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The Direct Peroxisome Proliferator-activated Receptor Target Fasting-induced Adipose Factor (FIAF/PGAR/ANGPTL4) Is Present in Blood Plasma as a Truncated Protein That Is Increased by Fenofibrate Treatment*

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Stéphane Mandard‡, Fokko Zandbergen‡, Nguan Soon Tan§, Pascal Escher¶, David Patsouris‡, Wolfgang Koenig‖, Robert Kleemann, Arjen Bakker‡‡, Frank Veenman‡, Walter Wahli§, Michael Müller‡, and Sander Kersten‡§§**

*From the ‡Nutrition, Metabolism and Genomics Group, Division of Human Nutrition, Wageningen University, 6700 EV, Wageningen, The Netherlands, §Center for Integrative Genomics, Université de Lausanne, Lausanne CH-1015, Switzerland, ¶Institute of Physiology, Pharmazentrum, University of Basel, Basel CH-4056, Switzerland, the ‖Department of Internal Medicine II-Cardiology, University of Ulm, Ulm D-89081, Germany, **Gaubius Laboratory, TNO-Prevention and Health, Leiden 2301 CE, The Netherlands, and the ‡‡Department of Human Biology, Maastricht University, Maastricht 6200 MD, The Netherlands*

The fasting-induced adipose factor (FIAF, ANGPTL4, PGAR, HFARP) was previously identified as a novel adipocytokine that was up-regulated by fasting, by peroxisome proliferator-activated receptor agonists, and by hypoxia. To further characterize FIAF, we studied regulation of FIAF mRNA and protein in liver and adipose cell lines as well as in human and mouse plasma. Expression of FIAF mRNA was up-regulated by peroxisome proliferator-activated receptor α (PPARα) and PPARβ/δ agonists in rat and human hepatoma cell lines and by PPARγ and PPARβ/δ agonists in mouse and human adipocytes. Transactivation, chromatin immunoprecipitation, and gel shift experiments identified a functional PPAR response element within intron 3 of the FIAF gene. At the protein level, in human and mouse blood plasma, FIAF was found to be present both as the native protein and in a truncated form. Differentiation of mouse 3T3-L1 adipocytes was associated with the production of truncated FIAF, whereas in human white adipose tissue and SGBS adipocytes, only native FIAF could be detected. Interestingly, truncated FIAF was produced by human liver. Treatment with fenofibrate, a potent PPARα agonist, markedly increased plasma levels of truncated FIAF, but not native FIAF, in humans. Levels of both truncated and native FIAF showed marked interindividual variation but were not associated with body mass index and were not influenced by prolonged semistarvation. Together, these data suggest that FIAF, similar to other adipocytokines such as adiponectin, may partially exert its function via a truncated form.

Obesity, defined as excess body fat, is associated with numerous secondary ailments, including hypertension, dyslipidemia, and insulin resistance, and is therefore an important health concern. As the prevalence of obesity is rising, there is an increasing interest in understanding the metabolic behavior of adipose tissue.

Since the discovery of leptin in 1994 (1), it has become clear that fat tissue not merely serves to store excess energy but also has an important endocrine function (2). Over the past few years, several factors secreted by white adipose tissue (WAT),[1] aptly named adipocytokines, have been identified and characterized, including resistin (also known as FIZZ 3 or ADSF (for adipocyte secreted factor)) (3–5), adiponectin (also known as adipoQ or ACRP30 (for adipocyte complement-related protein 30)) (6–8), acylation-stimulating protein (C3ades-Arg) (9), plasminogen activator inhibitor-1 (10), renin angiotensin system (11), metallothioneins (12), and the inflammatory cytokines interleukin-6, tumor necrosis factor-α, and tumor growth factor-β (13, 14). They have been implicated in a variety of different processes, ranging from blood pressure control to lipid metabolism and insulin sensitizing. Consequently, it has been tempting to attribute many of the clinical abnormalities associated with obesity, including insulin resistance, to altered secretion of particular adipocytokines.

Recently, we and others identified a new gene encoding the secreted fasting-induced adipose factor (FIAF), also known as PGAR (for PPARγ angiopoietin-related protein), ANGPTL4 (for angiopoietin-like protein 4), or HFARP (for hepatic fibrinogen/angiopoietin-related protein) (15–17). Several nonexclusive functions for FIAF have so far been proposed. Expression of FIAF is dramatically up-regulated during hypoxia in both endothelial cells and cardiomyocytes (18, 19), leading to the suggestion that FIAF may be involved in angiogenesis, in analogy with two other proteins that carry an angiopoietin/fibrinogen-like domain, angiopoietin-1 and angiopoietin-2. Subsequently, it was found that FIAF is able to induce a strong proangiogenic response in the chicken chorioallantoic membrane assay (19). FIAF has also been proposed to act as an apoptosis survival factor in vascular endothelial cells (16).

The closest relative of FIAF is angiopoietin-like protein 3 (ANGPTL3). KK/Snk mice, a mutant strain of KK obese mice,

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§§ To whom correspondence should be addressed: Nutrition, Metabolism and Genomics Group, Division of Human Nutrition, Wageningen University, P. O. Box 8129, 6700 EV Wageningen, The Netherlands. Tel.: 31-317-48-57-87; Fax: 31-317-48-33-42; E-mail: sander.kersten@wur.nl.

[1] The abbreviations used are: WAT, white adipose tissue; PPAR, peroxisome proliferator-activated receptor; SEAP, secreted alkaline phosphatase; FIAF, fasting-induced adipose factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ChIP, chromatin immunoprecipitation; PPRE, PPAR response element; RT, reverse transcriptase; IBMX, isobutylmethylxanthine.

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carry a mutation in the gene for ANGPTL3, resulting in low plasma triglyceride and free fatty acid levels (20). The low plasma triglyceride levels are possibly due to elevated lipoprotein lipase activity, which was reported to be inhibited by ANGPTL3 *in vitro*, whereas the low plasma free fatty acid levels may be connected to the impaired stimulatory effect of ANGPTL3 on adipose tissue lipolysis (21, 22). Similar to ANGPTL3, there is evidence that FIAF also may inhibit lipoprotein lipase activity and hereby influence plasma levels of triglycerides (18, 23), thus connecting FIAF to lipid metabolism.

Expression of FIAF in liver and WAT was originally found to be up-regulated by the nuclear hormone receptors PPARα and PPARγ (15, 17, 23). PPARs are ligand-activated transcription factors that mediate the effects of fibrates (PPARα) or thiazolidinediones (PPARγ) on DNA transcription (24, 25). PPARα is mainly expressed in brown adipose tissue and liver and plays an important role in the hepatic fatty acid oxidation, whereas PPARγ is the master regulator of adipogenesis. However, it is still unclear whether FIAF is a direct PPAR target gene, with a functional PPAR response element in its promoter.

In order to close in on the potential function of FIAF, we studied the regulation of FIAF mRNA and protein expression *in vitro* and in human blood plasma. Our main conclusions are that FIAF is a classical PPAR target gene in both humans and rodents and that FIAF protein is mainly present in blood plasma in a truncated form, whose levels show a large interindividual variability. Plasma levels of the truncated form of FIAF are increased by treatment with fenofibrate.

MATERIALS AND METHODS

Chemicals—Wy14643 was obtained from ChemSyn Laboratories. Rosiglitazone was from Alexis. Recombinant human insulin (Actrapid) was from Novo Nordisk. SYBR Green was from Eurogentec. Dulbecco's modified Eagle's medium, fetal calf serum, calf serum, and penicillin/streptomycin/fungizone were from BioWhittaker Europe (Cambrex Bioscience). Otherwise, chemicals were from Sigma.

Primary Human Adipocyte Differentiation—Isolation of stromal vascular cells was done as follows. Subcutaneous and visceral adipose tissues were obtained during gastric restriction surgery. Adipose tissue was collected in phosphate-buffered saline and cut into 3 × 3-mm pieces with scissors. The 3 × 3-mm pieces were further processed with a scalpel. Next, the pieces of adipose tissue were digested in DMEM-high glucose containing 4% bovine serum albumin and 2 mg/ml collagenase. 1–2.5 g of adipose tissue was digested in 5 ml of this solution at 37 °C on a shaking platform for 2 h. Next, the digest was transferred to a 5-ml syringe and gently pressed over a 500-μm sterile pore size disposable nylon mesh. Stromal vascular cells were separated from adipose cells by centrifugation (1 min, 170 g). Adipose cells were removed, and the stromal vascular cells were precipitated by centrifugation (5 min, 350 g). Red blood cells were lysed by resuspending the cell pellet in 10 ml of red cell lysis solution (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). After 5 min, stromal vascular cells were spun down (5 min, 350 g) and resuspended in DMEM/F-12 containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin and further referred to as complete medium. Approximately 10^7 cells were plated in a 75-cm^2 flask. After 48 h, the medium was replaced by differentiation medium consisting of complete medium (instead of 10% FCS, 2% FCS was used) plus 15 mM $NaHCO_3$, 15 mM HEPES, 33 μM biotin, 17 μM panthothenate, 200 pM T3, 1 μM dexamethasone, 500 nM insulin, 4 μg/ml transferrin, and 10 μM $cPGI_2$. After an average period of 30 days of differentiation, extraction of total RNA was performed.

SGBS Cell Line Culture and Induction of Adipogenesis—The culture of the SGBS cells and their induction into mature human adipocytes were performed exactly as previously published (26).

3T3-L1 Adipogenesis Assay—3T3-L1 fibroblasts were amplified in DMEM plus 10% calf serum and plated for final differentiation in DMEM plus 10% FCS. Two days after reaching confluence (which was day 0), the medium was changed, and the following compounds were added: isobutyl methylxanthine (0.5 mM), dexamethasone (1 μM), and insulin (5 μg/ml). On day 3, the medium was changed to DMEM plus 10% FCS and insulin (5 μg/ml). On day 6, the medium was changed to DMEM plus 10% FCS, which was changed every 3 days.

Isolation of Total RNA and RT-PCR—Total RNA was extracted from cells or tissue with Trizol reagent (Invitrogen) following the supplier's protocol. 3–5 μg of total RNA was treated with DNase I amplification grade (Invitrogen) and then reverse-transcribed with oligo(dT) using Superscript II RT RNase H (Invitrogen).

Real Time Quantitative PCR—Primers were designed to generate a PCR amplification product of 100–200 bp. Only primer pairs yielding unique amplification products without primer dimer formation were subsequently used for real time quantitative PCR assays. The following primer pairs were used: mFIAF (forward), 5′-GTTTGCAGACTCAGCTCAAGC-3′; mFIAF (reverse), 5′-CCAAGAGGTCTATCTGGCTCTG-3′; hFIAF (forward), 5′-CGTACCCTTCTCCACTTGGG-3′; hFIAF (reverse), 5′-GCTCTTGGCGCAGTTCTTG-3′; mβ-Actin (forward), 5′-CTGACTGACTACCTCATGAAGATCCT-3′; mβ-Actin (reverse), 5′-CTTAATGTCACGCACGATTTCC-3′; hβ-Actin (forward), 5′-CTTCCTGGGCATGGAGTC-3′; hβ-Actin (reverse), 5′-GCCAGGGTACATGGTGGT-3′; rPPARα (forward), 5′-CACCCTCTCTCCAGCTTCCA-3′; rPPARα (reverse), 5′-GCCTTGTCCCCACATATTCG-3′; rPPARβ (forward), 5′-AACGAGATCAGCGTGCATGTG-3′; rPPARβ (reverse), 5′-TGAGGAAGAGGCTGCTGAAGTT-3′; rPPARγ (forward), 5′-ATGGAGCCTAAGTTTGAGTTTGCT-3′; rPPARγ (reverse), 5′-GGATGTCCTCGATGGGCTTCA-3′; hPPARα (forward), 5′-CTGGAAGCTTTGGCTTTACG-3′; hPPARα (reverse), 5′-TGTCCCCGCAGATTCTACAT-3′; hPPARβ (forward), 5′-ACAGCATGCACTTCCTTCCA-3′; hPPARβ (reverse), 5′-TCACATGCATGAACACCGTA-3′; hPPARγ (forward), 5′-GAGCCCAAGTTTGAGTTTGC-3′; hPPARγ (reverse), 5′-CAGGGCTTGTAGCAGGTTGT-3′.

PCR was carried out using Platinum *Taq* polymerase (Invitrogen) and SYBR Green on an iCycler PCR machine (Bio-Rad). Expression was related to actin, which did not change under any of the experimental conditions studied.

Transactivation Assay—A 350-nucleotide fragment surrounding the putative PPRE within intron 3 of the human or mouse FIAF gene was PCR-amplified from human or mouse genomic DNA (mouse strain C57/B6) and subcloned into the KpnI and BglII sites of pTAL-SEAP (Clontech). This reporter vector was transfected into human hepatoma HepG2 cells together with an expression vector for PPAR (mPPARα, mPPARβ/δ, or mPPARγ in pSG5) in the presence or absence of Wy14643 (50 μM), L165041 (5 μM), or rosiglitazone (5 μM), respectively. Transfections were carried out by calcium phosphate precipitation. A β-galactosidase reporter was co-transfected to normalize for differences in transfection efficiency. Secreted alkaline phosphatase activity was measured in the medium 48 h post-transfection via the chemiluminescent SEAP reporter assay (Roche Applied Science). β-Galactosidase activity was measured in the cell lysate by standard assay using 2-nitrophenyl-β-D-galactopyranoside as a substrate.

Chromatin Immunoprecipitation (ChIP)—Pure-bred wild-type or PPARα null mice on a sv129 background were used. Mice were fed by gavage with either Wy14643 (50 mg/kg/day) or vehicle (0.5% carboxymethylcellulose) for 5 days. Alternatively, mice were fasted or not for 24 h. After the indicated treatment, mice were sacrificed by cervical dislocation. The liver was rapidly perfused with prewarmed (37 °C) phosphate-buffered saline for 5 min followed by 0.2% collagenase for 10 min. The liver was diced and forced through a stainless steel sieve, and the hepatocytes were collected directly into DMEM containing 1% formaldehyde. After incubation at 37 °C for 15 min, the hepatocytes were pelleted, and ChIP was done using mouse PPARα-specific antibodies as previously described (27). Sequences of primers used for PCR were 5′-TCTGGGTCTGCCCCCACTCCTGG-3′ (forward) and 5′-GTGTGTGTGTGGGATACGGCTAT-3′ (reverse). Control primers used were 5′-AGTAACTTTGACAGGAACCAGGGGTC-3′ (forward) and 5′-TTTGGACTGGGAACTTAGCTTAGTTG-3′ (reverse).

3T3-L1 cells were differentiated as described above. After cell lysis and sonication, the supernatant was diluted 20-fold in re-chIP dilution buffer (1 mM EDTA, 20 mM Tris-HCl, pH 8.1, 50 mM NaCl, 1% Triton X) prior to incubation with mouse PPARγ antibody. The remainder of the assay was carried out as described previously (27).

Gel Shift—hRXRα and hPPARα proteins were generated from pSG5 expression vectors using the TnT coupled *in vitro* transcription/translation system (Promega). The following oligonucleotides were annealed: hFIAF-PPRE, 5′-TCGGGGAAAGTAGGGGAAAGGTCGTGG-3′ and 5′-CTGGGCCAGCGACCTTTCCCCTACTTTCCC-3′; mFIAF-PPRE, 5′-TCGGGGAAAGTAGGAGAAAGGTCACTGG-3′ and 5′-CTGGCCAGTGACCTTTCTCCTACTTTCCC-3′; for specific competition malic enzyme PPRE, 5′-TCGCTTTCTGGGTCAAAGTTGATCCA-3′ and 5′-CTGGTGGATCAACTTTGACCCAGAAAG-3′; for nonspecific competition ETS, 5′-TGGAATGTACCGGAAATAACACCA-3′ and 5′-TGGTGTTATTTCCGGTACATTCCA-3′. Oligonucleotides were annealed and labeled by Klenow filling (Roche Applied Science) using Redivue [α-^{32}P]dCTP

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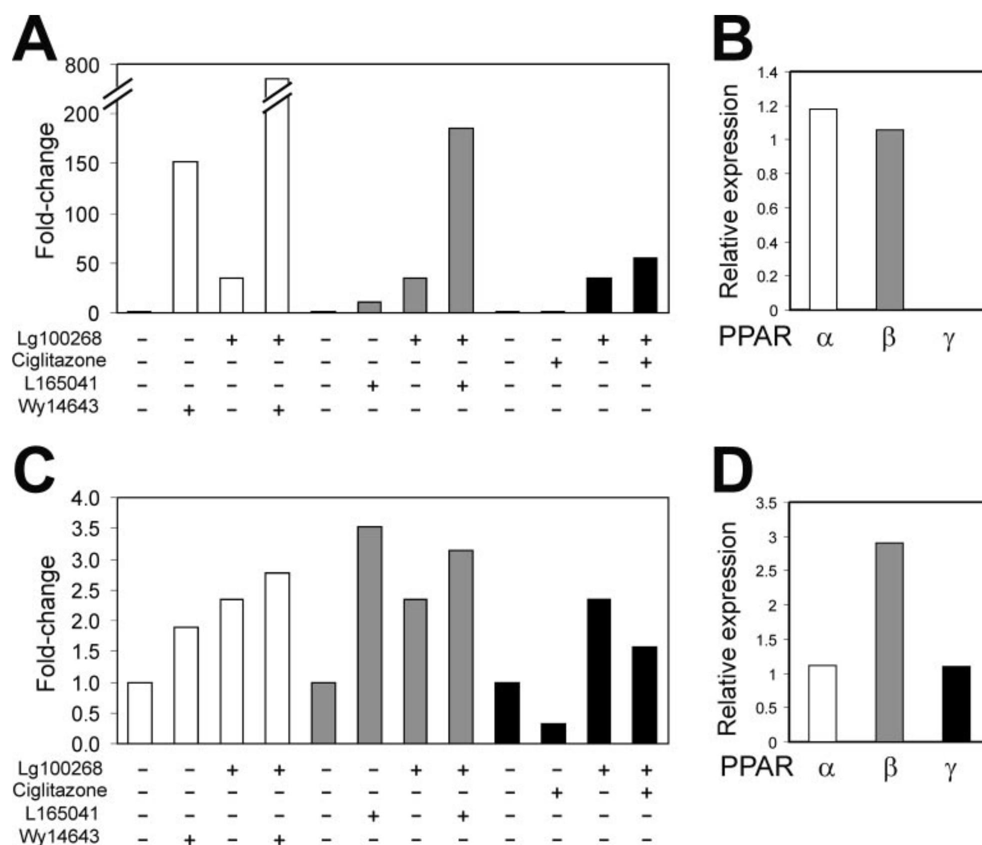

FIG. 1. **PPARα and PPARβ agonists induce FIAF gene expression in both rat hepatoma FAO and human hepatoma HepG2 cell lines.** *A*, FAO cells were treated for 24 h with different agonists selective for each PPAR isotype, Wy14643 (5 μM, PPARα), L165041 (7.5 μM, PPARβ), and ciglitazone (25 μM, PPARγ). The synthetic RXR agonist Lg100268 (5 μM) was used in combination with the different PPAR agonists. Expression was determined by quantitative RT-PCR analysis. *B*, relative expression of PPARs in FAO cells as determined by quantitative RT-PCR. *C*, HepG2 cells were treated for 48 h with PPAR or RXR agonists at the same concentrations as for FAO cells except for Wy14643 (50 μM). Data shown are representative results from three independent experiments in three different batches of HepG2 cells. *D*, relative expression of PPARs in HepG2 cells.

(3000 Ci/mmol) (Amersham Biosciences). *In vitro* translated proteins (0.5–0.8 μl/reaction) were preincubated for 15 min on ice in 1× binding buffer (80 mM KCl, 1 mM dithiothreitol, 10 mM Tris-Cl, pH 7.4, 10% glycerol plus protease inhibitors) in presence of 2 μg of poly(dI·dC), 5 μg of sonicated salmon sperm DNA, and competitor oligonucleotides in a final volume of 20 μl. Then 1 ng (1 ng/μl) of radiolabeled oligonucleotide was added, and incubation proceeded for another 10 min at room temperature. Complexes were separated on a 4% polyacrylamide gel (acrylamide/bisacrylamide, 37.5:1) equilibrated in 0.5× TBE at 25 mA.

Western Blot—The mouse polyclonal antibody used was directed against the epitope CQGPKGKDAPFKDSE located in the N-terminal part of the mouse FIAF protein. The human polyclonal antibody used was directed against the epitope CQGTEGSTDLPLAPE also located in the N-terminal part of the human FIAF protein. The peptide affinity-purified antibodies were generated in rabbit and ordered via Eurogentec's customized antibody production service. Western blotting was carried out using an ECL system (Amersham Biosciences) according to the manufacturer's instructions. The primary antibody was used at a dilution of 1:1000 (mouse) or between 1:2000 and 1:5000 (human), and the secondary antibody (anti-rabbit IgG, Sigma) was used at a dilution of 1:8000. All incubations were performed in 1× Tris-buffered saline, pH 7.5, with 0.1% Tween 20 and 5% dry milk, except for the final washings, when milk was omitted.

Human Subjects—In experiment 1, blood was taken from 16 young adults after an overnight fast. In experiment 2, blood was taken after an overnight fast from 28 subjects before and after a 4-week treatment with 250 mg of micronized fenofibrate daily. In experiment 3, blood was taken after an overnight fast from 20 men (body mass index ranging 22.7 to 39.8). Samples were from a published study (28). In experiment 4, blood was taken after an overnight fast from 22 subjects before and after a 46-day semistarvation program (2.1 MJ/day). Samples were from a published study (29). All human experiments were approved by the medical ethics committee of Wageningen University, Maastricht University, or the University of Ulm.

RESULTS

FIAF Expression Is Regulated by All Three PPARs—Previous studies have indicated that expression of FIAF/PGAR/ANGPTL4 is up-regulated by PPARα and PPARγ in mice. Several genes are known that are targets of PPARα in mice but not in humans (30, 31). To investigate whether expression of FIAF is under control of PPARα in other species, rat hepatoma FAO cells were treated with the PPARα agonist Wy14643 (Fig. 1*A*). According to real time quantitative PCR, FAO cells express relatively high levels of PPARα as well as PPARβ/δ, whereas PPARγ mRNA was below our detection limit (Fig. 1*B*). Basal expression of FIAF in FAO cells was extremely low but was dramatically increased by Wy14643, either alone or in combination with the RXR agonist Lg100268 (Fig. 1*A*). The synthetic PPARβ/δ agonist L165041 also strongly increased FIAF mRNA, suggesting that PPARβ/δ stimulates FIAF gene expression too. Finally, the PPARγ agonist ciglitazone had little effect on rat FIAF gene expression, which may be explained by the low expression of PPARγ mRNA in these cells.

To examine whether the human FIAF gene is also up-regulated by PPARs, human hepatoma HepG2 cells were treated with PPAR agonists (Fig. 1*C*). HepG2 cells express all three PPARs, with PPARβ/δ being the most abundant (Fig. 1*D*). Similar to what was observed in FAO cells, although with much more modest -fold inductions, FIAF mRNA was increased by Wy14643 and Lg100268, either alone or used in combination (Fig. 1*C*). The PPARβ/δ agonist L165041 also induced FIAF mRNA, but no additional effect of Lg100268 was observed. In contrast to PPARα and PPARβ/δ agonists, the PPARγ agonist

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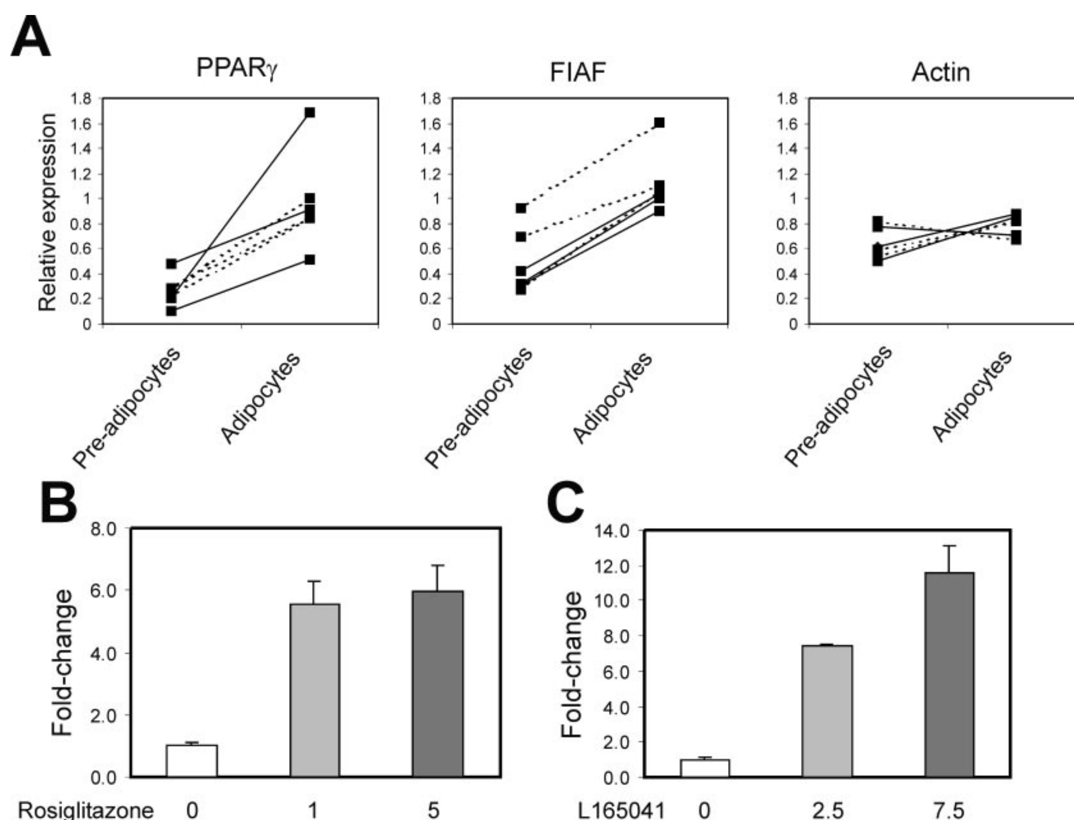

FIG. 2. **FIAF is a probable PPARγ target in human adipocytes.** Stromal vascular cells were isolated from subcutaneous (*solid line*) and visceral (*dotted line*) adipose tissue from three subjects and put into culture. Cells were differentiated into adipocytes by incubation with adipogenic medium. Expression is expressed relative to glyceraldehyde-3-phosphate dehydrogenase. Human SGBS differentiated adipocytes (day 13) were treated for 40 h with different concentrations of rosiglitazone (*B*) or L165041 (*C*). Concentrations are indicated in μM. FIAF gene expression was determined by quantitative RT-PCR.

ciglitazone reduced FIAF expression, which was maintained in the presence of Lg100268. Taken together, these results indicate that FIAF is up-regulated by PPARα and PPARβ/δ, but probably not by PPARγ, in human and rat hepatoma cells.

To better examine regulation of human FIAF expression by PPARγ, we turned to primary human preadipocytes. Upon stimulation with a mixture of hormones, these cells can be differentiated into adipocytes. Stromal vascular cells from both subcutaneous and visceral adipose tissue were isolated and induced to differentiate into adipocytes. Expression of FIAF was higher in adipocytes *versus* preadipocytes in all three subjects with cells from both subcutaneous and visceral origin (Fig. 2*A*). A similar induction of expression was observed for PPARγ, suggesting that FIAF is up-regulated by PPARγ during human adipocyte differentiation. In differentiated human SGBS adipocytes, both rosiglitazone and L165041 caused an induction of FIAF mRNA (Fig. 2, *B* and *C*, respectively), indicating that both PPARγ and PPARβ/δ regulate FIAF expression in human adipocytes. Together, these data suggest that FIAF is a PPARγ and possibly a PPARβ/δ target gene in human adipocytes.

FIAF Is a Direct Target Gene of PPAR—To unequivocally determine FIAF as a direct target gene of PPARs, direct binding of PPAR to the FIAF promoter needs to be demonstrated. Comparative analysis of the hFIAF and mFIAF gene sequence upstream of the transcription start site did not reveal any conserved stretches of DNA that might harbor a PPRE. Transactivation studies with several kilobases of the immediate upstream sequence from both the mouse and human FIAF gene did not yield any significant activation of a reporter gene, suggesting that the responsive element may be located elsewhere. While scanning the FIAF gene sequence for PPREs, a putative PPRE was identified in a conserved region of intron 3 of the human and mouse FIAF gene (AGG(G/A)GAAAGGTC(G/A)) that differed little from the consensus PPRE (Fig. 3*A*). To determine whether this PPRE binds PPAR *in vitro*, gel shift experiments were carried out with *in vitro* translated/transcribed PPARα and RXRα. For both the human and mouse PPRE, a retarded complex was only observed in the presence of both PPARα and RXRα (Fig. 3*B*), indicating that this complex represents a PPARα/RXRα heterodimer. The complex disappeared in the presence of an excess of cold specific oligonucleotide but not nonspecific oligonucleotide. Similar results were observed for PPARγ (data not shown). These data indicate that PPAR is able to bind to the human and mouse PPRE within intron 3 *in vitro*.

To assess whether the PPRE within intron 3 is able to mediate PPAR-dependent transactivation, a 350-nucleotide fragment surrounding the human or mouse PPRE was cloned in front of the thymidine kinase promoter followed by a SEAP reporter. In HepG2 cells, co-transfection of the reporter vector with a PPARα, PPARβ/δ, or PPARγ expression vector increased SEAP activity, which was further enhanced by the addition of ligand (Fig. 3*C*). In this assay, PPARα seemed to be the most potent activator, followed by PPARβ/δ and PPARγ. These data suggest that the PPRE identified in intron 3 of the FIAF gene is able to mediate PPAR-dependent transactivation.

Finally, to find out whether PPARα and PPARγ are bound to this sequence *in vivo*, ChIP was performed using antibodies against PPARα or PPARγ. In human HepG2 cells, binding of PPARα to the sequence spanning the putative PPRE within intron 3 was enhanced by Wy14643 (Fig. 4*A*). No immunoprecipitation was observed with preimmune serum, and no amplification was observed for a control sequence. In mice, treatment

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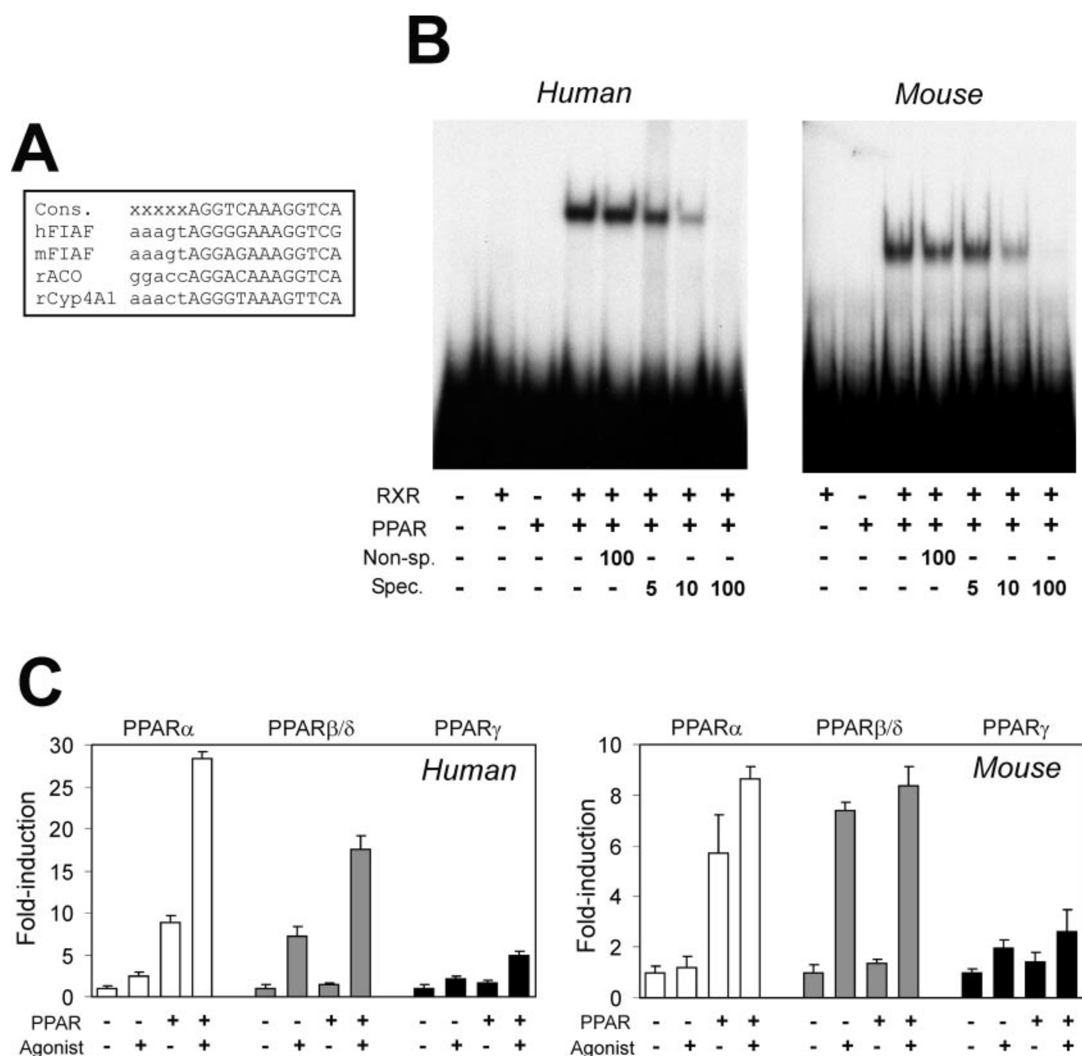

FIG. 3. **FIAF up-regulation by PPARα is mediated by a PPRE present in intron 3.** *A*, alignment of the PPRE present in intron 3 of the human and mouse FIAF gene with known PPREs. *Cons*., consensus. *B*, binding of the PPAR/RXR heterodimer to putative PPRE as determined by gel shift. A double-stranded response element containing the human (*left*) or mouse (*right*) FIAF PPRE was incubated with *in vitro* transcribed/translated hPPARα and hRXRα, and binding complexes were resolved on a 6% nondenaturing polyacrylamide gel. -Fold excess of specific (malic enzyme PPRE) or nonspecific (ETS oligonucleotide) cold probe is indicated. *C*, HepG2 cells were transfected with a SEAP reporter vector containing a 350-nucleotide fragment of intron 3 of the human (*left*) or mouse (*right*) FIAF gene and a PPAR expression vector. SEAP activity was determined in the medium 24–48 h post-transfection.

with Wy14643 enhanced binding of PPARα to the PPRE sequence in liver, which was not observed in PPARα null mice (Fig. 4*B*). Similarly, fasting enhanced binding of PPARα to the PPRE sequence, which was not observed in the PPARα null mice (Fig. 4*C*). With respect to PPARγ, previous data had shown that FIAF is up-regulated during mouse 3T3-L1 adipogenesis (17), indicating that it may be a direct PPARγ target gene. Using ChIP, we observed binding of PPARγ to the PPRE sequence in differentiated 3T3-L1 adipocytes but not in preadipocytes (Fig. 4*D*). These data clearly demonstrate that PPARα and PPARγ bind to the intronic sequence harboring the PPRE *in vivo*. Thus, FIAF can be formally classified as a direct PPAR target gene in human and mouse.

FIAF Protein Is Processed during Mouse Adipocyte Differentiation—The increased level of FIAF mRNA in primary differentiated adipocytes *versus* preadipocytes, regardless of the fat depot, indicates that FIAF is up-regulated during human adipocyte differentiation. Indeed, it was observed that FIAF mRNA increases during human SGBS adipocyte differentiation, displaying a dramatic up-regulation during early differentiation that diminished during prolonged differentiation (Fig. 5*A*). According to Western blot using an antibody that recognizes human FIAF (Fig. 5*B*), the mRNA expression profile of FIAF was mirrored at the protein level, with some delay (Fig. 5*C*). In the Western blot, a single band at the expected molecular mass (~45 kDa) was observed.

In accordance with previous studies by Yoon *et al.* (17), an increase in FIAF mRNA during prolonged mouse 3T3-L1 adipogenesis was observed (Fig. 5*D*). However, we also observed that FIAF expression transiently peaks at day 3 of differentiation, reaching a level exceeding that of fully differentiated adipocytes. This effect could be attributed to IBMX, since incubation of confluent 3T3-L1 cells with only IBMX, which does not induce adipocyte differentiation, markedly increased FIAF mRNA (Fig. 5*D*, *inset*). IBMX is removed from the medium from day 3 onwards, explaining the precipitous drop in FIAF mRNA at day 4.

Whereas FIAF protein directly followed FIAF mRNA expression during human adipocyte differentiation (Fig. 5, *A* and *C*), a remarkable protein expression pattern was observed for mouse adipocyte differentiation (Fig. 5, *D* and *E*). In parallel with FIAF mRNA, with a delay of 1 day, native FIAF protein rose during early differentiation and peaked at day 4, 1 day after the maximal FIAF mRNA level. Thereafter, its level de-

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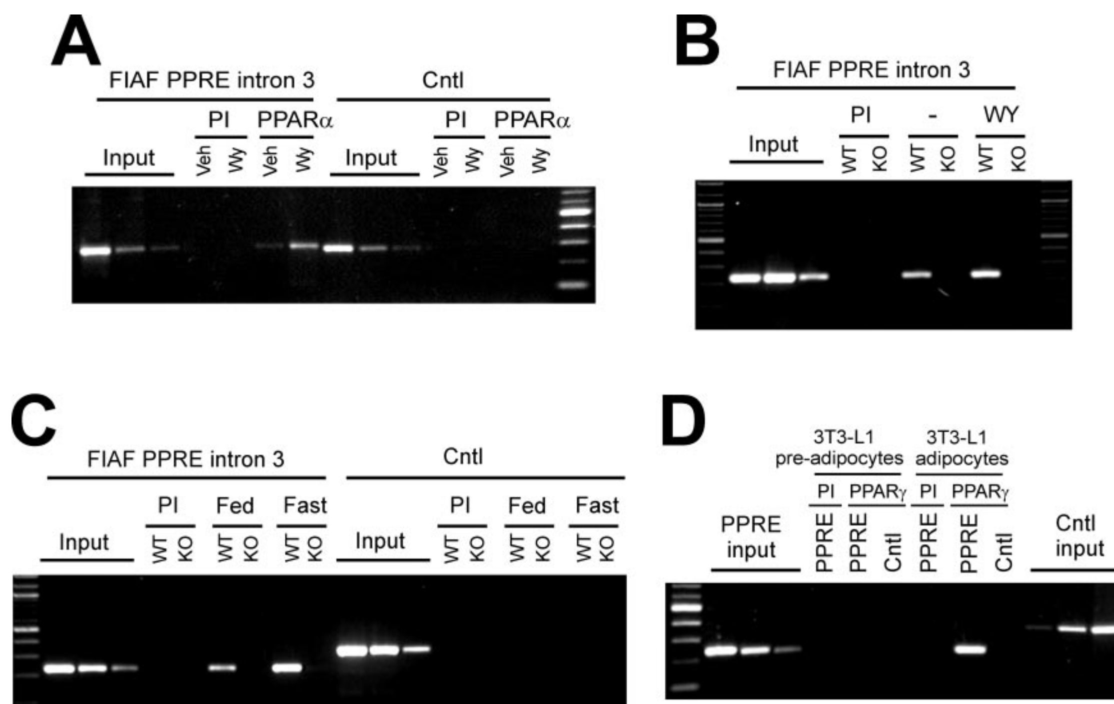

FIG. 4. **PPARα and PPARγ bind to the PPRE within intron 3 of the FIAF gene *in vivo*.** Chromatin immunoprecipitation of PPRE within intron 3 of the FIAF gene using antibodies against PPARα or PPARγ. The gene sequence spanning the putative PPRE and a random control sequence were analyzed by PCR in the immunoprecipitated chromatin of HepG2 cells treated with Wy14643 (*A*), livers of wild-type and PPARα null mice treated or not with WY14643 (*B*), livers of fed or fasted wild-type and PPARα null mice (*C*), or 3T3-L1 preadipocytes and adipocytes (*D*). Preimmune serum was used as a control. *PI*, preimmune serum; *Cntl*, random control sequence; *Veh*, vehicle; *WT*, wild type; *KO*, knock-out.

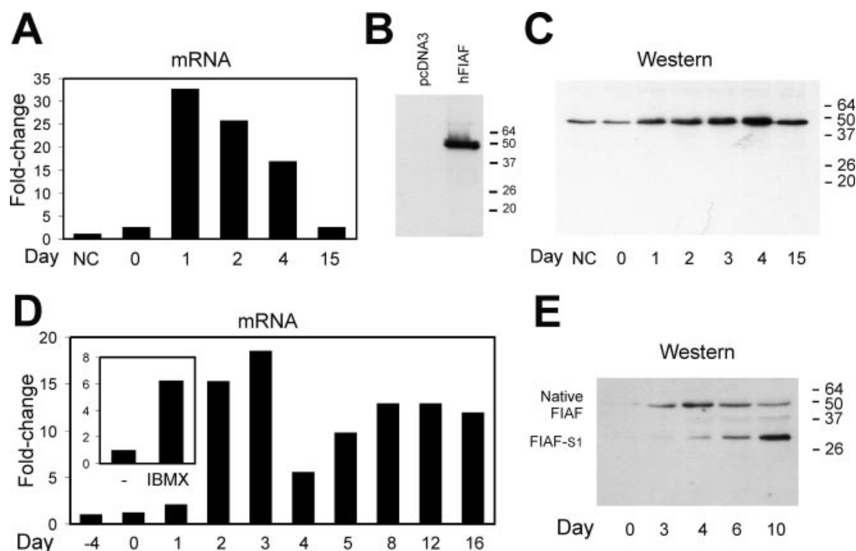

FIG. 5. **FIAF protein is processed during 3T3-L1 but not SGBS adipogenesis.** *A*, hFIAF mRNA expression during human SGBS adipogenesis as assessed by quantitative RT-PCR. *B*, Western blot of medium of HEK293 cells transfected with empty pcDNA3.1 or pcDNA3.1 expressing human FIAF using an anti-hFIAF antibody. *C*, hFIAF protein expression during SGBS adipogenesis as assessed by Western blot using anti-hFIAF antibody. 10 μg of protein (supernatant 16,000 × *g*) was loaded per lane. *NC*, nonconfluent. *D*, mFIAF mRNA expression during mouse 3T3-L1 adipogenesis as assessed by quantitative RT-PCR. *Inset*, effect of IBMX on mFIAF expression in 3T3-L1 preadipocytes. Confluent 3T3-L1 cells were treated for 3 days with IBMX (0.5 mM). *E*, mFIAF protein expression during 3T3-L1 adipogenesis as assessed by Western blot using anti-mFIAF antibody. 40 μg of protein (supernatant 16,000 × *g*) was loaded per lane.

creased (Fig. 5*E*). Interestingly, in the same immunoblot, an additional band with a molecular mass of about 32 kDa appeared at day 4 and further increased at days 6 and 10. Thus, the upper band, representing native FIAF, follows FIAF mRNA during early differentiation, whereas the lower band follows FIAF mRNA during prolonged adipocyte differentiation, suggesting it is derived from FIAF. We hypothesized that this band represents a truncated form of FIAF, which is observed in mouse but not human adipocytes.

If this is correct, it would be expected that the abundance of truncated FIAF would mirror the FIAF mRNA expression data in 3T3-L1 adipocytes treated with synthetic PPAR and RXR agonists. Indeed, induction of FIAF mRNA by the RXR agonist Lg100268 and by rosiglitazone in differentiated 3T3-L1 adipocytes was associated with an increased abundance of the lower molecular weight band (Fig. 6, *A* and *B*). Similarly, in livers of mice treated with Wy14643, which results in up-regulation of FIAF mRNA (Fig. 6*C*), the abundance of the lower molecular weight band was increased in parallel, providing compelling evidence that this band represents a truncated form of FIAF. Hereon, this form of FIAF is referred to as FIAF-S1 (for FIAF small form 1).

Because FIAF was initially found to be a protein secreted into the blood plasma, we set out to determine whether the same was true for FIAF-S1. Interestingly, besides native FIAF and FIAF-S1, another immunoreactive form of slightly higher molecular weight (about 2–3 kDa) was also detected, which we named FIAF-S2 and which was by far the most abundant (Fig. 6*D*). Preincubation of the mouse FIAF antibody with its peptide epitope completely abolished all three forms. Notice that in Fig. 6*E* native FIAF is barely visible because the blot was exposed for less time. It is not inconceivable that FIAF-S2 might rep-

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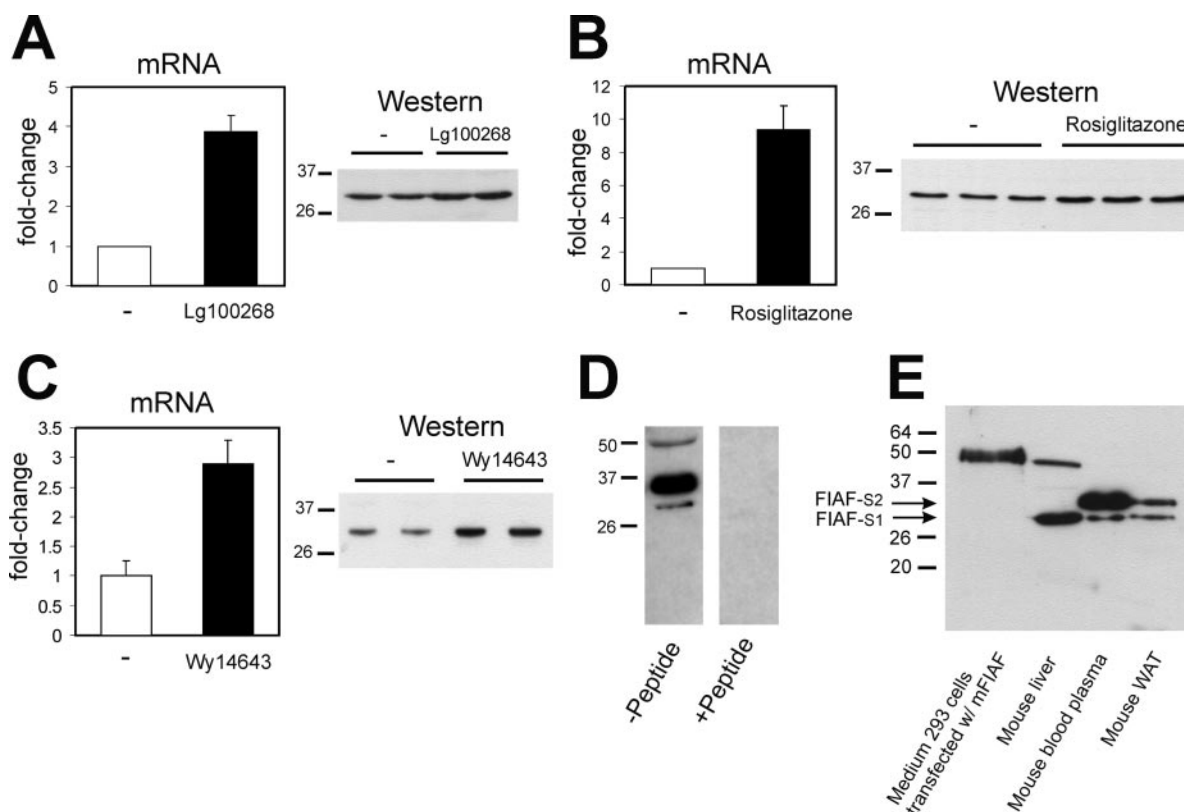

FIG. 6. **The truncated form of FIAF is up-regulated in concert with FIAF mRNA by synthetic RXR/PPAR agonists.** mFIAF protein (30 μg of total protein loaded per lane) and mRNA expression in differentiated 3T3-L1 adipocytes (day 10) after treatment for 48 h with 5 μM Lg100268 (*A*) or rosiglitazone (*B*) as assessed by Western blot and quantitative RT-PCR are shown. *C*, mFIAF protein (12 μg of total protein loaded per lane) and mRNA in liver of mice fed WY14643 (0.1%) for 5 days. *D*, Western blot of mouse plasma using anti-mFIAF antibody in the absence or presence of the peptide epitope. *E*, presence of truncated mFIAF variants in mouse white adipose tissue, blood plasma, and mouse liver. The position of native mFIAF is shown by transfection of pcDNA3 expressing mFIAF into HEK293 cells. Medium was collected, precipitated using trichloroacetic acid, and prepared for SDS-PAGE.

resent a phosphorylated or glycosylated form of FIAF-S1. Both FIAF-S1 and FIAF-S2 were well detected in mouse WAT, whereas only native FIAF and FIAF-S1 were detected in mouse liver (Fig. 6*E*). Together, these data suggest that FIAF is present in truncated forms in mouse blood plasma.

FIAF-S1 and FIAF-S2 Are Present in Human Blood Plasma—To establish that FIAF is also present in truncated forms in human plasma, Western blot was carried out on human blood plasma using an anti-hFIAF antibody. Almost copying the picture of mouse blood plasma, in human plasma both native human FIAF protein at 50 kDa but also two bands of lower molecular weight were observed, probably corresponding to FIAF-S1 and FIAF-S2 (Fig. 7*A*). Incubation with the peptide epitope caused the complete disappearance of all bands. The molecular weight of the putative FIAF-S1 and FIAF-S2 in human was lower than that of the same species in mice. Omission of dithiothreitol in the SDS-sample buffer led to the appearance of a very high molecular weight immunoreactive complex, suggesting that FIAF forms oligomers or possibly a high molecular weight complex involving other plasma proteins (Fig. 7*B*). Omission of dithiothreitol also slightly increased the mobility of native FIAF and FIAF-S2. Levels of putative FIAF-S2 after an overnight fast were very reproducible within subjects (not shown) but extremely variable between subjects (Fig. 7*C*). Levels of native FIAF also differed markedly between subjects but to a somewhat lesser extent. Together, these data indicate that FIAF is circulating in blood in several forms of different sizes at different concentrations.

Levels of FIAF-S2 in Human Blood Plasma Are Increased by Fenofibrate—Our data indicate that human FIAF mRNA is up-regulated by PPARα agonists in human hepatoma cells. If the lower molecular weight band in the immunoblot blot of human plasma indeed represents truncated FIAF protein, its level would be expected to increase after treatment with PPARα agonists. To find out whether this is true, plasma levels of putative FIAF-S2 were assessed by Western blot in 28 subjects before and after treatment with fenofibrate, a potent PPARα agonist (Fig. 7, *D* and *E*). In 24 of the 28 subjects, levels of FIAF-S2 rose after fenofibrate treatment, whereas four individuals showed a decrease or no change. The mean increase was 84.5% $\pm$ 20.1 (S.E.) (paired Student's t test, $p < 0.0001$). Levels of native FIAF did not respond or only slightly responded to fenofibrate treatment. These data suggest that FIAF is mainly present in human blood plasma in a truncated form (FIAF-S2), whose level is increased by fenofibrate treatment.

Fenofibrate, which primarily acts on liver, influences plasma levels of FIAF-S2 but not native FIAF. At the same time, human SGBS adipocytes only produce native FIAF. This raises the possibility that human liver mainly produces FIAF-S2, whereas human WAT mainly synthesizes native FIAF. In agreement with this notion, we only detect FIAF-S2 in human liver, and native FIAF in human WAT (Fig. 8*A*).

Despite WAT possibly being the main contributor to native FIAF in plasma, plasma levels of native FIAF did not respond significantly in a group of 22 subjects undergoing a 46-day semistarvation program (2.1 MJ/day), although they lost an average of 12 kg (Fig. 8*B*, representative results of four subjects). The same was true for FIAF-S2. Also, no association was observed between body mass index and plasma levels of native FIAF or FIAF-S2 in a group of individuals with varying body mass index (22.7–39.8) (Fig. 8, *C* and *D*).

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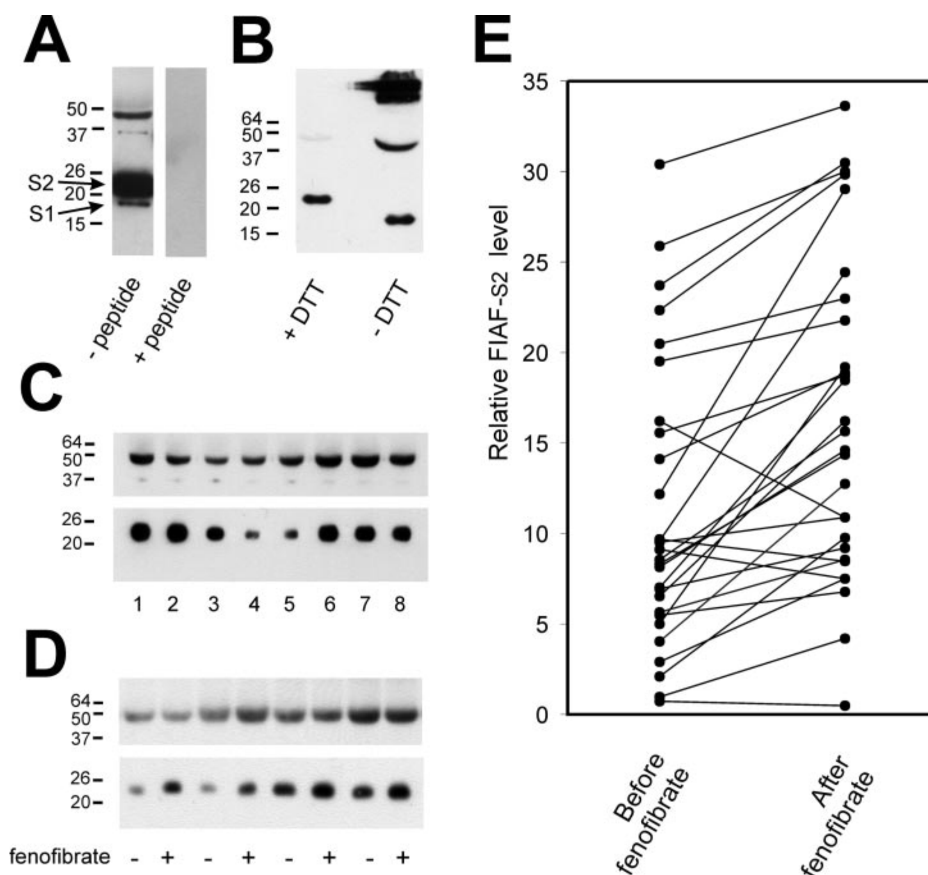

FIG. 7. **Plasma levels of hFIAF-S2 are increased by fenofibrate treatment.** *A*, Western blot of human blood plasma with anti-hFIAF antibody in the absence and presence of peptide epitope. *B*, Western blot of human blood plasma with anti-hFIAF antibody, in the presence and absence of dithiothreitol (*DTT*). The time of exposure was less in *B versus A*, which explains why FIAF-S1 is not visible. *C*, interindividual variation in plasma levels of native hFIAF and hFIAF-S2, as determined by Western blot. The part of the blot showing native hFIAF was exposed for a longer time. *D*, effects of fenofibrate treatment on plasma levels of native hFIAF and hFIAF-S2, as determined by Western blot. Four representative subjects are shown. The part of the blot showing native hFIAF was exposed for a longer time. *E*, quantitation of the effect of fenofibrate on plasma FIAF-S2 levels in 28 subjects.

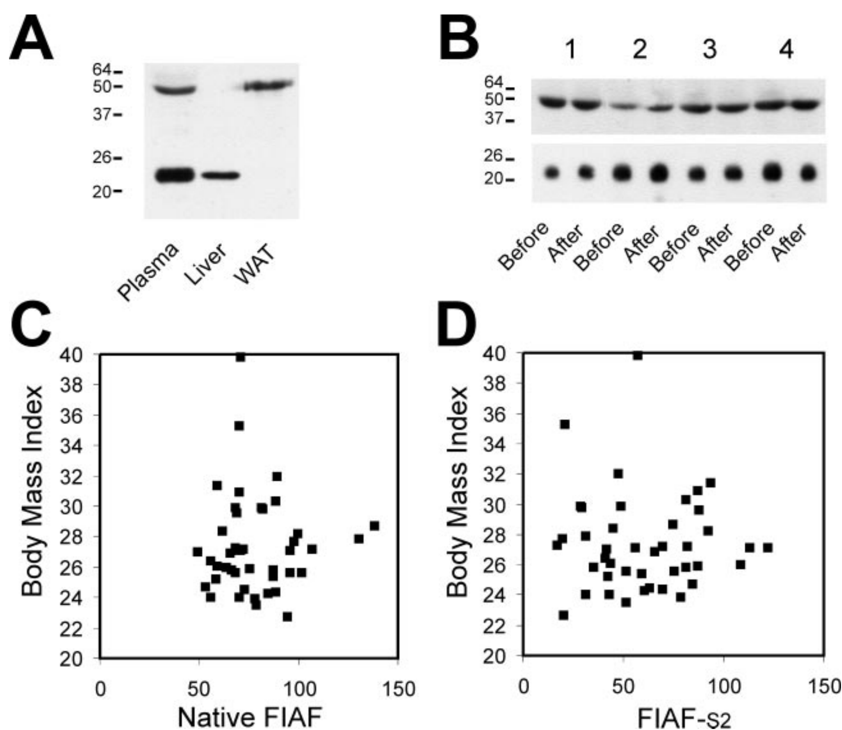

FIG. 8. **Native FIAF and FIAF-S2 are produced by adipose tissue and liver, respectively, yet plasma levels are not influenced by semistarvation or associated with body mass index.** *A*, presence of native hFIAF and hFIAF-S2 in human liver, plasma, and white adipose tissue samples according to Western blot using anti-hFIAF antibody. *B*, plasma levels of native hFIAF and hFIAF-S2 before and after 46 days of semistarvation, as determined by Western blot. Four representative subjects are shown. The part of the blot showing native hFIAF was exposed for a longer time. Association between plasma levels of native hFIAF (*C*) or hFIAF-S2 (*D*) and body mass index.

DISCUSSION

In the past decade, it has become clear that adipose tissue not merely serves to store energy but also has an important endocrine function, secreting an array of proteins that include leptin, resistin, adiponectin/ACRP30/adipoQ, interleukin-6, and tumor necrosis factor-α. These so called adipokines or adipocytokines are involved in numerous processes and have been particularly studied as potential mediators of the link between obesity and obesity-related metabolic abnormalities, with special emphasis on insulin resistance (2).

An adipocytokine that received a lot of publicity lately is adiponectin. A special property of adiponectin is that it is cleaved to generate a smaller product called globular adiponectin, which is probably the physiologically active form (32).

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According to our data, FIAF may also become proteolytically processed to generate a protein of 20–35 kDa, the exact size of which depends on the species and probably on glycosylation. Alternatively, FIAF-S could be generated through alternative splicing of FIAF mRNA, by differential initiation start sites, or by some unknown mechanisms. However, neither RT-PCR experiments using different primers, Northern blots, nor RNase protection provided any evidence of the generation of an additional mRNA. This suggests that FIAF-S1 and FIAF-S2 are generated by proteolytic processing. Considering that native FIAF is glycosylated, FIAF-S1 and FIAF-S2 may represent different glycosylated forms (15, 16). Our data also indicate that human liver mainly synthesizes FIAF-S2, whereas human WAT seems to produce native FIAF exclusively. This suggests that FIAF-S2 and native FIAF in plasma may originate from different tissues. This is supported by the observation that fenofibrate, which mainly acts on liver, increases plasma levels of FIAF-S2 but not native FIAF. In mice, the contribution of various tissues to plasma FIAF is less transparent.

Proteolytic processing of prohormone precursor proteins is a common theme in endocrinology. Numerous protein and peptide hormones, including insulin, glucagon, and adipocytokines such as tumor necrosis factor-α and adiponectin, are proteolytically cleaved to generate the smaller functional form of the protein. The most common processing recognition site in prohormones consists of a doublet of basic amino acids (33), which is recognized by subtilisin-like proprotein convertases, although other types of motifs are also possible. Carboxypeptidase E is responsible for the removal of carboxyl-terminal basic residues exposed by the endoproteases (34). Interestingly, in the primary structure of FIAF, two conserved adjacent arginines could be identified, which might represent proteolytic recognition sites. Digestion around this site (Arg^{229} and Arg^{230} in hFIAF) would be compatible with the size of fragments FIAF-S1 and FIAF-S2. Recently, it was found that expression of the proprotein convertases PACE4, PC7, and furin increases during 3T3-L1 adipocyte differentiation, when processing of native FIAF to FIAF-S1 becomes apparent (35). Consequently, it is conceivable that these enzymes participate in the processing of FIAF in 3T3-L1 adipocytes.

Besides being proteolytically processed, adiponectin also forms higher order oligomers, which may have a different functional activity than monomeric adiponectin. Resistin has been shown to self-associate as well (36, 37), which again may influence functional activity. According to our data, FIAF may also be present in human blood plasma as higher order oligomers, although the exact composition of the observed higher molecular weight complex(es) remains to be determined. Similar to adiponectin and resistin, oligomerization of FIAF may influence functional activity.

Very recently, Ono *et al.* reported that ANGPTL3 is cleaved *in vivo*, and, similar to our observations for FIAF (or ANGPTL4), is present in mouse blood plasma in several forms of around 30 kDa (38). Interestingly, it was found that the resulting N-terminal fragment is probably responsible for the plasma triglyceride-raising effect of ANGPTL3. Furthermore, while our manuscript was in preparation, data were published showing that recombinant FIAF protein is truncated and forms oligomers in HEK293 cells and *in vivo* (39). No data were provided on endogenous FIAF, in contrast to the present paper. Although details about the site of truncation seem to be different between the two papers, together they suggest that proteolytic processing, and perhaps oligomerization, may be important for FIAF function. Thus, proteolytic processing may be common among members of this protein family and may serve to regulate functional activity.

Previous studies have indicated that, at least in mouse, FIAF mRNA is most highly expressed in white adipose tissue (15). According to our data, human WAT mainly produces native FIAF. The lack of a significant association between body mass index and plasma levels of native FIAF and the absence of an effect of prolonged weight loss on native FIAF suggest that either the size of WAT has little impact on the total amount of native FIAF released from WAT into blood plasma or that adipose tissue may not be the primary source for native FIAF in human plasma.

Experiments in mice have shown that both hepatic and adipose expression of FIAF are elevated by fasting (15). With respect to FIAF in plasma, levels of native FIAF were found to be elevated after fasting (15), whereas levels of FIAF-S2 or FIAF-S1 did not seem to be affected.[2] Preliminary data indicate that the fasting-induced up-regulation of FIAF mRNA in adipose tissue may not be observed in mice of the FVB strain. With regard to humans, it is unclear whether fasting causes up-regulation of FIAF mRNA in liver and WAT. Levels of FIAF in plasma do not appear to be influenced by short term fasting (data not shown) or long term semistarvation. Thus, the term fasting-induced adipose factor may not aptly describe the behavior of FIAF in several species.

Previously, we and others have demonstrated that in mice FIAF is up-regulated by both PPARα and PPARγ (15, 17). Here it is shown that this regulation also occurs in humans, in contrast to many other PPARα target genes. Furthermore, besides PPARα and PPARγ, PPARβ is similarly able to induce FIAF expression in hepatocytes and adipocytes. It is also shown that up-regulation of FIAF expression by PPARs is, at least partly, mediated by a PPRE present in intron 3. Via chromatin immunoprecipitation on livers of fasted and fed or Wy14643-treated mice, direct *in vivo* binding of PPARα to intron 3 was demonstrated, which was enhanced by fasting and by Wy14643. Furthermore, binding of PPARα to the same sequence was enhanced by Wy14643 in human HepG2 cells. Finally, binding of PPARγ to the sequence could be demonstrated in differentiated 3T3-L1 adipocytes but not preadipocytes. Thus, FIAF can be added to the list of direct PPAR target genes. Although the presence of a functional PPRE within an intron is remarkable, it is not completely uncommon. Indeed, recently the presence of a functional PPRE within intron 3 of the rat peroxisomal thiolase B gene was demonstrated (40).

Plasma levels of FIAF-S2 are increased by fenofibrate treatment. Inasmuch as there is evidence that FIAF is involved in lipid metabolism, it can be speculated that the effects of synthetic PPARα agonists on plasma lipid levels may be partially mediated via changes in FIAF expression. Further studies are necessary to ascertain the potential of FIAF as a target for treatment of various forms of dyslipidemia.

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The Direct Peroxisome Proliferator-activated Receptor Target Fasting-induced Adipose Factor (FIAF/PGAR/ANGPTL4) Is Present in Blood Plasma as a Truncated Protein That Is Increased by Fenofibrate Treatment

Stéphane Mandard, Fokko Zandbergen, Nguan Soon Tan, Pascal Escher, David Patsouris, Wolfgang Koenig, Robert Kleemann, Arjen Bakker, Frank Veenman, Walter Wahli, Michael Müller and Sander Kersten

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