Structural and multifunctional properties of cardiac fatty acid-binding protein: from fatty acid binding to cell growth inhibition

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Introduction

The cardiac fatty acid-binding protein is one type of a family of nonenzymic proteins [1, 2] that bind hydrophobic ligands. The localization of these fatty acid-binding proteins (FABPs) is mainly cytosolic with concentrations up to ~5% [3] of soluble proteins. A closer inspection of the subcellular distribution by means of immunohistochemical methods and e.l.i.s.a. [4], however, reveals a type-specific compartmentation of FABPs, e.g. the cardiac-type FABP is associated with myofibrils and mitochondrial matrix, whereas in hepatocytes the so-called hepatic-type FABP is associated with the endoplasmic reticulum and the outer membrane of mitochondria [5]. Interestingly, both FABP types are also found inside the nucleus [4, 5]. From the abundance of FABP in tissues with ‘active’ lipid metabolism and the ability to bind fatty acids with high affinity, an involvement of these proteins in fatty acid metabolism was inferred. Already in the early days of FABP research a variety of studies related to this field was undertaken even though the proteins were often not available in pure form and structurally not characterized. With the advent of detailed structural information functions were proposed in a more rationalized manner for the nonenzymic FABPs, e.g. roles in signal transduction and in regulation of cell growth.

Dissecting the structure of cardiac fatty acid-binding protein

The cDNA for bovine heart FABP [6] codes for a 132 amino acid protein with a high proportion of β-sheet, as predicted from secondary structure calculations. Plasma desorption mass spectrometry indicates a molecular mass of 14679 ± 10 Da for the purified protein [7] in good agreement with that deduced from the cDNA, if one takes into account that the protein is N-terminally blocked by an acetyl group. The latter finding was corroborated by mass analysis of N-terminal peptides. In many cases FABP’s occur as isoforms that differ in their charge; for example cardiac FABP from bovine heart has pl 4.9- and pl 5.1-isoforms. A complete protein sequence determination of the purified isoforms pin-pointed the molecular basis for this heterogeneity to a single asparagin/aspartate exchange in position 98 [7]. The pl 5.1-isoform with asparagine in this position was co-linear to the cDNA (Fig. 1α) and the question arose, whether the two charge isoforms are translated from different mRNAs or result from post-translational deamidation, be it spontaneous or enzymic. Analysis by isoelectric focusing of immunoprecipitated [135]methionine-labelled products translated in vitro from total mRNA of bovine heart and from positive hybrid selected cardiac FABP-mRNA on the one hand showed two proteins that co-migrated with the authentic pl 4.9- and pl 5.1-isoforms. On the other hand translation of recombinant mRNA derived from the cDNA encoding the pl 5.1-isoform produced indeed the pl 5.1-isoform only (N. Bartetzko & F. Spener, unpublished work). The result of these experiments demonstrates that each isoform is encoded by its specific mRNA and precludes post-translational deamidation. Interestingly, only the pl 4.9-isoform is found in the mitochondrial matrix. Whether a specific import mechanism is responsible for this phenomenon is currently under investigation.

Tertiary structures have been solved for intestinal-type FABP [8] and for myelin P2 protein [9], the latter also a member of the FABP-family. The three dimensional structure of the intestinal FABP/fatty acid complex at 0.12 nm resolution [8] reveals the ligand fatty acid held inside the protein and an interaction of the fatty acid’s carboxyl group with Arg106. Modification experiments with phenylglyoxal, an arginine-specific reagent, indicate that in the case of cardiac FABP this basic amino acid interacts with the ligand fatty acid as well (T. Börchers & F. Spener, unpublished work). Recently, tertiary structures of a further two members from this protein family were published, that of cardiac FABP from bovine heart [10] (Fig. 1β) and that of murine adipocyte FABP [11]. Common to all structures is a highly conserved structural motif that consists of two β-sheets, each built from five antiparallel β-strands and one being interrupted by two short α-helices. The barrel-like β-sheets provide the environment for the fatty acid bound in the fatty acid-binding protein; dissociation constants are in...
Lipid-Binding Proteins

Structure of the cardiac type FABP

(a) Primary structures of cardiac FABP from bovine heart and of MDGI from bovine mammary gland. Differences are marked by an asterisk. (b) Schematic representation of tertiary structure of cardiac FABP. Amino acids of interest that are discussed in the text are marked by an arrow. P, phosphorylation site; FA, interaction with fatty acids; M, mutagenesis to convert cardiac FABP to MDGI. The shaded β-strand represents the sequence (Thr10-Gln131) of the inhibitory peptide.

![Figure 1](image)

the range $10^{-6}$ to $10^{-7}$ M [12, 13]. The functional aspect of fatty acid binding by this protein will be treated in the next section.

With regard to structure–function relationships three points are worthwhile mentioning. Firstly, the α-helices from positions 15 to 35 form an α-helix/turn/α-helix motif well known for a class of DNA-binding proteins. Taking into account that cardiac FABP is present in the nucleus, one may speculate over a role for this protein in gene regulation. Work is in progress to study the import of cardiac FABP and of mutants lacking the α-helix/turn/α-helix motif into the nucleus and their interaction with DNA. Secondly, a consensus sequence for tyrosine phosphorylation is present around Tyr10, which may indicate that cardiac FABP is a target in signal transduction. Thirdly, a stunning 96% homology of cardiac FABP to a 'mammary derived growth inhibitor' is noticed. The latter two findings are discussed in more detail in the last two sections.

Modulation of fatty acid metabolism

Among the functions that have been proposed for FABP and that we may call functions of the first generation are: (i) to serve as a pool for solubilized fatty acids, (ii) to promote the cellular uptake of fatty acids and their utilization, (iii) to protect enzymes from detergent effects of fatty acids, (iv) to modulate enzyme activities and (v) to facilitate targeted transport of fatty acids to specific metabolic pathways (Fig. 2b). Here we shall discuss the modulation of acyl-CoA ligase and of enzymes of the β-oxidative system in bovine heart mitochondria.

The functional aspect of compartmentation of cardiac FABP in the cytosol and mitochondrial matrix of myocytes was tested under the following working hypothesis: under conditions of low carbohydrate supply the heart cell gains 75% of the energy needed from the oxidation of fatty acids. Thus upon uptake of fatty acids from the blood into the cell, they partition into the cytosol for transport to the outer mitochondrial membrane, where they are activated by acyl-CoA ligase, a prerequisite for import of the fatty acid into the mitochondrial matrix and subsequent degradation via β-oxidation. In this intracellular path cardiac FABP may intervene in a 2-fold manner: firstly, binding the fatty acid in the cytosol by FABP promotes a targeted transport and/or modulates the activity of the acyl-CoA ligase, and secondly, the FABP in the matrix may modulate fatty acid oxidation. This rationale was tested experimentally in vitro with intact and broken mitochondria, respectively (J. Hassink, H. Rüdel & F. Spener, unpublished work).

Under optimized conditions intact mitochondria were monitored for acyl-CoA ligase activity with 14C-labelled palmitic, oleic and arachi-
Modulation of enzymic activities by cardiac FABP

(a) Modulation of activation of palmitic acid — O — oleic acid — O — and arachidonic acid — by mitochondrial acyl CoA-ligase in the presence of cardiac FABP. Incubation conditions: 27 μM fatty acid, 0.4 mM MgCl₂, 2 mM ATP, 0.5 mM EDTA, 1 mM KCN, 50 μg mitochondrial protein in 0.22 ml 150 mM Tris/HCl (pH 8.0, 37°C). (b) Model for the promotion of targeted transport of fatty acids by cardiac FABP. M, plasma and intracellular membranes, respectively; E, membrane-bound enzyme.

Unreacted fatty acids were removed by solvent extraction and the amount of newly synthesized acyl-CoAs in the aqueous phase was determined by liquid scintillation counting (Fig. 2a). In the absence of cardiac FABP the activity of acyl-CoA ligase is similar for palmitate and arachidonate, but for oleate is approximately 50% higher. In the presence of increasing amounts of cardiac FABP, on the one hand a concomitant pronounced decrease of oleoyl-CoA and arachidonoyl-CoA formation is seen. Already at 40 μM FABP arachidonoyl-CoA synthesis is almost abolished. On the other hand the inhibition of palmitic acid activation is less effective. Taking into account the dissociation constants for cardiac FABP (pI 4.9-isof orm) in complex with palmitic acid (0.88 μM), oleic acid (0.27 μM) and arachidonic acid (0.24 μM) (G. Jagschies & F. Spener, unpublished work) as determined by the Lipidex method [14], it is obvious that the high affinity of cardiac FABP for oleate was overcompensated by the high specificity of the enzyme for this substrate. The substrate specificity of the acyl-CoA ligase and the affinity of cardiac FABP for individual fatty acids explain the preferred channelling of palmitic and oleic acid into mitochondria, while arachidonate is retained in the cytosol. As shown in the model (Fig. 2b) the modulating effect of cardiac FABP on acyl-CoA ligase is indirect and based on individual partition equilibria for fatty acids between the enzyme-containing mitochondrial membrane and cardiac FABP. This indirect action of cardiac FABP corresponds with earlier experiments, where we could not detect direct interaction of this protein with mitochondrial membranes [4, 13], contrary to the situation in liver, where hepatic FABP is associated with the outer membrane of bovine liver mitochondria [5, 13].

The functional significance of mitochondrial localization of cardiac FABP for the degradation of acyl-CoAs was studied by incubating broken mitochondria with palmitoyl-CoA and oleoyl-CoA as substrates, again in the absence and in the presence of varying amounts of FABP. The assay system is based on the reduction of dichlorophenolphthalein by acyl-CoA dehydrogenases [15] and was optimized in the presence of KCN to inhibit mitochondrial respiration. With a substrate concentration of 10 μM acyl-CoA we observed in the presence of up to 20 μM cardiac FABP a decrease in the reaction rate of about 50%. When the acyl-CoA concentration was varied at a concentration of FABP fixed at 10 μM, the rate of oxidation at 5–10 μM acyl-CoA was lower, but at elevated acyl-CoA concentrations, up to 30 μM, the rate of oxidation...
was higher than that without cardiac FABP in the assay system. One explanation is that the concentration of 'free' acyl-CoA, which inhibits the acyl-CoA dehydrogenase is lower in the presence of cardiac FABP. It is possible that the FABP presented under physiological conditions to the mitochondrial matrix modulates the activities of the \( \beta \)-oxidative enzymes. Under anoxic conditions, when the concentration of acyl-CoA is heart rises, cardiac FABP may prevent the enzymes from being inhibited.

**Phosphorylation of cardiac fatty acid-binding protein**

Studies on the phosphorylation of proteins in 3T3-L1 cells revealed the adipocytic FABP as cellular target of the insulin receptor tyrosine kinase [16, 17]. Insulin-dependent phosphorylation occurred at Tyr\(^{19} \), which is part of the well-known consensus sequence for tyrosine kinases, Asn-Phe-Asp-Asp-Tyr, a sequence also present in the first \( \alpha \)-helix domain of cardiac FABP. It was thus conceivable that cardiac FABP takes part in insulin-dependent signal transduction, as insulin receptors are located in the plasma membrane of heart muscle cells as well. Our experimental model comprised a primary culture of adult rat heart myocytes treated with ortho-vanadate and phenylarsineoxide to inhibit phosphatase action and, concomitantly, to allow for accumulation of phosphorylated species (S. U. Nielsen & F. Spener, unpublished work). Myocytes were isolated via Langendorff perfusion of rat heart and after incubation with \(^{32}\)P phosphate in the presence of insulin, cytosolic proteins were separated by two-dimensional gel electrophoresis and blotted onto PVDF-membranes. Autoradiography of blots revealed several \( \sim 15 \) kDa proteins among phosphoproteins, yet only one of these could be precipitated specifically by affinity purified polyclonal antibodies. As expected, this spot did not match with either of the two isoforms of rat cardiac FABP observed after immunostaining, as the addition of the negatively charged phosphate moiety results in the pl of the phosphoprotein being slightly more acidic than that of the minor isomer. The amount of rat cardiac FABP actually phosphorylated was estimated to be clearly below \( \sim 0.5\% \) of total cardiac FABP in accordance with data on the phosphorylated adipocyte FABP, which indicate a stoichiometry for phosphorylation of 0.2\% [17]. In experiments where insulin was omitted during incubation with \(^{32}\)P phosphate, phosphorylated cardiac FABP was detected in autoradiograms only in minute amounts, if at all. This does not prove but certainly is in support of our hypothesis that cardiac FABP is an intracellular target of the insulin receptor tyrosine kinase. Further support came from the identification of the phosphorylated amino acid. For this purpose rat cardiac FABP was purified from radioactively labelled myocytes to near homogeneity in one step by immunoaffinity chromatography and submitted to tryptic peptide mapping. T.l.c. analysis together with radiosequencing of the major phosphopeptide clearly established Tyr\(^{19} \) as substrate for the kinase.

The low degree of phosphorylation observed in vivo precludes an effect on the gross flow of fatty acids, as may be deduced from the recent finding that phosphorylation of adipocytic FABP virtually abolishes fatty acid binding [18]. Together with a 10-fold reduction in \( K_\text{m} \) for phosphorylation of lipiddated adipocytic FABP compared with the apo FABP [19], an insulin-dependent cycling of fatty acids from the plasma membrane to the endoplasmic reticulum for eventual utilization was suggested. Considering the 500-fold excess of unphosphorylated FABP, however, that reportedly also has the potential to target fatty acids between membranes [20, 21] this catalytical model appears rather unlikely. Nevertheless, it is tempting to speculate that phosphorylation of FABP provides an early intermediate in the signalling chain from activated insulin receptor to enzymes of lipid metabolism. A possible role for FABP in this transduction is the sensing of the intracellular fatty acid concentration. Whether the phosphorylated FABP interacts with other intermediates of the signalling chain or directly with some specific regulatory elements of genes for enzymes of lipid metabolism [22] is not known at present.

**Inhibition of cell growth**

In the course of studies on mammary gland morphogenesis and the role of regulatory polypeptides controlling growth, differentiation and regression of the mammary epithelium, a factor was isolated from bovine lactating mammary gland with inhibitory action on various normal and transformed mammary epithelial cell lines [23]. This factor, named mammary derived growth inhibitor (MDGI), surprisingly shares with cardiac FABP from bovine heart a very high sequence identity. In Fig. 1(a) the seven amino acids that differ in the primary structure of both proteins are marked. Due to its binding capacity for fatty acids [24], its immunological cross-reactivity, its localization in 2D-gels and its abundance, MDGI may be regarded as an isoform of cardiac FABP. However, the
growth inhibitory action is not connected to binding of ligands, as a synthetic peptide spanning amino acids Thr\textsuperscript{121}-Gln\textsuperscript{131} (shaded in Fig. 1b) also inhibits growth of Ehrlich ascites mammary tumor cells, although two orders of magnitude less effectively than the whole protein [25]. Bovine heart cardiac FABP has no inhibitory effect in this proliferation assay despite its similarity to bovine MDGI (Fig. 3). To exclude the possibility that the manner of purification affects the inhibitory activity or that minute impurities of authentic MDGI were responsible for the biological action, we employed recombinant proteins in the comparison of MDGI and cardiac FABP. Efforts to clone a cDNA coding for MDGI from lactating bovine mammary gland were not successful, in fact, only a cDNA encoding the PI 5.1-isoform of cardiac FABP was obtained [26]. Thus, by site directed mutagenesis [27], cardiac FABP was sequentially transformed to MDGI. From corresponding cDNAs highly productive expression vectors based on the pET system [28] were constructed and the respective recombinant FABP, mutants and eventually the recombinant MDGI were overexpressed in \textit{Escherichia coli} strain BL21 (DE3). Recombinant proteins comprised up to 40\% of cytosolic proteins and were purified to homogeneity by a combination of cation exchange chromatography and gel filtration (A. Kromminga, A. G. Lexius, R. Grosse & F. Spener, unpublished work). The antiproliferative capacity of these proteins is summarized in Fig. 3. Recombinant FABP has no effect on growth of Ehrlich ascites tumor cells, whereas the first mutant, in which Thr\textsuperscript{121} of cardiac FABP is exchanged against Val\textsuperscript{132} of MDGI inhibits growth of tumor cells by \( \sim 20\% \) at a concentration of \( 10^{-8} \) M. Both recombinant and authentic MDGI inhibit cell growth at this concentration by \( \sim 40\% \). These data suggest that the C-terminal amino acids of MDGI carry the signal for the inhibition of proliferation of Ehrlich ascites tumor cells. Indeed, a short stretch of five amino acids (Arg-Val-Tyr-Glu-Lys), characteristic for many growth factors, is present in the active peptide.

MDGI resembles the adipocyte FABP in as much as both are expressed in a differentiation dependent manner. Expression of the proteins is highest in terminally differentiated tissue. MDGI is virtually absent in virgin mammary gland, scarce in pregnant mammary gland but abundant in the lactating tissue [13, 26].

**Conclusion**

FABPs are more and more understood at the level of primary structure: the molecular origin of isoforms, the mechanism of ligand binding and even tertiary structures are being unravelled. Yet the physiological function of FABP \textit{in vivo} still remains enigmatic and a more diverse role in cellular function than previously appreciated has to be envisaged as discussed in the last two sections. Progress in our understanding of FABP may arise from studies of the regulation of the protein at the genomic level [29], e.g. promotor analysis, and from transfection experiments either with sense or antisense constructs.

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Labelling of an 88 kDa adipocyte membrane protein by sulpho-N-succinimidyldi-long-chain fatty acids: inhibition of fatty acid transport

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Introduction
Di-isothiocyanodisulphonic acid (DIDS) is a potent irreversible inhibitor of oleate transport in rat adipocytes. Gel electrophoresis of plasma membranes isolated from rat adipocytes which had been incubated with tritiated dihydro DIDS demonstrated a prominent peak of radioactivity associated with membrane components migrating like a protein of 80000 to 90000 implicating it in the membrane transport of fatty acids (FAs). However, two concerns limited data interpretation. The first one related to the lack of specificity of the compound which, at the concentrations required to produce inhibition of FA uptake, labelled multiple membrane proteins. Secondly, since DIDS is a bifunctional reagent, the labelled proteins could have represented cross-linked products of smaller ones.

We have synthesized monofunctional non-