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3T3 fibroblasts transfected with a cDNA for mitochondrial aspartate aminotransferase express plasma membrane fatty acid-binding protein and saturable fatty acid uptake

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ABSTRACT **To explore the relationship between mitochondrial aspartate aminotransferase (mAspAT; EC 2.6.1.1) and plasma membrane fatty acid-binding protein ($FABP_{pm}$) and their role in cellular fatty acid uptake, 3T3 fibroblasts were cotransfected with plasmid pMAAT2, containing a full-length mAspAT cDNA downstream of a Zn^{2+}-inducible metallothionein promoter, and pFR400, which conveys methotrexate resistance. Transfectants were selected in methotrexate, cloned, and exposed to increasing methotrexate concentrations to induce gene amplification. Stably transfected clones were characterized by Southern blotting; those with highest copy numbers of pFR400 alone (*pFR400*) or pFR400 and pMAAT2 (*pFR400/pMAAT2*) were expanded for further study. [^{3}H]Oleate uptake was measured in medium containing 500 μM bovine serum albumin and 125–1000 μM total oleate (unbound oleate, 18–420 nM) and consisted of saturable and nonsaturable components. *pFR400/pMAAT2* cells exhibited no increase in the rate constant for nonsaturable oleate uptake or in the uptake rate of [^{14}C]octanoate under any conditions. By contrast, V_{max} (fmol/sec per 50,000 cells) of the saturable oleate uptake component increased 3.5-fold in *pFR400/pMAAT2* cells compared to *pFR400*, with a further 3.2-fold increase in the presence of Zn^{2+}. Zn^{2+} had no effect in *pFR400* controls ($P > 0.5$). The overall increase in V_{max} between *pFR400* and *pFR400/pMAAT2* in the presence of Zn^{2+} was 10.4-fold ($P < 0.01$) and was highly correlated ($r = 0.99$) with expression of $FABP_{pm}$ in plasma membranes as determined by Western blotting. Neither untransfected 3T3 nor *pFR400* cells expressed cell surface $FABP_{pm}$ detectable by immunofluorescence. By contrast, plasma membrane immunofluorescence was detected in *pFR400/pMAAT2* cells, especially if cultured in 100 μM Zn^{2+}. The data support the dual hypotheses that mAspAT and FABPpm are identical and mediate saturable long-chain free fatty acid uptake.**

Cellular uptake of long-chain free fatty acids (FFAs), formerly considered a purely passive process (1, 2), has been shown to exhibit kinetic properties of facilitated transport in hepatocytes (3–5), adipocytes (6–8), and other cells (9–12) but not in fibroblasts (13). Five distinct plasma membrane proteins have been identified as putative mammalian FFA transporters (14–19). The first to be isolated, plasma membrane fatty acid-binding protein ($FABP_{pm}$) (14), has been purified from mouse and rat hepatocytes, adipocytes, jejunal enterocytes, and cardiac myocytes (8–11, 14, 20) and identified in *Xenopus laevis* oocytes (21). Evidence of a role for $FABP_{pm}$ in cellular FFA uptake includes selective inhibition of FFA uptake by $FABP_{pm}$ antibodies (4, 8–11, 13, 21), liposome reconstitution studies (22), and parallel, progressive expression of $FABP_{pm}$ on the cell surface and saturable FFA uptake during differentiation of 3T3-L1 fibroblasts to an adipocyte phenotype (13). We recently reported that $FABP_{pm}$ was related to mitochondrial aspartate aminotransferase (mAspAT; EC 2.6.1.1) (23). Although this was initially disputed (24), further studies have clearly established that the two proteins are identical (25). We now report that transfection of 3T3 fibroblasts with a plasmid containing a full-length mAspAT cDNA (26) under the control of a metallothionein promoter (27) confers Zn^{2+}-dependent expression of cell surface $FABP_{pm}$ antigen and saturable FFA uptake in otherwise nonexpressing cells.

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MATERIALS AND METHODS

Transfection. Plasmid pMAAT2 (Fig. 1) was constructed by excising an *Eco*RI/*Bgl* II fragment including the Zn^{2+}-inducible mouse metallothionein promoter (27) from plasmid pMK-FcRI (gift of Jay Unkeless) and cloning it into the *Eco*RI/*Bam*HI-digested site of pGEM3-Z (Promega). A 2-kb *Hin*dIII fragment from plasmid pBSKSAAT-2, containing a full-length cDNA for rat mAspAT (26), was cloned downstream of the promoter. Plasmid pFR400 contains a cDNA for a mutant murine dihydrofolate reductase (DHFR) gene that encodes a product that confers methotrexate (MTX) resistance (28), under transcriptional control of the simian virus 40 promoter. pFR400 acts as a dominant selectable marker after transfection into murine fibroblasts and is amplifiable by MTX selection (29). 3T3 cells were transfected by $Ca_3(PO_4)_2$-DNA coprecipitation (30). Cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with dialyzed 10% fetal bovine serum. After cotransfection with pMAAT2 and pFR400 (31) or pFR400 alone, cells were selected for MTX resistance by supplementing the medium with 0.05 μM MTX. To amplify transfected sequences, the MTX concentration was increased by 0.05 μM weekly until it reached 0.50 μM. After initial MTX selection, individual clones were isolated with a cloning cylinder and expanded.

Identification of Transfectants and Cell Cloning. Once cells had grown to confluence, DNA was isolated from individual clones (31), digested with *Pst* I, and blotted onto nylon membranes (32). The filters were probed with pFR400, stripped, and rehybridized to pMAAT2. Clones containing the highest copy numbers of pFR400 alone and of pFR400 and pMAAT2 were expanded for subsequent experiments. Clones were maintained in culture under selective pressure with 0.5 μM MTX. The medium for some experiments was supplemented with 100 μM $ZnSO_4$ (see below). After preliminary studies documented that Zn^{2+} effects were maximal by 6 days

Abbreviations: FFA, long-chain free fatty acid; BSA, bovine serum albumin; $FABP_{pm}$, plasma membrane fatty acid-binding protein; mAspAT, mitochondrial isoform of aspartate aminotransferase; *pFR400*, 3T3 fibroblasts transfected with plasmid pFR400; *pFR400/pMAAT2*, 3T3 fibroblasts doubly transfected with plasmids pFR400 and pMAAT2; $\bar{\nu}$, oleate:BSA molar ratio(s); MTX, methotrexate; DHFR, dihydrofolate reductase.

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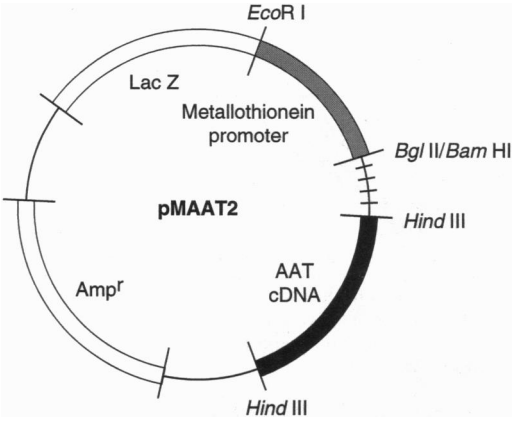

FIG. 1. Plasmid pMAAT2, containing a full-length cDNA for mAspAT under the control of Zn^{2+}-sensitive metallothionein promoter. See text for details.

and stable for at least 30 days, subsequent experiments were conducted on cells cultured in Zn^{2+} for 6–14 days.

Uptake Studies. [^{3}H]Oleate uptake was studied in cell clones transfected with pFR400 alone (*pFR400*) or with pFR400 and pMAAT2 (*pFR400/pMAAT2*). Cells were harvested from confluent monolayers by incubation for 10 min at 37°C in Joklik's modified medium containing 2 mM EDTA (33). They were then gently aspirated, washed three times with cold Hanks' buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HH), resuspended in HH ($2.5–3.5 \times 10^6$ per ml), counted, and assessed for viability using trypan blue. Preparations were used only if ≥90% of cells excluded the dye. Initial uptake rate (0–30 sec) was determined at oleate: bovine serum albumin (BSA) molar ratios ($\bar{\nu}$) from 0.25:1 to 2:1 in medium containing 500 μM BSA by a modification (10, 13) of the method of Abumrad *et al.* (6). Unbound oleate concentrations were computed (34) using published binding constants (35), as previously validated experimentally (36). All experiments were done in triplicate. Data fitting was conducted as described (5) and results are expressed as mean ± SE.

Antibody Inhibition Studies. Monospecific polyclonal antisera to rat liver $FABP_{pm}$ were prepared and characterized as described (13). The titer of the antiserum employed in these studies was 1:32 by immunoprecipitation (13). To study the effects of anti-$FABP_{pm}$ on [^{3}H]oleate uptake, *pFR400* and *pFR400/pMAAT2* cells were incubated for 60 min on ice at a dilution of 1:3 with either anti-$FABP_{pm}$ or preimmune rabbit serum. Subsequently, uptake of [^{3}H]oleate was determined from solutions containing 500 μM BSA ($\bar{\nu}$ = 1:1) as described above. Percent inhibition of uptake by anti-$FABP_{pm}$ was determined by comparison with results obtained with the preimmune serum control. To establish the specificity of the antibody, effects of anti-$FABP_{pm}$ on the initial uptake velocities of 2-deoxy[^{3}H]glucose and [^{14}C]octanoate were determined as described (13).

Studies with Plasma Membranes. Plasma membranes from transfected 3T3 fibroblasts were isolated according to Thom *et al.* (37). 3T3 plasma membranes purified in this way are enriched 5-fold in phosphodiesterase (38), while succinate dehydrogenase (39) is reduced to ≤15% of its activity in cell homogenates (13). Aspartate aminotransferase activity in plasma membranes was determined with a kit (Sigma Diagnostics: procedure 58-UV) in the presence of digitonin (23). Membrane protein was measured by the biuret reaction (40). After SDS/PAGE-Western blotting and autoradiography (20), membrane proteins were quantitated by scanning densitometry as the area under the densitometric curve (OD × mm). Expression of $FABP_{pm}$ antigen on the surface of control and transfected 3T3 fibroblasts was determined with rabbit anti rat-$FABP_{pm}$ as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for detection (14). Preimmune rabbit serum served as a control.

RESULTS

Transfection. Of 11 clones stably transfected with pFR400 alone (*pFR400*), Southern blots identified *pFR400* clones 6 and 11 as those with the highest pFR400 copy numbers (data not shown). Similarly, of 15 clones transfected with pFR400 and pMAAT2 (*pFR400/pMAAT2*), clones 7 and 12 had the most copies of both plasmids (Fig. 2). These four clones were used in subsequent experiments.

Uptake Studies. [^{3}H]Oleate uptake was faster in doubly transfected *pFR400/pMAAT2* cells than in *pFR400* cells at all oleate:BSA ratios studied (Fig. 3). Culture in the presence of Zn^{2+} had no effect on oleate uptake in *pFR400* cells but produced an appreciable further increase in [^{3}H]oleate uptake velocity in the *pFR400/pMAAT2* cell lines. Using established criteria for goodness of fit (5), the [^{3}H]oleate uptake data were best described as the sum of a saturable (Michaelis–Menten) and a linear function of the unbound oleate concentration, as previously reported for 3T3-L1 cells (13). In *pFR400* cells

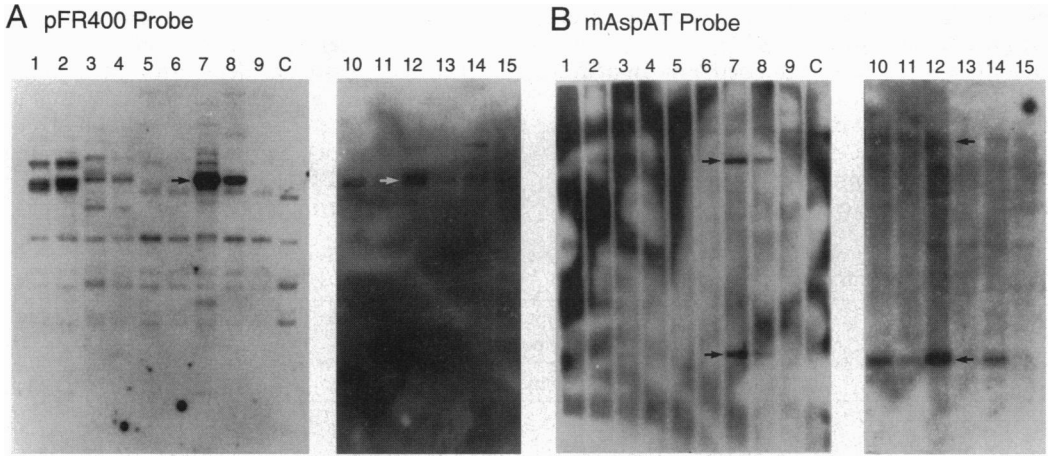

FIG. 2. Southern blot analysis of 15 cell clones derived from dual transfection of 3T3 cells with the plasmids pFR400 and pMAAT2. Lanes 1–15 contain digested DNA samples from individual *pFR400/pMAAT2* clones 1–15, respectively, which emerged after MTX selection. Lane C is from untransfected 3T3 cells. (*A*) Membranes were probed with ^{32}P-labeled pFR400; arrows indicate intense bands hybridizing to pFR400. (*B*) Same membranes, rehybridized to plasmid pMAAT2. Arrows identify bands with intense hybridization with pMAAT2. Clones 7 and 12 carry DNA homologous to both plasmids and were used for subsequent experiments.

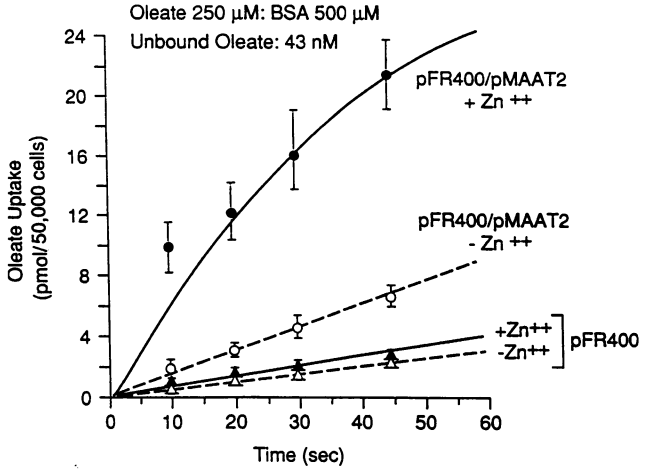

FIG. 3. Effect of culture in the presence of Zn^{2+} on [^{3}H]oleate uptake by 3T3 cells transfected with plasmid pFR400 alone or with pFR400 and pMAAT2. Cumulative uptake is expressed as a function of time. Error bars are ± SE.

neither the V_{max} nor the K_m for oleate uptake was influenced by culture in the presence of 100 μM Zn^{2+} (Table 1). However, there was a 3.3-fold increase in the V_{max} for [^{3}H]oleate uptake in *pFR400/pMAAT2* cells compared to *pFR400* in the absence of Zn^{2+}, with a further 3.1-fold increase in the the presence of Zn^{2+}. The 10.4-fold increase in V_{max} between *pFR400* cells without Zn^{2+} and *pFR400/pMAAT2* cells cultured in the presence of Zn^{2+} was highly significant ($P < 0.01$). K_m values for oleate uptake in *pFR400* and *pFR400/pMAAT2* cells, cultured with or without Zn^{2+}, did not differ. Transfection with pMAAT2 or culture in the presence of Zn^{2+} had no effect on the rate constant for nonsaturable FFA uptake, which was 0.16 ± 0.11 μl/sec per 50,000 cells in *pFR400* cells without Zn^{2+} and 0.14 ± 0.10 in *pFR400/pMAAT2* in the presence of Zn^{2+} ($P > 0.5$). Uptake of 0.5 mM octanoate by *pFR400/pMAAT2* cells also did not differ from that in *pFR400* (29.6 ± 1.7 vs. 24.7 ± 2.5 fmol/sec per 50,000 cells; $P > 0.1$) and was not affected by the presence of Zn^{2+} in the medium ($P > 0.2$).

Antibody Inhibition. Antisera to rat $FABP_{pm}$ had no effect on [^{3}H]oleate uptake velocity in *pFR400* cells. By contrast, such antisera selectively inhibited [^{3}H]oleate uptake by ≈61% in *pFR400/pMAAT2* cells ($P < 0.001$). The antibody effect was specific for FFA; the uptake rates of 2-deoxy[^{3}H]glucose and [^{14}C]octanoate were unaffected (Table 2).

Plasma Membrane Studies. By scanning densitometry of Western blots of membrane extracts (Fig. 4), plasma membranes from *pFR400/pMAAT2* cells contained a mean of 2.2 times more $FABP_{pm}$ than those from *pFR400* cells; a further 2.5-fold increase was observed in *pFR400/pMAAT2* cells cultured in Zn^{2+}. [^{3}H]Oleate uptake V_{max} and plasma membrane $FABP_{pm}$ were highly correlated ($r = 0.99$; $P < 0.01$). mAspAT enzymatic activity paralleled the changes in $FABP_{pm}$ expression: activity in *pFR400/pMAAT2* cells cultured in Zn^{2+}, 0.082 ± 0.010 unit/mg of membrane protein, was 2.1-fold that measured in the same cells cultured without Zn^{2+} (0.039 ± 0.004 unit/mg; $P < 0.01$). In indirect immunofluorescence studies with fixed, permeabilized cells, untransfected 3T3 fibroblasts, *pFR400* cells, and *pFR400/pMAAT2* cells all exhibited intracellular staining, principally in mitochondria; plasma membranes of the 3T3 and *pFR400* cells were essentially negative (Fig. 5*A*). To more clearly distinguish plasma membrane from intracellular fluorescence, live, nonpermeabilized cells were examined. Intracellular staining was no longer observed, and little or no plasma membrane staining was seen in either untransfected 3T3 or *pFR400* cells (Fig. 5 *B* and *C*). By contrast, plasma membrane staining was readily observed in *pFR400/pMAAT2* cells cultured without Zn^{2+}, and stronger staining was seen in *pFR400/pMAAT2* cells cultured in 100 μM Zn^{2+} (Fig. 5 *D* and *E*). No immunofluorescence was seen with preimmune serum. The plasma membrane localization of $FABP_{pm}$/mAspAT antigen was also indicated by laser confocal scanning microscopy (Fig. 5*F*).

Table 1. Stable transfection of 3T3 fibroblasts with pMAAT2 and culture with Zn^{2+}: Effect on oleate uptake

Parameter	*pFR400* Without Zn	*pFR400* With Zn	*pFR400/pMAAT2* Without Zn	*pFR400/pMAAT2* With Zn
V_{max}, fmol/sec per 50,000 cells	97 ± 24	91 ± 16*	323 ± 85†	1011 ± 197‡
K_m, nM of unbound oleate	78 ± 25	81 ± 21*	115 ± 44	114 ± 36*

*Compared to same cell line, without Zn^{2+} (P = not significant).
†Compared to *pFR400* with or without Zn^{2+} ($0.1 > P > 0.05$).
‡Compared to same cell line, without Zn^{2+} ($P < 0.05$); compared to *pFR400* with or without Zn^{2+} ($P < 0.01$).

DISCUSSION

Extensive evidence, cited above, suggests that $FABP_{pm}$ plays a role in facilitated FFA uptake. The finding that $FABP_{pm}$ was related to mAspAT (23) was unexpected. This observation met with considerable skepticism (24), not the least within our own laboratory. It is now clear, however, that the two proteins, one purified from highly enriched plasma membranes (14) and the other from isolated mitochondria (25, 41), are identical (23, 25, 42). Immunohistochemical data and specific immunoprecipitation of radiolabeled cell surface proteins (23) establish that the presence of mAspAT in plasma membranes is not simply an artifact of cell fractionation procedures.

The present studies demonstrate that transfection of 3T3 fibroblasts with a plasmid containing a full-length mAspAT cDNA under the control of a metallothionein promoter confers a Zn^{2+}-dependent increase in the expression of plasma membrane $FABP_{pm}$ antigen and saturable FFA uptake in these otherwise minimally expressing cells. The data argue

Table 2. Effect of anti-$FABP_{pm}$ on uptake of test substances by 3T3 fibroblasts stably transfected with pFR400 with or without pMAAT2

Substance	Conc., mM	Initial uptake velocity, fmol/sec per 50,000 cells: Preimmune	Anti-$FABP_{pm}$	% inhibition	P
pFR400/pMAAT2					
[^{3}H]Oleate	0.5*	247 ± 83	112 ± 33	61.0	<0.001
2-Deoxy[^{3}H]glucose	2	137 ± 28	119 ± 24	13.0	>0.5
[^{14}C]Octanoate	0.5	24 ± 4	25 ± 10	—	>0.5
pFR400					
[^{3}H]Oleate	0.5*	69 ± 16	64 ± 6	7.2	>0.5

*Unbound oleate concentration = 120 nM.

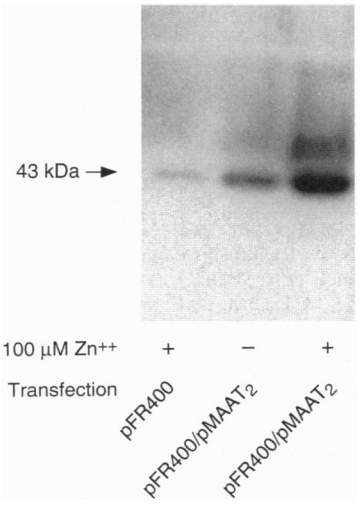

FIG. 4. Western blots of plasma membrane extracts from *pFR400* cells cultured in Zn^{2+} (left lane) and of *pFR400/pMAAT2* cells cultured without (center lane) and with (right lane) Zn^{2+}. Ten micrograms of protein was applied to each lane. The 43-kDa $FABP_{pm}$/mAspAT antigen was detected with a monospecific polyclonal anti-$FABP_{pm}$ and quantitated by autoradiography/scanning densitometry. An 8-hr exposure is shown. Results, in arbitrary densitometric units (OD × mm), are as follows: left lane, 0.86; center lane, 1.84; right lane, 4.63. In scans of a 24-hr exposure of the same membrane or of autoradiograms from studies involving 2 or 8 μg of protein per lane, relative intensities of the bands from *pFR400/pMAAT2* cells with or without Zn^{2+} compared to *pFR400* cells with Zn^{2+} were within ±20% of those illustrated. The smear in the right-hand lane above the 43-kDa band is an artifact that can be reproduced by serial addition of increasing amounts of highly purified $FABP_{pm}$ or mAspAT to extracts of plasma membranes prior to electrophoresis and Western blotting.

strongly that the increased V_{max} for FFA uptake observed in *pFR400/pMAAT2* cells is the direct result of the increased expression of $FABP_{pm}$/mAspAT in the plasma membrane and is not secondary to alterations in intracellular metabolism. (*i*) V_{max} and the plasma membrane expression of $FABP_{pm}$/mAspAT are extremely highly correlated. (*ii*) Although transfection with pMAAT2 and subsequent culture in Zn^{2+}, which activates the plasmid's metallothionein promoter, together produce a >10-fold increase in the V_{max} for the saturable component of FFA uptake, these perturbations produced no change in the rate constant reflecting nonsaturable, passive FFA uptake. If the observed increase in FFA uptake was due to accelerated intracellular metabolism rather than membrane events, there should have been a corresponding increase in this rate constant. (*iii*) The uptake rate of octanoate, which enters cells passively but then shares a number of major intracellular metabolic pathways with FFA, was unchanged by transfection with pMAAT2 or by culture with Zn^{2+}. (*iv*) Although antibodies to mAspAT do not penetrate nonpermeabilized, viable 3T3 cells either before or after transfection, such antibodies stain mAspAT epitopes on the plasma membrane and selectively inhibit FFA uptake in transfected cells. Although these experiments confirm that $FABP_{pm}$/mAspAT on the plasma membrane functions as a facilitator of FFA uptake, the protocol employed undoubtedly resulted in a generalized overexpression of mAspAT and, conceivably, nonphysiologic sorting to the plasma membrane. However, a similarly increased expression of $FABP_{pm}$/mAspAT on the plasma membrane, with only a minor increase in mitochondrial mAspAT, occurs physiologically during differentiation of 3T3-L1 fibroblasts to an adipocyte morphology and is accompanied by a parallel increase in FFA uptake V_{max} (13, 42). The V_{max} for oleate uptake achieved in *pFR400/pMAAT2* cells is approximately half of that achieved in differentiated 3T3-L1 adipocytes (13). Hence, the present study and the earlier ones in

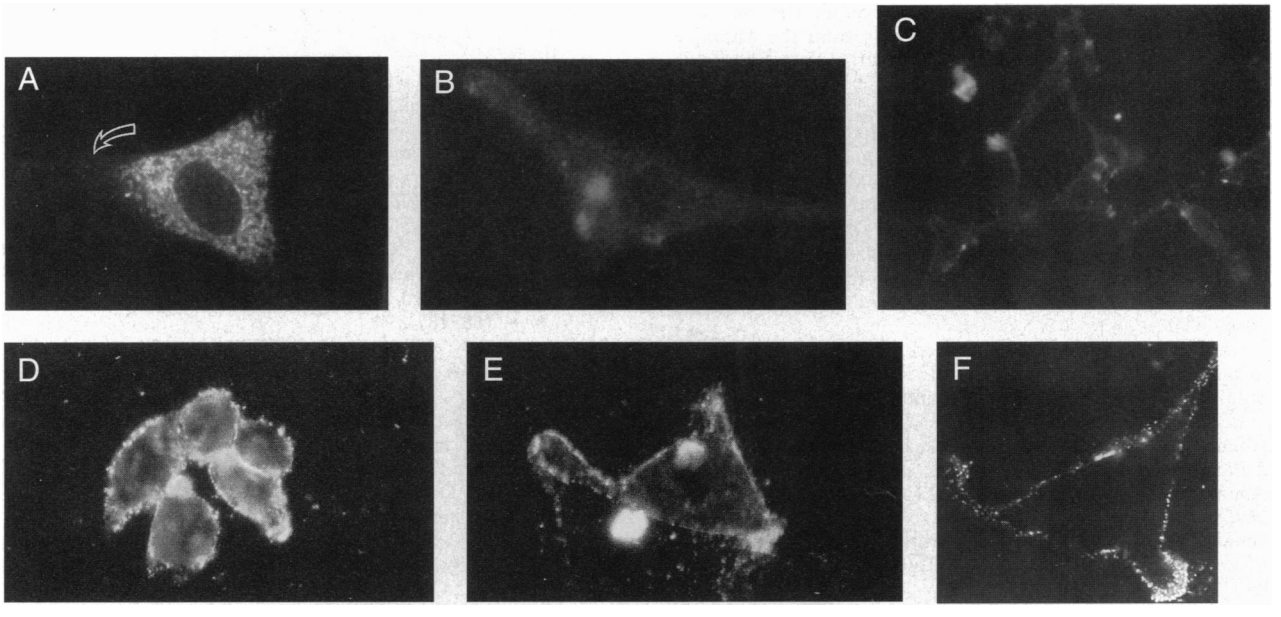

FIG. 5. Immunofluorescence studies employing anti-$FABP_{pm}$ as primary antibody. *A–E* were taken with a Nikon Optiphot fluorescence photomicroscope, using Ektachrome film (ASA 400). (*A*) Typical fixed, permeabilized *pFR400* cell. (×180; exposure, 60 sec.) Although all fixed/permeabilized cell lines exhibited intracellular immunofluorescence, principally in mitochondria, in untransfected 3T3, and in *pFR400* cells, the plasma membrane (curved arrow) showed little or no staining. (*B*) Analogous photomicrograph of a live, nonpermeabilized *pFR400* cell. (×180; exposure, 270 sec.) Neither intracellular nor plasma membrane staining is observed. (*C*) Nonpermeabilized *pFR400* cells. (×90; exposure, 510 sec.) At this very long exposure, some cells show very faint plasma membrane staining. (*D*) Nonpermeabilized *pFR400/pMAAT2* cells cultured in 100 μM Zn^{2+}. (×90; exposure, 45 sec.) Most such cells exhibit evident plasma membrane immunofluorescence. (*E*) Typical nonpermeabilized *pFR400/pMAAT2* cell cultured in 100 μM Zn^{2+}. (×180; exposure, 60 sec.) Higher-power view emphasizes plasma membrane localization of antigen. Compare with panel *B*. (*F*) Nonpermeabilized *pFR400/pMAAT2* cell cultured in 100 μM Zn^{2+}. (×180.) This photomicrograph, taken with a Leica confocal laser scanning microscope, model CLSM, equipped with SCANWARE software, clearly localizes the antigen to the plasma membrane.

3T3-L1 cells collectively provide strong support for the dual hypotheses that mAspAT and $FABP_{pm}$ are identical and play an important role in cellular FFA uptake.

How $FABP_{pm}$/mAspAT is synthesized, how it undergoes regulated sorting to the plasma membrane, how it attaches there, and how it binds FFA are crucial unanswered questions. $FABP_{pm}$/mAspAT isolated from plasma membranes lacks the leader sequence with which pre-mAspAT is initially synthesized (23, 26). Comparison of the mAspAT gene (43) with its cDNA (44) had led us to postulate that the protein on the plasma membrane might be the product of an alternative splice that replaced the mitochondrial leader encoded by exon I with a different signal peptide (42). The present experiments, conducted with a cDNA encoding the full-length preprotein including the mitochondrial leader, refute this hypothesis and suggest that plasma membrane $FABP_{pm}$/mAspAT is initially translated as pre-mAspAT and then undergoes post-translational cleavage of the leader sequence.

Four other plasma membrane proteins have been proposed as FFA transporters in different tissues, of which the best characterized thus far are fatty acid transporter (FAT) (18) and fatty acid transporting protein (FATP) (19). In contrast to $FABP_{pm}$/mAspAT, which is abundantly expressed on the plasma membranes of many cell types involved in FFA absorption and disposition, including the hepatocyte, FAT and FATP are most strongly expressed in adipose and cardiac muscle cells and little, if at all, in liver. In cells such as adipocytes that simultaneously express $FABP_{pm}$/mAspAT, FAT, and FATP, it remains to be established whether they represent separate, parallel pathways for FFA uptake or work coordinately as components of a single transport system, as occurs in bacteria (45).

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