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Vectorial Acylation in *Saccharomyces cerevisiae*

Fat1p AND FATTY ACYL-CoA SYNTHETASE ARE INTERACTING COMPONENTS OF A FATTY ACID IMPORT COMPLEX*

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In *Saccharomyces cerevisiae* Fat1p and fatty acyl-CoA synthetase (FACS) are hypothesized to couple import and activation of exogenous fatty acids by a process called vectorial acylation. Molecular genetic and biochemical studies were used to define further the functional and physical interactions between these proteins. Multicopy extragenic suppressors were selected in strains carrying deletions in *FAA1* and *FAA4* or *FAA1* and *FAT1*. Each strain is unable to grow under synthetic lethal conditions when exogenous long-chain fatty acids are required, and neither strain accumulates the fluorescent long-chain fatty acid C_1-BODIPY-C_{12} indicating a fatty acid transport defect. By using these phenotypes as selective screens, plasmids were identified encoding *FAA1*, *FAT1*, and *FAA4* in the *faa1*Δ *faa4*Δ strain and encoding *FAA1* and *FAT1* in the *faa1*Δ *fat1*Δ strain. Multicopy *FAA4* could not suppress the growth defect in the *faa1*Δ *fat1*Δ strain indicating some essential functions of Fat1p cannot be performed by Faa4p. Chromosomally encoded *FAA1* and *FAT1* are not able to suppress the growth deficiencies of the *fat1*Δ *faa1*Δ and *faa1*Δ *faa4*Δ strains, respectively, indicating Faa1p and Fat1p play distinct roles in the fatty acid import process. When expressed from a 2μ plasmid, Fat1p contributes significant oleoyl-CoA synthetase activity, which indicates vectorial esterification and metabolic trapping are the driving forces behind import. Evidence of a physical interaction between Fat1p and FACS was provided using three independent biochemical approaches. First, a C-terminal peptide of Fat1p deficient in fatty acid transport exerted a dominant negative effect against long-chain acyl-CoA synthetase activity. Second, protein fusions employing Faa1p as bait and portions of Fat1p as trap were active when tested using the yeast two-hybrid system. Third, co-expressed, differentially tagged Fat1p and Faa1p or Faa4p were co-immunoprecipitated. Collectively, these data support the hypothesis that fatty acid import by vectorial acylation in yeast requires a multiprotein complex, which consists of Fat1p and Faa1p or Faa4p.

Biological membranes are complex in both their protein and lipid compositions. This complexity is essential and contributes to the barrier function of the membrane and to selectively regulated transport of molecules into and out of the cell. Unlike hydrophilic molecules such as sugars and amino acids, hydrophobic fatty acids are able to dissolve in the membrane, and as a consequence, the processes governing their regulated movement across membranes are likely to be quite distinct. Recent investigations into the problem of fatty acid transport have intensified due to findings that exogenous fatty acids influence a number of important cellular functions, including signal transduction and transcriptional control. To date, several distinct membrane-bound and membrane-associated proteins have been identified as components of fatty acid import systems in eukaryotic cells. Most notable among these are fatty acid translocase (FAT,[1] the murine homologue to CD36) (1, 2), fatty acid transport protein (FATP) (3), and fatty acyl-CoA synthetase (3–6). FAT was identified following protein modification using sulfo-*N*-succinimidyl oleate (7), whereas FATP and fatty acyl-CoA synthetase were both identified using expression cloning (3). Both FAT and FATP have been claimed to be fatty acid transport proteins (1, 8, 9). Despite these claims, there is controversy surrounding the classification of FAT/CD36 and FATP as *bona fide* integral membrane-bound fatty acid transporters (10). Indeed, there are gnawing questions as to whether these proteins actually function as components of a fatty acid delivery system (*i.e.* FAT/DC36) or as components of a utilization driven fatty acid import system (*i.e.* FATP), which also includes fatty acyl-CoA synthetase (2, 4, 8, 10, 11). In this regard, proteins identified as required for fatty acid transport may function not as transport proteins *per se* but in an alternative manner, perhaps by promoting selectivity and specificity of fatty acid delivery to downstream metabolic events.

The best characterized fatty acid transport system is that found in *Escherichia coli* (4). In this case, the specific integral outer membrane protein, FadL, is required for long-chain fatty acid binding and transport across that membrane. The fatty acid ligands must then traverse the bacterial periplasmic space and the inner membrane. No inner membrane proteins have been identified that are required for this process. On the basis of studies defining the energetics of fatty acid transport, we suggested protonated fatty acids flip across the inner membrane and are subsequently abstracted from the inner membrane concomitant with activation by fatty acyl-CoA synthetase (12). In this manner, exogenous fatty acids are metabolically trapped as CoA thioesters upon transport, which

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[1] The abbreviations used are: FAT, fatty acid translocase; FATP, fatty acid transport protein; PBS, phosphate-buffered saline; C_1-BODIPY-C_{12}, 4,4-difluoro-5-methyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-dodecanoic acid.

TABLE I
Yeast strains used in this study

Name	Relevant genotype	Complete genotype (Ref.)
YB332	Wild type	*MATa ura3–52 his3Δ200 ade2–101 lys2–801 leu2–3,112* (30)
YB497	*faa1Δ*	*MATa ura3–52 his3Δ200 ade2–101 lys2–801 leu2–3,112 faa1Δ::HIS3* (30)
YB524	*faa4Δ*	*MATa ura3–52 his3Δ200 ade2–101 lys2–801 leu2–3,112 faa4Δ:LYS2* (30)
LS2020	*fat1Δ*	*MATa ura3–52 his3Δ200 ade2–101 lys2–801 leu2–3,112 fat1Δ::G418* (this study)
YB525	*faa1Δfaa4Δ*	*MATa ura3–52 his3Δ200 ade2–101 lys2–801 leu2–3,112 faa1Δ::HIS3 faa4Δ:LYS2* (31)
LS2086	*faa1Δfat1Δ*	*MATa ura3–52 his3Δ200 ade2–101 lys2–801 leu2–3,112 faa1Δ::HIS3 fat1Δ::G418* (this study)
LS2087	*faa4Δfat1Δ*	*MATa ura3–52 his3Δ200 ade2–101 lys2–801 leu2–3,112 faa4Δ:LYS2 fat1Δ::G418* (this study)
LS2089	*faa1Δfaa4Δfat1Δ*	*MATa ura3–52 his3Δ200 ade2–101 lys2–801 leu2–3,112 faa1Δ::HIS3 faa4::LYS2 fat1Δ::G418* (this study)

in turn generates a concentration gradient further driving the system. Overath and colleagues (5) coined the term "vectorial acylation" to describe this process at the time they identified the structural gene for the *E. coli* fatty acyl-CoA synthetase (*fadD*). This postulate was initially expanded by Frerman and Bennett (6) and subsequently by our laboratory (4, 12) as the underlying mechanism driving long-chain fatty acid transport in bacteria. Although at the time the model of vectorial acylation was proposed the bacterial fatty acid transporter FadL had not been identified, our subsequent studies have clearly shown that both FadL and fatty acyl-CoA synthetase are required for fatty acid transport in *E. coli*.

By using the yeast *Saccharomyces cerevisiae* as a model eukaryotic system, we have recently shown the fatty acyl-CoA synthetases Faa1p or Faa4p function in the fatty acid transport system presumably by activating exogenous fatty acids concomitant with transport (11). This finding presents somewhat of a conundrum as we have also shown that long-chain fatty acid import in yeast requires Fat1p, the yeast orthologue of the murine FATP1 (13). One of the central questions we are now faced with is to determine the mechanisms by which Fat1p and fatty acyl-CoA synthetase (Faa1p and/or Faa4p) work in concert to promote fatty acid import. A similar situation appears to be operational in murine adipocytes, where there are data supporting a functional association of mmFATP1 with fatty acyl-CoA synthetase (3, 15). We suggest vectorial acylation is one general mechanism of fatty acid import, which functions to promote the regulated import and metabolic trapping of exogenous long-chain fatty acids.

In our prior investigations into fatty acid import in yeast, we used reverse genetic approaches to demonstrate this process requires the yeast orthologue of mmFATP (Fat1p) and fatty acyl-CoA synthetase (Faa1p or Faa4p) (11, 13, 14). Despite the information gleaned from these studies, there are no data demonstrating these proteins function cooperatively in a physical complex, and there is no information as to whether there are additional proteins involved in mediating the regulated import of exogenous long-chain fatty acids. In the present work, we sought to identify additional components required for fatty acid transport and to confirm the importance of Fat1p and fatty acyl-CoA synthetase (Faa1p and Faa4p) by using a genetic approach. A valuable molecular-genetic method for the identification of participants in multicomponent cellular processes is the selection of plasmid-encoded multicopy extragenic suppressors (16). The rationale behind this approach is that the altered phenotype resulting from a deficiency in one participant can be suppressed by overexpression of another participant required for the same process (16). In this manner, we sought to identify plasmid-encoded multicopy extragenic suppressors of the deficiency in fatty acid import caused by deletion of *FAT1* and/or *FAA1* and *FAA4*. We report that plasmids encoding Fat1p, Faa1p, and Faa4p were identified in a screen for multicopy extragenic suppressors of the transport and activation deficiency of a *faa1Δ faa4Δ* strain, and plasmids encoding only Fat1p and Faa1p were identified as multicopy extragenic suppressors of the transport deficiency of a *faa1Δ fat1Δ* strain. Additional biochemical evidence is provided demonstrating Fat1p and acyl-CoA synthetase interact in a physical complex. This work establishes for the first time a genetic, physical, and functional linkage between Fat1p and fatty acyl-CoA synthetase and substantiates the hypothesis that these proteins, perhaps exclusively, are required for long-chain fatty acid transport in yeast.

EXPERIMENTAL PROCEDURES

Strains, Media, and Materials—The *S. cerevisiae* strains used in this study are listed in Table I. The *fat1Δ*::G418 mutation was introduced by transformation of the strain of interest with linear DNA generated by amplification of the kanamycin resistance cassette (resulting in G418 resistance) using oligonucleotides complementary to both *FAT1* and the cassette as described (17). The oligonucleotide for the coding strand was 5′-CACTGTCAAGAAGGGCAAGAAGGCAGCAGTATGGCTTGGGCATAGGCCACTAGTGGATCTG-3′, and the oligonucleotide for the template strand was 5′-CCACTGGATCATTCGTAAGTGATCCTGAAACAAACCATTCAGCAGCTGAAGCTTCGTACGC-3′. Chromosomal replacement of the native gene was confirmed by Southern analysis of chromosomal DNA from the transformants by comparison to DNA obtained from the parental strain. Yeast strains were transformed by the lithium acetate method (18).

YPDA consisted of 1% yeast extract, 2% peptone, 2% dextrose, and 20 mg/liter adenine hemisulfate. Yeast-supplemented minimal media contained 0.67% yeast nitrogen base (YNB), 2% dextrose, adenine (20 mg/liter), uracil (20 mg/liter), and amino acids as required (arginine, tryptophan, methionine, histidine, and tyrosine (20 mg/liter); lysine (30 mg/liter); and leucine (100 mg/liter)). To assess growth when fatty-acid synthase was inhibited, cells were grown on YNBD or YPDA plates supplemented with 45 μM cerulenin and 100 μM oleic acid unless otherwise indicated. Growth in liquid culture and on plates was at 30 °C.

Yeast extract, yeast peptone, and yeast nitrogen base were obtained from Difco. Oleic acid was obtained from Sigma. ^{3}H- or ^{14}C-labeled fatty acids were from PerkinElmer Life Sciences and American Radiochemicals. C_1-BODIPY-C_{12} was purchased from Molecular Probes. Enzymes required for all DNA manipulations were from Promega, Invitrogen, New England Biolabs, U. S. Biochemical Corp., or Roche Molecular Biochemicals. Anti-V5 antibody and anti-T7 antibodies were purchased from Invitrogen and Novagen, respectively. Anti-Pma1p was the gift of Dr. Günther Daum (Technische Universität Graz, Graz, Austria).

Complementation of faa1Δ faa4Δ and faa1Δ fat1Δ Using Multicopy Extragenic Suppression—Cells of the *faa1Δ faa4Δ* strain or *faa1Δ fat1Δ* strain were rendered competent using lithium acetate as noted above, transformed with a yeast multicopy library in YEp24, and transformants selected on YNBD containing the appropriate supplements but lacking uracil (19). Thirty thousand individual Ura$^+$ transformants were selected from the library and were screened for growth following replica plating on YPD plates containing 45 μM cerulenin and 100 μM oleic acid (YPD-CER-OLE). Transformants that were able to grow on YPD-CER-OLE were colony-purified on the same media and phenotypes validated on YPD-CER-OLE. Plasmids were isolated from those that retained positive growth on all three media and retransformed into the *faa1Δ faa4Δ* and *faa1Δ fat1Δ* strains. Additionally, the same plasmids were propagated in the *E. coli* strain DH5α, purified using QiaPrep columns (Qiagen), and sequenced using two plasmid-specific primers flanking the insert (upstream, 5′-GGAGCCACTATCGACTACGC-3′; downstream, 5′-CCTGTGGCGCCGGTGATG-3′) using an Applied Biosystems automated fluorescence DNA sequencer. The sequences obtained were compared with the *Saccharomyces* genome data base for identification.

Assessment of Fatty Acid Import Capacity—Fatty acid import was

assessed using confocal laser scanning microscopy to detect accumulation of the fluorescent long-chain fatty acid analogue 4,4-difluoro-5-methyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-dodecanoic acid (C_1-BODIPY-C_{12}) as described previously (13). Following growth under selective conditions, cells were harvested, washed with phosphate-buffered saline (PBS) and resuspended in 0.1 volume of PBS. All steps were performed at room temperature. Washed cells were incubated with 10 μM C_1-BODIPY-C_{12} for 60 s, washed with PBS containing 50 μM fatty acid-free bovine serum albumin (two times), PBS, resuspended in PBS, and visualized on an NORAN-OZ confocal laser scanning microscopy, interfaced with a Nikon Diaphot 200 inverted microscope equipped with a PlanApo ×60, 1.4 NA oil-immersion objective lens. The instrument settings for brightness, contrast, laser power, and slit size were optimized for the brightest sample to ensure that the confocal laser scanning microscopy was set for its full dynamic range. The same settings were used for all subsequent image collections.

Quantification of Fatty Acyl-CoA Synthetase Activities—Cells were grown from overnight cultures in YNBD (with appropriate supplements) and grown to A_{600} of 1.0. Following growth, cells were harvested by centrifugation, washed twice with PBS, and resuspended to a density of 1.2×10^9 cells/ml in 200 mM Tris-HCl, pH 8.0, 4 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol, 0.01% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM pepstatin A, and 8 μM leupeptin. The cells were lysed by vigorously vortexing the cell suspension containing glass beads for 1 min, 5 times at 0 °C. Samples were clarified by centrifugation (1,500 × *g*, 5 min, 4 °C), and supernatants were used to assess fatty acyl-CoA synthetase activities as described (20). The reaction mixtures contained 200 mM Tris-HCl, pH 7.5, 2.5 mM ATP, 8 mM $MgCl_2$, 2 mM EDTA, 20 mM NaF, 0.01% Triton X-100, fatty acid dissolved in 10 mg/ml α-cyclodextrin (final concentrations of fatty acids were 50 μM), 0.5 mM coenzyme A, and cell extract in a total volume of 0.5 ml. The reactions were initiated by the addition of coenzyme A, incubated at 30 °C for 20 min, and terminated by the addition of 2.5 ml of isopropyl alcohol, *n*-heptane, 1 M H_2SO_4 (40:10:1). The radioactive fatty acid was removed by organic extraction using *n*-heptane. Acyl-CoA formed during the reaction remained in the aqueous fraction and was quantified by scintillation counting. Protein concentrations in the cell extracts were determined using the Bradford assay and bovine serum albumin as a standard (21). The values presented represent the average from at least three independent experiments performed in duplicate. All experiments were subjected to analysis of variance (StatView, SAS Institute, Inc.).

Negative Dominance of Mutant Fat1p Over Fatty Acyl-CoA Synthetase—The sequence encoding the C-terminal 125 amino acids (residues 545–669) of Fat1p was cloned in-frame to the T7 epitope tag of the yeast expression vector YEpGALSET983 to generate YEpDB213. The resulting ^{T7}Fat1p^{125C} fusion was expressed under the control of the *GAL10* promoter. To test for negative dominance, YEpDB213 was transformed into YB332. Cells transformed with the vector (YEpGALSET983) served as a control. The cells were pre-grown in YNBD (without leucine) overnight. The culture was harvested by centrifugation and resuspended to a cell density of 0.1 A_{600} in 50 ml of YNB containing 2% galactose and 2% raffinose to induce expression of ^{T7}Fat1p^{125C}. When the density reached 1.0 A_{600}, cells were harvested by centrifugation, washed once in PBS, and resuspended in 1 ml of breaking buffer (200 mM Tris, pH 8.0, 4 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol, 0.01% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM pepstatin A, and 8 μM leupeptin). The cells were lysed by vortexing with glass beads and assayed for long-chain acyl-CoA synthetase activity as detailed above.

Two-hybrid Analysis of Fat1p and Faa1p—The yeast two-hybrid system was used to test Faa1p-Fat1p interaction (22). The bait plasmid vector used was pEG202; the trap plasmid vector was pJG4-5, and the reporter plasmid was pSH18-34T. To generate the full-length Faa1p-bait fusion protein, the coding sequence of *FAA1* was amplified using the upstream primer 5′-AGACCCATGGATGGTTGCTCAATATACCG-3′ and the downstream primer 5′-AAATGTTGGCGGCCGCAGACGAACTATAAACGGC-3′. The amplified DNA fragment was cleaved with *Nco*I and *Not*I and ligated into pEG202 cleaved with the same enzymes. For the trap plasmids, a single primer was used to amplify DNA at the 3′ end of the gene including the termination codon encoding amino acid 669, 5′-GAACATCCTCGAGTAATTTAATTGTTTGTGC-3′, whereas unique primers were used to amplify DNA at the 5′ ends. These included 5′-TTTTTAGCGCGCAATACTAAAGGCACTCCG-3′ to generate a peptide from amino acids 169 to 669 of Fat1p (Fat1p^{500C}) and 5′-GAAGATGAATTCACGGCCAGTAACAAAGAAC-3′ to generate a peptide from amino acids 544 to 669 of Fat1p (Fat1p^{125C}). The amplified DNA fragments were digested with the appropriate restriction enzymes and ligated into pJG4-5.

To test interaction, the Faa1p bait plasmid and the target trap plasmid of interest were transformed into yeast strain W303B carrying the reporter plasmid pSH18-34T. The reporter plasmid pSH18-34T contains the *lacZ* gene encoding β–galactosidase driven by a promoter controlled by eight LexA operators. To maintain each plasmid, transformants were selected and maintained on YNBD media lacking uracil (for pSH18-34T), histidine (for the pEG202-derived bait), and tryptophan (for the pJG4-5-derived traps). Expression of β-galactosidase activity was measured using the liquid assay employing *o*-nitrophenyl β-D-galactopyranoside as substrate as described previously (23). For these experiments, cells were grown overnight in YNBD (without uracil, histidine, or tryptophan) and subcultured to A_{600} 0.02–0.1 in 10 ml of YNB containing 2% galactose and 2% raffinose. Growth was continued until the A_{600} reached 0.5–1.0, at which time was stopped by placing the cultures on ice. Aliquots of cells (1 ml) were harvested by centrifugation (14,000 rpm for 3 min). The cell pellets were resuspended in 200 μl of 0.1 M Tris, pH 7.5, containing 0.05% Triton X-100. The sample was frozen on dry ice and stored at −80 °C prior to assay. All experiments defining β-galactosidase activities were performed in duplicate at least five times as described previously (23); the data were analyzed using paired *t*-tests against cells containing the bait (Faa1p), the trap vector (pJG4-5), and the reporter (StatView, SAS Institute, Inc.).

Co-immunoprecipitation of Fat1p and Faa1p or Faa4p—To identify a protein complex containing Fat1p and Faa1p or Faa4p, plasmids were constructed expressing each protein fused to a peptide tag, which is recognized by a commercially available antibody. Full-length Fat1p tagged with a T7 epitope was constructed in the vector YEpGALSET983 to generate plasmid YEpDB204. The coding sequence of *FAT1* was amplified using the upstream primer 5′-GCGGAGCTCATGTCTCCCATACAGGTTGTTG-3′ and the downstream primer 5′-CGCGGTACCATGCTCTAATGGAAAGGTAC-3′. The amplified DNA fragment was cleaved with *Sac*I and *Kpn*I and ligated into YEpGALSET983 cleaved with the same restriction enzymes. Expression clones encoding full-length Faa1p or Faa4p tagged at the C terminus with a V5 epitope were obtained from Invitrogen (GeneStorm™ clones pYES2/YOR317w and pYES2/YMR246w, respectively).

The plasmid pair encoding the proteins of interest (*e.g.* ^{T7}Fat1p and ^{V5}Faa1p or ^{V5}Faa4p) was transformed into the *fat1*Δ *faa1*Δ strain to test Fat1p-Faa1p interaction or the *fat1*Δ *faa4*Δ strain to test Fat1p-Faa4p interaction. The cells were pre-grown in YNBD without leucine and uracil to maintain both plasmids; cells were subsequently subcultured to 0.1 A_{600} in 75 ml of YNB containing 2% galactose and 2% raffinose (without leucine and uracil) to induce expression of the epitope-tagged target proteins. When the cell density reached 1.0 A_{600}, the cells were harvested, washed once with PBS, and resuspended in 1.5 ml of lysis buffer containing 50 mM Tris, pH 7.5, and 150 mM NaCl. The cells were lysed by vortexing with glass beads on ice as detailed above. The glass beads were pelleted by centrifugation (2,000 rpm, 2 min, 4 °C). The supernatant was removed to a new tube, and Triton X-100 was added to a final concentration of 1%, and the mixture was incubated on ice for 45 min. The sample was clarified by centrifugation (4,000 rpm, 15 min, 4 °C). The resultant supernatant was split into three 0.5-ml aliquots (~0.7 mg/ml); 2 μg of anti-T7 or 2 μg of anti-V5 antibodies was added to the first two, and an equal volume of lysis buffer was added to the third as a control (protein A-Sepharose bead control). The samples were incubated with gentle rotation overnight at 4 °C. Protein A-Sepharose beads (50 μl of 50% slurry) were added to each sample, which were then incubated for 2 h with gentle rotation at 4 °C. The protein A-Sepharose beads (containing the antigen-antibody complex) were pelleted by centrifugation (1,000 rpm, 1 min, 4 °C) and subsequently washed 5 times in 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100. The final pellets containing the protein A-Sepharose beads/antigen-antibody complex were resuspended in 70 μl of SDS sample buffer. Samples were boiled 5 min, and the proteins from 5 μl of the cell lysate or 15 μl of the immunoprecipitated sample were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred to nitrocellulose for immunoblotting. Tagged proteins were detected using the appropriate antibody (anti-V5, anti-T7, or anti-Pma1p) as detailed in the figure legends.

RESULTS

Identification of Multicopy Suppressors of Synthetic Lethality Imposed by Cerulenin on faa1Δ faa4Δ or faa1Δ fat1Δ Strains—When yeast cells are grown on media containing the fatty-acid synthase inhibitor cerulenin, they become auxotrophic for long-chain (C_{14}–C_{18}) fatty acids. Supplementation of the media with

TABLE II
Characterization of plasmids encoding multicopy suppressors of strains deficient in fatty acid import and long-chain acyl-CoA synthetase activity

Recipient strain	No. clones screened[a]	No. colonies selected[b]	ORF identified[c]	Percentage of total selected
faa1 Δ *faa4*Δ	30,000	161	*FAA1*	76
			FAT1	17
			FAA4	1
			Unknown	6
*faa1*Δ *fat1*Δ	40,000	90	*FAA1*	56
			FAT1	41
			FAA4	0
			Unknown	3

[a] Transformants were initially selected as uracil prototrophs upon transformation with the yeast YEp24 chromosomal DNA library (19).
[b] Uracil prototrophs were replica-plated to YNBD containing 45 μM cerulenin and 100 μM oleate.
[c] Open reading frames (ORF) were identified by restriction enzyme analysis and DNA sequencing.

100 μM oleate is sufficient to restore growth to wild-type strains. However, cells carrying deletions in *FAT1* or *FAA1* and *FAA4* are not viable on media containing cerulenin despite the addition of fatty acids. For *fat1*Δ strains, we have shown previously (13) this phenotype is due to a defect in the ability to import fatty acids and not due to depressed levels of long-chain fatty acyl-CoA synthetase activities. Strains carrying deletions in the genes encoding the fatty acyl-CoA synthetases Faa1p and Faa4p have a similar phenotype, which we hypothesize is due to a specific coupling between Fat1p-mediated fatty acid transport and Faa1p/Faa4p-mediated fatty acid activation (11). The esterification of the exogenous fatty acid to coenzyme A is required for all subsequent metabolic processes.

In an attempt to identify genes that could functionally replace *FAT1* or *FAA1* and *FAA4*, we screened a yeast genomic multicopy library for clones, which suppressed the cerulenin-induced lethality of an *faa1*Δ *faa4*Δ strain (deficient in long-chain fatty acyl-CoA synthetase activity) and an *faa1*Δ *fat1*Δ strain (deficient in fatty acid import and with reduced long-chain fatty acyl-CoA synthetase activity). Primary transformants were selected on YNBD plates lacking uracil and subsequently were replica-plated to YNBD plates containing cerulenin and oleate. Plasmids were isolated from colonies that grew on the selective media, and the individual plasmid encoded suppressors verified by retransformation. The identities of the inserts were determined by restriction enzyme analysis and by sequencing using plasmid-specific primers flanking the site of insertion. In both screens, multiple isolates of each plasmid-borne suppressor were identified indicating all possible suppressing clones available in this genomic library had been identified (Table II). As expected, because both strains carried a deletion in *FAA1,* most of the plasmids identified in either strain encoded the fatty acyl-CoA synthetase Faa1p. A surprising result was that *FAT1* was identified at high frequency, whereas *FAA4* was identified in only two cases in the screen using the *faa1*Δ *faa4*Δ strain. *FAA4* was not identified as a multicopy suppressor in the *faa1*Δ *fat1*Δ strain. Subsequent analyses of the *faa1*Δ *fat1*Δ strain transformed with a YEp24 plasmid derivative encoding Faa4p (YEpDB133) verified this fatty acyl-CoA synthetase could not substitute for *FAA1* and *FAT1* in this strain. Three plasmids identified as multicopy suppressors using these screens were chosen for further characterization. They were YEpDB02 encoding Faa1p, YEpDB133 encoding Faa4p, and YEpDB17 encoding Fat1p (Table II; Fig. 1).

We noted that several plasmids isolated from the colonies listed in the "other" category in Table II did not confer the suppressor phenotype upon re-transformation. Therefore, we presume the phenotype was associated with an undefined chromosomally encoded suppressor.

The Multicopy Suppressors Alleviate Fatty Acid Import Defects—In an effort to determine whether fatty acid import was restored by the plasmid-encoded suppressors, we monitored the accumulation of the fluorescent fatty acid analogue C_1-BODIPY-C_{12} using confocal laser scanning microscopy in wild-type strains and transformants of the *faa1*Δ *faa4*Δ and *faa1*Δ *fat1*Δ strains. Wild-type cells import C_1-BODIPY-C_{12} quickly (within 30 s) by a process that is essentially irreversible, which we suspect reflects metabolic activation (14). Previous work from our laboratory (13, 14) has shown a deletion within *FAT1* severely restricts C_1-BODIPY-C_{12} accumulation. Deletion of *FAA1* alone appears to decrease, but not completely eliminate, accumulation of C_1-BODIPY-C_{12}. Deletion of *FAA4* has essentially no effect. In contrast, when both *FAA1* and *FAA4* are deleted, the accumulation of C_1-BODIPY-C_{12} is restricted in a manner similar to that observed in *FAT1* mutants (11). As illustrated in Fig. 2, the accumulation of C_1-BODIPY-C_{12} was restored in the *faa1*Δ *faa4*Δ strain harboring *FAT1*, *FAA1,* or *FAA4* on a multicopy plasmid. These results point out that *FAT1* is a true multicopy suppressor. Only when expressed from a 2μ plasmid can *FAT1* compensate for deletions in *FAA1* and *FAA4*. These data support the notion that Fat1p and Faa1p or Faa4p form a functional network facilitating the import and activation of exogenous fatty acids, and in wild-type cells each functions in a distinct yet coordinate manner. It is important to note that *FAA4* on a multicopy plasmid (YEpDB133) did not restore in C_1-BODIPY-C_{12} accumulation in the *faa1*Δ *fat1*Δ strain, whereas both *FAA1* and *FAT1* did (Fig. 2). These results indicated that Faa1p, Faa4p, and Fat1p have overlapping, yet distinct roles. Of particular importance was the finding that Faa1p and Fat1p appeared to be functionally linked. Table III summarizes both the phenotypes and fatty acid transport profiles of the mutant strains alone and transformed with the selected multicopy suppressor plasmids. These findings are consistent with data obtained on the mmFATP1 and fatty acyl-CoA synthetase, which are proposed to form a functional complex (15).

*Deficiencies in Long-chain Acyl-CoA Synthetase Activity in faa1*Δ *faa4*Δ *and faa1*Δ *fat1*Δ *Strains Can Be Compensated by Multicopy FAT1*—The identification of *FAT1* as a multicopy suppressor in experiments using the *faa1*Δ *faa4*Δ and *faa1*Δ *fat1*Δ strains demonstrated that in high copy *FAT1* alone as well *FAA1* alone could compensate for the defects with regard to importing exogenous long-chain fatty acids. These data imply that under these conditions Fat1p contributed an enzymatic activity to promote the unidirectional transport of exogenous long-chain fatty acids. Previously, we have shown that deletion of *FAT1* does not reduce long-chain fatty acyl-CoA synthetase activities measured using whole cell extracts, whereas deletion of *FAA1* and *FAA4* reduced these activities ~95% (Table IV) (11, 13). Likewise, when *FAT1* is cloned into a centromeric plasmid (a pRS316 derivative designated pDB102) (14) and transformed into the *faa1*Δ *faa4*Δ strain, long-chain fatty acyl-CoA synthetase activities are not substan-

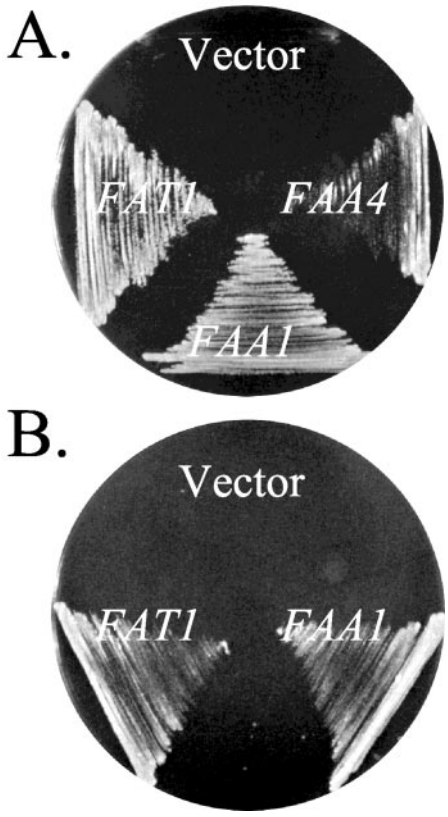

FIG. 1. ***FAA1, FAA4,* and *FAT1* are multicopy suppressors of the synthetic lethality of *faa1Δ faa4Δ* and *faa1Δ fat1Δ*.** Cells were streaked YNBD plates containing 100 μM oleate and 45 μM cerulenin, and the cultures were incubated for 48 h at 30 °C. The host strain was either YB525 (*faa1Δ faa4Δ*) (*A*) or LS2086 (*faa1Δ fat1Δ*) (*B*) carrying the vector, YEp24, or the plasmids encoding *FAA1* (YEpDB02), *FAA4* (YEpDB133), or *FAT1* (YEpDB17).

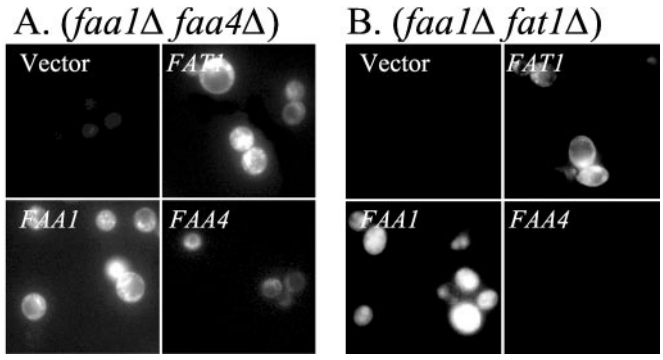

FIG. 2. **Fatty acid import in the *faa1Δ faa4Δ* strain (*A*) and *faa1Δ fat1Δ* (*B*) containing the indicated multicopy suppressor plasmids monitored by following the accumulation of the fluorescent long-chain fatty acid C_1-BODIPY-C_{12}.** Shown are the following: YEp24 (vector control), *FAA1* on plasmid YEpDB02, *FAA4* on plasmid YEpDB133, and *FAT1* on plasmid YEpDB17.

tially elevated, and no complementation was observed on YPD-CER-OLE plates (data not shown).

Our laboratory and others (14, 24) have shown that Fat1p has intrinsic very long-chain (C_{22}–C_{26}) fatty acyl-CoA synthetase activity. Indeed, when we first characterized the *FAT1* gene, we noted Fat1p shared similarities to the adenylate-forming family of enzymes, which includes the fatty acyl-CoA synthetases (13). We reasoned that when expressed from a high copy number plasmid, *FAT1* would result in sufficient long-chain fatty acyl-CoA synthetase activity to promote growth of the *faa1Δ faa4Δ* and *faa1Δ fat1Δ* strains under the synthetic lethal conditions used in this study. To test this idea, we measured fatty acyl-CoA synthetase activities in total cell extracts from the parental strain and strains harboring the multicopy suppressor plasmids using oleate ($C_{18:1}$) as a substrate (Table IV). Extracts prepared from the *faa1Δ faa4Δ* strain harboring YEpDB17 (encoding Fat1p) had ~4-fold higher oleoyl-CoA synthetase activity compared with the strain carrying the vector YEp24, which was 30% of the level obtained for the wild-type strain. This modest increase in oleoyl-CoA synthetase activity correlated with a 3-fold increase in protein level estimated using Western blot analysis of cellular extracts employing a Fat1p-specific antibody and analyzed using NIH Image analysis software. The same strain transformed with YEpDB02 and YEpDB133 (encoding Faa1p and Faa4p, respectively) had 10- and 2-fold oleoyl-CoA synthetase activities, respectively, compared with the same control cells. It is unclear why increased dosage of *FAA4* had such a limited impact on total oleoyl-CoA synthetase activity. This may be due to protein instability as noted for the purified enzyme (26) or due to regulatory parameters poorly defined at the present time. In the case of the *faa1Δ fat1Δ* strain, we noted similar results. Most notable among these was the finding that YEpDB17 (*FAT1*) resulted in oleoyl-CoA synthetase activities, which were increased 6-fold over the same strain harboring the plasmid vector (Table IV).

As noted above, Fat1p has been shown to confer very long-chain fatty acyl-CoA synthetase activity. Therefore, we also measured fatty acyl-CoA synthetase activities in the same cell extracts from above using the very long-chain fatty acid, lignoceric ($C_{24:0}$) as substrate (Table IV). As expected, expression of *FAT1* from YEpDB17 increased these activities just over 4-fold, whereas expression of *FAA1* from YEpDB02 or *FAA4* from YEpDB133 had no significant effect on total cellular very long-chain fatty acyl-CoA synthetase activities. Similar results were obtained for the *fat1Δ faa1Δ* and *fat1Δ* host strains (Table IV).

Fat1p and Fatty Acyl-CoA Synthetase Form a Physical Complex—In previous work, we provided independent evidence that Fat1p (13) and Faa1p or Faa4p (11) are each required for fatty acid import in yeast. The results of the multicopy suppressor analyses detailed above extended these results to include a functional dependence of fatty acid transport on both Fat1p and fatty acyl-CoA synthetase (Faa1p or Faa4p). Indeed, these data provided evidence suggesting Fat1p and Faa1p or Fat1p and Faa4p interact to coordinately facilitate fatty acid transport. The results from the multicopy suppressor screen are consistent with the notion that, at least in yeast, no other proteins participate in this process. Yet this experimental approach did not address whether Fat1p and fatty acyl-CoA synthetase form a physical complex. To address this question, we employed three different experimental strategies as follows: 1) negative dominance of mutant Fat1p over fatty acyl-CoA synthetase; 2) yeast two-hybrid analyses to investigate the hypothesized physical linkage between Fat1p and Faa1p or Faa4p; and 3) co-immunoprecipitation of Fat1p and a cognate fatty acyl-CoA synthetase.

Often when two proteins physically interact to form a functional complex, inactivation of one protein due to a mutation will result in a reduction in activity for the partner protein. This phenomenon is called negative dominance. Long-chain acyl-CoA synthetase activity in yeast is primarily contributed by Faa1p (≥95%). Therefore, we reasoned that the overexpression of nonfunctional Fat1p would result in a reduction of long-chain acyl-CoA synthetase activity if the proteins physically interact to facilitate vectorial acylation. For these experiments, we expressed a peptide derived from Fat1p made up of the C-terminal 125 amino acids (residues 545–669; ^{T7}Fat1p^{125C}).

TABLE III
Characteristics of yeast strains with mutations in FAT1 and the FAA genes

	Growth on YNBD-OLE-CER[a]	Fatty acid transport capacity[b]
Relevant genotype		
Wild type	1	+++
*faa1*Δ	3	+
*faa4*Δ	1	+++
*fat1*Δ	4	−
*faa1*Δ *faa4*Δ	4	−
*faa1*Δ *fat1*Δ	4	−
*faa4*Δ *fat1*Δ	4	−
*faa1*Δ *faa4*Δ *fat1*Δ	4	−
Multicopy suppressors		
*faa1*Δ *faa4*Δ/YEp24	4	−
*faa1*Δ *faa4*Δ/YEpDB02 (*FAA1*)	1	+++
*faa1*Δ *faa4*Δ/YEpDB133 (*FAA4*)	3	+
*faa1*Δ *faa4*Δ/YEpDB17 (*FAT1*)	2	++
*faa1*Δ *fat1*Δ/YEpDB02 (*FAA1*)	2	++
*faa1*Δ *fat1*Δ/YEpDB133 (*FAA4*)	4	−
*faa1*Δ *fat1*Δ/YEpDB17 (*FAT1*)	2	++
*fat1*Δ/YEp24	4	−
*fat1*Δ/YEpDB17 (FAT1)	1	+++

[a] Growth was scored by comparison to the wild-type strain. 1, positive growth after 24 h; 2, growth between 24 and 48 h; 3, growth between 48 and 96 h; 4, no growth after 96 h at 30 °C.
[b] Fatty acid accumulation monitored using C_1-BODIPY-C_{12}, visualized using confocal microscopy and scored relative to the wild type: wild-type, +++; ++, slightly reduced from wild type; +, visible accumulation but down dramatically; −, no visible accumulation.

TABLE IV
Fatty acyl-CoA synthetase activities in yeast strains with mutations in FAA1, FAA4, and/or FAT1 alone and transformed with multicopy suppressor plasmids

Relevant genotype	Fatty acyl-CoA synthetase activity	
	$C_{18:1}$	$C_{24:0}$
	pmol/min/mg protein (± S.E.)[a]	
Wild type	3,388.96 (382.17)	41.29 (4.12)
*faa1*Δ	258.70 (36.36)	42.67 (5.49)
*faa4*Δ	866.58 (110.55)	43.42 (5.99)
*fat1*Δ	3,338.67 (358.26)	19.85 (2.37)
*faa1*Δ *faa4*Δ	190.00 (49.67)	25.11 (1.75)
*faa1*Δ *fat1*Δ	190.05 (34.80)	10.09 (2.60)
*faa4*Δ *fat1*Δ	626.03 (53.66)	16.69 (3.45)
*faa1*Δ *faa4*Δ *fat1*Δ	133.86 (42.56)	10.32 (2.97)
*faa1*Δ *faa4*Δ/YEp24	267.57 (29.05)	36.95 (2.88)
*faa1*Δ *faa4*Δ/YEpDB02 (*FAA1*)	2,716.49 (310.61)	41.86 (3.89)
*faa1*Δ *faa4*Δ/YEpDB133 (*FAA4*)	480.54 (52.97)	35.99 (4.35)
*faa1*Δ *faa4*Δ/YEpDB17 (*FAT1*)	992.23 (102.84)	161.33 (25.59)
*faa1*Δ *fat1*Δ/YEpDB02 (*FAA1*)	4,109.75 (571.68)	26.38 (2.88)
*faa1*Δ *fat1*Δ/YEpDB133 (*FAA4*)	303.33 (65.44)	20.43 (2.12)
*faa1*Δ *fat1*Δ/YEpDB17 (*FAT1*)	1,549.73 (126.99)	29.68 (22.33)
*fat1*Δ/YEp24	ND	18.77 (2.29)
*fat1*Δ/YEpDB17 (*FAT1*)	ND	175.50 (32.58)

[a] Data from at least three independent experiments performed in duplicate. ND, not determined.

This peptide derived from Fat1p was non-functional in transport and activation, yet when analyzed using SDS-PAGE it formed a dimer, which was stable to boiling, suggesting it might contain a protein-protein interaction domain (data not shown). The expression of ^{T7}Fat1p^{125C} significantly reduced oleoyl-CoA synthetase activity (compared with vector control) (Fig. 3*A*). The reduction in activity was correlated with expression of the ^{T7}Fat1p^{125C} peptide, detected using a Western blot following expression with anti-T7 antibodies (Fig. 3*B*). Under these conditions, the ^{T7}Fat1p^{125C} peptide is expressed at levels nearly 10-fold higher when compared with native Fat1p (data not shown). These data are consistent with the proposal that Fat1p and fatty acyl-CoA synthetase form a functional complex.

Another method, which has become standard to evaluate protein-protein interactions, is the yeast two-hybrid system. These experiments employed plasmids encoding a bait protein, which consisted of a fusion between full-length Faa1p and the DNA binding domain of bacterial LexA and several trap proteins, which consisted of protein fusions containing full-length Fat1p and peptides derived from Fatp1 and the Gal-driven activation domain. In this system a third reporter plasmid contains the DNA-binding site of LexA in the promoter region driving expression of *lacZ* (encoding β-galactosidase), which is dependent on specific protein-protein interactions between the bait (bound to the DNA binding site) and the trap (fused to activation domain, which interacts with yeast RNA polymerase II). As shown in Table V, positive interactions between full-length Faa1p and either full-length Fat1p or two peptides carrying C-terminal fragments of Fat1p (Fat1p^{500C} and Fat1p^{125C}) were found when compared with the trap vector control alone. The peptide, which conferred negative dominance to fatty acyl-CoA synthetase activity (^{T7}Fat1p^{125C}) detailed above, also results in a positive interaction with Faa1p using the yeast two-hybrid system.

Additional evidence for specific protein-protein interactions between Fat1p and Faa1p or Faa4p was obtained using co-immunoprecipitation. As detailed under "Experimental Procedures," Fat1p was tagged with a T7 epitope (^{T7}Fat1p), and the fatty acyl-CoA synthetases were tagged with a V5 epitope (^{V5}Faa1p and ^{V5}Faa4p). Following growth, extracts were prepared from cells expressing ^{T7}Fat1p and ^{V5}Faa1p or ^{V5}Faa4p and immunoprecipitated using anti-T7 or anti-V5 antibodies.

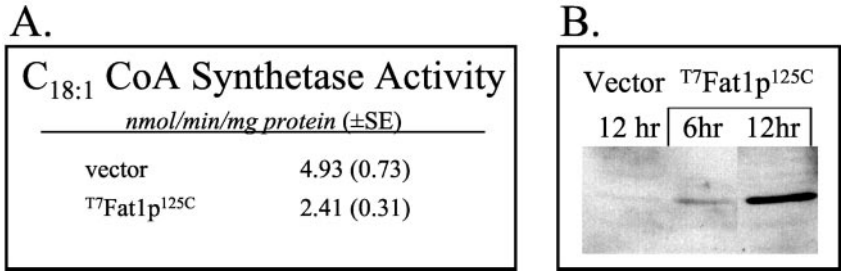

FIG. 3. **Negative dominance of mutant Fat1p over fatty acyl-CoA synthetase.** Extracts were prepared from wild-type cells carrying a plasmid encoding ^{T7}Fat1p^{125C} under the control of a galactose-inducible promoter and used to assess oleoyl-CoA synthetase activity (*A*) and peptide expression (*B*) using a Western blot probed with anti-T7 antibody.

TABLE V
Yeast two-hybrid analyses of Faa1p-Fat1p interaction

Bait[a]	Trap[a]	β-Galactosidase activity	p[c]
		Miller units (± S.E.)[b]	
Faa1p	Trap vector alone	8.81 (0.85)	
Faa1p	Fat1p	13.34 (0.91)	0.0021
Faa1p	Fat1^{500C}	16.08 (1.64)	0.0015
Faa1p	Fat1^{125C}	13.55 (0.82)	0.0029

[a] Bait, full-length Faa1p fused to the DNA binding domain of LexA. Trap, either full-length Fat1p or C-terminal peptides containing the number of amino acids as listed fused to the B42AD activation domain.
[b] Data from at least five independent experiments performed in duplicate.
[c] Significance defined using a paired *t* test (comparing strains expressing the Faa1p-Fat1p partners and the control expressing only the Faa1p bait plasmid and the trap vector plasmid).

The presence of the second protein in the complex was detected by Western blot analyses using the reciprocal antibody. The data presented in Fig. 4 showed that ^{V5}Faa1p and ^{V5}Faa4p are co-immunoprecipitated with ^{T7}Fat1p whether the precipitating antibody was anti-T7 directed against Fat1p or anti-V5 directed against one of the Faa proteins. In our control experiments using protein A-Sepharose beads alone (Fig. 4) or using an unrelated antibody (c-Myc) (not shown), we did not pull down the Fat1p-fatty acyl-CoA synthetase complex. Additionally, to test for nonspecific protein-protein interactions, we probed the immunocomplex using an antibody against Pma1p, an unrelated plasma membrane protein (25). No co-immunoprecipitation of Pma1p with Fat1p, Faa1p, or Faa4p was detected (Fig. 4). These data are in agreement with the results of the multicopy suppressor analysis, negative dominance, and yeast two-hybrid data presented above and fully support the notion that Fat1p and Faa1p or Faa4p form a physical complex, which we suggest is crucial to the process of vectorial esterification of exogenous long-chain fatty acids.

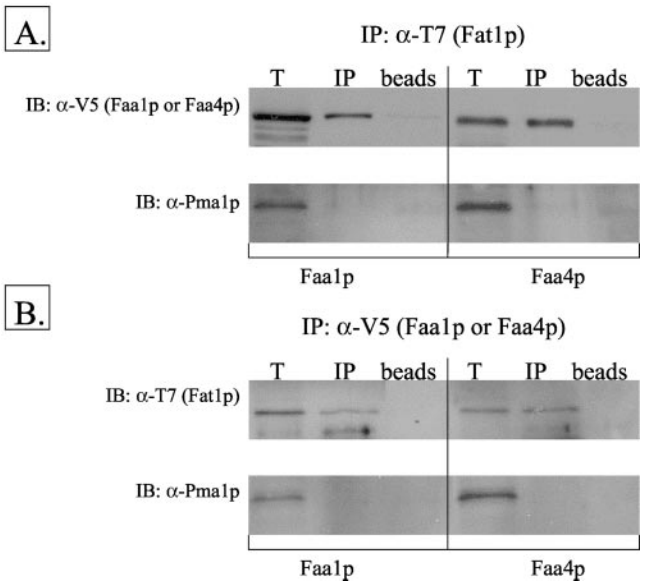

FIG. 4. **Co-immunoprecipitation of Fat1p and Faa1p or Faa4p.** *A*, anti-T7 antibody (*α-T7*) was used to pull down full-length ^{T7}Fat1p in extracts prepared from cells co-expressing ^{T7}Fat1p and ^{V5}Faa1p or ^{V5}Faa4p as indicated. The proteins were separated by SDS-PAGE, and subsequent Western blots were probed with anti-V5 (*α-V5*) antibody to detect ^{V5}Faa1p or ^{V5}Faa4p as shown. *B*, similarly, anti-V5 was used as the precipitating antibody to pull down ^{V5}Faa1p or ^{V5}Faa4p following co-expression of ^{T7}Fat1p and ^{V5}Faa1p or ^{V5}Faa4p, and the resultant blot was probed with anti-T7. *IB*, antibody used in the immunoblot; *T*, total cell extract; *IP*, samples immunoprecipitated with the indicated antibody; *Beads*, protein A-Sepharose alone without an immunoprecipitating antibody. Anti-Pma1p was used as a control protein specific to a yeast plasma membrane protein but unrelated to Fat1p, Faa1p, or Faa4p.

DISCUSSION

When long-chain fatty acids are supplied in the growth media, *S. cerevisiae* transports these compounds into the cell by a process, which requires Fat1p and the fatty acyl-CoA synthetase Faa1p. Even though Faa1p and Faa4p have been suggested to be functionally redundant, previous results and those presented here show that Faa1p, rather than Faa4p, plays a more distinct role in fatty acid import (11, 26). Importantly, the experiments reported here provide substantial genetic and biochemical evidence that Fat1p and fatty acyl-CoA synthetase (Faa1p or Faa4p) form a physical complex required to facilitate fatty acid import. These data are consistent with the hypothesis that the fundamental mechanism driving the accumulation of exogenous fatty acids within the cell is vectorial acylation whereby exogenous fatty acids are metabolically trapped as acyl-CoA thioesters.

Until this time, the physical and functional association of FATP and fatty acyl-CoA synthetase has been inferential (3, 11, 15). The present studies indicate that in the natural environment when fatty acids are limiting as, for example, occurs during hypoxia, Fat1p and Faa1p are each required for fatty acid import. Whereas each protein fulfills a separate function, the activities are coordinated and facilitated by a physical interaction. The former conclusion is based on the observation that in single copy neither gene can substitute for the other. The distinct functions for Fat1p and Faa1p were apparent in enzymatic analyses of acyl-CoA synthetase specificity and activity and in our fatty acid transport studies. In multicopy, Faa1p can substitute for Fat1p, and in turn, Fat1p can substitute for Faa1p in potentiating fatty acid import. Thus the apparent increase in accumulation of C_1-BODIPY-C_{12} when either of these genes is overexpressed appears related to the

essential role of long-chain fatty acyl-CoA synthetase activity in import and utilization rather than to a transport function *per se*. Thus utilization creates a diffusional gradient dependent upon the acyl-CoA synthetase Faa1p (and to a more limited extent Faa4p) but not Fat1p. The role of Fat1p in fatty acid import appears to be distinct from Faa1p and essential only at limiting fatty acid concentrations ($\leq$500 μM) such as might occur when cells are growing under hypoxic conditions in the natural environment. We suggest this mechanism of fatty acid transport by vectorial acylation exemplifies a system common to eukaryotes including mammalian cells that functions through FATP and a cognate fatty acyl-CoA synthetase.

There is substantial data showing Fat1p plays a role in long-chain fatty acid import yet has intrinsic very long-chain (C_{22}–C_{26}) fatty acyl-CoA synthetase activity (14, 24). This presents somewhat of a dilemma. Our results are consistent with the notion that the specificity of the fatty acid import system in yeast is for long-chain fatty acids as opposed to very long-chain fatty acids. Addition of very long-chain fatty acids to the growth media of yeast strains defective in very long-chain fatty acid synthesis does not alleviate the growth defect, suggesting the very long-chain fatty acids cannot be trafficked from an exogenous source to the site of metabolic utilization (27). Yet Fat1p is a central component of the long-chain fatty acid import system in yeast, being required both under anaerobic conditions and under cerulenin-induced conditional lethality, where exogenous long-chain fatty acids are required for growth (14). We have provided evidence recently (28) that the very long-chain fatty acyl-CoA synthetase activity intrinsic to Fat1p can be distinguished from fatty acid import in specific mutant alleles of *FAT1* with single amino acid substitutions. Additionally, the specificity of Fat1p-dependent import is for long-chain fatty acid substrates, whereasFat1p-dependent fatty acyl-CoA synthetase activity is for very long-chain substrates (14, 24). The deletion of *FAA1* encoding the major long-chain fatty acyl-CoA synthetase decreases fatty acid import nearly 3-fold; therefore, we suggest this enzyme is primarily responsible for activating fatty acids from an exogenous source and therefore contributes to the specificity of the import system (11).

The ability of *FAT1* encoded within a high copy number episome to suppress the phenotype on YNBD containing oleate and cerulenin of a *faa1*Δ *faa4*Δ strain and the corresponding ability of plasmid-encoded Faa1p to suppress the same phenotype of the *faa1*Δ *fat1*Δ strain is consistent with a functional interrelationship between Fat1p and Faa1p in long-chain fatty acid import. However, multicopy suppression might also result from alterations in intracellular metabolism and regulation distinct from the coupled transport/activation process when fatty acid import or fatty acyl-CoA synthetase activity is highly elevated by comparison to activities contributed by a single copy of the native gene. As detailed in these studies, we did not observe oleoyl-CoA synthetase activities comparable with or exceeding wild-type levels for either strain expressing *FAT1* or *FAA4* in high copy. In the case of the *faa1*Δ *faa4*Δ strain (wild type for *FAT1*) transformed with YEpDB17 (*FAT1*), there was sufficient oleoyl-CoA synthetase activity (albeit only ~30% wild-type), which appeared to drive the coupled import/activation process. On the other hand in the *faa1*Δ *fat1*Δ strain transformed with YEpDB133 (*FAA4*), there was detectable oleoyl-CoA synthetase activity (~10% wild type), but this was not sufficient to overcome the block as a consequence of a deletion in *FAT1*. By comparison, the *faa1*Δ *fat1*Δ strain transformed with YEpDB02 (*FAA1*) had robust oleoyl-CoA synthetase activity (~121% wild type), which was sufficient to overcome the block due to the *fat1*Δ deletion. Therefore, we believe the suppression is caused by overexpression of one of the partners in the import process, Fat1p or Faa1p.

Although we did not identify new partners in the fatty acid trafficking pathway by selecting multicopy suppressors, these results are of particular significance because they confirmed by using a powerful genetic approach the importance of an interaction between Fat1p and Faa1p in fatty acid import. Indeed, with one note of caution based on the suppressors presumed to be chromosomally encoded, these studies indicate these two proteins may be the only components mediating this process in yeast. Our present results parallel the previous work of Schaffer and Lodish (3) that identified independent clones encoding murine FATP1 and a fatty acyl-CoA synthetase using a functional cloning strategy. Functional cloning requires, in essence, overexpression of the protein target in a manner analogous to our studies using multicopy suppression. The murine FATP1 and fatty acyl-CoA synthetase each were identified and shown to function to promote the accumulation of C_1-BODIPY-C_{12} (3). By analogy, we have shown that Fat1p and Faa1p, when expressed from a 2-μm plasmid also function to promote the accumulation of C_1-BODIPY-C_{12}. The murine FATP1 also has intrinsic very long-chain acyl-CoA synthetase activity (29). Likewise, we and others (14, 24) have shown yeast Fat1p is a very long-chain acyl-CoA synthetase. Previously, we have shown (14) murine FATP1 complements the biochemical phenotypes associated with the *fat1*Δ strain in yeast indicating that the yeast and the mouse proteins are functionally equivalent. Collectively, these data support the notion that the fatty acid import mechanism working through Fat1p (or FATP) and fatty acyl-CoA synthetase is primarily through the esterification of the fatty acid with CoA, which results in metabolic trapping. Our working hypothesis is that Fat1p functions to increase fatty acid binding to the membrane, which in turn potentiates diffusion across the membrane. The fatty acid is subsequently metabolically activated concomitant with abstraction from the membrane by the Faa1p-Fat1p complex thereby generating a concentration gradient, which further drives the import process.

The present work demonstrates for the first time a physical interaction between Fat1p and Faa1p or Faa4p. In each series of experiments (*i.e.* negative dominance, two-hybrid analyses, and co-immunoprecipitation), the full-length proteins and C-terminal peptides of Fat1p resulted in positive interactions. One outcome from these experiments suggests the protein-protein interaction domain of Fat1p is localized at least in part to the C-terminal 125 residues. At present, we have no data localizing an interaction domain within Faa1p or Faa4p. Those experiments are currently underway.

Fatty acid transport in *S. cerevisiae* is tightly coupled to utilization and is primarily dependent upon the products of two genes, Fat1p and Faa1p. These proteins function in concert to couple fatty acid import to fatty acid activation and metabolic utilization, a process first described in bacteria as vectorial acylation. Due to the functional conservation of these proteins in higher eukaryotes, yeast provides a valuable, genetically tractable model system useful to further elucidate the mechanisms that underpin fatty acid import in eukaryotic systems.

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