The isolation and characterisation of a \textit{Saccharomyces cerevisiae} gene (\textit{LIP2}) involved in the attachment of lipoic acid groups to mitochondrial enzymes

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Abstract

Lipoic acid is an essential cofactor for a variety of mitochondrial enzymes. We have characterised a gene from \textit{Saccharomyces cerevisiae} which appears to encode a protein involved in the attachment of lipoic acid groups to the pyruvate dehydrogenase and glycine decarboxylase complexes. The predicted protein product of this gene has significant identity to the lipoyl ligase B of both \textit{Escherichia coli} and \textit{Kluyveromyces lactis}. A strain harbouring a null allele of this \textit{S. cerevisiae} gene is respiratory deficient due to inactive pyruvate dehydrogenase, and is unable to utilise glycine as a sole nitrogen source. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Lipoic acid is an important sulphur-containing coenzyme that plays a role as a biological antioxidant \cite{1} and is essential for the function of a variety of multi-enzyme complexes that catalyse oxidative decarboxylation reactions \cite{2}. It is attached by an amide linkage to carbon six of a specific lysine side chain in the E2p subunit of pyruvate dehydrogenase (PDH), the E2o subunit of \textit{\alpha}-ketoglutarate dehydrogenase (KGDH) and the H-protein subunit of glycine decarboxylase (GDC) \cite{3–5}. This results in freely mobile lipoyl lysyl side arms that function as carriers of reaction intermediates between active sites. Highly specific lipoic acid ligases are required to attach lipoate to the appropriate lysine residues of each corresponding apoprotein.

In prokaryotes lipoic acid is obtained from the extracellular environment or synthesised internally \cite{6–10}. In \textit{Escherichia coli} the LpLA enzyme preferentially utilises free lipoic acid in the growth medium. It converts lipoic acid to lipoyl-AMP in an ATP-dependent manner and then attaches the lipoyl groups to dependent apoproteins through its ligase activity. In the biosynthetic pathway LipA inserts sulphur groups into octanoate to form lipoate and an unidentified carrier then presents the lipoyl groups to the LipB ligase for attachment to dependent apoproteins. Some redundancy has been observed between LpLA and LipB function. Protein-bound lipoate is detected in a \textit{lipB}-null mutant, albeit at low levels, but is undetectable in a \textit{lipB}–\textit{lpLA} double mutant grown in lipoate-free medium. Furthermore, overexpression of \textit{lpLA} on a multi-copy plasmid complements the leaky growth phenotype of a \textit{lipB}-null strain. However, phenotypes associated with a \textit{lpLA}-null mutant are poorly complemented by overexpression of \textit{lpB}. It has therefore been proposed that the unidentified carrier presenting lipoyl groups to \textit{lipB} can also be utilised by \textit{lpLA} \cite{8}.

Little is known about lipoate metabolism in eukaryotes, but all proteins that contain lipoic acid and exhibit lipoyl protein ligase activity are situated in the mitochondria \cite{11–13}. In humans with defective PDH, KGDH and branched
chain 2-oxo acid dehydrogenase activity there are low levels of lipoic acid in the E2 subunits of these enzymes [14], and patients suffering from non-ketotic hyperglycinemia show deficient levels of lipoic acid in the H-protein subunit of GDC [15]. Human lipoyl transferase has been isolated and characterised and was reported to attach lipoyl groups to the 2-oxo acid dehydrogenase complexes and the H-protein of the glycine cleavage system [16]. However, the molecular details of lipoic acid acquisition, biosynthesis and attachment to dependent apoproteins remain unclear. In contrast genetic analysis of fungi has led to the identification of the involvement of a wider range of genes. In Kluyveromyces lactis a gene has been described which is analogous to lipB in E. coli [18]. Strains with a null allele of this gene (lipBΔ) display a respiratory deficient and growth was not restored by addition of lipoic acid, suggesting that the gene encodes a lipoyl ligase. In addition lipBΔ mutants cannot utilise glycine as a sole nitrogen source due to ineffective GDC activity. In Saccharomyces cerevisiae LIP5 is analogous to lipB in E. coli [19]. Strains with a null allele of this gene (lip5Δ) display a respiratory deficiency phenotype and undergo mitochondrial DNA deletions at high frequency. Four individual S. cerevisiae complementation groups have also been reported that result in defects in lipoic acid metabolism [19], but the corresponding genes have not been identified. We have identified a gene that complements the respiratory deficiency and hydrogen peroxide sensitivity phenotypes of a mutant S. cerevisiae strain defective in reductive iron uptake. Analysis at the DNA level, biochemical assays and the predicted protein sequence suggest that this gene plays a similar role to the lipB gene of E. coli and K. lactis.

2. Materials and methods

2.1. Strains and growth conditions

The S. cerevisiae strain used in this study was S150-2B (MATα, leu2-3, 112; his3Δ; trpl-289; ura3-52) [20]. Strain MM1-6 was a UV-mutagenised derivative of S150-2B that displayed defective cell surface ferric reductase activity and other associated phenotypes including respiratory deficiency, sensitivity to hydrogen peroxide, and sensitivity to high concentrations of copper. Yeast extract–peptone medium was used for growth on different carbon sources with 3% (w/v) glucose (YPD), glycerol (YPG), ethanol (YPE), or sodium succinate (YPS) added. For selection of transformants synthetic glucose medium (SD) was used (0.67% (w/v) yeast nitrogen base without ammonium sulphate) and yeast nitrogen base with ammonium sulphate (SDNH) added. For solid media, agar was added at 2% (w/v). For selection of transformants synthetic glucose medium (SD) was used (0.67% (w/v) yeast nitrogen base without ammonium sulphate) and yeast nitrogen base with ammonium sulphate (SDNH) added. For solid media, agar was added at 2% (w/v). For selection of transformants synthetic glucose medium (SD) was used (0.67% (w/v) yeast nitrogen base without ammonium sulphate) and yeast nitrogen base with ammonium sulphate (SDNH) added. For solid media, agar was added at 2% (w/v).

1% molten soft agarose. This was used as a top layer to SD medium and either 10 µl of hydrogen peroxide (8.8 M) or cupric chloride (1 M) was dispensed onto a nylon disc placed at the centre of the plate. The lack of growth forming a 'halo' around the disc was measured after 5 days incubation. For glycine medium (SDGly) 0.67% (w/v) yeast nitrogen base without ammonium sulphate was used with glycine added at 1.5% (w/v). All cultures were grown at 30°C and monitored using a haemocytometer at 0.5-h intervals. For solid media, agar was added at 2% (w/v).

2.2. Cloning, sequencing and disruption of the rescuing open reading frame (ORF)

Strain MM1-6 was transformed with a genomic library [22] and screened on SDHP medium. A single colony with restored growth was used for further study. Plasmid DNA (pR16c) was isolated from the colony and the genomic DNA insert determined by sequence analysis using primers 5′-CGATCATGCGACCACA-3′ and 5′-AC-GATGCCGTCGGCGTA-3′. Alignment of the resulting sequence with the S. cerevisiae database (http://genome-www.stanford.edu/Saccharomyces/) revealed a 3588-bp fragment of chromosome XII (Fig. 1a) containing two intact hypothetical ORFs YLR238w (1434 bp) and YLR239c (984 bp). Restriction with EcoRI and subsequent religation of pR16c liberated two fragments (1414 bp and 1095 bp) to remove all but 148 bp at the 3′ end of the YLR238w ORF and leave YLR239c intact (pR16c2; Fig. 1b). Strain MM1-6 was then transformed with plasmid pR16c2 and screened on SDHP medium. Growth was

![Fig. 1. DNA manipulations of the rescuing genomic clone. (a) Shows the portion of genomic DNA that was originally isolated through the ability to rescue the hydrogen peroxide sensitive and respiratory deficient phenotypes of strain MM1-6. (b) Restriction of pR16c with EcoRI and subsequent religation of the vector enabled YLR239c to be isolated in order to confirm that this ORF was responsible for mutant rescue. (c) Primers containing BamHI sites were used in an inverse PCR reaction to delete 586 bp from the 3′ end of the YLR239c ORF. A disruption cassette was then inserted as a BamHI fragment containing URA3 as a prototrophic marker.](FEMSLE9-9928-9-5-01.png)
restored, indicating that ORF YLR239c was responsible for complementing the hydrogen peroxide sensitivity phenotype of strain MM1-6. This phenotype was believed to be due to abnormal intracellular iron concentrations and therefore associated with the defective ferric reductase activity of the mutant strain.

To disrupt the rescuing ORF, a 1364-bp EcoRI–HindIII fragment from pR16c2, containing YLR239c, was cloned into the EcoRI–HindIII site of pUC19 to construct pYO1. Primers including BamHI sites (5’-GCGGTATCG-GATCGGCGG-3’ and 5’-CGTGAGACGGATCCCTCC-CC-3’) were designed and inverse PCR used to delete 586 bp from the 3’ end of YLR239c. A 1144-bp BamHI fragment carrying URA3 [23] was then introduced to construct pYD2 (Fig. 1c). Disruption of the chromosomal YLR239c ORF was achieved by transformation of strain S150-2B with the 1921-bp EcoRI–HindIII disruption cassette from pYD2. Allele replacement was confirmed by digestion with PsiI and Southern blot analysis (data not shown).

2.3. Enzyme assays

A modification of the method described by Dickinson et al. was used to assay PDH and malate dehydrogenase activity [24]. Cell extracts were obtained from stationary phase cells (≈1.5–2 × 10^8 ml^-1) and the protein content estimated by the Bradford assay. Enzyme activity was monitored continuously at 366 nm through the production of reduced 3-acetylpyridine adenine dinucleotide after the addition of pyruvic or malic acid (30 μM). Malate dehydrogenase activity was assayed to ensure that cell extract mitochondrial enzymes were functional as this enzyme is required in the citric acid cycle but does not require lipo-ylation for activity.

3. Results and discussion

3.1. Isolation of strain MM1-6

Strain MM1-6 was isolated during an attempt to find novel genes involved in the regulation of reductive iron uptake by S. cerevisiae. A UV-mutagenised population of the parental strain was analysed for defective cell surface ferric reductase activity using a colourimetric plate assay [25]. Strain MM1-6 displayed constitutive ferric reductase activity irrespective of the iron concentration in the growth medium. Several unsuccessful attempts were made to clone the rescuing gene using this assay so a search was made for other associated phenotypes to use as alternative screens. MM1-6 was found to be sensitive to hydrogen peroxide and high levels of copper, and possessed a respiratory deficiency, forming petite colonies, with severely impaired growth on medium containing glycerol or ethanol as the sole carbon sources. These pheno-types are typical of a strain with defective mitochondria and have previously been observed in S. cerevisiae mutants defective in reductive iron uptake [26]. Iron and copper are required for mitochondrial function, are toxic at high levels and, with hydrogen peroxide, are capable of generating destructive oxygen radicals. Therefore, these defects were thought to be due to either directly deficient delivery of either metal to the mitochondria, or indirectly as a result of incorrect intracellular levels.

3.2. Genetic analysis of mutant strain MM1-6

Preliminary genetic analysis of 10 tetrads indicated that the defective ferric reductase activity was the result of a single gene mutation and the hydrogen peroxide sensitivity and copper sensitivity appeared to cosegregate with this. Hydrogen peroxide medium (SDHP) was used as a screen and a colony with restored growth on this medium was isolated (see Section 2). However, the ORF responsible for mutant rescue on SDHP (YLR239c) did not restore growth on high copper medium (YPC) and did not complement the mutant ferric reductase activity. In contrast, growth on glycerol (YPG) and ethanol (YPE) medium was rescued (data not shown). These findings were inconsistent with a single gene mutation and so further genetic analysis of MM1-6 was performed.

More precise analysis of the 10 tetrads confirmed that copper sensitivity cosegregated with the defective ferric reductase as previously. However, the hydrogen peroxide sensitivity (previously classified to cosegregate 2:2 with the defective ferric reductase) revealed three phenotypes, mutant, wild-type and intermediate, suggesting that more than one gene mutation may be involved. The respiratory deficiency segregated 2:2 in one tetrad, 4:0 in four tetrads and 3:1 in five tetrads (wild-type to mutant). An important observation was that if a colony was respiratory incompetant it was also mutant for ferric reductase activity and copper sensitivity. Therefore it appeared that in fact two separate genes were contributing to the hydrogen peroxide sensitivity and respiratory deficiency in strain MM1-6, one was YLR239c and the second an unidentified gene responsible for the defective ferric reductase activity and copper sensitivity.

3.3. Sequence analysis of YLR239c

Analysis of the SwissProt database revealed that YLR239c encodes a predicted 37.2-kDa protein that is a ‘probable lipoate protein ligase B (primary accession number Q06005)’. Sequence analysis of the predicted protein using PSORT (http://psort.nibb.ac.jp/) revealed a mitochondrial targeting sequence at residues 49–54 (QRIPFQ), with 87.6% probability of localising to the mitochondrial matrix space and most likely cleaved between residues 51 and 52. Using the ExPASy database (http://www.expasy.ch/) a Blastp search revealed signifi-
cant identity to numerous characterised and putative lipoate ligases from different species, including the LipB protein of *K. lactis* [14], a putative lipoate protein ligase B from *Schizosaccharomyces pombe* and the LipB protein of *E. coli* [8,9]. Analysis of the *S. cerevisiae* putative lipoyl ligase protein sequence using MOTIFS from the GCG package (Wisconsin package version 9.1, Genetics Computer Group, Madison, WI, USA) revealed a LipB signature sequence (RGGQVTFHGPGQIVIY) at residues 162–177 found in all characterised prokaryotic and eukaryotic ligases of this type. We had isolated the YLR239c ORF through the functional rescue of phenotypes associated with oxidative stress and respiratory incompetence. This was consistent with the gene encoding a lipoate ligase as lipoic acid plays a role in the cell as an antioxidant and is required in eukaryotes by mitochondrial enzymes [1,11–13]. Due to these analogies we have named the YLR239c putative ORF *LIP2*.

3.4. A lip2-null mutant displays a growth defect on glycine medium and a respiratory deficiency that can be rescued by ethanol.

A lip2-null strain was constructed as outlined in Section 2. Growth of the lip2-null strain (S150-2B::lip2ΔURA3) and parental strain was compared in glucose (YPD) and glycerol (YPG) liquid medium. In YPD no significant difference in growth rate was observed, but in YPG the mutant strain exhibited a doubling time approximately twice that of the parental strain (S150-2B::lip2ΔURA3 = 400 ± 15 min; S150-2B = 227.5 ± 7.5 min). The inability to efficiently utilise a non-fermentable carbon source suggested that the lip2-null allele was affecting aerobic metabolism. Rescue of lipoate ligase mutants through the addition of both ethanol and sodium succinate to the growth medium has been previously reported [17]. Growth of S150-2B::lip2ΔURA3 was rescued on medium...
containing both ethanol and sodium succinate (YPE) but the addition of ethanol alone (YPE) was sufficient to rescue growth of the mutant strain (Fig. 2a). Growth was not rescued on medium containing sodium succinate as a sole carbon source (YPS). These findings suggested that the lip2-null allele was affecting PDH activity. In addition, slow growth on YPG was not rescued by addition of 3% lipoic acid suggesting that LIP2 is not required for synthesis of lipoic acid itself (data not shown). Another enzyme complex known to rely upon the incorporation of lipoic acid is GDC [4,5]. Strain S150-2B::lip2ΔURA3 displayed a growth defect when grown on medium containing glycine as a sole nitrogen source (SDGly; Fig. 2b) suggesting that the activity of this enzyme is also affected. To investigate the effect of a lip2-null allele on PDH activity we performed biochemical assays. No detectable PDH activity was observed in strain S150-2B::lip2ΔURA3 using the assay described in Section 2 (Table 1).

The S. cerevisiae LIP2 gene was isolated through the functional rescue of a respiratory deficient mutant strain that was sensitive to hydrogen peroxide. Genetic, functional, biochemical and sequence analyses of this gene support its putative role as a lipoil ligase. Both a K. lactis lipb-null mutant [17] and a S. cerevisiae lip2-null mutant display severely impaired growth on medium containing glycine as a sole nitrogen source, indicating that LipBp and Lip2p are involved in attaching lipoic acid groups to the H-protein subunit of GDC in each organism. Impaired growth is also observed in both mutants on medium containing glycerol as a sole carbon source. However, the two organisms differ in that the addition of ethanol alone to the growth medium restored growth to a S. cerevisiae lip2-null mutant, whereas both ethanol and sodium succinate were needed to rescue the growth of a corresponding K. lactis mutant. Addition of ethanol would circumvent the need for PDH activity and the addition of both ethanol and succinate would circumvent the need for both PDH and KGDH. Therefore these observations may mean that KGDH is in fact active in the S. cerevisiae lip2 mutant with LIP2p only being involved in the attachment of lipoic acid to PDH and not to KGDH. Alternatively S. cerevisiae may be more efficient than K. lactis at bypassing the need for KGDH activity by the utilisation of the glycolytic cycle. Consistent with this is the observation that kgd1 mutants, defective in the structural gene encoding KGDH activity, can grow on ethanol as a sole carbon source [27]. Using biochemical assays we have confirmed that PDH activity is affected in the S. cerevisiae lip2-null mutant. Therefore in summary we conclude that the S. cerevisiae lip2 gene encodes a lipoic acid ligase that is utilised by both the GDC and the PDH complex, but may or may not be utilised by the KGDH complex. It should be noted that as in K. lactis [17] additional enzymes with similar function to Lip2p may be present in S. cerevisiae as growth of the lip2-null mutant is detected in both glycerol and glycine medium after prolonged incubation. These findings will help elucidate the molecular details of lipoic acid acquisition, biosynthesis and attachment to dependent apoproteins in eukaryotes.

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References


Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>PDH activity (ΔA₃₆₆ min⁻¹ mg⁻¹)</th>
<th>Malate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>S150-2B</td>
<td>0.031 ± 0.014</td>
<td>0.65 ± 0.25</td>
</tr>
<tr>
<td>S150-2B::lip2ΔURA3</td>
<td>0.000</td>
<td>0.80 ± 0.22</td>
</tr>
</tbody>
</table>

Crude extracts were prepared from the lip2-null mutant and parental strain as described in Section 2. Specific enzyme activity was calculated from the change in absorbance at 366 nm per min per mg of protein (ΔA₃₆₆ min⁻¹ mg⁻¹). Malate dehydrogenase activity was monitored to ensure mitochondrial enzymes in the extract were functional. The experiment was performed twice and the data shown are the average of the two readings.


