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I-FABP expression alters the intracellular distribution of the BODIPY C16 fatty acid analog

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Abstract

To investigate the structure–function relationships of intestinal fatty acid-binding protein (I-FABP) in cellular fatty acid (FA) trafficking, we compared the distribution of a fluorescent FA analog (BODIPY FL C16) in Cos-1 cells transiently transfected with the wild type protein (wt I-FABP) to that of a variant deleted of the alpha helical domain (HL I-FABP). In vector-only cells, BODIPY fluorescence was distributed throughout the cytoplasm. In the absence of added FA, wt I-FABP was found largely in the perinuclear region with some cytoplasmic staining as well. Addition of BODIPY FL C16 to transfected cells showed that the fluorescent FA was essentially completely colocalized with the protein in the cytoplasmic and perinuclear regions as well as in cytoplasmic clusters that are not observed in the absence of wt I-FABP. For HL I-FABP, the distribution of the protein in the absence of FA was diffusely cytoplasmic, in marked contrast to the wt protein. Addition of BODIPY led to less extensive colocalization than that observed for wt I-FABP. In particular, no localization to the perinuclear region was found. Organelle colocalization studies showed that both proteins colocalized with mitochondria and endoplasmic reticulum/golgi markers, but little with a lysosomal marker. The perinuclear localization for wt I-FABP and BODIPY did not show colocalization with any of the markers tested. Taken together, these results indicate that I-FABP binds FA *in vivo* and that the helical domain may be important for targeting I-FABP to a perinuclear domain but not, perhaps, to the endoplasmic reticulum, golgi apparatus or mitochondria.

Keywords

BODIPY FL C16; I-FABP; Fatty acid; Fatty acid binding protein; Fluorescence

Introduction

Long chain fatty acids (LCFA) are well known as essential components of phospholipids, triglycerides and cholesteryl esters, and major substrates in energy metabolism reactions. Furthermore, during the last decade increased attention has been paid to their ability to bind nuclear transcription factors, casting a new light on their role in regulating the expression of specific genes [1]. This plurality of actions has led to the hypothesis that, within cells, LCFA function could be linked to their specific routing toward defined subcellular destinations. Such a key role in intracellular LCFA trafficking seems likely for the members of the fatty acid binding protein (FABP) family [2]. FABPs are ubiquitously expressed small proteins that share similar three-dimensional structures and are known to bind LCFA and in some cases other lipophilic ligands with a high affinity. Thus far, nine tissue-specific cytoplasmic FABPs have been identified in mammals and their roles in LCFA binding and transport have been investigated [3]. In addition, some members of the FABP family have been shown to be involved in cell growth and proliferation and have been found to cooperate with specific nuclear receptors of the PPAR family [4–6]. In intestinal cells that have to deal with particularly abundant amounts of fatty acids during the digestion phase, two FABPs are largely and equally expressed: FABP1 (or liver-FABP, L-FABP) that is also expressed in the liver and to a lesser extent elsewhere and FABP2 (or intestinal-FABP, I-FABP), which is specifically expressed in fully differentiated proximal absorptive enterocytes [7]. Another

member of the FABP family, the ileal lipid binding protein (ILBP) is expressed more distally along the small intestinal tract and is thought to bind primarily bile acids [8].

I-FABP and L-FABP have been extensively studied, and a large amount is known regarding their structural features and binding characteristics [9]. In vitro studies of fatty acid transport have demonstrated different fatty acid transfer mechanisms for I- and L-FABP, with the helical domains of the proteins being critical for determining the rate and mechanism of ligand transfer [3, 10]. Although it is hypothesized that these FABPs are important in intracellular transport of FA, their precise functions as well as the reason why a single cell type, the proximal enterocyte, contains more than one FABP are only beginning to be understood [7, 11, 12]. Studies of *Fabp2*^{-/-} mice found that different changes occurred in male and female animals and that an adaptive response in L-FABP and ILBP protein concentrations did not readily account for the gender dimorphism [13, 14]. In addition to studies in gene ablated mice, functional analysis of FABPs is being examined at the cellular level, where fluorescent lipid analogs such as NBD- or BODIPY-labeled fatty acids have been used to probe the functions of FABPs [15, 16]. By permitting simultaneous examination of the intracellular distribution of LCFA and FABPs, this approach in theory represents a powerful tool to study the intracellular roles of FABPs in intracellular lipid trafficking.

In the present studies, we overexpressed wt I-FABP in cells that do not spontaneously express any known FABP form. Our results demonstrate that the subcellular pattern of distribution of the BODIPY LCFA analog is linked to the expression of the I-FABP, and vice-versa. In addition, by using a variant of I-FABP deleted of 17 residues corresponding to the alpha helical domain of the protein [17], the so-called “helix-less” I-FABP, we address a role of this domain in intracellular fatty acid trafficking.

Materials and methods

Materials

BODIPY FL C16, MitoTracker, LysoTracker, and BO-DIPY TR ceramide were obtained from Molecular Probes (Invitrogen, Cergy Pontoise, France). Oleic acid and bovine serum albumin (BSA) were from Sigma (Saint Quentin Fallavier, France). Cos-1 cell lines were obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France). Rhodamine, Texas Red and FITC-labeled goat anti rabbit antibodies were from Sigma. Rabbit antibodies to purified rat I-FABP were generated by affinity Bioreagents (Golden, CO, USA). p-MON-HL I-FABP plasmid was kindly provided by Drs. John Monsey and David Cistola (Washington University, St Louis, MO, USA) [17].

Recombinant plasmids and production of recombinant protein

Rat wt I-FABP cDNA was amplified by PCR from rat jejunum RNA after conversion into cDNA by reverse transcription. Amplified wt I-FABP cDNA was then cloned into pSG5 vector (Promega, Charbonnières, France). Rat HL I-FABP sequence was amplified from p-MON-HL and subcloned into pSG5. In order to produce the recombinant protein for a Western blotting standard, the coding sequence of HL I-FABP was cloned under the control

of the bacterial *lac Z* promoter in pQE 60 vector (Qiagen, Courtabœuf, France) so that the coding sequence was ended by a 6xHis-tagged sequence. The tagged recombinant HL I-FABP protein was produced in *E. coli* according to supplier's recommendations. Purification of the tagged recombinant protein was achieved using HisLink protein purification resin (Promega, Charbonnières, France). The purified rat wt I-FABP protein was produced as previously described [16].

Cell culture and transfection assays

Transient transfections were performed in Cos-1 cells. Cells were cultured at 37°C under 5% CO₂ in DMEM supplemented with 10% (v/v) fetal calf serum, 4 mM L-glutamine, 100 µg/ml streptomycin, and 100 i.u./ml penicillin. One day before transfection, cells were treated with 0.05% trypsin, 0.53 mM EDTA, and then replated in six-well plates (1.3×10^5 cells per well). After 24 h, Cos-1 cells were transfected with 1 µg of pSG5-wt I-FABP, pSG5-HL I-FABP, or empty vector using the JetPEI (Polyplus transfection, Strasbourg, France) method. Cells were transfected in serum containing medium, 24 h after transfection the medium was changed and serum was withdrawn. Cells were harvested 48 h after transfection.

Immunofluorescence and subcellular localization of I-FABP

Cos-1 cells were cultured on 14 mm diameter glass coverslips placed in 6-well plates, and transient transfections were performed as described above. Forty-eight hours after the transfection, cells were washed with 0.1 M PBS, pH 7.2, incubated for 30 min at 37°C with or without 1 µM BODIPY FL C16 in 0.1% DMSO or with 150 µM oleic acid bound to 50 µM BSA, or with organelle-specific fluorescent probes (500 nM MitoTracker or 100 nM LysoTracker or 2.5 µM BODIPY TR ceramide), and then briefly rinsed with 0.1 M PBS pH 7.2 at 37°C. Cells were then fixed with 4% paraformaldehyde for 15 min at 37°C and rinsed in 0.1 M PBS pH 7.2 (3×5 min). The Image-iT FX signal enhancer (Molecular Probes) was added for 1 h at room temperature and cells were rinsed with 0.1 M PBS pH 7.2. They were incubated with primary anti I-FABP (diluted 1/2,000 in 0.1 M PBS pH 7.2, 0.1% casein) for 3 h at room temperature, and then overnight at 4°C. Cells were rinsed at room temperature in the same buffer before being incubated either with rhodamine-labeled, Texas Red-labeled, or with FITC-labeled secondary antibodies (diluted 1/2,000 in the same buffer) for 3 h at room temperature and then overnight at 4°C. The cells were washed with the same buffer before mounting in Prolong gold antifade DAPI reagent (Molecular Probes) and stored at room temperature protected from light until viewing.

Microscopy

Cells were viewed on a Leica Dialux 20 microscope and the images were taken with the Leica DC 100 camera. Fluorescence images were observed using Leitz Ploem Opak incident fluorescent illuminator with filters corresponding to the fluorochromes used.

Western blotting

Forty-eight hours post transfection, cells were lysed with reporter lysis buffer (Promega) and total proteins were quantified using the BCA Protein Assay (Perbio Science, France). About

10–50 µg of total proteins were fractionated by SDS-PAGE using 15% polyacrylamide gel and transferred onto PDVF membrane. Nonspecific binding sites were blocked using 5% solution of defatted milk proteins in PBS, 0.1% Tween. The membrane was probed with primary antibodies against I-FABP (diluted 1/10,000) followed by incubation with alkaline phosphatase conjugated secondary antibodies (diluted 1/5,000). The enzyme activity was detected using a colorimetric reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) as substrates. Purified recombinant rat wt I-FABP and HL I-FABP proteins were used as standards. Duplicate cultures were used for Western blot analysis and immunocytochemistry.

Results

Expression levels of the transfected proteins

Both wt I-FABP and HL I-FABP were recognized by the antibodies raised against I-FABP. However, to ensure that estimations of expression levels were not due to any differences in each of the proteins' affinity to the anti-I-FABP antibodies, protein concentrations of wt I-FABP and HL I-FABP were estimated using standard curves with recombinant wt I-FABP and HL I-FABP, respectively. Western blot analysis shows that both proteins were expressed at high levels, corresponding to 1–2% of total cellular proteins (Fig. 1). These levels fit well within the range of those physiologically observed inside the intestinal cell [3, 18].

Localization of wt I-FABP, BODIPY FL C16, and HL I-FABP in Cos-1 cells

To investigate the role of I-FABP in fatty acid trafficking within the cell, the cellular distribution of the fluorescent fatty acid BODIPY FL C16 was studied, either in the presence or absence of the protein.

In the absence of I-FABP, i.e., in vector-only transfected cells, the BODIPY FL C16 exhibited a diffuse cytoplasmic distribution (Fig. 2a). In cells expressing wt I-FABP, in the absence of BODIPY FL C16, the protein distribution was largely perinuclear with some cytoplasmic staining as well (Fig. 2b). When the protein and the BODIPY FL C16 were present together, the protein was detected not only in the cytoplasmic and perinuclear areas but also in cytoplasmic clusters that were not observed in the absence of any FA (Fig. 3a). In addition, as shown in Fig. 3a, b, the protein and the BODIPY FL C16 colocalized extensively in the cytoplasmic and the perinuclear areas as well as in cytoplasmic clusters. When oleate was used rather than the BODIPY FL C16, the same distribution of wt I-FABP was observed.

As noted above, we have hypothesized that the helix-turn-helix domains of the FABPs are important for their fatty acid transport functions. To address the question of the structure–function relationship for I-FABP, the helix less variant was transfected into Cos-1 cells and the distribution of the protein and the fluorescent fatty acid were compared to those of wt I-FABP. As shown in Fig. 2c, in the absence of BODIPY FL C16, the HL I-FABP was localized throughout the cytoplasm without any particular pattern of distribution. In the presence of BODIPY FL C16, the protein remained primarily cytoplasmic and was only

occasionally observed in the perinuclear region (Fig. 3c). In the HL I-FABP cells, the BODIPY fatty acid was localized in the cytoplasm as well as in cytoplasmic clusters but not in the perinuclear area (Fig. 3d). Thus in contrast to the native I-FABP, colocalization of the HL I-FABP and BODIPY FL C16 was less extensive, the specific cytoplasmic clusters stained with the fatty acid were never colocalized with the protein, and no colocalization in the perinuclear region was found.

Colocalization of I-FABP and subcellular organelles

The presence of FA appears to be necessary for the shift of the FABP to cytoplasmic clusters. It was therefore of interest to know which subcellular organelle(s) was the target of the protein. Using organelle-specific fluorescent probes in the presence of non-fluorescent oleic acid, it was found that both wt I-FABP and HL I-FABP colocalize with MitoTracker, a probe for mitochondria (Fig. 4a, b), and with BODIPY TR ceramide, a probe for the endoplasmic reticulum and golgi apparatus (Fig. 4c, d). Neither protein showed colocalization with LysoTracker, a probe for lysosomes (Fig. 5a, b).

Figure 4a shows that wt I-FABP colocalized with mitochondria, showing colocalization also with some cytoplasmic clusters. Colocalization was also observed for wt I-FABP and probes for endoplasmic reticulum and golgi apparatus (Fig. 4c). It is noteworthy that the above mentioned perinuclear colocalization for wt I-FABP and BODIPY FL C16 did not show colocalization with any of the markers tested. By contrast, although HL I-FABP colocalized with mitochondria (Fig. 4b) and with endoplasmic reticulum and golgi apparatus (Fig. 4d), some cytoplasmic localizations of the protein did not colocalize with any of the markers tested.

Discussion

The aim of this work was to investigate the role of I-FABP in fatty acid trafficking within the cell. Intestinal absorptive cells express two FABPs, I-FABP, and L-FABP; however, at present there is no cell model of mature enterocytes that expresses physiologically relevant levels of both FABPs. Caco-2 cells, for example, which are widely viewed as most enterocyte-like insofar as lipid assimilation is concerned, nevertheless express physiologic levels of L-FABP and extremely low levels of I-FABP [19, 20]. In addition, I-FABP overexpression in cells spontaneously expressing L-FABP results in altered L-FABP expression, which modifies the proportion of the two proteins [10, 21]. Thus we chose, as a first approach, to use a Cos cell model to obtain levels of I-FABP that are in the range of those observed in vivo in order to study the effects of I-FABP expression on fatty acid distribution in the cell. We showed previously that the fatty acid, BODIPY FL C16, binds to I-FABP with high affinity and that within the time-scale of the experiments used here it was found to be poorly or not metabolized in cultured cells [16].

In the present studies, we monitored the steady-state distribution of the FA analog BODIPY FL C16 after a 30 min incubation with Cos-1 cells. We found that the intracellular distribution of the fatty acid analog is strongly linked to the presence of the I-FABP protein. When Cos-1 cells are transfected with vector alone and do not express I-FABP, the FA analog was localized diffusely in the cytoplasm. In cells expressing wt I-FABP, in contrast,

the BODIPY distribution was found to be mostly perinuclear. This shift in FA localization thus seems to be linked to the presence of the wt I-FABP protein. Colocalization of BODIPY C16 with L-FABP was shown previously [16] in Caco-2 cells which, as mentioned above, physiologically express this protein. Although no endogenous FABP expression has been reported in Cos cells, we cannot rule out the possibility that the BODIPY distribution could be the result of an as-yet unidentified endogenous FABP or other FA binding/transport protein. In any case, expression of I-FABP results in a specific redistribution of long chain fatty acid within these cells.

The effect of I-FABP on localization of BODIPY FL C16 supports a role for the protein in the directed intra-cellular trafficking of unesterified fatty acid. Alpers et al. [22], using a rat intestinal explant system, demonstrated a marked redistribution of both L-FABP and I-FABP from an apical localization in the fasted state to the entire cytoplasm following a 3 h high fat feeding, and suggested a role for I-FABP, in particular, in the compartmentation of apically delivered fatty acids in the enterocyte. Our results support this hypothesis, indicating a potential targeting function for I-FABP in directing long chain FA to specific subcellular sites of utilization. The results show that BODIPY FL C16 is taken up by Cos cells transfected with I-FABP and that in the presence of I-FABP the FA analog is targeted to sites for metabolic pathways such as beta-oxidation and esterification into phospholipids and triacylglycerols. The similar data obtained in Caco-2 cells, demonstrating that BODIPY C16 was taken up by the cells and colocalized with L-FABP as well as markers for the mitochondria and endoplasmic reticulum/golgi apparatus [16], imply that the two FABPs may have certain overlapping functions in enterocyte fatty acid metabolism. However, no study has directly compared the localization of both I-FABP and L-FABP at the single cell level, in the absence and presence of free fatty acid. Interestingly, our results also cast light on the fact that targeting FA to the perinuclear area is dependent on the presence of I-FABP within the cell. Since we never observed I-FABP within the nucleus, a question that remains to be addressed is whether the FA, either bound or not to the protein, might enter the nucleus and participate in regulation of gene expression, as has been suggested for other members of the FABP family [4, 23].

As for all members of the FABP family, I-FABP exhibits a tertiary structure consisting of two short alpha helices and 10 antiparallel beta strands, organized in two beta sheets that delimit the ligand-binding pocket [9]. In vitro studies of FA transfer from I-FABP to membranes have shown the transfer process to be of a collisional type, in which FA movement occurs during protein-membrane interactions [3]. This collisional process appears to be dependent on the presence of the alpha helical domain [24–26]. By contrast, during in vitro transfer of FA from the HL I-FABP, the collisional process is almost completely ablated, and transfer is largely of a diffusional type [26]. The role played by the alpha helical domain of I-FABP in a cellular milieu was examined by the HL I-FABP transfection experiments. The colocalization of HL I-FABP with the FA analog, though not complete, indicates that FA can be bound by the beta sheet structure of the protein and that the alpha helical domain is not essential for ligand binding. This agrees with in vitro structural and binding studies showing that the helix-turn-helix domain of I-FABP was not required to maintain the integrity of the ligand binding cavity, as HL I-FABP displayed similar beta barrel topology and FA-binding characteristics as the wt protein [17, 27]. The

microscopic studies demonstrated that, unlike the largely perinuclear localization of wt I-FABP in the absence of BODIPY FL C16, the HL I-FABP resided almost entirely in the cytoplasm. In addition, compared to what was found in wt I-FABP transfected cells, FA remains in the cytoplasm of HL I-FABP transfected cells where it colocalizes to some extent with probes for mitochondria, endoplasmic reticulum, and golgi apparatus, but FA was mostly not targeted to the perinuclear area. Thus, the alpha helical domain may be important for targeting I-FABP to a perinuclear domain but perhaps not to endoplasmic reticulum, golgi, or mitochondria. Nevertheless, it is worth considering that Cos-1 cells may not contain the appropriate targets for I-FABP localization, which may be present in intestinal absorptive cells.

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Abbreviations

BODIPY FL	4,4-difluoro-5,7-dimethyl-4-bora-3a
C16	4-azido-3-indacene-3-hexadecanoic acid

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Fig. 1.

Western blot analyses for the detection of wt I-FABP and HL I-FABP in Cos-1 transfected cells, using polyclonal anti rat I-FABP. **a** Lane 1: purified rat wt I-FABP protein 25 ng, lane 2: 250 ng, lane 3: 500 ng, lane 4: lysate from wt I-FABP transfected cells 10 µg, lane 5: 30 µg, lane 6: lysate from vector only transfected cells 30 µg. **b** Lane 1: lysate from vector only transfected cells 50 µg, lane 2 and 3: lysates from HL I-FABP transfected cells 50 µg, lane 4: purified rat HL I-FABP protein 250 ng, lane 5: 500 ng. MW lanes: PageRuler Prestained Protein Ladder (Fermentas)

Fig. 2.

a Intracellular distribution of BODIPY FL C16 (1 μ M, 30 min) in vector-only transfected cell; **b** Immuno-localization of wt I-FABP in wt I-FABP transfected cells in the absence of BODIPY FL C16; **c** Immuno-localization of HL I-FABP in HL I-FABP transfected cells in the absence of BODIPY FL C16. **b** and **c**: 48 h post transfection cells were fixed then incubated with primary antibody (rabbit polyclonal anti rat I-FABP), followed by Texas Red-labeled goat anti-rabbit antibody. Bar = 10 μ m, all pictures were taken at the same magnification

Fig. 3. Immuno-localization of wt I-FABP or HL I-FABP (red) and distribution of fluorescent BODIPY FL C16 (green) in wt I-FABP transfected cells (**a, b**) and in HL I-FABP transfected cells (**c, d**). 48 h post transfection, cells were incubated with BODIPY FL C16 (1 μ M, 30 min), fixed then incubated with primary antibody (rabbit polyclonal anti rat I-FABP), followed by Texas Red-labeled goat anti-rabbit antibody. Bar = 10 μ m, all pictures were taken at the same magnification

Fig. 4.

Colocalization (orange) of the protein (green) and mitochondria (**a** and **b**) or endoplasmic reticulum/golgi (**c** and **d**) in wt I-FABP (**a**, **c**) or in HL I-FABP (**b**, **d**) transfected cells in the presence of oleic acid. 48 h post transfection cells were incubated with 150 μ M oleic acid, 50 μ M BSA together with MitoTracker (500 nM, **a** and **b**) or with BODIPY TR ceramide (2.5 μ M, **c** and **d**), which both gave a red fluorescence, for 30 min, fixed then incubated with primary antibody (rabbit polyclonal anti rat I-FABP) followed by FITC-conjugated goat anti-rabbit antibody. Bar = 10 μ m, all pictures were taken at the same magnification with an immersion-oil objective

Fig. 5.

Localization of Lysosomes (**a**), and immuno-localization of the HL I-FABP (**b**) in HL I-FABP transfected cells in the presence of oleic acid. Comparable images can be seen in wt I-FABP transfected cells. 48 h post transfection cells were incubated with 150 μ M oleic acid, 50 μ M BSA together with red fluorescent LysoTracker (100 nM, **a** and **b**) for 30 min, fixed then incubated with primary antibody (rabbit polyclonal anti rat I-FABP) followed by FITC-conjugated goat anti-rabbit antibody. Bar = 10 μ m, all pictures were taken at the same magnification with an immersion-oil objective