Effects of intestinal fatty acid-binding protein overexpression on fatty acid metabolism in Caco-2 cells

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Abstract **Intestinal fatty acid-binding protein (I-FABP) is a cytosolic protein expressed at high levels (up to 2% of cytosolic proteins) in the small intestine epithelium. Despite cell transfection studies, its function is still unclear. Indeed, different effects on fatty acid metabolism depending on the cell type and the amount of I-FABP expressed have been reported. Furthermore, a decrease in fatty acid incorporation has been unexpectedly obtained when I-FABP reached 0.72% of cytosolic proteins in fibroblasts (**Prows et al. **1997.** ***Arch. Biochem. Biophys.*** 340: **135). In the present study, the effect of a high level of I-FABP similar to amounts present in the small intestine was investigated in the human colon adenocarcinoma cell line, Caco-2. After transfection with human I-FABP cDNA, a clone expressing 1.5% I-FABP and unchanged level of liver FABP was selected. These cells, which had a lower rate of proliferation as compared with mock-transfected cells, developed the typical morphological characteristics of differentiated enterocytes. Incubation of differentiated cells with [^{14}C]palmitate showed a 34% reduction ($P < 0.01$) of fatty acid incorporation, whereas the relative distribution of radiolabel into triglycerides was not affected. A nonsignificant 21% reduction of fatty acid incorporation was observed with another clone expressing 10-fold less I-FABP. In conclusion, a high level of I-FABP expressed in a differentiated enterocyte model inhibited fatty acid incorporation, by a mechanism which remains to be defined.**—Darimont, C., N. Gradoux, E. Persohn, F. Cumin, and A. De Pover. **Effects of intestinal fatty acid-binding protein overexpression on fatty acid metabolism in Caco-2 cells.** ***J. Lipid Res.*** **2000.** 41: **84–92.**

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Fatty acid-binding proteins (FABP) are small (14 kDa) cytosolic proteins that bind long-chain fatty acids with submicromolar affinities (1, 2). They are highly concentrated in cells that actively metabolize fatty acids, representing 1–5% of cytosolic proteins (3). Thus, it is generally agreed that their primary function is to facilitate the intracellular transport and metabolism of fatty acids. There are tissue-specific types, e.g., in adipose tissue (A-FABP), heart and skeletal muscle (H-FABP), liver (L-FABP), and small intestine (I-FABP) (3). The presence of two types, L-FABP and I-FABP, in enterocytes is intriguing. L-FABP has two distinct fatty acid-binding sites instead of one for I-FABP (4) and can bind amphiphilic ligands (5). I-FABP is mainly expressed in the villus tips, while L-FABP is localized more deeply in the crypts (6, 7). In enterocyte cultures, different patterns of expression and regulation have been reported for these two types (8). Furthermore, experiments on liposomes suggest that I-FABP transfers fatty acids to membranes more efficiently than L-FABP, presumably by a collisional mechanism (9). However, in spite of direct studies in transfected cells (10–15), the role of I-FABP in fatty acid uptake and metabolism is still unclear.

In 1995, Baier et al. (16) reported the discovery in the Pima Indian population of a Thr/Ala substitution at codon 54 of I-FABP leading to increased fatty acid binding. In a transfection study, Caco-2 cells expressing very low amounts of the Thr54 protein (0.02% of cytosolic proteins) secreted more triglyceride than cells expressing the Ala54 protein (10). The Caco-2 cell line, derived from human colon adenocarcinoma, develops the characteristics of differentiated intestinal epithelial cells and is considered as a good model of lipoprotein secretion (17). However, the presence of large amounts of L-FABP in Caco-2 cells requires an evaluation of possible interactions between the two types, e.g., on protein expression. More recently, cell lines were chosen in transfection studies for the absence of endogenous intestinal and liver types. Mouse L cell fibroblasts transfected with the rat I-FABP cDNA had increased triglyceride and cholesteryl ester synthesis (11, 12). In contrast, in the rat intestinal hybrid cells "hB-RIE 380i-neg," which were unable to differentiate, transfection with the rat I-FABP cDNA induced a slight increase in radiolabeled oleic acid incorporation into diacylglycerol only (15). In both cell lines, which expressed similar amounts of I-FABP (about 0.35% of cytosolic pro-

Abbreviations: I-FABP, small intestine-specific fatty acid-binding protein; L-FABP, liver-specific fatty acid-binding protein.

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teins), fatty acid uptake was not affected (11, 12, 14, 15). To complicate matters, in L cells expressing larger amounts (0.72%), fatty acid uptake decreased (13). These observations suggest that the effect of I-FABP may differ according to the cell line and the amount of protein expressed, thus emphasizing the need for obtaining transfected enterocytes producing amounts similar to those present in small intestine epithelium.

The goal of the present study was to examine the effects of a high expression of I-FABP, similar to levels found in small intestine epithelium (up to 2% of cytosolic protein), on fatty acid incorporation and metabolism in Caco-2 cells. For this purpose, Caco-2 cells were stably transfected with human I-FABP cDNA. Two clones expressing respectively 0.12% ("IFABP1") and 1.5% of I-FABP ("IFABP2") were obtained. The latter showed L-FABP levels similar to those of control cells and appeared, therefore, suitable for studying the effects of a high level of I-FABP on palmitic acid metabolism. The results show a significant reduction of palmitic acid incorporation into IFABP2 cells, but not in IFABP1 cells.

MATERIALS AND METHODS

Material

pSF-neo and pSF-SMC/sCD23 vectors were kindly provided by Dr. F. Asselbergs (Novartis, Switzerland). Taurocholate and palmitic acid were purchased from Fluka (Zurich, Switzerland) and Sigma Chemical Co. (St. Louis, MO), respectively. [^{14}C]palmitic acid (55 Ci/mol) was obtained from Amersham (Zurich, Switzerland).

Cell cultures

Cell cultures were performed essentially as described previously (8). Caco-2 cells were subcultured on plastic plates (Falcon, Beckton Dickinson, Switzerland) coated with 30 μg collagen I per mL PBS (bovine skin type-I collagen, Roche Molecular Biomedicals) in a 10% fetal calf serum (FCS) medium (Dulbecco's modified Eagle's medium containing 4.5 g/L glucose and 4 mm glutamine, supplemented with 40 μg/mL gentamycin, 1% nonessential amino acids, and 10% FCS). For differentiation, cells were seeded on 24.5-mm (uncoated) polycarbonate Transwell-Clear filter inserts (0.4 μm pore size; Costar, Cambridge, MA) at a density of 4×10^5 cells/well in the 10% FCS medium. Cell differentiation was evaluated at day 17 by measuring the activities of brush border membrane enzymes, the sucrase-isomaltase and alkaline phosphatase, as previously described (8). For fatty acid transport experiments, the integrity of the monolayer was checked with phenol red.

Cell transfection

The cDNA coding for human intestinal [Ala54]-FABP was obtained as previously described (8) and was subcloned in the NcoI/BamHI sites of pSF-SMC/sCD23 (18). Caco-2 cells were transfected using a commercially available polycationic transfection system (Superfect Transfection Reagent, Qiagen, Switzerland). Cells were incubated for 3 h with 5 μg pSF-SMC/sCD23 plus 5 μg pSF-neo which expresses resistance to neomycin and its analogue G418 (geneticin®; Life Technologies; Switzerland), complexed with 50 μl of the Superfect transfection reagent. The DNA-containing medium was removed and cells were cultured in a fresh 10% FCS medium. After 48 h, 300 μg/mL G418 was added to the culture medium and viable clones were grown, isolated with cloning rings, and expanded. For a control cell line, only the pSF-neo was used for transfection. Transfected cells were subcultured and differentiated following the same protocol described above, with the exception that the media were supplemented with G418.

Western blot analysis of Caco-2 cells lysates

The quantitative analysis by Western blot of I-FABP and L-FABP concentrations in cell monolayer lysates was performed using sensitive and specific rabbit anti-human I- and L-FABP antisera as previously described (8).

Determination of cell number and DNA content

In order to evaluate cell proliferation, cells seeded at low density (20,000 cells/dish) on 60-mm diameter dishes were counted and DNA was quantified from day 1 to 6 after seeding. After a brief trypsination, cells were resuspended in a 10% FCS medium and directly counted using Coulter Counter® (Coulter Electronics Ltd.). DNA quantification was performed as described by Labarca and Paigen (19). Briefly, after 2 min centrifugation at 1000 *g*, cell homogenates made in 0.05 m $NaPO_4$ and 2 m NaCl, pH 7.4, were incubated with 20 μl of 10 mg/mL Hoechst 33258 reagent (Serva, Heidelberg, Germany). Fluorescence was immediately read at 365 nm for excitation and 450 nm for emission in a spectrofluorometer (Fluorolite 1000, Dynatech Laboratories), and the amount of DNA in samples was determined from a DNA standard curve.

Electron microscopy

Cell cultures were immersion fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 m cacodylate buffer, pH 7.4, containing 0.1 m saccharose. After postfixation with 1% OsO_4 in 0.1 m cacodylate buffer, pH 7.4, for 1 h at 4°C, the cell cultures were dehydrated in graded ethanol solutions, and embedded in Epon. Ultrathin sections of the three cell types (wild-type cells, mock-transfected cells, and cells overexpressing I-FABP) from selected cell monolayer blocks were counterstained with uranyl acetate and lead citrate and examined with a Philips CM 10 transmission electron microscope.

Determination of intracellular triacylglycerol mass

Cells were scraped from the inserts in 1 mL of ice-cold 0.2 m NaOH neutralized with HCl. Lipids were extracted with chloroform–methanol 2:1 (v/v), and the chloroform phase was washed once with methanol–water 1:1 (v/v) to remove excess of free glycerol, and completely evaporated under nitrogen. The residue was taken up in 20 μL isopropanol, and triacylglycerol concentration was determined using a commercially available enzymatic assay (GPO-Trinder, Sigma). This enzymatic assay transformed triglycerides into glycerol, which was then measured by coupled enzyme reactions catalyzed by glycerol kinase, glycerol phosphate oxidase, and peroxidase which led to a final product absorbing light at 540 nm in direct proportion to triglyceride concentration.

Fatty acid metabolism

Palmitic acid incorporation into lipid metabolites and their secretion in the basolateral medium were studied on cells at day 17 after confluence. Cells were incubated at 37°C for 15, 60 and 120 min with [^{14}C]palmitic acid–sodium taurocholate micelles on the apical side only (100 μm palmitic acid and 10 μm [^{14}C]palmitic acid in Dulbecco's modified Eagle's medium, supplemented with 8 mm sodium taurocholate, 40 μg/mL gentamycin, 1% nonessential amino acids). Incubation was terminated by removal of the apical and basolateral media, and the two compartments were washed twice with 0.75 mL of ice-cold PBS, and combined

with the media. Cells were scraped into 1 mL of ice-cold PBS and sonicated for 1 min. Fifty μl aliquots were diluted in scintillation fluid (Irgascint® A300; Novartis, Basel), and radioactivity was quantified using an LKB Wallac 1214 RackBeta liquid scintillation counter. Fatty acid incorporation was determined from the specific radioactivity of the incubation medium.

To determine fatty acid metabolites, lipids were extracted from cell lysates and basolateral media according to the method of Bligh and Dyer (20). Thin-layer chromatography was used to determine [^{14}C]palmitic acid incorporation into lipid metabolites. Lipid extracts were spotted on thin-layer chromatography plates (20 cm × 20 cm, Silica, Merck, Germany), and developed in hexane–diethylether–acetic acid 70:30:1. Radioactivity associated with lipids was measured by using a digital autoradiograph (Berthold, Germany).

Statistical analysis

All statistical analyses were performed on values by Fisher's LSD using the StatView software package. P values < 0.05 were considered significant. Data are presented as means ± SEM.

RESULTS

Expression of FABP types in transfected Caco-2 cells

Caco-2 cells were transfected with human [Ala54]-I-FABP cDNA. Two clones producing different amounts of I-FABP were obtained ("IFABP1" and "IFABP2" cells). The amounts of FABP proteins in IFABP1, IFABP2, and mock-transfected cells (control cells) were quantitatively measured by Western blot in confluent (day 0) and in differentiated cells (day 17). Results are reported in **Table 1**. The expression of I-FABP and L-FABP in control cells was similar to that previously reported for wild-type Caco-2 cells (8). In control cells, I-FABP was expressed only during differentiation and represented 0.016% of cytosolic proteins at day 17. In I-FABP transfected cells, both undifferentiated and differentiated, I-FABP represented about 0.15% (IFABP1) and 1.5% (IFABP2) of cytosolic proteins. In all cell types, L-FABP was already present at day 0. Between day 0 and day 17, it increased from 0.2% to 1.2% (control cells), from 3.8% to 5.8% (IFABP1), and from 0.4% to 1.8% (IFABP2). Thus, the high level of I-FABP in IFABP2 cells was associated with unchanged L-FABP level in comparison with control cells. In IFABP1 cells, L-FABP was very high already at day 0. For these reasons, the IFABP2 clone appeared the more suitable for studying the effect of I-FABP overexpression in Caco-2 cells. Its characteristics are described in details below.

Proliferation and differentiation of transfected cells

Cell proliferation was studied by measuring DNA contents and cell numbers. **Figure 1** shows that, already at day 3 after seeding, DNA content (Fig. 1A) and cell number (Fig. 1B) were significantly lower in IFABP2 than in mock-transfected cells. At day 6 after seeding, DNA synthesis and cell number were reduced by factors of 2 and 1.5, respectively. These results suggest that I-FABP overexpression in Caco-2 cells may induce an inhibition of cell proliferation.

Because a negative effect on cell proliferation could accelerate differentiation, the activities of two brush border enzymes, alkaline phosphatase and sucrase-isomaltase, known as differentiation markers for enterocytes, were measured from day 0 to 24 after confluence. Alkaline phosphatase activity increased slowly during differentiation and was not significantly different between IFABP2 and control cells (**Fig. 2A**). However, while sucrase-isomaltase activity strongly increased during the differentiation of control cells, it was completely abolished in IFABP2 cells (Fig. 2B). Similar results on sucrase-isomaltase and alkaline phosphatase activities were obtained with IFABP1 cells at day 17 (sucrase-isomaltase: 1.08 ± 0.55 mU/mg protein; alkaline phosphatase: 216 ± 29 mU/mg protein). These data show that alkaline phosphatase and sucrase-isomaltase were differentially affected by I-FABP overexpression. For this reason, the state of IFABP2 cells differentiation was examined by electron microscopy.

Morphology of differentiated transfected Caco-2 cells

To determine whether cells overexpressing I-FABP presented the same morphologic characteristics as control and wild-type Caco-2 cells, they were examined by electron microscopy. **Figure 3** (A and C) shows that, under our culture conditions on filter (day 17), wild-type and control cells were oriented in a polarized manner. They showed typical brush border microvilli on the apical surface, tight junctions at the lateral apical surface, desmosomes at the lateral surface, and basal-lateral foldings of the cell membrane with intercellular spaces. IFABP2 cells presented the same morphological characteristics as control and wild-type cells (**Fig. 4A and B**). Analysis of different views of several preparations did not show significant change in microvillus height between each cell types. Con-

TABLE 1. I-FABP and L-FABP amounts in undifferentiated and differentiated transfected cells

	Control Cells		IFABP1 Cells		IFABP2 Cells	
	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17
			μg/mg protein			
I-FABP	ND	0.16 ± 0.03	1.71 ± 0.91[b]	1.45 ± 0.18[a]	15.18 ± 3.72[b,d]	14.40 ± 1.96[b,c]
L-FABP	2.17 ± 0.50	12.66 ± 1.21	38.33 ± 7.58[b]	58.68 ± 9.02[b]	4.34 ± 1.22[d]	18.67 ± 1.68[a,c]

At days 0 and 17 after confluence, mock-transfected cells (control) and cells over-expressing I-FABP (IFABP1 and IFABP2) were harvested, and I-FABP and L-FABP were quantified in cell homogenates by Western blot analysis. Data are the mean ± SEM of values obtained in at least five separate experiments; ND, not detected.

[a] $P < 0.05$ and [b] $P < 0.01$ as compared to control cells.

[c] $P < 0.05$ and [d] $P < 0.01$ as compared to IFABP1 cells.

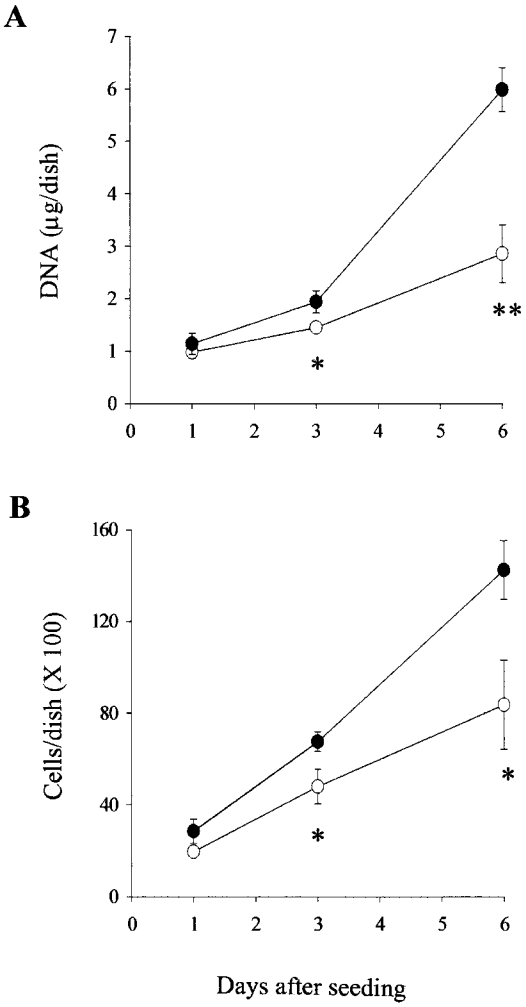

Fig. 1. Growth of transfected cells. DNA content (A) and cell number (B) were measured in control (●) or IFABP2 (○) cells harvested at days 1, 3 and 6 after seeding, as described under Materials and Methods. Data are the means ± SEM of values obtained in four separate experiments. Values statistically different from mock-transfected cells are indicated by * ($P < 0.05$) or ** ($P < 0.01$).

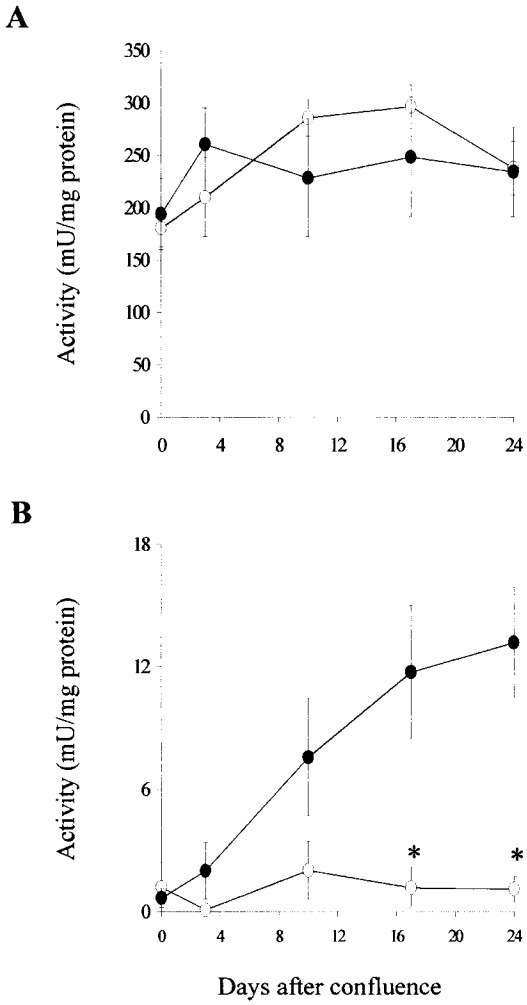

Fig. 2. Differentiation of transfected cells. Alkaline phosphatase (A) and sucrase-isomaltase (B) activities were measured during differentiation of control (●) or IFABP2 (○) cells, as described under Materials and Methods. Data are the means ± SEM of values obtained in at least three separate experiments. Values statistically different from mock-transfected cells are indicated by * ($P < 0.05$) or ** ($P < 0.01$).

trol and IFABP2 cells as well as wild-type cells contained a considerable amount of glycogen (Figs. 3A, 3C, and 4A), as previously reported for wild-type Caco-2 cells (17).

Furthermore, analysis of cell monolayers on horizontal sections showed that the number of lysosomes was strongly increased in control and in IFABP2 cells as compared with wild-type Caco-2 cells (Figs. 3B, 3D, and 4C). Some of these lysosomes were filled with an electron-opaque material of more or less homogenous appearance, whereas others often contained concentrically arranged membranes, giving them the aspect of lamellar bodies (Fig. 4D). The same accumulation of lamellar bodies was also observed in confluent (day 0) control and IFABP2 cells (not shown).

In addition, some Caco-2 and control cells, and many of the IFABP2 cells, contained lipid droplets in the basal part of their cell body (Fig. 4A). However, measurements of cellular triglycerides in cell homogenates showed similar amounts in control and IFABP2 cells at day 24 after confluence (82.1 ± 8.7 μg/mg protein versus 61.3 ± 10.1 μg/mg, respectively; n = 8). In wild-type cells, the amount of triglycerides was 33.5 ± 3.2 μg/mg (n = 3).

Palmitic acid metabolism

In order to study fatty acid incorporation, esterification, and secretion, transfected cells were incubated for 15, 60, or 120 min with 110 μm [^{14}C]palmitic acid at day 17 after confluence. The effect of I-FABP on palmitate metabolism was studied in cells expressing a 10-fold (IFABP1) and 100-fold (IFABP2) higher level of I-FABP than control cells. **Figure 5** shows that [^{14}C]palmitic acid incorporation strongly increased from 15 to 120 min incubation. It was significantly lower at 60 and 120 min in IFABP2, but not in IFABP1, than in control cells (34 and 21% inhibition at 120 min, respectively). Analysis of [^{14}C]palmitic acid distribution in lipids showed that the proportions of phospholipids and unesterified fatty acid decreased with incubation

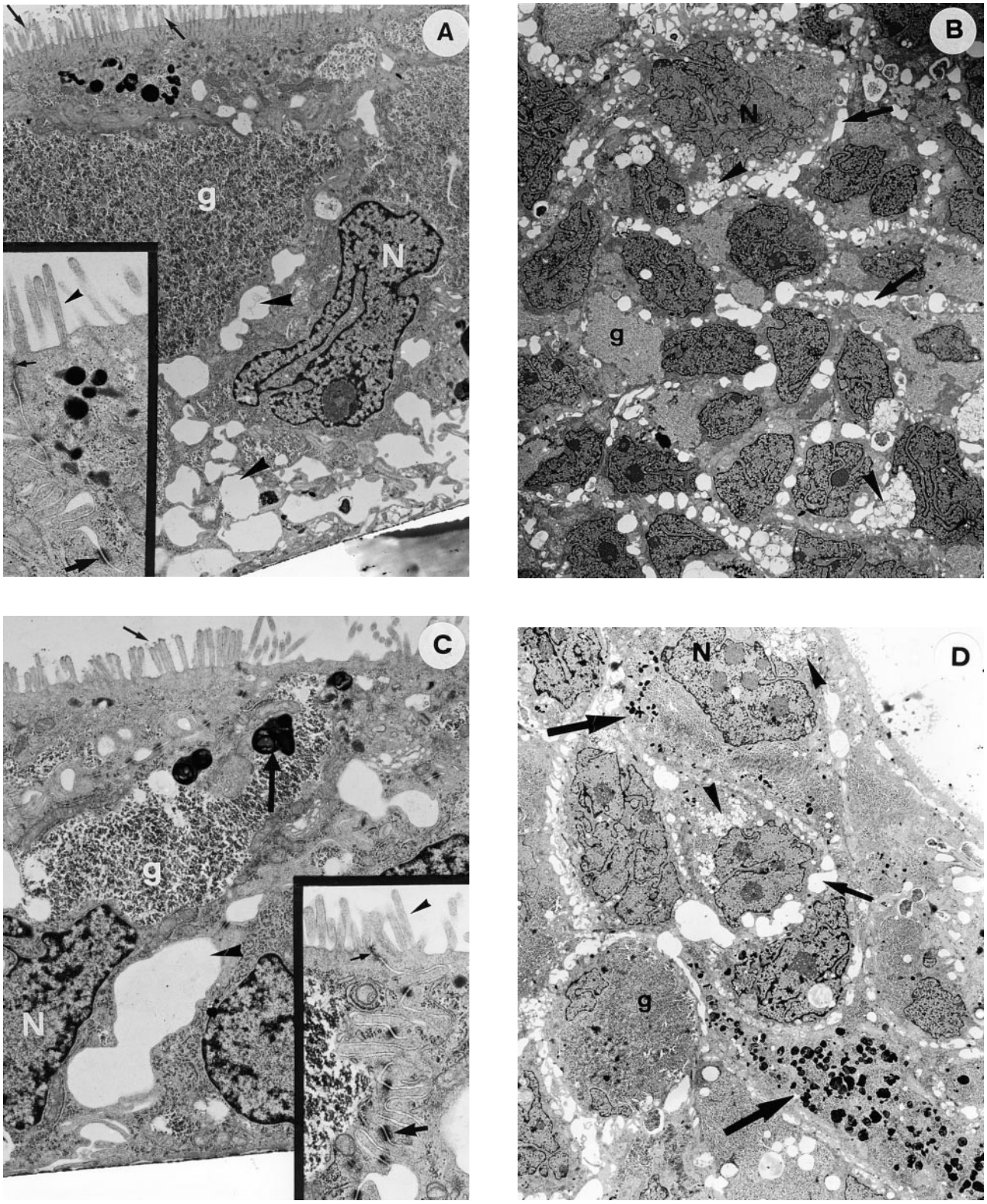

Fig. 3. Transmission electron micrograph of wild-type Caco-2 cells and mock-transfected cells differentiated on filters (f). Cells were cultured as described under Materials and Methods and fixed for microscopy on day 16. A+B: wild type Caco-2 cells; C+D; mock-transfected cells. A+C: cross-section showing cells from the basal part on the filter to the cell surface. Note the brush border microvilli (arrows) and the baso-lateral membrane foldings with intercellular spaces (arrowheads). Insets: High magnification showing tight junctions (small arrows), desmosomes (large arrows), and microvilli (arrowheads). B+D: Horizontal section. Cells are cut in the basal third. Intercellular spaces (arrows), lipid vacuoles (arrowheads). Note the normal lysosomes in wild-type Caco-2 cells (B) and the increased number of altered lysosomes (= lamellar bodies) in transfected cells (D; large arrows). Nucleus (N), glycogen (g). A, 4290 ×, inset, 12650 ×; B, 1320 ×; C, 7271 ×; inset, 12650 × D,. 1320 ×.

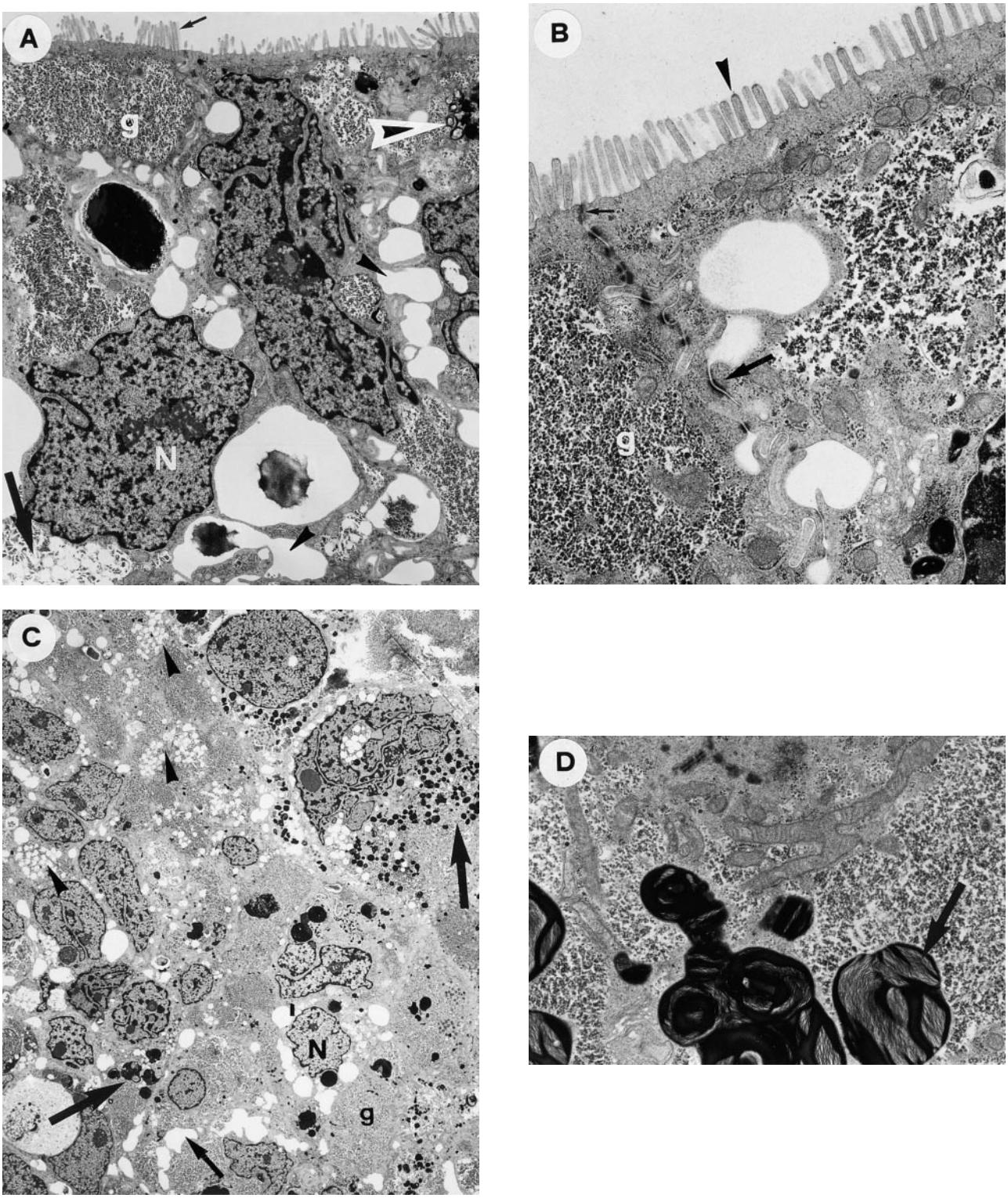

Fig. 4. Transmission electron micrograph of I-FABP-transfected cells differentiated on filters. IFABP2 cells were cultured and fixed as described in Fig. 3. A: Cross-section showing cells from the basal part on the filter to the cell surface. Note the brush border microvilli (small arrow), the baso-lateral membrane foldings with intercellular spaces (large arrowheads), the myeloid body (small arrowhead), and the lipid droplets in the basal part of the cells (large arrow). B: High magnification showing tight junctions (small arrows), desmosomes (large arrows) and microvilli (arrowheads). C: Horizontal section. Cells are cut in the basal third. Intercellular spaces (arrow), lipid vacuoles (arrowhead). D: Note the lamellated structure of altered lysosomes (= lamellar bodies) in transfected cells (large arrows). Nucleus (N), glycogen (g). A, 4290 ×; B, 12650 ×; C, 1320 × D, 13200 ×.

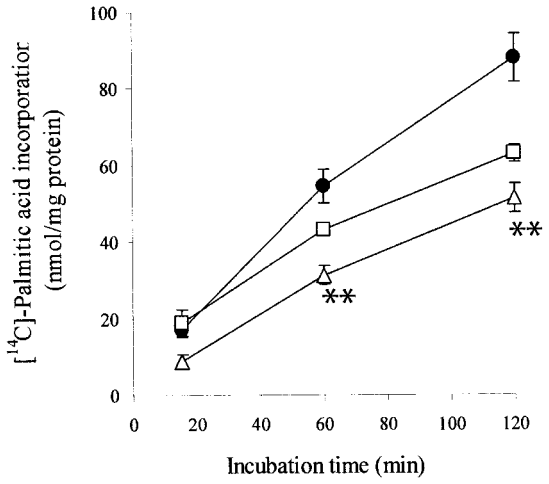

Fig. 5. Palmitic acid incorporation into differentiated transfected cells. At day 17 after confluence control (●), IFABP1 (□), and IFABP2 (△) monolayers were incubated 15, 60, or 120 min in the presence of 110 μm [^{14}C]palmitic acid complexed with 8 mm taurocholate. Total fatty acid incorporation was calculated as described under Materials and Methods. Data represent the means ± SEM of values obtained in at least three separate experiments, and values statistically different from mock-transfected cells are indicated by ** ($P < 0.01$).

time in favor of diglycerides and especially triglycerides (**Table 2**). Similar patterns of lipid synthesis were obtained in control, IFABP1, and IFABP2 cells. However, the proportion of diglycerides was increased significantly at 60 and 120 min in IFABP2 cells as compared with control cells, by 3.3- and 1.6-fold, respectively.

In the same experiments, basolateral lipid secretion was measured in each cell type after 120 min incubation with [^{14}C]palmitic acid. Total lipid secretion was slightly decreased in IFABP2 cells as compared with IFABP1 and control cells (2.02 ± 0.11, 3.56 ± 0.90, and 4.57 ± 0.27 nmol/mg protein, respectively). Analysis of [^{14}C]palmitic acid distribution in basolateral medium showed a lower percentage of secreted triglyceride with IFABP2 cells as compared with IFABP1 and control cells (2.8 ± 0.7%, 10.73 ± 2.9%, and 9.2 ± 4.7%, respectively). However, these differences in secretion between cell types were not statistically significant.

DISCUSSION

The role of I-FABP in fatty acid metabolism is still unclear. Surprisingly, previous studies have reported no increase or even decreases in fatty acid transport in I-FABP transfected fibroblasts (11–14) and non-differentiated epithelial cells (15), which did not contain endogenous I- and L-FABP. These unexpected results might be related to the type of cells used or to the amounts of protein expressed, which were lower than in the intestinal epithelium. In the present study, Caco-2 cells were stably transfected with human I-FABP cDNA, and two clones expressing different amounts of I- and L-FABP were isolated. In clone IFABP2, the intestinal type represented 1.5% of cytosolic proteins, which is similar to the amounts reported in rodent and human small intestine (21, 22). This is a significant improvement over previous attempts of cell transfection with I-FABP cDNA, giving 0.02% of cytosolic proteins in Caco-2 cells (10), 0.35% (11, 12, 14) and 0.72% (13) in L-cell fibroblasts, and about 0.30% in hBRIE 380i-neg cells (15). In the IFABP1 clone, we found 0.12% of intestinal type. In both IFABP1 and IFABP2 cells, high levels of L-FABP were found, thus excluding that decreases in fatty acid incorporation could be due to L-FABP down-regulation.

Previous reports indicate that FABP types could play an important role in the regulation of cell proliferation. L-FABP has been shown to stimulate the proliferation of rat hepatocytes (23), hepatoma cell lines (24), and mouse L-cell fibroblasts (25), while H-FABP inhibited proliferation of transfected mammary epithelial cells (26) and yeast (27). In the present study, we observed for the first time a marked retardation of growth in I-FABP transfected Caco-2 cells (IFABP2), suggesting an antiproliferative property of this type. In a previous study on transfected L cell fibroblasts, which did not express L-FABP, this effect was not observed (12). Therefore, it is tempting to speculate that in Caco-2 cells I-FABP may reverse the mitogenic effect of L-FABP (which is expressed in undifferentiated cells). Because the presence of linoleic acid in the culture medium

TABLE 2. Distribution of [^{14}C]palmitic acid in lipid classes in differentiated transfected cells

Lipid class	Control Cells Incubation Time (min)			IFABP1 Cells Incubation Time (min)			IFABP2 Cells Incubation Time (min)		
	15	60	120	15	60	120	15	60	120
	%			%			%		
PL	61.8 ± 7.0	64.2 ± 11.4	56.0 ± 3.15	53.8 ± 1.8	55.4 ± 1.0	44.3 ± 1.6	59.2 ± 1.8	57.4 ± 0.8	53.8 ± 2.7
DG	2.5 ± 0.1	1.6 ± 0.8	3.3 ± 0.3	4.4 ± 0.8	5.0 ± 0.5	3.9 ± 0.3	2.9 ± 0.4	5.4 ± 0.5[a]	5.2 ± 0.3[a]
FA	22.9 ± 6.1	10.2 ± 4.5	10.3 ± 2.3	13.5 ± 2.0	7.8 ± 2.4	6.7 ± 1.2	16.4 ± 3.1	10.4 ± 3.4	6.3 ± 1.1
TG	9.5 ± 1.3	19.2 ± 5.3	26.8 ± 2.1	19.1 ± 2.2	26.4 ± 1.0	36.6 ± 1.8	13.7 ± 2.1	20.3 ± 2.1	27.9 ± 2.6

After 15, 60, and 120 min incubation with 110 μm [^{14}C]palmitic acid complexed with 8 mm taurocholate, differentiated (day 17) control, I-FABP1, and I-FABP2 cells were homogenized. Lipids were extracted and analyzed by TLC. Data represent the percentages of radioactivity of each lipid class read on the plates and are the mean ± SEM of values obtained in at least three separate experiments; abbreviations: PL, phospholipids; DG, diglyceride; FA, fatty acid; TG, triglyceride.

[a] $P < 0.05$ as compared to control cells.

was required for L-FABP-induced cell growth in transfected hepatocytes (24), the inhibitory effect of I-FABP may result from a competition with L-FABP for linoleic acid binding. Thus, the effect of I-FABP may reveal the regulatory role of L-FABP in enterocyte proliferation, but one cannot exclude that it is independent of L-FABP.

The antiproliferative effect of I-FABP led us to examine its possible role in cell differentiation by measuring the activities of two brush-border enzymes, i.e., alkaline phosphatase and sucrase-isomaltase, generally used as markers of enterocyte differentiation. Although the alkaline phosphatase activity was not affected by I-FABP overexpression, sucrase-isomaltase activity was almost completely abolished. Nevertheless, electron microscopy revealed that cells presented the main characteristics of differentiated enterocytes. Furthermore, L-FABP expression, which can be considered as a differentiation marker, increased considerably during the 2 weeks after cell confluence, in both I-FABP-transfected and control cells. Therefore, it appears that the effect of I-FABP transfection on sucrase-isomaltase activity may be specific of this enzyme. Interestingly, specific decreases in sucrase-isomaltase have been previously observed in Caco-2 cells treated with epidermal growth factor (28) and with collagen (8). The mechanisms involved in sucrase-isomaltase inhibition remain unclear.

The high level of I-FABP expressed in our IFABP2 Caco-2 cells, without decrease in L-FABP, and their ability to fully differentiate made them suitable for studying the effect of a large amount of I-FABP on fatty acid metabolism. In these cells, we show that [^{14}C]palmitic acid incorporation was significantly decreased as compared with control cells. In previous studies, the role of I-FABP was investigated in stably transfected mouse L cell fibroblasts (11–14) and undifferentiated rat hybrid intestinal epithelial hBRIE 380i-neg cells (15), which did not contain endogenous I- and L-FABP. In both transfected cells, where I-FABP represented about 0.35% of cytosolic proteins, the uptakes of [^{3}H]oleic acid and of the fluorescent fatty acid probe, NBD-stearate, were not affected. However, intracellular diffusion of the fatty acid probe was accelerated in L cells suggesting a role for I-FABP in fatty acid intracellular transport (14). Interestingly, when I-FABP was expressed at a higher level in L cells (0.72%), *cis*-parinaric and [^{3}H]oleic acid incorporation decreased (13). Furthermore, in another study on transfected pluripotent mouse embryonic stem cells, I-FABP enhanced NBD-stearate uptake and diffusion in undifferentiated but not in differentiated cells (29). Thus, our results on transfected Caco-2 cells are consistent with these previous findings. In the present study, a smaller and nonsignificant inhibition of palmitic acid incorporation was observed in IFABP1 cells expressing 10-fold less I-FABP than IFABP2 cells (21% instead of 34% inhibition at 120 min incubation, respectively). These results indicate that the inhibition of fatty acid incorporation observed in I-FABP2 cells was not due to the selection of a particular clone from a heterogenous Caco-2 cell population. Furthermore, it emphasizes the importance of I-FABP expression level on the regulation of fatty acid incorporation.

The mechanism of this inhibitory effect is unknown. However, the very low sucrase-isomaltase activities in both IFABP1 and IFABP2 cells suggest that overexpression of this protein in Caco-2 cells may lead to alterations of the cell membrane, thus possibly affecting passive and active fatty acid transport. Future investigations in transfected cells should examine the expression of membrane fatty acid transporters such as FABPpm which is expressed in Caco-2 cells and might play an important role in fatty acid uptake (30).

[^{14}C]palmitic acid distribution into lipids showed no difference between IFABP1, IFABP2, and mock-transfected Caco-2 cells, except for a small increase in diacylglycerol in IFABP2 cells. This observation is in accordance with a previous study on the intestinal cell line, hBRIE 380i-neg (15), but contrasts with studies on transfected L cell fibroblasts showing increases in [^{3}H]oleic acid incorporation into triglycerides (11, 12). This discrepancy emphasizes the importance of using enterocytes for studying the role of I-FABP in lipid metabolism, perhaps because enterocytes develop enzyme activities different or higher than nonenterocytes, as previously discussed (15). However, although Caco-2 cells develop the characteristics of well-differentiated enterocytes, they have been reported to contain low activities of monoacylglycerol acyltransferase, a limiting enzyme in the 2-monoacylglycerol pathway of triglyceride synthesis which is predominant in the small intestine (31). For this reason, triglyceride synthesis proceeds only through the glycerol 3-phosphate pathway in Caco-2 cells. We have verified that addition of 2-monoacylglycerol did not increase fatty acid metabolism in transfected cells (data not shown). Therefore, one cannot exclude that I-FABP is specifically involved in the 2-monoacylglycerol pathway in small intestine epithelium, as previously suggested (31).

As a note of caution, it is necessary to mention that electron microscopy revealed the presence of lamellar bodies in both I-FABP and control cells, but not in wild-type Caco-2 cells. These bodies may indicate the existence of phospholipidosis, a phospholipid storage disorder which has been reported in several cell lines treated with cationic amphiphillic drugs like aminoglycosides (32). G418, which is commonly used as a selective agent in transfection experiments, belongs to the aminoglycoside family. Therefore, the presence of G418 in our culture medium may be responsible for the induction of lamellar bodies, and perhaps phospholipidosis. In the previous studies of I-FABP transfected cells (10–15), G418 concentrations higher than in the present study were used, but the morphology of the cells was not inspected. It is unknown whether G418 could interfere with the results. However, the effect we observed between I-FABP and control cells can be reasonably related to the presence of I-FABP and not to phospholipidosis because both cells presented similar morphologic alterations. To exclude any possible interference, a transfection method that does not induce lamellar bodies or phospholipidosis should be selected.

We conclude that I-FABP overexpression in this enterocyte model reduced fatty acid incorporation and affected

certain cell characteristics, but did not play a major role in esterification.

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REFERENCES

1. Ockner, R. K., J. A. Manning, R. B. Poppenhausen, and L. Ho. 1972. A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium, and other tissues. *Science.* **177:** 56–58.
2. Richieri, G. V., R. T. Ogata, and A. M. Kleinfeld. 1994. Equilibrium constants for the binding of fatty acids with fatty acid-binding proteins from adipocyte, intestine, heart, and liver measured with the fluorescent probe ADIFAB. *J. Biol. Chem.* **269:** 23918–23930.
3. Bass, N. 1988. The cellular fatty acid binding proteins: aspects of structure, regulation, and function. *Int. Rev. Cytol.* **111:** 143–184.
4. Thompson, J., N. Winter, D. Terwey, J. Bratt, and L. Banaszak. 1997. The crystal structure of the liver fatty acid binding protein. A complex with two bound oleates. *J. Biol. Chem.* **272:** 7140–7150.
5. Maatman R. G. H. J., H. T. B. van Moerkerk, I. M. A. Nooren, E. J. J. van Zoelen, and J. H. Veerkamp. 1994. Expression of human liver fatty acid-binding protein in *Escherichia coli* and comparative analysis of its binding characteristics with muscle fatty acid-binding protein. *Biochim. Biophys. Acta.* **1214:** 1–10.
6. Shields, H. M., M. L. Bates, N. M. Bass, C. J. Best, D. H. Alpers, and R. K. Ockner. 1986. Light microscopic immunocytochemical localization of hepatic and intestinal types of fatty acid-binding proteins in rat small intestine. *J. Lipid Res.* **27:** 549–557.
7. Halldén, G., and G. W. Aponte. 1997. Evidence for a role of the gut hormone PYY in the regulation of intestinal fatty acid-binding protein transcripts in differentiated subpopulations of intestinal epithelial cell hybrids *J. Biol. Chem.* **272:** 12591–12600.
8. Darimont, C., N. Gradoux, C. Cumin, H. P. Baum, and A. De Pover. 1998. Differential regulation of intestinal and liver fatty acid-binding proteins in human intestinal cell line (Caco-2): role of collagen. *Exp. Cell. Res.* **244:** 441–447.
9. Storch, J., F. M. Herr, K. T. Hsu, H. K. Kim, H. L. Liou, and E. R. Smith. 1996. The role of membranes and intracellular binding proteins in cytoplasmic transport of hydrophobic molecules-fatty acid-binding proteins. *Comp. Biochem. Phys.* **115:** 333–339.
10. Baier, L. J., C. Bogardus, and J. C. Sacchettini. 1996. A polymorphism in the human intestinal fatty acid binding protein alters fatty acid transport across Caco-2 cells. *J. Biol. Chem.* **271:** 10892–10896.
11. Prows, D. R., E. J. Murphy, and F. Schroeder. 1995. Intestinal and liver fatty acid binding proteins differentially affect fatty acid uptake and esterification in L-cells. *Lipids.* **30:** 907–910.
12. Prows, D. R., E. J. Murphy, D. Moncecchi, and F. Schroeder. 1996. Intestinal fatty acid-binding protein expression stimulates fibroblast fatty acid esterification. *Chem. Phys. Lipids.* **84:** 47–56.
13. Prows, D. R., and F. Schroeder. 1997. Metallothionein-IIA promoter induction alters rat intestinal fatty acid binding protein expression, fatty acid uptake, and lipid metabolism in transfected L-cells. *Arch. Biochem. Biophys.* **340:** 135–143.
14. Murphy, E. J. 1998. L-FABP and I-FABP expression increase NBD-stearate uptake and cytoplasmic diffusion in L cells. *Am. J. Physiol.* **275:** G244–G249.
15. Holehouse, E. L., M-L. Liu, and G. W. Aponte. 1998. Oleic acid distribution in small intestinal epithelial cells expressing intestinal-fatty acid binding protein. *Biochem. Biophys. Acta.* **1390:** 52–64.
16. Baier, L. J., J. C. Sacchettini, W. C. Knowler, J. Eads, G. Paolisso, P. A. Tataranni, H. Mochizuki, P. H. Bennett, C. Bogardus, and M. Prochazka. 1995. An amino acid substitution in the human intestinal fatty acid binding protein is associated with increased fatty acid binding, increased fat oxidation, and insulin resistance. *J. Clin. Invest.* **95:** 1281–1287.
17. Pinto, M., S. Robine-Leon, M. D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. J. HaffenFogh, and A. Zweibaum. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell.* **47:** 323–330.
18. Asselbergs, F. A. M., C. Gandor, and R. Widmer. 1996. Gene cassettes for directional insertion at the *Sfi*I cleavage site in the SV40 replication origin of mammalian expression vectors. *Anal. Biochem.* **243:** 285–288.
19. Labarca, C., and K. Paigen. 1980. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102:** 344–352.
20. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
21. Bass, N. M.,, J. A. Manning, R. K. Ockner, J. I. Gordon, S. Seetharam, and D. H. Alpers. 1985. Regulation of the biosynthesis of two distinct fatty acid-binding proteins in rat liver and intestine. *J. Biol. Chem.* **260:** 1432–1436.
22. Sakai, Y. 1990. Quantitative measurement of liver fatty acid binding protein in human gastrointestinal tract. *Jpn. J. Gastroenterol.* **87:** 2594–25604.
23. Sorof, S. 1994. Modulation of mitogenesis by liver fatty acid binding protein. *Cancer Metastasis Rev.* **13:** 317–336.
24. Keler, T., C. S. Barker, and S. Sorof. 1992. Specific growth stimulation by linoleic acid in hepatoma cell lines transfected with the target protein of a liver carcinogen. *Proc. Natl. Acad. Sci. USA.* **89:** 4830–4834.
25. Jefferson, J. R., D. M. Powell, Z. Rymaskzewski, J. Kukowska-Latallo, J. B. Lowe, and F. Schroeder. 1990. Altered membrane structure in transfected mouse L-cell fibroblasts expressing rat liver fatty acid-binding protein. *J. Biol. Chem.* **265:** 11062–11068.
26. Yang, Y., E. Spitzer, N. Kenney, W. Zschieshe, M. Li, A. Kromminga, T. Muller, F. Spener, A. Lezius, and J. H. Veerkamp. 1994. Members of the fatty acid-binding protein familly are differentiation factors for the mammary gland. *J. Cell. Biol.* **127:** 1097–1109.
27. Scholz, H., S. D. Kohlwein, F. Paltauf, A. Lezius, and F. Spener. 1990. Expression of a functionally active cardiac fatty acid-binding protein in the yeast, *Saccharomyces cerevisiae*. *Mol. Cell. Biochem.* **98:** 69–74.
28. Cross, H. S., and A. Quaroni. 1991. Inhibition of sucrase-isomaltase expression by EGF in the human colon adenocarcinoma cells Caco-2. *Am. J. Physiol.* **261:** C1173–C1183.
29. Atshaves, B. P., W. B. Foxworth, A. Frolov, J. B. Roths, A. B. Kier, B. K. Oetama, J. A. Piedrahita, and F. Schroeder. 1998. Cellular differentiation and I-FABP protein expression modulate fatty acid uptake and diffusion. *Am. J. Physiol.* **274:** C633–C644.
30. Trotter, P. J., S. Y. Ho, and J. Storch. 1996. Fatty acid uptake by Caco-2 human intestinal cells. *J. Lipid Res.* **37:** 336–46.
31. Trotter, P. J., and J. Storch. 1993. Fatty acid esterification during differentiation of the human intestinal cell line Caco-2. *J. Biol. Chem.* **368:** 10017–10023.
32. Halliwell, W. H. 1997. Cationic amphilic drug-induced phospholipidosis. *Toxicol. Pathol.* **25:** 53–59.