Structural and functional characterization of the mouse fatty acid translocase promoter: activation during adipose differentiation

Lydia TEBOUL*[1,2], Maria FEBBRAIO†, Danielle GAILLARD*, Ez-Zoubir AMRI*, Roy SILVERSTEIN† and Paul A. GRIMALDI*

*INSERM U470, Centre de Biochimie, Parc Valrose, UFR Sciences, Université de Nice-Sophia Antipolis, 06108 Nice, France, and †Cornell University Medical College, 1300 York Avenue, New York, NY 10021, U.S.A.

Fatty acid translocase (FAT/CD36) is a cell-surface glycoprotein that functions as a receptor/transporter for long-chain fatty acids (LCFAs), and interacts with other protein and lipid ligands. FAT/CD36 is expressed by various cell types, including platelets, monocytes/macrophages and endothelial cells, and tissues with an active LCFA metabolism, such as adipose, small intestine and heart. FAT/CD36 expression is induced during adipose cell differentiation and is transcriptionally up-regulated by LCFAs and thiazolidinediones in pre-adipocytes via a peroxisome-proliferator-activated receptor (PPAR)-mediated process. We isolated and analysed the murine FAT/CD36 promoter employing $C_2C_{12}N$ cells directed to differentiate to either adipose or muscle. Transient transfection studies revealed that the 309 bp upstream from the start of exon 1 confer adipose specific activity. Sequence analysis of this DNA fragment revealed the presence of two imperfect direct repeat-1 elements. Electrophoretic mobility-shift assay demonstrated that these elements were peroxisome-proliferator-responsive elements (PPREs). Mutagenesis and transfection experiments indicated that both PPREs co-operate to drive strong promoter activity in adipose cells. We conclude that murine FAT/CD36 expression in adipose tissue is dependent upon transcriptional activation via PPARs through binding to two PPREs located at -245 to -233 bp and -120 to -108 bp from the transcription start site.

Key words: adipocyte, myocyte, PPAR, PPRE.

INTRODUCTION

Fatty acid translocase (FAT), also named CD36 [1], is an 88 kDa cell-surface glycoprotein expressed in a variety of cell types, including adipocytes, enterocytes, cardiac myocytes, mammary epithelial cells, monocytes/macrophages, capillary endothelial cells and platelets [2]. FAT/CD36 is a member of the class B family of scavenger receptors [3,4], which recognizes native and modified lipoproteins, and which has also been characterized as a receptor for thrombospondin, collagens and apoptotic cells [5]. We have shown by ectopic expression of FAT/CD36 in fibroblasts that this protein functions as a high-affinity membrane receptor/transporter for long-chain fatty acids (LCFAs) [6]. FAT/CD36 expression is subject to developmental regulation as well as nutritional regulation. For example, FAT/CD36 expression increases during differentiation of monocytes or megakaryocytes [7] and is induced during pre-adipocyte differentiation [8]. Furthermore, FAT/CD36 expression is up-regulated in the small intestine and heart by high-fat diets [9,10]. Up-regulation of FAT/CD36 expression has also been observed in heart, skeletal muscle and adipose tissue of diabetic animals [10–12]. Finally, FAT/CD36 gene transcription is induced strongly by LCFAs and thiazolidinediones in pre-adipocytes [13], C_2C_{12} myoblasts and satellite cells from newborn mice [14], and by oxidatively modified low-density lipoprotein in monocytes [15].

Strong evidence supports the hypothesis that lipid-activated nuclear receptors, also called peroxisome-proliferator-activated receptors (PPARs), play an important role in FAT/CD36 gene expression during adipose differentiation, in response to LCFAs in various cell types, and in monocytes exposed to oxidatively modified low-density lipoprotein. PPARs are members of the nuclear hormone receptor superfamily and, after ligand activation, regulate the expression of genes containing specific response elements, called peroxisome-proliferator-responsive elements (PPREs) which are direct repeats-1 (DRs-1) [16]. Functional PPREs have been characterized in the promoters of genes involved in LCFA metabolism, such as acyl-CoA oxidase [17], liver fatty acid-binding protein (FABP) [18], acyl-CoA synthetase [19], lipoprotein lipase [20], adipocyte lipid-binding protein (ALBP) [21] and fatty acid transport protein [22]. We have demonstrated previously that ectopic expression of the PPARδ isoform (FAAR) in fibroblasts confers fatty acid responsiveness to FAT/CD36 gene expression [13]. Given the role of FAT/CD36 in LCFA transport, and its regulated expression in response to fatty acids and high-fat diets in tissues that also express PPARδ, we hypothesized that this transcription factor may play a role in FAT/CD36 gene expression. Based on the same type of analysis, we hypothesized that the adipose-specific PPARγ isoform, which plays a direct role in adipogenesis [23], was also involved in FAT/CD36 gene activation during adipose differentiation. In view of the potentially important physiological roles of FAT/CD36 protein in adipose tissue and other lipid-metabolizing tissues and in lipid accumulation by monocytes/macrophages (which may be the pathological initiation of atherosclerosis [24]), we initiated a study of the molecular mechanisms underlying the activation of FAT/CD36 gene expression during adipose-cell differentiation, as well as its up-regulation in response to LCFAs.

Characterization of the human CD36 gene promoter has shown that specific monocyte expression derives from the 158 bp upstream of exon 1 [25]. In studies utilizing a muscle/adipose bipotential cell system [14], we found that the human CD36 gene promoter as defined by Vega and co-workers [25] was not activated during adipose differentiation and was not responsive

Abbreviations used: ALBP, adipocyte lipid-binding protein; DR-1, direct repeat-1; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate; FABP, fatty acid-binding protein; FAT, fatty acid translocase; LCFA, long-chain fatty acid; PPAR, peroxisome-proliferator-activated receptor; PPRE, peroxisome-proliferator-responsive element; RSV, Rous sarcoma virus; RXR, retinoid X receptor.

[1] Present address: Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, U.K.

[2] To whom correspondence should be addressed, at the Institute of Cancer Research (e-mail lteboul@icr.ac.uk).

to PPARs in this tissue. We have also isolated and characterized the 5′-flanking region of the murine FAT/CD36 gene. Our data demonstrate that transcription of the mouse FAT/CD36 gene in adipose cells is controlled primarily by two imperfect DR-1 elements, which are binding sites for PPAR/retinoid X receptor (RXR) heterodimers, and these act co-operatively to drive high activity of the promoter.

EXPERIMENTAL

Materials

Culture media were obtained from Life Technologies. Bovine serum and other chemical products were purchased from Sigma Aldrich. Radioactive materials, the random-priming kit and Hybond membranes were from Amersham Pharmacia Biotech. Luciferin was from Promega. BRL 49653 was a kind gift from SmithKline Beecham Pharmaceuticals (Welwyn Garden City, Herts., U.K.).

Cell culture

$C_2C_{12}N$ is a subclone established in our laboratory from the C_2C_{12} cell line [14]. $C_2C_{12}N$ cells were plated at a density of $2 \times 10^3/cm^2$ and were grown in Dulbecco's modified Eagle's medium supplemented with 8 % bovine serum, 200 units/ml penicillin and 50 μg/ml streptomycin (hereon referred to as standard medium). Media were changed every other day and confluence was reached within 5 days. BRL 49653 was dissolved at a concentration of 50 mM in DMSO and added immediately to standard medium to obtain the indicated final concentration. This medium was prewarmed at 37 °C for 15 min and then added to the cells. Control experiments were performed to exclude any effects of DMSO. After confluence, cells were maintained in standard medium supplemented with 5 μM BRL 49653 to induce adipose differentiation, or maintained in medium containing 3 % bovine serum to trigger myotube formation [14].

Cloning of the human CD36 gene promoter

The human CD36 promoter, published by Vega and co-workers [25], encompasses nucleotides −273 to +19. It was synthesized from human genomic DNA (generously provided by G. Pages, Centre Antoine Lacassagne, Nice, France) by PCR using the following primers: 5′-CTGGCCTCTGACTTACTTGGAATG-GGA-3′ and 5′-GTCCTACACTGCAGTCCTCATTACATA-3′ [26]. The human CD36-luciferase vector was engineered by cloning the promoter in the correct orientation into pGL2-Basic (Promega). The construct was verified by sequencing.

Cloning of mouse FAT/CD36 gene regulatory sequences

Murine FAT/CD36 genomic sequences were cloned from a mouse strain 129/Sv genomic phage library (Stratagene), using a 551 bp probe derived from the murine cDNA (generously provided by G. Endemann, SciosNova, Mountain View, CA, U.S.A.) beginning at cDNA bp 2 and ending at bp 553 (an *Eco*RI–*Ava* II restriction fragment). A phage containing a 13 kb insert was identified and, after restriction-enzyme analysis, Southern blotting and dideoxy sequencing, proved to be genomic FAT/CD36, based on the similarity between exon sequences and the published cDNA sequence. This phage contained approx. 2 kb of sequence 5′ of exon 1.

Site-directed mutagenesis

Mutations were performed directly in the various luciferase constructs by the method described by Viville [27], using the oligonucleotides 5′-GTTCTCCAGCTTCGAACTTACTTGG-AT-3′ and 5′-TAACTAGCACGAAATTGGAACTTTTTTCT-3′ for PPRE-G and PPRE-J, respectively (mutated bases are underlined; PPRE-G and PPRE-J are defined as the sequences at positions −245 to −233 bp and −120 to −108 bp from the transcription start site, respectively). Three mutant constructs were engineered: pLuc G-mPPRE-G (mutation in the 5′ PPRE), pLuc G-mPPRE-J (mutation in the 3′ PPRE) and pLuc J-mPPRE-J (mutation in the 3′ PPRE). The constructs were verified by sequencing.

Transient transfection and expression assays

Transfections were performed by DOTAP {*N*-[1-(2,3-dioleoyl-oxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate} lipofection (Roche Molecular Biochemicals). Undifferentiated (75–80 % confluent) adipose- or myotube-differentiated $C_2C_{12}N$ cells were suspended by trypsinization in standard medium at a density of 2×10^5 cells/ml. The cell suspensions were mixed with DOTAP solution containing super-coiled DNA plasmids. Typically, 1 μg of DNA (100 ng of pSV-β-galactosidase and 900 ng of luciferase construct) was used for 5 μl of DOTAP and for 1 ml of final transfection volume. The cell/DNA mixture was seeded into the wells of 24-well plates (0.5 ml/well) and incubated for 6 h at 37 °C. After washing with fresh medium, cells were cultured for 36 h in the indicated media. Luciferase and β-galactosidase activities were determined in the same cell extract using an LKB 1250 luminometer and commercial kits (Luciferase Assay kit; Promega) and Galacton kit (Tropix, Bedford, MA, U.S.A.). Luciferase activities were determined in triplicate and normalized to β-galactosidase activities.

Stable transfections

Ob1771 cells [13] were grown to 50 % confluence and co-transfected by the DOTAP method with pSV-neo (a neomycin-resistant expression vector) and FAT/CD36 plasmids at a ratio of 1:20. After 2 days, cells were maintained in standard medium supplemented with G418 (Sigma Aldrich). After 3–4 weeks, clones were isolated, grown independently and tested for luciferase activity.

Northern blotting

RNA samples were prepared and analysed as described previously [13]. Blots were subjected to digital imaging (FujixBAS 1000).

Electrophoretic mobility-shift assays

Transcription factors were synthesized *in vitro* using rabbit reticulocyte lysates (Promega), and the quality of the *in vitro*-translated proteins was verified by SDS/PAGE. Nuclear extracts were prepared as described previously [13]. To study the FAT/CD36 PPREs, synthetic double-stranded oligonucleotides spanning nucleotides −250 to −224 (PPRE-G, 5′-GTTCTCTGGC-CTCTGACTTACTTGGAT-3′) and −125 to −101 (PPRE-J, 5′-TAGCAGTGAAGTGTGACTTTTTTCT-3′) of the mouse FAT/CD36 gene 5′ upstream regulatory sequence were used. For competition experiments, double-stranded oligonucleotides with a mutation in the DR-1 sequence were used (mutated PPRE-G, 5′-GTTCTCCAGCTTCGAACTTACTTGGAT-3′; mutated PPRE-J, 5′-TAACTAGCAGCAAATTGGAACTTT-TTTCT-3′). Gel mobility-shift assay buffer (20 μl) contained 10 mM Tris/HCl (pH 8.0), 100 mM KCl, 10 % glycerol, 1 mM dithiothreitol, 1 μg of poly[d(I-C)] and 2 μl of *in vitro*-synthesized

PPAR and/or RXR proteins, unprogrammed lysate or 10 μg of nuclear-extract proteins. After a 10 min incubation on ice, 0.5 ng of ^{32}P-labelled oligonucleotide was added and the incubation continued for 15 min. DNA–protein complexes were resolved on a 5% polyacrylamide gel in 45 mM Tris/HCl (pH 8.0)/45 mM boric acid/1 mM EDTA. Gels were dried and subjected to digital imaging (FujixBAS 1000).

RESULTS

Human CD36 gene promoter is not active in adipose cells

Human CD36 5′-flanking sequence was cloned by PCR using human genomic DNA and specific primers [26]. This DNA fragment was subcloned in the correct orientation into the pGL2-Basic vector, and the activity of this reporter construct was tested by transient transfection in undifferentiated $C_2C_{12}N$ cells or in $C_2C_{12}N$ cells induced to differentiate into either adipocytes or myotubes (Figure 1A). In these experiments, a Rous sarcoma virus (RSV) luciferase construct and a promoterless pGL2-Basic vector were used as positive and negative controls, respectively. The level of FAT/CD36 mRNA expression in these cells was checked by Northern-blot analysis (Figure 1B). In accordance with our previous data, FAT/CD36 mRNA was undetectable in growing undifferentiated $C_2C_{12}N$ cells, and expressed at very lowlevels in myotube-differentiated cells maintained for 5 days post-confluence in low-serum-containing media. In contrast, there was a strong accumulation of FAT/CD36 mRNA in cells induced to adipose differentiation, obtained after 5 days of culture in standard media supplemented with 5 μM BRL 49653. As shown in Figure 1(C), the level of expression of the human CD36 gene promoter construct remained comparable with that of the promoterless control pGL2-Basic vector. This suggested that the human CD36 promoter as described previously [26] was not active in $C_2C_{12}N$ cells, whatever their state of differentiation. These observations prompted us to isolate and characterize the mouse FAT/CD36 gene promoter.

Cloning of the mouse FAT/CD36 gene promoter

Murine FAT/CD36 genomic sequences were cloned from a phage library using a cDNA probe encompassing bp +2 to +553. A phage containing a 13 kb insert was identified, and restriction-enzyme analysis, Southern blotting and sequencing confirmed it to be genomic FAT/CD36. A series of overlapping subclones of this phage was prepared in the vector pBluescript II (Stratagene), and those that had reactivity with cDNA probes were sequenced. Exons 1–3 were present in the original 13 kb clone, which was described previously [28].

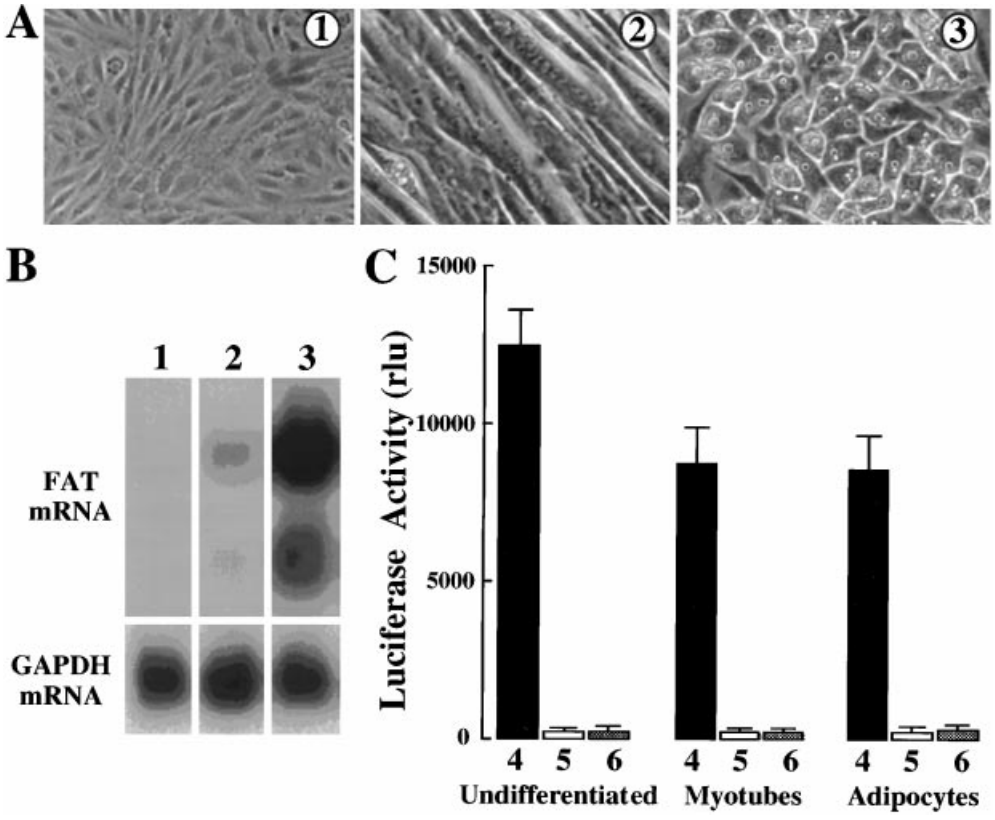

Figure 1 Lack of activity of the human CD36 gene promoter in $C_2C_{12}N$ cells

(**A**) Morphology of undifferentiated (1), myotube-differentiated (2) and adipose-differentiated (3) $C_2C_{12}N$ cells. Magnification $\times$100. (**B**) Northern-blot analysis of FAT/CD36 mRNA expression in $C_2C_{12}N$ cells. Total RNA (20 μg/lane) from undifferentiated (lane 1), myotube-differentiated (lane 2) and adipose-differentiated (lane 3) cells was analysed by Northern blotting as described in the Experimental section. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (**C**) RSV luciferase (lanes 4), promoterless luciferase constructs (lanes 5) or human CD36 promoter-luciferase (lanes 6) were transiently transfected together with the pSV-β-galactosidase reporter vector into undifferentiated, myotube- or adipocyte-differentiated 5 day post-confluent $C_2C_{12}N$ cells. After 6 h, the transfection medium was replaced and cells were maintained for 48 h in medium containing 8% bovine serum for undifferentiated cells, 3% bovine serum for myotubes or 8% bovine serum supplemented with 5 μM BRL 49653 for adipocytes. Luciferase and β-galactosidase activities were determined 48 h later and presented as described in the Experimental section. Results are the means $\pm$ S.D. from three independent experiments. rlu, relative luciferase units.

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mFAT    CTGGCCTCTGACTTACTTGGATGGGAACATGGCCAAAAAAAAAAAAAAAAA   -197
hFAT    CTGGCCTCTGACTTACTTGGATGGGAA-ATAGCCAAAAAAAAAAAAAAA--   -227

mFAT    AAAAAGACTGCTGAGTATTTCAAATGTAGGTGATGGGTCTTCACCAGAA-    -148
hFAT    --------TGCTGAATATCTCAGATATAGGTAATGGGTCTTCACCAGAAC    -185

mFAT    ------ATAGACCCTTGTGAGCTTAACTAGCAGTGAAGTGTGACTTTTTT   -104
hFAT    ATAAAAATAGACCCTTATTAGCCA---TATCAGTAATGTGCTGGGTGGGG   -138

mFAT    CTTTCTCTTCTTTTTTTCAATTCCCTTGGCAACCTACCACACAATTAGCAT   -54
hFAT    GATTTTTTT-TTTCTTTCAATTCCTCTGGCAACAAACCACACACTGGGAT    -89

mFAT    CTGACATTGAA----------------TTTTTTAAAGCTCG--------    -29
hFAT    CTGACACTGTAGAGTGCTTTCTCTTCTCTTTTTTTGGGGGGGGAGGGGG    -49

mFAT    ------------TTTCAACTCTCACACACATAAGTAGTAG              -1
hFAT    TGTGGTTGCATATTTAAACTCTCACGCATTTATGTAAT                -1
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Figure 2 Comparison of human and mouse FAT/CD36 proximal promoter regions

We compared FAT (−1 to −246) of the mouse (m) sequence with FAT (−1 to −273) of the human (h) sequence. Identical bases are shown in bold. All numbering is relative to the transcriptional start site. PPRE-G and PPRE-J are underlined.

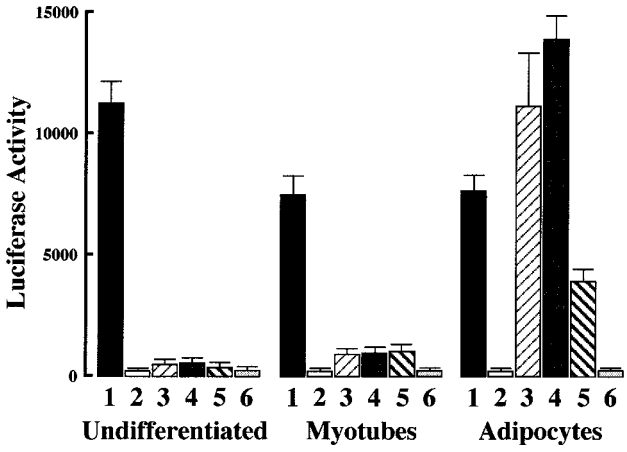

Figure 3 Identification by progressive deletion analysis of the minimal FAT/CD36 promoter able to confer adipose-specific expression

RSV luciferase or FAT/CD36 promoter constructs containing various 5′-flanking fragments of the mouse FAT/CD36 gene were transiently transfected into undifferentiated, myotube or adipose $C_2C_{12}N$ cells. Cell-culture conditions and biochemical assays were performed as described for Figure 1(C). Results are the means ± S.D. from five separate experiments. 1, RSV luciferase; 2, pGL2-Basic; 3, pLuc E (−555 to +6); 4, pLuc G (−309 to +6); 5, pLuc J (−170 to +6); 6, pLuc K (−81 to +6).

The entire region 5′ to exon 1 of mouse FAT/CD36 was subcloned into pBluescript and sequenced multiple times in both orientations. The sequence immediately upstream of exon 1 did not contain a TATA or CCAAT box, was surprisingly rich in T, and contained one CpG dinucleotide. Within the first 100 bp 5′ of exon 1, we found an Oct-4 site and a core-binding-factor motif (CBF) [25]. In other reports, CBF and Oct-2 have been linked to transcriptional regulation of the FAT/CD36 gene [29]. Approx. 270–280 bp upstream of exon 1 the sequence became GC rich and contained two sites for Sp1, and at approx. 375 bp upstream of exon 1 there was a CCAAT motif. Two potential full binding sites for PPAR were located at −245 and −120 (Figure 2).

Identification of the minimal mouse FAT/CD36 promoter driving adipose-specific expression

To locate the transcriptional regulatory sequences of the mouse FAT/CD36 gene involved in adipose-specific expression, a nested set of fragments covering the FAT/CD36 promoter from approx. −2 kb to +6 was prepared and ligated into the pGL2-Basic vector. These constructs were transiently transfected into proliferating undifferentiated $C_2C_{12}N$ cells or $C_2C_{12}N$ cells induced to differentiate into either myotubes or adipocytes. Preliminary experiments demonstrated that there was no difference in expression in constructs containing sequence upstream of −555 (results not shown). We thus concentrated on the region −555 to +6. As shown in Figure 3, the FAT/CD36 promoter constructs were not expressed in undifferentiated $C_2C_{12}N$ cells, and were only weakly active in myotubes when compared with the positive control, the RSV luciferase vector. In contrast, in adipocytes, the pLuc E and pLuc G FAT/CD36 constructs containing respectively 555 and 309 nt of the promoter region exhibited luciferase activity greater than that of the RSV luciferase vector. Both constructs exhibited 60–80-fold more activity in adipose cells than in undifferentiated growing cells. Deletion of the region between −309 and −170 (pLuc J construct) resulted in a 3-fold decrease of promoter activity in adipocytes, whereas the pLuc K construct starting at −81 was without any activity.

We have reported previously [14,30] that thiazolidinediones exert their adipogenic effects only in non-terminally differentiated myoblasts and that FAT/CD36 gene expression could not be induced by these compounds in fully differentiated $C_2C_{12}N$ myotubes. Furthermore, continuous exposure to inducers was not required to maintain the adipose phenotype in $C_2C_{12}N$ cells [14,30]. As shown in Figure 4, we observed a similar regulation for the pLuc G and pLuc J promoter constructs: (i) exposure to BRL 49653 on days 5–7 after myotube differentiation did not affect luciferase expression of either construct, and (ii) the luciferase expression mediated by the pLuc G and pLuc J promoters was not reduced when adipose cells were maintained for 2 days (days 5–7) in standard media. Taken together, these observations indicated that the −309 to +6 promoter region is sufficient to direct adipose-specific expression of the FAT/CD36

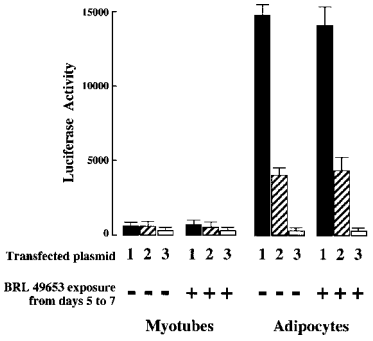

Figure 4 Activation of FAT/CD36 promoter constructs during adipose differentiation

$C_2C_{12}N$ cells were differentiated into myotubes or adipocytes as described in the Experimental section and then transfected with the indicated constructs and pSV-β-galactosidase at days 5 post-confluence. Myotubes or adipocytes were maintained for the next 2 days in the presence or absence of 5 μM BRL49653. Luciferase activities were determined as for Figure 1(C) and are the means $\pm$ S.D. from three separate experiments. 1, pLuc G (-309 to $+6$); 2, pLuc J (-170 to $+6$); 3, pLuc K (-81 to $+6$).

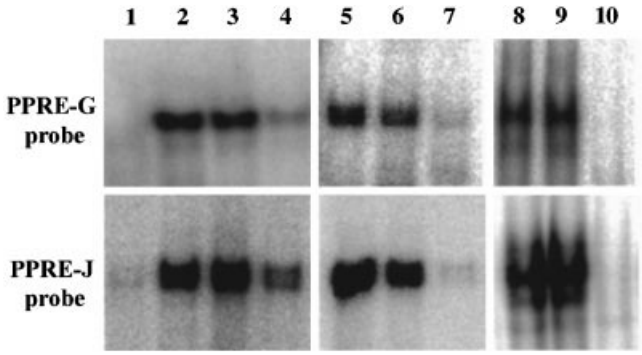

Figure 5 Electrophoretic mobility-shift assay of the potential PPREs with PPARγ– and PPARδ–RXR heterodimers and with $C_2C_{12}N$ nuclear extracts

Unprogrammed lysate (lane 1), PPARγ–RXR (lanes 2–4), PPARδ–RXR (lanes 5–7) or undifferentiated $C_2C_{12}N$ nuclear extract (lanes 8–10) were incubated with the indicated FAT/CD36 PPRE probe and analysed by electrophoretic mobility-shift assay in the absence (lanes 1, 2, 5 and 8) or presence of a 50-fold molar excess of wild-type (lanes 4, 7 and 10) or mutated (lanes 3, 6 and 9) PPRE-G or PPRE-J.

gene and reproduce the salient features of regulation of the endogenous gene. Furthermore, two separate elements located in the fragments -309 to -170 and -170 to -81 appeared to be important for high-level expression in adipose cells.

Two PPAR-responsive elements in the regulatory sequences of the mouse FAT/CD36 gene are involved in expression during adipose differentiation of $C_2C_{12}N$ cells

Analysis of the sequence of the mouse FAT/CD36 promoter revealed the presence of two imperfect DR-1 elements [16], corresponding to bp -245 to -233 (defined as PPRE-G) and -120 to -108 (defined as PPRE-J). The ability of these potential regulatory elements to specifically bind PPAR/RXR heterodimers was analysed by electrophoretic mobility-shift assay using *in vitro*-translated PPARγ, PPARδ and RXRα. As has been described previously for PPREs [13], there was no significant binding of either PPARγ or PPARδ in the absence of RXR to

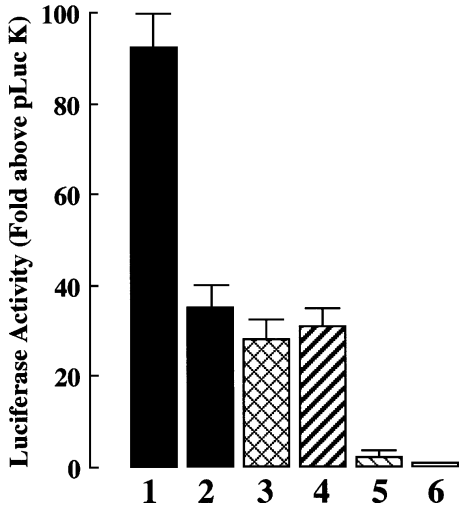

Figure 6 Effect of PPRE mutations on FAT/CD36 promoter activity in adipose-differentiated $C_2C_{12}N$ cells

Cells were cultured and transfected with the indicated constructs as for Figure 1. pSV-β-galactosidase was used as the transfection reporter. Luciferase activities were determined as for Figure 1 and are the means $\pm$ S.D. from three separate experiments. 1, pLuc G; 2, pLuc G-mPPRE-G; 3, pLuc G-mPPRE-J; 4, pLuc J; 5, pLuc J-mPPRE-J; 6, pLuc K (-81 to $+6$).

oligonucleotides encompassing the DR-1-like sequences, PPRE-G and PPRE-J (results not shown). In contrast, in the presence of RXRα there was binding of both PPARγ and PPARδ to both sequence elements, as shown by the appearance of a slower-mobility protein–DNA complex (Figure 5). Both PPREs also bound proteins from undifferentiated $C_2C_{12}N$ nuclear extracts (Figure 5). The pattern of the retarded bands appeared to be more complex than those generated by *in vitro*-translated proteins. This complexity could be the consequence of the presence in the nuclear extract of different proteins, i.e. various PPAR isoforms, able to bind the PPREs. This binding was specific, since competition with a 50-fold molar excess of unlabelled oligonucleotide abolished binding and complex formation, whereas competition with an oligonucleotide with mutations in the DR-1 sequences (see the Experimental section) did not (Figure 5). These data suggested that the DR-1-like sequences present in the FAT/CD36 promoter are binding sites for PPAR/RXR heterodimers.

To evaluate the functional significance of these putative PPREs in adipose-specific induction of FAT/CD36 gene expression, the core five nucleotides of the two DR-1-like elements from the FAT/CD36 promoter were mutated. Three mutant constructs were engineered: pLuc G-mPPRE-G (mutation in the 5′ PPRE), pLuc G-mPPRE-J (mutation in the 3′ PPRE) and pLuc J-mPPRE-J (mutation in the 3′ PPRE). Competition experiments in electrophoretic mobility-shift assays showed that the introduced mutations disrupted DNA affinity for the PPAR–RXR complex (Figure 5).

Wild-type and mutated luciferase constructs were transfected in undifferentiated cells or in $C_2C_{12}N$ cells differentiated into either myotubes or adipocytes. The PPRE mutations did not affect the low level of expression of the pLuc G and pLuc J constructs in both undifferentiated cells and myotubes (results not shown). In contrast, in adipocytes, the mutations dramatically decreased the expression of both pLuc G and pLuc J (Figure 6). Mutations in the distal and proximal PPREs both decreased luciferase expression of pLuc G equally, by about

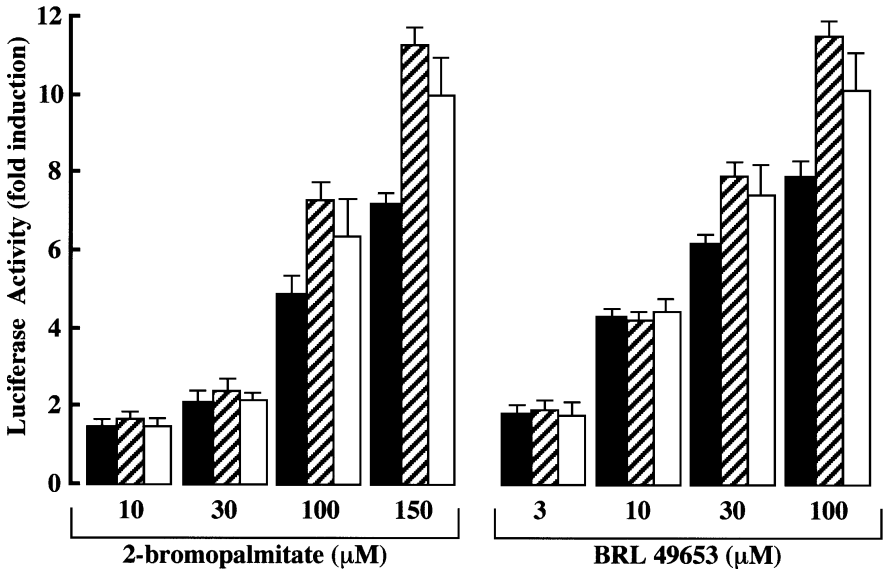

Figure 7 FAT/CD36 promoter activity in stably transfected Ob1771 pre-adipose cells

Cells from three Ob1771-pLuc G independent clones (black, hatched and white bars) were maintained for 2 days after confluence in standard medium containing the indicated concentrations of 2-bromopalmitate or BRL 49563. The value obtained for each clone with cells maintained in standard medium without any supplementation was set at 1. Results are the means ± S.D. from three independent experiments.

70%, to a level comparable with that of pLuc J. Mutation of the PPRE of the pLuc J construct almost completely abolished the activity of the promoter. These results demonstrated that the PPREs (and by extension the PPARs) played a crucial role in the regulation of FAT/CD36 gene expression in adipose cells, and that the two PPREs present in the mouse FAT/CD36 promoter co-operated to drive high-level expression in adipose cells.

Activity of the FAT/CD36 promoter in Ob1771 pre-adipose cells

The activity of pLuc G was next investigated in a pre-adipose cell context by stable transfection of Ob1771 cells [13]. To this end, cells were co-transfected with a neomycin-resistant expression vector (pSV-neo) together with the pLuc G construct. After 3 weeks of selection in G418-containing media, 13 individual clones were isolated. Cells were grown to confluence and then maintained for 48 h in standard media in the absence or presence of 2-bromopalmitate, a potent inducer of FAT/CD36 gene expression in pre-adipocytes [13]. The level of luciferase activity varied by clone, and both low- and high-expressing clones were identified. This expression heterogeneity most probably resulted from different plasmid-integration events (in terms of both number and location) in transfected cells. Despite this high variability in basal expression amongst the Ob1771-pLuc G clones, exposure to 100 μM 2-bromopalmitate resulted in reproducible and significant induction of luciferase activity (6.44 ± 0.8-fold) when compared with cells maintained in standard medium. The response to increasing concentrations of 2-bromopalmitate or BRL 49653 was further investigated in three independent Ob1771-pLuc G clones. As shown in Figure 7, luciferase activity was induced in a dose-dependent manner by both PPAR activators, leading to a maximal induction of 11.5-fold for 150 μM 2-bromopalmitate and 12-fold for 100 μM BRL 49653. The levels of activity induction of the pLuc G construct achieved in this dose–response experiment by 2-bromopalmitate and BRL 49653 were reminiscent of FAT/CD36 mRNA accumulation in pre-adipose cells [13].

DISCUSSION

This report describes the first structural and functional characterization of the mouse FAT/CD36 promoter. We are confident that this is indeed the murine FAT/CD36 promoter because it was contained within a phage clone that also contained FAT/CD36 exons 1–3, which matched the published murine cDNA sequence. Computer-assisted analysis of this 5′-flanking region of mouse FAT/CD36 gene did not reveal a TATA box. Promoters without TATA boxes were first described for housekeeping genes, but it is now well established that numerous genes with hormonal regulation and restricted tissue expression have such promoters. As in many of these genes, GC-rich sequences were found further upstream and a consensus binding site for Sp1 was noted, which may serve as the promoter for murine FAT/CD36. The human CD36 promoter is reported to contain a TATA element (TATTTAAA) at −20, and a CAAT box (CAAT) at −120. Both these reported elements differ from the consensus sequence, however, and no functional studies have been published to date.

More relevant to adipocyte expression is the presence of two PPREs. Members of the PPAR family have previously been shown to be involved in FAT/CD36 expression regulation [13,15], and this article constitutes the first report of functional PPREs in the FAT/CD36 gene.

It is somewhat surprising that the human promoter was completely without activity in our studies (Figure 1), because the more distal PPRE is conserved in the published human sequence (Figure 2). It is possible that the human promoter needs other upstream and/or downstream sequences to be active, or contains a strong repressor element. A study of the human promoter demonstrated that 5′ sequence from bp −158 to +43 is sufficient to drive monocyte-specific transcription of the CD36 gene [25].

More recently, however, in murine macrophage cell lines, expression was only achieved with co-transfection of PPAR/RXR-expressing constructs [31], raising the possibility that the amount of these factors is crucial for promoter activity.

Transient transfection of 5′ deletion constructs of the murine FAT/CD36 promoter in the myotube/adipocyte bipotential cell system, $C_2C_{12}N$, revealed that the region from −309 to +6 contained all the information necessary for strong expression in adipose cells. These same constructs were only weakly active in fibroblasts or myotubes (Figures 3 and 4). Deletion of sequences from −309 to −170 resulted in a 3-fold decrease in promoter activity in adipose cells. However, this −170 bp promoter still retained greater activity in adipocytes than in undifferentiated or myogenic $C_2C_{12}N$ cells. Moreover, reminiscent of endogenous FAT/CD36 regulation, the activities of the pLuc G (−309 to +6) and pLuc J (−170 to +6) constructs were increased during the course of adipose differentiation. Further deletion from −170 to −81 resulted in a 30-fold decrease in promoter activity and led to a level of expression comparable with that of the promoterless control luciferase construct, suggesting that the 5′ border of the minimal promoter is located in this portion of DNA.

An interesting feature of FAT/CD36 gene regulation is its induction by fatty acids and high-fat diets in a variety of tissues and cell types, including pre-adipocytes [13], small intestine [9] and cardiomyocytes [10]. In pre-adipocytes, we showed that transcription of the FAT/CD36 gene is strongly up-regulated by fatty acids and thiazolidinediones by a mechanism involving lipid-activated nuclear receptors, also called PPARs [13]. Transient transfection of NIH-3T3 fibroblasts with the −309 bp FAT/CD36-luciferase construct demonstrated that both PPARγ and PPARδ were capable of transactivation of the reporter plasmid upon thiazolidinedione or prostacyclin treatment, respectively (results not shown). In Ob1771 cells stably transfected with the pLuc G construct, a strong parallel was observed between regulation of luciferase activity and that of FAT/CD36 gene expression (Figure 7 and [13]). Taken together, these observations and the sequence data suggested strongly the existence of PPAR-responsive elements in the FAT/CD36 promoter. These responsive elements were unequivocally identified by biochemical analysis and site-directed mutagenesis. The murine FAT/CD36 promoter contained two imperfect direct repeats of a hexamer, similar to PPREs identified in other genes regulated via PPAR activation, such as acyl-CoA oxidase [17] and ALBP [21]. Electrophoretic mobility-shift assays indicated that both murine FAT/CD36 PPREs could bind both PPARγ– and PPARδ–RXR heterodimers (Figure 5). Mutations introduced in these PPREs, which disrupted binding of PPAR–RXR heterodimers, demonstrated that both regulatory elements were involved in the induction of gene expression during the adipose differentiation process. Disruption of either the distal or proximal PPRE resulted in a 70% decrease in FAT/CD36 promoter activity, leading to a level of expression comparable with that of pLuc J (−170 to +6), which contained only the proximal PPRE (Figure 6). Disruption of this PPRE in the pLuc J construct almost completely abolished the activity of the promoter in adipose $C_2C_{12}N$ cells. These data strongly support the crucial role of PPARs in the transcriptional control of FAT/CD36 expression in adipocytes. Such a direct implication of PPARδ in the control of FAT/CD36 gene expresssion by LCFAs was recently demonstrated in the Ob1771 cell line. We reported [32] that expression in these pre-adipose cells of a dominant-negative PPARδ mutant, which specifically inhibits PPARδ-mediated transcription, resulted in a striking reduction of fatty acid-induced FAT/CD36 mRNA. Furthermore, an increase in PPARδ concentration in the same cells led to an improvement of FAT/CD36 gene expression in response to LCFA treatment [32]. As members of the PPAR family are also expressed in intestine and heart [13], in which the FAT/CD36 gene is up-regulated by high-fat diets and fatty acids [9,10], it is possible that a similar mechanism of regulation may be occurring in these tissues as well.

FAT/CD36 plays an important role in fatty acid homoeostasis, as supported by recent evidence obtained with FAT/CD36-null mice [28] or FAT/CD36-overexpressing animals [33]. Furthermore, LCFAs act as signalling molecules for adipose-tissue development, probably by activating PPARs. It could be proposed that FAT/CD36 controls adipocyte differentiation by regulating the levels of PPAR activators, fatty acids themselves [34] or arachidonate metabolites [35]. In this view, the direct implication of PPARs in FAT/CD36 transcriptional regulation described in this paper represents a positive-feedback loop in the process of adaptation of the adipose-tissue mass to nutritional status. Interestingly, a similar positive-feedback loop has been proposed [36–38] for the adipocyte and liver FABPs (A-FABP, also called aP2 or ALBP, and L-FABP), which are also transcriptionally controlled by PPARs and implicated in the activation of the nuclear receptors. It has been demonstrated that A-FABP and L-FABP are translocated to the nucleus, interact with the PPARs and could in this way provide the activators, i.e. LCFAs or synthetic molecules, directly to the nuclear receptor [36–38].

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