Enolase and Glycolytic Flux Play a Role in the Regulation of the Glucose Permease Gene RAG1 of Kluyveromyces lactis

Marc Lemaire1 and Micheline Wésolowski-Louvel

UMR 5122 Microbiologie et Génétique, Université Claude Bernard Lyon 1, 69622 Villeurbanne, France

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ABSTRACT

We isolated a mutant, rag17, which is impaired in glucose induction of expression of the major glucose transporter gene RAG1. The RAG1 gene encodes a protein 87% identical to S. cerevisiae enolases (Eno1 and Eno2). The Kleno null mutant showed no detectable enolase enzymatic activity and has severe growth defects on glucose and gluconeogenic carbon sources, indicating that K. lactis has a single enolase gene. In addition to RAG1, the transcription of several glycolytic genes was also strongly reduced in the ΔKleno mutant. Moreover, the defect in RAG1 expression was observed in other mutants of the glycolytic pathway (hexokinase and phosphoglycerate kinase). Therefore, it seems that the enolase and a functional glycolytic flux are necessary for induction of expression of the Rag1 glucose permease in K. lactis.

IN most strains of Kluyveromyces lactis, the glucose uptake system relies on two nonredundant glucose transporters: a low-affinity permease encoded by RAG1 (Wésolowski-Louvel et al. 1992a) and a high-affinity permease encoded by HGT1 (Billard et al. 1996). HGT1 is constitutively expressed (Billard et al. 1996); expression of RAG1 is activated in the presence of high concentrations of glucose (Chen et al. 1992; Wésolowski-Louvel et al. 1992a). 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 cells have the Rag− phenotype: they cannot grow on 5% glucose in the presence of antimycin A, which blocks respiration (Goffrini et al. 1989; Wésolowski-Louvel et al. 1992b).

To date, studies of Rag− mutants have identified three key components that are involved in the positive regulation of RAG1 expression: the glucose sensor Rag4 (Betina et al. 2001), hexokinase Rag5 (Prior et al. 1993), and casein kinase I Rag8 (Blaisonneau et al. 1997).

In this report we present the characterization of another gene in K. lactis implicated in RAG1 regulation: RAG17 (Kleno) coding for enolase.

Materials and Methods

Yeast strains and growth conditions: Yeast strains 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 (YP) or a specified carbon source. Minimal medium containing 0.7% yeast nitrogen base without amino acids (Difco) and 2% glucose was supplemented with auxotrophic requirements. The Rag phenotype was tested on 5% complete glucose medium supplemented with 5 μM antimycin A. For G418 medium, YPG plates were supplemented with geneticin (200 μg/ml; Life Technologies). 5-FOA medium was prepared according to Boeke et al. (1984). The media for plates were solidified by the addition of 2% Bactoagar (Difco). Escherichia coli XL1-blue was used as a cloning host and DNA propagation and was grown in LB medium.

Genetics methods have been described previously (Wésolowski-Louvel et al. 1982; Goffrini et al. 1989).

Yeast transformation: Replicative transformation of K. lactis was performed by electroporation. For integrative transformation of K. lactis, the procedure described by Dohmen et al. (1991) was followed. Replicative and integrative transformation of Saccharomyces cerevisiae were standard.

Construction of deletion strains: One-step gene deletions using kanMX4 or HIS3 selection markers bearing PCR-generated long flanking homology (LFH-PCR; Wach 1996) was used to construct the mutant strains MWK3, MLK43, MLX702, and MLX703 (Table 1). The primers used for the KIENO LFH-PCR synthesis were P5′ KIENO (5′-CAGGTCACAATCCAGGCC ACC-3′), P5′ L. KIENO (5′-CCGTTCACCCTGACCGTGG CATGTTTTTTTG-5′), P3′ L. KIENO (5′-GCTCGAGATCCATCATC GATGATATTGACTGTCACCAAC-3′), and P3′ KIENO (5′-AGCTGACGTCGACGACG-3′). The primers used for the KIPG LFH-PCR synthesis were P5′ KIPG (5′-AGCTGACGTCGACGACG-3′), P5′ L. KIPG (5′-GGGGATCCCTGACGACG-3′), and P3′ KIPG (5′-ACGAGCTGACGTCGACGACG-3′). The primers used for the ScENO2 LFH-PCR synthesis

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1 Corresponding author: Unité Microbiologie et Génétique UMR 5122 CNRS/UCBL/INS, Université Claude Bernard, Bâtiment Lwoff, 43, Blvd. du 11 Novembre 1918, 69622 Villeurbanne Cedex, France. E-mail: mlemaire@univ-lyon1.fr

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were P5’ ScENO2 (5'-ATCTCAGCTATCCGGTTCGCACTGC-3') and P3’ ScENO2 (5'-GGATCCAAGCCTGACATGACGTAAGCA TGTATTGTGTGTTAGATGTA-3'). P5’ ScENO2 (5'-AAACAGGC TGCAGATCTTGTAGATATTTAATACTAAGATTT-3') and P3’ ScENO2 (5'-GTACGTGATAGGATGATGCAA-3') were used in all primer combinations. For all the P5’ and P3’ primers, the KmMX4 or His3 MX6 regions are in boldface type. All correct integrations were verified by Southern blot or PCR.

In S. cerevisiae, the ScENO2 gene was first disrupted in the diploid strain MLY701 by using a kanMX4 or a HIS3MX6 disruption cassette, yielding MLY702 and MLY703 strains, respectively (Table 1). After metoic analysis of these two diploid strains, the ΔSceno2 haploid strains MLY704 (ΔSceno2::KmMX4) and MLY708 (ΔSceno2::His3MX6) were obtained. The double mutant strain ΔSceno1 ΔSceno2 was then constructed by crossing the MLY708 strain with the ΔSceno1 strain Y07286 (Table 1), yielding the MLY713 diploid.

In K. lactis, the KIENO and KIPKG genes were disrupted in the MW270-7B strain using the corresponding KmMX4 disruption cassettes, yielding MWK3 and MLK43 strains, respectively.

Plasmid constructions: The ENO2 gene of S. cerevisiae was first PCR amplified from MLY701 genomic DNA by using the Pfx polymerase (Invitrogen, San Diego) and the P5’ ScENO2/P3’ ScENO2 primers. After phosphorylation with T4 poly-nucleotide kinase (New England Biolabs, Beverly, MA), the ScENO2 PCR product was cloned into the CEN-URA3 vector pRS416 (Sikorski and Hieter 1989) linearized with SmaI, yielding pML180. This plasmid could complement the slow growth of the ΔSceno2 mutant (Table 1) and thus contained a functional copy of ScENO2. The 2.9-kb XhoI-NotI fragment of pML180, containing ScENO2, was then cloned into the CEN TRP1 vector pRS414 (Sikorski and Hieter 1989) digested with XhoI and NotI, yielding pML190. The ScENO2 was also introduced into a K. lactis centromeric vector by cloning the XhoI-XhoI 2.9-kb fragment of pML180 between the XbaI and SalI sites of the CEN URA3 pCX18 vector (Chen 1996), yielding pML187.

The K. lactis KIENO gene was cloned into a S. cerevisiae vector by subcloning the 5.5-kb BamHI fragment of pMW1 (see below) into the BamHI site of pRS414, yielding pML183.

Cloning and sequencing of RAG17/KIENO gene: The RAG17 gene was cloned by in vivo complementation of rag17 mutation (strain MWK2) with a K. lactis genomic library made in the Kcp91 vector (Prior et al. 1993). Of 5000 Ura+ transformants, 4 were found to be RAG17+. The complementing plasmids extracted from these four transformants and amplified in E. coli showed that three of them carried the same plasmid, pMW1 containing a 7.5-kb insert (Figure 1), and one a plasmid with a 10-kb insert overlapping with a pMW1 insert.

A 2856-bp fragment containing the entire RAG17/KIENO gene was sequenced on both strands.

Preparation of yeast RNA and probes: Total RNA was extracted from cells grown to an OD600 of 2–3. Poly(A)+-enriched mRNA was obtained using mRNA Separator (CLONTECH, Palo Alto, CA). Several gene probes used were restriction fragments: RAG6 probe was a 1.3-kb EcoRI-Sall fragment (Bianchi et al. 1996); HGT1 probe was a 1.75-kb EcoRI-HindIII fragment containing the HGT1 gene (Billard et al. 1996). Other probes were obtained by PCR amplification using either K. lactis genomic DNA or the cloned genes as template. The oligonucleotides used were: 0487 (5'-GGGCTGTTCTTGACGTTCC-3') and 0369 (5'-GACGTAACCAGTACGTTAGACGTTGAATTTGTAATGATGTA-3') for RAG1; RAG2- up (5'-GTCAAGTACGTTACGTCGAC-3') and RAG2-down (5'-CAAGATTACAGCAGTATGTAATGTATGGTAATGATGTA-3') for RAG2; p2E1/9 (5'-GGGCTGTGTGTTTAATTAGATACTTTAATTAAACTAAG-3') and p2E1/5 (5'-GGGCTGTGTGTTTAATTAGATACTTTAATTAAACTAAG-3') for RAG4; 470 (5'-GGTAGGTTATTTGACGTAATGTA-3') and 471 (5'-GTACGTGATAGGATGATGCAA-3') for RAG5.

RESULTS

Isolation of the KIENO gene and deduced amino acid sequence of its product: In K. lactis, gene replacement by homologous recombination can be accomplished, but at lower frequencies compared to S. cerevisiae. Usually, the gene disruption cassette recombines at ectopic sites in the genome. While attempting to construct a rag4::URA3 gene disruption (Betta et al. 2001), we identified a RAG4+ mutant that is not allelic to rag4, although RAG1 transcription is highly reduced in this mutant. The mutation is also not allelic to rag5 and rag8 mutations, both of which affect genes that positively regulate the transcription of the RAG1 gene (Chen et al. 1992). We named the mutation rag17-1 (strain MWK1 in Table 1). The precise position of the URA3 insertion in the gene is not known.

RAG17 was isolated from a CEN-based K. lactis plasmid library by complementation of the Rag− phenotype of the rag17-1 mutation (materials and methods). The partial nucleotide sequence of the DNA fragment in the complementing plasmid (Figure 1 and materials and methods) revealed the presence of three ORFs: one encodes a protein 40% identical to the Fmo of S. cerevisiae, a flavin-containing monoxygenase involved in protein folding (Suh et al. 1999); another encodes a protein highly similar to the enolases of S. cerevisiae, Enol1 and Enol2 (Holland et al. 1981); the third encodes a protein 28% identical to Rax1 of S. cerevisiae, a protein implicated in bud site selection (Chen et al. 2000). Because of the Rag− phenotype of the mutation, the best candidate for RAG17 was the ORF that encodes the glycolytic enzyme enolase (KIENO). The predicted protein (437 amino acids) is 88 and 87% identical to ScEno2.
TABLE 1
Yeast strains

<table>
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<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>K. lactis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW270-7B</td>
<td>MATa uraA1-1 metA1-1 leu2</td>
<td>Billard et al. (1996)</td>
</tr>
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<td>PM4-4B</td>
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<td>Goffrini et al. (1991)</td>
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<td>PM6-7A</td>
<td>MATa uraA1-1 adeT-600</td>
<td>Chen et al. (1992)</td>
</tr>
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<td>MKW1</td>
<td>Isogenic to MW270-7B except KlENO::URA3 (rag17-1)</td>
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</tr>
<tr>
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<td>Isogenic to MW270-7B except KlENO::ura3</td>
<td>This work</td>
</tr>
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<td>Isogenic to MW270-7B except KlENO::URA3</td>
<td>This work</td>
</tr>
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<tr>
<td>MKW12</td>
<td>Isogenic to PM6-7A except KlpΔ1::URA3 (ΔKlpΔc)</td>
<td>Bianchi et al. (1996)</td>
</tr>
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<td>S. cerevisiae</td>
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</tr>
<tr>
<td>Y07286</td>
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<td>BM64-1A</td>
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<td>Euroscarf</td>
</tr>
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<td>Euroscarf</td>
</tr>
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<td>Isogenic to MLY701 except ENO2::enoΔ2 Δ1::kanMX4</td>
<td>This work</td>
</tr>
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<td>Isogenic to MLY701 except ENO2::enoΔ2 Δ1::HIS3</td>
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<td>MLY704</td>
<td>Meiotic segregant of MLY702 diploid: MATa ura3-1 trp1Δ2 leu2-3,112 his3-11 ade2-1 can1-100 enoΔ1::kanMX4</td>
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<td>Diploid issued from Y07286 × MLY708 cross</td>
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</tr>
<tr>
<td>MLY714</td>
<td>MLY713 diploid transformed with pML180 (ScENO2/pRS416)</td>
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<tr>
<td>MLY719</td>
<td>MLY714 Meiotic segregant: MATa ura3-1 trp1Δ2 leu2-3,112 his3-11 ade2-1 YGR254w::kanMX4 enoΔ2 Δ1::HIS3 + pML180 (ScENO2/pRS416)</td>
<td>This work</td>
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a YGR254w, ScENO1.

and ScEno1 of S. cerevisiae, respectively, and 64% identical to human α-enolase (Swiss-Prot accession P06733).

A PCR-based gene deletion cassette for RAG17/KIENO (Figure 1; Table 1; see MATERIALS AND METHODS for details) was used to disrupt the gene in the MW270-7B strain. Southern blot analysis confirmed the KIENO disruption in some G418R transformants, such as MKW3 (data not shown). We also demonstrated that the KIENO...

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**Figure 1.** (A) Restriction map of recombinant plasmid pMW1 and localization of the three ORFs (large arrows) identified by nucleotide sequences. Small arrows indicate the direction of DNA sequencing. (B) Restriction map of the RAG17/KIENO locus. (C) Disruption cassette of the KIENO gene with kanMX4 marker (open box) to construct the MKW3 strain (Table 1). The solid boxes indicate genomic sequences (see MATERIALS AND METHODS).
locus is modified in the original rag17-1 mutant (MWK), suggesting an ectopic integration of the rag4::URA3 gene disruption cassette in the KlENO gene. Like the rag17-1 mutation, the KlENO null mutation leads to a Rag⁻ phenotype. The allelism of KlENO with rag17-1 was confirmed by the absence of complementation of the Kleno null mutation (strain MWK3) by the rag17-1 mutation (MW352-2D) in a diploid constructed by crossing these two mutants. Thus, the cloned KlENO gene indeed corresponds to the RAG17 locus.

**Growth phenotype of the Kleno mutant:** Growth of the Kleno null mutant is severely reduced on media containing either glucose or glycerol as the sole carbon source (Figure 2). In addition, the mutant strain is unable to grow on ethanol as carbon source. It is noteworthy that the Kleno mutant can grow slowly on complete glucose medium: it exhibits a 4.5-fold increase of doubling time as compared to wild-type cells (450 min vs. 110 min; data not shown). Better growth of mutant cells on complete medium is probably supported by other carbon sources (e.g., amino acids) in this medium.

**Enolase activity of Kleno mutant and Kleno expression:** No detectable enolase activity is present in the ΔKleno mutant, regardless of the substrate used (glycerol or glucose; Figure 3A). This strongly suggests that KlENO is the single enolase-encoding gene in *K. lactis*. Presence of the KlENO sequence in a single copy and absence of other related sequences in the genome was confirmed by low-stringency Southern blotting (data not shown). In contrast, in *S. cerevisiae*, which possesses two enolase genes, enolase activity is still detectable in ΔSceno1 or ΔSceno2 single mutants grown on glucose or on glycerol. It was not possible to assay enolase activity in the double-mutant ΔSceno1ΔSceno2 since it is inviable on glucose or glycerol media (see below).

**KlENO transcription was examined by a Northern blot analysis.** The level of the KlENO transcript is slightly higher (twofold) when the cells are grown on 2% glucose (Figure 3B) than when they are cultivated on 2% glycerol. This result is consistent with the increased level of enolase activity detected in glucose-grown cells as compared to glycerol-grown cells (Figure 3A). In the null mutant, no transcript could be detected whatever the carbon source used. These results demonstrate that *K. lactis* possesses the single gene KlENO coding for an enolase and expressed under glycolytic as well as neoglucogenic conditions.

**The KlENO gene complements the ΔSceno1 ΔSceno2 mutations of S. cerevisiae:** The nucleotide sequences of the two enolase-encoding genes of *S. cerevisiae*, ScENO1 and ScENO2, are >90% identical. The major difference between the two genes is in their 5' noncoding sequence. Therefore, they are expressed differently: the gluconeogenic gene ScENO1 is constitutively expressed (COHEN et al. 1987); the glycolytic gene ScENO2 is induced by glucose (McALISTER and HOLLAND 1982; COHEN et al. 1986). The Sceno1 null mutation has no phenotype (McALISTER and HOLLAND 1982); the ΔSceno2 mutant grows more slowly on glucose than does the wild-type strain (NIEDENTHAL et al. 1999). Meiotic analysis of the diploid MLY713 (ScENO2::HIS3 ScENO1

**Figure 2.—Growth phenotype of the Kleno null mutant strain.** The wild-type strain MW270-7B (KlENO), and the isogenic mutant strain MWK3 (ΔKleno) were streaked onto single colonies on 2% glucose, 2% glycerol, and 2% ethanol minimal plates. The photographs were taken after 3 days of incubation at 28°C.
ΔSceno1::kanMX4) showed that no G418\(^{R}\) His\(^{+}\) spores are viable on YP medium containing either 2% glucose or 2% glycerol, but viable G418\(^{R}\) His\(^{+}\) spores could be obtained on YP medium containing 0.1% glucose + 2% ethanol. Under those conditions the ΔSceno1ΔSceno2 mutant probably obtains energy by respiration, which is derepressed at low concentrations of glucose.

To test if the KlENO gene can complement the growth defect of ΔSceno1ΔSceno2, we sporulated the MLY713 diploid (Table 1) carrying a CEN-URA3 plasmid containing the ScENO2 gene. A meiotic segregant of a complete tetrad, MLX719 (Ura\(^{+}\) His\(^{+}\) G418\(^{R}\) Trp\(^{-}\)), was transformed in parallel with a CEN-URA3 plasmid carrying either KlENO or ScENO2 (Table 1). In both cases the transformants were able to grow on 5-FOA medium, which counterselects for Ura\(^{+}\) cells (Figure 4). As a control, the same strain was transformed with the empty pRS414 plasmid and found unable to lose the plasmid carrying ScENO2 (pML180; i.e., this strain cannot grow on 5-FOA medium). We conclude that KlENO restores viability to the ΔSceno1ΔSceno2 mutant of S. cerevisiae. Thus, KlENO is a functional homolog of the ENO genes of S. cerevisiae. The reciprocal heterologous complementation was confirmed: ScENO2, cloned in a K. lactis centromeric vector (pML187; see MATERIALS AND METHODS), complements the Rag\(^{-}\) phenotype of the Kleno mutant (data not shown).

**KlENO is required for expression of genes encoding glucose permeases and glycolytic genes and their regulators:** Northern blot analysis presented in Figure 5 shows that the disruption of KlENO results in a severe reduction of transcript levels of both glucose transporter genes, RAG1 and HGT1. However, the transcription of HGT1 was less affected than that of RAG1. The transcription of the hexokinase (RAG5, KlHXK) and pyruvate decarboxylase gene (RAG6, KlPDC) is also impaired in the mutant. In contrast, the phosphoglucose isomerase gene (RAG2, KlPGI) is not affected (Figure 5).

The reduction of the transcription of genes encoding glycolytic enzymes and glucose transporters could result from a direct effect on these genes or it could be indirect, possibly through effects on the expression of genes encoding their regulators. One of these regulators—the glucose sensor, a positive regulator of RAG1—is encoded by RAG4 (Betina et al. 2001). Another one, SCK1, codes for a helix-loop-helix type DNA-binding transcription factor, homologous to SGCl of S. cerevisiae, which is required for full expression of glycolytic genes and of the glucose carrier gene RAG1 (Lemaire et al. 2002). KIGCR1 and KIGCR2 of K. lactis are the orthologs of the positive regulatory genes GCR1 and GCR2 of S. cerevisiae (Haw et al. 2001; Neil et al. 2004). The KIGCR1 and KIGCR2 genes, like SCK1, appear to positively control glucose transport.
the expression of glycolytic genes and RAG1 (Neil et al. 2004). The amount of each of these transcripts was decreased compared to the wild-type strain in the enolase mutant strain (Figure 6). The transcript levels of these genes were reduced approximately five- to sevenfold in the mutant relative to the reference KIACT and KIAAC transcripts (data not shown).

A robust glycolytic flux is necessary for the full expression of RAG1: KIENo is the second glycolytic gene controlling the RAG1 regulation to be identified. The RAG5 gene, coding for the single hexokinase in K. lactis, was already known to be required for the full expression of RAG1 (Prior et al. 1993). Enolase and hexokinase are suspected to harbor both catalytic and regulatory functions (Bisson and Fraenkel 1983; Entian and Fröhlich 1984; Prior et al. 1993; Feo et al. 2000; Subramanian and Miller 2000), but our data suggest a more general hypothesis: the overall glycolytic flux may regulate glucose transport.

To investigate this hypothesis, we analyzed the expression of RAG1 in several mutants that are defective for different steps of the glycolytic or fermentation pathways (Figure 7A): Δkhlxk (hexokinase), Δklpgi (phosphoglucoisomerase), Δklpgk (phosphoglycerate kinase), ΔKleno (enolase), and Δklpdc (pyruvate decarboxylase). The results showed that RAG1 expression is significantly reduced in Δkhlxk, Δklpgi, and ΔKleno mutants (Figure 7B) in which the glycolytic flux is blocked. However, the Δklpdc mutant, which can bypass the glycolytic block through the pentose phosphate pathway (Jacoby et al. 1993; Gonzalez Siso et al. 1996), has little or no effect. The Δklpdc mutation that blocks the first step of fermentation following glycolysis has no impact on RAG1 transcription. These findings suggest that glycolytic flux is required for full expression of RAG1.

Interestingly, regulation of glucose uptake by glycolytic flux in S. cerevisiae has been suggested (Bisson et al. 1993). We tested whether the activation of HXT1 (most closely related to RAG1) expression is impaired in the enolase mutant of S. cerevisiae grown on glucose. No effect on expression of an HXT1-LacZ fusion was observed in the single enol1 or enol2 mutants (data not shown). This negative result is not necessarily conclusive, because both single mutants retain some enolase activity (Figure 3A). Unfortunately, this experiment cannot be performed with the double-mutant enol1 enol2 since this mutant cannot grow on glucose (see Figure 2).

A reduced growth rate does not affect RAG1 transcription: The glycolysis block in the Kleno mutant leads to a severe growth defect (Figure 2). The Klhxk and Klpgk mutants, but not the Klpgi and Klpdc mutants, show a similar growth defect (Goffrini et al. 1991; Prior et al. 1993; Bianchi et al. 1996; data not shown). Thus, it remained possible that the RAG1 transcriptional defect in the Klhxk, Klpgk, and Kleno mutants could be due to their reduced growth rate rather than to their reduced glycolytic capability. To investigate this possibility, a wild-type strain (MW270-7B, KIENO) was grown on glucose. These genes were reduced approximately five- to severalfold in dividing and nondividing cells. These gene, coding for the single hexokinase in K. lactis, was already known to be required for the full expression of RAG1 (Prior et al. 1993). Enolase and hexokinase are suspected to harbor both catalytic and regulatory functions (Bisson and Fraenkel 1983; Entian and Fröhlich 1984; Prior et al. 1993; Feo et al. 2000; Subramanian and Miller 2000), but our data suggest a more general hypothesis: the overall glycolytic flux may regulate glucose transport.

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DISCUSSION

We have shown that the KIENO gene, which encodes enolase, the glycolytic enzyme that catalyzes the reversible conversion of 2-phosphoglycerate to phosphoenolpyruvate, is required for normal regulation of expression of RAG1, encoding the low-affinity glucose permease in K. lactis. Glycolytic enzymes have been extensively studied and characterized at the structural and biochemical level. Recently, interest in glycolytic enzymes has been revived due to their implications in other biological pathways. In S. cerevisiae, the hexokinase PII, encoded...
by the \textit{HXK2} gene, is involved in glucose repression (Randez-Gri \textit{et al.} 1998). In fact, nuclear localization of Hxk2 appears to be glucose regulated, and it interacts \textit{in vivo} with Mig1, the transcriptional repressor of many glucose-repressed genes (Ahuatzi \textit{et al.} 2004). In addition, in \textit{K. lactis} and \textit{S. cerevisiae}, hexokinase is also required for full glucose induction of \textit{RAG1} and \textit{HXT} gene expression (Prior \textit{et al.} 1993; Özcan and Johnston 1999). Several pieces of evidence suggest that enolase is a multifunctional protein playing a crucial role in transcriptional and physiological processes (reviewed in Pancholi 2001). For example, enolase has been identified as a heat-shock protein in \textit{S. cerevisiae} (Iida and Yahara 1985) and has been found to have transcriptional regulatory functions in larger eukaryotes. In the latter case, these functions occur through binding of enolase to certain gene promoters: \textit{c-myc} (Feo \textit{et al.} 2000; Subramanian and Miller 2000) or \textit{STZ/ZAT10}, encoding a transcriptional repressor in Arabidopsis (Lee \textit{et al.} 2002).

The finding that the expression of \textit{RAG1} as well as several genes encoding glycolytic enzymes is affected in the \textit{ΔKleno} mutant suggests that enolase could play a regulatory role in \textit{K. lactis} in addition to its catalytic activity. However, we have not yet demonstrated a direct role for enolase in the transcriptional regulation of \textit{RAG1}. In addition to enolase it was already known that induction of \textit{RAG1} expression by glucose is dependent on hexokinase activity (Prior \textit{et al.} 1993). Since the loss of any one of the glycolytic steps tested, except that of phosphoglucoisomerase, severely reduces \textit{RAG1} transcription, we believe that glucose metabolism generates a signal that induces \textit{RAG1} expression. We cannot, however, exclude the possibility that enolase has a general regulatory function on other genes.

The defect in \textit{RAG1} transcription in mutants blocked in glycolysis suggests the existence of regulatory mechanisms that prevent expression of genes encoding glucose transporters if a functional glycolytic pathway cannot be maintained. Milkowski \textit{et al.} (2001) previously showed that the absence of glucose transporters (hence glucose uptake) impaired the induction of \textit{KHT1/RAG1} expression by high levels of glucose. Altogether, these data suggest that an intracellular glucose-sensing mechanism relying on glucose metabolism through glycolysis may ensure optimal glucose uptake by activating expression of the gene encoding the low-affinity glucose transporter. This intracellular pathway presumably collaborates with the extracellular glucose-sensing mechanism operating through the Rag4 glucose sensor in the cell membrane (Betina \textit{et al.} 2001). In \textit{E. coli}, the expression of \textit{ptsG}, encoding the major glucose transporter IIC\textit{Bc} \textit{E. coli}, also requires glycolytic flux (Kimata \textit{et al.} 2001). Whatever the mechanism, regulation of glucose uptake by glycolytic flux seems to have been conserved from bacteria to yeasts.

We have a few clues to the mechanism by which glycolytic flux regulates expression of \textit{RAG1} and glycolytic genes. The inactivation of \textit{Kleno} leads to a severe reduction in expression of the regulatory genes \textit{KIGCR1}, \textit{KIGCR2}, \textit{SCK1}, and \textit{RAG4} (Figure 6). Thus, the effects of enolase on expression of genes encoding glycolytic enzymes and glucose transporters may be indirect. \textit{KIGCR1}, \textit{KIGCR2}, and \textit{SCK1} are required for the full expression of glycolytic genes (Lemaire \textit{et al.} 2002; Neil \textit{et al.} 2004). The \textit{KIGcr1/KIGcr2} complex directly regulates glycolytic gene expression through binding of KIGcr1 to glycolytic gene promoters (Neil \textit{et al.} 2004). An interaction between Sck1 and \textit{K. lactis} glycolytic promoters is also probable since its \textit{S. cerevisiae} ortholog

![Figure 7.—Northern blot analysis of \textit{RAG1} in mutant affected at different steps of glycolysis (A). Approximately 5–10 \( \mu \)g of total RNA extracted from cells grown on 2% glucose was loaded in each slot. Electrophoresis conditions were as in Figure 3. The probes used to detect \textit{RAG1} and 18S transcripts are described in MATERIALS AND METHODS. (B) The strains MW270-7B, PM4-4B, and PM6-7A are the isogenic wild-type strains of the different mutants used. \( \Delta Hkh1 \), MWK11 strain; \( \Delta Kpgi \), MWK12 strain; \( \Delta Kpgf \), MWK43 strain; \( \Delta Kleno \), MWK3 strain; \( \Delta Kleno \), MWK3 strain. These strains are described in Table 1. 18S mRNA was used as an internal standard. The hybridization signals have been quantified with a phosphoimager (MATERIALS AND METHODS) and numbers below the panel indicate the ratio of \textit{RAG1}:18S.](image-url)
The MW270-7B (KlENO) strain was first grown in 50 ml YP medium containing 2% glycerol to an OD$_{600}$ of 2. Cells were harvested, washed, and resuspended in sterile cold water and aliquots (~15 OD$_{600}$) were diluted in 50 ml YPG (2% glucose) or YPG containing 5 μM antimycin A, 5 mM KCN, or 100 μg/μl G418. Cultures were kept agitated at 28°C during 10 hr and OD$_{600}$ was checked at 0, 3, 6, and 10 hr.

(B) Northern blot analysis of RAG1, KIACT, and 18S transcript levels. Total RNA was extracted from the YPG culture at t = 0 (shift on glucose) and from the four cultures at t = 10 hr. Electrophoresis conditions and quantification were done as described in the Figure 6 legend. The probes used to detect RAG1, KIACT, and 18S transcripts are described in MATERIALS AND METHODS. KIACT and 18S mRNA was used as internal standards and numbers below the corresponding panels indicate the ratio of RAG1:KIACT or RAG1:18S.

**Figure 8.**—Effect of the growth rate on RAG1 transcription. (A) Inhibition of K. lactis growth by antimycin A, KCN, or G418. The MW270-7B (KlENO) strain was first grown in 50 ml YP medium containing 2% glycerol to an OD$_{600}$ of 2. Cells were harvested, washed, and resuspended in sterile cold water and aliquots (~15 OD$_{600}$) were diluted in 50 ml YPG (2% glucose) or YPG containing 5 μM antimycin A, 5 mM KCN, or 100 μg/μl G418. Cultures were kept agitated at 28°C during 10 hr and OD$_{600}$ was checked at 0, 3, 6, and 10 hr. (B) Northern blot analysis of RAG1, KIACT, and 18S transcript levels. Total RNA was extracted from the YPG culture at t = 0 (shift on glucose) and from the four cultures at t = 10 hr. Electrophoresis conditions and quantification were done as described in the Figure 6 legend. The probes used to detect RAG1, KIACT, and 18S transcripts are described in MATERIALS AND METHODS. KIACT and 18S mRNA was used as internal standards and numbers below the corresponding panels indicate the ratio of RAG1:KIACT or RAG1:18S.

**LITERATURE CITED**


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