Activation of fatty acids to their coenzyme A derivatives is necessary for subsequent metabolism. Very long-chain fatty acids, which accumulate in tissues of patients with X-linked adrenoleukodystrophy, are activated by very long-chain acyl-CoA synthetase (VLCS) normally found in peroxisomes and microsomes. We identified a candidate yeast VLCS gene (FAT1), previously identified as encoding a fatty acid transport protein, by its homology to rat liver peroxisomal VLCS. Disruption of this gene decreased, but did not abolish, cellular VLCS activity. Fractionation studies showed that VLCS activity, but not very-long-chain acyl-CoA synthetase activity, was reduced to about 40% of wild-type level in both 27,000 × g supernatant and pellet fractions. Separation of organelles in the pellet fraction by density gradient centrifugation revealed that VLCS activity was associated with peroxisomes and microsomes but not mitochondria. FAT1 deletion strains exhibited decreased growth on medium containing dextrose, oleic acid, and cerulenin, an inhibitor of fatty acid synthesis. FAT1 deletion strains grown on either dextrose or oleic acid medium accumulated very long-chain fatty acids. Compared with wild-type yeast, C22:0, C24:0, and C26:0 levels were increased approximately 20-, 18-, and 3-fold in deletion strains grown on dextrose, and 2-, 7-, and 5-fold in deletion strains grown on oleate. Long-chain fatty acid levels in wild-type and deletion strains were not significantly different. All biochemical defects in FAT1 deletion strains were restored to normal after functional complementation with the FAT1 gene. The level of VLCS activity measured in both wild-type and deletion yeast strains transformed with FAT1 cDNA paralleled the level of expression of the transgene. The extent of both the decrease in peroxisomal VLCS activity and the very long-chain fatty acid accumulation in the yeast FAT1 deletion model resembles that observed in cells from X-linked adrenoleukodystrophy patients. These studies suggest that the FAT1 gene product has VLCS activity that is essential for normal cellular very long-chain fatty acid homeostasis.

Metabolism of fatty acids by either catabolic or anabolic pathways requires initial activation to their coenzyme A (CoA) thioesters, a reaction catalyzed by acyl-CoA synthetase (1). Differences in substrate chain length specificity and subcellular localization distinguish the various acyl-CoA synthetases present in cells from all species examined, including animals, yeast, bacteria, and plants (1). Long-chain fatty acids (C12–C20) are activated by long-chain acyl-CoA synthetases (LCSs) found in mitochondria, peroxisomes, and microsomes (1), reflecting the primary roles of these organelles in fatty acid β-oxidation (mitochondria and peroxisomes) and complex lipid synthesis (microsomes). In contrast, activation of very long-chain fatty acids (VLCFA; C22 or longer) by very long-chain acyl-CoA synthetase (VLCS) takes place only in peroxisomal and microsomal subcellular fractions of rat (2), human (3), and yeast (4) cells. Lack of mitochondrial VLCS prevents this organelle from catalyzing VLCFA via β-oxidation, thus relegating this function to the peroxisome.

VLCFA β-oxidation is impaired in cells from patients with the progressive neurodegenerative disease X-linked adrenoleukodystrophy (XALD), resulting in elevated plasma and tissue concentrations of saturated VLCFA (5). Biochemical studies revealed that VLCS activity was decreased in peroxisomal, but not microsomal, subcellular fractions of fibroblasts from XALD patients (3, 6). However, further investigation of the molecular defect in XALD revealed that key steps in peroxisomal VLCFA metabolism are still poorly understood. The product of the gene defective in XALD (ALDP) is not VLCS, but rather is a peroxisomal membrane protein whose existence was previously unknown (7). Studies in many laboratories, including our own (8), clearly demonstrated that XALD patients have mutations in the ALD gene, and complementation studies confirmed that transfection of XALD cells with the ALD gene restores their ability to catalyze VLCFA (9, 10). While studies of XALD suggest that ALDP is required for VLCFA activation, the biochemical relationship between ALDP and VLCS has not yet been elucidated. Thus, to further our understanding of peroxisomal VLCFA activation, we are investigating this process in yeast.

Yeast (Saccharomyces cerevisiae) is an ideal model organism for the study of peroxisomal fatty acid β-oxidation, since they lack the mitochondrial β-oxidation pathway found in higher animals. They have been used as a model system for the study of peroxisomal fatty acid β-oxidation, since they lack the mitochondrial β-oxidation pathway found in higher animals.
valuable for identification of proteins subsequently shown to be defective in human disorders of peroxisome biogenesis (11–16). We previously reported that, as in mammalian tissues, peroxisomes and microsomes isolated from the yeast *Pichia pastoris* contain VLCs activity (4). The yeast *Saccharomyces cerevisiae* has been used by Gordon and co-workers to identify and characterize four fatty acid activator (FAC1–4) genes that encode acyl-CoA synthetases (17, 18). They examined the fatty acid substrate specificity of the protein products of three of these FAC genes (Faa1p, Faa2p, and Faa3p) in detail; while Faa3p weakly activated VLCFAs, Faa1p and Faa2p had no detectable activity with these substrates (17). Faa4p was reported to be functionally interchangeable with Faa1p and thus is not likely to be a VLCs (18). Based on growth studies of yeast mutants in which all four FAC genes were disrupted, Gordon and colleagues concluded that at least one other FAC gene exists in *S. cerevisiae* (18).

Recently, Hashimoto and co-workers purified an enzyme with VLCs activity from rat liver peroxisomes (rVLCs) (19) and subsequently cloned and sequenced its cDNA (20). We identified an open reading frame on *S. cerevisiae* chromosome II (YBR041W; locus FAT1) with homology to the rVLCs amino acid sequence. Both rVLCs and the FAT1 gene product, Fat1p, are homologous to mouse fatty acid transport protein (mFATP), described by Schaffer and Lodish (21). Faegeman et al. (22) independently identified the FAT1 (fatty acid transporter 1) gene by virtue of the homology of Fat1p to mFATP. These investigators found that deletion of FAT1 resulted in decreased rates of cellular uptake of labeled long-chain fatty acid, decreased cellular uptake of fluorescent fatty acids, and decreased uptake and incorporation of labeled oleic acid into cellular phospholipids (23). Although they noted amino acid sequence similarities between Fat1p and acyl-CoA synthetases (17, 18), they examined the fatty acid substrate specificity of the protein products of three of these FAC genes (Faa1p, Faa2p, and Faa3p) in detail; while Faa3p weakly activated VLCFAs, Faa1p and Faa2p had no detectable activity with these substrates (17). Faa4p was reported to be functionally interchangeable with Faa1p and thus is not likely to be a VLCs (18). Based on growth studies of yeast mutants in which all four FAC genes were disrupted, Gordon and colleagues concluded that at least one other FAC gene exists in *S. cerevisiae* (18).

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2 P. A. Watkins, unpublished observations.
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RESULTS

Identification of FAT1 as a Candidate S. cerevisiae VLCS Gene—A cDNA library from the liver of a rat treated with the peroxisome proliferator Gemfibrozil was screened, and a 660-bp fragment containing a 220-amino acid open reading frame thought to be the carboxyl terminus of rat VLCS was cloned as described under “Experimental Procedures.” The sequence of this fragment was 100% identical to that of authentic rat liver VLCS, which was subsequently published by Uchiyama et al. (20). The 220-amino acid sequence was compared with all open reading frames of the complete S. cerevisiae genome using the BLAST algorithm. Only one likely candidate for a homologous yeast protein was identified (smallest sum probability = 9e-24). The gene encoding this protein was identical to that recently reported by Faergeman et al. (22), which they designated FAT1 by virtue of its homology to mFATP.

While preparing expression constructs to investigate the biochemical properties of Fat1p, the product of the FAT1 gene, we detected an error in the sequence as contained in the Saccharomyces Genome Data Base. The published sequence contained a CpC dinucleotide insertion at bp 1857 and 1858, yielding an open reading frame of 623 amino acids. The corrected nucleotide sequence of FAT1 and its predicted amino acid sequences are shown in Fig. 1. The FAT1 gene encodes a protein of 669 amino acids with 33.7% identity and 57.2% similarity to rVLCS. Although the signal that targets rVLCS to peroxisomes is not certain, the protein ends in the carboxyl-terminal tripeptide -LKL (20) and may be a functional variant of the well characterized -SKL peroxisome targeting signal 1 (38). Fat1p ends in the carboxyl-terminal tripeptide -IKL, which has been shown to function as a peroxisome targeting signal 1 in yeast (28). To investigate the function of Fat1p, PCR-mediated gene disruption was used to replace the FAT1 gene with the HIS selectable marker as described under “Experimental Procedures.”

VLCS and LCS Activity of FAT1 Deletion Strains—In order to identify mutants in which the FAT1 gene had been disrupted and to ascertain whether Fat1p had VLCS activity, seven HIS prototrophs were assayed for their ability to activate a VLCFA, lignoceric acid (C24:0), to its CoA derivative. Spheroplasts were prepared from wild-type yeast and these candidate FAA1 deletion strains. Spheroplast homogenates from four strains in which FAA1 gene disruption was confirmed by PCR (see “Experimental Procedures”) also had decreased ability to activate lignoceric acid, suggesting that Fat1p has VLCS activity that was indeed disrupted. In these strains, LCS activity was similar to that of wild-type. Data from the two strains chosen for further study, designated fat1Δ-1 and fat1Δ-2, are shown in Table I.

The acyl-CoA synthetase activity of wild-type S. cerevisiae was assayed using a VLCFA substrate, lignoceric acid, was more than 100-fold less than that measured using a long-chain fatty acid, palmitate (C16:0) (Table I), in agreement with our previous observations in the yeast Pichia pastoris (4). In the FAT1 deletion strains, VLCS activity was reduced to <30% of wild-type level. Residual VLCS activity in deletion strains was clearly detectable; radioactivity in VLCFA assays of these strains was ≥2-fold higher than in enzyme-free control reactions. In contrast, LCS activity of FAT1 deletion strains was relatively unaffected (Table I). These findings indicate that Fat1p is responsible for activation of a considerable fraction of

Fatty Acid Analysis—Yeast grown in either YPD (250 A600) or YPOLT (80 A600) were harvested by centrifugation, washed three times with deionized water, suspended in 0.5 ml water, and subjected to sonic disruption. After centrifugation, the concentration of the protein by the method of Lowry et al. (21), total lipids were extracted by a modification of the method of Folch et al. (36), and total fatty acids were quantitated by gas chromatography as their methyl esters using the method of Moser and Moser (37).
cellular VLCFAs; however, it appears that Fat1p is not the only enzyme with VLCs activity.

**Growth of FAT1 Deletion Strains on Fatty Acids**—Growth of wild-type, FAT1 deletion, and FAT1 deletion complemented with FAT1 cDNA strains on YPD plates supplemented with 500 μM oleic acid was comparable at both 24 and 30 °C; in contrast, growth of the FAT1 deletion strain was markedly reduced at 24 °C and somewhat reduced at 30 °C when cerulenin, an inhibitor of fatty acid synthase (39), was added to the oleic acid growth medium (data not shown). This growth phenotype is similar to that reported previously for FAT1 deletion mutants (22). Transformation of FAT1 deletion strains with FAT1 cDNA restored growth to normal (data not shown).

**FAT1 Gene Deletion Strains Accumulate VLCFAs**—In XALD and in peroxisome biogenesis disorders, failure of peroxisomes to activate VLCFAs results in their accumulation in tissues (5). To determine whether deletion of the FAT1 gene affected VLCFA accumulation in yeast, fatty acid analysis was performed on wild-type, fat1D-1, and fat1D-2 strains grown in both YPD medium and in YPOLT medium to induce peroxisomes. As shown in Fig. 2, the cellular concentration of saturated fatty acids with chain lengths greater than or equal to C22 was increased in FAT1 deletion strains grown on YPD. The concentrations of saturated fatty acids containing 14–20 carbons were significantly increased in FAT1 deletion strains as compared to wild-type strains.

| Strain       | C160 | C240 | C24/C16 |
|--------------|------|------|---------|
| **wt**       | 29.8 | 0.27 | 0.0089  |
| **fat1D-1**  | 29.6 | 0.08 | 0.0026  |
| **fat1D-2**  | 27.6 | 0.08 | 0.0030  |

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similar in both wild-type and deletion strains (data not shown). When wild-type yeasts were grown on YPOLT instead of YPD, concentrations of saturated fatty acids, both VLCFA (Fig. 2) and long-chain fatty acids (data not shown), were 5–10-fold higher. As in YPD-grown yeast, FAT1 deletion strains grown on YPOLT had increased concentrations of VLCFA, particularly C24:0 and C26:0 (Fig. 2), relative to wild-type yeast grown under the same conditions. Long-chain fatty acid concentrations were similar in YPOLT-grown wild-type and FAT1 deletion strains.

To verify that elevations in VLCFA concentrations were specific and not the result of an overall increase in fatty acids in these cells, we calculated the cellular content of these fatty acids as a percentage of total fatty acids. As shown in Fig. 3, the increase in cellular content of VLCFA in FAT1 deletion strains was similar to the increase in cellular concentration of VLCFA (Fig. 2) in both YPD- and YPOLT-grown yeast. YPD-grown deletion strains preferentially accumulated behenic acid (C22:0) and lignoceric acid (C24:0), which were 20- and 18-fold higher in fat1Δ-1 and fat1Δ-2 strains than in the wild-type strain. YPOLT-induced deletion strains, on the other hand, mainly accumulated lignoceric acid and hexacosanoic acid (C26:0), which were 7- and 5-fold higher than in wild-type. To verify that the elevation in VLCFA content of FAT1 deletion strains was due solely to the lack of Fat1p, we transformed FAT1–1 cDNA (pJFL3302) into the fat1Δ-1 and fat1Δ-2 strains. Wild-type and deletion strains were also transformed with the vector alone. After growth on YPOLT, the cellular content of saturated VLCFA was determined. As shown in Fig. 4, C22:0, C24:0, and C26:0 were all restored to wild-type levels in deletion strains transformed with FAT1–1 cDNA. When deletion strains were transformed with FAT1 cDNA (pJFL3307), similar results were obtained (data not shown). These results strongly suggest that Fat1p is required for utilization of VLCFAs in S. cerevisiae and that this protein plays a significant role in regulating VLCFA metabolism.

VLC Activity of Both Organelle Pellet and Supernatant Fractions Is Reduced upon Deletion of FAT1 and Is Restored by Expression of FAT1 cDNA—We previously demonstrated that in the yeast P. pastoris, VLC activity was present in both 27,000 × g microsome-rich supernatant and organelar pellet fractions (4). When homogenates of S. cerevisiae spheroplasts were subjected to the same procedure, VLC activity was also found in both the peroxisome- and mitochondria-rich organelle pellet and the cytosol- and microsome-rich supernatant (Table II). As expected, these fractions also exhibited high LCS activity. In wild-type yeast, the organelle pellet fraction contained about 60% of the total cellular LCS activity and about 30% of the total VLC activity (data not shown). To determine whether Fat1p was responsible for VLCFA activation in supernatant, organelle pellet, or both, fat1Δ-1 and fat1Δ-2 strains were also fractionated. In these deletion strains, the specific activity of VLC was decreased in both the organelle pellet and the supernatant fractions. The relative decrease was greater in the microsome-rich supernatant than in the organelle pellet. The LCS activity of the fat1Δ-1 strain was more variable and slightly lower than in either the wild-type or the fat1Δ-2 strain; the cause of this variability is unknown.

To verify that the decrease in VLC activity in the FAT1 deletion strains was caused by lack of Fat1p, we assayed fractions prepared from wild-type, fat1Δ-1, and fat1Δ-2 strains that were transformed with either FAT1–1 cDNA (pJFL3302) or the vector alone. As shown in Table III, expression of FAT1–1 cDNA (on the low copy number CEN vector) in the wild-type strain resulted in a slight increase in VLC activity in the organelle pellet. In deletion strains expressing Fat1p, VLC
activities for the number of experiments indicated in parentheses. C16:0 or C24:0 as substrate was measured as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.”

**Table II**

Acyl-CoA synthetase activity in organelle pellet and supernatant fractions of wild-type and FAT1 deletion strains

Spheroplasts were prepared from oleate-grown wild-type (wt) and FAT1 deletion yeast strains, homogenized, and fractionated into an organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either C16:0 or C24:0 as substrate was measured as described under “Experimental Procedures.” Data are presented as mean ± S.D. of specific activities for the number of experiments indicated in parentheses.

| Activation | C16:0 | C24:0 |
|------------|-------|-------|
| Organelle pellet |       |       |
| wt (n = 6)  | 72.5 ± 33.1 | 0.31 ± 0.14 |
| fat1Δ-1 (n = 5) | 49.6 ± 36.9 | 0.13 ± 0.10 |
| fat1Δ-2 (n = 5) | 69.4 ± 22.8 | 0.18 ± 0.11 |
| Supernatant |       |       |
| wt (n = 6)  | 25.6 ± 11.2 | 0.30 ± 0.13 |
| fat1Δ-1 (n = 5) | 20.7 ± 9.3 | 0.09 ± 0.08 |
| fat1Δ-2 (n = 5) | 25.8 ± 18.9 | 0.09 ± 0.05 |

**Table III**

Restoration of VLCS activity in FAT1 deletion strains by functional complementation with FAT1-1 cDNA

Wild-type (wt) or FAT1-deletion yeast strains were transformed with either the CEN vector pRS416 or the vector containing FAT1-1 cDNA (pJFL3302) as described under “Experimental Procedures.” Spheroplasts were prepared and fractionated into an organelle pellet and a 27,000 × g supernatant as described under “Experimental Procedures.” Acyl-CoA synthetase activity with either C16:0 or C24:0 as substrate was measured as described under “Experimental Procedures.”

**Fig. 4.** FAT1-1 cDNA restores cellular VLCFA content to wild-type (WT) levels in FAT1-deletion strains. Wild-type and deletion strains were transformed with either the CEN vector alone (pRS416) or the vector containing FAT1-1 cDNA (pJFL3302). After growth on YPOLT medium, fatty acids were analyzed as in Fig. 2. The content of each fatty acid was calculated as a percentage of total cellular fatty acids. For wild-type yeast transformed with vector alone, the content of C22:0, C24:0, and C26:0 was 0.046, 0.076, and 0.091%, respectively. Results are expressed as a percentage of wild-type fatty acid content. FAT1Δ1 plus vector; FAT1Δ1 plus pJFL3302; FAT1Δ-2 plus pJFL3302.

Activity in the organelle pellet was similar to the activity in this fraction from vector-transformed wild-type (Table III). VLCS activity of supernatant fractions from deletion strains transformed with FAT1-1 cDNA was restored to about 75% of wild-type activity (Table III).

Overexpression of VLCS Activity in Wild-type and FAT1 Deletion Strains—To verify that Fat1p catalyzes VLCS activity, we overexpressed FAT1 cDNA in yeast strains. pSJW981, a high copy plasmid containing FAT1 cDNA, was transformed into both the wild-type and fat1Δ-2 strains. As shown in Fig. 5, a 4-fold increase over wild-type VLCS activity was observed in the wild-type strain transformed with pSJW981. The VLCS activity of the pSJW981-transformed deletion strain was nearly 3-fold higher than wild-type level (Fig. 5). Comparison of the VLCS activities of both the wild-type strain and fat1Δ-2 strain transfected with the low copy CEN plasmid (Table III) versus the high copy 2μ construct (Fig. 5) showed a correlation between levels of VLCS activity and the level of expression of the transgene. These results indicate that Fat1p indeed catalyzes VLCS activity.

**VLCS Is Not Induced by Peroxisome Proliferation—** Treatment of rats with peroxisome proliferators such as Gemfibrozil was found to increase the specific activity of peroxisomal VLCS.1 In yeast, peroxisome proliferation and the synthesis of several peroxisomal β-oxidation enzymes are induced by growth on medium containing a fatty acid such as oleic acid as primary carbon source (40, 41). A 21–24-hp oleate response element has been identified in the 5′-untranslated region of the genes encoding these proteins; however, Faergerman et al. (22) reported that there was no consensus oleate response element in the 5′-untranslated region of the FAT1 locus. We wanted to ascertain directly whether oleate-induced peroxisome proliferation in S. cerevisiae would increase VLCS activity and the level of FAT1 mRNA. When wild-type yeast were grown in oleic acid-containing medium, we did not observe an increase in VLCS activity over that of dextrose-grown yeast (data not shown). To estimate relative FAT1 mRNA levels, total RNA was isolated from spheroplasts prepared from yeast grown in either medium. When equal amounts of RNA were used for reverse transcriptase-catalyzed cDNA synthesis, no increase in FAT1 message in oleate-grown cells was detected by PCR (Fig. 6), consistent with the lack of an upstream oleate response element. In contrast, there was a marked increase in mRNA for EDS92, a gene encoding a peroxisomal enoyl-CoA hydratase that contains an oleate response element (Fig. 6).

**VLCS Activity of Fat1p in the Organelle Pellet Fraction Is Peroxisomal—** Because peroxisome induction by oleate in wild-type yeast failed to increase either FAT1 message (Fig. 6) or VLCS activity (data not shown), we questioned whether the VLCS activity in the organelle pellet was associated with peroxisomes or mitochondria. Although fatty acid β-oxidation does not take place in mitochondria, VLCFA activation for complex lipid synthesis or elongation could take place in this organelle. To address this question, we fractionated the organelle pellet...
from wild-type and gene deletion strains by density gradient centrifugation. As shown in Fig. 7, C and D, peroxisomes and mitochondria were well separated in both wild-type and FAT1 deletion strains. VLCs activity was associated mainly with peroxisomal gradient fractions, with a small amount of activity found in a low density fraction near the top of gradients (Fig. 7B). Mitochondrial fractions had very low VLCs activity, only 10% of that in the peroxisomal fraction, and was not appreciably decreased in the gene deletion strain. Overall, our results indicate that VLCs activity in S. cerevisiae appears to be catalyzed, at least in part, by Fat1p.

**FIG. 6.** FAT1 is not induced by growth on oleic acid. Wild-type yeasts were grown either on YPD (lanes B and D), or on YPOLT to induce peroxisomes (lanes C and E). Total RNA was isolated, and reverse-transcription of an equal amount of RNA was performed as described under “Experimental Procedures.” Results are presented as a percentage of wild-type VLCs activity and are the average of two independent experiments.

![Image](https://example.com/image.png)

**DISCUSSION**

In this report, we provide evidence that the *S. cerevisiae* FAT1 gene product, Fat1p, has VLCs activity. While several yeast enzymes with acyl-CoA synthetase have been reported, Fat1p is the first described that appears to play a significant role in VLCFA metabolism. Four yeast acyl-CoA synthetases were previously described by Gordon and colleagues (17, 18). These investigators also predicted the existence of at least one additional fatty acid activation gene that directs fatty acids to the $\beta$-oxidation pathway after they found that strains in which all four FAA genes were disrupted were viable on media containing fatty acids as sole carbon source (18). Faa1p, Faa2p, and Faa4p were very active toward medium- to long-chain fatty acids but showed no VLCs activity. Faa3p, expressed in bacteria as a His$_6$ fusion protein, weakly activated fatty acids over a broad range of chain lengths from 12 to 24 carbons. Although Faa3p was the only one of this group that demonstrated any activity toward VLCFAs, the activity of the recombinant enzyme with fatty acids of all chain lengths was very low. Studies to determine whether Faa3p or any of the other known yeast acyl-CoA synthetases are responsible for the residual VLCs activity in FAT1 deletion strains are in progress.

Deletion of the FAT1 gene resulted in decreased growth on rich medium containing both dextrose and oleic acid when an inhibitor of fatty acid synthesis, cerulenin, was present. This result is in agreement with findings reported by Faergeman et al. (22), who independently identified the FAT1 gene by virtue of the homology of Fat1p to mFATP, a mouse protein thought to be involved in fatty acid transport (21). In their study, FAT1 deletion strains showed evidence of defective fatty acid uptake into cells and decreased incorporation of labeled oleic acid into cellular phospholipids (22). However, when we quantitated total fatty acids in yeast grown on either dextrose- or oleate-containing medium, no difference in the concentrations of C14:0, C16:0, or C18:0 were found in wild-type, FAT1-deficient strains (Fig. 7C). FAT1 gene deletion caused yeast to accumulate VLCFAs when grown on either dextrose or oleic acid as primary carbon source, suggesting that the primary role of Fat1p is in VLCFA metabolism. Furthermore, it is possible that the biochemical effects in deletion mutants ascribed to defects in fatty acid transport result from perturbation of the cell membrane lipid composition by increased VLCFA concentrations. Alterations in membrane properties by VLCFAs have been described in model membranes (42), erythrocyte membranes (43), and adrenocortical cell membranes (44).

Our results clearly indicate that FAT1 deletion strains are unable to effectively utilize VLCFA. Accumulation of VLCFA could result from overproduction, failure to incorporate these fatty acids into complex lipids, failure to degrade excess fatty
acids by peroxisomal β-oxidation, or a combination of these processes. When yeast are grown on rich medium containing oleate, the cellular long-chain fatty acid content is 5–10-fold higher than when cells are grown on dextrose medium (data not shown), and VLCLA would be expected to arise primarily from chain elongation. However, when cells are grown in dextrose medium, fatty acids (including VLCLA) for biosynthesis of complex lipids could originate either from de novo synthesis or from lipids present in the yeast extract component of the medium. In either case, failure to activate VLCLA, either for incorporation into complex lipids or for degradation via β-oxidation, could result in significant accumulation within yeast cells. Furthermore, if Fat1p, in addition to its role in VLCLA activation also functions to transport VLCLA across organelar membranes, lack of this protein could similarly result in cellular accumulation of VLCLA.

Because of the association of defective peroxisomal fatty acid metabolism with human neurodegenerative diseases, we wanted to verify that yeast Fat1p was an acyl-CoA synthetase and to determine its subcellular location. Faergeman et al. (22) reported that deletion of the FAT1 gene did not decrease acyl-CoA synthetase activity in sonicated cell extracts when myristate (C14:0), palmitate (C16:0), or oleate (C18:1) was supplied as substrate. Similarly, we found no decrease in LCS activity in yeast spheroplast homogenates using palmitate as substrate (Table I). In contrast, total yeast VLCS activity as well as that of peroxisomes and a microsome-rich 27,000 × g supernatant fraction was significantly decreased when the FAT1 gene was deleted, indicating that Fat1p has VLCS activity (Tables I and II). The only other yeast acyl-CoA synthetase whose subcellular distribution has been studied is Faa2p, which was found to be mainly associated with peroxisomes (45). Faa2p, which had no VLCS activity, was found to be most active toward medium chain length saturated fatty acids (C10:0–C14:0) (17). The subcellular distribution of Faa1p, Faa3p, and Faa4p is unknown.

Decreased peroxisomal VLCS activity and elevated cellular VLCLA levels are consistent biochemical features of XALD. As reported here, deletion of the gene encoding the only S. cerevisiae protein that shares significant homology with mammalian VLCS produces a similar biochemical phenotype. However, the gene defective in XALD encodes ALDP, a protein of the ATP-binding cassette membrane transporter superfamily, and not VLCS (7). ALDP and at least three other structurally related proteins of this family are found in mammalian peroxisomal membranes (46–48). Yeasts contain only two peroxisomal proteins belonging to this family, Pxa1p (49) and Pxa2p (50) (also known as Pat2p and Pat1p, respectively (45)), which have been proposed as functional homologs of ALDP. Deletion of either the PXA1 or PXA2 genes resulted in decreased growth on medium containing oleate as primary carbon source (45, 49, 50), whereas no growth phenotype of FAT1 deletion strains was evident under the same conditions. Shani et al. (50) found that Pxa1p and Pxa2p function as a heterodimer. Hettema et al. (45) concluded that these two yeast ATP-binding cassette transporter proteins functioned to transport long-chain fatty acyl-CoAs into peroxisomes for catabolism via β-oxidation. Furthermore, they concluded that the acyl-CoAs transported by these proteins were activated by acyl-CoA synthetases located on organelles other than peroxisomes. They further concluded that Pxa1p and Pxa2p were not required for transport into peroxisomes of medium chain free fatty acids, which were subsequently activated inside the peroxisome by Faa2p. Preliminary data indicate that, in contrast to deletion of FAT1, deletion of PXA1 or PXA2 did not increase cellular C22:0 or C24:0 concentrations significantly.3 Thus, it appears that the functional relationship between VLCS and ATP-binding cassette transporters may differ between yeasts and humans and introduces the possibility that Fat1p both activates and trans-

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3 N. Shani, J.-F. Lu, and D. Valle, submitted for publication.
ports peroxisomal VLCFAs. Further studies to probe the relationship among Fat1p, Pxa1p, and Pxa2p and to determine whether Fat1p also functions as a peroxisomal transporter are currently in progress.

VLCSs and mFATP appear to belong to the same protein family. rVLCS shares 40% amino acid identity and 73% similarity with mFATP (20). Faergeman et al. (22) noted that Fat1p and mFATP contain several regions of high sequence similarity, including a consensus sequence (amino acids 255–268 of Fat1p; see Fig. 8, Motif 1) common to all known acyl-CoA synthetases and other proteins (e.g. insect luciferases and plant coumarate CoA ligase) with an AMP-binding domain (51). Another region of sequence similarity between Fat1p and mFATP consists of eight invariant amino acid residues in a 25-amino acid consensus sequence (consisting of eight invariant amino acid residues in a 25-amino acid consensus sequence) is partially contained in motif 2; residues 19–25 of the signature motif are residues 1–7 of motif 2. Conserved residues 4 and 5 of motif 2 (DR) are invariant amino acids of the signature motif. However, only amino acids 1, 7, and 8 (DXXXGXD) of the signature motif were also present in rVLCS, a protein known to possess acyl-CoA synthetase activity, indicating that the signature motif may require only five invariant residues. Notably, the DXXXGXD sequence was also present in all enzymes of the VLCS/FATP group (data not shown), suggesting that all of these proteins could have acyl-CoA synthetase activity.

Yeasts have proven to be extremely valuable in the study of peroxisomes and peroxisomal lipid metabolism. This organism has been extensively used to identify and characterize many proteins required for peroxisome biogenesis; indeed, protein defects in 6 of the 11 recognized complementation groups of human peroxisome biogenesis disorders were elucidated by virtue of their homology to proteins first identified in yeast (11–16). In this study, we have demonstrated that deletion of the FAT1 gene results in VLCFCA accumulation, a cellular phenotype resembling that found in XALD. The finding that VLCS activity was not totally abolished in FAT1 gene deletion strains suggests that a known (Pxa1p-4p) or unknown acyl-CoA synthetase is responsible for the residual activity. This conclusion is in concert with the observation that in XALD, peroxisomal β-oxidation of VLCFA is reduced (20–30% of control) but not decreased to the same extent as seen in the disorders of peroxisome biogenesis (<10% of control) (54). The nature of the enzyme(s) responsible for residual VLCS activity in yeast and the elucidation of similar enzyme(s) in humans are the subject of current investigation.

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Yeast Very Long-chain Acyl-CoA Synthetase

18219

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