

## Cellular uptake of long-chain fatty acids: role of membrane-associated fatty-acid-binding/transport proteins

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**Abstract.** The critical importance of long-chain fatty acids in cellular homeostasis demands an efficient uptake system for these fatty acids and their metabolism in tissues. Increasing evidence suggests that the plasma-membrane-associated and cytoplasmic fatty-acid-binding proteins are involved in cellular fatty acid uptake, transport and metabolism in tissues. These binding proteins may also function in the fine tuning of cellular events by modulating the metabolism of long-chain fatty acids implicated in the regulation of cell growth and various cellular functions. Several membrane-associated fatty-acid-binding/transport proteins such as plasma membrane fatty-acid-binding protein (FABP<sub>pm</sub>, 43 kDa), fatty acid translocase (FAT, 88 kDa) and fatty acid transporter protein (FATP, 63 kDa) have been identified. In the feto-placental unit, preferential transport of maternal plasma arachidonic and docosa-

hexaenoic acids across the placenta is of critical importance for fetal growth and development. Our studies have shown that arachidonic and docosahexaenoic acids are preferentially taken up by placental trophoblasts for fetal transport. The existence of a fatty-acid-transport system comprising multiple membrane-binding proteins (FAT, FATP and FABP<sub>pm</sub>) in human placenta may be essential to facilitate the preferential transport of maternal plasma fatty acids in order to meet the requirements of the growing fetus. The preferential uptake of arachidonic and docosahexaenoic acids by the human placenta has the net effect of shunting these maternal plasma fatty acids towards the fetus. The roles of plasma membrane-associated binding/transport proteins (FABP<sub>pm</sub>, FAT and FATP) in tissue-specific fatty acid uptake and metabolism are discussed.

**Key words.** Plasma membrane fatty-acid-binding protein (FABP<sub>pm</sub>); fatty acid translocase (FAT); fatty acid transporter protein (FATP); free fatty acid (FFA); fatty acid uptake; long-chain polyunsaturated fatty acids (LCPUFAs); long-chain fatty acids (LCFAs).

### Introduction

Long-chain fatty acids (LCFAs) play important roles in cell homeostasis [1–4]. Fatty acids serve as a metabolic energy source, building blocks for membrane lipids and cellular signalling molecules such as eicosanoids [2–6]. In addition, fatty acids may directly or indirectly interact with membranes, transporters, ion channels, enzymes or hormone receptors and thus regulate various cell functions [2–6]. Essential fatty acids (EFAs) and their long-chain polyunsaturated fatty acid (LCPUFA) derivatives are of critical importance in cell growth and development [2–6]. They are also increasingly being recognised as important intracellular mediators of gene expression [7]. Because of the fundamental role of EFA

and LCPUFA as structural elements and functional modulators, it has been hypothesised that the EFA/LCPUFA status of tissue or cells is an important determinant of health and disease [2–6]. The multiple roles of fatty acids suggest that careful regulation of all aspects of their disposition, including cellular uptake and subsequent intracellular transport, are critical for the maintenance of cellular integrity. However, the very property that makes LCFAs well suited to be components of membranes, i.e. their acyl chain hydrophobicity, complicates the process of transporting them from sites of intestinal absorption, hepatic synthesis and lipolysis to sites of utilisation. The insolubility of fatty acids in the aqueous environment requires specific

trafficking mechanisms to deliver fatty acids for cellular needs [8]. The uptake of fatty acids has long been considered to be a passive process, involving partitioning of the fatty acid molecule into the lipid bilayer of the plasma membrane, but recent studies from various laboratories clearly demonstrate the presence of different lipid-binding proteins both in the cytosol as well as in the cell membranes, and their involvement in the uptake and intracellular transport of these water-insoluble molecules [9–12]. These binding proteins may play a role in the transport or targeting of lipids in the cell or in the plasma, and may also interact directly or indirectly by modulation of the free ligand concentration with various cellular processes. Lipid-binding proteins belong to several unrelated families of proteins, and many of these molecules have not been well characterised [9–12]. Several reviews have focused on cytoplasmic lipid-binding proteins, i.e. cytoplasmic fatty-acid-binding protein (FABP), cellular retinoic acid-binding proteins (CRABPs), cellular retinol-binding proteins (CRBPs),  $\alpha$ -tocopherol-binding proteins (TBPs), and acylCoA-binding protein (ACBP) [9–14]. This review will concentrate on the membrane-associated fatty-acid-binding/transport proteins and their important roles in fatty acid uptake and metabolism, especially in the feto-placental unit. The primary purpose of the review is to provide an overview of the crucial steps involved in the cellular processes responsible for selective uptake and retention of LCFAs.

### Cellular fatty acid uptake in the feto-placental unit

The placental uptake of maternal plasma LCPUFA is critically important in fetal growth and development [15, 16]. In fetal development the deposition of LCPUFA in the fetus is rapid during growth. A failure to accomplish a specific component of brain growth due to inadequacy of LCPUFA of critical membrane lipids may lead to irreversible damage [15]. Fetal brain and retina are very rich in the LCPUFAs, arachidonic acid, 20:4n – 6 (ARA) and docosahexaenoic acid, 22:6n – 3 (DHA), and a sufficient supply of these fatty acids during the last trimester of pregnancy and in the neonatal period is of great importance [15–17]. Many studies have shown that levels of LCPUFAs are higher in fetal than in maternal circulation [15, 18, 19], but the underlying biochemical mechanisms controlling this phenomenon are not fully understood. Studies of fatty acid composition in fetal and maternal plasma have shown that at birth, linoleic acid, 18:2n – 6 (LA), represents about 10% of the total fatty acids in cord plasma compared with 30% in maternal plasma. Surprisingly, however ARA concentration in cord plasma is twice ( $\sim 10\%$ ) that observed in the mother ( $\sim 5\%$ ). Similarly,

$\alpha$ -linolenic acid, 18:2n – 3 (ALA), concentration in the newborn (0.3%) is half that in the mother (0.6%), whereas DHA concentration is double (3 vs. 1.5%) [5, 18, 19]. This situation in which the relative plasma concentration of the n – 3 and n – 6 LCPUFAs exceeds that of their precursors is specific to the newborn and is never observed in adults. This is clearly an extremely favourable situation for the development of the newborn, especially at a time when high quantities of ARA and particularly of DHA are needed by the brain and retina.

Placental transport of LCPUFAs from the maternal plasma is crucial for fetal growth and development because fetal synthesis of LCPUFAs is thought to be very low [15, 19]. Also, since human placental tissue lacks both the  $\Delta 6$  and  $\Delta 5$  desaturase activities [20], any LCPUFAs in the fetal circulation must primarily be derived from the maternal plasma.

In the materno-fetal unit, free fatty acids (FFAs) are the main class of naturally occurring lipids transferred across the placenta, irrespective of species or the source from which they originate in the maternal circulation [15, 21]. The uptake of FFAs was long considered to be a passive process, involving partitioning of the fatty acid molecules into the plasma membrane. Many studies have demonstrated that FFAs can traverse synthetic phospholipid/cholesterol membranes at rates that substantially exceed rates of cellular uptake, leading to the argument that there is, therefore, no need for a specific, facilitated uptake mechanism to meet cellular FFA requirements [22, 23]. Since 1981, several groups have clarified the influence of albumin binding on the uptake kinetics of FFAs, making more detailed studies of the uptake processes feasible [8, 24, 25]. Once it became clear that FFA uptake occurred principally from the very small, unbound ligand pool in plasma and not from the albumin-bound compartment, it was then quickly reported that a major component of FFA uptake in certain cell types, including hepatocytes, adipocytes, and cardiac and skeletal myocytes, exhibited all the kinetic properties of facilitated transport processes, i.e. trans-stimulation, cis-inhibition and countertransport [24–29]. All of these observations suggested the presence of a membrane fatty acid uptake protein or transporter. This was further pursued by isolation of membrane fatty-acid-binding protein (FABP<sub>pm</sub>) from rat liver [25]. FABP<sub>pm</sub> was subsequently also found in intestine, heart and other tissues [24, 25]. There is now evidence for the involvement of several membrane-associated fatty-acid-binding/transport proteins in the uptake of FFAs into a variety of mammalian cells such as hepatocytes, adipocytes, cardiomyocytes and jejunal mucosal cells [24, 25]. Although LCFAs move very rapidly across artificial protein-free lipid bilayers [22, 23], fatty-acid-binding or -transport

proteins may be required in biological membranes to (selectively) promote cellular uptake of LCFAs from a complex environment [30, 31]. Five proteins have been suggested to mediate LCFA uptake into cells. Four candidate LCFA transporters, FABP<sub>pm</sub> (43 kDa) [25, 32], 56-kDa renal FABPs [33], caveolin [34] and fatty acid translocase (FAT, 88 kDa) [35] were identified by their ability to bind fatty acids. The fifth, fatty-acid-transport protein (FATP, 63 kDa) was first identified by Schaffer and Lodish using an expression-cloning strategy [36].

### Plasma membrane fatty-acid-binding protein

Much of the evidence for the existence of plasma membrane FABP was derived from detailed binding and kinetic studies using plasma membranes [25]. Long-chain fatty acids such as oleate bind in a saturable manner to freshly isolated hepatic sinusoidal plasma membranes. Binding to membranes has a  $K_d$  value of approximately  $10^{-8}$  M, and heat denaturation of membranes greatly reduces binding, suggesting the existence of high-affinity fatty-acid-binding sites [25, 26, 37]. In several cell types, oleate uptake velocity is a function of the unbound oleate concentration in the presence of physiological concentrations of albumin [25]. After the observation that oleate bound specifically to liver plasma membranes, Stremmel et al. succeeded in isolating a membrane FABP using affinity chromatography [26, 37]. The protein was a single polypeptide of approximately 43 kDa, with no attached carbohydrate, and an isoelectric point of the order of 9.1 [37]. This membrane protein is located as an extrinsic molecule at the outer surface of the cell membrane since it is salt extractable and reacts with its specific antibody in intact cells. The purified protein co-chromatographed with a variety of long-chain fatty acids but not with other organic anions such as bilirubin, bromosulphophthalien or taurocholate [25]. It also did not co-chromatograph with phosphatidylcholine or fatty acid esters such as cholesterylolate. Antibodies to the 43-kDa protein did not cross-react with cytosolic or serum proteins and selectively inhibited the heat-sensitive binding of oleate to liver plasma membranes [25, 37]. This protein was finally designated as plasma membrane fatty-acid-binding protein (FABP<sub>pm</sub>) [25]. Proteins isolated by similar methods from heart, adipose tissue, and intestine cross-react with the liver FABP<sub>pm</sub>, suggesting that the protein is present in almost all tissues [25]. However, whether these proteins all represent the same FABP<sub>pm</sub> or whether tissue-specific types exist awaits the cloning of complementary DNAs (cDNAs).

Purified FABP<sub>pm</sub> and the mitochondrial isoform aspartate aminotransferase (mAspAT) (EC 2.6.1.1) are iden-

tical in terms of their amino acid sequences, isoelectric point and fatty-acid-binding activity [38]. The mAspAT is a well-characterised enzyme which plays an important role in amino acid metabolism and provides a major route of importation of reducing equivalents into mitochondria through participation in the malate:aspartate shuttle. Antibodies raised to both of these proteins have shown considerable cross-reactivity. Anti-rat mAspAT reacts by both immunodiffusion and immunoblotting against hepatic FABP<sub>pm</sub> and vice versa. Anti-FABP<sub>pm</sub> and anti-mAspAT produced similar inhibitory effect on the hepatocellular uptake of radiolabelled oleate, whilst having no effects on the uptake of either bromosulphothalein or taurocholate [38, 39]. Stable transfection of 3T3 fibroblasts with a cDNA for mAspAT conferred saturable fatty acid uptake by these transfected cells [40]. All these studies indicate therefore that these two proteins are identical. Exposure of cultured HepG2 cells to ethanol increased plasma mAspAT levels [41]. This indicates that the increased plasma mAspAT in alcoholics may be due to upregulation of mAspAT messenger (mRNA) and of mAspAT synthesis by ethanol. Increased mAspAT-mediated fatty acid uptake may therefore contribute to alcoholic fatty liver.

The mechanism of FFA uptake by FABP<sub>pm</sub> is still not well established because it is a peripheral membrane protein; its role may be to capture unbound FFAs for transfer to other integral membrane fatty acid transporters (as described below) for fatty acid transport across the cell membranes.

### Fatty acid translocase

The first evidence for the existence of fatty acid translocase (FAT) in mammalian cells was presented by Abumrad et al. [42] during their investigations into fatty acid uptake by adipocytes. As a similar effect was also seen with 4,4-diisothiocyano-2,2'-stilbenedisulfonate (DIDS, a compound known to inhibit the transport of inorganic and monocarboxylate anions in many cell types), adipocytes were incubated with radiolabelled DIDS, and the membrane proteins from the subsequent homogenate subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Most of the radioactivity bound was associated with a 80–90-kDa protein [27]. FAT was also identified by covalent labelling with sulfo-*N*-succinimidylolate (SSO) [43]. FAT is composed of a highly glycosylated polypeptide chain with an apparent molecular mass of 88 kDa which was cloned recently in adipocytes [44]. This 472-amino-acid (53-kDa) protein is substantially glycosylated (10 predicted N-linked glycosylated sites). FAT is present on the surface of human placenta, platelets, endothelial cells, monocytes, erythrocytes and other cell types [44–49]. FAT from rat

shows high sequence similarity (85%) to the human leukocyte differentiation antigen CD36 (glycoprotein IV), a receptor protein present among others in monocytes and platelets and perhaps involved in adhesion phenomena and intracellular signalling [44–49]. Unlike other membrane FABPs, FAT or CD36 is a multifunctional protein and has a number of putative ligands including FFAs, collagen, thrombospondin and oxidised low-density lipoprotein (LDL) [45–51]. A series of studies have shown that CD36 is involved in FFA uptake [44–49]. CD36 (FAT) is strongly induced during adipocyte differentiation and in diabetes, and the induction is paralleled with an increase in FFA uptake by the cells [44–49].

Recently, we have shown that CD36/FAT is involved in arachidonic acid uptake by human platelets [50]. It is possible that increased expression of CD36 may make platelets hyperactive. FAT is the prototype of a small family of single-polypeptide membrane glycoproteins that also includes LIMPII (a lysosomal membrane protein), rodent SR-B1 and CLA-1 (CD36 and LIMPII analogous receptor), the human homolog of SR-B1 [51, 52]. CD36 or FAT is predicted to have two trans-membrane domains at either ends of the molecule with short amino-terminal and carboxy-terminal cytoplasmic domains and a large, multiply N-glycosylated extracellular loop [53]. The configuration of CD36 or FAT suggests that it might effectively promote fatty acid uptake. Most of the protein is extracellular and forms a hairpin structure composed of small hydrophobic pockets [35]. CD36/FAT is fatty acetylated with myristate linked by an amide bond to an amino-terminal glycine, and palmitate linked by thioester bonds to at least one of the two cysteines located at the junction of the carboxyl-terminal trans-membrane and cytoplasmic domains [54]. CD36/FAT can associate with caveolae in transfected CHO cells or cultured Y1-BS1 adrenocortical cells [54]. Macrophages have, however, functional CD36/FAT despite their lack of caveolae. Thus, the association with caveolae may not be essential for CD36 function. The human gene for CD36 is located on chromosome 7.

CD36 or FAT appears to be a multifunctional protein that interacts with collagens type I and IV, oxidised LDL, fatty acids and anionic phospholipids. Recently it has been shown that LDL, high-density lipoprotein (HDL) and very low density lipoprotein (VLDL) can all bind CD36 with high affinity, although the apparent affinity for oxidised LDL is about threefold greater than that of native LDL [55]. Studies using monoclonal antibodies specific for different parts of the molecule, as well as domain swapping in COS cells transfected with CD36, showed that amino acid residues 155–183 of human CD36 were important for binding of oxidised LDL [56]. CD36 or FAT binds long-chain fatty acids

but has no preference for particular fatty acids [57]. Thus, binding was reversible and distinct from the palmitoylation of the protein known to occur on an extracellular domain. Comparison of the predicted secondary sequence of CD36 or FAT with that of human muscle FABP suggested that a potential binding site for the fatty acid on CD36 may exist in its extracellular segment between residues 127 and 279 [57].

The spontaneously hypertensive rat (SHR) is insulin resistant and a good model of these human syndromes. Quantitative trait loci for SHR defects in glucose and fatty acid metabolism, hypertriglyceridaemia and hypertension map to a single locus on rat chromosome 4. cDNA microarrays, congenic mapping and radiation hybrid mapping were used in combination to identify a defective spontaneously hypertensive rat gene, CD36 or FAT, at the peak of linkage to these quantitative trait loci. In the spontaneously hypertensive rat, CD36 cDNA contains multiple sequence variants caused by unequal genomic recombination of a duplicated ancestral gene [58]. The encoded protein product is undetectable in spontaneously hypertensive rat adipocyte plasma membrane. Transgenic mice overexpressing CD36 have decreased blood lipid levels compared with controls. Therefore, CD36 deficiency underlies insulin resistance, defective fatty acid metabolism and hypertriglyceridaemia in SHR and may be important in the pathogenesis of human insulin-resistance syndromes.

In addition, CD36 is physically associated with protein kinases of the src family which play important roles in the control of cellular differentiation. Further studies are clearly required to understand these complex regulations. In that respect, gene disruption experiments would be helpful to characterise the respective roles of these proteins, i.e. the peroxisome proliferator-activated receptor (PPAR) and LCFA-induced proteins in the control of cell proliferation and differentiation of various fatty acids.

#### **Fatty-acid-transporter protein**

Although FAT or CD36 binds LCFA and might be involved in signal transduction after fatty acid binding, it is still not clear whether it is a transport protein. The only candidate for an LCFA transporter for which functionality has been directly demonstrated is the fatty-acid-transporter protein (FATP). In adipocytes, FATP of molecular mass 63 kDa was implicated in the trans-membrane transport of FFA [37]. FATP is thought to facilitate bidirectional LCFA movement across the plasma membrane [31]. FATP is an integral plasma membrane protein with four to six predicted membrane-spanning regions, with the highest levels found so far in skeletal muscle, heart and fat cells, and

lower levels in brain, kidney, lung and liver [37]. Interestingly FATP is suggested to act in concert with fatty acylCoA synthetase (FACS), an enzyme that prevents efflux of the incorporated fatty acids by their conversion into acylCoA derivatives and hence rendering fatty acid uptake unidirectional. FATP shows a small 11-amino acid region of similarity to FACS, leading to the hypothesis that this common region might reflect a common function, such as affinity for binding sites [36]. These long-chain fatty acylCoA esters act both as substrates and intermediates in various intracellular functions. Cytoplasmic acylCoA binding protein (ACBP) binds long-chain fatty acylCoA esters with high affinity and is believed to play an important role in intracellular acylCoA transport and pool formation and therefore also for the function of fatty acylCoA esters as metabolites and regulators of cell function [9–11].

FATP mRNA is present in low levels in 3T3-L1 preadipocytes; it is upregulated 5–7-fold as a consequence of adipose conversion, whereas insulin downregulates FATP mRNA levels 10-fold in cultured adipocytes [31, 37, 59]. Regulation of FATP expression by insulin is rapid, reversible and exerted at the transcription level [59]. In animal studies, FATP mRNA levels in murine adipose tissue increased 11-fold during fasting, consistent with the role for insulin as a negative regulator of FATP gene expression [59]. Berk et al. recently demonstrated that FATP mRNA levels were increased 5-fold in insulin-resistant Zucker rats when compared with control animals [60]. Inhibitory action of insulin on FATP transcription is mediated through a cis-acting phosphoenolpyruvate carboxykinase-like element located at the region –1353 and –1347 [61].

The FATP gene is located on chromosome 8 and is close to the genes encoding lipoprotein lipase and uncoupling protein-1 (UCP-1) [61]. The IYSGTTGXPK motif common to all FATPs in divergent species is similar to domains in other proteins postulated or known to form adenylated intermediates [62]. This observation suggests that FATP may facilitate uptake of LCFA via an ATP-dependant mechanism. FATP-mediated uptake of LCFA was diminished in the face of cellular depletion of ATP. Mutation of the central serine in the IYSGTTGXPK motif dramatically decreased FATP function [62]. Additionally, FATP interacts directly with ATP, and serine 250 is important for this interaction. This residue is critical for LCFA transport function, probably due to a role in ATP binding. In addition to insulin, expression of FATP is regulated by other factors such as PPAR ligands. Since FATP is likely to be responsible for the increased LCFA necessary to sustain this increased  $\beta$ -oxidation, the tissue-selective effects of various PPAR activators and ligands on FATP and FACS provide insight in the relationship between FFA uptake and triglyceride synthesis and

$\beta$ -oxidation of fatty acids [63, 64]. The PPAR $\alpha$ -specific activators (fibrates) and the PPAR $\gamma$ -specific antidiabetic thiazolidinedione (BRL 49653) have tissue-specific effects on FATP and FACS gene expression. Fibrate treatment induced FATP and FACS expression most effectively in liver, whereas BRL 49653, the high-affinity ligand for PPAR $\gamma$ , had no effect on liver but induced FATP and FACS expression in adipose tissue [63]. The induction of these proteins was at the level of transcription and was associated with changes in cellular FFA uptake. In obesity, the adipose tissue-specific increase in FFA uptake may have the net effect of shunting plasma FFA away from tissues where they would be oxidised as fuel and towards adipose tissue where they are stored as fat [60]. The observations that tissue-specific physiological alterations in FFA uptake occur in obesity raises the possibility that equally tissue-specific pharmacological manipulations could reverse these changes. However, pursuit of this approach should logically be based on an understanding of the factors that regulate FFA transport in the relevant tissue. Furthermore, a striking parallelism exists between the induction by fibrates of a number of genes involved in fatty acid transport in the liver. For instance, mRNAs for lipoprotein lipase, FACS and FATP genes are all induced after fibrate treatment in the liver [63]. Recently, it has been demonstrated that the FATP gene possesses a peroxisome proliferator response element (PPRE) and is upregulated by activators of PPAR $\alpha$  and PPAR $\gamma$ , thereby linking the activity of the protein to the expression of its gene [65]. The demonstration of the inducibility of the FATP and FACS genes by PPAR $\gamma$  ligands has important implications for adipocyte physiology. PPAR $\gamma$ -mediated activation of FATP and FACS expression in cells of the adipogenic lineage may be responsible for adipocyte differentiation and induce development of obesity as observed in case of thiazolidinedione [66, 67]. In this context, it is worth noting that PPAR $\gamma$ -mediated effects of compound BRL49653 on FATP and FACS expression may act in concert with induced lipoprotein lipase expression, and the reduced levels of leptin mRNA and protein levels [67, 68]. Thus, BRL 49653 contributes to the increase in caloric intake by enhancing energy storage in the adipocytes [67, 68]. Interestingly, FATP and FACS are not coordinately regulated in perirenal and epididymal adipose tissue stores. Further studies are required to determine whether the role of FATP is a consequence of, or the cause of, the physiological differences between the adipose tissue stores. FATP and FACS mRNA levels can be regulated in a tissue-specific fashion by PPAR $\alpha$  activators and PPAR $\gamma$  ligands. In adipose tissue the increase in FATP, FACS and lipoprotein lipase production [69] after treatment with thiazolidinediones would therefore enhance the clearance of plasma triglycerides and provide the

preadipocytes with additional fatty acids which can further stimulate the trans-activation capacity of PPAR or which can be stored in the form of triglycerides. In the liver, the enhanced production of FATP and FACS after treatment with fibrates, together with the increase in  $\beta$ -oxidation and reduced production of apoCIII, may contribute to the hypolipidemic action of PPAR ligands. This tissue-selective induction of FATP and FACS gene transcription by activators of different PPARs demonstrates the feasibility of the development of highly specific PPAR subtype-specific agonists and antagonists, which can be used therapeutically.

#### **Presence of multiple membrane fatty acid transporters in tissues**

It has been suggested that specific membrane-associated and cytoplasmic fatty-acid-binding proteins cooperate in the uptake and retention of LCFA by various cells. It is difficult to know whether expression of a specific membrane protein is involved in LCFA uptake or whether the multiple membrane proteins that bind LCFA are coordinately upregulated. For example, in intestinal microvillous membranes FAT and cytoplasmic FABPs (I-FABP and L-FABP) are responsive to the dietary content of LCFA [70, 71]. Coexpression of FAT and H-FABP in muscle tissue as well as coexpression and association of both proteins in mammary gland have been reported [72]. In the intestine, FAT was coexpressed with the intestinal and liver type FABP [70]. FABP<sub>pm</sub>, FAT and FATP are present in a number of tissues including adipose, muscle, heart and so on [71–73]. The metabolic significance of this observation is unclear at present. When red and white muscles are compared, FATP mRNA and FTA mRNA abundance scale roughly with the LCFA oxidative capacities of these tissues [73–75]. This suggests that the lower LCFA oxidation rates of white muscle compared with red muscle may be due, in part, to the lower LCFA transport capacities of the white muscles [75]. However, which of the transporters is most critical is not known; alternatively the transporter may function in a cooperative manner to facilitate the movement of LCFA across the membranes. Knowledge of the tissue distribution of these proteins putatively involved in the LCFA uptake process can provide insight into the possibility that they may function in tandem to efficiently transport LCFA across cell membranes. Additionally, LCFA transport and esterification may also be coupled in mammalian cells to promote efficient LCFA import. Codistribution of FATP and FACS at the adipocyte plasma membrane and the synergistic effects of the two proteins on LCFA uptake in NIH 3T3 cells suggest that these proteins function coordinately to facilitate efficient LCFA im-

port in adipocytes [76]. In contrast to FATP, which acts in FFA transport, FACS prevents the efflux of the imported FFA by converting them into acylCoA derivatives, which can subsequently be used in both anabolic and catabolic pathways. The ability of cells to esterify LCFA directly at the plasma membrane has implications for the directionality of LCFA movement across the plasma membrane and for the mechanism of intracellular trafficking.

#### **Identification of placental membrane-associated fatty-acid-binding/transport proteins**

The placenta comprises highly specialised trophoblast cells, which arise from the embryo and differentiate to perform specialised functions. These functions include invasion of the uterine wall, nutrient and waste transport, metabolism, evasion of the maternal immune system, and cytokine and hormone production. The ability of fatty acids to cross the placenta is crucial for proper fetal growth and development, brain development, and cardiovascular and lung development [16]. Although passive diffusion does occur for some nutrient transfer, the fetal requirement for these nutrients is so great that passive diffusion alone is not adequate. Specific nutrient carriers, or transport proteins are, therefore, located in the placenta that act to facilitate transfer and meet the increased nutrients demand of the fetus during gestation.

In order to examine the role of the placenta in the processes responsible for the preferential accumulation of LCPUFAs in the fetus, we studied the uptake of various LCFAs by isolated human placental membranes, and human placental choriocarcinoma (BeWo) cells [16]. We investigated whether the placenta is capable of preferentially transporting LCPUFAs from the maternal circulation, and, if so, what biochemical processes are involved in preferential LCPUFA transfer from mother to the fetus.

#### **Placental plasma membrane fatty-acid-binding protein (p-FABP<sub>pm</sub>)**

The mechanisms involving preferential uptake of maternal LCPUFAs by the placenta was first examined in our laboratory by determining the fatty-acid-binding characteristics of human placental membranes using four different radiolabelled fatty acids [77]. The total FFA concentration in the plasma of pregnant mothers during the last trimester of pregnancy is around 0.75 mM, of which saturated fatty acids are 35%, monounsaturated fatty acids 41%, EFA (LA and ALA) 20% and LCPUFAs 4%, whereas the albumin concentration is 34.2 g/l [76]. This gives an approximate molar ratio of FFA to

albumin of 1:1. In this study, the molar ratio between albumin and fatty acid was 1:1. The binding of EFA/LCPUFA to human placental membranes was highly reversible compared with that of oleic acid, 18:1n-9 (OA). In addition, OA binding was inhibited very strongly by LCPUFA [ARA,  $\gamma$ -linolenic acid (18:3n-6, GLA), and EPA], followed by the relevant parent EFA (LA and ALA). The lack of strong inhibition of the binding of EFA/LCPUFA to placental membranes by OA suggests the existence of stronger affinities for EFA/LCPUFA compared with OA [77]. Eicosapentaenoic acid (EPA) and its eicosanoid metabolites are growth inhibitory as they reduce the availability of ARA and its metabolites by competing at cyclooxygenase/lipoxygenase and desaturation/elongation pathways of EFA metabolism. However, EPA has very little inhibitory effect on ARA binding to human placental membranes [66]. In contrast to EPA, its parent fatty acid, ALA inhibited the binding of both ARA and LA strongly. The competition experiments also suggested that the binding sites have heterogeneous binding affinities for different fatty acids. Binding sites had a strong preference for LCPUFA, the order of efficiency of competition being  $AA > > > LA > ALA > > > OA$  [77].

Evidence for the involvement of membrane protein in fatty acid uptake came from trypsin-treated placental membranes, which showed a decrease in specific [ $^{14}C$ ] fatty acid binding compared with that of untreated membranes [77]. The presence of an FABP<sub>pm</sub> in the placenta was then investigated. The presence of FABP<sub>pm</sub> both in sheep and human placental membranes was demonstrated [78, 79]. A 40-kDa protein which binds only long-chain fatty acids was then isolated and purified from human placental membranes [79]. The apparent molecular mass of the protein was determined by gel permeation chromatography and by SDS-PAGE. The pI value and the amino acid composition of human placental protein are different from those of hepatic or gut FABP<sub>pm</sub> [79]. In addition, unlike ubiquitous FABP<sub>pm</sub> [25], placental FABP<sub>pm</sub> did not have AspAT activity [80]. Therefore, despite having a

similar size and membrane location (both are peripherally membrane bound protein), p-FABP<sub>pm</sub> and ubiquitous FABP<sub>pm</sub> differ both in structure and function. We therefore designated the placental FABP<sub>pm</sub> as p-FABP<sub>pm</sub> [80]. Table 1 summarises the membrane-associated fatty-acid-binding/transport proteins which have been characterised to date.

Preincubation of placental membranes with polyclonal antiserum against the p-FABP<sub>pm</sub> inhibited the binding of fatty acids to a degree which varied depending on the type of fatty acid [77]. Antibody-mediated inhibition was much more acute for EFA/LCPUFA binding than OA binding, indicating that p-FABP<sub>pm</sub> is preferentially involved in placental EFA/LCPUFA uptake. Fatty-acid-binding activity, PAGE radiobinding assay and Western blot analysis of human placental membranes clearly demonstrated that the p-FABP<sub>pm</sub> is exclusively located in the microvillous membranes [81]. Since p-FABP<sub>pm</sub> may be responsible for preferential LCPUFA uptake, its location in the maternal facing side of the placenta favours the unidirectional flow of maternal LCPUFAs to the fetus.

To further elucidate the mechanisms of preferential transfer of maternal plasma LCPUFAs across the human placenta, direct binding of the purified p-FABP<sub>pm</sub> with these fatty acids was investigated [82, 83]. p-FABP<sub>pm</sub> bound only 14.58% of the total fatty acids when the protein was incubated with a mixture of fatty acids possessing the exact composition and concentration as the maternal plasma FFA pool during the last trimester of pregnancy. Almost 98% of the ARA and 87% of DHA present in the mixture bound to the protein which was much higher than for OA and LA (21 and 13%, respectively) [82]. Binding of palmitic and  $\alpha$ -linolenic acid was nil or insignificant. In contrast, under identical experimental conditions, human serum albumin (HSA) bound 36% of the total fatty acids but did not show any preference for particular fatty acids [82]. Radiolabelled fatty acid binding to these proteins revealed that p-FABP<sub>pm</sub> had higher affinities ( $K_d$ ) and binding capacities ( $B_{max}$ ) for LCPUFAs compared with other fatty acids. The apparent  $B_{max}$  values for OA, LA,

Table 1. Plasma membrane fatty-acid-binding/transport proteins.

Name	Size (kD)	year	Sites	Similarities	Proof of function	References
FABP <sub>pm</sub>	43	1985	Liver, adipose, cardiac muscle, intestine, endothelium, placenta	MAspAT	Antibody inhibition, gene expression	[26]
FABP <sub>pm</sub>	56	1987	Kidney, cardiac muscle	?	not reported	[33]
FABP <sub>pm</sub>	22	1991	Adipose	? caveolin	not reported	[34]
FAT	88	1993	Liver, adipose, cardiac muscle, intestine, endothelium, placenta	CD36	inhibition by protein labelling	[35]
FATP	63	1994	Liver, adipose, cardiac muscle, intestine, endothelium, placenta	FACS	gene expression	[36]
p-FABP <sub>pm</sub>	40	1995	Placenta	LIMPII?	antibody inhibition	[78]

Table 2. Effect of fatty acid addition on oleic and linoleic acids uptake by BeWo and HepG2 cells\*.

Radiolabelled fatty acid + unlabelled fatty acid	BeWo cells	HepG2 cells (nmol/mg protein)
	Fatty acid uptake	
[ <sup>3</sup> H]Oleic acid, 18:1n-9	5.36 ± 0.14	22.89 ± 0.34
[ <sup>3</sup> H]Oleic acid, 18:1n-9 + linoleic acid, 18:2n-6 (-10 fold)	1.41 ± 0.15†	8.67 ± 0.48‡
[ <sup>14</sup> C]Linoleic acid, 18:2n-6	6.72 ± 0.50	27.78 ± 1.59
[ <sup>14</sup> C]Linoleic acid, 18:2n-6 + oleic acid, 18:1n-9 (-10 fold)	5.40 ± 0.30	16.83 ± 1.89‡
[ <sup>14</sup> C]Linoleic acid, 18:2n-6 + α-linolenic acid, 18:3n-3 (-10 fold)	1.14 ± 0.09§	17.49 ± 0.362‡

\*  $x \pm \text{SEM}$  of three separate experiments in which triplicate determinations were performed [F. M. Campbell, A. K. Dutta-Roy, unpublished]. Competition between [<sup>3</sup>H]oleic acid or [<sup>14</sup>C]linoleic acid for uptake by BeWo and HepG2 cells was carried out in the presence of and absence of a 10-fold excess of unlabelled fatty acid. The statistical significance was determined using a two-tailed Student's *t* test, *P* values equal or less than 0.05 were considered significant. For experimental details, see [45]. † *P* > 0.0001; ‡ *P* < 0.005; § *P* < 0.001 vs. control (no inhibitors).

ARA and DHA were  $2.0 \pm 0.14$ ,  $2.1 \pm 0.17$ ,  $3.5 \pm 0.11$ ,  $4.0 \pm 0.10$  mol/mol of p-FABP<sub>pm</sub> whereas the apparent *K<sub>d</sub>* values were  $1 \pm 0.07$ ,  $0.73 \pm 0.04$ ,  $0.45 \pm 0.03$  and  $0.4 \pm 0.02$  μM, respectively [83]. In the case of HSA, the *K<sub>d</sub>* and *B<sub>max</sub>* values for all fatty acids were around 1 μM and 5 mol/mol of protein, respectively. These data provide direct evidence for the role of p-FABP<sub>pm</sub> in preferential sequestration of maternal LCPUFA by the placenta for transport to the fetus [83].

In order to elucidate further the mechanisms by which fatty acids are taken up by the placenta, the uptake of OA, LA, ARA and DHA by cultured human placental choriocarcinoma (BeWo) cells was examined [80, 84]. Fatty acid uptake by BeWo cells was temperature dependent and exhibited saturable kinetics. OA was taken up least and DHA the most by these cells. Moreover, competitive studies of fatty acid uptake by BeWo cells also indicated preferential uptake compared with OA in the order of DHA, AA and LA. When fatty acid uptake was compared between BeWo cells and HepG2 cells, EFA/LCPUFAs are taken up by BeWo cells preferentially over OA. However, no such discrimination was observed in experiments with HepG2 cells (table 2). These two cells types are derived from organs with specialised roles in lipid metabolism, i.e. liver (HepG2) is involved in a multiplicity of lipid metabolic roles including synthesis, esterification, lipoprotein export and oxidation for energy, and the placenta (BeWo) is primarily concerned with sequestration of maternal EFAs/LCPUFAs and transporting these to the fetus.

Western blot analyses demonstrated the presence of p-FABP<sub>pm</sub> in BeWo cells which was absent from HepG2 cells, confirming our previous observation that the placental protein is different from the hepatic protein [80]. Furthermore, pretreatment of BeWo cells with anti-p-FABP<sub>pm</sub> antibodies inhibited most of the uptake of DHA (64%) and AA (68%), whereas OA uptake was inhibited only 32% compared with the controls treated with pre-immune serum [80]. The order of fatty acid uptake inhibition by the antibodies was DHA > ARA > > > ALA > LA > > > OA, indicating that the p-FABP<sub>pm</sub> is involved in preferential uptake of LCPUFAs by these cells, similar to what was observed with human placental membranes [77]. Together, these results demonstrate the preferential uptake of EFAs/LCPUFAs by BeWo cells which is most probably mediated via the p-FABP<sub>pm</sub>.

These results clearly demonstrate that the p-FABP<sub>pm</sub> may be involved in the preferential uptake of LCPUFAs by these cells. Studies on the distribution of radiolabelled fatty acids in the cellular lipids of BeWo cells showed that DHA was incorporated mainly in the triacylglycerol fraction, followed by the phospholipid fractions, whereas for ARA the reverse was true. The preferential incorporation of DHA into triacylglycerol suggests that triacylglycerol may play an important role in the placental transport of DHA to the fetal circulation.

#### Identification and location of FAT and FATP in human placenta

In addition to p-FABP<sub>pm</sub>, the presence of FAT/CD36 and FATP was demonstrated in human placenta using both pure trophoblast cells and placental membrane preparations [45]. FAT and FATP are present in both the placental membranes, microvillous and basal membranes, whereas p-FABP<sub>pm</sub> is present only in microvillous membranes [45]. Location of FAT and FATP on both sides of the bipolar placental cells may allow bidirectional flow of all FFAs (nonessential fatty acids, EFAs and LCPUFAs) across the placenta, whereas the exclusive location of p-FABP<sub>pm</sub> on the maternal side may favour the unidirectional flow of maternal LCPUFAs to the fetus by virtue of its preference for these fatty acids. However, further work is necessary to understand the mechanistic roles of these proteins in trophoblasts which may provide insight into lipid transport and metabolism in the placenta. Although the role of these membrane proteins in complex FFA uptake and metabolism in the human placenta is yet to be understood, studies in other tissues suggest that these proteins alone or in tandem may be involved in effective uptake of FFAs.



### Other proteins and enzymes involved in fatty acid transport across the placenta

The presence of cytoplasmic H-FABP and L-FABP in human placental trophoblasts [45] indicates that complex interactions of these proteins with membrane-binding proteins may be essential for effective fatty acid transport across the placenta. There are significant differences in the properties and regulation of these two FABP types. H-FABP binds only long-chain fatty acids, whereas L-FABP binds heterogeneous ligands such as bile salts, heme, PPAR, selenium, lysophosphatidic acid and eicosanoids (see review [16]). By virtue of its binding of eicosanoids, L-FABP may play a critical role in eicosanoid synthesis in the feto-placental unit [16]. Differential effects of FABP types on fatty acid uptake and esterification were also demonstrated using transfected mouse L-cell fibroblasts. L-FABP increases fatty acid uptake and targets fatty acids for esterification into phospholipids. Although there are differences in the function and binding activity of L-FABP and H-FABP types, their individual and coordinated roles and expression in trophoblasts are yet to be established. It is possible that a complex interaction between these proteins may be required for the effective uptake of maternal FFA by the placenta and their subsequent transport to the fetus.

Plasma FFAs are not only the sources of fatty acid for cellular uptake [16, 85, 86]. Lipases associated with vascular endothelium or in the placenta may play an important role in providing FFA from circulating triacylglycerol. Placental lipase activities were dramatically increased during the last trimester of pregnancy, whereas there was an overall decrease in lipoprotein lipase activity, resulting in a subsequent decrease in triacylglycerol storage [16, 87]. Consequently, with decreased triacylglycerol hydrolysis for maternal storage, and an increase in placental lipolytic activity, the resulting availability of suitable substrate (triacylglycerol) is utilised by placental lipase for the provision of FFA for fetal transport. Triacylglycerol hydrolase activities of human placenta have been characterised in detail in our lab [88]. Homogenates of placenta exhibited three distinct triacylglycerol hydrolase activities with pH optima 4.5, 6.0 and 8.0. On further fractionation, placental cytosol exhibited both acid cholesterol ester hydrolase (pH 4.5) and hormone-sensitive lipase (pH 6.0) activities, whereas purified placental microvillous membranes exhibited two distinct triacylglycerol hydrolase activities: a minor activity at pH 8.0 and a second major activity at pH 6.0. Triacylglycerol hydrolase activity at pH 8.0 of microvillous membranes appeared to be lipoprotein lipase (consistent with criteria such as serum stimulation and salt inhibition), whereas at pH 6.0 the activity was unique in that it was almost

abolished by serum but was not affected by high NaCl concentrations. In contrast to lipoprotein lipase, this enzyme activity was only partially released from the membranes by heparin treatment, was inactivated by serum and was unaffected by the presence of high concentrations of NaCl. The triacylglycerol hydrolysing activity at pH 6.0 represented up to 80% of the total triacylglycerol hydrolysing capability of microvillous membranes. This unique triacylglycerol hydrolase activity at pH 6.0 may be involved in the packaging and/or releasing FFAs from the placenta, whereas lipoprotein lipase, which is mostly present in placental macrophages, may be responsible for hydrolysis of maternal plasma lipoproteins.

Both lipoprotein lipase and p-FABP<sub>pm</sub> are located only in microvillous membranes; this location at the same side (microvillous membrane) of bipolar cells is necessary for these proteins to work in tandem in placental sequestration of maternal LCPUFAs from circulating triglycerides for transport to the fetus. In fact, the discovery of a p-FABP<sub>pm</sub> and other membrane-binding proteins (FAT and FATP) in the human placenta provides us with a unique opportunity to study the role of the placenta in supply of EFAs/LCPUFAs in both physiological and clinical conditions such as intrauterine growth retardation, small for gestation age, diabetic pregnancies and prematurities. Future studies on the coordinate expression and relationships among these proteins (p-FABP<sub>pm</sub>, FAT, FATP, L-FABP, H-FABP and PL) in the placenta are required in order to understand their roles in the preferential uptake of maternal LCPUFAs. At the moment, the roles of H-FABP and L-FABP are not well established in the placenta, but they could play important roles by controlling the metabolic fate of fatty acids (i.e. oxidation or esterification) and thus modulation of cell growth and development (via PPAR $\gamma$ ). Since fatty acids play nutritional, metabolic and functional roles in mammalian systems, their diverse effects may depend on their specific transport to the tissue and subsequent metabolism. Figure 1 summarises putative roles of the plasma-membrane-associated and cytoplasmic fatty-acid-binding/transport proteins in placental fatty acid uptake and metabolism. In addition, the role of ACBPs in transporting acylCoA esters and their hydrolysis by acylCoA hydrolases to provide FFAs for PPAR $\gamma$ -induced cell proliferation and differentiation of placental cells [unpublished observation, A. K. Dutta-Roy et al.].

Plasma FFA composition and concentration are dynamic and depend on many factors, including state of pregnancy, obesity, insulin resistance, other metabolic and dietary conditions [89]. Therefore, plasma FFA uptake and internalisation by living cells are highly regulated to meet specific requirements of particular cells or tissues, involving a sequence of steps: dissocia-

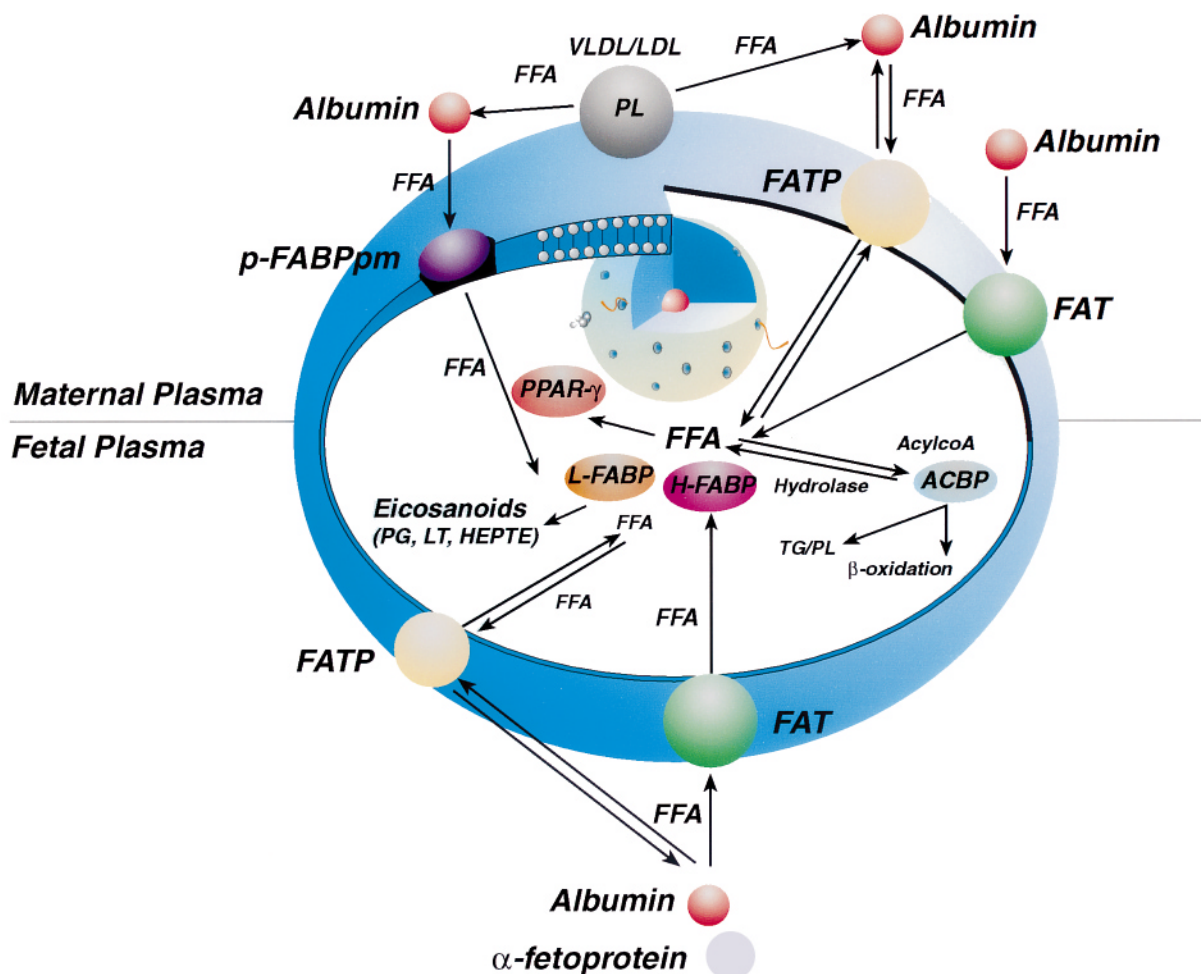


Figure 1. Schematic diagram of the putative roles of the placental plasma-membrane-associated and cytoplasmic FABPs in fatty acid uptake and metabolism in the placenta. Location of FAT and FATP on the both sides of the bipolar placental cells and lack of specificity for particular types of fatty acids suggests they may transport all FFAs (nonessential, EFAs and LCPUFAs) in both directions, i.e. from the mother to the fetus and vice versa, whereas p-FABP<sub>pm</sub> preferentially sequesters maternal plasma LCPUFAs. Cytoplasmic FABPs may be responsible for transcytoplasmic movement of FFAs to their sites of esterification,  $\beta$ -oxidation or to the fetal circulation via placental basal membranes. VLDL, very low density lipoproteins; PL, placental lipase, (pH optima 6.0); PPAR $\gamma$ ; peroxisome proliferator-activated receptor- $\gamma$ ; ACBP, acylCoA-binding protein (adapted from [45] with permission).

tion of FFAs from albumin, transport across the plasma membranes (via p-FABP<sub>pm</sub>, FAT, and FATP and FACS) and transport within the cytoplasm and its metabolism via several proteins (FABPs, ACBP, acyl-CoA hydrolase and PPAR $\gamma$ ). A focus for future studies will be to further establish the complex interrelationships of the many multiple proteins influencing cellular LCFA uptake and retention.

**Acknowledgements.** This paper is dedicated to Prof. B. R. Roy, ex-director of the Central Food Laboratory, Calcutta, India, a great teacher and humanist, on the occasion of his 75th birthday. This work was supported by the Scottish Executive Rural Affairs Department (SERAD).

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