Role of Snf1p in Regulation of Intracellular Sorting of the Lactose and Galactose Transporter Lac12p in Kluyveromyces lactis†

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Received 30 July 2004/Accepted 4 February 2005

The protein kinase Snf1/AMPK plays a central role in carbon and energy homeostasis in yeasts and higher eukaryotes. To work out which aspects of the Snf1-controlled regulatory network are conserved in evolution, the Snf1 requirement in galactose metabolism was analyzed in the yeast Kluyveromyces lactis. Whereas galactose induction was only delayed, K. lactis snf1 mutants failed to accumulate the lactose/galactose H⁺ symporter Lac12p in the plasma membrane as indicated by Lac12-green fluorescent protein fusions. In contrast to wild-type cells, the fusion protein was mostly intracellular in the mutant. Growth on galactose and galactose uptake could be restored by the KHT3 gene, which encodes a new transporter of the HXT subfamily of major facilitators. These findings indicate a new role of Snf1p in regulation of sugar transport in K. lactis.

The heterotrimeric protein kinase Snf1p is a central regulator of carbon homeostasis in yeasts and is structurally and functionally related to the mammalian AMP-activated protein kinase. An ancient function that emerges for this class of conserved kinases is a key role in adaptation to energy limitation. Energy-consuming anaerobic pathways are downregulated under these conditions and energy is generated by induction of catabolism (reviewed in references 6, 17, and 18). In the yeast Saccharomyces cerevisiae, Snf1p is essential for the reprogramming of gene expression under carbon limitation. The derepression of glucose-repressed genes has been studied extensively and serves as a model of Snf1p signaling to the nucleus (7).

Two classes of genes that are upregulated in response to Snf1p have been described. The first class is controlled by transcription factors Cat8p and Sip4p, which bind to carbon source-responsive elements and are targets of the Snf1p kinase. Among them are genes involved in gluconeogenesis, the glyoxalate cycle, carbon transport, and acetyl-coenzyme A synthesis (19, 20). The second class is subject to glucose repression via the Mig1p-Ssn6-Tup1 repressor complex. Mig1p is a target of Snf1p and Snf1-mediated phosphorylation regulates cellular compartmentation of Mig1p (11) and interaction with Ssn6-Tup1 (31). Among the Mig1p-regulated genes are those involved in the utilization of alternative sugars, of which the Suc2 gene and Gal4p-controlled galactose regulon (GAL regulon) have been studied most extensively (7, 26).

Saccharomyces cerevisiae has an exceptional preference for fermentation metabolism, and it is not clear in how far its strategies of carbon regulation and Snf1p signaling can serve as a model for other organisms. Even among yeasts, great differences in physiology exist, like the Crabtree effect, the Kluyver effect, and the petite character, that have been used for taxonomic classifications. Comparative studies between the petite-negative, Crabtree-negative yeast K. lactis, for which genome sequencing has been completed, and S. cerevisiae (petite-positive, Crabtree-positive) are being undertaken to provide insight into the genetic basis of such differences in physiology.

Given the broader substrate spectrum and the limited utilization of glucose under aerobic conditions in K. lactis (4), we are interested in elucidating the conserved aspects of the Snf1p-controlled regulatory networks. As shown before, homologues of the Snf1p-controlled transcription factors Cat8p, Sip4p, and Mig1p exist in K. lactis. However, their contribution to the regulation of carbon and energy metabolism differs from that in S. cerevisiae (8, 9, 15, 27). For example, the gluconeogenesis genes and the sucrose-degrading inulinase gene are subject to glucose repression, but neither K. lactis Cat8p nor K. lactis Mig1p is involved. K. lactis Mig1p contributes to the regulation of the GAL regulon, which is highly conserved between S. cerevisiae and K. lactis (13), but a K. lactis mig1 mutation has only a minor influence on expression of the lactose metabolism genes LAC12 and LAC4, which are part of the GAL regulon (LAC/GAL regulon) (14). Moreover, a mig1 mutation does not suppress the K. lactis Snf1p requirement for growth on galactose as is the case in S. cerevisiae (13).

Among the genes of the GAL regulon, only K. lactis GAL1 contains a Mig1p binding site. Through this site, each gene in the regulon is affected by a mig1 mutation, since K. lactis Gal1p is responsible for transcriptional activation of the regulon (13, 30). Gal1p flips the transcriptional switch in response to galactose by binding to and inactivating the Gal4p inhibitor Gal80p (41). Thus, derepression of GAL1 in a K. lactis mig1 mutant can partially overcome glucose repression, as does elevated expression of GAL4 (13, 28, 39). However, growth on galactose is still impaired in the K. lactis snf1 mutant strain. In particular, lactose permease activity, encoded by LAC12, was virtually undetectable in this mutant, and the permease deficiency could not be overcome by deletion of MIG1 (13). Growth on lactose is only weakly impaired, probably because even a small amount of glucose released from lactose by hydrolysis is sufficient to support growth of K. lactis at a high rate. Since Lac12p also

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† Supplemental material for this article may be found at http://ec.asm.org/.

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functions as a galactose permease (12, 32), its low activity may explain the poor growth of a *K. lactis snf1* mutant on galactose.

Here we have addressed the question of how *K. lactis* Snf1p exerts its function on the *LAC/GAL* regulon. We show that transcriptional regulation is only partly responsible for the galactose deficiency of a *K. lactis snf1* mutant. A major influence is exerted by a limitation of galactose uptake due to an influence of the *snf1* mutation on the intracellular sorting of the lactose-galactose permease Lac12p in the mutant. Overexpression of a so-far-uncharacterized hexose transporter, Kht3p, can partially circumvent this limitation.

### MATERIALS AND METHODS

**Strains and growth conditions.** The strains used in this work are listed in Table 1. Plasmid pD802 was used for deletion of *GAL80* in *JS91R/D80* as described (34). An AattII-ClaI fragment of plasmid pLAC12-EGFP was integrated at random chromosomal positions of strains JA6 and JS91R to create strains JA6-LAC12GFP and JS91R-LAC12GFP, respectively. Cells were grown in synthetic complete medium (0.67% yeast nitrogen base with amino acids and nucleobases) (23) with the indicated carbon sources. The same medium lacking uracil was used for deletion of a HindIII fragment, resulting in plasmid pKHT3, which comprises the *KHT3* open reading frame with 429 bp of 5' untranslated and 1,151 bp of 3' untranslated region. The *LAC12* plasmid pBK52 was isolated from the same library and contains the *LAC12* gene (including 1,201 bp upstream and 1,653 bp downstream of the *LAC12* open reading frame). The C-terminal end of *LAC12* was fused to GFP by replacing a DraI-EcoRI fragment in pBK52 with the Smal-EcoRI fragment from pEFGP (Clontech), giving plasmid pLAC12-EGFP. The GFP-KIGAL80 gene fusion and the GFP-KIGAL1 fusion are expressed from the *S. cerevisiae ADH1* promoter on pE1-based multicopy plasmids.

**Determination of β-galactosidase activity.** β-Galactosidase activity was determined in crude extracts prepared in β-Gal buffer (5% glycerol, 10 mM KCl, 5 mM Tris-HCl, pH 7.8). The enzyme assay was carried out at 30°C in β-Gal buffer containing 40 μg of o-nitrophenyl-galactoside (ONPG) per ml. The reaction was followed photometrically (16).

**Northern analysis.** For RNA isolation, cultures were grown overnight in SC medium. After harvesting by centrifugation, cells were resuspended in fresh medium containing the indicated carbon sources and cultivated for 4 more hours, harvested, and disrupted by glass beads. The RNA was isolated by a hot phenol method. Northern analysis was carried out as described (1). Generally, 15 μg of RNA sample was loaded per lane. RNA was denatured in loading buffer containing 6% formaldehyde. Digoxigenin-labeled probes were prepared by random priming or PCR labeling with a DNA labeling kit (Roche). The digoxigenin-labeled bands were detected by chemiluminescence with diosodium-3-[4-methoxy-ysinyl]-1,2-dioxetane-3,2′- (5-chlorotri cyclo(3.3.1.1^3^)dec an)-4-yl)phenyl phosphate (Roche).

**Nucleotide sequence accession number.** The accession number for *KHT3* is AJ5750581.

### RESULTS

**LAC12 transcription is only weakly impaired in a *K. lactis snf1* mutant strain.** The Gal4p-regulated *LAC12* gene, which is divergently transcribed with the β-galactosidase gene *LAC4*, is essential for the uptake of lactose in *K. lactis* and has a major impact on galactose transport, although additional galactose transporters exist, at least in some strains (3, 12). To address the question of whether impaired induction of *LAC12* is responsible for the growth deficiency on galactose of a *K. lactis snf1* mutant, we compared mRNA levels in wild-type and mutant cells shifted from glucose to galactose medium by Northern analysis. RNAs were isolated from cultures grown on glucose and shifted to fresh medium containing 2% glucose or galactose for 4 h. With a single probe, both, the *LAC12* and *LAC4* transcripts could be detected. As shown in Fig. 1, there is clearly induction of both genes in the mutant as well as in the wild type. RNA levels are somewhat lower in the *K. lactis Δsnf1* mutant, but it seems unlikely that this reduction explains the poor growth on galactose and the complete absence of lactose transport activity (13).

**Constitutive induction of the *LAC/GAL* regulon in a *K. lactis gal80* mutant is not sufficient to sustain full growth on galac-
Isolation of a new galactose transporter gene, \textit{KHT3}, as a multicyclic suppressor on galactose of a \textit{K. lactis snf1} mutant. To understand the role of Snf1p in galactose utilization, multicyclic suppressors of the poor growth of a \textit{K. lactis snf1} mutant on galactose were isolated. \textit{K. lactis} strain JSD1 (\textit{snf1}Δ) was transformed with a KEp6-based multicopy genomic library and plated on selective galactose medium (2%). Nine transformants that showed improved growth were characterized further. Eight of these contained the \textit{K. lactis SNF1}, gene as confirmed by PCR, whereas one clone did not give a signal with \textit{K. lactis SNF1}-specific primers. The plasmid recovered from this clone contained an insert of 5.3 kb, from which a 2.1-kb HindIII fragment could be deleted without affecting the complementing activity.

The DNA sequence of the remaining insert revealed an open reading frame of 528 codons related to the fungal \textit{HXT} hexose transporter family of major facilitators, which was named \textit{KHT3} (for \textit{Kluyveromyces lactis} hexose transporter). Overexpression of \textit{KHT3} in a \textit{K. lactis snf1} mutant strain resulted in significantly improved growth on galactose (Fig. 3A). In order to investigate whether Kht3p is a galactose transporter, we expressed \textit{KHT3} on a high-copy-number plasmid in a \textit{lac12} mutant strain. This mutant is not able to grow on lactose and galactose. Multiple copies of \textit{KHT3} were able to suppress this phenotype on galactose but not on lactose, indicating that Kht3p can function as a galactose transporter (Fig. 3B).

\textit{Lac12-GFP} fails to accumulate in the plasma membrane in \textit{K. lactis snf1} mutants. The isolation of a galactose transporter in our genetic screen, together with the finding that \textit{LAC12} mRNA is detected in a \textit{K. lactis snf1} mutant, indicated that

\textbf{FIG. 2.} Influence of a \textit{gal80} mutation on the \textit{K. lactis snf1} mutant phenotype. (A) Epistasis analysis of \textit{K. lactis gal80} and \textit{S. cerevisiae snf1} mutations on \textit{LAC4} gene expression. LAC4-encoded β-galactosidase activity was determined in congenic \textit{K. lactis} strains JA6 (wild type), JSD1R (\textit{Δsnf1}), JA6/D802R (\textit{gal80Δ2}) and JSD1D80 (\textit{Δsnf1 gal80Δ2}) after a shift from glucose to fresh glucose or galactose (2%) medium for 7 h. (B) Serial dilutions (1:100) of the same strains were spotted on SC plates containing 2% glucose or galactose. The plates were incubated for 2 days at 30°C.

\textbf{FIG. 3.} \textit{KHT3} functions as a galactose transporter. (A) Influence of \textit{KHT3} overexpression on growth of a \textit{K. lactis snf1} mutant on galactose. The mutant strain JSD1R (\textit{Δsnf1}) transformed with plasmid pKHT3 or the empty vector KEp6 was tested for growth on SC galactose (2%) medium as in Fig. 2. (B) Influence of \textit{KHT3} overexpression on the growth deficiency on galactose and lactose of a \textit{Δlac12} mutant strain. Strain DL12R (\textit{Δlac12}) was transformed with pKHT3 and the empty vector. Growth of the resulting transformants was compared to that of vector-transformed strain JA6 as in Fig. 2B.
**K. lactis** Snf1p is required for Lac12p-mediated galactose uptake at a posttranscriptional step. To be able to detect the Lac12 protein inside the cell, GFP was fused to the C terminus of Lac12p. The fusion protein expressed from its own promoter on a high-copy-number plasmid was able to complement the Gal\(^{-}\)/H11002 Lac\(^{-}\)/H11002 phenotype of the lac12 mutant, indicating that it can substitute for wild-type Lac12p. In wild-type cells, GFP fluorescence was mainly detected in the plasma membrane. Only in some cells was additional fluorescence observed in the vacuole. In striking contrast, for the **K. lactis** snf1 mutant strain we found the opposite distribution: in most cells GFP was localized in the vacuole and only weak fluorescence could be detected in the plasma membrane (Fig. 4).

As controls, the empty vector, GFP-Gal80p, and GFP-Gal1p were expressed in the **K. lactis** snf1 mutant. No fluorescence could be observed with the empty vector, whereas the latter two fusion proteins showed cytoplasmic and nuclear staining, respectively, which was not affected by the snf1 mutation (Fig. 4 and data not shown). By Western analysis with anti-GFP antibodies, all fusion proteins could be detected, but Lac12-GFP was very labile, and we were unable to quantitatively compare the wild-type and **K. lactis** snf1 mutant strains (see supplemental Fig. S1).

Since, due to plasmid loss, the cell population was highly heterogeneous, the **LAC12-GFP** fusion was integrated in the chromosome of the wild-type and snf1 **K. lactis** strains. These strains were used to analyze whether the difference in Lac12-GFP localization reflects a difference in Lac12-GFP turnover or in intracellular sorting. Cells were grown in glucose and shifted to YNB galactose medium, and samples were analyzed at intervals for GFP fluorescence. The GFP signal in these strains was much weaker and could not be detected before 4 h after the shift in the wild type and about 1 to 2 h later in the mutant. Interestingly, at the early time points in both strains, Lac12-GFP was detected in intracellular structures (Fig. 5, top). These structures were more numerous and less distinct in the mutant, and plasma membrane staining was much weaker. At later time points, the fluorescence of the plasma membrane increased in both strains but remained much weaker in the mutant (enhanced in Fig. 5). Most strikingly, intracellular membranes were no longer observed in 95% of wild-type cells, whereas they were very pronounced in 80 to 90% of the mutant cells. We thus conclude that the snf1 mutation prevents the accumulation of Lac12-GFP in the plasma membrane either by affecting a late step in the sorting process or by enhancing endocytosis or both.
In this work, we analyzed the function of the Snf1 kinase in the regulation of galactose metabolism in *K. lactis*. Our data indicate that an impairment of galactose uptake is the primary cause of the galactose deficiency in a *K. lactis snf1* mutant. A multicopy suppressor screen in the *snf1* mutant resulted in isolation of the *KHT3* gene. The Kht3 protein of 528 amino acids belongs to the yeast hexose transporter subfamily of major facilitators. Overexpression of *KHT3* is able to suppress the growth defect on galactose not only of an *snf1Δ* mutant but also of a *lac12Δ* mutant lacking the lactose/galactose transporter Lac12p. It can also support growth on galactose in an *S. cerevisiae* strain lacking all 20 hexose transporters (Wiedemuth and Breunig, unpublished data). We thus concluded that Kht3p functions as a galactose transporter. Since multiple copies of the *KHT3* gene are required to support slow growth on galactose in the *snf1* and the *lac12* mutants, Kht3p apparently works inefficiently as a galactose transporter, and the physiological role of Kht3p remains to be determined. In any case, Kht3 does not seem to require Snf1p to function and can thus circumvent the galactose uptake deficiency in the mutant.

It has been shown previously that Snf1p controls Lac12-mediated sugar uptake (13). Our data indicate that the lactose and galactose transport deficiency is not caused by a lack of *LAC12* transcription. Induction of the *LAC/GAL* regulon is delayed but not abolished in the *K. lactis snf1* mutant and a *gal80* mutation, which causes constitutive expression of the regulon, does not suppress the growth deficiency. These findings indicated that Snf1p was required at a posttranscriptional step. In fact, a Lac12-GFP fusion protein expressed from its own promoter in single copy could readily be detected in the mutant strain, suggesting that Snf1p affects the activity and not the synthesis of the transporter.

Apparently, Lac12p fails to accumulate in the plasma membrane of the *K. lactis snf1* mutant. In contrast to congenic wild-type cells, in which the Lac12-GFP fusion was properly sorted to the plasma membrane, a high proportion of the fusion protein was located in intracellular vesicles in the mutant. This altered intracellular distribution may explain the observed growth limitation on galactose.

When shifted from glucose to galactose, the fusion protein could first be detected in intracellular vesicles before it arrived in the plasma membrane. At this stage, the difference between the wild type and the mutant was less pronounced. The difference became more apparent upon prolonged induction, when the mutant failed to show the plasma membrane staining observed in the wild type. We thus favor the view that *K. lactis* Snf1p activity is required to redirect or stabilize the protein in this compartment.

Regulation of transporter targeting by nutrients is a common phenomenon. Addition of glucose to galactose-grown *S. cerevisiae* cells stimulates monoubiquitination of the major galactose transporter Gal2p, followed by internalization, delivery to the vacuole, and degradation (23, 24). Routing of the general amino acid permease Gap1p depends on the quality of the nitrogen source. On a rich nitrogen source, newly synthesized Gap1 is sorted directly from the Golgi to the vacuole instead of to the plasma membrane. Addition of ammonium ions to a culture grown on a poor nitrogen source leads to endocytosis of plasma membrane-bound Gap1p (10, 21, 33, 35). Both pathways to the vacuole require ubiquitination.

To our knowledge, we have shown here for the first time that intracellular localization of a sugar transporter is affected by an *snf1* mutation. However, several findings are compatible with an involvement of Snf1p in the regulation of transporter activity. For example, the *S. cerevisiae* maltose permease Mal61p requires Snf1p at a posttranscriptional step, since no maltose transport activity is measurable in an *snf1* mutant strain when the *MAL61* gene is expressed constitutively (25).

At present, we can only speculate about the function of Snf1p in sugar transport regulation. The fact that *KHT3* but not *LAC12* in multicopy can suppress the galactose uptake deficiency of a *K. lactis snf1* mutant indicates that Lac12p is more sensitive to the influence of Snf1 than other transporters.

The complex role of *K. lactis* Snf1p in the adaptation of cells to carbon limitation resembles that of the Npr1 serine/threonine protein kinase in adaptation to nitrogen limitation. Besides regulating transcription, Npr1 controls the post-Golgi sorting and degradation of amino acid permeases (29). Snf1 was found associated with the small GTPase Arf1p and its GTPase exchange factor Sec7p, both of which are implicated in vesicle transport (22). In *S. cerevisiae*, one form of the heterotrimeric Snf1 complex is associated with the vacuole (36).

We were unable to express Lac12p at sufficiently high levels in *S. cerevisiae* to reach unambiguous conclusions about the influence of *snf1* in a heterologous host. Moreover, the inability to grow *S. cerevisiae* *snf1* mutants under derepressing conditions complicates the analysis. *K. lactis snf1* can grow readily on lactose and also slowly on galactose and other carbon sources, a fact that is helpful in unraveling the complex regulatory network controlled by Snf1. The data shown here have revealed a new aspect of regulation of carbon utilization by Snf1p in addition to its influence on transcription and enzyme activity.

**ACKNOWLEDGMENTS**

We thank Bob Dickson (University of Kentucky) and Micheline Węsolsol-Louvel (Lyon) for strains and plasmids and Karin Sorge for technical assistance. Konstanze Wiedemuth is gratefully acknowledged for construction of the GFP-Gal80 and GFP-Gal1 fusion plasmids.

This work was supported by DFG grants Br921/5-1 (FOG 466/1-1) and Br921/6-1 to K.D.B.

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