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The Role of the *Saccharomyces cerevisiae* Lipoate Protein Ligase Homologue, Lip3, in Lipoic Acid Synthesis

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Abstract

The covalent attachment of lipoate to the lipoyl domains (LDs) of the central metabolism enzymes pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (OGDH) is essential for their activation and thus for respiratory growth in *Saccharomyces cerevisiae*. A third lipoate-dependent enzyme system, the glycine cleavage system (GCV), is required for utilization of glycine as a nitrogen source. Lipoate is synthesized by extraction of its precursor, octanoyl-acyl carrier protein (ACP) from the pool of fatty acid biosynthetic intermediates. Alternatively, lipoate is salvaged from previously modified proteins or from growth media by lipoate protein ligases (Lpls). The first Lpl to be characterized, LplA of *Escherichia coli*, catalyzes two partial reactions: activation of the acyl chain by formation of acyl-AMP, followed by transfer of the acyl chain to LDs. There is a surprising diversity within the Lpl family of enzymes, several of which catalyze reactions other than ligation reactions. For example, the *Bacillus subtilis* Lpl homologue LipM is an octanoyltransferase that transfers the octanoyl moiety from octanoyl-ACP to GCV. Another *B. subtilis* Lpl homologue, LipL, transfers octanoate from octanoyl-GCV to other LDs in an amidotransfer reaction. Study of eukaryotic Lpls has lagged behind studies of the bacterial enzymes. We report that the Lip3 Lpl homologue of the yeast *S. cerevisiae* has octanoyl-CoA: protein transferase activity and discuss implications of this activity on the physiological role of Lip3 in lipoate synthesis.

Key Words/Phrases

Lipoic Acid; Lipoate Protein Ligase; Octanoyl-CoA: Protein Transferase; Respiration; Tricarboxylic Acid Cycle; Mitochondrial Integrity

INTRODUCTION

Since the initial demonstration, purification and synthesis of lipoic acid in the early 1940s [Reed, 2001], much has been learned about the synthesis of this essential respiratory cofactor in prokaryotes [Cronan, 2008; Cronan *et al.*, 2005]. In *Escherichia coli*, lipoate is assembled on the lipoyl domains (LDs) of the lipoate-dependent enzyme systems from the eight carbon fatty acid, octanoate, in two steps. First, an octanoyltransferase (LipB) transfers an octanoyl moiety from the acyl carrier protein (ACP) of fatty acid synthesis to the LDs [Jordan and Cronan, 2003]. A thioester-bound acyl-enzyme intermediate is formed in the process [Zhao *et al.*, 2005]. Then lipoyl synthase (LipA) catalyzes replacement of single hydrogen atoms at carbons 6 and 8 with sulfur atoms using radical SAM chemistry [Booker

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et al., 2007]. Alternatively, exogenous lipoate or octanoate can be directly attached to LDs by lipoate protein ligase (LplA) via an acyl-AMP intermediate.

Although the LipA sulfur insertion reaction is conserved in all characterized lipoate synthesis pathways, there are three well-characterized variations to the *E. coli* pathway in the other reactions. In the lipoate auxotrophic archeon *Thermoplasma acidophilum*, the lipoate protein ligase is composed of two separately encoded subunits LplA and LplB that together catalyze formation of the acyl-AMP intermediate [Christensen and Cronan, 2009; Posner *et al.*, 2009]. LplB was found to be dispensable for the second half reaction, namely ligation of the acyl moiety to the acceptor lysine on the LDs [Christensen and Cronan, 2009]. The Gram-positive bacterium *Bacillus subtilis* lacks a LipB homologue, but encodes three LplA homologues, although only LplJ has the ligase function of *E. coli* LplA [Martin *et al.*, 2011]. One of the two remaining homologues (LipM) is an octanoyltransferase that specifically transfers octanoate from ACP to a small protein, GcvH [Christensen and Cronan, 2010], whereas LipL, the third homologue, is an amidotransferase that transfers the octanoyl moiety from GcvH to the LDs of pyruvate dehydrogenase (PDH), the branched chain oxoacid dehydrogenase, and oxoglutarate dehydrogenase (OGDH) [Christensen *et al.*, 2011b; Martin *et al.*, 2011]. LipL is also present in *Listeria monocytogenes* along with two other LplA homologues that are ligases. LplA1 specifically modifies GcvH whereas the LD specificity of LplA2 is undetermined [Christensen *et al.*, 2011a]. The lipoate scavenged by *L. monocytogenes* LplAs might come directly from the host environment or might be released from host lipoylated-peptides by the action of the lipoamidase(s) encoded by this bacterium.

The diversity in prokaryotic lipoate attachment mechanisms, summarized in Fig. 1, is surprising given the simple structure of the coenzyme. The situation in eukaryotes is not well understood and may contribute further enzymatic diversity. Early studies of the bovine LplA-homologue (LipT; for lipoyltransferase) demonstrated that the enzyme was unable to form the acyl-AMP intermediate [Fujiwara *et al.*, 1994]. However LipT was able to transfer the acyl chain from exogenously provided acyl-AMP to LDs *in vitro* giving rise to the proposal that mammalian lipoate attachment requires a “lipoate-activating enzyme”. Accordingly, Fujiwara *et al.* (2001) purified an enzyme from bovine liver that synthesized lipoyl-GMP *in vitro* which in turn could be utilized by LipT to modify LDs [Fujiwara *et al.*, 2001]. However this putative lipoate-activating enzyme is one of many poorly characterized medium chain fatty acyl-Coenzyme A (CoA) ligases that have extremely broad substrate specificities [Kasuya *et al.*, 2006; Vessey *et al.*, 2000], and thus the physiological relevance of its *in vitro* lipoyl-GMP forming activity is unclear.

In the yeast, *Saccharomyces cerevisiae*, there are at least three lipoate-dependent enzyme systems; PDH, OGDH and the glycine cleavage system (GCV). PDH catalyzes the oxidative decarboxylation of pyruvate, the endpoint of glycolysis, to acetyl-CoA and thereby links cytosolic glycolysis and mitochondrial respiration. The acetate moiety of acetyl-CoA is condensed with oxaloacetate to form citrate, the first intermediate of the tricarboxylic acid (TCA) cycle. Mitochondrial acetyl-CoA is also the building block for mitochondrial fatty acid (FA) biosynthesis which produces octanoyl-ACP, the precursor of lipoate [Hiltunen *et al.*, 2010]. OGDH catalyzes the oxidative decarboxylation of the TCA cycle intermediate, 2-oxoglutarate, to succinyl-CoA, a precursor of several amino acids, and the source of succinate which is the entry point to the respiratory chain. GCV is involved in cleavage of glycine to ammonia and C1 units and is essential for utilization of glycine as a sole source of nitrogen. All constituents of these enzyme systems, as well as those of mitochondrial fatty acid biosynthesis, are encoded by nuclear genes and have mitochondrial targeting sequences. Their functions are necessary for mitochondrial maintenance and respiratory growth [Contamine and Picard, 2000].

Studies by Schonauer *et al.* (2009) showed that Lip2, Lip5, Lip3 (the LipB, LipA and LplA homologues respectively) as well as Gcv3 (the GcvH homologue) are all required for endogenous lipoate synthesis and attachment [Schonauer *et al.*, 2009]. Respiratory growth of a *lip2* strain, which contains a wild type copy of the *lip3* gene, is not supported by exogenous lipoate supplementation [Chen, 1997; Marvin *et al.*, 2001], suggesting that *S. cerevisiae* lacks a lipoate scavenging pathway. On the other hand, early studies by Sulo and Martin [Sulo and Martin, 1993] showed that even though lipoate did not support the respiratory growth of *lip5* strains, the cells contained a substantial level of protein-bound lipoate when grown on lipoate-supplemented media, suggesting the presence of a scavenging mechanism in *S. cerevisiae*. We sought to further investigate the role of Lip3 in lipoate synthesis in *S. cerevisiae*. We show that Lip3 has octanoyl-CoA: protein transferase activity and present a model for lipoate synthesis in *S. cerevisiae*.

MATERIALS AND METHODS

General molecular biology techniques

Genomic DNAs were extracted using the Promega Wizard genomic DNA purification kit. Plasmids were prepared using either the Qiagen minispin or midi kits. PCR was conducted using either *Taq* (New England Biolabs) or *Pfu* (Invitrogen) polymerase, and appropriately designed primers from IDT (see Table S1). Restriction enzymes and ligases were from New England Biolabs. DNA sequencing was conducted by the Core Sequencing Facility of the Carver Biotechnology Center of the University of Illinois, Urbana Champaign.

Yeast suspensions for PCR were prepared by suspending a colony in 30 μ l of 0.2% SDS and boiling at 96° for 5 min, then cooling on ice. The suspension was then cleared at 3000xg for 1 min, and 10 μ l of supernatant was diluted with 67 μ l of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA.

Strains and plasmids

All *E. coli* strains, yeast strains and plasmids used in this study, as well as their relevant genotypes are listed in Table S2.

Plasmid pFH21 was made by digesting PCR-amplified *lip3* with BspHI and HindIII and ligating the product to NcoI and HindIII digested pBAD24. The *lip3* gene used was a synthetic gene composed of codons optimized for expression in *E. coli*. Plasmid pFH23 was constructed by moving the ClaI-HindIII fragment containing *lip3* and the arabinose promoter from pFH21 into pACYC177. Site-directed mutagenesis of the *lip3* gene in plasmid pFH23 was conducted using *pfu*Ultra HF from Invitrogen and appropriately designed primer sets per the manufacturer's recommendations. Vector pFH88 was made by PCR amplification of the *fadK* gene from pRK33 using primers fadK-for and fadK-rev. The PCR product was digested with NcoI and HindIII and ligated to pBAD322C treated with the same restriction enzymes.

To construct plasmid pFH72 the last 360 codons of the *lip3* gene were PCR amplified from a *S. cerevisiae* cell suspension using primers pPICNTlip3-for (which included codons for 6X His tag) and pPIClip3-rev. The PCR product was digested with BamHI and EagI and ligated into pPIC3.5K treated with the same restriction enzymes. Plasmid pFH38 was constructed by ligation independent cloning [Eschenfeldt *et al.*, 2009] of a synthetic *gcv3* gene altered to have *E. coli* optimized codons into pMCSG7.

Vector pFH46 is a pUC-type vector which carries a synthetic 126 bp fragment encoding the first 42 amino acids of the predicted mitochondrial targeting sequence of *S. cerevisiae* Coq3. The sequence was flanked by a BamHI cut site upstream and KpnI and PstI sites

immediately downstream. The *lplA* and *lipB* genes were PCR amplified from *E. coli* total genomic DNA using primers mtlplA-for and mtlplA-rev and mtlipB-for and mtlipB-rev, respectively. *B. subtilis lipL* was amplified from plasmid pQC32 using primers mtlipL-for and mtlipL-rev. The amplified genes were ligated into the KpnI and PstI sites of pFH46 and their sequences verified. The result of this step was to introduce a mitochondrial targeting sequence at the N-termini of LplA, LipB and LipL. The resulting plasmids were then digested with BamHI and PstI and the appropriate DNA fragments were ligated to the *E. coli*-*S. cerevisiae* shuttle vector pCM188 treated with the same restriction enzymes.

E. coli strains were transformed by heat shock treatment [Seidman *et al.*, 1997]. To construct a *fadD* gene replacement in strain FH306, a P1 phage stock was grown on strain RMK75 and the lysate was used to transduce FH306 to chloramphenicol resistance following the method of Miller (1992) [Miller, 1992]. The resulting strain, FH307 failed to grow on oleate as sole carbon source.

S. cerevisiae strains were transformed using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method of Gietz and Woods (2002) [Gietz and Woods, 2002]. Transformants were selected on glucose synthetic complete uracil dropout medium (see below). Strain FH553 was made by linearizing pFH72 with SalI and transforming the linear plasmid into *Pichia pastoris* strain GS115 by electroporation as described by the supplier. Transformants having chromosomal integration of the plasmid were selected on glucose synthetic complete histidine dropout medium.

Growth conditions

Yeast strains were generally grown at 30° on rich media consisting of yeast peptone (YP) containing 1% yeast extract and 2% peptone with either 2% glucose, 3% glycerol or 3% ethanol as carbon sources. Selection for transformants was done on glucose synthetic complete media missing either histidine or urea [Trecos, 1993]. Methanol (1%) was used to induce protein expression in *P. pastoris* strain FH553.

E. coli strains were grown at 37° on Luria-Bertani (LB) [Miller, 1992] or glucose minimal E media (GMM) [Vogel and Bonner, 1956]. Supplements were added to the following concentrations: acetate 5 mM, succinate 5 mM, lipoate 7.5 ng ml⁻¹, octanoate 50 µM, arabinose 0.2%, and glucose 0.4%. Antibiotics were used at the following concentrations (in µg ml⁻¹): sodium ampicillin, 100; spectinomycin sulfate, 50; kanamycin sulfate, 50; and chloramphenicol, 20.

Growth curves

Strains were inoculated in 5 ml of minimal E supplemented with arabinose, acetate, succinate and appropriate antibiotics and grown two nights at 37°. The cells were collected and washed three times with minimal E and diluted to initial OD600 of about 0.01 in 300 µl of growth medium. Growth was followed at 37° on a Bioscreen C automated growth curve analysis system set to continuous very strong intensity shaking for aeration. The means and standard deviations of 3 replicates are presented.

Assays of PDH and OGDH activities

The procedure described in Hermes and Cronan (2009) was followed with a few differences. The cultures were in 50 ml LB supplemented with arabinose, acetate and succinate and either lipoate, octanoate, both, or neither as indicated. Cell pellets were lysed using BugBuster Master Mix from Novagen per the manufacturer's protocol. The means and standard deviations of 3 repeat experiments are presented. *p*-values were calculated using Student's T-test, using a two-tailed distribution of probability for two independent samples

assuming equal variance. Significance was assigned for conditions whose p -values were smaller than 0.05.

Detection of in vivo lipoylation by western blotting

About 150 μ g of total protein was fractionated on 12% SDS gels and then blotted onto a polyvinylidene difluoride membrane using a standard protocol [Gallagher, 2010]. Lipoylated domain was detected using rabbit anti-lipoyl protein primary antibody from Calbiochem and goat anti-rabbit antibody from Roche.

Protein expression and purification

In our hands Lip3 consistently formed inclusion bodies when over expressed in *E. coli* regardless of the strain background and the numerous expression conditions attempted. Fusions to maltose binding protein (MBP) were soluble, but inactive, and Lip3 aggregated upon cleavage of the solubility tag. Purification under denaturing conditions followed by refolding produced inactive protein. We therefore resorted to expressing and purifying Lip3 from the yeast *P. pastoris*. Strain FH553 was cultured in YP glycerol overnight at 30°. The cells were collected and resuspended to OD₆₀₀ of 0.5 in 400 ml YP methanol in a two-liter flask and protein production was allowed to proceed overnight. The cells were collected, freeze-thawed and resuspended in breaking buffer (50 mM sodium phosphate (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 5% glycerol). Zymolase was added and the suspension was incubated at 30° for an hour. The extract was cleared by centrifugation. Hexahistidine tagged Lip3 was partially purified using Ni-NTA from Qiagen following the manufacturer's recommendations, and verified using western blotting with anti-his antibody. Protein concentrations were measured at 280 nm using the extinction coefficient 55810 M⁻¹cm⁻¹ predicted by the program ProtParam [Gasteiger E., 2005]. We obtained about 5 μ g of protein from 400 ml of culture.

To express hexahistidine-tagged apo Gcv3, strain FH437 was inoculated in LB supplemented with glucose, acetate, succinate, spectinomycin and ampicillin. At OD₆₀₀ of 0.8, protein production was induced overnight with arabinose. Gcv3 was purified under denaturing conditions with 8 M urea using Ni-NTA chromatography. The protein was refolded by gradually removing the urea by dialysis. Protein concentration was measured at 280 nm using the extinction coefficient 26930 M⁻¹cm⁻¹ (ProtParam) [Gasteiger E., 2005]. Expression and purification of the *E. coli* LD was conducted as described previously [Hermes and Cronan, 2009]

Gel shift assay of Lip3 activity

A 20 μ l reaction mixture contained 10 mM sodium phosphate buffer (pH 7), 0.3 mM dithiothreitol, about 650 nM Lip3, and either 6 μ M apo Gcv3 or 15 μ M apo LD. To test for ligase activity 5 mM MgCl₂, 5 mM ATP and 1 mM octanoate were added to the reaction. Alternatively 100 μ M octanoyl-CoA (from Sigma) was added. The reaction was run for 12 hours at 37°, after which 15 μ l was loaded in a 20% native polyacrylamide gel and separated by electrophoresis.

RESULTS

Expression of wild type Lip3 permits growth of an *E. coli* $\Delta lipB \Delta lplA$ strain on octanoate, but not lipoate supplemented media

E. coli strain QC146 is deleted for the *lipB* and *lplA* genes which renders it defective in both synthesis and scavenge of lipoate. Growth of strain QC146 on GMM is strictly dependent on supplementation with acetate and succinate which, respectively, bypass the PDH- and OGDH-catalyzed steps required for TCA cycle function [Herbert and Guest, 1968]. We

tested the ability of Lip3 to support the growth of strain QC146 either on unsupplemented GMM (i.e., to complement the *lipB* defect), or on GMM supplemented with either lipoate or octanoate (i.e., to complement the *lplA* defect). Strain QC146 containing pFH23, a plasmid that expresses Lip3, failed to grow on either unsupplemented or lipoate supplemented GMM but grew on octanoate supplemented GMM (Fig. 2A). The strain also grew on GMM containing a mixture of lipoate and octanoate (data not shown) which indicated that lipoate neither inhibited nor supported growth of strain QC146 harboring pFH23.

Members of the lipoate protein ligase (Lpl) family have a signature catalytic lysine that is essential for activity. From the sequence alignment of Lip3 with several other Lpl family homologues [Schonauer *et al.*, 2009], it appeared that K249 was the Lip3 catalytic site lysine residue. Replacing K249 with arginine had deleterious effects on the ability of Lip3 to complement growth of QC146 (Fig. 2B), whereas replacing K249 with alanine completely eliminated activity (Fig. 2B).

The *E. coli* $\Delta lipB \Delta lplA$ strain expressing wild type Lip3 contained active and lipoylated PDH and OGDH complexes

To confirm that growth of strain QC146 containing the Lip3 expression plasmid pFH23 on octanoate supplemented media was due to activation of PDH and OGDH, the activities of the complexes were assayed in extracts of this strain. The strain was grown in LB medium containing acetate, succinate, arabinose (to induce Lip3 expression) supplemented with either lipoate, octanoate or left unsupplemented. Strain QC146 containing the empty vector grew on these media due to the presence of acetate and succinate (see above). As expected the extracts of the vector containing strain had no detectable activity of either PDH or OGDH even when both lipoate and octanoate had been added to the cultures (Fig. 3A). On the other hand, extracts of strain QC146 containing the Lip3 expression plasmid grown without lipoate or octanoate, or with lipoate only had traces of PDH and OGDH activities (Fig. 3A). The trace activities were probably due to trace amounts of octanoate in LB medium which can be utilized by Lip3 to activate PDH and OGDH. Addition of octanoate to the cultures of strain QC146 containing pFH23 resulted in significantly higher PDH and OGDH activities (Fig. 3A).

To further confirm PDH and OGDH activation in strain QC146 carrying pFH23, cell extracts were assayed for lipoylated PDH and OGDH by western blotting using a commercial anti-lipoyl LD antibody. A faint band corresponding to SucB (the LD-containing subunit of OGDH) but no band for AceF (the LD-containing subunit of PDH) was observed in the extracts from unsupplemented and lipoate supplemented cultures (Fig. 3B, lanes 1 and 2). The SucB band was much more prominent in the extract from the octanoate-supplemented culture and an AceF band was also present in this extract (Fig. 3B, lane 3). We did not expect to detect lipoyl-GcvH (the LD of GCV) in this experiment because in *E. coli* GCV is expressed only in the presence of glycine.

Complementation of the *E. coli* $\Delta lipB \Delta lplA$ strain by expression of Lip3 requires a host-encoded acyl-CoA synthetase

The finding that Lip3 selectively utilized octanoate, but not lipoate, *in vivo* was surprising. What was more puzzling was the requirement for octanoate supplementation because we had earlier shown that a pool of intracellular free octanoate is present in *E. coli* [Hermes and Cronan, 2009]. This pool is sufficient for activation of PDH and OGDH either when LplA was expressed from a strong promoter or when a mutant enzyme having a lower K_m for octanoate was present. We therefore reasoned that the actual substrate for Lip3 may not free octanoate *per se* but the much rarer species, octanoyl-CoA. We argued against octanoyl-ACP as the substrate since this is a standard intermediate of fatty acid (and lipoate) synthesis

and is thus constitutively present. If octanoyl-CoA was the Lip3 substrate, then a fatty acid-CoA synthetase would be required to convert octanoate to octanoyl-CoA. Hence, growth of the *E. coli lipB lplA* strain carrying the pFH23 Lip3 expression plasmid on octanoate-supplemented GMM would depend on the presence of the *fadD* fatty acid acyl-CoA synthetase of aerobic β -oxidation. To test this premise, we deleted *fadD* and found that the resulting strain (strain FH307) was unable to grow on octanoate-supplemented GMM (Fig. 4A). Growth of the *E. coli lipB lplA* strain carrying the Lip3 plasmid was slow and it seemed likely that this was due to the fact that octanoate is a poor substrate for FadD. The acyl-CoA synthetase of the anaerobic β -oxidation pathway FadK has preference for short chain fatty acid substrates [Morgan-Kiss and Cronan, 2004]. However *fadK* is only expressed during anaerobiosis [Morgan-Kiss and Cronan, 2004]. Hence we placed the *fadK* gene downstream of an arabinose promoter and this plasmid was introduced into strain QC146 carrying the Lip3 expression plasmid pFH23. The resulting strain grew much better on octanoate-supplemented GMM than strain QC146 carrying only pFH23 (Fig. 4B), thus providing further support for the hypothesis that the Lip3 substrate is octanoyl-CoA (or is derived from it) rather than free octanoate.

Lip3 formed octanoyl-LD and octanoyl-Gcv3 *in vitro* using octanoyl-CoA as acyl donor

Lip3 activity *in vitro* was tested using an electromobility shift assay. Attachment of octanoate to the lysine residue of the LD or Gcv3 proteins results in loss of a positive charge which gives more rapid migration of the modified protein in native electrophoresis gels relative to the unmodified protein. Lip3 was unable to modify either LD or Gcv3 when provided with free octanoate, ATP and $MgCl_2$ (Fig. 5 and data not shown). When octanoyl-CoA was used as acyl donor, Lip3-catalyzed modification of both LD and Gcv3 was seen (Fig. 5, lanes 1 and 5). In contrast to the implications of its *in vivo* activity, Lip3 also catalyzed modification of Gcv3 and LD *in vitro* with octanoyl-ACP as substrate (Fig. S1). No amidotransfer activity was detected for Lip3 *in vitro* (Fig. S1B). The extreme insolubility of Lip3 (see MATERIALS AND METHODS) precluded further characterization of Lip3 *in vitro*.

Lip3 functions downstream of Lip2 in *S. cerevisiae*

As stated earlier lipoate synthesis in *S. cerevisiae* is required for mitochondrial maintenance and respiration. When presented with glucose, *S. cerevisiae* preferentially grows fermentatively even in the presence of oxygen. Fermentative growth does not require mitochondria and therefore to test mitochondrial function (an indicator of lipoate synthesis) *S. cerevisiae* must be grown on a nonfermentable carbon source such as ethanol. We tested growth of *S. cerevisiae* strains carrying deletions of the *lip2*, *lip3* or *gcv3* genes after transformation with either an empty vector or a plasmid encoding a mitochondrially targeted gene (*E. coli lipB*, *E. coli lplA*, or *B. subtilis lipL*) on rich ethanol media.

The wild type parent, strain BY4741, grew well on rich medium containing ethanol in place of glucose (Fig. 6) whereas the *lip2*, *lip3* and *gcv3* mutant strains transformed with the empty vector failed to grow, even when the medium was supplemented with octanoate (Fig. 6, panel A). Expression of mitochondrially targeted *E. coli* LplA allowed growth of all three mutant strains (Fig. 6, panel B) indicating that the mutant strains expressed PDH and OGDH albeit in their inactive (apo) forms. Moreover, these data indicate that Gcv3 *per se* is not required for respiration but is required for downstream lipoylation of PDH and OGDH.

Mitochondrially targeted *B. subtilis* LipL failed to complement the *lip2* mutant strain but complemented the *lip3* mutant strain (Fig. 6, panel D). This suggests that Lip2 is the primary source of octanoyl-Gcv3, the LipL substrate. *E. coli* LipB produces octanoyl-Gcv3 as indicated by its ability to complement a *lip2* strain (Fig. 6, panel C). However, the inability

of LipB to complement the *gcv3* strain indicates that *E. coli* LipB cannot efficiently modify *S. cerevisiae* PDH and OGDH. Finally, the inability of LipB to complement the *lip3* strain indicates that *lip3* acts downstream of the octanoyl-Gcv3 formed by either Lip2 or LipB.

Interestingly, the *lip2* strain expressing LipL exhibited slight growth on ethanol when octanoate was provided (Fig. 6, panel D). This suggests that under these conditions, Lip3 (or another as yet unknown enzyme) produces some octanoyl-Gcv3 which is presented to LipL for modification of PDH and OGDH. The *gcv3* strain expressing LipB also grew weakly on octanoate-supplemented ethanol media. This suggests that in the absence of competition with Gcv3 and in the presence of excess octanoate Lip3 can modify PDH and OGDH, albeit poorly.

DISCUSSION

Several studies of transgenic mice and clinical observations of human disease cases have underscored the essential role of lipoate synthesis in health [Mayr *et al.*, 2011; Smith *et al.*, 2012; Yi and Maeda, 2005]. Lipoate is an essential cofactor for the respiratory enzyme systems, PDH and OGDH. *S. cerevisiae* mutants defective in lipoate synthesis are thus unable to grow on nonfermentable carbon sources such as ethanol. These strains form characteristically small colonies, called *petites*, when grown on glucose, because the colonies stop growing upon exhaustion of glucose. In contrast colonies of wild type *S. cerevisiae* continue to grow by utilization of the ethanol produced by glucose fermentation [Tzagoloff and Dieckmann, 1990]. Petite mutants frequently exhibit complete loss or extensive deletions of mitochondrial DNA [Contamine and Picard, 2000].

There remain several gaps in our understanding of lipoate synthesis in *S. cerevisiae*. Sulo and Martin [Sulo and Martin, 1993] isolated and characterized Lip5, the gene encoding the *S. cerevisiae* LipA homologue. Interestingly, they showed that even though *lip5* strains form petite colonies, the cells contained a substantial level of protein-bound lipoate when grown on lipoate-supplemented media. This observation suggests the presence of a scavenging mechanism in *S. cerevisiae* similar to that of *E. coli* LplA, *B. subtilis* LplJ or the *T. acidophilum* LplA/B complex. Why then are *lip5* strains respiratory deficient? It is possible that the lipoate scavenge pathway modifies only one of the two respiratory enzyme systems, while the endogenous lipoate synthesis pathway targets both systems. The lipoate scavenging enzyme of *S. cerevisiae* has yet to be identified since this study shows that Lip3, the only known Lpl homologue to date in the yeast, is not involved in lipoate scavenge.

A complete set of type II fatty acid biosynthesis genes, including a gene encoding ACP, has been characterized in *S. cerevisiae* mitochondria [Hiltunen *et al.*, 2010]. Mitochondrial fatty acid biosynthesis produces octanoyl-ACP, the substrate for the *S. cerevisiae* LipB homologue, Lip2. The *lip2* genes of both *S. cerevisiae* and *Kluyveromyces lactis* have been cloned and disrupted. Although the Lip2 proteins of the two yeast species have almost identical amino acid sequences, the *S. cerevisiae lip2* mutant strain was respiratory deficient whereas the *K. lactis lip2* strain was not [Chen, 1997; Marvin *et al.*, 2001]. Thus the differences in the effects of *lip2* deletion could reflect diversity in lipoate synthesis and/or utilization between the two organisms. We cloned *S. cerevisiae lip2* and showed that it complements an *E. coli lipB lplA* mutant strain on unsupplemented GMM (Fig. S2A). Crude *E. coli* extracts containing Lip2 had octanoyltransferase activity and lacked ligase activity (Fig. S2B). It was necessary to confirm that Lip2 is indeed an octanoyltransferase because octanoyltransferases, amidotransferases and lipoate ligases cannot be distinguished from one another by examination of their amino acid sequences. Members of the three groups share minimal amino acid sequence identity yet have similar structural folds [Christensen *et al.*, 2011a; Ma *et al.*, 2006; Reche, 2000]. A case in point is LipM which has

the sequence of a lipoate ligase but is an octanoyltransferase rather than a ligase [Christensen and Cronan, 2010]. Whereas octanoyltransferases lack an accessory domain, ligases may contain the accessory domain at either the C-terminus of the same protein such as the case with *E. coli* LplA [Fujiwara *et al.*, 2010; Fujiwara *et al.*, 2005], or at the N-terminus, such as the case for *Streptomyces coelicolor* LplA [Christensen and Cronan, 2009], or the accessory domain may be encoded by a completely separate gene, such as the *T. acidophilum* LplB [Christensen and Cronan, 2009; McManus *et al.*, 2006; Posner *et al.*, 2009].

A third and unexpected protein involved in *S. cerevisiae* lipoate synthesis is Gcv3. Gcv3 is a lipoylated protein that is a subunit of the GCV complex of single carbon metabolism together with Gcv1, Gcv2 and Lpd1. Work by Schonauer *et al.* [Schonauer *et al.*, 2009] suggests that lipoylation of Gcv3 is required for lipoylation of PDH and OGDH. They showed that in a *gcv3* strain there is no detectable lipoyl-PDH or OGDH. This was true of *S. cerevisiae* strains containing a complete deletion of *gcv3* and of strains encoding a mutant Gcv3 that lacked the lysine residue modified by lipoate attachment. Deletion of the other GCV subunits had no effect on lipoylation of Gcv3, PDH or OGDH [Schonauer *et al.*, 2009]. The requirement of this apparent “checkpoint” in lipoate synthesis is unclear. Perhaps *S. cerevisiae* uses lipoyl-Gcv3 as a sensor for nutritional standing before proceeding with activation of respiratory enzymes. Furthermore the operation of this putative checkpoint is unclear. Schonauer and coworkers showed that in a *lip2* strain there is no detectable lipoylation of either Gcv3 or the PDH and OGDH, whereas in a *lip3* strain Gcv3 becomes lipoylated [Schonauer *et al.*, 2009]. One possible explanation for this is that Lip2 specifically modifies Gcv3, whereas Lip3 modifies PDH and OGDH once Gcv3 lipoylation is complete. In an *in vitro* assay we noticed that when provided with a mixture of Gcv3 and LD, Lip3 modifies only Gcv3 (data not shown). This suggests that Gcv3 has priority for modification in *S. cerevisiae* whether catalyzed by either Lip2 or Lip3. Active GCV might be needed at times when active PDH and OGDH are not. Indeed, when wild type *S. cerevisiae* was grown on glucose the sole lipoylated protein detected was Gcv3 (Fig. S3). In contrast the same strain grown under respiratory conditions (on glycerol in place of glucose) contained lipoylated Gcv3, PDH and OGDH (Fig. S3).

Note that we were able to bypass the lipoyl-Gcv3 checkpoint to some extent in a *gcv3* strain by expression of mitochondrially-tagged versions of either *E. coli* LplA or *E. coli* LipB in the presence of excess free octanoate (Fig. 6). The lipoyl-Gcv3 checkpoint could also be bypassed in a *lip2* strain upon expression of *B. subtilis* LipL in the presence of excess free octanoate (Fig. 6). The effects of checkpoint bypass on the physiology of *S. cerevisiae* merits further study.

The last known enzyme in the lipoate synthesis pathway of *S. cerevisiae* is Lip3. The substrate for Lip3 had not been identified prior to this work. Lip3 was shown to utilize octanoyl-CoA to activate PDH and OGDH. This substrate could come either directly from peroxisomal fatty acid β -oxidation or from an acyl-ACP:CoA transferase (Fig. 1). Free octanoate and octanoyl-Gcv3 were not substrates for Lip3 *in vitro*.

The simplest model which integrates our results with the reported lipoyl-Gcv3 checkpoint is presented in Fig. 7. In this model Lip2 specifically modifies Gcv3 using octanoyl-ACP as substrate. When all the Gcv3 has been octanoylated, octanoyl-ACP accumulates. An octanoyl-ACP:CoA transferase then transfers the octanoyl moiety to CoA, providing the substrate used by Lip3 to modify PDH and OGDH. The exact role of octanoyl-Gcv3 in the lipoylation pathway of *S. cerevisiae* remains an open question. It is not clear why a *gcv3* mutant does not grow on ethanol even though wild type Lip3 is present and octanoyl-ACP is expected to accumulate in this mutant. Perhaps octanoyl-Gcv3 is required for expression,

modification or mitochondrial targeting of either Lip3 of the octanoyl-ACP:CoA transferase. It is worth noting that a second distinct role for Gcv3 in RNA processing has been reported [Schonauer *et al.*, 2008].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Diversity of lipoate attachment mechanisms. In the biosynthetic pathways (dashed box), octanoyl-ACP (1) is extracted from the pool of fatty acid biosynthesis intermediates by an octanoyltransferase (LipB in *E. coli*, LipM in *B. subtilis*, or Lip2 in *S. cerevisiae*) which transfers the octanoyl moiety to the LDs (e.g., that of the glycine cleavage system; black box). This is followed by sulfur insertion by a lipoate synthase (designated LipA in prokaryotes and Lip5 in *S. cerevisiae*). The LipL amidotransferases transfer the lipoyl moiety from lipoyl-GCV (3) to pyruvate dehydrogenase (white box), 2-oxoglutarate dehydrogenase and the branched chain oxoacid dehydrogenase (not shown).

The lipoate ligase salvage pathways make use of free lipoate (5). LplA from *E. coli*, LplJ from *B. subtilis* and the LplA/B complex from *T. acidophilum* form lipoyl-PDH (4) for example. In *L. monocytogenes* LplA1 specifically modifies GCV, and the role of LplA2 is unknown (not shown). Also shown in the figure are other sources for octanoyl-ACP from free octanoate (6) and octanoyl-CoA (7) of fatty acid -oxidation. Lipoamidases produce free lipoate by cleaving the coenzyme from degraded proteins.

Figure 2.

Lip3 complementation of the *E. coli lipB lplA* strain QC146. **A:** Growth curves of strain QC146 carrying the Lip3 expression plasmid, pFH23, on either unsupplemented GMM or GMM supplemented with lipoate, octanoate, or a mixture of acetate and succinate as indicated. **B:** Replacing the Lip3 active site lysine (K249) with arginine or alanine greatly reduces or entirely eliminates the ability of Lip3 to support growth of QC146 on octanoate-supplemented GMM.

Figure 3.

Detection of active lipoylated PDH and OGDH complexes in the *E. coli* *lipB* *lplA* strain QC146 expressing Lip3. (A) PDH and OGDH were assayed in extracts of strain QC146 carrying either the empty vector (I) or a plasmid that expressed Lip3 under arabinose control (II – IV). Growth medium was supplemented with lipoate or octanoate, both lipoate and octanoate or left unsupplemented as indicated. The highest levels of PDH and OGDH activities were observed in extracts of strain QC146 carrying the Lip3 expression plasmid pFH23 when octanoate was present in the growth media (IV). Symbols: * this result is significant relative to that of condition II (p -value = 0.003), and condition III (p -value = 0.02). † this result is significant relative to that of condition II (p -value = 0.0001), and condition III (p -value = 0.0005). The differences in PDH and OGDH activities between conditions II and III are not significant (not shown) (B) Western blot analysis using anti-lipoyl LD antibody of extracts of the strain QC146 carrying pFH23 cultured in LB containing arabinose, acetate and succinate with either lipoate (lane 2), octanoate (lane 3) or neither (lane 1). Whereas lipoylated SucB (the LD of OGDH) was detected in all three extracts, lipoylated AceF (the LD of PDH) was detected only in cells grown the presence of octanoate (3).

Figure 4.

A: Lip3 complementation of the *E. coli lipB lplA* strain QC146 on octanoate-supplemented GMM requires the presence of the *fadD* encoded acyl-CoA synthetase. Shown are GMM plates unsupplemented (left plate), supplemented with octanoate (central plate), or with succinate and acetate (right plate). The top sector of each plate contains strain QC146 carrying a plasmid which expresses Lip3 whereas in the bottom sector of each plate the same strain carried a *fadD* deletion. **B:** Overexpression of the FadK acyl-CoA synthetase amplifies Lip3 complementation of the *E. coli lipB lplA* strain QC146. Growth curves on octanoate-supplemented GMM of QC146 carrying either the empty vector, a Lip3 expression plasmid or plasmids expressing both Lip3 and FadK as indicated.

Figure 5.

In vitro detection of Lip3 octanoyl transferase activity by an electrophoresis mobility gel shift assay. Left gel: Lip3 reaction with LD with octanoyl-CoA (lane 1) or with ATP, octanoate and MgCl_2 (lane 2). Right gel: Lip3 activity with Gcv3 and octanoyl-CoA (lane 5). Lanes 3 and 4 were loaded with octanoyl-LD and apo-Gcv3 standards, respectively. Lip3 was active with both LD and Gcv3 when octanoyl-CoA was provided (lanes 1 and 5, respectively).

Figure 6. Growth phenotypes of *S. cerevisiae* *lip2*, *lip3* and *gcv3* strains transformed with different plasmids. All strains were derivatives of BY4741 and were grown on YP glucose, YP ethanol or YP ethanol supplemented with octanoate. Plates were incubated at 30° for up to four days. Panel A: the mutant strains transformed with empty vector. Panel B: the mutant strains transformed with pFH56 expressing mitochondrially targeted *E. coli* LplA. Panel C: the mutant strains transformed with pFH57 expressing mitochondrially targeted *E. coli* LipB. Panel D: the *lip2* and *lip3* strains transformed with pFH58 expressing mitochondrially targeted *B. subtilis* LipL.

Figure 7.

Proposed pathway for lipoate synthesis/ attachment in *S. cerevisiae* which accounts for the observed octanoyl-Gcv3 checkpoint. Left: Lip2 specifically modifies Gcv3 using octanoyl-ACP from mitochondrial FA biosynthesis. Right: When all Gcv3 is octanoylated, octanoyl-ACP accumulates. An octanoyl-ACP: CoA transferase transfers the octanoyl moiety to CoA, providing substrate for Lip3 to modify PDH and OGDH. Lip5 catalyzes the sulfur insertions on the octanoyl moiety once it is attached to an LD (not shown).