Compartmentalization of metabolic pathways in Candida albicans: a matter of transport
Strijbis, K.

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Chapter 7

Investigations into the function and subcellular distribution of glutathione reductase in *Candida albicans*

Karin Strijbis¹, Wouter Visser² and Ben Distel¹

Departments of Medical Biochemistry¹ and Genetic Metabolic Diseases²
Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam
Abstract

The tripeptide glutathione plays a central role in detoxification of reactive oxygen species and reduction of cellular disulfide bonds by serving as a cofactor for a variety of glutathione-dependent enzymes. Oxidized glutathione (GSSG) is converted to reduced glutathione (GSH) by glutathione reductase (Glr1), an enzyme that is conserved from bacteria to higher eukaryotes. Glr1 is protective against reactive oxygen species (ROS) in the yeast *Saccharomyces cerevisiae*, while the protein is essential in the fission yeast *Schizosaccharomyces pombe* (13, 29). Reactive oxygen species are produced in different cellular locations and therefore all organelles must contain antioxidant systems. Here we present biochemical evidence suggesting that Glr1 of *Candida albicans* may localize to three different compartments: the cytosol, mitochondria and peroxisomes. We present a model to explain triple targeting from the single *GLR1* gene that involves alternative translational start sites, a mitochondrial targeting sequence (MTS) and a conserved N-terminal peroxisomal targeting signal 2 (PTS2). The *C. albicans* *GLR1* gene was found to encode all Glr1 activity in the cell and to be essential for normal growth.
Introduction

Glutathione (γ-glutamylcysteinylglycine) is an abundant tripeptide that plays an important role in maintaining the intracellular redox state by detoxification of reactive oxygen species (ROS) and reduction of cellular disulfide bonds (15). The redox-active thiol group of reduced glutathione (GSH) reacts with ROS, disulfides and metals, yielding oxidized glutathione (GSSG). The enzyme glutathione reductase (Glr1) mediates reduction of GSSG to GSH and uses NADPH as a reducing equivalent (6). The pentose phosphate pathway is the main source of NADPH in the cell and this pathway is functionally linked to Glr1 (Fig. 1). Different classes of enzymes like glutathione peroxidases (Gpx), glutaredoxins (Grx) and glutathione S-transferases (Gst and Gtt) are dependent on GSH as an electron donor for a variety of functions. Although Glr1 is conserved from bacteria to higher eukaryotes, the dependence of different organisms on this enzyme varies. In the yeast *Saccharomyces cerevisiae* Glr1 is required for protection against oxidative stress (13), *Escherichia coli* Glr1 mutants have no phenotype and they maintain a highly reduced glutathione pool (30), while Glr1 of *Schizosaccharomyces pombe* is an essential gene (19).

![Figure 1. Glutathione-dependent oxidation-reduction cycle](image)

**Figure 1. Glutathione-dependent oxidation-reduction cycle**

Schematic representation of the various roles of glutathione-dependent enzymes Gpx, Grx, Gst and Gtt in the decomposition of H₂O₂ and the reduction disulphide bonds in oxidized proteins. All enzymes use reduced glutathione (GSH) as a reducing agent generating oxidized glutathione (GSSG). Glr1 reduces GSSG back to GSH by using NADPH as an electron donor. The pentose phosphate pathway produces most NADPH in the cell. Abbreviations: Prot₁ox: oxidized proteins, Prot₁red: reduced proteins, Gpx: glutathione peroxidases, Grx: glutaredoxin, Gst: glutathione S-transferase, Gtt: glutathione S-transferase, GSH: reduced glutathione, GSSG: oxidized glutathione, Glr1: glutathione reductase.

The respiratory chain in mitochondria is a major site of ROS production in the cell. *S. cerevisiae* Glr1 was shown to localize both to mitochondria and cytosol by in- or exclusion of a N-terminal mitochondrial targeting sequence (MTS), a mechanism that relies on alternative translational start sites of the *GLR1* gene (23). GSH is synthesized in the cytosol and transported to the mitochondria by an anion carrier (8, 20), but GSSG is unable to exit the compartment (22). An essential process in the mitochondrial matrix is the generation of iron-sulphur (Fe-S) clusters, which are prosthetic groups found in various proteins of all living organisms (4). This process was shown to be directly dependent on glutathione, because mitochondrial glutaredoxin Grx5 of *S. cerevisiae* is required for maturation of Fe-S clusters (27). In absence of *S. pombe* mitochondrial Glr1,
activity of the oxidation-sensitive Fe-S protein aconitase was completely lost, illustrating the direct effect of a decreased GSH pool in mitochondria (16, 29).

The matrix of the peroxisome, the organelle that is named for its production of peroxide, harbours several H$_2$O$_2$-producing oxidases. Acyl-CoA oxidase, the first enzyme of the fatty acid β-oxidation pathway, D-amino acid oxidase that converts D-amino acids and alcohol oxidase involved in methanol metabolism all produce H$_2$O$_2$ as by-product. Peroxisomal catalase (Cta1) is the main H$_2$O$_2$-decomposing enzyme in the peroxisomes, but some reports also point at a peroxisomal glutathione-dependent system. Glutathione peroxidases activity has been detected in *C. boidinii* and rat liver peroxisomes (14, 28) and a glutathione S-transferases (Gto1; an omega class glutathione S-transferases) was shown to be peroxisomal in *S. cerevisiae*. Glutathione peroxidases activity has been detected in *C. boidinii* and rat liver peroxisomes (14, 28) and a glutathione S-transferases (Gto1; an omega class glutathione S-transferases) was shown to be peroxisomal in *S. cerevisiae*. Gto1 is targeted to the peroxisomes by a peroxisomal targeting signal 1 (PTS1) and a gto1Δ mutant displayed a growth defect on oleic acid (3). Although *S. cerevisiae* Glr1 is not predicted to contain any peroxisomal targeting signals, the enzyme was found to localize to the peroxisomes (32). The function of peroxisomal Glr1 however is unclear, because β-oxidation of oleate (C18:1) and myristate (C14:0) and number and size of peroxisomes was comparable between the glr1Δ and the wild type strain (32). The different phenotypes of the *S. cerevisiae* gto1Δ (growth defect on oleate) and glr1Δ (no growth defect on oleate) mutant strains might suggest that other protective mechanisms are upregulated to compensate for the lack of Glr1.

The human fungal pathogen *Candida albicans* is a commensal yeast that resides in the gastrointestinal tract. Candida infections mainly occur in immunocompromised hosts, indicating the essential role of the immune system in infection prevention (17). Upon detection of the pathogen, cells of the innate immune system initiate phagocytosis of the yeast cells. The ingested cells are exposed to a range of reactive oxygen species generated by NADPH oxidase complex of the phagocyte. To survive this oxidative burst, the pathogen requires a competent defence system, but the contribution of glutathione-dependent enzymes in this response is currently unknown. The *C. albicans* glutaredoxin Grx2 was shown to contribute to virulence in the mouse model of infection, but this could be (partially) due to a hyphae-formation defect of the grx2Δ/Δ strain (7). Another virulence-related trait of *C. albicans* is the ability to form biofilms. Genes involved in GSH synthesis are upregulated during early biofilm formation and this lead to the speculation that glutathione might play a role in the acquired drug resistance of biofilms (21). Glutathione is thought to be an essential metabolite in *C. albicans*, as disruption of glutathione synthesis by deletion of the γ-glutamylcysteine synthetase (GCS1) gene resulted in complete glutathione auxotrophy on minimal glucose medium and an increase of apoptotic markers (1).

Here we have studied the subcellular localization of *C. albicans* Glr1 and addressed its role in defence against oxidative stress. The *C. albicans* genome contain a single GLR1 gene that has two in frame ATG start codons, similar to *S. cerevisiae* GLR1 (23). In yeast dual targeting of Glr1 to mitochondria and the cytosol is regulated at the level of translation by a so called "leaky scanning" mechanism (23). In *C. albicans*, hypothetical translation from the first AUG would yield a protein that is predicted by the program MitoprotII (9) to contain a MTS and to be targeted to mitochondria, whereas translation from the second
AUG would result in a protein without a putative MTS that is predicted to remain in the cytosol. However, the predicted translation product starting at the first ATG also contains a putative peroxisomal targeting signal 2 (PTS2; -RLTQLSRQL-) that overlaps with the MTS (Fig. 2). The N-terminal PTS2 in Glr1 complies with the consensus sequence of R[L/V/I][H/Q][L/A] (where X is any amino acid) (18, 24) and is almost identical to that of known PTS2 proteins Thiolase (-RLNQLSGQL-) and Gnd1 (-RLSILSKQL-) (Chapter 5).

In this study we have employed fluorescence microscopy and subcellular fractionation experiments to investigate the subcellular distribution of *C. albicans* Glr1. The data suggest that in H₂O₂-stressed cells Glr1 may have a triple localization and is targeted to the cytosol, mitochondria and peroxisomes. Furthermore we show that a *glr1Δ/Δ* strain exhibits strongly reduced growth in all conditions tested, indicating that Glr1 is required for normal growth in *C. albicans*.

**Figure 2. Targeting signals and alternative start sites of *C. albicans* Glr1**
Schematic drawing of Glr1 depicting the class I/II oxidoreductase domain, a dimerization domain and the N-terminal domain that contains two putative start sites (AUG; boxed), a putative peroxisomal targeting signal (PTS2; boxed and underlined) and a mitochondrial targeting signal (MTS). Translation initiation from the first AUG would result in a protein that contains both a mitochondrial targeting signal (MTS) and a peroxisomal targeting signal (PTS2), while translation initiation from the second AUG would result in a protein without any targeting signal that would presumably remain in the cytosol.

**Materials and Methods**

**Media and culture conditions**

*C. albicans* strains were grown at 28°C unless otherwise stated. For routine non-selective culturing of *C. albicans* strains YPD + Uri (2% bactopeptone, 1% yeast extract, 2% glucose and 80 μg/ml uridine) was used. *C. albicans* transformants were selected and grown on minimal solid medium containing 0.67% Yeast Nitrogen Base (YNB) w/o amino acids (DIFCO), 2% glucose and amino acids as needed (20 μg/ml arginine, 20 μg/ml histidine, 80 μg/ml uridine). For subcellular fractionation, enzyme assays or immunoblot analysis, strains were pregrown for 16 hours on minimal glucose medium (YNB with 2% glucose), inoculated into at OD₆₀₀ 0.2 in YNB 0.3% glucose medium and grown for 8 hours. Finally, the strains were inoculated at OD₆₀₀ 0.005 into rich oleate medium (YPO; 2% bactopeptone, 1% yeast extract, 0.12%/0.2% oleic acid/Tween80) and grown for 16 hours.
Table I. Strains used in this study

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<td>BWP17</td>
<td>wildtype auxotroph</td>
<td>ura3∆::imm434/ura3∆::imm434 his1∆::hisG/his1∆::hisG arg4∆::hisG/arg4∆::hisG</td>
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<tr>
<td>CPK05</td>
<td>BWP17 prototroph</td>
<td>ura3∆::imm434/ura3∆::URA3 his1∆::hisG/his1∆::HIS1 arg4∆::hisG/arg4∆::ARG4</td>
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<td>CKS11</td>
<td>Glr1-6xMyc</td>
<td>ura3∆::imm434/ura3∆::imm434 his1∆::hisG/his1∆::hisG arg4∆::hisG/arg4∆::hisG GLR1/GLR1-6xMyc-URA3</td>
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<td>CKS19</td>
<td>Glr1-3xHA</td>
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<td>CKS02</td>
<td>Glr1-GFP</td>
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<td>CKS10</td>
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</tr>
<tr>
<td>CKS31</td>
<td>glr1Δ/Δ + GLR1</td>
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<td>This study</td>
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Spot test

Cells were pregrown on minimal glucose medium, transferred to 0.3% glucose medium, spun down and washed twice with water. Cells were resuspended to a concentration of about 2.7 x 10⁷ cells/ml (OD₆₀₀: 1.0), serially diluted (1:10 dilutions) and four microliters of each dilution was spotted onto agar plates. Plates contained YNB + 2% glucose, YPD, YPD + 1 or 4 mM H₂O₂ and YPD + 1 or 2.5 mM Paraquat (N,N’-Dimethyl-4,4’-bipyridinium dichloride; Sigma). The pictures were taken after 3 days of incubation at 28°C.

Strains and plasmids

C. albicans strains used in this study are listed in Table I, plasmids used in this study are listed in Table II and primers are listed in Table III. To construct a knockout strain of the C. albicans GLR1 gene (orf19.4147), disruption cassettes were made by PCR with primers KS09 and KS10 on plasmids pFA-HIS1 and pFA-ARG4 (12). Primers KS12 and KS13 were used in a second PCR reaction to increase the yield. The obtained disruption cassettes were subsequently transformed to BWP17 and correct integration of the cassettes was checked by PCR. Correct integration of both cassettes resulted in strain glr1Δ/Δ ura3-. Primers KS55 and KS56 were used in a reaction on C. albicans genomic DNA to PCR the GLR1 gene including a 800 bp promoter region. The PCR product was cloned BamHI/SacI into pLUBP (26), sequenced and named pLUBP-GLR1. The empty pLUBP and pLUBP-GLR1 plasmids were linearized with XhoI/PacI and transformed to the glr1Δ/Δ ura3- strain to create the prototrophic glr1Δ/Δ and glr1Δ/Δ + GLR1 strains. A new set of pFA plasmids was constructed for C-terminal tagging with 3xHA and 6xMyc epitopes in C. albicans. Plasmid pFA-3xHA-URA3 was constructed as follows: plasmid pFA6a-3HA-kanMX6 was used as a template to PCR the 3xHA tag with primers c3HAF and R, thereby introducing a 3x-Gly-Ala linker sequence at the 5’ site of the tag. The PCR product was cloned PstI/AscI into pFA-GFP-URA3 (12). The pFA-6xMyc-URA3 was constructed as follows: plasmid pFA6a-13Myc-kanMX6 was used as template in a PCR reaction with primers 6myc-F and R, resulting in a PCR product that contained a 6xMyc sequence. That PCR product...
was cloned SacI/BamHI into pAsk26.1, resulting in pAsk47. Primers cMycF and R were used in a PCR reaction on pAsk47, thereby introducing restriction sites and a 3x-Gly-Ala linker sequence at the 5’ site of the tag. This PCR product was also cloned PstI/AscI into pFA-GFP-URA3. For the tagging of Glr1 with GFP, 3xHA or 6xMyc, primers KS11 and KS10 were used in a PCR reaction on plasmids pFA-GFP-URA3, pFA-3xHA-URA3 or pFA-6xMyc-URA3. The yield of the reactions was increased by a second PCR with primers KS14 and KS13. The three PCR products were transformed to BWP17 resulting in strains Glr1-GFP, Glr1-3xHA and Glr1-6xMyc. Correct integration of the constructs and epitope tag of Glr1 was confirmed by PCR and immunoblot analysis with the α-GFP, α-HA or α-Myc antibodies, respectively.

Table II. Plasmids used in this study

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<td>(2)</td>
</tr>
<tr>
<td>pFA6a-13Myc-kanMX6</td>
<td>Template for pAsk47</td>
<td>(2)</td>
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<tr>
<td>pAsk26.1</td>
<td>Vector for pAsk47</td>
<td>A. Kragt, unpublished</td>
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<tr>
<td>pAsk47</td>
<td>Template for pFA-3xHA-URA3</td>
<td>A. Kragt, unpublished</td>
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<td>pFA-3xHA-URA3</td>
<td>C-terminal 3xHA tagging</td>
<td>This study</td>
</tr>
<tr>
<td>pFA-6xMyc-URA3</td>
<td>C-terminal 6xMyc tagging</td>
<td>This study</td>
</tr>
<tr>
<td>pFA-ARG4</td>
<td>Disruption with ARG4</td>
<td>(12)</td>
</tr>
<tr>
<td>pFA-HIS1</td>
<td>Disruption with HIS1</td>
<td>(12)</td>
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<tr>
<td>pLUBP</td>
<td>URA3 complementation</td>
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<tr>
<td>pLUBP-GLR1</td>
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Transformation

*C. albicans* was transformed using a modified lithium acetate protocol (33). The heat shock was carried out at 44°C for 15 minutes.

Glutathione reductase enzyme assay

Glutathione reductase activity was measured as previously described in the academic thesis of W. F. Visser (32). Glutathione reductase activity was measured at 37°C by monitoring the absorption at 340 nm over a time span of 5 minutes using a reaction mixture with the following components: 1 mM EDTA, 50 mM Tris buffer pH 7.2, 0.1% (v/v) Triton X-100, 0.1 mM NADPH, 5 mM GSSG and the sample to be analyzed. The change in absorbance of a solution containing no sample was recorded and subtracted from all measurements to obtain the total glutathione reductase activity.

Fluorescence microscopy

The Glr1-GFP strain was inoculated in YPD with 2.5 mM Paraquat, 4 mM H₂O₂ or without additions and grown for 3 hours. 100 nM Mitotracker Deep Red FM (Invitrogen/Molecular Probes) dissolved in DMSO was added to the growing cultures and mitochondrial staining was induced for 30 minutes. Cells were harvested by centrifugation and washed twice with PBS. Cells were embedded in low melting point agarose (Boehringer-Mannheim) and photographed using an Axiophot 2 microscope (Carl
Zeiss) equipped with a CoolSnap HQ digital camera (Photometrics). Image processing was performed using Imago-Pro Express software (Media Cybernetics).

**Subcellular fractionation and density gradient analysis**

The subcellular fractionation of *C. albicans* strains was performed as previously described (Chapter 2) and the fractions of the Nycodenz gradient were analyzed for the presence of enzymatic activity of the peroxisomal marker 3-hydroxyacyl-CoA dehydrogenase (3HAD) (34) and the mitochondrial marker Fumarase (31).

**Immunoblotting**

Gradient fractions or protein extracts were separated on a SDS-polyacrylamide gel (10%) and blotted to nitrocellulose membrane using a semi-dry system. Antibodies used were directed against *S. cerevisiae* catalase (Cta1), *S. cerevisiae* thiolase (Thiol), *S. cerevisiae* Hexokinase (Hxk1), *S. cerevisiae* glutathione reductase (Glr1), GFP, HA (mAb12CA5) and Myc (9b11, Cell Signaling). The Glr1 antibody was a kind gift of Caryn Outten, John Hopkins institute, USA.

### Table III. Primers used in this study

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### Results

**C. albicans Glr1 activity localizes to the cytosol and organelles**

The *C. albicans* wild type strain was grown on YPD and on YPD with 4 mM H$_2$O$_2$ to apply oxidative stress. Protein extracts of both cultures were analyzed for Glr1 activity. The total Glr1 activity was found to be four times higher in wild type strain grown in the presence of H$_2$O$_2$ compared to the wild type growing on YPD without any additions (Fig. 3A). To determine the subcellular distribution of Glr1, the protein was
C-terminally tagged at the chromosomal location with a 6xMyc epitope tag. Tagging of Glr1 at the C-terminus is expected not to interfere with the function of the predicted N-terminal targeting signals. A total homogenate (H) of cells grown on YPO was obtained by enzymatic removal of the cell wall and osmotic lysis of spheroplasts. The organellar pellet (P), containing mainly mitochondria and peroxisomes, was separated from the cytosolic supernatant (S) by differential centrifugation and all fractions were analyzed for the presence of Glr1-Myc, peroxisomal catalase, peroxisomal thiolase and cytosolic hexokinase by immunoblot analysis. Peroxisomal thiolase fractionated predominantly in the P fraction, while the distribution of catalase was equal between P and S fractions, suggesting some leakage of the peroxisomal enzymes due to damage of the organelles. We did not take along a mitochondrial marker in this experiment, but generally mitochondrial proteins are found solely in the P fractions. About 70% of the Glr1-6xMyc fusion protein was found in the S fraction, together with the cytosolic marker Hexokinase, while about 30% was associated with the organellar pellet fraction (Fig. 3B). This experiment shows that Glr1-6xMyc is mainly cytosolic, but that a considerable amount of the protein co-fractionates with organelles.

The organelle-associated Glr1 predominantly localizes to mitochondria
To investigate the organellar distribution of C. albicans Glr1 in more detail we performed density gradient analysis on organellar pellets obtained from the wild type strain grown on YPO (to induce peroxisomes) or YPO with a low or high concentration of H_2O_2 (to induce Glr1 activity). Gradient fractions were analyzed for the enzyme activities of the peroxisomal marker 3HAD, the mitochondrial marker Fumarase and Glr1. Additionally the distribution of Glr1 in the gradients was also determined by immunoblot analysis with an antibody directed against S. cerevisiae Glr1. Analysis of the gradients showed that Glr1 activity clearly colocalizes with the mitochondrial peak in all three conditions (Fig. 4A, B, D). No clear association of Glr1 activity with peroxisomes was found on YPO. Glr1 activity was not elevated in the peroxisomal fractions of the YPO with 1
mM H₂O₂ gradient, but immunoblot analysis of the gradient fractions revealed a minor Glr1 signal associated with the peroxisomes. The peroxisomal and mitochondrial peak fractions of the 1 mM H₂O₂ gradient were analyzed by immunoblot analysis in more detail and compared to TCA lysates of the wild type strain, glr1Δ/Δ mutant and a strain expressing 3xHA-tagged Glr1 incubated with 4 mM H₂O₂ (Fig. 4C). The mitochondrial fraction showed a clear Glr1 signal and a weak Glr1 band of slightly higher molecular weight was detectable in the peroxisomal fraction. The gradient of cells incubated with 4 mM H₂O₂ revealed a small peak of Glr1 activity that colocalized with the peroxisomal marker 3HAD (Fig. 4D). As no Fumarase activity was found in the peroxisomal peak fraction, these results may suggest that during high H₂O₂ stress conditions Glr1 is also targeted to peroxisomes.

**Figure 4. Organelle-associated Glr1 predominantly localizes to mitochondrial**

(A): Nycodenz density gradient of the organellar pellet of the wild type strain grown on YPO. The gradient was divided into 12 fractions and in each fraction enzymatic activity of the peroxisomal marker 3HAD, the mitochondrial marker Fumarase and Glr1 was determined. Underneath the gradient: immunoblot analysis of all fractions with α-Glr1. (B): Nycodenz density gradient of organellar fraction of the wild type grown on YPO in the presence of 1 mM H₂O₂ for one hour. Absolute activity of Glr1 in the mitochondrial peak fraction is 7x higher than in the gradient without H₂O₂. Underneath the gradient: immunoblot analysis of all fractions with α-Glr1. (C): Detailed immunoblot analysis with the α-Glr1 antibody of peroxisomal and mitochondrial peak fractions of the 1 mM H₂O₂ gradient along with TCA lysates of the wild type, glr1Δ/Δ and Glr1-3xHA strain incubated with 4 mM H₂O₂. (D): Nycodenz density gradient of subcellular fractionation experiment on wild type grown for 16 hours in the presence of 4 mM H₂O₂.

**Glr1 is upregulated and predominantly localizes to the cytosol after addition of H₂O₂**

Glr1 was C-terminally tagged with GFP and 3xHA by insertion of tagging cassettes in the chromosomal locus. The Glr1-3xHA strain was used to determine Glr1 induction
after addition of 2.5 mM Paraquat or 4 mM $\text{H}_2\text{O}_2$. Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is widely used as an agent for superoxide production in mitochondria (10). Samples were taken 0, 30 and 180 minutes after addition of the oxidative agents. The levels of Glr1-3xHA were very low at time point zero and addition of Paraquat resulted in only a slight upregulation after 180 minutes. Upon addition of $\text{H}_2\text{O}_2$, however, Glr1-3xHA levels were increased within 30 minutes (Fig. 5A). The Glr1-GFP strain was used to analyse Glr1 induction and localization after addition of $\text{H}_2\text{O}_2$. Fluorescence of the Glr1-GFP construct was barely detectable in the cells grown on YPD without additional oxidative stress (Fig. 5B). The Glr1-GFP strain that was grown in the presence of $\text{H}_2\text{O}_2$ for 3 hours showed a very bright fluorescent signal that was distributed throughout the cell indicating a predominantly cytosolic localization of the fusion protein (Fig. 5C).

**C. albicans GLR1 encodes all Glr1 activity and is essential for normal growth**

We constructed a glr1 null strain to investigate the overall contribution of Glr1 to oxidative stress defence in *C. albicans*. Construction of the double knockout was complicated by the slow growth of the mutant after disruption of the second allele. We measured total Glr1 activity in lysates of the glr1 null grown on YPD without or with 4 mM $\text{H}_2\text{O}_2$. No Glr1 activity was detected above background levels in the glr1 null strain in either condition, showing that the single *C. albicans* GLR1 gene encodes all measurable Glr1 (data not shown). We complemented the glr1 null strain with the wild type GLR1 gene and tested growth of the strains on minimal and rich glucose medium either in the presence or absence of Paraquat, a compound that mainly leads to production of superoxide radicals in mitochondria (10), or $\text{H}_2\text{O}_2$, that is thought to result in general oxidative stress in all compartments. Growth of the glr1 null strain was very slow compared to wild type and complemented strain in all tested conditions, but
especially on minimal glucose medium and in the presence of 2.5 mM Paraquat. High levels of \( \text{H}_2\text{O}_2 \) also lead to reduced growth of the \( glr1 \) null mutant, but a growth reduction was also observed for the wild type under these conditions (Fig. 6). The relatively severe phenotype of the \( glr1 \) null strain in the presence of Paraquat compared to the wild type strain might indicate that the contribution of \( C. albicans \) mitochondrial Glr1 is particularly important to cope with increased intra-mitochondrial ROS levels.

**Discussion**

Here we have addressed the function of Glr1 of \( C. albicans \) and studied its subcellular distribution. Microscopic and immunoblot analysis showed that Glr1 normally is expressed at relatively low levels, but that extracellular addition of \( \text{H}_2\text{O}_2 \) results in a strong increase of Glr1 levels within 30 minutes (Fig. 3A and 5A). Biochemical fractionation experiments showed that Glr1 is localized both to the cytosol and to the organellar pellet (Fig. 3B). Further separation of the organellar pellet on density gradients revealed that Glr1 is predominantly localized to mitochondria, with some peroxisomal association in cells stressed with \( \text{H}_2\text{O}_2 \) (Fig. 4).

While the peroxisomal localization of Glr1 remains to be firmly established, it is interesting to note that Glr1 contains a putative PTS2 in its N-terminus that overlaps with the predicted MTS (Fig. 2). The \( C. albicans \) \( GLR1 \) gene contains two in-frame ATG codons that potentially lead to translation of two gene products: a longer protein starting at the first AUG harbouring both the MTS and PTS2 and a shorter product lacking both signals that is predicted to be cytosolic. Dual targeting of identical proteins to mitochondria and an other compartment was shown to be associated with relatively low MTS parameters (11). The longer \( C. albicans \) Glr1 has a high MTS parameter (0.9795, MitoprotII, reference 9) and therefore equal distribution between mitochondria and another compartment is predicted to be unlikely. Our results confirm that the MTS overrules the (overlapping) PTS2 because Glr1 is mainly located in the mitochondrial fractions under the conditions that we have tested. However, it is possible that the PTS2 is more favoured under specific conditions that require high levels of peroxisomal Glr1.
The \textit{C. albicans} GLR1 gene structure is very similar to that of \textit{S. cerevisiae} GLR1 (23), except that the latter does not contain any obvious peroxisomal targeting sequences. Despite the absence of a PTS the \textit{S. cerevisiae} Glr1 has been reported to be equally distributed between peroxisomes and mitochondria (32). How peroxisomal targeting of Glr1 is achieved in \textit{S. cerevisiae} is currently unknown. Whether the difference in Glr1 localization is caused by different oxidative requirements of \textit{C. albicans} and \textit{S. cerevisiae} peroxisomes remains to be investigated.

The exact contribution of Glr1 to peroxisomal metabolism, if any, remains to be established with more certainty. We have previously shown partial peroxisomal localization of the NADPH-producing dehydrogenases of the pentose phosphate pathway (Chapter 5) indicating that NADPH is available in the peroxisomal matrix to provide Glr1 with the necessary reducing power. It seems likely that peroxisomal glutathione system would be involved in ROS detoxification and/or repair of oxidized proteins in the reactive environment of the peroxisomal matrix. The effect of absent Glr1 might not be immediately apparent, because the \textit{S. cerevisiae} glr1Δ mutant did not have a direct β-oxidation defect (32). The \textit{S. cerevisiae} Gto1 is an omega class glutathione S-transferase (GST) that localizes to peroxisomes (3). Omega class GSTs have low activity against standard GST substrates, but are active as redox regulators of thiol groups using GSH as reductant (5, 35). Peroxisomal Gto1 is hypothesized to be involved in redox regulation of peroxisomal cystathionine beta-lyase (Str3), which partakes in sulphur amino acid metabolism (3). In \textit{C. albicans} the ortholog of Stre3 (orf19.2092; -PKL\textsubscript{COOH}) and additionally the ortholog of cystathionine beta-synthase (Cys4; orf19.2263; -PKL\textsubscript{COOH}) are predicted to be peroxisomal proteins (our observations). We therefore hypothesize that peroxisomal glutathione-based protection could also be involved in sulphur amino acid metabolism in \textit{C. albicans}. Further experiments are required to address this issue.

The glr1 null strain that we constructed showed a severe growth defects on all media tested and seemed to be especially sensitive to Paraquat, a compound that causes ROS production in the mitochondria. High levels of H\textsubscript{2}O\textsubscript{2} affected growth of all the tested strains, including the wild type (Fig. 5). We showed that Glr1 is upregulated only slightly after addition of Paraquat, but is highly induced in the presence of H\textsubscript{2}O\textsubscript{2} (Fig. 4). The essential contribution of Glr1 during growth in the presence of Paraquat seems to contrast with the minor upregulation under these conditions. Although Glr1 activity is completely lost in the glr1 null strain, there might still be some reduced glutathione present in this strain because the glutathione biosynthesis pathway directly produces GSH. Previously it was shown that apoptosis was induced by disruption of a \textit{C. albicans} gene involved in glutathione biosynthesis (\textit{GCS1}) (1). Although the phenotypes of the \textit{gcs1} and \textit{glr1} mutants convincingly show that reduced glutathione is required for normal growth in \textit{C. albicans}, the exact cause of the growth defect remains to be identified. A good candidate protein that could be inactivated by oxidation in the absence of Glr1 would be the Fe-S cluster protein aconitase. This oxidation-sensitive protein, which is essential for aerobic growth was shown, to be completely inactivated in the \textit{S. pombe} glr1 mutant (29).
References

transcription profiling of the early phase of biofilm formation by *Candida albicans*. Eukaryot Cell 4:1562-1573.


