Disruption of the Saccharomyces cerevisiae Homologue to the Murine Fatty Acid Transport Protein Impairs Uptake and Growth on Long-chain Fatty Acids*

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The yeast Saccharomyces cerevisiae is able to utilize exogenous fatty acids for a variety of cellular processes including β-oxidation, phospholipid biosynthesis, and protein modification. The molecular mechanisms that govern the uptake of these compounds in S. cerevisiae have not been described. We report the characterization of FAT1, a gene that encodes a putative membrane-bound long-chain fatty acid transport protein (Fat1p). Fat1p contains 623 amino acid residues that are 35% identical and 54% with similar chemical properties as compared with the fatty acid transport protein FATP described in 3T3-L1 adipocytes (Schaffer and Lodish (1994) Cell 79, 427–436), suggesting a similar function. Disruption of FAT1 results in 1) an impaired growth in YPD medium containing 25 µM cerulenin and 500 µM fatty acid (myristate (C14:0), palmitate (C16:0), or oleate (C18:1)); 2) a marked decrease in the uptake of the fluorescent long-chain fatty acid analogue boron dipyrromethene difluoride dodecanoic acid (BODIPY-3823); 3) a reduced rate of exogenous oleate incorporation into phospholipids; and 4) a 2–3-fold decrease in the rates of oleate uptake. These data support the hypothesis that Fat1p is involved in long-chain fatty acid uptake and may represent a long-chain fatty acid transport protein.

Exogenous long-chain fatty acids represent an important class of hydrophobic compounds that serve as substrates for lipid biosynthesis, protein modification, and β-oxidation. While the mechanism that facilitates the uptake of these compounds into eukaryotic cells is not completely understood, information gleaned over the past 15 years is consistent with a facilitated, lipid-mediated process in higher eukaryotic cells. Three gen-

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1 The abbreviations used are: FAT, fatty acid translocase; FABPpm, membrane-bound fatty acid-binding protein; FATP, fatty acid transport protein; BODIPY, boron dipyrromethene difluoride dodecanoic acid; SMM, supplemented minimal media; bp, base pair(s); PBS, phosphate-buffered saline; CER, cerulenin.
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Johnson et al. (30). Plates were then incubated at 24, 30, or 37 °C for 72 h. Growth in liquid YPD with or without cerulenin and fatty acid was monitored over a 4-h period. All experiments were repeated at least three times.

Expression of FAT1—Yeast genomic DNA was purified from strain W303a as detailed in Kaiser et al. (32). For disruption, the FAT1 gene was amplified by thermocycling from genomic DNA using sets of specific primers; 5'-ACCCGGATCCGAAAAATTCTGGTATCT-3' (upstream) and 5'-CACCGGATCTCCTACTGATGTTGAAAC-3' (downstream), both containing BamHI sites (underlined). The HIS3 gene was amplified from pPH3 (obtained from David Nelson, University of Tennessee, Memphis) using the upstream primer 5'-AGGGATCTCCCTTCTTTAAACCCAGCGGTGTGC-3' and the downstream primer 5'-TACCGGATCCCTGACCCATATCCACAAA-3', both containing EcoRI sites (underlined). The 1549-bp fragment containing the coding sequence of FAT1 was cloned into the BamHI site of pACYC177 to generate pNjF1. The 480-bp EcoRI fragment within FAT1 was replaced by the 1346-bp EcoRI fragment containing the HIS3 gene to generate pNjF2. The strain W303a was rendered competent using lithium acetate using standard procedures and transformed with linearized pNjF2 according to Kaiser et al. (32). Clones were selected on minimal plates lacking histidine supplementation. His+ isolates were selected and colony-purified on minimal plates without histidine. Several isolates were obtained. The disruption of the FAT1 gene with HIS3 was confirmed using DNA amplification of genomic DNA. One such isolate, shown to contain a disruption in FAT1, designated W303a-fat1Δ-1 (fat1Δ) was selected for all further studies.

The expression of FAT1 was evaluated in strain W303a grown in YPD containing 500 μM oleate. Total RNA was purified using the RNeasy kit as recommended by the supplier (Qiagen). Reverse transcriptase amplification was performed on 1 μg of total RNA using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase as described by the manufacturer (Boehringer-Mannheim). Controls received no Moloney murine leukemia virus reverse transcriptase. First-strand cDNA was amplified using primers derived from the sequences corresponding to the C termini of Fat1p or histone H4 (used as an internal control).

Fatty Acid Transport—Fatty acid transport was performed essentially as described by Knoll et al. (19). Cells (W303a and W303a-fat1Δ-1) were grown in SMM at 24 or 30 °C to midlog phase (A600 = 1.0), collected by centrifugation, washed once in phosphate-buffered saline (PBS), and resuspended in 1/10 of the original volume in PBS. 200 μl of cells (1 × 107 cells) were preincubated for 10 min at 24 or 30 °C in SMM, and the assay was initiated by the addition of [9,10-3H]myristate, [9,10-3H]palmitate, or [9–10-3H]oleate at the concentrations indicated. All fatty acids were prepared as ethanol stocks. At the defined time points, the reactions were terminated by the addition of 10 ml of ice-cold PBS. The cells were immediately filtered through a Whatman GFB filter, washed three times with ice-cold PBS, and air-dried. The amount of cell-associated radioactivity was determined by scintillation counting. Background counts were subtracted from the experimental samples. Evaluating the amount of radioactivity on control filters with no cells. The final data were expressed in nmol of cell-associated fatty acid/min/108 cells. All transport data were analyzed using EnzymeKinetics software (version 1.0.4; Trinity Software). The data presented represents the mean from at least three independent experiments.

Metabolic Labeling of Cellular Lipids—W303a and W303a-fat1Δ-1 cells were grown in SMM at 30 °C to midlog phase (A600 = 1.0). One ml of cells were transferred to a tube containing 50 μM [9,10-3H]oleate (specific activity of 1 Ci/mmol, 50 μCi/ml). At the time points indicated, 1 volume of 10% ice-cold trichloroacetic acid was added to stop fatty acid metabolism. The cells were immediately collected by centrifugation (15,000 × g) and resuspended in 100 mm Tris-HCl, pH 7.5. After neutralization, oleate and unsaturated fatty acids were recovered as described by Bligh and Dyer (33). Samples were analyzed using high performance thin layer chromatography as described by Knoll et al. (19).

Analysis of Acyl-CoA Synthetase Activities—Yeast cells (FAT1 and fat1Δ) as noted for transport, harvested by centrifugation, washed twice with 100 mM distilled water without cerulenin to a density of 1.2 × 105 cells/ml in 10 mM Tris-HCl, pH 7.5, and lysed by three cycles of sonication at 0 °C. Acyl-CoA synthetase activities were determined in sonicated cell extracts as described by Kameda and Nunn (34). The reaction mixtures contained 200 mM Tris-HCl, pH 7.5, 2.5 mM ATP, 8 mM MgCl2, 2 mM EDTA, 20 mM NaF, 0.1% Triton X-100, 10 μM [3H]oleate, [3H]palmitate, or [3H]myristate, 0.5 mM coenzyme A, and cell extract in a total volume...
Identification of FAT1—While the processes that mediate fatty acid uptake in yeast are not defined, it was reasonable to predict that a homologue of one or more of the three putative fatty acid transport proteins from higher eukaryotic cells may be required. We compared the sequence of FAT, mitochondrial aspartate aminotransferase (plasma membrane FABPpm), and FATP to open reading frames within the *Saccharomyces cerevisiae* data base. We were unable to find open reading frames within the yeast data base that had significant homology to FAT. While seven different reading frames on five different chromosomes were found to have homology to mitochondrial aspartate aminotransferase, it was difficult to assess which if any of these open reading frames represented the FABPpm homologue in yeast. Of these comparisons, the FATP identified in 3T3-L1 adipocytes appeared to be the most promising candidate. We identified an open reading frame on yeast chromosomal FATT homologue in yeast. Of these comparisons, the FATP identified in 3T3-L1 adipocytes appeared to be the most promising candidate. We identified an open reading frame on yeast chromosome II encoding a 623-amino acid protein with 33% sequence identity and 54% similarity to FATP (Fig. 1A). We have designated this open reading frame Fat1p and the structural gene encoding this protein as *FAT1*.

The sequence similarities and identities exist throughout the lengths of Fat1p and FATP and thus suggest they may have similar structures and functions. Three segments of these two proteins are nearly identical. The first segment, between amino acids 255 and 268 of Fat1p, contains a consensus sequence (LIYSGTTGLPK) common to members of the AMP-binding protein family including the family of acyl-CoA synthetases (36, 37). The second and third regions of high sequence similarity include amino acids 255 and 268 of Fat1p, containing a consensus sequence (LIYSGTTGLPK) common to members of the AMP-binding protein family including the family of acyl-CoA synthetases (36, 37). The second and third regions of high sequence similarity include amino acids 324–399 and 491–544, respectively, and are restricted to Fat1p and FATP. Using the algorithms of Kyte and Doolittle (38), we compared the hydropathy profiles of Fat1p and FATP (Fig. 1B). These analyses demonstrated that both proteins have comparable profiles and in particular predicted that Fat1p, like FATP, contains at least four potential membrane-spanning segments. Fat1p also has four potential N-linked glycosylation sites. One of these glycosylation sites (at amino acid residue 534) is identical to that predicted for FAT. On the basis of these comparisons, we propose that Fat1p represents the yeast homologue of the murine long-chain fatty acid transport protein FATP.

Examination of the DNA flanking the Fat1p coding sequence identified two potential TATA boxes. The first is found 301 bases upstream from the presumptive translational initiation codon, and the second is 119 bases upstream. Of the two, the first (TATATAA) is predicted to be a very strong binding site for TFIIID (39). There were also two potential polyadenylation sites, AATAAAG(CG)14–22 (64) and AATAAAN14–22CA (14–22 nucleotides without consensus features), found 286 and 294 bases 3′ relative to the presumptive translational termination codon. Examination of the 5′ upstream region indicated that expression of the gene may be controlled by at least three regulators. There are three potential CCAAT elements located beginning at -846, -882, and -982 and two potential overlapping glucose repression ele-
Diluted in yeast nitrogen base, and 2 CGGN15/18CCG. There were no elements of this type identified (43–45). Each contains the minimal consensus sequence for UASINO, which is CATGTGAAAT (41). These elements are found in many genes whose product is required for phospholipid and fatty acid biosynthesis. Several genes encoding yeast peroxisomal proteins and the 9α-acyl-CoA desaturase are activated or repressed, respectively, after growth in media containing oleate (42). Several laboratories have identified DNA elements important for response to oleate growth in media containing oleate (42). Several laboratories identified four regions upstream of FATT1 at −369, −520, −727, and −835, which conform to this consensus sequence. It is not known at this time whether any of these DNA segments contribute to the expression or regulation of FATT1.

Disruption and Expression of the Gene Encoding Fat1p in S. cerevisiae—On the basis of the sequence similarities noted above, we predicted that Fat1p, like FATP, was involved in the uptake of long-chain fatty acids. To determine the function of Fat1p in this process, we disrupted FATT1 in S. cerevisiae by the replacement of a 480-bp internal EcoRI fragment (within the open reading frame of FATT1) with a 1.346-kilobase pair fragment encoding the yeast HIS3 gene. This construction was crossed onto the chromosome in the yeast strain W303a (his3). We identified four regions upstream of FATT1 at −369, −520, −727, and −835, which conform to this consensus sequence. It is not known at this time whether any of these DNA segments contribute to the expression or regulation of FATT1.

Disruption of FATT1 Impairs Growth on YPD Containing Cerulenin and Oleate—We initially tested whether the fat1Δ strain was phenotypically distinct from the parental FATT1 strain. Fat1p and fat1Δ cells were grown to midlog phase and diluted in yeast nitrogen base, and 2 × 10^6 cells were plated on cerulenin-containing media supplemented with oleate, palmitate, or myristate. The cultures were incubated at 24, 30, and 37 °C for 72 h. As predicted, both strains were unable to grow on YPD plates containing 25 μM cerulenin (YPD/CER) due to the inhibition of fatty acid synthesis. Growth of the FATT1 strain could be rescued at all temperatures by the addition of 500 μM myristate, palmitate, or oleate to the YPD/CER plates (YPD/CER/MYR, YPD/CER/PAL, or YPD/CER/OLE, respectively). The fat1Δ strain was viable on both YPD/CER/MYR and YPD/CER/PAL, although there was an apparent decrease in growth when compared with the wild type. The growth of the fat1Δ strain on YPD/CER/OLE was reduced dramatically when grown at 24, 30, and 37 °C when compared with the wild type.

To evaluate these observations further, growth of the FATT1 and fat1Δ strains was monitored at 30 °C in liquid YPD with and without cerulenin and fatty acid (Fig. 2). These data were in agreement with the phenotypic data noted above. The growth rate of the FATT1 strain while depressed with the addition of cerulenin was able to be rescued by the addition of fatty acid (myristate, palmitate, or oleate). The growth of the fat1Δ strain was reduced even when supplemented with fatty acid when compared with the isogenic FATT1 strain. This reduction was particularly notable with the oleate supplementation, which paralleled the observations made on YPD agar plates containing oleate and cerulenin.

Use of the Fluorescent Fatty Acid BODIPY-3823 to Monitor Fatty Acid Uptake—To visualize in a more direct way that fatty acid uptake was reduced in the fat1Δ strain of S. cerevisiae, we evaluated the uptake of a fluorophore-labeled long-chain fatty acid analog BODIPY-3823, employing confocal laser scanning microscopy. Pagano et al. (46) demonstrated that the spectral properties of several BODIPY-labeled ceramide analogues are highly dependent upon the concentration of the probe in lipid vesicles as characterized by a shift in the emission maximum from green (515 nm) to red (620 nm) with increasing concentrations. Typical confocal scanning micrographs of FATT1 and fat1Δ cells labeled with BODIPY-3823 are shown in Fig. 3. When the cells were observed in the green channel only, the wild-type strain appeared highly fluorescent, whereas the disrupted strain showed limited fluorescent labeling (Fig. 3, A and D, respectively). When the same cells were viewed using the red channel (Fig. 3, B and E), the difference between the FATT1 and fat1Δ strains was even more dramatic. When labeled cells were viewed in the red and green channels (>520 nm), wild-type cells were yellow-orange in color, in contrast to the fat1Δ cells, which appeared pale yellow-green (Fig. 3, C and F). These results are consistent with the conclusion that a disruption of FATT1 reduces the cell’s ability to take up the fluorescent long-chain fatty acid BODIPY-3823.

Incorporation of Exogenous Fatty Acids into Phospholipids in the fat1Δ Strain—To distinguish fatty acid transport from metabolic utilization, we evaluated the distribution of fatty acids when supplied exogenously to the intracellular fatty acid and phospholipid pools. We predicted that overall rate of uptake
and incorporation of exogenous long-chain fatty acids into the phospholipid pool in the fat1Δ strain would be reduced while the distribution of incorporated long-chain fatty acids among the phospholipid classes would be the same as the wild-type. To test these predictions, we monitored the time-dependent incorporation of exogenous [3H]oleate into cellular lipids using one-dimensional high performance TLC as detailed under "Experimental Procedures." The initial uptake and incorporation of [3H]oleate into total lipids was notably reduced in the fat1Δ strain (Fig. 4). Furthermore, the level of free fatty acid was markedly decreased when compared with the wild-type (Fig. 4A). At the later time points, the differences in incorporation of exogenous oleate were still striking. We noted that while oleate incorporation was reduced in the mutant, the pattern of incorporation into the various classes of phospholipids remained the same, arguing that the enzymatic machinery required for lipid biosynthesis was still intact. These data imply that Fat1p must necessarily operate prior to the incorporation of exogenous oleate into the phospholipid pool. We interpret these data to suggest a defect in the uptake of oleate prior to metabolic utilization.

As noted above, Fat1p shares amino acid sequence similarities with the acyl-CoA synthetases; we therefore evaluated acyl-CoA synthetase profiles in the FAT1 and fat1Δ strains using oleate, palmitate, and myristate as substrates (Table I). Acyl-CoA synthetase activities using all three fatty acid substrates were comparable, although the fat1Δ strain had higher levels of oleyl-CoA synthetase activity. On the basis of these data, we conclude that the observed decrease in the uptake of BODIPY-3823 and the incorporation of exogenous oleate into the phospholipid pool observed for the mutant strain were not the consequence of decreased acyl-CoA synthetase activity.

**Fatty Acid Transport Profiles in the fat1Δ Strain**—The transport of fatty acid (oleate, palmitate, and myristate) was evaluated in FAT1 and fat1Δ cells following growth in SMM. The data gleaned from these types of assays must be evaluated with caution, since they measure both fatty acid transport and subsequent metabolic utilization. As transport precedes utilization, we routinely measured levels of cell-associated fatty acid for 90 s following the initiation of the reaction and thus interpret the data in terms of uptake. We found that the uptake of oleate was linear for the first 90 s although reduced in the fat1Δ strain when compared with the wild type. Fig. 5 illustrates the substrate-dependent uptake of oleate at 30 and 24 °C in both the FAT1 and fat1Δ strains. Using the program EnzymeKinetics (Trinity Software), the calculated maximal transport rates
data indicated that the growth rates on the fat1Δ strain on YPD/CER with palmitate and myristate were also reduced when compared with the isogenic FAT1 parental strain. We therefore tested the rates of uptake in the FAT1 and fat1Δ strains using 100 μM palmitate or myristate as substrate (Table II). These data demonstrated that the levels of palmitate and myristate uptake were also decreased in the fat1Δ strain when compared with the wild type (20% and 47% decrease, respectively). Although these data demonstrate that fatty acid uptake is compromised in the fat1Δ strain, it is clear that an additional transport component remains operational.

**DISCUSSION**

The present work describes the identification of the fatty acid transport protein Fat1p in the yeast *S. cerevisiae*. Disruption of the FAT1 structural gene results in a marked decrease in 1) cell growth on YPD containing oleate, palmitate, or myristate and cerulenin, 2) the uptake of the fluorescent long-chain fatty acid BODIPY-3823, 3) the uptake and incorporation of exogenous oleate into the phospholipid pool, and 4) the rates of long-chain fatty acid uptake. We hypothesize that Fat1p represents the yeast homologue of the murine fatty acid transport protein FATP.

Fat1p was identified on the basis of amino acid similarity to the murine fatty acid transport protein FATP and is encoded within a structural gene located on chromosome II. Fat1p and FATP are remarkably similar proteins in that they 1) are of comparable length (623 and 646 amino acid residues, respectively) and calculated molecular mass (71,700 and 71,200 daltons, respectively), 2) have comparable calculated isoelectric points (8.14 and 8.32, respectively), and 3) share 54% amino acid sequence similarity and 33% amino acid sequence identity. It is predicted using the algorithms of Kyte and Doolittle (38) that each protein has four potential membrane-spanning segments. The hydropathy profiles of Fat1p and FATP are remarkably alike, suggesting these two proteins span the membrane in similar ways. Fat1p has four potential glycosylation sites, while FATP has three. Both proteins are members of the family of AMP-binding proteins on the basis of sequences conserved in adenylate-forming enzymes. In addition to Fat1p and FATP, this family of enzymes includes the CoA synthetases from mammals, yeast, and bacteria. On the basis of these conserved sequences, it is tempting to postulate that fatty acid transport proteins FATP and Fat1p and the acyl-CoA synthetases from mammals, yeast, and bacteria.

In the present study, a deletion in the FAT1 gene has been generated to evaluate the role of Fat1p in the uptake and metabolism of fatty acids. Strains carrying a deletion of FAT1 are phenotypically asymptomatic unless the cells are grown on cerulenin, thereby blocking fatty acid biosynthesis. Under these conditions, wild-type cells overcome growth inhibition by supplementation with long-chain fatty acids. However, the fat1Δ strain was unable to be rescued by fatty acid supplementation. These results indicate that deletion of FAT1 causes a block in one of the steps in fatty acid utilization including fatty acid transport, activation by acyl-CoA synthetase, or synthesis...
Fatty acid transport in yeast is a complex process that contains both protein-mediated and diffusion components. The protein-mediated component appears to be operational at low or physiological levels of long-chain fatty acids and thus must play a role in governing the accessibility of exogenous long-chain fatty acids for metabolic utilization. The murine fatty acid transporter, FATP, is induced during adipogenesis and, in addition to fat cells, is expressed at high levels in cardiac and skeletal muscle (14). In this regard, FATP plays a pivotal role in regulating available long-chain fatty acid substrates from exogenous sources in tissues undergoing high levels of β-oxidation or triglyceride synthesis. The identification of Fat1p in S. cerevisiae as the homologue to the murine FATP is especially significant, since this model system will allow us to specifically address the mechanism of long-chain fatty acid transport across the plasma membrane in eukaryotic cells. Like FATP, Fat1p functions to mediate the uptake of exogenous long-chain fatty acids and thus may play a pivotal role in regulating accessibility of these hydrophobic compounds prior to metabolic utilization. We hypothesize that Fat1p acts to facilitate long-chain fatty acid transport in S. cerevisiae by a saturable, high-affinity process. It is clear that a second mechanism for the uptake of exogenous long-chain fatty acids remains operative in fat1Δ derivatives. The efficiency of uptake using this alternative pathway is severely compromised. We suggest that, under physiological conditions, Fat1p functions primarily when long-chain fatty acids are limiting and required for growth to facilitate the efficient uptake of these hydrophobic compounds into the cell.

The connection between the uptake and metabolism of long-chain fatty acids in eukaryotic cells is largely unresolved. However, a murine isoform of acyl-CoA synthetase, when expressed in COS7 cells, increases the rate of oleate uptake, suggesting a role for this enzyme in the transport of long-chain fatty acids (14). In E. coli, the acyl-CoA synthetase must necessarily operate in conjunction with the outer membrane-bound long-chain fatty acid transport protein FadL to mediate the efficient uptake of long-chain fatty acids across the bacterial cell envelope (18, 47, 48). In the context of transport, the activity of acyl-CoA synthetase of E. coli is described as “vectorial acylation” (49). Our present data are consistent with the postulate that Fat1p of S. cerevisiae acts to facilitate the uptake of exogenous long-chain fatty acids and is distinct from the acyl-CoA synthetases. The activity of Fat1p must result in an increase in the intracellular pool of long-chain fatty acids. The cell, in turn, may respond to this increased pool by activating these compounds to long-chain acyl-CoA thioesters via acyl-CoA synthetase.

In the context of yeast physiology, Fat1p may play a significant role in the uptake of exogenous unsaturated long-chain fatty acids under anaerobic conditions or conditions were oxygen is limiting. Under these conditions, the desaturase involved in the synthesis of unsaturated fatty acids is dysfunctional, resulting in the formation of unsaturated long-chain fatty acids. While the present study has not evaluated the requirement of Fat1p under anaerobic conditions, the use of cerulenin to block fatty acid biosynthesis coupled with long-chain fatty acid rescue provides compelling evidence suggesting an important role for Fat1p in the uptake of these hydrophobic compounds under conditions where fatty acid synthesis is compromised. This work represents a valuable foundation to further explore the problem of long-chain fatty acid transport in eukaryotic cells. The use of S. cerevisiae as a model system is of great utility as transport can be evaluated at the genetic level in addition to the biochemical and physiological levels.

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TABLE II
Fatty acid transport profiles at 30 °C of FAT1 and fat1Δ strains using 1H]oleate (C16:0), 1H]palmitate (C16:0), and 1H]myristate as substrates

| Strain | Cell-associated fatty acid | C14:0 | C16:0 | C18:1 |
|--------|---------------------------|-------|-------|-------|
| FAT1   |                           | 2.99 ± 0.05 | 3.11 ± 0.12 | 7.56 ± 1.03 |
| fat1Δ  |                           | 1.59 ± 0.46 | 2.47 ± 0.18 | 5.33 ± 1.01 |

* S.E. is from three independent experiments.
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