasizing the
tight control of the glucose induction mechanism by this path-
way. In addition, glycolysis also seems to exert a positive con-
trol on the glucose induction of RAG1, but the underlying
mechanism remains unidentified. We are currently investigat-
ing further this pathway. Importantly, it will be interesting to
determine whether this intracellular pathway is connected to the
Rag4-KIRgt1 pathway or controls a factor not identified yet.

A significant insight gained from our study is that, in K.
lactis, a single protein, Sms1, is involved with repression by
KIRgt1, whereas two corepressor proteins, Std1 and Mth1, are
involved in this process in S. cerevisiae (8, 13, 18, 31). The
MTH1 and STD1 genes are regulated by glucose at the tran-
scriptional level in an opposite way, whereas both proteins are
degraded in the presence of glucose (8, 12, 13). In both yeasts,
SMS1 and STD1 genes are induced by glucose and regulated by
KIRgt1 and ScRgt1, respectively, whereas their gene products,
Sms1 and Std1, are degraded in response to glucose. The
similarity of K. lactis SMS1 and S. cerevisiae STD1 regulation,
together with their syntonic relationship, indicate that SMS1 is
a likely STD1 ortholog. This also suggests that the glucose-
dependent regulation of these two genes, involving the Rgt1
repressor, was the ancestral state of regulation. In contrast, in
S. cerevisiae, glucose reduces MTH1 expression via a repression
mediated by Mig1 and Mig2 (12, 13), a regulation that is
missing in K. lactis.

The presence of the duplicated gene pair STD1 and MTH1
in the S. cerevisiae genome results from the WGD event (45).
Actually, a single-copy gene ortholog of STD1/MTH1 genes is
present in the non-WGD species like K. lactis, whereas there is
a duplicated gene pair in the genome of the post-WGD species
like S. cerevisiae. A very interesting result is that the S. cerevi-
siae genes did not complement the Δsms1 mutation, which was
surprising regarding the functional similarity between the Snf3/
Rgt2 and Rag4 signaling pathways. Thus, Sms1, Std1, and
Mth1 may have evolved as species-specific key components
within these pathways. However, this absence of complemen-
tation of the Δsms1 mutation certainly is not due to a lack of
interaction with the Rag4 sensor or KIRgt1 repressor, since
both Std1 and Mth1 could interact with Rag4 and KIRgt1 in
our two-hybrid assay. Moreover, despite the fact that ScRgt1 is
functional in K. lactis (our unpublished data), its coexpression
together with either Std1 or Mth1 failed to suppress the Δsms1
mutation. We believe that Std1/Mth1 failed to reach their
repressor partner (Rgt1 or KIRgt1) in the nucleus, suggesting
supplementary control on Sms1/Std1/Mth1 factors. Interest-
ingly, Pasula et al. recently reported that Std1 and Mth1 were
regulated by the glucose-controlled Snf1 kinase (30), which is known to regulate the nucleocytoplasmic shuffling of several regulators. Whatever the mechanism underlying the sophisticated Sms1/Std1/Mth1 regulation, this absence of complementation and the K. lactis model represent an excellent tool to investigate further this novel insight as well as yeast cell adaptation to glucose concentration.

ACKNOWLEDGMENT

M. Hnatova was the recipient of a fellowship from the Ministére de la Recherche de France.

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