A Hexose Transporter Homologue Controls Glucose Repression in the Methylotrophic Yeast *Hansenula polymorpha*

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Peroxisome biogenesis and synthesis of peroxisomal enzymes in the methylotrophic yeast *Hansenula polymorpha* are under the strict control of glucose repression. We identified an *H. polymorpha* glucose catabolite repression gene (*HpGCR1*) that encodes a hexose transporter homologue. Deficiency in GCR1 leads to a pleiotropic phenotype that includes the constitutive presence of peroxisomes and peroxisomal enzymes in glucose-grown cells. Glucose transport and repression defects in a UV-induced *gcr1-2* mutant were found to result from a missense point mutation that substitutes a serine residue (Ser85) with a phenylalanine in the second predicted transmembrane segment of the Gcr1 protein. In addition to glucose, mannose and trehalose fail to repress the peroxisomal enzyme, alcohol oxidase in *gcr1-2* cells. A mutant deleted for the *GCR1* gene was additionally deficient in fructose repression. Ethanol, sucrose, and maltose continue to repress peroxisomes and peroxisomal enzymes normally and therefore, appear to have *GCR1*-independent repression mechanisms in *H. polymorpha*. Among proteins of the hexose transporter family of baker’s yeast, *Saccharomyces cerevisiae*, the amino acid sequence of the *H. polymorpha* Gcr1 protein shares the highest similarity with a core region of SmfIP, a putative high affinity glucose sensor. Certain features of the phenotype exhibited by *gcr1* mutants suggest a regulatory role for Ger1p in a repression pathway, along with involvement in hexose transport.

If provided with a mixture of carbon substrates, yeast preferentially utilizes the one that supports the fastest growth rate. This is achieved by several coordinated regulatory mechanisms of metabolic adaptation. They include: (i) the induction of enzymes involved in the metabolism of a preferred substrate and (ii) repression and/or inactivation of enzymes involved in the metabolism of less preferred carbon sources. Carbon source-triggered repression (or catabolite repression) generally affects expression of the corresponding target genes at the transcriptional level. Among co-repressor substrates in *Saccharomyces cerevisiae*, glucose is best known (for review see Refs. 1 and 2). The main targets of glucose repression in *S. cerevisiae* are enzymes of gluconeogenesis and the glyoxylate cycle, mitochondrial enzymes involved in oxidative phosphorylation, and enzymes involved in transport and metabolism of alternative carbon substrates, such as galactose, sucrose, and maltose. Despite extensive studies and a growing number of genes known to be involved in glucose repression in this and other species, its actual mechanism, especially in the early stages of sensing and signal transduction, is not fully understood.

In methylotrophic yeasts, unique peroxisomal and cytosolic enzymes of methanol metabolism are under strict control of repression by various carbon substrates, e.g. glucose and ethanol (3, 4). Glucose and ethanol also trigger inactivation of peroxisomal enzymes by a process that involves the autophagic degradation of whole peroxisomes by vacuolar proteases (5–7). Previously, in the methylotrophic yeasts *Pichia methanolica* (formerly *P. pinus*) and *Candida boidinii*, we and others showed that glucose and ethanol repression and degradative inactivation of peroxisomal enzymes are controlled by separate carbon source-specific sets of regulatory genes (4, 8, 9). One complementation group of *P. methanolica* mutants defective in glucose repression was found to be deficient in phosphofructokinase activity. We proposed that this enzyme has a second signaling function in repression that is distinct from its catalytic activity (8).

The methylotrophic yeast *Hansenula polymorpha* (syn. *Pichia angusta*) is an important organism for biotechnological use, e.g. heterologous gene expression, and for basic research on peroxisome biogenesis and degradation (7, 10). There are several reports describing *H. polymorpha* mutants defective in glucose repression (11–13). Kramarenko *et al.* (11) demonstrated that glucose has to be phosphorylated in order to cause repression in *H. polymorpha*, and mutants impaired in activities for hexo- or glucokinases are insensitive to repression. However, the molecular nature of other mutations that impair glucose repression in *H. polymorpha* has not been determined (12, 13).

In a previous report, we described the isolation and characterization of *H. polymorpha* mutants in a gene named *GCR1* that are defective in glucose repression (14). The abbreviations used are: GCR, glucose catabolite repression; AO, alcohol oxidase; TM, transmembrane domain; P_{AO}, promoter of the *AO* gene; EGF, enhanced green fluorescent protein; PTS, peroxisomal targeting signal; ORF, open reading frame.

The authors chose to use the name of the gene as it appeared in their original reports (14, 15), despite the fact that it coincides with the name of a different *S. cerevisiae* GCR1 gene, involved in the translational activation of glycolytic proteins.
cells of ger1 mutants exhibit pleiotropic alterations in cellular metabolism, namely: (i) the constitutive synthesis of the peroxisomal enzymes, alcohol oxidase (AO) and catalase, and the constitutive presence of peroxisomes; (ii) a decrease in growth rate; and (iii) a decrease in levels of glycolytic intermediates but wild-type levels of activity for each of the primary glycolytic enzymes. In addition, and unlike in wild-type cells, cytosolic enzymes required for methanol metabolism (formaldehyde and formate dehydrogenases) and α-glucosidase are not repressed in ger1 mutants grown in medium containing glucose along with either methanol or maltose, respectively. When shifted from methanol to glucose medium, AO is not inactivated in ger1 mutants. We suggested that a glucose transport defect might be responsible for the phenotypes displayed by our ger1 mutants (14, 15). Here, we further characterize the phenotype of ger1 mutants, describe the cloning and sequence analysis of the GCR1 gene and its product, Ger1p, the construction of a GCR1 deletion strain, and the identification of a missense mutation in a UV-induced mutant, ger1-2. Finally, we discuss the possible involvement of Ger1p in glucose sensing of repression in H. polymorpha.

EXPERIMENTAL PROCEDURES

Strains, Media, and Microbial Techniques—H. polymorpha strains used are listed in Table I. Auxotrophic strains AS8 (leu10), kindly supplied by Dr. P. Sudbery (University of Sheffield), and leu1–1 (both derived from NCTC495), were used in this study as the wild-type strains as indicated. The cells were grown at 37 °C in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or a minimal medium (0.17% w/v) yeast nitrogen base without amino acids (Difco, Detroit, MI) with 0.5% w/v ammonium sulfate as a nitrogen source. Concentration of carbon sources was 1% if not indicated otherwise. Amino acids were added to a final concentration of 50 μg/ml as required. For solid media, g/ml as required. For solid media, 1:10 in each case) by electroporation into H. polymorpha.

| Strain          | Genotype                  | Ref.   |
|-----------------|---------------------------|--------|
| AS8             | leu10                      | 14     |
| leu1–1          | –                         | 16     |
| met6            | –                         | 16     |
| leu1–1 met6     | –                         | This study |
| ade1–1          | –                         | 16     |
| leu1–1 ade11    | –                         | This study |
| gcr1-2          | gcr1-2 leu10              | 14     |
| gcr1-2          | gcr1-2 leu1-1             | This study |
| ade11 (GFP-PTS1)| gcr1-2 ScLEU2/Pmax-GFP-PTS1:ScLEU2 leu1-1 | This study |
| wt (GFP-PTS1)   |                           |        |
| gcr1-2 (GFP-PTS1)|                           |        |

of the methionine initiator ATG of GCR1 was amplified by PCR using plasmid pOS22 as a template with Vent DNA polymerase (NEB, Beverly, MA). The primers for this PCR, SO58 and SO57 (5′-ATCCTGGATCCCTCTTACGTTGTA-3′), specific for adjusted HpARS region on pYT3, and plasmid pOS22 as a template (see main text). Double stranded DNA sequencing was performed with gene-specific 18–21 bp primers. Oligonucleotides were purchased from Integrated DNA Technologies (Corvalle, IA). DNA sequencing was performed at the Oregon Regional Primate Research Center, Molecular Biology Core Facility (Beaverton, OR). For analysis of DNA and amino acid sequences, MacVector software (IBI, New Haven, CT) was used. Sequence alignments were performed using the ClustalW version 1.6 algorithm (22). The BLAST Network Service of the National Center for Biotechnology Information (Bethesda, MD) was used to search for amino acid sequence similarities.

To localize a putative mutation in the ger1-2 mutant allele, a number of pOS22 fragments comprised of different portions of GCR1 ORF were analyzed for ability to functionally complement the corresponding ger1-2 leu1-1 mutant. The fragments were isolated after digestion of pOS22 with selected restriction enzymes, and as PCR products, with pOS22 as a template and primers: SO53 (5′-ATCGAAGCTTCCCTT-3′), SO56 (5′-ATCCTGGATCCCTCTTACGTTGTA-3′), SO56 (5′-AA-CACCATGCAATGTCGAG-3′). The fragments were co-transformed along with plasmid pYT3 (molar concentration ratio of fragment versus plasmid was ~1:10 in each case) by electroporation into ger1-2 leu1-1 strain. Transformants were selected for leucine prototrophy on minimal YNB plates with sucrose. Colonies were further replicated on 1-Glc plates, and high, 55 μm glucose plates (h-Glc), for AO colony assay. To isolate the mutated ger1-2 gene and identify the mutation, the total genomic DNAs of ger1-2 leu1-1, and original ger1-2 leu10 mutant were extracted and used as templates in PCR reactions with TaqDNA polymerase (Invitrogen, Life Technologies, Inc.), primers SO58 (5′-CCCA-AGCTTTAAACGGAGTAAATCCT-3′) and SO72. Resulting 2.75-kb fragments were sequenced with the same GCR1–specific primers as used for sequencing of the wild-type gene. To exclude potential PCR amplification mistakes, two independently amplified fragments were sequenced in both directions. In addition, the wild-type fragment isolated by anologous PCR from leu10 parental strain was sequenced and served as a control. Nucleotide sequences were aligned using MacVector software to identify the site of a putative mutation.

Construction of a GCR1 Deletion Strain—A vector capable of deleting most of the HpGCR1 ORF was constructed in two steps. In the first step, a 0.5-kb fragment containing sequences just 5′ of the methionine

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Table I

H. polymorpha strains used in this study

All strains were derived from H. polymorpha NCYC 495. The genotype of a strain is not shown when it coincides with the strain’s name. In biochemical experiments, ger1-2 leu1-1 and the isogenic wild-type leu1-1 strains were utilized throughout this study, except that strains ger1-2 leu10 and isogenic AS8 (leu10) were used in glucose uptake experiments, and mixed substrate utilization experiments (referred to in text as ger1-2 and a wild type, respectively).
Fig. 1. Nucleotide and amino acid sequences of the *H. polymorpha* GCR1 gene and its product. A, twelve predicted membrane-spanning segments (TM 1–12) are numbered and underlined. A putative uORF is underlined, a potential TATA box is shown in bold, and potential binding sites for a Mig1p-like repressor protein are shown in italics in the GCR1 5′-region. B, hydrophobicity profile derived from the predicted amino acid sequence of Gcr1p according to the method of Eisenberg et al. (37) with a window size of 17 amino acids. Hydropathy values are on the y-axis, and the residue numbers are on the x-axis.
HindIII and XbaI, releasing a 3.9-kb fragment comprised of ScLEU2 flanked by GCR1 5’ and 3’ sequences and transformed into the leu1-1 met6 strain by electroporation. This double auxotrophic hybrid was isolated from the spore progeny of a diploid strain resulting from crossing of leu1-1 and met6. Transformants were selected and analyzed for Ger phenotype as described in the main text. To confirm deletion of the GCR1 gene, genomic DNAs were isolated from several Gcrleu1-1 formed into GAAGCTTATGGTGAGCAAGGGCGAG-3’ PMOX was linearized in the unique StuI site in the GTTTATAACTTTGCGGTTGATTGGGCG-3’ terminus (EGFP-PTS1). As the next step, the EGFP-PTS1 0.8-kb fragment was inserted into the unique HindIII site of pET1 down-flanked by ScLEU2 leu1-1 met6. Two sets of primers hybridized to sequences in the 5’ and 3’ flanking regions of the GCR1 ORF, present in both, the wild type and a deletion allele. Second primer, SO72, hybridized to sequences in the 5’ region of the wild-type GCR1 ORF that were absent in the gcr1 allele. The other set of primers contained the same 3’ flanking sequence primer SO56 and a second primer complementary to a sequence in ScLEU2, SO90 (5’-TAAGAAGATCGTCGTTTTGCC-3’). The first set of primers produced a 1.3-kb long fragment with only the wild-type genomic DNA as template, while the second set generated a 1.9-kb long fragment with genomic DNAs of each of the putative candidate gcr1-deletion transformants as template, but not with wild-type DNA. One transformant, gcr1 Δ:ScLEU2 leu1-1 met6, was utilized throughout this study as a gcr1 deletion strain.

Construction of Strains with Fluorescently Labeled Peroxisomes—pOS18, an E. coli-H. polymorpha shuttle vector capable of expressing a peroxisome-targeted red shifted form of the green fluorescent protein (EGFP-PTS1) under control of the H. polymorpha AO promoter (P_{MOX}) was constructed. As a first step, plasmid pOGP2-3 was constructed by introducing an adapter fragment encoding the last nine amino acids and stop codon of Pichia pastoris Pex8p (including the PTS1 sequence, C-terminal AKL) into a pEGFP-C3 vector (Clontech Laboratories, Inc., Palo Alto, CA) as described (23). It resulted in a chimera encoding EGFP with the last nine amino acids of Pex8p fused in-frame to its C terminus (EGFP-PTS1). At the next step, the EGFP-PTS1 0.8-kb fragment was amplified from pOGP2 by PCR with primers SO40 (5’-GT-GAAGCCTTATGGTGACCCGGCAG-3’) and SO2 (5’-AGCTACCG-GTTTATAACCTTCCGGGTGATTGGCCG-3’) that introduced HindIII flanking sites immediately 5’ and 3’ of the EGFP fusion gene. The fragment was inserted into the unique HindIII site of pET1 (18) downstream of P_{MOX}, in required orientation to produce pOGP3. The latter was linearized in the unique SacI site in the P_{MOX} sequence and transformed into leu1-1 wild type and gcr1-2 leu1-1 strains. Isolated prototrophic transformants were grown on methanol plates to induce P_{MOX}, and fluorescence was examined directly in yeast colonies with fluorescent microscope. To identify stable integrants, individual fluorescent clones were examined for mitotic stability by repeated shifting from selective methanol minimal to non-selective YPD medium. Two strains, WT (GFP-PTS1), a wild-type, and gcr1-2 (GFP-PTS1) (Table I, Fig. 2) were further utilized in this study. To isolate a gcr1 Δ GFP-PTS1-expressing strain, ade11 (GFP-PTS1) wild-type strain was isolated first in the same way as described above, by transforming leu1-1 ade11 with pOS18. The resulting strain was crossed with a gcr1 Δ met6-null mutant, and recombinant prototrophic gcr1 Δ (GFP-PTS1) was isolated from spore progeny as a fluorescent clone on glucose medium also unable to grow on 1-Glc plates.

Biochemical Methods—Preparation of crude cell free extracts was performed as described previously (23). AO activity was measured in cell-free extracts as described (24), and expressed as micromoles of product/min/mg of protein. Protein concentration in culture media with the alcohol oxidase-based enzymatic kit Alcotest as described (26).

Glucose Uptake Assays—For glucose transport assays, cells were grown on 1% glucose YNB medium until mid-logarithmic phase and harvested at a cell density of 1–1.5 mg dry weight/ml. Cells were washed twice by centrifugation in distilled water at 3,000 × g. Sugar transport was measured at 20 °C, starting with the addition of 0.1 ml of

| Glucose | AO activity | 5 mM | 25 mM | 55 mM |
|---------|-------------|------|-------|-------|
| Wild type | 0 | 0 | 0 | 0 |
| gcr1-2 | 2.0 | 2.4 | 2.5 | 2.5 |
| gcr1 Δ | 1.0 | 1.0 | 1.5 | 1.5 |

**TABLE II**

Effect of extracellular glucose concentration on AO activity in gcr1 mutants

- FIG. 2 Alignment of selected amino acid sequence regions of *H. polymorpha* Gcr1p, *S. cerevisiae* Snf3p and Rgt2p, *K. lactis* Rag4p, and *N. crassa* Rec0p. A, transmembrane domain regions. The serine residue (S85) altered in the gcr1-2 mutant to phenylalanine by a missense mutation is shown in bold and indicated by an asterisk. Predicted membrane spanning segments designated as TM 1–12 are shown with an overline. B, C-terminal regions. Conserved amino acid residues are shown with black shaded areas indicating identical residues, and light gray areas indicating similar residues.

For glucose transport assays, cells were grown on 1% glucose YNB medium until mid-logarithmic phase and harvested at a cell density of 1–1.5 mg dry weight/ml. Cells were washed twice by centrifugation in distilled water at 3,000 × g. Sugar transport was measured at 20 °C, starting with the addition of 0.1 ml of...
Effect of carbon source on AO activity and growth rate in wild-type and gcr1 strains

| Carbon source | Fructose | Mannose | Xylose | Maltose | Trehalose | Sucrose |
|---------------|----------|---------|--------|---------|-----------|---------|
|               | d.t. AO  | d.t. AO | d.t. AO| d.t. AO | d.t. AO   | d.t. AO |
| Wild type     | 2.6 0    | 2.6 0   | 15     | 15      | 4.6 0     | 6.6 0   |
| gcr1-2        | 2.6 0    | 5.5 140 | 28     | 120     | 4.6 0     | 11.3 95 |
| gcr1Δ         | 6.2 55   | 5.8 90  | 30     | 98      | 4.6 0     | 15.0 66 |

* Carbon source, 1% w/v each.

The uniformly labeled [14C]glucose or [14C]fructose in a final volume of 0.2 ml in 0.1 M potassium phosphate buffer (pH 6.0). Cell concentration was 50 mg/ml. Ten seconds later, transport was stopped by adding 8 ml of ice-cold 0.8 M glucose in the same buffer essentially as described (27). Samples were immediately filtered under vacuum and washed twice with 10 ml of ice-cold glucose solution. The treatment of control samples differed in that the cold glucose was added first to the cells and labeled sugar. Thereafter, the reaction was kept at 0°C. Samples were transferred to scintillation vials with 2 ml of scintillation liquid. A portion of the reaction mixture served as a reference to determine the total radioactivity. The radioactivity was measured with a liquid scintillation counter (Rac-Beta 1219, LKB). The final glucose concentration ranged from 0.5 to 50 mM. The glucose consumption rate (V_{cm}) was expressed as grams per hour per gram of dry weight.

**Electron and Fluorescence Microscopy**—Cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously (28). Immunolabeling was performed on ultrathin sections of Unicryl-embedded cells using specific antibodies against H. polymorpha AO and goat anti-rabbit antibodies conjugated to 15 nm gold particles (Amersham Biosciences) according to the instructions of the manufacturer. Fluorescence microscopy was performed essentially as described (23).

**RESULTS**

**Cloning and Sequence Analysis of the GCR1 Gene**—The GCR1 gene was isolated by functional complementation of a gcr1-2 leu1-1 mutant (see “Experimental Procedures”) with an H. polymorpha genomic DNA library (18). To clone the gene, we made use of the severe growth defect of the mutant at low extracellular glucose concentrations (14, 15). Library transformants were selected simultaneously for leucine prototrophy and for restored ability to grow on low glucose (5 mM) agar medium (l-Glc^-). Four transformants displaying a Leu^- l-Glc^- phenotype were further examined for AO activity in colonies grown on high (55 mM) glucose plates. All displayed a wild-type phenotype, i.e. full repression of AO synthesis by glucose (Aog^-). To isolate the GCR1 gene, total DNA was extracted from the transformants, and, after transformation of the total genomic DNA preparations into E. coli and amplification, four plasmids were recovered. All four were able to transform the gcr1-2 leu1-1 strain to Leu^+, l-Glc^-, and Aog^- phenotypes at high efficiency, suggesting that the plasmids most likely each harbored the complementing GCR1 gene. Restriction mapping of these four plasmids revealed identical 2.0-kb PstI and 1.2-kb SalI fragments in the genomic DNA inserts in each. Both restriction fragments were found to originate from within an ~3.3-kb long region of genomic DNA present in one of the plasmids, named pOS22. Subsequent sequence analysis of this fragment revealed a single open reading frame (ORF) of 1,623 bp, the putative GCR1 gene, predicted to encode a polypeptide of 541 amino acids (Fig. IA). This ORF was subsequently shown to be GCR1 (see below).

A search of the protein databases revealed significant sequence similarity between the deduced amino acid sequence of Ger1p and a number of proteins belonging to the large family of hexasaccharide transporters from different organisms (30, 31). The protein with the strongest similarity was AmMst-1p from the fungus Amanita muscaria (48% identity, 65% similarity) (GenPept accession no. CAB06078). Ger1p was also found to share 44% identity and 62% similarity with a core region of 478 amino acids from the S. cerevisiae high affinity glucose sensor, Snf3p (32, 33). Other proteins with strong similarity included:
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S. cerevisiae Rgt2p (34), Kluyveromyces lactis Rag4p (35), and Neurospora crassa Reo3p (36). Gcr1p exhibited less than 36% identity to other proteins of the hexose transporter family. Gcr1p is predicted to contain twelve membrane-spanning domains (TM) (Fig. 1B). These TMs are characteristic of the hexose transporters and related carriers (30, 31). Alignment of the putative Gcr1p homologues showed that the TMs were also the most conserved regions in the primary sequences (Fig. 2A).

Gcr1p and its putative homologues, ScSnf3p, ScRgt2p, KlRag4p, and NcRco3p, had in common several unique conserved amino acid residues not found in other hexose transporters. They included Gcr1p tyrosine residues Tyr739 and Tyr826, with Tyr826 predicted to be the target of a tyrosine protein kinase (38). An interesting feature of Gcr1p is that its hydrophilic region between TM6 and TM7, is larger relative to the other homologues and consists of 80 amino acid residues, whereas this region in the other proteins ranges from 71 to 74 amino acids. In addition, Gcr1p lacks a C-terminal extension present in its putative yeast homologues. However, a short sequence of amino acids exhibiting similarity to the so-called “glucose-sensing” domains of ScSnf3p, ScRgt2p, and KlRag4p is present in the Gcr1p C terminus (Fig. 2B) (33). The consensus sequence from this region is (M/L)G(I/L)X4(K/R)G, with two glycine residues (Gly520 and Gly527 in Gcr1p) that are conserved in each protein. Such a conserved amino acid sequence is not found at the C termini of other hexose transporters. In the promoter region of the GCR1 gene, a small ORF of 93 bp was identified at positions -146 to -84 bp upstream of the GCR1 translational start site (Fig. 1A). Small uORFs with distinctive sequences are also found in the ScSnf3 and NcRco3 promoter regions and have been proposed to play regulatory functions in the expression of the associated ORFs (36). A potential TATA box for the GCR1 gene is located at position -58 bp. Also, four hypothetical binding sites for a putative Mig1-like repressor protein exist at -24, -89, -105, and -335 bp upstream of the GCR1 translational start codon (Fig. 1A). The consensus sequence for the four sites is (A/G)/(A/G)AAAN(C/G)TGGGG, which corresponds well to that suggested for ScMig1p and found also in the ScSnf3 promoter region (39).

**Construction of a GCR1 Deletion Strain**—To confirm that the identified ORF was the GCR1 gene, a deletion mutant was constructed by the gene replacement method. For this, plasmid pOS29 was constructed in which 1479 bp of the GCR1 coding sequence (encoding amino acid residues 1–493) were replaced by a fragment containing the S. cerevisiae LEU2 gene as described under “Experimental Procedures.” This gcr1A::ScLEU2 allele was released with two restriction enzymes on a 3.9-kb DNA fragment and transformed into H. polymorpha leu1-1 met6. Leu+ transformants were selected on a sucrose-containing medium without leucine and subsequently analyzed for typical Gcr- (l-Glc+, Aog+) phenotypes. Total genomic DNA was isolated from several transformants unable to grow on l-Glc medium (l-Glc-) and displaying high AO activity on h-Glc plates (Aog+). With this DNA used as a template, PCR analysis indicated a correctly targeted chromosomal integration of the gcr1A::ScLEU2 fragment (not shown). Subsequently, the gcr1A::ScLEU2 leu1-1 met6 strain was crossed with a gcr1-2 leu1-1 strain and prototrophic diploid cells were examined for the Gcr- phenotype. All were l-Glc- and Aog+. Additionally, after sporulation, 1,000 spore products were grown on sucrose plates, then replica plated onto l-Glc plates. No colonies were observed. Together, these results demonstrated that the gcr1-2 and gcr1A alleles were tightly linked and most probably mutant alleles of the same gene.

**Cloning of the gcr1-2 Mutant Allele**—The phenotype of the gcr1-2 mutant was similar but not identical to that of a gcr1A strain. We determined the molecular nature of the gcr1-2 mutation by isolating the mutated gene from genomic DNA of gcr1-2 by PCR, and sequencing (see “Experimental Procedures” for details). A point mutation was identified that caused a transition from C to T at position 254 of the GCR1 ORF. In addition, only those fragments of pOS22 that contained the N-terminal part of the wild-type GCR1 gene including nucleotide 254, were able to rescue our gcr1-2 mutation when integrated into the mutant genome, thus confirming the 5' location of the site of the mutation in the gene (not shown). The mutation resulted in the substitution of a semi-conserved serine residue (Ser135) with a phenylalanine (Fig. 2A). At this position, only one of three amino acid residues is found in hexose transporters: alanine, glycine, or, in a majority of proteins, serine.
Effect of Carbon Substrates on AO Repression and Peroxisome Biogenesis in gcr1 Mutants—Defects in the GCR1 gene lead to synthesis of the peroxisomal enzyme AO in glucose-grown cells (Table II and Ref. 14). The level of AO induction in glucose-grown mutant cells was comparable or higher relative to wild-type cells induced by methanol under analogous conditions (1.7 units/mg). Remarkably, the AO-repression defect was more pronounced in the missense gcr1-2 mutant relative to the gcr1/H9004 mutant (Table II). The defects in repression were associated with the presence of AO-containing peroxisomes in mid-exponential glucose-grown cells of gcr1 mutants (Fig. 3 and Ref. 14).

Both gcr1-2 and gcr1Δ cells were impaired in the repression of AO synthesis in response to a number of other carbon substrates that are strong AO repressors in the wild-type strain (Table III). This defect was accompanied by retarded growth of both gcr1 mutants on these substrates relative to the wild-type strain. In addition to glucose, other sugars that no longer repressed AO in gcr1 mutants included mannose, xylose, and trehalose. Substrates that continued to normally repressed AO synthesis were sucrose, maltose, and ethanol. Remarkably, we observed that repression in response to fructose was defective in the deletion strain but was normal in the gcr1-2 mutant (Table III).

To confirm that the AO repression defect in gcr1 mutants on different carbon sources corresponded to altered transcriptional regulation of the AO promoter (PMox), we constructed strains with fluorescently labeled peroxisomes. These strains expressed peroxisome-targeted enhanced green fluorescent protein (EGFP-PTS1) under control of PMox. As expected, microscopic examination revealed fluorescent spots in wild-type and gcr1 cells with GFP-PTS1 labeled peroxisomes and FM64-labeled vacuoles. Time points indicated on the top correspond to methanol-induced cells (0), and those after 2 and 4 h of glucose adaptation.

Ultrastructural Studies of Peroxisome Degradation in gcr1 Mutants—When shifted to fresh glucose medium, methanol-grown cells of the wild-type strain exhibit a fast decrease in AO
activity due to the selective autophagic degradation of peroxisomes (termed pexophagy) (5). We addressed the question as to whether deficiency in the GCR1 gene also affects the pexophagic process. We observed a decrease in AO specific activity and AO protein levels in gcr1 cells upon glucose adaptation, but residual AO levels were higher in the gcr1 mutants relative to wild type (Fig. 5A). However, these data do not demonstrate a direct involvement of Gcr1p in pexophagy since in our gcr1 strains, de novo peroxisome synthesis occurs due to the defect in glucose repression.

A time course examination of cell morphology revealed clear signs that pexophagy in gcr1 mutants proceeds. Some peroxisomes were observed sequestered by additional membrane layers typical for initial stages of macroautophagic pexophagy degradation in H. polymorpha (Fig. 5B) (7). Also, in gcr1 cells with fluorescently labeled peroxisomes, the pexophagic process was evident upon glucose adaptation. Shortly after the shift, GFP fluorescence was observed in vacuoles, while in methanol-growing cells it is confined to peroxisomes (Fig. 5C). These data led to the conclusion that Gcr1p is not directly involved in pexophagy. Both gcr1 mutants continued to exhibit normal wild-type pexophagy degradation in response to ethanol (not shown).

Glucose Uptake and Consumption in H. polymorpha gcr1-2—The glucose repression defect in the gcr1-2 mutant was accompanied by retarded growth on glucose (14). Since glucose phosphorylation activity was normal in gcr1-2 cells, we suggested that a defect in glucose transport might be the primary cause of mutant catabolite repression deficiency (14, 15). In further investigations of this phenotype, we have determined that the rate of glucose consumption by the gcr1-2 mutant relative to the wild-type strain was decreased at all extracellular sugar concentrations (Fig. 6). The relative difference between the two strains was most pronounced at low extracellular glucose concentrations (e.g. at 5 mM the rate was 3.5-fold slower in the mutant relative to wild type), but this effect diminished with increasing glucose concentrations (e.g. at 55 mM the rate was only 1.3-fold slower than wild type) (Fig. 6). Using [14C]glucose, we observed that the kinetics of glucose uptake in the gcr1-2 mutant closely matched that of glucose consumption (Fig. 6). In contrast, fructose was transported and consumed by the mutant at wild-type rates (not shown).

Consistent with a relative decrease in the rate of glucose transport at low extracellular glucose concentrations (Fig. 6), the gcr1-2 mutant was able to grow on methanol or maltose media containing 1 mM, but not 10 mM 2-deoxyglucose (not shown). Either concentration of 2-deoxyglucose, a toxic glucose analogue capable of exerting a repression effect, completely blocked growth of wild-type cells in these two media.

Effect of Extracellular Glucose Concentration on AO Repression in gcr1 Mutants—The deficiency of the gcr1-2 mutant in AO repression is not a function of extracellular glucose concentration and, consequently, glucose uptake. In cells incubated in high-glucose (55 mM) medium, where the transport defect in the gcr1-2 mutant is less pronounced, AO activity was the highest (Table II). In low-glucose (5 mM) medium, where glucose uptake in the gcr1-2 mutant is severely impaired, AO activity was lower. A similar pattern of AO activity levels relative to glucose concentration was also displayed by our gcr1Δ strain. In the wild-type strain, any of these glucose concentrations was sufficient to completely repress AO synthesis (Table II).

Growth of the gcr1-2 Mutant in Glucose/Methanol Mixtures—Consistent with the classical catabolite repression paradigm (29), a wild-type strain of H. polymorpha utilizes glucose first when incubated in a glucose/methanol mixture, while enzymes of methanol utilization remain repressed. In the gcr1-2 mutant, we observed that the utilization of these carbon sources was reversed, i.e. glucose consumption initiates following exhaustion of methanol from the medium (Fig. 7). Furthermore, methanol, when administrated as a pulse to mid-exponential glucose-grown gcr1-2 cells, transiently blocked growth and glucose utilization of the strain. After a lag-period of several hours, gcr1-2 cells resumed growth, utilizing methanol as carbon substrate (not shown). Addition of methanol to glucose-grown wild-type cells did not affect the growth and substrate utilization pattern. Consistent with our previous data, fructose remained the preferred substrate in the gcr1-2 mutant grown in fructose/methanol mixtures (not shown).

DISCUSSION

The selection procedure utilized to isolate the gcr1 mutants presumed a pleiotropic phenotype. One mutant phenotype was the ability to grow on methanol in the presence of 2-deoxyglucose. Growth on methanol in the presence of this compound requires deregulation of genes encoding enzymes and other proteins essential for methanol metabolism (5). Another phenotype is insensitivity to the accumulation of toxic phosphorylated derivatives of 2-deoxyglucose, e.g. by reducing the rate of glucose uptake or early steps in glucose catabolism. In this report, we demonstrate that the gcr1-2 mutant is deficient both in glucose repression and glucose uptake, and that the GCR1 gene encodes a protein homologous to other hexose transporters. The most remarkable feature of gcr1 mutants is the constitutive presence of AO-containing peroxisomes in glucose-grown cells. Such a phenotype confirms that the H. polymorpha AO gene is controlled primarily at the transcriptional level by repression/depression mechanisms and that full expression of the gene is not dependent on a methanol induction mechanism (see also Ref. 4).

We demonstrated that deficiency in GCR1 does not block autophagic peroxisome degradation upon adaptation of methanol-grown cells to glucose. Nevertheless, gcr1 mutants are capable of preferentially utilizing methanol in the presence of glucose. This fact suggests that glucose metabolism is required for pexophagy to proceed. The gcr1 mutants may serve as a unique model to study molecular mechanisms of peroxisome homeostasis. An intriguing question is how the fate of pre-existing and newly formed peroxisomes is regulated in the mutants under pexophagy-triggering conditions.

A gcr1Δ mutant retains the capability to grow well on elevated concentrations of glucose, suggesting that other sugar transporter(s) facilitate the uptake of glucose in the absence of...
An H. polymorpha Hexose Transporter Gene Involved in Glucose Repression

Ger1p. Two kinetically distinct glucose transport systems have been described in *H. polymorpha*: a high and a low-affinity system (40). Our data suggest that Ger1p could be primarily involved in high-affinity glucose transport, as growth of ger1 mutants at glucose concentrations of less than 5 mM is severely hampered. However, to determine whether Ger1p is, in fact, a functional glucose transporter, further studies are required.

For *S. cerevisiae* hexose transport mutants, glucose uptake capacity determines the strength of the repression signal (41, 42). However, in our *H. polymorpha* ger1-2 mutant, the defect in AO repression did not correlate with a concentration-dependent glucose transport capacity, as AO levels were higher in cells fed with higher concentrations of glucose. Thus, it is possible that Ger1p somehow functions directly in AO repression, rather than just as a sugar carrier. The phenomenon of methanol inhibition of glucose utilization in the absence of methanol, rather than just as a sugar carrier. The phenomenon of possible that Gcr1p somehow functions directly in AO repression did not correlate with a concentration-dependent glucose transporter, further studies are required.

In conclusion, we have identified and characterized a gene of the *H. polymorpha* Hexose Transporter system (43). For *ScSnF3p*, this region is functionally essential for glucose sensing (43). A unique short amino acid sequence at the C terminus of the Ger1p C terminus exhibits similarity to the putative C-terminal "glucose-sensing" domains of *ScSnF3p*, *ScRgt2p*, and *KIRag4p* (Fig. 2B). Although, this similarity is too low to conclude that Ger1p plays a role in glucose sensing from sequence comparison alone, the concept that Ger1p is a sensor protein that affects glucose transport via a signaling mechanism similar to *ScSnF3p* and *ScRgt2p* is a reasonable working hypothesis.

The mutation in ger1-2 is predicted to result in the substitution of a serine residue for a phenylalanine at position 85 in the amino acid sequence of Ger1p. This amino acid change resides in the second predicted transmembrane segment (see Fig. 2A), which overlaps with a leucine zipper motif in many hexose transporters (30). The leucine zipper motif has been proposed to be essential for hetero- or homo-oligomerization of hexose transporters (30). It was also shown that the glucose-sensing function of *ScSnF3p* requires the presence of at least one of the *HXT* gene products, suggesting their possible interaction (44). Although this putative leucine zipper motif appears to be rather degenerate in Ger1p and its closest homologues, Ger1<sup>85Fp</sup> may be unable to form oligomeric structures or to correctly interact with other downstream components involved in signaling for repression. Alternatively, a mutation in the second TM may lead to cytoplasmic mislocalization of Ger1p, as demonstrated for one of the *ScSnF3p* mutants, namely in *snf3<sup>7-2</sup>* (45). This question will be addressed in further studies.

The second membrane-spanning segment of hexose transporters is not thought to be involved in formation of a channel pore (31).

Remarkably, the glucose repression defect in the ger1-2 mutant is more profound than in the *ger1*Δ strain. In addition, the ger1-2 strain is not defective in fructose-mediated repression, while the *ger1*Δ strain is. Similarly, certain mutants in *ScSNF3* and *NcRCO3* also exhibit phenotypes different from their respective null mutant strains. For example, an rco3<sup>1</sup> mutant was able to conidiate within the agar of solid medium, while the deletion mutant, rco3<sup>2</sup>, like the wild-type strain, could not. Other glucose-repressible phenotypes were the same between *N. crassa rco3* mutants (36). These results can be explained if one assumes that the peculiar and distinct phenotypes of the missense mutants are the result of an abnormal function conferred on the protein, whereas null phenotypes result from the abolition of all functions of that protein.

Results with the ger1 mutants suggest the existence of specific *GCR1*-dependent repression pathway(s) in *H. polymorpha* for sucrose, maltose and ethanol. In both the missense ger1-2 and *ger1*Δ mutants, these substrates continue to repress AO synthesis and to support a wild-type growth rate on these substrates. In contrast, trehalose, mannose, fructose, and xylose seem to have *GCR1*-dependent repression and transport mechanisms. This observation suggests that, in *H. polymorpha*, hydrolysis of a disaccharide to hexose residues precedes the transport step for trehalose, but not maltose and sucrose, a conclusion that is consistent with other reports (40).

The sugar-specific phenotype of ger1 mutants provides an opportunity for their exploitation as hosts for *P<sub>MOX</sub>*-directed expression of heterologous proteins (7), as demonstrated in this report with *EGFP-PTS1*. Such an expression system would combine the advantages of the strong regulatable *P<sub>MOX</sub>* with the utilization of convenient sugar substrates (i.e., sucrose and glucose, respectively) for growth and induction of ger1-based production strains, while avoiding the use of toxic and flammable methanol. We have also successfully utilized the glucose repression-deficient mutants derived from ger1<sup>1</sup>, for AO production in glucose medium (46, 47).

In conclusion, we have identified and characterized a gene essential for glucose repression in *H. polymorpha*. This gene, *GCR1*, encodes a hexose transporter homologue. We have also demonstrated that deletion of this gene, or a point mutation that causes a single amino acid substitution relieves repression triggered by several but not all sugar substrates. Our data implicate Ger1p in an early stage of the repression mechanism, functioning either in glucose transport or glucose signaling.

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