YDL142c encodes cardiolipin synthase (Cls1p) and is non-essential for aerobic growth of *Saccharomyces cerevisiae*

Gabriele Tuller[a], Claudia Hrastnik[a], Georg Achleitner[a], Ursula Schiefthaler[b], Franz Klein[b], Günther Daum[a,*]

[a]*Institut für Biochemie und Lebensmittelchemie, Technische Universität Graz, Petersgasse 12/2, A-8010 Graz, Austria*
[b]*Institut für Botanik, Universität Wien, Rennweg 14, A-1030 Wien, Austria*

Received 25 November 1997

Abstract The unassigned open reading frame YDL142c was identified to code for cardiolipin synthase, Cls1p. A *cls1* deletion strain is viable on glucose, galactose, ethanol, glycerol and lactate containing media, although the growth rate on non-fermentable carbon sources is decreased. Mitochondria of the *cls1* mutant are devoid of cardiolipin but accumulate the cardiolipin precursor phosphatidylglycerol when grown on non-fermentable carbon sources. Specific activity of phosphatidylglycerolphosphate synthase in *cls1* is reduced to 30–75% of the wild-type level. Amounts of mitochondrial cytochromes and activity of cytochrome *c* oxidase, however, are not affected in the *cls1* deletion strain. Collectively, these data indicate that cardiolipin is not essential for aerobic growth of *Saccharomyces cerevisiae*.

Key words: Cardiolipin; Phosphatidylglycerol; Phosphatidylglycerolphosphate; Mitochondria; Cytochrome *c* oxidase; Yeast

1. Introduction

Cardiolipin (CL), a phospholipid mainly present in the inner mitochondrial membrane of eukaryotes [1] is generally regarded to be an essential component of mitochondrial function. In the yeast *Saccharomyces cerevisiae* CL is an activator of cytochrome *c* oxidase [2] and probably involved in the import of proteins into mitochondria [3]. In higher eukaryotes, CL is an effector of the cytochrome P-450-dependent cholesterol side-chain cleavage enzyme [4], and activates cytochrome *c* oxidase [5] and the mitochondrial phosphate carrier protein [6].

Synthesis of CL in yeast (for reviews see [7,8]) requires three sequential reaction steps. First, phosphatidylglycerolphosphate synthase (Pgs1p) catalyzes the synthesis of phosphatidylglycerolphosphate (PtdGro-P) from CDP-diacylglycerol and glycerol-3-phosphate. Then, PtdGro-P phosphatase (PGPP) dephosphorylates PtdGro-P yielding phosphatidylglycerol (PtdGro) that is finally converted to CL by cardiolipin synthase (Cls1p) with CDP-diacylglycerol as a co-substrate. Reactions of this biosynthetic pathway in yeast have been characterized [9–12], but none of the genes encoding the respective proteins have been cloned nor have the enzymes been isolated.

During the European Functional Analysis Network (EUROFAN) Program [13] a large number of open reading frames of the yeast *S. cerevisiae* were disrupted by a standard technique [14], and null mutants were constructed and characterized. One of the open reading frames studied, YDL142c, has significant homology to genes encoding PtdGro-P synthase of various prokaryotic microorganisms. Here we present biochemical evidence that a yeast mutant with a deletion of YDL142c is defective in the formation of CL thus identifying this open reading frame as *CLS1*.

2. Materials and methods

2.1. Construction of a cls1 deletion strain

The open reading frame YDL142c was replaced by the KanMX4 marker using a PCR-mediated one step gene replacement strategy [14]. Two hybrid primers, P1: 5′-GGCCTGGTAGCATAGTTTGGTCCCTAATAATTTAGTCAATG*GTACGCTGCAGGTCGACGGATCCCC*-3′ and P2: 5′-GGCCTTTTGAAAGTCCTGTTTTCTATCTTCGAAATCCTCCTCGG*TATCATCGATGAATTCGAGCTCGTT*-3′, consisting of 41 bp and 44 bp of the YDL142c flanking sequence at the 5′ end and 25 bp of KanMX4 flanking sequence at the 3′ end (italic) were used to synthesize a linear DNA fragment using cosmid 2A7 (provided by L. Baron and Y. Legros) as a template. Positions 4 to 716 of the YDL142c open reading frame (total length 852 bp) were replaced thus deleting 84% of the gene at the 5′ end; the number of remaining original open reading frame nucleotides on the 5′ end was 3, and on the 3′ end 137 including the stop codon. This construct was directly used for transformation of the diploid wild-type strain FY1679 (MAT **a**/α, *ura3-52*/*ura3-52*, *TRP1*/*trp1Δ63*, *LEU2*/*leu2Δ1*, *HIS3*/*his3Δ200*). The replacement was verified twice by Southern blot analysis using two different restriction enzymes (*Hpa*I or *Eco*RI) to digest the genomic DNA. A 1.36 kb *Hpa*I/*Sac*I fragment in the 5′ region of the open reading frame was used as a probe. Upon tetrad dissection the deletion showed Mendelian 2:2 segregation as monitored by the KanMX4 marker. A haploid deletion strain (Mat **a**, *ura3-52*, *trp1Δ63*, *leu2Δ1*, *his3Δ200*, *cls1::KanMX4*) named *cls1* in the following grew on YPD plates similar to the isogenic haploid wild-type strain FY1697 (Mat **a**, *ura3-52*, *trp1Δ63*, *leu2Δ1*, *his3Δ200*, *CLS1*).

2.2. Strains, culture conditions, and isolation of mitochondria

Haploid and diploid wild-type *S. cerevisiae* FY1679, and the haploid *cls1* deletion strain (see above) were cultivated under aerobic conditions at 30°C on YP media containing 2% glucose, galactose, ethanol, glycerol or lactate as carbon sources. Spheroplasts and mitochondria were prepared as previously described [15].

2.3. Analytical procedures

CL synthase activity was determined essentially as described by Tamai and Greenberg [12] with some minor modifications. In brief, the assay contained 100 mM Tris-HCl, pH 9.0, 0.037% Triton X-100, 20 mM $MgCl_2$, 0.1 mM CDP-diacylglycerol and 0.2 mM [^{14}C]PtdGro (final specific activity 4×10^3 dpm/nmol) in a total volume of 0.35 ml. The assay was started by addition of 150–300 μg mitochondrial protein. The incubation was carried out at 37°C, and samples of 0.1 ml were taken after 0, 10 and 20 min. The reaction was stopped by addition of 3 ml chloroform/methanol (1:2, v/v) and 0.7 ml 1% perchloric acid, lipids were extracted for 1.5 h and washed three times with 2 ml 1% perchloric acid. Radioactively labeled products formed during the in vitro assay were separated by thin-layer chromatography [11] using chloroform/methanol/glacial acetic acid (65:25:8; per vol.) as a developing solvent. The products were localized by radio-scanning using a Tracemaster 20 Automatic TLC-Linear Analyzer (Bert-

PII S0014-5793(97)01525-1

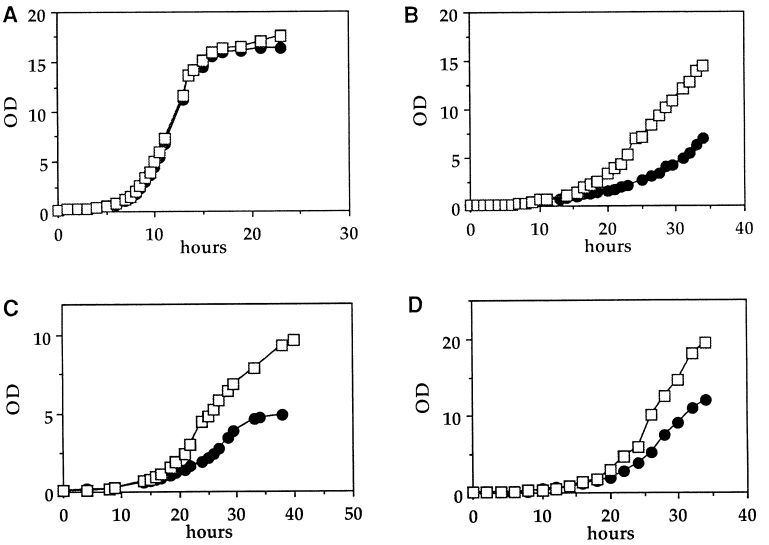

Fig. 1. Growth characteristics of the *cls1* mutant. The *cls1* deletion strain (●) and the corresponding haploid wild-type strain FY1679 (□) were grown on liquid media containing 2% glucose (A), 2% ethanol (B), 2% glycerol (C), or 2% lactate (D), respectively. Growth was monitored by measurement of optical density (OD) at 600 nm.

hold). For quantification bands were scrapped off the TLC plate and radioactivity was measured by liquid scintillation counting.

Radioactively labeled PtdGro used for the assay described above was isolated from a 500 ml culture of the *cls1* deletion strain that was pre-grown for 24 h on YP-glycerol medium and incubated for 30 min with 0.1 mCi [^{14}C]palmitate (specific activity 60 mCi/mmol). Cells were disintegrated with glass beads and [^{14}C]-labeled lipids were extracted by the procedure of Folch et al. [16]. [^{14}C]PtdGro was separated from other lipids by preparative thin-layer chromatography using the solvent system described above and recovered from TLC plates by extraction in chloroform/methanol (1:2; v/v).

PtdGro synthase activity was measured as described by Kelly and Greenberg [11]. The assay mixture contained 0.05 M MES·HCl, pH 7.0, 0.06% Triton X-100, 0.1 mM $MnCl_2$, 0.2 mM CDP-diacylglycerol and 0.5 mM [^{14}C]glycerol-3-phosphate (final specific activity 8×10^3 dpm/nmol) in a total volume of 0.35 ml. The assay was started by addition of 150–600 μg mitochondrial protein. Incubations were carried out at 30°C, and samples of 0.1 ml were taken after 0, 5 and 10 min. Lipids were extracted with 3 ml chloroform/methanol (1:2; v/v) and 0.7 ml 1% perchloric acid as described above. Radioactively labeled products formed during the in vitro assay were separated by thin-layer chromatography [10] using chloroform/methanol/glacial acetic acid/water (25:15:2:4; per vol.) as a developing solvent. The products were localized by radio-scanning and quantified by liquid scintillation counting.

Lipids of mitochondria were extracted by the procedure of Folch et al. [16]. Individual phospholipids were separated by two-dimensional thin-layer chromatography on Silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol/25% NH_3 (65:35:5; per vol.) as first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as second developing solvent. Phospholipids were visualized on TLC plates by staining with iodine vapor, scraped off the plate, and quantified by the method of Broekhuyse [17].

Protein was quantified by the method of Lowry et al. [18] using bovine serum albumin as standard. Spectrophotometric quantification of mitochondrial cytochromes was carried out by the method of Watson et al. [19] using a Hitachi U2310 double beam spectrophotometer. Enzymatic activity of cytochrome *c* oxidase was measured as described by Mason et al. [20].

3. Results and discussion

Screening of databases revealed significant homology of the yeast open reading frame YDL142c to genes of prokaryotic microorganisms encoding PtdGro-P synthases. Biochemical experiments carried out with a yeast mutant bearing a large deletion of YDL142c, however, demonstrated that this open reading frame did not encode yeast PtdGro-P synthase, but instead encodes CL synthase, Cls1p.

A *cls1* mutant constructed by deletion of YDL142c is viable on solid media with either glucose or glycerol as carbon source. The deletion strain is not temperature-sensitive, not affected by high salt concentration in the medium, and does not exhibit a petite growth phenotype on solid media. In liquid media the *cls1* mutant exhibited growth rates similar to the corresponding wild-type haploid strain FY1679 when glucose (Fig. 1A) or galactose (data not shown) were used as

Table 1
Enzymatic activities of cardiolipin synthase and phosphatidylglycerolphosphate synthase in mitochondria of the *cls1* deletion strain grown on different carbon sources

Carbon source	Specific activity (nmol/min×mg)			
	Cardiolipin synthase		Phosphatidylglycerolphosphate synthase	
	FY1679	*cls1*	FY1679	*cls1*
Glucose	0.023 ± 0.009	n.d.	0.28 ± 0.03	0.09 ± 0.01
Ethanol	0.160 ± 0.020	n.d.	2.90 ± 0.14	2.10 ± 0.02
Glycerol	0.093 ± 0.006	n.d.	2.00 ± 0.07	0.90 ± 0.14
Lactate	0.081 ± 0.011	n.d.	1.65 ± 0.05	0.90 ± 0.05

Mean values of three independent measurements are shown. F1679 is the haploid wild-type strain corresponding to *cls1*.
n.d., not detectable.

Table 2
Phospholipid composition of mitochondria of the *cls1* deletion strain grown on different carbon sources

	% of total mitochondrial phospholipids							
	Glucose		Ethanol		Glycerol		Lactate	
	FY1679	*cls1*	FY1679	*cls1*	FY1679	*cls1*	FY1679	*cls1*
PA	1.7 ± 0.5	2.5 ± 0.3	1.5 ± 0.10	3.0 ± 0.6	0.4 ± 0.1	3.7 ± 0.3	1.7 ± 0.3	6.9 ± 1.5
LysoPtdEtn	1.5 ± 0.1	2.4 ± 1.1	1.8 ± 0.80	1.4 ± 0.9	0.1 ± 0.1	1.0 ± 0.1	0.7 ± 0.3	0.5 ± 0.1
LysoPL	3.5 ± 0.3	3.0 ± 0.5	1.9 ± 0.6	1.2 ± 0.5	0.9 ± 0.2	0.8 ± 0.1	1.5 ± 0.1	2.2 ± 0.3
PtdSer	3.3 ± 0.5	4.5 ± 0.4	2.0 ± 0.3	3.1 ± 0.4	2.0 ± 0.3	1.3 ± 0.2	2.3 ± 0.8	2.6 ± 0.5
PtdIns	20.5 ± 0.9	20.2 ± 0.8	10.8 ± 1.2	12.9 ± 1.9	11.9 ± 1.1	9.0 ± 1.0	10.9 ± 0.5	13.0 ± 1.8
PtdEtn	22.7 ± 2.7	28.7 ± 1.7	29.0 ± 1.6	35.8 ± 1.4	25.6 ± 2.5	35.7 ± 1.5	26.4 ± 0.8	32.0 ± 1.5
DMPtdEtn	6.1 ± 0.3	6.1 ± 1.8	0.71 ± 0.3	1.1 ± 0.4	n.d.	n.d.	0.26 ± 0.2	n.d.
PtdCho	33.4 ± 0.8	32.5 ± 1.9	38.2 ± 1.8	33.1 ± 1.7	42.3 ± 2.2	35.1 ± 2.5	39.8 ± 0.6	31.0 ± 2.2
PtdGro	n.d.	n.d.	n.d.	8.3 ± 0.4	n.d.	13.3 ± 1.1	n.d.	11.9 ± 1.1
CL	7.2 ± 0.2	n.d.	15.0 ± 1.1	n.d.	17.0 ± 1.3	n.d.	15.7 ± 0.9	n.d.

PA, phosphatidic acid; LysoPtdEtn, lysophosphatidylethanolamine; LysoPL, lysophospholipids; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdEtn, phosphatidylethanolamine; DMPtdEtn, dimethylphosphatidylethanolamine; PtdCho, phosphatidylcholine; PtdGro, phosphatidylglycerol; CL, cardiolipin. Mean values of three independent measurements are shown. n.d., not detectable.

carbon sources. In contrast, the deletion strain grew markedly slower than wild-type on ethanol (Fig. 1B), glycerol (Fig. 1C) or lactate (Fig. 1D). These results indicate that YDL142c is not an essential gene, but deletion of *CLS1* affects aerobic metabolism on non-fermentable carbon sources.

Enzymatic analysis showed that CL synthase activity is missing in mitochondria of the *cls1* mutant (Table 1). In wild-type, specific activity of Cls1p depends on the carbon source. Cultivation of yeast on non-fermentable carbon sources resulted in significantly higher CL synthase activity than growth on glucose. Similarly, specific activity of PtdGro-P synthase in mitochondria of wild-type cells also depends on the carbon source (Table 1). These results are in agreement with data previously presented by Gaynor et al. [21] who showed that in yeast grown on non-fermentable carbon sources the activity of PtdGro-P synthase is increased. The level of CL present in mitochondria of these cells was significantly higher than in cells grown on glucose, which is in line with our data obtained with wild-type cells (Table 2). In mitochondria of *cls1* the specific activity of PtdGro-P synthase in vitro was only 30 to 75% of the wild-type control depending on the carbon source used for cultivation. In wild-type and in the *cls1* mutant, however, in vitro reaction products formed in the PtdGro-P synthase assay were the same, namely PtdGro-P and PtdGro. Both wild-type and mutant enzyme activities strictly depend on CDP-diacylglycerol as a co-substrate and are equally inhibited by 5 mM Mg^{2+}. The decrease of PtdGro-P synthase activity is not due to the lack of CL which may act as a possible activator, because addition of CL to the assay mixture had no stimulatory effect (data not shown). It is most likely that the expression of PtdGro-P synthase is reduced in the *cls1* mutant as a consequence of the lack of CL synthase.

Lipid analysis of isolated mitochondria revealed that the *cls1* strain lacked detectable amount of CL regardless of the carbon source on which cells were cultivated (Table 2). In mitochondria of *cls1* grown on non-fermentable carbon sources lack of CL was partially compensated for by increased levels of PtdGro. When *cls1* was grown on glucose mitochondria did not contain detectable amounts of PtdGro. Under these conditions the CL biosynthetic pathway seems to be repressed as suggested by decreased PtdGro-P synthase activity in mitochondria (see Table 1). In wild-type cells PtdGro was not detectable under all growth conditions indicating that in vivo PtdGro-P synthase is the rate-limiting step in the biosynthesis of CL. The lack of CL caused by deletion of the *CLS1* gene generally led to increased amounts of PtdEtn and phosphatidic acid (PA) and a decreased level of PtdCho in mitochondria.

Cellular growth, consumption of nutrients and energy production of *S. cerevisiae* cultivated under aerobic conditions largely depend on a functioning respiratory chain. Cytochrome *c* oxidase (cytochrome *aa3*) is the component of this pathway that is most sensitive to changes in the lipid composition of mitochondrial membranes. It has been suggested that CL is essential for cytochrome *c* oxidase activity in yeast [2]. The fact that the *cls1* deletion strain can grow under various conditions (see above) despite the lack of detectable CL rather suggests that this phospholipid is not absolutely required for mitochondrial respiration. In mitochondria of the *cls1* mutant, amounts of cytochrome *c*, cytochrome *aa3* (cytochrome *c* oxidase) and cytochrome *b* were similar to wild-type (Table 3). The activity of cytochrome *c* oxidase was not affected by the lack of detectable CL in mitochondria of *cls1* (Table 3).

Conclusively, deletion of the yeast *CLS1* gene does not lead to gross cellular defects. Other lipids, most likely PtdGro, or PtdEtn and PA, whose levels are increased in mitochondria of the *cls1* mutant strain, may thus at least in part functionally replace CL. Aerobic growth of yeast cells, however, seems to profit from the presence of CL, because growth rates of the *cls1* deletion strain were markedly reduced on non-fermentable carbon sources compared to wild-type (see Fig. 1). Reduced growth of *cls1* on ethanol, glycerol or lactate may thus reflect an increased requirement of CL for respiration.

While this work was in progress, Dowhan and coworkers (manuscript in preparation) identified, in an approach differ-

Table 3
Mitochondrial cytochromes of the *cls1* deletion strain

	Amounts of cytochromes (nmol/mg protein)	
	FY1679	*cls1*
Cytochrome *c*	0.36 ± 0.04	0.34 ± 0.05
Cytochrome *aa3*	0.07 ± 0.01	0.06 ± 0.01
Cytochrome *b*	0.18 ± 0.02	0.23 ± 0.03
	Specific activity (μmol/min × mg protein)	
Cytochrome *c* oxidase	0.10 ± 0.02	0.12 ± 0.02

Mitochondria were prepared from cells grown on 2% ethanol. Mean values of two independent measurements are shown.

ent from ours, the yeast genes *PGS1*, encoding PtdGro-P synthase, and *CLS1*, encoding CL synthase. In contrast to *cls1* mutants, yeast strains bearing a *PGS1* deletion are not viable on non-fermentable carbon sources. A *pgs1* mutant exhibits major dysfunction of mitochondria due to the lack of PtdGro and CL that cannot be replaced by other lipids. During preparation of this manuscript, Jiang et al. [22] reported identification of *CLS1* and the growth phenotype of a *cls1* deletion strain. Results obtained by Dowhan and coworkers and Jiang et al. [22] are similar to ours confirming that CL is largely dispensable for cellular growth, aerobic metabolism and membrane integrity of the yeast *S. cerevisiae*.

Acknowledgements: We are grateful to M. Haßlacher and A. Leber for their help in computer analysis, and to R. Schneiter and S.D. Kohlwein for critically reading the manuscript. W. Dowhan's helpful comments and sharing with us unpublished results are appreciated. This work was financially supported by the Eurofan project BIO4-CT95-0080, the Austrian Ministry of Science and Transportation (project 950080), and the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (project 12076).

References

[1] Daum, G. (1985) Biochim. Biophys. Acta 822, 1–42.
[2] Trivedi, A., Wearring, A.V., Kohlwein, S.D., Paltauf, F. and Tustanoff, E.R. (1988) in: Integration of Mitochondrial Function (Lemasters, J.J., Hackenbrock, C.R., Thurman, R.G. and Weterhoff, H.V., Eds.), pp. 87–94, Plenum Publ. Corp., New York.
[3] Endo, T., Eilers, M. and Schatz, G. (1989) J. Biol. Chem. 264, 2951–2956.
[4] Pember, S.O., Powell, G.L. and Lambeth, J.D. (1983) J. Biol. Chem. 258, 3198–3206.
[5] Robinson, N.C. (1993) J. Bioenerg. Biomembr. 25, 153–163.
[6] Kadenbach, B., Mende, P., Kolbe, H.V., Stipani, L. and Palmieri, F. (1982) FEBS Lett. 139, 109–112.
[7] Paltauf, F., Kohlwein, S.D. and Henry, S.A. (1992) in: The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression, pp. 415–500, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[8] Schlame, M. and Greenberg, M.L. (1997) Biochim. Biophys. Acta 1348, 201–206.
[9] Carman, G.M. and Belunis, C.J. (1983) Can. J. Microbiol. 29, 1452–1457.
[10] Kelly, B.L. and Greenberg, M.L. (1994) Gene 147, 111–114.
[11] Kelly, B.L. and Greenberg, M.L. (1990) Biochim. Biophys. Acta 1046, 144–150.
[12] Tamai, K.T. and Greenberg, M.L. (1990) Biochim. Biophys. Acta 1046, 214–222.
[13] Oliver, S. (1996) Trends Genet. 12, 241–242.
[14] Wach, A., Brachat, A., Pöhlmann, R. and Philippsen, P. (1994) Yeast 10, 1793–1808.
[15] Daum, G., Boehni, P.C. and Schatz, G. (1982) J. Biol. Chem. 257, 13028–13033.
[16] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509.
[17] Broekhuyse, R.M. (1968) Biochim. Biophys. Acta 260, 449–459.
[18] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[19] Watson, K., Houghton, R.L., Bertoli, E. and Griffiths, D.E. (1975) Biochem. J. 146, 409–416.
[20] Mason, T.L., Poyton, R.O., Wharton, D.C. and Schatz, G. (1973) J. Biol. Chem. 248, 1346–1356.
[21] Gaynor, P.M., Hubbell, S., Schmidt, A.J., Lina, R.A., Minskoff, S.A. and Greenberg, M.L. (1991) J. Bacteriol. 173, 6124–6131.
[22] Jiang, F., Rizavi, H.S. and Greenberg, M.L. (1997) Mol. Microbiol. 26, 481–491.