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The 51 kDa FADS3 is Secreted in the ECM of Hepatocytes and Blood in Rat

Hélène Blanchard, Nathalie Boulier-Monthéan, Philippe Legrand, and Frédérique Pédrono*

Laboratoire de Biochimie et Nutrition Humaine, INRA USC 2012, Agrocampus Ouest F-35042, Rennes, France

ABSTRACT

The fatty acid desaturase (*Fads*) cluster is composed of three genes encoding for the Δ5- and Δ6-desaturases and FADS3. The two former proteins are involved in the fatty acid biosynthesis; the latter one shares a high sequence identity but has still no attributed function. In a previous work performed in rat, we described three isoforms of FADS3 expressed in a tissue-dependent manner. In the present study, we demonstrated a specific subcellular targeting depending on the isoform. In cultured hepatocytes, which mainly expressed the 51 kDa protein, FADS3 was unexpectedly present in the cytosolic fraction, but was also secreted in the extracellular matrix on fibronectin-containing fibers. The secretion pathway was investigated and we determined the presence of exosome-like vesicles on the FADS3-stained fibers. In parallel, FADS3 was detected in blood of hepatic vessel, and particularly in serum. In conclusion, this study demonstrated a very specific intra- and extracellular location of FADS3 in comparison with the Δ5- and Δ6-desaturases, suggesting a unique function for this putative desaturase, even if no activity has been yet identified neither in the extracellular matrix of hepatocytes nor in serum. J. Cell. Biochem. 115: 199–207, 2014.

KEY WORDS: FADS3; subcellular localization; extracellular matrix; Δ5-desaturase; hepatocytes; rat

Fatty acid (FA) desaturases catalyze the introduction of a double bond in the carbon chain of FA. The Δ5- and Δ6-desaturases are key enzymes in the biosynthesis of long chain polyunsaturated fatty acids (PUFA) of the n−6 and n−3 series, converted from their essential precursors which must be obtained from the diet. These two enzymes are respectively encoded by the *Fatty acid desaturase* (*Fads*) *1* and *2* genes, localized within the *Fads* cluster, together with a third gene called *Fads3* [Marquardt et al., 2000]. *Fads3* is expressed in various tissues in human [Marquardt et al., 2000], baboon [Park et al., 2009], and rat [Pedrono et al., 2010], with a specific mRNA distribution compared to *Fads1* and *Fads2*. For example, the two latter genes are highly expressed in the rat liver, whereas the *Fads3* expression in this tissue remains particularly weak. On the contrary, the highest expression is surprisingly found in the lung and white adipose tissue [Pedrono et al., 2010].

So far, *Fads3* has no described function, but gene polymorphism studies carried out in human showed that several single nucleotide polymorphisms (SNP) located within the gene were associated with variations in triglyceride, HDL- and LDL-cholesterol plasma levels [Aulchenko et al., 2009; Kathiresan et al., 2009; Blanchard et al., 2010]. This gene was also identified as a candidate for familial combined hyperlipidemia [Plaisier et al., 2009]. Taken together, these studies underlined the correlation of *Fads3* with lipid levels, showing its putative involvement in lipid metabolism and confirming once again its specificity compared to *Fads1* and *Fads2*, which SNP were clearly associated with plasma PUFA levels [Schaeffer et al., 2006; Malerba et al., 2008; Koletzko et al., 2011]. Transcriptomic studies also provided information on the putative physiological implication of the *Fads3* gene. For example, *Fads3* was overexpressed after an oxidative stress induction in vitro [Chicault et al., 2006] and in vivo [Vasu et al., 2007], suggesting a possible role in regulation of oxidative balance. In relation with this hypothesis, *Fads3* was highly expressed in the premature aging mouse model XPD^{TTD} [Park et al., 2008], as well as in the liver from 12-year-old compared to 1-year-old dogs [Kil et al., 2010].

While *Fads1* and *Fads2* encode for the well-known Δ5- and Δ6-desaturases, it was only recently shown that *Fads3* was translated into proteins in murine and human organs [Pedrono et al., 2010]. Different isoforms of FADS3 were thus described depending on the tissues. For instance in rat, a 75 kDa protein was observed specifically in the lung, whereas a short 37 kDa protein was particularly abundant in the muscle. Importantly, a 51 kDa isoform was identified in various

*Correspondence to: Frédérique Pédrono, Laboratoire de Biochimie et Nutrition Humaine, Agrocampus Ouest, 65 rue de Saint Brieuc, 35042 Rennes cedex, France. E-mail: frederique.pedrono@agrocampus-ouest.fr
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tissues, probably translated from the classical splicing mRNA and therefore displaying a front-end FA desaturase structure. The N-terminal moiety of the amino acid sequence would contain a cytochrome b5-like domain, characterized by a HPGG motif that was shown to be essential for the desaturase activity, as described by Guillou on the Δ6-desaturase [Guillou et al., 2004]. The C-terminal moiety of FADS3 is likely to correspond to a desaturase domain containing three histidine boxes (^{186}HDLGH, ^{223}HFQHH, and ^{387}QIEHH in rat) characteristic of front-end FA desaturases [Sperling et al., 2003]. Thus, the FADS3 protein could be a new FA desaturase, even if no enzymatic activity has for now been identified in experiments based on overexpression of recombinant FADS3 in various mammalian cells such as HepG2, HEK293 [Stroud et al., 2009], COS7, SH-SY5Y [Pedrono et al., 2010], or in yeast [Brenna et al., 2010]. These negative reports emphasized the necessity of developing alternative methods to improve our knowledge about the native FADS3 protein and to better understand its function. Therefore, the present study aimed at describing the subcellular location of FADS3 isoforms in rat tissues and cultured hepatocytes by using two specific antibodies [Pedrono et al., 2010]. This work was completed with desaturation assays to determine the FADS3 enzymatic activity.

MATERIALS AND METHODS

ANIMALS AND CHEMICALS

Rats were purchased from the breeding center Elevage Janvier (Le Genest Saint Isle, France). The experimental procedure was in compliance with recommendations of the 2003/65/CEE European directive for animal experimentation. Tissue harvesting was performed as described elsewhere [Pedrono et al., 2010]. For hepatocyte primary culture, Williams′E medium, gentamycin, insulin and dexamethasone were from Sigma-Aldrich (Saint Quentin Fallavier, France), fetal bovine serum (FBS) from Lonza (Levallois Perret, France), penicillin and streptomycin from Eurobio (Courtaboeuf, France) and primaria™ dishes from BD Biosciences (Le pont de Claix, France). Chemicals, protease inhibitor cocktail, Hoechst 33342 and the Nuclei E/Z kit were obtained from Sigma-Aldrich. Solvents for lipid analysis were from Thermo Fisher Scientific (Illkirch, France). FA were purchased from Sigma-Aldrich and phospholipids from Avanti Polar Lipids (Alabaster). Radiolabeled FA and phospholipids were provided by American Radiolabeled Chemicals (St. Louis).

CELL CULTURE

Primary hepatocytes were obtained from Sprague Dawley adult rats (7–8 weeks old) fed ad libitum with a standard rodent chow (Special Diets Services, Saint Gratien, France) and fasted overnight before experimentation. Rats were anesthetized with two successive injections of pentobarbital (41 mg/kg each, Sigma-Aldrich) and hepatocytes were then isolated by a collagenase perfusion [Legrand and Bensadoun, 1991]. Cells were seeded in primaria dishes with a density of 4.2×10^5 cells/cm^2 in Williams′E medium supplemented with 7% FBS, 26 mM $NaHCO_3$, 12.5 mM HEPES, 15 μM bovin serum albumin (BSA), 50 UI/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml gentamycin, 1 μM insulin, and 1 μM dexamethasone. After plating, cells were maintained in a humidified incubator at 37°C with CO_2 5% in air; the medium was changed 4 h after seeding and depleted in serum. Hepatocytes were isolated using accutase® (Sigma-Aldrich) whereas extracellular matrix (ECM) was collected as described below. In some experiments, cells were treated with brefeldin A (BFA, 10 μg/ml), which was added 4 h after the plating and incubated for 48 h. The treatment efficiency was assessed by measuring the albumin content in the culture medium.

SUBCELLULAR FRACTIONATION

Subcellular fractionation was performed on rat liver, heart and lung and in cultured hepatocytes in order to determine the FADS3 protein localization. The fresh organs needed to be previously grinded in a Potter-Elvehjem homogenizer. All samples were then homogenized with two series of 20 strokes in a Dounce homogenizer interrupted by 5 min incubation on ice. The total fraction was collected after addition of a protease inhibitor cocktail. The homogenization was performed in two different buffers at the same time. The nuclear and mitochondrial fractions were obtained with the Nuclei E/Z kit according to the supplier instructions. Briefly, nuclei were isolated after two successive centrifugations (500*g*, 5 min at 4°C). From the resulting supernatant, mitochondria were finally pelleted (11,000*g*, 30 min at 4°C). The cytosolic and microsomal fractions were separated in a sucrose buffer (10 mM KH_2PO_4, 40 mM Na_2HPO_4, $2H_2O$, pH7.4 with 0.25 M sucrose) [D′Andrea et al., 2002]. Post-mitochondrial supernatant (PMS) was obtained after centrifugation (11,000*g*, 30 min at 4°C) and was then submitted to ultracentrifugation (100,000*g*, 1 h at 4°C) to isolate microsomes from the cytosol. The nucleus, mitochondrion and microsome pellets were resuspended in radio immunoprecipitation assay (RIPA) buffer made from phosphate buffered saline (PBS: 4 mM NaH_2PO_4, 0.94 mM Na_2HPO_4, 150 mM NaCl, pH7.4) with 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 1 mM ethylene diamine tetraacetic acid and 10 mg/ml aprotinin.

EXTRACELLULAR MATRIX ISOLATION

ECM was isolated from hepatocyte primary cultures by a modification of the sodium deoxycholate procedure described by Hedman et al. [1979]. After three washes in PBS, cells were dissociated from ECM with slow shaking (twice for 10 min on ice) in 10 mM Tris buffer pH8 containing 0.5% sodium deoxycholate. The isolated ECM was finally washed twice with 2 mM Tris buffer pH8, scraped off from the dishes, pelleted (600*g*, 5 min, 4°C) and suspended in RIPA.

SERUM FRACTIONATION

Serum lipoproteins were separated on an iodixanol gradient (Optiprep™, Sigma-Aldrich) by using a rapid method modified from Graham et al. [1996]. Blood was collected from the abdominal aorta and centrifuged (1,700*g*, 15 min at 20°C) to isolate the serum. Then, Optiprep™ was added to the serum (12%, v/v) and 2 ml of this solution were laid on 400 μl of iodixanol 20% in PBS and covered by 400 μl of PBS. After ultracentrifugation (350,000*g*, 3.5 h at 4°C), successive fractions (250 μl) were collected from the top (lowest density) to the bottom (highest density). Each fraction was characterized by a lipoproteinogramm (Sébia, Evry, France) coupled with a triglyceride and cholesterol assay (Biomerieux, Craponne, France).

IMMUNOBLOTTING

Proteins were identified by Western blot after SDS–PAGE. FADS3 isoforms were detected with either anti-NtermFADS3 or anti-CtermFADS3; these antibodies recognize specifically the amino- and carboxyl-terminal sequences of the recombinant and native rat FADS3 as previously published [Pedrono et al., 2010]. FADS3 expression was compared to the Δ5-desaturase as the protein profile is very similar. The rat Δ5-desaturase expression was analyzed with a polyclonal antibody (anti-Δ5D) targeting a specific peptide (^{100}QSSFEPTKNKALTDE) and produced in rabbit by a 28-day immunization protocol (Eurogentec, Angers, France), as performed for the FADS3 antibodies. The purified anti-Δ5D was incubated overnight at 4°C at 1 μg/ml in Tris buffered saline (TBS: 20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20 and 5% BSA. Other primary antibodies were used as recommended by the manufacturers: anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), anti-actin and -lamin A from Sigma-Aldrich; anti-voltage dependent anion channel (VDAC) from Ozyme (Saint-Quentin Yvelines, France); anti-tumor necrosis factor-receptor 1 (TNFR1), anti-CD-40, anti-acyl-CoA synthetase family member 2 (ACSF2) and anti-fibronectin from Santa Cruz (Heidelberg, Germany); anti-calreticulin, anti-*trans* golgi network 38 (TGN38) from BD Biosciences; anti-albumin from Thermo Fisher Scientific and anti-tumor susceptibility gene 101 (TSG101) from AbCam (Paris, France). Anti-NADH cytochrome b5 reductase (NCB5R) was kindly given by Dr. Borgese (University of California, USA). Anti-rab7 and anti-flotillin were kind gifts from the SeRAIC unit (Dominique Lagadic-Gossmann, University of Rennes 1, France). Primary antibodies were coupled to HRP-conjugated anti-IgG and peroxidase activity was determined by chemiluminescent detection using Immobilon reagents (Millipore, Molsheim, France). The apparent molecular mass of proteins was determined using a standard curve constructed with the Kaleidoscope marker (Bio-Rad, Marnes La Coquette, France) in 10% SDS–PAGE.

IMMUNOFLUORESCENCE ON CULTURED HEPATOCYTES

Immunofluorescence was realized on primary hepatocytes seeded at 10^6 cells/well in 12-well plates and cultured without FBS for 24 h on glass cover slips. At first, nuclei were stained with Hoechst 33342 at 2 μM in PBS for 15 min at 37°C. Cells were then fixed in 4% paraformaldehyde in PBS for 30 min at 4°C and permeabilized with PBS containing 0.2% Triton X-100 and 1% BSA for 1 h at room temperature (RT). Anti-NtermFADS3, anti-CtermFADS3, anti-Δ5D and mouse anti-GAPDH (1 μg/ml), mouse anti-fibronectin (1/200), mouse anti-TSG101 (1/200), goat anti-TNFR1 and goat anti-CD-40 (1/100) were incubated in PBS containing 1% BSA for 1.5 h at RT. Purified rabbit, mouse or goat IgG (Sigma-Aldrich) were used at 1 μg/ml as negative control. Alexa 594-conjugated anti-rabbit IgG (Santa Cruz) and Alexa 488-conjugated anti-mouse (Cell Signaling Technology, Danvers) or anti-goat IgG (Life Technologies, Saint-Aubin, France) were selected as secondary antibodies and incubated at 1/1,000 each for 1.5 h at RT in PBS with 1% BSA. Finally, fluorescence was captured by a DMRXA Leica epifluorescence microscope (magnification 100×; specified in legend if different) associated with a COHU high performance CCD camera using Metavue software (Dominique Lagadic-Gossmann, Microscopy-Rennes Imaging center, IFR 140, Université de Rennes 1, France).

LIVER IMMUNOSTAINING

Immunohistochemical staining was assessed on snapfrozen liver. Briefly, histological measurements were performed at −20°C on 7 μm sections cut on a Leica CM3050S cryostat at the Histopathology Facility H^2P^2 (Alain Fautrel, Biogeneouest, Université de Rennes I, France). Sections were fixed with acetone (−20°C, 10 min) and permeabilized in 0.1% triton X-100 (16 min). They were then incubated with anti-NtermFADS3 (20 μg/ml, 37°C for 1 h). Immunostaining was conducted on a Discovery XT automated system using an OmniMap anti-rabbit polymer detection kit (Ventana Medical Systems, Illkirch, France).

GLYCOSYLATION ASSAY

The putative attachment of FADS3 to glycosaminoglycans was tested using Glycoprofile II (Sigma-Aldrich) and protein deglycosylation mix (New England BioLabs, Evry, France) according to the supplier instructions. Briefly, cultured hepatocytes and isolated ECM were digested with the peptide: N-glycosidase F (PNGase F) to remove the N-linked carbohydrates or with an exoglycosidase mix containing O-glycosidase, neuraminidase, β1,4-galactosidase and β-N acetylglucosaminidase to remove the O-linked carbohydrates. The shift in gel migration was tested out by Western blot using the anti-NtermFADS3 antibody. In parallel, RNAseB and fetuin were used as positive controls and the deglycosylation was confirmed by a Bio-Safe™ Coomassie staining (Bio-Rad).

DESATURASE ACTIVITY

The enzymatic activity was determined on PMS and ECM of cultured hepatocytes and serum. PMS was collected from hepatocytes in culture, which were sonicated and then centrifuged (10,000*g*, 30 min at 4°C) in a sucrose buffer. Δ5- and Δ6-desaturase activities were assayed in a 500 μl reaction medium containing cofactors as described in a previous study [Guillou et al., 2004]. (1-^{14}C)20:3 n−6 and (1-^{14}C)18:3 n−3 (60 μM, 10 mCi/mmol) were respectively used as substrates for the Δ5- and Δ6-desaturases and incubated for 20 min at 37°C. Radiolabeled FA were also incubated with the isolated ECM and serum in phosphate buffer in order to identify an extracellular enzymatic activity. In that case, FA were used either in ethanol solution (3‰, v/v) or complexed with albumin. For complexation, FA were saponified with 0.1% KOH in ethanol (112 μl/mmol of FA) during 30 min at 70°C and dissolved in Williams′E containing 1% BSA. After sonication, complexes were allowed to form overnight at RT under continuous shaking. In addition, 18:0 and 18:2 n−6 esterified at the *sn−2* position of phospholipids were respectively incubated using 1,2-(1-^{14}C)distearoyl-*sn*-glycero-3-phosphocholine and 1, palmitoyl-2, (1-^{14}C)linoleoyl-*sn*-glycero-3-phosphocholine. After incubation, the FA profiles were analyzed by HPLC coupled with liquid scintillation counting as described elsewhere [D′Andrea et al., 2002].

PROTEOMIC ANALYSIS OF ECM

The ACSF2 acyl-CoA synthetase identification was performed by LC/MS/MS at the UMR1253 proteomic platform (INRA, Science et Technologie du Lait et de l′Oeuf, Rennes, France). Briefly, proteins from isolated ECM were separated by SDS–PAGE and in-gel digested by trypsinolysis [Dherbecourt et al., 2010]. Peptides were fractionated

by nanoLC on a reverse-phase column C18 PepMap 100 (LC packings, 3 μm, 100 Å, 75 μm i.d. × 15 cm). Mass spectra were performed using a hybrid quadrupole time of flight (Q.TOF) mass spectrometer Qstar XL (MDS Sciex, Toronto, Canada). Data were submitted to MASCOT for identification [Jardin et al., 2012].

STATISTICAL ANALYSIS

Each experiment was performed at least three times. *P*-Values were calculated using the Student test for two-group comparisons.

RESULTS

SUBCELLULAR LOCALIZATION OF FADS3 IN TISSUES

We previously demonstrated a tissue-specific profile, and different FADS3 isoforms [Pedrono et al., 2010]. In the present study, we investigated the subcellular location of FADS3 in various tissues selected according to the isoform occurrence. In the lung, the 75 kDa protein was mainly detected in the mitochondrial fraction (which also contained lysosomes and peroxysomes), and to a lesser extent in the microsomal fraction (Fig. 1A). On the contrary, the 51 and 37 kDa isoforms of FADS3 were both observed specifically in the cytosolic fraction in the liver, heart and skeletal muscles, as well as the hepatic 33 kDa protein (non-detected in some animals). This result contrasts with the microsomal localization of the 55 kDa-Δ5-desaturase (Fig. 1B), as well as the Δ6-desaturase (data not shown). In parallel, the consistency of the fractionation was demonstrated by characterizing the fractions with specific markers, showing in particular the absence of contamination of the cytosolic fraction by endoplasmic reticulum (Fig. 1C). In conclusion, our results highlighted the cytosolic localization of FADS3 in the liver, underlining a great specificity of the protein as compared to the microsomal Δ5-desaturase.

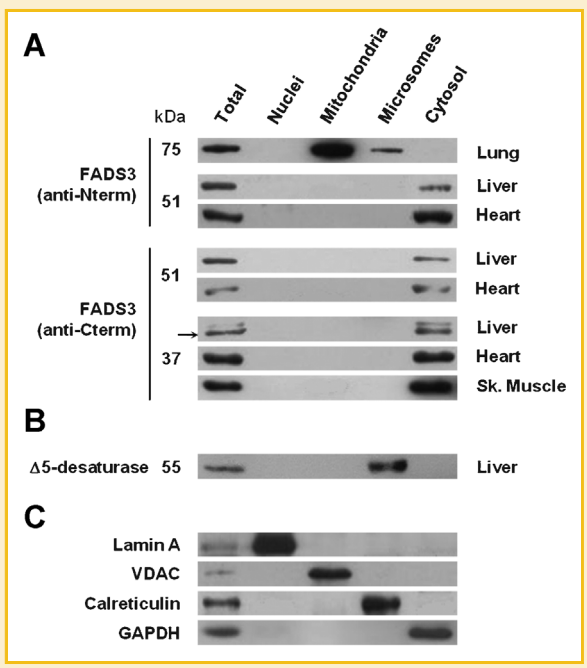

Fig. 1. FADS3 localization in subcellular organelles in rat tissues. Subcellular fractionation was performed by differential centrifugation on homogenized tissues. A: FADS3 was detected by Western blot with the anti-NtermFADS3 and anti-CtermFADS3 antibodies. The arrow indicates the 33 kDa protein. B: The 55 kDa isoform of the Δ5-desaturase was visualized with the anti-Δ5D antibody. C: Lamin A, VDAC, calreticulin and GAPDH were respectively used to characterize the nuclei, mitochondria, endoplasmic reticulum in microsomes and cytosol; only liver homogenates are presented here. VDAC, voltage dependent anion channel; GAPDH, glyceraldehyde 3-phosphate dehydrogenase, Sk muscle, skeletal muscle.

SUBCELLULAR LOCALIZATION OF FADS3 IN PRIMARY HEPATOCYTES

The 51 kDa isoform is considered as the native and potentially active protein, so that the study focused on this isoform, which is specifically abundant in hepatocytes. We usually conducted experiments on cultured cells within 24 h after cell seeding. Therefore we analyzed the time-sequential expression of FADS3 in our experimental conditions (Fig. 2A). Our results showed that the protein content was significant until 48 h of culture, with a maximum level at 4 h corresponding to the culture medium renewal, and then declined at 72 h. On the contrary, the actin content was increased as compared to the albumin used as control.

When subcellular fractions were analyzed in hepatocytes, as well as in tissues, FADS3 was identified in the cytosolic fraction of cultured cells, while the Δ5-desaturase was distinctively localized in microsomes (Fig. 2B). We confirmed by immunofluorescence that FADS3 was present in the cytoplasm of primary hepatocytes with a partial colocalization with GAPDH (Fig. 2C). In addition, the fluorescent staining revealed an extracellular location of FADS3, detected on extracellular fibrils constituting the matrix surrounding

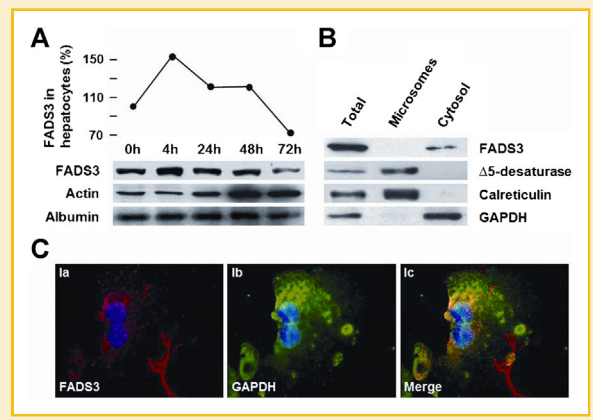

Fig. 2. Subcellular localization of FADS3 in primary hepatocytes. A: The time-sequential expression of FADS3 was determined in hepatocytes after isolation and seeding. The 51 kDa isoform of FADS3, specifically found in this cell type, was detected with the anti-NtermFADS3 antibody. The protein abundance was compared to actin and albumin. B: Subcellular fractionation was performed by differential centrifugation on 24 h cultured hepatocytes. The 51 kDa isoform of FADS3 was vizualized with the anti-NtermFADS3 antibody and compared to the 55 kDa-Δ5-desaturase identified with the anti-Δ5D antibody. Calreticulin and GAPDH were respectively used as specific markers of the endoplasmic reticulum and cytosol. C: Cultured hepatocytes were stained with the anti-NtermFADS3 (Ia) and anti-GAPDH (Ib). FADS3 was simultaneously observed with GAPDH (Ic) by merging the signals. Hoechst dye was used to visualize nuclei (in blue); magnification 100×.

cells (Figs. 2C and 3A). This observation contrasts with the intracellular targeting of the Δ5-desaturase (Fig. 3A), which represents the second main difference between FADS proteins.

As fibronectin is a major component of ECM, it was used as a marker of the extracellular structure (Fig. 3B). Simultaneous staining of FADS3 (Ia and IIa) and fibronectin (Ib and IIb) showed that the two proteins were located on the same fibers (Ic and IIc). This result was obtained both with the anti-NtermFADS3 (I) and the anti-CtermFADS3 (II). Comparison of isolated ECM and PMS (containing microsomes and cytosol) showed that FADS3 was located in both samples, whereas the Δ5-desaturase was only found in PMS (Fig. 3C). Non contamination was confirmed by specific markers.

SECRETION PATHWAY OF FADS3 IN PRIMARY HEPATOCYTES

BFA was used as an inhibitor of protein secretion in cultured hepatocytes [Fujiwara et al., 1988]. The FADS3 content was reduced in ECM with BFA treatment indicating that the protein was secreted by primary hepatocytes and targeted in ECM but not present in the culture medium (Fig. 4A).

As FADS3 is supposed to be a membrane-bound protein according to its amino acid sequence, we examined if any secretory organelle could contaminate the cytosolic fraction containing the protein. We therefore analyzed the presence of small vesicles by comparing microsomal and cytosolic samples (Fig. 4B). Protein markers for *trans*-golgi vesicles, late endosomes and membrane rafts were found in the microsomal fraction, whereas CD-40, TSG101 and TNFR1, markers for microvesicles, exosomes and exosome-like vesicles respectively, were identified in the cytosolic samples, underlining a membrane contamination in the latter fraction. We then investigated the possible occurrence of FADS3 within these organelles by immunofluorescence on cultured hepatocytes (Fig. 4C). Our results showed that CD-40 (Ib) and TNFR1 (IIIb) were also present on extracellular fibers, on the contrary of TSG101 (IIb). We found that extracellular FADS3 and TNFR1 could be present on the same fibers (IIIc), whereas FADS3 and CD-40 were observed on distinct extracellular fibers (Ic). In cells, no colocalization was observed

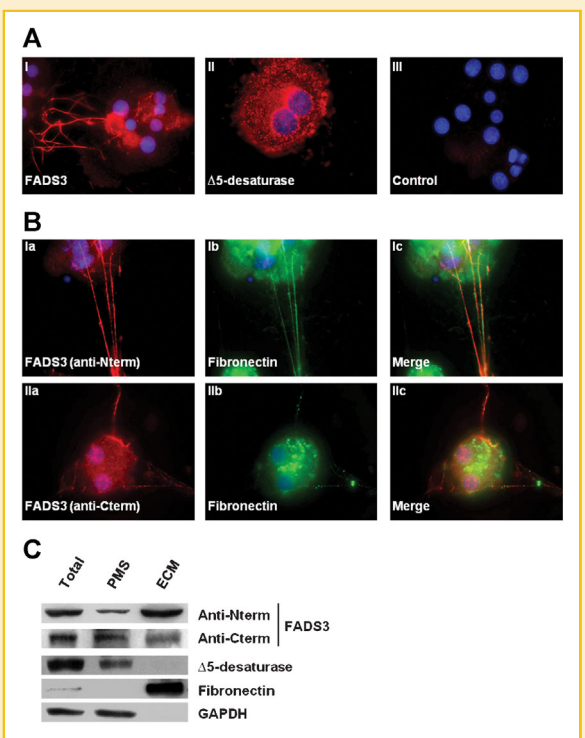

Fig. 3. FADS3 occurrence in the extracellular matrix of cultured hepatocytes. A: Immunofluorescence staining showed an extracellular localization of the 51 kDa isoform of FADS3 identified with the anti-NtermFADS3 antibody (I; magnification 63×). The Δ5-desaturase (II; magnification 125×) and purified rabbit IgG (III; magnification 63×) were used as controls. Nuclei were stained with Hoechst. B: FADS3 was detected both with the anti-NtermFADS3 (Ia) and the anti-CtermFADS3 (IIa) antibodies. FADS3 and the fibronectin (Ib and IIb) were simultaneously observed by merging the signals (Ic and IIc); magnification 100×. C: The FADS3 location was tested on isolated ECM by Western blot using the anti-NtermFADS3 and anti-CtermFADS3 in comparison with the 55 kDa-Δ5-desaturase visualized with the anti-Δ5D antibody. The PMS obtained from cultured hepatocyte fractionation (characterized by GAPDH) was compared to the ECM (characterized by fibronectin). ECM, extracellular matrix; PMS, post-mitochondrial supernatant.

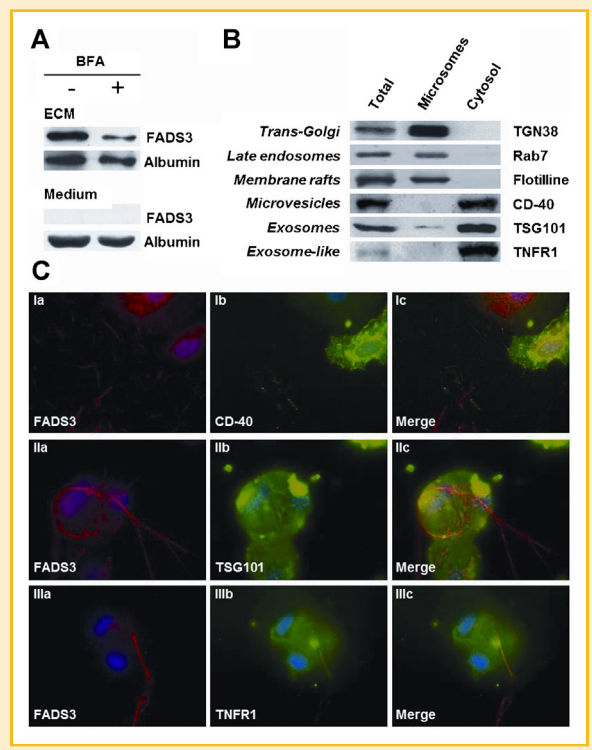

Fig. 4. Secretion pathway of FADS3 in cultured hepatocytes. A: Cultured hepatocytes were treated with BFA for 48 h. The extracellular amount of FADS3 (51 kDa) was assessed in using the anti-NtermFADS3 and normalized with albumin in isolated ECM and culture medium. B: The purity of the cytosolic fraction obtained from cultured hepatocytes was checked by Western blot in using different antibodies targeting membrane contaminants. C: Immunofluorescence stainings were performed to further characterize the secretion pathway of FADS3 in cultured hepatocytes. Anti-CD-40 (Ib), anti-TSG101 (IIb), and anti-TNFR1 (IIIb) were specifically used with anti-NtermFADS3 (Ia, IIa, IIIa) to visualize microvesicles, exosomes and exosome-like vesicles; magnification 100×. BFA, brefeldin A; TSG101, tumor susceptibility gene 101; TNFR1, tumor necrosis factor-receptor 1.

between FADS3 and CD-40, TSG101 or TNFR1. Moreover FADS3 was not detected in lipid droplets (result not shown).

As various proteins located in the ECM are glycoproteins such as fibronectin, the assessment of N/O-linked glycosylation of FADS3 was carried out using a digestive enzyme cocktail. No shift on the gel migration appeared between enzyme-treated and non-treated samples and the same profile was observed for cultured hepatocytes and ECM (Fig. 5). Our results thus demonstrated that FADS3 did not bear any N- or O-linked glycosylation motifs and presented no glycosaminoglycan attachment sites.

ANALYSIS OF A PUTATIVE FA DESATURATION CAPACITY IN THE ECM

We further searched for an enzymatic activity in the ECM, considering FADS3 as a putative front-end FA desaturase, needing NCB5R for the protein complex formation associated with partners for the active substrate supply. NCB5R was identified as a member of the Δ9-desaturation complex [Heinemann and Ozols, 2003] and is also considered to be involved in the Δ5- and Δ6-desaturation. We demonstrated on cultured hepatocytes that NCB5R was present in the PMS, as well as in the ECM (Fig. 6A). We also identified in ECM a synthetase of acyl-CoA involved in the regular FA substrate activation, recently discovered, and named ACSF2 [Watkins et al., 2007]. The proteomic analysis resulted in a MASCOT score of 307.11, a percent coverage of 12.20 and a peptide number that contributed in the calculation of the protein average ratio ($P < 0.05$) of 4. The ACSF2 occurrence was confirmed by Western blot in the mitochondrial fraction and ECM of cultured hepatocytes (Fig. 6B). These new findings support the hypothesis of an extracellular desaturation activity, potentially catalyzed by FADS3, using NCB5R as a catalytic partner, and on acyl-CoA as substrates. Thus, we performed desaturation assays in ECM (Fig. 6C). As a control, we used the PMS in which ^{14}C18:3 n−3 was converted into ^{14}C18:4 n−3 by the Δ6-desaturase and ^{14}C20:3 n−6 into ^{14}C20:4 n−6 by the Δ5-desaturase. In contrast, no desaturation product was measured when the same substrates were incubated with ECM as free FA or albumin FA complex. Negative results were obtained with various desaturase substrates like ^{14}C18:0, ^{14}C18:1 n−9, and ^{14}C18:2 n−6. In parallel, ^{14}C18:0 or ^{14}C18:2 n−6 esterified on phosphatidylcholine, considered as another potential substrate form, were incubated and we similarly found no desaturation activity (results not shown).

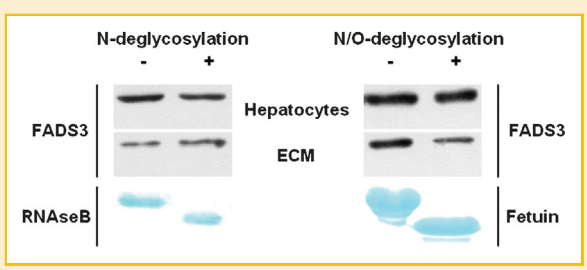

Fig. 5. Glycosylation of FADS3 from cultured hepatocytes. N- and O-glycosylation of FADS3 (51 kDa) was tested by enzymatic digestion on cultured hepatocytes and isolated ECM. FADS3 was detected using the anti-NtermFADS3 antibody. RNAseB and fetuin were used as controls and the gel shift was confirmed by Coomassie staining.

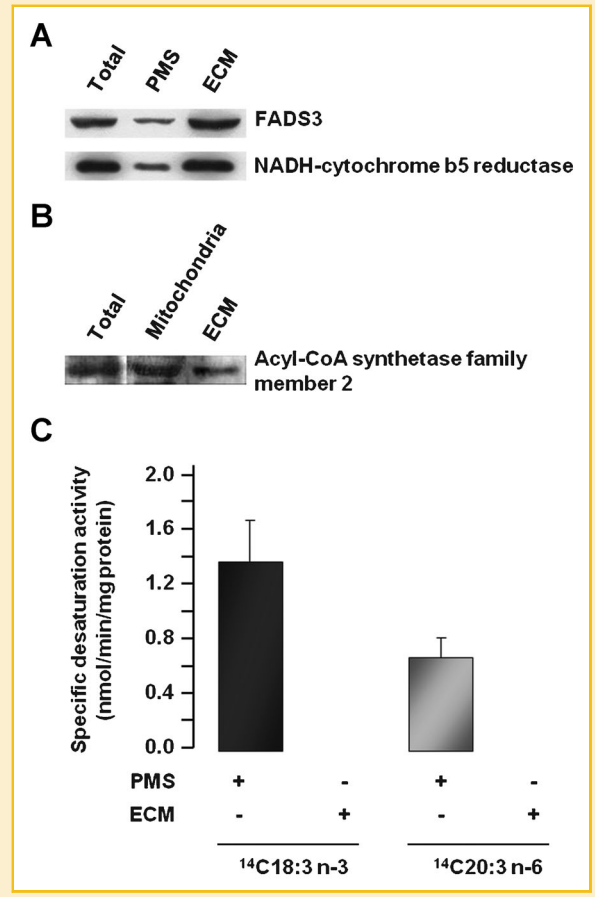

Fig. 6. Desaturation activity assay in ECM of cultured hepatocytes. A: The NCB5R occurrence was analyzed by Western blot on cultured hepatocytes, PMS and ECM. B: The proteomic analysis of ECM underlined the presence of a mitochondrial acyl-CoA synthetase named ACSF2, which occurrence was confirmed by Western blot using a specific anti-ACSF2. C: The desaturation of ^{14}C18:3 n−3 and ^{14}C20:3 n−6 was assayed in PMS and ECM by incubation of radiolabeled substrates as free ethanol diluted FA, followed by HPLC analyses and scintillation counting. Results were expressed as mean ± SEM (N = 3 in triplicates). NCBR5R, NADH cytochrome b5 reductase; ACSF2, acyl-CoA synthetase family member 2.

PLASMA SECRETION OF FADS3

Hepatocytes display a secretory function described for a large variety of proteins, such as albumin or apolipoproteins associated with lipoproteins. Therefore, we investigated whether FADS3 could be released in plasma and linked to lipoproteins. Immunostaining of liver section (Fig. 7A) showed that FADS3 was secreted in the extracellular matrix surrounding hepatocytes (I) on the contrary of the Δ5-desaturase detected inside the cells (III). We also showed that FADS3 was present in blood vessels (II).

To confirm this result, rat serum was fractionated on a density gradient, and each fraction was identified by a lipoproteinogram associated with cholesterol and triglyceride assays (Fig. 7B). VLDL were characterized by a low density and a high triglyceride amount (fractions 1–3), whereas we identified HDL thanks to their high density and cholesterol rate (fractions 6–9). Finally, LDL were distinguished since they migrated very little and were found in

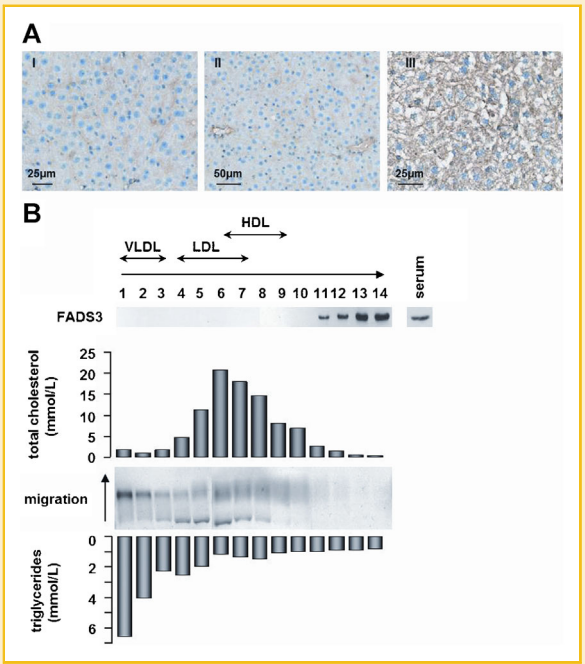

Fig. 7. FADS3 in rat blood. A: Liver sections were stained by using anti-Nterm-FADS3 (I and II) and anti-Δ5D (III). The extracellular location of FADS3 was vizualized surrounding hepatocytes (I) and in blood vessels (II), unlike the intracellular Δ5-desaturase (III). B: Rat serum fractionation was performed by ultracentrifugation on an iodixanol gradient. The resulting fractions were characterized by a lipoproteinogram coupled with triglyceride and cholesterol assays. FADS3 was detected by Western blot with the anti-NtermFADS3 antibody.

intermediate density fractions (fractions 4–7). We detected FADS3 in rat serum, but only in high density fractions containing soluble proteins. Additional results showed that FADS3 was detected in red blood cells but not in leukocytes (data not shown). Consequently, FADS3 was secreted in blood without apparent linkage with lipoproteins.

DISCUSSION

The present study aimed at describing the subcellular location of FADS3 in rat, in comparison with the Δ5-desaturase. We first showed that the subcellular localization of FADS3 depends on the isoform and de facto on the tissue. We then focused on the 51 kDa isoform which is specifically present in cultured hepatocytes, and observed its secretion within the ECM and its presence in rat serum.

Consistently with a previous work [Pedrono et al., 2010], we found that FADS3 displayed different isoforms, the main one detected at 51 kDa and the others identified at 37 kDa and 75 kDa, depending on the tissue. While the Δ5-desaturase is targeted in endoplasmic reticulum, our results demonstrated that FADS3 was not a microsomal protein in the liver. We detected the main 51 kDa isoform, as well as the short ones, in the cytosolic fractions in different tissues. This outcome contrasts with the hypothesis of a microsomal location of FADS3 formulated according to its sequence homology with the Δ5- and Δ6-desaturases and the prediction of transmembrane domains in the primary sequence. In cultured hepatocytes, we also found the protein in the cytosolic fraction, which contained membrane contaminants such as microvesicles, exosomes, and exosome-like vesicles. Nevertheless, the immunostaining suggested that FADS3 was not located in these organelles in the cytoplasm of hepatocytes. In addition to this unexpected subcellular location, our study demonstrated that FADS3 was secreted in the ECM of cultured hepatocytes. This finding was specific of hepatocytes as FADS3 could not be detected in the ECM of the other cultured hepatic cells, that is, Küpffer cells or HTC (result not shown). No signal peptide was identified by the SignalP 3.0 software [Bendtsen et al., 2004], which emphasizes that FADS3 may be secreted through another pathway than the prevalent endoplasmic reticulum/Golgi dependent one. Other secretion processes have been described, such as transport of molecules to the extracellular space by exosomes [Simons and Raposo, 2009]; however, this hypothesis can be ruled out because FADS3 did not colocalize with the TSG101 exosomal marker. Additionally, FADS3 was not identified by a recently published proteomic analysis of rat hepatocyte exosomes [Conde-Vancells et al., 2008]. Another unconventional pathway consists of a direct translocation of the protein from the cytosol to the extracellular space involving an interaction between the secreted protein and membrane phosphatidylinositol 4,5-bisphosphate [Nickel, 2005]. This allows for example the fibroblast growth factor 2 to cross the membrane and reach the ECM [Temmerman et al., 2008]. The extracellular occurrence of FADS3 can also be supported by a putative peptide C-terminal amidation on the amino sequence detected by the SMART server [Islam et al., 2007; Letunic et al., 2009], targeting the protein to secretory granules. FADS3 was detected on the same fibers than those stained with TNFR1, known to be in exosome-like vesicles [Islam et al., 2007]. Circulating TNFR1 exosome-like vesicles were recently found in human plasma, which would be consistent with the detection of FADS3 in hepatic vessel and particularly in serum [Zhang et al., 2008]. Based on these hypotheses, further experiments will be necessary to elucidate the FADS3 secretion pathways in the ECM, especially those involving exosome-like vesicles.

In comparison with other tissues, the ECM produced by the liver in vivo is not abundant [Martinez-Hernandez and Amenta, 1993], even though Stamatoglou showed that hepatocytes in primary cultures were able to secrete an extensive ECM rich in fibronectin and fibrin [Stamatoglou et al., 1987]. The ECM was at first studied for its structural function, but recent works underlined that the matrix was also involved in cell migration and differentiation, cell signaling and other metabolic functions [Hynes, 2009]. For example, the secreted phospholipase A2 (sPLA2), bound to glycosaminoglycans, catalyzes the hydrolysis of extracellular phospholipids within the ECM [Sartipy et al., 1998]. We demonstrated that FADS3 was not N/O-glycosylated, suggesting a different attachment along the fibronectin fibrils. Our immunostaining observations also supported a vesicular localization of FADS3, indicating a membrane anchorage through the predicted transmembrane domains. Further investigations are needed to clarify the specific localization of FADS3 in the ECM and thus its function in hepatocytes, which is potentially linked with triglyceride and cholesterol secretion suggested by gene polymorphism analyses in human [Aulchenko et al., 2009; Kathiresan et al., 2009; Plaisier et al., 2009].

Despite the lack of investigation as regards extracellular lipid metabolism, FADS3 could display a FA desaturase activity in ECM or extracellular vesicles. The catalytic reaction described for the Δ9-desaturase requires both the NCB5R and the cytochrome b5 [Strittmatter et al., 1974], whereas the Δ5- and Δ6-desaturases can be active with NCB5R alone as they contain a fused cytochrome b5-like domain, also present in the FADS3 primary sequence [Guillou et al., 2004]. The hypothesis of an ECM specific FA desaturase is sustained by the presence of NCB5R and supported by ACSF2, a non-conventional fatty acyl-CoA synthetase recently discovered and which biological function has to be explored [Watkins et al., 2007]. In our experimental conditions, no desaturation activity was identified by in vitro incubations of isolated ECM with various substrates for the Δ9-, Δ6-, and Δ5-desaturases, suggesting that FADS3 does not act as an alternative desaturase within the ECM under our experimental procedures. As established for $sPLA_2$ [Sartipy et al., 1998], the FADS3 activity could be regulated by a specific glycosaminoglycan composition, which was possibly not optimum in our cultured hepatocyte model. FADS3 might also be secreted within the ECM as a proenzyme, without becoming a mature protein in our work conditions. It is also possible that FADS3 product could be an end signal for a specific physiological situation that was not mimicked in our experimental surroundings. In serum, which is devoid of NCB5R (result not shown), the lack of catalytic activity may suggest that FADS3 would only be carried towards the targeted organ. However, if FADS3 displays an enzymatic function, it is now essential to explore the putative substrate diversity and to modulate the supply forms in further experiments. Finally, it remains essential to understand to what extent the liver could be a source or a target organ for this desaturase-like protein.

Very little information is available in the literature concerning the *Fads3* gene and no protein function has yet been described. In spite of its classical desaturase structure, the FADS3 specificity compared to the Δ5- and Δ6-desaturase is now clear. In this study, we showed that the extracellular localization ranks among these specificities. FADS3 was secreted specifically by hepatocytes in the ECM where it was located on fibronectin-containing fibers. The protein was also present in rat serum, suggesting that a new approach should be developed to identify its physiological role in considering the organism scale rather than the cellular model.

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