The activity of the glyoxylate cycle in peroxisomes of *Candida albicans* depends on a functional β -oxidation pathway: evidence for reduced metabolite transport across the peroxisomal membrane

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The glyoxylate cycle, a metabolic pathway required for generating C₄ units from C₂ compounds, is an important factor in virulence, in both animal and plant pathogens. Here, we report the localization of the key enzymes of this cycle, isocitrate lyase (Icl1; EC 4.1.3.1) and malate synthase (Mls1; EC 2.3.3.9), in the human fungal pathogen *Candida albicans*.

Immunocytochemistry in combination with subcellular fractionation showed that both Icl1 and Mls1 are localized to peroxisomes, independent of the carbon source used. Although Icl1 and Mls1 lack a consensus type I peroxisomal targeting signal (PTS1), their import into peroxisomes was dependent on the PTS1 receptor Pex5p, suggesting the presence of non-canonical targeting signals in both proteins. Peroxisomal compartmentalization of the glyoxylate cycle is not essential for proper functioning of this metabolic pathway because a *pex5Δ/Δ* strain, in which Icl1 and Mls1 were localized to the cytosol, grew equally as well as the wild-type strain on acetate and ethanol. Previously, we reported that a *fox2Δ/Δ* strain that is completely deficient in fatty acid β -oxidation, but has no peroxisomal protein import defect, displayed strongly reduced growth on non-fermentable carbon sources such as acetate and ethanol. Here, we show that growth of the *fox2Δ/Δ* strain on these carbon compounds can be restored when Icl1 and Mls1 are relocated to the cytosol by deleting the *PEX5* gene. We hypothesize that the *fox2Δ/Δ* strain is disturbed in the transport of glyoxylate cycle products and/or acetyl-CoA across the peroxisomal membrane and discuss the possible relationship between such a transport defect and the presence of giant peroxisomes in the *fox2Δ/Δ* mutant.

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INTRODUCTION

The glyoxylate cycle, present in bacteria, yeasts and plants, allows utilization of non-fermentable carbon sources such as acetate, ethanol and fatty acids by converting the produced acetyl-CoA into C₄ compounds that can be used for the biosynthesis of macromolecules (Kornberg & Krebs, 1957). This function has been confirmed through the analysis of bacterial and fungal mutants lacking either isocitrate lyase (Icl1; EC 4.1.3.1) or malate synthase (Mls1;

EC 2.3.3.9), the key enzymes of the glyoxylate cycle. Loss of Icl1 or Mls1 results in the inability of these mutants to grow on acetate, ethanol or fatty acids (Hartig *et al.*, 1992; Lorenz & Fink, 2001; McKinney *et al.*, 2000; Munoz-Elias & McKinney, 2005; Pellicer *et al.*, 1999). There is a renewed interest in the glyoxylate cycle that is based on recent observations suggesting a key role for this metabolic pathway in survival of bacterial and fungal pathogens within macrophages, phagocytic cells of the innate immune system that form the first line of defence against microbial infections. Whole-genome DNA microarrays have shown an upregulation of glyoxylate cycle genes and concomitant induction of genes associated with gluconeogenesis when *Candida albicans*, a human fungal pathogen, is internalized by macrophages (Lorenz *et al.*, 2004). Deletion of the *ICL1*

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Abbreviations: Icl1, isocitrate lyase; Mls1, malate synthase; PEX, peroxisome biogenesis gene/protein; PTS1, peroxisomal targeting signal 1; TCA, tricarboxylic acid.

gene resulted in a mutant *C. albicans* strain that showed reduced virulence in a mouse model, providing strong support for the requirement of the glyoxylate cycle for pathogenesis of this fungus (Lorenz & Fink, 2001). Similarly, the persistence of *Mycobacterium tuberculosis* in macrophages is dependent on a functional glyoxylate cycle (McKinney *et al.*, 2000; Munoz-Elias & McKinney, 2005). For both *C. albicans* and *M. tuberculosis* it has been suggested that the organism relies heavily on lipid catabolism for survival within the human host (Boshoff & Barry, 2005; Lorenz & Fink, 2002). However, we have recently shown that fatty acid metabolism is not essential for virulence of *C. albicans*, indicating that *in vivo* the acetyl-CoA that feeds the glyoxylate cycle may be derived from other, simple, carbon sources (Piekarska *et al.*, 2006). In support of this, Ramírez & Lorenz (2007) recently reported that the ability to utilize different non-fermentable carbon sources contributes to the virulence of *C. albicans*. Since the metabolism of these carbon compounds requires a functional glyoxylate cycle and this metabolic pathway is absent from mammals, Icl1 and Mls1 are potential targets for the development of drugs to combat bacterial and fungal infections.

The glyoxylate cycle consists of five enzymic activities, two of which are unique to the cycle, namely Icl1 and Mls1, while the other three activities, citrate synthase (EC 2.3.3.1), aconitase (EC 4.2.1.3) and malate dehydrogenase (EC 1.1.1.37), are shared with the tricarboxylic acid (TCA) cycle and are often carried out by isoenzymes. In the *C. albicans* genome, single genes exist for Icl1 (orf19.6844), Mls1 (orf19.4833) and citrate synthase (orf19.4393), whereas there are three encoding malate dehydrogenase (orf19.7481, orf19.4602 and orf19.5223) and two for aconitase (orf19.6385 and orf19.6632). Studies in fungi have shown that the key enzymes of the glyoxylate cycle, Icl1 and Mls1, are often compartmentalized in peroxisomes, the other enzymic steps being extra-peroxisomal (either cytosolic or mitochondrial) (Hikida *et al.*, 1991; Maeting *et al.*, 1999; Tanaka & Ueda, 1993; Titorenko *et al.*, 1998; Valenciano *et al.*, 1996). *Saccharomyces cerevisiae* seems to be an exception to this rule: Icl1 is a cytosolic enzyme (McCammon *et al.*, 1990) and Mls1 is localized either to the peroxisome or to the cytosol depending on the growth conditions (Kunze *et al.*, 2002). These data in conjunction with the observation that *S. cerevisiae* pex mutants lacking functional peroxisomes can grow on ethanol and acetate as sole carbon source imply that the glyoxylate cycle can function in the cytosol, at least in *S. cerevisiae*.

The import of proteins into peroxisomes is mediated by two cycling receptors: Pex5p and Pex7p (reviewed by Purdue & Lazarow, 2001). In the cytosol, Pex5p binds to proteins carrying a type I peroxisomal targeting signal (PTS1), which is a carboxyl-terminal tripeptide with the consensus sequence S/A/C-K/R/H-L/M (Gould *et al.*, 1989; Swinkels *et al.*, 1992). However, depending on the species and protein under study the carboxyl-terminal tripeptide

may deviate from the consensus, and residues just upstream of it also can contribute to Pex5p binding and peroxisomal targeting (Lametschwandtner *et al.*, 1998; Purdue & Lazarow, 1996). Proteins delivered to the peroxisome via the Pex7p receptor possess a PTS2, a bipartite amino acid motif with the consensus sequence R/K-L/V/I-x₅-H/Q-L/A (Gietl *et al.*, 1994; Glover *et al.*, 1994b) present in the amino terminus of these proteins. A number of proteins can be imported into peroxisomes independent of a recognizable PTS1 or PTS2 (Elgersma *et al.*, 1995; Klein *et al.*, 2002; Ozimek *et al.*, 2006; Small *et al.*, 1988). Subsequent studies have shown that, although the targeting signal is as yet not characterized, Pex5p mediates the peroxisomal sorting of these proteins (Elgersma *et al.*, 1995; Klein *et al.*, 2002; Ozimek *et al.*, 2006). Finally, some proteins can reach the organelle by association with another subunit or protein that possesses a functional targeting signal, a process referred to as 'piggy backing' (Glover *et al.*, 1994a; McNew & Goodman, 1994; Yang *et al.*, 2001). The PTS1 and PTS2 pathways converge at the peroxisomal membrane, where the cargo-loaded receptors dock onto a complex consisting of Pex13p and Pex14p (and Pex17p in *S. cerevisiae*). Subsequently, the cargo is released and translocated across the peroxisomal membrane, after which the receptors recycle back to the cytosol for a new round of import (Purdue & Lazarow, 2001). Although the early steps in peroxisomal protein import are rather well defined, very little is known about the events following docking.

Here we have studied the localization of the two key enzymes of the glyoxylate cycle, Icl1 and Mls1, in the human fungal pathogen *C. albicans* and show that both enzymes are localized to peroxisomes in a Pex5p-dependent way. We also provide evidence that the glyoxylate cycle no longer functions in giant peroxisomes of the fatty acid β -oxidation mutant *fox2 Δ /* Δ . The activity of the glyoxylate cycle in the *fox2 Δ /* Δ mutant is restored when Icl1 and Mls1 are relocated to the cytosol through deletion of the *PEX5* gene in this strain. Our data show that the glyoxylate cycle can function in the cytosol also in *C. albicans* and suggest that the giant peroxisomes of the *fox2 Δ /* Δ strain are impaired in metabolite transport across the membrane. Possible mechanisms that may explain the reduced peroxisomal metabolite transport are discussed.

METHODS

Strains, media and culture conditions. *C. albicans* and *S. cerevisiae* strains used in this study are listed in Table 1. *C. albicans* deletion strains are derivatives of BWP17 (Wilson *et al.*, 1999). Gene deletions in *S. cerevisiae* were constructed in BJ1991 (Jones, 1977). The solid minimal medium used for selection and growth of both *C. albicans* and *S. cerevisiae* transformants contained 0.67% (w/v) yeast nitrogen base (YNB) w/o amino acids (Difco), 2% (w/v) glucose, 2% (w/v) agar, amino acids (20–30 μ g ml⁻¹) and either 80 μ g uridine ml⁻¹ (*C. albicans*) or 20 μ g uracil ml⁻¹ (*S. cerevisiae*) as needed. For transformations using the dominant selection marker *SAT1*, a 24 h incubation under non-selective conditions was applied prior to

Table 1. Yeast strains used in this study

Strain	Genotype	Source or reference
SC5314	Wild-type	Gillum <i>et al.</i> (1984)
BWP17	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG</i>	Wilson <i>et al.</i> (1999)
CPK5 (BWP17 prototroph)	<i>ura3Δ::imm434/ura3Δ::URA3 his1Δ::hisG/his1Δ::HIS1 arg4Δ::hisG/arg4Δ::ARG4</i>	Piekarska <i>et al.</i> (2006)
CPK1	<i>ura3Δ::imm434/ura3Δ::URA3 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG pex5Δ::HIS1/pex5Δ::ARG4</i>	Piekarska <i>et al.</i> (2006)
CEM16	<i>ura3Δ::imm434/ura3Δ::URA3 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG fox2Δ::ARG4/fox2Δ::HIS1</i>	Piekarska <i>et al.</i> (2006)
CPK2	<i>ura3Δ::imm434/ura3Δ::URA3 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG pex13Δ::ARG4/pex13Δ::HIS1</i>	
CPK3	<i>ura3Δ::imm434/ura3Δ::URA3::PEX5 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG pex5Δ::HIS1/pex5Δ::ARG4</i>	Piekarska <i>et al.</i> (2006)
CEM15	<i>ura3Δ::imm434/ura3Δ::URA3::FOX2 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG fox2Δ::ARG4/fox2Δ::HIS1</i>	Piekarska <i>et al.</i> (2006)
CPK4	<i>ura3Δ::imm434/ura3Δ::URA3::PEX13 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG pex13Δ::ARG4/pex13Δ::HIS1</i>	Piekarska <i>et al.</i> (2006)
CMD3	<i>ura3Δ::imm434/ura3Δ his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG icl1Δ::HIS1/icl1Δ::ARG4</i>	Piekarska <i>et al.</i> (2006)
CMD8	<i>ura3Δ::imm434/ura3Δ::URA3 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG icl1Δ::HIS1/icl1Δ::ARG4</i>	Piekarska <i>et al.</i> (2006)
CMD6	<i>ura3Δ::imm434/ura3Δ::URA3::ICL1 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG icl1Δ::HIS1/icl1Δ::ARG4</i>	Piekarska <i>et al.</i> (2006)
CJB8	<i>ura3Δ::imm434/ura3Δ his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG fox2Δ::ARG4/fox2Δ::HIS1 pex5Δ::URA3/pex5Δ::SAT1</i>	This study
BJ1991	<i>MATα leu2 trp1 ura3-251 prb-1122 pep4-3 gal2</i>	Jones (1977)
Scfox2Δ	BJ1991; <i>fox2Δ::LEU2</i>	This study
Scicl1Δ	BJ1991; <i>icl1Δ::KanMX</i>	This study

replica plating on YPD + Uri [2 % (w/v) bactopectone, 1 % (w/v) yeast extract, 2 % (w/v) glucose and 80 μg uridine ml⁻¹] with 200 μg nourseothricin ml⁻¹ (clonNAT, Werner Bioagents). Plates used for spot assays had the same composition and contained 2 % (w/v) glucose, 2 % (v/v) ethanol, 2 % (v/v) glycerol, 2 % (w/v) sodium citrate or 2 % (w/v) sodium acetate (pH 5.0) as a carbon source. The

liquid medium used for culturing of the cells for total protein isolation, subcellular fractionation and immunoelectron microscopy contained 0.5 % (w/v) potassium phosphate buffer, pH 6.0, 0.3 % (w/v) yeast extract, 0.5 % (w/v) peptone and, as a carbon source, either 0.1 % (v/v) oleate and 0.2 % (v/v) Tween 40, or 2 % (v/v) ethanol. All yeast strains were grown at 28 °C unless otherwise stated.

Table 2. Primers used in this study

Primer	Sequence (5'–3')	Description
CaPex5Δ-F-FA	TAATTTTGGATTTCGAAAATTTGTTATTTTCTTTCTCCCATATCATTATC-AAAGAAGAAGCTTCGTACGCTGCAGGTC	Disruption primer
CaPex5Δ-R-FA	ACAGCTAGAAAAATAATAATTTGCAATTTTGCCATAAATACATACCCTATA-TACTTAGAATCTGATATCATCGATGAATTCGAG	Disruption primer
PEX5-F extension	GATTGGTCTGGACTATAATTTTGGATTTCGAAAA	Extension primer for disruption
PEX5-R extension	AGTCATTTTCTCATTACAGCTAGAAAAATAATAAT	Extension primer for disruption
CaPex5F	CCACTTTATCATTTCTCGGTGG	Diagnostic primer
CaPex5R	GTTGGTGGTAGGAAGCGTTG	Diagnostic primer
Verif-Ca-U4	ACGAATCAATGGCACTACAGC	Diagnostic primer
Verif-Ca-U5	GATTAGATGATAAAGGTGATGG	Diagnostic primer
JB12-SAT1-inR	TTCGTTGGCAATAAATCTTGG	Diagnostic primer SAT internal
JB13-SAT1-inF	AGCACAGGATGACGCCTAAC	Diagnostic primer SAT internal
A5	TTTAATGTGCGATGGGTTCA	Diagnostic primer ARG internal
A4	GTAAGATATTTGGTCAGTTATCTGG	Diagnostic primer
H4	GAAATGGCCTCCCCTACCACAG	Diagnostic primer HIS internal
H5	GGACGAATTGAAGAAAGCTGG	Diagnostic primer

Gene disruptions and plasmid constructions. For gene deletions in *C. albicans*, a PCR-based procedure with primers carrying 60–70 bp regions of homology to the 5' and 3' flanking sequences of the ORF was used (Wilson *et al.*, 1999). Primers are listed in Table 2. The construction of the *pex5Δ/Δ* (CPK1), *fox2Δ/Δ* (CEM16), *pex13Δ/Δ* (CPK2) and *icl1Δ/Δ* (CDM8) strains and their complemented derivatives (CPK3, CEM15, CPK4 and CDM6, respectively) has been described before (Piekarska *et al.*, 2006). To delete the *PEX5* gene in the *fox2Δ/Δ* strain CPK12, two disruption cassettes were made by PCR with primer pair CaPex5Δ-F-FA and CaPex5Δ-R-FA in combination with extension primers (PEX5-F extension and PEX5-R extension) on plasmids pFA-URA and pFA-SAT1, respectively (Gola *et al.*, 2003). The latter plasmid contains the dominant marker *SAT1* that confers resistance to nourseothricin. Sequential transformation of these cassettes into CPK12 resulted in strain CJB8 (*fox2Δ/Δ pex5Δ/Δ*). All *C. albicans* disruption strains were made prototrophic by transformation of a linearized pLUBP vector, which contains the complete *URA3* gene and the flanking *IRO1* gene. The *S. cerevisiae* *icl1Δ* and *fox2Δ* strains were generated by one-step PCR-mediated gene disruption using *KanMX* and *LEU2*, respectively (Gueldener *et al.*, 2002). All constructed strains were verified by diagnostic PCR and Southern blotting.

Spot assay. Cells were pre-grown in minimal liquid medium containing 0.3 % glucose for at least 24 h, washed with water and resuspended at a concentration of $\sim 2.7 \times 10^7$ cells ml⁻¹ (OD₆₀₀ 1). Cells were serially diluted (1:10 dilutions) in water and 5 µl aliquots of each dilution were applied to agar plates. Plates were incubated at 28 °C for 3 days (glucose) or 5 days (acetate, ethanol, citrate and glycerol).

Subcellular fractionation of *C. albicans*. For subcellular fractionation, *C. albicans* cells were pre-grown in minimal 0.3 % glucose medium for at least 24 h, transferred to 200 ml rich oleic acid or ethanol medium and grown to the late exponential phase. Subcellular fractionation was performed essentially as previously described (Aitchison & Rachubinski, 1990; Kamiryo *et al.*, 1982). Briefly, cells were collected by centrifugation, washed three times with water and converted to spheroplasts with Zymolyase 100T (0.25 mg per g cells) in buffer Z (5 mM MOPS pH 7.2, 0.5 M KCl and 10 mM Na₂SO₃). Spheroplasts were collected by centrifugation, resuspended in buffer F [5.5 mM MOPS pH 7.2, 5 % (w/v) Ficoll 400, 0.6 M sorbitol, 0.5 mM EDTA, 0.1 % (v/v) ethanol] and homogenized in a grinding vessel (Potter–Elvehjem) by 20 down-and-up strokes with a tight-fitting pestle. The homogenate was centrifuged for 10 min at 1000 g and the resulting post-nuclear supernatant (H) was fractionated into an organellar pellet (P) and cytosolic supernatant (S) fraction by centrifugation for 20 min at 20 000 g.

Antibodies and immunoblotting. Antibodies against *S. cerevisiae* thiolase and catalase and their cross-reactivity with the corresponding peroxisomal proteins in *C. albicans* have been described before (Piekarska *et al.*, 2006). Polyclonal rabbit antibodies against *S. cerevisiae* Mls1 (Kunze *et al.*, 2002), *S. cerevisiae* Zwfl (glucose-6-phosphate dehydrogenase) (Sigma Aldrich) and *Ashbya gossypii* Icl1 (Schmidt *et al.*, 1996) were used at dilutions of 1:1000, 1:500 and 1:10 000, respectively. Immunoreactive complexes were visualized with horseradish peroxidase-coupled goat-anti-rabbit IgG and the ECL system of Amersham Biosciences.

Enzyme assays. Preparation of cell-free extracts and enzyme assays were performed essentially as described by de Jong-Gubbels *et al.* (1995) with the following modifications. Extracts were freshly prepared and the assays were carried out in a UVIKON 820 double-beam spectrophotometer (Kontron) at room temperature. The malate synthase assay was started with acetyl-CoA instead of glyoxylate.

Morphometric analysis of peroxisomes. Images were acquired with an SIS MegaviewII camera of randomly selected cells at a magnification of $\times 5500$ (100 pixels = 0.44 µm). Using QWin (Leica) in an interactive mode, all peroxisomal structures in a cell as well as the whole cell were traced to determine their cross-sectional areas. The mean peroxisome area was calculated by dividing the total peroxisome area by the total number of peroxisomes counted.

Miscellaneous. The following procedures were performed according to published methods: nucleic acid manipulations (Sambrook *et al.*, 1989), *C. albicans* transformation (Walther & Wendland, 2003), preparation of whole-cell protein extracts (Elgersma *et al.*, 1996), SDS-PAGE and immunoblotting (Bottger *et al.*, 2000), and immunoelectron microscopy (Gould *et al.*, 1990).

RESULTS

Icl1 and Mls1 are localized to peroxisomes in a Pex5p-dependent manner

Both Calcl1 and CaMls1 lack a typical PTS1 or PTS2 motif in their primary sequence. To determine their subcellular distribution, we performed biochemical fractionation and immunoelectron microscopy experiments with *C. albicans* cells grown on two different non-fermentable-carbon sources: ethanol and oleic acid. The choice of the two media was based on a previous study in *S. cerevisiae* in which it was shown that the compartmentalization of Mls1 may vary with the carbon source used (Kunze *et al.*, 2002). First, we assessed the specificity and cross-reactivity of the antibodies used in this study by immunoblotting of total protein extracts (Fig. 1a). The antibody raised against *Ashbya gossypii* Icl1 reacted with a single protein band with an apparent molecular mass of 62 kDa in wild-type *C. albicans* and *S. cerevisiae* cells, while this band was absent in the *Calcl1* deletion strain. Similarly, the *S. cerevisiae* Mls1 antibody recognized a single protein band in both *S. cerevisiae* and *C. albicans* lysates with a molecular mass of about 61 kDa. No band was present in the extracts of *S. cerevisiae* *mls1Δ* cells, confirming the specificity of the antibody. Next, we carried out subcellular fractionation experiments to determine the distribution of Mls1 and Icl1 in *C. albicans* cells grown in ethanol- or oleic acid-containing media (Fig. 1b and c). In addition to wild-type cells we used *pex5Δ/Δ* and *pex13Δ/Δ* cells as controls. The *pex5Δ/Δ* mutant lacking Pex5p is specifically disturbed in the peroxisomal import of PTS1 proteins whereas in the *pex13Δ/Δ* strain both PTS1 and PTS2 import pathways are blocked. Differential centrifugation of a homogenate (H), obtained by osmotic lysis of spheroplasts, resulted in an organellar pellet fraction (P) containing mitochondria and peroxisomes, and a cytosolic supernatant fraction (S). Equal portions of each fraction were analysed by immunoblotting with the Mls1 and Icl1 antibodies. As a control, antibodies against thiolase (a PTS2 protein) and catalase (a PTS1 protein) were used, both of which are proven peroxisomal proteins in *C. albicans* (Piekarska *et al.*, 2006). In both oleic acid- and ethanol-grown wild-type cells Mls1 and Icl1 were predominantly present in the pellet fraction. In the *pex5Δ/Δ* mutant both proteins completely mislocalized to the supernatant fraction, independently of the carbon source used.

Fig. 1. Subcellular distribution of Icl1 and Mls1 in oleic acid- and ethanol-grown *C. albicans* strains. (a) The specificity of the antibodies to *A. gossypii* Icl1 (left panel) and *S. cerevisiae* Mls1 (right panel) was determined by immunoblotting using total protein extracts of the indicated *C. albicans* and *S. cerevisiae* strains. Equal amounts of total protein were loaded in each lane. (b, c) Oleic acid-grown (b) or ethanol-grown (c) wild-type (BWP17), *pex5Δ/Δ* and *pex13Δ/Δ* strains were subjected to subcellular fractionation as described in Methods. Equivalent volumes of the homogenate (H), organellar pellet (P) and supernatant (S) fractions were electrophoresed on SDS-polyacrylamide gels, followed by transfer to nitrocellulose. The blot was probed with antibodies to proteins indicated on the right. Cta1, catalase; thio, thiolase. The asterisk indicates a cross-reactive non-specific band.

Comparable results were obtained with the *pex13Δ/Δ* strain. The relatively large amounts of the peroxisomal marker proteins thiolase and catalase in the cytosolic fraction of wild-type cells suggested that during the fractionation procedure some peroxisomes are disrupted and matrix proteins leak out, a phenomenon that is quite often seen because peroxisomes are very fragile organelles.

To firmly establish the subcellular localization of Icl1 and Mls1, we performed immunoelectron microscopy (Fig. 2). The Icl1 and Mls1 antibodies almost exclusively labelled peroxisomal profiles in oleic acid- and ethanol-grown wild-type cells. In *pex5Δ/Δ* cells cultured on oleic acid gold particles were only found in the cytosol with both antibodies, substantiating the cytosolic localization of Icl1 and Mls1 in this strain inferred from the subcellular fractionation experiments of Fig. 1. Anti-thiolase labelling of *pex5Δ/Δ* cells showed that the PTS2 protein thiolase is still imported in peroxisomal remnants, as can be seen by

the gold particles decorating membranous structures in these cells. These data confirm the PTS1-specific import defect in this mutant strain. Similar results were obtained with the *pex5Δ/Δ* strain grown on ethanol (results not shown). Together, these data suggest that Icl1 and Mls1 are peroxisomal in oleic acid- and ethanol-grown cells and imply that Pex5p mediates their import into peroxisomes.

The glyoxylate cycle can function both in the peroxisome and in the cytosol

Next we addressed the question whether a peroxisomal localization of Icl1 and Mls1 is required for proper functioning of the glyoxylate cycle. To this end, the capacity of wild-type cells to grow on solid minimal ethanol- or acetate-containing media was compared to that of the *pex5Δ/Δ* and *icl1Δ/Δ* mutant strains (Fig. 3a). The *pex5Δ/Δ* mutant grew equally as well as the wild-type strain on acetate and ethanol, while the *icl1Δ/Δ* strain was unable to grow on either carbon source. Growth on glucose and glycerol (a non-fermentable C_3 carbon source) was similar for all strains.

These results suggest that the glyoxylate cycle can function both in the peroxisome and in the cytosol.

Growth of the fatty acid β -oxidation mutant *fox2Δ/Δ* on non-fermentable carbon sources can be restored by relocation of Icl1 and Mls1 to the cytosol

Recently, we reported that a *fox2Δ/Δ* mutant, which lacks the peroxisomal multifunctional enzyme and is completely deficient in peroxisomal fatty acid β -oxidation but has no PTS1 or PTS2 import defect, showed a strongly reduced growth on acetate- and ethanol-containing media (Piekarska *et al.*, 2006 and Fig. 3a). In contrast, growth of the *fox2Δ/Δ* mutant on glucose or on glycerol was comparable to that of wild-type cells (Fig. 3a). The addition of acetic acid or ethanol in the same concentrations to plates containing glucose did not affect growth of the *fox2Δ/Δ* mutant compared to glucose-only plates, suggesting that the strongly reduced growth on acetate and ethanol is due to the inability of the mutant to metabolize C_2 carbon sources efficiently and is not caused by toxic effects of these compounds (results not shown). This growth phenotype of the *C. albicans fox2Δ/Δ* strain was rather unexpected because the *S. cerevisiae fox2Δ* mutant is not deficient for growth on acetate or ethanol (Fig. 3b and data not shown).

Since it has been shown that in *S. cerevisiae* Mls1 and Icl1 are both cytosolic in ethanol- and acetate-grown cells (Kunze *et al.*, 2002) we suspected a correlation between the peroxisomal compartmentalization of these key enzymes of the glyoxylate cycle in *C. albicans* and the inability of the *fox2Δ/Δ* mutant to grow on C_2 carbon sources. To test this hypothesis we disrupted both alleles of the *PEX5* gene in the *fox2Δ/Δ* strain and assessed

Fig. 2. Peroxisome morphology and subcellular localization of Icl1 and Mls1 in wild-type, *pex5Δ/Δ* and *fox2Δ/Δ* cells. Electron microscopy analysis was performed on wild-type cells (a–c, g–i), *pex5Δ/Δ* cells (d–f) and *fox2Δ/Δ* cells (j–o) grown on medium containing oleic acid (a–f, j–l) or ethanol (g–i, m–o). Cryosections were labelled with primary antibodies specific for Mls1 (a, d, g, j and m), Icl1 (b, e, h, k and n) or thiolase (c, f, i, l and o) and immunogold particles coupled to protein A. P, peroxisome; M, mitochondrion. Bars, 0.5 μm.

Fig. 3. Growth characteristics of wild-type and mutant strains on different carbon sources. Cells were pre-grown on 0.3% glucose-containing medium, washed, resuspended at a concentration of about 2.7×10^7 cells ml^{-1} and serially diluted (1 : 10 dilutions). Aliquots (5 μl) of each dilution were spotted onto plates. The picture was taken after 3 days (glucose) or 5 days (acetate, ethanol, glycerol and citrate) of incubation at 28 °C.

growth of the double deletion strain on glucose, ethanol and acetate. Deletion of the *PEX5* gene in the *fox2Δ/Δ* strain completely restored its ability to grow on acetate, while growth on ethanol was partially restored (Fig. 3a). Ramírez & Lorenz (2007) reported recently that the *fox2Δ/Δ* mutant is not only unable to utilize acetate and ethanol (C_2 carbon sources) but also grows poorly on the C_6 carbon source citric acid. A similar phenotype was observed for a strain lacking Icl1, indicating that glyoxylate cycle function is also required for the utilization of citrate. Growth assays confirmed the poor citrate utilization phenotype of the *fox2Δ/Δ* mutant, which could be partially restored by deletion of the *PEX5* gene (Fig. 3a). Taken together, these results suggest that in the *fox2Δ/Δ* strain the glyoxylate cycle cannot function optimally in the peroxisomal compartment and that glyoxylate function can largely be restored by deletion of Pex5p and relocation of Mls1 and Icl1 to the cytosol.

Icl1 and Mls1 protein levels and activities are not reduced in the *fox2Δ/Δ* strain

One possible explanation for the strongly reduced growth of the *fox2Δ/Δ* cells on ethanol, acetate and citrate is that the glyoxylate cycle enzymes Icl1 and Mls1 are less stable or less active in peroxisomes of the mutant strain. To test this, protein levels and activities of Icl1 and Mls1 were determined in ethanol- and acetate-grown wild-type, *pex5Δ/Δ*, *fox2Δ/Δ*, *fox2Δ/Δ* complemented, *fox2Δ/Δ pex5Δ/Δ* and *icl1Δ/Δ* cells. Immunoblot analysis revealed that Icl1 and Mls1 were present at similar levels in wild-type, *fox2Δ/Δ*, *fox2Δ/Δ* complemented and *fox2Δ/Δ pex5Δ/Δ*

Δ , independent of the carbon source used (Fig. 4). In the *pex5Δ/Δ* strain grown on ethanol Icl1 levels appeared to be reduced, a phenotype that was also observed, although to a lesser extent, in acetate-grown *pex5Δ/Δ* cells. Icl1 was absent in the *icl1Δ/Δ* strain, confirming the mutant phenotype. Quantification of Icl1 and Mls1 enzyme activities in protein extracts of these six strains largely confirmed the immunoblot results (Table 3). Ethanol- and acetate-grown *pex5Δ/Δ* cells exhibited reduced Icl1 activity, amounting to about 60% and 40%, respectively, of that measured in wild-type cells while Mls1 activities were not reduced in this mutant strain. These data show that neither protein levels nor enzyme activities of Icl1 and Mls1 are reduced in the *fox2Δ/Δ* strain.

fox2Δ/Δ cells grown on oleate or ethanol have significantly larger peroxisomes than wild-type cells

During growth on ethanol or acetate the cytosolically formed acetyl-CoA must be transported into peroxisomes to enter the glyoxylate cycle, whereas products of this shunt, i.e. succinate and malate, have to leave the peroxisome and are shuttled to the cytosol/mitochondria. Similarly, the utilization of citrate may require the glyoxylate cycle for the generation of C_4 compounds and thus shuttling of metabolites across the peroxisome membrane. Since the levels and activities of the key enzymes of the glyoxylate cycle, Icl1 and Mls1, are not reduced in the *fox2Δ/Δ* strain, the observed phenotype of the mutant could be explained by defective peroxisomal membrane transport of (a) metabolite(s). Previously, it has

Fig. 4. Protein abundance of Icl1 and Mls1 in wild-type and mutant strains grown on ethanol or acetate. Equal amounts of total protein extracts of the indicated strains were analysed by immunoblotting with antibodies against Icl1, Mls1 or Zwfl (glucose-6-phosphate dehydrogenase). Zwfl served as loading control.

been observed in yeast, mammals and plants that mutants with a deficiency in peroxisomal β -oxidation exhibit a changed peroxisome size and abundance (Chang *et al.*, 1999; Germain *et al.*, 2001; Rylott *et al.*, 2006; Smith *et al.*, 2000). These observations prompted us to investigate peroxisome morphology in the *fox2Δ/Δ* strain (Fig. 2). Immunoelectron microscopy revealed that peroxisomes in oleic acid grown *fox2Δ/Δ* cells appeared more irregular in shape and larger in size than peroxisomes of wild-type cells. Morphometric analysis showed that wild-type cells did not contain peroxisomes larger than $0.6\ \mu\text{m}^2$ whereas many peroxisomes in *fox2Δ/Δ* cells had a surface area larger than $1.0\ \mu\text{m}^2$ (Fig. 5). The mean peroxisome area in *fox2Δ/Δ* cells was twice that of wild-type cells ($0.264\pm0.321\ \mu\text{m}^2$ and $0.126\pm0.090\ \mu\text{m}^2$ for *fox2Δ/Δ* and wild-type, respectively; $P<0.0001$). The enlarged peroxisomes were still capable of importing peroxisomal matrix proteins, as shown by the peroxisomal localization of the PTS2 enzyme thiolase and the glyoxylate cycle enzymes Icl1 and Mls1 (Fig. 2). Similarly, peroxisomes of *fox2Δ/Δ* cells grown on ethanol were considerably larger in size than those of wild-type cells and contained all three peroxisomal enzymes

(Figs 2 and 5). Again, morphometric analysis showed that the mean area of peroxisomes in *fox2Δ/Δ* cells was about 2.5 times that of wild-type cells ($0.162\pm0.121\ \mu\text{m}^2$ for *fox2Δ/Δ* and $0.062\pm0.040\ \mu\text{m}^2$ for wild-type; $P<0.0001$). However, in contrast to what was observed for cells grown on oleic acid, peroxisomes in ethanol-grown *fox2Δ/Δ* cells were regular in shape and appeared as oval-shaped structures as normally seen in wild-type cells. These results show a clear increase in peroxisome size in the *fox2Δ/Δ* mutant, which may contribute to its inability to efficiently metabolize carbon sources that require a functional glyoxylate cycle.

DISCUSSION

In most eukaryotic organisms that utilize the glyoxylate cycle, i.e. plant seedlings, yeasts and filamentous fungi, the metabolic pathway is compartmentalized in peroxisomes. In this paper we report the localization in *C. albicans* of the two key glyoxylate cycle enzymes, Icl1 and Mls1. In subcellular fractionation experiments both proteins

Table 3. Icl1 and Mls1 enzyme activities in total protein extracts of wild-type and mutant strains

Enzyme activities are in mU (mg protein)⁻¹. The results are means of two independent experiments that varied by <15% from the mean.

Strain	Icl1		Mls1	
	Acetate	Ethanol	Acetate	Ethanol
Wild-type	226.7	255.7	27.6	30.6
<i>pex5Δ/Δ</i>	86.15	146.2	46.5	38.6
<i>fox2Δ/Δ</i>	190.2	301.7	45.3	61.1
<i>fox2Δ/Δ pex5Δ/Δ</i>	192.3	173.9	42.3	35.8
<i>fox2Δ/Δ+FOX2</i>	206.1	216.8	33.7	30.6
<i>icl1Δ/Δ</i>	0	0	64.4	59.0

Fig. 5. Peroxisome size in wild-type and *fox2Δ/Δ* cells grown on different carbon sources. Morphometric analysis of peroxisomes was performed (see Methods) on wild-type and *fox2Δ/Δ* cells, grown on medium containing oleic acid (top panels) or ethanol (bottom panels). The number of peroxisomes counted on the micrographs for each condition was 69 (wild-type, oleate), 104 (*fox2Δ/Δ*, oleate), 80 (wild-type, ethanol) and 106 (*fox2Δ/Δ*, ethanol). On the y-axis the percentage of total peroxisome surface area occupied by the peroxisomes of each category is given. The maximum sizes of peroxisomes in each category are depicted on the x-axis.

co-fractionate with peroxisomal marker enzymes, and their peroxisomal localization was confirmed by immunoelectron microscopy (Figs 1 and 2). Targeting of Icl1 and Mls1 to peroxisomes appeared to be dependent on the PTS1 receptor Pex5p although neither protein has a PTS1 consensus sequence, which was defined as S/A/C-K/R/H-L/M (Gould *et al.*, 1989; Swinkels *et al.*, 1992). Peroxisomal targeting of proteins in the absence of a typical PTS1 has been reported before and these studies revealed that residues upstream of the carboxyl-terminal tripeptide could play a critical role in the targeting event, with a preference for a lysine (or arginine) at the -4 position immediately preceding the non-consensus PTS1 (Lametschwandner *et al.*, 1998; Mullen *et al.*, 1997; Purdue & Lazarow, 1996). A conserved lysine residue is present at the -4 position in Icl1 from *C. albicans* (KAKA_{COOH}), *Candida tropicalis* (KAKV_{COOH}) castor bean (KARM_{COOH}) and *Yarrowia lipolytica* (KSKL_{COOH}), although the latter two also contain a PTS1 that complies with the consensus sequence. In all four organisms Icl1 appears to be peroxisomal (Gao *et al.*, 1996; Tanaka & Ueda, 1993; Titorenko *et al.*, 1998) whereas in *S. cerevisiae* Icl1 is cytosolic, which is in line with the absence of both a consensus PTS1 and a lysine at -4 (GVKK_{COOH})

(McCammon *et al.*, 1990; Taylor *et al.*, 1996). Whether the carboxyl-terminal four amino acids in *C. albicans* Icl1 play a role in peroxisomal targeting of the protein remains to be established, in particular since it has been shown that castor bean Icl1 can be targeted to peroxisomes in a Pex5p-dependent way when the carboxyl-terminal 19 amino acids are deleted (Parkes *et al.*, 2003). These data suggest the presence of alternative non-PTS1 targeting sequences in castor bean Icl1.

C. albicans Mls1 also lacks a typical PTS1 (ERL_{COOH}) and no lysine or arginine is present at the -4 position, whereas in most other organisms, e.g. *Neurospora crassa*, *Aspergillus nidulans*, *S. cerevisiae* and *Brassica napus*, a consensus PTS1 is found (either SKL_{COOH} or SRL_{COOH}). The carboxyl-terminal tripeptide ERL_{COOH} is also found in *C. tropicalis* Mls1 and the peroxisomal location of the protein has been firmly established (Hikida *et al.*, 1991). A glutamic acid at the -3 position is also present in Mls1 of the closely related species *Debaryomyces hansenii* (-EKL_{COOH}), suggesting that the PTS1 consensus of Mls1 in these species deviates from the established one and that negatively charged amino acids at the -3 position may be tolerated. Although *S. cerevisiae* Mls1 contains a typical PTS1 (-SKL_{COOH}), the

localization of this enzyme is not always peroxisomal but depends on the carbon source used: in oleic acid-grown cells Mls1 is peroxisomal whereas in ethanol-grown cells it is cytosolic (Kunze *et al.*, 2002). It has been suggested that the carbon source-dependent localization of Mls1 may be advantageous to the cell, the enzyme being localized in the compartment where the acetyl-CoA, one of its substrates, is formed, i.e. peroxisomal on oleic acid and cytosolic on ethanol or acetate. Such a carbon source-dependent localization was not seen for Mls1 or Icl1 in *C. albicans*: both enzymes were found in peroxisomes under all conditions tested (Figs 1 and 2). Nevertheless, a peroxisomal compartmentalization of Mls1 and Icl1 is not absolutely required for proper functioning of the glyoxylate cycle since a *pex5Δ/Δ* strain, which mislocalizes both enzymes to the cytosol, showed wild-type growth rates on ethanol and acetate (Fig. 3) despite the reduced activity of Icl1 on these carbon sources (Table 3).

Next, we asked whether there is a correlation between the peroxisomal compartmentalization of the key enzymes of the glyoxylate cycle in *C. albicans* and the inability of the β -oxidation mutant *fox2Δ/Δ* to grow efficiently on C_2 carbon sources (Fig. 4 and Piekarska *et al.*, 2006; Ramírez & Lorenz, 2007). We showed that the growth inhibition is not caused by toxicity of acetate or ethanol, and mitochondrial functions appeared to be normal in the *fox2Δ/Δ* mutant, strongly suggesting that it cannot efficiently metabolize C_2 compounds. In support of this we found acetate metabolism in the *fox2Δ/Δ* mutant to be reduced to 60 % of that of the wild-type strain using ^{14}C -labelled acetate as substrate (data not shown). In addition to the growth deficiency on C_2 compounds (and oleate), the *fox2Δ/Δ* mutant uses citrate (C_6) poorly (Ramírez & Lorenz, 2007). The fact that the *icl1Δ/Δ* strain shows a similar growth defect on citrate implies that the glyoxylate cycle function is also required for the utilization of this C_6 compound. We suggest that (iso)citrate must enter the glyoxylate cycle to allow the cell to generate C_4 units (succinate/malate), which can be used to replenish the TCA cycle and/or function as precursors for gluconeogenesis. By deleting the *PEX5* gene in the *fox2Δ/Δ* mutant, growth on ethanol and citrate could be restored partially and growth on acetate completely, consistent with our hypothesis that the observed growth phenotype of the *fox2Δ/Δ* strain on these carbon compounds may be caused by peroxisomal compartmentalization of the glyoxylate cycle. Ramírez & Lorenz (2007) have recently suggested that the different growth phenotypes of the *C. albicans* and *S. cerevisiae fox2* null strains may be explained by differences in the regulatory networks governing carbon metabolism in both fungi. However, our data now strongly suggest that the different compartmentalization of glyoxylate cycle enzymes in both organisms may provide an explanation for these growth differences.

It is currently unclear why the glyoxylate cycle cannot function optimally in peroxisomes of the *C. albicans fox2Δ/Δ* strain. We show here that the impaired glyoxylate cycle function in the *fox2Δ/Δ* mutant is not caused by reduced

levels or activity of the key enzymes Icl1 and Mls1 (Fig. 4, Table 3). Therefore, we favour the hypothesis that the transport of metabolites (glyoxylate cycle substrates/products and/or acetyl-CoA) across the peroxisomal membrane is affected in the *fox2Δ/Δ* strain, thereby reducing the overall rate of non-fermentable carbon metabolism. We consider two possible mechanisms: direct inhibition of peroxisomal transport proteins by activated fatty acids (acyl-CoAs) or an indirect mechanism caused by the increase in size and/or changed morphology of peroxisomes. A consequence of blocking fatty acid β -oxidation by disruption of the *FOX2* gene is the accumulation of acyl-CoAs inside the cell. Even in the absence of exogenously added fatty acids such an accumulation of acyl-CoAs is likely to occur because fatty acid β -oxidation is also required for the turnover of endogenous membrane lipids. Since the peroxisome is the sole site of fatty acid β -oxidation in fungi it is conceivable that the acyl-CoA levels inside peroxisomes increase. The inhibitory effect of (long-chain) acyl-CoAs on mitochondrial carrier proteins is well documented (Morel *et al.*, 1974) and we speculate that peroxisomal metabolite transporters may be inhibited in a similar way in the *fox2Δ/Δ* mutant. It is also possible that, due to their amphipathic nature, the acyl-CoAs insert into the peroxisomal membrane, thereby changing its physical properties and transport capabilities. An alternative mechanism is based on simple physics: when the size of the organelle increases, the surface (=membrane) area-to-volume ratio decreases, and thus large peroxisomes have relatively less membrane surface available for transport processes than small peroxisomes. Similar suggestions were put forward by Kiel *et al.* (2005) to explain the increase of penicillin production in *Penicillium chrysogenum* upon overexpression of Pex11p. Penicillin biosynthesis in *P. chrysogenum* occurs partially in peroxisomes and partially in the cytosol. Overexpression of Pex11p in this organism resulted in massive proliferation of small tubular-shaped peroxisomes and a 2.5-fold higher penicillin production while the level of the penicillin biosynthesis enzymes remained unchanged, suggesting that an increase in membrane-to-volume ratio may increase the transport capacity of the organelles. Further experiments are required to distinguish between these possibilities.

In conclusion, our results provide an explanation for the unexpected growth phenotypes of the *C. albicans fox2Δ/Δ* strain on non-fermentable carbon sources and strongly support the existence of peroxisomal metabolite transporters. Identification of such transporters may now be feasible using *C. albicans* as a model system.

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