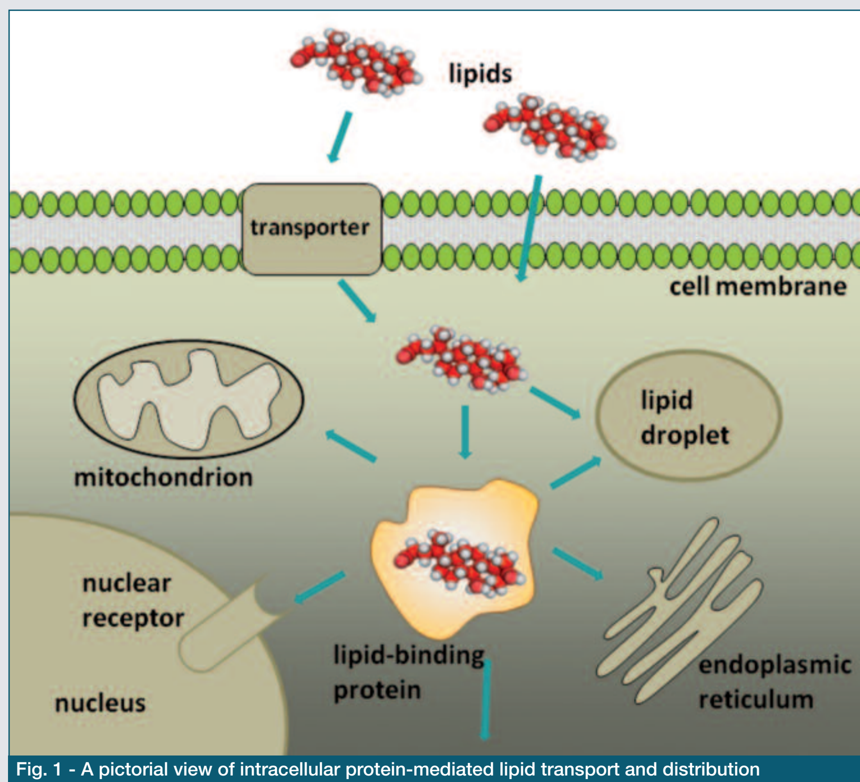

Fig. 1 - A pictorial view of intracellular protein-mediated lipid transport and distribution

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CRITICAL REVIEWS

PROTEIN-MEDIATED LIPID TRANSPORT: FROM MOLECULAR RECOGNITION TO DRUG DESIGN

Lipids are essential for many biological processes and crucial in the pathogenesis of several diseases. The transport of such poorly water-soluble metabolites is often mediated by lipid-binding proteins. Understanding the chemistry of ligand binding to intracellular lipid carriers sets the basis for the development of new drugs.

Lipids are commonly known to serve as metabolic and energy storage units or as architectural components of cells. However, the idea that lipids are bioactive molecules is experiencing a renaissance due to increased understanding of lipid structure-function relationships. Bioactive lipids involved in intracellular biochemical pathways have been directly linked to obesity, inflammation and diabetes and indirectly to numerous other diseases, such as cancer. Thus, those compounds that we call lipids are deeply integrated into the entire fabric and chemistry of cell biology because of their unique properties and structures.

At the heart of the renewed interest in lipid chemistry and biochemistry is an attempt to answer to two simple questions: how do lipids move during biological activity and how do they interact with other molecules? The answers to these questions are often interconnected and need to be found bearing in mind the chemical properties of these biomolecules. To develop their function, lipids must be transported

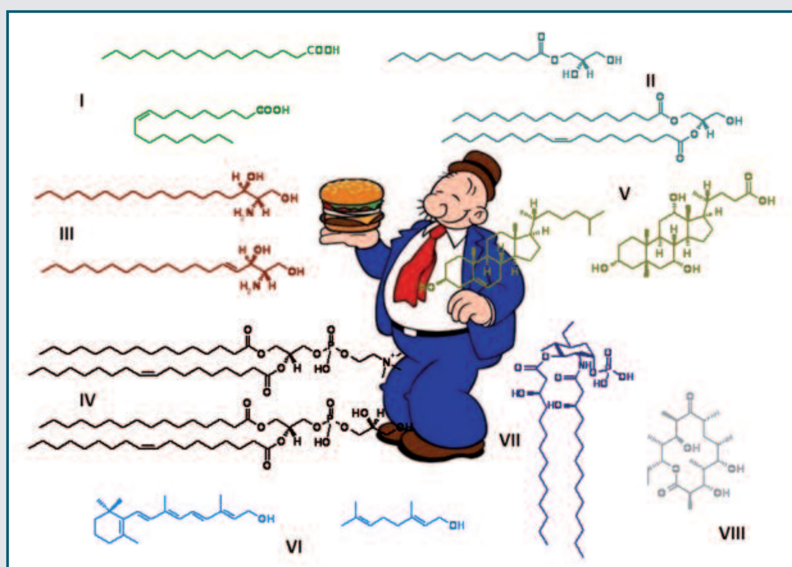

Fig. 2 - Chemical diversity of lipid molecules. Representative members of eight lipid categories defined on the basis of structure and biosynthesis are displayed

through aqueous compartments within the cell (Fig. 1) as well as in the blood and tissue spaces, posing unique problems relating to their insolubility in water. An effective way to circumvent this obstacle is provided by soluble proteins interacting with lipids, which can dramatically increase the total amount of such molecules in the aqueous phase. For example, the solubility of long-chain fatty acids at physiological pH is in the order of 1-10 nM [1]. Albumin, a protein occurring in plasma and interstitium at a concentration of 300-600 μM, can accommodate up to 1-2 mM fatty acids [2]. Likewise, fatty acid-binding proteins (FABPs) are abundantly present in the soluble cytoplasm of cells and can accommodate up to 150-300 μM fatty acids [3]. Consistent with this, of the total amount of fatty acids in plasma only less than one percent is present free in solution [4]. Thus, albumin and FABPs act as extracellular and intracellular buffers, respectively, for fatty acids and other lipids.

Along with fatty acids, other metabolites with low solubility include phospholipids, hydrophobic bile acids, retinoids, coenzyme A-esters, cholesterol, thyroid and steroid hormones, and a wide variety of exogenous drugs and toxins. All these compounds are either bound by specific carrier proteins, or are part of larger lipid-protein complexes referred to as lipoproteins. These proteins provide mobile hydrophobic binding sites that allow hydrophobic or amphipathic molecules to penetrate into and across aqueous layers. The cytoplasm contains a large number of soluble binding proteins. These include fatty acid-, bile acid-, retinol-, phospholipid-, thyroid hormone-, sterol-, heme-binding proteins, and a diverse group of glutathione S-transferases with overlapping specificities [5]. Albumin serves a similar function outside the cell by carrying long chain fatty acids and numerous organic anions across plasma layers. During the last decade a growing amount of evidence has been presented that the various proteins interacting with lipids also modulate their biological action or metabolism.

Therefore, the notion arises that for better understanding the action of lipids it is essential to have detailed knowledge on their interactions with cognate binding proteins [6]. Among the lipid carrier proteins, serum albumin has long been known to possess a unique capability to bind a variety of endogenous and exogenous compounds. This protein has also been recognized to have a strong impact on drug delivery, being able to control the free, active concentration of a drug, and to affect its absorption, metabolism, distribution, and excretion. On the other hand, intracellular lipid chaperones known as FABPs have also displayed ability to bind a variety of exogenous molecules and have been proposed as therapeutic targets due to their central role in lipid-mediated biological processes and systemic metabolic homeostasis. An overview of the ligand binding features of FABPs is here provided, as this knowledge substantially contributes to answer the fundamental questions about lipid translocation and because it potentially opens the way to the development of a broad range of novel therapeutic reagents based on lipophilic molecular scaffolds.

Structural chemistry and function of lipids and intracellular lipid binding proteins

Lipids constitute a class of structurally diverse compounds (Fig. 2) that may originate entirely or in part by carbanion-based condensations of thioesters (fatty acids, polyketides, etc.) and/or by carbocation-based condensations of isoprene units (prenols, sterols, etc.) [7]. Eight lipid categories have been proposed, including fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, prenols, saccharolipids, and polyketides. For the purpose of the present review, only two categories are considered in the following. The fatty acyl structure represents the major lipid building block of complex lipids. Among these, the fatty acids (FAs) function both as an energy source and as signals for metabolic regulation. Furthermore, FAs, particularly linoleic and arachidonic acids, can be metabolized into a diverse family of bioactive lipid mediators called eicosanoids, which may function as pro- and anti-inflammatory mediators.

The second considered category is that of the sterols. These compounds, of which cholesterol and its derivatives are the most widely studied, constitute an important component of membrane lipids. The steroids have different biological roles as hormones and signaling molecules. Additional classes within the sterols category are the bile acids (BAs), which in mammals are primarily derivatives of cholan-24-oic acid, and their conjugates [8]. The presence in bile acid molecules of a number of hydroxyl groups and the side chain structure supporting a carboxylic acid group confers them peculiar physical-chemical characteristics, which have made them very attractive building blocks in the design of novel antibiotics [9], chiral templates [10], drug targeting vehicles [11], scaffolds for combinatorial chemistry [12], new surfactants [13], and others. Among the most important physiological properties of bile salts, lipid transport by solubilization and the excretion of choles-

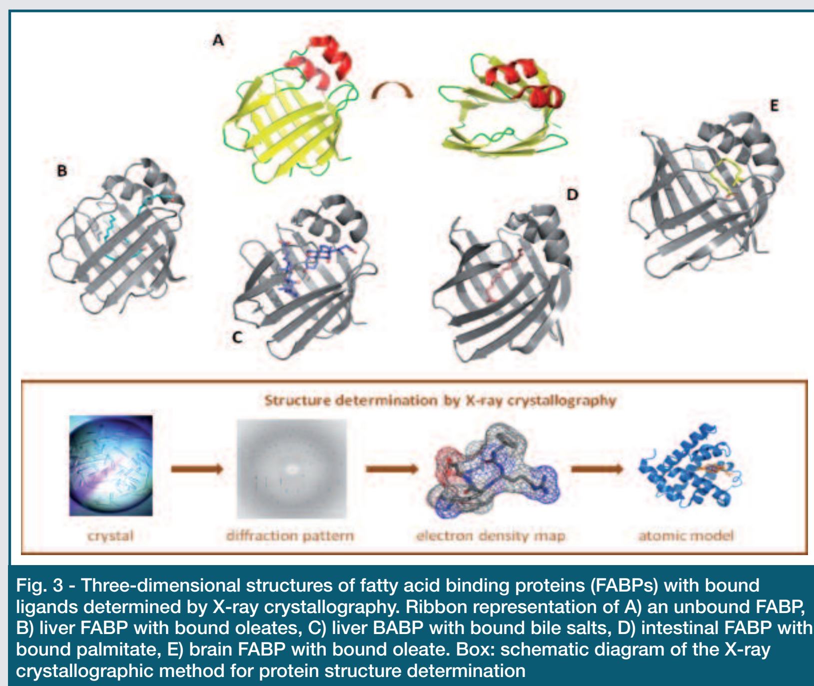

Fig. 3 - Three-dimensional structures of fatty acid binding proteins (FABPs) with bound ligands determined by X-ray crystallography. Ribbon representation of A) an unbound FABP, B) liver FABP with bound oleates, C) liver BABP with bound bile salts, D) intestinal FABP with bound palmitate, E) brain FABP with bound oleate. Box: schematic diagram of the X-ray crystallographic method for protein structure determination

terol into the intestinal tract can be mentioned [8]. Dedicated bile salt transporters in hepatocytes and enterocytes are responsible for the unidirectional transport of bile salts in the enterohepatic cycle [14].
Both the FAs and BAs can be bound reversibly and specifically by the intracellular FABPs, a group of abundantly expressed small proteins, found across many living species [15]. Crystallography and/or nuclear magnetic resonance (NMR) studies have revealed a similar tertiary structure of FABPs from intestine, heart, adipocyte, muscle, liver, epidermis, and brain [6, 15] (Fig. 3). They are composed of ten antiparallel β strands (βA-βJ) that form a sort of β clam surrounding a central water-filled cavity. The interior of the cavity is determined by the sidechains of both hydrophobic and polar amino acids which confer specificity of ligand binding. FABPs have been implicated in fatty-acid import, storage and export as well as cholesterol and phospholipid metabolism [15, 16]. Recently, the FABPs have been shown to be central to lipid-mediated processes and related metabolic and immune response pathways [17]. In a broader context, FABPs could be viewed as lipid chaperones that escort lipids and dictate their biological functions [18]. It is recognized that these proteins bear considerable potential as therapeutic targets for a range of associated disorders, including obesity, diabetes and atherosclerosis.

Structural and dynamic basis of binding

On the basis of amino acid sequence, FABPs can be divided into 3 subfamilies: (1) liver-(L-)type FABP, liver and ileal bile acid binding protein (L-BABP, I-BABP) ; (2) heart-(H-), brain-(B-), epidermal-(E-), myelin-(M-), and adipocyte-(A-)type FABP; and (3) intestinal-(I-)type FABP. This kind of clustering reflects also different binding abilities of the proteins [19].
L-FABP and BABPs are closely related based on sequence homology and both stand out because of their unusual ligand binding specificities [20]. L-FABP, which has been reported to bind a broad range of ligand molecules including heme, bilirubin and certain eicosanoids, is the only FABP that forms a complex with two FA molecules at the same time [21]. One ligand molecule is located at the bottom of the protein cavity in a bent conformation. The second ligand molecule adopts a rather linear shape, with the solvent-exposed carboxylate end sticking out of the fatty acid portal.
BABPs bind FA with lower affinity compared to the other FABPs, while they display preferential binding of BAs. Until today, no high-resolution structure of fully complexed mammalian I-BABP has been reported. On the other hand, few L-BABP [22] and I-BABP [23] structures from non-mammalian vertebrates are available. The stoichiometry of ligand binding for bile acids is mostly of two ligands inside the cavity per protein molecule. In the more general case, the ligand-binding cavity contains, together with hydrophobic residues, several hydrophilic side-chains able to establish extensive networks of hydrogen bonds with ordered water molecules and with ligand carboxylates or hydroxyl groups.
FABP subfamily 2 comprises the largest number of different types of fatty acid binding proteins. These proteins generally bind only a single fatty acid molecule in a U-shaped conformation [24-26]. The carboxy-

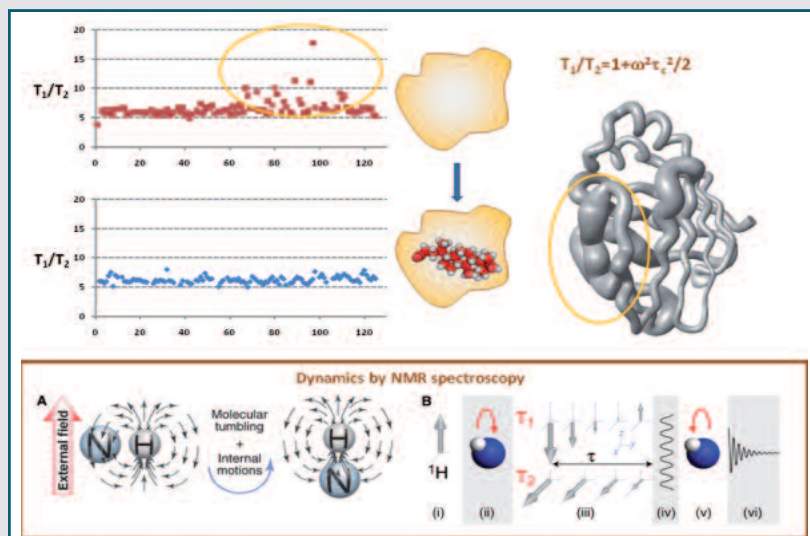

Fig. 4 - Backbone mobility of FABPs as derived from NMR spectroscopy experiments. Nuclear spin relaxation is commonly measured for every amide ^{15}N in isotopically enriched proteins. In several cases the FABP structures are stabilized by lipid binding, showing reduced backbone flexibility in the holo forms. Box: A) Illustration of the orientation-dependent magnetic field experienced by an amide ^{15}N nucleus due to the directly bonded proton; B) Schematic representation of an amide ^{15}N relaxation experiment: (i) equilibrium ^{1}H magnetization, (ii) ^{1}H to ^{15}N transfer, (iii) relaxation delay, (iv) indirect ^{15}N chemical shift detection, (v) ^{15}N to ^{1}H transfer, and (vi) direct ^{1}H chemical shift detection. T_1 and T_2 are the longitudinal and transverse relaxation time constants, τ_c is the correlation rate constant for overall molecular tumbling

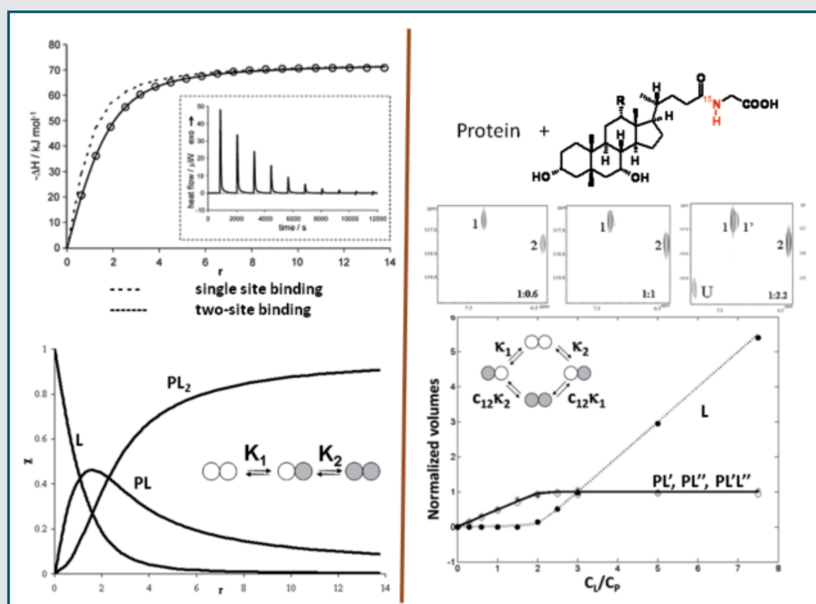

Fig. 5 - FABP/lipid binding models. Left: isothermal titration calorimetry proved useful for the description of stepwise binding models and evaluation of thermodynamic parameters. Right: isotopic labeling of the lipid ligand has been exploited to derive site-specific binding site occupancies, monitored by ^{1}H-^{15}N heteronuclear NMR, that allowed to describe a cooperative two-site biding of bile salts to a BABP. 1, 2, and U denote ligand bound to site 1, 2, and unbound, respectively. K_1 and K_2 are macroscopic binding constants. κ_1, κ_2, and c_{12} are the microscopic binding constants and cooperativity factor

late group is coordinated via an extensive hydrogen-bonding network to key arginine and tyrosine residues, while the ω-tail is located close to a phenylalanine at the fatty acid portal.

I-FABP (subfamily 3) is rather singular in both sequence and ligand binding characteristics [24]. The bound fatty acid adopts a slightly bent conformation, reverse in direction to the second fatty acid in L-FABP. Thus, the solvent-inaccessible carboxylate group is located deep inside the protein cavity directly coordinated to the side-chain of a buried arginine.

The above-described structural details are based largely on X-ray crystallographic results (Fig. 3), as well as on several high-resolution NMR solution structures. Structural studies in solution have the advantage that dynamic properties of the protein structure, e.g. flexibility of loop structures, different backbone conformational states or side-chain mobility, can be identified more easily (Fig. 4). In particular, ^{15}N nuclear spin relaxation studies in combination with hydrogen/deuterium exchange experiments are currently applied to investigate differences in the backbone dynamics of various FABP types, since local conformational disorder and differences in protein stabilities have hinted at a direct correlation between protein dynamics and ligand binding behaviour. In short summary, these data on protein dynamics have led to the following results: (1) the protein structures are stabilized by ligand binding, showing reduced backbone flexibility in the holo forms; (2) differences in the backbone dynamics of various FABPs can be correlated to preferences for specific ligands and their relative binding affinities; (3) in the portal region, the backbone structures generally display an increased conformational variability, which is reflected by either decreased order parameters (derived from ^{15}N relaxation data) or increased hydrogen/deuterium exchange. These observations suggest that during ligand exit/entry, the portal region undergoes a conformational change, allowing the ligand to pass through the portal.

Thermodynamics of protein-ligand adducts

A detailed knowledge of the tertiary structures of different FABP types provides useful insights into the overall structural features, conformational changes upon ligand binding and binding stoichiometry. Within the functional scope assumed for FABPs, however, the question always arises, which ligand binds best to which FABP type.

All FABPs bind saturated and unsaturated long-chain (≥14-C) FAs. Dissociation constants (K_d) appear to be in the nano- to micromolar range. Several analyses have been reported based on fluorescence measurements of FA binding to FABPs in the presence of acrylodated intestinal fatty acid-binding protein. These studies have led to the generalization that affinities tend to increase with increasing ligand hydrophobicity. In addition, isothermal titration calorimetry (ITC) has also been applied extensively to investigate FABP/FA interactions (Fig. 5). ITC measurements with orthologous L-FABPs allowed to trace differences in affinities and thermodynamic parameters back to protein structure. As an example, ITC binding experiments clearly indicated preference of B-FABP for polyunsaturated fatty acids, especially for docosahexaenoic acids, which can be explained on the basis of the crystal structures. The docosahexaenoic acid is buried inside the cavity in a helical, low energy conformation where the double bonds of the fatty acid show π-π interactions between themselves and with Phe104 and Met115. As a general conclusion of all interactions studies, it seems likely that differences in binding affinity of paralogous FABPs for a distinct fatty acid structure are related to functional uniqueness in the cell where they are expressed.

BABPs are FABPs which display preferential binding of BAs compared to other lipids. Almost invariably, the stoichiometry of binding is of 1:2 when considering the number of molecules occupying the internal cavity [22, 23, 27]. A favorable chemistry of BAs has made possible a detailed characterization of the interactions with their protein carriers, revealing fascinating features in terms of energetics and specificity. Compared to long-chain FAs, BAs are generally more water-soluble in monomeric form, allowing for more reliable estimation of dissociation constants. Particularly, physiologically relevant modifications such as glycine conjugation may determine increased solubility and ionization even at lower pH conditions. Furthermore, amidation can be readily achieved also with standard organic chemistry methods, allowing the introduction of isotopically enriched glycines that provide very convenient labels to be detected in high resolution NMR spectroscopy assays. As an example, a dilute solution of a ^{15}N-enriched isotopomer

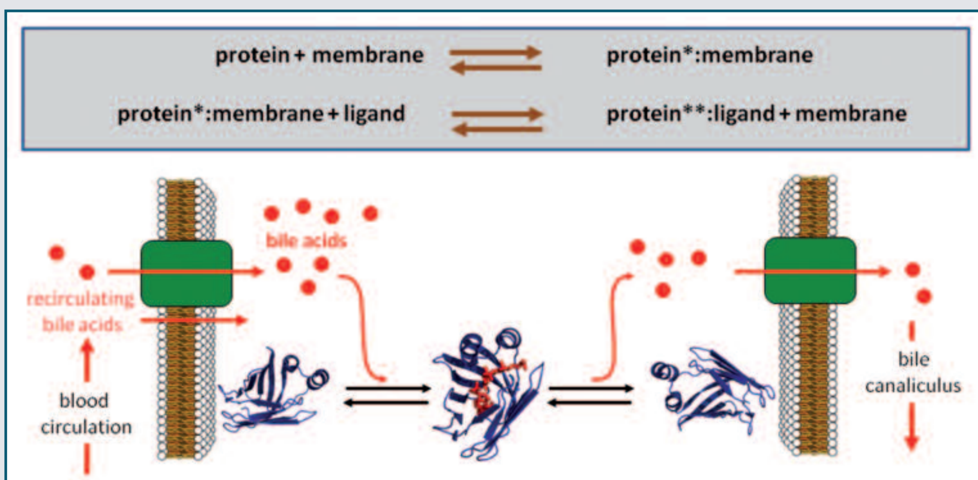

Fig. 6 - A model of directional transport of bile salts. L-BABP is normally associated with the membrane in an activated form displaying partial loss of structure. Bile salts are able to promote dissociation from the membrane. The establishment of competitive equilibria between ligand and membrane in the presence of concentration gradients inside the cell would allow the translocation of bile salts from one membrane to another

of glycocholic acid generates a single H-N cross-peak in a heterocorrelated two-dimensional spectrum. In the presence of an (unlabelled) interacting protein, the peak position changes as a consequence of an altered electronic environment of the bile salt amide group, thus reporting on the ligand bound to a specific site on the macromolecule. In case of strong protein-ligand affinities, it generally occurs that the chemical exchange rate is slow compared to the resonance frequency difference of the free and bound molecules. This occurrence implies that in the presence of both species two distinct signals are observed with intensities proportional to the corresponding concentrations. Therefore, the population fractions of all interacting species at equilibrium can be evaluated and probed against simulated data to validate binding models. Indeed, ^{13}C, and/or ^{15}N-enriched glycine-conjugated cholic and chenodeoxycholic acids were used in two-dimensional heteronuclear NMR titration studies to monitor the occupancy of each binding site in both I-BABP and L-BABP [28, 29] (Fig. 5). These investigations highlighted the superb resolving power of NMR to detect recognition events in a site-specific manner making it possible to assess the occurrence of microscopic cooperativity. Human I-BABP and chicken L-BABP displayed a very remarkable binding behavior, interacting with glycocholic acid and glycochenodeoxycolic acid, respectively, with modest intrinsic affinity and a high degree of positive cooperativity (the intrinsic cooperativity factor exceeding 10^3, possibly the largest value ever measured for a ligand-protein interaction). The structural basis for the observed cooperativity is proposed to result from allostery, where the binding of the first ligand is energetically communicated to the second site through a conformational change in the protein. The latter can be again assessed by NMR using reversed labeling schemes as those proposed to monitor binding site occupancies: the position changes of ^{15}N-labelled-protein amide signals on addition of unenriched ligand allow detecting protein structural rearrangements in an aminoacid-specific manner.

Most forms of FABP also seem to be able to reduce the free energy of dissociation of the lipid from the membrane, thereby catalyzing the rate of dissociation. Without binding proteins, the lipid must move from the hydrophobic core of the membrane to the hydrophilic environment of the cell, making this process thermodynamically unfavorable. Interaction with membranes has been proposed to be a key step enabling ligand release [30]. For example, studies on L-BABP suggest that L-BABP is normally associated with the membrane in an activated form displaying partial loss of structure, while addition of bile salts is able to promote dissociation from the membrane [31]. The establishment of competitive equilibria between ligand and membrane in the presence of concentration gradients inside the cell would allow the translocation of bile salts from one membrane to another (Fig. 6).

Lipidic drugs and therapeutic targeting of FABPs

Interest in lipid/FABP interactions has grown during the past few years essentially for two reasons: FABPs are considered obligate carriers of lipophilic drugs in the cytoplasm and they have been proposed as pharmaceutical targets.

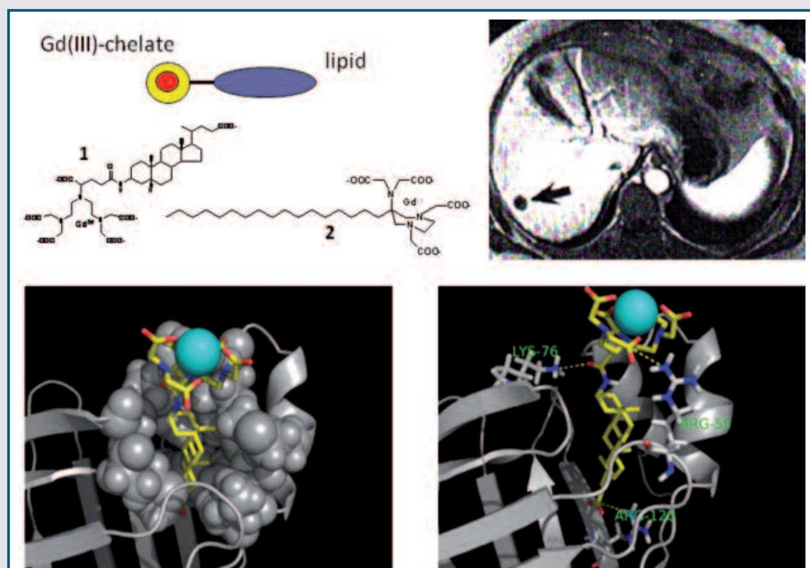

Fig. 7 - Lipid-functionalized gadolinium chelates are ligands to BABPs. Gadolinium chelates are important contrast agents commonly used in magnetic resonance imaging. Functionalization of the chelates (top left) allows control of drug availability, distribution and contrasting power through modulation of protein-drug interactions. Hepatospecific contrast agents have been realized which are not internalized by tumor cells (dark spot in top right image). Improvement of drug design takes advantage from high resolution structures of protein-drug adducts (bottom figures)

As far as the first motivation is concerned, it is expected that the barriers faced by lipophilic drug compounds during distribution in the body are similar to those faced by endogenous lipophilic molecules. Because a major limiting factor in the development of clinically useful drugs is the poor and variable drug absorption, the study of protein-mediated lipid transport constitutes a critical step in the effective design of drug candidates with optimal absorption characteristics. A diverse set of lipophilic drugs have been shown to bind to I-FABP and L-FABP with moderate-to-high affinities, confirming the expectation that compounds displaying chemical properties similar to FAs or BAs constitute potential ligands for these carriers [32]. The interaction between I-FABP and the lipophilic compounds bezafibrate, ibuprofen, and nitrazepam has been investigated [33], showing that each drug participates in specific interactions within the cavity and that a number of common contacts is established with residues also involved in fatty acid binding. Similar results were obtained with L-FABP, which additionally resulted able to bind a large number of structurally diverse compounds in different binding modes [34].

A further application of the acquired knowledge on FABP/lipid interactions has been recently proposed for the development of hepatospecific contrast agents (CAs) for magnetic resonance imaging (MRI). The latter has become a commonly used diagnostic tool as it offers a powerful way to map structure and function in soft tissues by sampling the amount, flow, and environment of water protons in vivo. The intrinsic contrast can be increased by the use of CAs in both clinical and experimental settings, a large part being constituted by gadolinium(III) complexes because of their favorable electronic and magnetic properties. There is growing interest in the development of CAs displaying tissue specificity and high intrinsic relaxivity (defined as the relaxation enhancement of water protons in the presence of the paramagnetic complex at 1 mM concentration). These properties can be obtained with an appropriate functionalization of the chelate allowing the drug to be actively transported to the target tissue and to specifically interact with a biomacromolecule. Binding of CAs to large, slowly-tumbling molecules increases the compound's relaxivity, resulting in enhanced sensitivity of the MR measurement. A number of potential CAs were designed by conjugating bile acid moieties to gadolinium chelating units such as DTPA or DOTA, making it possible to achieve specific liver tissue uptake [35] (Fig. 7). Indeed, active molecular transport in hepatocytes may be conveniently realized by exploiting the enterohepatic circulation machinery, whose key steps at both the hepatocytes and the enterocytes are mediated by a receptor system, an intracellular protein carrier, and an exit system [36]. Noticeably, two related Gd-DTPA-based conjugates of cholanoic and deoxycholic acid were shown to display differential cellular uptake between human healthy model cells and hepatoma cell lines. The reason for this is the absence of expression of a bile salt membrane transporter in diseased cells. In order to set the basis for the rational design of more efficient hepatospecific CAs, the structural details of the interaction between the above compounds and a putative intracellular protein binder were investigated. By use of relaxometric measurements and high resolution NMR, L-BABP was shown to be able to bind a single ligand molecule with a significantly low dissociation constant [37] and, very importantly, to determine an up to three-fold increase in the compound's relaxivity. The identified protein "hot spots" defined a binding site located at the protein portal region, partially overlapped with one bile salt binding epitope [38].

The potential of FABPs as pharmaceutical targets can be immediately appreciated from the here outlined central role in intracellular lipid trafficking. As an example, A-FABP and E-FABP act at the interface of metabolic and inflammatory pathways. The creation of pharmacological agents to modify FABP function may therefore provide tissue-specific or cell-type-specific control of lipid signalling pathways, inflammatory responses and metabolic regulation, thus offering a new class of multi-indication therapeutic agents [18]. A recent study reported the development of an isoform-specific and biologically active synthetic A-FABP inhibitor, and demonstrated that chemical inhibition of A-FABP could be a potential therapeutic strategy against insulin resistance, diabetes, and fatty liver disease [39]. The orally active small-molecule BMS309403, a rationally designed, potent and selective inhibitor of A-FABP, interacts with the fatty-acid binding pocket within the interior of A-FABP to inhibit binding of endogenous fatty acids. Results of X-ray crystallography studies identified the specific interactions of BMS309403 with key residues within the fatty-acid binding pocket as the basis of its high in vitro binding affinity and selectivity for A-FABP over other FABPs [40].

In a recent investigation, it was suggested that certain FABPs mediate intracellular transport of endocannabinoid anandamide (arachidonoyl ethanolamide, AEA), an uncharged neuromodulatory lipid that belongs to a family of signaling molecules collectively termed endocannabinoids [41]. The biological actions of AEA are tightly controlled through its enzymatic synthesis and degradation. Similar to many neurotransmitters, AEA is inactivated through its cellular uptake and subsequent

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catabolism. FABPs are thought to enable the lipophilic molecule to traverse the cytosol in order to reach the enzyme fatty acid amide hydrolase (FAAH) for subsequent hydrolysis. The role of FABPs as intracellular carriers may well extend beyond the endocannabinoid AEA. The structural similarity between AEA and related N-acylethanolamines (e.g., palmitoylethanolamide and oleoylethanolamide) suggests that FABPs may likewise mediate their intracellular delivery to inactivating enzymes such as FAAH. From a health-related viewpoint, understanding the mechanism(s) of endocannabinoid inactivation may lead to new treatments for a variety of disorders, including addiction, pain, inflammation, and appetite regulation. Similar to FAAH inhibitors, compounds that target FABPs may attenuate the clearance and inactivation of AEA in a tissue and cell-type-specific manner, thereby raising its levels at cannabinoid receptors and enhancing the endocannabinoid tone.

Conclusions

During the past decade, there has been the realization that lipids not only display structural and storage functions but are involved as bioactive molecules in an ever-expanding list of biological activities. Most of such functions require a finely tuned network of molecular interactions as well as efficient means of shuttling between aqueous compartments. Within cells, the fatty acid binding proteins have a central role in lipid-mediated processes and lipid transport. There are underexplored opportunities to exploit the multifaceted chemical space of protein-lipid interactions for the development of novel drugs with broad applications in biomedicine.

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RIASSUNTO

Trasporto lipidico mediato dalle proteine: dal riconoscimento molecolare alla progettazione di farmaci

I lipidi sono essenziali in molti processi biologici e determinanti nella patogenesi di molte malattie. Il corretto funzionamento ed il trasporto di questi metaboliti poco solubili in acqua richiedono spesso la partecipazione di proteine specifiche. La comprensione della chimica che governa l'interazione tra i lipidi ed i loro trasportatori proteici intracellulari costituisce una base per lo sviluppo di nuove molecole con svariate applicazioni in ambito biomedico.