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Transmembrane Movement of Exogenous Long-Chain Fatty Acids: Proteins, Enzymes, and Vectorial Esterification

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INTRODUCTION

Exogenous fatty acids and fatty acid derivatives influence a wide variety of cellular processes including fatty acid and phospholipid synthesis, organelle inheritance, vesicle fusion, protein export and modification, enzyme activation or deactivation, cell signaling, membrane permeability, bacterial pathogenesis, and transcriptional control (13, 25, 49, 92, 134). The processes governing the transport of fatty acids from the extracellular milieu across the membrane are distinct from those underpinning the transport of hydrophilic substrates such as sugars and amino acids. Investigations into fatty acid transport must address three central issues, which are unique to this process: (i) the low solubility of fatty acids under aqueous conditions; (ii) the physical and chemical parameters of fatty acids, which allows them to readily partition into a lipid bilayer; and (iii) the identification of membrane-bound and membrane-associated proteins, which are likely to play pivotal roles in this process. In addition, diversity of lipid and protein species in various biological membranes must be a central consideration for investigations directed at defining the biochemical mechanisms governing fatty acid transport.

The transport of exogenous long-chain fatty acids into the cell is a highly regulated process, suggesting protein involvement (58, 59, 62, 95). Cell types with high levels of fatty acid metabolism (either degradation or storage) transport exogenous fatty acids at higher apparent rates than do to those with low levels of lipid metabolism (1–3, 52, 75). In a number of cell types the process of fatty acid transport is inducible and commensurate with the expression of specific sets of proteins thought to participate in this process (9, 16, 17, 48, 49, 64). The process of fatty acid transport is protease sensitive and can be blocked through protein modification and the use of specific antibodies. Furthermore, fatty acid transport can be disrupted by the introduction of specific mutations in genes encoding membrane-bound and membrane-associated proteins that are likely to be involved in this process (14, 26, 27, 69, 127, 128,

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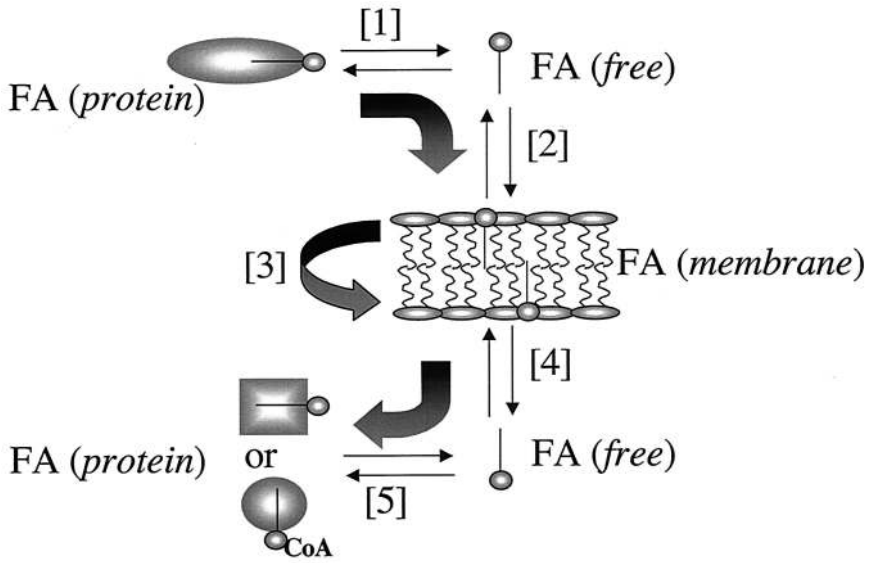

FIG. 1. Steps in fatty acid transport. As detailed in the text, this process is divided into five discrete steps, which can be kinetically defined (steps 1 to 5). Sites of potential protein involvement are highlighted by the thick arrows: fatty acid delivery to the membrane (steps 1 and 2), fatty acid translocation across the membrane (step 3), and fatty acid abstraction or removal from the membrane (steps 4 and 5).

137). Since fatty acids can readily partition into and flip between the two surfaces of a membrane, the roles played by these proteins in this process present something of a challenge. For example, these proteins may function to regulate fatty acid transport by contributing a specific activity at the levels of fatty acid delivery to the membrane (binding), transmembrane movement (or flip), or downstream metabolism. The use of genetically tractable systems (*Escherichia coli* and *Saccharomyces cerevisiae*) provides the genetic and biochemical tools necessary to discern how these proteins function to facilitate long-chain fatty acid transport.

FATTY ACIDS AND BIOLOGICAL MEMBRANES

The process of fatty acid transport across a membrane proceeds through five kinetically distinct phases defined as steps 1 to 5 (Fig. 1). From the standpoint of thermodynamic considerations, steps 2 and 4 are reversible and simply involve the transfer of the fatty acid into or out of the membrane. The binding of exogenous free fatty acids into the membrane (step 2) is fast and exhibits saturation kinetics, which is dependent on the number of fatty acid binding sites within the membrane (on both leaflets). The number of fatty acid binding sites may be an inherent property of the membrane alone or in combination with specific proteins that enhance this process. The rate at which this step occurs is also dependent on the equilibrium kinetics between the fatty acid in the bound state and in the free state (step 1). Step 3 involves the transmembrane movement of fatty acids from the exoplasmic face of the membrane to the cytoplasmic face. For fatty acids in the uncharged form (or protonated), most thermodynamic data suggest that this step is very fast ($t_{1/2}$ = ms; [85]), although there is one report suggesting that this step is rate limiting (89). There is general consensus that the transmembrane flip of fatty acid anions is slow ($t_{1/2} > 2$ s [85–88]). Using both model and biological membranes, Hamilton and colleagues have shown uncharged fatty acids flip between the exoplasmic face and the cytoplasmic face of the membrane thereby obviating the need for a specific protein to promote transmembrane movement (65–68, 85–88, 138). In addition, there are several thermodynamic studies which demonstrate that the movement of fatty acids between the two membrane surfaces is a diffusive process (65, 138). Step 4 involves the movement of fatty acids out of the membrane, and, while this can occur spontaneously, it is likely to be protein dependent and influenced by downstream metabolism (see below). Step 1 represents the equilibrium between fatty acid in a bound form and a free form. The bound form of the fatty acid may be part of a mixed micelle, as would be case in the intestine or in a protein-bound form as in a capillary bed. Step 5 is linked to the intracellular metabolism, which drives this process forward. This may involve the binding of fatty acids to intracellular fatty acid binding proteins or may involve downstream metabolism, which includes complex lipid and triglyceride synthesis and β-oxidation. It is envisioned that three fundamental steps represent potential sites for protein involvement in the process of fatty acid transport: (i) fatty acid delivery to the membrane (steps 1 and 2), (ii) the transmembrane flip of fatty acids (particularly for fatty acid anions) (step 3), and (iii) the movement of fatty acids out of the membrane prior to metabolism (steps 4 and 5).

As noted above, the binding and transmembrane flip of fatty acids to and across the membrane is a rapid process, particularly for uncharged fatty acids (87). It is clear that the transmembrane flip of fatty acids is a fundamental biophysical parameter that must be considered in defining the mechanism of fatty acid transport, but this process is not equivalent to diffusion. If diffusion is the fundamental driving force behind fatty acid transport, the rate will be slow and essentially uniform for all cell types. There is abundant evidence showing that different cell types transport exogenous fatty acids at differing rates,

which implies that proteins must necessarily be involved in this process. The questions of how and at which step in the process proteins are involved represent the experimental challenge.

Fatty Acid Transport Defined

Given that fatty acids bind to and flip between the two membrane leaflets, it is imperative to define the fatty acid transport process. The five steps described above for fatty acid transport are consistent with both protein and diffusional components. For the purposes of discussion, fatty acid transport is defined as the net movement of the fatty acid from the outside of the cell to the inside of the cell — or, more simply stated, the movement of the fatty acid from the extracellular space into the intracellular cytosolic compartment. Fatty acid transport cannot be defined simply in terms of the flip of the fatty acid from one leaflet of the membrane to the other, although this is a central component of the overall process.

The focus of this review is the net movement of exogenous fatty acids across the membrane, with a specific focus on the role of fatty acid transport proteins and fatty acyl coenzyme A (CoA) synthetases (FACS; fatty acid CoA ligase [AMP forming] [EC 6.2.1.3]). There is considerable evidence showing that the fatty acid transport proteins FadL (from gram-negative bacteria) and Fat1p (the yeast orthologue of mammalian fatty acid transport proteins [FATP]) function in concert with FACS as components of a fatty acid transport apparatus, which results in concomitant transport and activation to CoA thioesters by a process described as vectorial esterification.

MODEL SYSTEMS TO INVESTIGATE FATTY ACID TRANSPORT

The use of two model systems to investigate the process of fatty acid transport has provided fundamental insights into the underlying biochemical mechanism of fatty acid transport. Fatty acid transport is a saturable process in both *E. coli* and *S. cerevisiae*; in addition, specific proteins which participate in this process have been identified and characterized (18–29, 48–50, 54, 55, 77, 78, 93, 94, 138, 140, 141). Specific mutant strains of *E. coli* and *S. cerevisiae* have been identified that are unable to accumulate exogenous fatty acids, making these genetically tractable model system excellent models to investigate the biochemical principles essential to this process (25, 26, 92, 93, 137, 140). These different mutations result in specific and distinctive phenotypes, which have allowed the identification of individual protein components of their respective fatty acid transport systems.

Genetic Foundations of Fatty Acid Transport

Microbial model systems are particularly useful to investigate complex metabolic processes, since screens can be developed to select for mutations that confer specific phenotypes directly related to that process. In the context of fatty acid transport and activation in bacterial and yeast systems, mutant strains defective in both fatty acid transport and/or fatty acid activation have been identified that result in specific and distinguishing phenotypes (e.g., see references 23, 27, 55, 93, 94, 137, and 140).

The seminal work of Peter Overath in the late 1960s described the fatty acid degradation (*fad*) regulon of *E. coli* (110). These studies demonstrated that this regulon contains genes involved in fatty acid activation and β-oxidation and are under coordinate regulation by a common transcription factor. A central tenet of this work was the proposal that fatty acid transport into the cell was tightly linked to FACS-mediated fatty acid activation to the CoA ester. Furthermore, these investigators showed that FACS was required for mediating the induction of the genes of the *fad* regulon (110). This enzyme was both membrane associated and cytosolic, suggesting that it moves into the membrane in response to a specific signal. This early work suggested that this process was analogous to vectorial phosphorylation, and the researchers coined the term "vectorial acylation" to describe this process (110).

These early studies relied on classical bacterial genetics to identify and map the genes involved in fatty acid activation and degradation. Subsequent studies using molecular genetics have expanded our understanding of these coordinately regulated genes. Included in these later studies include those which identified the gene for the outer membrane-bound fatty acid transporter (FadL) and demonstrated that long-chain fatty acyl CoA is the effector molecule regulating the DNA binding activity of the transcription factor FadR (44, 47, 132, 133). A recent review by DiRusso et al. (49) provides a complete discussion of the *fad* regulon in *E. coli* and specific information on the coordinate regulation of the genes involved in fatty acid biosynthesis and fatty acid import, activation, and β-oxidation by FadR.

The fatty acid transport system in *E. coli* is presumed to be common to gram-negative bacteria. A number of FadL and FACS orthologues have been identified both experimentally and by sequence comparisons (e.g., see reference 49). Most notable are those described in *Enterobacter cloacae* and *Haemophilus influenzae* (112, 135). An orthologue of the FACS FadD has been experimentally defined in *Mycobacterium tuberculosis* (113).

More recently, a second model system, *S. cerevisiae*, has been used to investigate the process of fatty acid transport in eukaryotic cells. Yeast requires exogenous unsaturated long-chain fatty acids when grown under anaerobic conditions due to the O_2 requirement of Δ^9 fatty acid desaturase (126). In addition, a conditional auxotrophy for exogenous long-chain fatty acids occurs when fatty acid synthase is blocked using the antibiotic cerulenin (54, 77, 78). Under both of these conditions, mutants have been selected that are unable to grow despite addition of long-chain fatty acids to the growth media. This approach has been used to identify and characterize two genes encoding two isoforms of FACS (*FAA1* and *FAA4*), which are involved in the activation of exogenous long-chain fatty acids (51, 55, 77, 78, 90, 91), and one gene that encodes the yeast orthologue of the mammalian fatty acid transport protein (*FAT1*) (50, 54, 140).

PROTEINS IMPLICATED IN FATTY ACID TRANSPORT

A number of proteins which are hypothesied to play pivotal roles in fatty acid transport have been identified and characterized in both prokaryotic and eukaryotic cell types (Table 1). On the basis of current understanding, the process of fatty acid

TABLE 1. Proteins Involved in long-chain fatty acid transport

Protein	Method of identification	Features	Reference(s)
FAT-CD36	Affinity labeling with sulfo-*N*-succinimidyl-oleate	85,000 M_r plasma membrane-bound glycoprotein; also serves as a receptor of oxidized LDL	5, 70
$FATP_{pm}$	Oleate affinity chromatography	Identical to mitochondrial aspartate aminotransferase	17, 122
FATP	Expression cloning	Plasma membrane-bound protein; has intrinsic very-long chain FACS activity; multiple isoforms	74, 115
FadL	Genetic knockout; functional studies on purified protein	45,000 *M*, outer membrane protein restricted to gram-negative bacteria	108
FACS	Genetic knockout; expression cloning; functional studies on purified enzyme	Widely distributed (including isoforms) with an M_r of ~65,000; functions to activate exogenous fatty acids concomitant with transport	55, 110

transport is governed by two general mechanisms: (i) direct fatty acid transport across the membrane and (ii) fatty acid transport coupled to esterification to CoA thioesters. The candidate fatty acid transporters FAT and $FABP_{pm}$ appear to contribute to this process via the first mechanism, while FATP and FadL appear to operate in concert with FACS through the second mechanism.

Fatty Acid Translocase

The first candidate eukaryotic fatty acid transport protein to be identified was fatty acid translocase (FAT) (1–5, 12, 37, 75). This 85,000 M_r protein was selected on the basis of its ability to bind the fatty acid analogue sulfo-*N*-succinimidyl-oleate (SSO) and the anion inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) (1, 70). Analysis of a cDNA clone of the gene encoding FAT revealed that it is a 472-amino-acid protein with a predicted molecular weight of 52,466 and is the rat homologue of CD36, a glycoprotein first described in human platelets and lactating mammary epithelium (4). FAT/CD36 has been found in myocardial membranes, where it is also implicated in the transmembrane transport of long-chain fatty acids (106, 129, 130). This protein is a member of a broad family of scavenger receptors and has been reported to act as a receptor for thrombospondin, collagen, oxidized low-density lipoprotein (LDL), anionic phospholipids, and *Plasmidium falciparum* in addition to fatty acids (reviewed in reference 4). There is some indication that CD36 deficiency contributes to the etiology of hereditary hypertrophic cardiomyopathy (106, 130). Genetic linkage studies suggest that a deficiency of FAT/CD36 is associated with hypertriglyceridemia and hyperinsulinemia in the spontaneously hypertensive rat (SHR) (6, 69). FAT/CD36 may also facilitate the transduction of signals responsible for the stimulation of enzymes catalyzing the conversion of arachidonic acid into different bioactive metabolites (4). In genetically obese (*ob*/*ob*) mice FAT mRNA levels are 15-fold higher in liver and 60 to 80% higher in adipose tissue of *ob*/*ob* mice than of their control littermates, (96) and FAT is induced by lipids and peroxisomal proliferator activated receptor agonists (101, 107, 117). More recent data have shown that CD36 is recruited to the membrane from intracellular sites in response to insulin (31). The most informative data on FAT/CD36 came from studies of transgenic overexpressing and knockout mice. The overexpression of FAT/CD36 in transgenic (MCK-CD36) mice results in slightly lower body weight than that of control littermates, reduced levels of triglycerides (LDL fraction), and elevated levels of circulating fatty acids (73). Mice with engineered deletions in the gene encoding FAT/CD36 are viable yet have a significant decrease in binding and uptake of oxidized LDL in peritoneal macrophages. These animals also have significant increases in fasting levels of cholesterol (high-density lipoprotein [HDL] fraction), nonesterified free fatty acids, and triacylglycerol (LDL fraction) (37, 56). Each of these phenotypes is consistent with alteration in lipid-trafficking pathways. There is some information which suggests that FAT may function in concert with the intracellular fatty acid binding proteins (FABP). If this is indeed the case, FATP may function as an intracellular sink for fatty acids following transport and thus act to drive their net accumulation in the cell (4).

Fatty Acid Binding Protein—Membrane Bound

A second putative fatty acid transporter identified in mammalian cells, FABP—membrane bound ($FABP_{pm}$), is identical to mitochondrial aspartate amino transferase (mAspAT) (14, 17, 76, 122–125). NIH 3T3 fibroblasts transfected with a full-length mAspAT cDNA under the control of the Zn^{2+}-inducible metallothionein promoter express $FABP_{pm}$ in the presence of Zn^{2+} (17). Expression correlates with a commensurate increase in oleate uptake, and oleate uptake can be selectively inhibited by antisera to $FABP_{pm}$ (76, 123, 125). More recently, this protein has been identified in the placenta, where it is proposed to participate in the uptake of long-chain and polyunsaturated fatty acids fatty acids required for fetal development (32–34). FAT/CD36 and FATP are also expressed in the placenta, perhaps indicating some type of cooperative interaction between these three proteins (e.g., see reference 52).

The role of $FABP_{pm}$ in long-chain fatty acid transport is, however, somewhat controversial. Intestinal epithelial cells take up long-chain fatty acids by a saturable process and express $FABP_{pm}$. In this case, pretreatment of these cells with anti-$FABP_{pm}$ sera does not inhibit long-chain fatty acid uptake, arguing that, at least in this cell type, there is a distinct component involved in the transport process (131). In another study, *Xenopus laevis* oocytes were injected with poly(A)$^+$ RNA isolated from liver cells and an increase in long-chain fatty acid transport could be measured, suggesting that a protein(s) expressed from the cDNA was responsible for the apparent increase in transport. However, when the cDNA for mAspAT ($FABP_{pm}$) was injected alone, there was no increase

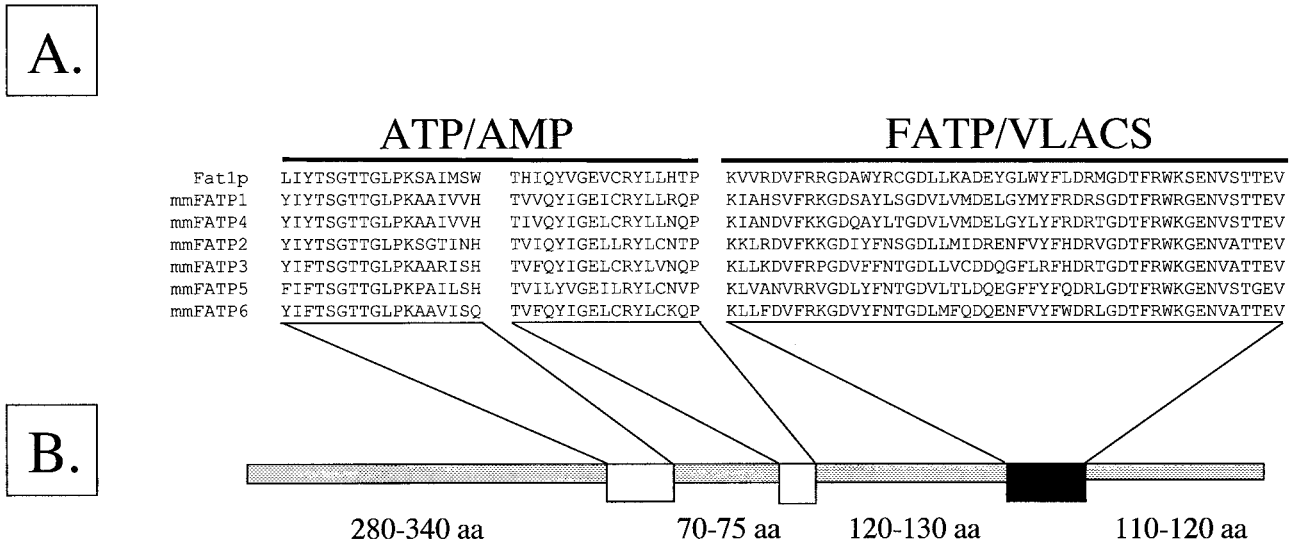

FIG. 2. Domain organization of the FATP family of proteins. (A) Amino acid sequence alignments of the ATP-AMP motif (common to all members of the adenylate-forming superfamily of enzymes) and the FATP-VLACS motif (restricted to the FATP family) from Fat1p and the six murine FATP orthologues. (B) Cartoon showing the approximate positions of the two elements of the ATP-AMP (white rectangles) and the FATP-VLACS (black rectangle) motifs in the FATP family of proteins. aa, amino acids.

in long-chain fatty acid uptake, although the protein was detected (57).

Fatty Acid Transport Protein

In 1992, two mouse proteins were identified using expression-cloning techniques, which, following transfection of Cos7 cells, resulted in an increased level of fluorescent fatty acid accumulation. The first was FATP, and the second was FACS (see below) (115). The use of expression cloning demonstrated a direct physiological role for both of these proteins in the net accumulation of fatty acids across a biological membrane. A number of different isoforms of FATP have subsequently been identified experimentally in mice, rats, humans, and yeast (e.g., mmFATP1, through mmFATP6 in mice) (54, 61, 74, 119–121). Members of the FATP family have also been identified experimentally or by sequence comparisons in nonmammalian systems including *Caenorhabditis elegans*, *Drosophila melanogaster*, *S. cerevisiae*, and *M. tuberculosis* (54, 74). The first mouse isoform identified (mmFATP1) has 646 amino acid residues and an apparent molecular weight of 63,000. Hydropathy profiles predict that mmFATP1 contains one to four membrane-spanning segments; recent experiments employing epitope tagged forms have confirmed the presence of at least one transmembrane domain (95). Immunofluorescence studies of epitope-tagged mmFATP1 support the prediction this is an integral membrane protein localized, at least in part, to the plasma membrane (95).

The yeast FATP orthologue, Fat1p, has 35% sequence identity to mmFATP1 and mmFATP4 (50, 54, 74). Fat1p, like several mmFATP isoforms, plays a role in the transport of long-chain fatty acids across the plasma membrane and in the activation of very long-chain fatty acids (36, 50, 136). Two subdomains within these proteins, in particular, have a significant level of identity (70 to 80%), which serves as distinguishing sequence elements or motifs (Fig. 2): (i) the ATP-AMP binding motif (common to all adenylate-forming enzymes) and (ii) the FATP-VLACS motif, which may be involved in contributing to fatty acid specificity (and generally is restricted to the FATP and very long-chain acyl-CoA synthetase [VLACS] families). Some sequence identities within these motifs (particularly the ATP-AMP motif) are also shared among the greater superfamily of FACSs (see below). The finding these proteins belong to the superfamily of adenylate-forming enzymes was noted in the initial characterization of Fat1p, which suggested an enzymatic activity (54). Indeed, subsequent studies have demonstrated that increased expression of three isoforms of the murine FATP (mmFATP1, mmFATP2, and mmFATP4) and yeast Fat1p results in increased VLACS activities (36, 38, 50, 71, 72, 136, 140). Data from the Schaffer laboratory has shown an mmFATP1 allele carrying a single amino acid substitution in the predicted ATP-AMP binding region fails to transport fatty acids (127, 128). One interpretation of these data is that the formation of an acyl-adenylate intermediate is required for transport.

The Long-Chain Fatty Acid Transport Protein FadL

Of the proteins characterized to date, only the long-chain fatty acid transport protein FadL, found in gram-negative bacteria, fulfills all the criteria that defines an integral membrane-bound fatty acid transporter. (i) FadL is localized in the outer membrane, where it is proposed to span the membrane 20 times and form a β-barrel specific for the transmembrane movement of long-chain fatty acids (42). (ii) Bacterial strains with a deletion of the *fadL* gene cannot grow on long-chain fatty acids as a sole carbon and energy source and cannot transport long-chain fatty acids across the cell envelope, yet

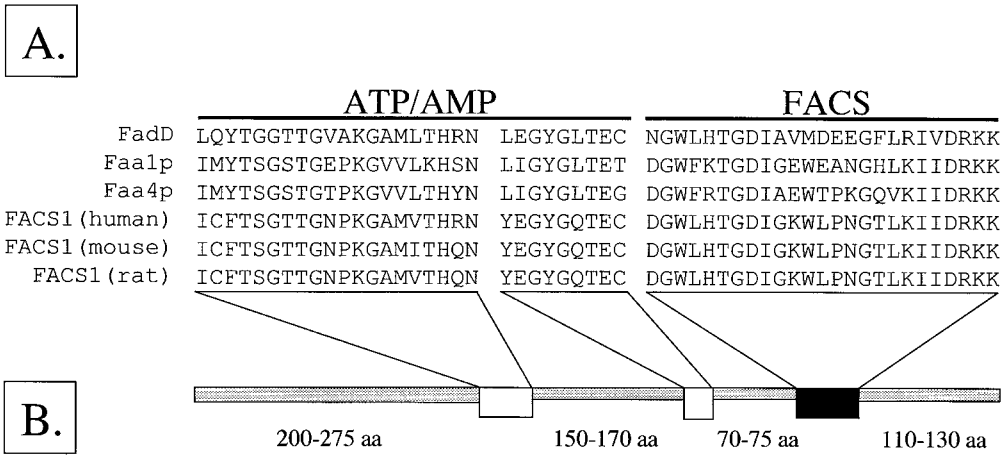

FIG. 3. Domain organization of the FACS family of enzymes. (A) Amino acid sequence alignments of the ATP-AMP motif (common to all members of the adenylate-forming superfamily of enzymes noted in Fig. 2) and the FACS motif (restricted to the FACSs) from FadD, yeast (Faa1p and Faa4p), human, mouse, and rat. (B) Cartoon showing the approximate positions of the two elements of the ATP-AMP (white rectangles) and FACS (black rectangle) motifs. aa, amino acids.

they retain their ability to β-oxidize long-chain fatty acids in vitro (18, 99, 108). (iii) Mutational analyses have defined specific amino acid residues and subdomains of FadL, which distinguish the long-chain fatty acid binding and transport activities intrinsic to the protein (26, 93, 94). (iv) Fatty acid binding to FadL has been demonstrated both in vivo and in vitro (24). In the *E. coli* model system, fatty acid import not only is dependent on the membrane-bound transporter, FadL, but also requires the FACS, FadD (see below) (23, 49, 110). In this regard, long-chain fatty acid transport is described as vectorial acylation since the imported fatty acid becomes metabolically trapped by esterification with Co A (110).

Fatty Acyl-CoA Synthetase

As noted above, the early work of Overath and colleagues was consistent with the hypothesis that FACS plays a central role fatty acid transport (110). FACS activity can be measured in both membrane and soluble fractions in gram-negative bacteria, suggesting that this enzyme moves between the cytosol and plasma membrane to facilitate the vectorial esterification of exogenous fatty acids (110). Indeed, more recent studies suggest that this enzyme is recruited to the plasma membrane, but no information has been gleaned about the underlying mechanism (99).

FACS catalyzes the formation of fatty acyl CoA by a two-step process proceeding through the hydrolysis of ATP to yield pyrophosphate. A central feature of catalysis is the formation of an adenylated intermediate, which is enzyme bound (63). This activation step involves the linking of the carboxyl group of the fatty acid through an acyl bond to the phosphoryl group of AMP. Subsequently, a transfer of the fatty acyl group to the sulfhydryl group of Co A occurs, releasing AMP. The reaction proceeds via a Bi-Uni, Uni-Bi Ter-molecular ping-pong mechanism with fatty acid, ATP, and CoA all serving as substrates (63).

$$\text{Fatty acid} + \text{ATP} \rightarrow \text{fatty acyl-AMP} + \text{PP}_i$$

$$\text{Fatty acyl-AMP} + \text{CoA} \rightarrow \text{fatty acyl-CoA} + \text{AMP}$$

The FACS, are part of a large family of proteins referred to as the ATP-AMP binding proteins. A common feature of enzymes in this family is that they all form an adenylated intermediate as part of their catalytic cycle. This group of enzymes is diverse in catalyzing the activation of a wide variety of carboxyl-containing substrates, including amino acids, fatty acids, and luciferin. Sequence comparison of members of the ATP-AMP binding protein family has identified two highly conserved sequence elements (YTSGTTGXPKGV and GYGXTE) that comprise the ATP-AMP signature motif, shared with the FATP family noted above (Fig. 3). The first sequence is generally 125 to 130 residues upstream from the second. A third, less highly conserved element that is thought to contribute to ATP-AMP binding overlaps the FACS signature (70 to 75 residues downstream from the second), which is involved in both catalysis and specificity of the fatty acid substrate (see below).

A second, more highly conserved sequence element DGWLHTGDIGXWXPXGXLKIIDRKK is common to all FACSs but is not highly conserved in the larger family of ATP-AMP binding proteins (Fig. 3). This sequence has been defined as the FACS signature motif (27). There are a number of notable features within the FACS signature motif. (i) This region contains two invariant glycine residues (at positions 2 and 7) and a highly conserved glycine at position 16. Therefore, it is reasonable to predict that this region adopts a similar tertiary structure in all FACSs. (ii) This region contains an additional six residues that are invariant in the FACSs: Trp at position 3, Thr at position 6, Asp at position 8, Asp at position 22, Arg at position 23, and Lys at position 25. (iii) The consensus sequence predicts an aspartic acid residue at position 1. However, in the bacterial enzyme, this is an asparagine, and conversion of the asparagine to alanine has no effect on enzyme activity, indicating that the presence of the carboxylate is not crucial for activity. (iv) The residue in the fourth position is hydrophobic and is either a leucine, a methionine, or phenylalanine. (v) This region of the enzyme contains hydrophobic residues (either leucine, isoleucine, or valine) at positions 4, 9,

18, 20, and 21. These residues, in addition to tryptophan residues at position 3, may comprise part of a fatty acid binding pocket. (vi) There is a preference for basic residues at positions 19 and 24 in addition to those at positions 22 and 25 (27).

Emerging evidence is consistent with the notion that FACS also plays a pivotal role in fatty acid transport in eukaryotic systems. In the expression cloning experiments that identified mmFATP1, a second, independent (and often overlooked) clone encoding FACS was also isolated, suggesting that this enzyme is also a component of the fatty acid transport system in murine adipocytes (115). In fact, in the original work describing mmFATP1, Schaffer and Lodish did suggest that these two proteins function in concert to facilitate long-chain fatty acid uptake by a process analogous to that defined for *E. coli* FadL and FadD (115). More recent work has shown that mouse FACS isoform 1 is localized at the plasma membrane, suggestive of its role in activating exogenous long-chain fatty acids (60). In yeast, there is a strict requirement for FACS (Faa1p or Faa4p) in the fatty acid transport process (55, 141).

VECTORIAL ACYLATION: ONE MECHANISM OPERATIONAL IN FATTY ACID TRANSPORT

In this review, we specifically focus on the fatty acid transport systems that function through a coupled fatty acid transport-activation mechanism referred to as vectorial acylation. As detailed above, this mechanism is likely to be operational in all eukaryotic and prokaryotic cell types, which allows this process to be highly regulated to meet the needs of the cell. For bacteria and yeast, current evidence suggests that this is the predominant mechanism driving exogenous long-chain fatty acid transport.

THE BACTERIAL PARADIGM

The cell envelope of gram-negative bacteria represents a formidable barrier for long-chain fatty acids. It is composed of two structurally and functionally distinct membranes (78, 79, 102–105, 111, 114). The outer membrane is composed of an external layer of lipopolysaccharide and an internal layer of phospholipid. The external lipopolysaccharide layer is refractory toward hydrophobic compounds, thereby providing a protective shield for the cell, while the internal phospholipid layer is associated with a layer of peptidoglycan. Outer membrane proteins involved in the acquisition of nutrients fall into three general classes: (i) nonspecific porins, (ii) substrate-specific porins, and (iii) high-affinity, substrate-specific transport proteins (102–104). The inner membrane is a more typical phospholipid bilayer and contains proteins involved in nutrient transport, energy production, and phospholipid biosynthesis (78, 79). The two membranes are separated by the aqueous periplasmic space, which is rich in proteins (some of which function in nutrient transport) and membrane-derived oligosaccharides (7, 8). For *E. coli* to utilize exogenous long-chain fatty acids, these compounds must first traverse the three layers of the cell envelope.

In *E. coli*, two genes are required for the transport of exogenous long-chain fatty acids: *fadL*, encoding the outer membrane protein FadL, and *fadD*, encoding the inner membrane-associated FACS FadD (Fig. 4) (49). Deletion of either *fadL* or *fadD* results in an inability of cells to grow on minimal plates containing fatty acids of any chain length. In cells harboring a mutation in both *fadL* and *fadR* (encoding the fatty acid-responsive transcriptional regulator), cells grow on plates containing medium-chain fatty acids but not long-chain fatty acids. This implies that FadL is specific for long-chain fatty acids while the *E. coli* FACS is involved in activating fatty acids of different chain lengths (18, 21, 108). Studies of the purified FACS FadD have borne this out: this enzyme can activate fatty acids with chain lengths varying from $C_{6:0}$ to $C_{20:4}$, with the highest specificity for $C_{14:0}$ to $C_{18:0}$ fatty acid substrates (82, 83). The periplasmic protease Tsp is also required for optimal levels of transport, although its precise role in this process remains undefined (10). There is also evidence supporting the existence of a specific fatty acid/H^+ cotransporter in the inner membrane; the structural gene encoding this protein has not been identified (81, 84).

Energetics of Fatty Acid Transport in Gram-Negative Bacteria

The long-chain fatty acid transport system in *E. coli* is partially shock sensitive, suggesting that a precise chemical composition (pH, periplasmic protein, etc.) is required, requires ATP generated by either substrate-level or oxidative phosphorylation, and requires the proton electrochemical gradient across the inner membrane for maximal proficiency (11).

The transport of long-chain fatty acids requires ATP generated through substrate-level or oxidative phosphorylation. This apparently reflects the ATP requirement of FACS as a component of this transport apparatus. Many transport systems that require ATP are protein-dependent ABC transporters, which function together with a periplasmic binding protein, an inner membrane-bound protein(s), and an ATPase protein. The ATP requirement in the bacterial long-chain fatty acid transport system is distinct and reflects the formation of a fatty acyl adenylate intermediate during the catalytic cycle FACS (63). The process of long-chain fatty acid transport in bacteria is also linked to the proton electrochemical gradient across the inner membrane. This is evidenced by an appropriate decrease in long-chain fatty acid transport rates when cells are treated with protonophores prior to assay.

The long-chain fatty acid transport system in *E. coli* requires both intracellular ATP pools and an energized inner membrane and thus has features common to both types of classically defined transport systems (11). Figure 4 illustrates a model of how this transport system is energized. Long-chain fatty acids traverse the outer membrane via FadL, pass through the periplasmic space, and partition into the inner membrane. If, as suggested Hamilton and coworkers (87), long-chain fatty acids traverse the membrane via diffusion (flip) in the protonated form, then the energized membrane may act to acidify the periplasmic space, resulting in the protonated form of the long-chain fatty acid. Once the long-chain fatty acid partitions into the inner membrane, FACS functions to abstract these compounds from the membrane, concomitant with activation to CoA thioesters. The β-oxidation of long-chain fatty acids following activation provides the respiratory substrates required for the maintenance of an energized membrane and intracellular pools of ATP and thus provides sufficient meta-

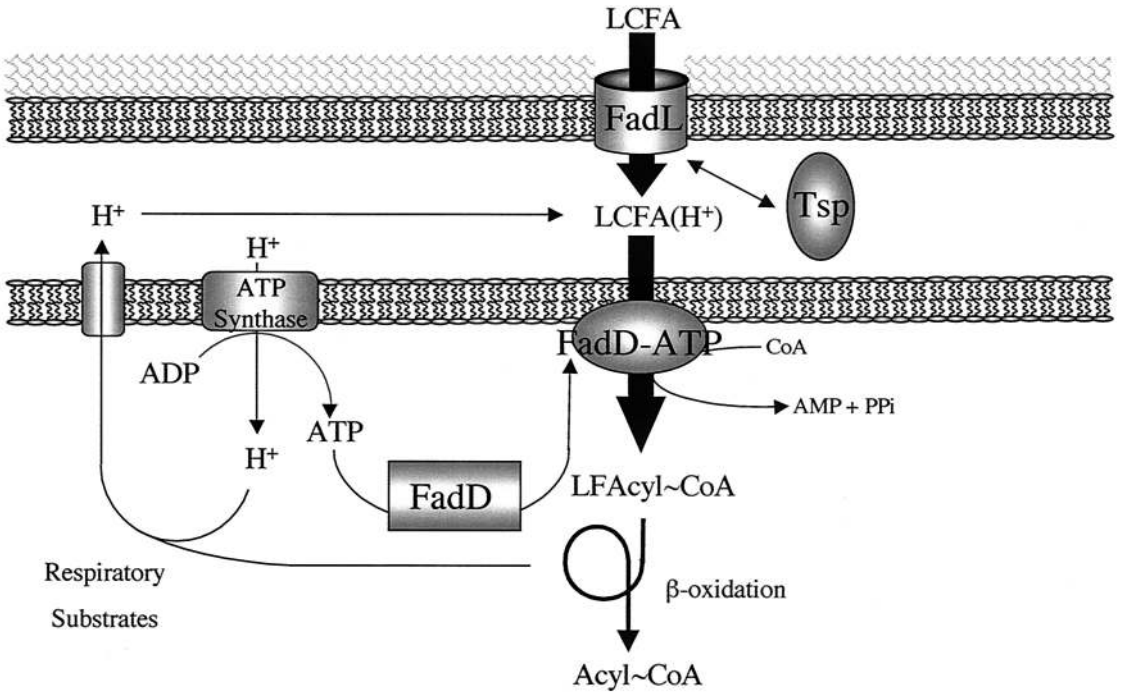

FIG. 4. Components and energetics underlying the transport of long-chain fatty acids (LCFA) across the cellular envelope of *E. coli*. Long-chain fatty acids traverse the outer membrane by way of the specific transport protein FadL. Once they traverse the outer membrane, they enter the periplasmic space, where they are protonated allowing them to partition into the inner membrane. The proton electrochemical gradient across the inner membrane contributes the protons in the periplasmic space. FACS (FadD) responds to the intracellular pools of ATP and in a primed form (FadD-ATP) is hypothesized to sense the free fatty acid [LCFA(H^+)] in the inner membrane. In the membrane-bound form, FadD catalyzes the formation of long-chain fatty acyl-CoA (LCFacyl CoA), rendering the process of transport unidirectional. Respiratory substrates for the generation of the electrochemical gradient across the inner membrane under conditions where cells are grown on long-chain fatty acids come from ATP synthetase and β-oxidation. Adapted from reference 11 with permission.

bolic energy for the efficient uptake of exogenous long-chain fatty acids across the cellular envelope.

The Fatty Acid Transporter FadL

The process of fatty acid transport in *E. coli* was originally described in work characterizing the *fadD* gene, encoding FACS (110). On the basis of kinetic data, these early studies predicted that at least one additional protein is also involved in the facilitated transport of long-chain fatty acids in *E. coli*. Nunn and Simons identified and mapped the *fadL* gene, encoding the long-chain fatty acid transport protein FadL, confirming this prediction (108). Subsequent studies describing the kinetics of fatty acid transport in *E. coli* are consistent with the postulate that FadL is specifically involved in the transport of long-chain fatty acids and requires the carboxylate of the fatty acid for ligand binding (21, 100, 109). FadL is predicted to span the membrane 20 times as antiparallel β-strands forming a β-barrel (Fig. 5) (42).

How does FadL mediate long-chain fatty acid binding and transport? Long-chain fatty acid binding to FadL is predicted to result in a conformational change, thereby exposing the transport channel and facilitating transport across the outer membrane. Several mutations within *fadL*, including *fadLH3*, have been identified, which suggest that this protein undergoes conformational change on fatty acid binding (Fig. 5B) (93, 94). The mechanism promoting the movement of fatty acids across the outer membrane via the FadL channel is not known, but on the basis of data generated from a collection of *fadL* mutants, it is presumed to involve both hydrophobic and charged amino acid residues within the carboxyl-terminal region of the protein (94).

The Fatty Acyl-CoA Synthetase FadD

E. coli contains a single FACS (FadD) with broad chain length specificity toward saturated, unsaturated, and polyunsaturated fatty acids (82, 83). This enzyme is essential for the activation of exogenous long-chain fatty acids destined for β-oxidation and plays an essential role in the regulation of the transcription factor FadR. The *E. coli* FACS has considerable similarities to other FACSs and, more broadly, to the superfamily of adenylate-forming enzymes. As noted above, this family of enzymes contains two conserved sequence elements: the ATP-AMP signature (involved in ATP binding) and the FACS signature (involved in fatty acid binding and specificity) (Fig. 3).

The *E. coli* FACS FadD contains two sequence elements, which comprise the ATP-AMP signature motif (^{213}YTGGTT GVAKGA^{218} and ^{356}GYGLTE^{361}). A series of alanine substitutions were generated corresponding to the ATP-AMP signature motif site to evaluate the role of this highly conserved

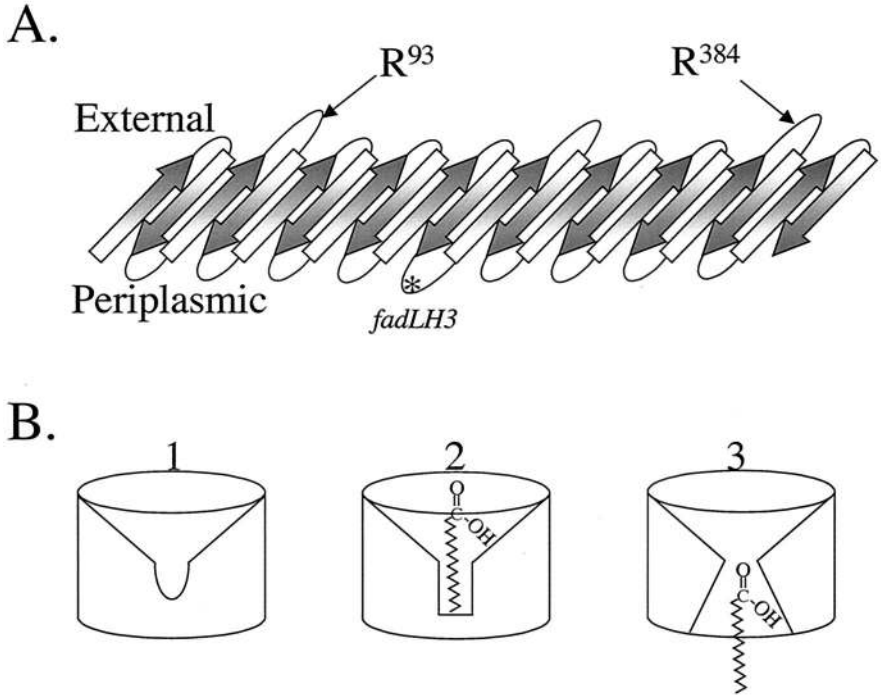

FIG. 5. Predicted topology and functional predictions of the of the long-chain fatty acid transport protein FadL. (A) Cartoon showing the 20 antiparallel β-strands of FadL, which are presumed to form a fatty acid-specific β-barrel within the outer membrane; noted are R^{93} and R^{384}, which are externally exposed, and the location of the insertion mutation *fadLH3*, a 2-amino-acid insertion in a periplasmic exposed loop that causes the FadL channel to be in an open conformation. (B) Cartoon illustrating ligand-induced conformational change in FadL, thereby facilitating long-chain fatty acid movement across the outer membrane. 1, FadL in a closed conformation; 2, FadL-specific binding of long-chain fatty acids requires both the acyl chain and the carboxylate of the fatty acid; 3, long-chain fatty acid induced conformational change allowing the fatty acid to traverse the membrane and enter the periplasmic space. Reprinted from reference 42 with permission.

region in enzyme function and fatty acid transport (137). Two major classes of *fadD* mutants were identified, both of which depressed enzyme activity: (i) those with 25 to 45% of wild-type activity and (ii) those with 10% of wild-type activity or less. The defect in the first class results in catalytic insufficiency, although several mutant forms also have a reduced affinity for ATP. Both classes of *fadD* mutations result in biochemical phenotypes that also reduce or essentially eliminate the transport of exogenous long-chain fatty acids, supporting the hypothesis that FACS functions in the vectorial movement of exogenous fatty acids across the plasma membrane by acting as a metabolic trap resulting in the formation of acyl-CoA esters.

Alanine-scanning mutagenesis has allowed for the molecular dissection of specific roles of the amino acid residues within the FACS signature motif noted above. These studies demonstrated that the FACS signature motif contains specific amino acids essential for catalytic activity and specify the fatty acid binding site within the enzyme (27). Three distinct classes of *fadD* mutations were identified on the basis of growth characteristics, FACS profiles using oleate, myristate, and decanoate as substrates, and studies using purified wild-type and mutant forms of the enzyme. (i) Only one substitution (*fadD*^{N431A}) resulted in wild-type FACS activity profiles (Fig. 3). (ii) Ten mutations abolished or greatly diminished enzyme activity. (iii) Seven mutations resulted in altering fatty acid chain length specificity. The finding that specific mutations resulted in altering fatty acid chain length specificity is consistent with the hypothesis that this region of the enzyme is specifically required for fatty acid binding.

Subsequent studied have clearly shown that the region of the enzyme corresponding to the FACS motif is involved in fatty acid binding. The affinity-labeled long-chain fatty acid 9-*p*-[^{3}H]azidophenoxy nonanoic acid (APNA) specifically modifies a region adjacent to and including the FACS signature of the *E. coli* enzyme (28). This work provided the first experiment-based data identifying the carboxyl-containing substrate binding domain within the adenylate-forming family of enzymes. As noted below, the predicted structural model for the *E. coli* FACS suggests that the FACS motif lies within a cleft separating two distinct domains of the enzyme and is adjacent to a region that contains the AMP-ATP signature motif, which, together, are likely to represent the catalytic core of the enzyme.

Structural Considerations of FACS

While several members of the ATP-AMP binding protein family have been crystallized and their structures have been resolved, the structure of FACS has not yet been defined. Using the crystallographic information for two enzymes containing the ATP-AMP signature motif (firefly luciferase [39] and the phenylalanine activating subunit [PheA] of gramicidin synthetase 1 [40]), a three-dimensional model for the *E. coli* FACS FadD has been proposed (Fig. 6) (28). This model

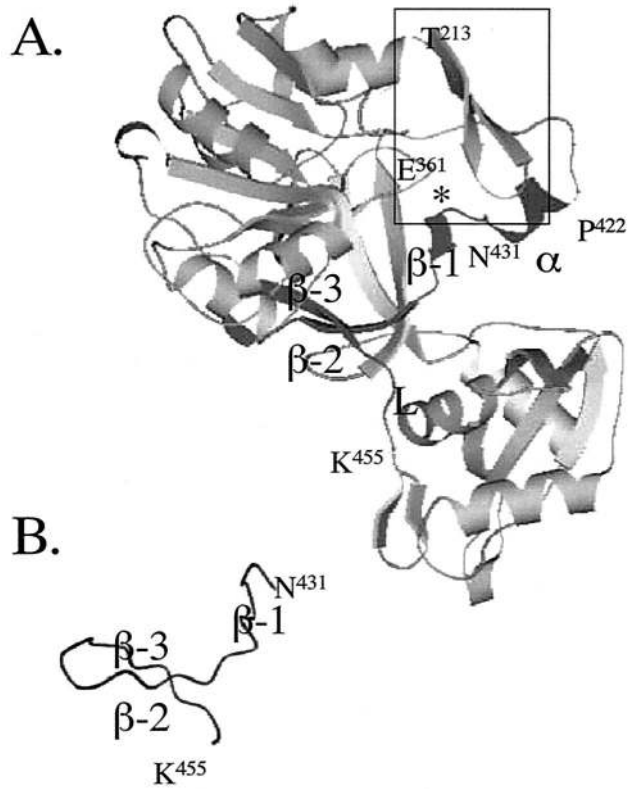

FIG. 6. (A) Model of the *E. coli* FACS developed using the Swiss-Model Protein Modeling Server and visualized using MolScript. The predicted structure begins with residue T^{213} of the native enzyme. Residues that comprise the peptide modified with APNA and continuing into the FACS signature motif are indicated (P^{422} to K^{455}). α denotes the predicted α-helix upstream from the FACS signature motif; β-1, β-2, and β-3 denote the β, β-turn-β structure of the FACS signature motif (Fig. 3); and L denotes the linker between the large N-terminal and small C-terminal domains of the enzyme. The *boxed* region denotes the β-loop β structure that comprises the first sequence element of the ATP-AMP signature motif, while the residue identified by an asterisk is E^{361} (within the second sequence element of the ATP-AMP signature motif), which is conserved in all adenylate-forming enzymes. (B) α-Carbon tracing of the FACS signature motif (N^{431} to K^{455}) highlighting the β,β-turn-βstructure, which is proposed to contribute to the fatty acid binding pocket within the enzyme. Reproduced from reference 28 with permission.

predicts that the region identified as the FACS signature, which is hypothesized to specify fatty acid binding, forms a β,β-turn-β structure, which is on the same face of the enzyme as elements that comprise the ATP-AMP signature motif and are presumed to specify ATP binding. The region of the enzyme identified using affinity labeling identified a peptide, beginning with P^{422}, adjacent to and contiguous with the FACS signature, confirming the hypothesis regarding the fatty acid binding domain. On the basis of the predicted structure of this enzyme, the region bound by β-1 and β-2 of the FACS signature motif contributes to a cavity that is likely to represent the fatty acid binding site. On the basis of this information, it seems likely that the region of FACS which includes the cleft separating the two domains of the enzyme represents the catalytic core of the enzyme. It is worth speculating that on ligand (ATP and fatty acid) binding, the two domains of the enzyme become juxtaposed to facilitate the formation of the fatty acyl adenylate.

The conservation of residues within the two elements of the ATP-AMP signature motif by all adenylate family members indicates that these regions contribute to the binding of ATP and/or to the formation of the enzyme adenylated reaction intermediate, since ATP is the one substrate common to all members. The crystal structure of firefly luciferase reveals that the N-terminal domain comprises the major portion of the molecule and consists of a distorted antiparallel β-barrel and two β-sheets flanked on either side by α-helices. Based on the predicted three-dimensional model of the FACS FadD from *E. coli*, the majority of these residues are clustered in a cleft separating two domains of the enzyme (28). The crystal structure of PheA complexed with AMP shows an Mg^{2+} bridge between the invariant glutamate of the second sequence element of the ATP-AMP signature and the O-1 phosphate of AMP (40). Changing this glutamate to alanine in the FACS FadD results in complete loss of enzyme activity, which then results in an inability to transport long-chain fatty acids (137).

As noted above, many members of the adenylate-forming family of enzymes contain conserved sequence elements that also overlap the FACS signature sequence. Of particular note is a highly conserved aspartate at position 438, which lies at the beginning of the FACS signature. Using the crystal structure of PheA complexed with AMP as a guide, is seems plausible this residue functions to position the ribose ring of AMP. In PheA, the carboxyl group of this aspartate forms specific H-bonding interactions with the two hydroxyls of the ribose moiety of AMP (40). A mutant form of the FACS FadD, $FadD^{D438A}$, has no acyl-CoA synthetase activity with oleate and decanoate as substrates, in contrast to the native enzyme (27). These data imply that Asp^{438} is critical for catalysis and contributes to fatty acid substrate specificity. The loss of the carboxylate may affect the orientation of contiguous residues, thereby modifying the geometry of the binding cleft. Indeed, site-directed mutagenesis studies of the adenylate-forming enzyme TycA support the hypothesis that a hydrogen bond donor is required at this position to stabilize the nucleotide. In TycA, replacement of the comparable Asp with Arg reduces ATP-PP_i exchange to 78% of the wild-type levels while replacement with Ser reduces this activity by only 12% (35).

FATTY ACID TRANSPORT AND ACTIVATION IN YEAST: EVIDENCE FOR A MULTICOMPONENT COMPLEX

As detailed above, fatty acid transport in yeast requires Fat1p (the orthologue to the mammalian FATPs) and FACS (either Faa1p or Faa4p). These data suggest that, as in the bacterial system, the process of fatty acid transport is driven by the esterification of fatty acids as a result of either Faa1p or Faa4p. The role of Fat1p is, however, quite distinct from the bacterial outer membrane protein FadL. Strains defective in *FAT1* have wild-type FACS activities, which is consistent with the notion that the activity of Fat1p precedes that of either Faa1p or Faa4p.

The phenotypes in yeast strains defective for fatty acid transport are more complex than those defined in bacteria, in part due to the difficulty of growing yeast on minimal fatty acid plates. As noted above, two conditions exist where growth of

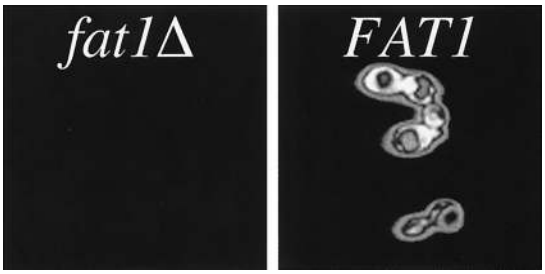

FIG. 7. Patterns of C^1-BODIPY-C^{12} uptake in *fat1*Δ and wild-type (FAT1) cells of *S. cerevisiae*. Reproduced from reference 50 with permission.

yeast requires supplementation of exogenous fatty acids to the growth media, providing a screen to select for mutants defective in fatty acid transport and activation. The first involves anaerobic conditions, where the cells require exogenous unsaturated long-chain fatty acids due to inactivity of the O_2-requiring Δ^9 fatty acid desaturase. The second is a conditional auxotrophy when fatty acid synthase is inhibited with the antibiotic cerulenin (51, 54). Using these screening conditions, three genes have been identified as components of the fatty acid transport system in *S. cerevisiae*. Strains defective in *FAT1* (encoding Fat1p) or in both *FAA1* and *FAA4* (encoding the FACSs Faa1p and Faa4p) are unable to grow under anaerobic conditions or on media containing cerulenin, even with long-chain fatty acid supplementation (54, 55).

Role of Fat1p

Disruption of *FAT1* (encoding Fat1p) results in five phenotypes expected for cells with restricted ability to import fatty acids. These mutant cells fail to grow on media containing the fatty acid synthesis inhibitor cerulenin even when the long-chain fatty acid oleate is supplied in the growth media. These cells are also unable to grow when cultured under hypoxic conditions when they are auxotrophic for unsaturated fatty acids. Yeast cells containing a *FAT1* deletion fail to accumulate the fluorescent long-chain fatty acid analogue C_1-BODIPY-C_{12} and have a greatly diminished capacity to transport exogenous long-chain fatty acids (Fig. 7). Furthermore, the utilization of exogenous fatty acids in β-oxidation and phospholipid biosynthesis is also diminished in mutant cells by comparison with wild type. These data attest to the physiological importance of Fat1p in the transport of exogenous long-chain fatty acids. As noted above, the deletion of *FAT1* does not result in decreasing FACS activities when decanoate, myristate, and oleate are used as substrates (54).

Work by DiRusso et al. (50) has shown that yeast Fat1p and murine FATP1 are functionally equivalent. Each of the mutant phenotypes noted above is eliminated when the mutant strains are transformed with either a clone encoding the yeast Fat1p or an expression clone encoding the murine FATP.

In addition to playing a central role in fatty acid transport, there are data showing that Fat1p is involved in very long-chain (C_{22} to C_{26}) fatty acid metabolism (29, 42, 136). Fat1p and several other members of the FATP family have intrinsic very long-chain FACS activity, suggesting that these enzymes are involved in intracellular fatty acid trafficking and, more specifically, in very long-chain fatty acid metabolism. Strains deficient in *FAT1*, for example, accumulate very long-chain fatty acids and have reduced very long-chain FACS activities. These findings present something of a dilemma in how to specifically reconcile both observations within the framework of yeast fatty acid metabolism. The specificity of the fatty acid transport system in yeast appears to be toward long-chain fatty acids as opposed to very long-chain fatty acids. Given the rarity of very long-chain fatty acids in the natural environment, it seems unlikely that a cell would evolve an import system specifically for these compounds. It is unknown whether the very long-chain FACS activity intrinsic to Fat1p is required for fatty acid import or whether these two activities are distinct. The current understanding of this protein favors independent functions for two reasons. First, the specificity of Fat1p-dependent import is for long-chain fatty acid substrates, while Fat1p-dependent FACS activity is for very long-chain substrates (50, 54). Second, deletion of *FAA1* encoding the major long-chain FACS decreases fatty acid import nearly threefold, which suggests that this enzyme is primarily responsible for activating fatty acids from an exogenous source and therefore dictates the specificity of the import system (55). As detailed below, there is emerging evidence supporting the functional association of Fat1p and Faa1p in mediating the regulated transport of exogenous long-chain fatty acids.

Directed Mutagenesis of *FAT1* and Functional Organization of Fat1p

As noted above, members of the FATP family have amino acid similarities and identities to the FACSs and the greater superfamily of adenylate-forming enzymes, the hallmark of which is the ATP-AMP signature motif. The FATP protein family is distinguished from the FACSs and other adenylate-forming enzymes by containing additional sequence elements common only to these proteins designated the FATP-VLACS signature motif (Fig. 2). To test the hypothesis that regions of sequence identity between the FATP family and the greater FACS family define the common ATP-AMP motif and sequence identities common only to members of the FATP family define the transport functions, a library of *fat1* alleles with alanine substitutions within each region have been generated and characterized (Fig. 8) (140). The residues replaced with alanine in the ATP-AMP signature motif included Y^{256}, S^{258}, and T^{260}. Two of the resultant mutant proteins, $Fat1p^{Y256A}$ and $Fat1p^{T260A}$, had reduced VLACS activities, which correlated with reductions in long-chain fatty acid transport. However, $Fat1p^{S258A}$ is unique because it has lost detectable VLACS activity but retained the ability to transport long-chain fatty acids, indicating that ATP binding and formation of the acyl adenylate may be separated from the transport function, while it is essential for catalysis. Additional alanine substitutions were generated at F^{325}, L^{353}, N^{372}, or T^{398} in Fat1p. Each of these amino acids lies in areas conserved within the FATP family but more divergent when compared with the long-chain FACS family. These amino acid residues overlap the ATP-AMP motif shown in Fig. 2. By comparison with the FACS FadD, which has been more extensively characterized (27, 28), these amino acid residues are likely to be positioned in the nucleotide binding pocket of the protein. As expected, substi-

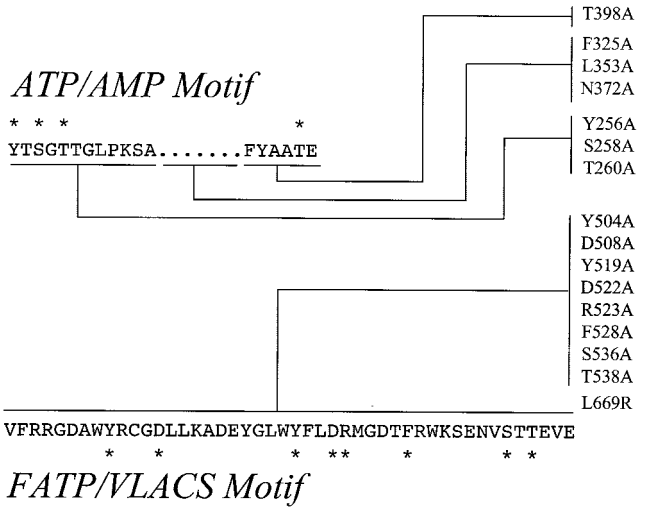

FIG. 8. Directed mutagenesis of FAT1. Asterisk indicate the residues within the ATP-AMP and FATP-VLACS signature motifs (Fig. 2) within Fat1p subjected to mutagenesis. Highlights of these mutants (140) are presented in the text.

tution of each of these highly conserved residues resulted in decreased VLACS activity. They also resulted in decreased growth under selective conditions, which was correlated with decreased long-chain fatty acid transport (140).

Regions within Fat1p that contain the FATP-VLACS motif and sequences toward the carboxyl end of the protein are highly conserved among members of the FATP and very long-chain FACS families. These regions are likely to specify functional domains for promoting fatty acid binding specificity and fatty acid transport: functions that are unique and define this family. Preliminary analyses of eight amino acid substitutions constructed in Fat1p within these regions support this proposal (140). Three of the residues replaced with alanine were absolutely conserved between yeast Fat1p and the five murine FATP isoforms: D^{508}, D^{522}, and R^{523} (140). Of these, Fat1p^{D522A} and Fat1p^{R523A} had greatly reduced VLACS and transport activities. Fat1p^{D508A} was unique in that it retained the transport function but lost VLACS activity. Five other targeted amino acids were characterized because they were identical in Fat1p, mmFATP1, and mmFATP4 but were different in one or more of the other murine FATP isoforms. Phenylalanine is found in mmFATP2, mmFATP3, and mmFATP5 at the position corresponding to Y^{504} of Fat1p (and mmFATP1 and mmFATP4). Replacement of Y^{504} with alanine reduced but did not eliminate any of Fat1p functions. Y^{519} (of Fat1p) is conserved in all murine isoforms except mmFATP5, which contains a histidine residue. Replacement of this residue with alanine severely decreased all functions associated with Fat1p. F^{528} and L^{669} are conserved in all isoforms of mmFATP but mmFATP3, which contains isoleucine and arginine at the respective positions. Replacement of either of these residues in Fat1p (Fat1p^{F528A} and Fat1p^{L669R}) eliminated fatty acid transport activity, and while VLACS activity was reduced, it was not eliminated. The results obtained for these two mutants and for Fat1p^{S258A} and Fat1p^{D508A} are very valuable because they demonstrate that the fatty acid transport activity could be experimentally separated from the VLACS activity.

The Fatty Acyl-CoA Synthetases Faa1p and Faa4p

It is difficult to dissociate fatty acid transport from metabolic utilization. In bacteria, yeast, and higher eukaryotes, one mechanism promoting fatty acid transport is proposed to involve the coupling of transport with activation to CoA thioesters. The concomitant transport and activation result in activated fatty acids, which are metabolized very quickly. As noted above, this is clearly the case in the bacterial paradigm. In yeast and higher eukaryotes, the same fundamental process is likely to be operational. The FACSs encoded within yeast *FAA1* or *FAA4* (Faa1p and Faa4p, respectively) play a central role in fatty acid transport (55).

As detailed above, the process of long-chain fatty acid transport is likely to include diffusion of the fatty acid across the membrane, where there is flip of the uncharged fatty acid from the outer leaflet to the inner leaflet (65). However, it is clear from a number of studies that there is a need for a sink to establish a concentration gradient from the outside to the inside or the fatty acid would remain trapped in the membrane (4, 62, 65, 89). In yeast, the formation of the acyl-CoA thioester catalyzed by Faa1p or Faa4p represents the sink that governs transport.

In yeast, fatty acid import is restricted in strains carrying a deletion in *FAT1* as well as in strains carrying deletions in both *FAA1* and *FAA4*. The *fat1*Δ and *faa1*Δ *faa4*Δ strains have indistinguishable phenotypes when grown on YPD containing oleate and cerulenin or under anaerobic conditions (54, 55). In addition, strains carrying deletions in both *FAA1* and *FAA4* fail to accumulate C^1-BODIPY-C^{12} (Fig. 9). This information implies that minimally Fat1p and either Faa1p or Faa4p are components of a metabolic system linking fatty acid import and utilization. Several studies have shown that Faa1p, as opposed to Faa4p, is the predominant FACS involved in this process. Four notable results have led to this conclusion. (i) Faa1p functions as the major FACS within the cell during the logarithmic phase growth. (ii) Fatty acid import is markedly reduced in Faa1p-deficient cells. (iii) Formation of oleoyl-CoA from oleate supplied exogenously is reduced in the *faa1*Δ strains. (iv) The levels of β-oxidation are severely depressed in strains containing a deletion in *FAA1* compared to the levels in the wild type and strains containing a deletion in *FAA4*.

Interaction of Fat1p and FACS

The studies described above show that exogenous long-chain fatty acids enter the yeast cell by a process that requires Fat1p and either the FACS Faa1p or Faa4p (54, 55). Prior to metabolic utilization, exogenous fatty acids must be activated to their CoA thioesters. In yeast, the FACS Faa1p accounts for approximately 95% of the myristoyl- and palmitoyl-CoA synthetase activity while Faa4p accounts for approximately 2% of the activity toward these substrates (77, 90). Deletion of *FAT1* or *FAA1* and *FAA4* impairs growth on media supplemented with oleate and cerulenin and under anaerobic conditions. Furthermore, deletion of both *FAA1* and *FAA4* prevents the incorporation of exogenously supplied fatty acids into phos-

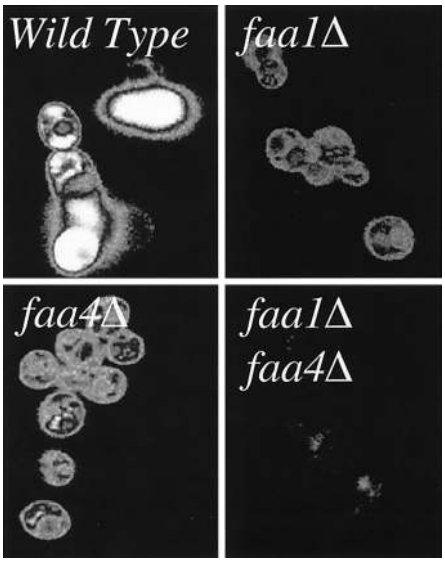

FIG. 9. Patterns of C^{1}-BODIPY-C^{12} uptake in wild-type, *faa1*Δ, *faa4*Δ and *faa1*Δ *faa4*Δ cells of *S. cerevisiae*. Reprinted from reference 54 with permission.

pholipids while deletion of *FAT1* reduces the rate of incorporation (55). Under conditions where Faa1p is inactive or expression of *FAA1* is reduced, Faa4p partially compensates for the loss of function (55, 77, 90).

More recent molecular genetic and biochemical studies further define the functional and physical interactions between these Fat1p and Faa1p or Faa4p. Multicopy extragenic suppressors were selected in strains carrying deletions in *FAA1* and *FAA4* or *FAA1* and *FAT1*. In the first strain, plasmids encoding *FAA1, FAT1*, and *FAA4* were identified, while in the second strain, plasmids encoding *FAA1* and *FAT1* were identified. In the latter case, multicopy *FAA4* identified in the *faa1*Δ *faa4*Δ strain could not suppress the growth defect in the *faa1*Δ *fat1*Δ strain, indicating that some essential functions of *FAT1* cannot be performed by this FACS. Chromosomally encoded *FAA1* does not suppress the growth deficiency of a *fat1*Δ *faa1*Δ strain, nor does chromosomally encoded *FAT1* complement the growth defect of a *faa1*Δ *faa4*Δ strain, indicating that these proteins play distinct roles in the fatty acid transport process (141). When expressed from a 2μm plasmid, Fat1p has significant oleoyl-CoA synthetase activity, which indicates that vectorial esterification and metabolic trapping is the driving force behind import. Evidence of a physical interaction between Fat1p and Faa1p comes from three independent biochemical approaches (141). (i) a C-terminal peptide of Fat1p deficient in fatty acid transport exerts a dominant negative effect against long-chain FACS activity. (ii) Protein fusions employing Faa1p as the bait and portions of Fat1p as the trap are active when tested using the yeast two-hybrid system. (iii) Coexpressed, differentially tagged Fat1p and Faa1p or Faa4p are coimmunoprecipitated (Fig. 10). 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 (Fig. 11).

FATTY ACID TRANSPORT AND BIOLOGICAL MEMBRANES

The transport and activation of exogenous long-chain fatty acids allows the cell to use these compounds in lipid biosynthesis, protein acylation, organellar biogenesis, and energy production. The mechanisms underlying this process appear to be complex and involve a combination of diffusion (transmembrane flip) and specific membrane-bound proteins and fatty acid-activating enzymes. It is clear that one fundamental mechanism that is operational in most cell types is vectorial acylation. Long-chain fatty acid transport is highly regulated and linked to downstream metabolism and, at least in the case of gram-negative bacteria, the energized state of the cell. We are now confronted with the challenge of defining how these specific membrane-bound and membrane-associated proteins function in concert to facilitate the specific and regulated transport of long-chain fatty acids using well-defined genetic model systems.

Long-Chain Fatty Acid Transport and Activation in Bacteria: Implications in Early States of Infection

The genes required for acquisition of long-chain fatty acids in *E. coli* are regulated at the level of transcription (46, 49). Under high-nutrient conditions, the genes required for fatty acid transport, activation, and β-oxidation are repressed by the global transcription factor FadR. When the cell encounters long-chain fatty acids, FadL and FACS function to transport and activate these compounds into intracellular pools of long-chain acyl-CoA. These compounds represent the effectors that, when present at sufficiently high intracellular concentrations, bind to FadR, resulting in the derepression of the genes involved in fatty acid transport, activation, and β-oxidation. This initiates a cascade of events that allow the cell to utilize long-chain fatty acids as a carbon and energy source. DNase 1 footprinting has identified two FadR binding sites for both the *fadL* (encoding the transporter) and *fadD* (encoding FACS) genes (23, 45). Both the *fadL* and *fadD* genes are normally expressed at basal levels under high-nutrient growth conditions. In the presence of long-chain fatty acids as the primary carbon source, these two genes become induced two- to threefold (45, 49). In addition to being regulated through long-chain fatty acyl-CoA–FadR-mediated process, the *fadL* gene is regulated in response to changes in osmolarity through OmpR while the *fadD* gene is also regulated by catabolite repression through cyclic AMP (cAMP)-cAMP receptor protein (49). These multiple levels of transcriptional regulation of *fadL* and *fadD* must be necessary for the cell, given the physiological consequences of high levels of long-chain fatty acyl-CoA. In addition to regulation at the level of transcription, FACS is apparently regulated at both the levels of translational initiation (23) and membrane association (137). The long-chain fatty acid transport system is also responsive to the energized state of the cell, adding yet another dimension of regulation (11).

Given the multiple levels of regulation, which we have defined for the long-chain fatty acid transport system in *E. coli*, one might question why such a system has evolved. The answer must lie in the opportunistic nature of these organisms. The

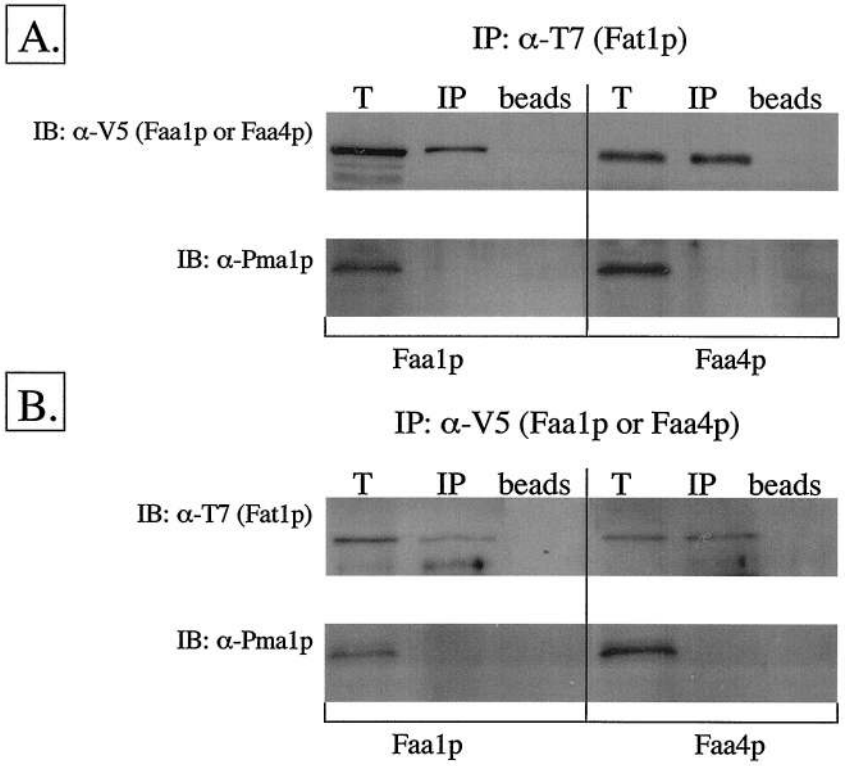

FIG. 10. Coimmunoprecipitation of Fat1p and Faa1p or Faa4p indicate Fat1p and a cognate FACS form a functional complex at the plasma membrane. (A) Anti-T7 antibody (α-T7) was used to pull down full-length ^{T7}Fatlp in extracts prepared from cells coexpressing ^{T7}Fatlp and ^{V5}Faa1p or ^{V5}Faa4p as indicated. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 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 coexpression of ^{T7}Fat1p and ^{V5}Faa1p or ^{V5}Faa4p, and the resultant blot was probed with anti-T7. IP, immunoprecipitating antibody; IB, antibody used in the immunoblot. Lanes: 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. Reprinted from reference 141 with permission.

outer membrane of *E. coli* and other gram-negative bacteria has an outer leaflet of lipopolysaccharide, which renders it refractory toward hydrophobic compounds (including long-chain fatty acids). Long-chain fatty acids represent important sources of metabolic energy and carbon for macromolecular synthesis and therefore must be specifically and efficiently transported across the cell envelope. Since FadL and FACS are present at basal levels under nutrient-rich conditions, they can function to specifically transport and activate long-chain fatty acids when they are encountered in the environment. Under conditions where high levels of long-chain fatty acids are encountered in the environment, there is a specific induction of the fatty acid transport apparatus, which occurs by way of long-chain fatty acyl-CoA–FadR-mediated derepression of the *fadL* and *fadD* genes.

The transport of long-chain fatty acids in gram-negative bacteria also appears to have pathophysiological implications. Using in vivo expression technology, Mahan et al. have shown that the *fadB* gene is specifically induced during early infection of mice by *Salmonella enterica* serovar Typhimurium (98). The *fadB* gene is part of the *fadBA* operon, which encodes the β-oxidation multienzyme complex. The expression of the genes involved in β-oxidation of long-chain fatty acids (including *fadBA*) is controlled at the level of transcription by FadR in response to intracellular long-chain fatty acyl-CoA. High-level expression of FadL and FACS is dependent on FadR-mediated transcriptional control. The finding that *fadB* is induced implies that the intracellular levels of long-chain fatty acyl-CoA rise, which must necessarily be the result of the concerted activity of FadL and FACS in the transport and activation of exogenous long-chain fatty acids. High concentrations of long-chain fatty acids, including arachidonate, are found in the extracellular inflammatory milieu as the result of phagocytosis and the action of specific classes of phospholipases. The transport, activation, and β-oxidation of long-chain fatty acids such as arachidonic acid by gram-negative bacteria would be predicted to result in the suppression of a local inflammatory response. This may provide the cells with an advantage during the early stages of colonization as well as with a means of detoxification of high concentrations of long-chain fatty acids that may be encountered under such conditions. Therefore, expression of *fadB* along with the other fatty acid transport, activation, and degradative genes may contribute to the metabolism of bactericidal or proinflammatory host fatty acids. There is emerging evidence that the FACS FadD and exogenous fatty acids also function in HilA-dependent activation of

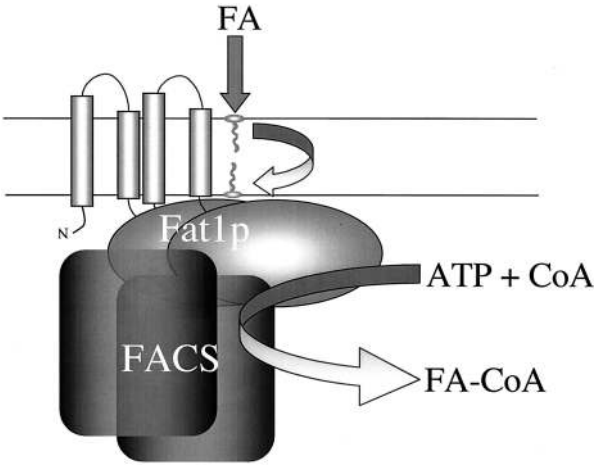

FIG. 11. Vectorial acylation in yeast. Cartoon showing the interaction between Fat1p and FACS (either Faa1p or Faa4p), which function to promote the coupled transport and activation of exogenous fatty acids. Fatty acids (FA) partition into the plasma membrane and flip between the membrane surfaces. It is unclear whether Fat1p enhances this process. Fat1p (illustrated as a dimer within the membrane) and a cognate FACS (illustrated as a dimer) form a complex, which functions to abstract the fatty acid from the membrane concomitant with esterification to CoA thioesters (FA-CoA) for further metabolism.

the expression of *S. enterica* serovar Typhimurium invasion genes. A Tn *5* insertion was identified in the *fadD* gene, which reduced the expression of *hilA*. These data suggest that *hilA* is regulated by a FadD-dependent, FadR-independent mechanism, suggesting that fatty acid derivatives may act as intracellular signals to regulate *hilA* expression (96). Work from Spector's laboratory also suggests the involvement of exogenous fatty acids in *S. enterica* serovar Typhimurium, particularly as related to the starvation stress response. Three carbon starvation-inducible *lacZ* fusions were identified in the *fadF* gene, which encodes an acyl-CoA dehydrogenase specific for medium- and long-chain fatty acids (118), suggesting that fatty acid trafficking in bacteria changes on initiation of the starvation stress response. It is not yet known whether this also translates into changes in fatty acid transport and/or activation.

A present challenge is to define the mechanism by which FadL and FACS interact to facilitate transport. If the hypothesis that FACS partitions into the inner membrane in response to fatty acid ligands is correct, part of the challenge is to define how FadL delivers fatty acids to the inner membrane and how this signal facilitates membrane association. Given the pathophysiological implications of fatty acid metabolism in enterotoxigenic gram-negative bacteria noted above, an additional challenge is to define whether this process provides cells with a protective advantage during early stages of inflammation.

Fatty Acid Transport and Trafficking in Yeast

In yeast, long-chain fatty acids imported by Fat1p are converted to CoA thioesters by the FACSs Faa1p or Faa4p (54, 55). The long-chain fatty acyl-CoAs are incorporated into phospholipids, used as a substrate in protein acylation and as carbon and energy sources. Intracellular long-chain acyl-CoAs are presumed to bind to acyl-CoA binding protein (ACBP), which acts to buffer the cell from the detergent properties of these compounds as well as providing a means of intracellular targeting (116). While this scheme of fatty acid trafficking appears to be reasonably simple, there are a number of unanswered questions. For example, we do not yet understand the precise roles of the activating enzymes Faa1p and Faa4p. Are they essentially redundant, or are they differentially compartmentalized or expressed? ACBP is presumed to function as a long-chain fatty acyl-CoA pool former, but can this protein also target these compounds to specific sites of utilization? Lastly, it is possible that other proteins interact with ACBP or bind long-chain fatty acyl-CoA directly to specify intracellular targeting.

β-Oxidation of long-chain fatty acids occurs exclusively in the peroxisomes of yeast (73). Growth of yeast in the presence of long-chain fatty acids induces the proliferation of peroxisomes and results in the activation of genes whose protein products are involved in β-oxidation (73). For example, the *POX1* gene (encoding fatty acid oxidase) and the *POX2* and *POX3* genes (encoding a multifunctional enzyme complex made up of a trifunctional polypeptide subunit and a subunit with thiolase activity) are specifically induced in the presence of long-chain fatty acids. Therefore, these compounds or a derivative thereof must signal the transcriptional apparatus to specifically induce genes required for peroxisomal proliferation and β-oxidation. On the other hand, growth in the presence of long-chain fatty acid oleate results in the repression of *OLE1*, encoding fatty acid desaturase. In this regard, the intracellular trafficking of these compounds must be highly regulated to maintain cellular homeostasis.

On the basis of work identifying Fat1p as a fatty acid transporter and work demonstrating that Faa1p and Faa4p are involved in the activation of exogenous long-chain fatty acids, there must be a flow of long-chain fatty acid metabolites proceeding from Fat1p at the plasma membrane to intracellular sites of metabolic utilization. If this is the case, ACBP must play a pivotal role in regulating the flux of long-chain fatty acyl-CoA within the cell. In fact, work from Knudsen's laboratory demonstrated that the disruption of the ACBP structural gene (*ACB1*) alters acyl-CoA metabolism (116). The current challenge is to define how these different proteins function in the flow of these fatty acid metabolites proceeding from transport and activation. Central to this is understanding (i) how Fat1p, Faa1p, and Faa4p function to transport and activate long-chain fatty acids and (ii) whether ACBP is specifically involved in intracellular targeting. At the level of the peroxisome, certain details of long-chain fatty acid targeting are now starting to be resolved. For example, two independent pathways describing long-chain fatty acid transport across the peroxisomal membrane have been defined (73). The first is specific for long-chain fatty acyl-CoAs and requires Pat1p (Pxa2p) and Pat2p (Pxa1p). The second is specific for medium-chain fatty acids, requires the fatty acyl-CoA synthetase Faa2p, and is apparently independent of Pat1p and Pat2p. Pat1p and Pat2p were initially identified as peroxisomal integral membrane proteins, each of which has six or seven predicted transmembrane domains and an ATP binding cassette (ABC) characteristic of ATP binding protein transporters. These two proteins have sequence similarities to the protein product of the human adrenoleukodystrophy gene (ABCD1) and three

related peroxisomal ABC half-transporters (ABCD2 to ABCD4). Deletion of either Pat1p or Pat2p impairs the growth of yeast on the long-chain fatty acids palmitate and oleate but not myristate. The conclusion that these two proteins are involved in fatty acid transport into the peroxisome is based on studies showing that a deletion of either gene reduces β-oxidation of whole cells but not of cell extracts (73). A third putative peroxisomal fatty acid transporter, Fat2p (Psc60p), has been cloned by reverse genetics by selection for peroxisomal proteins induced by oleic acid (30). Although no functional studies of Fat2p have been reported, we have found that this protein has 23% amino acid identity and 45% amino acid similarity to Fat1p (C. C. DiRusso and P. N. Black, unpublished data). Like Fat1p, Fat2p contains a presumptive ATP-AMP signature motif characteristic of the enzymes, which form adenylated intermediates as part of their catalytic cycle (including the FACSs) (30). Yet, unlike these enzymes, Fat2p has no measurable FACS activity. Deletion of Fat2p has no apparent effect on peroxisomal induction and proliferation or β-oxidation of long-chain fatty acids, which indicates either that the protein is not required for these functions or that there is an additional protein(s) that serves the same function.

Lessons Applied to Mammalian Systems

The studies using the bacterial and yeast model systems to investigate the process of fatty acid transport can be directly applied to understanding related processes in mammalian systems. It is apparent that in both bacteria and yeast, fatty acid transport occurs by a process that links transport with activation to CoA thioesters. This process promotes efficiency in the delivery of fatty acids to intracellular sites of utilization (including lipid synthesis, β-oxidation, and transcriptional control).

In mammalian systems, this process is likely to be more complex, in part due to the presence of other putative fatty acid transport proteins (e.g., FATP, FAT-CD36, and $FABP_{pm}$) and intracellular fatty acid binding proteins. In addition, there are multiple isoforms of both FATP and FACS, which provides additional complexity to the process. While the relationships between the FATP isoforms and FAT-CD36 and $FABP_{pm}$ have not yet been defined, is seems likely that a number of FATP isoforms may work in conjunction with a cognate FACS to facilitate the coupled fatty acid import-activation process. In the context of intracellular fatty acid homeostasis, this may function in membrane lipid turnover or complex lipid synthesis. Other evidence showing differential distribution suggests more specific roles for the different FATP isoforms, perhaps in either tissue specificity, developmental specificity, or substrate specificity. The finding that mmFATP1 functions in yeast suggests that this model system is ideally poised to allow us to more completely understand the process of fatty acid transport in eukaryotic systems and the process of vectorial esterification as a fundamental biochemical mechanism, promoting fatty acid transport.

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