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 (Ser499) 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 Snf3p, a putative high affinity glucose sensor. Certain features of the phenotype exhibited by gcr1 mutants suggest a regulatory role for Gcr1p 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 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 triggering of glycolytic enzymes.

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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).

<table>
<thead>
<tr>
<th>Strain</th>
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</tr>
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<td>met6</td>
<td></td>
</tr>
<tr>
<td>leu1-1 met6</td>
<td></td>
</tr>
<tr>
<td>ade1-1</td>
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<tr>
<td>gcr1-2</td>
<td></td>
</tr>
<tr>
<td>gcr1-2 ade11 (GFP-PTS1)</td>
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</tr>
<tr>
<td>wt (GFP-PTS1)</td>
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<td>gcr1-2 (GFP-PTS1)</td>
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<tr>
<td>gcr13 (GFP-PTS1)</td>
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**Experimental Procedures**

**Strains, Media, and Microbial Techniques—**H. polymorpha strains are listed in Table I. Auxotrophic strains AS8 (leu10), kindly supplied by Dr. P. Sudbery (University of Sheffield), and leu1-1 (both derived from NCYC495), 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, agar was added to 2% (w/v). Sporulation/matting media and techniques were essentially as described (16). Absorbance was determined at 600 nm and yeast cells density calculated as mg dry weight per ml using a calibration curve. Cultivation of Escherichia coli DH5α and standard recombinant DNA techniques were performed essentially as described (17).

Cloning and Sequence Analysis of the GCR1 Gene and Ger1-2 Mutant Allele—For the GCR1 gene cloning, a recombinant mutant strain ger1-2 leu1-1 was isolated from spore progeny after crossing the original ger1-2 leu10 mutant (14) with leu1-1 wild-type strain. To isolate the GCR1 gene, the mutant ger1-2 leu1-1 was transformed with a H. polymorpha genomic pYT3-based DNA library carrying ScLEU2 gene as a selectable marker (18) by the electroporation method (19). Following selection for leucine prototrophy on YND plates with low, 5 mM glucose (l-Glc), marker (18) by the electroporation method (19). Following selection for genomic pYT3-based DNA library carrying gene, the mutant strain. The prototrophic transformants were analyzed for carbon sources was 1% if not indicated otherwise. Amino acids were supplied by Dr. P. Sudbery (University of Sheffield), and leu10

The prototrophic transformants 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'-ATGCAAGGTCCTGT-GAC-3'), SO56 (5'-TTCTCTGCTCACCATGGATT-3'), and SO72 (5'-AA-CACATGCAAATGTCGAG-3'). The fragments were co-transformed along with plasmid pYT3 (molar concentration ratio of fragment versus plasmid was ~1:10 in each case) by electropermeation 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 mM glucose plates (h-Glc), for AO colony assay. 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'-ATGCAAGGTCCTGT-GAC-3'), SO56 (5'-TTCTCTGCTCACCATGGATT-3'), and SO72 (5'-AA-CACATGCAAATGTCGAG-3'). The fragments were co-transformed along with plasmid pYT3 (molar concentration ratio of fragment versus plasmid was ~1:10 in each case) by electropermeation 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 mM 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 SO53 (5'-CCCA-ACGGTTAAAACGAGTAATCCT-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 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'-ATGCAAGGTCCTGT-GAC-3'), included restriction sites for HindIII and PstI, respectively. The 5'-flanking fragment was inserted into HindIII/PstI-digested plasmid pPT1 (18), carrying ScLEU2 gene as a selectable marker, to create pOS24. For the second step, a 1.2-kb Sall-Sall fragment of pOS22 composed of sequences from the 3' terminus of GCR1, beginning at nucleotide 1480 of the ORF and adjusted 3'-flanking region of ~1.1 kb was inserted in the correct orientation into Sall-digested pOS28 to create pOS29. The latter plasmid was digested with

**Table I**

<table>
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<tr>
<td>ade11</td>
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</tr>
<tr>
<td>gcr1-2</td>
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<td>gcr1-2 ade11 (GFP-PTS1)</td>
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<td>wt (GFP-PTS1)</td>
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<td>gcr1-2 (GFP-PTS1)</td>
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</tr>
<tr>
<td>gcr13 (GFP-PTS1)</td>
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</tbody>
</table>
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.
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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 leu1-1 and met6. Transformants were selected and analyzed for Ge phenotype as described in the main text. To confirm deletion of the GCR1 gene, genomic DNAs were isolated from several Gcr strains pregrown in YNB medium with sucrose and incubated with selected concentrations of glucose for 18 h. Data are mean values from three independent experiments. AO activity is expressed in units/mg of protein.

**Glucose Uptake Assays**—Preparation of crude cell free extracts was performed as described previously (23). AO activity was measured in cell-free extracts of H. polymorpha strains pregrown in YNB medium with sucrose and incubated with selected concentrations of glucose for 18 h. Data are mean values from three independent experiments. AO activity is expressed in units/mg of protein.

**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, or in permeabilized whole cells (8), and expressed in micromoles of product/min/g dry weight. Protein concentration was determined by the method of Lowry (26). Extracellular glucose concentration was measured with a glucose oxidase-based enzymatic kit Diagluc (UBT Ltd., Lviv, Ukraine), and methanol 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

**Table II**

<table>
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<th>Glucose</th>
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<th>5 min</th>
<th>25 min</th>
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<td>Wild type</td>
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<td>0</td>
</tr>
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<td>2.4</td>
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<td>gcr1Δ</td>
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<td>1.0</td>
<td>1.0</td>
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**Fig. 2** Alignment of selected amino acid sequence regions of H. polymorpha Gcr1p, S. cerevisiae Snf3p and Rgt2p, K. lactis Rag4p, and N. crassa Rec1p. 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.
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 ($V_{\text{glc}}$) was expressed as grams per hour per gram of dry weight.

**Electron and Fluorescence Microscopy**—Cells were fixed and prepped 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 (29).

**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 Leu+ 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. 1A). 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 hexose 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:

**TABLE III**

**Effect of carbon source on AO activity and growth rate in wild-type and gcr1 strains**

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<th>Carbon source</th>
<th>d.t. AO</th>
<th>d.t. AO</th>
<th>d.t. AO</th>
<th>d.t. AO</th>
<th>d.t. AO</th>
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<td>0</td>
<td>2.6</td>
<td>0</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>gcr1-2</td>
<td>2.6</td>
<td>0</td>
<td>5.5</td>
<td>140</td>
<td>28</td>
<td>120</td>
</tr>
<tr>
<td>gcr1Δ</td>
<td>6.2</td>
<td>55</td>
<td>5.8</td>
<td>90</td>
<td>30</td>
<td>98</td>
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* Carbon source, 1% w/v each.

The glucose consumption rate ($V_{\text{glc}}$) 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 ($V_{\text{glc}}$) was expressed as grams per hour per gram of dry weight.
S. cerevisiae Rgt2p (34), Kluyveromyces lactis Rag4p (35), and Neurospora crassa Repo3p (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, KfRag4p, and NcRco3p, had in common several unique conserved amino acid residues not found in other hexose transporters. They included Gcr1p tyrosine residues Tyr<sup>296</sup> and Tyr<sup>306</sup>, with Tyr<sup>306</sup> 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 KfRag4p is present in the Gcr1p C terminus (Fig. 2B) (33). The consensus sequence from this region is (M/L)G(L/I)X<sub>4</sub>(K/R)G, with two glycine residues (Gly<sup>320</sup> and Gly<sup>327</sup> 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 ScSnf3p and NcRco3p 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/A(N/C/G)TG(GG, which corresponds well to that suggested for ScMig1p and found also in the ScSnf3p 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 *gcr1Δ:*ScLEU2 allele was released with two restriction enzymes on a 3.9-kb DNA fragment and transformed into *H. polymorpha* leu1-1 met6. Leu<sup>+</sup> transformants were selected on a sucrose-containing medium without leucine and subsequently analyzed for typical *Gcr<sup>−</sup>* (1-Glc<sup>−</sup>, Aog<sup>−</sup>) phenotypes. Total genomic DNA was isolated from several transformants unable to grow on 1-Glc medium (1-Glc<sup>−</sup>) and displaying high AO activity on h-Glc plates (Aog<sup>−</sup>). With this DNA used as a template, PCR analysis indicated a correctly targeted chromosomal integration of the *gcr1Δ:*ScLEU2 fragment (not shown). Subsequently, the *gcr1Δ:*ScLEU2 *leu1-1 met6* strain was crossed with a *gcr1−* strain and prototrophic diploid cells were examined for the *Gcr<sup>−</sup>* phenotype. All were 1-Glc<sup>−</sup> and Aog<sup>−</sup>. Additionally, after sporulation, −1,000 spore products were grown on sucrose plates, then replica plated onto 1-Glc plates. No colonies were observed. Together, these results demonstrated that the *gcr1−* and *gcr1Δ* alleles were tightly linked and most probably mutant alleles of the same gene.

**Cloning of the gcr1− Mutant Allele**—The phenotype of the *gcr1−* mutant was similar but not identical to that of a *gcr1Δ* strain. We determined the molecular nature of the *gcr1−* mutation by isolating the mutated gene from genomic DNA of *gcr1−* 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−* 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 (Ser<sup>256</sup>) with a phenylalanine (Fig. 24). At this position, only one of three amino acid residues is found in hexose transporters: alanine, glycine, or, in a majority of proteins, serine.

**Fig. 4. Fluorescence microscopy images of wild-type, gcr1−, and gcr1Δ strains expressing peroxisome-targeted EGFP-PTS1 fusion protein on different carbon substrates.** Left panels (A–E), mid-exponential cells were analyzed for the presence of fluorescent peroxisomes with an excitation light wavelength of 490 nm. Right panels (A′–E′), the same field of cells in visible light. The same fluorescence intensity setting was used for all images.
Effect of Carbon Substrates on AO Repression and Peroxisome Biogenesis in ger1 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 ger1 mutants on these substrates relative to the wild-type strain. In addition to glucose, other sugars that no longer repressed AO in ger1 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 ger1 mutants on different carbon sources corresponded to altered transcriptional regulation of the AO promoter (P\textsubscript{AO}), we constructed strains with fluorescently labeled peroxisomes. These strains expressed peroxisome-targeted enhanced green fluorescent protein (EGFP-PTS1) under control of P\textsubscript{AO}. As expected, microscopic examination revealed fluorescence in all glucose-grown wild-type cells, as well as in ger1 mutant cells grown on sugars that failed to repress AO synthesis (Fig. 3). Cells of the gcr1-2 mutant grown on fructose, of both ger1 missense and deletion mutants grown on either sucrose, maltose, or ethanol, as well as cells of the wild-type strain grown on the above sugars or ethanol, did not exhibit fluorescence (not shown). It appears that Ger1p is selectively involved in repression of P\textsubscript{AO} triggered only by a subset of carbon substrates.

Ultrastructural Studies of Peroxisome Degradation in ger1 Mutants—When shifted to fresh glucose medium, methanol-grown cells of the wild-type strain exhibit a fast decrease in AO

FIG. 5. Peroxisome degradation in gcr1 mutants. A, Western blot analysis of AO degradation. Cells pregrown in YPS medium were induced in methanol medium for 19 h and shifted to fresh glucose medium. Aliquots were taken at time points 0, 2, 4, and 6 h to measure AO activity and for Western blotting for AO. Equal amounts of protein were loaded per lane. The initial AO activity (time point 0) for the wild-type strain was 2.77 units/mg, gcr1-2; 1.5 units/mg and Δgcr1, 0.78 units/mg. The residual AO specific activity in time point 6 was for the wild-type strain, 1.5%; gcr1-2, 46%; and Δgcr1, 35% of the corresponding initial activity. B, electron microscopic image of Δgcr1 cell 2 h after transfer from methanol medium to fresh glucose medium. Additional membrane wrappingautophagic peroxisome (specific for macropexophagy) is indicated by arrow. P, peroxisome; V, vacuole; N, nucleus; M, mitochondrion. C, combined fluorescent microscopic images of the wild type and Δgcr1 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.

FIG. 6. Glucose uptake and utilization as a function of extracellular glucose concentration. Glucose uptake (open symbols, dashed lines) and consumption from the medium (solid symbols and lines) were measured in wild-type (circles) and gcr1-2 (triangles) strains as described under “Experimental Procedures.” The glucose consumption rate (V\textsubscript{Glc}) is given in grams per hour per gram of cells (dry weight).

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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 peroxisome 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 peroxisome 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-deoxymethanol (not shown). Either concentration of 2-deoxymethanol, 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 en-

**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

**Fig. 7. Growth and carbon substrate consumption in glucose and methanol medium mixture. Kinetics of growth (squares), glucose (circles), and methanol (triangles) consumption in wild-type (solid symbols) and gcr1-2 (open symbols) strains grown in batch culture. Initial glucose and methanol concentrations were 55 mM and 1% v/v respectively.**
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 ger1-2 mutant, also suggests a regulatory function for Ger1p. We hypothesize that the glucose transport deficiency in the ger1-2 mutant alone is not sufficient to cause this regulatory effect. Alternatively, this phenomenon may be a common physiological feature of *H. polymorpha* mutants deficient in glucose repression (13), and may result from altered carbon fluxes through glycolysis and gluconeogenesis pathways.

All closely related potential orthologues of Ger1p are integral proteins of the plasma membrane and are involved in glucose sensing and/or glucose repression. This sensing/repression function and sequence similarity separates these transporters into their own subgroup within the hexose transporter family (30). The closest *GCR1* homologues in *S. cerevisiae*, ScSNF3, and ScRGT2, are thought to encode high and low affinity glucose sensors, respectively, that are non-functional as sugar carriers. Both gene products are involved primarily in the regulation of glucose transport via differential induction of other hexose transporters in response to changes in extracellular glucose concentrations, but are not thought to act directly in the repression pathway (33, 34). Two other close Ger1p homologues, *K. lactis* Rag4p and *N. crassa* Rco3p, seem to be directly involved in a signaling mechanism for repression, in addition to sugar transport (35, 36). For instance, NcRco3p regulates catabolite repression of conidiation genes, the synthesis of several repressible enzymes, as well as glucose transport in this fungus (36). Like ScSNF3 and ScRGT2, NcRco3p is expressed at a low level (36). However, there are no conclusive data, as to whether it is a functional transporter or a sensor protein.

HpGer1p, in contrast with the other putative sensor homologues, ScSnf3p, ScRgt2p, NcRco3p and KIRag4p, lacks an elongated protein-specific C-terminal region (43). For ScSnf3p, this region is functionally essential for glucose signaling (33). A unique short amino acid sequence at 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, Ger1p***S** 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 *snf3Δ*72 (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*Δ mutant was able to conidiate within the agar of solid medium, while the deletion mutant, *rco3*Δ, 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*-independent repression pathway(s) in *H. polymorpha* for sucrose, maltose and ethanol. In both the missense *ger1* 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* _Mox_ -directed expression of heterologous proteins (7), as demonstrated in this report with EGF-P-PTS1. Such an expression system would combine the advantages of the strong regulatable *P* _Mox_ 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*, 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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