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REVIEW

Role of Fatty Acid Binding Proteins and Long Chain Fatty Acids in Modulating Nuclear Receptors and Gene Transcription

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Abstract Abnormal energy regulation may significantly contribute to the pathogenesis of obesity, diabetes mellitus, cardiovascular disease, and cancer. For rapid control of energy homeostasis, allosteric and posttranslational events activate or alter activity of key metabolic enzymes. For longer impact, transcriptional regulation is more effective, especially in response to nutrients such as long chain fatty acids (LCFA). Recent advances provide insights into how poorly water-soluble lipid nutrients [LCFA; retinoic acid (RA)] and their metabolites (long chain fatty acyl Coenzyme A, LCFA-CoA) reach nuclei, bind their cognate ligand-activated receptors, and regulate transcription for signaling lipid and glucose catabolism or storage: (i) while serum and cytoplasmic LCFA levels are in the 200 μM–mM range, real-time imaging recently revealed that LCFA and LCFA-CoA are also located within nuclei (nM range); (ii) sensitive fluorescence binding assays show that LCFA-activated nuclear receptors [peroxisome proliferator-activated receptor-α (PPARα) and hepatocyte nuclear factor 4α (HNF4α)] exhibit high affinity (low nM K_ds) for LCFA (PPARα) and/or LCFA-CoA (PPARα, HNF4α)—in the same range as nuclear levels of these ligands; (iii) live and fixed cell immunolabeling and imaging revealed that some cytoplasmic lipid binding proteins [liver fatty acid binding protein (L-FABP), acyl CoA binding protein (ACBP), cellular retinoic acid binding protein-2 (CRABP-2)] enter nuclei, bind nuclear receptors (PPARα, HNF4α, CRABP-2), and activate transcription of genes in fatty acid and glucose metabolism; and (iv) studies with gene ablated mice provided physiological relevance of LCFA and LCFA-CoA binding proteins in nuclear signaling. This led to the hypothesis that cytoplasmic lipid binding proteins transfer and channel lipidic ligands into nuclei for initiating nuclear receptor transcriptional activity to provide new lipid nutrient signaling pathways that affect lipid and glucose catabolism and storage.

Keywords L-FABP · ACBP · PPARα · HNF4α · CRABP · Fatty acid · Transgenic mice

Abbreviations

LCFA	Long chain fatty acids
L-FCFA-CoA	Long chain fatty acyl Coenzyme A
L-FABP	Liver fatty acid binding protein
A-FABP	Adipocyte FABP
H-FABP	Heart FABP
K-FABP	Keratinocyte FABP
I-FABP	Intestinal FABP
B-FABP	Brain FABP
ACBP	Acyl CoA binding protein
CRABP	Cellular retinoic acid binding protein
PPAR	Peroxisome proliferator-activated receptor
HNF4α	Hepatocyte nuclear factor-4α
RA	Retinoic acid
RXR	Retinoic acid X receptor
SCP-2	Sterol carrier protein-2
WT	Wild-type

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Introduction

Long chain fatty acids (LCFA) have been known for some time to serve as components of biological membranes and are also used for metabolic fuel. LCFA are not only precursors of signaling molecules; they are also endogenous ligands [1–3] for nuclear receptors that initiate transcription of multiple genes involved in LCFA (β-oxidation, lipoprotein) and glucose metabolism. Concomitantly, LCFA down-regulate genes involved in insulin signaling (e.g. protein tyrosine phosphatase) (rev. in [4–6]). Abnormal activation of the nuclear peroxisome proliferator-activated receptors (PPARs) contributes to lipotoxicity associated with obesity, insulin resistance, type 2 diabetes, and hyperlipidemia (rev. in [5, 7]).

Mammals have evolved both short/rapid acting and long/slower mechanisms to control metabolism. Rapid allosteric control and posttranslational modification activate/deactivate enzymes or alter protein stability within seconds to minutes [5]. Most effective for longer-lasting control (hours to days) is transcriptional regulation of at least two types of receptors [8]: (i) hydrophilic ligands (e.g. diazapines) bind with cell surface membrane receptors, activating cascades of second messengers, which transmit signals to transcription factors that control expression of target genes; and (ii) in contrast, hydrophobic ligands (fatty acids, retinoic acid, vitamins, hormones) must actually enter the cell and be transported to the nucleus, interacting with intracellular and nuclear receptors that serve as nutrient sensors [8, 9]. These hydrophobic ligands act as modulators of transcription factors, exerting regulatory functions directly at the gene level [8]. Because nuclear receptors such as PPAR isoforms (α, β, γ), HNF4α, thyroid receptor (T_3R), glucocorticoid receptor (GR), liver X receptor (LXR), retinoic acid X receptor (RXR), and others regulate transcription of genes involved in lipid and glucose metabolism [5, 9–13], there is great interest in discovery of pharmaceutical antagonists of these receptors to reduce/prevent the deleterious effects of LCFA and LCFA-CoA in obesity, diabetes mellitus, and cardiovascular disease. This focus has led to important discoveries that certain hydrophobic xenobiotics and metabolites/analogues of LCFA including LCFA-CoA are high-affinity ligands that inhibit transcriptional activity of their respective nuclear receptor.

However, the identity of endogenous ligands for PPARα and HNF4α has been elusive. Clearly, dietary LCFA regulate activity of PPARα and HNF4α to induce transcription of genes encoding enzymes and proteins of LCFA and carbohydrate metabolism in cells [14–20] and animals [21], but until recently it was not clear whether LCFA themselves versus their metabolites LCFA-CoA exhibit the requisite characteristics generally acknowledged as hallmarks of physiologically significant ligands: (i) presence in the nucleus, but at levels sufficiently low not to saturate the nuclear receptors, (ii) high affinity binding, with K_ds in the range of physiological concentrations of the ligands in nuclei; (iii) ligand-induced conformational change in the nuclear receptor; and (iv) ligand-induced alterations in coregulator recruitment to the nuclear receptor [7, 22–25]. Further, until recently little was known regarding how hydrophobic ligands [e.g. LCFA, LCFA-CoA, retinoic acid (RA)] could be transported to the nucleus.

Since elevated levels of LCFA and LCFA-CoA are characteristic of several chronic metabolic disorders, including obesity, diabetes mellitus, hyperlipidemia, and cardiovascular disease [26], it is important to resolve mechanism(s) that regulate intracellular and nuclear LCFA/LCFA-CoA concentrations for optimal LCFA and glucose metabolic homeostasis [27]. This review focuses on recent evidence supporting one hypothesis, "exemplified for liver, that transfer and channeling of LCFA to PPARα in the nucleus can be mediated by L-FABP" (Fig. 1).

Dietary LCFA Activate Nuclear Receptors PPARα and HNF4α: Functional Data

Early studies of PPARα and HNF4α ligand specificity illustrate the difficulty in determining the nature of the endogenous ligands of these ligand-activated nuclear receptors. High-fat diets increase PPARα-activated gene expression, especially of L-FABP and β-oxidative enzymes, stimulating LCFA β-oxidation, regardless of whether dietary LCFA are polyunsaturated,

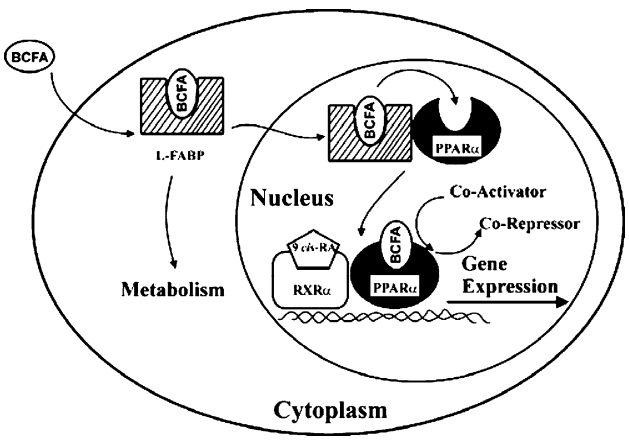

Fig. 1 Selective cooperation between L-FABP and PPARα in branched-chain fatty acid signaling to the nucleus. BCFA, branched-chain fatty acid; L-FABP, liver fatty acid binding protein; RXRα, retinoic acid X receptor α; 9 *cis*-RA, 9 *cis*-retinoic acid

monounsaturated, or saturated [13, 16, 21]. While straight-chain LCFA (unsaturated > saturated) stimulate PPARα transactivation [14, 16, 18, 23, 28], they are relatively non-selective and effective on all three PPAR isoforms (α, β, γ) [29]. In contrast, branched-chain LCFA (pristanic acid, phytanic acid) selectively activate only PPARα [29], particularly in liver [30], and more effectively than any other dietary LCFA or synthetic peroxisome proliferator drugs [29, 31]. Plasma of healthy individuals contains <10 μM phytanic and <3 μM pristanic acid, levels that activate PPARα in cultured cells [29]. In patients with peroxisomal disorders (Refsum, Zellweger, and other peroxisomal abnormalities), plasma phytanic and pristanic acid levels rise to 8,000 and 80 μM, respectively [29, 32].

While together these data suggest that the LCFA activate PPARα, nutritional studies and activation assays do not discriminate whether dietary LCFA are themselves PPARα ligands, induce endogenous PPARα ligands, or are metabolized to active PPARα ligands [16]. Early studies with radioligand binding assays indicated that saturated LCFA and VLCFA (except for arachidonic acid) were very poorly or not bound by PPARα (see below). However, this apparent contradiction of dietary saturated LCFA and very long chain fatty acids (VLCFA) directly activating PPARα [21] was recently resolved by showing that metabolites of saturated LCFA and VLCFA (LCFA-CoA and VLCFA-CoA) are the actual high-affinity PPARα ligands [2, 3]. Mouse studies lend further support for the potential importance of LCFA-CoA in regulating PPARα: (i) peroxisome proliferators induce expression of acyl-CoA oxidase (AOX) and bifunctional protein (BPE), the first two enzymes of the β-oxidation pathway [33], and increase the level of CoA thioesters of peroxisome proliferators [34, 35]; (ii) 2-bromopalmitate, a general inhibitor of fatty acid thioesterification, inhibits bezafibrate induction of peroxisomal proliferation [36]; (iii) AOX gene ablation increases serum VLCFA, and since VLCFA-CoA are not β-oxidized in AOX-null mice, they accumulate and hyperactivate PPARα [37]. Conversely, ablation of the adrenoleukodystrophy gene (prevents VLCFA and/or VLCFA-CoA from being transported to peroxisomes) increases VLCFA (but not VLCFA-CoA) levels and does not alter PPARα activity [38]. These data show that gene alterations resulting in high acyl-CoA elicit PPARα activation, while gene alterations leading to reduced acyl-CoA result in PPARα inactivation.

With HNF4α activation, dietary saturated fatty acids increase plasma levels of lipid rich lipoproteins (VLDL, LDL, HDL) and their constituent apolipoproteins, while dietary unsaturated fatty acids decrease plasma levels of these lipoproteins and their apolipoproteins [39]. This fatty acid nutrient regulation of serum lipids and lipoproteins has been ascribed to transcriptional modulation of HNF4α activated genes encoding apolipoproteins (AI, AII, B, CIII) [40, 41]. Dietary fatty acids modulate HNF4α transactivation in a highly selective manner, consistent with LCFA-CoA representing the activating ligand rather than LCFA (rev. in [42–45]). Mutations in HNF4α form the basis of mature onset diabetes of the young (MODY-1), while agonist LCFA-CoA ligands rescue MODY-1 mutants of HNF4α in transactivation assays [45].

In summary, the above data show that dietary LCFA regulate the PPARα- and HNF4α-mediated transcription of genes involved in LCFA and glucose metabolism. However, it was not clear from earlier studies whether LCFA and their acyl CoA represented the endogenous ligands of these nuclear receptors. For LCFA and LCFA-CoA to represent physiologically significant endogenous ligands they must meet the classical criteria of ligand-activated nuclear receptors (summarized in Table 1). These criteria provide a mechanistic framework accounting for current observations, and establish LCFA and LCFA-CoA as physiologically relevant ligands for these nuclear receptors.

LCFAs and LCFA-CoAs must be Present Within the Nucleus

Serum LCFA levels exceed the solubility of LCFA (1–100 μM) by several orders of magnitude: 200–600 μM under normal conditions, 1 mM under fasting, and up to 8 mM in Refsum's disease, diabetes, cancer, and sepsis (rev. in [29, 32, 46]). Because LCFA are potent detergents and thus toxic at higher concentrations, serum LCFA are almost completely bound by albumin such that the concentration of free unbound LCFA available for uptake into cells is quite low, 2–67 nM, depending on the assay (rev. in [46]). Still, cultured cells can generally take up unbound LCFA from media very rapidly ($t_{1/2}$ of minutes) into the cytoplasm (rev. in [46]). Because of their potent detergent action at high concentration, once taken up the LCFA are rapidly (sec to min) converted to LCFA-CoA by fatty acyl

Table 1 Essential criteria for LCFA or LCFA-CoA activated nuclear receptors: PPARs and HNF4α

	Criterion
1	LCFA or LCFA-CoA levels in nuclear envelope and nucleoplasm are low (nM range)
2	Nuclear receptors have high affinity (nM K_ds) for LCFAs or LCFA-CoAs
3	LCFA or LCFA-CoA binding alters nuclear receptor conformation
4	LCFA or LCFA-CoA binding alters cofactor recruitment

CoA synthase activity at the plasma membrane—a process that can be facilitated by certain fatty acid binding proteins in the cytoplasm (rev. in [46, 47]). While LCFA-CoA are less potent detergents than LCFA, cellular LCFA-CoA levels are generally 10–100 fold lower than LCFA levels because the LCFA-CoA are more rapidly utilized for metabolism (esterification, oxidation), regulation of receptor/enzyme activity, and/or transport to nuclei to potentially regulate transcriptional activity of LCFA-CoA dependent nuclear receptors (rev. in [27, 48, 49]).

However, to be physiologically significant ligands and regulators of nuclear receptors, these LCFA and/or their LCFA-CoA metabolites must: (i) be able to enter nuclei and (ii) nucleoplasmic concentrations of these ligands must be in the same affinity range as the nuclear receptors for these ligands. Whether these ligands enter nuclei was originally addressed by purifying nuclei through subcellular fractionation, in which it was determined that LCFA and LCFA-CoA were detectable [50–54]. However, such experiments could not discriminate contributions from contaminating adherent endoplasmic reticulum (ER) fragments and ligand binding to nuclear membranes, versus actual distribution into nucleoplasm. These potential problems were resolved by real-time confocal and/or multiphoton imaging of a naturally-occurring fluorescent, slowly metabolizable LCFA (*cis*-parinaric acid) as well as non- and poorly-metabolizable synthetic fluorescent LCFA (NBD-stearic acid, BODIPY-C16, BODIPY-C12) and a non-hydrolyzable fluorescent synthetic LCFA-CoA (BODIPY-C16-S-S-CoA) in intact living cells [55–57]. While much of the fluorescent LCFA and LCFA-CoA was present outside the nucleus, significant amounts also colocalized with a nucleic acid dye such that the intracellular distribution was of the order: outside the nucleus $\gg$ nuclear membrane $>$ more dense structures within the nucleoplasm $>$ diffuse nucleoplasm [56]. Thus LCFA and LCFA-CoA are indeed present in nuclei and, more importantly, distributed throughout the nucleoplasm of both fixed and live cells.

Secondly, measurement of LCFA and LCFA-CoA concentrations within the nucleus was initially also addressed by chemical analysis of nuclei isolated by subcellular fractionation, in which nuclear LCFA and LCFA-CoA concentrations were measured in the high μM range (rev. in [55, 56]). Such experiments could not discriminate contributions due to esterase activity on LCFA-CoA and more complex lipids to release LCFA, redistribution of LCFA and LCFA-CoA from other intracellular sites, or differences in the proportion of LCFA and LCFA-CoA in the nuclear envelope versus nucleoplasm. In later experiments, real-time confocal and multiphoton imaging of naturally-occurring and synthetic fluorescent LCFA and LCFA-CoA in living cells measured nucleoplasmic LCFA levels at 39–69 nM [55, 56] and nucleoplasmic LCFA-CoA levels at $<$10 nM [56], levels consistent with earlier calculations [48, 49].

PPARα and HNF4α must Exhibit Specific, High Affinity (nM K_ds*) for LCFA and/or LCFA-CoA*

While LCFA are currently considered putative, physiologically significant endogenous ligands of PPARα, earlier data did not support this hypothesis. As initially measured by radioligand competition and indirect binding assays, PPARα had a low affinity for unsaturated LCFA (10–50 μM K_ds), and did not bind saturated LCFA or LCFA-CoA [15, 17]. These μM K_ds would be several orders of magnitude higher than nuclear LCFA and LCFA-CoA concentrations. Unanswered was whether LCFA or LCFA-CoA alter conformation of PPARα and/or alter coactivator binding, both hallmarks of ligand-activated nuclear receptors (rev. in [1, 2]). More recent data utilizing new assays not requiring separation of bound from free lipidic ligands (LCFA, LCFA-CoA) showed that radioligand binding assays produce significant levels of non-specific binding [15] and underestimate PPARα affinities for LCFA [46] and LCFA-CoA [58, 59]. The newer fluorescence-based assays showed that PPARα has high affinity (1–20 nM K_ds) for unsaturated LCFA, saturated LCFA-CoA, unsaturated LCFA-CoA, unsaturated and saturated VLCFA-CoA, and branched-chain BCFA-CoA, as well as similar or somewhat weaker affinity for fibrates and branched-chain fatty acids (both PPARα agonists) [1–3, 60]. In contrast, saturated LCFA, saturated VLCFA, and unsaturated VLCFA (PPARα agonists) as well glitazones (PPARγ agonists) do not or only weakly bind to PPARα [2, 3, 61, 62]. LCFA-CoA binding to PPARα did not require hydrolysis of LCFA-CoA to the free acid form (LCFA), since PPARα bound non-hydrolyzable *S*-hexadecyl-CoA (S-C16-CoA) with high affinity (K_d = 10 nM) [2, 3].

In the case of HNF4α, earlier studies on the HNF4α ligand binding domain (LBD) suggested that HNF4α did not contain a ligand binding site for either LCFA or LCFA-CoA [63]. Indeed, initial studies using radioligand competition and indirect binding assays showed that the full-length HNF4α had low affinity for unsaturated LCFA-CoA (1–4 mM K_ds) and did not bind saturated LCFA [24, 42, 64]. Further, LCFA, but not LCFA-CoA, was bound by truncated HNF4α ligand binding domain (LBD) constructs that were missing both the N-terminal domains and more importantly the C-terminal F domain (52.5% of the protein) [64]. This reversal of ligand specificity was primarily due to deletion of the C-terminal F domain since deletion of the N-terminal domains had little effect on ligand affinity or specificity [64]. Confirming the effect of C-terminal F-domain truncation on

ligand specificity, X-ray crystallography of truncated HNF4α constructs detected only bound LCFA [64]. Since LCFA-CoA are very unstable under conditions necessary for crystallization of HNF4α (i.e. several weeks at room temperature) [65], lack of the terminal F domain, instability of LCFA-CoA under crystallization conditions, and the fact that HNF4α exhibits thioesterase activity [65, 66] could account for LCFA-CoA not being observed by x-ray crystallography [24, 65, 67, 68]. Interestingly, the LCFA binding site identified by X-ray crystallography is located within a classic LBD [66–68], has weak affinity for LCFA [24, 45, 64] and does not alter its conformation in response to LCFA binding [24, 66–68]. More recent fluorescence data have shown that both unsaturated and saturated LCFA-CoA are bound in a second site with high affinity (i.e. low nM K_ds) by full-length HNF4α [24, 45, 64, 66]. LCFA-CoA binding was subsequently confirmed by mass spectrometry of full-length HNF4α [66]. Mass spectrometry also detected a second ligand binding site which was discrete from the LCFA-CoA binding site, bound only LCFA, and bound LCFA in this site were not displaced by adding exogenous LCFA [66]. While X-ray crystallography studies also showed that the bound LCFA could not be displaced by exogenous LCFA, it is not known if the LCFA binding site determined by mass spectrometry is identical to that determined by X-ray crystallography. Since LCFA bound to HNF4α did not alter structure, and LCFA that are poorly metabolized to LCFA-CoA do not affect HNF4α transactivation [42, 45], the physiological function if any of the LCFA bound within the HNF4α second ligand site remains to be shown.

In summary, PPARα (exhibits high affinity for unsaturated LCFA and nearly all LCFA-CoA and VLCFA-CoA, but not saturated LCFA or VLCFA. Thus select LCFA and all LCFA-CoA/VLCFA-CoA satisfy the high affinity and location requirements for physiologically significant ligands that activate PPARα [1]. These data help to explain why dietary or exogenous saturated LCFA and both saturated and unsaturated VLCFA alter transcriptional activity of PPARα even though they are weak ligands. In contrast, select LCFA-CoA (but not unsaturated or saturated LCFA) satisfy the high affinity requirements for ligands that enhance or inhibit full-length HNF4α transactivation [45, 66]. What remains to be shown, however, is whether LCFA or LCFA-CoA directly bind with PPARα or HNF4α in nuclei of living cells.

LCFA and LCFA-CoA Binding must Alter PPARα and HNF4α Conformation

Recent studies by circular dichroism, quenching of intrinsic aromatic amino acid fluorescence, and protease susceptibility have all demonstrated that LCFA and/or LCFA-CoA alter the conformation of PPARα and HNF4α (rev. in [2, 3, 66]). All high affinity (but not low/no affinity) endogenous or synthetic ligands alter PPARα conformation [2, 3]. Likewise, high affinity (but not low/no affinity) endogenous or synthetic ligands alter conformation of full-length HNF4α [24, 64] as well as HNF4α constructs retaining the C-terminal F-domain, which is the largest F domain (near 80aa) of any nuclear receptor examined to date [64]. Finally, circular dichroism studies of HNF-4α in the absence and presence of various acyl-CoA ligands demonstrated that saturated versus polyunsaturated acyl-CoA differentially altered HNF-4α secondary structure conformation [64], suggesting that different lipid ligands could modulate HNF-4α activity by inducing conformational changes in the structure of HNF-4α [64].

LCFA and LCFA-CoA Alter Cofactor Recruitment to PPARα and HNF4α

It has been shown that high affinity (but not low/no affinity) endogenous or synthetic LCFA and LCFA-CoA alter PPARα coactivator binding [2, 3]. Conversely, select LCFA-CoA but not LCFA alter DNA binding and/or coactivator recruitment to HNF4α (rev. in [42, 43, 45]).

Potential Roles of LCFA and LCFA-CoA Binding Proteins in Regulating Nuclear Receptors

Fatty acid binding proteins represent a large family of soluble proteins that bind LCFA and, in most cases LCFA-CoA, with high affinity (rev. in [46]). Although these proteins were generally named after the tissue in which they were first discovered, most occur in multiple tissues. For example, liver fatty acid binding protein (L-FABP) is expressed at highest amounts in liver and intestine, as well as in lower quantities in kidney and several other tissues (rev. in [4, 46, 69–75]). All tissues examined to date express one or more FABP (L-FABP, adipose A-FABP, intestine I-FABP, heart H-FABP, brain B-FABP), often at a relatively high level, representing as much as 2–5% of cytosolic protein and concentrations of 200–1,000 μM depending on genetic status [76], sex (rev. in [46, 69]), physiological status (rev. in [46]), and induction by peroxisome proliferators (rev. in [46]). In addition, all tissues examined contain the ubiquitous acyl CoA binding protein (ACBP), usually present at several fold-lower levels than the FABPs in the same tissue (rev. in [27, 48, 49, 77, 78]). ACBP and various FABPs are generally expressed at highest levels in tissues that exhibit the highest LCFA metabolic activity, such as liver and heart (rev. in [27, 48, 49, 77, 78]). ACBP in combination with select FABPs may

regulate the nuclear concentration of LCFA and LCFA-CoA by one or more of the following mechanisms.

ACBP and FABPs have Ligand Affinities in the Same Range as those of Nuclear Receptors

To effectively transport and donate bound LCFA and/or LCFA-CoA, ACBP and FABPs must have affinities in the same range or slightly weaker than those exhibited by nuclear receptors, such as PPARα and HNF4α described previously, and indeed this parallel affinity is the case. For example, native liver L-FABP exists in two isoforms which exhibit high affinity for LCFA (K_ds of 8–60 nM, depending on the isoform) and LCFA-CoA (K_ds of 14–110 nM, depending on the isoform) [59]. L-FABP binds unsaturated LCFA with 2–3 fold higher affinity than saturated LCFA, while affinities for both types of LCFA-CoA are more similar (rev. in [59, 79]). ACBP exhibits a higher affinity, but only for LCFA-CoA (K_ds of 0.6–7 nM) [48, 49, 80]. ACBP binds LCFA-CoA with the following order of affinities: saturated > unsaturated > polyunsaturated [80, 81]. However, it must be noted that some studies based on titration microcalorimetry, a method that uses relatively high concentrations of proteins and ligands (e.g. 130,000 nM), yields K_ds of 1–10 nM for ACBP (rev. in [48, 49, 82]) while K_ds for L-FABP are 1,000 nM [83]. The reasons for the discrepancy are not clear but may be related to the fact that fluorescence binding assays use very low concentrations of proteins and ligands (e.g. 180 nM) below the critical micellar concentration of the LCFA-CoA ligand [59, 80], while titration microcalorimetry uses relatively high concentrations of proteins and ligands (e.g. 130,000 nM) above the critical micellar concentration of LCFA-CoA ligand (rev. in [48, 49, 82]). While LCFA-CoA levels above the critical micelle concentration apparently do not affect calculation of binding affinities for proteins with very high affinities such as with ACBP for LCFA-CoA, titration microcalorimetry with lower LCFA-CoA binding affinity proteins such as L-FABP results in underestimation of the binding affinity by one to two orders of magnitude. These data indicate that while ACBP is a higher LCFA-CoA affinity protein than L-FABP, the difference is not 1,000-fold, but rather in the range of 10–50 fold depending on the ligand. Further, the physiological significance of both proteins in LCFA-CoA metabolism was confirmed by a variety of studies indicating that not only ACBP, but also L-FABP [27, 79, 84] enhance acyl transferase enzymes (GPAT, ACBP) in vitro (rev. in [85]). Additionally, studies with livers of gene targeted mice indicate that, while ACBP alters both fatty acyl CoA pool size and acyl chain distribution [81], L-FABP alters cytosolic fatty acyl CoA binding capacity, albeit not fatty acyl CoA pool size [86]. Thus, both L-FABP and ACBP exhibit physiologically significant LCFA-CoA affinities, slightly weaker or in the same range as PPARα and HNF4α-consistent with these proteins being able to effectively donate bound LCFA and LCFA-CoA to the nuclear receptors.

FABPs Enhance LCFA Uptake into the Cell: Role in LCFA-CoA Pool Size

Studies with transfected cells overexpressing FABPs (L-FABP, I-FABP, A-FABP, H-FABP) and with cultured primary hepatocytes from L-FABP null mice show that FABP enhance LCFA uptake 1.5–5 fold (rev. in [46, 79, 87–92]). FABPs may enhance LCFA uptake either by acting as acceptors from plasma membrane LCFA transport/translocase proteins and/or by enhancing intracellular metabolism of LCFA (rev. in [46, 79, 87–92]).

Since FABPs enhance LCFA uptake, they may thereby increase intracellular LCFA pool size and thus increase intracellular availability of LCFA for targeting to the nucleus. Consistent with this possibility are: (i) FABPs can extract LCFA from membranes and increase their solubility in aqueous buffer (rev. in [46, 79, 87–89]). (ii) The LCFA binding sites of native L-FABP isolated from liver are 76% occupied by LCFA (rev. in [79]). (iii) Studies with L-FABP gene-ablated mice indicate that L-FABPs accounts for 90% of the cytosolic LCFA binding capacity in liver (rev. in [46, 93]). Despite these findings, however, studies with gene-targeted mice indicate that L-FABP gene ablation does not decrease liver LCFA pool size [93] or LCFA-CoA pool size [86]. L-FABP gene ablation did alter LCFA-CoA acyl chain distribution [86]. The lack of effect on LCFA and LCFA-CoA pool sizes may be explained by the fact that L-FABP enhances the activities of both catabolic (oxidative) and anabolic (esterification) pathways of LCFA metabolism—thereby maintaining the respective pool sizes. For example, tissue levels of FABPs correlate with LCFA oxidative activity [94–97] and L-FABP expression enhances LCFA (via LCFA-CoA) oxidation with isolated liver mitochondria [98, 99], in transfected cells overexpressing L-FABP [92], and in wild-type as compared to L-FABP null mice [86, 91, 93, 100–102]. Furthermore, L-FABP stimulates LCFA esterification (via LCFA-CoA) with isolated liver microsomes [79, 85, 89, 103], in transfected cells overexpressing L-FABP (rev. in [46, 104]), and in wild-type as compared to L-FABP null mice [86, 91, 93, 102]. In contrast to L-FABP, ACBP increases LCFA-CoA pool size by extracting LCFA-CoA from membranes (rev. in [81]), in yeast overexpressing ACBP [105], and in transgenic mice overexpressing ACBP [81]. Interestingly, ACBP also enhances LCFA catabolic (oxidation) and anabolic (esterification) pathways of LCFA metabolism in vitro [58, 81, 87, 106, 107] and in vivo [81].

In summary, since tissue total (cytosol plus membranes) LCFA-CoA concentrations are generally 10–100 fold lower than LCFA concentrations, these studies indicate that hepatic L-FABP and ACBP levels are sufficient to extract almost all available LCFA and LCFA-CoA from membranes and from cytosol (rev. in [46, 48, 49, 79]). Furthermore, ACBP expression increases LCFA-CoA pool size, while L-FABP expression does not alter either LCFA or LCFA-CoA pool size, likely due to its higher affinity for LCFA-CoA as compared to L-FABP.

FABPs Enhance LCFA Intracellular Trafficking

Studies with a variety of cultured cell lines overexpressing L-FABP [46, 108–112] and with hepatocytes from wild-type and L-FABP gene-ablated mice [91] indicate that several members of the FABP family (L-FABP, I-FABP) enhance LCFA intracellular diffusion/trafficking. There have been no published reports, to our knowledge, examining the effect of FABPs or ACBP on LCFA-CoA diffusion/transport through the cytoplasm. Thus, based on the finding that FABPs facilitate intracellular transport and trafficking of bound LCFA, the possibility that FABPs may facilitate LCFA trafficking to/into nuclei for interaction with nuclear receptors must be considered. By analogy it may be predicted that the LCFA-CoA binding proteins (e.g. L-FABP, ACBP) may likewise facilitate trafficking of this ligand into nuclei.

FABPs Enhance LCFA and LCFA-CoA Distribution into Nuclei, both Nuclear Membrane and Nucleoplasm

LCFA binds to purified nuclei only in the presence of L-FABP [113]. However, such in vitro studies do not discriminate whether L-FABP only transfers bound LCFA to the cytoplasmic face of the nuclear envelope or actually facilitates LCFA transport into nuclei. This issue was recently addressed by use of confocal and multiphoton microscopy of naturally-occurring, slowly-metabolizable fluorescent LCFA (*cis*-parinaric acid) and non- or very poorly-metabolizable synthetic fluorescent LCFA (NBD-stearic acid, BODIPY-C16, BODIPY-C12) and LCFA-CoA (BODIPY-C16-S-S-CoA) [55–57]. Real-time imaging of these probes in live L-cell fibroblasts overexpressing L-FABP and in cultured primary hepatocytes from L-FABP null mice demonstrated that L-FABP enhances distribution of: (i) LCFA into nuclei, both into nucleoplasm and within the nuclear envelope, and (ii) LCFA-CoA into nuclei, primarily diffuse in nucleoplasm with less within the nuclear membrane. Thus, L-FABP distributes LCFA and LCFA-CoA to and into nuclei, but unknown is whether L-FABP cotransfers bound LCFA/LCFA-CoA through the nuclear envelope into the nucleoplasm (Fig. 2, Pathway 1), or just to the nuclear envelope for subsequent release and diffusion of LCFA/LCFA-CoA into the nucleoplasm (Fig. 2, Pathway 2). Nuclear pores are sufficiently large to accommodate diffusion not only of the LCFA and LCFA-CoA ligands (<1 kDa) but also of 14 kDa L-FABP complexes with these ligands (rev. in [56]).

FABPs, ACBP, and CRABP-2 Enter Nuclei (Fig. 2, Pathway 1): Role of Ligands

Several members of the FABP family have been detected in nuclei and nucleoplasm, as shown by confocal imaging of living cells expressing GFP-tagged FABPs, indirect immunofluorescence confocal imaging of fixed cells, and immunogold electron microscopy of fixed cells: L-FABP in nuclei of hepatocytes as well as transfected cells (L-cells, ES cells) [56, 85, 114], H-FABP in cardiac myocytes as well as in transfected CV1 and 3T3-L1 cells [115, 116] K-FABP in transfected CV1, 3T3–L1, and COS1 cells [116, 117], and CRABP-2 in MCF7 cells [118, 119]. In L-cell fibroblasts overexpressing L-FABP (0.4% of cytosolic protein, 10 fold lower than in liver), L-FABP exhibited punctate and clustered distribution in the nucleoplasm [56]. However, when transfected into adipocytes, L-FABP is not detected in nuclei—suggesting that nuclear localization of some members of the FABP family may be cell type specific (rev. in [4]). Furthermore, the localization of FABPs in nuclei as well as cytoplasm is specific for select members of the FABP family, since another member of the FABP family, CRABP-1, is not detected in nuclei [118].

Similarly, a member of a separate LCFA-CoA binding protein family, i.e. ACBP, has also been detected in nuclei. Immunofluorescence microscopy, confocal microscopy and immunogold electron microscopy detect significant amounts of ACBP in nuclei of fixed transfected cells (CV1, 3T3-L1, COS7) overexpressing ACBP [117, 120, 121], rat and mouse hepatoma cells that normally express high amounts of ACBP [120, 121], as well as normal rat and mouse liver hepatocytes [120, 121]. This pattern of distribution was also detected by immunofluorescence confocal microscopy of endogenous ACBP in mouse hepatoma cells (Fig. 3e–h). To assure that this distribution was not due to a fixation or immunolabeling artifacts, a recently developed approach was used for real-time imaging of ACBP in living cells [122]. Purified recombinant ACBP was chemically labeled with small (<1 kDa) fluorescent tags such as Cy5, incorporated into living cells, and intracellular distribution was examined by confocal microscopy (Fig. 3e–h) [123]. ACBP (Fig. 3e) and the nuclear marker Hoechst 33342 (Fig. 3f) were then simultaneously imaged through

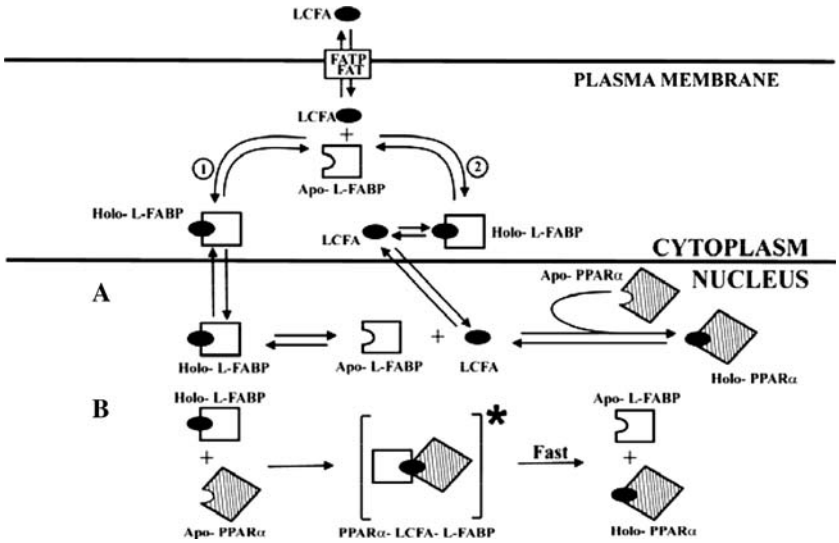

Fig. 2 Mechanisms of LCFA transfer from L-FABP to PPARα within the nucleus. Suggested pathways by which L-FABP may deliver LCFAs to PPARα: (*A*) diffusional and (*B*) collisional/complex

separate photomultipliers. When only colocalized pixels were shown (Fig. 3g), it was apparent that ACBP was significantly colocalized with the DNA binding dye in nuclei of living cells. A pixel fluorogram indicated that 22% of ACBP was colocalized with nuclei (Fig. 3f). These results in living cells confirm the findings of immunofluorescence and immunogold labeling of fixed cells and indicate that significant amounts of ACBP are distributed to nuclei.

While these data clearly show that many members of the FABP family can enter nuclei, it is less clear whether ligands enhance FABPs targeting/transport into the nucleoplasm (Fig. 2, Pathway 1). Some fluorescence imaging studies have shown that ligands enhanced distribution of GFP-A-FABP, GFP-K-FABP, and GFP-CRABP-2 (but not GFP-CRABP-1) into nuclei of living COS1 cells [116, 118]. In contrast, another study using immunolabeling fluorescence microscopy and confocal microscopy showed that ligands did not alter the distribution of ACBP, A-FABP, and K-FABP into nuclei of fixed transfected CV1 or 3T3–L1 cells [117]. The reasons for the discrepancy are not known, but may relate to the cell type, FABP expression level, use of live versus fixed cells, use of GFP vs antibodies to decorate the FABPs, or other as yet unresolved factors.

In summary, several members of the FABP family as well as ACBP are present at significant levels in nuclei and nucleoplasm—consistent with these small sized proteins being able to enter nuclei through the nuclear pores. A cluster of basic amino acids in some FABPs (e.g. C-terminal region of A-FABP and K-FABP) and a cluster of lysine residues (far C-terminal region) of ACBP, both apparent only in the respective three-dimensional folded protein structures, have been suggested to resemble a nuclear localization signal [117]. However, the fact that yeast ACBP does not contain such a region, but nevertheless is significantly localized to nuclei suggests that additional as yet unresolved factors are involved [117]. Finally, several, but not all, studies indicate that ligands can enhance the distribution of the FABPs into nuclei under at least some conditions—suggesting cotransport of FABP-ligand complexes through the nuclear pores into the nucleoplasm.

FABPs Directly Bind Nuclear Receptors

Growing evidence indicates that multiple members of the FABP family interact directly with nuclear receptors, especially PPARs and RAR (Table 2). For example, co-immunoprecipitation, immunofluorescence colocalization, and transactivation assays (despite some disagreement regarding enhancement or inhibition of transactivation [116, 117]) suggest that several members of the FABP family interact with nuclear receptors to transfer bound ligands: CRABP-2 with RAR [116, 118, 119, 124]; A-FABP and K-FABP with PPARγ and β isoforms, respectively, but not PPARα [116, 117]; H-FABP with PPARα [116]; ILBP with farnesoid X receptor (FXR), an interaction augmented by bound bile acid [125]. Likewise, transactivation, coimmunoprecipitation, two-hybrid assay, and immunofluorescence colocalization indicate that L-FABP binds with both PPARα (Fig. 2, L-FABP/PPARα complex) and PPARγ [18, 56, 126]. However, such indirect assays do not provide direct proof of physical association between the FABP family of proteins with their respective nuclear receptors. The finding that L-FABP does not contain an LXXLL domain characteristic of proteins bound by PPARα [18] suggests that L-FABP either interacts with PPARα through an unidentified domain, or that these

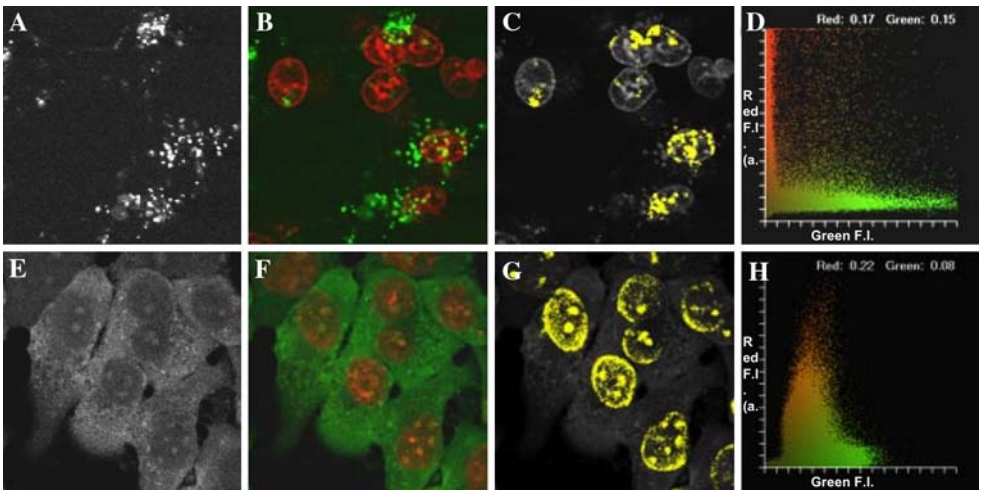

Fig. 3 Intracellular localization of exogenous ACBP in living COS-7 cells, and endogenous ACBP in fixed mouse hepatoma cells. **a–d** Live COS-7 cells were incubated with Cy5-ACBP/Pep-1 and counterstained with nuclear marker Hoechst 33342 before LSCM imaging. **a** Fluorescence image of Cy5-labeled ACBP. **b** Fluorescence image overlay of Cy5-ACBP (*red*) and nuclear marker (*green*). **c** Colocalized pixels are shown in *yellow*. **d** Fluorograph of colocalization analysis of image in panel **b**. **e–h** Mouse hepatoma cells were preincubated with nuclear marker Hoechst 33342, then fixed and labeled with primary antibody against ACBP and Texas-Red-labeled secondary antibody. **e** Indirect immunofluorescence image of ACBP. **f** Overlay of fluorescence images from ACBP (*green*) and nuclear marker (*red*). **g** Colocalized pixels from panel **f** are shown in *yellow*. **h** Fluorograph of colocalization analysis of image in panel **f**

Table 2 Cytoplasmic LCFA and LCFA-CoA binding proteins and nuclear receptors

Cytoplasmic protein	Acronym	Nuclear receptor
FABP family		
Liver FABP	L-FABP	PPARα, PPARγ
Heart FABP	H-FABP	PPARα
Adipocyte FABP	A-FABP	PPARγ
Keratinocyte FABP	K-FABP	PPARβ
Cellular retinoic acid binding protein-2	CRABP-2	RAR
Ileal lipid binding protein[a]	ILBP	FXR
ACBP family		
Acyl CoA binding protein	ACBP	HNF4α

Cytoplasmic LCFA and LCFA-CoA binding proteins enter nuclei, bind with nuclear receptors with high affinity for LCFA/LCFA-CoA, and thereby regulate transcriptional activity of nuclear receptors

[a] Interaction augmented by bound bile acid

assays do not demonstrate direct molecular interaction. With the latter possibility, positive results with the above assays could arise from L-FABP interacting with intermediary protein(s) that in turn bind to PPARα, L-FABP enhancing release of endogenous PPARα ligands [16], and/or L-FABP enhancing formation of additional LCFA metabolite(s) which binds PPARα as exemplified by LCFA-CoAs [2, 3, 47, 127–129].

Despite these reservations, several recent studies have provided molecular details of FABP/nuclear receptor interaction, including intermolecular distance between the proteins, affinity of the proteins for each other, conformational changes upon interaction, and the mechanism(s) whereby ligand cargo is transferred between these proteins. Physical interaction between purified CRABP-2 and RAR proteins was indicated by coimmunoprecipitation [118]. Likewise, physical interaction between purified A-FABP and PPARγ as well as between K-FABP and PPARβ has been shown by coimmunoprecipitation [116]. Circular dichroism has shown that interaction of purified L-FABP and PPARα alters protein conformation [57]. Likewise, a fluorescence resonance energy transfer (FRET) demonstrated that purified L-FABP binds PPARα with high affinity [57]. Finally, FRET between purified L-FABP and PPARα showed these proteins interacting with very close intermolecular distance, i.e. a few angstroms [57]. This was confirmed by immunofluorescence and immunogold electron microscopy. Although L-FABP overexpressed in transfected L-cell fibroblasts has been detected in nuclei, most of this distribution appeared distinct from that of PPARα [56]. However, subsequent double immunogold electron microscopic observations in cultured primary hepatocytes indicate that significant amounts of L-FABP and PPARα in the punctuately distributed regions of the nucleoplasm are in close proximity [57].

In summary, both indirect (coimmunoprecipation, two hybrid, transactivation) and direct (circular dichroism, FRET-based K_ds, FRET-based intermolecular distance in fixed cells, immunogold EM) binding assays show that FABPs interact with select nuclear receptors (PPAR isoforms, RAR, and HNF4α). The ligand-dependence of these

interactions remains to be elucidated. Taken together, these studies indicate that the FABPs may selectively cooperate with the respective nuclear receptors in providing a signaling pathway for LCFA metabolism. However, molecular mechanisms and physiological impact of these interactions remain to be resolved.

ACBP Directly Binds Nuclear Receptors: HNF4α

ACBP, a member of a separate LCFA-CoA binding protein family, interacts directly with the nuclear receptor HNF4α (Table 2). A model similar to that for the L-FABP/PPARα interaction (Figs. 1, 2) is illustrated in Fig. 4. In this model, ACBP interacts with the ligand binding domain of HNF4α to elicit a conformational change and/or transfer bound LCFA-CoA ligand to elicit a conformational change which in turn alters coregulator recruitment and transactivation. Whether other LCFA-CoA binding proteins compete with ACBP for binding and differentially modulating transcriptional activity of this nuclear receptor is not known (Fig. 4). This model is supported by both indirect and direct interaction assays.

Indirect assays such as coimmunoprecipitation, mammalian two hybrid, and transactivation indicate that ACBP

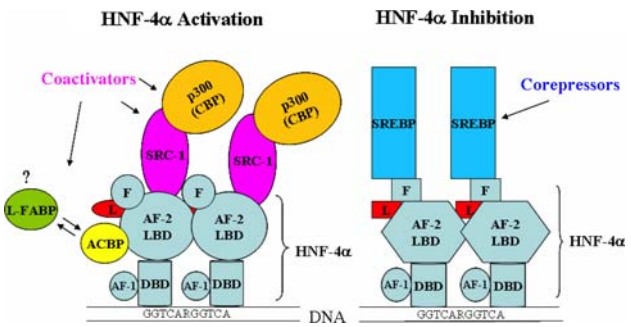

Fig. 4 Suggested modulation of HNF-4α transactivation by specific acyl-CoA ligands, and acyl-CoA-binding proteins ACBP and L-FABP. The DNA sequence shown in the diagram is a specific response element in promoters of HNF-4α target genes. AF-1, DBD, AF2, LBD, F are HNF-4α activation function 1 (ligand-independent), DNA-binding domain, activation function-2 (ligand-dependent), ligand binding domain, and F-regulatory domain at the C-terminus of HNF-4α molecule, respectively. L, ACBP, L-FABP are denotations for ligands (saturated-round shape, or polyunsaturated-square shape), acyl-CoA binding protein and liver fatty acid binding protein, respectively. SRC-1/p300 are complexes of coactivators which contribute to increasing the transcription when recruited by HNF-4α to the target gene promoter. SREBP-1 is a transcription factor reported to negatively regulate HNF-4α transcription activity. In this model, it is assumed that depending on the structure of HNF-4α ligand or on the interaction of HNF-4α with ligand binding proteins like ACBP and L-FABP, the conformation of HNF-4α is significantly altered—thereby resulting in recruiting of either coactivator complexes (such as SRC-1/p300) or corepressor transcriptional factors like (SREBP-1)

directly interacts with HNF4α [120]. It has been reported that HNF4α transactivation is stimulated by saturated acyl-CoAs like C14:0- and C16:0-CoA, and inhibited by polyunsaturated C18:2-, C18:3-, C20:5-CoA thioesters [130]. Thus, depending on the LCFA-CoA species bound to ACBP, the type of ACBP/LCFA-CoA complex may regulate the type of interaction/activation observed.

While such indirect assays do not provide direct proof of physical association between ACBP with this nuclear receptor, perhaps the strongest direct evidence to date that these lipid binding proteins may physically interact with nuclear receptors has been obtained for the ACBP/HNF4α complex [120, 122]: (i) interaction of pure ACBP and HNF4α proteins significantly altered the protein secondary structure; (ii) HNF4α bound ACBP with high affinity (K_d ~60–110 nM); (iii) the intermolecular distance between HNF4α and bound ACBP determined by FRET was 73 Å; (iv) double immunofluorescence labeling confocal microscopy FRET demonstrated an intermolecular distance of 53Å between HNF4α and bound ACBP in fixed cells; (v) double immunogold electron microscopy showed an intermolecular distance of 43 Å between HNF4α and bound ACBP [120]. These molecular interactions were specific, since ACBP did not interact with/bind β-galactosidase (a cytosolic enzyme), Sp1 (a coregulator of nuclear receptors) and GR (glucocorticoid receptor in the nucleus). However, recent evidence from our laboratory indicates that HNF-4α interacts with multiple LCFA-CoA binding proteins, not just ACBP, as demonstrated by in vitro FRET between fluorescent tagged L-FABP and HNF4α as well as between ACBP and HNF4α (not shown).

In summary, both indirect (coimmunoprecipation, yeast two hybrid, mammalian two hybrid, transactivation) and direct (circular dichroism, FRET-based K_ds, FRET-based intermolecular distance in fixed cells, immunogold EM) binding assays show that ACBP interacts with select nuclear receptors (HNF4α). Again, the ligand-dependence of these interactions remains to be elucidated. Taken together, these studies indicate that ACBP may also selectively cooperate with a nuclear receptor (HNF4α), analogous to FABPs cooperating with nuclear receptors (PPARs), in providing a signaling pathway for LCFA metabolism.

Interaction Between Nuclear Receptors (HNF4α, PPARα) and ACBP/FABP in Liver

If FABP/ACBP directly channel ligands to nuclear receptors, this could represent a significant mechanism of gene regulation. This direct association of ACBP with HNF-4α would ensure HNF-4α binding to LCFA-CoA while precluding the availability of LCFA-CoA for interaction with

PPARα. Since LCFA-CoA are endogenous, high-affinity ligands of both HNF-4α [24] and PPARα [2, 3], this would shift the balance of transcriptional regulation to HNF-4α. The binding of saturated LCFA-CoA to HNF-4α would result in increased HNF-4α activity [24] and decreased PPARα activity (due to lack of availability for binding and activation) [2], while unsaturated LCFA-CoA would decrease HNF-4α activity and possibly increase or decrease PPARα activity. Similarly, L-FABP binding to either PPARα or HNF-4α could provide a direct route of transport for both LCFA and LCFA-CoA, while ensuring that these ligands interacted exclusively with one nuclear receptor and preventing their interaction with the other.

Moreover, interaction of ACBP with HNF-4α, both in the presence and absence of ligands, directly stimulates HNF-4α transactivation [120], while ACBP inhibits PPARα transactivation [62, 117]. Since HNF-4α and PPARα both regulate downstream transcription through the binding of similar DR1 sequences [43, 131], ACBP may again shift the transcriptional control to HNF-4α, rather than PPARα, by allowing preferential DNA binding by HNF-4α. Given that HNF-4α and PPARα compete for the same co-activators and co-repressors (rev in [132]), cross-talk between these nuclear receptors could represent a pertinent tool for maintaining energy homeostasis in the liver, and FABP/ACBP may function to regulate this cross-talk.

Interaction of Proteins within Nuclear Receptor/ Coregulator Complexes: HNF4α, Coactivators, and Corepressors

The interaction of HNF-4α with ACBP (Fig. 4) and PPARα with L-FABP (not shown) is thought to elicit downstream alterations in coactivator and corepressor protein association (Fig. 4). To date, however, there are very few if any data directly demonstrating the individual interactions between specific proteins in such complexes. A first approach to study multi-protein nuclear receptor complexes by triple immunolabeling confocal fluorescence microscopy and FRET (fluorescence resonance energy transfer) was applied to HNF4α. Rat hepatoma cells in culture were demonstrated to express detectable amounts of HNF-4α, coactivators of HNF4α (SRC-1, p300) and corepressors of HNF4α (SREBP-1) by Western blotting (not shown). Rat hepatoma cells were fixed and labeled with primary antibodies (rabbit anti-HNF-4α, mouse anti-p300, mouse SBEBP-1, goat anti-SRC-1) and with fluorescent dye-labeled secondary antibodies (i.e. FITC-anti-mouse IgG, Cy3-anti-rabbit IgG, Cy5-anti-goat IgG). If three proteins are closely bound into a complex, by this technique it would be possible to detect Cy5 sensitized emission at 680 nm by excitation of FITC at 488 nm, as excitation of FITC at 488 nm could result in FRET from FITC to Cy3, which in turn would be followed by FRET from Cy3 to Cy5. Two examples demonstrate the feasibility of this approach.

First, triple immunolabeling confocal FRET imaging of fixed cells demonstrated specific physical complex formation between HNF4α and coactivators (SRC-1 and p300) in fixed cells (Fig. 5). Excitation of FITC/p300 at 488 nm, resulted in: (i) a fluorescence image with emission at 530 nm from p300 molecules inside hepatoma cells (Fig. 5a); (ii) a FITC → Cy3 FRET image with emission at 600 nm, from HNF-4α molecules (Fig. 5b) due to close interaction of HNF-4α with p300; (iii) a Cy3 → Cy5 FRET image with emission at 680 nm from SRC-1 molecules closely associated to HNF-4α molecules (Fig. 5c). Images obtained with excitation of Cy3 at 568 nm and Cy5 at 647 nm are shown in Fig. 5d–i; by direct excitation of Cy3/HNF-4α both HNF-(α and SRC-1 molecules were detected, being associated within a FRET distance of less than 100 Å.

Second, triple immunolabeling confocal FRET imaging of fixed cells demonstrated that, in contrast to the coactivator p300, the corepressor SREBP-1 was not detected within a complex of HNF4α with SRC-1 (Fig. 6). Thus, excitation of FITC/SREBP-1 resulted in a fluorescence emission image of SREBP-1 (Fig. 6a), a FRET image of Cy3/HNF-4α (Fig. 6b) but not a further FRET image of Cy5/SRC-1 (Fig. 6c) suggesting that the cells contained HNF-4α molecules that were complexed with SREBP-1, but these complexes did not contain SRC-1. Excitation of Cy3/HNF-4α resulted in emission fluorescence image of HNF-4α (Fig. 6e) and FRET image of SRC-1, indicating that a subpopulation of HNF-4α molecules were associated with SRC-1; the fact that no FITC → Cy3 → Cy5 FRET image from SREBP-1 → HNF-4α → SRC-1 (Fig. 6a–c) was detected in panel C even though there were HNF-4α/SRC-1 complexes, strongly indicates that SREBP-1 was not associated with HNF-4α that was complexed with SRC-1. This was in agreement with previous publications reporting SREBP-1 as a negative coregulator of HNF-4α, by reporter gene assays [133].

These triple-immunolabeling confocal imaging FRET studies illustrate for the first time the direct physical proximities of coactivators and corepressors with a nuclear receptor (HNF4α) involved in fatty acid and glucose metabolism. What remains to be done is to apply these approaches to resolve the effects of the LCFA-CoA binding proteins (ACBP, FABPs) on these intermolecular interactions.

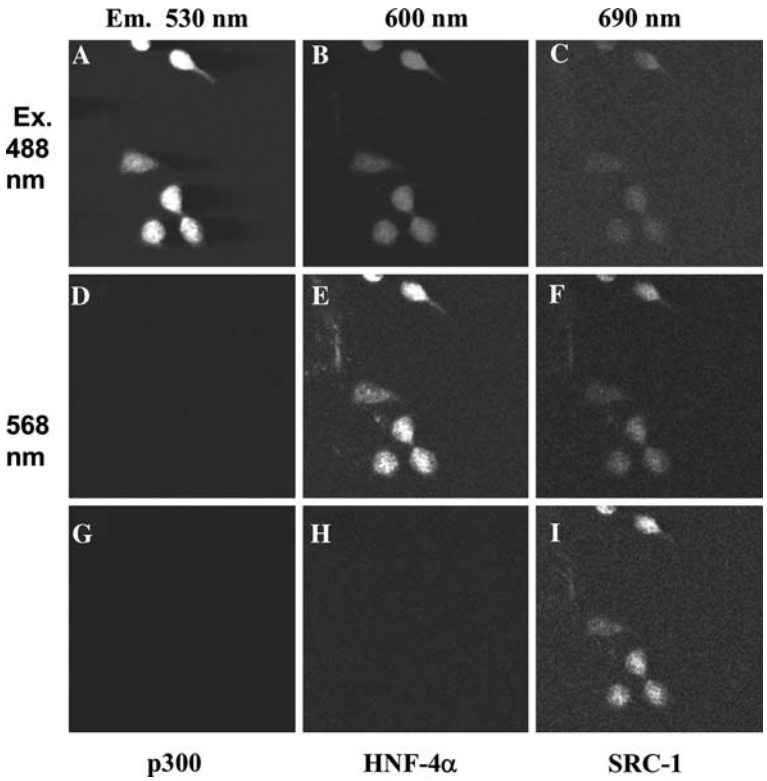

Fig. 5 Interaction of HNF-4α with coactivators p300 and SRC-1. T-7 cells were labeled with primary antibodies against p300, HNF-4α and SRC-1 and secondary antibodies conjugated to FITC, Cy3 and Cy5, respectively. **a** FITC/p300 detected with excitation at 488 nm; **b** Cy3/HNF-4α detected with excitation of FITC at 488 nm, due to FRET; **c** Cy5/SRC-1 detected with excitation of FITC at 488 nm, due to double FRET. **d–f** show images in *green*, *red* and *blue* channels, respectively, when excited at 568 nm (for Cy3); fluorescence Cy5 in F is due to FRET; **g–i** show fluorescence images in channels *green*, *red* and *blue* channels, when excited at 647 nm (for Cy5)

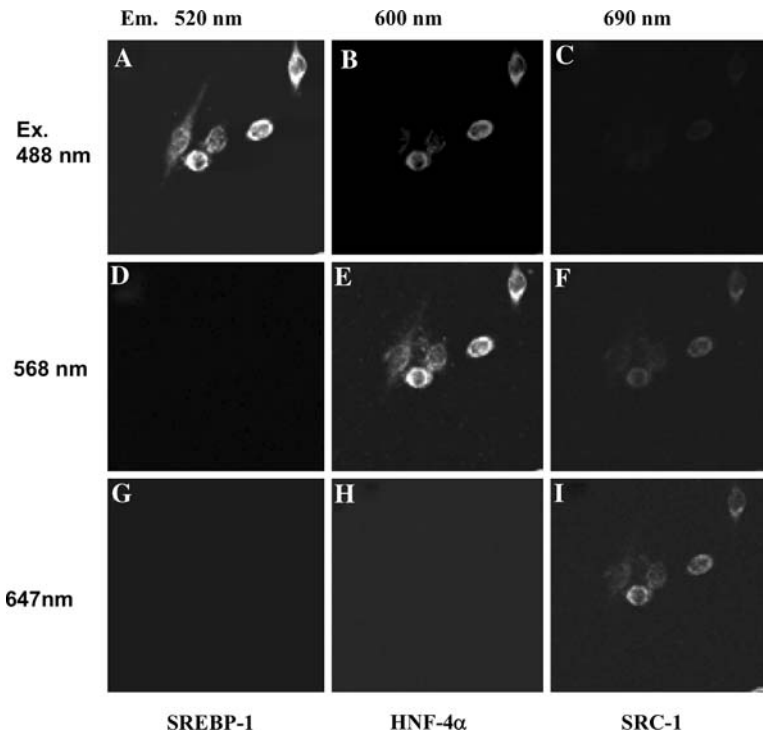

Fig. 6 Interaction of HNF-4α with coactivator SRC-1 and corepressor SREBP-1. T-7 cells were labeled with primary antibodies against SREBP-1, HNF-4α and SRC-1 and secondary antibodies conjugated to FITC, Cy3 and Cy5, respectively. **a** FITC/SREBP-1 detected with excitation at 488 nm; **b** Cy3/HNF-4α detected with excitation of FITC at 488 nm, due to FRET; **c** Cy5/SRC-1 detected with excitation of FITC at 488 nm, due to double FRET. **d–f** show images in *green*, *red* and *blue* channels, respectively, when excited at 568 nm (for Cy3); fluorescence of Cy5 in F is due to FRET; **g–i** show fluorescence images in channels *green*, *red* and *blue* channels, when excited at 647 nm (for Cy5)

Direct Channeling of Ligands between FABPs and Nuclear Receptors

Several studies with purified proteins indicate that by binding to nuclear receptors, different FABPs may directly channel bound ligands to the receptors. For example, CRABP-2 transfers bound retinoic acid (a poorly soluble nuclear regulatory ligand) to the nuclear receptor RAR in vitro [116] via transient collisional interactions (rev. in [4, 118, 119, 124]). Likewise, A-FABP directly channels

bound ligands to PPARγ, but K-FABP does not [116]. However, complexes of these proteins all appeared to be transient collisional interactions. Whether this is the case for interactions between other FABPs or ACBP with nuclear receptors remains to be shown.

Summary/Conclusions

The past few years have seen great advances in our understanding of potential contributions of FABPs and ACBP to nuclear signaling mediated through PPARs, RAR, and HNF4α. The overall picture that is emerging is illustrated by Fig. 2, Pathway 1. In this proposed scheme, FABPs and ACBP enhance uptake of lipidic ligands (LCFA, RA, and/or LCFA-CoA), bind these ligands with high affinity in the cytoplasm, cotransport this cargo to nuclei and through the nuclear pores into the nucleoplasm, form complexes with nuclear receptors exhibiting even higher affinity for the respective ligands, and directly channel this cargo to the respective nuclear receptors to regulate receptor activation. Thus, the FABPs and ACBP may act as nutrient sensors [1, 4, 5]. For example, LCFA-mediated PPARα activation enhances L-FABP transcription (rev. in [23]) while PPARα gene ablation reduces L-FABP expression, especially during fasting [13, 134, 135]. These data suggest that by transporting LCFA (or LCFA-CoA) to PPARα in the nucleus, L-FABP may in part regulate its own expression (rev. in [5, 18]). These data suggest that other FABPs and ACBP may also in part regulate their own expression.

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