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Impaired biosynthesis of the non-bilayer lipids phosphatidylethanolamine or cardiolipin does not affect peroxisome biogenesis and proliferation in Saccharomyces cerevisiae

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Abstract

The non-bilayer forming lipids cardiolipin (CL) and phosphatidylethanolamine (PE) modulate membrane curvature, facilitate membrane fusion and affect the stability and function of membrane proteins. Yeast peroxisomal membranes contain significant amounts of CL and PE. We analysed the effect of CL deficiency and PE depletion on peroxisome biogenesis and proliferation in Saccharomyces cerevisiae. Our data indicate that deletion of CRD1, which encodes cardiolipin synthase, does not affect peroxisome biogenesis or abundance, both at peroxisome repressing (glucose) or inducing (oleate) growth conditions. Analysis of strains deficient in one of the three PE biosynthesis pathways (psd1, psd2 or the triple deletion strain eki1 cki1 dpl1) revealed that in all three strains peroxisome numbers were reduced upon growth of cells on oleic acid, whereas the psd1 strain also showed a reduction in peroxisome abundance upon growth on glucose. Because PE is an intermediate of the phosphatidylcholine (PC) biosynthesis pathway, PE depletion affects PC formation. PC however can be synthesized by an alternative pathway when choline is supplemented to the growth medium. Because the addition of choline resulted in suppression of the peroxisome phenotypes in phosphatidylserine decarboxylase mutant strains, we conclude that peroxisome biogenesis and proliferation are not crucially dependent on CL or PE.

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1. Introduction

Peroxisomes are cell organelles present in almost all eukaryotic cells. Enzymes localized in peroxisomes are involved in a variety of metabolic processes including β-oxidation of fatty acids and hydrogen peroxide detoxification [1,2].

The main phospholipid constituents of Saccharomyces cerevisiae peroxisomal membranes are phosphatidylcholine (PC) (48%), phosphatidylethanolamine (PE) (23%) and phosphatidylinositol (16%) [3]. In addition, these membranes contain substantial levels (up to 7%) of cardiolipin (CL) [3]. A similar composition was reported for peroxisomal membranes of the yeast Pichia pastoris [4].

PC has a cylindrical shape and is a typical lipid of biological membranes. Recent studies indicated that depletion of PC results in disturbed peroxisomal membrane fluidity in S. cerevisiae [5]. CL and PE have an inverted cone shape and are non-bilayer forming lipids. Such lipids have been reported to modulate membrane curvature, facilitate membrane fusion [6,7], affect binding of peripheral membrane proteins and formation of membrane protein complexes [8]. In mitochondria CL and PE play crucial roles in various aspects of organelle function and dynamics [7,9–12]. So far, the functions of CL and PE in the peroxisomal membrane are unknown.

In S. cerevisiae CL biosynthesis occurs solely in mitochondria and requires among others the CRD1 gene (Fig. 1A) [13,14]. The biosynthesis of PE is more complex and involves 3 pathways which occur in different cell compartments (Fig. 1B) [15]. Phosphatidylserine (PS) can be decarboxylated by phosphatidylserine decarboxylase 1 (Psd1) at the inner mitochondrial membrane [16,17] or by phosphatidylserine decarboxylase 2 (Psd2) in vacuole/Golgi membranes [18]. Additionally, PE can be formed by...
incorporation of ethanolamine through the Kennedy pathway localized to microsomes [19]. Ethanolamine utilized in this route can be taken up from the growth medium or be released within the cell by phospholipases, which is followed by phosphorylation by ethanolamine kinase 1 (Eki1) [20]. Ethanolamine can also be phosphorylated by choline kinase 1 (Cki1) [21]. Additionally, ethanolamine phosphate can derive from degradation of sphingolipid PE can be methylated to form PC. Alternatively, PC can be formed by incorporation of choline via the Kennedy pathway. An intermediate of this pathway—ethanolamine phosphate (Etn-P)—can also derive from degradation of sphingolipids. PE can be methylated to form PC. Alternatively, PC can be formed by incorporation of choline via the Kennedy pathway.

The role of both non-bilayer lipids, CL and PE, on peroxisome biogenesis or proliferation. Our data indicate that CL and PE are not crucial for peroxisome biogenesis or proliferation. Our data indicate that CL and PE are not crucial for peroxisome biogenesis or proliferation.

### 2. Materials and methods

#### 2.1. Strains and growth conditions

The S. cerevisiae strains used in this study are listed in Table 1. S. cerevisiae cells were grown at 30 °C in mineral medium [24] containing 0.5% glucose or a mixture of 0.1% glucose, 0.1% oleic acid and 0.05% Tween 80. Culture medium was supplemented with leucine (30 μg/ml), histidine (20 μg/ml), uracil (30 μg/ml) and lysine (30 μg/ml).

### 2.2. Cloning and construction of yeast strains

The plasmids and primers used in this study are listed in Table 2 and Table 3.

Plasmid pTDH3_GFPSKL was constructed by amplification of the GFP_SKL open reading frame with additional HindIII/SalI sites in a PCR reaction using primers TDH3_GFPSKL_F and TDH3_GFPSKL_R and plasmid pHIPX7_eGFPSKL as a template, followed by HindIII/SalI digestion and ligation with HindIII/SalI digested

### Table 1

<table>
<thead>
<tr>
<th>S. cerevisiae</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (WT)</td>
<td>BY4742 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>crd1</td>
<td>BY4742 CRD1::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>psd1</td>
<td>BY4742 PSD1::kanMX6</td>
<td>This study</td>
</tr>
<tr>
<td>psd2</td>
<td>BY4742 PSD2::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>eki1 cki1 dpl1</td>
<td>BY4742 EKI1::kanMX6 CKI1::HPH DPL1::NAT</td>
<td>This study</td>
</tr>
<tr>
<td>psd1 psd2</td>
<td>BY4742 PSD1::kanMX6 PSD2::NAT</td>
<td>This study</td>
</tr>
<tr>
<td>WT GFP-SKL</td>
<td>WT PmHIS4-GFP-SKL</td>
<td>This study</td>
</tr>
<tr>
<td>crd1 GFP-SKL</td>
<td>crd1 PmHIS4-GFP-SKL</td>
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</tr>
<tr>
<td>psd1 GFP-SKL</td>
<td>psd1 PmHIS4-GFP-SKL</td>
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<tr>
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<tr>
<td>psd1 psd2 GFP-SKL</td>
<td>psd1 psd2 PmHIS4-GFP-SKL</td>
<td>This study</td>
</tr>
</tbody>
</table>

* All S. cerevisiae strains listed here require leucine, lysine, histidine and uracil for growth.
Table 1
Primer name
ScPSD1_del_F GCCAGTTAAGAACGCCTTGGCGCAAGGGAGGACGCTCCTCATGGGGAGGACAGCTGAAGCTTCGTACGC
ScPSD1_del_R CAGCTATGTTAGTTTAACTTCCTCGATTTTTCAATTTGAAGAGTTTTTCACGTTTTCGACACTGGATGG
ScPSD2_del_F GTATCAATTGGTAAAGAATCCTCGATTTTCAGGAGCATCCAACGAAGCCCACACACCATAGCTTCAA
ScPSD2_del_R TACTCATCTGGACATTTGATTCTATATAGCGTTTTCGACACTGGATGG
ScEKI1_del_F TACCTCTGATATAATTTTCTTTACGCATAGGCCACTAGTGGATCTG
ScEKI1_del_R ACTGAGCTACAGATTTTCTTTACGCATAGGCCACTAGTGGATCTG
ScCKI1_del_F ACTGCTACAGATTTTCTTTACGCATAGGCCACTAGTGGATCTG
ScCKI1_del_R GAACTCTGATATAATTTTCTTTACGCATAGGCCACTAGTGGATCTG
ScDP1_del_F TACCTCTGATATAATTTTCTTTACGCATAGGCCACTAGTGGATCTG
ScDP1_del_R GAACTCTGATATAATTTTCTTTACGCATAGGCCACTAGTGGATCTG
TDH3_GFPSKL_F TGACGTCGACTTCTCTTATATTTTCTTTACGCATAGGCCACTAGTGGATCTG
TDH3_GFPSKL_R TGACGTCGACTTCTCTTATATTTTCTTTACGCATAGGCCACTAGTGGATCTG

Table 2
Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>pUG6</td>
<td>contains gentamicin resistance cassette (kanMX6); amp^R</td>
<td>[39]</td>
</tr>
<tr>
<td>pENTR-221-HPH</td>
<td>pENTR-221 containing hygromycin marker; kan^R</td>
<td>[40]</td>
</tr>
<tr>
<td>pENTR-221_NAT</td>
<td>pENTR-221 containing nourseothricin marker; kan^R</td>
<td>[40]</td>
</tr>
<tr>
<td>pHIPX7_eGFPSKL</td>
<td>contains eGFPSKL under the control of H. polymorpha P^Hpol promoter; contains S. cerevisiae LEU2 gene for complementation of leucine auxotrophy; kan^R</td>
<td>[41]</td>
</tr>
<tr>
<td>pTDH3_mcherrysfGFP</td>
<td>contains tandem protein (mcherry + sfGFP) expressed from S. cerevisiae P^Hpol promoter; contains zeocine resistance cassette; amp^R</td>
<td>Sanjeev Kumar (unpublished)</td>
</tr>
<tr>
<td>pTDH3_GFPSKL</td>
<td>contains eGFPSKL under the control of S. cerevisiae P^Hpol promoter; contains zeocine resistance cassette; amp^R</td>
<td>this study</td>
</tr>
</tbody>
</table>

pTDH3_mcherrysfGFP. Transformed E. coli DH5α were selected on LB agar plates supplemented with 100 µg/ml ampicillin.

Transformation of S. cerevisiae cells was performed by the Li-Ac method [25]. Selection of yeast transformants was performed on YPD agar plates supplemented with 200 µg/ml hygromycin B, 100 µg/ml gentamycin, 200 µg/ml zeocine or 100 µg/ml nourseothricin.

S. cerevisiae psd1 deletion strain was obtained by replacement of

Fig. 2. Intracellular localization of GFP-SKL in S. cerevisiae cells impaired in the biosynthesis of CL or PE. Fluorescence microscopy of WT and the indicated mutants producing GFP-SKL, growing exponentially in medium containing 0.5% glucose or a mixture of 0.1% oleate and 0.1% glucose. Scale bar represents 5 µm.
nucleotides +1 to +1185 from the start codon of the PSD1 gene (YNL1696) by the gentamycin resistance cassette. To this end, primers ScPSD1_del_F and ScPSD1_del_R were used to amplify the gentamicin resistance cassette using plasmid pUG6 as the template. The obtained PCR product was used for transformation of WT (BY4742) cells.

The psd1 psd2 double deletion strain was constructed by deletion of PSD2 (YGR170W) in psd1 cells. Nucleotides +1 to +3180 from the start codon of PSD2 were replaced by a nourseothricin resistance cassette. To this end, primers ScPSD2_del_F and ScPSD2_del_R were used to amplify the nourseothricin resistance cassette using plasmid pENTR-221_NAT as the template. The corresponding deletion cassettes were amplified by PCR using primers ScEKI1_del_F and ScEKI1_del_R and pUG6 plasmid, primers ScCKI1_del_F and ScCKI1_del_R and pENTR-221_HPH plasmid, primers ScDPL1_del_F and ScDPL1_del_F and pENTR-221_NAT plasmid, respectively.

2.3. Fluorescence microscopy and image analysis

Fluorescence microscopy images were captured using a Carl Zeiss confocal microscope LSM510 equipped with photomultiplier tubes (Hamamatsu Photonics). The GFP signal was visualized using a 488 nm argon ion laser (Lasos) and a 500–550 nm bandpass emission filter. Images were acquired using ZEN 2012 software (Carl Zeiss). Before analysis, S. cerevisiae cells were fixed for 1 h with 4% formaldehyde in 0.1 M sodium phosphate buffer pH 7.2. The presented fluorescence microscopy images represent maximum intensity projections in the GFP channel. Additionally, bright field images were false coloured in blue to mark cell edges.
Peroxisome quantification in S. cerevisiae was performed manually by counting GFP spots in individual cells throughout the Z-stacks.

3. Results

3.1. Reduction in PE biosynthesis or CL depletion do not have major effects on peroxisomes biogenesis or proliferation in S. cerevisiae

We started our studies with the analysis of the effect of CL deficiency and reduced PE levels. To this purpose we used an S. cerevisiae mutant lacking cardiolipin synthase (crd1), which results in a complete block in CL biosynthesis [26]. In addition we studied three mutants (psd1, psd2 and eki1 cki1 dpl1) (Fig. 1B), in which one of the three PE biosynthetic pathways is blocked, leading to significantly reduced PE levels [23]. Cells were grown on defined mineral medium lacking yeast extract to prevent the presence of lipid precursors (ethanolamine, choline) or lyso-lipids, including lyso-phosphatidylethanolamine [27–29].

As shown in Fig. 1C, all mutant strains grew similar as the wild-type (WT) control on medium containing glucose. However, when cells were grown on medium containing oleate, the psd1 strain showed an increased doubling time, but the final optical densities (OD600) of all strains were the same (Fig. 1D).

Fluorescence microscopy analysis of WT and mutant cells exponentially growing on glucose or oleic acid containing media showed that the matrix marker protein GFP-SKL was properly imported in peroxisomes in all strains (Fig. 2). Quantification of peroxisome numbers revealed that crd1 cells displayed similar numbers of peroxisomes as WT control cells both upon growth on glucose or oleate (Fig. 3A, B). Interestingly, deletion of PSD1 resulted in a reduction of peroxisome numbers both on glucose and oleate (Fig. 3A, B). Deletion of PSD2 or EKI1 CKI1 DPL1 had no effect on peroxisome abundance in glucose-grown cells, whereas the numbers were slightly decreased upon growth on oleate (Fig. 3B).

3.2. Addition of choline to the growth medium suppresses the impact of phosphatidyserine decarboxylase deficiency on peroxisome proliferation

PE may also be methylated to form phosphatidylcholine (PC) (Fig. 1B). Thus impaired PE biosynthesis indirectly affects the formation of PC. To allow PE independent formation of PC, media were supplemented with choline, which can be used in the Kennedy pathway for the formation of PC (Fig. 1B). This largely suppressed the growth defect of psd1 cells in oleate containing medium (Fig. 4A, compare Fig. 1D). Concomitantly, no significant reduction in peroxisome numbers was observed anymore upon growth of psd1 cells on glucose or oleic acid (Fig 4B, C). Previously, the simultaneous deletion of PSD1 and PSD2 was shown to enhance the depletion of cellular PE relative to that observed in PSD1 or PSD2 single deletion strains [23]. Growth of psd1 psd2 cells in medium containing glucose or oleate supplemented with choline was not altered relative to psd1 cells (data not shown). Furthermore, at these conditions peroxisome abundance was not reduced (Fig 4B, C).

These data indicate that the effect of impaired PE synthesis on peroxisomes and oleic acid growth is likely to be associated with hampered synthesis of PC.

4. Discussion

CL has been detected in peroxisomal membranes isolated from S. cerevisiae, P. pastoris and castor bean [3–5,23,30]. However, here we show that a complete block in CL biosynthesis (in crd1 cells) does not affect peroxisome formation, function (reflected in normal growth on oleate) or proliferation. Our data are consistent with studies in plants which revealed no effect of CL deficiency on peroxisome morphology [31]. Our observations raise the question as to why CL is present in yeast peroxisomal membranes. Possibly, CL deficiency is compensated by other non-bilayer forming lipids, such as PE. In mitochondria CL and PE are playing partially overlapping roles and only a simultaneous block in mitochondrial PE and CL synthesis (by deletion of PSD1 and CRD1) is lethal [7,32]. Alternatively, the reported presence of CL in peroxisomal membrane fractions is due to contamination with other, CL containing membranes. Indeed, CL was not detected in peroxisomes isolated form Candida tropicalis [33] or rat liver [34,35]. Mitochondrial contamination could be due to artificial association of both organelles occurring during the peroxisome isolation procedure [36], but both organelles may also form physical contacts in vivo [37,38].

In contrast to CL, PE represents a major fraction of the
peroxisomal phospholipids, which is unlikely due to contamination by other organelles [3,4,33]. We show that deletion of PSD1 resulted in a growth defect on media containing oleic acid, suggesting a partial defect in peroxisome function. However, this growth defect was largely restored upon addition of choline to the medium, indicating that the growth problem was indirectly caused by a defect in PC biosynthesis (Fig. 1).

Similarly, the reduction in peroxisome numbers observed in the psd1 mutant was abolished by supplementation of the medium with choline. This is in line with recent data that indicate that peroxisome numbers in S. cerevisiae psd1 and psd1 psd2 cells grown on rich YPO (yeast extract, peptone, oleate) medium, which contains trace amounts of ethanolamine and choline [23]. Hence, we speculate that the decrease in peroxisome numbers in S. cerevisiae PE biosynthesis mutants in the absence of choline is indirectly caused by impaired PC formation, which is considered as essential in yeast [15].

Overall, our data indicate that unlike in mitochondria, a reduced content of cellular CL or PE does not affect peroxisome biogenesis or proliferation.

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