Properties and physiological significance of fatty acid binding proteins

Norbert H. Haunerland[a] and Friedrich Spener[b,*]

[a]*Department of Biological Sciences, Simon Fraser University, Burnaby, BC, Canada V5A 1S6*
[b]*Department of Biochemistry, University of Münster, Wilhelm-Klemm-Str. 2, 48149 Münster, Germany*
[*]*Correspondence address: Tel.: +49-251-83-33100; fax: +49-251-83-32132*
E-mail: spener@uni-muenster.de(F.S.)

Abbreviations

CRABP: cellular retinoic acid binding protein; CRBP: cellular retinol binding protein; FABP: fatty acid binding protein; I-BABP: intestinal bile acid binding protein; iLBP: intracellular lipid binding protein; ITC: isothermal titration calorimetry; PPAR: peroxisome proliferator activated receptor; PPRE: peroxisome proliferator responsive element; RAR: retinoic acid receptor; RARE: retinoic acid responsive element; RXR: retinoid X receptor.

1. Introduction

In 1978, Ockner et al. [1] discovered a small protein in the cytosol of certain rat tissues that bound fatty acids and consequently named it "fatty acid binding protein" (FABP). Since then, such FABPs have been found in many tissues of many different organisms which include mammals, fish, birds, and insects. Some of these proteins were originally characterized in a different context (organic anion binding protein, Z-protein) and only later were found to be FABPs. All FABPs are members of a large multigene family now called "intracellular lipid binding proteins" (iLBPs) with various functions in the transport and metabolism of their ligand fatty acids and other lipophilic ligands. Many excellent reviews have been published on different aspects of these proteins (for a recent review see Ref. [2]), which are remarkably conserved throughout the animal kingdom. While their roles in different cells, tissues, and organisms may vary, common features become apparent in the context of metabolic tasks and conditions. The purpose of this review is to summarize current knowledge about these proteins, and to provide insight into their roles in different organisms.

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2. FABPs as members of the iLBP family

FABPs as members of the iLBP family have traditionally been named after the tissue from which they were first isolated. Liver-type, heart-type, and intestinal-type FABP (L-FABP, H-FABP, I-FABP) have been the first to be discovered [1], and later the aP2 protein was recognized as adipocyte-type (A-) FABP [3]. With the increasing availability of ESTs and gene array data, it has become clear that most iLBPs are not confined to a single tissue. This, however, does not necessarily mean that they are un-specifically expressed, as tissues always contain different cell types. For example, heart tissue contains not only cardiomyocytes, but also significant amounts of epithelial and smooth muscle cells as well as some adipocytes. Moreover, even defined cells such as adipocytes express more than one FABP-type [4]. This is even more apparent when FABPs expressed in non-mammalian animals are considered: for example, the most prominent FABP-type expressed in shark liver [5] clearly belongs to the same subfamily (see below) as H-FABP, while the FABPs found in the livers of other fish species of chicken and are basic proteins, yet distantly related to the mammalian L-FABP [6].

In this review, the widely accepted nomenclature for FABP that is based on the tissue occurrence will be used. The numerical classification used by Genbank may be more accurate, but less intuitive. In Table 1 the classical names, alternative designations found in the literature and the GenBank designations are summarized, as is the occurrence of the proteins in tissues of mature animals.

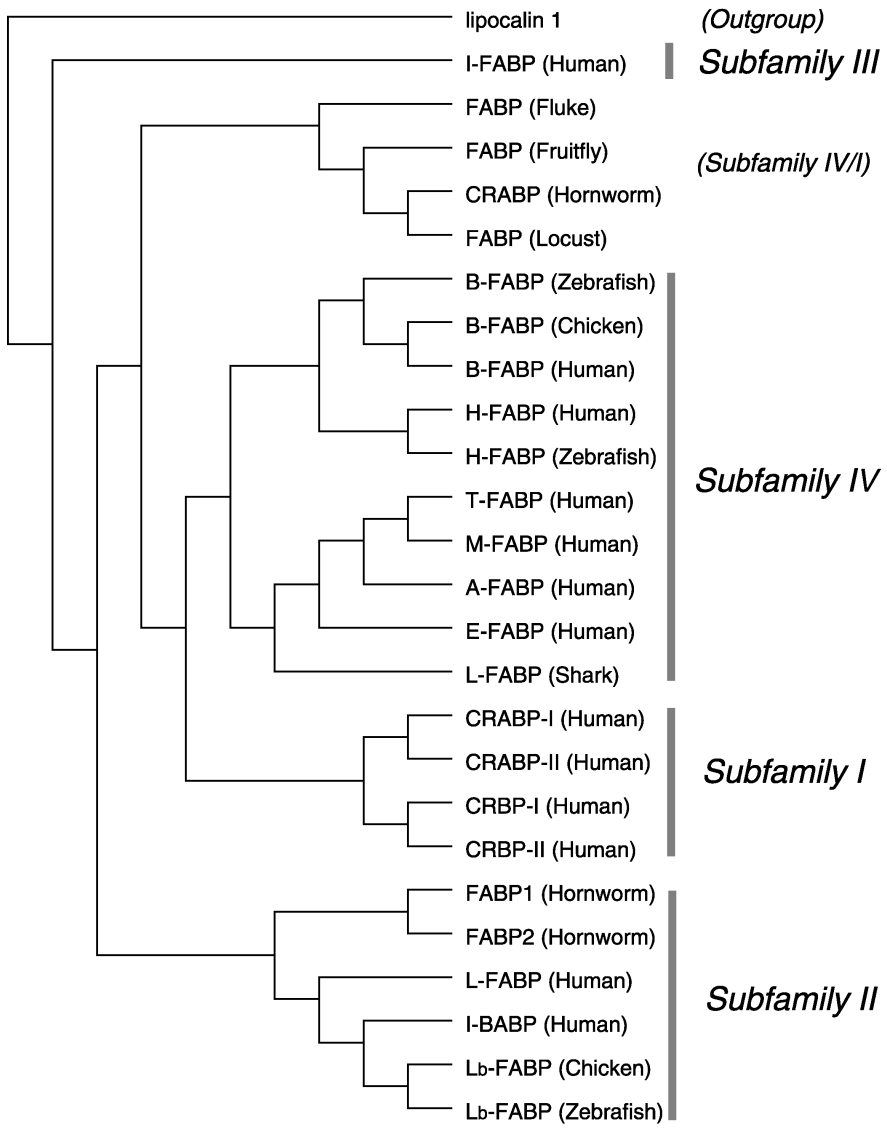
FABPs are expressed in vertebrate (mainly mammals, fish, birds) and invertebrate species. Pertaining to the latter, two FABPs are expressed in the midgut of the tobacco hornworm (*Manduca sexta*) [7] and believed to be involved in lipid digestion. The FABP from the flight muscle of locusts has been especially well characterized [8,9]. It is present in high concentration and shares many characteristics with its mammalian H-FABP counterparts. They have a high sequence homology to other insect proteins that have been identified only at cDNA levels, namely from the fruit fly (*Drosophila melanogaster*) [10] and the mosquito *Anopheles gambiae* [11]. A protein found in the brain of the tobacco hornworm, initially identified as a cellular retinoic acid binding protein (CRABP) [12], belongs to the same subfamily as H-FABP as well (see below). Surprisingly, FABPs have also been found to be prominent arthropod allergens, e.g. in the dust mites *Blomia tropicalis* [13] and *Acarus siro* [14]. In the fluke *Schistosoma mansoni* [15] and various other parasitic worms [16], FABPs are considered essential for lipid absorption, since these animals are unable to synthesize complex lipids de novo [17].

Given the wide distribution of iLBPs throughout the animal kingdom, it is apparent that they belong to an ancient gene family. Major gene duplications gave rise to the separate subfamilies. Multiple alignments of iLBP sequences and construction of phylogenetic trees by the Clustal W algorithm illustrate this relationship as shown in Fig. 1. Four major subfamilies for the mammalian proteins have been categorized based on this sequence homology and, in addition, on ligand binding characteristics [18] (see Table 1 and Fig. 1):

(I) The intracellular retinoid binding proteins [19] can be further subdivided into the cellular retinoic acid binding proteins (CRABP I and II) and the cellular retinol binding proteins (CRBP I and II).

Table 1
Nomenclature and expression pattern for intracellular FABPs

iLBP-type	Alternatives names	Gene name (human)	Mammalian expression	Non-mammalian expression
L-FABP (liver)		FABP1	Liver, intestine, kidney, lung, pancreas	
I-FABP (intestinal)		FABP2	Intestine	
H-FABP (heart)	M-FABP (muscle) MDGI	FABP3	Heart, skeletal muscle, kidney, lung, mammary, placenta, testis, stomach, ovary	Fish muscle, bird muscle, insect muscle, fish ovary
A-FABP (adipocyte)	ALBP aP2	FABP4	Adipose tissue	Fish muscle (?)
E-FABP (epidermal)	E-FABP KLBP mal1	FABP5	Skin, adipose tissue, lung, brain, heart, skeletal muscle, testis, retina, kidney	
I-BABP (intestinal)	ILBP Gastrotropin	FABP6	Ileum	
Brain FABP	B-FABP R-FABP	FABP7	Brain, neurons	Bird brain, retina
M-FABP (myelin)	mP2 Myelin P2	FABP8	Schwann cells	
T-FABP (testis)	T-FABP	FABP9	Testis	
Lb-FABP (liver basic) Midgut FABP	L-FABP	FABP10		Fish, chicken, iguana liver Insect midgut



Q6 Fig. 1. Phylogenetic tree for the iLBP family. Sequences for the vertebrate and invertebrate members of the iLBP gene family were aligned with Clustal W. The tree was constructed with the neighbor joining method, using lens lipocalin as an outgroup. For mammalian iLBPs only the human paralogs are shown. For the subfamily concept see Sections 2 and 3 in the text.

(II) L-FABP and I-BABP (intestinal bile acid binding protein) are closely related based on sequence homology and both stand out because of their unusual ligand binding specificities. L-FABP, which binds a broad range of ligand molecules (acyl-CoAs, heme, squalene, bilirubin and certain eicosanoids), is the only FABP that forms a complex with two fatty acid molecules at the same time [20–22].

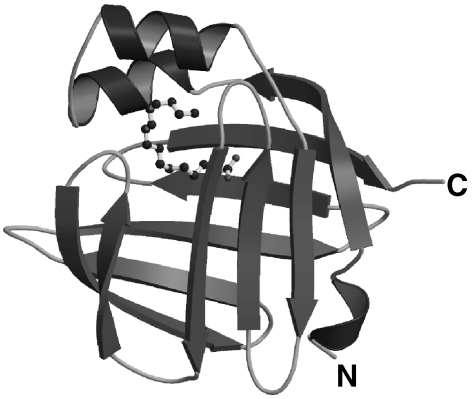
(III) I-FABP is rather singular in sequence characteristics and binds one fatty acid molecule.
(IV) This iLBP subfamily comprises the largest number of different types of FABPs, i.e. H-, A-, E- (epidermal-type), M- (myelin-type), T- (testis-type), and B- (brain-type) FABP. They all bind only a single fatty acid molecule.

Generally, the non-mammalian FABPs fall into one of the subfamilies as defined above and shown in Table 1 and Fig. 1, attesting to the considerable evolutionary conservation of this protein family. Various papers have discussed the phylogenetic relationship between the different members of the FABP family [3,23,24]. From phylogenetic analysis it is likely that a common ancestor gene branched out into two major families more than 900 million years ago, long before the vertebrate–invertebrate divergence. Thus, subfamily II includes not only L-FABP and I-BABP, but also the insect midgut FABPs. The FABP from insect muscle is assembled not only with the H-FABP expressed in mammalian heart and skeletal muscle cells, but also with the cellular retinoid binding proteins, since subfamilies I and IV are believed to have split after the vertebrate–invertebrate divergence [25].

3. Structure and conformation of FABPs and their ligands

The iLBPs are small proteins of 127–134 amino acids, whose expression in *E. coli* made available substantial quantities of recombinant protein for biophysicists and structural biologists to gain deeper insights into structure and binding properties of these proteins. Thus, three-dimensional structures have been determined by X-ray crystallography [22,26–30] and/or NMR [31–35] for all types of the mammalian iLBPs, with the exception of T-FABP. In addition, the crystal [36] and solution structure [37] of the chicken basic liver-type (Lb-) FABP are known. Of the invertebrate FABPs, the three-dimensional structures of a midgut FABP from tobacco hornworm [38] and of the H-FABP from desert locust [9] have been solved. From this wealth of data it has become clear that the tertiary structure of all iLBPs is highly conserved, despite the considerable differences in their primary structure. Sequence identities in this protein family range from 25% for some paralogous members to over 90% for some orthologs. The common structural feature is a 10-stranded β-barrel, made of two orthogonal antiparallel 5-stranded sheets that form the "clam"-shaped binding cavity [39]. The opening of this clam, considered the portal domain, is framed on one side with the N-terminal helix-turn-helix domain, a further common structural motif of all iLBPs (Fig. 2). The 10 antiparallel strands that form the barrel is the salient feature of iLBPs within the "calycin" superfamily of lipid binding proteins, whose other families, the avidins and lipocalins, are characterized by 8-stranded antiparallel barrels forming the binding cavity [40].

In the binding pocket of iLBPs the deprotonated carboxyl group of the bound ligand is generally buried inside the cavity for electrostatic interaction with one or two arginine residues, in addition to be hydrogen bonded by a tyrosine- or serine-OH and an ordered water molecule [27]. Nonetheless, important differences between individual iLBP-types exist, which influence binding kinetics and affinity as well as the mechanism of ligand



Q6 Fig. 2. Three-dimensional structure of *holo* E-FABP (with palmitic acid) [29]. All iLBPs have the characteristic β-barrel structure, in which 10 antiparallel β-strands form the "clam"-shaped ligand binding site, framed by the helix-turn-helix domain as part of the portal. In E-FABP, fatty acid is bound in a U-shaped conformation, characteristic for subfamily IV iLBPs.

transfer [18,41]. FABP-type specific affinities for fatty acids are due to different volumes of the binding cavities and to the amino acid side chains facing one side of the fatty acid's hydrocarbon chain directly, and indirectly the other side via ordered water molecules. This view is not uncontested, however (see Section 4).

A close-up inspection of protein structure and ligand conformation by crystallographic techniques fosters the above-mentioned subfamily concept for iLBPs:

(I) The conformation of the characteristic isoprenoid tail of the retinoid ligands is extended and the α-ionone ring located close to the helix-turn-helix domain, whereas the functional group is always deeply immersed into the binding cavity. Here Arg111 and132 and Tyr134 directly bind all-*trans* retinoic acid in the case of CRABP I and II (cellular retinoic acid binding proteins) [42] which is a scenario similar to that of straight-chain fatty acid binding in proteins of subfamily IV. In CRBP I and II (cellular retinol binding proteins), which bind either all-*trans* retinol or retinal, Gln108 interacts with the functional group of the ligand [43,44] and in CRBP III and IV, variants binding only retinol, Gln108 is replaced by His [45,46].

(II) Of the two fatty acids bound by L-FABP, one is coordinated in a bent conformation electrostatically via Arg122 and an extensive hydrogen-bonding network involving Ser124 and 39 located at the bottom of the protein cavity, which again is reminiscent of fatty acid binding in subfamily IV. The second fatty acid in L-FABP adopts a rather linear shape, with the acyl chain in the cavity extending down towards the center of the other fatty acid molecule and the carboxylate sticking out of the fatty acid portal, thus being solvent exposed and pH sensitive [22]. Interestingly, although I-BABP contains the respective residues (Arg121, Ser123 and 38), it binds fatty acid only weakly, instead of a bile acid molecule with high affinity. Again, the bulk steroid molecule is inside the cavity and the carboxylate group at the protein–solvent interface [47].

(III) The fatty acid bound by I-FABP adopts a slightly bent conformation, reverse in direction to the second fatty acid in L-FABP, thus the carboxylate group is located deep inside the protein cavity directly coordinated to the side-chain of Arg106 similar to the ligands' carboxylate bound by proteins belonging to subfamilies I and IV [26].

(IV) The FABP-types of this subfamily all bind only a single fatty acid molecule in a U-shaped conformation. While the carboxylate group is bound electrostatically and hydrogen bonded via Arg106 and 126 as well as Tyr128 (H-FABP numbering), the hydrocarbon chain is located close to Phe57 (Leu60 in E-FABP) at the fatty acid portal [27]. Several unique features in this iLBP subfamily have been reported only recently. First, human E-FABP contains six cysteine residues, of which C120 and C127 form a disulfide bridge inside the protein cavity [29]. Secondly, human B-FABP binds oleic acid in the common U-form conformation, but very long-chain docosahexaenoic acid (DHA) in a helical conformation [30]. It remains to be seen whether the latter is a consequence of chain-length, or not a specific feature for binding $n-3$ fatty acids. The three-dimensional structure of insect muscle FABP has been solved for the *apo*-protein only [9]. It is remarkably similar to mammalian H-FABP, although steric limitations seem to predict a somewhat different shape of the ligand in the binding pocket.

4. The binding and transfer of fatty acids by FABPs

As far as we know, the obvious task of FABPs is to bind fatty acids. A total of eight FABP-types are expressed in various mammalian tissues each carrying out distinct metabolic tasks. Is fatty acid binding to these FABPs a mere variation of a common structural "leitmotiv", with little consequence for binding affinities? Or do the small structural differences in the binding sites lead to binding selectivities for distinct fatty acid structures? It is not easy to decide which view is correct, and literature data on this aspect are somewhat controversial.

The ADIFAB reagent is a covalently modified I-FABP, with a fluorescent label that changes its emission maximum upon the binding of fatty acids [48,49]. On the one hand, data elaborated with this ADIFAB assay have been interpreted in terms of the "solubility hypothesis", which states in a first approximation that the solubility of a given fatty acid in the bulk aqueous phase drives its affinity for any FABP. The binding site of I-FABP is considered to act similar to a non-polar solvent, and hence its affinity for different fatty acids is mainly determined by the entropic contribution of the hydrophobic effect. Recently, however, thermodynamic parameters for ligand double bonds were incorporated into the calculation of dissociation constants to reflect physico-chemical properties of a given FABP binding site, in fact, the enthalpic contribution to binding. For all FABP-types and their ligand fatty acids tested so far, the values for K_ds found with the ADIFAB method are between 2 and 200 nM.

On the other hand, far greater variations in binding constants were found with other methods. The earliest assays used charcoal to remove unbound fatty acid from the solution and calculated binding constants from the ratio of charcoal- and protein-bound radioactivity [50]. Soon charcoal was replaced by a lipophilic dextrane derivative,

Lipidex 1000 [51]. This material has strong affinity to fatty acids at 37 °C, and can be used to delipidate FABP. At 0 °C, however, protein-bound fatty acids were shown to remain bound to FABP, while unbound fatty acids were adsorbed to Lipidex. Determination by this method afforded dissociation constants between 0.2 and 0.4 μM which are now considered too high, because of the low temperature and the time required to separate Lipidex from FABP [18]. More reliable values can be obtained by measuring dissociation constants without physically separating free from bound ligands, such as fluorescence-based methods like the ADIFAB assay. Another popular approach is isothermal titration calorimetry (ITC), which measures the heat absorbed or released upon binding of the ligand to the protein [18]. For mono- and polyunsaturated fatty acids, dissociation constants in the 10–300 nM range have been determined, whereas remarkably larger values were found for saturated fatty acids, for which the ADIFAB method suggests very strong affinity. The reasons for these discrepancies are not clear, but could be related to solubility problems. A comparison is shown in Table 2, taking the example of B-FABP.

It follows from this short discussion (for more details, see Ref. [18]) that absolute values of dissociation constants depend on the method used for their determination. Their relative values, however, are comparable from method to method, in particular for Lipidex and ITC data. Some of the latter can be explained on the basis of crystallographic studies [52]. Moreover, further insights into binding can be gained by inspecting the dynamic properties of FABPs through various NMR techniques, Fourier transform infrared spectroscopy and recent molecular dynamics calculations [18]. These studies lead to the following conclusions: (i) Differences in the backbone dynamics of various FABPs can be correlated to preferences for specific fatty acids and their relative binding affinities. (ii)

Table 2
Dissociation constants for human B-FABP/ligand complexes determined by the ADIFAB and ITC method

Ligand fatty acid class	K_d (nM)	
	ADIFAB, 37°C[a]	ITC, 30°C[b]
Saturated		
Palmitic acid	7	7100[c]
Stearic acid	2.3	13,500[c]
Monounsaturated		
Oleic acid	7	46.7 ± 1.4
Polyunsaturated $n-6$		
Linoleic acid	11	115 ± 19
Arachidonic acid	18	207 ± 19
Polyunsaturated $n-3$		
Docosahexaenoic acid	13	53.4 ± 4.1
α-Linolenic acid	21	27.5 ± 1.3

[a]Ref. [49].
[b]Ref. [30].
[c]By Lipidex assay and referenced to $K_d = 47\ nM$ for oleic acid as obtained by ITC.

The *apo*-conformation of the protein can adapt to a particular ligand fatty acid and is thus stabilized by reduced backbone flexibility in some *holo*-FABPs [53], even "structured" water molecules as part of the tertiary structure may add to this stability. (iii) In the portal region, the backbone structures generally display an increased conformational variability.

Finding the correct answer to the questions raised at the start of this section is not easy. Certainly, preferences for interactions of certain FABP-types with structurally defined fatty acid classes can be recognized, such as E-FABP with saturated fatty acids, I-FABP with saturated and monounsaturated fatty acids, H-FABP with $n-6$ polyunsaturated fatty acids, L-FABP with mono- and $n-3$ polyunsaturated fatty acids, and B-FABP with $n-3$ polyunsaturated fatty acids. This would have functional implications. A tenet to this statement is that all binding data published originate from in vitro assays that may not reflect the complexity seen within a cell in vivo. According to Weisiger [52], "free" unbound fatty acids in the aqueous cellular compartments originate from their spontaneous membrane-to-membrane transfer that is very slow and depends on the mean diffusional excursion (d_m) of a fatty acid from the membrane. The bulk of the "free" fatty acid molecules in the cell, however, is bound to membranes and to intracellular binding proteins, particularly FABPs. When intracellular transfer of fatty acids beyond d_m is needed, certain FABPs act as "membrane-inactive" binding proteins, and catalyze the diffusional transfer step by increasing fatty acid concentration in the soluble (= diffusible) pool; others act as "membrane-active" binding proteins that catalyze fatty acid dissociation from donor membranes and rebinding to acceptor membranes through FABP-membrane collisions. This intriguing concept received convincing support by elegant studies at the molecular level, which demonstrated that L-FABP and CRABP II belong to the membrane-inactive, non-collisional group, while all other FAPB-types investigated are membrane active and catalyze collisional transfer [54].

This collisional transfer of fatty acids from the FABP to zwitterionic and anionic membranes relies on interactions with positively charged amino acid residues in the helix-turn-helix motif and in turns belonging to the portal domain of respective FABPs [55–57]. Thus, modulation of fatty acid transfer rates in either direction depends on electrostatic interactions of the protein with membrane lipid or protein; additional hydrophobic interactions appear to be at work as well. If this concept is true, why does a cell need membrane-inactive FABP, such as L-FABP at all? It has been proposed that membrane-active FABPs would lose diffusional mobility and thus ability to catalyze efficient fatty acid transfer in cells densely packed with membranes that require efficient fatty acid transfer between membranes over some distance. Hepatocytes and enterocytes are such cell types, and both express L-FABP [58].

5. Metabolic actions of FABPs

In contrast to the very detailed knowledge of the structure and binding characteristics of FABPs, much less is known about their biological functions. The fact that they bind fatty acids suggests that these proteins participate in various aspects of lipid transport and metabolism. Many studies have demonstrated that FABPs modulate metabolic reactions in vitro, but this does not imply that similar effects occur in living cells. Given the poor

solubility of fatty acids in water, one can expect, for example, that the presence of FABP in a buffer increases the availability of fatty acid to enzymes, thus leading to increased metabolic rates in vitro. FABPs are believed to serve the following cellular tasks:

- uptake of fatty acids into the cell;
- formation of cytosolic pool for fatty acids to be rapidly utilized and, concomitantly, to avoid detergent effects on cellular proteins and structures;
- targeting of fatty acids to specific metabolic pathways and modulation of enzymatic activities;
- involvement in fatty acid signaling and gene regulation;
- affecting cellular growth and differentiation;

For the first three tasks indirect evidences are available and will be generally addressed first in this section, followed by a detailed account of the specific FABP-types. The other two tasks will be dealt with in Sections 7 and 8.

Uptake of fatty acids into the cell. The various mechanisms and accompanying phenomena of fatty acid uptake are being dealt with in more detail in Chapters 2, 4, 5, and 6 of this book. In these processes FABPs would be at the receiving end in the cytosol. But the need for such cellular proteins in mediating fatty acid uptake, however, remains controversial [59]. General experimental approaches have been transfection of immortalized cultured cells with a certain FABP and determination of fatty acid uptake either by radioactivity or fluorescence. Thus, L-FABP enhanced initial uptake of oleic acid into L-cell fibroblasts [60] as did A-FABP in transfected CHO-cells, but not a non-binding mutant [61]. When endogenous L-FABP concentrations were decreased by transfecting HepG2 cells with antisense L-FABP cDNA, fatty acid uptake decreased accordingly [62]. On the other hand, expression of L-FABP mRNA in oocytes of *Xenopus laevis* had no effect on fatty acid uptake [63] as had the transfection of L6 myoblast with A- and H-FABP [64]. By the same token transfection with I-FABP cDNA of rat hBRIE 380 cells, murine L-cell fibroblasts, and human Caco-2 cells did not change the uptake kinetics of fatty acids [65–68]. The effect of FABP on fatty acid uptake obviously differs with respect to FABP-type and/or cell-type. Reasons can be the unknown coupling of the uptake process to cellular utilization of the fatty acid incorporated and, of course, the unknown proportions of the mechanisms contributing to the translocation of the fatty acid through the membrane.

Cytosolic pool for fatty acids. Due to the amphipathic nature of fatty acids, their accumulation in large quantities would result in the formation of micelles in the cytosol and damage to cellular membrane structures. FABP may protect against such damage, especially in cells that encounter large fatty acid fluxes. The protein may also modulate the regulatory effects of fatty acids on enzymes or on nuclear transcription factors.

Cytosolic fatty acid transport and targeting. Given the poor solubility of fatty acids in aqueous media, protein-mediated transport of fatty acids may be necessary to achieve high fluxes of fatty acids within cells. Indeed, tissues that metabolize large amounts of fatty acids, such as muscle of adipose tissue, have a high FABP content. FABP increases the total concentration of fatty acids in the cytosol, and it may transport fatty acids more rapidly through the aqueous phase (see Section 4). The proteins may also deliver fatty

acids to specific intracellular compartments or enzymes, for example, to mitochondria for β-oxidation, or to acyl-CoA synthetases for esterification and subsequent storage as triglycerides.

It is difficult to conclusively determine how a particular FABP functions in a living cell, especially since many cells express more than one member of the FABP gene family. However, functional conclusions can be drawn from metabolic differences in cells, tissues, and animals with different FABP content. At the cellular level, such differences can be induced through the transfection of cell lines with various FABPs. FABP levels can also be modified through experimental conditions, such as diet, hormones, or exercise. More recently, dramatic progress with respect to functional aspects has come from gene disruption studies. Knock-out mice for L-FABP, H-FABP, I-FABP, A-FABP, and E-FABP have shed light at the different functions of these proteins, but also revealed that other members of the gene family may compensate at least partly for the loss of one particular FABP. Other cues were obtained from comparing FABP orthologs in different animals. This approach is especially useful for animals that have adapted to extreme rates of lipid metabolism. In assessing the potential functions of FABPs, it is important to distinguish between the individual members of this gene family, and to consider the metabolic functions of the tissues in which they are expressed. Depending on the tissue, fatty acids need to be directed to different compartments, or to different pathways. Data from experimentally modified animals or different, specially adapted species support functions of FABP in intracellular fatty acid trafficking, but the details of underlying mechanisms have yet to be determined.

L-FABP: Liver is a major place of biosynthesis and detoxification, and L-FABP has long been speculated to function in directing fatty acids or related metabolites to the appropriate sub-cellular compartments. It may increase fatty acid acylation rates by making fatty acid more accessible to acyl-CoA synthetase [69]. Circumstantial evidence for a transport function was obtained from comparative studies between hepatocytes from male and female rats. In female cells, where FABP expression is 20% higher than in males, the fatty acid diffusion rate was markedly increased [70]. Other studies have also demonstrated that L-FABP modulates the uptake of fatty acids. In L-FABP knock-out mice, hepatic uptake of fatty acids from the blood was reduced by 50%. This is most likely a direct consequence of the markedly reduced fatty acid binding capacity (−80%) in the cytosol of liver cells, which do not express any other FABP. The cells, however, maintained normal levels of non-esterified fatty acids, triglycerides, and total lipids [71]. Due to its wide range of ligands that includes xenobiotics, it has been suggested that L-FABP may also play a role in mitogenesis [72] (see Section 8).

I-FABP: Three different members of the FABP gene family are strongly expressed in the small intestine, albeit in different regions: cells of the proximal area of the small intestine express mostly L-FABP, while I-FABP is found in the medial region. The distal region expresses the intestinal bile acid binding protein (I-BABP). Since the small intestine is involved in dietary lipid absorption, it is plausible that these proteins mediate the uptake of lipids and their subsequent release into the bloodstream. The link between fatty acid uptake and I-FABP content is supported by various observations in cultured cells: Fatty acid uptake into undifferentiated stem cells was increased 1.7-fold following transfection with I-FABP, while the reduction of I-FABP levels in cultured enterocytes by

epidermal growth factor treatment resulted in reduced fatty acid uptake [73,74]. Other evidence supports a pivotal role of I-FABP in lipid absorption in vivo: A common mutation in this FABP gene doubles the affinity of I-FABP for fatty acids and results in increased fatty acid uptake, a finding that may explain why Pima Indians, a high incidence population group, are predisposed to type 2 diabetes [75,76]. However, targeted gene disruption of the I-FABP gene in knock-out mice did not impair their intestinal lipid absorption [77]. This, however, may be due to the overexpression of L-FABP in the intestine of these animals [78]. Like in other FABP knock-out models, an alternative FABP seems to compensate for the loss of I-FABP in the intestine of I-FABP null mice.

A-FABP: In adipocytes, free fatty acids are mostly incorporated into triacylglycerol for subsequent storage. A-FABP is therefore thought to direct fatty acids towards esterification at intracellular membranes where the long-chain acyl-CoA synthetases are located. Supporting data have been produced in experiments with primary and cultured adipocytes (reviewed in Ref. [79]). Alternatively, a role for A-FABP may arise during lipolysis, when free fatty acids are released from lipid droplets catalyzed by hormone sensitive lipase. As this enzyme is subject to feedback inhibition by fatty acid, it seems logical that rapid removal of fatty acids is required for efficient lipid mobilization. Indeed, A-FABP interacts directly with hormone sensitive lipase, making it possible to sustain rapid transport of fatty acids to the plasma membrane for export, or towards re-esterification at other organelles [80].

In order to study A-FABP function in vivo, a targeted disruption of its gene was generated in mice [81]. The mice appeared to be of normal phenotype, developed normally and were fertile. The morphology of adipocytes, and their fatty acid composition and uptake rates were unaltered. These findings, however, cannot be taken as indication that this FABP is not essential, as its loss greatly increased the expression of E-FABP in adipocytes, which normally makes up only 1% of total FABP in these cells [82]. While no changes in lipid metabolism were apparent in these animals when reared normally, differences were seen after diet-induced obesity. In contrast to wild-type mice, A-FABP null mice showed no increase in serum triglyceride levels, and remained sensitive to insulin. The concentrations of free fatty acid in the adipocytes were elevated, while lipolysis was reduced by 40% [83].

A-FABP is also expressed in macrophages which take up oxidized LDL and contribute to the development of atherosclerosis. Atherosclerotic lesions from hypercholesterolemic, ApoE-deficient mice contained high levels of A-FABP, and it has been demonstrated that oxidized LDL induces A-FABP expression. Double knock-out mice lacking both the ApoE and the A-FABP gene developed smaller lesions with fewer macrophages, indicating that macrophage A-FABP plays an important role in the formation of atherosclerotic lesions [84–86].

E-FABP: Epidermal FABP is the most universally expressed member of this gene family. It is the most abundant FABP in the skin. It may play a role in the maintenance of the water-permeability barrier of the epidermis, as suggested by recent studies with knock-out mice [87]. E-FABP null mice were of normal phenotype, and no differences were visible in histological examinations. No differences were seen in the epidermal fatty acid composition, but the basal *trans*-epidermal water loss was lower that that in wild-type animals. When the lipid barrier was damaged by acetone treatment, the recovery period

required to reach the basal level was much longer than in wild-type animals [88]. A significant increase in H-FABP expression was observed in the liver of neonatal mice, where E-FABP is normally strongly expressed [87]. Adipocytes of E-FABP knock-out mice showed a higher capacity for insulin-stimulated glucose transport; higher systemic insulin sensitivity was also observed [89]. In contrast, transgenic mice overexpressing E-FABP were less sensitive to insulin. The expression of E-FABP and A-FABP in adipocytes is interdependent: When E-FABP is overexpressed, the levels of A-FABP are reduced [90], while A-FABP knock-out mice reveal highly elevated levels of E-FABP expression [82].

B-FABP: This protein is found at its highest levels in developing brain [91]. The protein is expressed in glia cells, and its expression is regulated in response to interactions with neurons [92,93]. Unlike most other FABPs, B-FABP does not bind palmitic acid, but requires a longer hydrocarbon chain and a higher degrees of desaturation [94]. Its natural ligand appears to be DHA, the very long-chain fatty acid that is essential for the development of the nervous system. The expression of B-FABP in the brain coincides with its requirement for DHA, and therefore B-FABP is believed to be involved in the signaling pathways between developing neurons and glia cells [95]. B-FABP is also prominent in neural development of avian species, for example, in the neurogenesis of glial cells in chicken retina [96]. In contrast to the mammalian central nervous system, which is fully developed at maturity, the brain of birds shows significant levels of neurogenesis in the adult stage. The presence of B-FABP in adult bird brain, and its anatomical distribution lends credence to its role in neural migration and synaptic reorganization [97].

H-FABP: Perhaps, the clearest link between FABP and fatty acid metabolism is seen up to date for H-FABP. This protein is the only FABP expressed in various muscle tissues, in both vertebrates and invertebrate species [98,99]. The protein is highly conserved, even between insects and mammals, and is found in all muscles that metabolize fatty acids. A strong correlation exists between the fatty acid oxidation capacity of a muscle and its H-FABP content, as illustrated in Fig. 3. Smooth muscle that depends largely on carbohydrates possesses very low levels of this FABP, while the content in red muscles increased. With higher β-oxidation rates typical for various red muscles, equally increased levels of H-FABP can be found [100]. Cardiac tissue, which depends mostly on lipid for energy supply and encounters the highest β-oxidation rates of all mammalian muscles, also has the highest FABP content (up to 5% of all cytosolic proteins). This observation applies also to non-mammalian muscles, which need to sustain high metabolic rates for long periods: Approximately, 9% of all cytosolic proteins are H-FABP in flight muscles of the Western sandpiper, a migratory shorebird found along the Pacific coast of North and South America; this high FABP content again reflects the fatty acid oxidation rates sustained in these muscles [101]. Higher metabolic demands exist for migratory insects as well, which retrieve energy during endurance flights exclusively through β-oxidation [8]. A classical example is the flight muscle of desert locust, which oxidizes almost 1 μM of fatty acid per minute and gram tissue, as H-FABP makes up almost one-fifth of all soluble proteins.

In all these muscles, elevated levels of H-FABP expression have been observed as a consequence of endurance training or otherwise increased fatty acid utilization. For example, chronic electrical stimulation in rat soleus muscle led to a 30% increase in

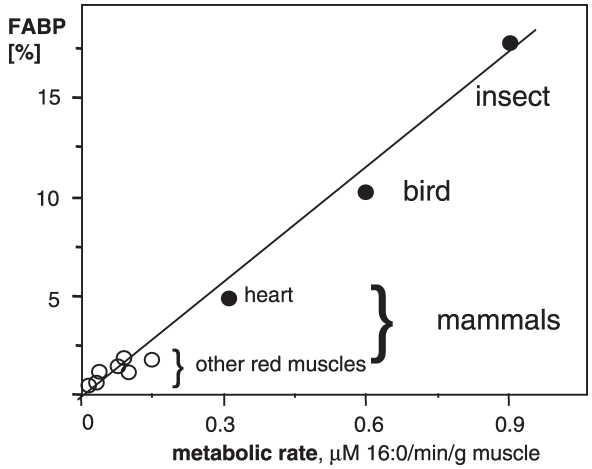


Fig. 3. Correlation between fatty acid oxidation capacity and FABP content in different muscles. Metabolic rates, expressed as the oxidation of μg of palmitate per minute and gram tissue, for mammalian muscles were taken from Ref. [100], for other muscles from Ref. [159]. FABP values for mammalian muscles were obtained from Refs. [100,160], for locust flight muscle from Ref. [8] and for sandpiper flight muscle from Ref. [161].

H-FABP expression [100], and in vivo experiments confirmed this finding: after 8 weeks of swimming, the concentration of H-FABP in rat skeletal muscle increases by 30%, though not in the heart [102]. Diets enriched with polyunsaturated fatty acids led to similar effects in skeletal muscle. In spite of the already extreme H-FABP content of locust flight muscles, its further expression still can be induced, both in response to exercise and to increased fatty acid supply alone [103]. As discussed in more detail below, H-FABP may act as a fatty acid sensor and modulates the expression of its own gene. This would assure that H-FABP levels are appropriate for the fatty acid transfer rates required to fuel muscle activity.

Studies in H-FABP knock-out mice confirm the importance of H-FABP for fatty acid transport and metabolism. The absence of H-FABP did not result in phenotypical differences, and the histology of skeletal and cardiac muscle appeared normal [104]. However, fatty acid uptake was reduced markedly in cardiac tissue (−80%) and isolated cardiomyocytes (−45%). Because of the impaired fatty acid uptake, cardiac muscle contraction in these animals relied on glucose oxidation, which can provide sufficient energy to resting animals [105]. Higher metabolic rates, however, could not be sustained. When exercised, H-FABP null mice fatigued quickly, a finding that lends support to the essential role of H-FABP in cardiac metabolism. Since no other FABPs are expressed in cardiac cells, a compensation mechanism as observed in other knock-out models may not be possible.

In contrast to vertebrates, fish appear to express both H-FABP and a protein more similar to A-FABP in their heart and skeletal muscle [106]. This is noteworthy because fish muscles also serve as the major lipid storage organ. The presence of A-FABP and

H-FABP would be consistent with distinct roles of these proteins in lipid metabolism: A-FABP could direct fatty acids towards storage, for example, during the early stages of migration when food intake exceeds the energy demand. H-FABP should be more prominent during spawning when vast quantities of energy are needed.

6. Regulation of FABP gene expression

From the functional data discussed above, it is not surprising that cells in tissues with prominent roles in fatty acid metabolism are especially rich in FABP. Moreover, FABP levels often increase as a consequence of increased fatty acid exposure. How is this achieved at the molecular level?

All FABPs share an identical gene structure of four conserved exons and three introns of variable size [4,107]. This overall gene structure is of ancient origin, as it is even found in non-mammalian species. The exon/intron boundaries are in identical positions in all FABPs, with the only exception that the second intron has been lost in several, but not all insect FABPs [108]. All FABP promoters contain a classical TATA box. The elements that control the tissue-specific expression of FABP are currently only poorly understood, but potential enhancer sequences have been characterized for several genes. These include two HNF1α regulatory elements in the L-FABP promoter [109], a fat-specific enhancer required for A-FABP expression in adipocytes [110], and several binding sites for members of the POU transcription factor family that control B-FABP expression [111]. A concise promoter region that contained an atypical MEF2 binding site was shown to be responsible for the muscle-specific expression of H-FABP [112].

Better understood is the up-regulation of various FABP genes by fatty acids. It has long been known that the induction of FABP expression in response to lipid-rich diet [113] or endurance training [114] is the result of increased intracellular concentrations of fatty acids, which in turn activate nuclear transcription factors [115,116]. The best known of such transcription factors are the subtypes of the peroxisome proliferators activated receptor (PPAR α, β, γ), so called because of their activation by xenobiotic peroxisome proliferators in rodents [117]. Long-chain fatty acids and certain eicosanoids are considered as their natural ligands. PPARs bind as heterodimers with the subtypes α, β, γ of the retinoid receptor RXR to direct-repeat elements (peroxisome proliferators response elements, PPREs) in the promoter region of the genes that they regulate.

While circumstantial evidence suggests that PPARs are involved in the regulation of various FABP genes, proof has been provided for A-FABP [118] and L-FABP [119] only. In reporter-gene and transactivation assays Tontonoz et al. [118] have shown that the murine A-FABP gene is regulated by the binding of PPARγ2 and RXRα to a direct-repeat element 5.2 kb upstream of the FABP gene. The expression of the rodent L-FABP gene in the liver is under the control of PPARα bound to a PPRE around 110 bp upstream of the transcriptional start site; interestingly, its expression in intestinal cells is controlled by PPARβ, which binds to the same response element as PPARα in the liver [120].

Several studies have demonstrated that treatment of muscle cells with the PPARα agonist Wy14,643 resulted in elevated FABP mRNA levels, and concluded that the H-FABP gene is also under the control of PPARα [121]. Although a direct-repeat sequence

reminiscent of a PPRE can be found in the distal promoter of rodent H-FABP genes, the involvement of this element could not be demonstrated. The absence of a functional PPRE in the human H-FABP promoter raises the possibility that PPARs may act indirectly through cross-talk with other nuclear receptors. Alternatively, the observed induction of gene expression by PPAR agonists could instead be a consequence of increased fatty acid uptake into the myocyte, caused by the induction of the membrane fatty acid transporter FAT/CD36 that is known to be controlled by PPARα [121]. While it has been proposed that transcription factors other than PPARs may be involved in fatty acid mediated gene control [122], such factors have not been extensively studied. To this end, insights can be obtained from invertebrates, which do not express PPARs [123], but the ortholog of H-FABP, which can be induced by fatty acids [103]. It is interesting to note that a different fatty acid response element (FARE) has been identified in the promoter of the H-FABP gene from locust muscle [108,124]. Unlike PPRE, the locust FARE is an IR-3 element, a palindromic sequence containing two hexanucleotide half-sites (AGTGGT, ATGGGA) separated by three nucleotides reminiscent of a steroid hormone response element. Reporter gene constructs containing the locust FABP promoter were expressed in rat myoblasts cells, and treatment with fatty acids resulted in a twofold increase in expression. Deletion of the element did not affect the basal expression rate, but completely eliminated induction by fatty acid. Nuclear proteins from rat myoblasts bound to the element in gel-shift experiments, but additional fatty acid was required to achieve the same effect with nuclear proteins from locust muscle [124]. Perhaps, higher concentrations of fatty acids are required in the latter tissue, because its large FABP content may prevent full access of a signaling fatty acid to the nuclear receptor.

The locust FARE appears to be conserved in evolution: similar elements can be found not only within the proximity of putative FABP genes from other insects (*D. melanogaster* and *A. gambiae*), but also in the promoters of all mammalian H-FABP genes. In the latter case, however, the hexanucleotide half-sites (consensus sequence AGAAGA and AGGTGA) are pointing outwards, forming an everted repeat sequence [125]. It remains to be seen whether these elements alone are responsible for the regulation of the H-FABP gene by a fatty acid, and which transcription factors are involved. In any case, it appears that indeed there is more than one way by which fatty acids can control gene expression.

7. The role of FABPs in fatty acid signaling and gene transcription

The induction of A- and L-FABP mRNA expression by fatty acids and retinoids, involving heterodimers of PPAR and RXR subtypes, is a paradigm for all genes having a PPRE. It follows the general scheme for gene activation by lipophilic ligands that bind to nuclear receptors of the steroid hormone receptor superfamily [126]. In A- and L-FABP expressing cells, fatty acids thus induce their own intracellular binding proteins, a finding that insinuates that these proteins may be the vehicles for targeted transfer of the hydrophobic activators into the nucleus, where they become agonists of transcription factors [126,127]. Other examples from the iLBP family include CRABP (subfamily I) and I-BABP (subfamily II). CRABPs transport retinoic acid to the nucleus, and their genes are under the control of retinoic acid response elements (RARE), which in turn are

activated by the complex of retinoic acid with RAR and RXR [128]. I-BABP is up-regulated by its ligand as well, via the farnesoid X receptor FXR, a nuclear receptor that is activated by bile acid [129].

The members of the iLBP family are well suited to deliver ligands into the nucleus: as small cytosolic proteins of ~15 kDa, FABPs may readily pass nuclear pores or enter by a specific recognition signal the nuclear compartment. Indeed, immunolabeling techniques allowed to detect nuclear localization of L-FABP in hepatocytes already in 1989 [130], of B-FABP in astrocytes [131], of A-FABP in 3T3-L1 adipocytes [132], and of H-FABP in mammalian [133] and insect myocytes [8]. In locust muscle, the cytosolic levels of FABP increase rapidly after adult ecdysis, and the nuclear levels were shown to increase proportionally. Thus, it is conceivable that FABPs transfer fatty acids to PPARs or other nuclear receptors, which in turn are activated to enhance transcription. While the ligand exchange could be simply a matter of fatty acid affinities between binding protein and nuclear receptor, recent studies point towards direct interactions between FABP and PPARs [134]. L-FABP and PPARα co-localize in the nucleus of mouse hepatocytes and, as shown in vitro, the binding protein interacts via protein–protein contacts with PPARα and γ. These contacts are required for the activation of gene expression in response to treatment of HepG2 cells with PPAR ligands, including long-chain fatty acids. Tan et al. [135] obtained similar results using the COS cell model: A-FABP and E-FABP interact directly with PPARγ and β, respectively, and co-expression of the binding protein and respective PPAR subtypes enhance gene activation. Moreover, it appears translocation of the FABP into the nucleus itself is a regulated process, with a massive import in response to ligand binding. The primary structures of FABPs do not carry nuclear import signals; therefore, other mechanisms must be operative. In the case of L-FABP, the negatively charged carboxylate group of the second fatty acid molecule at the surface of the *holo*-protein has been considered such a recognition signal [136,137].

While complete mechanistic details are not yet understood, it seems that FABPs act as fatty acid sensors and mediators in the regulation of gene expression, as illustrated in Fig. 4. This does not mean that the mechanism by protein–protein contacts is exclusive for the ligand to become agonist. Moreover, for reasons not yet known, conflicting data have been reported for the ligand dependence of these protein–protein contacts. On the one hand, the interaction of L-FABP with PPARα or γ has been shown to be independent of the presence of ligand [134]; on the other hand, A-FABP interacted with PPARγ and E-FABP with PPARβ only in the presence of ligand [138]. It is interesting to note the parallels between these FABPs and other iLBPs. It was found that CRABP II, but not CRABP I interacts with the retinoic acid receptor (RARα); this collisional contact leads to the transfer of all-*trans* retinoic acid from the binding protein to the nuclear receptor [139]. Although the affinity of 9-*cis* retinoic acid to CRABP II is much lower affinity than that of the *trans* isomer, it can be transferred by the same collisional mechanism to RXRα [140]. Therefore, L-, A-, E-FABP, and CRABP II appear to play complementary roles in gene regulation; protein–protein contacts are necessary between nuclear receptors and these binding proteins and thus can be addressed as co-activators of nuclear receptors [140].

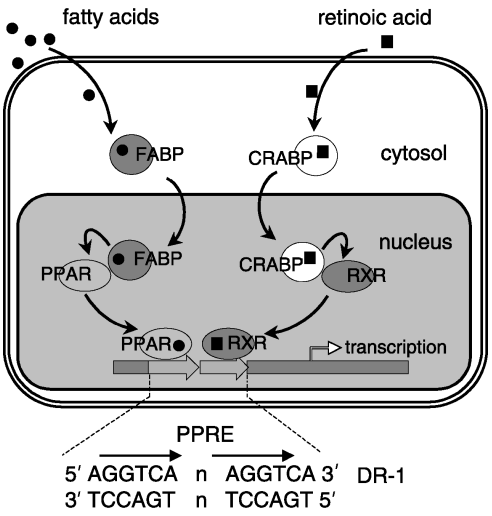


Fig. 4. The path of signaling fatty acids to the nucleus (bold arrows). Protein–protein contacts between iLBP (L-, A-, E-FABP, CRBP II) and the nuclear receptors are shown. The binding proteins deliver fatty acids and retinoic acid to the nucleus, where they are transferred by collision to their respective transcription factors (specific subtypes of PPAR and RXR). Nuclear receptor heterodimers then bind to PPRE for gene transcription.

8. Role of FABPs in cell growth and differentiation

Siding with the notion that FABPs target their lipophilic ligands, e.g. fatty acids or xenobiotics, to the nucleus to affect the cell cycle, we would expect either mitogenesis or growth arrest, the latter with or without differentiation. This modulation brought upon by the binding protein can be seen in the light of its cytosolic sensor function in signaling (Section 7), which may be operative only at low concentrations of the ligand [135]. However, if directed nuclear transport does not take place, the effect will be adverse in either direction, as FABP in a concentration-dependent manner would buffer the lipophilic ligands and prevent them from interacting with their nuclear targets.

L-FABP of subfamily II increased proliferation affected by mitogens and carcinogens in transfected liver and hepatoma cells [72,141]. Carcinogenic peroxisome proliferators became more potent in cells co-transfected by L-FABP, leading to higher cell proliferation rates due to targeting [142].

In contrast, FABPs of subfamily IV reveal growth inhibitory action, for which only a few other peptides are known such as interferons and transforming growth factor β. Thus, loss of A-FABP was correlated with progression of human bladder transitional cell carcinoma [143] and E-FABP, upon application to skin, reduced proliferation of melanoma cells, while normal skin fibroblasts were unaffected [144]. The gene product of a "mammary derived growth inhibitor-related inhibitor gene" (MRG), later identified as B-FABP, suppressed tumor growth in a nude mouse model and breast cancer cell proliferation after transfection with MRG [145,146]. Finally, transfection of MCF-7 cells, a human breast cancer line, with cDNA encoding bovine H-FABP reduced cell growth, in addition, the H-FABP producing transfectants reduced in vivo tumorigenicity [147]. At

present it is not clear whether or not growth inhibition is due to the FABP itself or to its putative ligand. But it is also tempting to speculate in the case of B-FABP that the high affinity-ligand DHA (Table 2) would exert the inhibitory effect.

The background of these observations during the last 15 years was the discovery of bovine "mammary derived growth inhibitor" (MDGI) in 1987 [Böhmer et al., JBC, 262]. It was soon recognized as a variant of H-FABP [148] and finally identified as a preparation of H-FABP contaminated with small amounts of closely related A-FABP [149]. MDGI was a potent inhibitor of epithelial proliferation in various mammalian organ and cell cultures [150]. MDGI, and H-FABP alone also showed anti-proliferation activity in breast cancer cells and H-FABP expression seemed to be reduced in malignant breast tumors [151]. When administered extracellularly, however, the anti-tumor activity of H-FABP was not due to a bound ligand, but could be mapped to a C-terminal fragment of the protein [152]. More details on MDGI-activities of FABPs can be found in a review published in 1998 [153].

In mammary gland organ culture, growth inhibition was associated with functional differentiation in the presence of MDGI or H-FABP; in fact, this differentiation is preceded by heavy expression of H-FABP in the mammary epithelial cells, which then promotes milk protein synthesis in the differentiated cells [154]. Based on this observation, it was argued that H-FABP acts as a differentiation factor. A-FABP as well was assumed originally to be such a factor as it was expressed in the course of differentiation from preadipocytes to adipocytes of both primary cells and the 3T3-L1 cell model. Yet it was soon recognized that the fatty acids themselves (transported by E-FABP in the preadipocyte?) are the trigger of differentiation and, as a result A-FABP and PPARγ among others are expressed. In fully differentiated adipocyte culture, removal of fatty acids from the medium and re-supplementation decreased and replenished A-FABP mRNA levels, respectively [155]. From today's perspective we can ascribe to A-FABP a carrier function in fatty acid signaling to the nucleus to interact with PPARγ and a transport function needed during the time of heavy triacylglycerol accumulation. Indeed, tissue-specific enhancer and proximal promoter regions of the A-FABP gene interact with adipogenic transcription factors in a time-dependent manner [156]. In line with this, H-FABP in C2C12 cells was induced upon differentiation from the myoblast to the myotube stage [157]. A careful follow-up study demonstrated later that E-FABP in myoblasts is down-regulated during differentiation, while H-FABP was induced at later stages of differentiation when energy retrieval in the cells shifts from glycolysis to β-oxidation, indicative of a metabolic transport function of the binding protein [158].

9. Outlook

Much progress has been made in the last decade in the study of the structure and binding behavior of the FABPs. Much of the current research activity is directed to understand the control of their gene expression, and the interactions of FABPs with other proteins in the cell. Undoubtedly, these studies will help to more fully understand the pleiotropic roles of these intracellular transport proteins, especially with respect to signal transduction, both at the molecular and the cellular level. It is the belief of the authors that

analysis of this conserved gene family in various organisms will continue to provide new insights into their regulatory functions.

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